Synthesis, biological evaluation and molecular docking studies of new amides of 4-chlorothiocolchicine as anticancer agents

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**ABSTRACT**

Colchicine belongs to a large group of microtubule polymerization inhibitors. Although the anti-cancer activity of colchicine and its derivatives has been established, none of them has found commercial application in cancer treatment due to side effects. Therefore, we designed and synthesized a series of six triple-modified 4-chlorothiocolchicine analogues with amide moieties and one urea derivative. These novel derivatives were tested against several different cancer cell lines (A549, MCF-7, LoVo, LoVo/DX) and primary acute lymphoblastic leukemia (ALL) cells and they showed activity in the nanomolar range. The obtained IC\textsubscript{50} values for novel derivatives were lower than those obtained for unmodified colchicine and common anticancer drugs such as doxorubicin and cisplatin. Further studies of colchicine and selected analogues were undertaken to indicate that they induced apoptotic cell death in ALL-5 cells. We also performed in silico studies to predict binding modes of the 4-chlorothiocolchicine derivatives to different β tubulin isotypes. The results indicate that select triple-modified 4-chlorothiocolchicine derivatives represent highly promising novel cancer chemotherapeutics.

**ARTICLE INFO**

InChIKeys:
IAKHMKGGTNLSZ-INIZCTEOSA-N
VKKQPIARGCFIIN-QFIPXVFZSA-N
ZAIALAYKTNLRMS-KRWDZBQOSA-N
ALELANHAGUYPLN-ZNNXBMFYSA-N
WWCFRPLZH1UYX-HNXXBMFYSA-N
BUCSUUCAHATWAO-ZDUSSCGKSA-N
UDHIXIPCPSDIE-HNXXBMFYSA-N
UNZGKZVQPLLE-INIZCTEOSA-N
JTYANOAOHUBBKB-IBGZPJMESA-N
HVBRWCZHIWFPQGV-HNXXBMFYSA-N
SSRFZIYVLACS-INIZCTEOSA-N

**Keywords:**
Anticancer activity
Tubulin inhibitors
Triple-modified colchicine, Amides
Mechanistic investigation

**1. Introduction**

Colchicine (1) is a plant alkaloid isolated from Colchicum autumnale [1]. It is used for the treatment of acute gout, familial Mediterranean fever, Behçet’s disease and pericarditis and other diseases [2–12]. Colchicine showed potent anti-inflammatory and anti-mitotic properties but its biological mechanism of action remained unknown until 1968, when Taylor et al. identified tubulin as its target [13]. While tubulin-targeting agents have made a considerable impact as effective anticancer drugs, the use of colchicine as an antitumor agent has been restricted because of toxicity. Therefore current efforts, including those in our research group, have been focused on the development of clinically applicable colchicine derivatives [14–35].

In 2011, Hiromitsu Takayama’s research group published results of their studies on C-4 halogen substituted colchicine derivatives, including 4-chlorocolchicine [26]. A year later they published extended results of 4-chlorocolchicine derivatives bearing an amide moiety in the C-7 position [27]. Also Kerekes et al. reported colchicine derivatives with diversified amide moieties in the C-7 position and a thiomethyl group in the C-10 position [36]. 4-chlorocolchicine and some of the double-modified derivatives exhibited strong antitumor activities. Encouraged by the previously reported results, we decided to extend the library of these potent and selective molecules by synthesizing a series of novel triple-modified amide and urea derivatives of 4-chlorothiocolchicine. Here we report the synthesis, molecular docking characterization and biological activity of these compounds against a variety of cancer cell lines. The results show that several of the 4-chlorothiocolchicine derivatives have properties superior to the parent compound, and as such represent important new leads and warrant further investigation as novel cancer drugs.
2. Results and discussion

2.1. Chemistry

Compounds 2–4 were obtained according to previously described procedures [26,30,36]. Detailed information is given in Supplementary data. Compounds 5–11 were synthesized in one pot reaction of compound 4 with respective acyl (5–10) or carbamoyl (11) chloride in the presence of triethylamine and 4-dimethylaminopyridine (DMAP) (see Scheme 1). The structures and purity of all products were confirmed using ESI-MS, FT-IR, $^1$H NMR and $^{13}$C NMR methods (see Supplementary data). The most characteristic shifts in $^{13}$C NMR amide carbon atom ($\delta$ 156.0 ppm) were observed for the signal of the urea spectra ($\delta$ 105–115 ppm) were observed in the range 167.0–177.0 ppm for the signal of the amide carbon atom (5–10), and the urea carbon atom shift (11) was observed at 156.0 ppm.

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

The seven triple-modified colchicine derivatives (5–11), other colchicine derivatives (2–4) and parent colchicine (1) were evaluated for their in vitro antiproliferative effect on primary acute lymphoblastic leukemia (ALL-5) cells and four human cancer cell lines including human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma (LoVo) and its doxorubicin-resistant subline (LoVo/DX), as well as on non-neoplastic murine embryonic fibroblasts (BALB/3T3). The data, expressed as IC$_{50}$ ± SD of the tested compound, are presented in Table 1 [37,38].

The best cytotoxic activity among triple-modified colchicine derivatives for the respective primary ALL-5 cells or cancer cell lines was as follows: 5 and 8–9 against ALL-5 (IC$_{50}$ = 2.3–5.2 nM); 5–9 against A549 (IC$_{50}$ = 11.1–28.8 nM), 5–9 against MCF-7 (IC$_{50}$ = 11.7–18.2 nM), 5–11 against LoVo/LoVo (IC$_{50}$ = 7.2–78.3 nM), and 5–11 against LoVo/DX (IC$_{50}$ = 34.5–619.0 nM). These IC$_{50}$ values are lower than those of parent colchicine (1) and standard anticancer drugs including doxorubicin and cisplatin for the respective cell types. Of note, 4-chlorothiocolchicine (2) and double-modified derivative (3) showed very good activity against primary ALL-5 cells (IC$_{50}$ = 2.5 and 3.6 nM, respectively), three of the tested cancerous cell lines (A549, MCF-7 and LoVo; IC$_{50}$ = 23.0–69.1 and 22.2 nM respectively) and doxorubicin resistant LoVo (LoVo/DX; IC$_{50}$ = 783.6 and 111.1 nM).

Importantly, the majority of the investigated compounds showed the highest activity towards ALL-5 cells. The exceptions were analogs 6, 7 and 10, which were more potent towards LoVo cell line (IC$_{50}$ = 9.8, 7.2 and 78.3 nM respectively). This is of relevance since ALL-5 are primary cells and as such exhibit properties more reflective of the tumor cell population compared to immortalized conventional cell lines which tend to deviate substantially from the respective parental cells.

The ability of the derivatives to overcome MDR mediated drug resistance was investigated by comparing effects in drug-sensitive LoVo versus drug resistant LoVo/DX cells. Resistance index (RI) values were calculated and summarized in Table 1. For the derivatives showing the lowest IC$_{50}$ values against LoVo/DX cell line (5, 7–9), calculated RI values were RI = 4.8–11.8. Thus the tested compounds were not able to efficiently overcome the drug resistance of the LoVo/DX cell line.

Comparison between the activity towards cancer cells and non-neoplastic murine fibroblasts (BALB/3T3) was made to calculate the Selectivity Index (SI) as an indication of the therapeutic potential of novel compounds (Fig. 1). Ideally, the drug should kill cancer cells of the patients without significantly affecting their healthy cells. Higher SI values correlate with better drug selectivity. Some general trends arise from these data. First, the majority of the triple-modified colchicine analogs showed beneficial SI values of > 1 on ALL-5 cells and A549, MCF-7 and LoVo cell lines. The exceptions were compounds: 10 towards ALL-5, A549 and MCF-7, and 11 towards A549 and MCF-7 (SI < 1). Second, with the exception of analogs 6, 7 and 10, the highest SI values were shown towards primary ALL-5 cells, and combined with very high antiproliferative activity make them lead candidates for further studies. However, it is important to note that in general, for compounds with decreased IC$_{50}$’s toward the cancerous cell lines, they also displayed a decreased IC$_{50}$ for the non-neoplastic BALB/3T3 cell line (Table 1), thus moderating benefit. A notable exception was 4-chlorocolchicine (2) which was more potent than colchicine towards all the cancerous cell lines but was similarly potent toward BALB/3T3 cells (Table 1), identifying compound 2 as a promising lead in future studies.

2.3. Contrasting effects on cell death and mitotic arrest in primary ALL-5 versus MCF-7 cells

Since colchicine (1) and its derivatives showed the highest activity towards primary ALL-5 cells, we undertook investigation of their...
cellular effects particularly with respect to cell death and cell cycle progression. In order to verify whether the compounds induced apoptotic cell death we assessed DNA content and fragmentation employing flow cytometry and propidium iodide staining. Cells with sub-G1 (<2N) DNA were considered to have undergone cell death. ALL-5 cells were treated with 1 or analogs 2, 3, 5, 7, 8 or 9 (characterized by lower IC50 values than 1, see Table 1) at concentrations equal to 5 × IC50 values for 24, 48 and 72 h. Cells incubated with vehicle (0.1% DMSO) for equivalent times served as a control.

For the representative full cytograms see Fig. S22 (Supplementary material). A graphical representation of cell cycle distribution from the mean of three such experiments is presented in Fig. 2A. Statistically significant percentage of cells with fragmented DNA was observed after 48 h of treatment further increasing after 72 h (Fig. 2A, red bars). Notably, analogues 2, 5, 7, 8 and 9 caused cell death more rapidly, indicated by significant sub-G1 DNA as early as 24 h of treatment. Of note, a decrease in the pool of cells in G1 phase of cell cycle (Fig. 2A, blue bars) occurred concomitant with the generation of cells with sub-G1 DNA (Fig. 2A, red bars). Notably, analogues 2, 5, 7, 8 and 9 caused cell death more rapidly, indicated by significant sub-G1 DNA as early as 24 h of treatment. Of note, a decrease in the pool of cells in G1 phase of cell cycle (Fig. 2A, blue bars) occurred concomitant with the generation of cells with sub-

Table 1

| Compound | ALL-5 | A549 | MCF-7 | LoVo | LoVo/DX | BALB/3T3 |
|----------|-------|------|-------|------|---------|----------|
| IC50 (nM) | IC50 (nM) | IC50 (nM) | IC50 (nM) | IC50 (nM) | RI | IC50 (nM) |
| 1 | 8.6 ± 0.2 | 125 ± 12 | 54.3 ± 27.5 | 108 ± 25 | 1,690 ± 275 | 15.7 | 139 ± 73 |
| 2 | 2.5 ± 0.6 | 46.1 ± 34.6 | 18.4 ± 4.0 | 69 ± 11.5 | 784 ± 277 | 11.4 | 138 ± 69 |
| 3 | 3.6 ± 1.5 | 11.1 ± 2.2 | 15.6 ± 2.2 | 11.9 ± 1.3 | 111 ± 45 | 5.1 | 70 ± 26 |
| 4 | 52.9 ± 21.4 | 147 ± 27 | 221 ± 22 | 98.1 ± 9.8 | 172 ± 49 | 1.8 | 270 ± 69 |
| 5 | 5.2 ± 1.9 | 125 ± 2.1 | 13.2 ± 1.2 | 7.6 ± 1.2 | 68 ± 5 | 8.9 | 125 ± 3.6 |
| 6 | 16.9 ± 2.9 | 288 ± 17.6 | 18.2 ± 4.5 | 9.8 ± 0.1 | 166 ± 26 | 17.0 | 78.6 ± 37.1 |
| 7 | 8.7 ± 1.1 | 11.1 ± 1.1 | 11.7 ± 2.0 | 7.2 ± 1.1 | 35 ± 8 | 4.8 | 14.3 ± 6.3 |
| 8 | 2.3 ± 0.1 | 12.2 ± 1.2 | 12.2 ± 1.2 | 7.2 ± 1.2 | 84 ± 7 | 11.7 | 11.5 ± 3.3 |
| 9 | 2.5 ± 1.3 | 12.6 ± 0.1 | 12.6 ± 0.1 | 7.7 ± 1.2 | 66 ± 23 | 8.6 | 38.4 ± 46.5 |
| 10 | 188 ± 37 | 476 ± 368 | 807 ± 149 | 78.3 ± 23.9 | 619 ± 106 | 7.9 | 83.6 ± 14.1 |
| 11 | 26.9 ± 1.5 | 112 ± 16 | 105 ± 6 | 36.5 ± 13.9 | 532 ± 179 | 14.6 | 88.1 ± 11.6 |
| Doxorubicin | 39 ± 7 | 258 ± 44 | 386 ± 118 | 92 ± 18 | 4,750 ± 990 | 51.6 | 166 ± 74 |
| Cisplatin | ——* | 6,370 ± 1,410 | 10,700 ± 750 | 4,570 ± 73 | 5,700 ± 630 | 1.3 | 3,900 ± 1,500 |

The IC50 value is defined as the concentration of a compound at which 50% growth inhibition is observed. The values shown are mean ± S.D. (n = 3 or more).

*Inhibition of proliferation did not exceed 50% at the highest concentration tested of 10 µM.

RI values indicate resistance index. RI was calculated for each compound using the formula: RI = IC50 for LoVo/DX/IC50 for LoVo cell line. When RI is 0–2, the cells are sensitive to the compound tested, RI in the range 2–10 means that the cell shows moderate sensitivity to the drug tested, RI above 10 indicates strong drug-resistance.

For the representative full cytograms see Fig. S22 (Supplementary material). A graphical representation of cell cycle distribution from the mean of three such experiments is presented in Fig. 2A. Statistically significant percentage of cells with fragmented DNA was observed after 48 h of treatment further increasing after 72 h (Fig. 2A, red bars). Notably, analogues 2, 5, 7, 8 and 9 caused cell death more rapidly, indicated by significant sub-G1 DNA as early as 24 h of treatment. Of note, a decrease in the pool of cells in G1 phase of cell cycle (Fig. 2A, blue bars) occurred concomitant with the generation of cells with sub-

![Selectivity Index (SI) values of tested compounds. SI was calculated for each compound using the formula: SI = IC50 for non-neoplastic cell line BALB/3T3/IC50 for respective cancerous cells or cell line. A beneficial SI > 1.0 indicates a drug with efficacy against tumor cells greater than the toxicity against non-neoplastic cells.](image-url)
G1 DNA (Fig. 2A, red bars) suggesting they were the primary source. Because colchicine (1) is known for its ability to induce mitotic arrest in established cancer cell lines [39], it was of interest to investigate this property among the analogs. We first examined ALL-5 cells, assessing 4 N DNA content as an indicator of mitotic arrest (Fig. 2A). Overall levels of mitotically arrested cells were low, with a maximum level of about 20%. This observation is in good agreement with our previous work, where we showed that other microtubule destabilizing agents such as vincristine and eribulin failed to cause mitotic arrest in ALL-5 cells, and tend instead to induce death directly in G1 phase [40]. In contrast, when we examined MCF-7 breast adenocarcinoma cells, treatment with 1 or its analogs caused characteristic mitotic arrest (Fig. 2B, red bars), indicated by relatively high levels of cells with 4 N DNA content (Fig. 2B, gray bars). In addition, the compounds did not induce sub-G1 DNA content in MCF-7 cells. Thus, similar to effects we observed previously with thiocolchicine urethanes, MCF-7 cells appear to be susceptible in M phase but apparently die through a mechanism that does not involve DNA fragmentation, possibly related to their lack of caspase-3 [41].

2.4. The effect of colchicine and 8 on PARP cleavage in primary ALL-5 cells

In the next step we examined poly (ADP-ribose) polymerase (PARP) cleavage by immunoblotting as an additional marker of apoptotic cell death for colchicine (1) and its most active triple-modified analog 8 (based on the lowest IC50 value in Table 1). The immunoblots are presented in Fig. 3A, and respective quantitation of band intensities in
PARP 116 kDa
85 kDa

GAPDH 37 kDa

| Compound | Harvest time (h) |
|----------|-----------------|
| vehicle  | 24              |
| 1        | 24              |
| 1        | 48              |
| 8        | 24              |
| 8        | 48              |
| DX       | 24              |

Fig. 3. (A) Cleavage of PARP. ALL-5 cells were treated with 43 nM compound 1, 11.5 nM compound 8, 0.2 µM doxorubicin (DX), or 0.1% DMSO (vehicle) for the times indicated, and extracts were prepared and subjected to immunoblotting for PARP. The intact (116 kDa) and cleaved (85 kDa) forms of PARP are shown. GAPDH was used as a loading control. (B) Bar diagram showing the fold changes of proteins normalized to GAPDH. Images were quantified by measuring the band intensity using ImageJ software. Data represented as mean ± S.D. of three independent determinations (n = 3); 24 h treatment was compared with 48 h for respective compound. **P < 0.005, *P < 0.05.

2.5. Molecular docking studies

Prediction of the binding modes of the 4-chlorothiocolchicine derivatives with different tubulin isotypes, namely, α-βI, α-βIIa, α-βIII, α-βIVb and α-βVI was carried out by first running molecular dynamics (MD) simulations of each isotype during 70 ns in order to generate representative structure(s) of the proteins. Identification of such structures was done by performing RMSD-based clustering analysis on all the structures generated over the last 30 ns of each MD trajectory (see Section 4.6). Next, docking was performed on every representative structure using Autodock Vina and DOCK 6.5 (see Section 4.7). All the poses generated by both programs were then rescored using the Vina scoring function. Table 2 shows the top binding pose of colchicine and the derivatives as predicted by the Vina scoring function for the α-βIIIA model which was found to produce the best results in terms of pIC50 predictions (see below). Active residues involving non-hydrophobic interactions with the ligand are also specified for each compound. Subsequent to docking, the best Vina scores were collected for each isotype-compound pair. Two-variable linear regressions using the mentioned scores as the first variable and the Morituguchi octanol-water partition coefficient (MLogP) as the second variable was carried out in an attempt to fit experimental pIC50 values. Table 3 depicts, for each isotype, the R² values computed from such linear regressions using the pIC50 values reported in Table 1. The highest R² (0.669) was obtained for primary acute lymphoblastic leukemia cells from Vina scores computed in the case of the βIIA tubulin isotype (see also Fig. 4). Some correlation was also found for MCF-7 cells still in the case of the βIIA isotype where the R² = 0.436. In the cell lines where good correlation was not found, especially LoVo/DX (which is a drug resistant type), additional factors may be playing a role in the compounds’ efficacy such as P-glycoprotein based drug resistance, off-target interactions of the compounds or membrane permeability issues which need to be addressed in detail in future studies.

3. Conclusions

Seven different and novel triple-modified colchicine derivatives (5–11) were synthesized with moderate yields. All of these compounds, together with unmodified colchicine and two conventional anticancer drugs, were tested for their anti-proliferative activity against several human cancer cell lines: A549, MCF-7, LoVo and LoVo/DX, as well as primary acute lymphoblastic leukemia ALL-5 cells. Some of the triple-modified colchicine derivatives were up to 10 × more potent than unmodified colchicine against both primary and established cancer cells. Effects of colchicine and selected 4-chlorothiocolchicine analogues on cell death and cell cycle progression were investigated. Interestingly, colchicine and its derivatives caused mitotic arrest in MCF-7 cells, but failed to do so in ALL-5 cells. Instead, they induced death of ALL-5 cells in interphase, consistent with previous findings. Based on our molecular docking studies we found a good correlation between experimental and computational results for ALL-5 cells and a moderate correlation for MCF-7 cells. Other factors, still to be defined, may be playing a role in the cell lines where correlation was poor. Nevertheless, several of the triple-modified derivatives described here have excellent potential as a novel cancer chemotherapeutics, and warrant additional mechanistic studies and investigation of their in vitro pharmacokinetic and pharmacodynamic properties.

4. Experimental section

4.1. General

All precursors and solvents for the synthesis were obtained from Sigma Aldrich (Merck KGaA, Saint Louis, MO, USA) and were used without further purification. CDCl₃, spectral grade solvent was stored over 3 Å molecular sieves for several days. TLC was performed on precoated plates (TLC silica gel 60 F254, Aluminium Plates Merck, Merck KGaA, Saint Louis, MO, USA) visualized by illumination with an UV lamp. HPLC grade solvents (without further purification) were used for flash chromatography (CHROMASOLV from Sigma Aldrich, Merck KGaA, Saint Louis, MO, USA). The elemental analysis of compounds was performed on Vario ELIII (Elementar, Langenselbold, Germany).

4.2. Spectroscopic measurements

The ¹H, ¹³C spectra were recorded on a Varian VNMR-S 400 MHz spectrometer (Varian, Inc., Palo Alto, CA, USA). ¹H NMR measurements
Table 2
Binding location and orientation of colchicine and its derivatives on α-βIIA tubulin as predicted by Autodock Vina scoring function and active residues interacting with each ligand via hydrogen bonding or π-interactions. Compounds with no specified active residues (N/A) were found to interact only via hydrophobic interactions.

| Compound | Binding pose | Vina Score | Active residues |
|----------|--------------|------------|-----------------|
| 1        |              | −8.90      | Leu255, Lys352  |
| 2        |              | −8.73      | N/A             |
| 3        |              | −8.85      | Leu255, Lys352  |
| 4        |              | −7.73      | Cys241, Leu255  |
| 5        |              | −7.89      | Asn101, Leu248  |
| 6        |              | −8.16      | N/A             |

(continued on next page)
of 2–11 (0.07 mol dm−3) in CDCl3 were carried out at the operating frequency 402.64 MHz. The error of the chemical shift value was 0.01 ppm. The 13C NMR spectra were recorded at the operating frequency 101.25 MHz. The error of chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of CDCl3. The 1H and 13C NMR spectra are shown in the Supplementary Materials.

The FT-IR spectra of 2–11 in the mid infrared region were recorded in KBr. The spectra were taken with an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe, Germany) equipped with a DTGS detector; resolution 2 cm−1, NSS = 64. The Happ-Genzel apodization function was used.

The ESI (Electrospray Ionisation) mass spectra were recorded also on a Waters/Micromass (Waters Corporation, 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). The sample was infused into the ESI source using a Harvard pump at a flow rate of 20 ml 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 $m/z = 100$ to $m/z = 1000$, as well as from $m/z = 200$ to $m/z = 1500$.

4.3. Synthesis

4.3.1. Synthesis of 4-chlorocolchicine (2)

A mixture of NCS (175 mg, 1.31 mmol) and colchicine (1) (500 mg, 1.25 mmol) in acetonitrile was stirred at RT under nitrogen atmosphere for the 72 h. Reaction time was determined by TLC. The reaction was quenched with saturated aqueous Na$_2$S$_2$O$_3$. The whole mixture was extracted four times with CH$_2$Cl$_2$, and the combined organic layers were dried over MgSO$_4$, filtered, and evaporated under reduced pressure. The residue was purified by CombiFlash$^*$ (EtOAc/MeOH, increasing concentration gradient) to give 2 (C$_{22}$H$_{24}$ClNO$_5$S, MW = 433.9 g/mol) with yield 75%.

4.3.2. Synthesis of 4-chlorothiocolchicine (3)

To a mixture of 2 (500 mg, 1.15 mmol) in MeOH/water (1/1, v/v, 5 ml), the sodium methanethiolate (solution 21% in H$_2$O, 0.87 ml, 2.3 mmol) was added. The mixture was stirred in RT for 72 h. Reaction time was determined by TLC. After that time the reaction mixture was quenched by the addition of water (150 ml). The whole mixture was extracted four times with CH$_2$Cl$_2$, and the combined organic layers were dried over MgSO$_4$, filtered, and evaporated under reduced pressure. The residue was purified by CombiFlash$^*$ (hexane/EtOAc (1/1), then EtOAc/MeOH, increasing concentration gradient) to give 3 (C$_{22}$H$_{24}$ClNO$_5$S, MW = 450.0 g/mol) as amorphous yellow solid with yield 37%.

4.3.3. Synthesis of 4-chlorodeacetylothiocolchicine (4)

Compound 4 was prepared from 3 by hydrolysis with 2 N HCl. To a solution of compound 3 (500 mg, 1.11 mmol) in MeOH (3 ml), the 2 N HCl solution (5 ml) was added. The mixture was stirred at 90 °C for 72 h. Reaction time was determined by TLC. After that time the reaction mixture was quenched by the addition of water (100 ml). The whole mixture was extracted four times with CH$_2$Cl$_2$, and the combined organic layers were dried over MgSO$_4$, filtered, and evaporated under reduced pressure. The residue was purified by CombiFlash$^*$ (EtOAc/MeOH, increasing concentration gradient) to give 4 (C$_{22}$H$_{24}$ClNO$_5$S, MW = 407.9 g/mol) with yield 88%.

4.3.4. General procedure for the synthesis of colchicine derivatives (5–11)

Compounds 5–11 were obtained directly from compound 4. To a solution of compound 4 (100 mg, 0.25 mmol) in tetrahydrofuran (THF, 5 ml) cooled to the 0 °C temperature, the following compounds were added: Et$_3$N (2 ml, 14 mmol) and DMAP (catalytic amount). The mixture was first stirred at 0 °C for a few minutes and then the solution of respective acyl chloride (5–10) or diethylcarbamoyl chloride (11) in THF (0.75 mmol in 2.5 ml) was added dropwise. The mixture was stirred at RT for the next 24 h. The solution was filtered to remove triethylamine hydrochloride. The THF was evaporated and the residue was purified by CombiFlash$^*$ (hexane/ethyl acetate, increasing concentration gradient) to give respective compounds as amorphous yellow solids with yield from 29% to 86% (5–11).

4.4. Cell lines and culturing conditions

Primary ALL-5 cells were derived from the bone marrow of a 37-year-old patient as previously described [45,46]. Although these cells can be cultured up to 6 months with no obvious change in their properties [45], in the present study they were exclusively used at low passage for all experiments, and are thus referred to as primary cells. Primary ALL-5 cells were routinely maintained at 37 °C in a humidified 5% CO$_2$ incubator in IMDM Modified (HyClone) media supplemented with 10 μg ml$^{-1}$ cholesterol, 6 mg ml$^{-1}$ human serum albumin, 2 mM l-glutamine, 2% v/v amphotericin-B/penicillin/streptomycin, 1 μg ml$^{-1}$ insulin, 200 μg ml$^{-1}$ apo-transferrin, and 50 μM β-mercaptoethanol, and were subcultured to maintain a density of 1–3 × 10$^6$ cells ml$^{-1}$. Human MCF-7 mammary gland adenocarcinoma cells originally isolated from a 69-year-old Caucasian woman with several characteristics of differentiated mammary epithelium were cultured in Eagle’s Minimum Essential Medium (EMEM) (30–2003, ATCC, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (FP-0500-A, Atlas Biologicals, USA), and 1% Penicillin/Streptomycin Solution 100x (30-002-Ci, Corning, USA). MCF-7 cell line was tested via short tandem repeat profiling in July 2018 by Genetica DNA Laboratories (Burlington, NC) and verified as authentic, giving a 100% match when compared to the known reference profile [47]. Both primary ALL-5 cells and MCF-7 cell line for cell cycle analysis were maintained in the Department of Biochemistry & Molecular Biology at University of Arkansas for Medical Sciences, USA.

The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), A549 and MCF-7 cell lines–from European Collection of Authenticated Cell Cultures (Salisbury, UK), LoVo cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and LoVo/DX by courtesy of Prof. E. Borowski (Technical University of Gdańsk, Gdańsk, Poland). All of the above listed cell lines were maintained in the Institute of Immunology and Experimental Therapy (IET), Wroclaw, Poland. Human lung adenocarcinoma cell line (A549) was cultured in the mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wroclaw, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan UT, USA) and 2 mM l-glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Human colon adenocarcinoma cell lines (LoVo) were cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (IET, Wroclaw, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan UT, USA) and 2 mM l-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA) and 10 μg/100 ml doxorubicin for LoVo/DX (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). Mouse embryonic fibroblast cells (BALB/3T3) were cultured in Dulbecco medium (Life Technologies Limited, Paisley, UK).
supplemented with 10% fetal bovine serum (GE Healthcare, Logan, UT, USA) and 2 mM glutamine (Sigma-Aldrich, Merck KGaA, Saint Louis, MO, USA). All cell culture media contained antibiotics: 100 U/ml penicillin and 100 μg/ml streptomycin (Polfa-Tarchomin, Warsaw, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO2. Cells were tested for mycoplasma contamination by mycoplasma detection kit for conventional PCR: Vener GeM Classic (Minerva Biolabs GmbH, Berlin, Germany) and negative results were obtained. The procedure is repeated every year or in the case of less frequently used lines after thawing.

4.5. Cell viability assays

4.5.1. SRB assay

Sulforhodamine B (SRB) assay was performed to assess cytotoxic activity of studied compounds towards adherent cell lines. Cells (10^4 per well) were seeded in 96-well plates (Sarstedt, Numbrecht, Germany) in appropriate complete cell culture media and after 24 h prior addition of tested compounds. Cells were subjected to the treatment with tested agents or cisplatin (Teva Pharmaceuticals Polska, Warsaw, Poland) or doxorubicin (Accord Healthcare Limited, Middlesex, UK) in the concentration range 100–0.1 μg/ml for 72 h. Treatment with DMSO (POCh, Gliwice, Poland) at concentrations corresponding to these present in tested agents' dilutions was applied as a control (100% cell viability). After 72 h of incubation with the tested compounds, cells were fixed in situ by gently adding of 50 μL per well of cold 50% trichloroacetic acid (TCA) (POCh, Gliwice, Poland) following incubation at 4 °C for one hour [37]. Next, wells were washed four times with water and air dried. 50 μL of 0.1% solution of sulforhodamine B (Sigma–Aldrich, Merck KGaA, Saint Louis, MO, USA) in 1% acetic acid (POCh, Gliwice, Poland) were added to each well and plates were incubated at room temperature for 0.5 h. Unbound dye was removed by washing plates four times with 1% acetic acid. Stained cells were solubilized with 10 mM Tris base (Sigma–Aldrich, Merck KGaA, Saint Louis, MO, USA). Absorbance of each solution was read at Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski VT, USA) at the 540 nm wavelength.

Results are presented as mean IC50 (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC50 values were calculated in Cheburator 0.4, Dmitry Nevzhay software (version 1.2.0 software by Dmitry Nevzhay, 2004–2014, http://www.cheburator.nezhay.com, freely available) for each experiment [38]. Compounds at each concentration were tested in triplicate in individual experiment and each experiment was repeated at least three times independently.

4.5.2. MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay was used to evaluate the effect of drugs on the viability of primary ALL-5 cells [48,49]. Cells (10^5/well) in 100 μL of complete IMDM Modified medium were seeded in 96-well plates (TPP, Switzerland) and treated with drugs at concentrations up to 10 μM for 120 h with control cells receiving vehicle (0.1% DMSO) alone. After treatment, 10 μL of MTT solution (5 mg/mL) was added to each well and the plate was incubated at 37 °C for 4 h in a humidified 5% CO2 incubator. Then 100 μL of 10% SDS in 0.01 M HCl was added to each well and the plate was incubated at 37 °C for a further 24 h. Absorbance was recorded at 540 nm using a BioTek Plate Reader. Inhibition of formation of colored MTT formazan was taken as an index of cytotoxicity activity. IC50 values were determined by non-linear regression analysis using GraphPad Prism 6 for Windows (GraphPad Software).

Selectivity index (SI) was calculated by dividing the IC50 value for BALB/3T3 cells by the IC50 value for individual cancer cell lines, and resistance index (RI) was calculated by dividing the IC50 for LoVo/DX cells by the IC50 for LoVo cells. The Resistance Index (RI) was defined as the ratio of IC50 for a given compound calculated for resistant cell line to that measured for its parental drug sensitive cell line (Table 1).

4.6. DNA content analysis

ALL-5 (1.5 × 10^4) and MCF-7 (0.2 × cells 10^4) were seeded in 100 mm Petri dishes (Corning, NY) and incubated in the presence of vehicle (0.1% DMSO) or compounds, at concentrations specified in the text, for 24, 48 or 72 h at 37 °C in a humidified 5% CO2 incubator. Cells were then washed with 1 ml phosphate-buffer saline (PBS), fixed with 1–3 ml of 70% ice-cold ethanol and stored at 4 °C prior to flow cytometric analysis. Cells were centrifuged, treated with 500 μl propidium iodide/RNase Staining buffer (BD Biosciences, San Jose, CA, USA) and stored in the dark for 1 h at RT. The stained cells were subjected to a FacsAria IIIu Flow Cytometer (BD Biosciences, San Jose, CA, USA) performed by UAMS Flow Cytometry Core Facility and data were analyzed using FlowJo software.

4.7. Western blot analysis

ALL-5 cells (15 × 10^6 cells/dish) were treated for 24 and 48 h with 1 and 5 at 5 × IC50 values concentration or with vehicle (0.1% DMSO) or DX (0.2 μM) for 24 h. Cells were washed in PBS and lysed in lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, EDTA-free complete protease inhibitor tablets (Roche), 20 μg ml−1 aprotonin, 50 μg ml−1 leupeptin, 10 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM Na2VO3, and 1 μM okadaic acid. Protein content was measured by Bradford assay and equal amounts (20 μg) were selected by electrophoresis using Mini-PROTEAN® pre-cast gels (Bio-Rad). Proteins were electrothermally transferred onto a PVDF membrane (Immobilon-FL, Merck Millipore) and next stained with Ponceau S to assess transfer efficiency and verify equal loading. The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% TWEEN-20 (TBS-T) for 1 h at RT and incubated overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology) against PARP (9532) (1:2500 dilution) and GAPDH (2118) (1:10000 dilution). After washing with TBS-T for 5 × 5 min the membrane was incubated with secondary HRP-conjugated goat anti-rabbit IgG (H + L) antibody (1:5000 dilution) (Bio-Rad) for 1 h at RT. After washing in TBS-T the membrane was exposed to ClarityTM Western ECL Substrate luminol enhancer solution and peroxide solution (Bio-Rad) for 5 min and visualized and quantified using Image J software.

4.8. Statistical analysis

Unpaired t test with Welch’s correction was performed for the significance and p values of < 0.05 were considered significant.

4.9. Homology modeling and molecular dynamics

A combination of different computational methods was used to investigate in silico the binding modes and binding energies of the colchicine derivatives for different isotypes of the human tubulin dimer. The 3D structures of the human isotypes were built from homology modeling by using the crystallographic structure of the bovine α-β tubulin isotype complexed with colchicine as a template (PDB ID: 1SAO) [50]. The dimer models were generated by the α subunit for α-ubulin (UniProt ID: Q7TU36) and different isotypes of the β-tubulin including β1 (UniProt ID: P07437), βIIIA (UniProt ID: Q13885), βIIB (UniProt ID: Q13509), βIVB (UniProt ID: P68371), βVI (UniProt ID: Q9H4B7) [51]. The latter isotypes of tubulin are the most commonly expressed ones in cancer cells. Homology modeling was performed using the Molecular Operating Environment (MOE) [52] by setting the number of generated models to 10 and by selecting the final model based on MOE’s generalized Born/volume integral (GB/VI) scoring...
function. During the modeling, cofactors including GTP, GDP, colchicine, and the magnesium ion located at the interface between α- and β-monomers were kept as part of the environment and included in the refinement step. The final model was protonated at neutral pH and minimized using a built-in protocol in the MOE package.

Subsequent to homology modeling, molecular dynamics (MD) simulations were run on the generated model of each isotype using Amber14 [53]. MD parameters, e.g., partial charges and force constants were set via the ff14SB force field for the protein while parameters for GTP, GDP and colchicine were obtained from the GAFF force field. The complex system was further solvated in TIP3P water using the Amber’s tLEaP program. Energy minimization of the structure was carried out in two steps, both using the steepest descent and conjugate gradient methods successively. First, 2000 cycles of energy minimization were performed on solvent atoms only, by restraining the protein–ligand complex. Next, minimization was run without the restraint for 5000 cycles. The structure was then equilibrated in an NVT ensemble during 20 ps and in an NPT ensemble during 40 ps setting the temperature to 298 K and the pressure to 1 bar. Finally, MD production was run for 70 ns (see Fig. S.23).

Clustering analysis of the last 30 ns of the generated MD trajectories was carried out using Amber’s CPPTRAJ program [54] to find the representative conformations of each tubulin isotype. Clustering was done via a hierarchical agglomerative algorithm using the RMSD of atoms in the colchicine binding site as a metric. An RMSD cut-off of 1.0 Å was set to differentiate the clusters. For each isotype, the representative structure of each cluster was used as a rigid target for the screening of the colchicine derivatives.

4.10. Docking simulations

Docking of the 4-chlorothiocolchicine derivatives was performed using two different docking software packages, namely, AutoDock Vina [55] and DOCK6.5 [56]. Since those programs are based on different methods for ligand placement and scoring, using both programs simultaneously normally increases the chance of generating the correct ligand pose [57]. Vina includes an iterated local search global optimizer as a searching method and a combination of knowledge-based and empirical potentials as a scoring function [55]. On the other hand, DOCK6.5 is based on the anchor-and-grow algorithm to generate ligand poses and makes use of a force-field-based potential to score them [56]. For our docking simulations, a cubic box with size 30.0 Å centered at the center of mass of the bound colchicine was considered. All cofactors, namely, GTP, GDP, colchicine, and the magnesium ion were removed during docking while the target was kept rigid. For every compound, docking was run separately on each of the tubulin representative structures obtained from clustering. The number of generated ligand poses was set to 10 for both AutoDock Vina and DOCK6.5, meaning that a maximum of 20 ligand poses was produced for every compound/protein structure pair. The ligand poses were eventually scored using AutoDock Vina’s scoring function. For every derivative, the pose with the best Vina score over all representative structures of each tubulin isotype was kept for further analysis, especially to investigate the correlation with experimental pIC50 values. Besides Vina scores, the Morighuchi octanol-water partition coefficient (MLoGP) of every compound was calculated using the ADMET Predictor 8.0 package (ADMET Predictor, Simulations Plus, Lancaster, CA, USA). Both Vina scores and MLoGP values were used as inputs to build a two-variable linear regression model for every tubulin isotype.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support by grant of the Polish National Science Centre (NCN) – No. 2016/21/B/ST5/00111 is gratefully acknowledged. GK gratefully acknowledges the doctoral scholarship “ETUDA” – No. 2018/28/T/ST5/00041 financed by the Polish National Science Centre (NCN). T.C.C. acknowledges support from Arkansas Breast Cancer Research Program. The authors thank Andrea Harris from the UAMS Flow Cytometry Core for performing flow cytometry experiments and assisting with interpretation, and Eric Siegel of the UAMS College of Medicine Biostatistics Department for assistance with data and statistical analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioprog.2020.103664. These data include MOL files and InChiKeys of the most important compounds described in this article.

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G. Klęgorowska, et al.

Biorganochemistry 97 (2020) 103664