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Synthesis, antiproliferative and antibacterial evaluation of C-ring modified colchicine analogues

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ABSTRACT

A series of 10 amine derivatives of colchicine have been obtained with high yields by modification at C(10)-OCH_3 position of C-ring and characterized by spectroscopic methods. *In vitro* cytotoxicity has been evaluated against four human tumour cell lines (HL-60, HL-60/vinc, LoVo, LoVo/DX), as well as antibacterial activity against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). From among the compounds tested the most active is colchicine derivative **2h** with bis(2-methoxyethyl)amine substituent which is active in nanomolar to submicromolar concentrations and is several times more cytotoxic than cisplatin and doxorubicin. This compound is also effective against the methicillin-resistant *Staphylococci* strains.

1. Introduction

Chemical modification of biologically active compounds of natural origin is one of the most efficient approaches in drug development. Among them, colchicine (**1**) (Scheme 1) the major alkaloid isolated from *Colchicum autumnale* L. (Liliaceae) is of particular interest as a starting compound. It participates in microtubule polymerization process and regulatory functions in living cells [1–5]. Anticancer activity of colchicine is the capacity of the tubulin–colchicine complex which binds to the ends of microtubules to physically prevent the elongation of the microtubule polymer [3–5]. Many of microtubule-targeting drugs have been discovered and developed in the synthesis to increase the activity [6,7]. The therapeutic potential of the colchicine binding site has been considered for chemotherapy applications. Thus, there is a need to develop colchicine site inhibitors (CSIs) as potential anticancer drugs with higher activity and lower toxicity.

Colchicine is a potent anticancer agent but its medical application in cancer chemotherapy is limited because of its relatively high toxicity, although colchicine is currently used in therapy [8,9], *i.e.* for gout [3,10,11], acute pericarditis [12,13] and Familial Mediterranean fever (FMF) [14,15].

Some derivatives of **1**, such as thiocolchicoside (Neoflax™, Muscoril™) show improved therapeutic properties and clinical significance as anti-inflammatory, analgesic and anticancer drug [16,17]. Though medicinal use of **1** is limited many attempts have been made to discover more effective and less toxic analogues of **1** by modifying the substituents of its basic structure. **1** is still a lead compound for the generation of potent anticancer drugs [18–22]. It was assumed that structural modification of colchicine could produce compounds with novel pharmacological activity [20–22].

The aim of this study was to examine the cytotoxicity and antimicrobial activity of 10 colchicine derivatives. They were newly synthesized and are not commercially available. In order to obtain compounds with better biological activities, in this study we synthesized a variety of novel colchicine derivatives by combining **1** and differently substituted primary and secondary aliphatic amines and tested their anticancer activities against a selected panel of human cancer cell lines. In the present study, the antiproliferative effect of ten colchicine derivatives (**2a**–**2j**) was tested *in vitro* using human promyelocytic leukaemia (HL-60) and its vincristine-

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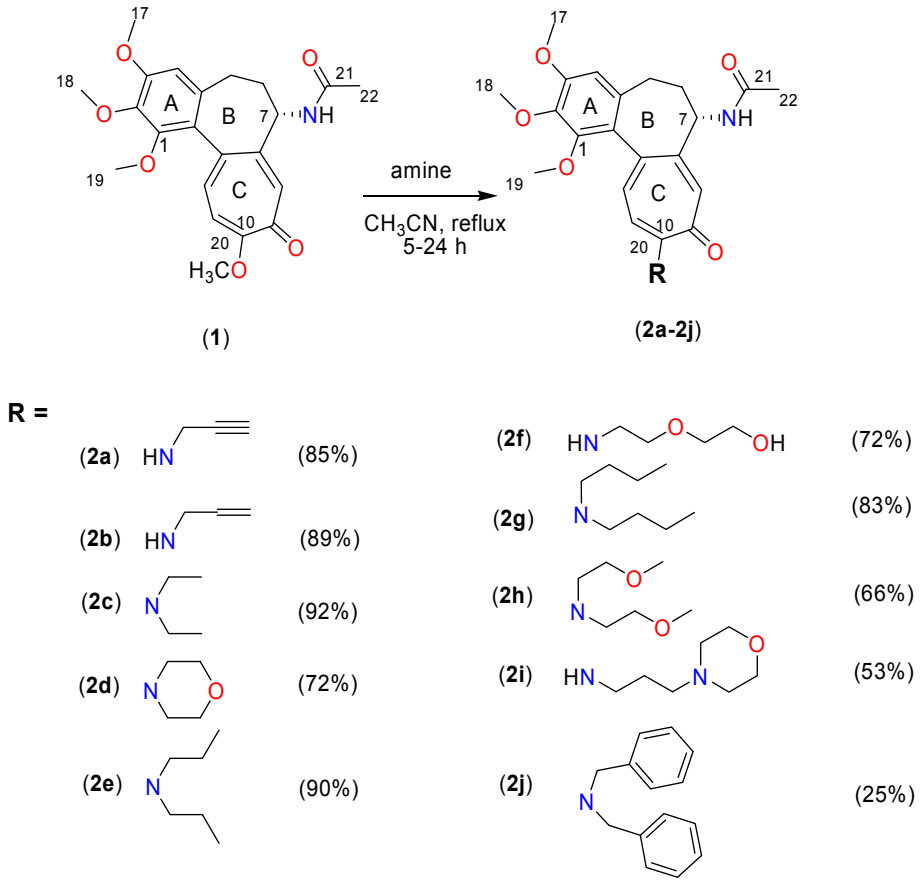

Scheme 1. Reaction and conditions: Colchicine (**1**), 15 equiv amines, acetonitrile, reflux, 5–24 h. Time for completion of the reaction at reflux as indicated by TLC. Yield of isolated and purified products in brackets.

resistant subline (HL-60/vinc), human colon adenocarcinoma cell line (LoVo) and doxorubicin resistant subline (LoVo/DX), and normal murine embryonic fibroblast cell line (BALB/3T3).

Antimicrobial activities of **1** and **2a**–**2j** were also tested *in vitro* against Gram-positive and Gram-negative bacteria and fungi, as well as against a series of clinical isolates of *Staphylococcus*.

2. Results and discussion

2.1. Chemistry

To investigate the effect of different amine substitutions at C-10 position of **1** on its bioactivity, ten amine derivatives (**2a**–**2j**) were synthesized using a simple reaction between **1** and the respective commercially available amine. Colchicine (**1**) was refluxed with respective amine in acetonitrile solution for 5–24 h to give colchicine derivatives (**2a**–**2j**) with good yields. The synthetic route of these target compounds is outlined in Scheme 1.

To facilitate the structural activity relationship analysis (SAR) we chose colchicine amine with different substituents such as: unsaturated alkyl chains (**2a** and **2b**), saturated alkyl chains (**2c**, **2e**, **2g**), alkyl chains containing oxygen atoms (**2f**, **2h**), alkyl chains containing morpholine ring (**2d**, **2i**), alkyl chains containing aromatic substituents (**2j**). All obtained compounds (**2a**–**2j**) were easily isolated in pure form after dry column vacuum chromatography on silica gel.

The lowest 25% yield was obtained for the reaction between **1** and dibenzylamine because it has highly sterically crowded molecule. The yields of the other reactions varied from 53% up to 92%.

The products of the regioselective one-pot reaction of **1** with respective amines through the substitution of OCH_3 group at C(10) position in the tropolone ring of **1**-ring process are very well identified using the spectroscopic methods. The structures of all products (**2a**–**2j**) were determined using the ESI-MS, FT-IR, ^1H and ^{13}C NMR methods and are shown in Supplementary data (Figs. S1–S8) and discussed below. The ^1H and ^{13}C NMR signals were assigned using two-dimensional spectra such as HETCOR, HMBC.

The characteristic signal of OCH_3 group at C(10) position of **1** in the ^{13}C and ^1H NMR spectra is observed at 56.1 ppm and as a singlet at 4.03 ppm, respectively. These signals vanish completely after the reaction of **1** with all used amines proving the substitution of OCH_3 group at C(10) position in the tropolone ring of **1**.

The connection of the tropolone ring moiety was carried out on the basis of two- and three-bond long-range correlation detected in the HMBC spectrum (Fig. S8). For example, for compound **2d**, the correlation of the proton from two groups C(22)H_2 as ddd at 3.57 and 3.32 ppm with the ^{13}C NMR signals at 169.7 ppm, assigned to C10 atom, implied the conclusion that the morpholine group was bonded by the C-10 atom as shown in Scheme 1.

3. Biological activity

3.1. In vitro determination of drug-induced inhibition of human cancer cell line growth

The synthesized colchicine derivatives (**2a**–**2j**) were evaluated for their *in vitro* antiproliferative effect on normal and cancer cells.

Each compound was tested on two human cancer cell lines displaying various levels of drug resistance, such as human promyelocytic leukaemia (HL-60) and its vincristine-resistant subline (HL-60/vinc), and human colon adenocarcinoma cell line (LoVo), and doxorubicin resistant subline (LoVo/DX). The antiproliferative effect was also studied on normal murine embryonic fibroblast cell line (BALB/3T3) for better description of cytotoxic activity of the compounds studied. The mean IC_{50} ± SD of the tested compounds are collected in Table 1. To evaluate the agent activity against the cells with MDR (multidrug resistance) phenotype, two drug resistant cancer cell lines *i.e.* HL-60/vinc and LoVo/DX were tested and the indexes of resistance (IR) were calculated (Table 1). The IR values indicate how many times more resistant is the subline in comparison to its parental cell line.

As shown in Table 1, some of the obtained derivatives (**2a**, **2b**, **2h**) showed little superior or comparable cytotoxic activity to colchicine against four tumour cells. The cytotoxic activity of these compounds against normal cell line (BALB/3T3) is the same as for colchicine. It is worth noting that the cytotoxic effect of compounds **2a**, **2b** and **2h** against BALB/3T3 cells is also comparable with the cytotoxic effect of commercially used anticancer drug like doxorubicin but their selectivity indexes are generally better (Table 2).

The extended losses of cytotoxic activity were observed for two derivatives **2f** and **2g** both against cancer and normal cells (Table 1).

The other compounds studied (**2c**–**2e**, **2g** and **2j**) showed moderate cytotoxic activity against HL-60 cancer cell line, which was worse than the activity of **1** and anticancer drugs such as doxorubicin and cisplatin. These compounds showed also weak activity against the other examined cancer cells, especially against the drug resistant sublines LoVo/DX and HL-60/vinc. The selectivity index (SI), an important pharmaceutical parameter that facilitates the estimation of possible future clinical development, was determined as the ratio of IC_{50} value for normal cell line (BALB/3T3) to IC_{50} value for the respective cancerous cell line. The bioactivity of each compound was evaluated on the basis of a combination of its IC_{50} value and the corresponding SI. Higher values of SI indicate greater anticancer specificity and SI greater than 3 was considered as highly selective.

The best selectivity index was obtained for compounds **2g** and **2j** which showed moderate cytotoxic activity against four tested human cancer cell lines, indicating that not only the most active compounds should be recognised as potential anticancer drugs. The preliminary structure–activity relationships (SARs) revealed that the size of the substituent at C(10) position in tropolone ring of **1** had an important impact on activity. The compounds with small amine substituents such as propargylamine (**2a**) and allylamine (**2b**) displayed more potent anticancer activities than those with a larger amine substituent. An interesting observation follows from a comparison of activity of compounds **2g** and **2h**. The size of the amine substituents in them is very similar but compound **2h** contains additionally two oxygen atoms. This small difference makes compound **2h** more active against cancer cells than **2g** but the former one has a worse selectivity index.

It is postulated that the cytotoxic effect of colchicine derivatives is related to tubulin clustering ability and the fact that it prevents microtubule polymerization. Therefore, the differences observed between compounds **2g** and **2h** should be connected with the differences in the formation of complexes between these compounds and tubulin. Similar conclusions are also obvious for the other compounds studied. The most cytotoxic compounds

Table 2
The calculated selectivity index (SI) which represents IC_{50} for normal cell line/IC_{50} for cancerous cell line.

Compound	Calculated selectivity index SI			
	HL-60	HL-60/vinc	LoVo	LoVo/DX
1	22.5	0.18	1.5	0.18
2a	8.89	0.17	0.76	0.09
2b	2.96	0.21	1.45	0.13
2c	21.33	0.18	1.89	0.20
2d	2.36	0.12	0.58	0.12
2e	2.46	0.26	1.41	0.29
2f	24.44	–	0.80	–
2g	12.53	2.21	6.42	7.63
2h	17.78	0.19	0.80	0.11
2i	2.79	–	–	–
2j	6.83	3.40	2.81	5.68
Doxorubicin	4.09	0.20	1.20	0.03
Cisplatin	5.30	0.77	1.43	1.02

The SI (Selectivity Index) was calculated for each compounds using formula: SI = IC_{50} for normal cell line (BALB/3T3)/IC_{50} for respective cancerous cell line. A beneficial SI > 1.0 indicates a drug with efficacy against tumour cells greater than toxicity against normal cells.

Table 1
Antiproliferative activity of colchicine (**1**) and its derivatives (**2a**–**2j**) compared with antiproliferative activity of standard anticancer drugs doxorubicin and cisplatin.

Compound	HL-60	HL-60/vinc		LoVo	LoVo/DX		BALB/3T3
	IC_{50} (μM)	IC_{50} (μM)	IR	IC_{50} (μM)	IC_{50} (μM)	IR	IC_{50} (μM)
1	0.008 ± 0.0005	1.00 ± 0.33	125	0.12 ± 0.01	0.98 ± 0.05	8.16	0.18 ± 0.03
2a	0.018 ± 0.005	0.95 ± 0.14	52.77	0.21 ± 0.05	1.77 ± 0.12	8.43	0.16 ± 0.02
2b	0.054 ± 0.016	0.75 ± 0.14	13.88	0.11 ± 0.002	1.20 ± 0.02	10.91	0.16 ± 0.01
2c	0.66 ± 0.2	80.05 ± 11.00	121.3	7.46 ± 0.52	71.56 ± 5.85	9.59	14.08 ± 1.00
2d	6.20 ± 1.14	117.5 ± 32.5	18.96	25.03 ± 7.08	125.2 ± 36.6	5.00	14.64 ± 1.65
2e	5.76 ± 2.39	54.24 ± 18.32	9.42	10.09 ± 0.81	48.87 ± 13.35	4.84	14.18 ± 2.43
2f	4.95 ± 0.99	uv[a]	–	150.8 ± 12.8	uv[a]	–	120.9 ± 39.3
2g	4.29 ± 1.36	24.29 ± 5.43	5.67	8.37 ± 0.78	7.04 ± 0.34	0.84	53.74 ± 5.43
2h	0.009 ± 0.001	0.86 ± 0.01	95.55	0.20 ± 0.06	1.52 ± 0.32	7.60	0.16 ± 0.014
2i	47.60 ± 22.44	uv[a]	–	uv[a]	uv[a]	–	133.0 ± 42.4
2j	5.98 ± 2.97	12.04 ± 3.73	2.01	14.53 ± 8.04	7.20 ± 1.95	0.50	40.88 ± 12.96
Doxorubicin	0.044 ± 0.038	0.88 ± 0.26	20	0.15 ± 0.06	5.46 ± 1.56	36.40	0.18 ± 0.07
Cisplatin	1.00 ± 0.23	6.87 ± 1.63	6.86	3.70 ± 1.20	5.20 ± 0.93	1.41	5.30 ± 2.93

The IC_{50} value is defined as the concentration of a compound that corresponds to a 50% growth inhibition.
Human promyelocytic leukaemia (HL-60) and its vincristine-resistant subline (HL-60/vinc); human colon adenocarcinoma cell line (LoVo) and doxorubicin resistant subline (LoVo/DX); normal murine embryonic fibroblast cell line (BALB/3T3).
Data are expressed as the mean ± SD.
The IR (Index of Resistance) indicates how many times a resistant subline is chemoresistant relative to its parental cell line. When IR is 0–2 the cells are sensitive to tested compound; IR of the range 2–10 means the cell show moderate sensitivity to a drug and IR above 10 indicates strong drug resistance.
[a] uv – Unavailable in range of concentrations used, IC_{50} could not be calculated due to weak antiproliferative activity of compound across the tested concentrations.

synthesized in this work (**2a**, **2b** and **2h**) are active in nanomolar to submicromolar concentrations and are several times more cytotoxic than cisplatin and doxorubicin.

Selective inhibition of cancer cell growth is a major challenge. The target of colchicine, and probably its analogues are microtubules, therefore the toxicity of such agents towards cancer and normal cells is related to the proliferation ratio of a particular cell. An example of colchicine analogue rejected from phase II studies despite of encouraging results from preclinical studies, due to associated cardio-toxicity in humans is the allocolchicine derivative, ZD 6126 [23]. Therefore the emphasis is put on the search for colchicine derivatives showing selectivity towards cancer cells [24]. Regardless of the high cytotoxicity of **2h**, we would like to stress out the interesting profile of activity of **2g**. This compound, selective against cancer cells, is also not a good substrate for MDR pumps and will be considered as a candidate for further studies concerning structure optimization.

3.2. Antimicrobial screening

In contrast to the anticancer activity, the antimicrobial activity of colchicine derivatives has been much poorer recognised, although the interest in development of new antibacterial compounds is still growing. Up to date, no evaluation of antibacterial activity and especially the activity against antibiotic-resistant *Staphylococcus* has been carried out. Therefore, the antimicrobial activities of colchicine (**1**) and its synthesized derivatives (**2a**–**2j**) were tested *in vitro* against typical Gram-positive cocci, Gram-negative rods and yeast-like organisms, as well as against a series of clinical isolates of *Staphylococcus*: methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE). Further evaluation was then carried out for the compounds showing reasonable growth inhibition zones (Giz) to determine their minimal inhibitory concentration (MIC) values using the twofold serial dilution method.

Table 3
Antibacterial activity of selected colchicine derivatives (**2g**, **2h**) against hospital strains methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus aureus* (MIC (μg/cm^3)).

Staphylococcus strains	Compounds		
	2g	**2h**	**Ciprofloxacin**
Reference strains			
S. aureus NCTC 4163	32	128	0.25
S. aureus ATCC 25923	32	64	0.5
S. aureus ATCC 6538	32	128	0.25
S. aureus ATCC 29213	32	256	0.5
S. epidermidis ATCC 12228	32	256	0.25
S. epidermidis ATCC 35984	64	256	0.125
Hospital strains methicillin-resistant *Staphylococcus epidermidis*			
459/11	32	256	16
460/11	32	512	0.125
461/11	32	512	0.25
466/11	64	256	2
467/11	32	256	16
468/11	64	256	16
469/11	64	256	8
470/11	64	512	0.125
488/11	64	256	16
489/11	32	256	0.25
Hospital strains methicillin-resistant *Staphylococcus aureus*			
452/11	32	256	32
456/11	32	128	32
462/11	32	256	64
514/11	32	256	32
522/12	32	256	32
537/12	32	256	64
572/12	32	128	64
573/12	64	128	32
585/12	32	128	64
586/12	64	128	64

Compounds **1**, **2a**–**2f** and **2i**–**2j** were practically inactive towards all microorganisms tested (Giz 10–12 mm and MIC ≥ 256 μg/cm^3).

Hospital strains of MRSA and MRSE were isolated from different biological materials from patients of the Warsaw Medical University Hospital. The other microorganisms used here were provided by the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland. The data concerning the antimicrobial activity of the compounds are summarized in Table 3. Colchicine as well as all of its derivatives were inactive against strains of *Candida* (*Candida albicans* and *Candida parapsilosis*) and Gram-negative (*Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*). Colchicine was also inactive against typical Gram-positive bacteria like *S. aureus*.

Among the colchicine derivatives tested (**2a**–**2j**), only its two derivatives (**2g**, and **2h**) showed moderate activity against typical Gram-positive bacteria (MIC = 32–256 μg/cm^3) (Table 3). The other compounds exhibited very low activity against Gram-positive bacteria (MIC ≥ 256 μg/cm^3). Promising results of microbiological tests performed on drug-susceptible strain of reference *Staphylococcus* made us check, whether these two compounds were also effective against the methicillin-resistant *Staphylococci* strain (MRSA and MRSE) (Table 3). Methicillin-resistant strains of *Staphylococcus* are resistant to all β-lactams and they are often resistant to some other widely used antibiotics and belong to the so-called multidrug resistant strains. MRSA and MRSE strains are the most important pathogens causing bacterial infections of the bloodstream, lower respiratory tract, and skin, and soft tissue in many developed countries. Hospitalized patients are at greatest risk, and have breaches of the skin barrier allowing entry of the pathogen. One of the few drugs effective in combating infections caused by MRSA is ciprofloxacin, which has been used by us as a reference drug (Table 3).

As shown in Table 3 compound, **2g** with dibutylamine amine substituent at C(10) atom shows a considerably better activity against hospital strains of MRSA and MSSA than compound **2h** with bis(2-methoxyethyl)amine indicating that the presence of two oxygen atoms has a strong influence on the antibacterial activity of colchicine derivatives.

4. Conclusion

In conclusion, the one-pot reaction of colchicine (**1**) with primary or secondary amines permitted the synthesis of new colchicine derivatives **2a**–**2j** with good yields. The advantages of the protocol include simple reaction workup, easily available starting materials and convenient isolation. The compounds **2a**–**2b** and **2g**–**2h** exhibited very good anticancer activity with good selectivity indicating the importance of amine substitution in C-10 position of colchicine. The highest anticancer activity exhibits compound **2h**, but also the interesting profile of activity has compound **2g**, which has lower anticancer activity than 2 h but has better selectivity index. Our studies clearly show that appropriate modification of the colchicine molecule and synthesis of its analogues can attenuate the toxicity which can be of great benefit particularly in cancer treatment.

In addition, chemical modification of colchicine has brought its two derivative compounds with novel antibacterial activity including activity against the methicillin-resistant *Staphylococci* strains.

5. Experimental

5.1. General

All precursors for the synthesis colchicine, amines and solvents were obtained from Aldrich or Fluka and were used as received

without further purification. $CDCl_3$ spectral-grade solvent weas stored over 3 Å molecular sieves for several days. TLC was carried out on precoated plates (TLC silica gel 60 F_{254}, Aluminium Plates Merck) and spots were detected by illumination with an UV lamp. All the solvent used in flash chromatography were of HPLC grade (CHROMASOLV from Sigma–Aldrich) and were used as received. The elemental analysis of **2a**–**2j** was carried out on Vario ELIII (Elementar, Germany).

5.2. Spectroscopic measurements

The ^1H, ^{13}C spectra were recorded on a Bruker Avance DRX 600 spectrometer. ^1H NMR measurements of **1**–**13** (0.07 mol dm^{-3}) in $CDCl_3$ were carried out at the operating frequency 600.055 MHz; flip angle, pw = 45°; spectral width, sw = 4500 Hz; acquisition time, at = 2.0 s; relaxation delay, d_1 = 1.0 s; T = 293.0 K and using TMS as the internal standard. No window function or zero filling was used. Digital resolution was 0.2 Hz per point. The error of the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at the operating frequency 150.899 MHz; pw = 60°; sw = 19,000 Hz; at = 1.8 s; d_1 = 1.0 s; T = 293.0 K and TMS as the internal standard. Line broadening parameters were 0.5 or 1 Hz. The error of chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of $CDCl_3$.

The ^1H and ^{13}C NMR signals were assigned using 2-D (COSY, HETCOR, HMBC) spectra shown in the Supplementary Materials. 2-D spectra were recorded using standard pulse sequences from Varian and Bruker pulse-sequence libraries. The FT-IR spectra of **2a**–**2j** in the mid infrared region were recorded in KBr.

The ESI (Electrospray Ionisation) mass spectra were recorded on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples were prepared in dry acetonitrile (5×10^{-5} mol dm^{-3}) with the addition of acetic acid. The sample was infused into the ESI source using a Harvard pump at a flow rate of 20 μl min^{-1}. The ESI source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages: 10 and 30 V. The source temperature was 120 °C and the desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 dm^3 h^{-1}. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from m/z = 300 to m/z = 750.

5.3. Synthesis

5.3.1. General procedure for the synthesis of colchicine derivatives (*2a*–*2j*)

A solution of colchicine (400 mg; 1 mmol) and 2-(2-aminoethoxy)ethanol (1.58 g; 15 mmol) was stirred at reflux for 24 h. Following evaporation, the mixture was dissolved in CH_2Cl_2 (5 ml) and purified chromatographically on silica gel (Fluka type 60) to give compound **2f** with yield 72% as a yellow powder. The exemplary 2D NMR spectra of compound 2f are included in the Supplementary material. ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm:174.9 (C=O), 169.9 (C=O), 154.1 (C), 152.6 (C), 151.4 (C), 150.8 (C), 141.2 (C), 139.0 (CH) 134.4 (C), 130.6 (C), 126.5 (C), 122.9 (C), 108.5 (CH) 107.0 (CH), 72.3 (OCH_2), 68.4 (OCH_2), 61.4 (OCH_3), 61.2 (OCH_3), 61.1 ($HOCH_2$), 55.9 (OCH_3), 52.4 (CH), 42.3 ($NH–CH_2$), 36.8 (CH_2), 29.8 (CH_2), 22.5 (CH_3). ^1H NMR (403 MHz, $CDCl_3$) δ ppm: 8.64 (dd, J = 20.90, 6.53 Hz, 1H), 7.58 (s, 1H), 7.58 (m, 1H), 7.45 (d, J = 11.21 Hz, 1H), 6.66 (d, J = 11.38 Hz, 1H), 6.55 (s, 1H), 4.73–4.65 (m, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.82 (t, J = 5.34 Hz, 2H), 3.76 (m, 2H), 3.64 (mz, 2H), 3.63 (s, 3H), 3.58 (dd, J = 10.69, 5.30 Hz, 2H), 3.29–3.02 (bs, 1H), 2.47 (dd, J = 12.55, 5.67 Hz, 1H), 2.25 (m, 2H), 1.97 (s, 3H).

5.4. Antiproliferative activity of colchicine and its derivatives

Four human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of colchicine and its derivatives: human acute promyelocytic leukaemia (HL-60) and its vincristine-resistant subline – (HL-60/vinc), human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/DX) respectively, and also normal murine embryonic fibroblast cell line (BALB/3T3). The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC Rockville, Maryland, USA), HL-60 cell line – from European Type Culture Collection by courtesy of Professor Spik and Dr Mazurier (Laboratory of Biological Chemistry USTL, Lille, France) and HL-60/vinc, LoVo and LoVo/DX by courtesy of Prof. E. Borowski (Technical University of Gdańsk, Poland). All the cell lines are maintained in the Institute of Immunology and Experimental Therapy (IIET), Wroclaw, Poland.

Human leukaemia cells were cultured in Iscove medium (IIET, Wroclaw) containing 10% foetal bovine serum, 2 mM L-glutamine (Sigma–Aldrich, Germany) and 1 μg/100 ml doxorubicin for HL-60/vinc (Sigma–Aldrich, Germany). Human colon adenocarcinoma cell lines were cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IIET, Wroclaw), supplemented with 5% foetal bovine serum (Thermo Fisher Scientific), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma–Aldrich, Germany) and 10 μg/100 ml doxorubicin for LoVo/DX (Sigma–Aldrich, Germany). Murine embryonic fibroblast cells were cultured in Dulbecco medium (Gibco), supplemented with 10% foetal bovine serum (Thermo Fisher Scientific) and 2 mM glutamine (Sigma–Aldrich, Germany). All culture media contained antibiotics: 100 U/ml penicillin and 100 μg/ml streptomycin (Polfa-Tarchomin, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO_2.

5.4.1. The antiproliferative assays in vitro

Twenty four hours before adding the tested compounds, all cell lines were seeded in 96-well plates (Sarstedt, Germany) in appropriate media with 10^4 cells per well. All cell lines were exposed to each tested agent at four different concentrations of the range 100 to 0.1 μg/ml for 72 h. Cells were also exposed to the reference drug cisplatin (Accord) and doxorubicin (Sigma–Aldrich, Germany). Additionally, all cell lines were exposed to ethanol (solvent used for tested compounds) (Sigma–Aldrich, Germany) at concentrations corresponding to those present in the tested agents' dilutions. For adherent cells, sulphorhodamine B assay was performed and MTT assay for leukaemia cells.

5.4.2. SRB

After 72 h of incubation with tested compounds, the cells were fixed *in situ* by gently adding 50 μl per well of cold 50% trichloroacetic acid TCA (Avantor, Poland) and were incubated at 4 °C for one hour. Then the wells were washed four times with water and air dried. Next, 50 μl of 0.2% solution of sulphorhodamine B (Sigma–Aldrich, Germany) in 1% acetic acid (Avantor, Poland) were added to each well and the plates were incubated at room temperature for 0.5 h. After incubation time, the unbound dye was removed by washing plates four times with 1% acetic acid, whereas the stain bound to cells was solubilized with 10 mM Tris base (Sigma–Aldrich, Germany). Absorbance of each solution was read at Synergy H4 photometer (BioTek Instruments, USA) at 540 nm wavelength.

5.4.3. MTT

Proliferation inhibition of leukaemia cells by tested compounds was measured by means of MTT assay. Thus, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution

(Sigma–Aldrich, Germany) were added to each well and the plates were left in cell incubator for four hours to allow the cells to metabolize yellow MTT to blue formazan. Then, the lysing mixture consisting of 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate (both from Sigma–Aldrich, Germany) and 275 ml of distilled water, was added in 80 μl volume per well. The plates were incubated for 24 h for the formazan crystals to be released from cells and dissolved and than absorbance of each well was read at Synergy H4 photometer (BioTek Instruments, USA) at 570 nm wavelength.

Results are presented as mean IC_{50} (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC_{50} values were calculated in Cheburator 0.4, Dmitry Nevozhay software for each experiment. Compounds at each concentration were tested in triplicates in single experiment and each experiment was repeated at least three times independently. Results are summarized in Table 1. The IR was defined as the ratio of IC_{50} for a given compound calculated for resistant cell line to that measured for its parental drug sensitive cell line (Table 1).

5.5. Antimicrobial activity of colchicine and its derivatives

The microorganisms used in this study were as follows: Gram-positive cocci: *S. aureus* NCTC 4163, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, Gram-negative rods: *E. coli* ATCC 10538, *E. coli* ATCC 25922, *E. coli* NCTC 8196, *P. vulgaris* NCTC 4635, *P. aeruginosa* ATCC 15442, *P. aeruginosa* NCTC 6749, *P. aeruginosa* ATCC 27863, *B. bronchiseptica* ATCC 4617 and yeasts: *C. albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019.

The other microorganisms used were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Antibacterial activity was examined by the disc-diffusion method under standard conditions using Mueller-Hinton II agar medium (Becton Dickinson) according to CLSI (previously NCCLS) guidelines [25]. Antifungal activities were assessed using Mueller-Hinton agar +2% glucose and 0.5 μg/cm^3 Methylene Blue Dye Medium [26].

Sterile filter paper discs (9 mm diameter, Whatman No 3 chromatography paper) were dripped with tested compound solutions (in EtOH) to load 400 μg of a given compound per disc. Dry discs were placed on the surface of appropriate agar medium. The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35 °C.

Minimal Inhibitory Concentration (MIC) was determined by the twofold serial microdilution method (in 96-well microtiter plates) using Mueller-Hinton Broth medium (Beckton Dickinson) according to CLSI guidelines [27]. The stock solution of the tested agent was prepared in EtOH and diluted in sterile water. Concentrations of the tested agents ranged from 0.0625 to 512 μg/cm^3. The final inocula of all studied microorganisms were 10^5 CFU/cm^3 (colony forming units per ml). Minimal inhibitory concentrations (the lowest concentration of the tested agent that prevents visible growth of a microorganism) were read after 18 h of incubation at 35 °C.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.11.037. These data include MOL files and InChiKeys of the most important compounds described in this article.

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