https://doi.org/10.7124/bc.000B2F
UDC 577.18, 579.61

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THE ANTI-*ACINETOBACTER BAUMANNII* THERAPEUTIC POTENTIAL OF 6-CHLORO-4-OXO-4H-CHROMENE-3-CARBONITRILE

Aim. *Acinetobacter baumannii is a Gram-negative opportunistic pathogen responsible for a wide spectrum of hospital-acquired infections. This bacterium has acquired resistance to nearly all existing antibiotics, including aminoglycosides, quinolones, broad-spectrum β-lactams, carbopenems, polymyxins and even colistin which is a last-line antimicrobial agent, providing the stimulus to search for novel antibiotics. The aim of this study is to identify novel anti-Acinetobacter baumannii agents among 4H-chromen-4-one derivatives.* ***Methods.*** *88 4H-chromen-4-one derivatives were tested by broth microdilution against five bacterial pathogens such as Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa and toward two fungal pathogens such as Candida albicans and Cryptococcus neoformans.* ***Results.*** *Among the derivatives we have found two active compounds – 6-Chloro-4-oxo-4H-chromene-3-carbonitrile (**1**) inhibiting growth of C. albicans (MIC = 0.5 mg/L) and C. neoformans (MIC = 0.25 mg/L) and 6-Chloro-3-(6-hydroxy-3-oxo-3H-benzofuran-2-ylidenemethyl)-chromen-4-one (**2**), inhibiting growth of C. neoformans (MIC = 16 mg/L). In addition, compounds **1** and **2**, at a concentration of 32 mg/L, inhibited the growth of A. baumannii ATCC 19606 by 24.8% and 23.7%, correspondingly. Compound **1** was revealed to inhibit the growth of multidrug resistant clinical isolate A. baumannii №144 with MIC value of 32 mg/L. Also, it was established that this compound is not cytotoxic toward HEK293 cells.* ***Conclusions.*** *Therefore, 6-Chloro-4-oxo-4H-chromene-3-carbonitrile can be promising candidate for further research and chemical optimization.*

Keywords: *Acinetobacter baumannii, 4H-chromen-4-one, antimicrobial activity, multidrug resistance.*

Citation: Volynets G.P., Gumeniuk M.I., Lukashov S.S., Sapelkin V.M., Bdzhola V.G., Bieda O.A., Prykhod'ko A.O., Pletnova L.V., Yarmoluk S.M. (2026) The anti-*Acinetobacter baumannii* therapeutic potential of 6-Chloro-4-oxo-4H-chromene-3-carbonitrile. *Biopolymers* & *Cell*, 1(42), 68—74. https://doi.org/10.7124/bc.000B2F

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Introduction

Acinetobacter baumannii is a common cause of hospital- and community-acquired infections, ranging from minor wound infections to severe, life-threatening diseases such as pneumonia, cystitis, pyelonephritis, meningitis, brain abscesses, bloodstream infections, *etc.* [1, 2]. This pathogen has developed resistance to almost all available antibiotics [3—6]. At present, the last line of defense for treating infections caused by multidrug-resistant *A. baumannii* is colistin, however, cases of resistance to this antibiotic have already been reported [7—11]. Notably, no new class of antibacterials has been introduced in the past 50 years for managing *A. baumannii* infections [12, 13], highlighting the urgent need for development of novel therapeutic options.

The derivatives of chromen-4-one possess a wide range of bioactive properties, including some compounds showing antimicrobial activity against Gram-positive and Gram-negative bacteria. For example, natural 4H-chromen-4-one derivative from marine *Streptomyces* demonstrates potent antibacterial activity toward *Bacillus subtilis* with MIC value of 0.25 mg/L and *Micrococcus luteus* with MBC value of 0.5 mg/L [14]. Several pyrimidine-containing 4H-chromen-4-one derivatives possess significant inhibitory activities *in vitro* against *Xanthomonas axonopodis pv. Citri*, *Xanthomonas oryzae pv. Oryzae* and *Ralstonia solanacearum* [15].

Antibacterial molecular action of 4H-chromen-4-one is not known. The most possible molecular mode of action is inhibition of an essential bacterial enzyme (e.g., DNA gyrase, DHFR, or another replicative/metabolic enzyme) since several chromene derivatives such as 2-imino-2H-chromene-3-carboxamide core was reported to inhibit DNA gyrase and DHFR [16]. Also, given the planar aromatic nature of chromenes, there is a possibility of DNA intercalation or binding.

The aim of this study is to identify novel antimicrobial agents among 4H-chromen-4-one derivatives.

Materials and Methods

Antibacterial assay

The bacterial strains *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 27853 were grown overnight at 37 °C in cation-adjusted Mueller-Hinton broth (CAMHB). Each overnight culture was diluted 40-fold in fresh CAMHB and incubated at 37 °C for 1.5—3 h to reach the mid-logarithmic growth phase. The mid-log cultures were then further diluted (CFU/mL determined via OD600 measurement) and dispensed into compound-containing wells to achieve a final cell density of 5×10^5 CFU/mL in a total volume of 50 μL. Plates were covered and incubated at 37 °C for 18 h without shaking. Bacterial growth inhibition was assessed by measuring absorbance at 600 nm (OD600) with a Tecan M1000Pro monochromator plate reader. For each well, the percentage of growth inhibition was calculated using the negative control (medium only) and the positive control (bacteria without inhibitors) from the same plate as reference points.

Antifungal assay

Fungi strains *Candida albicans* ATCC 90028 and *Cryptococcus neoformans var. grubii* ATCC 208821 were cultured on Yeast Extract-Peptone-Dextrose (YPD) agar at 30 °C for 3 days. A yeast suspension of 1×10^6 to 5×10^6 CFU/mL, determined by OD530, was prepared from five colonies. The suspension was diluted and dispensed into compound-containing wells to obtain a final fungal cell density of 2.5×10^3 CFU/mL in a total volume of 50 μL. Plates were covered and incubated at 35 °C for 36 h without shaking. Growth inhibition of *C. albicans* was determined by measuring absorbance at 530 nm (OD530). For *C. neoformans*, inhibition was evaluated by measuring the absorbance difference between 600 nm and 570 nm (OD600—570), after addition of resazurin (0.001% final concentration) and incubation at 35 °C for additional 12h. Absorbance was recorded with a Biotek Synergy HTX plate reader.

Different OD values were used to measure the growth of *C. albicans* and *C. neoformans* because of differences in their cell structure and growth rate. *C. albicans* has larger, non-encapsulated cells that scatter light strongly, while *C. neoformans* has a thick polysaccharide capsule that reduces light scattering and grows more slowly, requiring lower absorbance values and longer incubation for accurate measurement.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which microbial growth was fully suppressed, corresponding to an inhibition of ≥ 80%.

Antibacterial assay toward A. baumannii

A. baumannii bacterial suspensions were prepared from the 24-hour microbial cultures with sterile saline 0.9% at a cell density of 1.5 x 10^8 CFU/ml, corresponding to a 0.5 McFarland standard using a Densi-La-Meter (PLIVA-Lachema Diagnostika, Czech Republic). The serial dilution method was used to determine the MIC values of compounds **1** and **2**. In the tubes containing Mueller-Hinton broth with appropriate concentrations of investigated compounds **1** and **2**, standard antibiotic ciprofloxacin or DMSO, the microbial suspension (5 × 10^6 CFU/mL) was added. The tubes were incubated at 37 °C for 18—20 hours. Results were assessed visually by the presence or absence of turbidity in the broth. The drug concentration in the last tube (in a series of twofold dilutions of the sample) showing a clear medium was considered the MIC of the compound.

The minimum bactericidal concentration (MBC) was determined by plating 0.1 mL from tubes showing no visible growth onto Mueller-Hinton agar, followed by incubation at 37 °C for 24 h. The MBC was defined as the lowest concentration yielding no colony growth.

Cytotoxicity assay

Cytotoxicity of compound was evaluated using human embryonic kidney 293 (HEK293) cells. Cells were counted with a Neubauer haemocytometer and

Table 1. **Structures and antimicrobial activity (percentage growth inhibition) for compounds 1 and 2 at 32 mg/L**

№	Compound	*S. aureus* ATCC 43300	*E. coli* ATCC 25922	*K. pneumonia* ATCC 700603	*A. baumanii* ATCC 19606	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 90028	*C. neoformans var. grubii* ATCC 208821
1		16.9	24.8	14.7	24.8	5.0	98.2	103.7
2		11.6	−4.8	4.8	23.7	7.0	4.0	91.1

seeded into 384-well plates at a density of 5000 cells/well in 50 μL DMEM supplemented with 10% FBS. After incubation for 20 h at 37 °C in 5% CO_2, the cell viability was assessed by adding 5 μL of resazurin solution (final concentration 2.3 mg/L) and incubating for an additional 3 h. Fluorescence was measured (Ex 560/10 nm; Em 590/10 nm) using a Tecan M1000 Pro plate reader with automatic gain adjustment.

Cytotoxicity screening was conducted in duplicate (n = 2), with each replicate on different assay plates. The CC_{50} value (concentration causing 50% cytotoxicity) was calculated by fitting the inhibition data to a sigmoidal dose-response curve. The compounds exhibiting CC_{50} values greater than the highest tested concentration were considered inactive. The cytotoxic samples were classified as those with CC_{50} ≤ 32 μg/mL in both replicates.

Haemolytic assay

The haemolytic assay was performed by the Community for Open Antimicrobial Drug Discovery (CO-ADD) according to the method described earlier [17]. Human whole blood was washed three times with three volumes of 0.9% NaCl and resuspended in the same solution to a final concentration of 0.5×10^8 cells/mL, determined manually using a Neubauer haemocytometer. The suspension was then added to 384-well plates containing the test compounds to a final volume of 50 μL. The plates were shaken for 10 min and incubated for 1 h at 37 °C, followed by centrifugation at 1000 g for 10 min to pellet cells and debris. A 25 μL aliquot of the supernatant was transferred to a polystyrene 384-well assay plate, and haemolysis was quantified by measuring absorbance at 405 nm (OD405) with a Tecan M1000 Pro monochromator plate reader.

Results and Discussion

In order to find novel antimicrobial compounds the phenotypic screening of 88 4H-chromen-4-one derivatives, was performed by the CO-ADD toward five bacterial strains such as *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853 and toward two fungal strains such as *Candida albicans* ATCC 90028 and *Cryptococcus neoformans var. grubii* ATCC 208821.

Table 2. **The sensitivity of *A. baumannii* clinical isolate № 144 to antibiotics**

Antibiotic	Sensitivity
Aztreonam	R
Tigecycline	S
Ceftriaxone	R
Ceftazidime	R
Cefepime	R
Cefoperazone/sulbactam	R
Gentamicin	R
Amikacin	R
Netilmicin	R
Tobramycin	R
Levofloxacin	R
Moxifloxacin	R
Colistin	S
Meropenem	R
Imipenem	R
Cilastatin	R
Ertapenem	R
Chloramphenicol	R

R — resistance, S — susceptibility

Table 3. **Antibacterial activity of compounds 1 and 2 toward multidrug resistant *A. baumannii* strain № 144 (minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), mg/L)**

Compounds	MIC, mg/L	MBC, mg/L
Compound **1**	32	512
Compound **2**	1024	>1024
Ciprofloxacin	256	>1024
DMSO	1024	>1024

As a result, two active compounds possessing antimicrobial activity were identified (Table 1). It was found that the most active compound 6-Chloro-4-oxo-4H-chromene-3-carbonitrile (**1**) inhibits growth of *C. albicans* with MIC value of 0.5 mg/L and *C. neoformans* with MIC value of 0.25 mg/L. Other compound — 6-Chloro-3-(6-hydroxy-3-oxo-3H-benzofuran-2-ylidenemethyl)-chromen-4-one (**2**), inhibits only growth of *C. neoformans* with MIC value of 16 mg/L. The chemical synthesis and antifungal properties of compounds were previously patented by us [18]. During this phenotypic screening it was revealed that compounds **1** and **2** also inhibit growth of *A. baumannii* ATCC 19606 by 24.8% and 23.7%, correspondingly. The antimicrobial activity of compounds **1** and **2** toward five bacterial strains such as *S. aureus*, *E. coli*, *K. pneumonia*, *A. baumannii*, *P. aeruginosa* and toward two fungal strains such as *C. albicans* and *C. neoformans* is presented in the Table 1.

Next, we tested antimicrobial activity of the compounds **1** and **2** toward multidrug resistant clinical isolate *A. baumannii* № 144, which was isolated from endotracheal tube of a patient admitted to the intensive care unit in Ukraine. The sensitivity of *A. baumannii* № 144 isolate to antibiotics is presented in the Table 2.

The antibacterial activity of compounds **1** and **2** toward multidrug resistant strain *A. baumannii* № 144 in comparison with standard antibiotic ciprofloxacin is presented in the Table 3.

EUCAST resistance breakpoint for ciprofloxacin MIC values for *Acinetobacter spp.* is > 1.0 mg/L [19]. As it can be seen from the Table 1, the clinical test strain *A. baumannii* № 144 is completely resistant to ciprofloxacin.

Compound **1** exhibited measurable antibacterial activity against *A. baumannii* strain № 144, with a MIC value of 32 mg/L. Although this activity is lower than that observed for the clinically used antibiotics tigecycline (MIC ≈ 2 mg/L) and colistin (MIC ≈ 0.25—2 mg/L), to which the tested strain remained susceptible, the result indicates that compound 1 possesses an inherent antibacterial potential. The moderate potency, combined with the structural simplicity of the 4H-chromen-4-one scaffold, suggests that this compound could serve as a promising lead for further chemical modification and optimization aimed at improving activity against multidrug-resistant *A. baumannii*.

Cytotoxicity of compound **1** was evaluated using HEK293 cells. The CC_{50} (50% cytotoxic concentration) value for compound **1** exceeded 32 mg/L (the maximum concentration tested) in both replicates, indicating that it is non-cytotoxic.

Compound **1** was also evaluated for haemolytic activity. The CC_{50} value for compound **1** exceeded 32 mg/L (the maximum concentration tested), suggesting that it does not reveal haemolytic activity.

Conclusion

Therefore, 6-Chloro-4-oxo-4H-chromene-3-carbonitrile (**1**) which was earlier reported as a compound with strong antifungal activity, also inhibits growth of multidrug resistant clinical test strain *A. baumannii* № 144 with MIC value of 32 mg/L, does not reveal haemolytic activity and is not cytotoxic toward HEK293 cells.

Acknowledgements. Antimicrobial screening was performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Wellcome Trust (UK) and The University of Queensland (Australia).

Funding. This work was supported by IMBG Simons Foundation Grant for Ukrainian Institutions № SFI-PD-UKRAINE-00017453.

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Received: 15.10.2025
Accepted: 24.03.2026
Published: 14.04.2026

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ТЕРАПЕВТИЧНИЙ ПОТЕНЦІАЛ 6-ХЛОРО-4-ОКСО-4Н-ХРОМЕН-3-КАРБОНІТРИЛУ ПРОТИ *ACINETOBACTER BAUMANNII*

Мета. *Acinetobacter baumannii* — грамнегативний опортуністичний патоген, який спричиняє широкий спектр нозокоміальних інфекцій. Цей збудник набув стійкості майже до всіх відомих антибіотиків, включаючи аміноглікозиди, хінолони, бета-лактами широкого спектру дії, карбапенеми, поліміксини та навіть колістин, який є антибіотиком останньої лінії, що зумовлює необхідність пошуку нових антимікробних засобів. Метою дослідження є виявлення нових сполук із протимікробною активністю щодо *A. baumannii*. ***Методи.*** Протимікробну активність 88 похідних 4Н-хромен-4-ону досліджували методом серійних мікророзведень щодо п'яти бактеріальних патогенів (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*) та двох грибкових патогенів (*Candida albicans* і *Cryptococcus neoformans*). ***Результати.*** Серед досліджених сполук було виявлено дві активні**:** 6-хлор-4-оксо-4Н-хромен-3-карбонітрил (**1**), що інгібує ріст *C. albicans* (МІК = 0,5 мг/л) та *C. neoformans* (МІК = 0,25 мг/л), і 6-хлор-3-(6-гідрокси-3-оксо-3Н-бензофуран-2-іліденметил)-хромен-4-он (**2**), активний проти *C. neoformans* (МІК = 16 мг/л). Також було встановлено, що сполуки **1** та **2** при концентрації 32 мг/л знижують ріст *A. baumannii* ATCC 19606 на 24,8% та 23,7%, відповідно. Подальші дослідження антимікробної активності цих сполук щодо мультирезистентного клінічного ізоляту *A. baumannii* № 144 продемонстрували, що сполука **1** пригнічує його ріст зі значенням МІК = 32 мг/л. Водночас встановлено, що ця сполука не є цитотоксичною щодо клітинної лінії HEK293. ***Висновки.*** Отже, 6-хлор-4-оксо-4Н-хромен-3-карбонітрил є перспективним кандидатом для подальших біологічних досліджень та хімічної оптимізації.

Ключові слова**:** *Acinetobacter baumannii*, 4Н-хромен-4-он, антимікробна активність, мультирезистентність до антибіотиків.