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Short communication

Searching for new derivatives of neocryptolepine: Synthesis, antiproliferative, antimicrobial and antifungal activities

Katarzyna Sidoryk [a,*], Anna Jaromin [b], Jessica A. Edward [c], Marta Świtalska [d], Joanna Stefańska [e], Piotr Cmoch [a,f], Joanna Zagrodzka [a], Wojciech Szczepek [a], Wanda Peczyńska-Czoch [g], Joanna Wietrzyk [d], Arkadiusz Kozubek [b], Robert Zarnowski [c], David R. Andes [c], Łukasz Kaczmarek [a]

[a] Pharmaceutical Research Institute, Rydygiera 8, 01-793 Warsaw, Poland
[b] Department of Lipids and Liposomes, Faculty of Biotechnology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland
[c] Department of Medicine, Section of Infectious Diseases, 4125 Microbial Sciences Building, 1550 Linden Dr., University of Wisconsin-Madison, Madison, WI 53706, USA
[d] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 12 Weigla St., 53-114 Wroclaw, Poland
[e] Medical University of Warsaw, Department of Pharmaceutical Microbiology, 3 Oczki St., 02-007 Warsaw, Poland
[f] Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland
[g] Division of Organic Technology, Faculty of Chemistry, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland

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ABSTRACT

A series of novel amino acid and dipeptide derivatives of neocryptolepine were synthesized and tested for their antimicrobial, antifungal and antiproliferative activity *in vitro* against cancer cell lines (KB, A549, MCF-7, LoVo) and normal mice fibroblast cells (BALB/3T3). Biological evaluation revealed that almost all of the new compounds displayed high antiproliferative activity against the tested cells and moderate to potent antibacterial activities. Interestingly, these compounds were active against *Candida albicans* biofilms at doses significantly lower than those required against free-floating planktonic fungal cells. The most promising compounds are derivatives with glycine and L-proline as a substituent both at 2 and at 9 position of 5*H*-indolo[2,3-b]quinoline. In general, these new compounds (**2a**, **3a**, **6a** and **7a**) showed the highest dual action against cancer lines and infectious pathogenic microbes *in vitro*.

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1. Introduction

Many drugs used in modern medicine are either directly isolated from plants or synthetically modified from lead compounds of natural origin. Among numerous currently exploited medicinal African plant species, *Cryptolepis sanguinolenta* (Lindl.) Schltr. (Periplocaceae) has recently received greater attention from an immense number of researchers and pharmaceutical companies. This stemmed twining and scrambling shrub, which contains an orange-colored juice in the cut stem [1], has been used by some traditional herbalists in the treatment of broad symptoms of fever, urinary and upper respiratory tract infections, malaria, rheumatism, and venereal diseases [2,3].

Numerous pharmacological activities have been demonstrated for the extracts prepared either from entire plants or their individual fractions. Indeed, anti-malarial [4–8], anti-diabetic [9–11], anti-thrombotic [12], anti-inflammatory activities [13,14], and more recently described anti-androgenic and anti-spermatogenic properties with potential anti-aphrodisiac activity [15] have been experimentally determined. Simultaneously, the *C. sanguinolenta* extracts have aroused considerable interest because of their antimicrobial activities that might be potentially beneficial to human health [16–21]. The active components found in this plant are known to be the indoquinoline alkaloids, consisting mainly of the indole and the quinoline moieties. The major alkaloid of the roots, cryptolepine (5-methyl-5*H*-indolo[3,2-b]quinoline), is reported to possess intricate biological effects, while neocryptolepine (5-methyl-5*H*-indolo[2,3-b]quinoline, Fig. 1a) remains a minor alkaloid of *C. sanguinolenta*.

* Corresponding author.
E-mail addresses: k.sidoryk@ifarm.eu, sidorykk@gmail.com (K. Sidoryk).

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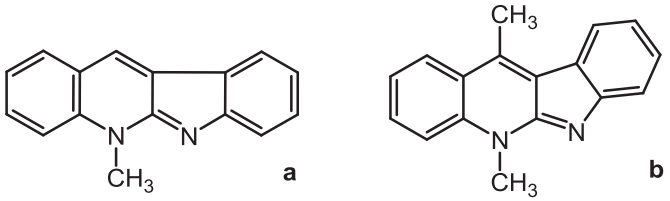
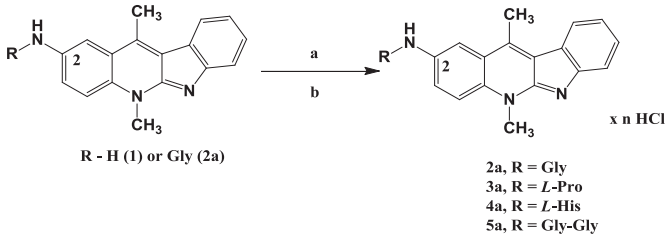


Fig. 1. Structures of neocryptolepine (**a**) and 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline (DiMIQ, **b**).



Reagents and conditions: (a) Boc-AA, TBTU, HOBt, DIPEA, DMF, r.t., 6-24 h; (b) 2.2 M HCl/methanol, r.t., 2h.

Scheme 1. General method for the synthesis of amino acid and dipeptide derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline substituted in position 2.

One of the reasons for the growing frequency of nosocomial infections is the increasing use of immunosuppressive agents in antitumor and transplant therapies, which leads to breakdown of the barrier between the gut and bloodstream in humans. This process quite often results in the formation of highly drug-resistant microbial biofilms, which is definitely disadvantageous for human health. The development of new anticancer drugs that would offer the efficacy of antimicrobial prophylaxis in clinical settings is therefore urgently needed, especially since the number of therapeutic options for cancer-accompanying infectious diseases remains relatively limited. There is also increasing awareness of the hazards related to the overuse of antibiotics and other toxic chemical agents that lead to multidrug resistance in pathogenic microorganisms. Overall, this has led to accelerated investigations on naturally occurring products and their chemically modified derivatives as new sources of anticancer agents that would have the potential of antimicrobial lock therapy in clinical practice.

In our previous studies we reported the syntheses of neocryptolepine derivatives containing an amino acid or a dipeptide at the C-9 position and their evaluation for antitumor activity [22]. It was shown that the derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b] quinoline, an analogue of neocryptolepine, with a substituted amino acid or dipeptide chain in position 9, displayed high antiproliferative activity *in vitro* and antitumor activity *in vivo*. The biological data revealed that the attachment of an amino acid moiety or a short peptide chain to 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline (DiMIQ, Fig. 1b) significantly improved its physicochemical properties, resulting in auspicious *in vivo* anticancer actions with relatively low hemolytic levels in the host. Research on the relationship between structures and antiproliferative activity of indolo[2,3-b]quinolines revealed that not only the nature of the substituents but also the position of the substituents in the core of indolo[2,3-b]quinolines played the crucial role for the cytotoxic activity of these derivatives [23,24]. For example, many of the alkylaminoalkyl derivatives of 5*H*-indolo[2,3-b]quinoline substituted at position C-2 displayed a higher antiproliferative activity than the same derivatives substituted at position C-9 [23]. Compounds of this family, especially amino acid derivatives of DiMIQ, displayed high anticancer activity, but their antimicrobial and antifungal activity have not been investigated yet.

In this paper, we describe synthesis of the new derivatives of neocryptolepine substituted with either an amino acid or a dipeptide chain at the C-2 position. These compounds were evaluated for their antiproliferative activity *in vitro* against normal (BALB/3T3) and several types of cancer cell lines (KB, MCF-7, A549 and LoVo), as well as further investigated as potential antimicrobials against the most common pathogenic bacteria and fungi that cause cancer- and transplant-associated infections in humans.

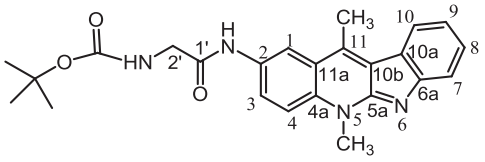
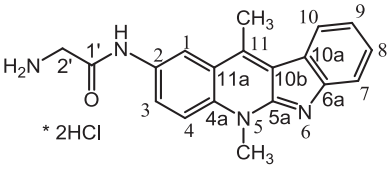
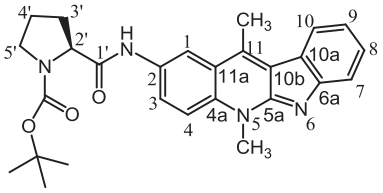
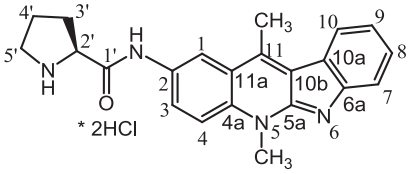
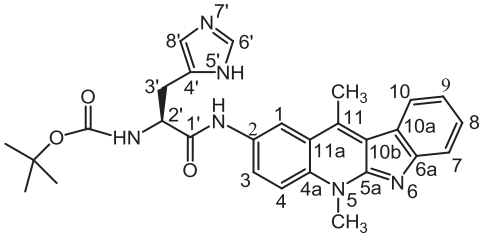
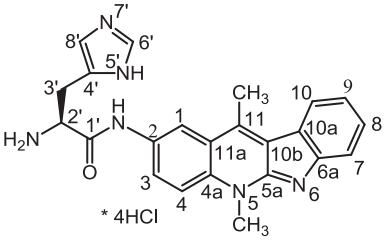
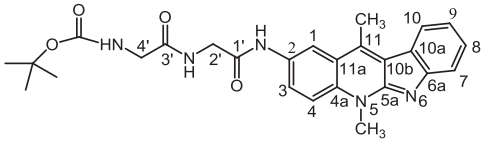
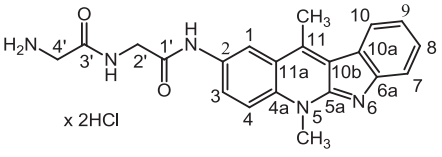
2. Results and discussion

2.1. Synthesis

The target compounds depicted in Scheme 1 were obtained by reacting the starting material 5,11-dimethyl-5*H*-indolo[2.3-b] quinolin-2-ylamine (**1**) with 1.3 molar equivalent of N^{α}-*tert*-butyloxycarbonyl-amino acid. The coupling was achieved using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling activator in the presence of *N*-hydroxybenzotriazole, and *N*,*N*-diisopropylethylamine [25]. The starting material **1** was not commercially available and was synthesized as described previously [26–29]. In all cases the crude compounds **2**–**4** were treated with water and $CHCl_3$, and the organic layer was separated, and washed successively with $NaHCO_3$ aq solution and NaCl aq solution. The extract was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The products **2**–**4** were then purified by flash chromatography with a mixture of chloroform and methanol. The pure fractions were crystallized from ethyl acetate (compounds **2** and **3**) or from diethyl ether (compound **4**). The yield of coupling reaction and the purification of compounds **2**–**4** ranged from 63 to 67%. In the next step Boc groups were removed by 2.2 M HCl in methanol. The deprotection gave the appropriate hydrochlorides **2a**–**4a** as the final products with good yields ranging from 85 to 95%. The peptide derivative of **1** was synthesized using the "step by step" method. *N*-(5,11-dimethyl-5*H*-indolo[2,3-b]quinolin-2-yl)glycylamide dihydrochloride (**2a**) was coupled with Boc-N^{α}-Gly by the TBTU method in DMF. The crude product **5** was then separated by extraction and purified by flash chromatography with a mixture of chloroform and methanol. The pure fraction was evaporated and the product was crystallized from the ethyl acetate and the yield after purification was 84%. The Boc removal of compound **5** with hydrogen chloride in methanol gave the hydrochloride **5a** with a good yield of 91%. The purity of the obtained final products (**2a**–**5a**) was assessed by the analytical C18 RP-HPLC method using acetonitrile-water as a mobile phase. The purity of all synthesized analogues was in the range 95–98%.

The structures and the yields of all the new amino acid and dipeptide derivatives of **1** are displayed in Table 1. The formation of compounds **2**–**5** and **2a**–**5a** was confirmed by the elementary analysis and ESI-MS spectra. To confirm structures of the synthesized compounds 1D and 2D NMR experiments (see Experimental section) were performed and ^{1}H NMR and ^{13}C NMR characteristics of these compounds are presented in the Experimental Section. The ^{1}H NMR spectra of compounds **2**, **4** and **5** showed a characteristic signal of nine protons of *tert*-butyl group as a singlet in the range 1.40–1.52 ppm. In the case of compound **3**, due to the presence of two rotamers [22], there are two singlets at δ 1.55 and 1.70 ppm (ca. 6.8 H and ca. 2.2 H, respectively). The aliphatic protons signals from the amino acid moiety were observed for all the compounds in ^{1}H NMR spectra. It is important, that the measurements of NMR had to be performed in a mixture of DMSO and D_2O (solubility of the most of new compounds in organic solvents was poor), and it led to a disappearance of several proton signals in the ^{1}H NMR spectra of compounds **2a**–**5a**. It was caused by their exchange with an excess of D_2O. In case of *N*-Boc protected

Table 1
The yields (in %) obtained for the amino acid and dipeptide derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline substituted in position 2.

Compound	Substituent in position 2	Structure	Yields (%)
2	**Boc-Gly**		**67**
2a	**Gly**		**95**
3	**Boc-*L*-Pro**		**63**
3a	***L*-Pro**		**90**
4	**Boc-*L*-His**		**65**
4a	***L*-His**		**85**
5	**Boc-Gly-Gly**		**84**
5a	**Gly-Gly**		**91**

compounds **2**–**5**, NH protons are usually non-exchangeable and that is why they were visible in ^{1}H NMR spectra as sharp signals. There was also one exception to the above-mentioned description. In compound **4**, which is a L-histidine derivative of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline, proton of the imidazole ring takes part in an exchange process between both imidazole nitrogen atoms. Therefore in ^{1}H NMR spectrum no presence of this proton was observed.

The attachment of the amino acids to 5,11-dimethyl-5*H*-indolo [2,3-b]quinoline clearly evidenced from the ^{13}C NMR spectra of these compounds which showed the characteristic carbonyl signal from amide above 160 ppm. In all cases, ^{13}C NMR spectra showed one carbonyl signal (C-1′) at δ 166.4 ppm for **2a**, 169.1 ppm for **3a**, 170.8 ppm for **4**, and 170.0 ppm for **4a**. In the dipeptide derivatives two carbonyl signals were observed: at δ 168.5 ppm (C-1′), and at δ 170.0 ppm (C-3′) for compound **5**, and at δ 168.6 ppm (C-1′), and at δ 170.2 ppm (C-3′) for compound **5a**. Moreover, in ^{13}C NMR spectra of Boc derivatives **4** and **5** the carbonyl signals from Boc group were observed at δ 155.3 ppm, and 156.0 ppm, respectively. They disappeared after deprotection.

For comparison purposes the ^{1}H/^{13}C NMR chemical shifts of 2-(**2a**) and 9-(**6a**) glycine containing neocryptolepine derivatives are presented in Table 1 in the Supplementary Material. To indentify the structures of both isomeric indoloquinoline **2a** and **6a**, the results of 1D/2D NMR experiments were carefully analyzed. At first, to distinguish both ^{1}H NMR spin systems (ABX and AA′BB′) in isomeric compounds **2a** and **6a**, NOE effects were used. The irradiation of the methyl group at ca. 2.82 ppm in case of both isomeric compounds (**2a** and **6a**) leads to NOE signal enhancements at 7.95 and 8.33 ppm for compound **2a** or 8.03 and 8.23 ppm for compound **6a**. Multiplicity of these signals and values of *J* (H–H) enable to differentiate both X protons (at 8.03 and 8.33 ppm) of the spin system ABX in isomeric **2a** and **6a**. Additionally, using results of ^{1}H–^{13}C *g*-HSQC/*g*-HMBC experiments for both **2a** and **6a**, complete ^{1}H/^{13}C NMR signal assignment was achieved. Analysis of these NMR results completely confirmed the structure of both isomeric neocryptolepines **2a** and **6a**. Similar procedure was performed for all other compounds **3**–**5** and **3a**–**5a** leading to a complete confirmation of their structures. Moreover, comparison of the ^{1}H/^{13}C NMR data for compounds **4** and **4a** is in a complete accordance with results presented by Bednarek and co-workers [30].

Additionally, we performed a gradient selected ^{1}H–^{15}N HMBC experiment for the most of the synthesized compounds. The ^{15}N NMR chemical shifts were presented in Experimental Section. Most of the ^{15}N results were obtained for these samples where the solubility was relatively high (compounds **3a**, **4** and **5**). In case of **2a**, **4a** and **5a** only N-5 signals were observed. Not all of the expected correlations were obtained. Especially the measurements of hydrochlorides **2a** and **5a** were difficult because of their very low solubility in a DMSO-D_2O mixture. The ^{15}N NMR chemical shifts obtained for protected compounds, which existed in the neutral form, are in agreement with data published by Bednarek and co-workers [30]. Formation of hydrochlorides (**2a**, **3a**, and **5a**) is connected with protonation of the nitrogen atom of the heteroaromatic core, most probably N-6. Comparison of the NMR data (^{13}C/^{15}N NMR chemical shifts) between neutral compounds and their hydrochlorides leads to several deshielding/shielding effects. Most significant are shielding increase of C5a and C6a cores (by ca. 8 and ca. 14 ppm, respectively) as well as deshielding of nitrogen N5 by ca. 10 ppm.

All synthesized compounds were also characterized by FT-IR spectra. Compared to the substrate, amine **1**, the Boc protected amino acid derivatives **2**–**5** revealed two additional strong absorption bands and one moderate absorption band coming from the introduced amino acid fragments. The first one, lying in the range 3299–3426 cm^{-1}, is characteristic of N–H amide stretching. The second one, located at 1669–1693 cm^{-1} and broad, is a superposition of two strong C=O absorption bands of amide and carbamate. Finally, the last moderate band at 1365–1367 cm^{-1} can be attributed to C–H aliphatic rocking. After Boc deprotection the obtained final products **2a**–**5a** showed the presence of the same bands in the ranges 3331–3457 cm^{-1}, 1681–1702 cm^{-1} and 1361–1369 cm^{-1}, respectively. However, the lack of carbamate carbonyl resulted in narrowing of the amide carbonyl band at 1681–1702 cm^{-1}. The remaining strong absorption bands, present in both series of compounds, are characteristic of their heteroaromatic nucleus.

2.2. Biological studies

2.2.1. Antiproliferative activity in vitro

The synthesis and antiproliferative activity of amino acid and dipeptide derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline, substituted in position 9, were described previously [22]. The structures of these compounds are summarized in Table 2.

All the synthesized compounds **2a**–**5a** were evaluated for their antiproliferative activity *in vitro* against the KB cells (cervix

Table 2
Structures of amino acid and dipeptide derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline, substituted in position 9.

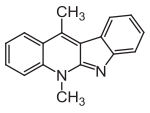
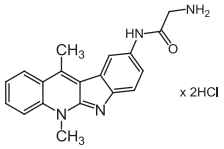
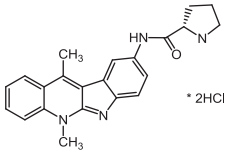
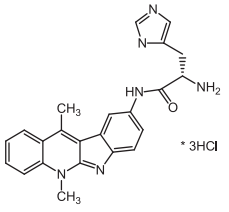
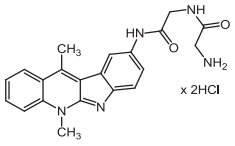
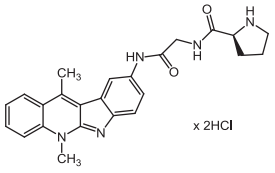
Compound	Substituent in position 9	Structure
DiMIQ	–	
6a	**Gly**	 x 2HCl
7a	**L-Pro**	 * 2HCl
8a	**L-His**	 * 3HCl
9a	**Gly-Gly**	 x 2HCl
10a	**Gly-L-Pro**	 x 2HCl

Table 3
In vitro antiproliferative activities of the hydrochloride amino acid and peptide derivative of DiMIQ against human cancer cell lines and normal mice fibroblasts. IC_{50} – compound concentration leading to 50% inhibition of cell proliferation; DiMIQ – referential compound; DOX – doxorubicin hydrochloride.

Compd.	Subst.	IC_{50} [μM]				
		BALB/3T3	A549	MCF-7	LoVo	KB
DiMIQ		5.77 ± 0.93	2.19 ± 0.48	1.54 ± 0.52	0.20 ± 0.40	1.14 ± 0.61
DOX		1.08 ± 0.03	0.33 ± 0.10	0.44 ± 0.16	0.11 ± 0.03	0.84 ± 0.03
2a	Gly	0.42 ± 0.02	0.08 ± 0.04	0.46 ± 0.09	0.06 ± 0.01	0.42 ± 0.11
3a	L-Pro	0.54 ± 0.11	0.12 ± 0.05	0.62 ± 0.05	0.07 ± 0.02	0.47 ± 0.17
4a	L-His	–	–	–	–	3.88 ± 0.41
5a	Gly-Gly	2.11 ± 0.20	1.44 ± 0.53	4.11 ± 0.44	1.17 ± 0.38	1.54 ± 0.01

carcinoma). The results of the studies on the antiproliferative activity of the amino acid and peptide derivatives of DiMIQ are summarized in Table 3. The tested compounds showed diverse activity against the KB cell line. The IC_{50} values of the amino acid derivatives **2a** and **3a** (values in the range 0.42–0.47 μM) were lower than IC_{50} of the reference compounds, DiMIQ and doxorubicin hydrochloride (DOX), which were 1.14 μM and 0.84 μM, respectively. The compounds **4a**, substituted with L-histidine, and **5a**, substituted with glycylglycine, had lower activity than the reference compounds and their IC_{50} values were 3.88 μM and 1.54 μM, respectively. Furthermore, the antiproliferative activity of the derivative that contained glycine at position C-2 (**2a**) was higher than the antiproliferative activity of the corresponding derivative substituted with the same amino acid in position C-9 (IC_{50} 0.67 μM [22]). No significant differences in activities of the compound derivatives either with L-proline (IC_{50} 0.47 μM, vs. IC_{50} 0.42 μM [22]) or L-histidine (IC_{50} 3.88 μM, vs. IC_{50} 3.46 μM [22]) were observed in this study. Strikingly, the activity of dipeptide derivatives of DiMIQ, which in fact contains glycylglycine in its skeleton at position C-2, was higher than the activity of the analogous dipeptide in position C-9 (IC_{50} 1.54 μM, vs. IC_{50} 4.65 μM [22]).

The selected compounds **2a**, **3a** and **5a** were subjected to further studies of their antiproliferative activity against the following cell lines: non-small cell lung cancer A549, breast cancer MCF-7, colon cancer LoVo, and the normal mice fibroblasts BALB/3T3. As outlined in Table 2, the majority of the prepared compounds showed high antiproliferative activity against all the tested cell lines. Interestingly, the glycine derivative of DiMIQ (**2a**) had the highest activity against A549 (IC_{50} 0.08 μM) and LoVo (IC_{50} 0.06 μM) cell line, and this activity was higher than the activity observed against the normal BALB/3T3 cell line (IC_{50} 0.42 μM). The L-proline derivative of DiMIQ (**3a**) was also active in the tested cell line systems with the highest activity against LoVo cell line (IC_{50} 0.07 μM), whereas its activity against the non-mutated normal BALB/3T3 cell line was noticeably lower (IC_{50} 0.54 μM).

Our experimental data presented in the current work showed no significant differences in determined toxicity levels between the tested C-2, and C-9 substituted amino acid and dipeptide DIMIQ derivatives. However, the tested C-9 derivatives displayed no differences in antiproliferative activity against normal and cancer cell lines whereas the C-2 derivatives displayed notable alterations in antiproliferative levels against both types of tested cell lines. This exciting finding indicates the presence of a distinct mechanism of action for this particular group of DiMIQ derivatives, which is determined by the type of substituent introduced and its structural localization. Indeed, derivatives substituted with an amino acid in position 2 and 9 had higher antiproliferative activities than derivatives containing peptides in their structures.

2.2.2. *Antimicrobial and antibiofilm activity in vitro*

Antimicrobial resistance has become an increasingly serious public health problem in a wide range of infectious diseases, especially in those associated with cancer and transplant therapies. It is, therefore, imperative to design novel and improve already existing antimicrobials that lead towards more efficient treatment of diseases caused by microbial pathogens. Strikingly, about 80% of all human-related infections involve microbial biofilms, which constitute loosely organized structured communities that are significantly more difficult to culture and more resistant to control strategies and host defenses than their planktonic counterparts.

The title compounds were tested for their *in vitro* antibacterial and antifungal activity by the twofold serial agar dilution method to determine their minimal inhibitory concentration (MIC) under standardized conditions according to CLSI guidelines [31,32].

The results of antimicrobial activity of the compounds are summarized in Table 4. The tested compounds were active

Table 4
In vitro antimicrobial activities of the amino acid and dipeptide derivatives of 5*H*-indolo[2,3-b]quinoline substituted in position 2 and 9 (na) – compounds having MIC value >300 μg/mL. Cipr. – Ciprofloxacin, Fluc. – Fluconazole.

Tested strain	MIC (μg/mL)											
	Gly (**2a**)	Pro (**3a**)	His (**4a**)	Gly-Gly (**5a**)	Gly (**6a**)	Pro (**7a**)	His (**8a**)	Gly-Gly (**9a**)	Gly-Pro (**10a**)	DiMIQ	Cipr.	Fluc.
S. aureus NCTC 4163	**12.5**	**12.5**	>200	>200	50	**12.5**	>200	>200	>200	100	0.25	–
S. aureus ATCC 25923	**12.5**	50	>200	>200	50	50	>200	>200	>200	200	0.5	–
S. aureus ATCC 6538	**12.5**	**12.5**	>200	>200	50	**12.5**	>200	>200	>200	100	0.25	–
S. aureus ATCC 29213	**12.5**	50	>200	>200	100	50	>200	>200	100	100	0.5	–
S. epidermidis ATCC 12228	**12.5**	**12.5**	>200	>200	25	**12.5**	>200	>200	100	100	0.25	–
B. subtilis ATCC 6633	**12.5**	**6.25**	>200	>200	**12.5**	**12.5**	>200	100	100	50	<0.125	–
B. cereus ATCC 11778	50	25	>200	>200	50	50	>200	>200	>200	100	1	–
E. hirae ATCC 10541	100	100	>200	>200	100	100	>200	>200	>200	200	4	–
M. luteus ATCC 9341	**6.25**	**6.25**	>200	>200	**6.25**	**6.25**	>200	200	50	50	2	–
M. luteus ATCC 10240	**12.5**	**6.25**	>200	>200	**6.25**	**6.25**	>200	200	50	50	1	–
C. albicans ATCC 10231	100	100	>200	>200	25	200	>200	200	200	**12.5**	–	1
C. albicans ATCC 90028	100	50	>200	>200	50	200	>200	200	200	**12.5**	–	1
C. albicans SN250	15.62	31.25	250	>500	15.62	200	125	200	200	**12.5**	–	–
C. parapsilosis ATCC 22019	100	50	>200	>200	25	200	>200	200	200	**12.5**	–	2

against Gram-positive bacteria and *Candida* sp. All examined derivatives were inactive against Gram-negative rods (data not shown). The observed inactivity phenomenon could be determined by multiple yet undefined factors, including (i) a putative inability of the tested compounds to cross over the Gram-negative cell wall structure; (ii) the lack of putative target receptors for DiMIQ derivatives in Gram-negative bacteria; or (iii) putative interactions with intracellular bacterial molecules that result in complex formation and drug deactivation. Compounds **2a**, **3a**, **6a** and **7a** showed higher activity against tested Gram-positive cocci in comparison with the reference compound DiMIQ. For most bacterial strains tested the MIC values of compounds **2a**, **3a**, **6a** and **7a** were between 6.25 and 12.5 μg/mL. An opposite observation was made with reference to the screened fungal *Candida* species. In this case DiMIQ showed significantly higher antifungal activity (MIC 12.5 μg/mL) than other tested compounds.

We selected *Candida albicans* SN250 strain, which appeared slightly more susceptible to the tested of neocryptolepine derivatives when compared to MIC values measured for the other fungal isolates examined in this study. First of all doses of the tested neocryptolepine derivatives required to inhibit growth were definitely lower for biofilms than for planktonic cell cultures that contained similar numbers of metabolically active cells. The concentrations of L-proline and glycine derivatives of 5*H*-indolo[2,3-b] quinoline substituted in position either 2 or 9 needed to decrease the burden of mature biofilms by 50% were lower for biofilm cell inhibition than for planktonic cell inhibition (Fig. 2). L-Histidine and both types of dipeptide derivatives of 5,11-dimethyl-5*H*-indolo[2,3-b]quinoline substituted in position either 2 or 9 were considerably less active against *C. albicans* biofilms and their resulting doses required to obstruct the burden of mature biofilm cells were significantly higher. It is also noteworthy that compounds **2a**, **6a**, and **7a** were most active in the tested *in vitro* fungal biofilm model, but levels of their antifungal action were still comparable to antifungal action of DiMIQ, which was used as a reference compound in this study.

The current medical treatment options for *C. albicans* biofilm-related infections are rather rare due to the intrinsic increased tolerance to antimicrobial drugs. Our striking findings showed the tested of neocryptolepine derivatives to be potent agents against *C. albicans in vitro* biofilms, which were active at doses significantly lower than those required against free-floating planktonic fungal cells. Since the examined neocryptolepine derivatives also possessed anticancer activities, there is a great potential of antifungal lock therapy for the prevention and treatment of biofilm-related infections in immunocompromised patients. This strategy lies within a novel trend recently observed in the antimicrobial research area towards the identification of natural products, plants, and their extracts that could be potentially used in other medical fields due to their numerous biological (e.g. anticancer) activities. Combining both anticancer and antimicrobial activities in one drug seems to be beneficial as a means to enhance efficacy in a variety of clinical settings. There are several foreseeable advantages to this anticancer/antimicrobial strategy that include a widened spectrum and potency of drug activity, more rapid treatment effects, and lowered dosing of drugs required for biofilm control and eradication.

Utilizing neocryptolepine derivatives as agents with different mechanisms of action may be a hallmark in future medical therapies in numerous medical disciplines, but it is also obvious that one has to be cautious of some combinations that may be antagonistic or clinically indifferent with additive side effects. Our results presented in this study demonstrate the proof-of-concept for the application of these neocryptolepine derivatives as anticancer and antimicrobial agents, and their further testing is warranted as a novel cancer/transplant-associated anti-*Candida* therapy. It is also apparent that the dual anticancer/antimicrobial action described here is an *in vitro* concept that is difficult to translate into clinical studies, which are definitely needed, but expensive and hard to perform.

Nevertheless, our study provides evidence of dual action of the tested neocryptolepine derivatives against cancer lines and infectious pathogenic microbes *in vitro*, which might be the first step in

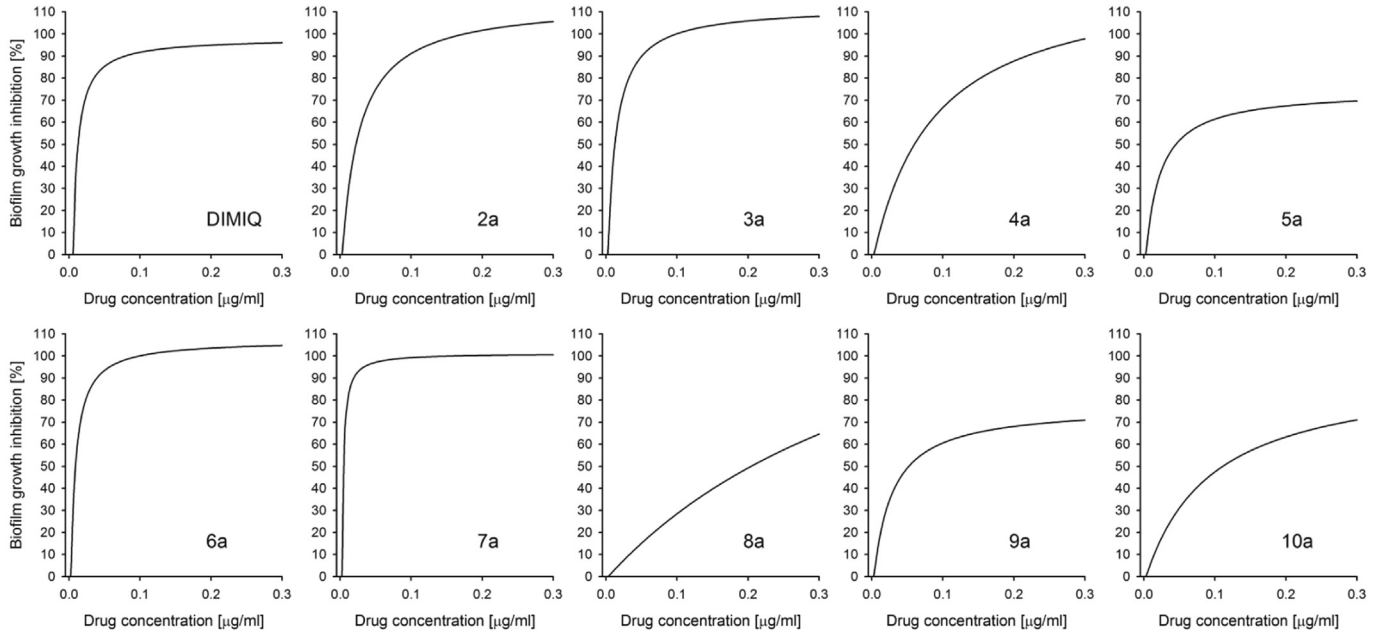


Fig. 2. Percentage reduction of SN250 biofilms after 24 h growth and 24 h treatment with amino acid and dipeptide derivatives at C-2 and C-9 position of 5,11-dimethyl-5*H*-indolo [2,3-b]quinoline.

establishing complex and efficient concurrent anticancer and antimicrobial therapy.

3. Conclusion

In conclusion, we synthesized a set of neocryptolepine derivatives substituted with an amino acid or a dipeptide either at C-2 or C-9 position that were further evaluated for antiproliferative and antimicrobial/antifungal activities. The examined chemicals exerted antiproliferative activity against selected cancer cell lines and possessed antimicrobial activity against pathogenic Gram-positive bacteria. Next, this investigation showed the tested neocryptolepine derivatives to be potent agents against *C. albicans in vitro* biofilms. Interestingly, some of these compounds were active against *C. albicans* biofilms at doses significantly lower than those required against free-floating planktonic fungal cells as shown in Table 4.

Our study provides evidence of dual action of the tested indoloquinoline derivatives against cancer cell lines and infectious pathogenic microbes *in vitro*, and demonstrates the proof-of-concept for the application of these derivatives as anticancer and antimicrobial/antifungal agents. Our findings also showed a great potential of antifungal lock therapy, which might be the first step in establishing complex and efficient concurrent anticancer and antimicrobial strategies for the prevention and treatment of biofilm-related infections in humans.

4. Experimental section

4.1. Chemistry

4.1.1. General

Melting points (m.p.) were determined using a Kofler-type apparatus and were uncorrected. The IR spectra were recorded with a Perkin–Elmer 1640 FTIR BX spectrophotometer in KBr pellets. The ^{1}H and $^{13}C/^{15}N$ NMR spectra of all compounds studied were measured in $CDCl_3$, DMSO-D_6 or a mixture of DMSO-D_6/D_2O using Varian-NMR-vnmrs500, Varian-NMR-vnmrs600 and Varian Gemini 200 spectrometers at a temperature of 298 K. Standard experimental conditions and standard Varian programs were used. To assign the structures under consideration the following 1D and 2D experiments were employed: the 1D selective NOESY, and 2D gradient selected COSY, $^{1}H–^{13}C$ HSQC and $^{1}H–^{13}C/^{1}H–^{15}N$ HMBC. The ^{1}H and ^{13}C NMR chemical shifts are given relative to the DMSO signal at 2.5 ppm (^{1}H NMR spectra) and 39.5 ppm (^{13}C NMR spectra), whereas nitromethane, whose chemical shift of the ^{15}N nucleus is 0.0 ppm, was used as a calibration standard for nitrogen ^{15}N NMR spectra. Concentration of all solutions used for measurements was about 10–20 mg of compounds in 0.6–0.8 cm^3 of solvent. The ESI-MS spectra were recorded on a PE Biosystems Mariner mass spectrometer. Progress of the reaction was monitored by thin layer chromatography (TLC) with Merck DC-Alufolien Kieselgel 60 F_{254}. The chemicals and solvent were purchased from Fluka Company. Column chromatography was performed on Merck silica gel 60 (230–400 mesh). Measurement of optical rotation was performed using a Jasco Digital Polarimeter P-2000; measurements of optical rotation were made at 589 nm at 20 °C. HPLC experiments were carried out on a: Waters HPLC system (Waters Assoc., Milford, MA, USA) consisting of a Multisolvent Delivery System 600E, a Photodiode Array Detector 2996, a Rheodyne Model 7725i injector and Chromatography Manager Empower 2 software for PC computations; and a Luna C-18 column (Phenomenex, USA) with 5 μm particles, 250 × 4.6 mm. Detection was performed using UV at $\lambda = 275$ nm; the compound concentration was about 2.0 mg/mL, and the injection volume was 20 μL. Method: the mobile phase consisting of A (H_2O + 0.1% TFA, v/v) and B (acetonitrile + 0.1% TFA, v/v) was used with a linear gradient from 20% B to 80% B for 20 min (**2a, 3a, 4a, 5a**). All key compounds were proven by HPLC method to show ≥95% purity.

4.1.2. General procedure for synthesis of compounds 2, 3 and 4

To a solution of N^{α}-protected L-amino acid (1 mM), TBTU (1 mM) and HOBt (1 mM) in DMF (3 mL), DIPEA (1.5 mM) was added and the mixture was stirred for 15 min at room temperature. Then the solution of 5,11-dimethyl-5*H*-indolo[2.3-b]quinolin-2-ylamine **(1)** (0.76 mM) in 2 mL DMF was added and the reaction mixture was stirred at room temperature for 6–24 h (TLC monitoring). After the reaction was completed the solvent was evaporated under reduced pressure at ca. 40 °C. The resulting oil was treated with water and $CHCl_3$, and the organic layer was separated, and washed successively with $NaHCO_3$ aq solution and NaCl aq solution. The extract was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness.

4.1.3. N^{α}-tert-Butyloxycarbonyl-N-(5,11-dimethyl-5H-indolo[2,3-b] quinolin-2-yl)glycylamide (2)

Compound **2** was obtained as a yellow solid from Boc-Gly-OH and **1**. The crude product **2** was crystallized from ethyl acetate to afford orange crystal; yield 213 mg (67%); m.p. (°C): 135–137; IR: 3426 (N–H_{amide}), 2977, 1688 (C=$O_{amide+carbamate}$), 1631 (C=$C_{aromatic}$), 1583 (C=$C_{aromatic}$), 1560, 1489 (ring stretching), 1466 (ring stretching), 1448 (ring stretching), 1366 (C–$H_{alifatic}$), 1240 (C–$H_{aromatic}$), 1169, 759 (C–$H_{aromatic}$), 741 (C–$H_{aromatic}$) cm^{-1}; ^{1}H NMR ($CDCl_3$): 8.58 (1H, m, NH), 8.43 (1H, d, $J = 2.2$ Hz), 8.14 (1H, d, $J = 7.7$ Hz), 7.74 (2H, m), 7.60 (1H, d, $J = 9.5$ Hz), 7.53 (1H, brd t, J ca 7.7 Hz), 7.24 (1H, brd t, J ca 7.3 Hz), 5.32 (1H, m, NH), 4.27 (3H, s), 3.99 (2H, d, $J = 6.2$ Hz), 3.05 (3H, s), 1.52 (9H, s); ESI-MS: 837.5 $(2M+H)^+$, 419.2 $(M+H)^+$; Anal. Calcd. for $C_{24}H_{26}N_4O_3 \times 2H_2O$ [454.52]: C 64.42, H 6.65, N 12.33 Found: C 64.08, H 6.61, N 12.46.

4.1.4. N-(5,11-Dimethyl-5H-indolo[2,3-b]quinolin-2-yl)glycylamide dihydrochloride (2a)

Product **2** (400 mg, 0.95 mM) was treated with 2.2 M HCl/CH_3OH (13 mM, 6 mL) and stirred for 2 h (TLC monitoring). The precipitated salt **2a** was collected by filtration and recrystallized from ethyl acetate; yield 400 mg (95%); m.p. (°C): 265; IR: 3401 (N–H_{amide}), 2951, 1699 (C=O_{amide}), 1640 (C=$C_{aromatic}$), 1615 (C=$C_{aromatic}$), 1579 (C=$C_{aromatic}$), 1537, 1499 (ring stretching), 1463 (ring stretching), 1361 (C–$H_{alifatic}$), 1323, 1251 (C–$H_{aromatic}$), 1198, 747 (C–$H_{aromatic}$) cm^{-1}; ^{1}H NMR (DMSO + D_2O): 8.33 (1H, d, $J = 2.3$ Hz, H-1), 7.96 (1H, m, H-4), 7.95 (1H, m, H-10), 7.86 (1H, dd, $J = 2.3$, $J = 9.3$ Hz, H-3), 7.29 (1H, m, H-8), 7.28 (1H, m, H-7), 7.19 (1H, m, H-9), 4.06 (3H, s, 5-C$\underline{H}_3$), 3.82 (2H, s, H-2′), 2.83 (3H, s, 11-C$\underline{H}_3$); ^{13}C NMR (DMSO + D_2O): 166.4 (C-1′), 148.9 (C-11), 146.5 (C-5a), 140.3 (C-6a), 136.2 (C-2), 132.7 (C-4a), 130.9 (C-8), 127.0 (C-3), 124.9 (C-10), 124.8 (C-9), 124.3 (C-11a), 120.9 (C-10a), 120.7 (C-10b), 118.6 (C-4), 116.0 (C-1), 113.5 (C-7), 42.2 (C-2′), 37.2 (5-C$\underline{H}_3$), 17.1 (11-C$\underline{H}_3$); ^{15}N NMR (DMSO + D_2O): −243.6 (N-5); ESI-MS: 319.3 $(M+H)^+$; Anal. Calcd. for $C_{19}H_{18}N_4O \times 3H_2O \times 2HCl$ [445.34]: C 51.24, H 5.88, N 12.58, Cl 15.92 Found: C 51.95, H 5.90, N 12.55, Cl 15.90; HPLC: 97.1%.

4.1.5. N^{α}-tert-Butyloxycarbonyl-N-(5,11-dimethyl-5H-indolo[2,3-b] quinolin-2-yl)-L-prolylamide (3)

Compound **3** was obtained as a red solid from Boc-*L*-Pro-OH and **1**. The crude product **3** was purified by chromatography on a silica gel column with chloroform-methanol 8:1 (v/v) and crystallized from ethyl acetate/diethyl ether to afford an orange solid; yield 220 mg (63%); m.p. (°C): 198–200; IR: 3302 (N–H_{amide}), 2974, 1669 (C=$O_{amide+carbamate}$), 1632 (C=$C_{aromatic}$), 1575 (C=$C_{aromatic}$),

1557, 1489 (ring stretching), 1467 (ring stretching), 1446 (ring stretching), 1365 (C–$H_{alifatic}$), 1238 (C–$H_{aromatic}$), 1162, 759, 741 (C–$H_{aromatic}$) cm^{-1}; ^1H NMR ($CDCl_3$) (two rotamers [22]): 9.97 (1H, m, NH), 8.44 (1H, n m), 8.13 (1H, brd d, J = 7.8 Hz), 7.81 (1H, dd, J = 9.3 Hz, J = 2.1 Hz), 7.72 (1H, brd d, J = 7.8 Hz), 7.52 (2H, brd t, J ca 7.6 Hz), 7.22 (1H, brd t, J = 7.6 Hz), 4.57 (1H, m), 4.20 (3H, s), 3.50 (2H, m), 3.06 (3H, s), 2.50 (1H, m), 2.02 (3H, m), 1.70 and 1.55 (9H [1:3.16], s and s); ESI-MS: 917.6 $(2M+H)^+$, 459.3 $(M+H)^+$; Anal. Calcd. for $C_{27}H_{30}N_4O_3 \times 2H_2O$ [494.58]: C 65.57, H 6.93, N 11.33 Found: C 65.83, H 6.53, N 11.19.

4.1.6. *N-(5,11-Dimethyl-5H-indolo[2,3-b]quinolin-2-yl)-L-prolylamide dihydrochloride (**3a**)*

Product **3** (180 mg, 0.32 mM) was treated with 2.2 M HCl/CH_3OH (13 mM, 6 mL) and stirred for 2 h (TLC monitoring). The precipitated salt **3a** was collected by filtration and recrystallized from ethyl acetate; yield 134 mg (90%); m.p. (°C): 270; IR: 3457 (N–H_{amide}), 2950, 1698 (C=O_{amide}), 1643 (C=$C_{aromatic}$), 1617 (C=$C_{aromatic}$), 1579 (C=$C_{aromatic}$), 1499 (ring stretching), 1459 (ring stretching), 1369 (C–$H_{alifatic}$), 1254 (C–$H_{aromatic}$), 1202, 747 (C–$H_{aromatic}$) cm^{-1}; ^1H NMR (DMSO + D_2O): 8.32 (1H, d, J = 2.4 Hz, H-1), 7.94 (1H, d, J = 9.3 Hz, H-4), 7.92 (1H, d, J = 8.0 Hz, H-10), 7.89 (1H, dd, J = 2.4 Hz, J = 9.3 Hz, H-3), 7.27 (1H, m, H-8), 7.25 (1H, m, H-7), 7.16 (1H, m, H-9), 4.40 (1H, dd, J = 7.0 Hz, J = 9.0 Hz, H-2′), 4.04 (3H, s, 5-CH_3), 3.36 (2H, m, H-5′), 2.83 (3H, s, 11-CH_3), 2.49 (1H, m, H-3'A), 2.06 (1H, m, H-3'B), 2.03 (2H, m, H-4′); ^{13}C NMR (DMSO + D_2O): 169.1 (C-1′), 149.2 (C-11), 146.8 (C-5a), 140.4 (C-6a), 136.1 (C-2), 133.1 (C-4a), 131.1 (C-8), 127.6 (C-3), 124.98 (C-10), 124.95 (C-9), 124.5 (C-11a), 121.0 (C-10a), 121.0 (C-10b), 118.7 (C-4), 117.0 (C-1), 113.7 (C-7), 61.6 (C-2′), 47.8 (C-5′), 37.2 (5-CH_3), 31.1 (C-3′), 25.2 (C-4′), 17.2 (11-CH_3); ^{15}N NMR (DMSO + D_2O): −243.8 (N-5), −251.2 (2-NH), −328.0 (N-6′); ESI-MS: 359.3 $(M+H)^+$; Anal. Calcd. for $C_{22}H_{22}N_4O \times 2H_2O \times 2HCl$ [467.39]: C 56.53, H 6.04, N 11.99, Cl 15.17 Found: C 56.65, H 6.10, N 11.89, Cl 15.16; HPLC: 97.1%; $[\alpha]_D^{20} = -2.12$ (c = 0.1, H_2O).

4.1.7. *N^{α}-tert-Butyloxycarbonyl-N-(5,11-dimethyl-5H-indolo[2,3-b]quinolin-2-yl)-L-histidylamide (**4**)*

Compound **4** was obtained as a red oil from Boc-*L*-His-OH and **1**. The crude product **4** was purified by chromatography on a silica gel column with chloroform-methanol 6:1 (v/v) and crystallized from diethyl ether to afford a red solid; yield 245 mg (65%); m.p. (°C): 166–168; IR: 3299 (N–H_{amide}), 2978, 1693 (C=$O_{amide+carbamate}$), 1633 (C=$C_{aromatic}$), 1574 (C=$C_{aromatic}$), 1489 (ring stretching), 1448 (ring stretching), 1366 (C–$H_{alifatic}$), 1250 (C–$H_{aromatic}$), 1168, 744 (C–$H_{aromatic}$) cm^{-1}; ^1H NMR (DMSO): 10.35 (1H, 2-NH), 8.60 (1H, d, H-1), 8.19 (1H, m, H-10), 8.06 (1H, m, H-3), 7.92 (1H, m, H-4), 7.67 (1H, m, H-6′) 7.57 (1H, m, H-7), 7.47 (1H, m, H-8), 7.18 (1H, m, H-9), 7.15 (1H, m, 2′-NH), 6.89 (1H, m, H-8′), 4.43 (1H, m, H-2′), 4.25 (3H, s, 5-CH_3), 3.03 (3H, s, 11-CH_3), 3.02 (1H, m, H-3'A), 2.94 (1H, m, H-3'B), 1.40 (9H, s, CH_3, Boc); ^{13}C NMR (DMSO): 170.8 (C-1′), 155.3 (CO, Boc), 154.1 (C-6a), 153.8 (C-5a), 140.1 (C-11), 134.8 (C-6′), 133.53 (C-2), 133.53 (C-4′), 132.5 (C-4a), 128.0 (C-8), 124.3 (C-10b), 124.1 (C-10a), 123.3 (C-10), 123.2 (C-3), 120.7 (C-11a), 119.2 (C-9), 116.72 (C-7), 116.72 (C-8′), 115.6 (C-4), 114.7 (C-1), 78.2 (C, Boc), 55.3 (C-2′), 32.8 (5-CH_3), 29.7 (C-3′), 28.2 (CH_3, Boc), 15.2 (11-CH_3); ^{15}N NMR (DMSO + D_2O): −254.2 (N-5), −251.3 (2-NH), −289.7 (2′-NH); ESI-MS: 997.6 $(2M+H)^+$, 499.3 $(M+H)^+$; Anal. Calcd. for $C_{28}H_{30}N_6O_3 \times 3H_2O$ [552.62]: C 60.86, H 6.57, N 18.06 Found: C 60.21, H 6.05, N 18.45.

4.1.8. *N-(5,11-Dimethyl-5H-indolo[2,3-b]quinolin-2-yl)-L-histidylamide tetrahydrochloride (**4a**)*

Product **4** (100 mg, 0.2 mM) was treated with 2.2 M HCl/CH_3OH (13 mM, 6 mL) and stirred for 12 h (TLC monitoring). The precipitated salt **4a** was collected by filtration and recrystallized from ethyl acetate; yield 170 mg (85%); m.p. (°C): 270; IR: 3401 (N–H_{amide}), 3004, 1702 (C=O_{amide}), 1638 (C=$C_{aromatic}$), 1616 (C=$C_{aromatic}$), 1585 (C=$C_{aromatic}$), 1465 (ring stretching), 1362 (C–$H_{alifatic}$), 1248 (C–$H_{aromatic}$), 1195, 832, 756 (C–$H_{aromatic}$) cm^{-1}; ^1H NMR (unprotected neutral form for NMR measurements in DMSO-D6): 8.67 (1H, m, H-1), 8.23 (1H, m, H-10), 8.13 (1H, m, H-3), 7.96 (1H, m, H-4), 7.61 (1H, m, H-6′), 7.58 (1H, m, H-7), 7.48 (1H, m, H-8), 7.18 (1H, m, H-9), 6.92 (1H, m, H-8′), 4.27 (3H, s, 5-CH_3), 3.82 (1H, m, H-2′), 3.09 (3H, s, 11-CH_3), 3.08 (1H, m, H-3'A), 2.85 (1H, m, H-3'B); ^{13}C NMR (unprotected neutral form for NMR measurements in DMSO-D6): 172.0 (C-1′), 155.1 (C-6a), 154.4 (C-5a), 139.7 (C-11), 134.9 (C-6′), 133.1 (C-2), 132.8 (C-4a), 128.0 (C-8), 124.7 (C-10b), 124.4 (C-10a), 123.3 (C-10), 123.1 (C-3), 120.7 (C-11a), 119.0 (C-9), 117.1 (C-7), 115.6 (C-4), 114.8 (C-1), 55.4 (C-2′), 32.7 (5-CH_3), 31.7 (C-3′), 15.2 (11-CH_3); ^{15}N NMR (DMSO): −254.9 (N-5); ESI-MS: 399.2 $(M+H)^+$, 200.3 $(M+2H)^{++}$; Anal. Calcd. for $C_{23}H_{22}N_6O \times 5H_2O \times 4HCl$ [670.84]: C 41.18, H 5.56, N 12.53, Cl 26.42 Found: C 41.76, H 5.06, N 12.54, Cl 26.45; HPLC: 98.4%; $[\alpha]_D^{20} = 17.4$ (c = 0.1, H_2O).

4.1.9. *N^{α}-tert-Butyloxycarbonyl-N-(5,11-dimethyl-5H-indolo[2,3-b]quinolin-2-yl)glycylglycylamide (**5**)*

To a solution of Boc-Gly (122 mg, 0.7 mM) in DMF (7 mL), TBTU (224 mg, 0.7 mM), HOBt (107 mg, 0.7 mM) and DIPEA (0.35 mL, 2 mM) were added at RT and the solution was stirred for 15 min. Then the *N*-(5,11-dimethyl-5*H*-indolo[2,3-b]quinolin-2-yl)glycylamide dihydrochloride (**2a**) (200 mg, 0.5 mM) was added to the solution and the reaction mixture was stirred for 24 h. After the reaction was completed the solvent was evaporated under reduced pressure at ca. 40 °C. The resulting oil was treated with water (10 mL) and chloroform (40 mL) and stirred for 5 min. The organic layer was separated, and washed successively with $NaHCO_3$ aq solution (30 mL) and NaCl aq solution (20 mL). The extract was dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by flash chromatography and crystallization from ethyl acetate to afford compound **5** as orange crystal, yield 200 mg (84%); m.p. (°C): 185–186; IR: 3350 (N–H_{amide}), 2979, 1685 (C=$O_{amide+carbamate}$), 1640 (C=$C_{aromatic}$), 1578 (C=$C_{aromatic}$), 1500 (ring stretching), 1464 (ring stretching), 1367 (C–$H_{alifatic}$), 1250 (C–$H_{aromatic}$), 1170, 750 (C–$H_{aromatic}$) cm^{-1}; ^1H NMR (DMSO): 10.5 (1H, m, 2-NH), 8.87 (1H, d, J = 2.2 Hz, H-1), 8.40 (1H, d, J = 8 Hz, H-10), 8.34 (1H, d, J = 9.4 Hz, H-4), 8.30 (1H, t, J = 5.8 Hz, 2′-NH), 8.21 (1H, dd, J = 2.2 Hz, J = 9.4 Hz, H-3), 7.70 (1H, d, J = 8 Hz, H-7), 7.65 (1H, m, H-8), 7.46 (1H, m, H-9), 7.13 (1H, t, J = 6.1 Hz, 4′-NH), 4.40 (3H, s, 5-CH_3), 4.01 (2H, d, J = 5.8 Hz, H-2′), 3.67 (2H, d, J = 6.1 Hz, H-4′), 3.20 (3H, s, 11-CH_3), 1.41 (9H, s, CH_3, Boc); ^{13}C NMR (DMSO): 170.0 (C-3′), 168.5 (C-1′), 156.0 (CO, Boc), 147.1 (C-11), 146.4 (C-5a), 140.5 (C-6a), 136.3 (C-2), 131.5 (C-4a), 129.4 (C-8), 125.3 (C-3), 124.1 (C-10), 123.3 (C-11a), 123.0 (C-9), 120.5 (C-10a), 120.1 (C-10b), 117.7 (C-4), 113.8 (C-1), 112.8 (C-7), 78.2 (C, Boc), 43.3 (C-4′), 42.8 (C-2′), 36.2 (5-CH_3), 28.2 (CH_3, Boc), 16.0 (11-CH_3); ^{15}N NMR (DMSO): −241.8 (N-5), −251.2 (2-NH), −276.0 (2′-NH), −301.8 (4′-NH); ESI-MS: 951.5 $(2M+H)^+$, 498.3 $(M+Na)^+$, 476.3 $(M+H)^+$; Anal. Calcd. for $C_{26}H_{29}N_5O_4 \times 2H_2O$ [511.57]: C 61.04, H 6.50, N 13.68 Found: C 61.00, H 6.49, N 13.50.

4.1.10. *N-(5,11-Dimethyl-5H-indolo[2,3-b]quinolin-2-yl)glycylglycylamide dihydrochloride (**5a**)*

Product **5** (150 mg, 0.31 mM) was treated with 2.2 M HCl/CH_3OH (13 mM, 6 mL) and stirred for 2 h (TLC monitoring). The precipitated salt **5a** was collected by filtration and recrystallized from ethyl acetate; yield 134 mg (91%); m.p. (°C): 210–212; IR: 3331 (N–H_{amide}), 3134, 1790 (weak), 1681 (C=O_{amide}), 1644 (C=$C_{aromatic}$), 1619 (C=$C_{aromatic}$), 1572 (C=$C_{aromatic}$), 1533, 1504 (ring stretching), 1432 (ring stretching), 1366 (C–$H_{alifatic}$), 1198, 1136, 795, 759 (C–$H_{aromatic}$), 705 cm^{-1}; ^1H NMR (DMSO + D_2O): 8.32 (1H, d, J = 2 Hz,

H-1), 7.98 (1H, d, $J = 8$ Hz, H-10), 7.93 (1H, d, $J = 9.5$ Hz, H-4), 7.83 (1H, dd, $J = 2.5$, $J = 9.5$ Hz, H-3), 7.34 (1H, m, H-8), 7.32 (1H, m, H-7), 7.23 (1H, m, H-9), 4.06 (3H, s, 5-C$\underline{H}_3$), 4.00 (2H, s, H-2′), 3.77 (2H, s, H-4′), 2.82 (3H, s, 11-C$\underline{H}_3$); ^{13}C NMR (DMSO + D_2O): 170.2 (C-3′), 168.6 (C-1′), 149.0 (C-11), 146.7 (C-5a), 140.5 (C-6a), 136.5 (C-2), 132.7 (C-4a), 131.1 (C-8), 127.3 (C-3), 125.1 (C-10), 125.0 (C-9), 124.4 (C-11a), 121.1 (C-10a), 120.9 (C-10b), 118.5 (C-4), 116.2 (C-1), 113.7 (C-7), 44.1 (C-2′), 41.6 (C-4′), 37.2 (5-C$\underline{H}_3$), 17.1 (11-C$\underline{H}_3$); ^{15}N NMR (DMSO + D_2O): −243.6 (N-5); ESI-MS: 376.1 $(M+H)^+$; Anal. Calcd. for $C_{21}H_{21}N_5O_2 \times 2HCl \times 2H_2O$ [484.38]: C 52.07, H 5.62, N 14.46, Cl 14.64 Found: C 52.14, H 5.98, N 14.39, Cl 14.60; HPLC: 95.1%.

4.2. Biological assays

4.2.1. Antiproliferative assay

4.2.1.1. Cell lines. Established *in vitro*, human cancer cell lines KB (cervix carcinoma), A549 (non-small cell lung cancer), MCF-7 (breast cancer), LoVo (colon) and normal mice fibroblasts (BALB/3T3) were used. All lines were obtained from American Type Culture Collection (Rockville, Maryland, USA) with the exception of LoVo by courtesy of Prof. E. Borowski (Technical University of Gdańsk, Poland). All the cell lines were maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

KB, A549, MCF-7 and LoVo cells were cultured in a mixture of RPMI 1640 and Opti-MEM (1:1) medium (Gibco, UK) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate (KB and LoVo), 5% fetal bovine serum (all from Sigma–Aldrich Germany). The culture of MCF-7 cells was supplemented with 0.01 mg/mL insulin (Sigma–Aldrich Germany). BALB/3T3 cells were cultured in Dulbecco medium (IIET, Wroclaw) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum. All culture medium was supplemented with 100 units/mL penicillin (Sigma–Aldrich, Germany), and 100 μg/mL streptomycin (Polfa, Tarchomin S.A., Poland). All cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere.

4.2.1.2. In vitro antiproliferative assay. Test solutions of the compounds tested (1 mg/mL) were prepared by dissolving the substances in 100 μl of water completed with 900 μl of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 10, 1, 0.1 and 0.01 μg/mL. Results were converted into μM concentrations.

Twenty-four hours before addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at a density of 10^4 cells per well in 100 μl of culture medium. The cells were cultured in RPMI 1640 and Opti-MEM (1:1) medium supplemented with 2 mM glutamine, penicillin (100 U/mL) (both from Sigma–Aldrich, Germany), streptomycin (100 μg/mL) (Polfa, Tarchomin, Poland) and 5% fetal bovine serum (Gibco, USA). The cell cultures were maintained at 37 °C in a humid atmosphere saturated with 5% CO_2.

4.2.1.3. SRB assay. The details of this technique were described by Skehan [33]. The antiproliferative assay was performed after 72-h exposure of the cultured cells to varying concentrations (from 0.01 to 10 μg/mL) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, POCh, Gliwice, Poland) on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (Sigma–Aldrich, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (Sigma–Aldrich, Germany) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Synergy H4 photometer (BioTek Instruments, USA). The background optical density was measured in the wells filled with culture medium, without the cells. Each compound in a given concentration was tested in triplicate in each experiment, which was repeated 3–5 times.

The results of cytotoxic activity *in vitro* were expressed as IC_{50} – the concentration of compound (in μg/mL and μM) that inhibits the proliferation rate of the tumor cells by 50% as compared to control untreated cells.

4.2.2. Antimicrobial assay

Minimal inhibitory concentration (MIC) was tested by the twofold serial dilution method using Mueller–Hinton II agar medium (Becton Dickinson) for bacteria and RPMI agar (Sigma) for *Candida*, according to CLSI guidelines [31,32]. Concentrations of tested agents in solid medium ranged from 3125 to 200 μg/mL. The final inoculum of all studied organisms was 10^4 CFU mL^{-1} (colony forming units per mL), except the final inoculum for *Enterococcus hirae* ATCC 10541, which was 10^5 CFU mL^{-1}. Minimal inhibitory concentrations were read after 18 h of incubation at 35 °C. Microorganisms used in this study were as follows: Gram-positive cocci: *Staphylococcus aureus* NCTC 4163, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *E. hirae* ATCC 10541, *Micrococcus luteus* ATCC 9341, *M. luteus* ATCC 10240; Gram-negative rods: *Escherichia coli* ATCC 10538, *E. coli* ATCC 25922, *E. coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *P. aeruginosa* NCTC 6749, *P. aeruginosa* ATCC 27863, *Bordetella bronchiseptica* ATCC 4617.

C. albicans ATCC 10231, *C. albicans* ATCC 90028, and *Candida parapsilosis* ATCC 90028 were used for testing antifungal activities of the compounds. Microorganisms were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

4.2.3. Antifungal susceptibility testing in in vitro C. albicans biofilms

Experiments designed to assess the impact of the selected neocryptolepine derivatives on *C. albicans* biofilm cell viability were performed in a 96-well format as previously described [34]. For the antifungal susceptibility assay, 100 μl of the inoculum (10^6 cells/mL in RPMI medium-MOPS) was added to each well of a 96-well flat-bottom plate. After 24-h incubation at 37 °C, the wells were washed with phosphate-buffered NaCl (PBS) three times to remove any nonadherent cells. Fresh medium and the tested chemicals were added followed by additional periods of 24-h long incubation. The concentrations of the tested chemicals ranged from 2.4 μg/mL up to 625.0 μg/mL. After 24 h of incubation at 30 °C, an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was performed and endpoints were determined spectrophotometrically at 492 nm as a measure of cell metabolic activity. Briefly, 90 μl of XTT (1 mg/mL) and 10 μl of phenazine methosulfate (320 μg/mL) were added to each well and the plate was incubated at 37 °C for 30 min. Absorbance at 490 nm was measured using an automated plate reader. Each of the above growth assays was performed in triplicate for each of the performed experiments.

Abbreviations

Boc, *tert*-butyloxycarbonyl group; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; HCl, hydrochloride acid; HOBt, *N*-hydroxybenzotriazole monohydrate; HPLC, high performance liquid chromatography;

IR, infrared spectroscopy; MS, mass spectrometry; NMR, nuclear magnetic resonance; SAR, structure activity relationship; TBTU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS, trimethylsilyl; $N-H_{amide}$, $N-H_{amide}$ stretching; $C{=}O_{amide+carbamate}$ and $C{=}O_{amide}$, C=O stretching, amide I band; $C{=}C_{aromatic}$, $C{=}C_{aromatic}$ stretching; ring stretching, heterocyclic compounds ring stretching; $C-H_{alifatic}$, $C-H_{alifatic}$ rocking; $C-H_{aromatic}$ (ca 1250 cm^{-1}), $C-H_{aromatic}$ bending, in-plane; $C-H_{aromatic}$ (ca 750 cm^{-1}), $C-H_{aromatic}$ bending, out-of-plane; brd, broadened; n, narrow

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.060.

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