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

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ABSTRACT

Colchicine is the major alkaloid isolated from the plant \textit{Colchicum autumnale}, which shows strong therapeutic effects towards different types of cancer. However, due to the toxicity of colchicine towards normal cells its application is limited. To address this issue we synthesized a series of seven triple-modified 4-bromothiocolchicine analogues with amide moieties. These novel derivatives were active in the nanomolar range against several different cancer cell lines and primary acute lymphoblastic leukemia cells, specifically compounds: 5 – 9 against primary ALL-5 (IC\textsubscript{50} = 5.3 – 14 nM), 5, 7– 9 against A549 (IC\textsubscript{50} = 10 nM), 5, 7 – 9 against MCF-7 (IC\textsubscript{50} = 11 nM), 5 – 9 against LoVo (IC\textsubscript{50} = 7 – 12 nM), and 5, 7 – 9 against LoVo/DX (IC\textsubscript{50} = 48 – 87 nM). These IC\textsubscript{50} values were lower than those obtained for unmodified colchicine and common anticancer drugs such as doxorubicin and cisplatin. Further studies revealed that colchicine and selected analogues induced characteristics of apoptotic cell death but manifested their effects in different phases of the cell cycle in MCF-7 versus ALL-5 cells. Specifically, while colchicine and the studied derivatives arrested MCF-7 cells in mitosis, very little mitotically arrested ALL-5 cells were observed, suggesting effects were manifest instead in interphase. We also developed an \textit{in silico} model of the mode of binding of these compounds to their primary target, β-tubulin. We conducted a correlation analysis (linear regression) between the calculated binding energies of colchicine derivatives and their anti-proliferative activity, and determined that the obtained correlation coefficients strongly depend on the type of cells used.

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1. Introduction

Natural products and their derivatives play an important role in many modern therapies and as a foundation for the development of new drugs. For example, for cancer drugs developed during the period from the 1940s to the end of 2014, of 175 small molecules approved, 49% were natural products or direct derivatives of natural products \textsuperscript{1,2}. Thus, chemical modification of biologically active compounds of natural origin is a proven and efficient approach to novel drug development. Among them, colchicine, an alkaloid and secondary metabolite extracted from plants of the genus \textit{Colchicum} e.g. \textit{Colchicum autumnale}, is one of the oldest therapeutic substances known to mankind \textsuperscript{6}. Colchicine is still used for the treatment of familial Mediterranean fever, Behcet’s disease, acute gout, chondrocalcinosis, and other types of microcrystalline arthritis because of its anti-inflammatory properties \textsuperscript{7-17}. Its primary target is β-tubulin where it forms complexes with tubulin dimers and copolymerizes into microtubule structures, suppressing microtubule dynamics and disrupting key cellular processes like mitotic spindle formation \textsuperscript{18-20}. Compounds which bind to the colchicine site on tubulin and inhibit microtubules (colchicine site inhibitors, CSIs) have been a rich source of new drugs particularly those with tumor vascular disrupting ability \textsuperscript{21-23}. The parent molecule colchicine has shown very high anticancer activity \textit{in vitro}, but its clinical potential for human tumors is limited because of its relatively high toxicity, which results from its accumulation in the gastrointestinal tract, as well as neurotoxicity \textsuperscript{5,23,24}. Many efforts have been made to optimize the structure of colchicine to generate less toxic and more bioavailable analogues \textsuperscript{25-46}. We have previously reported studies on 4-bromothiocolchicine derivatives with diversified carbamate substituents in the C-7 position \textsuperscript{30}. 
In order to further increase anti-cancer activity and decrease toxicity towards normal cells, in this study we investigated the impact of replacing the carbamate moiety with an amide. Previous independent work studied C-4 halogen substituted colchicine derivatives and 4-chloro colchicine derivatives bearing an amide moiety in the C-7 position. 4-Chlorocolchicine and some of the double-modified derivatives with trifluoroacetyl or propionyl substituents exhibited strong antitumor activities over broad effective dosage ranges in vivo, but their metabolic stabilities were poorer than that of colchicine. In the present study, we synthesized 4-bromothiocolchicine derivatives bearing a thiomethyl group in the C-10 position anticipated to increase molecular stability. Thiocolchicines do not undergo photochemical isomerization, while colchicine isomerizes, as well as harsher conditions can be used for deacetylation at the C(7) of thiocolchicines without any undesirable changes in their structures. We report here the synthesis and biological activity in normal and cancer cells of a series of novel triple-modified derivatives, with six different amide substituents and one urea moiety in the C-7 position.

2. Results and discussion

2.1. Chemistry

Seven novel triple-modified colchicine derivatives (5-11) were synthesized from 4-bromodeacetylthiocolchicine in a simple one-pot reaction with respective acyl chloride in the presence of triethylamine and 4-dimethylaminopyridine (DMAP) in tetrahydrofuran (THF). As shown in Scheme 1, 4-bromodeacetylthiocolchicine (1) and 4-chloro colchicine (2) and 4-thiocolchicines (3) and 4 -bromodeacetylthiocolchicine (4) were prepared according to previously described methods. The structures and purity of all products 2-11 were determined using ESI-MS, FT-IR, 1H-NMR and 13C-NMR methods as well as elemental analysis (for new previously unpublished compounds). The detailed synthetic procedures, spectroscopic characteristics, as well as 1H NMR and 13C NMR spectra, are shown in Supplementary Figures S1-S20. In the 13C-NMR spectra of 2, a resonance for the C-4 carbon atom of the A aromatic ring was observed at 113.5 ppm, while in 1 it was observed at 107.3 ppm. After the substitution of a thiomethyl group in the C-10 position, shifts of the 13C NMR signal for the C-20 carbon atom in compound 3 were observed at 15.2 ppm, while in unmodified 1 as well as 2 shifts of the signal for the C-20 carbon atom were observed in the range 56.1-56.5 ppm. In the 13C-NMR spectra of 4, signals for the acetyl group, which were observed at 170.0 and 22.9 ppm in compound 3, had disappeared. In amides (5–11), shifts of the signal for the amide carbon atom were observed in the range 167.0–176.9 ppm while in urea (11) the shift was observed at 155.9 ppm.

2.2. Growth inhibition of human cancer cell lines

The seven triple-modified colchicine derivatives (5-11), other colchicine derivatives (2-4) and colchicine (1) were evaluated for their in vitro anti-proliferative effect on primary acute lymphoblastic leukemia cells (ALL-5) and four human cancer cell lines, namely human lung adenocarcinoma (A549), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma cell line (LoVo), and its doxorubicin-resistant subline (LoVo/DX), as well as on normal murine embryonic fibroblast cell line (BALB/3T3). The data are expressed as IC50 (mean ± SD) of the tested compounds and are presented in Table 1. Some general trends emerged from these results. First, most of the triple-modified colchicine derivatives (except compound 10 with long alkyl chain connected to amide group) showed anti-proliferative activity in the nanomolar range. Second, the highest cytotoxic activity among triple-modified colchicine derivatives against respective cancerous cell lines was demonstrated by compounds 5–9 against primary ALL-5 cells (IC50 = 5.3 – 14 nM), 5 – 9 against A549 (IC50 = 10 nM), compounds 5, 7 – 9 against MCF-7 (IC50 = 11 nM), compounds 5 – 9 against LoVo (IC50 = 7 – 12 nM), and compounds 5, 7 – 9 against LoVo/DX (IC50 = 48 – 87 nM). Also, other derivatives (2 and 3) showed very good activity against primary ALL-5 cells (IC50 = 5.9 and 5.4 nM respectively) as did double-modified analogue (3) on three of the tested cancerous cell lines (A549, MCF-7 and LoVo; IC50 = 10 – 15 nM). These IC50 values are lower than those obtained for unmodified colchicine (1) and common anticancer agents such as doxorubicin and cisplatin. Third, the majority of the tested
values, towards primary ALL-5 cells than other cell lines, towards LoVo cell line (IC_{50} = 12, 7, 7, 24 nM, respectively).

Table 1
Anti-proliferative activity (IC_{50}), selectivity index (SI) values and resistance index (RI) values of colchicine (1) and its derivatives (2-11) compared with anti-proliferative activity of standard anticancer drugs doxorubicin and cisplatin.

| ALL-5 | A549 | MCF-7 | LoVo | LoVo/DX | BALB/3T3 |
|-------|------|-------|------|---------|----------|
| IC_{50}(nM) | SI | IC_{50}(nM) | SI | IC_{50}(nM) | SI | IC_{50}(nM) | SI | IC_{50}(nM) | SI | IC_{50}(nM) | SI |
| 1     | 8.6 ± 0.2 | 15.4 | 125.2 ± 12.5 | 1.1 | 54.3 ± 27.5 | 2.6 | 108.4 ± 25.0 | 1.3 | 1,694.3 ± 275.4 | 0.1 | 15.7 | 139.4 ± 72.6 |
| 2     | 5.9 ± 1.3 | 23.7 | 104.5 ± 8.4 | 1.4 | 26.6 ± 8.4 | 5.3 | 83.6 ± 20.9 | 1.7 | 1,554.0 ± 167.3 | 0.1 | 18.7 | 142.2 ± 73.2 |
| 3     | 5.4 ± 2.2 | 20.6 | 10.1 ± 0.2 | 10.3 | 14.8 ± 2.0 | 6.9 | 13.6 ± 4.0 | 7.4 | 134.9 ± 12.1 | 0.8 | 9.6 | 103.2 ± 89.0 |
| 4     | 91.4 ± 17.8 | 13.8 | 114.9 ± 6.6 | 11.0 | 178.4 ± 19.9 | 7.1 | 125.3 ± 44.2 | 10.1 | 700.0 ± 88.4 | 1.8 | 5.6 | 1,259.9 ± 795.8 |
| 5     | 5.3 ± 2.2 | 2.2 | 10.2 ± 1.1 | 1.1 | 11.4 ± 0.02 | 1.1 | 7.6 ± 0.02 | 1.6 | 71.2 ± 11.7 | 0.2 | 9.3 | 11.4 ± 0.02 |
| 6     | 14.0 ± 1.0 | 4.6 | 53.4 ± 33.5 | 0.8 | 48.9 ± 38.9 | 1.3 | 12.0 ± 3.7 | 5.3 | 265.8 ± 164.9 | 0.2 | 22.2 | 64.1 ± 8.5 |
| 7     | 5.8 ± 2.3 | 2.8 | 9.6 ± 1.0 | 1.7 | 11.4 ± 1.0 | 1.5 | 6.6 ± 1.0 | 2.4 | 47.9 ± 11.0 | 0.4 | 7.3 | 17.4 ± 8.1 |
| 8     | 8.5 ± 0.9 | 1.3 | 10.5 ± 1.1 | 1.1 | 11.2 ± 1.1 | 1.0 | 7.2 ± 1.1 | 1.6 | 87.2 ± 9.9 | 0.1 | 12.1 | 11.2 ± 1.1 |
| 9     | 8.6 ± 1.8 | 2.2 | 10.2 ± 1.1 | 2.0 | 11.5 ± 0.02 | 1.8 | 7.0 ± 1.1 | 2.9 | 75.9 ± 9.4 | 0.3 | 10.8 | 20.4 ± 7.3 |
| 10    | 196.0 ± 22.4 | 0.5 | 880.9 ± 65.2 | 0.1 | 956.7 ± 37.5 | 0.1 | 482.2 ± 168.8 | 0.2 | 866.6 ± 151.0 | 0.1 | 1.8 | 98.4 ± 15.3 |
| 11    | 55.3 ± 19.3 | 1.4 | 99.1 ± 10.9 | 0.8 | 97.3 ± 11.7 | 0.1 | 53.8 ± 8.4 | 1.5 | 621.3 ± 61.8 | 0.1 | 11.6 | 80.4 ± 20.2 |

DOX | 39.1 ± 7.0 | 4.3 | 258.0 ± 44.0 | 0.6 | 366.0 ± 118.0 | 0.4 | 92.0 ± 18.0 | 1.8 | 4,750.0 ± 990.0 | 0.03 | 51.6 | 166.0 ± 74.0 |
| CCPT | ----- | 6,367.0 ± 1,413.0 | 0.6 | 10,700.0 ± 753.0 | 0.4 | 4,370 ± 73.0 | 0.9 | 5,700.0 ± 630.0 | 0.7 | 1.3 | 3,900.0 ± 1,500.0 |

The IC_{50} value is defined as the concentration of a compound at which 50% growth inhibition is observed.

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

SI was calculated for each compound using the 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 tumor cells greater than the toxicity against normal cells.

The RI indicates how many times a resistant subtype is chemoresistant relative to its parental cell line. The RI was calculated for each compound using the formula: RI = IC_{50} for LoVo/DX / IC_{50} 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.

This is of significance since primary ALL cells are not transformed, immortalized or adapted to culture and are thus anticipated to exhibit properties more closely representative of the initial cancer cells than conventional cell lines. The viability curves for ALL-5 cells are presented in Supplementary Fig. S21.

The selectivity index (SI) is a ratio that measures the window between cytotoxicity and anticancer activity by dividing the given IC_{50} values for the normal BALB/3T3 cell line and respective IC_{50} values for cancerous cell lines. Higher SI values are desirable since they reflect efficacy with less toxicity. The precursor colchicine derivatives (compounds 2-4) had in general high SI values for each of the non-MDR cell types (Table 1). Data for A549, MCF-7 and LoVo were reported previously (REF) and data for ALL-5 are new and showed a similar trend. The triple-modified colchicine derivatives showed relatively lower SI values on primary ALL-5, A549, MCF-7 and LoVo cell lines (SI = 0.8 – 2.0). The exceptions were compound 10 with extremely low SI values (SI = 0.1 – 0.2), and compounds 5, 6, 7 and 9 with moderate SI values on primary ALL-5 cells (SI = 2.2, 4.6, 2.8 and 2.2 respectively) and LoVo cell line (SI = 5.3 (6), 2.4 (7) and 2.9 (9)). Importantly however, conventional anticancer agents like doxorubicin and cisplatin typically showed lower SI values than many of the colchicine analogs for a given cell type (Table 1). Overall, the results of Table 1 indicate that triple modified colchicine derivatives have greater potency than the parent colchicine for several different types of cancer cell.

To evaluate activity against the cells with an MDR (multidrug resistance) phenotype, one drug resistant cancer cell line, i.e. LoVo/DX, was tested and the resistance index (RI) values were calculated (Table 1), as described in Materials and Methods. Most of the derivatives were not able to overcome the drug resistance of the LoVo/DX cell line, with RI values ranging from 5.6 to 22.2. One exception was compound 10, with an RI value of 1.8, but this desirable property was offset by a poor SI value (Table 1).

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

Since colchicine (1) and its analogs showed favorable activity towards primary ALL-5 cells further mechanistic investigation was conducted. First, we studied whether the compounds induced apoptotic cell death, by assessing DNA content and fragmentation via flow cytometry. We employed propidium iodide staining in order to determine DNA content, and cells with sub-G1 (<2N) DNA were considered to have undergone cell death. ALL-5 cells were subjected to treatment with 1, six...
analogs characterized by IC\textsubscript{50} values lower than for parent molecule 1, and \textbf{DX}, at concentrations of ≤ x IC\textsubscript{50} values (Table 1), for 24, 48 or 72 h. Cells treated with vehicle (0.1% DMSO) for equivalent incubation times served as control. The full set of representative flow cytometric data is included in Supplementary Fig. S22A. A graphical representation of sub-G1 DNA content derived from the mean of three such experiments is shown in Fig. 1A. For all of the compounds, statistically significant DNA fragmentation was observed after 48 h of treatment and further increased after 72 h. However, for analogs 5, 7, 8 and 9, cell death was more rapidly induced, with significant DNA fragmentation observed as early as 24 h. Since parent molecule 1 is a well-known microtubule targeting agent (MTA)\textsuperscript{20}, it was of interest to determine whether the analogs retained this property. Therefore, cells with 4N DNA content, reflecting mitotic arrest, were quantitated after treatment (Fig. 1B; Supplementary Fig. S22A). In a population of untreated, asynchronous ALL-5 cells, about 10% have 4N DNA (Fig. 1B). When treated with starting compound 1 and its analogs this amount significantly increased after 24 h (exception 2) and further after 48 h (exception 3 and 7), to subsequently drop down after 72 h due to the growing pool of dead cells (Fig. 1A). Overall, levels of mitotic arrest were low, previous work where it has been shown that in response to microtubule destabilizing drugs, primary ALL-5 cells tend to be susceptible not only in M phase but also in G1 phase. Thus, the above observations are in good agreement with our previously published data, where other two microtubules destabilizers, namely vincristine and eribulin, failed to cause mitotic arrest in primary ALL-5 cells but instead induced death directly in G1 phase when treated with 100 nM or higher concentrations of these drugs \textsuperscript{55}. In contrast to ALL-5 cells, MCF-7 breast cancer cells showed only low levels of DNA fragmentation after treatment, but much higher levels of mitotic arrest, with a more rapid onset (Fig. 1C, 1D). This is consistent with MCF-7 cells: having a shorter doubling time versus ALL-5 cells (24 h versus 60 h, respectively) \textsuperscript{54}; exhibiting susceptibility in M phase not interphase; and lacking caspase-3 \textsuperscript{56}, and thus dying in a manner which does not involve DNA fragmentation. Similar results have been previously reported by us for a series of thiocholchicine urethanes \textsuperscript{33}. 

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ALL-5 (A and B) and MCF-7 (C and D) cells were treated with DMSO (vehicle), 1, its selected derivatives and \textbf{DX} for 24, 48 or 72 h and subjected to propidium iodide staining and flow cytometry. Percent of cells observed with sub-G1 DNA (left column) and extent of mitotic arrest (4N DNA, right column) was summarized. Data are presented as a mean ± SD (n = 3 for all ALL-5 time points and MCF-7 at 72 h; for MCF-7 at 24 and 48 h, n = 1) ****P ≤ 0.0001, ***P ≤ 0.001, **P < 0.01, *P < 0.05 control versus dose). See Supplementary Fig. S1 for a full set of representative cytograms.
2.4. The effect of colchicine and 5 on PARP cleavage in primary ALL-5 cells

Colchicine (1) and compound 5 as the most active triple-modified colchicine derivative based on IC_{50} value (Table 1) were selected for further studies examining poly (ADP-ribose) polymerase (PARP) cleavage by immunoblotting as an additional marker of apoptotic cell death. The immunoblots are shown in Fig. 2A, and quantitation of band intensities in Fig. 2B. Both 1 and 5 induced significant loss of 116 kDa PARP expression after 48 h vs. 24 h treatment. The characteristic 85 kDa degradation product significantly increased after 48 h treatment with 1, whereas this stayed at similarly high level after treatment with 5 at both 24 h and 48 h time points (Fig. 2A, top panel, and Fig. 2B). Treatment with DX was performed as a positive control, and loss of 116 kDa PARP and generation of the 85 kDa product was observed, as previously reported in both ALL-5 57,58 and HeLa cells 59. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a loading control (Fig. 2A, lower panel).

![Figure 2A](image1.png)

**Figure 2.** (A) Cleavage of PARP. ALL-5 cells were treated with 43 nM compound 1, 26.5 nM compound 5, 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 < 0.001, *P < 0.05.

2.5. Molecular docking studies

Prediction of the binding modes of the 4-bromothiocolchicine 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 for 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 4.9). As a second step, docking was performed on every representative structure using Autodock Vina and DOCK 6.5. All the poses generated by both programs were then rescoring using AutoDock Vina’s scoring function. Table 2 shows the top binding pose of colchicine and the derivatives as predicted by Vina scoring function for α-βI. 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 above-mentioned scores as the first variable and the Moriguchi 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 R^2 values computed from such linear regressions using the pIC50 values reported in Table 1. The best correlations were obtained for primary acute lymphoblastic leukemia cells where R^2 values were found to range between 0.359 and 0.525. The highest R^2 (0.525) was produced by using Vina scores computed on the most common tubulin isotype βI (see also Fig. 3). The second best yet poor correlation was obtained in the case of MCF-7 cells, where R^2 values were found around 0.2 and the best R^2 value was again obtained in the case of the βI isotype. 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 future studies.
software and active residues interacting with each ligand via hydrogen bonding, acidic, basic or pi-stacking interactions. Interaction diagrams show nearby residues within a 4.0 Å radius. Compounds with no specified active residues (N/A) were found to interact only via hydrophobic interactions.

| Compound | Binding pose | Vina Score | β1 Active residues |
|----------|--------------|------------|--------------------|
| 1        | ![Diagram 1](image1.png) | -8.57      | Met259, Lys352     |
| 2        | ![Diagram 2](image2.png) | -9.02      | Lys254             |
| 3        | ![Diagram 3](image3.png) | -8.80      | N/A                |
| 4        | ![Diagram 4](image4.png) | -7.54      | N/A                |
5  -7.22  Cys241
6  -8.16  Lys254, Leu255
7  -9.10  Asn258
8  -8.30  N/A
9  -8.00  Asp251
### Table 3

$R^2$ values obtained from two-variable linear regression models used to fit experimental pIC50s for different cell lines. Input variables for the regression models are MlogP and Vina scores obtained for each tubulin isotype.

| Cells         | $\beta I$ | $\beta IIA$ | $\beta III$ | $\beta IVB$ | $\beta VI$ |
|---------------|-----------|-------------|-------------|-------------|------------|
| ALL-5         | 0.525     | 0.359       | 0.414       | 0.418       | 0.313      |
| A549          | 0.125     | 0.067       | 0.069       | 0.085       | 0.031      |
| MCF-7         | 0.301     | 0.240       | 0.244       | 0.266       | 0.169      |
| LoVo          | 0.089     | 0.041       | 0.080       | 0.095       | 0.029      |
| LoVo/DX       | 0.000     | 0.007       | 0.006       | 0.000       | 0.068      |
| BALB/3T3      | 0.049     | 0.129       | 0.051       | 0.110       | 0.014      |

**Diagram:**

- **Legend:**
  - polar
  - acidic
  - basic
  - greasy
  - proximity
  - contour
  - sidechain acceptor
  - sidechain donor
  - backbone acceptor
  - backbone donor
  - ligand exposure
  - solvent residue
  - metal complex
  - solvent contact
  - metal/ion contact
  - receptor exposure
  - arene-arene
  - H arene-H
  - arene-cation

**Note:**

- Cell lines: $\beta£IIA$, $\beta III$, $\beta IVB$, $\beta VI$.
3. Conclusions

Seven novel triple-modified colchicine derivatives (5-11) were synthesized with moderate yields. All of the compounds were evaluated for their anti-proliferative activity against several standard cancer cell lines as well as primary acute lymphoblastic leukemia cells. We conclude that the introduction of different amide moieties in the C-7 position had an impact on the biological activity of colchicine derivatives. Several triple modified derivatives were more potent than colchicine when tested against primary cancer cells and established cancer cell lines (Table 1), in many cases by a factor of 10 or greater. These results strongly support the further development of triple-modified bromo-analogues of colchicine as anti-cancer agents. Further studies revealed that both colchicine (1) and its most potent derivatives induced characteristics of apoptotic cell death and induced contrasting effects on the progression of cell cycle in MCF-7 vs. ALL-5 cells. While 1 and studied analogues caused mitotic arrest in MCF-7 cells, such effect was not observed in ALL-5, showing premises of interphase cell death. Based on our molecular docking studies we found reasonably close correlation between the calculated binding affinity for the most common isotype of tubulin βI with the experimental IC₅₀ values for most of the cases, in particular for acute lymphoblastic leukemia cells. Other factors may be playing a role in the cell lines, where good correlation was not found. Those include p-glycoprotein based drug resistance, off-target interactions of the compounds or membrane permeability issues. Several of the newly synthesized compounds show excellent potential as a novel cancer chemotherapeutics. They show favorable activity comparing not only to their parent molecule, colchicine but also to standard chemotherapeutic agents such as doxorubicin and cisplatin. However, more studies are warranted in order to increase a clinical potential of this promising class of compounds.

4. Experimental section

4.1. General procedures

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 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 x
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 dm³ 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-bromocolchicine (2)

A mixture of N-bromosuccinimide (NBS, 279 mg, 1.57 mmol) and 1 (500 mg, 1.25 mmol) in acetonitrile was stirred at RT under nitrogen atmosphere for 72 h. The progress of the reaction was monitored by TLC. The reaction was quenched with saturated aqueous Na₂SO₃. The whole mixture was extracted four times with CH₂Cl₂, and the combined organic layers were dried over MgSO₄, filtered, and evaporated under reduced pressure.

The residue was purified by Combiflash® (EtOAc/MeOH, increasing concentration gradient) to give 2 as amorphous yellow solid with yield 80% (578 mg).

4.3.2. Synthesis of 4-bromothiocolchicine (3)

A mixture of compound 2 (578 mg, 1.57 mmol) in THF (0.66 mmol in 2.5 ml) was added dropwise. 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 (0.66 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 18% to 83% (5-11). The 1H and 13C NMR spectra of the compound 5-11 are shown in the Supplementary Materials.

Compounds 5-11 were obtained directly from 4. To a solution of compound 4 (100 mg, 0.22 mmol) in tetrahydrofuran (THF, 5 mL) cooled to 0 °C, the following compounds were added: Et₃N (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 (0.66 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 18% to 83% (5-11). The 1H and 13C NMR spectra of the compound 5-11 are shown in the Supplementary Materials.
### Journal Pre-proofs

H, 1H), /82 (dd, J = 8.4, 1.3 Hz, 2H), /5.2 (s, 1H), /7.6 (dd, J = 6.7, 3.9 Hz, 1H), 7.30 – 7.24 (m, 2H), 7.08 (d, J = 10.7 Hz, 1H), 4.86 – 4.79 (m, 1H), 4.00 (s, 3H), 3.98 (s, 3H), 3.72 (s, 3H), 3.30 (dd, J = 10.7, 3.2 Hz, 1H), 2.45 (s, 3H), 2.34 – 2.27 (m, 2H), 2.08 – 2.01 (m, 1H), 1.84 – 1.76 (m, 1H) ppm. 13C-NMR (101 MHz, CDCl3) δ 182.2, 167.0, 159.2, 151.2, 150.8, 150.5, 146.6, 137.3, 134.7, 133.5, 133.3, 131.6, 130.2, 128.6, 128.4, 127.1, 126.1, 113.6, 61.7, 61.5, 61.0, 52.4, 34.6, 29.1, 15.2 ppm. FT-IR (KBr pellet): 3339, 3058, 2934, 1606, 1600, 1553, 1529, 1488, 1463, 1410, 1348, 1230, 1196, 1154, 1083, 1020 cm⁻¹. ESI-MS (m/z): [M+H⁺] calcd 551, found 551, [M+2H⁺] calcd 553, found 553, [M+Na⁺] calcd 573, found 573, [M+2Na⁺] calcd 575, found 575. Anal. Caled. for C, 54.45; H, 5.67; Br, 14.49; N, 5.08; O, 14.51; S, 5.81; found: C, 54.31; H, 5.69; Br, 14.65; N, 5.14; S, 5.76.

#### 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.³⁶⁰ Although these cells can be cultured up to 6 months with no obvious change in their properties, 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% CO₂ 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 x 10⁶ 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-0050-A, Atlas Biologicals, USA), and 1% Penicillin/Streptomycin Solution 100x (30-002-C, 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.³⁶¹ 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), Wrocław, Poland. Human lung adenocarcinoma cell line (A549) was cultured in the mixture of OptiMEM and RPMI 1640 (1:1) medium (IIEET, 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 (IIEET, Wroclaw, Poland), supplemented with 5% fetal bovine serum (GE Healthcare, Logan, UT, USA), 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). Murine 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...
4.5. Cell viability assays

4.5.1. SRB assay

Sulforhodamine B (SRB) assay was performed to assess about cytotoxic activity of studied compounds towards adherent cell lines. Cells (10^4 per well) were seeded in 96-well plates (Sarstedt, Nümbrecht, 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 – 0.01 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 52. 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. Unbounded 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 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 Nevzhay software (version 1.2.0 software by Dmitry Nevzhay, 2004–2014, http://www.cheburator.nevozhay.com, freely available) for each experiment 53. 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-diphenyldihydropyridinium bromide (MTT)-based assay was used to evaluate the effect of drugs on the viability of primary ALL-5 cells 62,63. Cells (10^4/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 24 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. IC_{50} values were determined by non-linear regression analysis using GraphPad Prism 6 for Windows (GraphPad Software).
correlation with experimental pIC

isotype was kept for further analysis, especially to investigate the best Vina score over all representative structures of each tubulin

Vina's scoring function. For every derivative, the pose with the ligand poses was produced for every compound/protein structure

cofactors, namely, GTP, GDP, colchicine, and the magnesium ion were removed during docking while the target was kept rigid.

the center of mass of the bound colchicine was considered. All our docking simulations, a cubic box with size 30.0 Å centered at

makes use of a force-field-based potential to score them [7]. For the anchor-and-grow algorithm to generate ligand poses and

scoring function [6]. On the other hand, DOCK6.5 is based on a combination of knowledge-based and empirical potentials as a

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chance of generating the correct ligand pose

are based on different methods for ligand placement and scoring, performed using two different docking software packages,

of docked clusters was used as a rigid target for the screening of the clusters. For each isotype, the representative structure of each

trajectories was carried out using Amber's CPPTRAJ program

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 Figure S.23).

Clusterering analysis of the last 30 ns of the generated MD trajectories was carried out using Amber’s CPPTRAJ program69 to find the representative conformations of each tubulin isotype. Clusterering 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-bromothiocolchicine derivatives was performed using two different docking software packages, namely, AutoDock Vina66 and DOCK6.570. 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 pose71. 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 [6]. 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 [7]. 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 rescored 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, 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.

Bibliography

References and notes in the text should be indicated by superscript Arabic numerals that run consecutively through the paper and appear after any punctuation. Authors should ensure that all references are cited in the text and vice versa. Authors are expected to check the original source reference for accuracy. Journal titles should be abbreviated according to American Chemical Society guidelines (The ACS Style Guide; Dodd, J. S., Ed.: American Chemical Society: Washington, DC, 1997). Inclusive pagination is strongly recommended. See examples for journal articles,1 theses,2 books,3,4 and patents,5 shown in the References section.

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Colchicine is a potent cytotoxic drug. It is a natural product isolated from Colchicum autumnale. Colchicine is a tubulin poison that causes mitotic arrest in metaphase and prevents microtubule polymerization. The drug has been used for the treatment of gout and is still being studied for its anti-cancer properties.

Several studies have explored the design and synthesis of novel colchicine derivatives to enhance their anti-cancer activity. These derivatives have been evaluated for their ability to induce cell death in cancer cell lines.

The anti-proliferative activity of colchicine derivatives was validated with molecular docking simulation studies. These studies help to understand the molecular interactions between the drug and its target, which is crucial for the development of more effective anti-cancer agents.

Supplementary Material

Highlights

- Novel triple-modified colchicine derivatives were designed and synthesized as anti-cancer agents.
- Some of the compounds were active in the nanomolar range against different cell lines.
- The antiproliferative activity results were validated with molecular docking simulation study with the tubulin protein.
- Colchicine derivatives show favorable activity comparing to their parent molecule.

Conflict of Interest.
