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Antimicrobial screening of a historical collection of over 140 000 small molecules

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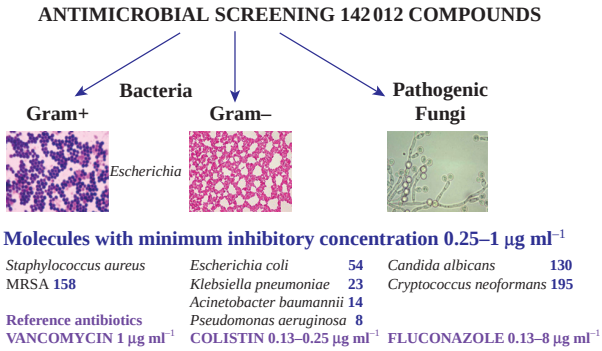
Most of the chemical compound collection (currently about 200 000), assembled at the N. D. Zelinsky Institute of Organic Chemistry over the past 30 years, has been screened for antimicrobial activity against five ESKAPE pathogens and two fungi at the University of Queensland on a charitable basis. A total of 2517 active molecules (MIC ⩽ 32 μg ml^{-1}) were found, of which about 10% are active at very low concentrations (MIC ⩽ 1 μg ml^{-1}). Structures of 142 012 compounds and experimental data on their antimicrobial activity are publicly available through the demo version of the CheD software and the public database of the Community for Open Antimicrobial Drug Discovery.



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The 'superbug crisis' is a global problem that requires a global solution. Although infectious diseases remain the second leading cause of death worldwide, the number of new antibiotics approved has fallen from 16 to only two per year over the past 25 years.[1] Moreover, the number of large pharmaceutical companies conducting antibiotic research has decreased from 18 in 1990 to four in 2020. The increase in multidrug-resistant bacteria and the decline in treatment efficacy have led to a 'superbug crisis' that costs more than 35 billion dollars a year in the United States alone.[2] The COVID-19 pandemic, complicated by a secondary bacterial infection, exacerbates the 'superbug crisis' and shows how dangerous a global pandemic can be. The antibiotic pipeline is broken.[3] Numerous publications show how few new antibiotics are under clinical development.[4–6] This shortage of new antibiotics is caused by a lack of financial viability,[7] the collapse of research in pharmaceutical companies and the depletion of the chemical diversity contained in pharmaceutical libraries, most of which have already been tested for antimicrobial activity.

In many countries, including Russia, hundreds of thousands of new compounds are synthesized annually, which remain unexplored due to the lack of modern robotic equipment, funding and joint research of chemists and biologists and other reasons. This communication informs about the methodology for the search for antimicrobial substances in connection with the possibility of free screening in international cooperation. This research has led to experimental databases on the antimicrobial and antifungal activity of more than 140 000 compounds against five key bacteria and two fungi which are publicly available.

Over the past 30 years, the N. D. Zelinsky Institute of Organic Chemistry has assembled a constantly growing collection of chemical compounds (currently about 200 000) for screening various types of biological activity.[8] Compounds are tracked using barcode storage in a stock room with an online database that allows searches by structural fragments for both molecules and associated NMR and mass spectra using the domestic CheD software.[9] This efficient program, running on a personal computer, allows one to store, retrieve and process chemical information. It was developed at the Institute of Physiologically Active Compounds of the Russian Academy of Sciences. The program can work as a stand-alone application or together with a specially written Web server application. It can also interact with some standard SQL servers such as Oracle, Interbase and MS SQL. CheD has an open architecture; thus, custom data types, controls and services can be added. The www-server chemical data retrieval application is easy and convenient to install on Windows platforms.

Chemists from the N. D. Zelinsky Institute of Organic Chemistry and biologists from the University of Queensland have joined forces to find new classes of antimicrobial molecules and lead generation[10,11] on a charitable basis. For this purpose, a non-profit initiative, the Community for Open Antimicrobial Drug Discovery (CO-ADD), was established in 2015 to conduct a global international search for new antimicrobial drugs from a previously untapped source: a repository of chemically diverse compounds designed and synthesized by academic chemists. The goal of CO-ADD is to 'crowdsource' new compounds to fight drug-resistant infections by reaching out to chemists who have many compounds on their shelves that were not designed as antibiotics and would not otherwise be tested for antimicrobial activity. CO-ADD provides unencumbered, free access to this testing, making it as easy as possible for chemists to participate in the

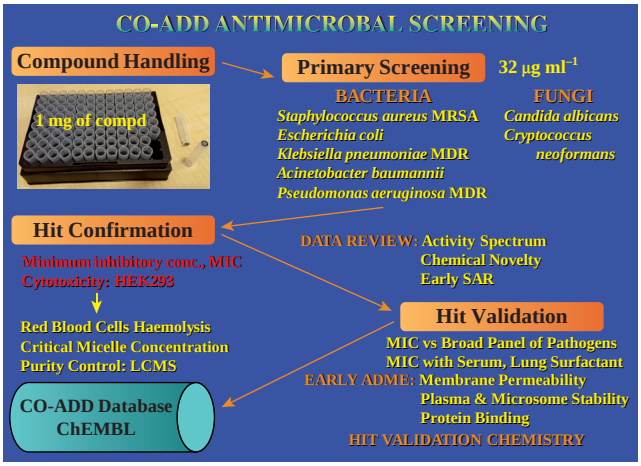

Figure 1 Flow chart for antimicrobial screening at the Institute for Molecular Bioscience, the University of Queensland.

discovery of antibiotics. CO-ADD's public good service nature was initially possible due to 5 years of operational funding from the Wellcome Trust (UK) and The University of Queensland (Australia). The Wellcome Trust is an independent international charity based in London (UK) that funds biomedical research with working capital of over 20 billion pounds sterling.[12]

To date, CO-ADD has tested more than 300 000 individual compounds from over 300 academic groups in 47 countries. CO-ADD antibacterial screening is a free service for academic research groups. The only requirement for submission is that all data (structures and screening results, whether positive or negative) will be made available in an open-access database[17] for use by the community, following a confidentiality period that scientists can publish or patent their positive hits. Contributors can publish their results with no CO-ADD authorship, though an acknowledgment is requested.[†]

CO-ADD is conducting initial screening against five key ESKAPE bacteria and two fungi of concern and is creating a growing collection of bacterial and fungal strains for subsequent assays, encompassing reference ATCC strains, a range of clinical isolates from around the world, multidrug-resistant strains and genetically modified strains. Key Gram-negative bacteria of concern include *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*, which, together with Gram-positive *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (MRSA), make up the ESKAPE pathogens that are responsible for two-thirds of all health care-associated infections.[13] Fungal screening which includes fungal resistance is of growing concern, yet there are fewer antifungals than antibacterials in drug development.[14]

The screening facility equipped with high-performance robots can test up to 2000 compounds per week.[15] Primary screening is performed at a single concentration (32 μg ml^{-1}) and provides initial activity data to select compounds for additional detailed screening (Figure 1). The active compounds from the primary screening were tested in dose-response antimicrobial assays to confirm their antimicrobial activity and against mammalian HEK-293 cells and human red blood cells to assess their cytotoxicity and hemolytic activity, respectively. Standard Minimum Inhibitory Concentration (MIC) testing is conducted using a broth microdilution assay in either 96-well plates or 384-well plates in a high-throughput format.

[†] CO-ADD kindly requests contributors to acknowledge CO-ADD when publishing work using the following statement: 'Antimicrobial screening was performed by CO-ADD (The Community for Open Antimicrobial Drug Discovery), funded by the Wellcome Trust (UK) and The University of Queensland (Australia).' and to quote the article from that reference.[10]

Table 1 Overall results of CO-ADD primary screening of N. D. Zelinsky Institute compounds for antimicrobial activity at 32 μg ml^{-1}.

Compounds submitted to screening	Number	Percentage (%)
Total tested	142 012	100
Active at 32 μg ml^{-1} against 5 bacteria and 2 fungi	2 517	1.77
Non-toxic to HEK-293 and human red blood cells	1 552	1.09

The next step in the CO-ADD process[18] is testing promising hit compounds against a broader panel of multidrug-resistant and extensively drug-resistant bacterial strains and clinical isolates and in the presence of various cofactors (such as serum or lung surfactant). This process of hit validation (see Figure 1), which occurs in agreement with the authors, is not considered in this communication. Screening Intellectual property and practical aspects are presented in Online Supplementary Material.

Over the years, CO-ADD has assessed 142 012 Zelinsky Institute compounds in Primary Screening against five ESKAPE pathogens and two CO-ADD fungi at the single concentration (32 μg ml^{-1}). Of these, 2 517 compounds (1.77% of the total number of submitted compounds) exhibited antimicrobial activity (Table 1). The microorganisms that had the highest 'hit' rates were the Gram-positive bacteria *S. aureus* (1 271) and two fungi, *C. albicans* (1 170) and *C. neoformans* (1 459) (Table 2).

The toxicity of all substances was investigated by testing their ability to affect the viability of a mammalian cell line (HEK-293, obtained from human embryonic kidneys) and their ability to cause hemolysis in red blood cells. Of the 2 517 active compounds, there were 1 552 (1.09% of the total library tested) 'non-toxic' substances that did not affect these cells at a concentration of <32 μg ml^{-1} (Table 1); that is, there was at least some selectivity between the concentration at which they inhibit the growth of microorganisms and the concentration of general cell toxicity.

The active compounds from the primary screening were then tested in antimicrobial dose-response assays to confirm their activity. The CO-ADD 384-well plate format utilizes optical density readouts, and the MIC (μg ml^{-1}) of active compounds is defined as the threshold concentration at which bacterial growth is inhibited by 80% after incubation for 18 h (Table 2). Statistically, substances acting on Gram-negative bacteria are much less common than those acting on Gram-positive bacteria, which is also confirmed in our research: active inhibitors of Gram-positive bacteria (0.11% of all compounds) were found about 1.8 times more frequently than those acting on Gram-negative bacteria (0.06%) (Table 2). This statistic is consistent with the fact that Gram-negative bacteria are generally more difficult to kill due to an additional permeability barrier (outer membrane) that reduces the permeation of the compounds and allows for more effective efflux of the permeable compounds.

Table 2 Distribution of N. D. Zelinsky Institute compounds with antimicrobial activity by the level of activity and strains of pathogens.

Type of micro-organism	Strain	MIC/ μg ml^{-1}	Hit number	Hit rate (%)	MIC/ μg ml^{-1}	Hit number	Hit rate (%)
Gram-positive bacterium	MRSA	⩽32	1271	0.9	⩽0.25–1	158	0.11
Gram-negative bacteria	*E. coli*	⩽32	258	0.18	⩽0.25–1	54	0.038
	K. pneumoniae	⩽32	89	0.063	⩽0.25–1	23	0.016
	A. baumannii	⩽32	379	0.27	⩽0.25–1	14	0.01
	P. aeruginosa	⩽32	86	0.061	⩽0.25–1	8	0.006
Fungi	*C. albicans*	⩽32	1170	0.82	⩽0.25–1	130	0.092
	C. neoformans	⩽32	1459	1.03	⩽0.25–1	195	0.137

Table 3 MIC values of clinical antibiotics used as controls in CO-ADD antimicrobial screening.

Microorganism	Clinical antibiotic	MIC/μg ml^{-1}
MRSA	Vancomycin·HCl[a]	1
E. coli	Colistin sulfate	0.125
K. pneumoniae	Colistin sulfate	0.25
A. baumannii	Colistin sulfate	0.25
P. aeruginosa	Colistin sulfate	0.25
C. albicans	Fluconazole	0.125
C. neoformans	Fluconazole	8

[a] Vancomycin is inactive (MIC > 32 μg ml^{-1}) against all Gram-negative strains.

Table 2 shows the number of compounds that inhibit the growth of each microbial strain at effective concentrations (⩽0.25–1 μg ml^{-1}) similar to those of clinical antibiotics used as a control on each plate during assays: vancomycin for Gram-positive bacteria, colistin for Gram-negative bacteria and fluconazole for fungi (their MICs are given in Table 3). The amount of these most potent compounds (MIC ⩽ 0.25–1 μg ml^{-1}) does not exceed 3–10% of the identified hit compounds (MIC ⩽ 32 μg ml^{-1}) for each microbial strain.

An important advantage of the Zelinsky Institute's library of compounds is a high degree of structural diversity, making it possible to find a significant amount of active substances in various biological tests. In Table 4, we present a comparison of screening results for two libraries of approximately the same size: the Zelinsky Institute's library and a library of commercially available 'drug-like' compounds that were screened during the initial establishment of CO-ADD, although the commercial library was tested against only one Gram-negative species (*E. coli*). The Zelinsky Institute's library contains 3.7 and 7.5 times more compounds that are highly active (⩽0.25–1 μg ml^{-1}) against Gram-positive and Gram-negative bacteria, respectively, which confirms CO-ADD's hypothesis that antimicrobial testing limited to 'drug-like' compounds may be one of the reasons why new antibiotics are not being discovered.

Most of the highly active compounds are found among many well-known and rare classes of heterocycles such as flavonoids, benzimidazoles, pyrroles, tetrazoles, triazoles, pyrazoles, thiadiazoles, bicyclic bis-ureas, 3*H*-1,2-dithiol-3-one, selenium-containing heterocycles, pyrazines and pyrimidines. It is interesting to note that among the nitro compounds and derivatives of furazans and furoxans, which are fragments of high-energy substances, many active structures have been found. Some of them are non-toxic. Highly active and non-toxic compounds are also found in the classes of the simplest salts of pyridinium and triphenylphosphonium with linear C_7–C_{10} hydrocarbon substituents.

All experimental data are presented in the antimicrobial database of the Zelinsky Institute, containing 142 012 compounds (together with duplicated results of antimicrobial tests), in a demo version of the CheD program, which can be downloaded free of charge on the specified website.[16] This database allows one to find any set of compounds using the search by structural fragment or activity (MIC) for each strain of bacteria and fungi and different types of toxicity (Figure S1, see Online Supplementary Materials). The latter function allows one to find compounds with a specific activity level. A screenshot of the antimicrobial activity of a compound recorded in the CheD program database[16] is presented in Figure S1.

Table 4 Statistics for two libraries of compounds screened for antimicrobial activity in CO-ADD.

Library of compounds	No. of screened compounds	Rate of non-toxic hits (%)	
		Against Gram-positive bacteria	Against Gram-negative bacteria
At Zelinsky Institute	142 012	0.11	0.06
'Drug-like' commercial, at CO-ADD	144 184	0.03	0.008

Further open access to the data is provided through CO-ADD's public database,[17] which contains all structures and activity data made publicly available by collaborators so far. The database currently contains activity data for 100 000 compounds as of January 2021, including preliminary data from both primary screening and hit confirmation, along with complete data to be processed and released in the future. The database allows the browsing of data with some filtering based on collaborator and activity as a compact summary display of data across multiple strains and cell lines (see Figure S2). Future improvements planned include the ability to search based on structure, although the database can be downloaded as either a structure definition file (SDF) or a comma-separated value (CSV) file containing the chemical structure as a SMILES string. A screenshot of a summary overview of compound structures and associated antimicrobial data in the CO-ADD open-access database[17] is presented in Online Supplementary Materials.

The data obtained will allow chemists searching for antibacterial molecules to find interesting classes of substances and experimentally compare the relationship between structure and activity to optimize them further and create new effective antibacterial agents. It is important to note that the databases contain 'negative' results for inactive compounds, often not included in published data. We hope that this will prevent unnecessary duplication of efforts by scientists re-synthesizing the same compounds, not knowing that they have already been prepared and tested. These databases also provide critical data for scientists building models to predict antimicrobial activity, as they contain a large number of compounds tested under standard conditions and include structural data for inactive compounds.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2021.07.015.

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