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CAGE ARYLSULFONAMIDES AND THEIR ANTIMICROBIAL PROPERTIES

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Abstract

Arylsulfonamides bearing (aza)norbornane, 3-azabicyclo[3.2.1]oct-6-ene and related motifs were selected and evaluated for antimicrobial activity toward five key ESKAPE pathogenic bacteria, one Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (ATCC 43300), four Gram-negative bacteria, *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853) and antifungal activity towards two pathogenic fungal strains *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* var. *Grubii* (H99; ATCC 208821). One compound with 4-nitrobenzenesulfonamide motif demonstrated high activity towards methicillin-resistant *Staphylococcus aureus* (ATCC 43300). The compound VP-4606 has low cytotoxicity towards pseudo-normal cells of HaCaT, Balb/c 3T3 lines, and mitogen-activated lymphocytes isolated from healthy adult peripheral blood of healthy donor up to 2.5 mM.

Keywords: nitrobenzenesulfonamides; (aza)norbornane; 3-azabicyclo[3.2.1]oct-6-ene; cytotoxicity; SwissADME tool

КАРКАСНІ АРИЛСУЛЬФОНАМІДИ ТА ЇХ АНТИМІКРОБНІ ВЛАСТИВОСТІ

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Анотація

Арилсульфонаміди, що містять (аза)норборнан, 3-азабіцикло[3.2.1]окт-6-ен та схожі фрагменти, були відібрані та протестовані на антимікробну активність до п'яти ключових ESKAPE патогенних бактерій, однієї грампозитивної бактерії, стійкої до метициліну *Staphylococcus aureus* (ATCC 43300), чотирьох грамнегативних бактерій, *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606) і *Pseudomonas aeruginosa* (ATCC 27853) і протигрибкову активність відносно двох патогенних штамів *Candida albicans* (ATCC 90028) та *Cryptococcus neoformans* var. *Grubii* (H99; ATCC 208821). Одна сполука з 4-нітрофенілсульфонамідним фрагментом продемонструвала високу активність щодо метицилін-резистентного *Staphylococcus aureus* (ATCC 43300). Сполука VP-4606 має низьку цитотоксичність щодо псевдонормальних клітин ліній HaCaT, Balb/c 3T3 та мітоген-активованих лімфоцитів, виділених із здорової дорослої периферичної крові здорового донора до 2.5 мМ.

Ключові слова: нітрофенілсульфонаміди; (аза)норборнан; 3-азабіцикло[3.2.1]окт-6-ен; цитотоксичність; інструмент SwissADME

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Introduction

In recent years, new infectious diseases have emerged to lead to psychological disorders and threaten people's life, thus affecting socio-economic stability [1–3]. A wide variety of pathogens and the constant emergence of new multidrug-resistant pathogenic strains complicate the treatment and prevention of infectious diseases, as for many of them, there are no successful pharmaceuticals or vaccines [4]. Therefore, the discovery of new antimicrobial drugs is an urgent issue.

Currently, significant progress in the use of cage compounds in the drug discovery has been made [5; 6]. Cage compounds are proposed as bioisosteres for arenes, to reduce the number of the aromatic rings in lead-like molecules [7]. The conformationally diversified compounds, such as piperidine or pyrrolidine alkaloids and proline, tend to show biological activities [8].

In this regard, the synthesis and chemistry of polycyclic cage-like heterocycles bearing (aza)norbornane and related motifs has attracted considerable attention in recent years both from our group and many others [9–20]. As an example, 2-azabicyclo[2.2.1]heptane (azanorbornane) derivatives have also been applied as convenient precursors in the stereoselective synthesis of monocyclic systems useful in medicinal chemistry [8]. Thus, cage-like molecules are attractive for drug discovery as promising antimicrobial agents. Antiviral agent Ledipasvir, diuretic Tripamide and antipsychotic Lurasidone even became marked drugs (Fig. 1).

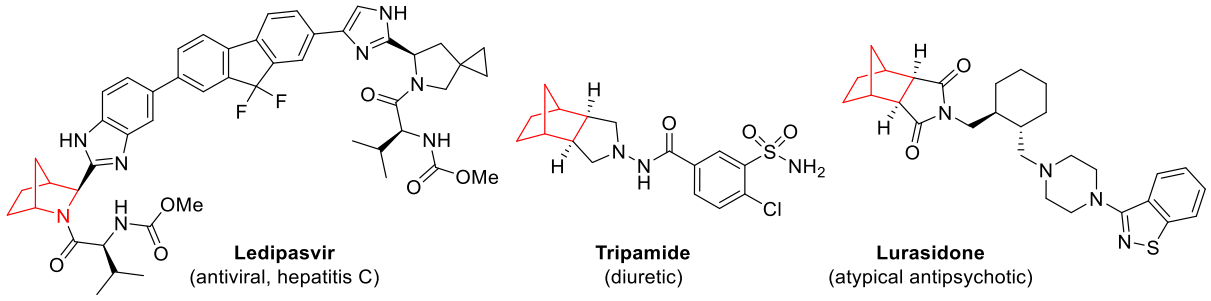


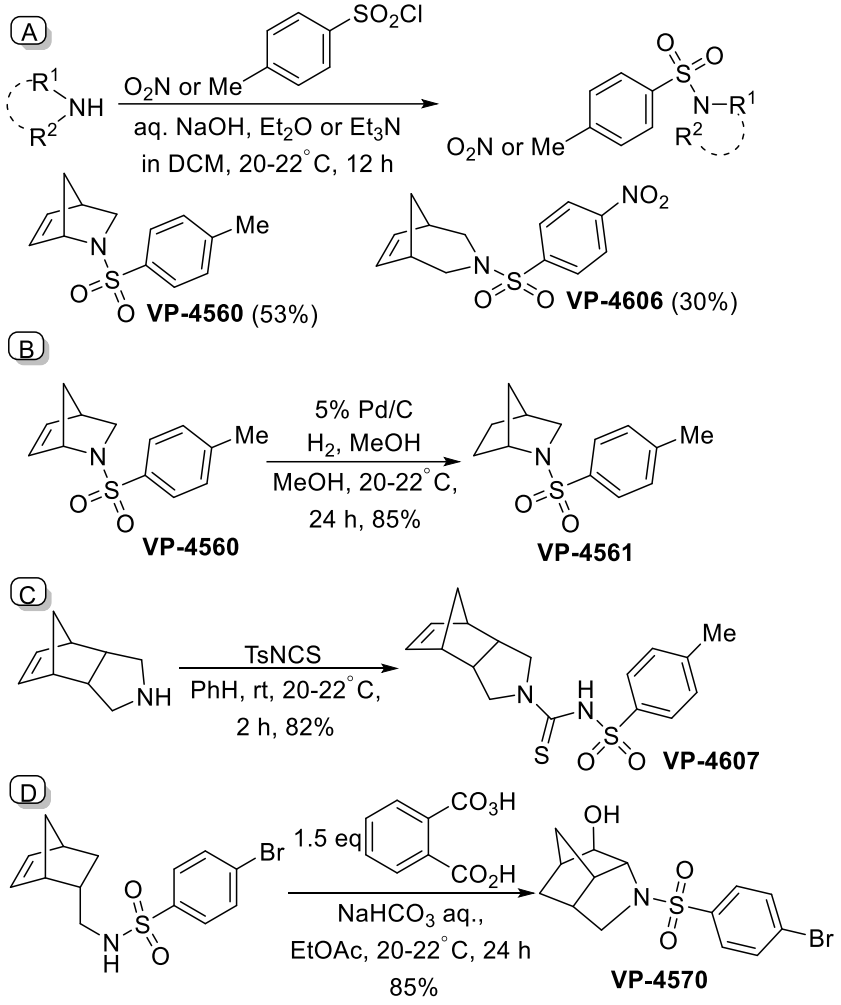
Fig. 1. A representative polycyclic cage-like drugs bearing (aza)norbornane motif (*showed in red*)

Results and discussion

Chemistry

A fragment-oriented approach was chosen for the design of (aza)norbornane compounds for screening for antimicrobial activity. In particular, it is proposed to combine (aza)norbornane with sulfonamide motif, which is a well-known pharmacophore for the discovery of antimicrobial agents [21–23]. Thus, based on previous works of our group a series of cage sulfonamides (**VP-4560, VP-4561, VP-4570, VP-4606** and **VP-4607**) were designed and synthesized as summarized in Scheme 1. At first, sulfonamides **VP-4560** [24] and **VP-4606** [25] were synthesized by sulfonylation of the appropriate cage-like amines with Et_3N in DCM or aq. NaOH in Et_2O (Scheme 1, **A**). The 2-azabicyclo[2.2.1]hept-5-ene **VP-4560** [24] was readily reduced to 2-azabicyclo[2.2.1]heptane **VP-4561** [26] via hydrogenation over palladium (Scheme 1, **B**). Tosyl-thiourea bearing 2,3,3a,4,7,7a-hexahydro-1*H*-4,7-methanoisoindole **VP-4607** was prepared by addition of *p*-toluenesulfonyl isocyanate to the corresponding amine in benzene (Scheme 1, **C**) [27]. Azabrendane **VP-4570** [28; 29] was synthesized via epoxidation followed by intramolecular heterocyclization of the appropriate amide (Scheme 1, **D**).

Chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET), play key roles in drug discovery and development. A high-quality drug candidate should not only have sufficient efficacy against the therapeutic target, but also show appropriate ADMET properties at a therapeutic dose. We performed *in silico* evaluation all the tested compounds using SwissADME tool [30]. Considering druglikeness we can conclude that all compounds match parameters for Lipinski, Ghose, Veber, Egan and Muegge rules. Lipophilicity in terms of Log $P_{octanol/water}$ for our set of compounds is in the range 1.18-2.60. All compounds are soluble or moderately soluble in water, have high gastrointestinal (GI) absorption and no alerts for PAINS filter (Table 1).



Scheme 1. Synthetic routes to the target compounds

Table 1

Druglikeness properties of the tested compounds through SwissADME tool

Code	Lipophilicity Log $P_{o/w}$	GI absorption	Bioavailability	PAINS filters
VP-4560	2.08	high	0.55	0 alert
VP-4606	1.18	high	0.55	0 alert
VP-4561	2.30	high	0.55	0 alert
VP-4607	2.60	high	0.55	0 alert
VP-4570	2.01	high	0.55	0 alert

Thus, our proposed methods for obtaining compounds are convenient, variable and allow rapid optimization of the structure, which is especially valuable in the modern drug discovery.

Biological activity

Antimicrobial screening. The primary screening against 5 key ESKAPE pathogens and 2 fungi were performed by the Community for Antimicrobial Drug Discovery (CO-ADD), funded by the Wellcome Trust (UK) and The University of Queensland Australia [31; 32]. All synthesized compounds were evaluated in concentration 32 μg/mL (approx. 100 μM) for their antimicrobial activity towards five pathogenic bacteria, methicillin-resistant *Staphylococcus aureus* (ATCC 43300) as Gram-positive bacteria and *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853) as Gram-negative bacteria, and antifungal activity towards two pathogenic fungal strains *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* var. *Grubii* (H99; ATCC 208821). The results of two parallel trials are presented in Table 2.

Journal of Chemistry and Technologies, 2022, *30*(1), 1-10

Table 2

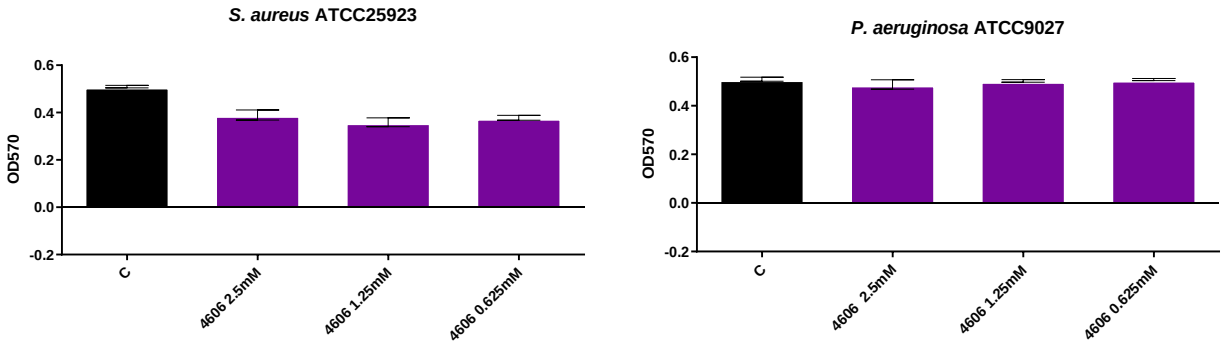
Preliminary screening of arylsulfonamides bearing (aza)norbornane motif

Compound	The percentage of growth inhibition in concentration 32 μg/mL, %						
	S. aureus	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. baumannii*	*C. albicans*	*C. neoformans*
VP-4560	-6.6; 4.6	-3.9; -7.9	-2.8; -4.5	-2.7; -4.7	3.6; 6.2	3.1; 5.4	-0.6; -14.2
VP-4606	**94.3; 97.2**	-0.9; 0.7	1.1; 2.9	-1.4; -2.5	13.2; 16.2	6.5; 9.9	-12.8; -14.0
VP-4561	17.9; 3.9	-5.7; 2.8	0.8; 3.5	-0.4; 5.6	3.5; 5.2	24.9; 28.7	-3.2; 3.1
VP-4607	0.5; 5.8	-3.0; -3.6	-0.9; -1.0	-1.2; -1.5	10.3; 10.6	4.4; 6.7	-30.7; -37.5
VP-4570	-11.2; 6.7	-6.5; 2.9	-5.5; 0.0	-3.8; 1.0	-1.8; -4.0	5.3; 9.9	-3.6; 2.3

It is important to underline the relationship between the structure and activity of the tested compounds. In all cases, compounds with the 4-methyl/bromo-benzenesulfonamide moiety showed low activity. However, compound **VP-4606** with electron acceptor nitro group showed excellent activity against the bacterium *Staphylococcus aureus* (ATCC 43300). At the same time, compound showed low activity towards Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853).

The minimal inhibitory concentration (MIC; μg/mL) measurements were performed for compound **VP-4606** with significant microbial growth inhibition toward *Staphylococcus aureus* (ATCC 43300), using ceftriaxone as a reference drug. However, the MIC of compound **VP-4606** was close to Ceftriaxone and at the same time, its cytotoxic concentration toward HEK-293 (ATCC CRL-1573) cells (CC_{50} = 11.6; 12.8 μg/mL) was lower.

Based on the primary screening results, we checked the antibacterial effect of the most active compound **VP-4606** towards *Staphylococcus aureus* ATCC25923 and *Pseudomonas aeruginosa* ATCC9027 using the MTT bacteria assays. The compound **VP-4606** demonstrated moderate activity toward Staphylococcus aureus ATCC25923 strain (Fig. 2, **A**) and was not active toward Pseudomonas aeruginosa ATCC9027 strain (Fig. 2, **B**) that indicating its selectivity to methicillin-resistant Staphylococcus aureus (ATCC 43300).



A) B)

Fig. 2. Antibacterial effect of studied compounds towards *Staphylococcus aureus* ATCC25923 (A) and *Pseudomonas aeruginosa* ATCC9027 (B). C – control data.

Cytotoxicity

Cytotoxicity of the most active compound **VP-4606** was further evaluated towards human keratinocytes of HaCaT line, murine fibroblasts of Balb/c 3T3 line, and human lymphocytes of healthy donor. Studied compound has low cytotoxicity towards pseudo-normal cells of HaCaT line and Balb/c 3T3 line (Fig. 3). Compound does not reach the CC_{50} value for HaCaT and Balb/c 3T3 cells up to 2.5 mM. At the highest dose of 2.5 mM, compound **VP-4606** inhibited the growth of HaCaT cells by 43.8 %, of Balb/c 3T3 cells – by 32.0 %. Solvent DMSO demonstrated similar toxicity towards HaCaT and

Balb/c 3T3 cells. Doxorubicin was used as a standard positive control drug. Doxorubicin showed higher cytotoxic effect than studied compound **VP-4606** towards HaCaT and Balb/c 3T3 cells with the CC_{50} value of 0.8 μM and 0.9 μM, respectively.

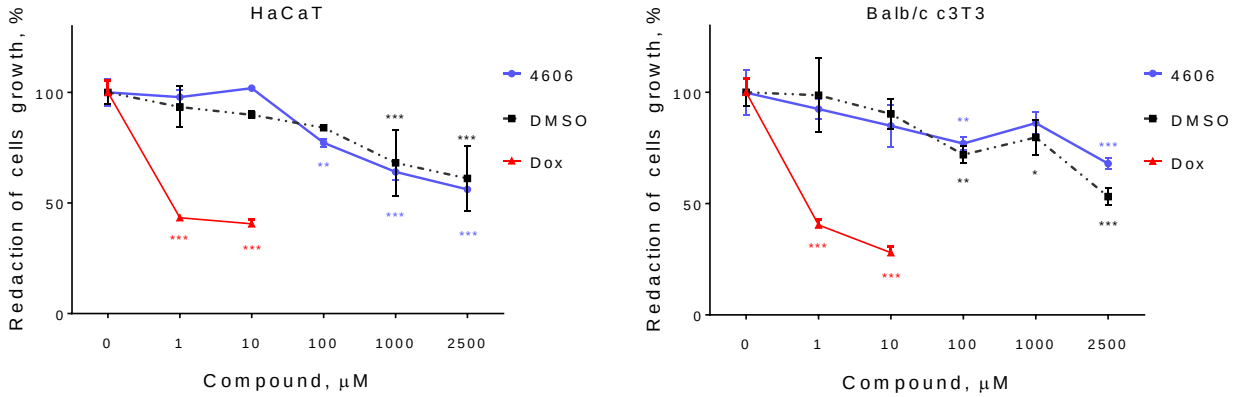


Fig. 3. Cytotoxicity of studied compounds towards human keratinocytes of HaCaT line, and murine fibroblasts of Balb/c 3T3 line. After a total experimental time (72 h), cell vitality was evaluated by the MTT assay. Dox – doxorubicin. * – P ≤ 0.05; – P ≤ 0.01;*** – P ≤ 0.001 (difference compared with the not treated control cells.**

Studied compound **VP-4606** demonstrated low toxicity towards mitogen-activated lymphocytes isolated from healthy adult human peripheral blood. This compound at the dose of 2.5 mM inhibited the growth of lymphocytes by 37.4 % (Fig. 4). The CC_{50} value of doxorubicin was 2.6 μM.

The destruction effect of compound **VP-4606** on red blood cells was also investigated. Red blood cells (RBC) are inactive against organic and non-organic compounds in blood stream. Compounds can directly interact with membrane of erythrocytes resulted in hemolysis of these cells and, thus, enhance the general toxicity of studied compound [33; 34]. Hemolysis value of human red blood cells (HC_{10}) > 32 **μ**g/mL (100 μM). One may consider that compound **VP-4606** has not direct toxic effect towards erythrocytes and lymphocytes of healthy donor (Fig. 4).

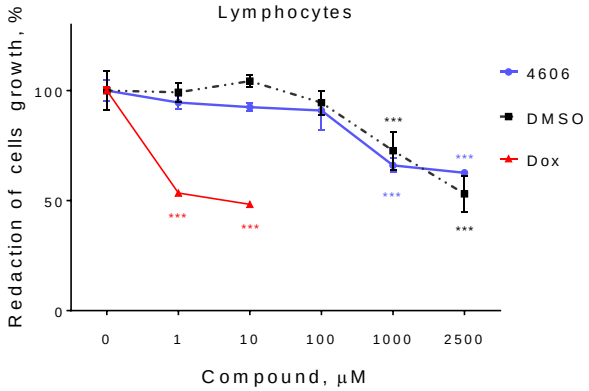


Fig. 4. Cytotoxicity of studied compound VP-4606 towards mitogen-activated lymphocytes isolated from healthy adult human peripheral blood. After a total experimental time (48 h), cell vitality was evaluated by the MTT assay. Dox – doxorubicin. * – P ≤ 0.001 (difference compared with the not treated control cells).**

Experimental Section

General

All chemicals used were of laboratory grade and used without further purification. The compounds were synthesized according to previously described synthetic procedures (**VP-4560** [24], **VP-4606** [25], **VP-4561** [26], **VP-4607** [27] and **VP-4570** [28, 29]). The full compounds characterization is given in cited papers. The spectral data for the most promising compound **VP-4606** (N-(4-nitrophenylsulfonyl)-3-azabicyclo[3.2.1]oct-6-ene) are as follows: m.p. 161-162°C, R_f 0.83 (Et_2O). IR spectrum (cm^{-1}): 3058, 1528, 1473, 1348, 1306, 1165, 730. ^1H

NMR ($CDCl_3$, 300 MHz), *δ*, ppm: 5.83 m (H^6, H^7), 3.51 d (H^{2x}, H^{4x}), 2.84 d (H^{2n}, H^{4n}), 2.64 m (H^1, H^5), 2.00 d (H^{8s}), 1.35 d (H^{8a}). ^{13}C NMR ($CDCl_3$, 75 MHz), *δ*, ppm: 133.38 (C^6, C^7), 46.61 (C^2, C^4), 41.12 (C^8), 38.02 (C^1, C^5). Mass-spectrum, *m/z* (I_{rel}, %), EI 70 eV: 66 (12), 79 (100), 108 (72), 122 (11), 186 (17), 229 (88), 243 (6), 294 (19). Found, %: N 9.58. $C_{13}H_{14}N_2O_4S$. Calc., %: N 9.52.

Compound preparation

Initially, the tests were carried out at a single compound concentration of 32 μg/mL in duplicate, to identify any active compound. Furthermore, a hit confirmation of the active compounds by a dose–response test, using eight concentrations at 1:2 dilutions, in duplicate, to determine the MIC against bacteria and yeasts, CC_{50} (concentration at 50 % cytotoxicity) against mammalian cells, and HC_{10} (concentration at which 10 % hemolysis is induced) against human red blood cells was performed. All substances were dissolved in DMSO to form a stock concentration of 10 mg/mL. Aliquots were diluted in water and 5 μL were dispensed into empty 384-well plates in duplicate for each strain and cell-assayed. As soon as cells were added to the plates, this gave a final compound concentration of 32 μg/mL, or in case of a serial dilution assay compound concentrations from 32 to 0.25 μg/mL, in both cases with a maximum DMSO concentration of 0.3 %.

Primary Antimicrobial assays via co-add [32]

The compounds have been investigated for activity towards one Gram-positive bacteria (*S. aureus* ATCC 43300 MRSA), four Gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603, *A. baumannii* ATCC 19606), and two yeasts (*C. albicans* ATCC 90028 and *C. neoformans* H99 ATCC 208821), and this research was performed by the Community for Open Antimicrobial Drug Discovery (CO-ADD).

All bacteria were overnight cultured in cation-adjusted Q14 Mueller–Hinton broth (CAMHB) at 37 °C. The resultant mid-log phase cultures were added to each well of the compound containing plates (384-well nonbinding surface plates-Corning 3640), giving a cell density of 5×10^5 CFU/mL (colony-forming units/mL). All plates were covered and incubated at 37 °C for 18 h without shaking. Inhibition of bacterial growth was determined measuring absorbance at 600 nm. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm, while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm, after the addition of resazurin (0.001 % final concentration) and incubation at 35 °C for additional 2 h. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references. Percentage growth inhibition of an individual sample is calculated based on Negative controls (media only) and Positive Controls (bacterial/fungal media without inhibitors). Negative inhibition values indicate that the growth rate (or OD600) is higher compared to the Negative Control (Bacteria/fungi only, set to 0 % inhibition). The growth rates for all bacteria and fungi have a variation of -/+ 10 %, which is within the reported normal distribution of bacterial/fungal growth. Any significant variation (or outliers/hits) is identified by the modified Z-Score, and actives are selected by a combination of inhibition value and Z-Score. Growth inhibition was evaluated as a percentage between untreated cells (positive growth control) and media only (negative growth control). Compounds with ≥ 80 % growth inhibition were selected as active compounds in the initial screening, and MIC was determined following EUCAST recommendations. Also, 80 % growth inhibition was used as a threshold for full inhibition.

Antimicrobial methods

Antibacterial effect was determined using MTT test. Experiments were conducted at 7.2. Subsequent bacterial culture in logarithmic phase of growth in Sabouraud medium, pH 7.2, was centrifuged 10 min at 500×g, sediment of bacteria was washed with sterile saline and resuspended in small volume of sterile saline. A defined volume of this suspension was introduced into Sabouraud medium with pH 7.2 for achievement of OD 0.4-0.6 at 590 nm (optical path 1.0 cm). 100 μL of each suspension were introduced into series of 1.5 mL Eppendorf tubes and thereafter inoculated with 10, 5 and 2 μL of tested sample solution were inoculated. Each point was repeated in triplicate. Tubes were incubated 4 h at 37 °C. Thereafter 10 μL of MTT solution (5 mg/mL) was introduced and incubation was continued for 1 h. Cells were harvested by centrifugation 5 min at 1,500×g, supernatant was discarded, small sediment was suspended in 1 mL of DMSO. After the incubation for 1 h at 37°C the OD of liquid was measured at 580 nm using

spectrophotometer ULAB 102 UV (Ukraine). The effect of CMC upon bacteria viability was compared with that in a control.

A suspension of *Candida sp* containing 10^7 cells/mL was prepared by suspending cells taken from the colonies grown on the Sabouraud agar, pH 5.8. Cells number was counted in Horyaev hemocytometric camera, since a size of *Candida* cells (2.5–4 μm) permitted doing that accurately. The tested CMC solution in 10, 5 and 2,5 μL volume was introduced into 3 round bottom Eppendorf tubes, and thereafter, 100 μL of *Candida* cells suspension was added. Two control tubes were prepared: at the start (time 0) and at the end (4 h) of incubation. The tubes were incubated for 4 h at 37 °C (except control 0 kept at 4 °C). 10 μL aliquote was withdrawn at the end of incubation from each tube after thorough mixing, diluted 10,000 fold with water and 0.2 mL of this dilution was distributed on the surface of Sabouraud agar medium, pH 5.8, in the Petri dish. They were incubated at 37 °C and after distinct formation of colonies (usually after 24 h) the image was scanned and colonies were counted with the aid of the Photoshop program. The number of colonies in control tube at 0 h time must be 200±50 per dish, in control after 4 h of incubation colonies number must be 1.5-2.5 fold higher. The experiment was abolished when the increase in number of colonies in control after the incubation was less than 1.5 fold. The effect of CMC sample upon the viability of *Candida* cells was expressed as a ratio of colony number.

The index lower than control (0-hour time) was considered as candidacidal effect, while higher than control (0-hour time) but less than in control after 4 h of incubation was classified as growth inhibition, and higher than in control after 4 h of incubation indicated a stimulation of growth [35].

Cytotoxicity assay toward HEK-293

HEK-293 (human embryonic kidney) ATCC CRL-1573 cells were counted manually in a Neubauer hemocytometer and then plated in 384-well tissue culture-treated plates (Corning 3712) containing the compounds to give a density of 5,000 cells/well in a final volume of 50 μL. Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum was used as growth media and the cells were incubated at 37 °C with the compounds for 20 h in 5% CO_2. Cytotoxicity (or cell viability) was determined by fluorescence, ex: 560/10 nm, em: 590/10 nm (F560/590), after the addition of 5 μL of 25 μg/mL resazurin (2.3 μg/mL final concentration) and after incubation at 37 °C for further 3 h in 5% CO_2. Tecan M1000 Pro monochromator plate reader was used for the fluorescence intensity measurement, using automatic gain calculation. CC_{50} (cytotoxic concentration) was calculated by means of curve fitting the inhibition values versus log (concentration) using a sigmoidal dose–response function, with variable fitting values for bottom, top, and slope.

Hemolysis assay

Human whole blood was washed three times with three volumes of 0.9 % NaCl and then resuspended in the same with a concentration of 0.5×10^8 cells/ml, as determined by manual cell count in a Neubauer hemocytometer with further addition of washed cells to the 384-well compound containing polystyrene plates (Corning 3657) for a final volume of 50 μL. The plates were incubated for 1 h at 37 °C after 10-min shaking on a plate shaker. The next step was centrifugation of plates at 1000g for 10 min to pellet cells and debris; 25 μL of the supernatant was then transferred to a polystyrene 384-well assay plate (Corning 3680). Hemolysis was defined by the supernatant absorbance at 405 nm (OD405) using a Tecan M1000 Pro monochromator plate reader. HC_{10} was established by curve fitting the inhibition values versus log (concentration) using a sigmoidal function with variable fitting values for top, bottom, and slope. The use of human blood (sourced from the Australian Red Cross Blood Service) for hemolysis trials was approved by the University of Queensland Institutional Human Research Ethics Committee (Approval Number: 2014000031).

Cells culture and cytotoxicity MTT assay

Human keratinocytes of HaCaT line and murine fibroblasts of Balb/c 3T3 line were obtained from a Collection at the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (Kyiv, Ukraine).

Cells were grown in the DMEM (Biowest, Nuaille, France) culture medium supplemented with 10 % of fetal bovine serum (Biowest, Nuaille, France) under standard conditions. *In vitro* evaluation of cytotoxic activity of the synthesized compounds in compare with doxorubicin, used as a reference control, towards HaCaT and Balb/c 3T3 cells was measured by the MTT test [36]. Briefly, cells were seeded for 24 h in 96-well microtiter plates at a concentration of 5,000 cells/well (100 μL/well); after that, cells were incubated for 72 h with various additions of the synthesized compounds or DMSO (1; 10; 100;

1,000; 2,500 μM), or Dox (1; 10 μM). MTT, which is converted to dark blue, water-insoluble formazan by the mitochondrial dehydrogenases, was used to determine viable cells according to the Sigma-Aldrich protocol. Formazan was dissolved in DMSO, and the results of the reaction were determined by an Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., Winooski, VT, USA).

The study protocol with human lymphocytes isolated from healthy adult human peripheral blood was approved by Ethics Committee of the Institute of Cell Biology of National Academy of Sciences of Ukraine (protocol #2 dated by January 27, 2019). Lymphocytes of human peripheral blood were isolated from blood consisting of anti-coagulant sodium heparin solution 10 U/mL (B.BRAUN MEDICAL, S.A., Spain) from a healthy adult donor on density gradient of Gradisol G (Polfa, Poland), as described [37]. The blood : Gradisol G mixture (1 : 1) was centrifuged at 400×g at room temperature for 30 min. The cells were washed in the phosphate buffered saline (PBS). The residual erythrocytes were removed from the lymphocytes population by the hypotonic lysis. Lymphocytes were cultured in the RPMI-1640 (Biowest, Nuaille, France) medium supplemented with 20% fetal bovine serum (Biowest, France) at 95 % air and 5 % CO_2, and 37 °C. The lymphocytes were activated using phytohemagglutinin-L (PHA-L, 1 μg/mL, Sigma-Aldrich, USA) mitogen and incubated for next 24 h before treatment with studied compounds.

The evaluation of the anti-proliferative activity *in vitro* of the studied compounds or DMSO (1; 10; 100; 1,000; 2,500 μM), or Dox (1; 10 μM) towards mitogen-activated lymphocytes (100,000 cells / 100 μL) of human peripheral blood was conducted on 48 h using MTT assay (EZ4U, Biomedica, Vienna, Austria). The optical density was measured with the Absorbance Reader at 490 nm with 630 nm as a reference wavelength. The redaction of cells growth (in percentages, %) was calculated as ratio of absorbance in treated cells relative to absorbance in control cells. The anti-proliferation activity of the studied compounds was expressed as an CC_{50} value (the concentration of sample that reduces the 50 % of cells growth).

Statistical Analysis

Z-Score analysis is done to investigate outliers or hits among the samples. The Z-Score is calculated based on the sample population using a modified Z-Score method which accounts for possible skewed sample population. The modified method uses median and median average deviation (MAD) instead of average and Standard deviation (SD), and a scaling factor [38]: M(i) = 0.6745 *(x(i) - median(x))/MAD). All screening is performed as two replicas (n=2), with both replicas on different assay plates, but from single plating and performed in a single screening experiment (microbial incubation). Two values are used as quality controls for individual plates: Z-Factor= 1-[3*(SD(Negative controls) + SD (Positive Controls)) /(average(Positive Controls)-average(Negative controls))].

Cytotoxicity data are presented as the mean (M) ± standard deviation (SD). Results were analysed and illustrated with GraphPad Prism (version 6; GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using two-way ANOVA with Dunnett's multiple comparisons test (cells growth inhibition). A *P*-value of <0.05 was considered as statistically significant.

Conclusion

Summarize the results of the antibacterial activity and toxicity, we found that the 4-nitrobenzenesulfonamide **VP-4606** bearing 3-azabicyclo[3.2.1]oct-6-ene cage fragment, has stronger antimicrobial effect on methicillin-resistant *Staphylococcus aureus* (ATCC 43300) Gram-negative bacteria, which favourably distinguishes it among synthetic antibacterial substances. The obtained results indicate the prospects for further studies of compound **VP-4606** and its derivatives as new hits for antimicrobial screening.

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