Pharmacological Reports 71 (2019) 357–360

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Short communication

Synthesis of novel spirooxindole-pyrrolidines and evaluation of their cytotoxic activity

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ARTICLE INFO

Article history:
Received 24 June 2018
Received in revised form 11 December 2018
Accepted 14 December 2018
Available online 15 December 2018

Keywords:
Spirooxindole-pyrrolidines
1,3-dipolar cycloaddition
Azomethine ylide
Cytotoxicity
HeLa

ABSTRACT

Background: A variety of spirooxindoles have demonstrated cytotoxic activity toward several cancer cell lines. This study investigates the cytotoxicity of five novel spirooxindole-pyrrolidines by using the Vero and HeLa cell lines.
Methods: Vero and HeLa cells were treated with the synthesized spirooxindoles, and the cytotoxicity was evaluated by using the AlamarBlue Cell Viability Reagent and live/dead assay.
Results: A series of poly-substituted pyrrolidines differing in nature and in substituent positions were obtained, with yields of 42–63%. Of the synthesized cycloadducts, 3-picolinoyl-4-(2,4-dichlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**4**) was the most cytotoxic (IC_{50} < 20 μg/ml for both cell lines). Besides, 3-picolinoyl-4-(2-chlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**1**) was three times more toxic to the HeLa cancer cell line (IC_{50} = 70 μg/ml) than it was to the Vero healthy cell line. The cytotoxicity of compounds **1** and **4** was confirmed with a live/dead assay. The cytotoxicity of a molecule was found to depend on the substitution nature on the benzene ring at the C-4 atom.
Conclusion: 3-Picolinoyl-4-(2-chlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**1**) can be used as a source for the synthesis of novel therapeutic agents against cancer.

Introduction

Reactions of 1,3-dipolar cycloaddition have huge synthetic potential, since they allow obtainment of complex heterocyclic spiro-systems, including natural compounds and alkaloids [1,2]. Alkaloids containing an indole fragment as part of their structure deserve special attention. Such compounds (including tryptamine, gramine, and many others) have various physiological activities, and some of them are used in medicine [3–5]. In addition, the spiro compounds themselves are no less important, because they have a wide range of biological activity (antiviral [6], anti-cancer [7], anti-bacterial [8,9], fungicidal [10], *etc.*). For example, spirooxo-3,3′-pyrrolidines (MI-77301, MI-43, MI-63) are now being clinically tested as anti-cancer drugs [11]. In this series, the key structural fragment responsible for the presence of bioactivity is the oxindole nucleus, linked to a spiropyrrolidine ring. Some compounds show high activity against the MCF-7 and MDA-MB 231 cancer cells when used at low concentrations and are toxic to the Vero and HEK-293 cell lines when used at very high concentrations [12]. To find new biologically active substances, we synthesized a series of novel spirooxindoles by using azachalcones as dipolarophiles in the 1,3-dipolar cycloaddition reaction.

Materials and methods

Apparatus and analysis

^{1}H-NMR and NOESY spectra were recorded at 25 °C on a Varian-400 spectrometer (400 MHz; Agilent Technologies, Santa Clara, CA, USA), by using DMSO-d_6 as a solvent and tetramethylsilane as an internal standard. ^{13}C-NMR spectra were recorded at 25 °C on a Varian-400 spectrometer (100 MHz; Agilent Technologies, Santa Clara, CA, USA), with DMSO-d_6 as a solvent and tetramethylsilane as an internal standard. Thin-layer chromatography (TLC) was done with Alugram Xtra Sil G 254 plates (Macherey-Nagel GmbH & Co. KG, Düren, Germany; ethanol-chloroform, 1:25). The melting points were measured in open capillaries. Elemental analyses were carried out on a Vario Micro cube Elementar CHNS analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). IR spectra

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https://doi.org/10.1016/j.pharep.2018.12.004
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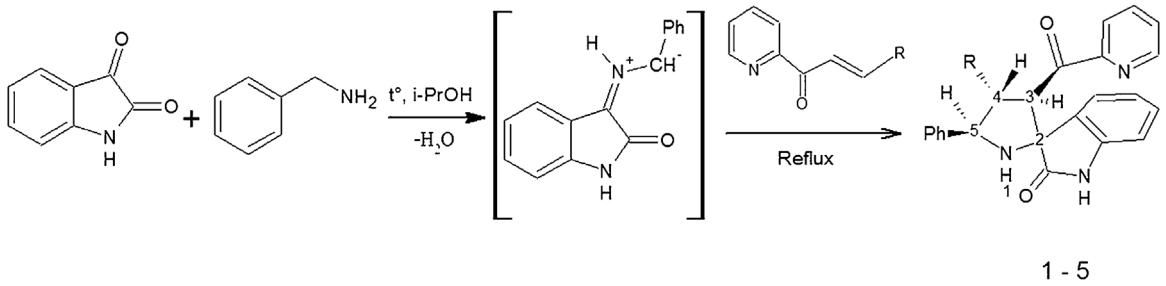


R = 2-Cl-C_6H_4 (1); R = 3-NO_2-C_6H_4 (2); R = 4-OMe-C_6H_4 (3);

R = 2,4-Cl,Cl-C_6H_3 (4); R = 2-OMe-C_6H_4 (5).

Scheme 1. Synthesis of target spirooxindoles (**1-5**).

were recorded on an FSM 1201 IR-Fourier spectrometer by using KBr pellets.

*General procedure for the synthesis of 4-aryl-3-picolinoyl-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one derivatives (**1-5**)*

A mixture of isatin (1.0 mmol), benzyl amine (1.0 mmol), and azachalcone (1.0 mmol) was refluxed for 1–2 h in absolute isopropyl alcohol (7–10 mL) with a drying tube. The reaction was monitored by TLC. After the reaction was over, the precipitate was isolated, washed with cold isopropyl alcohol, and dried *in vacuo*.

*Data of spirooxindoles **(1-5)***

*3-Picolinoyl-4-(2-chlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**1**)*

Beige powder; yield 54%; mp: 210–212 °C; IR (KBr pellets, ν_{max}/cm^{-1}): 1620, 1702 (C = O), 3186, 3330 (N-H); ^{1}H-NMR (400 MHz, DMSO – d_6), δ: 3.95 (d, 1H, *J* = 5.2 Hz, NH_{pyr}), 4.63–4.68 (t, 1H, H-4), 4.94–4.98 (m, 1H, H-5), 5.16 (d, 1H, H-3), 6.35–8.40 (m, 17H, ArH), 10.29 (s, 1H, NH_{isat}); ^{13}C-NMR (100 MHz, DMSO – d_6), δ: 49.93, 61.37, 67.94, 68.58, 109.09, 121.37, 125.65, 127.33, 127.83, 128.44, 128.88, 129.72, 130.30, 134.45, 137.43, 138.25, 141.22, 142.77, 148.69, 152.47, 182.53, 198.75. Anal. Calcd. for $C_{29}H_{22}ClN_3O_2$: C, 72.58; H, 4.59; N, 8.76. Found: C, 72.88; H, 4.96; N, 9.32.

*3-Picolinoyl-4-(3-nitrophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**2**)*

Beige powder; yield 48%; mp: 186–189 °C; IR (KBr pellets, ν_{max}/cm^{-1}): 1618, 1704 (C = O), 3180, 3337 (N-H); ^{1}H-NMR (400 MHz, DMSO – d_6), *δ*: 4.02 (d, 1H, *J* = 5.2 Hz, NH_{pyr}), 4.06–4.11 (t, 1H, H-4), 5.03–5.08 (m, 1H, H-5), 4.48 (d, 1H, H-3), 6.56–8.85 (m, 17H, ArH), 10.06 (s, 1H, NH_{isat}); ^{13}C-NMR (100 MHz, DMSO – d_6), δ: 53.31, 61.25, 67.79, 72.49, 109.74, 121.42, 121.71, 122.43, 122.74, 124.54, 127.29, 127.61, 128.10, 128.60, 129.53, 130.12, 135.32, 137.67, 138.87, 142.50, 143.11, 147.80, 149.54, 153.14, 180.60, 199.70. Anal. Calcd. for $C_{29}H_{22}N_4O_4$: C, 71.02; H, 4.49; N, 11.43. Found: C, 70.71; H, 4.41; N, 11.20.

*3-Picolinoyl-4-(4-methoxyphenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**3**)*

Beige powder; yield 42%; mp: 220–222 °C; IR (KBr pellets, ν_{max}/cm^{-1}): 1620, 1699 (C = O), 3176, 3332 (N-H); ^{1}H-NMR (400 MHz, DMSO – d_6), δ: 3.66 (s, 3H, 4-OMe), 3.83 (d, 1H, *J* = 5.2 Hz, NH_{pyr}), 3.89–3.94 (t, 1H, H-4), 4.97–5.05 (m, 2H, H-3, H-5), 6.35–8.42 (m, 17H, ArH), 10.24 (s, 1H, NH_{isat}); ^{13}C-NMR (100 MHz, DMSO – d_6), δ: 54.13, 55.28, 61.71, 67.29, 67.65, 109.00, 114.29, 121.27, 125.62, 127.63, 128.39, 128.77, 129.18, 129.61, 130.71, 137.49, 142.09, 142.76, 148.68, 152.59, 158.37, 182.52, 199.18. Anal. Calcd. for $C_{30}H_{25}N_3O_3$: C, 75.77; H, 5.30; N, 8.84. Found: C, 73.92; H, 5.20; N, 9.14.

*3-Picolinoyl-4-(2,4-dichlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**4**)*

Light **brown** powder; yield 53%; mp: 185–187 °C; IR (KBr pellets, ν_{max}/cm^{-1}): 1618, 1700 (C = O), 3178, 3332 (N-H); ^{1}H-NMR (400 MHz, DMSO – d_6), δ: 4.03 (d, 1H, *J* = 5.2 Hz, NH_{pyr}), 4.58–4.63 (t, 1H, H-4), 4.90–4.94 (m, 1H, H-5), 5.11 (d, 1H, H-3), 6.36–8.40 (m, 16H, ArH), 10.33 (s, 1H, NH_{isat}); ^{13}C-NMR (100 MHz, DMSO – d_6), δ: 49.65, 61.25, 67.91, 68.49, 109.14, 121.40, 125.61, 127.28, 127.83, 128.40, 128.96, 130.23, 131.17, 132.12, 135.32, 137.43, 141.01, 142.78, 148.68, 152.38, 182.50, 198.61. Anal. Calcd. for $C_{29}H_{21}Cl_2N_3O_2$: C, 67.70; H, 4.11; N, 8.17. Found: C, 67.15; H, 3.83; N, 7.94.

*3-Picolinoyl-4-(2-methoxyphenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**5**)*

Beige powder; yield 63%; mp: 201–203 °C; IR (KBr pellets, ν_{max}/cm^{-1}): 1622, 1706 (C = O), 3183, 3336 (N-H); ^{1}H-NMR (400 MHz, DMSO – d_6), δ: 3.43 (s, 3H, 2-OMe), 3.74 (d, 1H, *J* = 5.6 Hz, NH_{pyr}), 4.47–4.52 (t, 1H, H-4), 5.02–5.06 (m, 1H, H-5), 5.22 (d, 1H, H-3), 6.36–8.43 (m, 17H, ArH), 10.21 (s, 1H, NH_{isat}); ^{13}C-NMR (100 MHz, DMSO – d_6), δ: 47.69, 55.95, 59.75, 66.90, 67.98, 108.94, 112.19, 121.19, 125.78, 127.52, 127.96, 128.19, 128.43, 128.82, 130.54, 137.33, 142.31, 142.74, 148.67, 152.77, 158.21, 182.45, 199.19. Anal. Calcd. for $C_{30}H_{25}N_3O_3$: C, 75.77; H, 5.30; N, 8.84. Found: C, 75.07; H, 4.67; N, 8.73.

Procedure for the study of cytotoxic activity

The cytotoxic activity of the synthesized compounds was studied by using kidney epithelial cells of the African green monkey (Vero) and the cancer cell line HeLa (cells were provided by the Lab of Nanobiotechnology staff, IBPPM RAS, Saratov, Russia). The cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. Cell viability was tested with the AlamarBlue Cell Viability Reagent (a colorimetric test to assess cellular metabolic activity). The cells were seeded in a 96-well plate at a density of

Table 1
Concentrations of novel spirooxindoles **1-5** causing 50% inhibition of the respiratory activity of Vero and HeLa cell lines.

Product	R	Vero (μg/ml)	HeLa (μg/ml)
1	2-Cl-C_6H_4	>110	70
2	3-NO_2-C_6H_4	>110	>110
3	4-OMe-C_6H_4	>110	>110
4	2,4-Cl,Cl-C_6H_3	17	16
5	2-OMe-C_6H_4	>110	>110

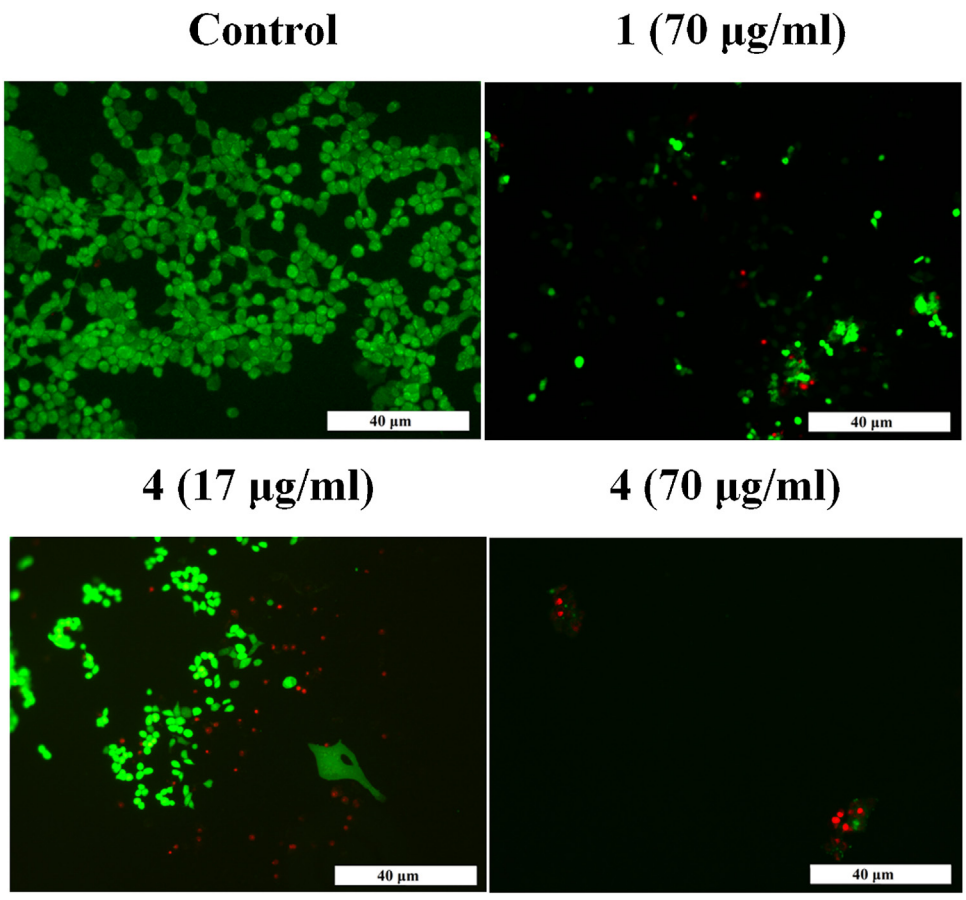


Fig. 1. Spirooxindoles **1** and **4** increased the death of HeLa cells. The live/dead data revealed an increase in cell death (red fluorescence) after treatment with the spirooxindoles, as compared to the control group (green fluorescence). Bars are 40 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

7×10^3 cells/ml in Dulbecco's modified Eagle's medium (DMEM) to form 70% of the monolayer content and were then incubated with toxicants (8–110 μg/ml test compound in DMSO solution) for 24 h. After the addition of 100 μl of an aqueous solution of Alamar Blue, the plates were incubated at 37 °C. After 2 h, the plates were read on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA) at a test wavelength of 530 nm and a reference wavelength of 600 nm. Absorbance in the absence of drugs was set as the 100% control. All experiments were conducted three times (each in duplicate), and mean values are reported.

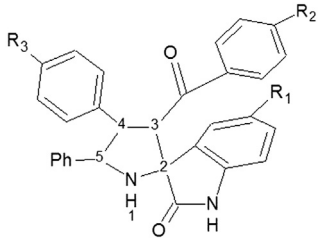
Live/dead assay

The live/dead cell viability assay (Invitrogen, USA) was conducted by following the manufacturer's instructions. Cells were treated with various concentrations of compounds **1** and **4** for 24 h. The live/dead assay was analyzed by multicolor imaging with a Leica DMI 3000B manual inverted microscope. Dead cells were detected by the red fluorescent signal (610 nm), while live cells were analyzed by green fluorescent light emission (527 nm).

Results and discussion

Chemistry

A series of spirooxindoles were synthesized by a one-step stereoselective reaction of 1,3-dipolar cycloaddition (Scheme 1). Azomethine ylide generated *in situ via* condensation of isatin and benzyl amine in boiling absolute isopropyl alcohol was used as a 1,3-dipole. α,β-Unsaturated ketones (azachalcones) obtained by the well-known method [13] served as dipolarophiles. For compound **5** as an example, it was found by NOESY spectroscopy that the reaction proceeded stereoselectively and mostly formed a single diastereomer. The diastereomer conformation was determined from the correlations of the C-3/C-5 protons (5.22/5.02, δ, ppm) and also by their interactions with the protons of the aromatic substituent at the C-4 position (5.22/7.29 and 5.02/7.29, respectively), which determines their *cis*-configuration.



6 a - c

R_1 = H, R_2 = H, R_3 = OMe (6a);

R_1 = H, R_2 = H, R_3 = NO_2 (6b);

R_1 = Cl, R_2 = OCH_2CH_2Piperidyl, R_3 = H (6c).

Fig. 2. Compounds 6a–c.

Table 2
Toxicity to the Vero cell line of known spirooxindoles **6a-c** and synthesized compounds **2**, **3**, and **5**.

Compound	6a	6b	6c	2	3	5
IC_{50} (μg/ml)	21	>25	>30	>110	>110	>110

Cytotoxic activity in vitro *and fluorimetric assay*

Primary screening *in vitro* of the obtained compounds **1-5** was carried out in the concentration range 8–110 μg/ml for the detection of a cytotoxic effect on the Vero healthy cells and the HeLa cancerous cells (Table 1). The most cytotoxic substance was cycloadduct **4**, with an IC_{50} of 16–17 μg/ml for both cell lines. At a concentration of 110 μg/ml of compound **4**, the growth of both lines was inhibited by nearly 90%. We attribute this effect to the presence of two chlorine atoms at 2,4-positions in the benzene ring at the C-4 atom of spirooxindole **4**. The presence of chlorine atoms determines the toxicity of the molecule and increases its lipophilicity. Compound **1**, containing one chlorine atom at position 2 in the benzene ring at C-4, proved to be much less toxic than compound **4**. The maximum inhibition of the respiratory activity of the HeLa cells by compound **1** was 50% at 70 μg/ml. The cytotoxic activity of this compound did not increase with increasing concentration. Interestingly, cycloadduct **1** at 110 μg/ml was not toxic for the Vero cells, because growth was inhibited by no more than 15%. The other spirooxindoles were non-toxic to both cell lines at all tested concentrations. An exception was compound **5**, which inhibited the growth of Vero cells by 40% when used at 35 μg/ml.

The cytotoxic effects of products **1** and **4** were also confirmed by fluorimetric assays with the HeLa cancer cell line (Fig. 1).

Comparison of the cytotoxicity (Fig. 2, Table 2) of compounds **1-5** with the bioactivity of the known structural analogs [12] **6a-c** showed that the presence of a -COPy fragment at the C-3 atom reduced the cytotoxicity of compounds **2**, **3**, and **5**. This is because the presence of a pyridine ring increases the polarity of the molecule and, accordingly, reduces its lipophilicity. As expected, the introduction of chlorine atoms into the benzene ring at the C-4 atom increased the cytotoxicity of the compounds, especially when two chlorine atoms were introduced. The **6c** analog showed [12] high activity toward the MCF-7 cancer cells (IC_{50} = 4 μg/ml) and low toxicity to the Vero healthy cells, possibly owing to the presence of a peripheral piperidyl substituent, which may be the main pharmacophore group.

Conclusion

A series of previously unknown spirooxindoles were obtained in good yield through the stereoselective reaction of 1,3-dipolar cycloaddition. Cytotoxicity tests of the novel compounds were carried out with the Vero healthy cells and HeLa cancerous cells. Comparisons were made between the cytotoxic activity of products **1-5** and that of known structure analogs **6a-c**. The cytotoxicity of a molecule was found to depend on the substitution nature on the benzene ring at the C-4 atom. 3-Picolinoyl-4-(2-chlorophenyl)-5-phenylspiro[indoline-3,2'-pyrrolidine]-2'-one (**1**) can be used as a source for the synthesis of novel therapeutic agents against cancer.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

The study was not supported by any specific grant.

Acknowledgment

We are grateful to T.E. Pylaev for help with cell culture experiments and to E.S. Avdeeva for technical assistance.

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