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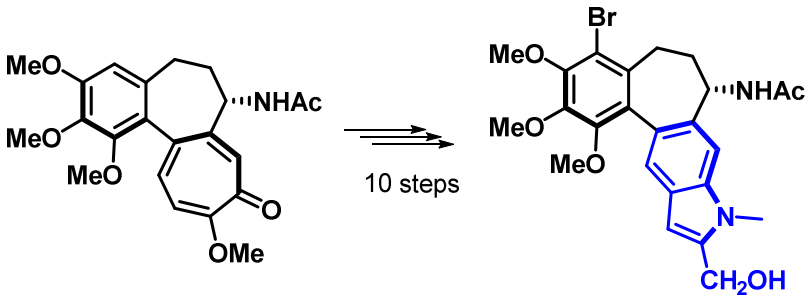
Graphical Abstract



- **IC_{50}** < 1 nM
- **G2/M** cell cycle arrest
- **25%** apoptosis induction
- lower destructive short-time effects in comparison with colchicine

Synthesis and biological evaluation of novel non-racemic indole-containing allocolchicinoids

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Abstract

Two novel indole-containing allocolchicinoids were prepared from naturally occurring colchicine exploiting the Curtius rearrangement and tandem Sonogashira coupling/Pd-catalyzed cyclization as the key transformations. Their cytotoxic properties, apoptosis-inducing activity, tubulin assembly inhibition and short-time cytotoxic effects were investigated. Compound **7** demonstrated the most pronounced anti-cancer activity: IC_{50} < 1 nM, cell cycle arrest in the G2/M phase, 25% apoptosis induction, as well as lower destructive short-time effects on HT-29 cell line in comparison with colchicine. Docking studies for prepared indole-derived allocolchicine analogs were carried out.

Key words: colchicine, heterocyclic allocolchicinoids, tubulin, apoptosis, cytotoxicity, short-time effects, antimitotics, colchicine site of tubulin.

1. Introduction

Tubulin proteins play an essential role in many cellular functions of eukaryotic cells such as cell proliferation, the development of cell shape, cell motility, and cell signaling [1]. Colchicine (**1**), isolated from *Colchicinum autumnale*, was the first tubulin-binding agent ever discovered [2]. For a long time, colchicine was considered as a perspective antitumor agent. However, its significant general toxicity even in therapeutic doses prevented its use in cancer therapy [3]. On the other hand, the tubulin-destabilizing properties of colchicine allow its use in clinical practice for the treatment of familial Mediterranean fever, Behcet's disease, as well as several types of microcrystalline arthritis [4]. Recently, colchicine was suggested as a drug for the treatment of cardiovascular diseases, such as acute pericarditis, atrial fibrillation caused by inflammation, and ischemic diseases [5]. These properties, obviously, are associated with the ability of colchicine to accumulate in the immune system cells, leading to the suppression of inflammatory reactions. Potentially, colchicine and its analogs may be useful in the treatment of autoimmune, allergic and neurodegenerative diseases, and in therapy of chronic infections [6, 7]. Therefore, the search for less toxic colchicine-binding site inhibitors [2c] with improved bioavailability and tissue distribution might lead to the elaboration of novel therapeutic agents, useful in the treatment of inflammatory, cardiovascular, autoimmune and oncological diseases.

Our group has recently synthesized a series of heterocyclic allocolchicine analogs of colchicine, containing indole (**2-4**) [8] or benzofurane (**5** and **6**) [9] fragments replacing the C-ring in the parent compound (Figure 1).

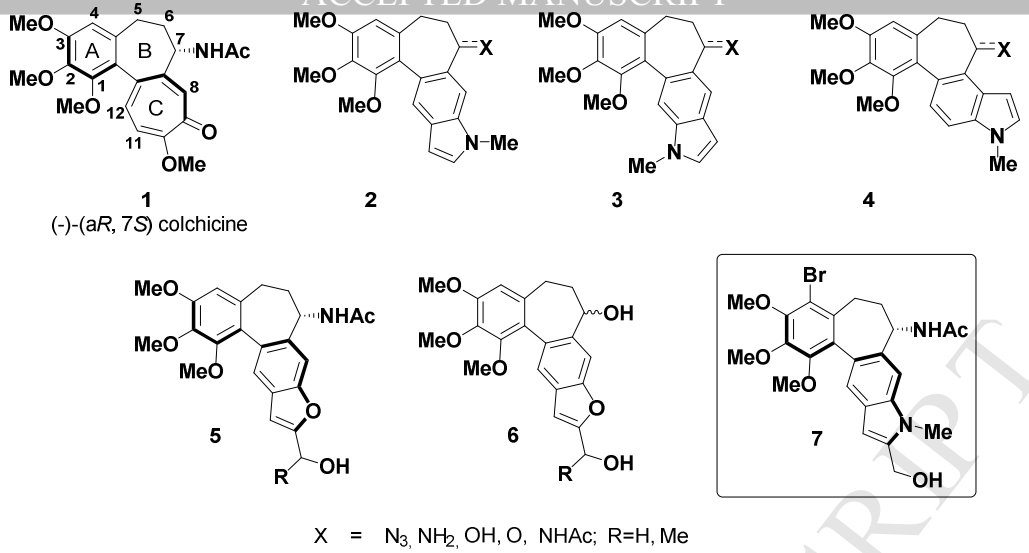


Figure 1. Colchicine and its heterocyclic analogues.

These molecules have demonstrated higher activity as tubulin polymerization and cell proliferation inhibitors compared to colchicine, while their general cytotoxicity was relatively low. Thus, furano-allocolchicinoids **5** inhibit tumor growth in mice without displaying symptoms of acute toxicity such as weight loss or neurological disorders, typical for colchicine poisoning.

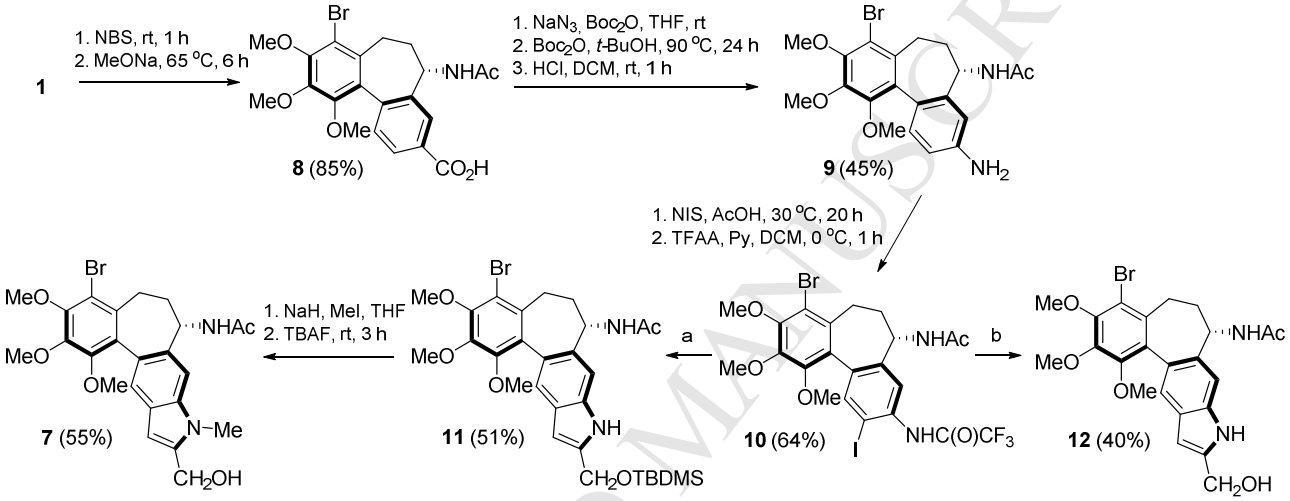
2. Results and discussion

2.1. Chemistry

Racemic pyrrolo-allocolchicinoids **2-4** were prepared previously in 11 steps starting from 3,4,5-trimethoxyphenylpropionic acid [8]. Herein, we report a conceptionally different ten-steps semi-synthetic approach (Scheme 1) towards non-racemic pyrrolo-allocolchicine **7** (Figure 1) starting from naturally occurring (-)-(a*R*, 7*S*)-colchicine **1**. Previously, we observed that the presence of a hydroxymethyl substituent either at C-7 of ring B (compounds **2-4**) [8a] or on the heterocyclic fragment (compounds **5** and **6**) [9] significantly increases the anti-proliferative activity. Thus, a similar 2''-hydroxymethyl substitution pattern in the pyrrole fragment was chosen for these new synthetic targets.

The bromination of colchicine (**1**) using NBS in TFA/AcOH mixture afforded 4-bromocolchicine in almost quantitative yield. It was transformed into 4-bromoallocolchicinic acid **8** by applying the previously described base-induced ring contraction [10] (85% yield over two

stages). Allocolchicinic acid **8** was subjected to the Curtius degradation yielding the corresponding aniline **9** in 45% yield. The followed α-iodination reaction (NIS/AcOH) and subsequent acylation gave allocolchicinoid **10** in 64% yield. Finally, the desired *N*-methylated pyrrolo-allocolchine **7** was prepared in 3 steps from the amide **10**. The TBDMS-protected indolyl-2-methanol **11** was obtained through tandem Sonogashira reaction/cyclization sequence in 51% yield [11], using *O*-protected propargyl alcohol. After the *N*-methylation of the indole nitrogen (NaH/MeI) and selective cleavage of the silyl group, compound **7** was isolated in 55% yield over two steps.



Scheme 1. The synthetic route to pyrrolo-allocolchicinoids **7** and **12**. *Reagents and conditions:* (a) HC≡C-CH_2-OTBDMS (1.2 equiv.), Pd(dppf)Cl_2 (0.05 equiv.), CuI (0.1 equiv.), DIPEA (3.0 equiv.), MeCN, 80 °C, 8 h; (b) HC≡C-CH_2OH (1.2 equiv.), Pd(OAc)$_2$ (0.05 equiv.), CuI (0.1 equiv.), PPh_3 (0.15 equiv.), AcOK (3.0 equiv.), MeCN, 80 °C, 8 h. See Experimental Section.

In order to determine the influence of substituents at the nitrogen atom on the biological activity, *N*-H pyrrolo-allocolchine **12** was also prepared, starting from amide **10** via two-step one-pot Sonogashira/Larock-type cyclization [11], using unprotected propargyl alcohol for the coupling stage. It has been demonstrated, that the presence of a halogen atom in the A-ring of colchicinoids slightly enhances their cell inhibitory activity [12], thus the presence of bromine in the final products was considered as positive.

2.2. Biology

2.2.1. Cytotoxicity

The cytotoxicity of the synthesized compounds towards human pancreatic adenocarcinomas COLO-357 and MiaPaCa-2, and human embryonic kidney cell line HEK293 was investigated *in vitro*. A tetrazolium-based assay was used to determine the drug concentration required to inhibit cell growth by 50% (IC_{50}) after incubation in the culture medium for 72 h. The calculated IC_{50} values are summarized in Table 1. Compound **12** was less active than colchicine. In contrast, *N*-methylated analog **7** inhibited cell proliferation already at subnanomolar concentrations, lower than of colchicine. We presume that the presence of alkyl on the *N*-1 of the pyrrolo-allocolchicine skeleton is essential for the antiproliferative activity of the prepared compounds.

Table 1. Cytotoxic properties of synthesized compounds, (IC_{50}, nM)[a].

Compound	**7**	**12**	Colchicine **1**
HEK293	6.4	160	32
COLO-357	0.3	3.5	1.3
MiaPaCa-2	<0.1	10	1.3

[a] Each drug concentration was tested in triplicate, and the SE of each point is <10%.

2.2.2. Cell cycle analysis

The main mechanism of the colchicine action is the inhibition of tubulin assembly, which leads to the block of cell proliferative cycle and subsequent apoptosis. We found that both new compounds demonstrate colchicine-specific tubulin interaction (Figures 2 and 3). When compared in the equal concentrations, the only difference found was the higher activity of compound **7** in cell cycle arrest (Figure 2, Table 2) expressed by the increased number of cells in G2/M phase, and higher percentage of cells in the apoptotic fraction.

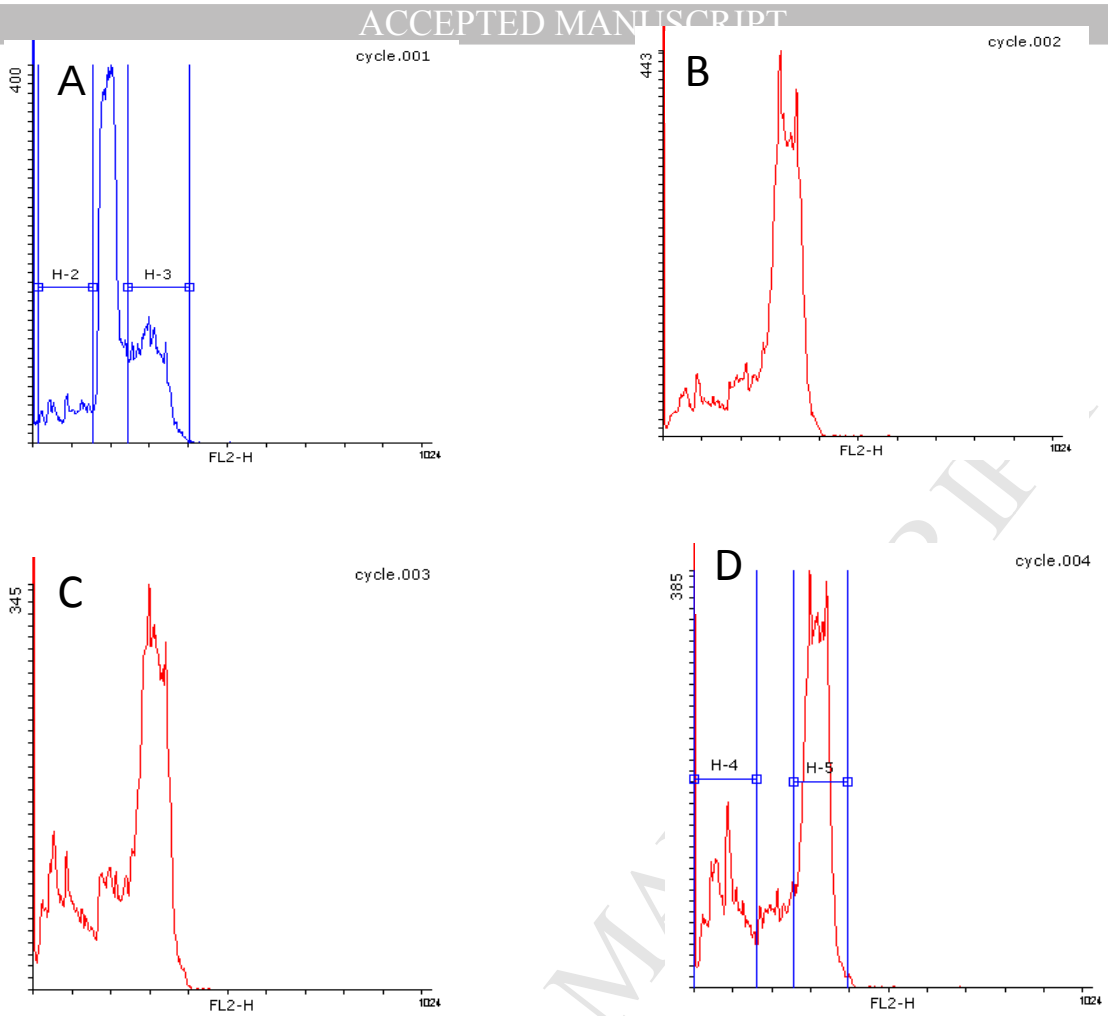


Figure 2. Cell cycle analysis of colchicine analogues. COLO-357 were incubated without (A) or with 5 μM colchicine **1** (B), **12** (C), or **7** (D) compounds for 72 h, permeabilized, stained with propidium iodide and analyzed by flow cytometry. Regions H2 and H4 correspond to apoptotic fraction, H3 and H5 – to G2/M cells. Quantitative results are shown in Table 2.

Table 2. Cell cycle inhibition induced by colchicine analogues in COLO-357 cells.

	Apoptosis	G	G2/M	G1/G2
Control	12.4	56.6	31	1.8
Colchicine	12.4	12.6	75	0.2
12	23	16	61	0.3
7	25	12	63	0.2

Similarly, a higher activity in tubulin assembly inhibition was found in cultures stained with anti-β-tubulin antibodies (Figure 3a-d).

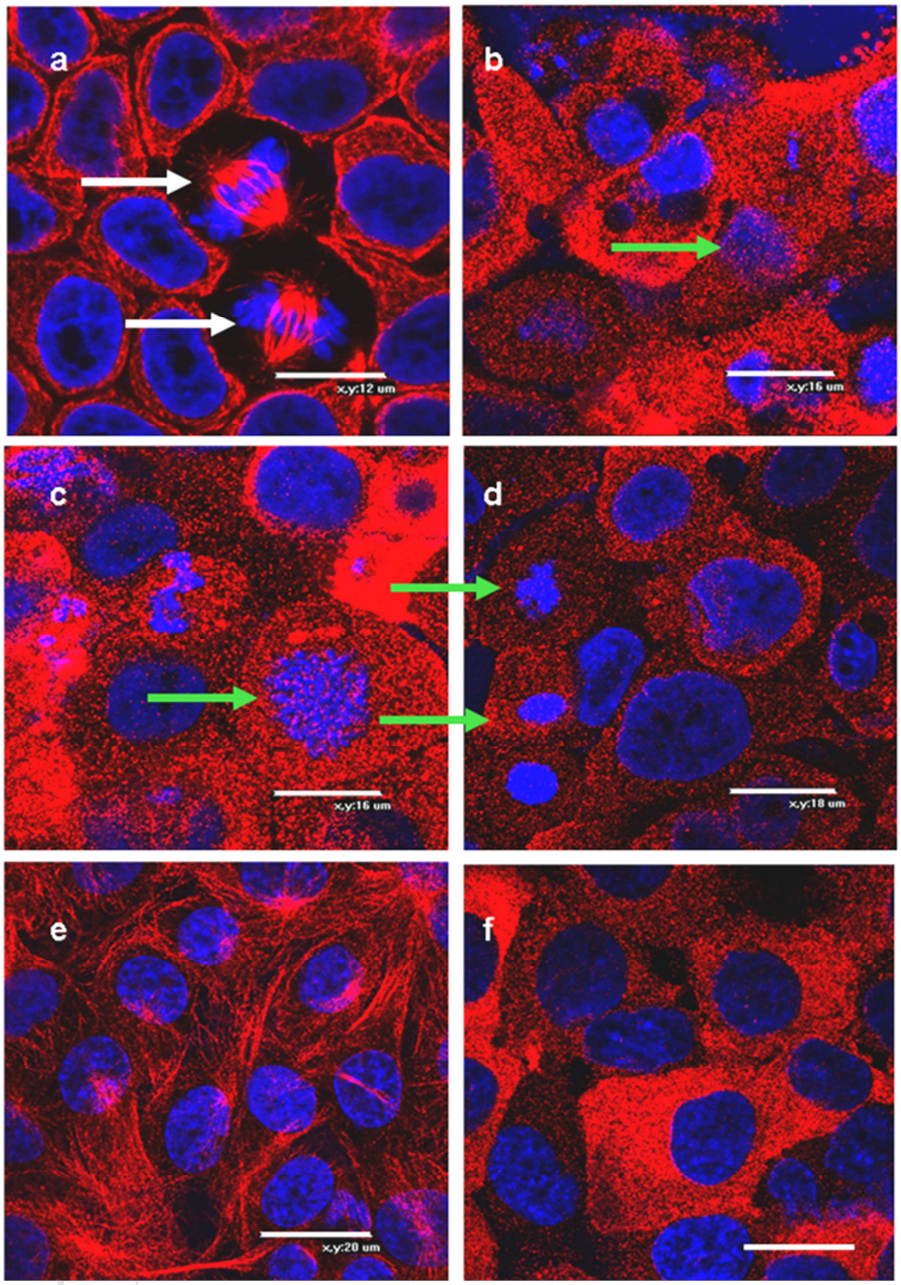


Figure 3. Tubulin organization in COLO-357 cells. COLO-357 were incubated without (a, e) or with 5 μM colchicine **1** (b), **12** (c), or **7** (d, f) compounds for 24 h (a-d) or 3 h (e-f), permeabilized, stained with anti-β-tubulin antibody (red), and analyzed by confocal microscopy. Mitotic spindles are shown with white arrows; rearranged chromosomes or apoptotic bodies - with green arrows. Nuclei are stained with Hoechst 33342 (blue). Scale bar 12-20 μm.

Tubulin assembly inhibition, resulting in the mitotic spindle block and G2/M cell cycle arrest, requires at least 20-24 h to proceed completely (see Figure 2A-D). However, colchicine and its analogs bind to tubulin much faster. Incubation of cells with compound **7** for 3 h already re-localized β-tubulin (Figure 3e-f). The cytotoxic effects of colchicine and its analogues *in vitro* are rather mild, and require 72 h to reach maximum 60-80 % inhibition. Short-time cell incubation with colchicine derivatives demonstrated that cytotoxic effect depended both on time and the cell type (Figure 4).

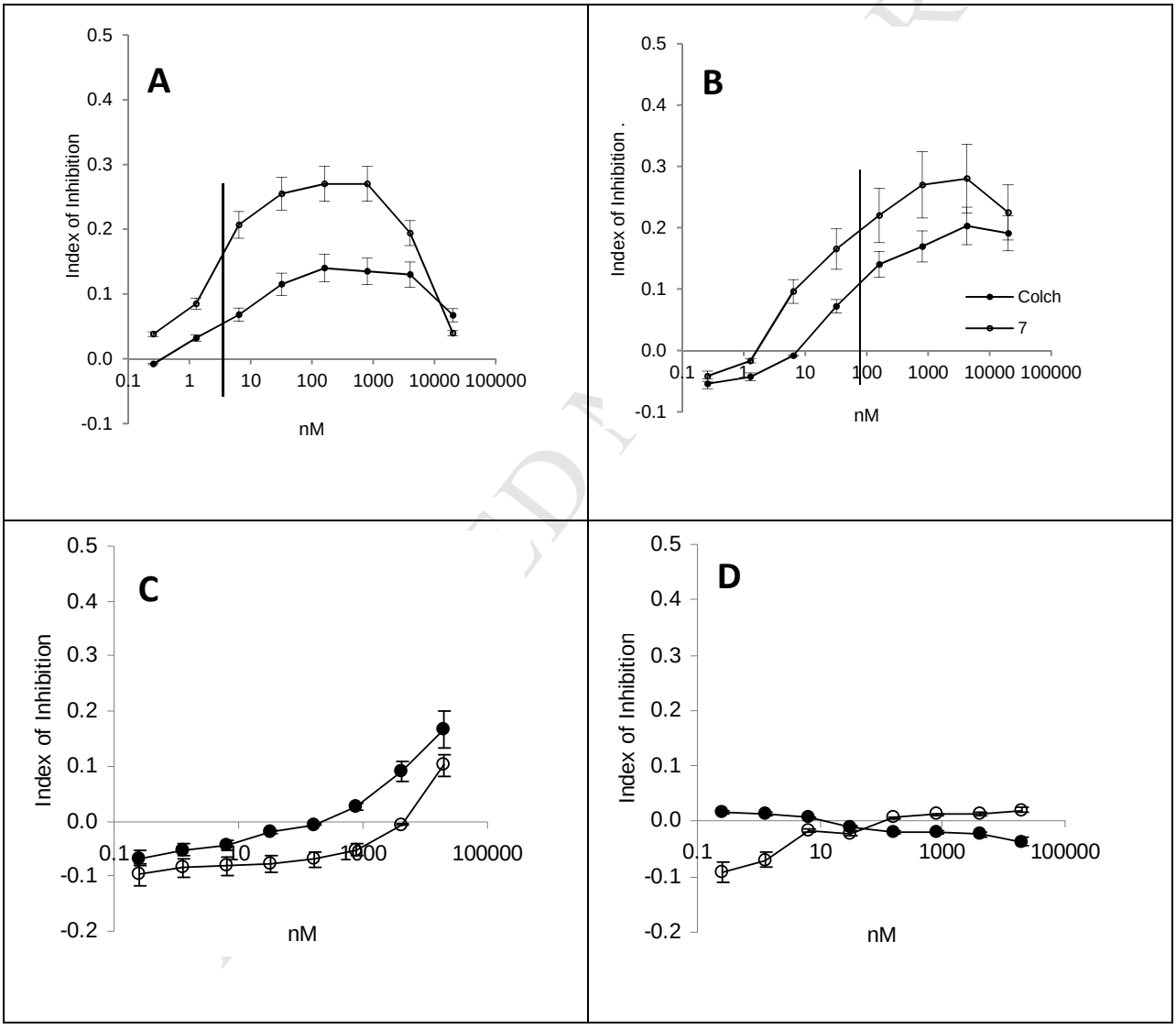


Figure 4. Effect of colchicine (**Colch**) and compound **7** short time incubation on cell proliferation. Human colon HT-29 (A), pancreatic COLO-357 (B), PANC-1 (C) carcinoma cells, and embryonic kidney HEK293 (D) cells were incubated with colchicine **1** or colchicinoid **7** for 24 h and their

proliferation was analyzed by MTT assay. Vertical lines in a and b show differences in the sensitivity of cells to the preparations.

Among all, colon cell line HT-29 was the most sensitive to colchicinoid **7**, followed by COLO-357 cells. At the same time, PANC-1 and HEK293 cells were not affected at that time (Figure 4). The sensitivity of cell lines to colchicinoids at early time points of incubation correlates with the cells division rates. These early effects may translate into systemic toxicity *in vivo*. Multiple studies demonstrated that colchicine and its analogues affect lysosomal/endosomal/autophagial pathways, induce endoplasmic reticulum (EPR) and mitochondrial stress, disrupt Golgi apparatus, and stimulate heart beating [13]. Other multiple effects on cell signaling have also been demonstrated [4e]. So far, it is not quite clear which pathway of colchicine activity is the most important for the systemic effects. Clinical studies have demonstrated that the most often adverse effects in humans are diarrhea and vomiting, causing loss of liquid [14].

Earlier, we have shown a hyperpolarization of mitochondrial and lysosomal membranes induced by allocolchicinoids in 3D cultures of pancreatic cancer cells [9b]. To understand the role of different cell compartment disturbances induced by colchicinoids, we compared short time effect on several organelles in colon cells HT-29. Minor differences between control and cultures treated with colchicine and compound **7** was found in Golgi apparatus, EPR, endosomes, membrane integrity (data not shown). The cell mitochondria responded to colchicine by significant increase in the membrane potential, their number, and relocation from perinuclear position to cytoplasm (Figure 5). The compound **7** also stimulated mitochondria movement, although the effect was less significant ($p<0.05$). Possibly, a higher specific activity of the compound **7** is responsible for this difference. Analysis of the lysosomal membrane potential demonstrated that the colon cells responded to colchicine decreasing it, while the compound **7** did not affect lysosomes at all (Figure 5g). A stronger effect of **7** was found against pancreatic tumor cells COLO-357 mitochondria (Figure 5h and 5i).

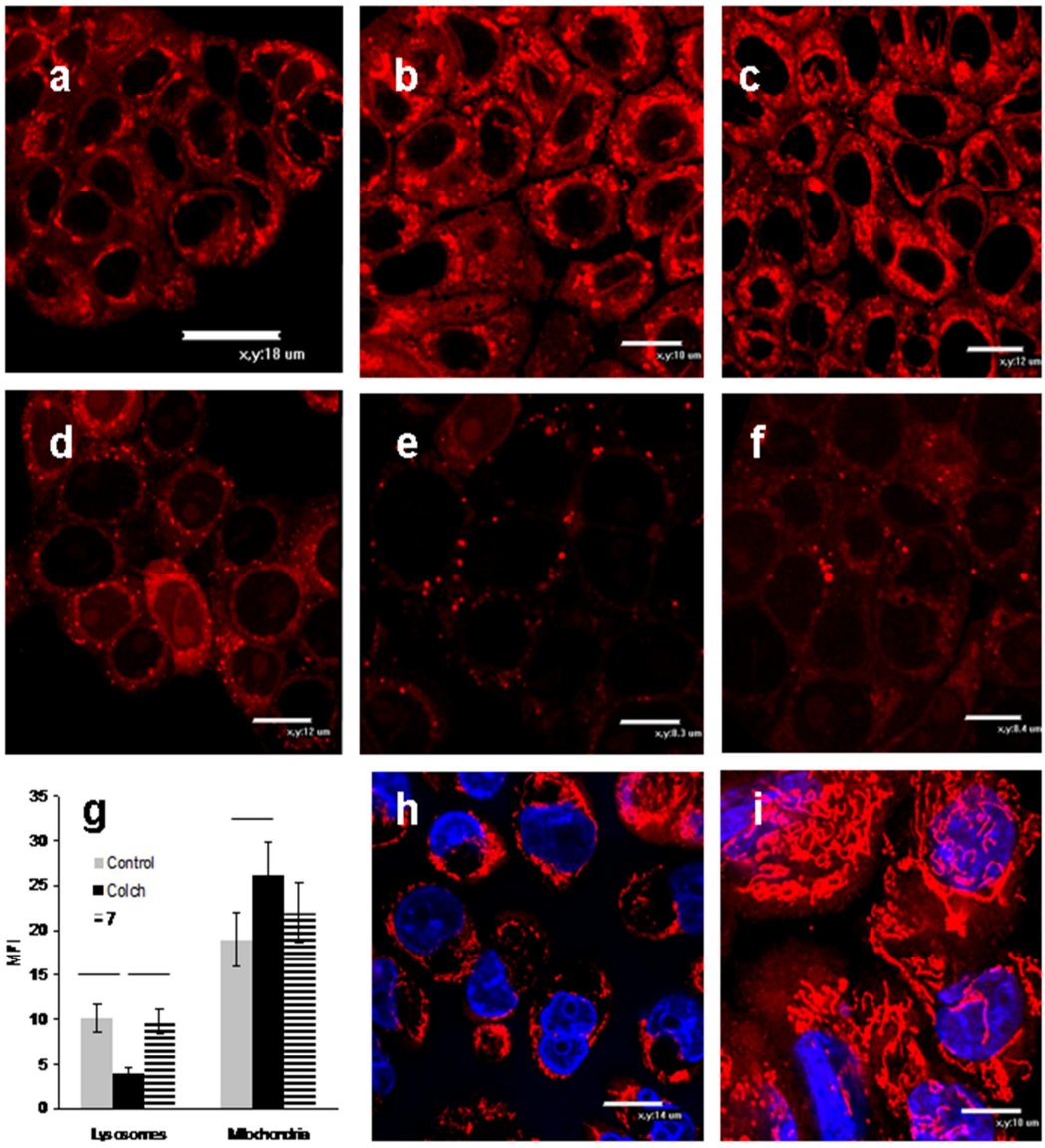


Figure 5. Short-time effect of colchicinoids on mitochondria and lysosomes. HT-29 (a-g) or COLO-357 (h, i) were incubated for 3 h without (a, d, h), with 5 µM colchicine (**1**) (b, e) or compound **7** (c, f, i), and stained with red mitochondrial (a-c, h, i) or lysosomal tracker (d-f). Nuclei in (h, i) are stained with Hoechst 33342 (blue). Scale bar 8-15 µm. g. Flow cytometry analysis of lysosomal and mitochondrial tracker staining in HT-29 cells.

Generally, it might be assumed that a lesser effect against colon cells of the new compound **7** will result in a lesser toxicity to gastrointestinal tract while preserving its antitumor potential.

2.3. Docking studies

In order to assess the ability of newly synthesized compounds for inhibition of intracellular tubulin functions, we carried out the docking study of complexes between compounds **7** and **12** and

tubulin dimer and estimated their binding energy in comparison with the colchicine-tubulin complex, studied earlier [15]. The calculated binding energy (ΔG_{bind}) of the most favorable complexes of **7** and **12** with tubulin are close to the values of the colchicine-tubulin complex (Table 3).

Table 3. Binding energies of the most favorable conformations of complexes between tubulin dimer and colchicinoids **1**, **7,** and **12**.

Compound	Binding energy, kcal/mol
Colchicine (**1**)	–8.66
7	–7.96 (–7.82[a])
12	–8.26 (–7.47[b])

[a] – binding energy of **12** in the conformation similar to the most favorable structure of **7**-tubuline complex;

[b] – binding energy of **7** in the conformation similar to the most favorable structure of **12**-tubuline complex.

The obtained geometry of colchicine-tubulin complex with the colchicine site of tubulin coincides with the one previously reported [15]. The geometries of most favorable conformations for compounds **7**, **12** in the colchicine site of tubulin are remarkably different from the colchicine structure (Figure 6A). On Figure 6B, a superposition of the most energetically favored conformation of **7** (ΔG = -7.96 kcal/mol), and the conformation of **12**, corresponding to a local minimum (ΔG = -7.82 kcal/mol) is presented.

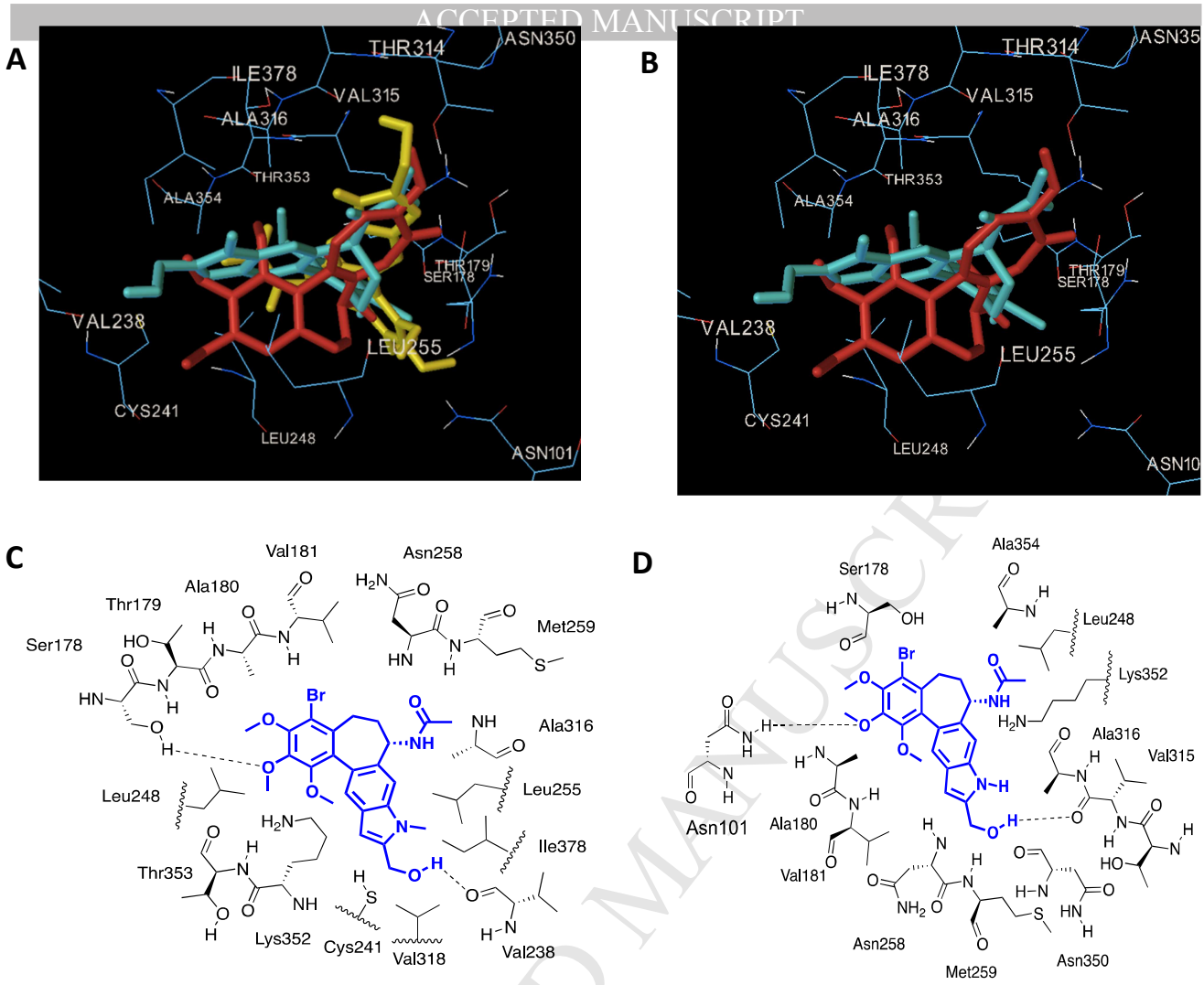


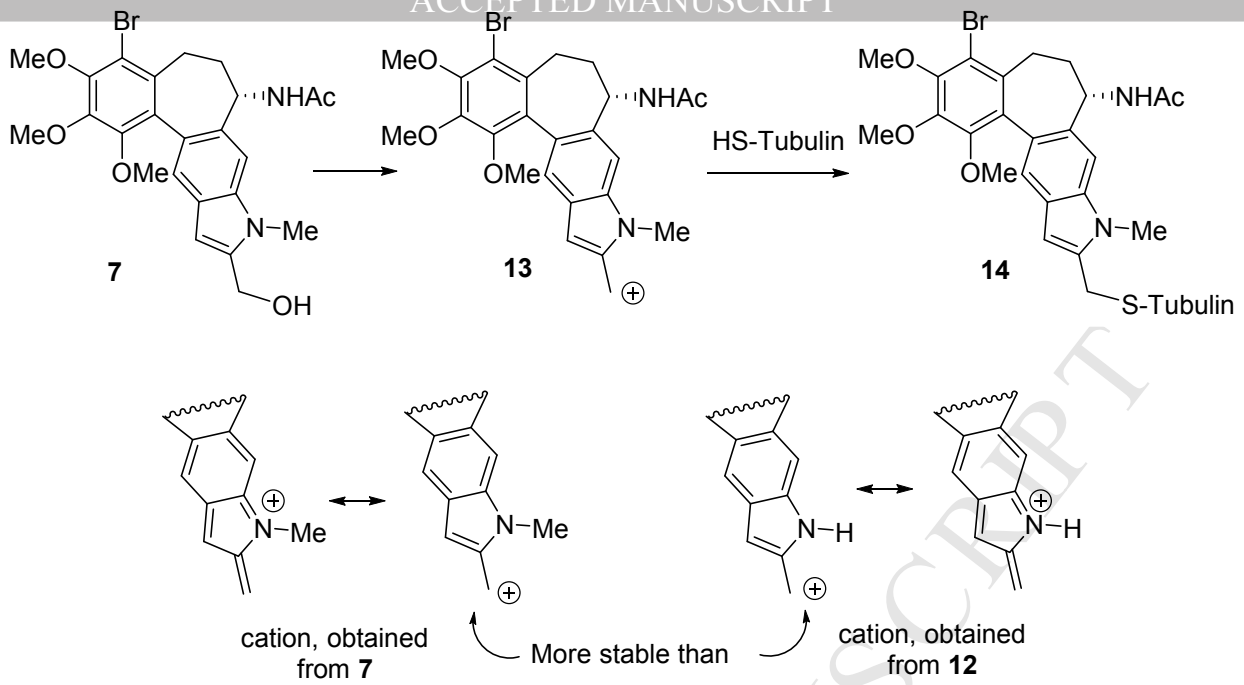
Figure 6. Accommodation of colchicinoids **1**, **7** and **12** in the colchicine site of tubulin. (A) *red* - colchicine **1**, *blue* - **7**, *yellow* - **12**. (B) colchicine **1** (*red*) and completely superposed pyrrolo-allocolchicinoids **7** and **12** (*blue*) in colchicine site of tubulin. Amino acids environment of **7** (C) and **12** (D).

In the case of the most favorable complex of pyrrolo-allocolchicinoid **12** with tubulin, the 2-methoxy (colchicine numeration) group in **A** ring contacts with *N*-H peptide group of Asn101α residue (hydrogen bond length is 1.95 Å) (Figure 6A and 6C). The acetamide group of aliphatic **B** ring is located in the region of the week polar contacts with Alaβ354 and Leuβ248 residues. The **C** ring (indole moiety) is placed in zone of contacts with Lysβ352, Asnβ258, Asnβ350, Alaβ316, Valβ315, Thrβ314, Metβ259 and Valα181, wherein hydroxymethyl fragment forms the hydrogen bonds with peptide C=O group of Valβ315 (1.90Å).

In the case of most favorable complex of colchicinoid **7** with tubulin (Figure 6B and 6D), 2-methoxy group in **A** ring contacts with OH-group of Ser-α178 residue (the hydrogen bond length is 1.79 Å). The acetamide fragment of aliphatic **B** ring, like in the case of compound **12**, is located in the field of week polar interactions (Lysβ352, Valα181, Asnβ258 and Metβ259 residues). The hydroxymethyl moiety of indole side chain forms H-bond with the peptide C=O group of Valβ238 (1.81 Å), while the *N*-Me substituent is arranged in the hydrophobic pocket constituted by residues of Leu255, Ile378 and Val318.

Although colchicinoid **7** is by two orders of magnitude more active than compound **12**, the difference of the calculated binding energies for these two molecules are close. To explain the significant difference in cytotoxicity of two compounds, we can speculate that allocolchicinoid **12**, in contrast to its analog **7**, is able to shape the intramolecular H-bond between *N*-H in pyrrole core and oxygen in hydroxymethyl group of the side chain. This intramolecular hydrogen bonding can be competitive with the "target" **12**/tubulin interactions, leading to serious decrease in activity. At the same time, due to the intermolecular hydrogen bonds, the molecules of **12** can form relatively stable dimeric associates having significantly lower membranotropicity in comparison with monomeric units of homolog **7**.

To explain very potent antimitotic activity of colchicinoids **2**-**7** (Figure 1, Scheme 1), containing hydroxyl groups in benzylic or pseudobenzylic positions, we hypothesized that these compounds are even under physiologic conditions capable to form a pseudobenzylic cation like **13** that immediately attacks the free cysteine fragments of tubulin, forming a covalent bond (Scheme 2).



Scheme 2. An illustration of hypothesis of possible covalent bounding of colchicinoid **7** to tubulin.

This idea is indirectly confirmed by the ability of furano-allocolchicinoids **5** to react readily with various thiols, leading to the corresponding sulfides in quantitative yields [16]. Our docking studies revealed the proximal location of the hydroxymethyl moiety of colchicinoid **7** to the Cys241 of tubulin. The *N*-methylindole fragment of **7** should form a more stable cation than the *N*-unsubstituted indole (compound **12**, Scheme 2). This might explain the differences in their biological activity. Experimental work directed at the proof of this concept is currently in progress.

3. Conclusions

Starting from naturally occurring colchicine, highly cytotoxic pyrrolo-allocolchicinoids **7** and **12** were synthesized in ten and eight steps consequently as non-racemic compounds. Allocolchicinoid **7** exhibits cytotoxic properties at sub-nanomolar concentration, and efficiently arrests the cell cycle in G2/M phase, thus increasing the number of cells undergoing apoptosis in comparison with colchicine. Short-time incubation of cells with **7** demonstrated that the cytotoxic effect strongly depends both on time and the cell type. It can be assumed that a lesser effect of **7** against some types of cells in comparison with colchicine (for example, against colon cells) could possibly result in a lesser toxicity to gastrointestinal tract while preserving its antitumor potential.

4. Experimental

4.1. Materials. ^{1}H NMR and ^{13}C NMR spectra were recorded on *Agilent DDR2 400* spectrometer at 25 °C. Chemical shifts (δ) are reported in ppm for the solution of compound in DMSO-d_6 with internal reference TMS and *J* values in Hertz. EI mass spectra (70 eV) were obtained on a *DSQ II mass-spectrometer (Thermo Electron Corporation)* with quadrupole mass-analyzer. *Optical rotation* $[\alpha]_D$ was measured on *JASCO P-2000* polarimeter at 19 °C and λ=589 nm (cuvette length: 1.0 dm, volume: 1.0 mL). Concentration is given in g/100 mL. Separation by column chromatography was performed using *Merck Kieselgel 60* (70 – 230 mesh). All reactions were performed with commercially available reagents («Aldrich», «Alfa Aesar», «Acros», «Serva»). Phenylacetylene was distilled prior to use under reduced pressure (b.p. 75 °C/80 mm Hg). Solvents were purified according to standard procedures. The petroleum ether refers to the fraction with distillation range 40 – 70 °C.

4.2. Synthesis of pyrrolo-allocolchicinoids

4.2.1. Synthesis of 1′-bromo-2′, 3′, 4′-trimethoxybenzo[5′, 6′:4, 5]1H-(aR, 1S)1-acetamido-6,7-dihydro-cyclohepta[3, 4-f]1H-2-hydroxymethylindole ***(12)***

Into a Schlenk flask with a stirring bar, were subsequently added compound **10** (60.0 mg, 0.090 mmol), palladium acetate (1.1 mg, 0.004 mmol), copper (I) iodide (1.7 mg, 0,009 mmol), triphenylphosphine (3.5 mg, 0.013 mmol) and potassium acetate (26.5 mg, 0.270 mmol). The flask was filled with argon. Acetonitrile (3.0 mL) was added under inert atmosphere. Then, propargylic alcohol (6.2 mg, 0.110 mmol) was added, the temperature was raised to 80 °C, and the mixture stirred for 8 h. The volatiles were removed under reduced pressure, and the residue was purified by column chromatography (petroleum ether – ethyl acetate – ethanol 15:1:1). The product was isolated as white solid, 40% yield; mp 144-145 °C. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 11.27 (s, 1H, NH-indole), 8.53 (d, *J* = 8.4 Hz, 1H, N<u>H</u>Ac), 7.47 (s, 1H, C5”-H), 7.33 (s, 1H, C4”-H), 6.47 (s, 1H, C3”-H), 5.18 (d, *J* = 12.8 Hz, 2H, <u>CH$_2$</u>OH), 4.52 – 4.47 (m, 1H, C1-H), 3.90 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.04 – 3.00 (m, 1H, C6-H), 2.06 – 1.93 (m, 2H, C7-H), 1.90 (s, 3H, C(O)<u>CH$_3$</u>), 1.86 – 1.79 (m, 1H, C6-H). ^{13}C NMR: (101 MHz, DMSO-d_6) δ 168.4, 150.1, 149.2, 145.9, 136.3, 134.6,

133.7, 133.6, 130.8, 125.9, 124.5, 121.3, 112.5, 105.7, 102.2, 61.2, 60.7, 60.3, 59.0, 48.4, 31.3, 22.7, 20.7. MS: m/z (%) = 412.94 (100%), 410.90 (57%), 472.90 (74%), 470.94 (53%), 473.94 (61%), 471.91 (73%), 428.90 (45%), 430.96 (34%), 488.02 (27%), 488.96 (10%), 490.81 (7%). Anal. Calcd for $C_{23}H_{25}BrN_2O_5$: C, 56.45; H, 5.15. Found: C, 56.39; H, 5.19. $[\alpha]^{19}_D$ = -28.2 (*c* = 0.59, Chloroform).

4.2.2 Synthesis of 1′-bromo-2′, 3′, 4′-trimethoxybenzo[5′, 6′:4, 5]1H-(aR, 1S)1-acetamido-6, 7-dihydrocyclohepta[3, 4-I]1H-2-tert-butyldimethylsilyloxymethylindole ***(11)***

Into a Schlenk flask with a stirring bar were placed compound **10** (80.0 mg, 0.120 mmol), palladium(diphenylphosphinoferrocene) dichloride (4.4 mg, 0.006 mmol), and copper (I) iodide (2.3 mg, 0.012 mmol). The flask was filled with argon. Acetonitrile (3.0 mL) was added under inert atmosphere. *N*,*N*-diisopropylethylamine (0.095 mL, 0.366 mmol) and *tert*-butyldimethyl(2-propynyloxy)silane (0.030 mL, 0.146 mmol) were added to the reaction mixture, the temperature was raised to 90 °C and kept for 8 h. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (petroleum ether-ethyl acetate-ethanol 5:1:1). The product was obtained as pale-beige oil in 51% yield. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 11.06 (s, 1H, NH-indole), 8.55 (d, *J* = 8.5 Hz, 1H, NHAc), 7.42 (s, 1H, C5”-H), 7.31 (s, 1H, C4”-H), 6.31 (s, 1H, C3”-H), 4.78 (s, 2H, **C**H_2OTBDMS), 4.48 – 4.41 (m, 1H, C1-H), 3.89 (s, 3H, OMe), 3.84 (s, 3H, OMe), 3.36 (s, 3H, OMe), 2.07 – 1.96 (m, 4H, C6-H, C7-H), 1.90 (s, 3H, C(O)CH_3), 0.89 (s, 9H, Si-Bu^t), 0.09 (s, 6H, SiMe_2). ^{13}C NMR: (101 MHz, DMSO-d_6) δ 168.7, 150.3, 149.2, 146.0, 139.0, 136.3, 133.9, 133.9, 131.1, 126.2, 124.3, 121.1, 112.6, 105.7, 99.5, 61.3, 60.9, 60.5, 58.8, 48.6, 36.8, 29.9, 26.0, 22.8, 18.2, -5.0. Anal. Calcd for $C_{29}H_{39}BrN_2O_5Si$: C, 57.70; H, 6.51. Found: C, 58.00; H, 6.55.

4.2.3 Synthesis of 1′-bromo-2′, 3′, 4′-trimethoxybenzo[5′, 6′:4, 5]1H-(aR, 1S)1-acetamido-6, 7-dihydro-cyclohepta[3, 4-f]1H-1-methyl-2-hydroxymethylindole ***(7)***

Pyrrolo-allocolchicinoid **11** (48.0 mg, 0.079 mmol) was treated with sodium hydride (60% in mineral oil, 0.190 mmol, 7.6 mg) and methyl iodide (17.7 mg, 0.125 mmol) in THF at 0 °C. The

temperature was raised to 65 °C. The mixture was stirred for 6 h, then the solvent was removed and the residue was filtered through a short pad of silica. The crude product was dissolved in 4.0 mL of THF, 0.045 mL of 1*N* TBAF in THF was added, and the mixture was stirred for 3 h. The volatiles were removed, and the product was isolated using column chromatography (petroleum ether – ethyl acetate – ethanol (15:1:1)). The product **7** was obtained as white solid in 55% yield (for 2 steps), mp 175-177 °C. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 8.51 (d, *J* = 8.3 Hz, 1H, NHAc), 7.46 (s, 1H, C5"-H), 7.37 (s, 1H, C4"-H), 6.38 (s, 1H, C3"-H), 5.22 (t, J = 5.0 Hz, 1H, OH), 4.64 (d, *J* = 5.1 Hz, 2H, CH_2OH), 4.60 – 4.56 (m, 1H, C1-H), 3.91 (s, 3H, OMe), 3.86 (s, 3H, OMe), 3.77 (s, 3H, NMe), 3.34 (s, 3H, OMe), 3.04 – 2.99 (m, 1H, C6-H), 2.10 – 2.05 (m, 1H, C6-H), 2.02 – 1.97 (m, 1H, C7-H), 1.93 (s, 3H, C(O)CH_3), 1.84 – 1.79 (m, 1H, C7-H). ^{13}C NMR: (101 MHz, DMSO-d_6) δ 168.5, 150.1, 149.1, 145.8, 140.7, 137.2, 133.8, 133.7, 130.2, 125.2, 124.3, 121.2, 112.5, 103.6, 100.1, 61.2, 60.7, 60.2, 55.5, 48.3, 37.0, 29.8, 29.7, 22.80. MS: m/z (%) = 502.08 (100%), 504.09 (91%), 443.09 (79%), 445.08 (78%), 431.10 (50%), 429.08 (39%) 414.06 (61%), 416.08 (60%), 401.07 (29%), 399.09 (28%). Anal. Calcd for $C_{24}H_{27}BrN_2O_5$: C, 57.26; H, 5.41. Found: C, 57.33; H, 5.44. $[\alpha]^{19}_{D}$ = -37.6 (*c* = 0.59, Chloroform).

4.3. Cell cultures. Pancreatic human cell lines COLO-357, MiaPaCa-2, PANC-1; nontransformed human embryonic kidney cell line HEK293, human colon cell line HT-29 were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), pen-strep-glut (all from PanEco, Moscow, Russian Federation). Adherent cells were detached using 0.05% trypsin-EDTA (PanEco, Moscow), counted and sub-cultured. Twenty-four hours before the assays, cells were seeded in the appropriate plates (96- or 24-well plates), adjusted to 3×10^5 cells/mL, and incubated overnight to achieve standardized growth conditions.

4.4. MTT-assay. Cytotoxic effect of colchicine and the compounds **7** and **12** was estimated by a standard 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) test as described earlier [9]. All the compounds were dissolved in dimethyl sulfoxide to 20 mM concentration and stored at -20 ^{o}C until the assay. Different dilutions of the new compounds from

20 μM to 0.1 nM were prepared separately and transferred in 100 μL to the plates with the cells. Non-treated cells served as controls. Plates were incubated for 72 h. For the last 6 h, 5 mg/mL of MTT was added in the amount of 10 μL to each well. After the incubation, culture medium was removed and 100 μL dimethyl sulfoxide was added to each well. Plates were incubated in a shaker for 15 min to dissolve the formed formazan product. Optical density was read on spectrophotometer Titertek (UK) at 540 nm. Results were analyzed by Excel package (Microsoft). Cytotoxic concentration giving 50% of the maximal toxic effect (IC_{50}) was calculated from the titration curves. The inhibition of proliferation (inhibition index, II) was calculated as [1 – ($OD_{experiment}$ / $OD_{control}$)], where OD was MTT optical density.

4.5. Confocal analysis. For confocal analysis, cells were grown overnight on sterile cover slips immersed into 6-well plates (Costar) in 200 μL of complete culture medium. Colchicine or new compounds **12** and **7** were dissolved in 4 mL of complete medium and added to the wells. After the incubation, cells were stained with different trackers. Wheat germ agglutinin (WGA)-AlexaFluor555, LysoTrackerRed, MitoTrackerRed, and Transferrin-AlexaFluot555 (all from Molecular Probes, Invitrogen, USA) were used to stain membranes and Golgi apparatus, lysosomes, mitochondria and endosomes accordingly. After the incubation with the trackers, cells were fixed with 1% paraformaldehyde, washed, and treated with Mowiol 4.88 medium (Calbiochem, Germany). Tubulin was identified by anti-tubulin antibody (SantaCruz, USA) followed by anti-mouse IgG-AlexaFluor555 (Molecular Probes, Invitrogen, USA) in cells permeabilized by 0.1% Triton X100 in PBS. Hoechst 33342 (Sigma) was used to visualize nuclei. Slides were analyzed using Eclipse TE2000 confocal microscope (Nikon, Japan).

4.6. Flow cytometry. Cell cycle was analyzed using PI-stained DNA. Cells from colchicinoid-treated cultures were collected at indicated time, trypsinized, washed in ice-cold PBS, fixed by the addition of 70% ethanol and left for 2 h at -20 ^{o}C. Thereafter, the cells were washed twice in PBS, stained with 50 μg/mL of propidium iodide (Sigma Chemical Co) in PBS, treated with 10 μg/mL of RNAse and analyzed by flow cytometry using FACScan device (BD, USA). Total 2000 events were

collected. The results were analyzed using Flowing 2.5.1 software (Finland). To analyze lysosomal and mitochondrial membrane potentials, cells were incubated with colchicinoids for 3 h, stained with the trackers for the last 30 min, detached by trypsin-EDTA and analyzed by flow cytometry. Total 20000 events were collected.

4.7. Docking study. Docking calculations were carried out using the AutoDock 4.2 program [17]. The default settings were used with the MM potential of MMFF94 type. The necessary hydrogen atoms, Kollman united atom charges, and solvation parameters were added using the AutoDockTools [17]. Grid maps of 30×30×30 $Å^3$ with 3Å mesh size were generated with AutoGrid [17]. Lamarckian genetic algorithm combined with the Solis-Wets [18] local optimization algorithm were used for the structure optimization. Each docking experiment was resultant from 200 that were to come to an end after a maximum of 2500000 energy evaluations. The geometry of the ligands (colchicine **1**, and of compounds **7** and **12)** was obtained on the basis of full geometry optimization at the DFT level (B3LYP/6-31G(d,p)) using the Gaussian 03 program [19].

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

The following is the supplementary data related to this article:

General information, syntheses and characterization of compounds, ^{1}H and ^{13}C NMR spectra, biological assays, docking study.

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Highlights:

- Two pyrrole-based allocolchicinoids were prepared from commercial colchicine
- Compounds exhibit excellent cytotoxicity towards epithelial cell lines
- **7** and **12** induced cell accumulation in the G2/M phase in 2D cultures
- **7** has lower destructive short-time effects on HT-29 cell line in comparison with colchicine