RSC Medicinal Chemistry

ROYAL SOCIETY OF CHEMISTRY

RESEARCH ARTICLE

View Article Online
View Journal

Cite this: DOI: 10.1039/d0md00060d

Allocolchicinoids bearing a Michael acceptor fragment for possible irreversible binding of tubulin†

Ekaterina S. Sazanova,[a] Iuliia A. Gracheva,[a] Diane Allegro,[b] Pascale Barbier,[b] Sébastien Combes,[cd] Elena V. Svirshchevskaya[e] and Alexey Yu Fedorov *[a]

Received 22nd February 2020,
Accepted 10th April 2020

DOI: 10.1039/d0md00060d

rsc.li/medchem

We describe an attempt to apply the concept of covalent binding towards the highly active allocolchicinoids selected on the basis of SAR analysis of previously synthesized molecules. To achieve the irreversible binding of the agent to the cysteine residues of the colchicine site of tubulin protein, we synthesized a number of new allocolchicinoids bearing the acceptor moiety. Some of the new derivatives possess cytotoxic activity against COLO-357, BxPC-3, HaCaT, and HEK293 cell lines in a low nanomolar range of concentrations. A substoichiometric mode of microtubule assembly inhibition was demonstrated. The most active compounds possess close to colchicine general toxicity on mice.

Introduction

Malignant neoplastic diseases have long been the second most common cause of death worldwide,[1] and the treatment of cancer is still a crucial aspect of modern medicine. In the case of usage of chemotherapeutic small molecules in cancer therapy, the main disadvantages include low selectivity of biological action and, as a result, high systemic toxicity. The decrease in therapeutic activity is also often associated with the emergence of the resistant tumor cells due to the activation of various anti-apoptotic pathways,[2] drug target mutations preventing drug binding,[3] increased expression of proteins that compensate for the loss of the drug target,[4] and in many cases drug resistance is a result of the functioning of the ATP-binding cassette proteins (P-gp and BCRP membrane pumps) removing xenobiotics from the cells.[5–8] Thereby, the search for the new synthetically available antimitotic agents with a high therapeutic index and improved pharmacokinetic parameters is a matter of current interests.

The activity and/or selectivity of low molecular weight therapeutic molecules can be increased by introduction a reactive functional group designed for covalent binding to specific sites in the target.[9]

Target covalent inhibitors (TCI) have gained popularity in the pharmaceutical sector in recent years as potentially more beneficial in efficiency and selectivity:[10] due to the high strength of the covalent bond, the inhibitor (electrophile) irreversibly attach to the target (nucleophile), leading to its inactivation. The most significant progress has been made in targeting the cysteine residues[11] due to their relatively low prevalence and tendency to act as a nucleophile. In recent years, several anticancer drugs have been introduced to the pharmacological market by FDA, the mechanism of action of which involves covalent binding to cysteine residues of various proteins[12–14] (Fig. 1).

Structures shown in Fig. 1a contain a Michael acceptor fragment (highlighted in green), due to which covalent binding to the free SH-group of protein cysteine residues is possible (Fig. 1b). Other functional groups capable of reacting *via* this mechanism include small strain cycles (electrophilic cyclopropanes, epoxides, four-membered β-lactams or β-lactones), aldehyde functions, amines, acetals, and others. Such approach enabled the creation of a number of drugs used in clinical practice for the treatment of various diseases, as well as molecules currently undergoing clinical trials.[15–19]

Herein we suggest a series of allocolchicine derivatives bearing Michael acceptor units enabling the compounds to be potentially capable of covalent interaction with tubulin, the key protein of cell division. In this case, the creation of covalent inhibitors may lead to a progress in drugs application against drug-resistant cancers. In the vast

[a] Department of Chemistry, N. I. Lobachevsky State University of Nizhny Novgorod, 23 Gagarin Avenue, 603950 Nizhny Novgorod, Russian Federation
[b] Institute of NeuroPhysiopathology (INP) – CNRS UMR 7051, Aix-Marseille University, 27 Boulevard Jean Moulin, 13385 Marseille, Cedex 5, France
[c] CRCM, CNRS, Inserm, Institut Paoli-Calmettes, Aix-Marseille University, 232 Boulevard de Sainte-Marguerite, 13009 Marseille, France
[d] DOSynth Platform, CRCM, Faculté de Pharmacie, Aix-Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille, Cedex 5, France
[e] Laboratory of Cell Interactions, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, 16/10 Miklukho-Maklaya Street, 117997 Moscow, Russian Federation

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0md00060d

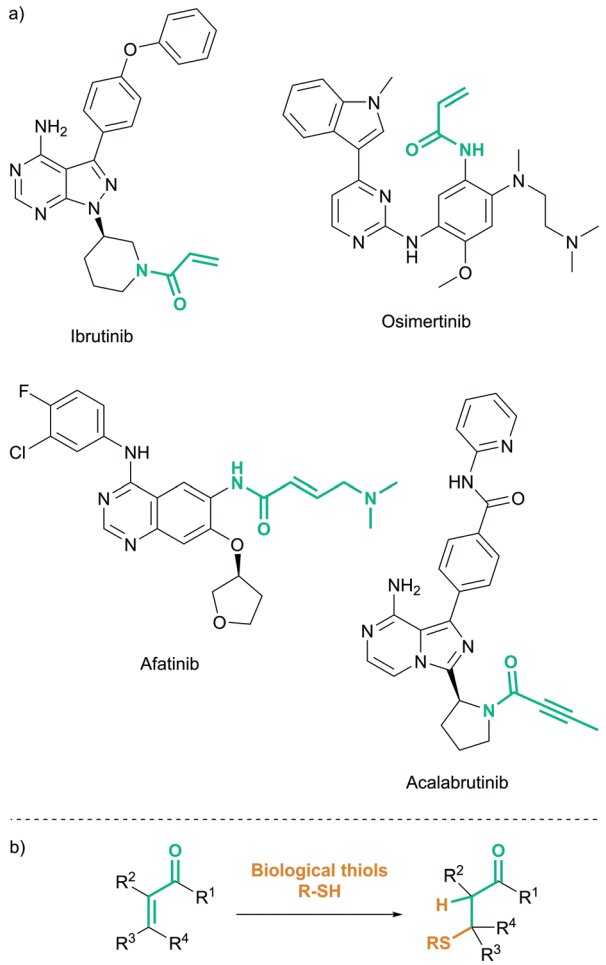


Fig. 1 Examples of covalent inhibitors in clinical practice (a), and illustration of Michael reaction with biological thiols (b).

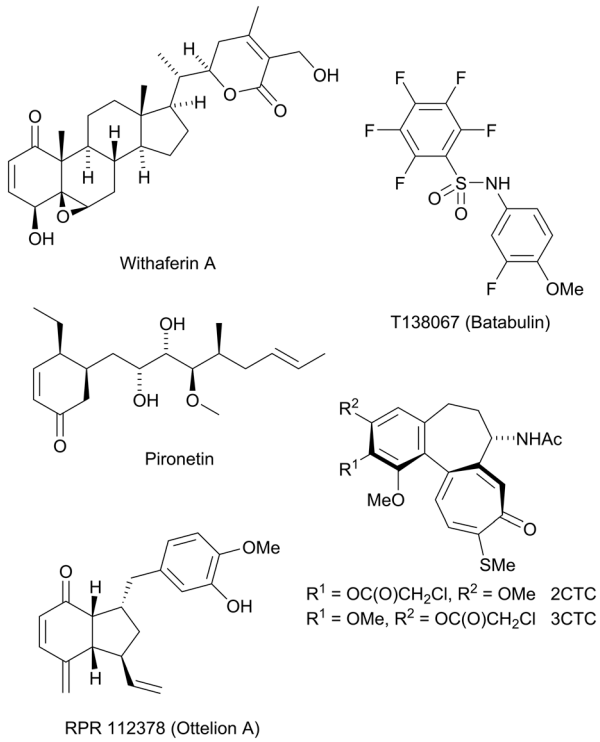


Fig. 2 Tubulin covalent inhibitors.

majority of cases, the interaction of tubulin with a ligand molecule is realized due to the formation of hydrogen bonds, ionic, Van der Waals and hydrophobic interactions at the protein binding site. Nevertheless, in the last decades, several molecules binding to tubulin *via* covalent bonds have been discovered[20,21] (Fig. 2). Withaferin A, a steroidal lactone found in plants of the *Solanaceae* family, was shown to possess anticancer activity in a variety of human cancer cells *in vitro* and *in vivo*, including due to the covalent binding to Cys303 of β-tubulin.[22] Pironetin, an α/β unsaturated lactone, originally isolated from fermentation solutions of *Streptomyces* species, contains the Michael system for binding to Cys316 of α-tubulin.[23] Ottelion A (PRR 112378), first isolated from the fresh water plant *Ottelia alismoides*, is a highly cytotoxic compound with IC_{50} values in the pM–nM range against a panel of 60 human cancer cell lines, and may covalently bond with Cys241 residue by a 1,6-Michael addition reaction.[24] Batabulin (T138067) has long been a promising inhibitor of tubulin, forming a covalent adduct by interaction with Cys239 *via* the nucleophilic substitution reaction in the pentafluorophenyl fragment,[25] and reached phase II clinical trials.[26]

Currently, the only examples of TCI among colchicine ligands are 2-demethyl-2-chloroacetyl thiocolchicine (2CTC) and 3-demethyl-3-chloroacetyl thiocolchicine (3CTC)[27] (Fig. 2), synthesized to study the morphology of the binding site. Studies with these derivatives showed the presence of interaction between colchicine and Cys241 at the boundary of the α,β-tubulin heterodimer.[28,29]

Results and discussion

Synthesis and investigation of acceptor properties

Acting as a mitotic poison, colchicine prevents the mitotic spindle assembly, that leads to a block in mitosis, and reduces cell motility.[30] Moreover, as an immunosuppressant, colchicine accumulates in the immune system cells, which brings about the suppression of inflammatory reactions.[31,32] As most other colchicine site ligands, this compound exerts a destructive effect on the blood supply to tumors, preventing the formation of new vessels or destroying already formed capillaries.[33] In addition, colchicine site ligands are a little susceptible to multidrug resistance associated with the alteration of tubulin isotypes. In the human body, there are 8 isoforms of α-tubulin and 7 isoforms of β-tubulin, their expression is tissue-specific. Changes in the expression of tubulin isotypes are characteristic of many types of cancer, and overexpression of βI, βII, βIII, βVIa and βV-isoforms correlates with the aggressive course of the disease, resistance to chemotherapy and, as a consequence, with low patient survival. The effectiveness of colchicine site ligands in turn does not depend on the composition of β-tubulin isotypes.[34,35]

Despite the unique combination of properties, colchicine has not found so far an application in antitumor clinical practice due to substantial and poorly controlled systemic toxicity even at therapeutic doses.[36] Numerous attempts of chemical modifications of colchicine aimed at reducing the side effects have been realized. Rather successful examples are presented mostly by synthetic allocolchicinoids made *via* converting a seven-membered cycle C to a six-membered and/or creation a fused heterocyclic ring D.[37–40] Having this in mind, and with the idea of the introduction of Michael acceptor units,[41–43] we designed structures of potential irreversible tubulin inhibitors with a selected number of attached fragments allowing them to react with target cysteine residues (Fig. 3).

For the synthesis of colchicinoid agents with expected capability of covalent binding to tubulin, we used colchicinoid intermediates previously described by us.[44] Commercial colchicine (**1**) was converted into deacetylallocolchicine **2** by a three-step cleavage of the acetamide group in 56% yield. In the next step, the amino group in **2** was acylated with glycolic acid or protected glycine under Steglich conditions to give allocolchicines **3** and **4** in good yields. In another route, colchicine (**1**) was converted to iodocolchinol **6** in 2 steps according to the known protocol[44] with 88% yield. Deacetylation of **6** by procedure similar to the cleavage of Ac-group of colchicine (**1**) led to deacetyliodocolchinol **7**. After the acylation of the amino group in **7** with glycolic acid or *N*-Boc-glycine under Steglich conditions, derivatives **8** and **9** were subjected to catalytic tandem Sonogashira coupling/Larock-type cyclization sequence with propargyl acetate and propargyl alcohol, respectively, to give furano-allocolchicinoids **10** and, after deprotection, **11**. Finally, the α,β-unsaturated carbonyl fragment was introduced into the structures of allocolchicinoids by reactions with a number of unsaturated acids under the Steglich conditions to afford the target derivatives **5a–j** and **12a**, **b** (Scheme 1).

To test the Michael-type reactivity of the synthesized compounds towards thiol nucleophiles, the reaction of compound **5c** with protected cysteine was performed (Scheme 2). The choice of compound as an example **5c** was based on a compromise between the activity (Table 1) and the double bond shielding. Under the given conditions,[45] the complete conversion of **5c** to *rac*-**13** was observed in 2 h. It demonstrated electrophilic nature of the compound **5c**.

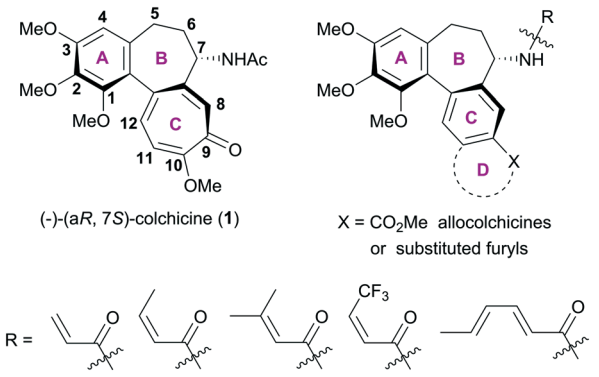


Fig. 3 Structure of colchicine and design of allocolchicine derivatives bearing Michael acceptor units.

Biological investigations

The *in vitro* cytotoxicity of the synthesized compounds **5a–j** and **12a**, **b** towards the human epithelial cell lines (COLO-357, BxPC-3, HaCaT, HEK293) and murine fibroblasts (L929) was investigated. A tetrazolium-based assay was used to determine the drug concentration required to inhibit cell growth by 50% after incubation in the culture medium for 72 hours. The calculated IC_{50} values are summarized in Table 1.

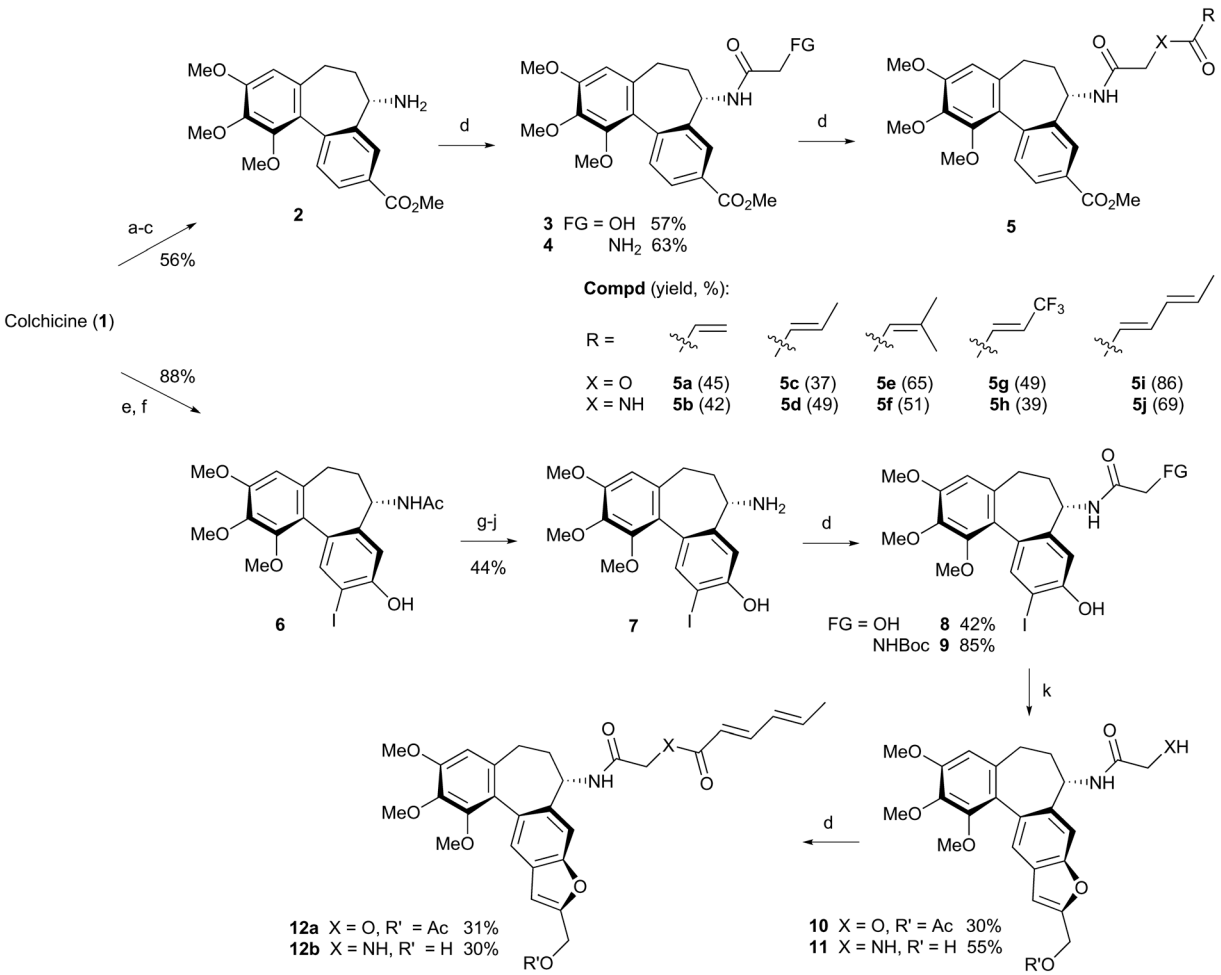
The experimental data show that the ester derivatives (**5a**, **5c**, **5e**, **5g**, **5i**, and **12a**) are more active than the compounds where the acceptor fragment is attached to the core molecule *via* the amide function (**5b**, **5d**, **5f**, **5h**, **5j**, and **12b**). All compounds obtained are active in the nanomolar concentrations against epithelial cell lines, while the **5i** derivative is active in concentrations of hundreds of picomoles.

Inhibition of the cell cycle in comparison with colchicine (**1**) was studied for the most active compounds **5c** and **5i** in COLO-357 and low sensitive L929 cells in the same concentrations of 5 μM. Derivatives **5c** and **5i** as well as colchicine (**1**) effectively induced cell accumulation in the G2/M phase corresponding to non-dividing cells, which steadily resulted in apoptosis (Fig. 4).

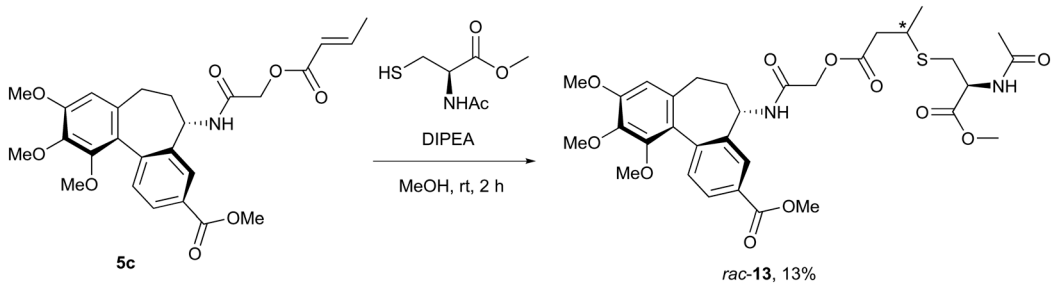
To visualize the full population of cells after **5c**, **5i** and colchicine (**1**) treatment, monolayer COLO-357 cultures were analyzed by confocal microscopy. All the studied compounds were found to effectively disrupt the mitotic spindle, which led to the scattering of chromosomes, and the inability of cells to divide (Fig. 5).

To define the therapeutic range of the most promising compounds, we determined intravenous LD_{50} and LD_{100} acute toxicity of **5c**, **5i** and **1** in C57BL/6 mice. The LD_{50}/LD_{100} for **5c** and **5i** were 6.5/9.8 and 6.1/8.4 mg kg^{-1}, respectively, *versus* 8.5/10.9 mg kg^{-1} for colchicine (**1**). Lethality was observed in 40–48 h for **5c** and in 24 h for **5i** after injections. Thus, the new compounds possess systemic toxicity close to colchicine.

In order to investigate is the cytotoxic effect of compounds of type **5** and **12** with Michael acceptor fragment a consequence of their strong binding to tubulin, we studied the effects of **5i** on microtubule formation. Fig. 6 shows the effects of colchicine (**1**) (panel A) as positive control, and **5i** (panel B), on the turbidimetry time course of microtubule assembly from pure tubulin. A clear inhibition was noted, and the rate of assembly as well as the final amount of microtubules was lower in the presence of ligands than in the control experiment. When the samples were cooled to 15 °C, the polymers depolymerized (data not shown). The insets of panel A and B of Fig. 6 show that the extent of inhibition



Scheme 1 Synthesis of target allocolchicine derivatives **5**, **12**. Reagents and conditions: (a) Boc_2O, DMAP, Et_3N, CH_3CN, reflux, 3 h; (b) MeONa, MeOH, 40 °C, 1 h; (c) TFA, CH_2Cl_2, r.t., 1 h; (d) glycolic acid (for compds **3**, **8**) or *N*-Boc-glycine (for **4**, **9**), or appropriate unsaturated acid (for **5a**–**d**, **f**–**j**, **12a**, **b**), DIC, NHS, Et_3N, CH_2Cl_2, rt, 16 h; then HCl, CH_2Cl_2, rt, 1 h in case of **4** and **9**; 3,3-dimethylacrylic acid, DCC, DMAP, CH_2Cl_2, rt, 16 h for compd **5e**; (e) 0.1 M HCl, AcOH, 100 °C, 3 h; (f) NaOH, I_2, KI, H_2O, 0–5 °C, 2 h; (g) MOMCl, DIPEA, CH_2Cl_2, 0–20 °C, 20 h; (h) Boc_2O, DMAP, Et_3N, CH_3CN, reflux, 26 h; (i) NaOMe (20 mol%), MeOH, rt, 1.5 h; (j) HCl, EtOH, rt, 20 h; (k) propargyl acetate (for 10) or propargyl alkohol (for **11**), $Pd(OAc)_2$ (0.05 equiv.), CuI (0.1 equiv.), AcOK (3 equiv.), Ph_3P (0.15 equiv.), CH_3CN, 70 °C, 10 h.



Scheme 2 Testing of reactivity of compd **5c** towards thiol nucleophiles.

by colchicine (**1**) and **5i**, respectively, increased monotonically with the mole ratio of the total ligand to total tubulin in the solution (*R*). In these figures, 50% inhibition occurred at a mole ratio of 0.27 mol (IC_{50} = 4.89 μM) of colchicine (**1**) per mol of tubulin and at a mole ratio of 0.37 mol (IC_{50} = 6.78 μM) of **5i** per mol of tubulin. Hence, compound **5i** demonstrates similar to colchicine substoichiometric mode of microtubule formation inhibition.

Summarizing the biological studies, it can be concluded that a surprisingly high activity, comparable to allocolchicine,[44] suggests that the acceptor fragment might perform its function in tubulin binding. However, non-

Table 1 *In vitro* cytotoxicity of the target compounds (IC_{50}, nM)[a]

Compound	COLO-357	BxPC-3	HaCaT	HEK293	L929
Colchicine (**1**)	16	16	3	16	2000
5a	16	16	16	16	>10 000
5b	400	80	80	400	>10 000
5c	16	16	3	3	2000
5d	80	80	80	80	>10 000
5e	16	3	3	16	2000
5f	80	16	16	16	2000
5g	16	16	16	16	2000
5h	80	16	16	80	2000
5i	3	3	0.6	3	80
5j	16	3	3	16	2000
12a	16	16	0.6	3	10 000
12b	400	400	400	400	2000
rac-**13**	80	16	16	16	—[b]

[a] IC_{50} concentration inducing 50% inhibition of cell growth. [b] Not determined.

specific interactions with other proteins or binding to non-protein targets might occur that causes significant systemic toxicity.

Conclusions

In this work, we investigated the strategy associated with the insertion of Michael acceptor fragments into colchicinoids to target an efficient binding of the agents to the cysteine residues of the colchicine-binding site of tubulin protein. Covalent binding to a CH_2SH-fragment of cysteine is possible at least in non-physiological conditions. For all compounds, high tubulin binding activity and low nanomolar inhibition concentration of epithelial cells proliferation, in the range of the best-known tubulin inhibitors, was demonstrated. Further work in this direction is currently underway.

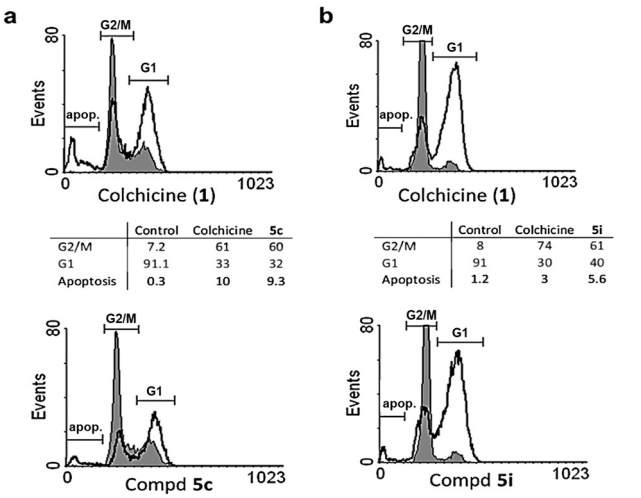


Fig. 4 Induction of cell cycle arrest by colchicine (**1**) and compds **5c** and **5i**. COLO-357 (column a) and L929 (column b) cells were treated for 72 h with 5 μM of colchicine (**1**), **5c** or **5i**, respectively; trypsinized, permeabilized, and stained with PI. The percentage distribution of cells is indicated in the tables. Control cells are shown in grey.

Experimental

General materials and methods

Commercially available reagents (Aldrich, Alfa Aesar, Acros, ABCR) were used without additional purification. Column chromatography was performed using Macherey-Nagel Kieselgel 60 (70–230 mesh). All ^1H, ^{13}C and ^{19}F NMR spectra were recorded at 25 °C in DMSO-d_6 on Agilent DD2 400 instrument. Chemical shifts (δ) are reported in parts per million (ppm) from TMS using the residual solvent resonance (DMSO-d_6: 2.50 ppm for ^1H NMR, 39.52 ppm for ^{13}C NMR). Standard abbreviations are used to indicate multiplicities.

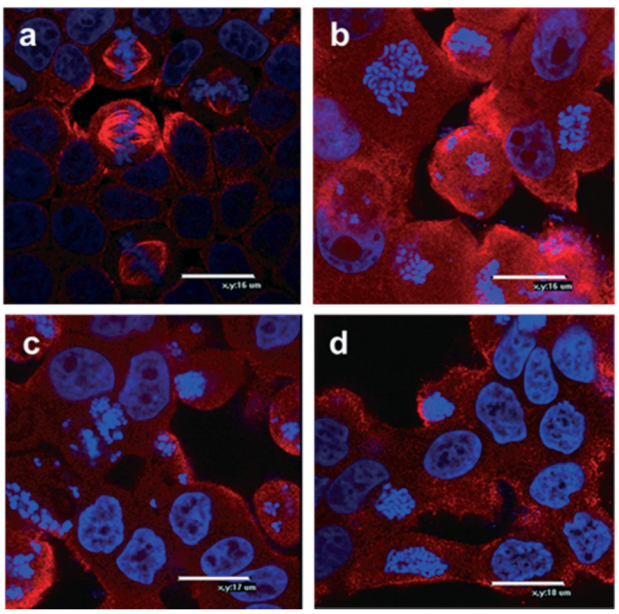


Fig. 5 Effect of colchicine (**1**) and compounds (**5c**) and (**5i**) on mitotic spindle formation and β-tubulin expression in pancreatic epithelial cells COLO-357. COLO-357 cells (seeded at 10^5/glass) were incubated for 72 h without (a) or with 5 μM of colchicine (**1**) (b), **5c** (c), or **5c** (d), fixed, stained with anti-βtubulin antibody (red) and nuclei stain Hoechst 33342 (blue). Scale bars correspond to 16–18 μm.

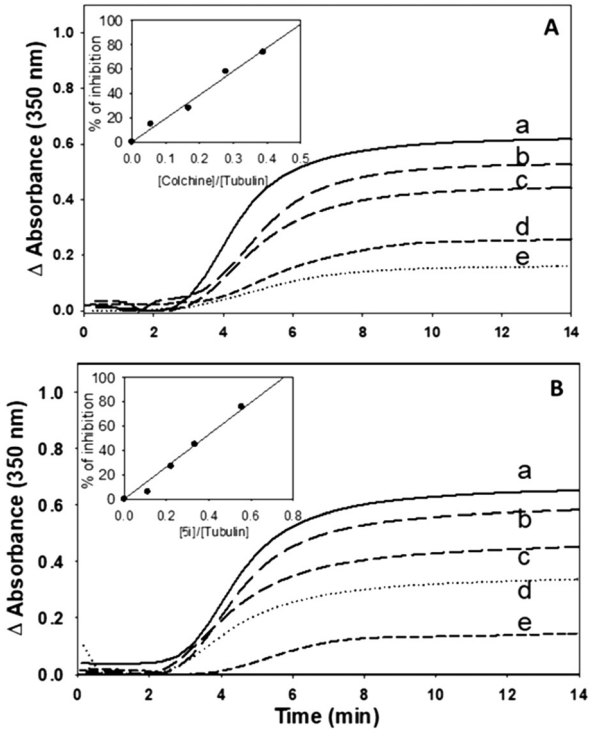


Fig. 6 Effect of ligands on the turbidity time course of *in vitro* microtubule assembly. The reaction was started by warming the solution from 15 to 37 °C. (A) Effect of various concentrations of colchicine (**1**) (a: 0 μM; b: 1 μM; c: 3 μM; d: 5 μM; and e: 7 μM) on tubulin at 18 μM in polymerization buffer. (B) Effect of various concentrations of **5i** (a: 0 μM; b: 2 μM; c: 4 μM; d: 6 μM; and e: 10 μM) on tubulin at 18 μM in polymerization buffer. The insets represent the percentage of assembly inhibition as a function of the mole ratio of the total ligand to total tubulin in the solution (*R*).

Allocolchicine atom numbering was used for signal assignment of allocolchicinoids. EI mass spectra (70 eV) were obtained on a DSQ II mass spectrometer (Thermo Electron Corporation) with a quadrupole mass analyzer. ESI mass spectra were recorder on a Polaris Q (Thermo Finnigan) mass spectrometer. MALDI mass spectra were obtained on a MALDI-TOF mass spectrometer Bruker Microflex LT. Combustion analysis was performed using an Elementar (Vario Micro Cube) apparatus. Optical rotation $[\alpha]_D$ was measured on JASCO P-2000 polarimeter at 20 °C and λ 589 nm (cuvette length: 1.0 dm, volume: 1.0 mL); concentration is given in g/100 mL. Solvents were purified according to the standard procedures. Petroleum ether (PE) used is the fraction with bp 40–70 °C. Compounds **3**, **4**, **10** and **11** were prepared according to the previously described procedures.[44] Atomic numeration is given only for NMR assignment, for details see ESI.†

General procedure for the synthesis of Michael acceptors 5a–d, f–j and 12a, b. Compound **3** (**4**) or **10** (**11**) (1 equiv.), corresponding α,β-unsaturated acid (1 equiv.), and NHS (1 equiv.) were dissolved in CH_2Cl_2 (20 mL per 1 mmol of starting materials). 1,3-Diisopropylcarbodiimide (DIC; 1.5 equiv.) and Et_3N (3 equiv.) were added, and the mixture was stirred for 16 h at room temperature under an inert atmosphere. The crude product obtained after solvent removal was purified by column chromatography using CH_2Cl_2/MeOH or PE/EtOAc/EtOH as eluent.

Methyl (5*S*, *aR*)-5-(2-(acryloyloxy)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5a). Purified by column chromatography (PE/EtOAc/EtOH 2 : 1 : 1); white solid (45%); m.p. 163 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (d, J = 7.5 Hz, 1H, NH), 7.13 (s, 1H, C8–H), 7.12 (d, J = 12.7 Hz, 1H, C10–H), 7.03 (d, J = 10.8 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 6.34 (dd, J = 17.3, 1.7 Hz, 1H, C5′–H), 6.21 (dd, J = 17.3, 10.2 Hz, 1H, C4′–H), 5.97 (dd, J = 10.2, 1.6 Hz, 1H, C5′–H), 4.61 (s, 2H, C2′H_2), 4.35 (dt, J = 13.1, 7.0 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.64–2.59 (m, 1H, C5H_2), 2.23 (td, J = 13.0, 7.0 Hz, 1H, C5H_2), 2.03 (dt, J = 12.2, 6.0 Hz, 1H, C6H_2), 1.88 (m, 1H, C6H_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.96, 165.99, 164.89, 163.54, 152.97, 150.43, 150.13, 140.76, 134.97, 134.44, 134.12, 132.24, 130.41, 127.79, 125.33, 112.08, 107.78, 62.19, 60.82, 60.68, 56.05, 55.85, 51.21, 35.71, 29.14; MS (MALDI-TOF): m/z = 470.0 $[M + H]^+$, 492.0 $[M + Na]^+$; elemental analysis calcd (%) for $C_{25}H_{27}NO_8$: C 63.96, H 5.80, N 2.98; found: C 64.19, H 6.03, N 3.10.

Methyl (5*S*, *aR*)-5-(2-acrylamidoacetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5b). Purified by column chromatography (PE/EtOAc/EtOH 2 : 1 : 1); white solid (42%); m.p. 254 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (d, J = 7.3 Hz, 1H, C7–NH), 8.27 (t, J = 5.8 Hz, 1H, C2′–NH), 7.12 (s, 1H, C8–H), 7.11 (d, J = 10.1 Hz, 1H, C10–H), 7.02 (d, J = 10.8 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 6.26 (dd, J = 17.1, 10.2 Hz, 1H, C4′–H), 6.05 (dd, J = 17.1, 2.2 Hz, 1H, C5′–H), 5.56 (dd, J = 10.2, 2.3 Hz, 1H, C5′–H), 4.34 (dt, J = 12.8, 7.0 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.60 (q, J = 5.5 Hz, 1H, C5H_2), 2.22 (m, 1H, C5H_2), 2.02 (dt, J = 12.8, 6.3 Hz, 1H, C6H_2), 1.90–1.82 (m, 1H, C6H_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.98, 168.12, 164.75, 163.53, 152.94, 150.50, 150.44, 140.74, 135.05, 134.36, 134.19, 131.45, 130.37, 125.41, 125.36, 112.07, 107.81, 60.79, 60.71, 56.05, 55.86, 51.42, 41.71, 35.71, 29.16; MS (MALDI-TOF): m/z = 491.0 $[M + Na]^+$; elemental analysis calcd (%) for $C_{25}H_{28}N_2O_7$: C 64.09, H 6.02, N 5.98; found: C 64.31, H 6.30, N 5.86.

Methyl (5*S*, *aR*)-5-(2-(((*E*)-but-2-enoyl)oxy)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5c). Purified by column chromatography (PE/EtOAc/EtOH 3 : 1 : 1); white solid (37%); m.p. 144 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (d, J = 7.4 Hz, 1H, NH), 7.13 (s, 1H, C8–H), 7.11 (d, J = 11.0 Hz, 1H, C10–H), 7.02 (d, J = 10.8 Hz, 1H, C11–H), 6.92 (dd, J = 15.6, 6.9 Hz, 1H, C5′–H), 6.77 (s, 1H, C4–H), 5.93 (dd, J = 15.6, 1.8 Hz, 1H, C4′–H), 4.56 (s, 2H, C2′H_2), 4.34 (dt, J = 13.7, 6.9 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.64–2.59 (m, 1H, C5H_2), 2.22 (td, J = 13.1, 7.1 Hz, 1H, C5H_2), 2.02 (dt, J = 12.6, 6.0 Hz, 1H, C6H_2), 1.93–1.87 (m, 1H, C6H_2), 1.85 (dd, J = 6.9, 1.7 Hz, 3H, C6′H_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.97, 166.22, 164.94, 163.54, 152.96,

150.43, 150.18, 146.12, 140.76, 134.98, 134.43, 134.14, 130.41, 125.35, 121.68, 112.09, 107.79, 61.89, 60.83, 60.70, 56.06, 55.86, 51.19, 35.69, 29.15, 17.75; MS (MALDI-TOF): *m*/*z* = 484.0 $[M + H]^+$, 506.0 $[M + Na]^+$; elemental analysis calcd (%) for $C_{26}H_{29}NO_8$: C 64.59, H 6.05, N 2.90; found: C 64.29, H 6.25, N 2.69.

Methyl (5*S*, *aR*)-5-(2-((*E*)-but-2-enamido)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5d). Purified by column chromatography (PE/EtOAc/EtOH 2 : 1 : 1); white solid (49%); m.p. 223 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.66 (d, J = 7.4 Hz, 1H, C7–NH), 8.04 (t, J = 5.8 Hz, 1H, C2′–NH), 7.12 (s, 1H, C8–H), 7.11 (d, J = 8.5 Hz, 1H, C10–H), 7.02 (d, J = 10.9 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 6.63–6.53 (m, 1H, C5′–H), 5.93 (dd, J = 15.3, 1.8 Hz, 1H, C4′–H), 4.33 (dt, J = 13.2, 6.7 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.50 (s, 3H, OMe), 2.63–2.57 (m, 1H, $C5H_2$), 2.22 (td, J = 13.0, 7.0 Hz, 1H, $C5H_2$), 2.01 (dp, J = 19.1, 6.4 Hz, 1H, $C6H_2$), 1.90–1.83 (m, 1H, $C6H_2$), 1.76 (dd, J = 6.9, 1.7 Hz, 3H, $C6'H_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.99, 168.34, 165.06, 163.53, 152.94, 150.54, 150.44, 140.74, 138.03, 135.07, 134.35, 134.20, 130.39, 125.57, 125.42, 112.07, 107.80, 60.79, 60.72, 56.06, 55.87, 51.40, 41.66, 35.71, 29.17, 17.34; MS (MALDI-TOF): *m*/*z* = 483.1 $[M + H]^+$, 505.1 $[M + Na]^+$; elemental analysis calcd (%) for $C_{26}H_{30}N_2O_7$: C 64.72, H 6.27, N 5.81; found: C 64.94, H 6.58, N 5.65.

Methyl (5*S*, *aR*)-9,10,11-trimethoxy-5-(2-(3-methylbut-2-enamido)acetamido)-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5f). Purified by column chromatography (PE/EtOAc/EtOH 2 : 1 : 1); white solid (51%); m.p. 159 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61 (d, J = 7.4 Hz, 1H, C7–NH), 7.86 (t, J = 5.9 Hz, 1H, C2′–NH), 7.12 (s, 1H, C8–H), 7.11 (d, J = 10.0 Hz, 2H, C10–H), 7.01 (d, J = 10.8 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 5.66 (s, 1H, C4′–H), 4.33 (dt, J = 13.0, 6.9 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.60 (q, J = 7.4, 6.8 Hz, 1H, $C5H_2$), 2.22 (td, J = 13.0, 7.1 Hz, 1H, $C5H_2$), 2.04 (s, 3H, $C5'CH_3$), 1.99 (dd, J = 12.6, 6.4 Hz, 1H, $C6H_2$), 1.86 (td, J = 11.9, 7.0 Hz, 1H, $C6H_2$), 1.75 (s, 3H, $C5'CH_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.97, 168.52, 166.06, 163.52, 152.93, 150.53, 150.43, 149.01, 140.73, 135.06, 134.31, 134.18, 130.40, 125.42, 118.70, 112.04, 107.78, 60.78, 60.70, 56.03, 55.85, 51.34, 41.43, 35.72, 29.17, 26.78, 19.29; MS (MALDI-TOF): *m*/*z* = 497.1 $[M + H]^+$, 519.1 $[M + Na]^+$; elemental analysis calcd (%) for $C_{27}H_{32}N_2O_7$: C 65.31, H 6.50, N 5.64; found C 65.02, H 6.68, N 5.88.

Methyl (5*S*, *aR*)-9,10,11-trimethoxy-5-(2-(((*E*)-4,4,4-trifluorobut-2-enoyl)oxy)acetamido)-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5g). Purified by column chromatography (PE/EtOAc/EtOH 3 : 1 : 1); white solid (49%); m.p. 150 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (d, J = 7.5 Hz, 1H, NH), 7.16 (d, J = 6.8 Hz, 1H, C5′–H), 7.13 (s, 1H, C8–H), 7.12 (d, J = 10.3 Hz, 1H, C10–H), 7.03 (d, J = 10.8 Hz, 1H, C11–H), 6.78 (dd, J = 15.9, 2.1 Hz, 1H, C4′–H), 6.78 (s, 1H, C4–H), 4.68 (s, 2H, $C2'H_2$), 4.36 (dt, J = 13.0, 6.9 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.63–2.58 (m, 1H, $C5H_2$), 2.24 (td, J = 13.0, 7.0 Hz, 1H, $C5H_2$), 2.03 (tt, J = 12.3, 6.7 Hz, 1H, $C6H_2$), 1.94–1.86 (m, 1H, $C6H_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.94, 165.52, 163.54, 163.00, 152.97, 150.43, 150.02, 140.76, 134.93, 134.48, 134.10, 131.14, 130.80, 130.40, 129.20, 125.31, 112.10, 107.78, 63.01, 60.81, 60.68, 56.05, 55.86, 51.25, 35.68, 29.12; ^{19}F NMR (376 MHz, DMSO-d_6) δ −64.05; MS (MALDI-TOF): *m*/*z* = 537.9 $[M + H]^+$; elemental analysis calcd (%) for $C_{26}H_{26}F_3NO_8$: C 58.10, H 4.88, N 2.61; found: C 58.42, H 5.17, N 2.44.

Methyl (5*S*, *aR*)-9,10,11-trimethoxy-5-(2-((*E*)-4,4,4-trifluorobut-2-enamido)acetamido)-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5h). Purified by column chromatography (PE/EtOAc/EtOH 3 : 1 : 1); white solid (39%); m.p. 191 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (t, J = 7.5, 7.1 Hz, 2H, 2*x*NH), 7.12 (s, 1H, C8–H), 7.12 (d, J = 10.6 Hz, 1H, C10–H), 7.02 (d, J = 10.8 Hz, 1H, C11–H), 6.85 (dd, J = 15.7, 1.9 Hz, 1H, C4′–H), 6.78 (s, 1H, C4–H), 6.76 (dd, J = 15.8, 2.0 Hz, 1H, C5′–H), 4.34 (dt, J = 13.0, 6.8 Hz, 1H, C7–H), 3.90 (dd, J = 7.9, 5.9 Hz, 2H, $C2'H_2$), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.60 (q, J = 8.3, 6.5, 4.9 Hz, 1H, $C5H_2$), 2.23 (td, J = 13.1, 7.0 Hz, 1H, $C5H_2$), 2.03 (dp, J = 12.6, 6.3 Hz, 1H, $C6H_2$), 1.87 (td, J = 12.2, 7.0 Hz, 1H, $C6H_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 178.41, 167.99, 163.98, 162.50, 153.39, 150.89, 150.82, 141.19, 135.45, 134.83, 134.61, 133.07, 133.01, 130.79, 125.84, 112.51, 108.25, 61.21, 61.15, 56.50, 56.30, 51.92, 42.46, 36.13, 29.59; ^{19}F NMR (376 MHz, DMSO-d_6) δ −63.37; MS (MALDI-TOF): *m*/*z* = 558.9 $[M + Na]^+$; elemental analysis calcd (%) for $C_{26}H_{27}F_3N_2O_7$: C 58.21, H 5.07, N 5.22; found: C 58.52, H 5.25, N 5.00.

Methyl (5*S*, *aR*)-5-(2-(((2*E*,4*E*)-hexa-2,4-dienoyl)oxy)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5i). Purified by column chromatography (CH_2Cl_2/MeOH 20 : 1); white solid (86%); m.p. 173 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (d, J = 7.4 Hz, 1H, NH), 7.27–7.19 (m, 1H, C5′–H), 7.13 (s, 1H, C8–H), 7.11 (d, J = 10.8 Hz, 1H, C10–H), 7.02 (d, J = 10.9 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 6.35–6.24 (m, 1H, C6′H), 5.89 (d, J = 15.2 Hz, 1H, C7′–H), 5.56 (d, J = 7.9 Hz, 1H, C4′–H), 4.57 (s, 2H, $C2'H_2$), 4.38–4.31 (m, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.60 (dd, J = 13.3, 5.9 Hz, 1H, $C5H_2$), 2.22 (td, J = 13.1, 7.2 Hz, 1H, $C5H_2$), 2.06–1.98 (m, 1H, $C6H_2$), 1.93–1.86 (m, 1H, $C6H_2$), 1.82 (d, J = 4.9 Hz, 3H, $C8'H_3$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.96, 166.26, 165.70, 163.53, 152.96, 150.42, 150.17, 145.75, 140.75, 140.50, 134.98, 134.42, 134.13, 130.42, 129.58, 125.34, 118.00, 112.08, 107.77, 60.82, 60.68, 56.05, 55.85, 51.19, 35.68, 29.14, 18.46; MS (EI) *m*/*z* (%) = 509.6 (100), 508.2 (48), 480.3 (20), 368.4 (9), 338.2 (14), 327.3 (9); elemental analysis calcd (%) for $C_{28}H_{31}NO_8$: C 66.00, H 6.13, N 2.75; found: C 65.72, H 6.25, N 2.98.

Methyl (5*S*, *aR*)-5-(2-((2*E*,4*E*)-hexa-2,4-dienamido)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5j). Purified by column chromatography (PE/EtOAc/EtOH 3 : 1 : 1); white solid (69%); m.p. 164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (d, J = 7.3 Hz, 1H, C7–NH), 8.12 (t, J = 5.7 Hz, 1H, C3′–NH), 7.13 (s, 1H,

C8–H), 7.11 (d, J = 10.9 Hz, 1H, C10–H), 7.01 (d, J = 11.5 Hz, 1H, C11–H), 6.99–6.92 (m, 1H, C5′–H), 6.77 (s, 1H, C4–H), 6.22–6.03 (m, 2H, C6′–H, C7′–H), 5.93 (d, J = 15.2 Hz, 1H, C4′–H), 4.33 (dt, J = 12.8, 6.9 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.50 (s, 3H, OMe), 2.61 (d, J = 6.2 Hz, 1H, C5H_2), 2.22 (td, J = 13.1, 7.2 Hz, 1H, C5H_2), 2.01 (tt, J = 12.7, 6.3 Hz, 1H, C6H_2), 1.86 (td, J = 12.0, 7.2 Hz, 1H, C6H_2), 1.77 (d, J = 6.4 Hz, 3H, C8′H_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.99, 168.34, 165.50, 163.53, 152.94, 150.52, 150.44, 140.75, 139.46, 136.75, 135.06, 134.34, 134.19, 130.39, 129.89, 125.42, 122.60, 112.06, 107.80, 60.78, 60.71, 56.05, 55.86, 51.41, 41.79, 35.72, 29.17, 18.25; MS (EI): m/z (%) = 508.9 (52), 508.1 (100), 507.3 (70), 479.7 (38), 355.6 (18), 328.2 (34), 313.5 (16), 223.6 (13), 209.4 (12); elemental analysis calcd (%) for $C_{28}H_{32}N_2O_7$: C 66.13, H 6.34, N 5.51; found: C 66.37, H 6.61, N 5.66.

1′,2′,3′-Trimethoxybenzo[5′,6′:5,4]1*H*-(*aR*, 1*S*)-1-((((2‴*E*,4‴*E*)-hexa-2‴,4‴-dienoyl)oxy)acetamido)-6,7-dihydrocyclohepta[3,2:*f*]-2″-acetoxymethylbenzofuran (12a). Purified by column chromatography (PE/EtOAc/EtOH 8 : 1 : 1); white solid (31%); m.p. 139 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.68 (d, J = 8.0 Hz, 1H, NH), 7.56 (s, 1H, C4″–H), 7.50 (s, 1H, C7″–H), 7.25 (m, 1H, C3‴–H), 7.00 (s, 1H, C3″–H), 6.80 (s, 1H, C4′–H), 6.30 (dd, J = 6.2, 2.9 Hz, 2H, C4‴–H, C5‴–H), 5.92 (d, J = 15.3 Hz, 1H, C2‴–H), 5.22 (s, 2H, 2″–CH_2OAc), 4.57 (dd, J = 11.2, 6.5 Hz, 1H, C1–H), 3.84 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.51 (s, 2H, 1-NHC(O)CH_2), 3.39 (s, 3H, OMe), 2.56–2.52 (m, 1H, C6–H), 2.21–2.13 (m, 1H, C6–H), 2.09 (s, 3H, OAc), 2.03–1.91 (m, 2H, C7H_2), 1.82 (d, J = 5.2 Hz, 3H, C6‴H_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.96, 166.23, 165.90, 153.96, 152.37, 152.17, 150.39, 145.67, 140.64, 140.44, 137.98, 134.66, 129.60, 129.02, 125.80, 124.44, 122.31, 118.13, 108.08, 107.08, 105.91, 69.79, 62.25, 60.62, 60.50, 57.97, 55.84, 37.93, 29.86, 20.58, 18.46; MS (EI): m/z (%) = 564.1 (27), 563.3 (64), 562.6 (100), 561.0 (74), 450.0 (94), 449.0 (54), 408.4 (33), 392.2 (45), 377.6 (34), 333.6 (26), 319.4 (14), 303.4 (11), 263.9 (10); elemental analysis calcd (%) for $C_{31}H_{33}NO_9$: C 66.06, H 5.90, N 2.49; found: C 65.91, H 5.71, N 2.32.

1′,2′,3′-Trimethoxybenzo[5′,6′:5,4]1*H*-(*aR*, 1*S*)-1-((((2‴*E*,4‴*E*)-hexa-2‴,4‴-dienoyl)amido)acetamido)-6,7-dihydrocyclohepta-[3,2:*f*]-2″-hydroxymethylbenzofuran (12b). Purified by column chromatography (PE/EtOAc/EtOH 4 : 1 : 1); white solid (30%); m.p. > 250 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.55 (d, J = 8.2 Hz, 1H, C1–H), 8.17 (t, J = 5.9 Hz, 1H, 1‴(O)–NH), 7.51 (s, 1H, C4″–H), 7.47 (s, 1H, C7″–H), 7.04–6.97 (m, 1H, C3‴–H), 6.79 (s, 1H, C3″–H), 6.75 (s, 1H, C4′–H), 6.20 (dd, J = 15.5, 10.3 Hz, 1H, C4‴–H), 6.13–6.04 (m, 1H, C5‴–H), 5.99–5.94 (m, 1H, C2‴–H), 5.45 (t, J = 5.9 Hz, 1H, OH), 4.58 (m, 1H, C1–H), 4.57 (d, J = 5.8 Hz, 2H, C2″–CH_2), 3.91–3.84 (m, 2H, NHCH_2), 3.84 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.38 (s, 3H, OMe), 2.60–2.52 (m, 1H, C6–H), 2.16 (tt, J = 12.0, 6.1 Hz, 1H, C6–H), 2.06 (dd, J = 12.5, 6.7 Hz, 1H, C7–H), 1.99–1.90 (m, 1H, C7–H), 1.79 (d, J = 6.5 Hz, 3H, C6‴H_3); ^{13}C NMR (101 MHz, DMSO-d_6): δ = 168.25, 165.63, 158.42, 153.76, 152.24, 150.41, 140.61, 139.41, 137.15, 136.73, 134.73, 129.91, 128.62, 126.22, 124.74, 122.70, 121.76, 108.04, 105.73, 103.21, 60.63, 60.41, 56.23, 55.83, 48.69, 42.11, 38.09, 29.96, 18.26. MS (ESI): m/z (%) = 520.2 (30), 380.3 (30), 368.3 (100), 352.3 (70), 337.3 (28), 321.3 (16), 250.3 (15); elemental analysis calcd (%) for $C_{29}H_{32}N_2O_7$: C 66.91, H 6.20, N 5.38; found: C 66.62, H 6.35, N 5.14.

Synthesis of methyl (5*S*, *aR*)-9,10,11-trimethoxy-5-(2-((3-methylbut-2-enoyl)oxy)acetamido)-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (5e). Compound **3** (60.0 mg, 0.144 mmol), DCC (59.4 mg, 0.288 mmol), and DMAP (43.9 mg, 0.360 mmol) were dissolved in CH_2Cl_2 (14 mL), then 3,3-dimethylacrylic acid (14.4 mg, 0.144 mmol) was added and the mixture was stirred for 16 h at room temperature under an inert atmosphere. The crude product obtained after solvent removal was purified by column chromatography, eluent PE/EtOAc/EtOH 3 : 1 : 1, to afford **5e** as a white solid (47.0 mg, 65%); m.p. 175 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (d, J = 7.4 Hz, 1H, NH), 7.13 (s, 1H, C8–H), 7.11 (d, J = 11.5 Hz, 1H, C10–H), 7.02 (d, J = 10.8 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 5.74 (m, 1H, C4′–H), 4.51 (d, J = 3.1 Hz, 2H, C2′H_2), 4.35 (dt, J = 11.4, 6.9 Hz, 1H, C7–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.51 (s, 3H, OMe), 2.60 (dd, J = 13.3, 6.0 Hz, 1H, C5H), 2.22 (td, J = 12.8, 7.1 Hz, 1H, C5H), 2.08 (d, J = 1.3 Hz, 3H, C5′–CH_3), 2.01 (dt, J = 12.5, 6.2 Hz, 1H, C6H, the other C6H-signal is under DMSO), 1.88 (d, J = 1.4 Hz, 3H, C5′–CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.96, 166.41, 164.91, 163.53, 158.10, 152.96, 150.42, 150.20, 140.75, 134.99, 134.41, 134.13, 130.44, 125.35, 114.91, 112.08, 107.78, 61.40, 60.82, 60.69, 56.05, 55.85, 51.14, 35.68, 29.16, 26.89, 19.97; MS (ESI): m/z (%) = 497.2 (29), 414.2 (19), 369.3 (42), 338.3 (62), 312.3 (100), 281.3 (59), 254.3 (28), 239.3 (18), 208.3 (11); elemental analysis calcd (%) for $C_{27}H_{31}NO_8$: C 65.18, H 6.28, N 2.82; found: C 65.34, H 6.47, N 3.07.

Synthesis of methyl (5*S*, *aR*)-5-(2-((3-((2-acetamido-3-methoxy-3-oxopropyl)thio)butanoyl)oxy)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5*H*-dibenzo[*a*,*c*]cyclohepten-3-carboxylate (*rac*-13). A solution of **5c** (70.0 mg, 0.145 mmol) and *N*-acetyl-L-cysteine methyl ester (28 μL, 0.159 mmol) in dry methanol (1 mL) was cooled to 0 °C. DIPEA (28 μL, 0.159 mmol) was added, and the reaction was stirred for 2 h. TLC indicated full conversion. The crude product obtained after solvent removal was purified by column chromatography, eluent PE/EtOAc/EtOH 3 : 1 : 1, to afford *rac*-**13** as a yellowish solid (12.0 mg, 13%); m.p. 172 °C; $[\alpha]_D$ −60.0° (c 0.5000 in $CHCl_3$, 20 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73 (d, J = 7.5 Hz, 1H, NHAc), 8.35 (d, J = 7.8 Hz, 1H, C7–NH), 7.12 (s, 1H, C8–H), 7.11 (d, J = 8.5 Hz, 1H, C10–H), 7.03 (d, J = 10.8 Hz, 1H, C11–H), 6.77 (s, 1H, C4–H), 4.53 (s, 2H, C2′H_2), 4.43–4.32 (m, 2H, C7–H, C2″–H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.78 (s, 3H, OMe), 3.62 (s, 3H, OMe), 3.52 (s, 3H, OMe), 3.18–3.06 (m, 2H, C5′–H, C3″–H), 2.91–2.77 (m, 2H, C3″–H, C5–H), 2.63 (m, 2H, C5–H, C4′–H), 2.23 (td, J = 13.0, 7.2 Hz, 1H, C4′–H), 2.02 (dt, J = 12.6, 6.3 Hz, 1H, C6–H), 1.90 (dd, J = 12.1, 7.2 Hz, 1H, C6–H), 1.83 (s, 3H, Ac), 1.23 (d, J = 6.8 Hz, 3H, C6′H_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.94, 171.13, 170.21, 169.36, 166.03, 163.53, 152.97, 150.41, 150.10, 140.75, 134.97, 134.46, 134.11, 130.42, 125.32, 112.09, 107.77, 62.20, 60.80,

60.68, 56.05, 55.85, 52.34, 52.02, 51.16, 45.65, 41.17, 36.16, 35.71, 31.32, 22.23, 20.99; MS (ESI): *m*/*z* (%) = 660 (1), 516 (10), 455 (22), 416 (12), 369 (26), 354 (55), 312 (100), 297 (55), 254 (24), 239 (17), 211 (13); elemental analysis calcd (%) for $C_{32}H_{40}N_2O_{11}S$: C 58.17, H 6.10, N 4.24; found: C 58.41, H 5.97, N 4.35.

Cell cultures

Cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), pen–strep–glut (all from PanEco, Moscow, Russian Federation). All cell lines used were routinely tested for mycoplasma. Adherent cells were detached using 0.05% trypsin–EDTA (PanEco, Moscow), counted and sub-cultured. Twenty-four hours before assays, cells were seeded in the appropriate plates (96- or 24-well plates), adjusted to 3×10^5 cells per mL, and incubated overnight to achieve standardized growth conditions.

MTT-assay

Cytotoxic effect of the allocolchicinoids was estimated by a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma) test as described earlier.[46] All the compounds were dissolved in DMSO to 20 mM concentration and stored at −20 °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^{-1} of MTT were added in the amount of 10 μL to each well. After the incubation, culture medium was removed and 100 μL of DMSO were added to each well. Plates were incubated at shaking 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.

Cell cycle analysis by flow cytometry

Cell cycle was analyzed using PI-stained DNA. COLO-357 and L929 cells 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 °C. Thereafter, the cells were washed twice in PBS, stained with 50 μg mL^{-1} of propidium iodide (Sigma Chemical Co) in PBS, 10 μg mL^{-1} of DNAse 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).

Confocal analysis

For confocal analysis, COLO-357 cells (10^5/glass) were grown overnight on sterile cover slips in 200 μL of complete culture medium in 6-well plates (Costar). Colchicine (**1**), compd **5c** or **5i** (5 μM) was dissolved in 4 mL of complete medium and added to the wells. Cells were cultivated for 72 h. After incubation, cells were fixed with 1% paraformaldehyde, permeabilized by 0.1% Triton X100 in PBS, washed, and treated with Mowiol 4.88 medium (Calbiochem, Germany). Tubulin was identified by anti-tubulin antibody (Santa Cruz, USA) followed by anti-mouse IgG-AlexaFluor555 (Molecular Probes, Invitrogen, USA). Hoechst 33342 (Sigma) was used to visualize nuclei. Slides were analyzed using Eclipse TE2000 confocal microscope (Nikon, Japan).

Animals

C57BL/6 mice were purchased from Pushchino Affiliation of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow. All mice were 6–8 weeks old and maintained in a minimal pathogen animal facility at the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow. All studies were conducted in an AAALAC accredited facility in compliance with the PHS Guidelines for the Care and Use of Animals in Research. The range of lethal concentrations was predetermined in the preliminary experiments. To conduct acute toxicity experiments mice (10 per group) received intravenously a single dose of colchicine or its derivatives. Four different doses were used: 4, 7, 10 and 13 mg kg^{-1}. Toxic effect was evident 24 post injection (ruffled fur, poor mobility, a decrease in body temperature). The lethality was registered 24 h post injection in 10 and 13 mg kg^{-1} groups and some lethality at 48 h for 4 and 7 mg kg^{-1}. Mice survived 48 h were alive 1 week after.

Preparation of lamb brain tubulin

Tubulin was extracted from lamb brains by ammonium sulfate fractionation and ion exchange chromatography and stored in liquid nitrogen.[47] Before use, aliquots of protein were chromatographed in drained spin columns (1 cm × 5 cm) of Sephadex G25, equilibrated with polymerization buffer (20 mM sodium phosphate, 3.4 M glycerol, EGTA 1 mM, $MgCl_2$ 10 mM, 0.1 mM GTP, pH 6.5), followed by passage through a gravity column of Sephadex G25 (1 cm × 10 cm) equilibrated with the same buffer. Protein concentration was measured spectrophotometrically with a Perkin-Elmer spectrophotometer Beckman DU70 at 275 nm with an extinction coefficient of 1.09 L g^{-1} cm^{-1} in guanidine hydrochloride in neutral aqueous buffer.

Microtubule assembly assay

Microtubule assembly was performed with 18 μM tubulin in polymerization buffer. The aliquots were incubated for 40 min at 4 °C prior to start the reaction by warming the samples at 37 °C in thermostated cuvettes. The mass of polymer formed was monitored by turbidimetry at 350 nm with a Jasco V-750 spectrophotometer. Samples containing the compound and their controls had less than 3% residual DMSO.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the Russian Science Foundation (project 19-13-00158).

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