**Uniwersytet Przyrodniczy w Poznaniu**

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# mgr inż. Jakub Michalski

## Wykorzystanie wybranych cieczy jonowych do zwalczania lekoopornych bakterii z gatunku *Pseudomonas aeruginosa*

The use of selected ionic liquids for the control of drug-resistant bacteria *Pseudomonas aeruginosa*

**PRACA DOKTORSKA**

**Dyscyplina naukowa: Rolnictwo i ogrodnictwo**

**Promotor: prof. UPP dr hab. Dorota Narożna**

**Promotor pomocniczy: dr inż. Tomasz Cłapa**

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Współautorom publikacji,
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## Spis treści

Wykaz publikacji naukowych wchodzących w skład pracy doktorskiej pod tytułem „Wykorzystanie wybranych cieczy jonowych do zwalczania lekoopornych bakterii z gatunku *Pseudomonas aeruginosa*”: ..... 5

Wykaz skrótów ..... 6

1. Streszczenie ..... 8

2. Abstract ..... 11

3. Wprowadzenie ..... 14

3.1. Antybiotykooporność ..... 14

3.2. *Pseudomonas aeruginosa* ..... 15

3.3. Ciecze jonowe ..... 17

4. Cel pracy ..... 22

5. Materiały i metody ..... 24

5.1. Szczepy *Pseudomonas aeruginosa* ..... 24

5.2. Ciecze jonowe ..... 24

5.3. Antybiotyki ..... 25

5.4. Przygotowanie kultur bakteryjnych ..... 25

5.5. Ocena antybakteryjnego działania cieczy jonowych ..... 25

5.5.1. Wyznaczanie wartości MIC ..... 26

5.5.2. Wyznaczanie wartości MBC ..... 26

5.6. Ilościowe oznaczanie piocyjaniny ..... 26

5.7. Pomiar aktywności dehydrogenaz ..... 27

5.8. Oznaczanie biofilmu bakteryjnego ..... 28

5.9. Ocena występowania efektu synergii ..... 29

5.10. Aktywność hemolityczna ..... 29

6. Omówienie uzyskanych wyników ..... 31

6.1. Minimalne stężenie hamujące oraz minimalne stężenie bakteriobójcze dla cieczy jonowych wobec *P. aeruginosa* – Publikacja 1 ..... 31

6.2. Zmiany aktywności dehydrogenaz bakteryjnych *P. aeruginosa* po traktowaniu cieczami jonowymi – Publikacja 1 ..... 32

6.3. Oznaczenie zawartości piocyjaniny w kulturach traktowanych cieczami jonowymi – Publikacja 1 ..... 33

6.4. Wyznaczenie minimalnego stężenia hamującego antybiotyków – Publikacja 3 . 34

6.5. Oznaczenie ilości i aktywności metabolicznej biofilmów bakteryjnych – Publikacja 3 ..... 35
6.6. Analiza zależnego od stężenia wpływu cieczy jonowych na tworzenie biofilmu przez *P. aeruginosa* – Publikacja 3 ..... 37
6.7. Ocena występowania efektów synergii w kombinacjach cieczy jonowych i antybiotyków – Publikacja 3 ..... 38
6.8. Analiza zmian wrażliwości *P. aeruginosa* na antybiotyki w obecności cieczy jonowych – Publikacja 3 ..... 40
6.9. Oznaczanie hemolitycznej aktywności cieczy jonowych – Publikacja 3 ..... 41
7. Podsumowanie i wnioski ..... 43
8. Literatura ..... 45
Oświadczenia współautorów ..... 57
Publikacje wchodzące w skład rozprawy doktorskiej ..... 66
Publikacja 1 ..... 67
Publikacja 2 ..... 80
Publikacja 3 ..... 96

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**Michalski J.**, Odrzygóźdź C., Mester P., Narożna D., Cłapa T., 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids,* 369.
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**Publikacja 3**

**Michalski J.**, Cłapa T., Syguda A., Narożna D., van Oostrum P., Reimhult E. 2024 Morpholinium- based ionic liquids as potent antibiofilm and sensitizing agents for the control of Pseudomonas aeruginosa. *Journal of Molecular Biology*, 436, 13.
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## Wykaz skrótów

**[2,4-D]** - 2,4-dichlorofenoksyoctan

**[4-CPA]** - 4-chlorofenoksyoctan

**[Chlopyralid]** - dichloropirydyno-2-karboksylan

**[Dec2Mor]** - 4,4-didecylomorfolina

**[DecEtMor]** - 4-decylo-4-etylomorfolina

**[Dikamba]** - 3,6-dichloro-2-metoksybenzoesan

**[MCPA]** - 4 chloro-2-metylofenoksyoctan

**[MCPP]** - (±)-2-(4-chloro-2-metylofenoksy)propionian

**AMK** - amikacyna

**ATM** - aztreonam

**CAZ** - ceftazydym

**CFP** - cefoperazon

**CIP** - cyprofloksacyna

**CST** - kolistyna

**CV** - fiolet krystaliczny (ang. *crystal violet*)

**EPS** – polimery zewnątrzkomórkowe (ang. *extracellular polymeric substances*)

**ESKAPE** - *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* i *Enterobacter spp.*

**EUCAST** - Europejski Komitet ds. Oznaczania Lekowrażliwości (ang. *European Committee on Antimicrobial Susceptibility Testing*)

**FICI** - indeks frakcyjnego stężenia hamującego (ang. *fractional inhibitory concentration index*)

**HAI** - zakażenia wewnątrzszpitalne (ang. *healthcare-associated infections*)

**HCl** - kwas solny

**HGT** - horyzontalny transfer genów (ang. *horizotnal gene transfer*)

**IL** - ciecz jonowa (ang. *ionic liquid*)

**jtk** - jednostka tworząca kolonię

**LPS** - warstwa lipopolisacharydowa (ang. *lipopolysaccharide structure*)

**LVX** – lewofloksacyna

**MBC** - minimalne stężenie bakteriobójcze (MBC ang. *minimum bactericidal concentration*)

**MDR** - wielolekooporny (ang. *multidrug-resistant*)

**MEM** - meropenem

**MIC** - minimalne stężenie hamujące (MIC ang. *minimum inhibitory concentration*)

**$OD_{600}$** - gęstość optyczna dla 600 nm

**OFX** - ofloksacyna

**PBS** - sól fizjologiczna buforowana fosforanami (ang. *phosphate buffered saline*)

**PCN** - piocyjanina

**PDR** - całkowicie oporny (ang. *pandrug-resistant*)

**PIP** - piperacylina

**ppm** - części na milion (ang. *parts per million*)

**rpm** - obroty na minutę (ang. *revolutions per minute*)

**TOB** - tobramycyna

**TSA-Y** - agarowe podłoże tryptonowo-sojowe z ekstraktem drożdżowym (ang. *tryptic soy agar with yeast extract*)

**TSB-Y** - pożywka tryptonowo-sojowa z ekstraktem drożdżowym (ang. *tryptic soy broth with yeast extract*)

**TTC** - chlorek 2,3,5-trifenylotetrazoliowy

**UE** - Unia Europejska

**WB** - krew pełna (ang. *whole blood*)

**WE** - erytrocyty (ang. *washed erythrocytes*)

**WHO** – Światowa Organizacja Zdrowia (ang. *World Health Organization*)

**XDR** - o rozszerzonej oporności (ang. *extensively drug-resistant*)

# 1. Streszczenie

Pojawienie się lekoopornych bakterii oraz rozprzestrzeniająca się antybiotykooporność stanowią jedne z najważniejszych wyzwań stojących przed współczesną medycyną. Przykładem antybiotykoopornej bakterii o dużym znaczeniu klinicznym jest oportunistyczny patogen *Pseudomonas aeruginosa*. *P. aeruginosa* może wywoływać groźne, nierzadko śmiertelne, infekcje układu oddechowego czy moczowego. Ponadto gatunek ten jest jednym z głównych czynników etiologicznych odpowiedzialnych za zakażenia na terenie placówek szpitalnych. Powszechne występowanie wśród *P. aeruginosa* lekoopornych szczepów skutkuje obniżeniem skuteczności antybiotykoterapii oraz wzrostem trudności w leczeniu infekcji. W związku z tym poszukuje się nowych, alternatywnych metod zwalczania tej bakterii. Grupę związków o potencjalnym zastosowaniu w walce z *P. aeruginosa* stanowią ciecze jonowe. Ciecze jonowe to niskotopliwe sole, które mogą wykazywać szereg aktywności biologicznych, w tym właściwości antybakteryjne.

Podstawowym celem niniejszej pracy była ocena antybakteryjnego potencjału morfoliniowych cieczy jonowych na bazie herbicydów w zwalczaniu lekoopornych szczepów *Pseudomonas aeruginosa*, a także określenie możliwości wykorzystania tych związków jako samodzielnych środków antybakteryjnych, oraz jako adiuwantów w antybiotykoterapii. Badania przeprowadzono na czterech szczepach *P. aeruginosa* o dużym znaczeniu klinicznym - PAO1, LES B58, 39016, UCBPP-PA14, dla zestawu 12 morfoliniowych cieczy jonowych zsyntetyzowanych na bazie herbicydów.

Przeprowadzone badania pozwoliły określić zdolność morfoliniowych cieczy jonowych do hamowania wzrostu i przeżywalności *P. aeruginosa*. Wykazano, że antybakteryjna aktywność morfoliniowych cieczy jonowych zależy od struktury kationu i objawia się jedynie dla związków z dwoma długimi podstawnikami alkilowymi. Dodatkowo pokazano, że ciecze jonowe hamujące wzrost bakterii wykazują też działanie bakteriobójcze. W badaniach zidentyfikowano szczep 39016 jako niewrażliwy na działanie cieczy jonowych, tym samym wykazując istnienie szczepowo-specyficznej oporności na ciecze jonowe wśród gatunku *P. aeruginosa*. Wykazano również, że ciecze jonowe mogą hamować aktywność metaboliczną bakterii w stężeniach

niższych niż MIC, a zjawisko to jest w dużej mierze niezależne od zdolności tych związków do hamowania wzrostu bakteryjnego.

W badaniach nad zmianami wirulencji zaobserwowano, że subinhibicyjne stężenia morfoliniowych cieczy jonowych hamują syntezę piocyjaniny, ważnego zewnątrzkomórkowego czynnika wirulencji. Tym samym jako pierwsi wskazaliśmy na potencjał cieczy jonowych do obniżania patogeniczności *P. aeruginosa* przez hamowanie syntezy czynników wirulencji. Dodatkowo obserwowany efekt inhibicji syntezy piocyjaniny był częściowo zależny od struktury kationu, ale nie wynikał on ze spadku gęstości hodowli bakteryjnych.

Przeprowadzone badania nad inhibicją biofilmów pozwoliły zidentyfikować cztery ciecze jonowe zdolne do hamowania tworzenia biofilmu przez wszystkie badane szczepy *P. aeruginosa*. Inhibicja występowała dla stężeń niższych od wartości MIC, co wskazuje na wysoki potencjał morfoliniowych cieczy jonowych w zwalczaniu biofilmu bakteryjnego. Dodatkowo zaobserwowano stymulujący wpływ bardzo niskich stężeń badanych związków na tworzenie biofilmu przez szczepy LES B58 i UCBPP-PA14, zgodnie z efektem hormetycznym. Z drugiej strony wykazano, że morfoliniowe ciecze jonowe z dwoma długimi podstawnikami alkilowymi mogą w wysokich stężeniach promować powstawanie biofilmu na skutek wzmożonej agregacji bakterii. Jak do tej pory nikt wcześniej nie obserwował wystąpienia podobnych, zależnych od stężenia, efektów indukcji tworzenia biofilmu dla cieczy jonowych.

W pracy wykazano też, że morfoliniowe ciecze jonowe mogą tworzyć razem z antybiotykami kombinacje o charakterze addytywnym lub synergicznym. Dodatnie interakcje występowały najczęściej w mieszaninach z kolistyną oraz kombinacjach zawierających ciecze jonowe z dwoma długimi podstawnikami alkilowymi. Dodatkowo jako pierwsi pokazaliśmy, że dodatek cieczy jonowych może prowadzić do uwrażliwienia opornych szczepów *P. aeruginosa* na działanie antybiotyków, jednoznacznie wskazują na użyteczność wykorzystania cieczy jonowych jako adiuwantów w antybiotykoterapii.

Z drugiej strony, przeprowadzone eksperymenty wykazały, że wszystkie badane związki posiadają wysoką aktywność hemolityczną, co istotnie ogranicza potencjalne zastosowania morfoliniowych cieczy jonowych w leczeniu infekcji wywoływanych przez *P. aeruginosa*, zawężając je do stosowania pozaustrojowego. Aktywność hemolityczna była wyższa dla związków z dwoma długimi podstawnikami alkilowymi i dobrze korelowała z zaobserwowaną zależnością między strukturą kationów, a zdolnością badanych związków do hamowania wzrostu *P. aeruginosa*. Uzyskane rezultaty dostarczyły dodatkowych dowodów na to, że antybakteryjny mechanizm działania morfoliniowych cieczy jonowych oparty jest na oddziaływaniu tych związków z błonami komórkowymi.

Podsumowując, w badaniach wykazano wysoki potencjał morfoliniowych cieczy jonowych na bazie herbicydów w zwalczaniu lekoopornych bakterii z gatunku *P. aeruginosa*, z uwzględnieniem wpływu tych związków na wzrost i przeżywalność bakterii, aktywność metaboliczną, syntezę czynników wirulencji oraz tworzenie biofilmu. Ponadto wykazano, że ciecze jonowe mogą zwiększać wrażliwość *P. aeruginosa* na antybiotyki, tym samym znajdując potencjalne zastosowanie jako adiuwanty w antybiotykoterapii. Z drugiej strony, zaobserwowana aktywność hemolityczna oraz promocja tworzenia biofilmu przez ciecze jonowe, w skutek występowania hormezy i agregacji komórek, wskazała na istnienie istotnych ograniczeń w stosowaniu badanych związków w zwalczaniu patogenów bakteryjnych.

## 2. Abstract

The emergence of drug-resistant bacteria and the spread of antibiotic resistance represent a significant challenge to modern medicine. An example of an antibiotic-resistant bacterium of high clinical relevance is the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* can cause severe and often fatal infections of the respiratory or urinary tract. Furthermore, this species is one of the primary etiological agents responsible for nosocomial infections. The prevalence of drug-resistant strains among *P. aeruginosa* has resulted in a reduction in the efficacy of antibiotic therapy and an increase in the difficulty of infection treatment. Accordingly, there is a need to identify alternative strategies for combating this bacterium. Ionic liquids, a class of low-melting salts with diverse biological activities, including antibacterial properties, represent a promising avenue for developing new approaches to combat *P. aeruginosa*.

The primary objective of this study was to evaluate the antibacterial potential of morpholinium-based ionic liquids with herbicidal anions against drug-resistant strains of *Pseudomonas aeruginosa* and to determine the potential for using these compounds as stand-alone antibacterial agents and as adjuvants in antibiotic therapy. The study was conducted on four strains of *Pseudomonas aeruginosa* of high clinical importance: PAO1, LES B58, 39016, and UCBPP-PA14. These were tested against a set of 12 morpholinium-based ionic liquids synthesized from herbicides.

The conducted studies have determined the inhibitory potential of morpholinium-based ionic liquids to inhibit the growth and survival of *P. aeruginosa*. It was demonstrated that the antibacterial activity of morpholinium-based ionic liquids is contingent upon the structure of the cation, exhibiting a pronounced effect only in compounds with two long alkyl substituents. Furthermore, it was demonstrated that ionic liquids with inhibitory effect also exhibit bactericidal activity. The study identified strain 39016 as insensitive to ionic liquids, thereby demonstrating the existence of strain-specific resistance to ionic liquids among *P. aeruginosa* species. It was also demonstrated that ionic liquids can suppress the metabolic activity of bacteria at concentrations below the MIC, that was largely independent of the ability of these compounds to inhibit bacterial growth.

The results of the virulence studies indicated that subinhibitory concentrations of morpholinium-based ionic liquids inhibit the synthesis of pyocyanin, an important secreted virulence factor. Thus, we were the first to identify the potential of ionic liquids to reduce the pathogenicity of *P. aeruginosa* by inhibiting the synthesis of its virulence factors. The observed inhibitory effect was found to be partly dependent on the structure of the cation, but was not due to a decrease in bacterial culture density.

The results of the biofilm inhibition studies have enabled the identification of four ionic liquids with the capacity to inhibit biofilm formation by all tested strains of *P. aeruginosa*. Inhibition was observed at concentrations below the MIC values, highlighting the potential of the morpholinium-based ionic liquids to combat bacterial biofilms. Conversely, a stimulating effect of low doses of test compounds on biofilm formation by LES B58 and UCBPP-PA14 strains was observed, indicating a hormetic effect. Moreover, it was demonstrated that high concentrations of morpholinium-based ionic liquids with two long alkyl substituents can facilitate biofilm formation due to enhanced bacterial aggregation. To date, no other studies have reported the occurrence of concentration-dependent induction of biofilm formation by ionic liquids.

The study also demonstrated that morpholinium-based ionic liquids can form either additive or synergistic combinations with antibiotics. Positive interactions were the most prevalent in mixtures with colistin and combinations comprising ionic liquids with two long alkyl substituents. Furthermore, we were the first to demonstrate that the addition of ionic liquids can result in the sensitisation of drug-resistant *P. aeruginosa*, thereby providing a clear evidence of the potential utility of ionic liquids as adjuvants in antibiotic therapy.

Conversely, the experiments demonstrated that all tested ionic liquids exhibit high haemolytic activity, which considerably limits the potential applications of morpholinium-based ionic liquids in the control of *P. aeruginosa* infections, confining them to extracorporeal utilisation. The degree of haemolytic activity was found to be higher for compounds with two long alkyl substituents and correlated well with the observed relationship between cation structure and the ability of the tested compounds to inhibit the growth of *P. aeruginosa*. The results provided further evidence that the

antibacterial mechanism of action of morpholinium-based ionic liquids is based on the interaction of these compounds with cell membranes.

In conclusion, the study demonstrated the high potential of morpholinium-based ionic liquids synthesized from herbicides for the control of drug-resistant *P. aeruginosa*, providing insight into the effects of these compounds on bacterial growth and survival, metabolic activity, synthesis of virulence factors and biofilm formation. Moreover, it was demonstrated that ionic liquids can enhance *P. aeruginosa* sensitivity to antibiotics, indicating a potential role for ionic liquids in antibiotic therapy as adjuvants. Conversely, the observed haemolytic activity and promotion of biofilm formation by ionic liquids, due to the occurrence of hormesis and cell aggregation, highlighted significant limitations of ionic liquids in their use against bacterial pathogens.

# 3. Wprowadzenie

## 3.1. Antybiotykooporność

Antybiotykooporność to pojęcie opisujące występującą wśród bakterii niewrażliwość na działanie antybiotyków. Oporność na antybiotyki można podzielić na dwa typy - naturalną i nabytą (Singh i in., 2020). Oporność naturalna stanowi wrodzoną niewrażliwość bakterii na dany antybiotyk lub grupę antybiotyków i jest cechą charakterystyczną dla danego gatunku. Ten typ oporności może wynikać ze strukturalnego zróżnicowania budowy komórki, obecności lub braku określonych receptorów komórkowych bądź enzymów, lub z istnienia różnic w przepuszczalności błon bakteryjnych, które warunkują gatunkową niewrażliwość na działanie danego związku (Kostyanev i Can, 2017). W przypadku oporności nabytej, bakterie pierwotnie podatne na konkretny ksenobiotyk nabierają cech oporności w wyniku zajścia spontanicznej mutacji lub pozyskania genów oporności od innych mikroorganizmów na drodze horyzontalnego transferu genów (HGT ang. *horizontal gene transfer*) (Reygaert, 2018). Nabywanie oporności jest jednym z podstawowych mechanizmów adaptacyjnych wykorzystywanych przez bakterie celem przystosowania się do niekorzystnych warunków środowiskowych, w tym do obecności środków antybakteryjnych (Munita i Arias, 2016).

Obserwowane w ostatnich dekadach wysokie tempo rozprzestrzeniania się antybiotykooporności wśród ludzkich patogenów stanowi jedno z najważniejszych wyzwań przed którym stoi współczesna medycyna (Chinemerem Nwobodo i in., 2022). Główną przyczyną tego zjawiska jest nadmierne stosowanie antybiotyków oraz środków dezynfekujących w rolnictwie, przemyśle i medycynie (Dadgostar, 2019). Konsekwencją nieodpowiedniego gospodarowania antybiotykami jest przedostawanie się tych związków do środowiska. Ekspozycja bakterii na antybiotyki w stężeniach niższych od dawek hamujących wzrost mikoorganizmów prowadzi do wystąpienia presji selekcyjnej, która promuje przeżywanie i wzrost szczepów opornych. Dodatkowo obecność niskich stężeń środków antybakteryjnych stanowi czynnik stresowy, który prowadzi do wzrostu częstości zachodzenia HGT i dalszego rozsiewania genów oporności w środowisku (Larsson i Flach, 2022).

Najpoważniejszym skutkiem postępującej lekooporności jest pojawienie się i rozprzestrzenienie szczepów wielolekoopornych, wykazujących szerokie spektrum niewrażliwości na antybiotyki. Ze względu na stopień nabytej oporności rozróżnia się 3 kategorie szczepów lekoopornych: wielolekooporne MDR (ang. *multidrug-resistant*), o rozszerzonej oporności XDR (ang *extensively drug-resistant*) i całkowicie oporne PDR (ang. *pandrug-resistant*). Szczepy MDR wykazują oporność na co najmniej jeden antybiotyk z trzech lub więcej grup antybiotyków. Szczepy XDR są wrażliwe na działanie co najwyżej dwóch grup antybiotyków. Szczepy PDR są niewrażliwe na wszystkie antybiotyki stosowane do zwalczania konkretnego gatunku bakterii (Magiorakos i in., 2012). Pojawienie się szczepów wielolekoopornych ma swoje daleko idące medyczne konsekwencje. Infekcje wywoływane przez lekooporne szczepy są trudne do wyleczenia i charakteryzują się niższą skutecznością antybiotykoterapii, wyższą śmiertelnością, dłuższym czasem hospitalizacji i wyższymi kosztami leczenia (Chinemerem Nwobodo i in., 2022). Szacuje się, że w samym roku 2022 infekcje wywołane przez lekooporne patogeny przyczyniły się do śmierci około 5 milionów osób, przy czym dla około 25% zgonów wystąpienie oporności było czynnikiem decydującym o śmierci (Antimicrobial Resistance Collaborators, 2022). W związku z potrzebą wyróżnienia najważniejszych bakterii związanych z problemem lekooporności utworzono grupę o akronimie ESKAPE, zawierającą sześć wysoce opornych patogenów o ogromnym znaczeniu klinicznym - *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* i *Enterobacter spp.* (Santajit i Indrawattana, 2016).

## 3.2. *Pseudomonas aeruginosa*

Należąca do ESKAPE *Pseudomonas aeruginosa* (inaczej pałeczka ropy błękitnej) to Gram-ujemna niefermentująca bakteria zdolna do wywoływania infekcji u ludzi. *P. aeruginosa* występuje powszechnie w glebie i zbiornikach wodnych, a także w mikrobiomie roślin i zwierząt (W. Wu i in., 2014). Pałeczka ropy błękitnej charakteryzuje się niskimi wymaganiami pokarmowymi, wykazując równocześnie tolerancję na szereg niekorzystnych czynników środowiskowych, takich jak wysokie zasolenie czy obecność dezynfektantów (Lyczak i in., 2000; W. Wu i in., 2014). Obie te cechy przekładają się na łatwość z jaką *P. aeruginosa* jest w stanie adaptować się do nieprzyjaznych środowisk i zasiedlać nisze ekologiczne o zróżnicowanym charakterze.

*P. aeruginosa* wykazuje dużą różnorodność genetyczną i zmienność międzyszczepową (Cullen i in., 2015; De Soyza i in., 2013). Dzięki niskiej przepuszczalności błon komórkowych i obecności licznych pomp wielolekowych *P. aeruginosa* wykazuje naturalną oporność na szeroki zakres antybiotyków, głównie z grupy antybiotyków β-laktamowych (Pang i in., 2019). Wysoka zdolność do tworzenia biofilmu również przekłada się na zwiększony potencjał adaptacyjny tego gatunku (Gellatly i Hancock, 2013).

*P. aeruginosa* jest zaliczana do grupy ludzkich patogenów oportunistycznych, czyli bakterii infekujących wyłącznie osoby z obniżoną odpornością lub znajdujące się w grupie ryzyka (Martinez, 2014). Pałeczka ropy błękitnej może powodować infekcje dróg oddechowych, infekcje układu moczowego, sepsę czy zapalenie opon mózgowych (Reynolds i Kollef, 2021). Wśród czynników zwiększających prawdopodobieństwo rozwinięcia się infekcji *P. aeruginosa* występują takie schorzenia jak mukowiscydoza, cukrzyca, choroby hemolityczne, nowotwory oraz obecność poparzeń (Restrepo i in., 2018). Oprócz tego, szczególnie podatni na zakażenie są pacjenci długotrwale przebywający na oddziałach intensywnej terapii oraz osoby poddawane cewnikowaniu lub intubacji (Merchant i in., 2018). *P. aeruginosa* syntetyzuje szereg czynników wirulencji m.in. ramnolipidy, alginiany, egzotoksyny czy enzymy zewnątrzkomórkowe, umożliwiające skuteczne infekowanie organizmu gospodarza (W. Wu i in., 2014). Wśród czynników wirulencji na wyróżnienie zasługuje piocyjanina, barwnik wytwarzany wyłącznie przez bakterie z gatunku *P. aeruginosa.* W trakcie infekcji piocyjanina prowadzi do indukcji stresu oksydacyjnego w komórkach gospodarza oraz wywołuje silną odpowiedź zapalną (Hall i in., 2016). Dodatkowo pośredniczy ona w mechanizmach oporności oraz w procesie tworzenia biofilmu (Muller i Merrett, 2014). Sam biofilm także stanowi jeden z podstawowych czynników wirulencji, zapewniając mikrorganizmom dodatkową ochronę przed działaniem niekorzystnych czynników zewnętrznych (Davies, 2003). Ponadto biofilm bakteryjny sprzyja zachodzeniu HGT i rozprzestrzenianiu się genów oporności (Abe i in., 2021; Muhammad i in., 2020).

*P. aeruginosa* stanowi jedną z najczęstszych przyczyn zakażeń wewnątrzszpitalnych (HAIs, ang. hospital associated-infections), czyli infekcji do których dochodzi na terenie placówek ochrony zdrowia (Sundermann i in., 2021). Gatunek ten

odpowiadał za około 11 % zapaleń płuc, 9 % zakażeń układu moczowego i 6,5 % zakażeń krwi występujących u pacjentów europejskich szpitali w latach 2022-2023 (European Centre for Disease Prevention and Control, 2024). Ponadto placówki ochrony zdrowia stanowią szczególnie przyjazne środowisko dla rozwoju i rozprzestrzeniania się antybiotykooporności. Przyczynia się to do powszechnego występowania lekoopornych szczepów wśród gatunku pałeczki ropy błękitnej. O skali zjawiska świadczy fakt, że około 31 % wszystkich klinicznych izolatów pozyskiwanych na terenie UE jest niewrażliwe na co najmniej jedną grupę antybiotyków, a 13 % stanowią szczepy o profilu MDR (European Centre for Disease Prevention and Control i World Health Organization, 2023). Dodatkowo infekcje wielolekoopornymi szczepami *P. aeruginosa* doprowadziły do 340 000 śmierci w 2022 roku, co stanowiło około 7% wszystkich zgonów spowodowanych przez antybiotykooporne patogeny (Antimicrobial Resistance Collaborators, 2022).

### 3.3. Ciecze jonowe

Ciecze jonowe (ang. *ionic liquids*) to złożone z jonów sole o temperaturze topnienia niższej niż 100°C (Lei i in., 2017). Choć istnieją przykłady cieczy jonowych o nieorganicznych kationach, najczęściej są one zbudowane z organicznego kationu sparowanego z anionem o organicznym lub nieorganicznym pochodzeniu (Tucker i in., 2017). Ciecze jonowe charakteryzują się niską prężnością par, wysoką stabilnością termiczną oraz szerokim oknem elektrochemicznym (Ong i in., 2011; Shah i in., 2013). Ponadto ciecze jonowe wykazują zdolność do rozpuszczania szerokiej gamy związków (Kubisa, 2004; Marullo i in., 2021), dzięki czemu znalazły one zastosowanie w rozlicznych dziedzinach chemii takich jak chemia katalityczna, koloidalna lub chemia granic fazowych, a także w technikach separacyjnych (Pendleton i Gilmore, 2015). Poprzez dobór odpowiedniego anionu i kationu możliwe jest nadanie cieczom jonowym pożądanych, ściśle ukierunkowanych właściwości, możliwych do przewidzenia już na etapie syntezy (Han i in., 2019). Co za tym idzie, specyficzne właściwości cieczy jonowych wynikające ze struktury jonów mogą być bardzo odmienne i obejmować swoim zakresem zróżnicowane własności fizyczne, chemiczne lub biologiczne (Shamshina i Rogers, 2023). Możliwość sparowania ze sobą kationów i anionów o unikalnych cechach doprowadziła do syntezy dwufunkcyjnych cieczy jonowych posiadających wysoce wyspecjalizowane właściwości (Choi i Nidetzky, 2022; Hough-

Troutman i in., 2009; Wilms i in., 2020). Dodatkowo ogromna liczba możliwych do uzyskania kombinacji kationu i anionu, szacowana na $10^{18}$, czyni z cieczy jonowych bardzo wszechstronną grupę związków o teoretycznie nieograniczonym potencjale (Pendleton i Gilmore, 2015).

Ciecze jonowe, które wykazują ukierunkowaną aktywność biologiczną są zaliczane do trzeciej generacji (Hough i in., 2007). Wśród aktywności biologicznych opisanych dla cieczy jonowych można wyróżnić m.in. działanie antybakteryjne, przeciwwirusowe, przeciwgrzybiczne, herbicydowe czy deterentne, a także funkcje przeciwzapalne, znieczulające lub przeciwzakrzepowe (Chantereau i in., 2019; Ferraz i in., 2020; Kaczmarek i in., 2018; Mir i in., 2024; Turguła i in., 2020). Biologicznie aktywne ciecze jonowe najczęściej zawierają kationy czwartorzędowych soli amonowych lub fosfoniowych, czy kationowe pochodne imidazolu, pirydyny, piperydyny, pirolidyny, chinoliny lub morfoliny (Egorova i in., 2017; Pernak i in., 2003). Kationy te mogą współtworzyć ciecze jonowe z szerokim wachlarzem anionów, począwszy od prostych anionów nieorganicznych takich jak aniony halogenkowe czy reszty kwasów tlenowych, przez aniony organiczne jak wodorosiarczan metylu, dicyjanamid czy trifluorometanosulfonian, aż po bardzo złożone pochodne związków aktywnych biologicznie takich jak herbicydy, antybiotyki czy substancje czynne (Ferraz i in., 2020; Mir i in., 2024; Nikfarjam i in., 2021; Wilms i in., 2020). Początkowo ciecze jonowe były uznawane za „zielone" związki o niewielkim wpływie na środowisko i organizmy żywe, w przeciwieństwie do powszechnie stosowanych rozpuszczalników organicznych. Z czasem jednak zaczęto coraz częściej obserwować toksyczne działanie tych związków, co pozwoliło na otwarcie nowych kierunków badań nad potencjalnym wykorzystaniem cieczy jonowych jako środków biobójczych (Pendleton i Gilmore, 2015).

Strukturalne zależności między budową a antybakteryjną aktywnością cieczy jonowych, wraz z uwzględnieniem sposobów oddziaływania z bakteriami, zostały szczegółowo opisane w pracy przeglądowej wchodzącej w skład niniejszej rozprawy (**Publikacja 2**; Michalski, Odrzygóźdź, i in., 2023). Antybakteryjne działanie cieczy jonowych jest ściśle powiązane z ich budową i zależy przede wszystkim od: rodzaju kationu; długości alkilowych łańcuchów bocznych podstawionych do kationu; liczby długich alkilowych podstawników kationu; rodzaju grup funkcyjnych podstawionych do kationu; rodzaju

anionu; oddziaływań między jonami w obrębie struktury związku (**Publikacja 2;** Michalski, Odrzygóźdź, i in., 2023; Gal i in., 2012; Mikkola i in., 2015; X. Wu i in., 2013). Podczas gdy zarówno rodzaj kationu, jak i anionu, wpływa na toksyczność cieczy jonowych, najważniejszą determinantą ich antybakteryjnych właściwości jest długość łańcuchów bocznych podstawionych do kationu (Anvari i in., 2016). Wykazano, że toksyczność cieczy jonowych rośnie wraz ze wzrostem długości łańcucha bocznego, a najsilniejsze właściwości antybakteryjne wykazują związki, których kationy posiadają podstawnik o długości od 10 do 14 atomów węgla (Pernak i in., 2003; Ranke i in., 2004). Podobny efekt zaobserwowano również w przypadku cieczy jonowych zbudowanych z anionów reszt kwasów tłuszczowych o zróżnicowanej długości (Gundolf i in., 2019). Co ciekawe, wzrost długości reszt alkilowych powyżej pewnego poziomu (punktu odcięcia; ang. *cut-off*) prowadzi do osłabienia antybakteryjnej aktywności (Kowalczyk i in., 2018; Stolte i in., 2007). Wynika to m.in. ze spadku rozpuszczalności, występowania efektów sterycznych czy agregacji cząsteczek (Ventura i in., 2012). Podobnie jak w przypadku długości pojedynczego podstawnika, rosnąca liczba długich alkilowych łańcuchów bocznych także prowadzi do wzrostu toksyczności cieczy jonowych (Drücker i in., 2017; Sommer i in., 2018).

Antybakteryjne działanie cieczy jonowych oparte jest na kilku mechanizmach. Podstawowy z nich zakłada sorpcję cieczy jonowych na powierzchni bakterii oraz ich inkorporację do błon komórkowych. W efekcie dochodzi do permeabilizacji błon i przerwania ich ciągłości, a następnie wycieku materiału cytoplazmatycznego, co prowadzi do śmierci komórki (**Publikacja 2;** Michalski, Odrzygóźdź, i in., 2023; Benedetto, 2023). Opisany mechanizm pokrywa się ze sposobem działania surfaktantów i związków powierzchniowo czynnych, które wykazują silne strukturalne podobieństwo z cieczami jonowymi (Pendleton i Gilmore, 2015). Szkodliwy wpływ cieczy jonowych na bakteryjne błony jest także pozytywnie skorelowany z długością oraz liczbą hydrofobowych łańcuchów bocznych (Cook i in., 2019; Drücker i in., 2017; Witos i in., 2017). Ponadto ciecze jonowe są w stanie wpływać na szereg fizykochemicznych właściwości błon biologicznych takich jak grubość, płynność, krzywizna, sztywności i elastyczność czy potencjał elektrokinetyczny (**Publikacja 2;** Michalski, Odrzygóźdź, i in., 2023; Benedetto, 2023). Z drugiej strony, bakterie mogą w sposób aktywny bronić się przed szkodliwym działaniem cieczy jonowych poprzez uruchomienie pomp błonowych lub zmianę kompozycji błon komórkowych np. zwiększając frakcję

nienasyconych kwasów tłuszczowych (Borkowski i in., 2018; Khudyakov i in., 2012). Antybakteryjna skuteczność cieczy jonowych zależy także od budowy bakterii. Wykazano, że gatunki Gram-ujemne są o wiele mniej wrażliwe na działanie cieczy jonowych w porównaniu z bakteriami Gram-dodatnimi (Busetti i in., 2010; Weyhing-Zerrer i in., 2017). Różnica ta wynika z obecności warstwy lipopolisacharydowej (LPS, *ang. lipopolysaccharide structure*) na powierzchni bakterii Gram-ujemnych oraz z różnic w budowie ściany komórkowej (Gundolf i in., 2018; Kowalczyk i in., 2018).

Inny mechanizm toksyczności cieczy jonowych oparty jest na ich oddziaływaniu z białkami. Ciecze jonowe mogą zarówno stabilizować strukturę białek, jak i prowadzić do ich denaturacji (Schindl i in., 2019). Denaturujący efekt jest warunkowany przez stężenie i strukturę cieczy jonowych, i zależy przede wszystkim od rodzaju użytego anionu (Jha i Venkatesu, 2016; Tarannum i in., 2019). Jednocześnie budowa kationu również wpływa na denaturujące właściwości cieczy jonowych (Jianliang i in., 2017; **Michalski**, Sommer, i in., 2023). W przypadku bakterii wykazano, że wysoka antybakteryjna skuteczność cieczy jonowych na bazie choliny i pochodnych imidazolu o krótkich łańcuchach bocznych jest skorelowana z ich właściwościami denaturującymi (Mester, Wagner, i in., 2015; Wang i in., 2021). Dodatkowo wskazano, że bakteriobójcze imidazoliowe ciecze jonowe z długimi łańcuchami bocznymi prowadzą do denaturacji białek bakteryjnych, jednocześnie nie wywołując zmian w błonach komórkowych (Mester i in., 2019).

Oprócz wpływu na błony i białka, ciecze jonowe mogą wywoływać zmiany w komórkach bakterii na poziomie transkryptomu, proteomu i metabolomu (Borkowski i in., 2018; Khudyakov i in., 2012). Wykazano, że ekspozycja na ciecze jonowe indukuje bakteryjną odpowiedź stresową i prowadzi do tworzenia reaktywnych form tlenu, a także wywołuje szereg zmian w aktywności enzymów odpowiedzialnych za ochronę bakterii przed stresem oksydacyjnym (Yu i in., 2016; Zhang i in., 2013). Oprócz tego, powstałe reaktywne formy tlenu najprawdopodobniej pośredniczą w wywoływaniu uszkodzeń bakteryjnego DNA, obserwowanych po ekspozycji mikroorganizmów na ciecze jonowe (Kowalczyk i in., 2018). Ciecze jonowe mogą również oddziaływać na biofilm bakteryjny, hamując jego tworzenie lub prowadząc do dyspersji dojrzałych biofilmów. Podobnie jak w przypadku badań nad toksycznością, wykazano że biofilmy tworzone przez bakterie Gram-dodatnie są bardziej podatne na

działanie cieczy jonowych (Duman i in., 2019). Dodatkowo przeciwbiofilmowa aktywność tych związków jest również silnie zależna od długości łańcuchów bocznych (Busetti i in., 2010). Hamujące biofilm działanie cieczy jonowych zostało opisane dla związków zawierających m.in. imidazoliowe, morfoliniowe czy pyrolidyniowe kationy (Florio i in., 2019; Venkata Nancharaiah i in., 2012). Potencjał cieczy jonowych do inhibicji tworzenia biofilmu jest w dużej mierze oparty na ich zdolności do utrudniania komórkom bakteryjnym adhezji do powierzchni (Venkata Nancharaiah i in., 2012). W związku z tym, ciecze jonowe mogą być wykorzystywane do syntezy materiałów lub powłok o właściwościach zapobiegających przyleganiu bakterii do podłoża (Anandkumar i in., 2020). Zdolność do eradykacji dojrzałych biofilmów wykazano m.in. dla imidazoliowych, piperydyniowych czy cholinowych cieczy jonowych (Anandkumar i in., 2020; Duman i in., 2019; Rita Pereira i in., 2021). Ciecze jonowe mogą także destabilizować strukturę biofilmu poprzez bezpośrednie oddziaływanie z elementami macierzy biofilmu, takimi jak zewnątrzkomórkowe DNA (**Publikacja 2;** Michalski, Odrzygóźdź, i in., 2023; Rita Pereira i in., 2021).

# 4. Cel pracy

*Pseudomonas aeruginosa* jest oportunistycznym patogenem, który jest odpowiedzialny za istotny procent zakażeń wewnątrzszpitalnych. Niektóre z jego izolatów klinicznych i ich subpopulacje wykazują wysoką przeżywalność w obecności szerokiego spektrum antybiotyków zalecanych przez Europejski Komitet ds. Oznaczania Lekowrażliwości EUCAST. W 2024 roku antybiotykooporne szczepy *P. aeruginosa* zostały przypisane przez Światową Organizację Zdrowia WHO do grupy patogenów o wysokim priorytecie dla prowadzenia badań nad ich zwalczaniem (World Health Organization, 2024). Znalezienie terapii skutecznie zwalczającej antybiotykooporne szczepy *P. aeruginosa* stanowi istotne wyzwanie, ponieważ wymaga opracowania nowych związków przeciwdrobnoustrojowych należących nie tylko do grupy antybiotyków. Jedną z obiecujących grup związków hamujących wzrost patogenów bakteryjnych są ciecze jonowe, które w połączeniu z antybiotykami mogą wzmacniać ich działanie wobec lekoopornych bakterii.

Głównym celem przeprowadzonych badań była ocena antybakteryjnego potencjału morfoliniowych cieczy jonowych na bazie herbicydów w zwalczaniu lekoopornych szczepów *Pseudomonas aeruginosa*, w kontekście wykorzystania tych związków jako samodzielnych środków antybakteryjnych oraz jako adiuwantów w antybiotykoterapii.

W ramach realizacji głównego celu badawczego zaplanowano cele szczegółowe:

1. charakterystyka antybakteryjnego wpływu morfoliniowych cieczy jonowych na bazie herbicydów na wzrost i przeżywalność wybranych czterech szczepów (PAO1, LES B58, 39016, UCBPP-PA14) *P. aeruginosa*,
2. ocena wpływu morfoliniowych cieczy jonowych na zjadliwość *P. aeruginosa* związaną ze zmianą poziomu syntezy niebiałkowego czynnika wirulencji, piocyjaniny,
3. określenie przeciwbiofilmowych właściwości morfoliniowych cieczy jonowych na bazie herbicydów oraz ocena ich wpływu na tworzenie i aktywność metaboliczną biofilmów pałeczki ropy błękitnej,
4. analiza występowania efektu synergicznego pomiędzy morfoliniowymi cieczami jonowymi na bazie herbicydów, a wybranymi antybiotykami, w zwalczaniu lekoopornych szczepów *P. aeruginosa*,

5. ocena aktywności hemolitycznej morfoliniowych cieczy jonowych na bazie herbicydów w stosunku do ludzkich erytrocytów.

# 5. Materiały i metody

## 5.1. Szczepy *Pseudomonas aeruginosa*

W badaniach wykorzystano cztery szczepy *Pseudomonas aeruginosa*: szczep modelowy PAO1, oraz szczepy LES B58, 39016 i UCBPP-PA14, o dużym znaczeniu klinicznym i badawczym. Szczepy bakteryjne zostały dobrane w taki sposób by jak najlepiej reprezentować biologiczną różnorodność występującą wśród gatunku *P. aeruginosa*. PAO1 jako szczep modelowy jest najczęściej wykorzystywanym izolatem w badaniach nad pałeczką ropy błękitnej. LES B58 stanowi szczep referencyjny w badaniach nad infekcjami dróg oddechowych pacjentów chorych na mukowiscydozę. Szczep UCBPP-PA14 jest obok PAO1 drugim najczęściej stosowanym w badaniach szczepem *P. aeruginosa*, wykorzystywanym głównie w kontekście studiów nad zjadliwością i czynnikami wirulencji *P. aeruginosa*. Szczep 39016 jest przedstawicielem *P. aeruginosa* odpowiedzialnym za trudne w leczeniu infekcje narządów wzroku. Genomy wszystkich wykorzystanych szczepów zostały sekwencjonowane, a ich sekwencje są powszechnie dostępne w publicznych bazach danych. Wybrane szczepy charakteryzują się znacznym zróżnicowaniem pod względem szybkości wzrostu bakteryjnego, wydajności tworzenia biofilmu, zdolności syntezy różnorodnych czynników wirulencji czy oporności na antybiotyki.

## 5.2. Ciecze jonowe

W badaniach wykorzystano zestaw 12 morfoliniowych cieczy jonowych zsyntetyzowanych na bazie herbicydów z grupy regulatorów wzrostu. W skład każdej z badanych cieczy jonowych wchodził jeden z dwóch kationów: kation 4,4-didecylomorfoliniowy $[Dec_2Mor]^+$ lub kation 4-decylo-4-etylomorfoliniowy $[DecEtMor]^+$. Kationy sparowane były z jednym z sześciu anionów pochodzenia herbicydowego: anionem 2,4-dichlorofenoksyoctanu $[2,4\text{-}D]^-$; anionem 4-chlorofenoksyoctanu $[4\text{-}CPA]^-$; anionem dichloropirydyno-2-karboksylanu $[Chlopyralid]^-$; anionem 3,6-dichloro-2-metoksybenzoesanu $[Dikamba]^-$; anionem 4-chloro-2-metylofenoksyoctan $[MCPA]^-$; lub anionem (±)-2-(4-chloro-2-metylofenoksy)propionianu $[MCPP]^-$. Pełna lista cieczy jonowych wraz ze wzorami strukturalnymi została zamieszczona w **Tabeli 1 w Publikacji 1**.

## 5.3. Antybiotyki

Do badania występowania synergii między cieczami jonowymi a antybiotykami, oraz w celu określenia profili antybiotykooporności badanych szczepów *P. aeruginosa*, wykorzystano 11 antybiotyków o zróżnicowanej strukturze i sposobie działania: amikacynę, aztreonam, cefoperazon, ceftazydym, cyprofloksacynę, kolistynę, lewofloksacynę, meropenem, ofloksacynę, piperacylinę oraz tobramycynę. Listę antybiotyków, wraz z zaznaczoną przynależnością do konkretnej grupy antybiotyków oraz przypisanym mechanizmem działania, przedstawiono w **Tabeli S2 materiałów dodatkowych dołączonych do Publikacji 3**.

## 5.4. Przygotowanie kultur bakteryjnych

Kultury *P. aeruginosa* prowadzono w pożywce tryptonowo-sojowej uzupełnionej 0,6% ekstraktem drożdżowym (TSB-Y, ang. *tryptic soy broth with yeast extract*). Szczegółowy skład pożywki był następujący (g/L): pepton sojowy, 3; D(+)-glukoza, 2,5; pepton kazeinowy, 17; fosforan dipotasu, 2,5; chlorek sodu, 5; ekstrakt drożdżowy, 6. pH pożywki wyrównano do 7,3±0,2. Do posiewów na podłożu stałym wykorzystywano pożywkę o tym samym składzie z dodatkiem 1,6 % agaru (TSA-Y, ang. *tryptic soy agar with yeast extract*). Płynne hodowle *P. aeruginosa* przygotowano przez przeniesienie pojedynczej kolonii bakteryjnej z podłoża stałego do pożywki płynnej. Tak przygotowane hodowle inkubowano przez 18 godzin w temperaturze 37 °C, z wytrząsaniem.

## 5.5. Ocena antybakteryjnego działania cieczy jonowych

Oceny antybakteryjnego działania cieczy jonowych i antybiotyków dokonano poprzez oznaczenie dwóch parametrów: minimalnego stężenia hamującego (MIC ang. *minimum inhibitory concentration*), które odpowiada najmniejszemu stężeniu substancji zdolnemu do zahamowania wzrostu mikroorganizmów; oraz minimalnego stężenia bakteriobójczego (MBC ang. *minimum bactericidal concentration*), które odpowiada najmniejszemu stężeniu substancji prowadzącemu do śmierci co najmniej 99,9% bakterii.

#### 5.5.1. Wyznaczanie wartości MIC

Wartości MIC cieczy jonowych i antybiotyków wyznaczano z wykorzystaniem metody seryjnych rozcieńczeń w płytkach 96-dołkowych. W skrócie, do dołków z pierwszego rzędu dodawano po 80 µL badanych związków, zaś pozostałe dołki uzupełniano 40 µL pożywki TSB-Y. Następnie, zaczynając od pierwszego rzędu przenoszono po 40 µL roztworu do kolejnych rzędów, wykonując serię dwukrotnych rozcieńczeń. Dołki w ostatnim rzędzie nie zawierały badanych związków i służyły jako próby kontrolne. Pożywkę TSB-Y, zawierającą 0,02 mg/mL resazuryny lub bez resazuryny, zaszczepiano płynnymi kulturami szczepów *P. aeruginosa* i dodawano do dołków (160 µL). Płytki inkubowano przez 18 godzin, w temperaturze 37°C z wytrząsaniem. W przypadku metody z resazuryną wzrost bakteryjny powodował zmianę koloru pożywki z ciemnoniebieskiego na różowy, na skutek redukcji barwnika przez bakterie. W przypadku metody bez resazuryny, wzrost bakteryjny określano poprzez pomiar gęstości optycznej hodowli ($OD_{600}$) na czytniku mikropłytek. Najniższe stężenie cieczy jonowej lub antybiotyku, dla którego nie zaobserwowano wzrostu kultury, zostało oznaczone jako MIC. Początkowa liczba bakterii w każdym dołku wynosiła $5 \times 10^5$ jtk/mL. Najwyższe badane stężenie w przypadku cieczy jonowych wynosiło 1024 mg/L (ppm).

#### 5.5.2. Wyznaczanie wartości MBC

Do wyznaczenia wartości MBC badanych związków wykorzystano mikropłytki z oznaczeń MIC. Z dołków, w których nie zaobserwowano wzrostu bakterii, pobierano po 10 µL hodowli i nanoszono je na płytki ze stałym podłożem TSA-Y. Płytki inkubowano przez noc w temperaturze 37 °C. Najniższe stężenie, dla którego nie zaobserwowano wzrostu kolonii bakteryjnych oznaczono jako MBC.

## 5.6. Ilościowe oznaczanie piocyjaniny

Wpływ ILs na syntezę piocyjaniny przez *P. aeruginosa* został zbadany po całonocnej inkubacji badanych szczepów z cieczami jonowymi w stężeniach równych ½ i ¼ MIC. W przypadku cieczy jonowych, których wartości MIC wykraczały poza sprawdzany zakres stężeń, jako ½ i ¼ MIC przyjęto dwa najwyższe stężenia - 1024 i 512 mg/L. Piocyjaninę oznaczono metodą spektrofotometryczną po uprzednim przeprowadzeniu dwuetapowej ekstrakcji chloroformem i roztworem HCl (0,2N). Próby przygotowano poprzez inokulację 3 ml pożywki TSB-Y płynnymi hodowlami *P. aeruginosa* do gęstości optycznej $OD_{600}$ = 0,01. Zaszczepione próby zawierające ½ i ¼ MIC cieczy

jonowych oraz próby kontrolne inkubowano przez 20 godzin w temperaturze 37 °C, z wytrząsaniem przy obrotach 150 rpm. Po inkubacji pobierano po 500 µL z każdej badanej próby i dwukrotnie rozcieńczono w pożywce TSB-Y, celem pomiaru gęstości optycznej hodowli ($OD_{600}$). Pozostałą część próby wirowano, a supernatant przenoszono do probówki zawierającej 1,5 mL chloroformu. Próby worteksowano, otrzymane emulsje wirowano przez 10 minut przy 10 000 rpm celem rozdzielenia faz. Fazę organiczną zawierającą piocyjaninę przenoszono do nowych probówek, dodawano do niej 750 µL 0,2N roztworu HCl, worteksowano i wirowano przez 2 minuty przy 10 000 rpm. Następnie zbierano fazę wodną i mierzono absorbcję prób dla fali o długości 520 nm.

## 5.7. Pomiar aktywności dehydrogenaz

Do określenia wpływu cieczy jonowych na ogólną aktywność metaboliczną i żywotność mikroorganizmów wykorzystano test TTC mierzący zdolność dehydrogenaz bakteryjnych do redukcji chlorku 2,3,5-trifenylotetrazoliowym (TTC). Pomiar aktywności metabolicznej *P. aeruginosa* został przeprowadzony po 20 godzinach inkubacji badanych szczepów z cieczami jonowymi w stężeniach równych ½ i ¼ MIC. W przypadku cieczy jonowych, których wartości MIC wykraczały poza sprawdzany zakres stężeń, jako ½ i ¼ MIC przyjęto dwa najwyższe stężenia - 1024 i 512 mg/L. W skrócie, do probówek dodawano po 1 mL pożywki TSB-Y zawierającej ciecze jonowe o końcowym stężeniu równym ½ i ¼ MIC, a następnie próby zaszczepiano płynnymi hodowlami *P. aeruginosa* do gęstości optycznej $OD_{600}$ = 0,01. Próby inkubowano przez noc w temperaturze 37 °C z wytrząsaniem, po czym dodawano do nich po 1 mL 1% roztworu TTC. Próby inkubowano ponownie przez 30 minut, w tych samych warunkach. Na tym etapie bezbarwny i rozpuszczalny TTC był redukowany przez aktywne metabolicznie komórki do czerwonego i nierozpuszczalnego w wodzie formazanu. Po inkubacji hodowle wirowano przez 2 minuty przy 14 000 rpm, a uzyskany osad rozpuszczano w 2 mL 96% etanolu. Po całkowitym rozpuszczeniu formazanu w etanolu próby ponownie wirowano w celu usunięcia osadu komórkowego. Absorbcję prób mierzono dla fali o długości 495 nm.

## 5.8. Oznaczanie biofilmu bakteryjnego

Wpływ cieczy jonowych na tworzenie biofilmu przez *P. aeruginosa* zbadano z wykorzystaniem barwienia fioletem krystalicznym (CV) i testu TTC. Barwienie fioletem krystalicznym pozwalało oznaczyć ilość uformowanej biomasy, natomiast test TTC pozwalał na ocenę aktywności metabolicznej komórek bakteryjnych w biofilmach. Studzienki płytki wielodołkowej zawierające 10 µL odpowiednich cieczy jonowych uzupełniono 190 µL pożywki TSB-Y zaszczepionej płynną hodowlą *P. aeruginosa*. Końcowa liczba bakterii w każdym dołku wynosiła $10^6$ jtk/mL, a końcowe stężenie ILs wynosiło ½ MIC. W przypadku cieczy jonowych, których wartości MIC wykraczały poza sprawdzany zakres stężeń, jako ½ MIC przyjęto najwyższe stężenie - 1024 mg/L. W każdej płytce znajdowały się trzy rodzaje prób kontrolnych: zaszczepiona kontrola pozytywna bez cieczy jonowych, kontrola negatywna z cieczami jonowymi w pożywce i kontrola negatywna z samą pożywką. Tak przygotowane płytki inkubowano w temperaturze 37°C przez 24 godziny. Po inkubacji mierzono gęstość optyczną hodowli za pomocą czytnika mikropłytek w celu oceny wzrostu bakterii. Po usunięciu pożywki biofilm bakteryjny trzykrotnie płukano roztworem PBS w celu usunięcia niezwiązanych komórek. Przemyte biofilmy były poddawane barwieniu fioletem krystalicznym lub inkubowane z TTC. W przypadku barwienia fioletem krystalicznym biofilm wybarwiano 0,1% roztworem fioletu krystalicznego przez 15 minut. Po usunięciu roztworu płytki płukano trzykrotnie roztworem PBS celem usunięcia niezwiązanego barwnika. Związany z biofilmem fiolet krystaliczny był rozpuszczany w 99,8% etanolu, a ilość wytworzonej biomasy określano spektrofotometrycznie dla fali o długości 570 nm. W teście TTC do dołków dodawano po 200 µL pożywki TSB-Y zawierającej 0,2 % TTC, a następnie inkubowano w temperaturze 37°C przez 5 h, w ciemności. Na tym etapie bezbarwny i rozpuszczalny TTC był redukowany przez aktywne metabolicznie komórki do czerwonego i nierozpuszczalnego w wodzie formazanu. Po inkubacji płytki wirowano, a osadzone kryształki formazanu rozpuszczano w 99,8% etanolu. Po ponownym zwirowaniu supernatant przenoszono na nową płytkę i mierzono absorbcję dla fali o długości 570 nm. Wpływ cieczy jonowych na tworzenie biofilmu wyrażono jako stosunek absorbcji prób traktowanych cieczami jonowymi do absorbcji nietraktowanej kontroli, korzystając ze wzoru:

$$Biofilm\ [\%] = \frac{A_{Próba\ z\ cieczą\ jonową} - A_{Pożywka\ z\ cieczą\ jonową}}{A_{Kontrola\ pozytywna} - A_{Pożywka}} \times 100\%$$

## 5.9. Ocena występowania efektu synergii

Występowanie synergii w kombinacjach cieczy jonowych i antybiotyków zbadano metodą szachownicy (ang. *checkerboard assay*). Aby zawęzić liczbę testowanych kombinacji początkowo zbadano jedynie mieszaniny dla których stężenia obu składników wynosiły ½ MIC. Do dalszych testów metodą szachownicy wybrano jedynie te kombinacje, które wykazały zdolność hamowania wzrostu bakterii podczas etapu wstępnego. W skrócie, mieszaniny cieczy jonowych i antybiotyków przygotowano w pożywce TSB-Y poprzez wykonanie serii seryjnych rozcieńczeń w płytkach 96-dołkowych. Następnie, wszystkie dołki zaszczepiono badanymi szczepami *P. aeruginosa* do końcowej liczby komórek $5 \times 10^5$ jtk/mL. Stężenia składników mieszanin wynosiły od ½ MIC do 1⁄64 MIC. Płytki inkubowano przez 24 godziny w temperaturze 37 °C z wytrząsaniem. Pomiaru gęstości optycznej hodowli dokonywano za pomocą czytnika mikropłytek przed rozpoczęciem i po zakończeniu inkubacji. Jako kryterium inhibicji wzrostu bakteryjnego przyjęto różnicę w gęstości optycznej $OD_{600}$ mniejszą niż 0,1. Dla prób z odnotowaną inhibicją obliczano indeks frakcyjnego stężenia hamującego (FICI, ang. *fractional inhibitory concentration index*) przy użyciu wzoru:

$$FICI = \frac{MIC_{Cieczy\ jonowej\ w\ kombinacji}}{MIC_{Samej\ cieczy\ jonowej}} + \frac{MIC_{Antybiotyku\ w\ kombinacji}}{MIC_{Samego\ antybiotyku}}$$

Występowanie efektu synergii określano na podstawie uzyskanych wartości FICI zgodnie z kryteriami: FICI ≤ 0,5 – synergia; 0,5 < FICI ≤ 1 – efekt addytywny. Wszystkie kombinacje, które wykazały działanie synergiczne lub addytywne, były sprawdzane w trzech powtórzeniach.

## 5.10. Aktywność hemolityczna

Aktywność hemolityczną cieczy jonowych oceniano dla krwi pełnej oraz dla samych erytrocytów. Ludzkie erytrocyty przygotowano poprzez odwirowanie krwi przy 800 g przez 10 minut i trzykrotne przemycie roztworem PBS (pH 7,4). Po płukaniu erytrocyty zawieszano w 2% PBS (v/v). W przypadku krwi pełnej procedura płukania została pominięta, a próbka rozcieńczona przed testem 1:1 roztworem PBS. Dwukrotne seryjne rozcieńczenie 10 µL cieczy jonowych przeprowadzono w mikropłytce do której dodano

90 µL krwi pełnej lub przemytych erytrocytów. Jako kontrolę pozytywną i negatywną zastosowano kolejno 1% Triton X-100 i roztwór PBS. Płytki inkubowano przez godzinę w temperaturze 37 °C, a następnie odwirowywano z prędkością 800 g przez 10 minut. Z każdej studzienki pobrano po 60 µl supernatantu i przeniesiono do nowej płytki. Absorbcję prób mierzono za pomocą czytnika mikropłytek dla fali o długości 570 nm. Wartości odczytane dla prób z Triton X-100 i PBS uznano za odpowiadające kolejno całkowitej hemolizie i brakowi hemolizy. Procent lizy erytrocytów mierzono według następującego wzoru:

$$Hemoliza\ [\%] = \frac{A_{570\ Próba\ z\ cieczą\ jonową} - A_{570\ Próba\ z\ PBS}}{A_{570\ Próba\ z\ Triton\ X-100} - A_{570\ Próba\ z\ PBS}} \times 100\%$$

# 6. Omówienie uzyskanych wyników

## 6.1. Minimalne stężenie hamujące oraz minimalne stężenie bakteriobójcze dla cieczy jonowych wobec *P. aeruginosa* – Publikacja 1

W celu określenia antybakteryjnych właściwości morfoliniowych cieczy jonowych dla każdego związku wyznaczono wartości MIC i MBC wobec czterech szczepów *Pseudomonas aeruginosa* - PAO1, LES B58, 39016, UCBPP-PA14. Zaobserwowano, że antybakteryjne działanie badanych cieczy jonowych jest silnie zależne od struktury kationu (**Publikacja 1, Fig. 2; Materiały dodatkowe, Tabela S1**). Ciecze jonowe 1-6 zawierające kation $[DecEtMor]^+$ nie wykazały zdolności do zahamowania wzrostu *P. aeruginosa* w badanym zakresie stężeń (max. 1024 mg/L (ppm)), za wyjątkiem związku [DecEtMor][Chlopyralid] (IL 3), który był w stanie doprowadzić do inhibicji wzrostu szczepów PAO1 i UCBPP-PA14 w najwyższym badanym stężeniu, oraz szczepu LES B58 w stężeniu 768 mg/L. Ciecze jonowe 7-12 z kationem $[Dec_2Mor]^+$ wykazały wysoką toksyczność wobec trzech badanych szczepów (PAO1, LES B58 i UCBPP-PA14), a ich wartości MIC mieściły się w zakresie od 11 do 75 mg/L. Szczep 39016 okazał się być wysoce oporny na działanie wszystkich badanych cieczy jonowych, wykazując jedynie niewielką podatność na działanie wysokich stężeń [$Dec_2$Mor][Chlopyralid] (IL 9) (średnia wartość MIC 853 mg/L) (**Publikacja 1, Fig. 2a; Materiały dodatkowe, Tabela S1**). Wyznaczone wartości MBC były zbliżone do wartości MIC i nie przekraczały ich dwukrotności (**Publikacja 1, Fig. 2b; Materiały dodatkowe, Tabela S1**).

Oba kationy wchodzące w skład badanych cieczy jonowych różniły się długością łańcuchów bocznych podstawionych do centralnego atomu azotu pierścienia morfoliniowego. Kationy $[Dec_2Mor]^+$ o wysokiej skuteczności antybakteryjnej zawierały dwa długie dziesięciowęglowe podstawniki, podczas gdy kationy $[DecEtMor]^+$ zawierały jedną grupę decylową i etylową. Tym samym wykazano, że zdolność badanych cieczy jonowych do zahamowania wzrostu *P. aeruginosa* zależy od liczby długich alkilowych podstawników przyłączonych do kationu. Uzyskane rezultaty zgadzają się z opisanym w literaturze trendem wzrastającej aktywności antybakteryjnej cieczy jonowych wraz ze zwiększeniem liczby długich łańcuchów bocznych podstawionych do kationu (Sommer i in., 2018). Analiza wartości MIC wskazała też na pozytywny wpływ anionu chlopyralidu na wzrost skuteczności badanych związków w hamowaniu wzrostu *P. aeruginosa*. Efekt anionu miał jednak

charakter drugorzędny w porównaniu do dominującej roli kationu. Ciecze jonowe hamujące wzrost bakterii, wykazały również wysoki potencjał bakteriobójczy, o czym świadczą wartości MBC zbliżone do wartości MIC. Zaobserwowany efekt kationu oraz chlopyralidu został później potwierdzony w badaniach nad przeżywalnością i hamowaniem wzrostu pięciu innych gatunków bakterii (*Listeria monocytogenes, Salmonella enterica, Escherichia coli, Lactococcus lactis* i *Pseudomonas syringae*) oraz w testach inhibicji aktywności polimerazy DNA (**Michalski**, Sommer, i in., 2023). Wskazuje to na uniwersalny charakter zależności strukturalnych warunkujących antybakteryjne działanie morfoliniowych cieczy jonowych. Odnotowana wysoka tolerancja szczepu 39016 wskazała na istnienie szczepowo specyficznej oporności na morfoliniowe ciecze jonowe bakterii *P. aeruginosa*. Choć przyczyny niewrażliwości szczepu 39016 nie zostały zidentyfikowane, to mogą mieć one związek z różnicami w budowie LPS, występującymi między szczepami, lub z obecnością specyficznych białek błonowych warunkujących oporność, takich jak niektóre pompy wielolekowe (Cullen i in., 2015; Mester, Gundolf, i in., 2015).

## 6.2. Zmiany aktywności dehydrogenaz bakteryjnych *P. aeruginosa* po traktowaniu cieczami jonowymi – Publikacja 1

W celu oceny wpływu cieczy jonowych na aktywności metaboliczną *P. aeruginosa* oznaczono zmiany aktywności bakteryjnych dehydrogenaz po traktowaniu cieczami jonowymi w stężeniach niższych niż MIC (**Publikacja 1, Fig. 3**). Wzrost aktywności metabolicznej odnotowano jedynie dla szczepu PAO1 po traktowaniu ¼ MIC cieczy jonowej [$Dec_2$Mor][Dikamba] (IL 10), natomiast obecność ¼ MIC związków [$Dec_2$Mor][2,4-D], [$Dec_2$Mor][4-CPA] i [$Dec_2$Mor][MCPA] (IL 7, 8 i 11) nie wywołała znaczących zmian u tego szczepu względem kontroli. Pozostałe ciecze jonowe prowadziły do znacznego spadku aktywności metabolicznej szczepów *P. aeurignosa*, a efekt inhibicji był wyraźnie mocniejszy dla ½ MIC. Niemniej, znaczne zahamowanie aktywności dehydrogenaz było widoczne już w obecności ¼ MIC cieczy jonowych. W przypadku szczepów PAO1 i LES B58 ciecze jonowe z kationem $[DecEtMor]^+$ powodowały silniejszą inhibicję niż związki z kationem $[Dec_2Mor]^+$. Największy spadek aktywności metabolicznej szczepu PAO1 zaobserwowano dla ½ MIC cieczy jonowych 1-6, natomiast szczepu LES B58 dla ½ MIC związków [DecEtMor][2,4-D], [DecEtMor][Chlopyralid] i [DecEtMor][MCPA] (IL 1, 3 i 5). Odwrotnie do tego, silniejszy spadek aktywności dehydrogenaz szczepu UCBPP-PA14 wystąpił

w obecności ½ MIC cieczy jonowych 7-12 z kationem $[Dec_2Mor]^+$. W przypadku szczepu 39016 wszystkie ciecze jonowe zmniejszały aktywność dehydrogenaz w zakresie od 85 do 97% dla obu badanych stężeń (**Publikacja 1, Fig. 3**).

Aktywność dehydrogenaz bakteryjnych stosowana jest jako miara ogólnej aktywności metabolicznej mikroorganizmów oraz ich żywotności (Kumar i Tarafdar, 2003). Uzyskane wyniki wskazują, że ciecze jonowe mogą ograniczać metabolizm *P. aeruginosa* nawet w stężeniach, które nie wpływają negatywnie na wzrost bakterii. Dodatkowo spadek aktywności metabolicznej był obserwowany zarówno dla związków o silnym działaniu antybakteryjnym, jak i tych niezdolnych do zahamowania wzrostu *P. aeruginosa*, co sugeruje, że charakter inhibicji metabolizmu bakteryjnego jest niezależny od wpływu cieczy jonowych na wzrost mikroorganizmów. Świadczy też o tym znaczny spadek aktywności metabolicznej odnotowany dla szczepu 39016, który charakteryzował się wysoką opornością na ciecze jonowe w trakcie oznaczania MIC i MBC (**Publikacja 1, Fig. 2; Materiały dodatkowe, Tabela S1**).

## 6.3. Oznaczenie zawartości piocyjaniny w kulturach traktowanych cieczami jonowymi – Publikacja 1

Aby określić potencjał cieczy jonowych do zmniejszania wirulencji *P. aeruginosa* wykonano ilościowe oznaczenia piocyjaniny wytwarzanej przez badane patogeny w obecności cieczy jonowych o stężeniach równych ½ i ¼ MIC (**Publikacja 1, Fig. 4**). Badane szczepy różniły się między sobą zdolnością do syntezy piocyjaniny. Najbardziej wydajnym producentem tego barwnika okazał się być szczep LES B58. Szczep PAO1, pomimo posiadania zdolności do syntezy piocyjaniny (zaobserwowanej w płytkach 96 dołkowych poprzez zmianę koloru pożywki na zielony), nie wytwarzał jej w warunkach wybranych do oznaczeń ilościowych. W związku z tym, testy inhibicji syntezy piocyjaniny zostały przeprowadzone jedynie dla szczepów LES B58, 39016 i UCBPP-PA14 (**Publikacja 1, Fig. 4**). Wszystkie badane ciecze jonowe prowadziły do spadku ilości piocyjaniny w zakresie od 35% do 100% względem kontroli. Jedynie w przypadku szczepu LES B58 traktowanego ½ MIC [DecEtMor][Chlopyralid] (IL 3) inhibicji piocyjaniny towarzyszył spadek gęstości kultur. W pozostałych przypadkach wzrost bakterii był porównywalny do prób kontrolnych. Inhibicja syntezy piocyjaniny była zależna od zastosowanego stężenia cieczy jonowej. Dla większość prób silniejsza inhibicja była obserwowana dla ½ MIC. Związki 7-12 z kationem $[Dec_2Mor]^+$ były w stanie całkowicie zahamować syntezę piocyjaniny w szczepach 39016

i UCBPP-PA14 w obu badanych stężeniach (**Publikacja 1, Fig. 4b,c**). Pozostałe ciecze jonowe prowadziły do znacznej, choć nie całkowitej, inhibicji. W przypadku szczepu LES B58 wszystkie badane związki hamowały syntezę piocyjaniny w podobnym stopniu (**Publikacja 1, Fig. 4a**).

W przeprowadzonym badaniu po raz pierwszy udało się wykazać zdolność cieczy jonowych do obniżania zjadliwości bakterii patogennych *P. aeruginosa* poprzez inhibicję syntezy czynników wirulencji, na przykładzie piocyjaniny. Uruchomienie syntezy piocyjaniny jest zależne od gęstości hodowli bakteryjnej (Schaber i in., 2004). Jednocześnie, tylko w jednej próbie zaobserwowano zahamowanie wzrostu bakteryjnego po traktowaniu cieczami jonowymi. Tym samym odnotowany spadek ilości piocyjaniny nie był związany ze śmiercią komórek, a wynikał bezpośrednio z oddziaływania cieczy jonowych na bakterie. Zaobserwowana inhibicja może być potencjalnie związana ze spadkiem aktywności metabolicznej mikroorganizmów wykazanym w testach z użyciem TTC.

## 6.4. Wyznaczenie minimalnego stężenia hamującego antybiotyków – Publikacja 3

Celem określenia profili antybiotykooporności badanych szczepów *P. aeruginosa* przeprowadzono testy wrażliwości na 11 antybiotyków o zróżnicowanej strukturze i mechanizmie działania (**Publikacja 3; Materiały dodatkowe, Tabela S2**). Oporność badanych szczepów określono na podstawie wartości granicznych MIC (ang. *MIC breakpoints*) podanych przez Europejski Komitet ds. Oznaczania Lekowrażliwości EUCAST (ang. *European Committee on Antimicrobial Susceptibility Testing*) dla gatunku *P. aeruginosa* (**Publikacja 3; Materiały dodatkowe, Tabela S3**). Największą oporność na antybiotyki wykazał szczep LES B58, który był oporny na działanie 9 z 11 użytych antybiotyków (amikacynę, aztreonam, ceftazydym, cyprofloksacynę, kolistynę, lewofloksacynę, ofloksacynę, piperacylinę i tobramycynę). Szczep PAO1 był najbardziej wrażliwy na działanie antybiotyków wykazując oporność jedynie na ceftazydym, kolistynę i piperacylinę. Szczepy 39016 i UCBPP-PA14 były niewrażliwe na działanie kolejno 6 i 5 testowanych antybiotyków w tym na aztreonam, ceftazydym, kolistynę i tobramycynę. Wszystkie badane szczepy były oporne na kolistynę i ceftazydym, oraz podatne na działanie meropenemu (**Publikacja 3; Materiały dodatkowe, Tabela S3**). Na podstawie uzyskanych wyników określono profile wielolekooporności badanych szczepów stosując klasyfikację zaproponowaną

dla *P. aeruginosa* przez Magiorakos i in., 2012. Szczepy LES B58, 39016 i UCBPP-PA14 zostały sklasyfikowane jako szczepy wielolekooporne/ o potencjalnie rozszerzonej oporności (MDR/ potencjalnie XDR), natomiast szczep PAO1 jako potencjalnie wielolekooporny (potencjalnie MDR) (**Publikacja 3; Materiały dodatkowe, Tabela S4**).

## 6.5. Oznaczenie ilości i aktywności metabolicznej biofilmów bakteryjnych – Publikacja 3

W celu określenia przeciwbiofilmowych właściwości cieczy jonowych wykonano ilościowe oznaczenia biofilmów wytworzonych przez *P. aeruginosa* w obecności cieczy jonowych oraz zbadano ich aktywność metaboliczną. Oznaczeń dokonano dla cieczy jonowych w stężeniach równych ½ MIC (**Publikacja 3, Tabela 1**). Badane szczepy różniły się między sobą wydajnością tworzenia biofilmu (**Publikacja 3, Fig. 1**). Spośród nich, szczep PAO1 wytwarzał średnio dwa razy więcej biofilmu niż pozostałe izolaty. Porównując mediany ilości wytworzonej biomasy, szczep UCBPP-PA14 okazał się być najsłabszym producentem biofilmu. Zaobserwowano, że morfoliniowe ciecze jonowe mogą hamować tworzenie biofilmu przez *P. aeruginosa* w stężeniach niższych niż MIC (**Publikacja 3, Fig. 2**). W przypadku szczepów LES B58 i UCBPP-PA14 wszystkie badane ciecze jonowe prowadziły do znacznego zahamowania ilości wytworzonego biofilmu, powodując jednoczesny spadek żywotności bakterii wchodzących w jego skład **(Publikacja 3, Fig. 2a,b**). Dla szczepu 39016 zaobserwowano zmniejszenie ilości biofilmu i zahamowanie jego metabolicznej aktywności po traktowaniu cieczami jonowymi 1-6 z kationem $[DecEtMor]^+$. Z kolei związki z kationem $[Dec_2Mor]^+$, za wyjątkiem $[Dec_2Mor][MCPA]$ (IL 11), istotnie zwiększały ilość wytworzonego biofilmu przez ten szczep (**Publikacja 3, Fig. 2c**).

Te same ciecze jonowe w badanych stężeniach powodowały zmętnienie pożywki, prowadząc do wysokich odczytów kontroli negatywnych podczas barwienia fioletem krystalicznym. W związku z tym, aby określić rolę badanych związków w indukcji tworzenia biofilmu przez szczep 39016 przeprowadzono dodatkowe testy agregacji. Wykazano, że wysokie stężenia cieczy jonowych z kationem $[Dec_2Mor]^+$ są w stanie indukować powstawanie agregatów w kulturach bakteryjnych (**Publikacja 3, Fig. 4**). Choć osady były obecne zarówno w kontrolach bakteryjnych bez dodatku cieczy jonowych jak i w sterylnych pożywkach zawierających badane związki, to wielkość powstałych osadów była zdecydowanie mniejsza w porównaniu do kultur bakteryjnych

traktowanych cieczami jonowymi (**Publikacja 3, Fig. 4**). Świadczy to o tym, że wysokie dawki cieczy jonowych z kationem $[Dec_2Mor]^+$ stymulują agregację komórek i powstawanie osadu bakteryjnego. Jako, że obecność osadu i procesy agregacyjne sprzyjają powstawaniu biofilmu bakteryjnego, obserwowany wzrost ilości biomasy szczepu 39016 można przypisać procesom agregacji i wytrącania stymulowanym przez badane związki. Równocześnie, te same ciecze jonowe prowadziły do spadku aktywności metabolicznej biofilmów szczepu 39016 (**Publikacja 3, Fig. 2c**). Choć uzyskane wyniki są w pozornej sprzeczności, ilość biofilmu nie musi odpowiadać jego aktywności metabolicznej, a biofilmy o wysokiej biomasie często charakteryzują się niską aktywnością metaboliczną (Haney i in., 2018; Meissner i in., 2013). Dodatkowo martwe oraz nieaktywne metabolicznie komórki wchodzące w skład biomasy, odgrywają ważną rolę w rozwoju biofilmów bakteryjnych. W rzeczywistości, biofilmy o wysokiej zawartości biomasy mogą charakteryzować się znaczną przewagą komórek martwych nad żywymi w swojej strukturze, w porównaniu do mniej obfitych biofilmów, dających niższe wartości w metodzie barwienia fioletem krystalicznym (Desai i in., 2019).

W przypadku szczepu PAO1 wykazano istotny statystycznie spadek ilości wytworzonego biofilmu dla związków [DecEtMor][2,4-D], [DecEtMor][4-CPA], [DecEtMor][Dikamba] i [DecEtMor][MCPP] (IL 1, 2, 4 i 6) (**Publikacja 3, Fig. 2d**). Zahamowanie aktywności metabolicznej zaobserwowano dodatkowo dla cieczy jonowych [DecEtMor][Chlopyralid] oraz $[Dec_2Mor]$[MCPP] (IL 3 i 12).

Zestawiając ze sobą wyniki testów inhibicji biofilmu zidentyfikowano cztery ciecze jonowe, [DecEtMor][2,4-D], [DecEtMor][4-CPA], [DecEtMor][Dikamba] i [DecEtMor][MCPP] (IL 1, 2, 4 i 6), zdolne do hamowania tworzenia biofilmu przez wszystkie badane szczepy *P. aeruginosa*. Co zaskakujące, wszystkie z czterech cieczy jonowych zostały wcześniej zidentyfikowane jako związki o niskim potencjale hamującym wzrost *P. aeruginosa* (**Publikacja 1, Fig. 2; Materiały dodatkowe, Tabela S1**). Dane literaturowe wskazują, że długość łańcuchów bocznych warunkuje przeciwbiofilmowe właściwości cieczy jonowych w podobnym stopniu co ich zdolność do inhibicji wzrostu mikroorganizmów (**Publikacja 2;** Michalski, Odrzygóźdź, i in., 2023). Jednakże przytoczone wnioski są oparte głównie na rezultatach testów inhibicji lub eradykacji biofilmu przeprowadzanych dla stężeń równych lub wyższych od MIC. Stąd raportowana wyższa skuteczność długołańcuchowych cieczy jonowych w zwalczaniu biofilmów wynika z niższych wartości MIC tych związków i stanowi

inny przejaw ich wysokiego potencjału do hamowania wzrostu bakteryjnego. W naszych badaniach wykorzystano stężenia niższe od MIC, stąd uzyskane wyniki nie stoją w sprzeczności z dostępnymi danymi.

## 6.6. Analiza zależnego od stężenia wpływu cieczy jonowych na tworzenie biofilmu przez *P. aeruginosa* – Publikacja 3

Cztery ciecze jonowe [DecEtMor][2,4-D], [DecEtMor][4-CPA], [DecEtMor][Dikamba] i [DecEtMor][MCPP] (IL 1, 2, 4 i 6), które wykazały zdolność hamowania tworzenia biofilmu u wszystkich badanych szczepów *P. aeruginosa*, zostały wybrane do badań nad wpływem stężenia cieczy jonowych na formowanie biofilmu. W przypadku szczepu LES B58 statystycznie istotny spadek ilości i aktywności metabolicznej biofilmów zaobserwowano tylko dla najwyższego badanego stężenia cieczy jonowych (**Publikacja 3, Fig. 3a,b**). Zmianom tym towarzyszył ponad 50% spadek gęstości optycznej kultur. Jednocześnie po traktowaniu bakterii cieczami jonowymi w stężeniach niższych niż 64 mg/L odnotowano wzrost ilości wytworzonego biofilmu (**Publikacja 3, Fig. 3a**). Podobnie, indukcję tworzenia biofilmu zaobserwowano dla szczepu UCBPP-PA14 przy stężeniu 16 mg/L (**Publikacja 3, Fig. 3c**). Najniższe stężenie skutecznie zmniejszające ilość i aktywność biofilmu formowanego przez szczep UCBPP-PA14 wynosiło 128 mg/L (**Publikacja 3, Fig. 3c,d**). Zarówno dla szczepu LES B58 jak i UCBPP-PA14 przyrostowi ilości biofilmu odnotowanemu dla niskich stężeń cieczy jonowych nie towarzyszył wzrost aktywności dehydrogenaz (**Publikacja 3, Fig. 3b,d**). W przypadku szczepów 39016 i PAO1 żadna spośród czterech badanych cieczy jonowych nie stymulowała tworzenia biofilmu przy niskich stężeniach (**Publikacja 3, Fig. 3e,g**). Najniższe stężenie hamujące tworzenie biofilmu przez szczep 39016 wyznaczono na - 512 mg/L dla [DecEtMor][2,4-D] (IL 1), 128 mg/L dla [DecEtMor][4-CPA] (IL 2) i 64 mg/L dla [DecEtMor][Dikamba] (IL 4) oraz [DecEtMor][MCPP] (IL 6) (**Publikacja 3, Fig. 3e**). Najniższe stężenie powodujące istotny statystycznie spadek aktywności metabolicznej biofilmów szczepu 39016 wyznaczono na - 64 mg/L dla [DecEtMor][2,4-D] (IL 1), 128 mg/L dla [DecEtMor][4-CPA] (IL 2) i 256 mg/ml dla [DecEtMor][Dikamba] (IL 4) oraz [DecEtMor][MCPP] (IL 6) (**Publikacja 3, Fig. 3f**). W przypadku szczepu PAO1 wszystkie cztery ciecze jonowe prowadziły do istotnego zahamowania ilości wytworzonego biofilmu już przy dawce 128 mg/L, chociaż najwyższy stopień inhibicji odnotowano dla stężenia 256 mg/L (**Publikacja 3, Fig. 3g**). Jednocześnie dla

wszystkich badanych stężeń zaobserwowano istotny statystycznie spadek aktywności metabolicznej biofilmów PAO1, przy czym stopień inhibicji malał wraz ze spadkiem stężenia (**Publikacja 3, Fig. 3h**). Dodatkowo zbadano wpływ stężeń cieczy jonowych 7-12 z kationem $[Dec_2Mor]^+$ na szczep 39016. Wzrost ilości biofilmu zaobserwowano jedynie dla najwyższego badanego stężenia, natomiast niższe dawki nie powodowały istotnych statystycznie zmian lub prowadziły do częściowej inhibicji tworzenia biofilmu (**Publikacja 3, Fig. 3i**). Tym samym uzyskane wyniki dostarczyły dodatkowych dowodów, że za indukcję tworzenia biofilmu przez szczep 39016 w obecności wysokich stężeń cieczy jonowych 7-12 odpowiada wspomniany efekt agregacji, który zanika wraz ze spadkiem stężenia.

Wykazano, że wybrane morfoliniowe ciecze jonowe zachowują aktywność przeciwbiofilmową nawet w stężeniach znacznie niższych od MIC, co świadczy o ich wysokim potencjale do zwalczania biofilmu tworzonego przez *P. aeruginosa*. Z drugiej strony, przyrost biofilmu szczepów LES B58 i UCBPP-PA14 po traktowaniu niskimi stężeniami cieczami jonowymi, wskazuje na wystąpienie efektu hormezy i stymulujące działanie niewielkich dawek cieczy jonowych na tworzenie biofilmu. Z uwagi na brak zmian aktywności metabolicznej biofilmów odnotowanej dla indukujących stężeń cieczy jonowych można wnioskować, że obserwowany wzrost biomasy to efekt przede wszystkim zwiększenia ilości zewnątrzkomórkowych polimerów EPS syntetyzowanych przez bakterie. Podobny efekt promocji biofilmu był już obserwowany dla niektórych antybiotyków czy nanocząstek (Cui i in., 2018; Iavicoli i in., 2021), lecz nie dla cieczy jonowych. Tym samym jako pierwsi wykazaliśmy występowanie efektu hormezy dla cieczy jonowych w kontekście ich wpływu na tworzenie biofilmów bakteryjnych.

## 6.7. Ocena występowania efektów synergii w kombinacjach cieczy jonowych i antybiotyków – Publikacja 3

W celu określenia współdziałania cieczy jonowych z antybiotykami badano wpływ 132 kombinacji cieczy jonowych i antybiotyków (użyto 12 cieczy jonowych i 11 antybiotyków) na hamowanie wzrostu czterech szczepów *P. aeruginosa*. Dla większości badanych kombinacji nie zaobserwowano dodatnich interakcji między cieczami jonowymi, a antybiotykami (**Publikacja 3, Fig. 5**). Co najmniej addytywny efekt odnotowano jedynie dla około 30% badanych mieszanin. Synergiczne współoddziaływanie było jeszcze rzadsze i zaobserwowano je tylko dla 11, 15 i 8 kombinacji wobec odpowiednio szczepu LES B58, UCBPP-PA14 i PAO1

(**Publikacja 3, Fig. 5a,b,d**). W przypadku szczepu 39016 synergia między składnikami była zdecydowanie częstsza i wystąpiła w 26 kombinacjach (**Publikacja 3, Fig. 5c**). Ciecze jonowe 7-12 z kationem $[Dec_2Mor]^+$ wchodziły w dodatnie interakcje z antybiotykami kilka razy częściej wobec szczepów 39016 i UCBPP-PA14 oraz nieznacznie częściej dla szczepu PAO1 (**Publikacja 3, Fig. 5b,c,d**). W przypadku szczepu LES B58 najwięcej skutecznych kombinacji zawierało ciecz jonową [DecEtMor][Chlopyralid] (IL 3) (**Publikacja 3, Fig. 5a**). Dla żadnego szczepu nie odnotowano dodatnich interakcji pomiędzy cieczami jonowymi, a cefoperazonem i ceftazydymem (**Publikacja 3, Fig. 6**). Dodatkowo nie zaobserwowano synergii dla żadnej kombinacji zawierającej ofloksacynę lub cyprofloksacynę (**Publikacja 3, Fig. 6b**). Z drugiej strony, wszystkie mieszaniny cieczy jonowych z kolistyną wykazywały dodatnie, często synergiczne, interakcje. Ponadto jedyne kombinacje, dla których zaobserwowano efekt synergii wobec wszystkich badanych szczepów *P. aeruginosa* zawierały właśnie kolistynę oraz jedną z następujących cieczy jonowych: $[Dec_2Mor]$[2,4-D], $[Dec_2Mor]$[4-CPA], $[Dec_2Mor]$[Dikamba] lub $[Dec_2Mor]$[MCPP] (IL 7, 8, 10 i 12) (**Publikacja 3, Fig. 6b**). Dodatnie interakcje występowały też często pomiędzy cieczami jonowymi a amikacyną, piperacyliną i aztreonamem, oraz rzadziej z tobramycyną i meropenemem (**Publikacja 3, Fig. 6**). Uwzględniając wszystkie wyniki, najsilniejszą synergię zaobserwowano dla kombinacji aztreonamu z $[Dec_2Mor]$[2,4-D], $[Dec_2Mor]$[4-CPA] i $[Dec_2Mor]$[MCPP] wobec szczepu 39016. (**Publikacja 3, Fig. 5c**).

Sumarycznie częstsze współdziałanie z antybiotykami związku [DecEtMor][Chlopyralid] oraz cieczy jonowych z kationem $[Dec_2Mor]^+$, może świadczyć o tym, że wyższa aktywność antybakteryjna cieczy jonowej sprzyja wystąpieniu co najmniej addytywnego efektu w kombinacji. Ponadto powszechne występowanie kolistyny mieszaninach skutecznie hamujących wzrost bakterii wskazuje na kluczową rolę rodzaju antybiotyku w powstawaniu synergii. Kolistyna wiąże się ze strukturą LPS bakterii Gram-ujemnych, co prowadzi do przerwania ciągłości błon komórkowych (Andrade i in., 2020). Wysoka skuteczność tego antybiotyku w kombinacjach z morfoliniowymi cieczami jonowymi może wynikać z połączonego działania obu związków na błony bakteryjne. Efekt współdziałania kolistyny z cieczami jonowymi był wcześniej opisany dla soli z imidazoliowym, pyrolidyniowym i piperydyniowym kationem (Florio i in., 2020). Ponadto wykazano, że dodatek cieczy jonowych do polimyksyny B, należącej do tej samej grupy antybiotyków co kolistyna, prowadzi do

zwiększonej permeabilizacji błon lipidowych (Hanna i in., 2017). Obserwowany w badaniach efekt synergiczny może również wynikać z ułatwionej penetracji komórek przez ciecze jonowe na skutek destabilizacji warstwy LPS przez antybiotyk (Cole i in., 2011).

## 6.8. Analiza zmian wrażliwości *P. aeruginosa* na antybiotyki w obecności cieczy jonowych – Publikacja 3

Wyniki badań synergii zestawiono z profilami antybiotykooporności badanych szczepów w celu oceny potencjalnej użyteczności stosowania morfoliniowych cieczy jonowych jako adiuwantów w zwalczaniu lekoopornych izolatów *P. aeruginosa*. Dla każdego lekoopornego szczepu *P. aeruginosa* udało się zidentyfikować co najmniej kilkanaście kombinacji cieczy jonowych z antybiotykami prowadzących do obniżenia oporności patogenu poniżej wartości granicznej MIC danego antybiotyku (**Publikacja 3, Tabela 2**). W najbardziej spektakularnych przypadkach dodatek cieczy jonowej prowadził do aż 64-krotnego spadku stężenia antybiotyku koniecznego do inhibicji wzrostu *P. aeruginosa*. W przypadku wszystkich szczepów opornych na kolistynę i piperacylinę znaleziono co najmniej jedną kombinację, dla której wartość MIC antybiotyku w mieszaninie spadła poniżej wartości granicznej. Ponadto część cieczy jonowych prowadziła do uwrażliwienia: szczepu LES B58 na działanie ofloksacyny, szczepu UCBPP-PA14 na cyprofloksacynę i lewofloksacynę, oraz szczepu 39016 na działanie aztreonamu, lewofloksacyny i tobramycyny. Związki [DecEtMor][Chlopyralid], [$Dec_2$Mor][4-CPA], [$Dec_2$Mor][Chlopyralid], [$Dec_2$Mor][Dikamba] i [$Dec_2$Mor][MCPP] (IL 3, 8, 9, 10 i 12) okazały się być najskuteczniejszymi adiuwantami, tworząc najwięcej kombinacji uwrażliwiających *P. aeruginosa* na antybiotyki spośród wszystkich badanych cieczy jonowych (**Publikacja 3, Tabela 2**).

Jako pierwsi wykazaliśmy, że ciecze jonowe mogą uwrażliwiać wielolekooporne szczepy z gatunku *P. aeruginosa* na działanie antybiotyków, tym samym wskazując na sensowność wykorzystania tych związków jako adiuwantów w antybiotykoterapii. Ponadto spadek oporności był widoczny dla antybiotyków o różnych mechanizmach działania, co wskazuje na uniwersalny charakter cieczy jonowych w roli adiuwantów.

## 6.9. Oznaczanie hemolitycznej aktywności cieczy jonowych – Publikacja 3

W celu oceny cytotoksyczności morfoliniowych cieczy jonowych wyznaczono ich aktywność hemolityczną dla ludzkich erytrocytów oraz krwi pełnej (**Publikacja 3, Fig. 7; Tabela 3**). Aktywność hemolityczna cieczy jonowych była zależna od struktury kationu podobnie jak w przypadku ich właściwości antybakteryjnych. Ciecze jonowe z kationem $[Dec_2Mor]^+$ (IL 7-12) prowadziły do całkowitej lizy erytrocytów w stężeniach wyższych lub równych 32 mg/L, za wyjątkiem związku [$Dec_2$Mor][MCPA] (IL 11), który maksymalną aktywność hemolityczną osiągał w stężeniu 512 mg/L i wyższym (**Publikacja 3, Fig. 7b**). Graniczne stężenie nieprowadzące do hemolizy wynosiło dla wspomnianego związku 128 mg/L oraz od 4 do 8 mg/L dla pozostałych cieczy jonowych z kationem $[Dec_2Mor]^+$ (**Publikacja 3, Tabela 3**). Ciecze jonowe z kationem $[DecEtMor]^+$ (IL 1-6) nie powodowały lizy erytrocytów w stężeniach równych 512 mg/L lub niższych, za wyjątkiem związku [DecEtMor][Chlopyralid] (IL 3), który prowadził do 100% hemolizy w stężeniu 1024 mg/L oraz nie wykazywał hemolitycznego działania w stężeniach równych 128 mg/L lub niższych (**Publikacja 3, Fig. 7a; Tabela 3**). Godne uwagi są rezultaty uzyskane dla krwi pełnej, które wskazały na zdecydowanie niższą aktywność hemolityczną badanych cieczy jonowych w porównaniu do wyników hemolizy samych erytrocytów (**Publikacja 3, Tabela 3**).

Występowanie różnic w testach hemolitycznych przeprowadzanych na erytrocytach i krwi pełnej było już obserwowane (Sæbø i in., 2023). Przyczyna takiego stanu rzeczy może leżeć w zwiększonej wrażliwości erytrocytów na lizę po przejściu procedury przemywania lub wynikać z obecności związków maskujących hemolityczne działanie cieczy jonowych we krwi pełnej (Sæbø i in., 2023; Schmidt i in., 2016). W związku tym, wyniki hemolizy dla krwi pełnej mogą być uznane za bardziej reprezentatywne dla działania cieczy jonowych w warunkach *in vivo*. Z kolei rezultaty dla czerwonych krwinek można przyjąć za lepiej oddające efekt cieczy jonowych na ludzkie błony komórkowe. Wszystkie ciecze jonowe posiadały aktywność hemolityczną, prowadząc do lizy ludzkich erytrocytów. Uzyskane rezultaty istotnie ograniczają potencjalne zastosowania morfoliniowych cieczy jonowych w leczeniu infekcji wywoływanych przez *P. aeruginosa*, zawężając ich użycie głównie do stosowania pozaustrojowego. Dodatkowo aktywność hemolityczna była wyższa dla cieczy jonowych o kationach z dwoma długimi podstawnikami i dobrze korelowała z zaobserwowaną zależnością

między strukturą kationów, a zdolnością badanych związków do hamowania wzrostu *P. aeruginosa*. Podobny związek między aktywnością hemolityczną a długością łańcuchów bocznych był już obserwowany dla cieczy jonowych (He i in., 2022). Jako, że erytrocyty wykorzystywane są do oceny działania ksenobiotyków na błony komórkowe, zaobserwowana korelacja pomiędzy budową kationu, aktywnością hemolityczną i aktywnością antybakteryjną, wskazuje że antybakteryjne właściwości morfoliniowych cieczy jonowych wynikają z ich oddziaływań z błonami bakteryjnymi.

## 7. Podsumowanie i wnioski

Rezultaty badań nad wpływem morfoliniowych cieczy jonowych na bazie herbicydów na antybiotkooporne szczepy bakteryjne z gatunku *Pseudomonas aeruginosa* pozwoliły na sformułowanie następujących wniosków:

1. morfoliniowe ciecze jonowe skutecznie hamują wzrost *P. aeruginosa,* a ich antybakteryjne właściwości zależą od liczby długich łańcuchów bocznych podstawionych do kationu. Wysoka skuteczność inhibicji charakteryzowała związki z kationem $[Dec_2Mor]^+$ z dwoma decylowymi podstawnikami.
2. wśród gatunku *P. aeruginosa* występuje szczepowo-zależna niewrażliwość na działanie morfoliniowych cieczy jonowych, a szczep 39016 charakteryzuje się wysoką opornością na działanie cieczy jonowych.
3. morfoliniowe ciecze jonowe hamują aktywność metaboliczną *P. aeruginosa* w stężeniach poniżej wartości MIC. Spadek aktywności metabolicznej indukowany przez ciecze jonowe nie zależy od ich wpływu na wzrost bakterii.
4. subinhibicyjne stężenia morfoliniowych cieczy jonowych hamują syntezę piocyjaniny, zmniejszając wirulencję *P. aeruginosa*. Zmiana ilości piocyjaniny nie wynika ze zmian gęstości hodowli bakteryjnych.
5. morfoliniowe ciecze jonowe mogą hamować tworzenie biofilmu przez *P. aeruginosa* w stężeniach poniżej wartości MIC. Zidentyfikowano cztery ciecze jonowe, [DecEtMor][2,4-D], [DecEtMor][4-CPA], [DecEtMor][Dikamba] i [DecEtMor][MCPP], zdolne do zmniejszenia ilości i aktywności metabolicznej biofilmu u wszystkich badanych szczepów *P. aeruginosa*.
6. morfoliniowe ciecze jonowe mogą indukować powstawanie biofilmu bakteryjnego w zależności od użytego stężenia. Niewielkie dawki cieczy jonowych 1, 2, 4 i 6 stymulują szczepy LES B58 i UCBPP-PA14 do tworzenia biofilmu, zgodnie z efektem hormetycznym. Wysokie stężenia cieczy jonowych z kationem $[Dec_2Mor]^+$ stymulują tworzenie biofilmu przez szczep 39016 na skutek agregacji bakterii.
7. morfoliniowe ciecze jonowe mogą tworzyć z antybiotykami kombinacje o addytywnym lub synergicznym działaniu wobec *P. aeruginosa*. Występowanie dodatnich interakcji koreluje z wysokim potencjałem antybakteryjnym cieczy jonowych i obecnością kationu $[Dec_2Mor]^+$. Obecność

kolistyny faworyzuje wystąpienie synergii najprawdopodobniej ze względu na pokrewny mechanizm działania obu związków.

8. morfoliniowe ciecze jonowe mogą zwiększać wrażliwość lekoopornych szczepów *P. aeruginosa* na działanie różnych grup antybiotyków, a dodatek niektórych cieczy jonowych prowadzi do obniżenia stężenia antybiotyku koniecznego do zahamowania wzrostu lekoopornych szczepów poniżej wartości granicznej MIC danego środka.
9. morfoliniowe ciecze jonowe wykazują zdolność do hemolizy. Aktywność hemolityczna jest zależna od rodzaju użytego kationu i dobrze koreluje z aktywnością antybakteryjną cieczy jonowych.

Podsumowując, w badaniach wykazano wysoki potencjał morfoliniowych cieczy jonowych na bazie herbicydów w zwalczaniu *P. aeruginosa*. Wskazano na zdolność cieczy jonowych do hamowania wzrostu i przeżywalności bakterii, obniżania aktywności metabolicznej, inhibicji syntezy czynników wirulencji oraz hamowania tworzenia biofilmu. Ponadto wykazano, że ciecze jonowe mogą zwiększać wrażliwość *P. aeruginosa* na działanie antybiotyków, szczególnie kolistyny, tym samym znajdując potencjalne zastosowanie jako adiuwanty w antybiotykoterapii. Z drugiej strony, zaobserwowana aktywność hemolityczna znacząco ogranicza potencjalne zastosowania morfoliniowych cieczy jonowych w leczeniu infekcji wywoływanych przez *P. aeruginosa*, zawężając je do stosowania pozaustrojowego. Dodatkowo występowanie zależnego od stężenia efektu indukującego powstawanie biofilmu może stanowić kolejną przeszkodę w stosowaniu badanych związków w zwalczaniu patogenów bakteryjnych.

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## Oświadczenia współautorów

Poznań, 05.07.2024

mgr inż. Jakub Michalski

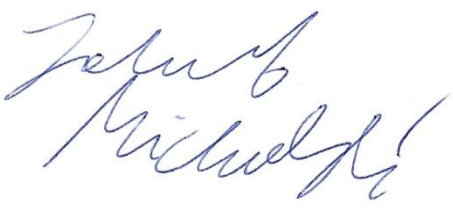
Wydział Rolnictwa, Ogrodnictwa i Biotechnologii,
Katedra Biochemii i Biotechnologii
Uniwersytet Przyrodniczy w Poznaniu, Poznań 60-637, Polska
email: jakub.michalski @up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Cłapa T., **Michalski J.**, Syguda A., Narożna D., van Oostrum P., Reimhult E. 2021 Morpholinium- based ionic liquids show antimicrobial activity against clinical isolates of Pseudomonas aeruginosa. *Research in Microbiology*, 172, 3. (Publikacja 1) polegał na wykonaniu wszystkich eksperymentów (za wyjątkiem syntezy cieczy jonowych i analiz chemicznych), analizie danych i interpretacji wyników, wykonaniu wykresów i współtworzeniu figur, napisaniu pierwszej wersji manuskryptu, współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu, udziale w przygotowaniu odpowiedzi na recenzje.

**Michalski J.**, Odrzygóźdź C., Mester P., Narożna D., Cłapa T., 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids*, 369. (Publikacja 2) polegał na przeprowadzeniu przeglądu literaturowego, wiodącym udziale w przygotowaniu pierwszej wersji tekstu, współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu, przygotowaniu odpowiedzi na recenzje

**Michalski J.**, Cłapa T., Syguda A., Narożna D., van Oostrum P., Reimhult E. 2024 Morpholinium- based ionic liquids as potent antibiofilm and sensitizing agents for the control of Pseudomonas aeruginosa. *Journal of Molecular Biology*, 436, 13. (Publikacja 3) polegał na współtworzeniu koncepcji badań, wykonaniu wszystkich eksperymentów, przeprowadzeniu analizy danych i interpretacji wyników, wykonaniu wykresów i figur, samodzielnym napisaniu pierwszej wersji manuskryptu, współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu, przeprowadzeniu dodatkowych analiz oraz przygotowaniu odpowiedzi na recenzje.



Poznań, 05.07.2024

prof. UPP. dr hab. Dorota Narożna

Wydział Rolnictwa, Ogrodnictwa i Biotechnologii,
Katedra Biochemii i Biotechnologii
Uniwersytet Przyrodniczy w Poznaniu, Poznań 60-637, Polska
email: dorota.narozna @up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

Cłapa T., Michalski J., Syguda A., **Narożna D.**, van Oostrum P., Reimhult E. 2021 Morpholinium- based ionic liquids show antimicrobial activity against clinical isolates of Pseudomonas aeruginosa. *Research in Microbiology*, 172, 3. (Publikacja 1) polegał na współtworzeniu koncepcji badań, opiece merytorycznej podczas wykonywania badań, współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu.

Michalski J., Odrzygóźdź C., Mester P., **Narożna D.**, Cłapa T., 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids*, 369. (Publikacja 2) polegał na współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu.

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Poznań, 05.07.2024

dr inż. Tomasz Cłapa

Wydział Rolnictwa, Ogrodnictwa i Biotechnologii,
Katedra Biochemii i Biotechnologii
Uniwersytet Przyrodniczy w Poznaniu, Poznań 60-637, Polska
email: tomasz.clapa@up.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

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Michalski J., Odrzygóźdź C., Mester P., Narożna D., **Cłapa T.**, 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids*, 369. (Publikacja 2) polegał na udziale w przygotowaniu pierwszej wersji tekstu, przygotowaniu większości figur, współudziale w redakcji tekstu.

Michalski J., **Cłapa T.**, Syguda A., Narożna D., van Oostrum P., Reimhult E. 2024 Morpholinium- based ionic liquids as potent antibiofilm and sensitizing agents for the control of Pseudomonas aeruginosa. *Journal of Molecular Biology*, 436, 13. (Publikacja 3) polegał na współtworzeniu koncepcji badań, współudziale w redakcji tekstu i opracowaniu końcowej wersji manuskryptu, zapewnieniu finansowania badań.

Tomasz Cłapa

Poznań, 05.07.2024

dr inż. Anna Syguda

Wydział Technologii Chemicznej,
Instytut Technologii i Inżynierii Chemicznej,
Zakład Technologii Chemicznej,
Politechnika Poznańska, Poznań 60-925, Polska
email: anna.syguda@put.poznan.pl

Oświadczam, że mój udział w przygotowaniu pracy:

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Michalski J., Cłapa T., **Syguda A.**, Narożna D., van Oostrum P., Reimhult E. 2024 Morpholinium- based ionic liquids as potent antibiofilm and sensitizing agents for the control of Pseudomonas aeruginosa. *Journal of Molecular Biology*, 436, 13. (Publikacja 3) polegał na przeprowadzeniu syntezy morfoliniowych cieczy jonowych wykorzystanych w badaniach.

Anna Syguda.

Vienna, 05.07.2024

Univ. Prof. Dr. Erik Reimhult

Institute of Colloid and Biointerface Science,
Department of Nanobiotechnology,
BOKU University, Vienna1190, Austria,
email:erik.reimhult@boku.ac.at

I declare that I have contributed to the articles:

Cłapa T., Michalski J., Syguda A., Narożna D., van Oostrum P., **Reimhult E**. 2021 Morpholinium- based ionic liquids show antimicrobial activity against clinical isolates of Pseudomonas aeruginosa. *Research in Microbiology*, 172, 3.(Article 1) by conceptualising the research, conceiving the methodology, reviewing and editing the final draft.

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Priv.-Doz. Dr. Peter van Oostrum

Institute of Colloid and Biointerface Science,
BOKU University, Vienna 1190, Austria,
email: peter.van.oostrum@boku.ac.at

I declare that I have contributed to the articles:

Cłapa T., Michalski J., Syguda A., Narożna D., **van Oostrum P**., Reimhult E. 2021 Morpholinium- based ionic liquids show antimicrobial activity against clinical isolates of Pseudomonas aeruginosa. *Research in Microbiology*, 172, 3. (Article 1) by conceptualising the research, designing the methodology, reviewing and editing the final draft.

Michalski J., Cłapa T., Syguda A., Narożna D., **van Oostrum P**., Reimhult E. 2024 Morpholinium- based ionic liquids as potent antibiofilm and sensitizing agents for the control of Pseudomonas aeruginosa. *Journal of Molecular Biology*, 436, 13. (Article 3) by designing the research, reviewing and editing the final draft.

Vienna, 05.07.2024

Dr Patrick Mester

Unit of Food Microbiology
University of Veterinary Medicine, Vienna 1210, Austria
email: patrick-julian.mester@vetmeduni.ac.at

I declare that I have contributed to the article:

Michalski J., Odrzygóźdź C., Mester P., Narożna D., Cłapa T., 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids*, 369. (Article 2) by visualizing and preparing some of the figures, reviewing and editing the final draft.

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dr inż. Cezary Odrzygóźdź

Instytut Chemii Bioorganicznej PAN w Poznaniu
ul. Noskowskiego 12/14
61-704 Poznań
email: codrzygozdz@gmail.com

Oświadczam, że mój udział w przygotowaniu pracy:

Michalski J., **Odrzygóźdź C**., Mester P., Narożna D., Cłapa T., 2023 Defeat undefeatable: Ionic liquids as novel antimicrobial agents. *Journal of Molecular Liquids*, 369. (Publikacja 2) polegał na udziale w przygotowaniu pierwszej wersji manuskryptu.

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## Publikacja 1

Cłapa T., **Michalski J.**, Syguda A., Narożna D., van Oostrum P., Reimhult E.
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Original Article

# Morpholinium-based ionic liquids show antimicrobial activity against clinical isolates of *Pseudomonas aeruginosa*

Tomasz Cłapa [a, *], Jakub Michalski [a], Anna Syguda [b], Dorota Narożna [a], Peter van Oostrum [c], Erik Reimhult [c]

[a] Poznań University of Life Sciences, Department of Biochemistry and Biotechnology, Dojazd 11, 60-632, Poznan, Poland
[b] Poznan University of Technology, Department of Chemical Technology, Berdychowo 4, 60-965, Poznan, Poland
[c] University of Natural Resources and Life Sciences, Department of Nanobiotechnology, Institute for Biologically Inspired Materials, Muthgasse 11-II, A-1090, Vienna, Austria

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ABSTRACT

*Pseudomonas aeruginosa* is a multi-drug resistant (MDR) pathogen. It is classified by WHO as one of the most life-threatening pathogens causing nosocomial infections. Some of its clinical isolates and their subpopulations show high persistence to many antibiotics that are recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Thus, there is a need for non-traditional classes of antibiotics to fight the increasing threat of MDR *P. aeruginosa*. Ionic liquids (IL) are one such promising class of novel antibiotics. We selected four strains of *P. aeruginosa* and studied the growth inhibition and other effects of 12 different ILs. We used the well-characterized *P. aeruginosa* PAO1 (ATCC 15692) as model strain and compared it to three other isolates from chronic lung infection (LES B58), skin burn infection (UCBPP-PA14) and keratitis infection (39016), respectively. The ILs consisted of either 4,4-didecylmorpholinium $[Dec_2Mor]^+$ or 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$ cations combined with different anions. We found that the ILs with 4,4-didecylmorpholinium $[Dec_2Mor]^+$ cations most effectively inhibited bacterial growth as well as reduced strain fitness and virulence factor production. Our results indicate that these ILs could be used to treat *P. aeruginosa* infections.



## 1. Introduction

*Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by multi-drug resistance (MDR) and the ability to utilize many different substrates as sources of energy. These bacteria are also known to evolve very fast and adapt to various environments. *P. aeruginosa* mostly infects patients with cystic fibrosis and those with compromised immune systems or patients that suffer from burns [1–4]. Furthermore, this pathogen is a carbapenem-resistant bacterium. Thus, its eradication is very difficult [5–7]. *P. aeruginosa* is one of the ESKAPE pathogens, an acronym for six bacteria that have evolved to resist many commonly used antibiotics. Their growing multi-drug resistance and virulence have led to that they are serious threats for nosocomial infections in a healthcare environment [8,9]. *P. aeruginosa* cannot be fully eradicated because of its resistance to a high number of antibiotics, good adaptation, and efficient protection system. An effective therapy against it presents a significant challenge for the scientific community and requires the development of novel types of antibiotic solutions. Generally, the increasing drug resistance in pathogenic microorganisms makes the search for new antimicrobial compounds extremely urgent [10]. One class of promising growth-inhibiting compounds is ionic liquids (ILs). ILs are characterized by low melting temperatures (typically <100 °C), non-flammability, high polarity due to dissociation of the salt that is combined with apolar moieties, chemical stability, and ionic conductivity [11–13]. The combination within one molecule of an extended hydrophobic moiety with an ionic charge makes ILs act as ionic surfactants. The majority of studies on ILs involve their degradation and chemical characteristics but do not consider environmental effects or interactions with living organisms [12]. However, ionic liquids have been shown to possess antimicrobial

* Corresponding author.
*E-mail addresses:* tomasz.clapa@up.poznan.pl (T. Cłapa), jak.michalski@onet.pl (J. Michalski), anna.syguda@put.poznan.pl (A. Syguda), dorna@o2.pl (D. Narożna), peter.van.oostrum@boku.ac.at (P. van Oostrum), erik.reimhult@boku.ac.at (E. Reimhult).

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properties, *e.g.*, growth inhibition and cytotoxic effects [14]. ILs were also recently combined with antibiotics, *e.g.*, β-lactam, to enhance their antibacterial activity [15].

Our study aims to investigate if and how ionic liquids influence the growth, viability, and production of virulence factor (pyocyanin) of four strains of *P. aeruginosa*. To best cover a wide diversity of *P. aeruginosa* species and represent various clinical sources, four relevant *P. aeruginosa* strains were selected. Twelve ionic liquids consisting of two different cations (4,4-didecylmorpholinium $[Dec_2Mor]^+$ and 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$) with six different anions are tested on the selected strains. The toxicity of the ILs is determined using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Changes in pathogenicity are measured with a pyocyanin inhibition assay, and the effect of the ILs on the viability of *P. aeruginosa* are evaluated with a dehydrogenase activity test.

## 2. Materials and methods

### 2.1. Ionic liquids

Twelve morpholinium ionic liquids with six types of herbicidal anions were used in the experiment. Each ionic liquid consisted of either a 4,4-didecylmorpholinium $[Dec_2Mor]^+$ or 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$ cation and one of following anions: 2,4-dichlorophenoxyacetate $[2,4\text{-}D]^-$; 4-chloro-2-methylphenoxyacetate $[MCPA]^-$; 3,6-dichloro-2-methoxybenzoate $[Dicamba]^-$; (±)-2-(4-chloro-2-methylphenoxy)propionate $[MCPP]^-$; 4-chlorophenoxyacetate $[4\text{-}CPA]^-$; or 3,6-dichloro-2-pyridinecarboxylate $[Clopyralid]^-$ (Table 1).

### 2.2. Synthesis of ILs

All morpholinium ionic liquids with herbicidal anions (HILs) were synthesized according to the procedures described by Ławniczak et al. [20]. Fig. 1. Presents a scheme for the synthesis of four ionic liquids that are new compounds. We provide the NMRs of the obtained morpholinium HILs in the next section and their basic properties in Table 2. The characteristics of the additional eight ionic liquids were presented earlier [20].

### 2.3. The results of $^1H$ and $^{13}C$ NMR and CHN analysis

$^1H$ NMR spectra of the newly synthesized morpholinium HILs were recorded on a Varian VNMR-S spectrometer at 400 MHz with tetramethylsilane as the standard; $^{13}C$ NMR spectra were recorded on the same instrument at 100 MHz. Elemental analyses were performed at Adam Mickiewicz University, Poznan (Poland). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, quart = quartet, q = quintet, m = multiplet.

*4-Decyl-4-ethylmorpholinium 3,6-dichloro-2-pyridinecarboxylate* **[DecEtMor][Clopyralid]** $^1H$ NMR ($D_2O$) $\delta$ ppm = 0.89 (t, $J$ = 6.9 Hz, 3H), 1.26 (t, $J$ = 5.4 Hz, 3H), 1.30 (m, 14H), 1.63 (q, $J$ = 8.7 Hz, 2H), 3.36 (t, $J$ = 8.7 Hz, 2H), 3.53 (quart, $J$ = 4.7 Hz, 2H), 3.55 (t, $J$ = 7.3 Hz, 4H), 4.04 (t, $J$ = 4.9 Hz, 4H), 7.26 (d, $J$ = 8.5 Hz, 1H), 7.82 (d, $J$ = 8.4 Hz, 1H); $^{13}C$ NMR $\delta$ ppm = 8.70, 16.03, 23.00, 24.81, 24.74, 28.10, 31.06, 31.45, 34.06, 54.58, 56.10, 59.68, 62.27, 126.17, 127.62, 142.82, 149.96, 158.88, 172.04. Anal. Calcd for $C_{22}H_{36}N_2O_3Cl_2$: C 59.04, H 8.13, N 6.26; Found: C 58.66, H 8.00, N 6.14.

*4-Decyl-4-ethylmorpholinium 4-chlorophenoxyacetate* **[DecEtMor][4-CPA]** $^1H$ NMR ($D_2O$) $\delta$ ppm = 0.92 (t, $J$ = 6.9 Hz, 3H), 1.23 (t, $J$ = 7.0 Hz, 3H), 1.31 (m, 14H), 1.55 (q, $J$ = 8.5 Hz, 2H), 3.24 (t, $J$ = 8.6 Hz, 2H), 3.40 (quart, $J$ = 4.7 Hz, 2H), 3.45 (t, $J$ = 7.1 Hz, 4H), 3.97 (t, $J$ = 4.9 Hz, 4H), 4.84 (s, 2H), 6.95 (d, $J$ = 9.0 Hz, 2H), 7.23 (d, $J$ = 9.1 Hz, 2H); $^{13}C$ NMR $\delta$ ppm = 8.71, 16.10, 23.07, 24.86, 28.27, 31.27, 31.62, 31.75, 34.12, 55.95, 59.70, 60.43, 62.25, 69.36, 118.42, 127.22, 131.47, 159.34, 176.62. Anal. Calcd for $C_{24}H_{40}NO_4Cl$: C 65.20, H 9.14, N 3.17; Found: C 65.69, H 9.46, N 3.28.

*4,4-Didecylmorpholinium 3,6-dichloro-2-pyridinecarboxylate* **[$Dec_2$Mor][Clopyralid]** $^1H$ NMR ($CDCl_3$) $\delta$ ppm = 0.88 (t, $J$ = 7.0 Hz, 6H), 1.31 (m, 28H), 1.71 (q, $J$ = 7.5 Hz, 4H), 3.59 (t, $J$ = 8.5 Hz, 4H), 3.77 (t, $J$ = 4.6 Hz, 4H), 4.08 (t, $J$ = 4.5 Hz, 4H), 7.58 (d, $J$ = 8.3 Hz, 1H), 7.11 (d, $J$ = 8.3 Hz, 1H); $^{13}C$ NMR $\delta$ ppm = 13.86, 21.44, 22.40, 26.07, 29.00, 31.58, 57.95, 58.84, 60.37, 122.71, 125.37, 139.39, 147.82, 158.36, 168.21. Anal. Calcd for $C_{30}H_{52}N_2O_3Cl_2$: C 64.37, H 9.38, N 5.01; Found: C 64.85 1, H 8.90, N 4.90.

*4,4-Didecylmorpholinium 4-chlorophenoxyacetate* **[$Dec_2$Mor][4-CPA]** $^1H$ NMR ($CDCl_3$) $\delta$ ppm = 0.88 (t, $J$ = 6.9 Hz, 6H), 1.25 (m, 28H), 1.64 (q, $J$ = 7.8 Hz, 4H), 3.51 (t, $J$ = 8.6 Hz, 4H), 3.67 (t, $J$ = 4.7 Hz, 4H), 3.99 (t, $J$ = 4.6 Hz, 4H), 4.41 (s, 2H), 6.87 (d, $J$ = 9.1 Hz, 2H), 7.17 (d, $J$ = 9.1 Hz, 2H); $^{13}C$ NMR $\delta$ ppm = 13.88, 21.39, 22.42, 26.10, 29.02, 29.20, 31.60, 31.65, 57.74, 58.89, 60.25, 67.82, 115.90, 124.65, 128.77, 157.57, 172.20. Anal. Calcd for $C_{32}H_{56}NO_4Cl$: C 69.33, H 10.20, N 2.53; Found: C 68.90 1, H 10.51, N 2.44.

### 2.4. Microorganisms and media

Four clinically relevant *P. aeruginosa* strains: PAO1 (ATCC 15692) as model strain and three other isolates from chronic lung infection (LES B58), skin burn infection (UCBPP-PA14), and keratitis infection (39016) were used in the experiments. All microorganisms were obtained from the Department of Biochemistry and Biotechnology, Poznań University of Life Sciences bacterial strains collection. All strains were cultured at 37 °C in tryptic soy broth medium (TSB; Sigma-Aldrich) supplemented with 0.6% yeast extract (TSB-Y).

### 2.5. Antimicrobial activity

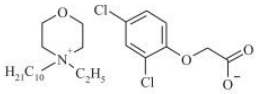
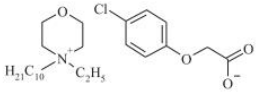
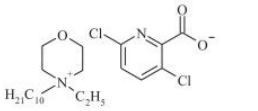
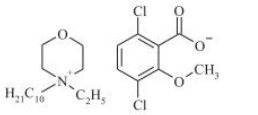
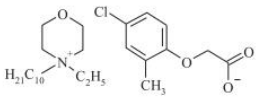
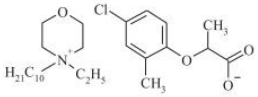
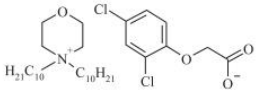
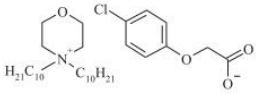
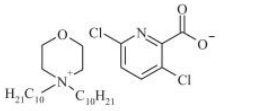
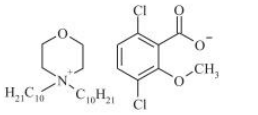
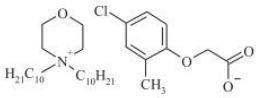
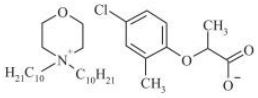
The minimum inhibitory concentration (MIC) of the tested ILs was determined using the microdilution method with resazurin dye as a growth indicator. The resazurin assay was chosen because some ILs added turbidity to the samples and therefore affected standard OD measurements of bacterial counts. Briefly, 80 μL of each ionic liquid was added to the first row of 96-well plate at a concentration of 5120 ppm. Afterward, 40 μL of sterile TSB-Y medium was distributed to empty wells, and serial dilutions were performed. Next, TSB-Y medium containing resazurin (0.02 mg/mL) was inoculated with overnight cultures of *P. aeruginosa* strains to the final concentration of $5 \times 10^5$ CFU/mL 160 μL of such prepared inoculum was added to all wells. Plates were incubated overnight at 37 °C with shaking. Bacterial growth was marked by a change of colour from dark blue to pink. The lowest concentration with no colour change observed was identified as the minimum inhibitory concentration (MIC).

After the MIC evaluation, the same bacterial cultures were used for the determination of minimum bactericidal concentration (MBC). From all wells where no bacterial growth was observed, 10 μL aliquots were transferred onto fresh agar plates containing TSB-Y medium. Plates were incubated overnight at 37 °C, and the lowest concentration with no colonies growth observed was taken as the MBC.

### 2.6. Dehydrogenase activity

A dehydrogenase activity assay based on 2,3,5-triphenyltetrazolium chloride (TTC) was performed to determine the microbial viability. Briefly, 1 mL of IL solution was made in TSB-Y medium at a final concentration equal to ½ and ¼ MIC. In case the

**Table 1**
List of morpholinium herbicidal ionic liquids examined in the study.

| Abbreviation | Acronym | Name | Chemical structure |
|---|---|---|---|
| IL-1 | [DecEtMor] [2,4-D] | 4-decyl-4-ethylmorpholinium 2,4-dichlorophenoxyacetate |  |
| IL-2 | [DecEtMor] [4-CPA] | 4-decyl-4-ethylmorpholinium 4-chlorophenoxyacetate |  |
| IL-3 | [DecEtMor] [Clopyralid] | 4-decyl-4-ethylmorpholinium 3,6-dichloro-2-pyridinecarboxylate |  |
| IL-4 | [DecEtMor] [Dicamba] | 4-decyl-4-ethylmorpholinium 3,6-dichloro-2-methoxybenzoate |  |
| IL-5 | [DecEtMor] [MCPA] | 4-decyl-4-ethylmorpholinium 4-chloro-2-methylphenoxyacetate |  |
| IL-6 | [DecEtMor] [MCPP] | 4-decyl-4-ethylmorpholinium (±)-2-(4-chloro-2-methylphenoxy)propionate |  |
| IL-7 | [$Dec_2$Mor] [2,4-D] | 4,4-didecylmorpholinium 2,4-dichlorophenoxyacetate |  |
| IL-8 | [$Dec_2$Mor] [4-CPA] | 4,4-didecylmorpholinium 4-chlorophenoxyacetate |  |
| IL-9 | [$Dec_2$Mor] [Clopyralid] | 4,4-didecylmorpholinium 3,6-dichloro-2-pyridinecarboxylate |  |
| IL-10 | [$Dec_2$Mor] [Dicamba] | 4,4-didecylmorpholinium 3,6-dichloro-2-methoxybenzoate |  |
| IL-11 | [$Dec_2$Mor] [MCPA] | 4,4-didecylmorpholinium 4-chloro-2-methylphenoxyacetate |  |
| IL-12 | [$Dec_2$Mor] [MCPP] | 4,4-didecylmorpholinium (±)-2-(4-chloro-2-methylphenoxy)propionate |  |

MIC of the examined compound could not be determined, the two highest tested concentrations were used (1024 and 512 ppm). The prepared tubes were inoculated with overnight cultures of microorganisms to reach the final $OD_{600}$ value equal to 0.01. This OD was required as starting point to achieve sufficient growth for a reliable analysis. Next, the samples were incubated overnight at 37 °C, with shaking. After incubation, 1 mL of 1% TTC solution was introduced into the tubes, and the samples were incubated for 30 min at 37 °C. At this stage, soluble and colourless TTC was metabolized by viable bacteria cells into insoluble, red-coloured formazan (TPF). Subsequently, cultures were centrifuged for 2 min at 14000 rpm. The precipitates were dissolved in 2 mL of 96% ethanol. Then samples

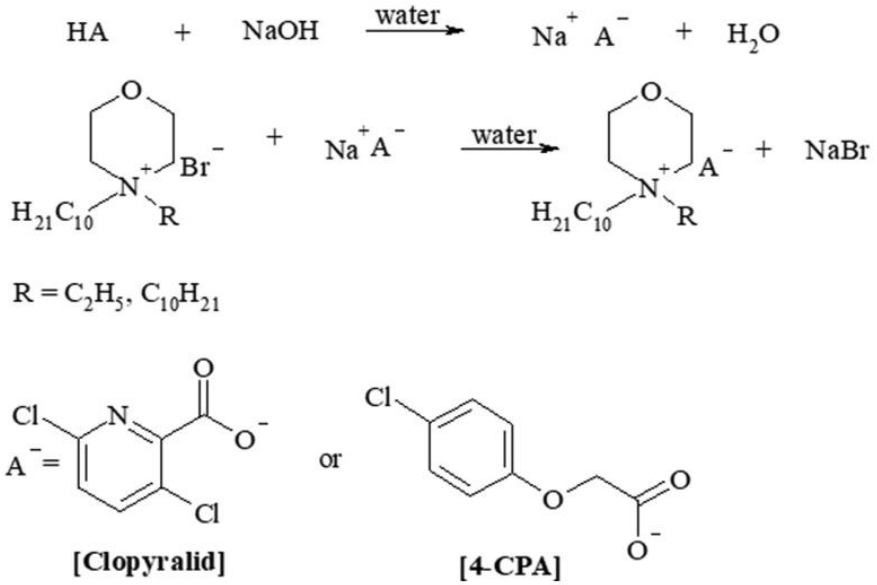
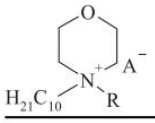



**Fig. 1.** Reaction scheme for the new morpholinium HILs.

**Table 2**
Properties of the new morpholinium HILs.




| R | $A^-$ | Abbreviation of HIL | Surfactant content[a] [%] | Yield [%] | State at 25 °C |
|---|---|---|---|---|---|
| $C_2H_5$ | Clopyralid$^-$ | [DecEtMor][Clopyralid] | 98 | 87 | liquid |
| $C_2H_5$ | 4-CPA$^-$ | [DecEtMor][4-CPA] | 97 | 82 | liquid |
| $C_{10}H_{21}$ | Clopyralid$^-$ | [$Dec_2$Mor][Clopyralid] | 97 | 90 | grease |
| $C_{10}H_{21}$ | 4-CPA$^-$ | [$Dec_2$Mor][4-CPA] | 96 | 99 | grease |

[a] ISO 2871−1:1988. "Surface active agents—Detergents—Determination of cationic-active matter content— Part 1: High-molecular mass cationic-active matter ", ISO 2871−2:1990. "Surface active agents—Detergents—Determination of cationic-active matter content— Part 2: Cationic-active matter of low molecular mass (between 200 and 500)".

were centrifuged again in order to pellet the cells. The absorbance of the supernatant was measured at a wavelength of 495 nm.

### 2.7. Pyocyanin quantification

Pyocyanin ($PCN^-$) was quantified spectrophotometrically after chloroform-HCl extraction. After pipetting 3 mL of sterile TSB-Y broth into conical tubes, ionic liquids were added to reach the final concentration equal to ½ and ¼ MIC. For samples of which the MIC could not be determined, the two highest tested concentrations were used (1024 and 512 ppm). Subsequently, overnight cultures of microorganisms were standardised to $OD_{600} = 1$ and then distributed to the test tubes in 1:100 dilution. A starting $OD_{600} = 0.01$ was required to achieve sufficient growth for a reliable analysis with this assay. A high cell density is a crucial factor in increasing the production of pyocyanin. These cultures were grown for 20 h at 37 °C, with shaking at 150 rpm. After incubation, a 500 μL aliquot of each sample was collected, diluted 2 times in medium broth, and its optical density was measured at 600 nm. The rest of the bacterial suspension was centrifuged for cell removal. The collected supernatant was mixed with 1.5 mL of chloroform and vortexed. After that, the emulsion was centrifuged for 10 min at 10.000 rpm, and the chloroform phase containing pyocyanin was transferred to new tubes. Next, 750 μL of 0.2 N HCl was added to samples, mixed vigorously, and centrifuged for 2 min at 10.000 rpm. The absorbance of the pink top layer containing pyocyanin was measured at a wavelength of 520 nm.

## 3. Results

### 3.1. Antimicrobial activity

Antimicrobial activity assays showed considerable differences in toxicity between the studied ionic liquids in relation to their chemical structure (Supplementary material, S1 Table, and Fig. 2.). ILs-1-6 with $[DecEtMor]^+$ cation embedded into the structure demonstrated no antimicrobial effect within the tested concentration range. [DecEtMor][Clopyralid] was only capable of inhibiting the growth of the PAO1, LES B58, and UCBPP-PA14 strains at concentrations of 1024, 768, and 1024 ppm, respectively. In contrast, ILs-7-12 with the $[Dec_2Mor]^+$ cation displayed high toxicity towards all studied strains with MIC values ranging from 11

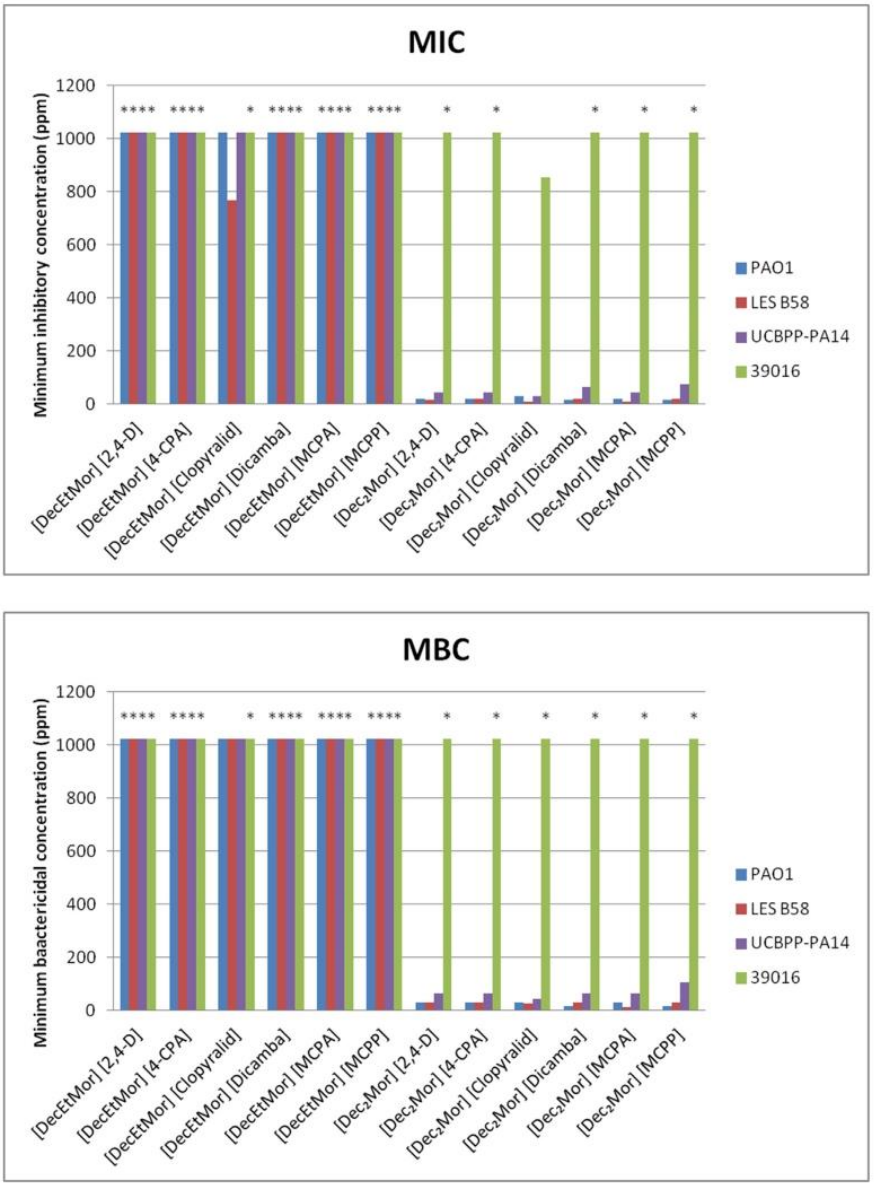



**Fig. 2.** Mean MIC (A) and MBC (B) values for four *P. aeruginosa* strains: PAO1, LES B58, 39016, and UCBPP-PA14; *No antimicrobial effect observed at 1024 ppm concentration.

to 75 ppm. The 39016 strain was found to be highly resistant towards all tested compounds, showing only slight susceptibility towards [$Dec_2$Mor][Clopyralid] at the highest tested concentrations. Ionic liquids that inhibited bacterial growth also exhibited bactericidal properties, with MBC values found within two-fold concentrations above the MIC values. Importantly, subjecting the *P. aeruginosa* strains to only the sodium salts with the herbicidal anions produced no antimicrobial effect (data not shown).

### 3.2. Dehydrogenase activity

Results obtained from the TTC assay are displayed in Fig. 3. An increase of dehydrogenase activity was observed only for the PAO1 strain treated with IL-10 at a concentration equal to ¼ MIC. In contrast, results obtained for the PAO1 strain incubated with IL-7, IL-8, and IL-11 at ¼ MIC concentrations and IL-10 at ½ MIC concentration were comparable to measurements made on the non-treated control. For all other samples, a considerable decrease of dehydrogenase activity was noted. This reduction varied between 25 and 97%. The observed inhibitory effect was more pronounced for cultures incubated with a higher concentration of IL and differed greatly for the individual strains and compounds. In the case of PAO1 and LES B58, the biggest decreases of dehydrogenase activity were noted for cultures treated with $[DecEtMor]^+$ ILs; ILs-1-6 at ½ MIC concentration for PAO1 and ILs-1,3,5 at ½ MIC concentration for LES B58. On the contrary, a similar rate of reduction regarding

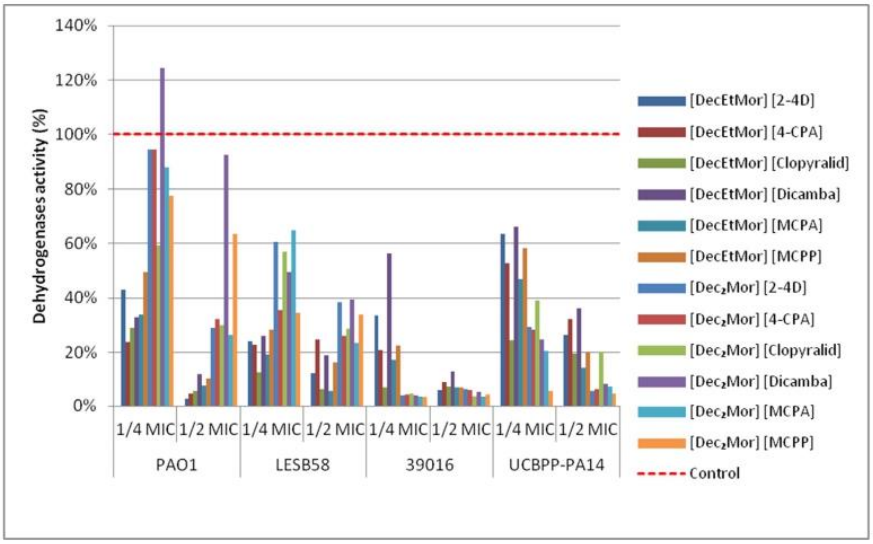



**Fig. 3.** Dehydrogenase activity of *P. aeruginosa* cultures treated with sub-inhibitory (½ and ¼ MIC) concentrations of ionic liquids in relation to non treated culture.

the UCBPP-PA14 strain was observed for compounds with $[Dec_2Mor]^+$ cation; ILs-7,8,10,11 at ½ MIC concentration and IL-12 at both concentrations tested. Strain 39016 was the most sensitive to the action of ionic liquids in the TTC assay. An 85–97% inhibition of dehydrogenase activity was observed for all ILs at a concentration of ½ MIC. The same level of reduction was shown for all $[Dec_2Mor]^+$ ILs already at ¼ MIC.

### 3.3. Pyocyanin production

The levels of pyocyanin biosynthesis inhibition in cultures treated with ILs at sub-inhibitory concentrations compared to the nontreated control are presented in Fig. 4. The strains differed significantly in pyocyanin production. The LES B58 strain turned out to be the most effective PCN- producer, and PAO1 strain showed no pyocyanin yield under the given conditions. A significant decrease in pyocyanin production was observed for all tested compounds, which varied from 35% up to 100%. A substantial loss in cell density was noted only for the LESB58 sample treated with IL-3 at ½ MIC concentration. For all other samples, the bacterial growth was comparable to that achieved in nontreated cultures (data not shown). Big differences in terms of pyocyanin inhibition rate were noticed with respect to the IL structure and bacterial strain. In the case of the 39016 and UCBPP-PA14 strains $[Dec_2Mor]^+$, ILs were extremely efficient inhibitors, fully suppressing PCN- production, whereas ILs with the $[DecEtMor]^+$ cation showed high but only partial pyocyanin inhibition. No such clear correlation between IL structure and the intensity of PCN- inhibition could be made for the LES B58 strain since all test compounds yielded a similar rate of suppression. The general trend was that the use of higher IL concentrations resulted in increased inhibition of pyocyanin. Exceptions to this trend were LES B58 treated with IL-4, 39016 treated with IL-3,4,6, and UCBPP-PA14 treated with IL-3, which showed the opposite dependence of the inhibition of pyocyanin on the IL concentration.

## 4. Discussion

The aim of this study was to examine the antibacterial and antivirulent properties of twelve morpholinium herbicidal ionic liquids towards *P. aeruginosa*. All examined strains are completely sequenced, sharing around 90% genetic identity [16]. The analysis of mobile gene pools showed that the chosen strains represent both major genetic clusters present among *P. aeruginosa* [17]. The selected strains also exhibit considerable differences in phenotype, both in terms of virulence and resistome pattern [16–19]. Ławniczak et al. [20] evaluated the toxicity of eight of the twelve HILs used in this study on microbiota isolated from different environmental niches. Also, the biodegradability was analysed. The authors demonstrated that ILs with $[Dec_2Mor]^+$ cation were more toxic than compounds with $[DecEtMor]^+$ moiety. This finding was confirmed in the present study. However, the differences in toxicity between ILs with distinct cations were far more pronounced against *P. aeruginosa* than against the microbiota in the previous study. This may be due to the higher resistance and adaptation ability exhibited by *P. aeruginosa* compared to the microorganisms that were tested previously. In this study, a high antimicrobial potency was exhibited by $[Dec_2Mor]^+$ ILs with two decyl groups bound to the morpholine ring, while no antimicrobial effect was observed for $[DecEtMor]^+$ ILs with only one decyl group attached to the cation. This observation corresponds well with the general trend that an increase of cation hydrophobicity increases ILs toxicity [21]. For instance, the elongation of the alkyl side-chain of morpholinium or alkylammonium cations increases the antimicrobial activity of ILs towards bacteria species like *Escherichia coli*, *Bacillus cereus*, or *Vibrio Fischeri* [22,23]. Additionally, a similar effect may be achieved after increasing the number of long alkyl chains on cation substituents [24]. This phenomenon is predominantly ascribed to cell membrane disruption caused by ionic liquids that act in a manner similar to cationic surfactants [25,26]. However, reports are indicating that some ionic liquids' antimicrobial properties are primarily related to protein denaturation rather than to membrane permeabilization, like in the case of imidazolium-based ILs [27]. Up to date, no scientific papers are specifying the mode of action of morpholinium-based ionic liquids towards microorganisms.

Ławniczak et al. [20] also reported a strong dependence of HILs toxicity on the type of anion. They observed that the $[MCPA]^-$ anion contributed to the highest toxicity, while ILs with the $[Dicamba]^-$ anion exhibited no toxic effect towards environmental microbiota.

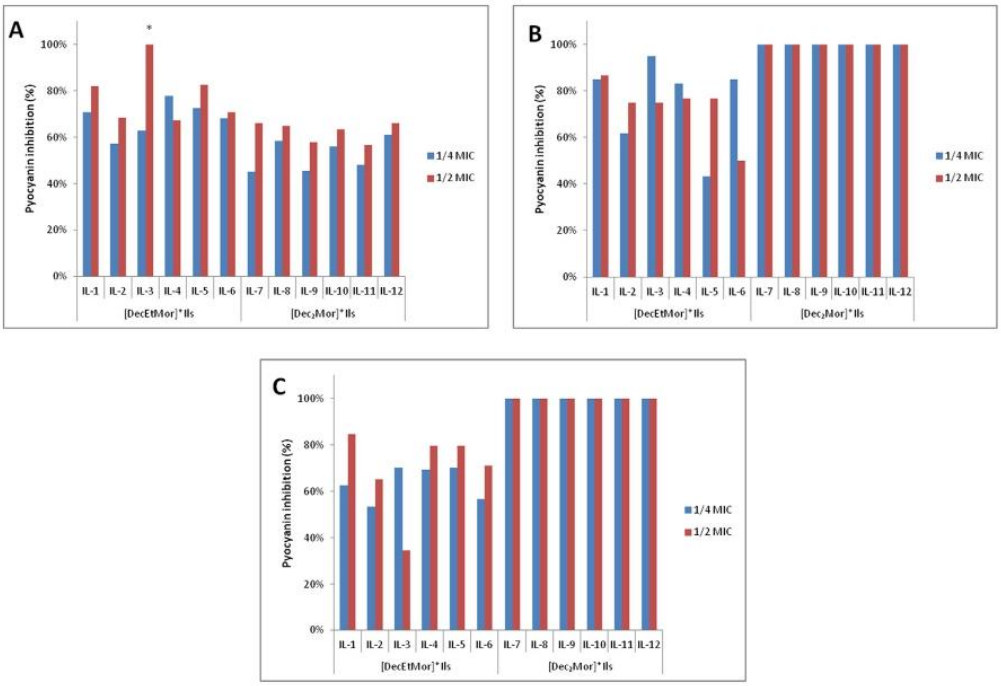



**Fig. 4.** Inhibition of pyocyanin production in *P. aeruginosa* (A) LES B58, (B) 39016, and (C) UCBPP-PA14 strains after treatment with sub-inhibitory (½ and ¼ MIC) concentrations of ionic liquids. The results are presented for each test compound as a percentage reduction of pyocyanin produced by treated cultures compared to nontreated control; *No pyocyanin yield was accompanied with a substantial loss of cell density.

$[MCPP]^-$ and $[2,4\text{-}D]^-$ anions demonstrated lower toxicity than $[MCPA]^-$. We did not observe this dependence. The anionic part seemed to have no significant influence on the antimicrobial activity of ILs towards *P. aeruginosa*, except for $[Clopyralid]^-$ which appeared to slightly increase the antimicrobial effect. Furthermore, also herbicides in the form of sodium salts showed no antimicrobial effect towards examined bacteria. We can, therefore, conclude that the antimicrobial effect of ILs on *P. aeruginosa* was primarily associated with an action of the cation.

Considerable differences in the susceptibility to ILs between the strains were also noted. *P. aeruginosa* strains are known to display high variability in their resistance to various antimicrobials. Many strain-dependent resistance mechanisms to antibiotics, such as the presence of specific efflux pumps, porins, or enzymes, have been described [28]. However, not much is known about strain-dependent tolerance to ionic liquids in *P. aeruginosa*. Extremely high tolerance to all tested HILs was observed for clinical isolate 39016, associated with severe keratitis. The 39016 strain is highly adapted to cause corneal infections. It is characterised by high twitching motility, which is coupled with the presence of a distinctive *pilA* gene encoding the main type IV pili protein [19]. Furthermore, contrary to other strains used in this study, isolate 39016 carries the type A allele of gene *fliC* encoding flagellin [29]. It also lacks three specific outer membrane porins: OprP, OprO and OpdD [30]. On top of that, Cullen et al. [18] showed that strain 39016 possesses O-antigen in its lipopolysaccharide (LPS) structure with a unique pattern that differs from O-antigen in LPS of other strains used in the present study. There are a few studies on *E. coli* species, indicating a strong influence of LPS structure on resistance towards alkylammonium and imidazolium ionic liquids [23,31,32]. According to those reports, strains lacking O-antigen, inner or outer core oligosaccharide in their LPS structure are characterized by higher membrane hydrophobicity and permeability. Therefore, such isolates are more susceptible to the action of ionic liquids with long or multiple alkyl chains. The LPS of *P. aeruginosa* were also reported to play a crucial role in resistance towards antibiotics causing bacterial bilayer disruption, like in the case of colistin [33]. Thus, we hypothesise that the distinctive LPS structure of isolate 39016 contributes to its increased tolerance towards tested ILs. Another important factor of resistance against some agents causing permeabilization of bacterial membranes is type IV pili [34]. Since strain 39016 carry a unique *pilA* gene, we speculate that this characteristic also contributes to the increased tolerance to morpholinium ionic liquids. These hypotheses require further studies in view of that a membrane-permeabilizing mode of action is not yet confirmed for ionic liquids with a morpholinium cation, although it is the most plausible mode of action.

A dehydrogenase activity assay was performed to study the effect of morpholinium herbicidal ionic liquids on *P. aeruginosa* metabolism. The TTC test, along with other tetrazolium salt-based methods, is commonly used to determine microbial activity and growth of microorganisms [35–37]. In this type of analysis, the ability of bacterial dehydrogenase to reduce TTC into formazan is examined. This ability reflects the metabolic activity and condition of bacterial cells [38]. In almost all cultures treated with ILs, a substantial decrease in the amount of TTC metabolised to formazan was observed. Thus, it was shown that the tested ionic liquids could inhibit bacterial metabolism, even at concentrations that do not affect the growth of the microorganisms. Interestingly, the same inhibitory effect was observed regardless of if the tested compounds exhibited high, minor, or no antimicrobial effect in the MIC assay. Surprisingly, isolate 39016 was the strain most susceptible to changes in the dehydrogenase activity despite that it showed extremely high resistance to all ILs during MIC evaluation. On the

one hand, this indicates that even when ionic liquids do not inhibit bacterial growth, the microorganisms are not unaffected and can be forced to downregulate their cell metabolism strongly. On the other hand, it demonstrates that bacterial cells could maintain their ability to proliferate even at a low metabolic rate.

Our results also suggest that the intensity of the described inhibitory effect is related to the chemical structure of ionic liquids. However, this connection is not observed for all the tested strains. $[DecEtMor]^+$ ILs most effectively inhibited bacterial metabolism of PAO1 and LES B58 strains, whereas, for UCBPP-PA14, a stronger inhibition was observed for $[Dec_2Mor]^+$ ILs. In the case of the 39016 strain, all ionic liquids were equally effective. These observations greatly differ from the MIC assay results, where a very clear distinction could be made between non-toxic $[DecEtMor]^+$ ILs and highly toxic ILs with $[Dec_2Mor]^+$ cation. Thus, the mechanism of action of morpholinium herbicidal ionic liquids on bacterial metabolism may be distinct from the mechanism affecting the growth of the microorganisms. Moreover, it seems that the strains' individual characteristics play a crucial role in the observed differences in metabolic response to ionic liquids. However, those traits were not identified in this study.

Morpholinium herbicidal ionic liquids appear as a limiting factor for *P. aeruginosa* pathogenicity, affecting the production of one of the main virulence factors, pyocyanin. Pyocyanin is a blue secondary metabolite produced exclusively by *P. aeruginosa* species [39]. It plays a crucial role in biofilm formation [40], resistance mechanisms [41], and pathogenesis [42,43]. It can also act as an antimicrobial agent, inhibiting the growth of competitive bacteria species [44]. Pyocyanin production is strain dependant and is under the control of a quorum sensing (QS) system [45]. The inhibitory effect on pyocyanin productivity has been described for plant extracts [46], natural compounds [47], quorum sensing inhibitors [48,49], and antibiotics [50]. The opposite effect was observed for subinhibitory concentrations of some antibiotics that were testes by Zhou et al. [51]. To date, no research was conducted to evaluate the influence of ILs on pyocyanin biosynthesis.

All strains used in this study are reported to produce pyocyanin [18,40,52]. However, the PAO1 strain showed no pyocyanin yield in our measurements, despite being recognized as a strong pyocyanin producer [18]. Interestingly, during the MIC assay performed on the 96-well plate, the ability of the PAO1 strain to produce pyocyanin was observed. That pyocyanin production efficiency might explain this strongly depends on several factors, including cell density, growth phase, culture duration [53,54], aeration conditions [55,56], and medium composition [57–59]. Optima for some of these parameters can differ between individual *P. aeruginosa* strains [53,60]. Most likely, the culturing method chosen for the pyocyanin quantification assay was not optimal to induce the production of the pigment by the PAO1 strain. That said, it was not possible to conduct pyocyanin quantification on 96-well plates due to the detection limit of the extraction method. At the same time, it was necessary to ensure equal conditions for all strains. Thus, we were unable to evaluate the antivirulence activity of the ILs on the PAO1 strain.

For the first time, the antivirulence of ionic liquids was investigated. All tested compounds were found to inhibit pyocyanin production at sub-MIC concentrations. The effectiveness of ILs was partially dependent on the chemical structure of the compounds and varied for individual strains. As one would expect, highly antimicrobial $[Dec_2Mor]^+$ ILs were proven better inhibitors than $[DecEtMor]^+$ ILs with low antimicrobial activity for two of the tested strains. However, pyocyanin biosynthesis in LES B58 cultures was inhibited to a similar extent by all compounds regardless of their structure. Therefore, it seems that *P. aeruginosa* vulnerability and potential defence mechanisms against the antivirulent action of HILs are strain-dependent. Since pyocyanin production is a quorum-sensing regulated process, it can be suppressed in low-density cultures. At the same time, a decrease of pyocyanin yield was not accompanied by a loss of cell density. Thus, the observed inhibition in pyocyanin production was not due to bacterial cell death. Another known mechanism of pyocyanin suppression includes the downregulation of QS genes. For instance, there are reports indicating that trans-cinnamaldehyde and salicylic acid downregulate key QS regulatory genes from the *las* and *rhl* operons [47] in parallel with inhibiting pyocyanin production. Studies on alkyl gallates also showed that PCN- suppression can be caused by the downregulation of the *phz1* and *phz2* operons, which directly mediate pyocyanin biosynthesis [49,61].

## 5. Conclusions

Our work demonstrates that ILs with a $[Dec_2Mor]^+$ cation are more toxic to *P. aeruginosa* strains than ILs with a $[Dec_2Mor]^+$ cation. However, very high growth tolerance for all tested ILs was observed for clinical isolate 39016 associated with severe keratitis. This might correlate with its fast adaptation to different environmental factors, LPS membrane structure, as well as its unique genes expressing Type IV pili. Further, we demonstrated that all strains decreased their metabolism when exposed to morpholinium HILs, even when no growth inhibition was observed. A decreased production of virulence factor (pyocyanin) was also observed in all strains, except for PAO1, for which the assay could not be established. As pyocyanin production is related to pathogenicity and quorum sensing, we conclude that exposure to morpholinium HILs decreases *P. aeruginosa* virulence. Thus, the tested ionic liquids exhibit a strong influence on the *P. aeruginosa* isolates, which render them interesting alternative antimicrobials. However, our study also highlights a strain-dependence in response to HILs, which strongly suggests that anti-growth and anti-virulence activities rely on different mechanisms. We suggest that the membrane interaction of the cation of our HILs is a plausible main mechanism of the antimicrobial activity. While the mechanisms of action are still unknown, research into the molecular basis of the anti-growth and anti-virulence mechanisms of these HILs is warranted to optimize further their action against *P. aeruginosa* and other ESKAPE pathogens. For medical or other wide-spread applications, the cytotoxicity of the most efficient of these compounds also should be further investigated. Such investigations on human cells are being undertaken.

## Ethical approval

Not required.

## Contribution to the article

These experiments were conceptualized by Tomasz Cłapa, Dorota Narożna, Peter Van Oostrum, and Erik Reimhult. These authors also conceived the methodology. Data curation was made by Jakub Michalski and Tomasz Cłapa. Jakub Michalski performed most analyses and was responsible for the first draft of the paper. Tomasz Cłapa, Dorota Narożna and Anna Syguda assisted in reviewing and editing of the first draft. Anna Syguda performed and documented the synthesis of the ionic liquid synthesis. Data visualization was made by Jakub Michalski and Tomasz Cłapa. The final draft of the paper was prepared by Tomasz Cłapa, Peter Van Oostrum, and Erik Reimhult. All authors have read and agreed to the published version of the manuscript.

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## Declaration of competing interest

The authors declare no conflicts of interest.

## Acknowledgement

Graphical abstract was Created with BioRender.com.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2021.103817.

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**S1 Table .** Antimicrobial effect of ionic liquids on four *Pseudomonas aeruginosa* strains: PAO1, LES B58, 39016 and UCBPP-PA14

| Ionic Liquid | MIC (ppm) | | | | MBC (ppm) | | | |
|---|---|---|---|---|---|---|---|---|
| | **PAO1** | **LES B58** | **39016** | **UCBPP-PA14** | **PAO1** | **LES B58** | **39016** | **UCBPP-PA14** |
| **IL-1** [DecEtMor][2,4-D] | >1024 (>1024; >1024) | >1024* (1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) |
| **IL-2** [DecEtMor][4-CPA] | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) |
| **IL-3** [DecEtMor][Clopyralid] | 1024 (1024; 1024) | 768 (256; 1024) | >1024 (>1024; >1024) | 1024 (1024; 1024) | 1024 (1024; 1024) | 1024 (1024; 1024) | >1024 (>1024; >1024) | 1024 (1024; 1024) |
| **IL-4** [DecEtMor][Dicamba] | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) |
| **IL-5** [DecEtMor][MCPA] | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) |
| **IL-6** [DecEtMor][MCPP] | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) | >1024 (>1024; >1024) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **IL-7** [$Dec_2Mor$][2,4-D] | 21 (16; 32) | 16 (16; 16) | >1024 (>1024; >1024) | 43 (32; 64) | 32 (32; 32) | 32 (32; 32) | >1024 (>1024; >1024) | 64 (64; 64) |
| **IL-8** [$Dec_2Mor$][4-CPA] | 21 (16; 32) | 21 (16; 32) | >1024 (>1024; >1024) | 43 (32; 64) | 32 (32; 32) | 32 (32; 32) | >1024 (>1024; >1024) | 64 (64; 64) |
| **IL-9** [$Dec_2Mor$][Clopyralid] | 32 (32; 32) | 11 (8; 16) | 853 (512; >1024) | 32 (32; 32) | 32 (32; 32) | 27 (16; 32) | >1024 (>1024; >1024) | 43 (32; 64) |
| **IL-10** [$Dec_2Mor$][Dicamba] | 16 (16; 16) | 21 (16; 32) | >1024 (>1024; >1024) | 64 (64; 64) | 16 (16; 16) | 32 (32; 32) | >1024 (>1024; >1024) | 64 (64; 64) |
| **IL-11** [$Dec_2Mor$][MCPA] | 21 (16; 32) | 11 (8; 16) | >1024 (>1024; >1024) | 43 (32; 64) | 32 (32; 32) | 13 (8; 16) | >1024 (>1024; >1024) | 64 (64; 64) |
| **IL-12** [$Dec_2Mor$][MCPP] | 16 (16; 16) | 21 (16; 32)[1] | >1024 (>1024; >1024) | 75 (32; 128) | 16 (16; 16) | 32 (32; 32) | >1024 (>1024; >1024) | 107 (64; 128) |

Mean MIC and MBC values (ppm) for triplicate with the span of measured values (lower limit; upper limit) are presented. *No effect observed for two out of three measurements

## Publikacja 2

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# Defeat undefeatable: Ionic liquids as novel antimicrobial agents

Jakub Michalski [a], Cezary Odrzygóźdź [b], Patrick Mester [c], Dorota Narożna [a], Tomasz Cłapa [a,*]

[a] Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, Dojazd 11, 60-632 Poznań, Poland
[b] Department of Molecular and Systems Biology, Institute of Bioorganic Chemistry, Polish Academy of Sciences, ul. Z. Noskowskiego 12/14, 61-704 Poznań, Poland
[c] Unit of Food Microbiology, Institute of Food Safety, Food Technology and Veterinary Public Health Department for Farm Animals and Veterinary Public Health University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria

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ABSTRACT

Emerging antimicrobial resistance and spread of drug-resistant pathogens is currently-one of the biggest threats posed to global health. Due to that, search of new antimicrobials replacing now used antibiotics has been given a very high priority. Ionic liquids; low-melting salts, thanks to their unique tunable nature and biological activity are considered as a group of candidate compounds to become antimicrobials of the future. This Review presents data collected on the effects of ionic liquids on pathogenic and non-pathogenic bacteria and viruses, focusing on the: (i) interactions between ionic liquids and key cell components including cell membranes, cell wall, proteins and nucleic acids; (ii) oxidative stress and metabolic dysregulation induced by ionic liquids; (iii) interactions of ionic liquids with bacterial biofilms; (iv) potential drawbacks related to the use of ionic liquids as antimicrobials.



**Contents**

1. Introduction . . . 1
2. Interactions between ionic liquids and free-living bacterial cells . . . 2
 2.1. Ionic liquids and the cell's surface, membranes and walls interactions . . . 2
 2.2. Ionic liquids and protein interactions . . . 4
 2.3. Intracellular damages and stress induced by ionic liquids . . . 5
 2.4. Influence on DNA interaction . . . 6
3. Interactions between ionic liquids and bacterial colonies . . . 7
 3.1. Ils antibiofilm activity . . . 7
 3.2. Ils and the cell matrix interactions . . . 8
4. Ionic liquids and viruses . . . 9
5. Conclusions and prospects . . . 10
 Declaration of Competing Interest . . . 10
 Acknowledgements . . . 10
 References . . . 10

## 1. Introduction

In February 2017, the World Health Organization (WHO) for the first time published a list of antibiotic-resistant "priority pathogens" that pose the greatest threat for human kind [1], including a growing number of problematic pathogens not vulnerable to antibiotics [2]. Thus, the WHO divided pathogens into three categories of: (i) critical, (ii) high and (iii) medium priority. It is known that bacteria can be intrinsically resistant to antibiotics, as well as possess molecular mechanisms to protect itself by e.g. mutations in chromosomal genes, horizontal gene transfer that includes transposons, integrons and plasmids or by highly effective efflux pump systems [3,4]. Furthermore, genes responsible for antibiotic resistance were encoded by microorganisms even before

* Corresponding author.
*E-mail address:* tomasz.clapa@up.poznan.pl (T. Cłapa).

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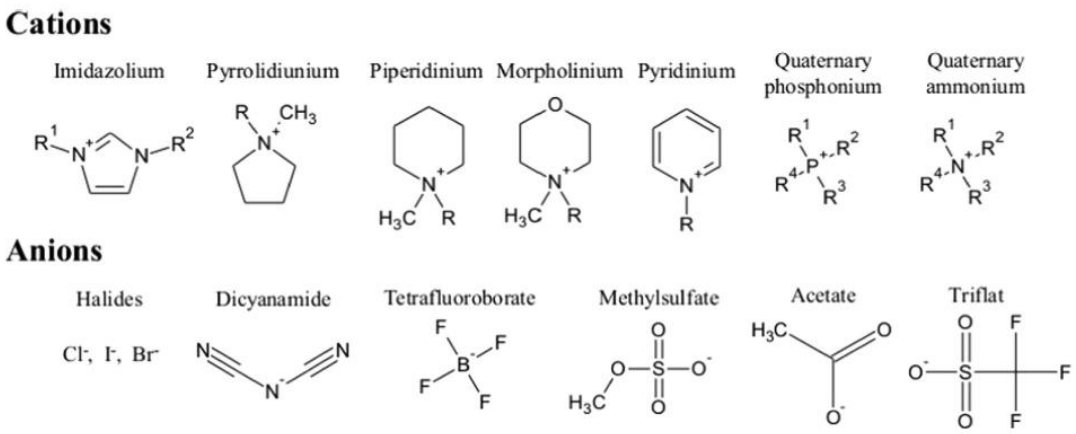


**Fig. 1.** Structures of typical cations and anions that commonly used in ionic liquids.

humankind appeared on the Earth and evolved during millennia. Thus it is not a surprise that those genes abundantly exist in natural environments, which are perceived as their reservoir [5,6]. Since antibiotic resistance is an old and natural phenomenon it is challenging to produce new and highly efficient antibiotics that will work effectively [7]. Thus, novel chemical compounds should be synthesized as new antimicrobial agents and tested for their antibacterial properties [8].

Ionic liquids (ILs) are relatively new and highly tunable chemical compounds with interesting properties, making them a promising 'tool' in the fight with pathogenic bacteria [9]. ILs can be characterized as salts with low melting temperature ($T_m$ < 100 °C), high thermal stability and negligible vapor pressure. Typical ionic liquids consist of an organic cation and an inorganic or organic anion. Because of the tunable nature of those compounds, it is possible to design up to $10^{18}$ unique ILs, each with different chemical, physical and biological properties, e.g. hydrophobicity or polarity, that can be modulated by varying IL architecture and ion composition [10–13]. Most of the publications that describe ILs and their biological interactions include imidazolium, pyridinium, piperidinium, quaternary phosphonium, pyrrolidinium, quinolinium, morpholinium and quaternary ammonium cations [14,15] (see Fig. 1).

IL cations are paired with anions ranging from simple inorganic halides, through more complex like triflate or dicyanamide anions [16], to very complex anionic compounds including for example antibiotics [17], herbicides [18] or active pharmaceutical ingredients [19]. Moreover, to introduce specific biological activity, IL cations can be functionalized with derivatives of e.g. natural compounds or commonly used drugs [20,21]. So far, several structure–activity-relationships (SARs) for IL toxicity on different living organisms have been reported [22]. The proposed mechanisms of toxicity effects are correlated with: (i) number of carbons in alkyl chain of cations; (ii) degree of cation side-chain functionalization; (iii) anion and cation nature; (iv) interactions between them [23–25]. While the reported toxicity of ILs reduced their perception as a "green" chemical class, the effects can have beneficial applications, especially as new antimicrobial agents.

Ionic liquids are promising chemical compounds that possess new properties, thus their interactions with living organisms, especially with bacteria, are very intriguing and suggest that ILs could be perceive as new antimicrobial agents. This Review discusses multiple interactions between various ionic liquids and bacteria (mostly pathogens), showing cell changes and cell response at the molecular level as well as whole-cell organism.

## 2. Interactions between ionic liquids and free-living bacterial cells

Bacteria behave differently as free-living organisms (planktonic cells) and aggregates or biofilms [26]. Thus, chemical compounds will act differently on those types of life organization structures. Because of highest cell surface accessibility in planktonic cells, comparing to other structures, the influence of ILs will be more direct.

It has been reported that the length of ionic liquid's alkyl chain is correlated with its toxicity; the longer side chain, the more toxic ILs get and the stronger their antimicrobial activity [27]. In general, ionic liquids containing 10 to 14 carbon atoms in the side-chain show the highest antibacterial activity and for such ILs their anions, most of the time, do not influence toxicity but can modulate it in some cases [15,28–30]. Interestingly, alkyl chains that consist of 14 carbon atoms seem to be the upper limit in terms of toxicity and for ILs that possess C > 14-alkyl chain length, toxicity is not increasing any more. This phenomenon is connected with high lipophilic properties exhibited by longer alkyl chains and is known as the "cut-off" effect [28,31]. Trying to explain this effect, it is necessary to consider (i) rate of the solubility of ionic liquids; (ii) steric effects, that are caused by the long alkyl chain, (iii) ILs aggregation and (iv) tested microorganisms, because of the differences in cell morphology [32].

### *2.1. Ionic liquids and the cell's surface, membranes and walls interactions*

Many ionic liquids have a similar structure to cationic-surfactants or pesticides. Therefore, the mode of action of ILs is thought to be by sorption on and disruption of negatively charged cell membranes, also leading to cytoplasmic leakage [8,33–36], or an increased membrane permeability of ILs leading to disruption of intracellular processes and proteins [37]. This effect is connected to the presence of alkyl or allyl chains that can interact with lipid bilayer and contributes to the increased hydrophobicity of ILs [38]. Molecular dynamics simulations (MDs) showed that ILs with longer side chains can incorporate into membrane through passive diffusion mechanism [21,39]. The absorbed IL cations are distributed mainly in the outer leaflet of the bilayer in a way that the more hydrophobic side chain of cation interacts directly with lipid chains of the membrane, while the more hydrophilic head group is located closer to the membrane surface level [40,41]. As a result, longer alkyl chains are correlated with stronger perme-

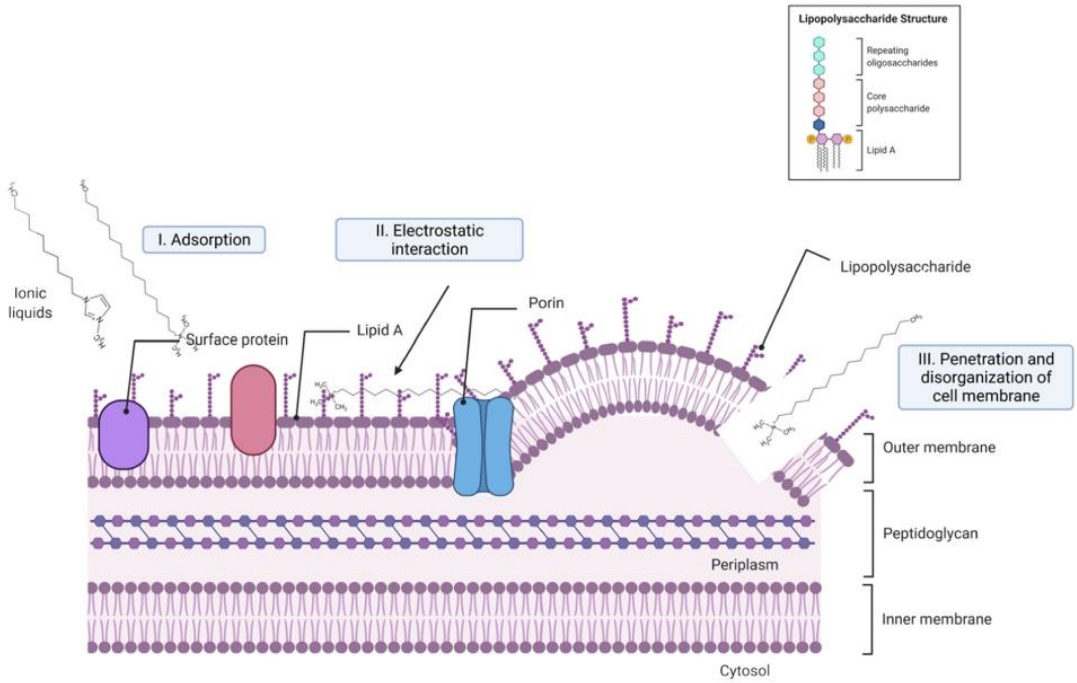


**Fig. 2.** Scheme of Gram-negative cell wall with membrane treated with ionic liquids.

ation of the phospholipid bilayer and an induction of this process [40]. In case of compounds with two long alkyl chains, IL cations was found to fuse with membrane, disrupt it or pass through it without damage, depending on the length of their chains (C15, C11 and C7 respectively) [42]. Moreover, ionic liquids are not only able to penetrate cell membrane but also disrupt proteins anchored there, leading to cell death [23,43].

There is abundant evidence that Gram-negative bacteria are less susceptible to the action of ionic liquids than Gram-positive species [21,22,44–46]. This difference is mainly attributed to the presence of outer membrane and lipopolysaccharide (LPS) layer in G- microorganisms [8]. Due to its hydrophilic nature, LPS can prevent large, hydrophobic compounds from passing through the membrane [8], see Fig. 2.

The importance of LPS in resistance against ionic liquids was demonstrated by Gundolf et al. (2018) and Kowalczyk et al. (2018). In those studies, *E. coli* strains with varied LPS structure were challenged by set of ionic liquids. As a result, it was found that wild type strains with fully developed LPS structure (with lipid A, inner core, outer core and O-antigen present) were the most resistant, while strains lacking one or more LPS components were more susceptible to ILs. In the latter study, ILs toxicity measured towards R4 strain with LPS modifications was comparable to the values obtained for Gram-positive *Bacillus cereus*; hence, presence of lipopolysaccharide layer contributes to the observed differences between G + and G- species in terms of their resistance against ionic liquids [47,48].

Furthermore, studies conducted on model lipid bilayers showed that ionic liquids are able to alter a number of physicochemical properties of membranes. ILs interaction with membranes can lead to the reduction in the thickness of lipid bilayers, with a concurrent increase of their electron density [49]. This effect is enhanced for model membranes with higher negative charge, which more accurately that simulate bacterial membranes [50]. It was demonstrated that ILs can increase phospholipid bilayers fluidity [18,51]. Such effect is due to the decrease of thermotropic gel-to-fluid phase transition temperature of the membrane and was accompanied with an increase of membrane's curvature [51]. Regarding membrane phases, Benedetto et.al (2014) showed that model bilayers in the fluid phase are able to incorporate more molecules of ionic liquid than membranes in the gel phase, and the absorption efficiency increases with temperature [52]. Moreover, the presence of IL cations in the bilayer structure can lead to a substantial decrease of membranes bending modulus and this effect is found to be stronger in higher temperatures; thus, highlighting the role of ionic liquids as membrane stiffeners [53]. A decrease in membrane elasticity and penetration resistance after exposure to ionic liquids has been reported for model membranes as well. These changes were correlated with the increased cell migration observed for eukaryotic cells, and were more pronounced for ILs with longer tails [54]. Membrane elasticity is a property that plays important role in microorganisms e.g. modulation of elasticity can affect bacteria cell division process [55]. Due to that, modification of this property is recognized as the mechanism of action of some antimicrobials [56]. Thus, the ability of ILs to alter those properties reveal another mechanism by which ILs can manifest their antimicrobial activity.

Besides, IL treatment may lead to a change of the membranes electrokinetic potential (zeta-potential; $\zeta$ potential) by absorbing with a thin layer on the outer membrane [23]. In consequence, the negative charge of bacterial bilayer can be weakened or even reversed to positive values [57]. Interestingly, reversion of the surface charge was observed only for ILs with cations exceeding 12 carbon atoms in the alkyl side chain [57–59]. That shows, once again, the relevance of alkyl chain length for ILs biological activity. Moreover, it was demonstrated that extraneous charge introduced by incorporation of cations into the bilayer can hinder activity of ion channels due to the action of repulsive forces [60]. Ionic liquids

can also affect hydrophobicity of bacterial surface, however not in a straightforward manner. Studies performed by Rita Pereira et al. (2021) showed that treatment with choline-based ILs can lead both to the decrease and increase of surface hydrophobicity of *Pseudomonas fluorescens* and *Bacillus cereus* respectively. Since *B. cereus* is Gram-positive and *P. fluorescens* is Gram-negative bacteria, varied response to ILs action observed for the cell surface hydrophobicity might be possibly connected with the structural differences between G + and G- microorganisms [61].

To prevent membrane breaching, bacteria can undergo changes in phospholipid bilayer composition [62]. Therefore, some effects observed in cells after ILs treatment are a result of bacterial stress response mechanisms. Gram-negative bacteria are known to counteract the increase of membrane fluidity, caused by stress factors, by increasing the share of trans fatty acids in their membranes [63]. Such increase of the unsaturated fatty acid fraction, with a concurrent decrease of saturated fatty acid presence, was noted for *Escherichia coli* after treatment with long chained quaternary alkylamonium ILs [59]. Similar increase of trans phospholipids share in bilayer was marked for ILs treated *Pseudomonas putida* [39]. In case of *Enterobacter lignolyticus*, 1-ethyl-3-methylimidazolium chloride [$C_2$mim]Cl was found to induce increment of cyclopropane fatty acids and to up-regulate genes involved in its synthesis [64]. Rearrangements of bacterial membranes observed in response to ILs do not limit itself only to the change of fatty acid composition, but also involve shift in membrane proteins. A study on *E. lignolyticus* showed a substantial down-regulation of genes encoding porins and siderophore transporters, paired with up-regulation of peptide, amino-acid and sugar transporters and some multi-drug efflux pumps after incubation with ionic liquids [64]. Decrease of the porin level lead to reduction of membrane permeability and efflux pumps can actively remove toxic substances from cell interior [62]. Thus, observed changes in the bacterial transcriptome is a part of protective mechanism against ILs action. In fact, the efflux pump from a major facilitator superfamily (MFS) encoded by *eilA* was found to be induced by [$C_2$mim]Cl and was recognized to very efficiently export [$C_2$mim]$^+$ cations outside the cell [65]. Similar results were also found for the G + pathogen *Listeria monocytogenes*, where QacH, a small multidrug resistance protein family (SMR) transporter, was shown to significantly increase the tolerance of bacterial cells against classic QAC based biocides as well as ILs with long alkyl side-chains [66]. Also efflux pumps from Resistance Nodulation-Division family (RND) have been identified as a resistance factor against ionic liquids in *E. coli* and *Salmonella enterica*. However, phosphonium- and imidazolium-based ILs with multiple side chains were not affected by the efflux pumps for *E. coli* and both species tested, respectively [67].

Finally, it has been proven that ionic liquids can target not only the bacterial cell membrane but the cell wall as well. Treatment with ILs composed of 1-vinyl-3-dodecyl imidazolium cation, that was characterized by high affinity towards crucial components of peptidoglycan, led to the loss of mechanical properties of the cell wall, and to its deformation or complete decomposition at higher concentrations [58]. Also, significant changes in cell morphology were observed for bacteria exposed to 1-ethylpiperazinium tetrafluoroborate, accompanied with cell wall and membrane destruction [68]. Interestingly, those changes were more pronounced in *E. coli* than in *S. aureus,* possibly indicating another difference between G- and G + species in terms of ILs mode of action.

### 2.2. Ionic liquids and protein interactions

Thanks to the joint effort of researchers from around the globe, with major contribution of Profesor Venkatetsu's group, knowledge of protein-ILs interactions has grown rapidly over the past two decades. Available data on the interaction of ionic liquids with proteins prove that ILs are a multipurpose agent for many applications. As is well known, ionic liquids have attracted a lot of attention due to their solubilization properties, being recognized as a group of very versatile solvents. Indeed, ionic liquids have been proven to efficiently dissolve a number of proteins, not only globular but also fibrous like keratin, which are not soluble in water and are generally considered difficult to dissolve in other organic solvents [69–72]. Aside from solvation ionic liquids can also stabilize solubilized proteins, helping them retain enzymatic activity and protecting them from thermal unfolding even at extreme temperatures [70,73]. That said, as shown by Jha and Venkatesu (2015), the same ionic liquid can can have a stabilizing or destabilizing effect on a given protein, depending on the concentration used [71]. Due to stabilizing effect of some ionic liquids, described compounds are hoped to find application in protein packaging for long-term storage [74–76]. Choline-based ionic liquids are particularly suitable for this purpose, where they have been found not only to protect proteins from stress factors and to preserve their enzymatic activity, but also to greatly enhance it [74,76]. Due to this property, ionic liquids can be also employed as buffers in enzymatic reactions, providing an increase in enzyme activity, but also counteracting the action of enzyme inhibitors like in case of EDTA-based chelating ionic liquids [77,78]. The beneficial activity of ionic liquids is not limited only to stabilization of native structure of proteins under normal condition. ILs can protect proteins from denaturation caused by various factors including high and low temperature, pH, urea, guanidinium chloride, antioxidants and hinder proteolytic activity of proteases [74,79–81]. Ionic liquids are also known for their ability to inhibit amyloid formation and influence amyloid properties such as size or electric potential [82–85]. Moreover, ILs are not only protective agent against denaturation, but they are also able to induce renaturation process, refolding denaturated proteins back to their native state [77,86,87]. Another interesting property of ionic liquids is their ability to promote crystallization of proteins. This ability can be found useful for X-ray crystallography especially in case of proteins that are difficult to crystallize [88,89]. Finally, interactions between ionic liquids and proteins can be exploited to design new kinds of protein-based biomaterials. Due to the increased protein stability and activity observed in IL environment, such materials could find application in biosensors design or be used for more efficient drug delivery [90–92].

The ability of ionic liquids to interact with proteins also involves negative interactions. Ionic liquids can act disruptively on proteins, leading to their denaturation and loss of enzymatic activity. As already mentioned, such behavior can be concentration dependant. For example, 1–allyl-3-methylimidazolium chloride [Amim][Cl] was found to stabilize hemoglobin at low concentration range, however further increase of IL concentration led to destabilization and aggregation of proteins [71]. A similar observation was made for propylammonium formate which denaturated lysozyme at high concentration although having stabilizing effect at low concentrations [77]. The destabilizing effect of ionic liquid also depends on the type of anion. For example, studies on collagen, α-chymotrypsin and casein showed that a set of 1-butyl-3-methylimidazolium ($C_4$mim) ionic liquids can be sorted by anion in a manner similar to Hofmeister series, according to their protein destabilizing properties [93–95]. That said, anions of ionic liquids do not always follow the order of Hofmeister series, indicating that more complicated interactions have to be considered to explain observed destabilizing effect of ILs [96,97]. Due to that, simulation techniques such as molecular docking and molecular dynamics were employed to give more insight into ILs-protein interactions. As a result, it has been shown that anions can destabilize proteins by binding with amino acids located in close neighborhood of

enzyme active site [96]. Additionally, weakly hydrated anions were found to denaturate proteins by causing dehydration of protein backbone after binding to polar and positively charged residues [98]. Also, thorough studies on a model protein GFP indicated crucial function of the triflate anion in inducing changes in GFP tertiary structure and in causing protein size contraction [99]. At the same time, IL cations can also affect protein structure and activity. As reported by O. Singh et al. (2020), while $[EtSO_4]^-$ and $[Et_2PO_4]^-$ anions were found to interact with lysozyme in a global manner, the imidazolium cation $[C_2mim]^+$ acted locally by binding with tryptophan Trp62; a crucial residue for lysozyme proper folding [100,101]. Additionally, length of the cation's side chain has influence on IL-protein interaction as well. Imidazolium-based ionic liquids with longer side chain were found to stronger inhibit activity of laccase and had greater impact on bovine serum albumin secondary structure [102,103]. On the contrary, other experiments indicated that, the shorter alkyl chain of alkylammonium cation, the more pronounced a destabilizing effect of ILs on hemoglobin and myoglobin is [104]. Thus, the effects of an ionic liquid on a given protein is a result of protein sequence, cation and anion structure, and interactions between them, combined altogether.

Focusing on microorganisms, it has been proven, that ionic liquids can destabilize bacterial proteins involved in defense against oxidative stress and responsible for immunity against bacteriocins [98,105,106]. On cellular level, increasing toxicity of choline-based ILs towards *Escherichia coli* was correlated with their increasing ability to destabilize protein structure [107] and for imidazolium ILs with short alkyl side chains toxicity followed the Hofmeister series [108]. In another study, Fourier transform infrared (FTIR) spectroscopy highlighted significant changes in protein spectral region in *E. coli* and *Staphylococcus aureus* treated with long-chained imidazolium ILs of high biocidal activity [109]. Since such changes have not been noted in lipid region (corresponding to bacterial membranes), those results suggest that the observed antimicrobial effect was primarily due to ILs-protein interaction [109]. Also, low tolerance of extreme halophilic archaea species towards $[C_2mim][Cl]$ and $[C_4mim][Cl]$ was assigned to destabilization of archeal acidic proteome induced by ionic liquid cations [110]. Additionally, as shown by Borkowski et al. (2018a), increasing concentration of quaternary ammonium ionic liquids can trigger changes in *E.coli* proteomic pattern [59]. Thus, bacterial response to ILs action can be observed at the whole proteome level as well. Furthermore, studies using model cell membranes revealed that long chained imidazolium ionic liquids can decrease the flow rate of ions through gramicidin A ion channel [60]. Since gramicidin A is an antibiotic causing membrane permeation [111,112], the described effect can be considered as beneficial to the bacteria; however, on the other hand, those results demonstrate that ionic liquids have potential to hinder activity of bacterial ion channels and possibly other membrane proteins, that are crucial for growth and survival of microorganisms. In view of the evidence above, it is clear that protein disrupting properties of ionic liquids can contribute to their overall toxicity towards bacteria (Fig. 3).

### 2.3. Intracellular damages and stress induced by ionic liquids

Another cytotoxic mechanism exhibited by ILs is induction of oxidative stress and generation of reactive oxygen species (ROS), that can directly damage DNA, proteins and the bacterial lipid membrane [113]. As it is known, cells can adapt to or even fight ROS by antioxidant defense enzymes, e.g., superoxide dismutase (SOD) and catalase (CAT). Both, SOD and CAT are known to detoxify $O_2^-$ and reduce $H_2O_2$, respectively [114]. However, during cell exposure to highly toxic ILs, antioxidant enzymes are not able to fully

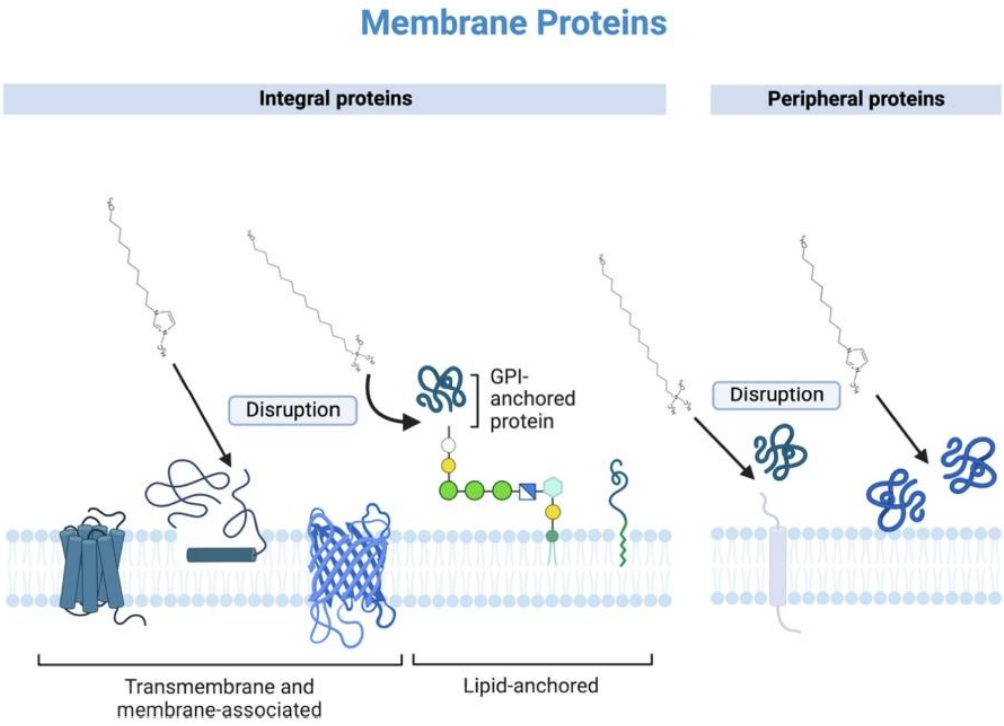


**Fig. 3.** Interactions of ionic liquids with proteins.

remove the reactive oxygen species. Thus, ROS can accumulate, causing more cell damages [115]. Furthermore, ROS generation can induce lipid peroxidation, by enhancing the oxidation of polyunsaturated fatty acids, and cause oxidative DNA damage [114,116,117]. Hydroxyl radical generated closely to DNA can attack DNA backbones and bases, leading to ss-DNA (single-strand DNA) production or nucleobases damage [118]. It has been reported for eukaryotic organisms that antioxidant response is a direct effect of time, exposure and concentration of ILs and also can change over time [119].

Induction of oxidative stress by ionic liquids was also observed for bacteria. J. Zhang et al. (2013) showed that short chained alkylimidazolium bromides can trigger changes in balance of redox reactants and antioxidases in a time- and dose-dependent manner. In that study, increased presence of SOD and CAT proteins alongside with NADH and FMN reactants was marked for *Vibrio qinghaiensis* after 20 h incubation with $[C_2mim][Br]$ and $[C_4mim][Br]$ ILs. At the same time, a decreased presence of tested compounds was observed for cultures incubated with ILs for only 2 h. No such changes were observed for ionic liquids with longer side chains, indicating the importance of alkyl chain length for the observed effect [120]. In another study on *Vibrio fisheri* by Z. Y. Yu et al. (2016), the role of anions in inducing oxidative stress response was highlighted. Exposure to $[C_2mim][BF_4]$ ionic liquid led to the increase of ROS and antioxidants like CAT, SOD, LPO (lipid peroxidases) and GSH (reduced glutathione) after 18 and 24 h at all concentration tested. For $[C_2mim][Cl]$ such changes were observed only at the two highest concentrations after 24 h, and at the second highest concentration after 18-hour incubation. At the same time, the highest concentration of $[C_2mim][Br]$ triggered decrease of ROS and antioxidants after the treatment. Thus, ILs potential to induce oxidative stress and bacterial response to it, is depending on the type of anion [121]. Additionally, some ionic liquids are designed particularly for triggering oxidative stress in bacteria, like in case of ILs based on chlorin e6 (Ce6) photosensitizer [58]. Photosensitizers are compounds that produce reactive oxygen species under light excitation [122]. Due to the enhanced penetration of chlorine e6 anions into cells within ionic liquid formulation, Ce6-IL were found more efficient against bacteria than Ce6 alone [58].

Ionic liquids do not necessarily have to lead to bacterial cell death. Some ILs are able to change or dysregulate cell metabolism by slowing it down, thus increasing bacterial generation time [123]. In this manner, ILs can affect ATP level in cells [121], hinder fermentation processes and inhibit production of e.g. gases [124] or secondary metabolites such as pyocyanin [125]. Also, transcriptome analysis shows varied effect of ILs on cell metabolism. In case of *Enterobacter lignolyticus* up-regulation of genes involved with carbohydrate and amino acid metabolism with a concurrent down-regulation of translation, nucleotide synthesis and biogenesis genes were observed. Also, a decrease in gene expression of cell motility and enterobactin biosynthesis genes was marked [64]. Thus, it may suggest that in face of ionic liquid stress, bacteria can stop replication process and suppress metabolism of non-essential pathways, redirecting cellular energy to the pathways connected with cell survival.

### 2.4. Influence on DNA interaction

When discussing ionic liquids effect on cells, the interaction with DNA has to be considered. Because of their ionic nature, ILs can evidently interact with negatively charged DNA, thus they can be helpful in nucleic acid-based studies. Ionic liquids have been proven to be a useful tool for efficient extraction of DNA from diverse biological material [126,127] and may even be used for extracting specific DNA sequences [128]. ILs can also be used to store nucleic acids and as a protective agent against DNA and RNA degradation [129,130]. Additionally, ionic liquids have been demonstrated to be a promising agent for gene delivery, used either alone or combined with other well established techniques [131].

It has been proven that ILs can affect DNA mechanical properties. For example, choline- and imidazolium- based ionic liquids led to the increase of DNA persistence length and stretch modulus, making it less likely to bend [132]. In consequence, higher rigidity caused DNA molecules to be less exposed to degrading action of DNase I, that was also observed for poly[3-butyl-1-vinylimidazolium L-proline salt] tested as a gene delivery vector [133]. Depending on their structure, ionic liquids can bind with DNA in multiple manners but for the most part, cation binding with DNA minor groove has been observed [134,135]. Binding occurs mainly through action of electrostatic forces between the cationic head group and DNA phosphate groups, and can be supported by hydrophobic interactions between cation's side chain and non-polar DNA bases [136,137] (Fig. 4).

As expected, strength of hydrophobic interaction with DNA increases with alkyl side chain length [138]. In case of alkylimidazolium bromides, an increase in intercalative binding with DNA was observed with increasing side chain length [139]. Additionally, Sunipa Sarkar & Chandra Singh (2020) presented a combined mode of binding exhibited by tetramethylguanidinium-based (TMG) ILs, involving both intercalation and interaction with DNA minor groove. In general, the role of anions in IL-DNA interaction is reported to be negligible; however, in the same study authors reported that highly hydrophobic anions can indirectly facilitate binding of cation to DNA [140]. By interacting with DNA, ionic liquids can influence its structure. For example, imidazolium- based ILs were found to preserve predominant B-form of double helix, preventing it from transition into A-form [138]. On the other hand, choline dihydrogen phosphate affected more exotic tertiary structures of DNA, leading to stabilization of DNA triplexes and i-motifs, and destabilizing the G-quadruplex form [141,142]. Moreover, changes in quaternary structure induced by ILs could be also observed with use of microscopy techniques [136,137]. Finally, some ILs are able to bind not only to dsDNA, but to free nucleobases as well [135].

DNA damage has been reported for cells challenged with ionic liquids; however, most reports on this subject focused on eukaryotes. For example, DNA fragmentation was marked for various type of human cell lines treated with different imidazolium ILs [143–145] and similar results were obtained for plant and insect cells [146,147]. Currently available data on the induction of DNA damage by ILs is quite limited for prokaryotes; nevertheless, Kowalczyk et al. (2018) has reported plasmid DNA fragmentation for *E. coli* bacteria challenged with theophylline-based quaternary alkylammonium ionic liquids (TILs). In this study, fragmentation of plasmid DNA was evident for strains treated with ILs having at least 12 carbon atoms in cation's side chain. At the same time, no significant changes were observed for short chained compounds. Interestingly, also no DNA damage was reported in *in vitro* tests, involving incubation of extracted DNA with TILs. Thus, damage of bacterial DNA was not the result of direct IL-DNA interaction, but most probably a consequence of oxidative stress induced by ionic liquids (see paragraph "*Intracellular damages and stress induced by ionic liquids*") [48].

Referring to the effects of ionic liquids on DNA, it is necessary to mention a massive potential drawback of using ILs as antimicrobials. It is known that bacteria can acquire resistance to antibacterial agents, like antibiotics, either by mutations or via horizontal gene transfer (HGT) [148]. HGT is a process of genetic information transfer between cells, mediated by mobile genetic elements (MGEs) such as plasmids, transposons, integrons or insertion sequences [149]. HGT can be promoted by antibiotics, disinfec-

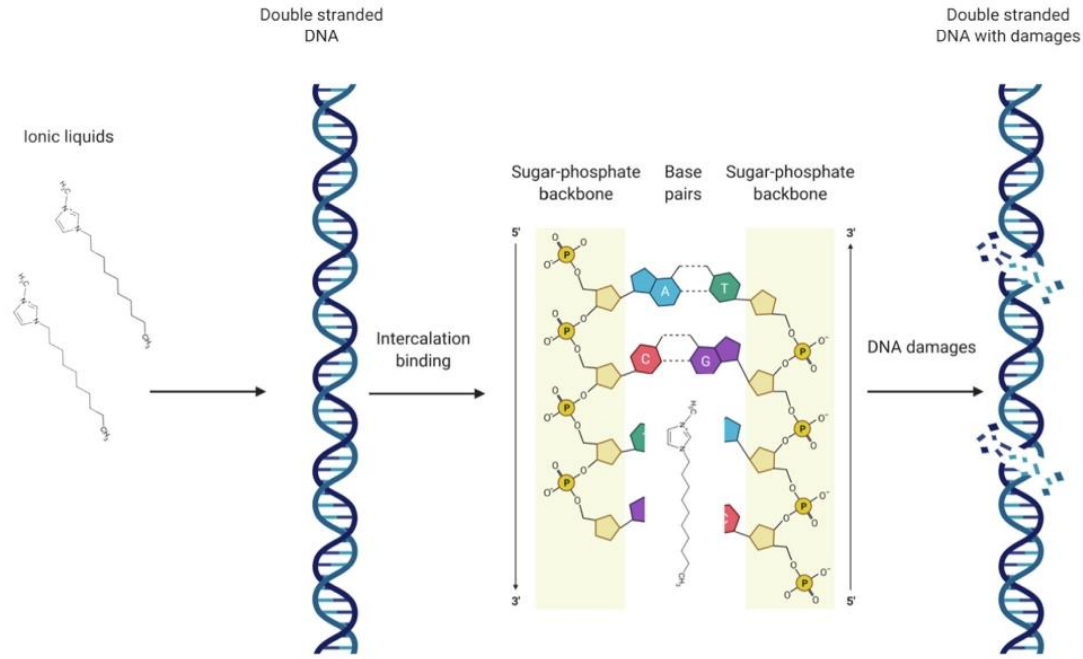


**Fig. 4.** Interactions of ionic liquids with DNA structure.

tants, metal ions, nanoparticles or preservatives, and lead to the spread of antibiotic resistance [150]. Luo et al. (2014) for the first time reported that increased dissemination of antibiotic resistance genes (ARGs) can be induced by ionic liquids. The authors observed augmented abundance of *sulI*, *sulII* and *intI* genes in genetic pool of freshwater microcosm, after addition of $[C_4mim][PF_6]$ ionic liquid. *SulI* and *sulII* genes encodes resistance to sulfonamides and *intI* gene is a part of the class I integrons. Higher copy numbers of tested genes was observed with increasing IL concentration (until exceeding toxic values). Additionally, DNA sequencing confirmed that horizontal transfer of *sulI* gene was mediated by class I integrons and frequent occurrence of integrons transfer was also proved in donor-recipient experiment. At the same time, increased cell membrane permeation, facilitating HGT, was noted. Thus, the observed effect of resistance genes propagation was primarily due to the action of ionic liquids [151]. Similarly, higher frequency of HGT was marked for plasmids. Interestingly, when bacteria were treated with imidazolium-based ILs of varying side chain length, plasmid transfer efficiency was lower for ILs with longer alkyl chain [152]. It was also found that Gram-negative bacteria were more likely to acquire transferred plasmids after IL treatment, compared to Gram-positive microorganisms [153]. This topic was further explored by Xiaolong Wang et al. (2020) who studied changes in the resistome of freshwater and sediment microcosm after exposure to ionic liquids. The authors reported a significant enrichment of ARGs already after 16 h of IL treatment and those changes were maintained up to 28 days. The increased abundance was marked for genes related to tetracycline, streptomycin, erythromycin and ampicillin resistance, with the greatest shift marked for the latest group. Additionally, the most considerable increase was observed for genes encoding efflux pumps, with a significant change noted for genes involved in drug inactivation and target modification as well. Dissemination of MGEs was also marked, including plasmids and integrons carrying resistance genes. At the same time, no immediate shift in bacterial populations was noted; therefore, observed resistome enrichment can be attributed to occurrence of HGT induced by ionic liquid [154]. In all described studies ARGs proliferation was induced by ILs at sub-inhibitory concentrations and, alarmingly, also pathogenic strains were found among enriched bacterial species [151–154]. Thus, ionic liquids should not be view as a "wonder drug", since as presented above they are not free of some disadvantages inherent in commonly used antiseptics.

## 3. Interactions between ionic liquids and bacterial colonies

A bacterial biofilm is a three-dimensional structure created by individual cells that stick to each other and to surface, forming a compact organization [155]. Due to the occurrence of intercellular interactions between cells, behaviors of bacteria observed within a biofilm are distinctly different from those in a free living state [156]. Consequently, biofilms find application in many areas like bioremediation, wastewater treatment, biofuels production or plant protection [157]. However, alongside with the benefits, biofilms are also connected to many problems in food industry, water distribution systems and healthcare, posing a threat to humans and other living organisms [157,158]. Due to the limited availability of antimicrobial solutions to all cells, microorganisms within biofilm show up to 1000 times increased resistance to antibiotics [159]. That makes infections caused by cell clusters particularly difficult to treat. Thus, during efficacy assessment of any novel biocide against microorganisms, it is necessary to investigate its antibiofilm activity.

### 3.1. Ils antibiofilm activity

Antibiofilm activity of ionic liquids is well described in scientific literature. Similar to cytotoxic effects, the difference between Gram-positive and Gram-negative bacteria can be observed. As a general trend, biofilms formed by G + bacteria seem to be more vulnerable to the action of ILs and are characterized by lower minimal biofilm eradication concentration (MBEC) values than that formed by G- species [21,44]. Furthermore, increase of ILs activity

with increasing cation side chain length and the aforementioned "cut-off" effect have been observed for antibiofilm properties as well [22,45]. Depending on the biofilm stage, antibiofilm compounds can show varying efficiency. Ionic liquids have already proven to be a potent biofilm formation inhibitor. For example long chained imidazolium-, morpholinium- and pyrrolidinium-based ILs showed over 90 % efficiency in preventing biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* species [34,160]. Also anthracene-based ILs effectively inhibited *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* biofilms at the earliest stages of its formation [161]. The capability of ionic liquids to inhibit biofilm formation depends mostly on their potential to impair bacterial adherence to the surface. This anti-adherence activity of ILs is mainly conditioned by the cation's ability to bind to the negatively charged bacterial membrane [34,162]. Due to their effective inhibition of biofilm formation, ionic liquids have already been used for synthesis of antifouling materials. In this manner, coating with imidazolium- and piperdinium-based ILs greatly improved antimicrobial properties of titanium and carbon steel, effectively hindering bacterial colonization [162]. Also, 1-ethylpyridinium docusate used as plasticizer enhanced PVC property to inhibit biofilm formation by *S. aureus* [163]. PVC infused with phosphonium docusate ionic liquids completely prevented adhesion of *P. aeruginosa* and *S. aureus* to the surface after 24 h incubation, while PVC coated with the most effective trioctyl(tetra decyl)phosphonium docusate maintained its antifouling activity after 7 days of incubation [164]. Alongside inhibiting biofilm formation, ionic liquids can act on mature biofilm as well. It has been shown that imidazolium-, phosphonium- or choline-based ILs lead to dispersion of well-established mature biofilms formed by clinically significant pathogens like *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* or *Enterococcus feacalis* [44,61,162,165].

### 3.2. Ils and the cell matrix interactions

Extracellular polymeric substances (EPS) are a key component of biofilms. It is produced by bacteria and consists of proteins, lipids, polysaccharides and free DNA. EPS builds and allows maintaining a stable biofilm structure [166], facilitates adhesion to the surface [159] and ensures increased resistance to the antibacterial agents [167]. Originally, it was considered that the intercellular matrix alone limits the diffusion of drugs into cells; however, it has been proven that its mere presence does not cause changes in drug distribution. Limited efficacy of antimicrobials towards biofilms is dictated by mechanism called diffusion–reaction inhibition which involves combination of processes like chelation, enzymatic degradation, precipitation etc. [156,168]. Another resistance factor of biofilms is the presence of cells in the viable-but-nonculturable state (VBNC state) [169]. VBNC bacteria are the most durable forms, characterized by low metabolic activity, unchanged cell wall structure, intact membranes and increased tolerance to stress conditions including antimicrobial agents [170]. Ionic liquids can be employed to overcome those biofilm related resistance mechanisms by being used as drug carriers or adjuvants. In a work presented by Takahashi et al. (2019) PLGA nanoparticles coated with chitosan, surfactant and 1 butyl 3 methylimidazolium hexafluorophosphate ionic liquid were proven efficient in removal of EPS matrix, also inducing formation of cracks at the boundary between cell wall and cytoplasmic membrane of bacterial cells [171]. In another study performed on a biofilm infected wound model, the IL choline geranate was shown to enhance biofilm penetration ability of the antibiotic ceftazidime. As a result, the use of a combination of choline geranate and ceftazidime led to > 98 % reduction of bacterial viability compared to > 95 % and 20 % viability reduction for treatment with respectively ionic liquid and ceftazidime alone [165], demonstrating the usefulness of ILs as drug delivery system in treatment of biofilm infections.

Ionic liquids can affect EPS structure directly, by disturbing the extracellular matrix [61]. The components that form EPS have the ability to create hydrogen bonds between themselves [172]. At the same time, hydrophobic ionic liquids can cause the EPS structure to relax, facilitating deep penetration of ILs into the aggregate (Fig. 5).

Depending on the bacterial strains, we can see the different zeta potential of the aggregate on the surface, but most often it is a negative value (-50 mV to −10 mV) [173]. Thus, ILs cations may destabilize the compact, communicable matrix structure by its positive

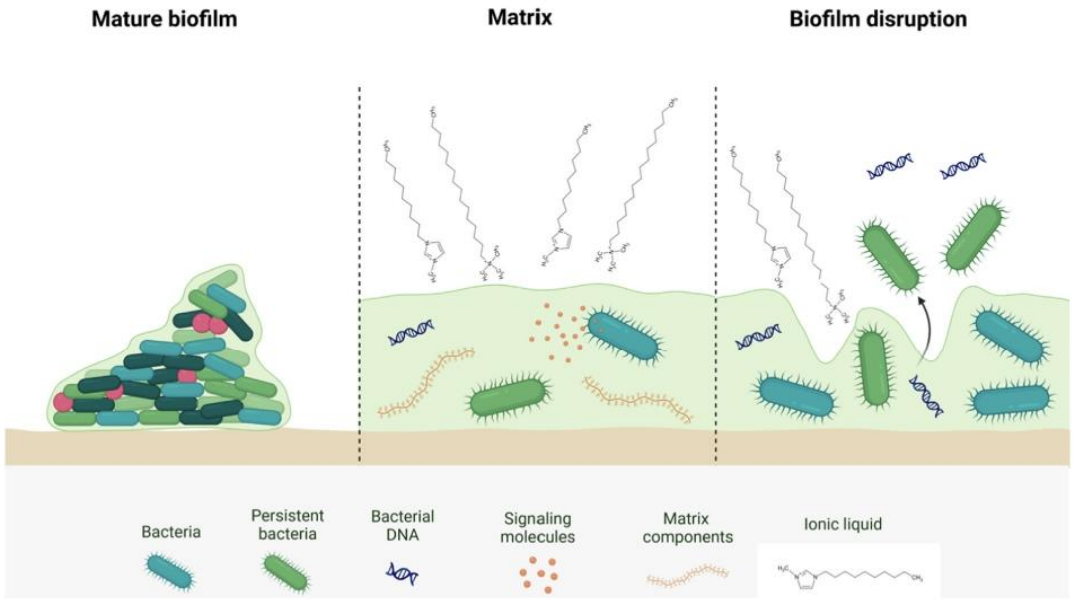


**Fig. 5.** Biofilm disruption after interaction with ionic liquids.

potential. Another site of action of ILs within the EPS matrix is extracellular DNA (eDNA). eDNA plays important role in stabilization of biofilm and is a part of gene-transfer mechanism [174]. Due to that, eDNA can be used as a target in biofilm removal. The first such approach was the use of DNase treatment [175], which is nowadays often co-administered with antibiotics [176]. DNase, despite the lack of lethal significance for the biofilm, allows antibiotics to penetrate deeply into the biofilm structure, and not to act only on the surface. However, in the case of eDNA-directed therapy, the age of the biofilm is crucial since older biofilm can maintain full functionality despite the use of combined therapy [177]. Ionic liquids through their positive charge are able to attract and destabilize the eDNA structure contained within the EPS matrix. As mentioned in the "*Influence on DNA interaction*" paragraph, choline-based ILs can destabilize G-quadruplex structures of DNA. Seviour et al. (2021) showed that G-quadruplexes can be found in biofilm formed by *Pseudomonas aeruginosa* and greatly contribute to the stability of eDNA network structure; therefore, ionic liquids have potential to control biofilm also by targeting G-quadruplex structures [178].

## 4. Ionic liquids and viruses

Despite the fact, that a large part of scientific literature on biological activity and applications of ionic liquids focuses on bacteria, there are a number of studies that concentrate solely on interactions between ILs and viruses. It has been proven before that ionic liquids can be successfully applied in virus research e.g. for extraction of virions from aqueous samples or for breaking down viral capsids prior nucleic acids extraction [126,179]. Additionally, some choline-based ionic liquids have been shown to stabilize inactivated foot-and-mouth disease virus particles (iFMDV), preventing them from dissociation [180]. Interestingly, this effect strongly depended on anion composition, since only ILs with $Cl^-$ and $SO_4^{2-}$ ions increased stability of viral particles, while ILs with $H_2PO_4^-$ anion showed opposite, destabilizing effect. Thus, ionic liquids can be used as protective agent in handling of virus particles.

Since low or limited efficacy of currently used disinfectants and antiseptics apply not only to resistant bacteria but to some viruses as well [181–183], studies focusing on virucidal potential of ILs are conducted. Up to date, there are few reports indicating virucidal properties of ionic liquids and much of currently available data focuses on the influence of ionic liquids on bacteriophages. Research conducted on P100, MS2 and Phi6 phages indicate that some of imidazole and alkylammonium ILs can reduce viral infectivity [184,185]. Interestingly, increase of ILs toxicity with an increase of side-chain number and length was noted also in case of bacteriophages. In other studies aforementioned "side-chain" and "cut-off" effects were marked for a set of pyrithione- and fatty acid-based ionic liquids as well [30,186]. That said, presence and intensity of described effects strongly depended on the tested phage species, being particularly manifested for enveloped Phi6 phage. This vulnerability of Phi6 phage may be explained by a presence of additional lipid bilayer surrounding its viral capsid, which is presumably the target of ionic liquids action [187].

Beside studies performed on phages, several research studies have been conducted on human and animal viruses. For example, it has been demonstrated that choline and geranate deep eutectic solvent (CAGE), which can be classified as an ionic liquid [188,189], leads to complete neutralization of human herpesviruses HSV-1 and HSV-2 at 1 % concentration [190]. Also, experiments performed on a set of quaternary ammonium salts (QASs) showed a similar or increased antiviral efficacy of some low-melting salts examined (with melting point below 100 °C) against varicella zoster virus (VZV), compared to the standard benzalkonium salts [191,192]. In another approach, antiviral properties of ILs were evaluated by viral enzyme inhibition assays. For

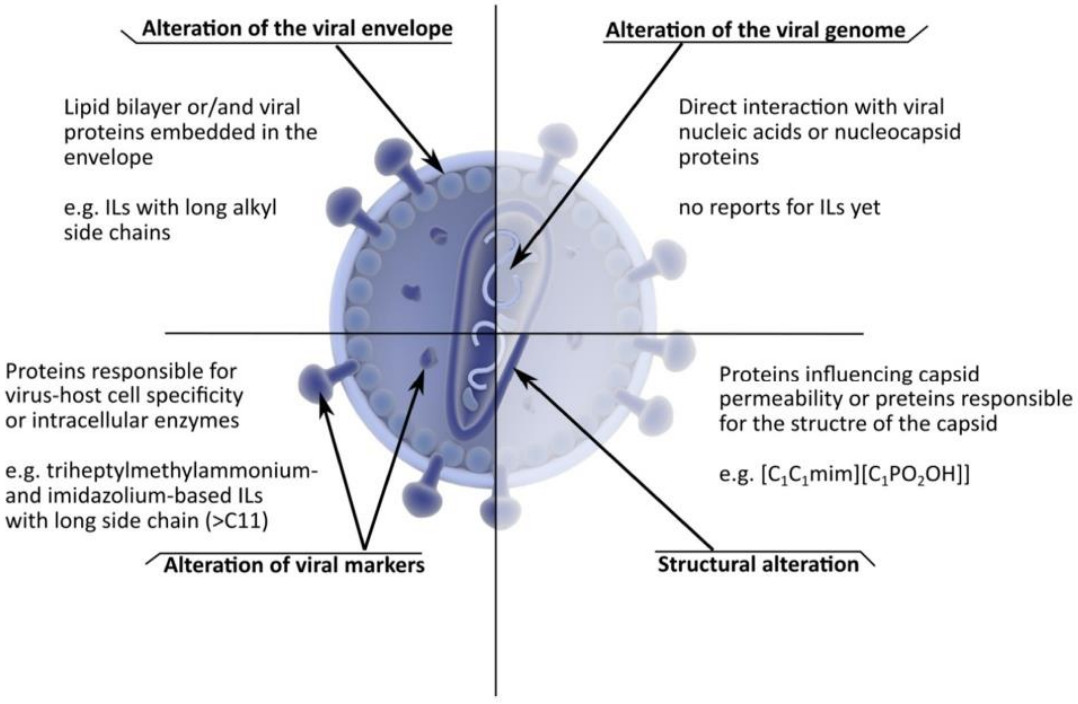


**Fig. 6.** Interaction of ionic liquids with virus particle and its nucleic acid.

example, triheptylmethylammonium- and imidazolium-based ionic liquids with long side chain (>$C_{11}$) were demonstrated to have an inhibitory effect on integrase enzyme of human immunodeficiency virus type 1 (HIV-1*a*) [193]. Surprisingly, also in the case of enzyme inhibition, the previously described "side chain" effect was observed.

Finally, it is worth to mention that ionic liquid technology can be applied to increase bioavailability of currently used antiviral drugs through alteration of their physicochemical properties like solubility. Such approach was proposed for favipiravir (FAV), a candidate drug for treating COVID-19 disease [194–196]. As a result, FAV ionic liquids were 78–125 times more soluble in water and were characterized by increased bioavailability, elimination half-life, peak blood concentration and absorption time compared to the original FAV formulation [197]. Moreover, also the pattern of drug distribution in treated mice differed between FAV and FAV ionic liquids. Thus, it shows that ionic liquids can be successfully used as antiviral agents (Fig. 6).

## 5. Conclusions and prospects

The antibacterial potential of ionic liquids has been repeatedly demonstrated in numerous scientific reports over past years, making ILs considered as a very promising antimicrobial agents. As discussed in This Review ionic liquids are able to influence microorganisms at multiple sites, that includes: interactions with bacterial membranes and walls, destabilisation of proteins and enzymes, dysregulation of cell metabolism, damaging DNA or disruption of higher organizational structures of bacteria like biofilms. Such diversity of modes of action can be further exploited in the development of novel IL based technologies used for fighting microorganisms and bacterial infections.

One of such applications assumes the use of ionic liquids as adjuvants in antibiotic therapies. Following this approach, it could be possible to increase the overall antimicrobial efficacy of antiseptic formulation by combining antibiotics and ILs that act on either the same or different target sites [198]. In another approach, the ease of designing new ionic liquids could be utilized for creating ILs based on active pharmaceutical ingredients (API-ILs), like antibiotics. While this approach has already been exercised in a number of ways by scientific community [199,200], due to the enormous number of possible combinations provided by IL technology, further development in this field is highly anticipated and encouraged.

Another prospective way to exploit ILs properties discussed in This Review is designing specialized antifouling materials that could found application in settings where biofilm is particularly undesirable factor [162–164]. Such solutions could be used for: finishing surfaces in hospitals and health-care facilities, creating less biofilm-prone installations dedicated to the food industry and water supply systems, or improving membrane filtration-based processes. The materials in question might even be used to produce medical devices that are especially susceptible to biofilm formation, like intubation tubes or joint prostheses. However, these latter examples would require a very careful approach to ensure safety of such technology for human health.

Also virucidal potential of ionic liquids has been addressed. In the face of the recent SARS–CoV-2 pandemic, the antiviral properties of ionic liquids should particularly draw the attention of scientists. Currently, the biggest challenge in this field is the lack of good IL toxicity structure activity relationships (SARs) suitable for viruses [184]. Although SARs developed for bacteria or eukaryotic cells work well for some types of viruses, most of viral groups do not fall into the same pattern. The development of one IL structure–activity model that would match all viruses or alternatively several SARs specific for different groups of viral particles would be a breakthrough in the research on ILs antiviral activity and should be given the highest priority.

That said the shortcomings of using ionic liquids as antimicrobials and some white spots in understanding of ILs-bacteria interactions cannot be ignored. As stressed in the text, ILs can induce the dissemination of antibiotic resistance genes [151–154]. This could be a massive drawback since such events increase the chance of spreading antibiotic resistance in the environment. At the same time, little is known about resistance mechanisms against ionic liquids present in bacteria, and thus whether exposure to ILs can facilitate the development and spread of such resistance [65–67]. Also, data on the effects of ILs on pathogen virulence is quite limited. New information obtained on that topic could either reveal another possible drawback of ionic liquids as antimicrobials or unlock their new applications. So far there are individual reports suggesting that ionic liquids can reduce bacterial virulence [125], making them a useful tool for future use as antivirulent agents applied alone or in combination with antibiotics; however, the data on this subject are not exhaustive and require further investigation.

## Data availability

No data was used for the research described in the article.

## Declaration of Competing Interest

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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## Publikacja 3

**Michalski J.**, Cłapa T., Syguda A., Narożna D., van Oostrum P., Reimhult E.
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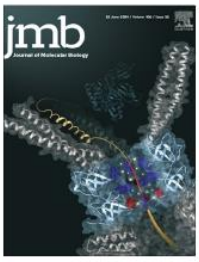
Research Article



# Morpholinium-based Ionic Liquids as Potent Antibiofilm and Sensitizing Agents for the Control of *Pseudomonas aeruginosa*

**Jakub Michalski [1], Tomasz Cłapa [1,*], Dorota Narożna [1], Anna Syguda [2], Peter van Oostrum [3] and Erik Reimhult [3]**

*1 - **Poznań University of Life Sciences,** Department of Biochemistry and Biotechnology, Dojazd 11, 60-632 Poznan, Poland*
*2 - **Poznan University of Technology,** Department of Chemical Technology, Berdychowo 4, 60-965 Poznan, Poland*
*3 - **BOKU University,** Department of Bionanosciences, Institute of Colloid and Biointerface Science, Muthgasse 11-II, A-1090 Vienna, Austria*

***Correspondence to Tomasz Cłapa:** tomasz.clapa@up.poznan.pl (T. Cłapa)*
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## Abstract

Rising antimicrobial resistance is a critical threat to worldwide public health. To address the increasing antibiotic tolerance, diverse antimicrobial agents are examined for their ability to decrease bacterial resistance. One of the most relevant and persistent human pathogens is *Pseudomonas aeruginosa*. Our study investigates the anti-biofilm and sensitizing activity of 12 morpholinium-based ionic liquids with herbicidal anions on four clinically relevant *P. aeruginosa* strains. Among all tested compounds, four ionic liquids prevented biofilm formation at sub-minimum inhibitory concentrations for all investigated strains. For the first time, we established a hormetic effect on biofilm formation for *P. aeruginosa* strains subjected to an ionic liquid treatment. Interestingly, while ionic liquids with 4,4-didecylmorpholinium $[Dec_2Mor]^+$ are more efficient against planktonic bacteria, 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$ showed more potent inhibition of biofilm formation. Ionic liquids with 4,4-didecylmorpholinium ($[Dec_2Mor]^+$) cations even induced biofilm formation by strain 39016 at high concentrations due to flocculation. Morpholinium-based ionic liquids were also shown to enhance the efficacy of commonly used antibiotics from different chemical groups. We demonstrate that this synergy is associated with the mode of action of the antibiotics.



## Introduction

Antimicrobial resistance (AMR) is one of modern medicine's greatest challenges. Nearly 5 million deaths in 2019 were caused by resistant bacterial strains. AMR was a decisive factor for the fatal outcome in about 25% of the cases.[1] The spread of AMR results in (i) a decrease in the effectiveness of antibiotics, (ii) an increase in treatment costs and length, and (iii) an increase in infection mortality.[2] Particularly concerning is the emergence of strains resistant to multiple groups of antibiotics, as in the case of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) phenotypes.[3] Various approaches are employed to overcome antimicrobial resistance, including the use of combination therapy or adjuvants designed to increase bacteria sensitivity to antimicrobial agents.[4]

A notable, clinically relevant example of a microorganism associated with the rise of AMR is *P. aeruginosa*. This bacterium is a non-opportunistic human pathogen recognised as a major etiological factor in healthcare-associated infections.[5] It contributes to approximately 6% of all deaths caused by MDR bacteria, and about 13% of its clinical isolates exhibit MDR phenotype.[1,6] *P. aeruginosa* can cause severe infections

of the urinary tract, respiratory tract, bloodstream, and meninges.[7] People with cystic fibrosis, patients with a suppressed immune system, or patients undergoing medical treatment using catheters or intubation tubes are particularly vulnerable to infections caused by this pathogen.[8–10] The high resistance and infecting effectiveness of *P. aeruginosa* are due to a wide selection of resistance factors, including active and passive mechanisms.[11] Perhaps the most powerful example of a passive resistance mechanism is biofilm formation.

Biofilms are three-dimensional surface-attached structures composed of bacteria connected and protected by an extracellular polymeric matrix of proteins, glycans, and DNA.[12] Microorganisms found in biofilms demonstrate increased survivability and high tolerance to adverse environmental factors, which include antibiotics. The behaviour and metabolic patterns of bacteria in biofilms differ from those of free-living bacteria.[13] Due to the increased tolerance, biofilms formed by pathogenic bacteria are a highly unwelcome and problematic issue in the food industry and medicine.[14] Bacteria in biofilms can be up to 1.000 times more resistant to antimicrobial agents, making it more challenging to eradicate pathogens.[15] In medical settings, biofilm formation is a factor responsible for increasing the infectivity of pathogens and the chronicity of infections.[14] Therefore, new chemical compounds targeting biofilms and reducing the antibiotic resistance of human pathogens, such as *P. aeruginosa,* in biofilms must be investigated. Ionic liquids (ILs) could serve this purpose.

Ionic liquids are salts with a melting temperature below 100 °C. ILs consist most often of organic cations and organic or inorganic anions.[16] They are characterized by low vapor pressure, high ionic conductivity, and high thermal stability.[17] Due to the roughly $10^{18}$ possible anion–cation combinations, ionic liquids are a group of incredibly versatile chemicals that can exhibit a wide range of diverse properties.[18] Their properties include biological activities, such as antimicrobial or virucidal activity.[19,20] Their antimicrobial properties were demonstrated for, e.g., quaternary ammonium-, imidazolium-, and quaternary phosphonium-based ionic liquids.[21] The inhibition of bacterial cell growth, hindrance of bacterial metabolism, or death of microorganisms manifests the antimicrobial activity of ionic liquids. In addition, ILs could also affect bacterial biofilms, inhibiting their formation or leading to the dispersion of mature biofilm.[22]

In this study, we investigated the antimicrobial potential of ionic liquids with the morpholinimum-based cations 4,4-didecylmorpholinium $[Dec_2Mor]^+$ and 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$ to inhibit bacterial biofilm formation of four clinically relevant *P. aeruginosa* strains: PAO1, LESB58, 39016, and UCBPP-PA14. Further, we investigated the potential use of these ionic liquids as adjuvants and sensitizers by testing their ability to form synergistic combinations with antibiotics used to treat *P. aeruginosa* infections. Finally, we estimated the toxicity of the IL at the relevant concentrations by a hemolytic assay.

## Results

### Antimicrobial activity of ionic liquids and antibiotics

MICs of ionic liquids were adopted from our previous study,[23] and the ½ MIC values that were used for the subsequent assays are presented in Table 1. MIC values obtained for antibiotics together with breakpoint values, are presented in Supplemental Table S3. The antimicrobial activity of ionic liquids was determined by the cation structure. ILs I-VI were found to be ineffective in inhibiting bacterial growth, whereas ILs VII-XII with the $[Dec_2Mor]^+$ cation showed strong antimicrobial activity against 3 out of 4 tested strains. Strain 39016 was found to be completely resistant to all tested ionic liquids.

Antibiotic susceptibility tests revealed great differences in the resistance phenotypes of tested strains (Supplemental Table S4). In agreement with the EUCAST (European Committee on Antimicrobial Susceptibility Testing)[79] MIC breakpoints, the LESB58 strain was the most resistant, showing resistance to 9 out of 11 antibiotics (no breakpoint for cefoperazone available). PAO1 was the most susceptible strain, resistant to 3 of 11 drugs tested. All tested strains were susceptible to meropenem and resistant to colistin and ceftazidime. Using the classification of multi-drug resistant strains of *P. aeruginosa* created by Magiorakos et al. 2012, LESB58, UCPP-PA14, and 39016 strains were classified as MDR/possible XDR strains, while the PAO1 strain was classified as possible MDR.

### Biofilm inhibition

During the study, all tested strains showed the ability to form a biofilm, as confirmed by crystal violet staining. However, the amount of biofilm formed varied significantly between the strains (Figure 1). PAO1 was the most effective biofilm former with a mean CV absorbance of 5.19, which was approximately twice that of all other strains (2.76; 2.44; and 2.32 mean value for LESB58, 39016, and UCBPP-PA14 strains, respectively). While the distribution for the UCBPP-PA14 strain was highly skewed, a comparison of medians revealed even greater differences between PAO1 and the other strains. The median CV absorbance of the UCBPP-PA14 strain was more than five times lower than the PAO1 median (0.871 vs. 4.38) and more than two times lower than for the 39016 and LESB58 strains (1.97 and 1.93 median values, respectively).

Table 1 ½ MIC values [μg/mL] of the ionic liquids used for the biofilm inhibition assay.

| ½ MIC [μg/mL] | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PAO1** | 1024[a] | 1024[a] | 512 | 1024[a] | 1024[a] | 1024[a] | 11 | 11 | 16 | 8 | 11 | 8 |
| **LESB58** | 512 | 1024[a] | 384 | 1024[a] | 512 | 1024[a] | 8 | 11 | 5 | 11 | 6 | 11 |
| **39016** | 1024[a] | 1024[a] | 1024[a] | 1024[a] | 1024[a] | 1024[a] | 1024[a] | 1024[a] | 427 | 1024[a] | 1024[a] | 1024[a] |
| **UCBPP-PA14** | 1024[a] | 1024[a] | 512 | 1024[a] | 1024[a] | 1024[a] | 21 | 21 | 16 | 32 | 21 | 37 |

Note: Concentrations were calculated based on the results reported by Cłapa et al. 2021.
a – MIC exceeded the tested concentration range; thus, the highest concentration tested was taken as the ½ MIC for biofilm inhibition.

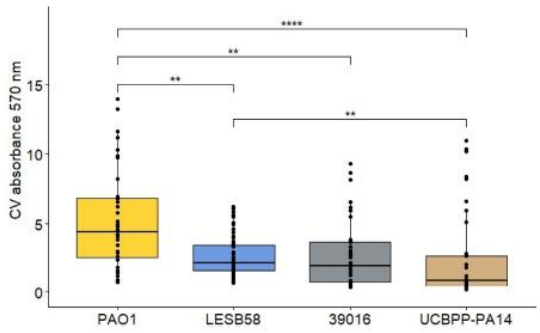


**Figure 1.** Biofilm formation by four *Pseudomonas aeruginosa* strains. Boxplot represents absorbances measured for bacterial biofilms after crystal violet staining. For each group: a median (horizontal black line), an interquartile range (colour box), upper and lower adjacent values (whiskers) and all measurements (black dots) are denoted. For group, Kruskal-Wallis test was performed followed by Dunn's test with Benjamini-Hochberg adjustment of p-value. Statistically significant differences are marked with asterisk (*$p$ < 0.05; ** $p$ < 0.01; *** $p$ < 0.001; **** $p$ < 0.0001).

The amount of biofilm formed by *P. aeruginosa* after ILs treatment was assessed by crystal violet staining and TTC assay in relation to the untreated control, as shown in Figure 2a-d. Crystal violet staining allows quantification of biofilm biomass and TTC assay was used to measure metabolic activity of bacteria within biofilms. All compounds tested were found to significantly decrease the amount of biofilm biomass formed by strains LESB58 and UCBPP-PA14 at ½ MIC. Depending on the ILs used, 8.3 to 3.5-fold and 10 to 3.2-fold reductions in CV absorbance were observed for each strain, respectively. Inhibition of biofilm measured by the TTC assay was less pronounced for these two strains; however, a significant decrease in biofilm viability was observed for all ILs tested at ½ MIC (Figure 2a-b), which corresponds well with the results obtained from CV staining.

The pattern observed for strain 39016 was considerably different. Compounds I–VI significantly inhibited biofilm formation, resulting in a 1.4- to nearly 3-fold reduction in biofilm biomass and a 3.5- to 5.2-fold reduction in biofilm metabolic activity (Figure 2c). At the same time, a surprising promotion of biofilm formation was detected with CV staining for ILs VII, VIII, IX, X, and XII. In this case, the most pronounced change was observed for compound VII, which led to a more than 4.6-fold increase in the amount of biofilm. The results obtained from TTC assay were opposite, indicating a statistically significant inhibition of bacterial viability within the biofilms for the same compounds (Figure 2c).

For the PAO1 strain, CV staining method showed a significant inhibition of biofilm formation only for compounds I, II, IV, and VI, resulting in 40%, 21%, 24%, and 38% of the control amount of biofilm biomass (Figure 2d), respectively. The other compounds caused insignificant changes in PAO1 biofilm biomass levels. These results agree with observations from TTC assay, except for IL III and XII that caused a statistically significant decrease in biofilm viability (Figure 2d). Comparing all the data collected from both assays, only ILs I, II, IV, and VI (all comprising the IL cation $[DecEtMor]^+$) were able to inhibit biofilm formation for all strains.

## Dose-dependent biofilm inhibition

Based on the initial results, dose-dependent biofilm inhibition was investigated for ILs I, II, IV, and VI (Figure 3a-h). For strain LESB58, biofilm inhibition was only observed at 512 μg/mL, with both methods used (Figure 3a-b). These changes were accompanied by an over 50% decrease in the optical density of the bacterial cultures (data not shown). In addition, a significant increase in biofilm formation was observed with CV staining at ILs concentrations below 64 μg/mL (Figure 3a). As for TTC test, no significant changes in dehydrogenases activity of LESB58 were observed at concentrations below 512 μg/mL (Figure 3b). The promotion of biofilm formation was also noted for the UCBPP-PA14 strain at a concentration of 16 μg/mL and, similarily to LESB58 results, TTC assay showed no increase in biofilm metabolic activity at the given dose, instead indicating no significant changes (Figure 3c-d). The lowest concentration that effectively inhibited biofilm formation of UCBPP-PA14 strain for all four ILs was 128 μg/mL; however, a decrease in biofilm viability was of

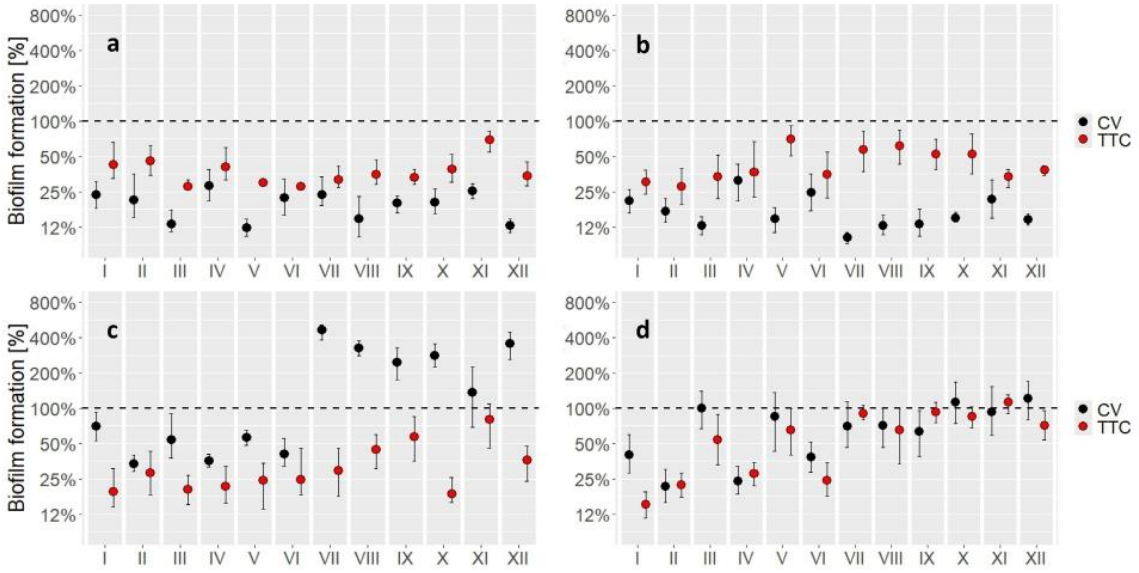


**Figure 2.** Relative biofilm formation [%] by *Pseudomonas aeruginosa* strains LESB58 (a), UCBPP-PA14 (b), 39016 (c) and PAO1 (d) after treatment with ionic liquids evaluated by crystal violet staining (CV) and TTC assay (TTC). Results are presented for ILs at ½ MIC or 1024 μg/mL when MIC could not be determined (Table 1). Each point represents the ratio of means of the treated sample to the untreated control for the given assay (black dots – CV staining; red dots – TTC assay). At least 9 replicates were performed for all samples. Error bars indicate 95% confidence intervals constructed using the bias-corrected and accelerated (BCa) method after bootstrapping with 10.000 resamples. Samples were considered significantly different from the control if the confidence interval for their ratio did not include 1.

lesser magnitude compared to the reduction in biomass at this dose (Figure 3c-d).

The lowest concentration that effectively inhibited biofilm formation of strain 39016 was 512 μg/mL for IL I, 128 μg/mL for IL II and 64 μg/mL for ILs IV and VI (Figure 3e). For the biofilm viability test, the lowest concentration that significantly reduced biofilm in strain 39016 was 256 μg/mL for IL IV and VI, 128 μg/mL for IL II and 64 μg/mL for IL I (Figure 3f). Formation of PAO1 biofilm biomass was significantly inhibited in the range of 128–102 4 μg/mL of all four ILs tested; however, the highest inhibition rate was observed at a concentration of 256 μg/mL, resulting in the formation of 12%, 9%, 12%, and 14% of the control biofilm for compounds I, II, IV, and VI, respectively (Figure 3g). Viability tests showed a significant inhibition of PAO1 biofilm for all tested concentrations of ILs I, II, IV and VI, but these changes were less pronounced with decreasing concentrations (Figure 3h).

The dose-dependent effect of [Dec2Mor] + ILs on strain 39016 was also investigated. CV staining showed an increase in biofilm formation at the highest concentrations (427 μg/mL for IL IX and 1024 μg/mL for the other compounds). The lower concentrations caused insignificant changes or even inhibited biofilm formation (Figure 3i). On the other hand, TTC assay showed a substantial decrease in biofilm viability at the highest concentrations of ILs VII-XII, and no change at lower concentrations, except for IL XI and XII, which caused a slight inhibition also at lower doses (Figure 3j). Since we observed turbidity after introducing high doses of ILs VII-XII into the medium and higher than normal absorbance for negative controls with these compounds after CV staining, we decided to perform additional aggregation tests. The aggregates formed at the bottom of the tubes were larger in samples with bacteria and [Dec2Mor] + ILs compared to the control sample with bacteria alone (Figure 4, top row). Small precipitates, which were not present in sterile medium, were also found in samples containing ionic liquids in TSB-Y and no bacteria (Figure 4, bottom row); however, they were much smaller than the precipitates formed in bacteria samples treated with ILs. This indicates that high doses of ILs VII-XII stimulate cell aggregation.

## Synergy effect

A total number of 132 antimicrobial combinations (12 ionic liquids x 11 antibiotics) were tested for each strain. The results obtained are summarized in the form of heat maps (Figure 5). The FICI for the combinations and concentrations for the mixture components are presented in the

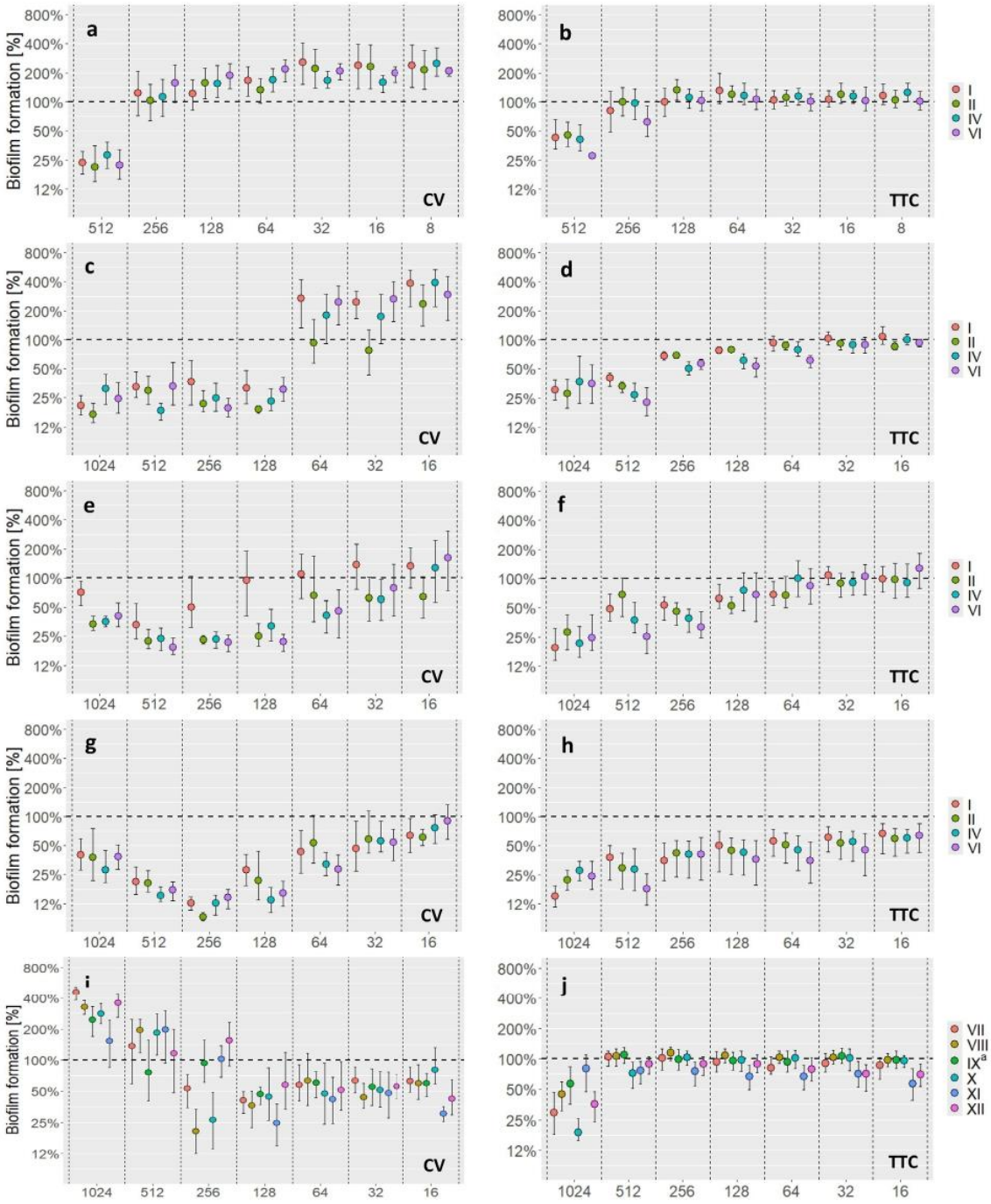


**Figure 3.** Dose-dependent changes in relative biofilm formation [%] evaluated by crystal violet staining (a, c, e, g, i) and TTC assay (b, d, f, h, j) for *Pseudomonas aeruginosa* strains LESB58 (a, b), UCBPP-PA14 (c, d), 39016 (e, f, i, j) and PAO1 (g, h), after treatment with decreasing concentrations of ionic liquids. Concentrations tested start at 512 μg/mL for LESB58 and 1024 μg/mL for three other strains, and are reduced 2-fold at each dilution step. Results are presented for: ILs I, II, IV and VI for all strains (a-h) and ILs VII-XII for strain 39016 (i, j). Each point represents the ratio of means of the treated sample to the untreated control. At least 9 replicates were performed for all samples. Error bars indicate 95% confidence intervals constructed using the bias-corrected and accelerated (BCa) method after bootstrapping with 10.000 resamples. Samples were considered significantly different from the control if the confidence interval for their ratio did not include 1. [a] IL IX concentrations are 427; 213.5; 106.7; 53.3; 26.6; 13.3; 6.7 μg/mL.

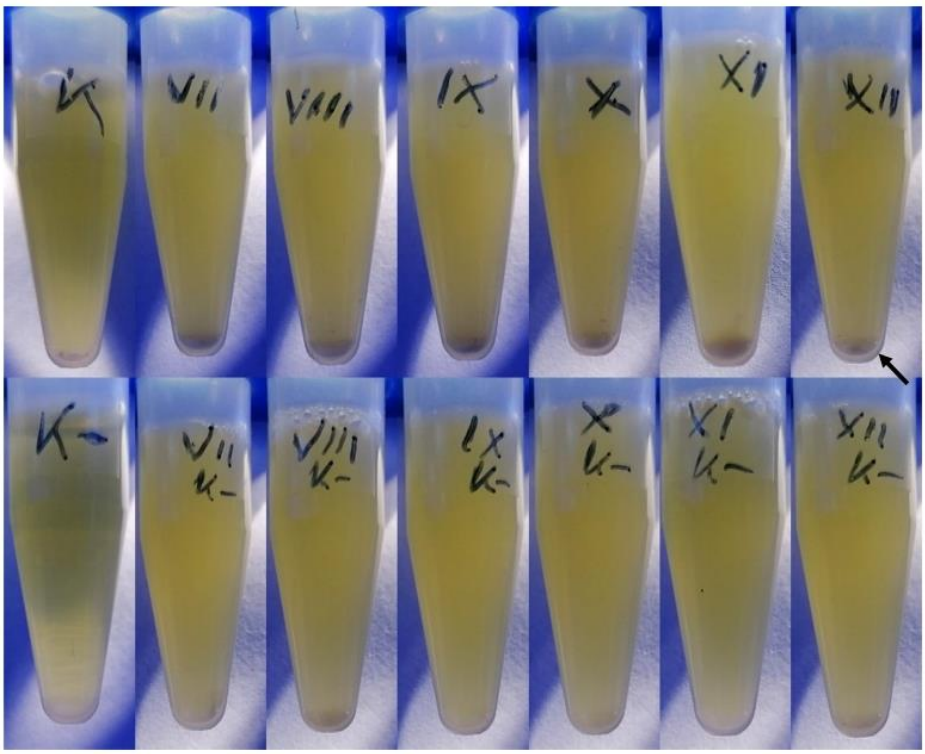


**Figure 4.** Cell aggregates of strain 39016 formed at high doses of ILs VII-XII. The overnight culture of strain 39016 was two times diluted in TSB-Y medium and ionic liquids were added to a final concentration of 427 μg/mL for IL IX and 1024 μg/mL for the other ILs tested. Pictures of cell aggregates formed at the bottom of the tubes were taken after 24 h incubation period. Top row: K, bacterial culture without ionic liquids; VII to XII, bacterial cultures with corresponding ionic liquids. Bottom row: K-, negative control with only sterile medium; VII K- to XII K-, sterile medium with corresponding ionic liquids. Black arrow indicates example of cell aggregates formed at the bottom of the tube.

Supplementary Files. Most of the combinations showed no effect of synergy or additivity. For LESB58, UCBPP-PA14, strain 39016 and PAO1 no interaction was observed in 96, 90, 84, and 91 of all tested combinations, respectively. For LESB58, UCBPP-PA14, and PAO1strains, synergy was rare, occurring in only 11, 15, and 8 of all combinations, respectively. Synergy was much more common for strain 39016 and was detected in 26 combinations.

Some differences between tested strains were also observed with respect to the IL structure. For strains 39016 and UCBPP-PA14, the amplification effect occurred much more frequently in combinations with ionic liquids VII-XII, while for the other two strains, the observed effect was distributed independently of the cation structure. For the LESB58 strain, IL III was much more prevalent in additive and synergistic combinations than other ILs.

Strong synergy, indicated by FIC indices $\leq 0.25$, was found only in colistin mixtures for LESB58, UCBPP-PA14, and PAO1 strains. Interactions of this magnitude were observed for 39016 for some IL combinations with colistin, aztreonam, and piperacillin. The lowest FICI was measured for aztreonam mixtures with IL VII, VIII, and XII, and was equal to 0.09.

For all combined strains, no effect was observed for the mixtures with cefoperazone and ceftazidime, and there was also a lack of synergy for formulations with ofloxacin and ciprofloxacin (Figure 6). All mixtures with colistin showed an additive or synergistic effect for all strains. At least additivity was also very common for combinations with amikacin, piperacillin, and aztreonam, and moderately common for mixtures with tobramycin and meropenem. However, synergy was mainly observed for IL-colistin combinations. Only four of all combinations show a synergistic effect in all tested strains, and these were colistin combined with the $[Dec_2Mor]^+$-containing VII, VIII, X, and XII ionic liquids.

Due to the addition of ionic liquids, a reduction in the concentration below the resistance breakpoints was achieved for some initially non-effective antibiotics (Table 2). This was observed for at least one combination of colistin and piperacillin in the strains that were initially resistant to those antibiotics. Sensitization of LESB58 to ofloxacin,

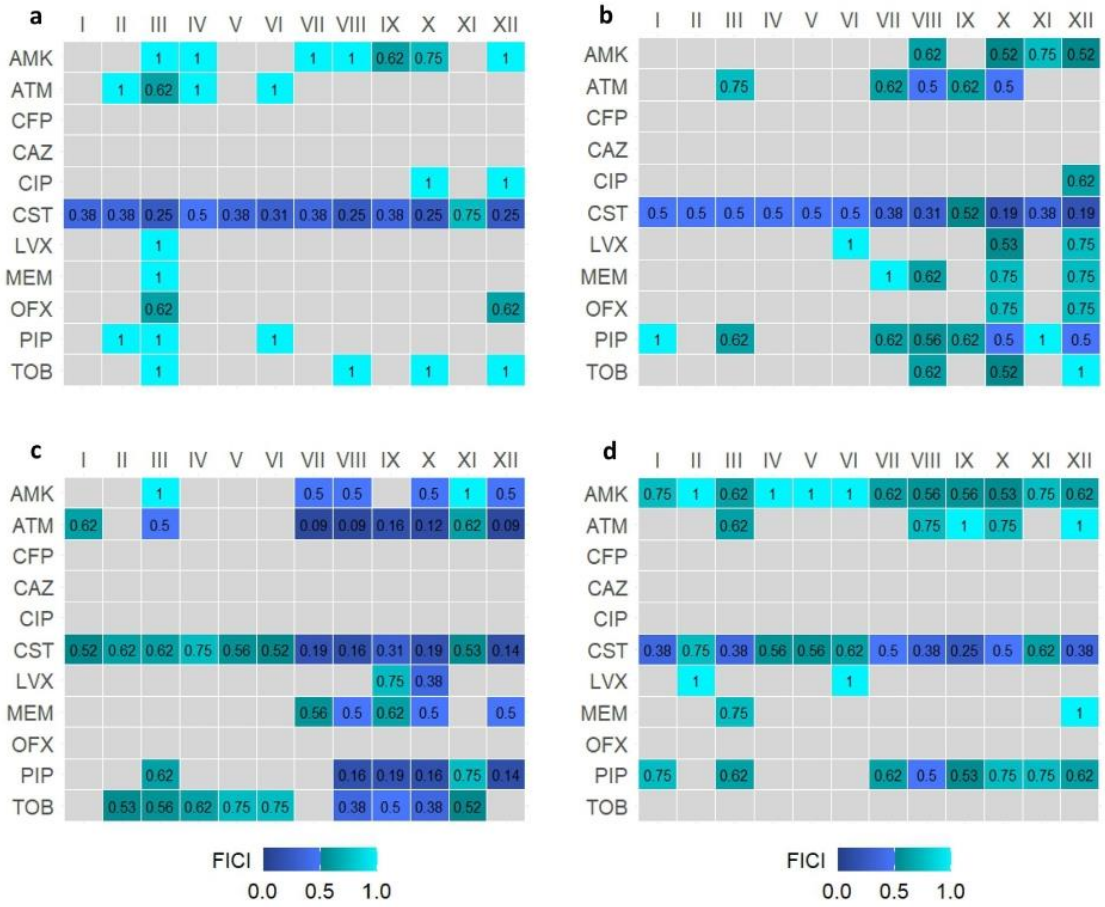


**Figure 5.** Results of the synergy assay for all IL combinations with antibiotics presented for four *P. aeruginosa* strains: LESB58 (a), UCBPP-PA14 (b), 39016 (c) and PAO1 (d). The ILs are arranged into columns and antibiotics into rows. FIC indices are given for combinations with additive ($0.5 < FICI \leq 1$) and synergistic ($FICI \leq 0.5$) effect, which are marked with turquoise and blue gradient, respectively. Combinations with no observed interaction are shown in grey. Each reported combination was confirmed in 3 separate replicates. AMK – amikacin; ATM – aztreonam; CFP – cefoperazone; CAZ – ceftazidime; CIP – ciprofloxacin; CST – colistin; LVX – levofloxacin; MEM – meropenem; OFX – ofloxacin; PIP – pieracillin; TOB – tobramycin.

UCBPP-PA14 to ciprofloxacin, strain 39016 to levofloxacin, and both UCBPP-PA14 and strain 39016 to aztreonam and tobramycin was also observed. Ionic liquids III, VIII, IX, X, and XII were the adjuvants most frequently present in combinations with this sensitizing effect, each being involved in at least nine of such combinations. The highest decrease in antibiotic inhibitory concentration was 64-fold (lower concentrations were not tested). It occurred in combinations of aztreonam with IL IX for strain 39016; colistin with IL III, VIII, X, or XII for LESB58; amikacin with IL X or XII, piperacillin with IL X, aztreonam with IL X, tobramycin with IL X, and colistin with IL X or XII for UCBPP-PA14. For the PAO1 strain, the highest decrease in antibiotic inhibitory concentration was 32-fold for the combination of colistin and IL III.

## Hemolytic activity

We show the hemolytic activity of the investigated ionic liquids in Figure 7. Following Amin and Dannenfelser (2006),[25] concentrations at which the hemolytic activity did not exceed 10% are considered non-hemolytic. The lowest non-hemolytic concentrations for each IL are listed in Table 3. All ionic liquids caused hemolysis of washed erythrocytes. ILs with the $[Dec_2Mor]^+$ cation demonstrated very high hemolytic activity, leading to total lysis of red blood cells at concentrations higher or equal to 32 μg/mL (Figure 7). The only exception was IL XI which caused nearly 100% hemolysis at a concentration greater or equal to 512 μg/mL. Lysis of less than 10% of washed erythrocytes was observed for compounds VII, VIII, IX, and X at 8 μg/mL, compound XI at 128 μg/mL, and

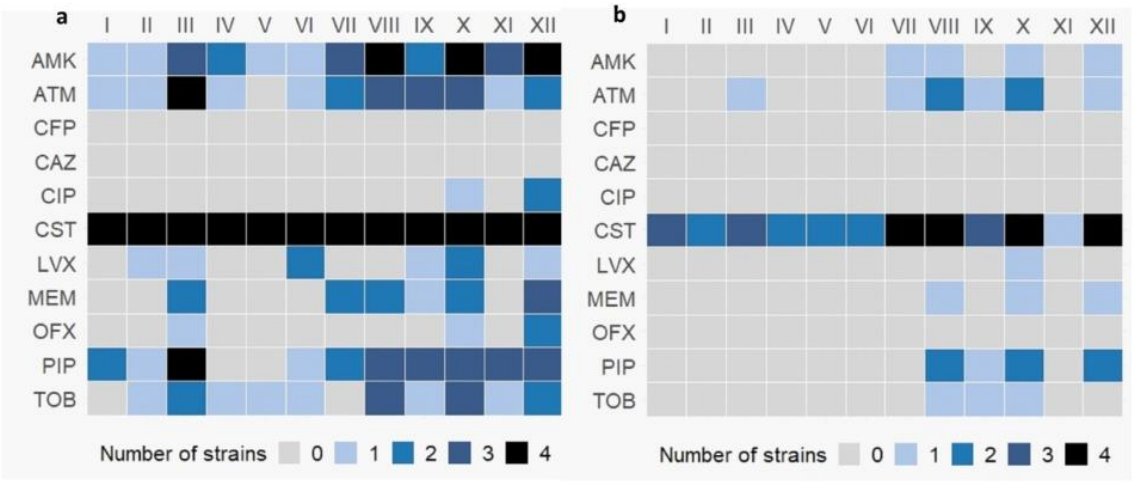


**Figure 6.** Results of the synergy assay summarized for all four *P. aeruginosa* strains combined. The ILs are arranged into columns and antibiotics into rows. The number of strains with at least additive (a) or synergistic (b) effect observed is marked for each combination. Combinations for which no interaction was observed in any strain are shown in grey. AMK – amikacin; ATM – aztreonam; CFP – cefoperazone; CAZ – ceftazidime; CIP – ciprofloxacin; CST – colistin; LVX – levofloxacin; MEM – meropenem; OFX – ofloxacin; PIP – piperacillin; TOB – tobramycin.

Table 2 Overview of sensitizing effect of ionic liquids on *Pseudomonas aeruginosa*.

| Strain | Antibiotic (MIC; MIC breakpoint) | Ionic liquid | Selected combinations (antibiotic μg/mL; IL μg/mL; FICI) |
|---|---|---|---|
| **PAO1** | Colistin (16.67; 4) | I, III, VI, VIII, IX, X, XII | III (0.52; 512; 0.531);IX (2.08; 4; 0.25) |
| | Piperacillin (18.67; 16) | I, III, VII, VIII, IX, X, XI, XII | III (2.33; 512; 0.625);VIII (4.67; 5.33; 0.5) |
| **LESB58** | Colistin (66.67; 4) | III, VI, VIII, X, XII | VIII, X, XIII (1.042; 10.67; 0.516) |
| | Ofloxacin (17.33; 4) | III, XII | XII (2.17; 10.67; 0.625) |
| | Piperacillin (21.33; 16) | II, III, VI | III (10.67; 384; 1) |
| **39016** | Aztreonam (26.67; 16) | I, III, VIII, IX, X, XI, XII | IX (0.42; 427; 0.516);VIII (0.83; 128; 0.09) |
| | Colistin (13.33; 4) | III, VII, VIII, IX, X, XI, XII | X (0.83; 512; 0.313);XII (1.67; 32; 0.141) |
| | Levofloxacin (2.25; 2) | IX, X | X (0.28; 512; 0.375) |
| | Piperacillin (32; 16) | III, VIII, IX, X, XII | XII (1; 512; 0.281);XII (4; 32; 0.141) |
| | Tobramycin (4; 2) | VIII, IX, X | VIII (0.5; 512; 0.375) |
| **UCBPP-PA14** | Aztreonam (26.67; 16) | III, VII, VIII, IX, X | X (0.42; 32; 0.516);VIII (6.67; 10.67; 0.5) |
| | Ciprofloxacin (1.33; 0.5) | XII | XII (0.17; 37.33; 0.625) |
| | Colistin (11.67; 4) | I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII | XII (0.18; 18.67; 0.266);X (0.73; 8; 0.188) |
| | Tobramycin (2.67; 2) | VIII, X, XII | X (0.04; 32; 0.516) |

Note: For each strain, all antibiotics whose MIC was decreased below the resistance breakpoint are listed together with all ILs found in combinations with such an effect. In the last column, one combination with the highest reduction in MIC of the antibiotic and one combination with the lowest FICI are highlighted.

compound XII at 4 μg/mL. The same hemolytic activity was observed for 128 μg/mL of IL III, and 512 μg/mL of ILs I, II, IV, V, and VI (Table 3). Hemolysis was observed considerably less frequently in whole blood samples. A 32-, 4- and 64-fold increase in the lowest non-hemolytic concentration was observed in whole blood samples for ILs VII-X, XI, and XII, respectively. Also, no hemolytic activity was observed in whole blood samples for compounds II, III, IV, and VI.

## Discussion

The selection of ionic liquids for the biofilm assay and synergy testing was based on their

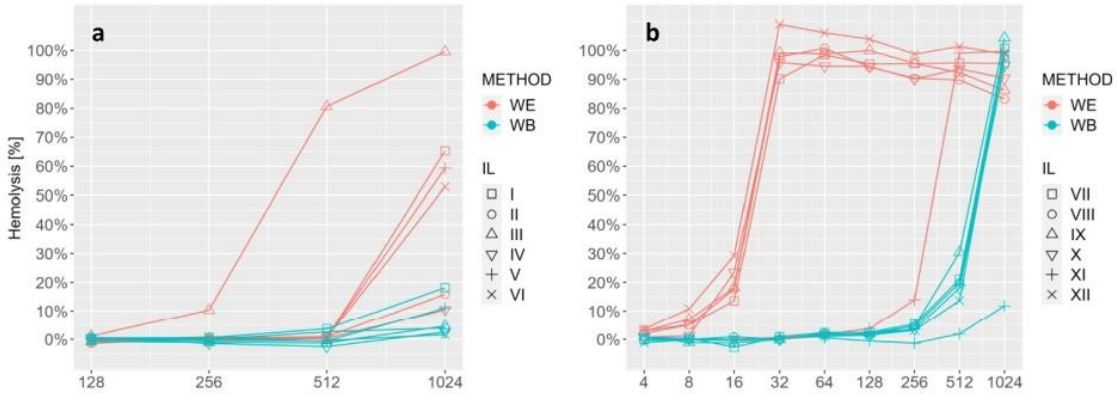


**Figure 7.** Hemolytic activity [%] of ILs I-VI (a) and VII-XII (b) measured for two sample types: WE- washed erythrocytes (red); WB – whole blood sample (blue). Cell lysis in 1% Triton X-100 and PBS was considered as total lysis and no lysis, respectively. Data presented are averaged from 3 separate replicates.

Table 3 The lowest non-hemolytic concentrations [μg/mL] of all ionic liquids.

| | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **WE** | 512 | 512 | 128 | 512 | 512 | 512 | 8 | 8 | 8 | 8 | 128 | 4 |
| **WB** | 512 | 1024 | 1024 | 1024 | 512 | 1024 | 256 | 256 | 256 | 256 | 512 | 256 |

Note: The lowest non-hemolytic concentrations was defined as the lowest concentration with hemolysis no greater than 10% following Amin and Dannenfelser (2006).[25] All experiments were done in triplicate.
WE – washed erythrocytes; WB – whole blood samples.

antimicrobial activity already described in the literature. Ławniczak et al. 2015 were the first to demonstrate the toxicity of eight of the ionic liquids presented in our work against various environmental microbiota. Expanding this research, we were able to show the efficacy of the described ionic liquids in the control of *P. aeruginosa* in one of our studies.[23] The results obtained in that study indicated a clear relationship between the structure of the cation and the IL potential to inhibit the growth of *P. aeruginosa*. The $[Dec_2Mor]^+$ cation, with two decyl chains as substituents, was identified as responsible for the antimicrobial activity of the tested ionic liquids. In another study, we extended this observation to five other bacterial strains: *Lactococcus lactis*, *Escherichia coli*, *Pseudomonas syringae*, *Salmonella typhimurium,* and *Listeria monocytogenes*. In addition, we proved that the same structure–activity relationship applies to the inhibition of DNA polymerase activity and two bacteriophages: Phi6 and P100.[19] The effect of a cation's alkyl chain length on the antimicrobial activity is one of the best-described structure–activity relationships for ionic liquids. Namely, the toxicity of ILs tends to increase with increasing alkyl chain length to a point where further increase in chain length becomes counter-effective.[27] Similarly, the antimicrobial activity of ionic liquids increases as the number of long alkyl substituents in the cation increases.[28]

Several ionic liquids, e.g., imidazolium- or piperidinium-based ionic liquids, have been shown to inhibit biofilm formation.[29,30] We showed that morpholinium-based ionic liquids can inhibit biofilm formation by *P. aeruginosa* species. Biofilm properties can be assessed in terms of the amount of biomass formed, biofilm metabolic activity or biofilm viability.[29,31,32] Inhibition was observed in terms of both total biofilm biomass assessed by crystal violet staining and biofilm metabolic activity measured by TTC assay. Furthermore, we identified four ILs (I, II, IV, and VI) that significantly reduced biofilm formation by all tested strains, also at concentrations several-fold lower than the MIC. Interestingly, all these compounds possess the $[DecEtMor]^+$ cation, which was ineffective in the growth inhibition assay. This might be surprising, especially since the aforementioned alkyl chain effect was observed for biofilms by other authors.[33–35] However, the alkyl chain effect in biofilms has been reported for biofilm inhibition and eradication assays with ILs at concentrations exceeding their MIC values. Thus, the mentioned effect may be a manifestation of the lowered MIC values of long-chained ILs. Our results do not contradict previous findings, as we used ionic liquids at sub-inhibitory concentrations.

Biofilm inhibition was mostly unrelated to changes in culture density, meaning the ILs did not reduce bacterial growth, only biofilm growth. However, ILs also can inhibit biofilm formation by inhibiting

bacterial growth. We observed that some changes in biofilm levels were accompanied by a substantial decrease in the optical density of the cell cultures. Thus, biofilm inhibition in these samples can be attributed to lower bacterial growth. For the majority of the samples with inhibited biofilm growth, other inhibitory mechanisms must be responsible for the observed reduction in biofilm formation. Ionic liquids can inhibit biofilm formation by preventing bacterial adhesion to surfaces.[30] Such anti-adherence properties may contribute to the antifouling effect of low-toxicity morpholinium-based ionic liquids. Cationic electrolytes can also bind to negatively charged bacterial cell wall, through electrostatic interactions.[36] ILs could similarly disrupt the formation of the extra-polymeric matrix that holds biofilms together and prevent the film from forming efficiently. Cationic ILs could complex strongly to generally anionic sugars and solubilize hydrophobic segments due to their detergent properties.

Hence, the investigated ILs are effective antibiofilm agents even at low sub-MIC concentrations. However, an analysis of the dose-dependent inhibition of biofilms reveals several interesting effects. First, a hormetic effect was observed for ILs I, II, IV, and VI against UCBPP-PA14 and LESB58 strains. Hormesis is an effect of low-dose stimulation and high-dose inhibition observed for organisms subjected to stress factors.[37] Due to the hormetic effect, low doses of some antimicrobials may lead to the unwanted induction of bacterial growth, resistance mechanisms, or synthesis of virulence factors.[38] Such effects have been observed for low doses of, e.g., antibiotics or disinfectants.[39,40] Hormesis has also been observed for short-chain ILs, stimulating bacterial growth and metabolic activity.[41] Hormetic induction of biofilm formation has been reported for factors such as antibiotics, nanoparticles, or bacteriophages.[38,42,43] Promotion of biofilm formation was only observed with crystal violet staining which binds to both cells and extracellular matrix produced by bacteria.[44] Since TTC assay showed no change in biofilm viability at these concentrations, these results suggest that the observed biofilm promotion was due to the increase in EPS production by the bacteria alone, rather than an increase in bacterial growth. Our study is the first to show that ILs with antibiofilm properties can exhibit hormetic induction of biofilm formation by *P. aeruginosa*. However, we also observed a non-hormetic stimulation of strain 39016 biofilm formation at the highest concentrations of ILs VII-XII tested, as measured by CV staining. The stimulatory effect was observed even though the initial concentrations of ILs used in the biofilm inhibition assay were higher than for the other strains due to the increased tolerance of strain 39016. The [$Dec_2$Mor] ILs tended to precipitate from the medium at high doses, which also resulted in high background absorbance. The presence of precipitates and surface roughness are known inducers of biofilm formation by microorganisms.[45,46] While the background absorbance was already factored into the biofilm formation calculations, high concentrations of [$Dec_2$Mor] ILs were also able to stimulate aggregation of cells. The aggregation process is known to be involved in biofilm formation.[47,48] Cell aggregates formed in the solution favor bacteria settlement on the surface to initiate biofilm development.[49] Hence, we attribute the observed increase in biofilm amount to the flocculation and precipitation processes. That said, observed increase in CV absorbance was accompanied by a significant decrease in cell viability of the biofilms. As the CV staining method does not discriminate between live and dead cells, part of the measured effect may be due to sedimentation of dead cells not involved in biofilm formation. On the other hand, dead cells play an important role in the development of bacterial biofilms.[50–52] In fact, strong biofilms with high biomass content can be characterized by a significant predominance of dead over living cells within their structure, compared to weak biofilms giving lower readings for CV staining.[53] Also, data shows that biofilm biomass do not correspond with metabolic activity, and biofilms with high biomass are often characterized by low metabolic activity.[54–56] Thus, the results of low metabolic activity detected by the TTC assay are consistent with elevated CV values for strain 39016 treated with [$Dec_2$Mor] ILs.

Synergistic interactions between morpholinium-based ionic liquids and antibiotics were confirmed for *P. aeruginosa* strains with different resistance phenotypes. Synergy was found to be rare, which is consistent with other studies on antibacterial drug interactions.[57–59] Such interactions have already been investigated for mixtures of imidazolium-, pyrrolidinium- and piperidinium-based ionic liquids with antibiotics.[60–62] We found that ionic liquids with [$Dec_2$Mor]$^+$ cations carrying two long alkyl chains are more often associated with additive and synergistic interactions. Similarly, Saraswat et al. 2020 found synergistic effects were more pronounced for ionic liquids with longer alkyl chains. The length and number of long alkyl chains are well-known determinants of the antimicrobial activity of ionic liquids, and, as mentioned, [$Dec_2$Mor] ILs exhibit a high antimicrobial activity.[23,27] In addition, a higher incidence of additivity was observed for formulations with IL III, characterized by high antibacterial potency compared to other representatives of [DecEtMor] ILs. This suggests that higher antimicrobial activity is associated with a higher likelihood of at least an additive effect between ILs and antibiotics. Additionally, the number of long alkyl chains is a good predictor of synergy.

Most synergistic events were observed for strain 39016, almost exclusively in mixtures with [$Dec_2$Mor] ionic liquids. This strain has been

previously characterised as highly tolerant to ionic liquids. However, ILs were still found to impair the strain's metabolic activity.[23] Thus, the higher incidence of synergy marked for strain 39016 may be due to the higher absolute concentrations of ILs used against it compared to other bacterial strains.

Ionic liquids and colistin were likely to form synergistic combinations. Our results are consistent with those of Florio et al. 2020, who demonstrated the emergence of a synergistic effect between colistin and three ionic liquids with different cation headgroups in Gram-negative bacteria. Colistin is a peptide antibiotic that belongs to the polymyxins group. Its mechanism of action is based on the binding to lipopolysaccharides (LPS) of Gram-negative bacteria, followed by disruption of membranes due to colistin incorporation.[63] Because biological membranes are also a target site for ionic liquids,[22] the observed synergistic effect between colistin and morpholinium-based ILs may be due to the increased permeation caused by the joint action of both compounds on the bacterial bilayer. Indeed, a study on lipid vesicle membranes has shown that the combined use of ionic liquids and polymyxins increases the permeation of lipid membranes, leading to membrane leakage.[64]

For three fluoroquinolone antibiotics (levofloxacin, ciprofloxacin, and ofloxacin), additive effects were very rare; we identified only one synergistic combination. Our results are consistent with the study by Chandrasekaran et al. 2016, showing that quinolones and drugs targeting the DNA supercoiling process are more likely to exhibit antagonistic or no effect with other antibacterials. The same work shows that the activity of aminoglycosides remains indifferent in combinations with other drug families or stress factors, including membrane targeting factors such as ILs.[65,66] Accordingly, we found that tobramycin and amikacin were very often involved in forming additive combinations but not synergistic ones. That said, aminoglycosides are known to create strong synergies with antibacterials such as β-lactams.[67] A synergy between the aminoglycoside representative, kanamycin, and imidazolium-based ILs has been reported but not universally observed for all bacterial species.[61] Aminoglycosides are concentration-dependent drugs.[68] Because the aminoglycosides target a small subunit of the bacterial ribosome, they must enter the cell to exert their bactericidal activity.[69] Since ionic liquids can increase membrane permeability, they should facilitate the penetration of antibiotics into the cell. Indeed, for some combinations with [$Dec_2$Mor] ILs associated with higher permeation and antimicrobial activity, we observed a substantial decrease in the amikacin and tobramycin concentrations required to show antimicrobial activity. Still, for most combinations, the amikacin and tobramycin MICs were at most halved. Strain-specific traits and insufficient permeation of bacterial membranes caused by ILs may explain why synergistic effects were rare for aminoglycosides.

A more variable response was observed for IL mixtures with β-lactam antibiotics. For two of the tested β-lactam antibiotics, ceftazidime, and cefoperazone, both of which are third-generation cephalosporins, no synergetic effect with ILs was observed. In contrast, piperacillin, aztreonam, and meropenem, representing penams (penicillins), monobactams, and carbapenems, all showed additive and synergistic effects with ILs, with meropenem performing slightly worse. All these antibiotics inhibit the synthesis of bacterial cell walls but differ in the chemical group fused to the β-lactam ring. A saturated five-membered, an unsaturated five-membered, or an unsaturated six-membered ring is fused to β-lactam in penams, carbapenams, and cephalosporins, respectively, while no such group is present in monobactams.[70] In view of our results, it is tempting to hypothesise that the presence of a six-membered ring or aromatic bonds in β-lactam antibiotics is unfavourable for the emergence of synergy with ILs. However, in the absence of a model to explain the molecular interactions between ionic liquids and β-lactams and given that the data obtained are limited to a single representative of each β-lactam subgroup, any conclusions drawn about the structure–activity relationship to interpret the observed differences between β-lactams in IL presence should be treated with caution.

For the first time, we could demonstrate that ionic liquids can sensitize MDR strains of *P. aeruginosa* to different groups of antibiotics. The most common strategies applied to decrease bacterial antibiotic tolerance involve using adjuvants. Adjuvants can reduce bacterial resistance by blocking intrinsic or active resistance mechanisms.[71] Another approach is combination therapy, in which two or more antibiotics, often with different modes of action, are used together to break pathogen resistance.[67] Sensitization of *P. aeruginosa* strains to antibiotics was reported for, e.g., aminoimidazol derivatives or polyaminofarnesyl derivatives.[72,73] We observed a decrease in the inhibitory concentration of antibiotics below the MIC for the representatives of all antibiotic groups when combined with ILs. The universality of this effect indicates that ILs' adjuvant action targets the passive resistance mechanisms of *P. aeruginosa*. This is reasonable since ionic liquids, e.g., act on bacterial membranes. Furthermore, the most pronounced synergistic effect was observed for combinations with colistin, which would be strongly aided by increased membrane permeation. Thus, there are strong indications that ionic liquids increase the efficacy of antibiotics through their adverse impact on bacterial membrane integrity.

Erythrocytes are model cells for assessing the effects of xenobiotics on membranes.[74] Hence,

we evaluated the hemolytic activity to assess the cytotoxicity of the investigated morpholinium-based ionic liquids. All ionic liquids caused hemolysis, indicating ionic liquid toxicity towards erythrocytes. This finding should be considered a significant limitation for using morpholinium-based ILs as adjuvants in treating bacterial infections. Interestingly, a huge difference in hemolysis rate was observed between washed erythrocytes and whole blood samples. Similar results were obtained by Sæbø et al. 2023[75], who reported significantly lower hemolysis in whole blood samples for antimicrobial peptides and mellitin. The washing procedure increases the osmotic fragility of erythrocytes, making them more susceptible to hemolysis.[76] Therefore, the results from the whole blood assay may be considered more representative of *in vivo* conditions.

The concentrations of ILs at which antibacterial or hemolytic activity, respectively, are observed should also be taken into account when considering using ILs as adjuvants. Low doses of ionic liquids did not result in hemolysis. Comparing concentrations, more than half of the effective IL combinations with antibiotics should be rejected due to the high hemolytic activity of the ionic liquids if we estimate their hemolytic activity on washed erythrocytes. However, the hemolytic activity in the more relevant whole blood samples paints a much more positive picture. Almost all additive or synergistic combinations contain ILs at non-hemolytic concentrations (Supplemental Figure S1 and S2). Hemolytic activity was correlated with increased antimicrobial activity. It was also considerably higher for ILs carrying cations with two long alkyl chains. This is consistent with previous reports showing that the hemolytic activity of ILs increases with increasing alkyl chain length.[77,78]

In conclusion, our work demonstrates that some morpholinium-based ionic liquids exert antibiofilm activity against various strains of *P. aeruginosa* at concentrations several times lower than their MICs. As biofilm formation is a major virulence factor, such results indicate the antivirulent potency of the investigated ILs. Interestingly, the morpholinium-based cation most effective as an anti-biofilm agent, $[DecEtMor]^+$, is not the same as the one most efficient at inhibiting bacterial growth and showing the highest hemolytic activity, i.e., $[Dec_2Mor]^+$. The importance of detailed investigations of the dose–response of ILs is further emphasized by the frequently observed hormetic effect or less frequently observed flocculation and precipitation. Both effects led to a dose-dependent stimulation of biofilm growth.

We also showed ILs' potential to sensitize *P. aeruginosa* to antibiotics. We found the most pronounced synergistic effects in combinations with colistin, exemplifying that ILs were most effective as adjuvants with antimicrobial agents sharing the same target. Still, synergies with antibiotics exhibiting several different modes of action suggest a universal application of ILs as adjuvants. On the flip side, the hemolytic activity of the ILs we tested could significantly restrain their use in treating infections and limit them primarily to extracorporeal use.

## Materials and Methods

### Ionic liquids and antibiotics

Twelve morpholinium-based ionic liquids with herbicidal derivatives as anions were used in the study. All ILs with their full names and abbreviations are listed in Supplemental Table S1. Each ionic liquid includes one of the two cations 4, 4-didecylmorpholinium $[Dec_2Mor]^+$ or 4-decyl-4-ethylmorpholinium $[DecEtMor]^+$. The cations were paired with one of the six anions: 2,4-dichlorophenoxyacetate $[2,4\text{-}D]^-$, 4-chlorophenoxyacetate $[4\text{-}CPA]^-$, 3,6-dichloro-2-pyridinecarboxy late $[Clopyralid]^-$, 3,6-dichloro-2-methoxybenzoate $[Dicamba]^-$, 4-chloro-2-methylphenoxyacetate $[MCPA]^-$, or (±)-2-(4-chloro-2-methylphenoxy)propio nate $[MCPP]^-$. Synthesis and chemical properties of all ILs were reported in previous papers.[23,26] The structures of the IL cations and anions are presented in Figure 8. Eleven antibiotics were used for synergy testing: amikacin, aztreonam, cefoperazone, ceftazidime, ciprofloxacin, colistin, levofloxacin, meropenem, ofloxacin, piperacillin, and tobramycin. All antibiotics were purchased from Pol-Aura and are listed in Supplemental Table S2. The stock solutions of antibiotics were prepared and stored in accordance with manufacturer recommendations.

### Microorganisms and media

All four *P. aeruginosa* strains used in the study: PAO1, LES B58, 39016, and UCBPP-PA14 were obtained from the Department of Biochemistry and Biotechnology, Poznań University of Life Sciences. All strains were cultured at 37 °C in TSB-Y medium, a tryptic soy broth medium (TSB; Sigma-Aldrich) supplemented with 0.6% yeast extract. Detailed media composition was as follows: papaic digest of soya, (3 g/L), D(+)-glucose (2.5 g/L), digest pancreatic of casein (17 g/L), di-potassium hydrogen phosphate (2.5 g/L), sodium chloride (5 g/L), and yeast extract (6 g/L). The media pH was adjusted to 7.3 ± 0.2.

### MIC assay

The minimum inhibitory concentration (MIC) was determined for all antibiotics using the microdilution method. Briefly, bacterial strains were grown overnight in TSB-Y medium at 37 °C. Antibiotics were added to the first row of the 96-well plate, and a 2-fold serial dilution was performed. TSB-Y medium was inoculated with *P.*

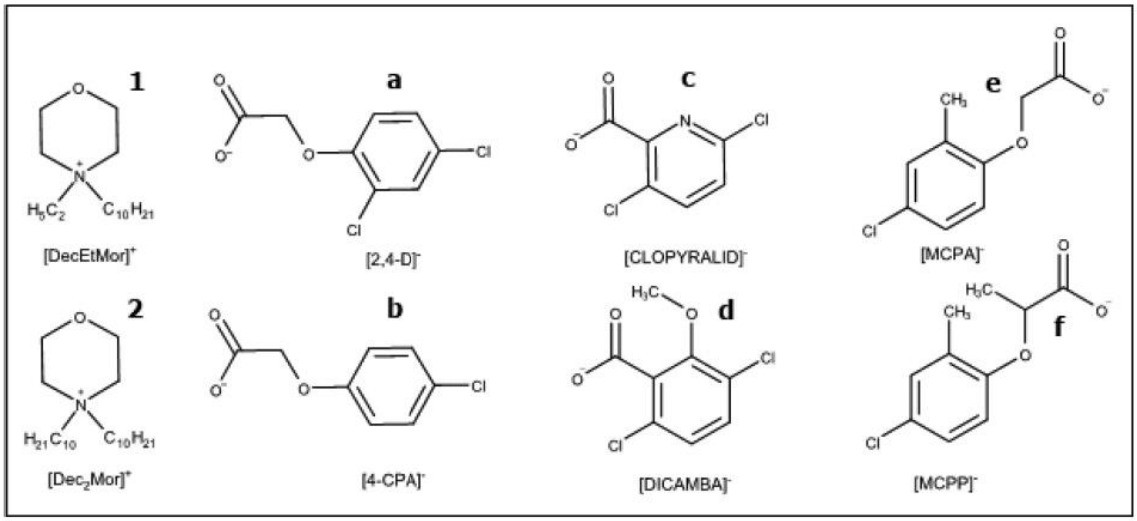


**Figure 8.** Chemical structures of cations and anions of morpholinium-based ionic liquids investigated in this study. The $[DecEtMor]^+$ cation is present in ILs I-VI and the $[Dec_2Mor]^+$ cation in ILs VII-XII: I – 1a; II – 1b; III – 1c; IV – 1d; V – 1e; VI – 1f; VII – 2a; VIII – 2b; IX – 2c; X – 2d; XI – 2e; XII – 2f.

*aeruginosa* strains and distributed to all wells to a final volume of 200 μL. The final bacterial concentration in each well was $5 \times 10^5$ CFU/mL. The plates were incubated overnight at 37 °C with shaking. The optical density (OD) of the samples was measured before and after incubation at 600 nm using a microplate reader. Samples with $OD_{600}$ values < 0.1 were considered to have no visible bacterial growth. The lowest antibiotic concentration with no visible growth was taken as the minimum inhibitory concentration. The MIC reported for each antibiotic is the mean of three separate experiments. The MICs for ionic liquids were taken from our previous study.[23]

## Biofilm inhibition assay

The biofilm inhibition assay was performed as described by Florio et al. 2019 and Sabaeifard et al. 2014 with some modifications. Briefly, wells of a 96-well microplate were filled with TSB-Y medium, *P. aeruginosa* overnight inoculums and ionic liquids in a total volume of 200 μL. The final concentration of bacteria in each well was equal to $10^6$ CFU/mL, and the final concentration of ILs was adjusted to ½ of the MIC value. The MIC values for tested ILs were taken from our previous study and half MICs used in the experiment are presented in Table 1.[23] Three types of control samples were included in each plate, a positive control without ionic liquids, a negative control with ILs in sterile medium, and a negative control with medium alone. Such prepared plates were incubated at 37 °C for 24 h. After incubation, OD600 of cultures was measured using a microplate reader to determine bacterial growth. Microplate wells were then emptied and washed three times with PBS to remove the unattached cells. Plates were air-dried and formed biofilms were subjected to either crystal violet (CV) staining to determine biomass or TTC assay to assess biofilm viability. For CV staining, biofilms were stained with 0.1% crystal violet solution for 15 min, followed by three washes with PBS to remove unbound dye. Bound crystal violet was dissolved in 99.8% ethanol and the amount of biofilm biomass formed in each well was quantified by measuring the absorbance of the dissolved crystal violet at 570 nm in a microplate reader. For the TTC assay 200 μL of 0.2% TTC prepared in TSB-Y medium was added to the wells with biofilm and the plates were incubated at 37 °C for 5 h in the dark. During this time, TTC was reduced by viable bacteria to red, water-insoluble formazan. After incubation, plates were centrifuged to pellet formazan crystals, which were dissolved in 200 μL of 99.8% ethanol. Microplates were centrifuged again and 100 μL of wells content were transferred to new plates for absorbance measurement. Absorbance was measured at 490 nm using a microplate reader. Relative biofilm formation was expressed as the ratio of the absorbance measured for the bacteria-seeded samples to the positive control without IL, using the formula:

$$Biofilmformation = \frac{A_{570Sample} - A_{570MediumwithILs}}{A_{570Positivecontrol} - A_{570Medium}} \times 100\%$$

All tested combinations were performed in at least 9 replicates. For selected compounds, a dose-dependent effect of ILs on biofilm formation was examined as well. In that case, a series of two-fold serial dilutions starting at ½ MIC was performed prior to the biofilm inhibition assay.

### Synergy testing

The synergistic effect of ionic liquid and antibiotic combinations was investigated with a checkerboard assay. All combinations were initially tested at ½ MIC concentration or 1024 μg/mL when MIC could not be determined (Table 1). Only the combinations that showed inhibition of bacterial growth were selected for further testing with a synergy checkerboard assay. Briefly, the mixtures of compounds tested were prepared in TSB-Y medium using the two-fold serial dilution method on a 96-well plate. All wells were then inoculated with bacterial strains at a final concentration of $5 \times 10^5$ CFU/mL. The range of the concentrations tested was from ½ MIC to $^1/_{64}$ MIC for each. Plates were incubated for 24 h at 37 °C with shaking. The optical density of the plates was measured before and after incubation at 600 nm using a microplate reader. The combinations with an $OD_{600}$ value < 0.1 were considered to have no visible bacterial growth, for which a fractional inhibition concentration index (FICI) was calculated using the formula:

$$FICI = \frac{MIC_{ILincombination}}{MIC_{ILalone}} + \frac{MIC_{Antibioticincombination}}{MIC_{Antibioticalone}}$$

The results obtained were interpreted as follows: a FICI $\leq$ 0.5 indicated synergy, whereas a FICI between 0.5 and 1 indicated additive effect (European Committee on Antimicrobial Susceptibility Testing 2000). All combinations reported to have a synergistic or an additive effect were done in triplicate.

### Hemolysis assay

The hemolytic activity of morpholinium-based ionic liquids was evaluated for washed erythrocytes and whole blood samples. Human blood was centrifuged at 800 g for 10 min and washed three times with PBS (pH 7.4) to prepare erythrocytes. After washing, erythrocytes were suspended in PBS at a final concentration of 2% (v/v). For whole blood samples, the washing procedure was skipped, and the samples were diluted with 1 vol of PBS prior to the assay. A two-fold serial dilution of 10 μL of ionic liquids was performed on the microplate, and 90 μL of either washed erythrocytes or whole blood was distributed into wells. 1% Triton X-100 and PBS were used as positive and negative controls, respectively. Plates were incubated for 1 h at 37 °C and then centrifuged at 800 g for 10 min. Subsequently, 60 μL of supernatant were collected from each well and transferred into a new plate. Sample absorbance was measured on a microplate reader at 570 nm. Hemolysis in Triton X-100 and PBS was considered as 100% and 0%, respectively, and the percentage of hemolysis was calculated using the following formula:

$$Hemolysis[\%] = \frac{A_{570ILsample} - A_{570PBS}}{A_{570TritonX-100} - A_{570PBS}} \times 100\%$$

### Statistical analysis

All biofilm samples were tested in at least nine replicates. Control samples were tested in at least 30 replicates. Only samples with $OD_{600} > 0.1$ were used for data analysis. Statistical significance of differences in biofilm formation between strains was tested using the Kruskal-Wallis test followed by Dunn's test with a Benjamini-Hochberg adjustment of the *p*-value. A value of $p < 0.05$ was considered statistically significant. Differences in biofilm formation were expressed as the ratio of the mean of the treated sample to the mean of the untreated control with a 95% confidence interval. Confidence intervals were constructed using the bias-corrected and accelerated (BCa) method after bootstrapping with 10.000 resamples. Samples were considered significantly different from the control if the confidence interval for their ratio did not include 100%.

## Author Contribution Statement

JM, TC, DN, PvO and ER conceived and designed research. AS was responsible for the synthesis of ionic liquids. JM conducted experiments and performed data collection and analysis. The first draft of the manuscript was written by JM. All authors assisted in reviewing and editing of the final draft. All authors read and approved the manuscript.

## Data Availability

The data generated during the current study are available from the corresponding author on reasonable request.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

## CRediT authorship contribution statement

**Jakub Michalski:** Investigation. **Tomasz Cłapa:** Writing – review & editing, Validation, Methodology, Funding acquisition, Conceptualization. **Dorota Narożna:** Conceptualization. **Anna Syguda:** Resources. **Peter van Oostrum:** Conceptualization. **Erik Reimhult:** Conceptualization, Data curation, Writing – review & editing.

## DECLARATION OF COMPETING INTEREST

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.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2024.168627.

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## Further reading

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**Supplementary material**

# Morpholinium-based ionic liquids as potent antibiofilm and sensitizing agents for the control of *Pseudomonas aeruginosa*

Jakub Michalski[1], Tomasz Cłapa[1*], Dorota Narożna[1], Anna Syguda[2], Peter van Oostrum[3], Erik Reimhult[3]

[1]Poznań University of Life Sciences, Department of Biochemistry and Biotechnology, Dojazd 11, 60-632 Poznan, Poland

[2]Poznan University of Technology, Department of Chemical Technology, Berdychowo 4, 60-965 Poznan, Poland

[3]University of Natural Resources and Life Sciences, Department of Bionanosciences, Institute of Biologically Inspired Materials, Vienna, Muthgasse 11-II, A-1090 Vienna, Austria

*Corresponding author: tomasz.clapa@up.poznan.pl

**Supplemental Table S1** List of all ionic liquids used in the study

| Abbreviation | Acronym | Name |
|---|---|---|
| **I** | [DecEtMor] [2,4-D] | 4-decyl-4-ethylmorpholinium 2,4-dichlorophenoxyacetate |
| **II** | [DecEtMor] [4-CPA] | 4-decyl-4-ethylmorpholinium 4-chlorophenoxyacetate |
| **III** | [DecEtMor] [Clopyralid] | 4-decyl-4-ethylmorpholinium 3,6-dichloro-2-pyridinecarboxylate |
| **IV** | [DecEtMor] [Dicamba] | 4-decyl-4-ethylmorpholinium 3,6-dichloro-2-methoxybenzoate |
| **V** | [DecEtMor] [MCPA] | 4-decyl-4-ethylmorpholinium 4-chloro-2-methylphenoxyacetate |
| **VI** | [DecEtMor] [MCPP] | 4-decyl-4-ethylmorpholinium (±)-2-(4-chloro-2-methylphenoxy)propionate |
| **VII** | [$Dec_2$Mor] [2,4-D] | 4,4-didecylmorpholinium 2,4-dichlorophenoxyacetate |
| **VIII** | [$Dec_2$Mor] [4-CPA] | 4,4-didecylmorpholinium 4-chlorophenoxyacetate |
| **IX** | [$Dec_2$Mor] [Clopyralid] | 4,4-didecylmorpholinium 3,6-dichloro-2-pyridinecarboxylate |
| **X** | [$Dec_2$Mor] [Dicamba] | 4,4-didecylmorpholinium 3,6-dichloro-2-methoxybenzoate |
| **XI** | [$Dec_2$Mor] [MCPA] | 4,4-didecylmorpholinium 4-chloro-2-methylphenoxyacetate |
| **XII** | [$Dec_2$Mor] [MCPP] | 4,4-didecylmorpholinium (±)-2-(4-chloro-2-methylphenoxy)propionate |

**Supplemental Table S2** List of all antibiotics used in the study with mechanism of action and drug family outlined

| Mechanism of action | Antibiotic group | Antibiotic |
|---|---|---|
| **Inhibition of DNA replication** | Fluoroquinolones | Ciprofloxacin |
| | | Levofloxacin |
| | | Ofloxacin |
| **Inhibition of protein synthesis** | Aminoglycosides | Amikacin |
| | | Tobramycin |
| **Inhibition of cell wall biosynthesis** | Monobactams | Aztreonam |
| | Cephalosporins | Cefoperazone |
| | | Ceftazidime |
| | Carbapenems | Meropenem |
| | Penicillins | Piperacillin |
| **Cell membranes disruption** | Polymixins | Colistin |

**Supplemental Table S3** Minimum inhibitory concentration (MIC) of eleven antibiotics determined for four Pseudomonas aeruginosa strains: PAO1, LESB58, 39016 and UCBPP-PA14

| Antibiotic | PAO1 | LESB58 | 39016 | UCBPP-PA14 | MIC breakpoint |
|---|---|---|---|---|---|
| **Amikacin** | 9.3 (4-16) | 125 R (125-125) | 10.7 (8-16) | 8 (8-8) | 16 |
| **Aztreonam** | 10.7 (8-16) | 250 R (250-250) | 26.7 R (16-32) | 26.7 R (16-32) | 16 |
| **Cefoperazone** | 12 (4-16) | 128 (128-128) | 32 (32-32) | 16 (16-16) | ND |
| **Ceftazidime** | 128 R (128-128) | 256 R (256-256) | 16 R (16-16) | 16 R (16-16) | 8 |
| **Ciprofloxacin** | 0.25 (0.25-0.25) | 7.5 R (7.5-7.5) | 0.5 (0.25-1) | 1.3 R (1-2) | 0.5 |
| **Colistin** | 16.7 R (10-20) | 66.7 R (50-100) | 13.3 R (10-20) | 11.7 R (5-20) | 4 |
| **Levofloxacin** | 2 (2-2) | 8.3 R (6.25-12.5) | 2.25 R (1-4) | 1.3 (1-2) | 2 |
| **Meropenem** | 2.1 (1.25-2.5) | 5 (5-5) | 2.1 (1.25-2.5) | 0.625 (0.625-0.625) | 2*/8 |
| **Ofloxacin** | 2 (2-2) | 17.3 R (16-20) | 2 (2-2) | 0.5 (0.5-0.5) | 4 |
| **Piperacillin** | 18.7 R (8-32) | 21.3 R (16-32) | 32 R (32-32) | 13.3 (8-16) | 16 |
| **Tobramycin** | 2 (2-2) | 21.3 R (16-32) | 4 R (4-4) | 2.7 R (2-4) | 2 |

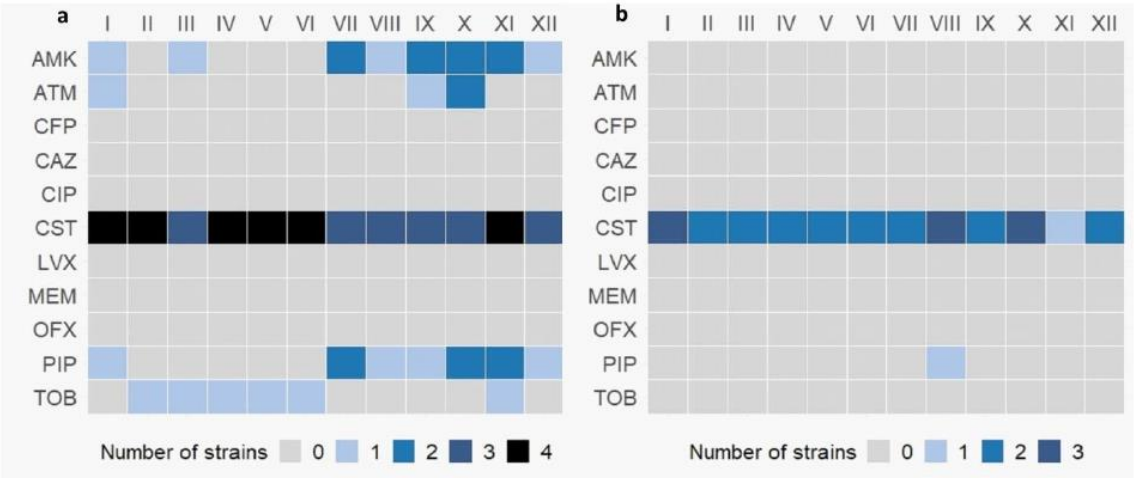
Note: MIC values [µg/mL] presented are the mean of three replicates. The lowest and highest MIC values obtained for each antibiotic are given in parentheses. MIC breakpoints given in the last column indicate the cut-off value for resistance to the corresponding agent. These values are taken from the European Committee on Antimicrobial Susceptibility Testing document "Breakpoint tables for interpretation of MICs and zone diameters" (European Committee on Antimicrobial Susceptibility Testing 2023)

* - indication for meningitis; R - strain resistant to the corresponding antibiotic; ND - no data available

**Supplemental Table S4** Worksheet for susceptibility profiling of four *Pseudomonas aeruginosa* strains

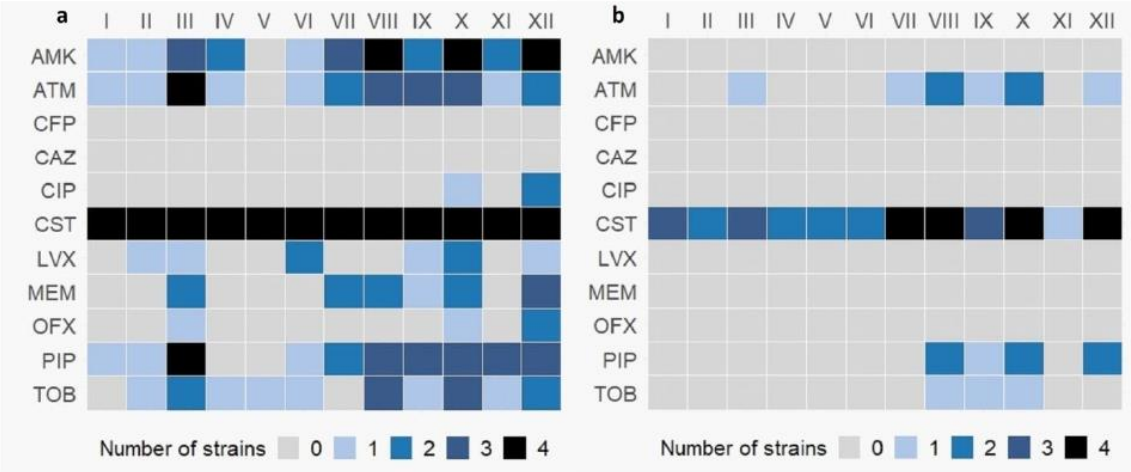
| Antimicrobial category | Antimicrobial agent | PAO1 | LESB58 | 39016 | PA14 |
|---|---|---|---|---|---|
| **Aminoglycosides** | Gentamicin | - | - | - | - |
| | Tobramycin | S | R | R | R |
| | Amikacin | S | R | S | S |
| | Netilmicin | - | - | - | - |
| **Antipseudomonal carbapenems** | Imipenem | - | - | - | - |
| | Meropenem | S | S | S | S |
| | Doripenem | - | - | - | - |
| **Antipseudomonal cephalosporins** | Ceftazidime | R | R | R | R |
| | Cefepime | - | - | - | - |
| **Antipseudomonal fluoroqinolones** | Ciprofloxacin | S | R | S | R |
| | Levofloxacin | S | R | R | S |
| **Antipseudomonal penicillins + β-lactamase** | Ticarcillin-clavula acid | - | - | - | - |
| | Piperacillin-tazobactam | R* | R* | R* | S* |
| **Monobactams** | Aztreonam | S | R | R | R |
| **Phosphonic acids** | Fosfomycin | - | - | - | - |
| **Polymyxins** | Colistin | R | R | R | R |
| | Polymyxin B | - | - | - | - |
| **Resulting resistance phenotype** | | **Not MDR/ possible MDR** | **MDR/ possible XDR** | **MDR/ possible XDR** | **MDR/ possible XDR** |

MDR - resistant to ≥1 agent in >3 antimicrobial categories; XDR - resistant to ≥1 agent in all but ≤2 categories; PDR: resistant to all antimicrobial agents listed (Magiorakos et al. 2012). R- resistant; S- susceptible; - not tested; *- result for piperacillin alone




**Supplemental Fig. S1** Results of the synergy assay summarized for all four *Pseudomonas aeruginosa* strains combined with some combinations excluded based on the results of hemolysis assay on washed erythrocytes. Ionic liquids are arranged into columns and antibiotics into rows. The number of strains with at least additive (a) or synergistic (b) effect observed is marked for each combination. Combinations for which no interaction was observed in any strain are shown in grey. AMK –

amikacin; ATM – aztreonam; CFP – cefoperazone; CAZ – ceftazidime; CIP – ciprofloxacin; CST – colistin; LVX – levofloxacin; MEM – meropenem; OFX – ofloxacin; PIP – pieracillin; TOB – tobramycin




**Supplemental Fig. S2** Results of the synergy assay summarized for all four *Pseudomonas aeruginosa* strains combined with some combinations excluded based on the results of hemolysis assay on whole blood samples. Ionic liquids are arranged into columns and antibiotics into rows. The number of strains with at least additive (a) or synergistic (b) effect observed is marked for each combination. Combinations for which no interaction was observed in any strain are shown in grey. AMK – amikacin; ATM – aztreonam; CFP – cefoperazone; CAZ – ceftazidime; CIP – ciprofloxacin; CST – colistin; LVX – levofloxacin; MEM – meropenem; OFX – ofloxacin; PIP – pieracillin; TOB – tobramycin

## References

European Committee on Antimicrobial Susceptibility Testing (2023) Breakpoint tables for interpretation of MICs and zone diameters

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18:268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x