Antitumor Activity of Furanoallocolchicinoid-Chitosan Conjugate

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Abstract

Colchicine irreversibly binds to tubulin, blocks microtubule formation, and inhibits cell division. However, its usage as an antitumor agent is limited due to its distribution to many tissues and low accumulation in the tumor. The increase in molecule weight can change colchicine biodistribution and decrease side effects. The aim of this work was to study in vivo and in vitro antitumor activity of colchicine-chitosan conjugate. A new allocolchicine derivative – furanoallocolchicinoid 3 was synthesized and conjugated to chitosan (4). Both 3 and 4 induced in vitro tubulin reorganization, cell cycle arrest, and inhibition of cell proliferation in 2D and 3D cultures. Antitumor effect of chitosan, 3, and 4 was studied in Wnt-1 breast tumor bearing mice. Conjugate 4 demonstrated significantly better tumor growth inhibition than 3 possibly as a result of a better accumulation in the tumor.

Keywords: Furanoallocolchicinoid; Chitosan; Multicellular tumor spheroids; Wnt-1 breast tumor

Introduction

Colchicine is a small 400 Da hydrophobic molecule which passively penetrates cells and irreversibly binds to α-tubulin by this mean preventing microtubule formation and cell division [1-4]. Modernly colchicine is approved by FDA for the treatment of acute gout and familial Mediterranean fever, Behcet disease, chondrocalcinosis, and other microcrystalline arthritides [5,6]. Potential indications include primary biliary cirrhosis, psoriasis, amyloidosis, various dermatitis, relapsing polychondritis, necrotizing vasculitis, Sweet’s syndrome, leukocytoclastic vasculitis [7-9]. Due to its extremely specific antimitic activity, colchicine can be a potent antitumor agent. High therapeutic doses (50-100 mg/m²) induce relatively mild side effects the commonest of which (5-10%) is general gastro-intestinal toxicity [10-12]. Earlier studies demonstrated that colchicine antineoplastic therapeutic index is narrow [11]. Besides, in rare cases on average well tolerated doses of colchicine can be lethal [10,13]. Modernly several clinical trials are being conducted to find safer protocols for mitotic inhibitors such as colchicine, vincristine, taxol, and taxols to treat advanced cancer [14-17].

Colchicine is a small hydrophobic molecule able to bind serum albumin and to accumulate in leukocytes [18,19]. Blood colchicine half-life was estimated as 17 hours and maximal blood concentration was found 2 h after injection [20]. Biodistribution studies were conducted 20 years ago by Mehta et al. using autoradiography [21] and recently by Erfani et al. [22]. The authors demonstrated that tumor/muscle ratio was around 4 at 1 h after injection [21-22]. This is relatively high however, liver, intestine, kidney, and heart accumulated colchicine at ratios 11, 5, 7, and 6 accordingly [21] showing that the major obstacle is a high accumulation of colchicine in non-target organs. Close data were obtained by Satpati et al. who showed tumor/blood and tumor/muscle ratios 0.14 and 1 at 1 h after injection that increased to 1 and 4 at 24 h accordingly [20]. Colchicine as well as other hydrophobic drugs is likely to have a high partition coefficient (log (P)). For example, paclitaxel log (P) was >4 as determined by Colby et al. using dialysis partitioning experiments [23]. Most anti-microtubules have distribution coefficient log (D) from 2 to 7 [24]. This means that colchicine partitions quickly into adjacent cell membranes (lipophilicity), primarily binding blood leucocytes and lately – endothelial and epithelial cells.

Unspecific tissue partition of colchicine may be reduced by decorating it with hydrophilic groups such as PEG [25]. Another way to sequester colchicine from non-target organs is to increase its molecular weight (MW) [25]. Indeed, the increase in the molecule size can improve the therapeutic index of anticancer agents via “enhanced permeability and retention effect” [26] due to an increased uptake of macromolecules by tumors.

Drug conjugates are intensively studied. The concept of “smart” antitumor drug conjugate was first proposed by Ringsdorf [27]. The Ringsdorf model implies a conjugation of the drug to a biocompatible polymer backbone and additionally its decoration with a targeting moiety able to deliver the conjugate to a particular tumor associated target. Basing on Ringsdorf model multiple drug conjugates were developed. PEG is one of the most popular polymers used to reduce hydrophobicity and to increase MW of many antitumor drugs including colchicinoids [28-32]. Besides, PEG also shields colchicine from interactions with plasma albumin. Of note, the only nanosized drug conjugate with anti-microtubule agent recommended for the treatment of metastatic breast cancer – Abraxane, is paclitaxel-albumin nanoparticles [33]. The results of in vivo studies demonstrate that drug conjugates better accumulate in tumors and induce fewer side effects [28-35].

However, PEG molecule possesses only one reactive group which can be used for conjugation. It makes it difficult to equip the conjugate with a targeting group as well as to immobilize several molecules of a drug on the long polymeric backbone.

Among many biodegradable and biocompatible polymers, chitosan and its derivatives are ones of the best. Chitosan is a biodegradable nontoxic polycation with multiple reactive groups easily used to obtain derivatives with a desired charge and hydrophobic properties.
[36]. Unmodified chitosan is soluble in diluted acids and aggregates at neutral pH. Substitution of amino groups for carboxyl ones increases chitosan solubility at neutral pH while hydrophobic chitosan derivatives are prone to form nanoparticles spontaneously in aqueous solutions [37,38].

This study is designed as a "proof-of-concept" one. Recently we have synthesized a series of potent allocolchicine analogues - furanoallocolchicinoids [39]. In this work we tried to obtained a conjugate of furanoallocolchicinoid 2 [39] with 40 kDa chitosan and to study its in vitro and in vivo activity.

## Materials and Methods

### Materials

Medium molecular weight (MW) chitosan with deacetylation degree 0.94 (Aladdin Chemistry Co., Ltd., Shanghai, China); N,N'-carboxymethylidimiazole (CDI) (Sigma); succinic anhydride (Pierce); 1-ethyl-3-[3-dimethylaminopropyl] N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Pierce); N-hydroxy succinimide (NHS) (Reanal) were used. Chitosan was purified by an extensive dialysis. All other chemicals were of analytical grade and used as received.

### Methods

#### Dynamic Light Scattering (DLS):
The average molecule size of 3 and 4 was determined using 90 Plus Particle Size Analyzer (Brookhaven, United States) in water (25.0 ± 0.1°C) at a scattering angle of 90° and wavelength of 661 nm using Big Particle Sizing Software. Zeta potential of NPs was determined in 10 mM KCl solution using identical Big Pal Zeta-Potential analyzer hard-ware and software.

#### MTT-assay:
Cytotoxic effect of 1-4 derivatives was estimated by a standard 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) test in 2 dimensional (2D) conditions as was described earlier [40,41]. To analyze the effect of the preparation in 3D conditions 96-flat bottom plates were coated with poly(2-hydroxyethyl methacrylate) (PolyHEMA, Sigma), cells were seeded and cultivated overnight to form 3D spheroids. Compounds 1-3 were dissolved in dimethylsulfoxide (DMSO) to 20 mM concentration and stored at -20°C until the assay. Solution of the conjugate was prepared in 10 mM KCl. Compounds 4 were dissolved in 20 µM of chitosan, which was an equivalent amount of chitosan in 10 µg/mouse of chitosan, which was an equivalent amount of chitosan in 200 µl of saline intravenously.

#### Cell cycle analysis:
Cell cycle was analyzed using propidium iodide stained DNA. Cells were incubated with colchicine derivatives for 72 h in 2D or 3D conditions, trypsinized, washed in ice-cold PBS, fixed with 70% cold ethanol and left for 2 h at -20°C. Important, trypsinization of multicellular tumor spheroids was incomplete. Addition of collagenase A (Roche, USA) at 0.3 units/ml to trypsin/EDTA solution helped to obtain single cell suspension. Thereafter, the cells were washed twice in PBS, stained with 50 µg/ml of propidium iodide (Sigma Chemical Co) in PBS, 10 µg/ml of DNAse and analyzed by flow cytometry using FACSscan device (BD, USA). Cell cycle analysis was repeated multiple times with the same results. The results were analyzed using WinMDI 2.8 software.

### In vivo experiments:
C57BL/6 mice were purchased from Pushchino Affiliation of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow. All mice were 6–8 wk old and maintained in 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. Wnt-1 tumor cells [42] (10^5/mouse) were obtained from a frozen stock, thawed and inoculated subcutaneously into the left inguinal mouse fat pad (MFP #4). The injection of cells in 100 µl of PBS was performed through the skin of anesthetized mice. Tumor size was measured with vernier calipers twice a week and calculated using the formula (W^2×L)/2, where W and L correspond to width and length of tumors. Before the treatment, mice were sorted to obtain comparable average volume of tumors (50–200 mm³) in all groups (n=4-5). Allocolchicine derivatives were injected in the dose 10 µg/mouse (400 µg/kg) of colchicine equivalent in 200µl of saline intravenously into the orbital sinus of mice at days 20, 25, 30, and 35. Control group was injected with saline only. Chitosan group was injected with 200 µg/mouse of chitosan, which was an equivalent amount of chitosan in sample 4. The experiment was terminated at day 40 when two control mice died.

### Experimental

#### Synthesis of allocolchicine derivatives
Furanoallocolchicinoid 2 (N-(115)-2-(Hydroxymethyl)-1,2,3-

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trimethoxy-6,7-dihydro-1H-benzo[5',6':5,4]cyclohepta[3,2-f]benzofuran-1-yl)acetamide) was synthesized as described earlier [39]. To obtain 4-(((1S)-1-acetamido-2,3,-trimethoxy-6,7-dihydro-1H-benzo[5',6':5,4]cyclohepta[3,2-f]benzofuran-1-yl)methoxy)-4-oxobutanoic acid) 3, sample 2 (50 mg, 0.12 mmol) and succinic anhydride (12 mg, 0.12 mmol) were dissolved in 2 ml of tetrohydrofuran under an inert atmosphere; then 17 µl of triethylamine (0.12 mmol) was added. The mixture was stirred for 2 h at 20°C and then 12 h at 70°C. After the reaction was completed (TLC control), the resulting solution was extracted with EtOAc, washed with brine and dried over Na2SO4. The solvent was removed under reduced pressure. The pure product 3 as white crystals (42 mg, 67%) was isolated by column chromatography, eluent petroleum spirit - ethyl acetate - ethanol (1:1:1).

1H NMR (400 MHz, CDCl3): δ 8.60 (d, J = 8.0 Hz, 1H), 7.61 (s, 1H), 7.45 (s, 1H), 6.85 (s, 1H), 6.75 (s, 1H), 5.26 (s, 2H), 4.75 (dd, J = 11.6, 6.3 Hz, 1H), 3.89 (d, J = 5.6 Hz, 6H), 3.46 (s, 3H), 2.70 – 2.59 (m, 4H), 2.54 (dd, J = 12.3, 5.2 Hz, 1H), 2.34 - 2.20 (m, 2H), 2.04 (s, 3H), 1.94 (td, J = 11.5, 6.5 Hz, 1H). 13C NMR (101 MHz, DMSO): δ 175.70, 172.96, 170.56, 154.64, 154.49, 153.52, 147.92, 140.46, 134.85, 130.35, 128.77, 127.91, 127.30, 109.03, 107.01, 106.83, 60.65, 60.65, 57.43, 56.79, 51.37, 37.31, 31.23, 30.99, 30.10, 22.75. Both 4- and 5-10 times higher than of comparable with colchicine 3, prepared at 1:5, 1:15, and 1:45 ratio was used. Finally, chitosan with MW 40 kDa was used to obtain conjugate 4.

The specific allocolchicinoid activity depended directly on the amount of immobilized groups (Figure S1, A-B). Concentration of 3 in 4 was determined by the comparison of IC50, calculated from inhibitory titration curves obtained in Colo-357 and MiaPaCa-2 cell lines (Figure S1, C-D). As it was shown earlier [39] activity of the compound 2 was 5-10 times higher than of 1, however the activity of the compound 3 containing -COOH group needed for chitosan conjugation, was comparable with colchicine 1 (Figure S1, C-D). Activity of conjugates 4 prepared at 1:5, 1:15, and 1:45 chitosan to structure, namely, 3 was conjugated to chitosan with different MW (200, 40, and 12 kDa) and at different chitosan to 3 ratios (1:5, 1:15, 1:45). Conjugate with chitosan 200 kDa aggregated during storage; the activity of conjugate with chitosan 12 kDa was low possibly due to a relatively short backbone of chitosan molecule to immobilize several molecules of the compound 3. Finally, chitosan with MW 40 kDa was used to obtain conjugate 4.

Scheme of synthesis, structures, NMR spectra and size distribution for the compounds 1-3 and de novo synthesized conjugate 4 are shown in Figure 1. Chitosan protons in NMR spectrum of the conjugate 4 interfered with the signals from 1-3 at <5 ppm (Figure 2), however peaks at >5 ppm were specific (shown with braces) for 1-3.
The size of 1-3 molecules was below the limit of DLS (<5 nm). Conjugation of 3 with chitosan at ratio 45 to 1 resulted in the size increase of 3 in at least up to 8 times. The sizes of chitosan and 4 molecules were determined by DLS and were comparable (28-40 nm). Unmodified chitosan, used to prepare 4, could be dissolved only in diluted acids as its ζ-potential was +30 mV, while the conjugate 4 was not soluble at acidic pH and was dissolved in PBS at neutral pH. When measured, ζ-potential of 4 appeared to be -4 mV which was a result of chemical modifications. Negative ζ-potential of the drug used for IV injections is preferential as positively charged substances interact with negatively charged cells and thus are more toxic [43]. Besides, positively charged chitosan derivatives and chitosan based nanoparticles poorly penetrate cells than negatively charged ones [44].

Functional activity of 4 was determined as described above using 5 different cell lines including W1204, a Wnt-1 related murine epithelial cell line [42]. Activity of 1-3 is shown averaged for 5 cell lines (Figure 3A), and individual titration curves for 4 are shown with thin lines while averaged data are shown with a bold one (Figure 3B). As shown above, specific activity of 2 was higher than of colchicine 1 while its derivation with -COOH group decreased it to the colchicine one (Figure 3A). Functional activity of 4 was determined as 0.8 mM which corresponded to 25 times dilution of 3 (20 mM) due to chitosan conjugation.

Functional activity of colchicinoids in vitro

 Functional activity of 1-4 was tested in 2D and 3D cultures using human epithelial cell line Colo-357 and murine epithelial cell line W1204 (Figure 3), which originates from Wnt-1 breast tumor used in this study to estimate in vivo activity of furanoallocolchicinoid conjugate 4. 3D cultures are believed to represent closer avascular in vivo tumors. Usually activity of antitumor drugs are lower in 3D cultures in comparison with 2D ones as it was shown for many antineoplastic drugs including antitubulins such as paclitaxel and vincristine [45]. However, 3D cultures were never tested to estimate sensitivity of cells to colchicinoids. The only publication on colchicine analogue E-combretastatin describes the penetration of the drug into 3D spheroids [46] while it does not compare cell sensitivity to the drug in 2D and 3D conditions. Thus, our data are the first study of colchicine, furanoallocolchicine and furanoallocolchicinoid-chitosan conjugate in 3D cultures.

Data for 2D and 3D cultures demonstrated that in both cases the cells were sensitive to 1-4 as was demonstrated by the dose-dependent curves. However, the cumulative cytostatic activity of 1-4 was 2-3 times

![Figure 3: Effect of preparations 1-4 on cell proliferation. A. Inhibition of cell proliferation by 1-3. Results are shown as average inhibitory indices (II) for the titration curves obtained for Colo-357, MiaPaca-2, HaCaT, EA.hy926, and W1203 cells. Vertical lines show IC50 values. B. Determination of effective furanoallocolchicinoid concentration in 4. Compound 4 was titrated v/v and incubated with Colo-357, MiaPaca-2, HaCaT, EA.hy926, or W1204 cells (thin lines). Average inhibitory curve (bold line) was used to estimate IC50 for 4. Final concentration of 3 in 4 was determined by comparison of IC50’s for 3 and 4. C-F. Colo-357 (C-D) and W1204 (E-F) were grown in 2D (C, E) and 3D (D, F) in perforated cells in control (A-B) and treated (C-F) cultures. Scale bars correspond to 13-15 µm.](image-url)
higher in 2D cultures (Figure 3C-F) as is evidenced by 80-90% maximal inhibition of cell proliferation in 2D cultures and only 30-40% - in 3D ones (Figure 3C-F, shown with horizontal lines). This was true both for human (Figure 3C-D) and murine (Figure 3E-F) cells. IC₅₀ also was ~5 times higher in 3D cultures. This result can be explained by the activation of multidrug resistance transporter P-glycoprotein in 3D conditions absent in 2D conditions [47].

No difference in the activity was found between 1-4 in 3D cultures while the compound 2 was 5 times more active in 2D cultures as it was shown earlier [39].

Effects of the compound 3 and the conjugate 4 on β-tubulin organization was studied in 2D and 3D cultures in Colo-357 cells using confocal microscopy. The results are shown for β-tubulin organization (Figures 4 and 5, A, C, E) and nuclei structures (Figures 4 and 5, B, D, F). Both 3 and 4 disrupted tubulin microtubules and blocked mitotic spindle formation in 2D and 3D conditions.

Colchicine is also known and a vascular contacts disrupting agent [48,49]. Moreover, it was shown that the disruption of microtubules with colchicine resulted in actin microfilament rearrangement in resting aortic endothelial monolayers [49]. To study whether it is true in epithelial in comparison with endothelial cells we also compared F-actin expression in 3D cultures after cell treatment with 3 and 4. It appeared that F-actin expression and arrangement was unaffected by the compound 3 and the conjugate 4 in both types of cells (Figure 6).

One of the basic feature of colchicinoid action is a cell cycling suppression. Incubation of epithelial cells with the compounds 1, 3, and 4 induced cell accumulation in G2/M phase and a significant apoptosis both in 2D and 3D cultures (Figure 7). Of note, murine cells W1204 proliferated less in 3D conditions (Figure 7, I-J) and changed DNA condensation in G1 phase (note the shift of M2 fraction in treated versus control cells). Besides, the number of cells in S-phase was also increased (Figure 7, J, arrow).

Antitumor activity of furanoallocolchicinoids in vivo

Chitosan is a nontoxic polymer. It did not induce tubulin reorganization or cell cycle inhibition (Figure S2) however it could affect tumor growth. To exclude its effect, an additional control group treated with an equivalent amount of chitosan was included in the study. The results demonstrated that the conjugate 4 was significantly (p<0.05) more effective than the compound 2, and both 2 and 4...
significantly inhibited the tumor growth in comparison with the control and chitosan groups (Figure 8).

Conclusion

The results of this study demonstrated that the decoration of small hydrophobic molecules could serve several important purposes: it makes the drug less lipophilic, increases the molecule size, shields the drugs from the instant contact with blood cells, and possibly decreases drug accumulation in non-target organs, resulting in an improved antitumor efficacy and possibly few side effects. These findings are in a good agreement with earlier published data [28-35]. Although in this work we did not study the biodistribution of 3 and 4 in tumor and non-target organs, the results of other groups show that conjugates of a small size such as antibody-drug ones demonstrate the same biodistribution as an antibody alone [50], while nanosized carrier accumulated better in the tumor than in non-target organs [51].

The polymers often used to decorate active substances such as PEG, PLA, liposomes, in many cases lack multiple reactive groups needed to load them with several moieties by this limiting the functional activities of the conjugates [33]. Among few biocompatible polymers possessing multiple reactive groups, chitosan is one of the best candidates for the development of drug delivery systems. Modernly chitosan is used in medicine with no side effects as wound healing films, hemostatic sponges, and hydrogels for regenerative medicine [52,53]. The obstacle in a wider clinical application of chitosan is its poorly standardized structure. Due to the availability of multiple reactive groups, chitosan interacts with its own side chains and plasma proteins via electrostatic, hydrophobic, Van-Der-Waals forces and hydrogen bonds [54]. This makes chitosan a flexible molecule. We showed that a direct conjugation of 3 to chitosan did not affect specific colchicine activity. Thus, no special degradable linkers were required. Taken into account rather simple synthesis of furanoallocolchicinoids 2 and 3, unlimited renewable sources and cheap production of chitosan, colchicine-chitosan conjugates can be considered as highly perspective medications for cancer treatment.

Conflict of Interest

There are no financial or other issues that might lead to conflict of interest.

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