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In silico and *in vitro* studies of a number PILs as new antibacterials against MDR clinical isolate *Acinetobacter baumannii*

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ABSTRACT

QSAR analysis of a set of previously synthesized phosphonium ionic liquids (PILs) tested against gram-negative multi-drug resistant clinical isolate *Acinetobacter baumannii* was done using the Online Chemical Modeling Environment (OCHEM). To overcome the problem of overfitting due to descriptor selection, five-fold cross-validation with variable selection in each step of the model development was applied. The predictive ability of the classification models was tested by cross-validation, giving balanced accuracies (BA) of 76-82%. The validation of the models using an external test set proved that the models can be used to predict the activity of newly designed compounds with a reasonable accuracy within the applicability domain (BA = 83-89%). The models were applied to screen a virtual chemical library with expected activity of compounds against MDR *Acinetobacter baumannii*. The eighteen most promising compounds were identified, synthesized and tested. Biological testing of compounds was performed using the disc diffusion method in Mueller-Hinton agar. All tested molecules demonstrated high anti-*A. baumannii* activity and different toxicity levels. The developed classification SAR models are freely available online at http://ochem.eu/article/113921 and could be used by scientists for design of new more effective antibiotics.

Keywords:

- Phosphonium ionic liquids (PILs)
- Antibacterial activity
- *Acinetobacter baumanii*
- Machine learning
- OCHEM

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1. Introduction

It is known that the persistent change of infectious human pathogens is connected with widespread and uncontrolled use of antibiotics, the lack of vaccination, a significant number of individuals with immune-mediated diseases, expansion of migration processes in society, development of new technologies, etc. In addition, a large number of resistant strains of microorganisms occur as a result of spontaneous mutations of the bacterial genome, alteration of the target or binding site, alteration of the metabolic pathway, drug inactivation or modification, etc.(Davies et al., 2010) Since bacterial resistance to antibiotics is inevitable, researchers must respond with innovative strategies to discover and develop novel drug candidates.

Gram-negative bacteria *Acinetobacter spp.* are one of the most dangerous multidrug-resistant (MDR) ESKAPE pathogens for the human population.(Rice, 2010) In particular, different kinds of hospital-acquired infections caused by opportunistic microbe *A. baumannii* have increased in recent years. It is known that *A. baumannii* isolates are able to form biofilms on the surfaces of surgical instruments, medical devices, implants, etc.(Asif et al., 2018) It should also be noted that *Acinetobacter* strains were widely detected in acute wound infections of patients in military conflicts in Iraq, Afghanistan, Ukraine, and other countries.(Khan et al., 2018; O'Toole et al., 2019)

A. baumannii strains are capable of colonising skin, mucosal and epithelial surfaces, and most often affect the respiratory, circulatory and urinogenital systems.(Doi et al., 2015; Eliopoulos et al., 2008) Senile age, alcoholism, heavy smoking, diabetes mellitus, severe injuries, extensive burns, maligned neoplasms, broad surgical interventions, radiation, hormonal and cytostatic therapy, newborns pathology, immunodeficiency syndrome, chronic obstructive pulmonary disease and renal disease are the risk factors of *A. baumannii* infections.(Visca et al., 2011) The mortality rate from the diseases of the blood system caused by MDR *A. baumannii* is 49%, and from resulting central nervous system damage is 70%. According to the literature, there are cases of *A. baumannii* infection as a result of procedures such as catheterization, lumbar puncture, myelography and ventriculography, intrathecal infusions or spinal anaesthesia, gastrointestinal endoscopy, etc.(Doi et al., 2015; Eliopoulos et al., 2008; O'Toole et al., 2019)

Acinetobacter spp. are usually highly resistant to a number of commonly used antibiotics, including ampicillin, carbenicillin, cefoxitin, gentamicin, chloramphenicol.(Seifert et al., 1993; Vila et al., 1993) The multidrug resistance of these bacteria is mediated by all of the major

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resistance mechanisms that are known to occur in bacteria. These include the modification of target sites (the methylation of ribosomal RNA), enzymatic inactivation (β-lactamases, aminoglycosidases, tetraciclinases), and the slowing of drug influx and direct active efflux.(Dijkshoorn et al., 2007) Thus, extensive research and the development of new antibacterials for the prevention or treatment of MDR *Acinetobacter* infection are highly relevant and necessary.

Phosphonium liquids/salts (PILs) are a promising class of pharmacologically active organophosphorus compounds. They are used for imaging and diagnosing tumors, and also as anticancer,(Madar et al., 2007) antioxidant,(Sheu et al., 2006) antiviral and antiparasitic agents.(Spivak et al., 2014) PILs are also used extensively to fight bacterial and fungal infections,(Trush et al., 2018) and additionally as biocides and antifouling agents.(Joseph et al., 2016) One of the advantages of phosphonium compounds is the high thermal and chemical stability they present due to the absence of acidic protons in their structure.(Fraser et al., 2009) Moreover phosphonium liquids, in general, are less toxic and more environmentally friendly compared to, for example, ammonium salts.(Kumar et al., 2009)

The main goal of this study was to investigate the anti-*A. baumanii* activity of a number of PILs based on Structure Activity Relationship studies(Dearden, 2016) derived using computational models developed by the On-line CHemical Database and Modeling Environment (OCHEM).(Tetko et al., 2017)

2. Methods and Materials

2.1. QSAR modeling

2.1.1. Data

A series of 263 compounds and their bioactivities against *A. baumannii* were collected from the literature.(Cheng et al., 2010; Singh et al., 2015; Sun et al., 2011) The compounds had MIC values ranging from 0.137 to 900 μM. All compounds in this set were unique. The collected data are freely accessible under the CC-BY license on the OCHEM.(Tetko et al., 2017)

2.1.2. On-line Chemical Database and Modeling Environment

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The OCHEM platform was used to develop public and freely accessible models for predicting the antibacterial activity of compounds. The resulting OCHEM models estimate standard mean errors for each prediction and allow users to decide whether the provided predictions are sufficiently accurate for their studies.

Methods. Well-known classical machine-learning methods such as Associative Neural Networks (ASNNs),(Tetko, 2008) k-Nearest Neighbors (kNNs), and WEKA-RF (Random Forest) (Breiman, 2001) were used to build classification QSAR models.

Descriptors. OCHEM supports multiple software packages for calculation of diverse molecular descriptors. For this study we used electrotopological (E-state)(Hall et al., 1995) descriptors and AlogPS,(Tetko et al., 2002) which were frequently the top-performing descriptors in our previous studies. AlogPS estimates lipophilicity and solubility of chemical compounds while E-state descriptors describe their electronic and topological characteristics.

Descriptor preprocessing. The unsupervised filtering of descriptors was used. Descriptors with fewer than two unique variables or with a coefficient of variance, less than 0.01 were excluded. Moreover, descriptors with a pairwise non-parametric Pearson's correlation coefficient $R > 0.95$ were grouped. Additionally, the Unsupervised Forward Selection (UFS) method(Whitley et al., 2000) was used to select a representative non-redundant set of descriptors for model development.

Model validation. Two validation protocols were used. First of all the initial set of 263 compounds was split by chance into training (210 compounds) and test (53) sets. Five-fold Cross-Validation (CV) with variable selection in each step of the analysis was used to estimate the accuracy of the models for the training set.(Tetko et al., 2006) The dataset of 210 compounds was divided into 5 subsets of equal size. Out of each 5-subsets a single subset was retained for validation while the remaining sets were used for training. For each subdivision, we first selected descriptors using the respective training set, developed the model, and then applied it to predict the excluded molecules from the respective validation set. Then the statistical coefficients over the combined five validation sets were computed. The prediction performance of the final model developed with 210 compounds was also tested using the test set of 53 compounds (see Table 1).

Estimation of prediction accuracy. The OCHEM estimates the applicability domain (AD) and the accuracy for each prediction, which are calibrated using CV.(Sushko et al., 2010) A probability of incorrect classification of molecules based on the standard deviation and average prediction of a model class (PROB-STD), which provided the best accuracy for classification

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models as described elsewhere,(Sushko et al., 2010) was used as a distance-to-model. The model AD corresponded to PROB-STD covering 90% of molecules within the training set.

A detailed description of used machine-learning methods, all selected descriptors, and validation procedures can be found in the *Supplementary materials* and the OCHEM manual.

2.2. Biology

2.2.1. Anti-A. baumannii activity

The antibacterial activity of the studied PILs was estimated against gram-negative MDR clinical isolate *A. baumannii* obtained from the Museum of Microbial Culture Collection of the P.L. Shupyk National Medical Academy of Postgraduate Education.

Antibacterial properties were determined by disc diffusion method in Mueller-Hinton agar.(Bauer et al., 1966) A final inoculum concentration of $1 \cdot 10^5$ colony-forming unit (CFU) per mL was established using a 0.5 McFarland turbidity standard and a subsequent dilution of 0.02 ml of the tested compounds was applied to standard paper disks (6 mm) which were placed on agar plate. All compounds were dissolved in 0.1% dimethyl sulfoxide solution (DMSO). The compound content on a disk was 0.3 μM. The studied bacterial culture was not sensitive to DMSO solvent, which was used as a negative control in the experiment. The known antibiotics Ampicillin, Oxacillin and Ceftriaxone were applied as positive control using the same experimental protocol.

The plates were incubated for 24 h at 37 °C. The tests were repeated three times. The antibacterial activity of the tested compounds was identified by measuring zone diameter of the growth inhibition, which indicates the degree of susceptibility or resistance of *A. baumannii* isolate against the test compounds. Inhibition zones were measured in millimeters. The compounds with growth inhibition zones > 15 mm were selected as active.

2.2.2. Acute Daphnia magna toxicity testing

In order to exclude the observed activity of compounds due to nonspecific toxicity, we evaluated the acute toxicity test of the investigated compounds to *Daphnia magna.* LD_{50} was estimated according to the procedures set out in the Organization for Economic Co-operation and

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Development Guideline 202.(OECD, 2014) *D. magna* neonates (6-24 h-old) were used for the controls and for the geometric series of concentrations of each test compound. The mortality of the neonates was observed after 48 h. The LD_{50} values in mg/L with their 95% confidence intervals (CI) were determined using Statistica 7 program. All experiments were run in triplicate. The sensitivity of *D. magna* to the reference toxicant potassium dichromate ($K_2Cr_2O_7$) was determined as well.

3. Results and discussion

3.1. Models

The activity data were collected from different sources and thus could have some variations due to differences across laboratory protocols. In order to decrease this variation we decided to develop classification models. All compounds were assigned to active (147 compounds with MIC $\leq$ 50 μM) and inactive (116, MIC > 50 μM) classes. The classification models were developed using the protocols described in section ***2.2***.

Table 1. Statistical coefficients calculated for classification models.

N	Method	Specificity (%)		Sensitivity (%)		Balanced Accuracy (%)	
		Training	Test	Training	Test	Training	Test
1	ASNN	73	85	78	88	76 ± 3	87 ± 4
2	WEKA-RF	84	81	82	85	81 ± 3	83 ± 5
3	kNN	80	92	82	86	81 ± 3	89 ± 4
4	Consensus[b]	83	81	83	85	82 ± 3	83 ± 5

[a]The training and test sets included 210 and 53 molecules, respectively. The cross-validation results are reported for the training set [b]The consensus model was built by averaging outputs of all individual models.

All models had similar performance in terms of sensitivity, specificity and balanced accuracy (BA) as summarized in Table 1 (see also **Fig. 1S** of the *Supplementary materials)*. The BAs for the training sets were in the range of 76-82 % (Table **1**). The compounds in the test sets were predicted with similar accuracies: *BA* = 83-89%. The statistical parameters for both training

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and test sets have large standard mean errors due to small dataset sizes. Despite some variations, the model performances for both these sets were not significantly different.

A consensus model was built as a simple average of the three models. Despite its statistical parameters being similar to those of individual models (see Table **1**), the consensus model was expected to be have a smaller variance. Moreover, the predictions of the consensus model were used to estimate its applicability domain.(Sushko et al., 2010)

3.2. Evaluation activity of new compounds

A virtual set of drug-like compounds was generated based on available synthetic blocks and reactions. It included 41 compounds with different substitution patterns (Table 2S in *Supplementary materials*,). 14 compounds predicted as inactive as well as three compounds predicted outside of the AD of the model were excluded. The remaining 24 compounds were evaluated for synthetic feasibility by organic chemists, as well as for absence of potential toxicity (mutagenicity, tumorigenicity, irritation and reproductive effectiveness) using the DataWarrior software (Datawarrior, 2019). As a result of this analysis, 18 compounds were selected for synthesis and testing (**Tables 1 and 1S** in *Supplementary materials*). The biological testing (Table 2) confirmed their biological activity.

3.3. *Chemistry*

Oxazoles **1-17** were synthesized according to the known methods(Abdurakhmanova et al., 2017; Brovarets et al., 2001; Van Meervelt et al., 1995) from the starting amides and trichloroacetaldehyde (*Schema 1*). Obtained *N*-(2,2,2-trichloro-1-hydroxyethyl)amides were treated with thionyl chloride to form *N*-(1,2,2,2-tetrachloroethyl)amides. These compounds were converted into phosphonium chlorides by reaction with triphenylphosphine running in benzene by heating to 70-80 °C. Sequential interaction of phosphonium salts with an excess of sodium hydrosulfide and alkyl iodide leads to the formation of 5-alkylthio-1,3-oxazol-4-ylphosphonium salts **1-17** (*Scheme 1*). The structures of all obtained phosphonium salts **1-17** were in accordance with data of elemental analysis, ^{1}H, ^{13}C, ^{31}P NMR, mass-spectrometry.

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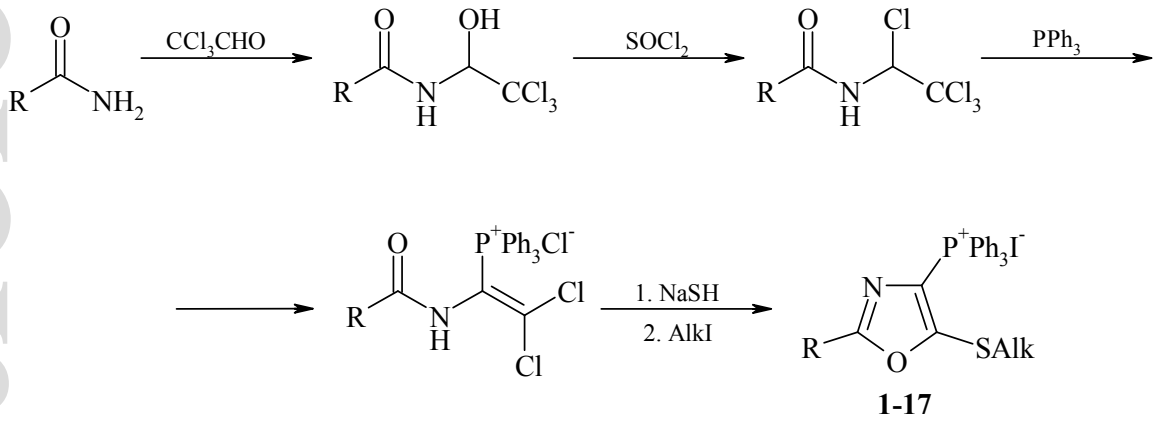

Scheme 1. General synthesis of compounds **1-17**
R = CH_3 (**5**, **8**, **11**), CH_2F (**9**), $C(CH_3)_3$ (**14**), C_6H_5 (**4**, **7**, **13**, **17**), 4-$CH_3C_6H_4$ (**15**), 2-ClC_6H_4 (**6**), 4-ClC_6H_4 (**1**, **3**, **16**), 2,4-ClC_6H_3 (**12**), furan-2-yl (**2**, **10**); AlkS = CH_3S (**3**, **7**, **14**), C_2H_5S (**2**, **6**, **8**, **9**, **13**, **15**, **16**), C_3H_7S (**1**, **5**), C_4H_9S (**4**), CH_2=$CHCH_2S$ (**10-12**,**17**).

Particular attention was paid to ^{13}C NMR data. These spectra were remarkable as signals of a 1,3-oxazole ring appear as doublets due to the spin-spin coupling with the phosphorus atom. Thus, C^5 and C^2atoms resonate at 156.4-174.2 ppm as doublets with coupling constants of 18.9-29.4 Hz. The signal of the C^4 is a doublet at 115.3-128.5 ppm with spin-spin coupling constants of 122.5-146.1 Hz.

The signal of phosphorus nuclei in ^{31}P NMR spectra of compounds is observed at 9.80-13.21 ppm.

The detailed description of the chemical synthesis of selected compounds (**Table 1S**) was provided in the *Supplementary materials*.

3.4. Biology

The results of biological activity are summarized in **Table 2**.

Table 2. Antibacterial activity and acute toxicity of the studied PILs

Compound	The inhibition zones diameters (mm) of MDR clinical isolate *A. baumannii* culture	Lethal doses of compounds to *D. magna* in 48-h acute toxicity bioassays		
		LD_{50} (mg/L)	95% confidence	Toxicity classification[a]

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			intervals	(by Passino and Smith)
1	29	0.12 ± 0.04	0.03-0.2	++++
2	30	2.0 ± 0.6	0.88-3.18	+++
3	24	0.11 ± 0.03	0.05-0.18	++++
4	28	0.15 ± 0.05	0.05-0.25	++++
5	30	2.8 ± 0.7	1.28-4.25	+++
6	27	0.18 ± 0.05	0.08-0.29	++++
7	29	0.36 ± 0.09	0.18-0.55	++++
8	28	3.1 ± 0.6	1.9-4.36	+++
9	25	9.4 ± 2	4.72-14.01	+++
10	29	1.54 ± 0.4	0.72-2.36	+++
11	28	2.8 ± 0.5	1.69-3.92	+++
12	22	0.06 ± 0.02	0.028-0.097	+++++
13	31	0.12 ± 0.04	0.032-0.19	++++
14	28	0.7 ± 0.2	0.4-1.06	++++
15	32	0.6 ± 0.2	0.33-0.93	++++
16	30	0.13 ± 0.04	0.046-0.22	++++
17	32	0.23 ± 0.05	0.11-0.34	++++
18	28	0.23 ± 0.05	0.12-0.34	++++
Ampicillinum	NA[b]	ND[c]	ND	ND
Oxacillinum	NA	ND	ND	ND
Ceftriaxone	15	ND	ND	ND

[a]Toxicity classification and LD_{50} range: "+" - practically harmless (100–1000 mg/L); "++" - slightly toxic (10–100 mg/L); "+++" - moderately toxic (1–10 mg/L); "++++" - highly toxic (0.1–1mg/L); "+++++" - extremely toxic (0.01–0.1mg/L). [b]NA – no activity; [c]ND – not determined.

All synthesized PILs exhibited high activity against MDR clinical isolate *A. baumannii* with diameters of inhibition zones ranging from 22 to 32 mm (Table 2) while Ampicillin, Oxacillin and

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Ceftriaxone antibiotics were inactive. The acute toxicity of PILs ranged from 0.063 to 9.36 mg/L. According to the D.R. Passino classification six (33%) compounds were classified as moderately toxic (1–10 mg/L), 11 (61%) were highly toxic (0.1–1 mg/L) and only one compound was extremely toxic (0.01–0.1 mg/L). However, despite the analysed compounds having different toxicity classes, all of them exhibited strong *in vitro* activity against *A. baumannii.* Thus their inhibitory activity was specific and was not connected to their toxicity.

4. Conclusion

We investigated antibacterial activity of new PILs. Firstly, predictive *in silico* models based on different machine learning techniques were built using the OCHEM platform. The created classification models demonstrated good stability, robustness and predictive power. Eighteen new compounds were synthesized and their activity against *A. baumanii* MDR isolate was evaluated. All PILs showed high activity against MDR clinical isolate *A. baumannii*. According to the acute toxicity results all tested PILs belonged to moderately and highly toxic classes, and only one substance was classified as extremely toxic. Therefore, the activity of the analyzed structures against *A. baumannii* was not related to their toxicity and these structures need to be further investigated as promising antibacterial agents. The developed models (http://ochem.eu/article/113921) can be used by scientific community to search for new antibiotics against MDR *A. baumannii* strains.

Declaration of interest

IVT is CEO of BIGCHEM GmbH, which licenses the OCHEM software. Other authors declare no conflict of interest.

Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/XXXX.

Data Availability Statement

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All data used in this article are publicly available under CC-BY license at http://ochem.eu/article/113921.

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