Synthesis of thicolchicine-based conjugates: investigation towards bivalent tubulin/microtubules binders

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Abstract: Four different hybrid compounds have been efficiently synthetized by conjugation of deacetylthiocolchicine with pironetin inspired derivatives. The modest bioactivity and the apparent absence of interaction with α-tubulin is explained by a posteriori in silico investigation that suggested a relevant distance between the thiocolchicine binding site and the proper pocket on the α-tubulin. The modest activity on resistant cells suggested that the lipophilic nature of the used linker renders the obtained compounds better substrates for p-Gp efflux pumps. The study better clarifies the design of hetero tubulin/microtubules-targeting bivalent compounds.

Keywords: bivalent binder, microtubules, pironetin, tubulin binder, thiocolchicine,

Introduction

Microtubules (MTs) are highly dynamic structures, diffused in eukaryotic cells, as well as in some bacteria.[1] They are fundamental components of the cytoskeleton and they play important roles in several essential cellular processes, such as cell division, locomotion, intracellular transport and cell shape definition. MTs are originated from the head-to-tail arrangement of α,β-tubulin heterodimers, leading to the formation of cylindrical assemblies. In physiological conditions, MTs are characterized by alternating phases of growth and shrinkage, a behaviour known as “dynamic instability”. This dynamism is essential for MTs correct functionality, in particular for the formation of the mitotic spindle during the mitosis process.[2] The perturbation of this dynamic behaviour can result in deleterious effects for the cells, halting the cell replication and inducing cellular apoptosis. Therefore, MTs represent very suitable drug target, for anticancer therapy and also for neurodegenerative diseases treatment, as recently emerged. In fact, MTs dynamic instability is essential in many neuronal activities, which are regulated by tubulin post translational modifications.[3] Depending on the mode of action, MT-targeting agents can be divided into two different families: MT-stabilizing agents and MT-destabilizing agents. In the last decade, both these classes have been successfully applied in the treatment of several cancer types,[4] and the search of new cytotoxic or neuroprotective agents is still of primary interest. Thus, our interest in the obtainment of new molecules able to target tubulin and MTs, prompted us to plan the synthesis of new tubulin/microtubules-targeting bivalent compounds. Bifunctional drugs, bearing chemical entities that are able to bind to separate sites of the protein, recently attracted the attention of the scientific community, thanks to their possible great pharmacological potential. The idea of conjugating different MTs binders into bivalent entities led to the obtainment of novel antimitotic agents, including taxoid-colchicine,[5] vinca alkaloids-taxoid hybrids,[6] colchicine–tubulizine,[7] colchicine-pironetin,[8] podophyllotoxin-thiocolchicine and hybrids merging histone deacetylase inhibitors with different tubulin binders.[9], [10] On one hand the use of too short or rigid linkers prevents the interaction with the desired binding site and leads to steric clashes. On the other hand, the use of a too long and flexible linker may lead to solubility and cell internalization problems.[11] As the linker seems to play a critical role tuning the bioactivity of these class of molecules, we planned the synthesis of new bivalent compounds, constituted by a well known β-tubulin binder (thiocolchicine) and a pironetin inspired compound (hybrid **1**, Figure 1) connected by linkers of different types and length, to investigate how their properties affect the binding capability.

Results and Discussion

For the first explorative studies a 10-atom all-carbon chain and a 24-atom pseudo-peptidic chain were taken into account. As active units, we choose thiocolchicine and hybrid compound **1** as models of β- and possible α-binder, respectively (Figure 1). Compounds **1** were recently designed in our laboratory, as the merging of two natural compounds, pironetin and dumetorine.[12] Pironetin is an α-tubulin binder, characterized by a δ-unsaturated lacton, which serves as Michael acceptor for the

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Cys316 residue inside α-tubulin binding site.[13] It is noteworthy that while several β-tubulin binders are currently available, only few molecules are known to bind α-tubulin.
The modification of pironetin structure, introducing the piperidine ring typical of dumetorine, resulted in the obtainment of an anchor point exploitable for the synthesis of bivalent compounds.

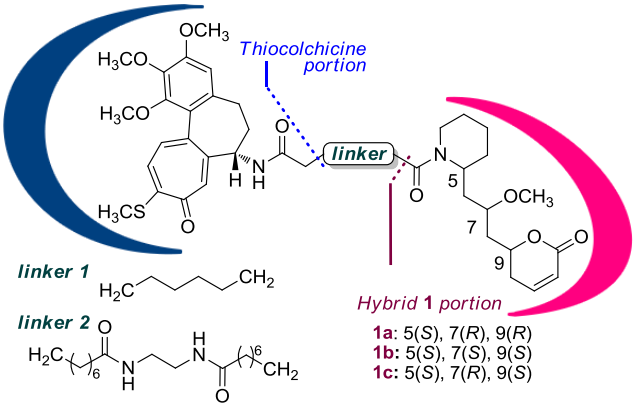
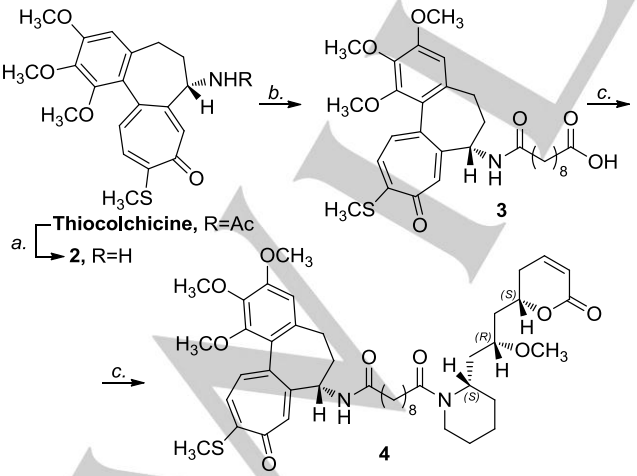


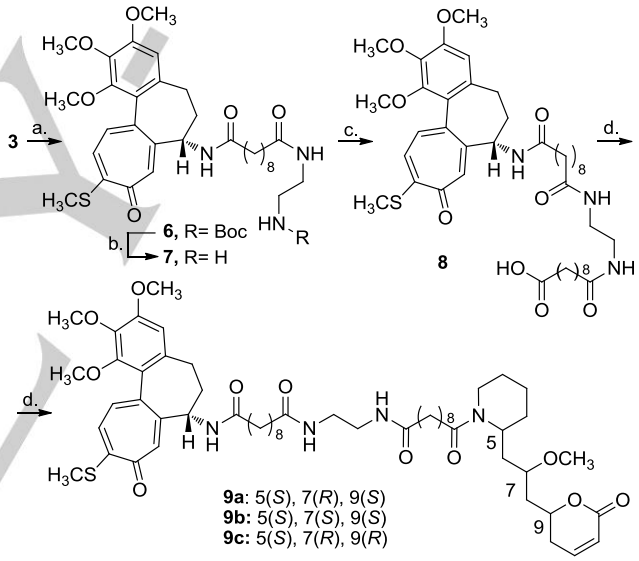
Figure 1. General scheme for the assembly of the conjugate compounds and representation of the employed specific components.

We took into consideration three of the eight prepared stereoisomers,[12] in particular **1a-c,** for the synthesis of the new conjugate compounds on the base of their efficacy in giving stable and pure compounds. First, *N*-deacetyl-10-thiocolchicine **2**, deriving by acidic hydrolysis of thiocolchicine,[14] was converted into compound **3**, exploiting a condensation with sebacic acid. A strict control over the amount of sebacic acid avoided the undesired reaction on both the carboxylic moieties. Reaction of **3** with compound **1a** in the presence of HATU and DIPEA, afforded compound **4**, characterized by a linker of 10 carbon atoms (Scheme 1).



Scheme 1. *Reagents and conditions:* a) HCl 2M, MeOH, 85°C, 3 days; b) sebacic acid, HATU, DIPEA, CH_2Cl_2, rt, 3h; c) **1a**, HATU, DIPEA, CH_2Cl_2, rt, 4 h.

The condensation reaction of compound **3** with **1b** and **1c** gave unexpectedly complex mixture of compounds probably due to the intrinsic instability of the desired compounds. The synthesis of conjugate compounds bearing a longer pseudo-peptidic linker was considered (Scheme 2). To this extent, the 10 carbon atoms chain of compound **3** was furtherly elongated, through the introduction of the *tert*-butyl 2-aminoethylcarbamate fragment **5**, obtained according to reported procedure.[15] The cleavage of the Boc protecting group, upon treatment with TFA led to compound **7**. The obtained free amino group, was exploited for a further reaction with sebacic acid. Under these conditions, the thiocolchicine derivative **8** was obtained as major product. Final compounds **9a-c** were accessed by condensation reaction between **8** and the proper stereoisomer **1**.



Scheme 2. *Reagents and conditions:* a) **5**, HATU, DIPEA, CH_2Cl_2, rt, 3h; b) TFA, CH_2Cl_2, 0°C to rt, 1h; c) sebacic acid, HATU, DIPEA, CH_2Cl_2, rt, 3h; d) **1a-c**, HATU, DIPEA, CH_2Cl_2, rt, 3h.

Biological data. We submitted compounds **4** and **9a-c** to preliminary biological tests, aimed at the elucidation of their activity as tubulin/microtubules targeting compounds. Firstly, they were tested on wild -type and resistant ovarian cancer cells lines (A2780 and A2780AD respectively). Cytotoxicity data (reported in Table 1) seem to suggest that our compounds do not interact through the pironetin-like unit. In fact, if this was the case, they would have accumulated inside the cells, avoiding the P-glycoprotein resistance like other MTs covalent binders usually do.[16] Compound **4** is almost as toxic as *N*-deacetyl-10-thiocolchicine **2** in non-resistant cells but 100 times less toxic in resistant cells; compounds **9a-c** displayed even less toxicity, and also in this case the trend is more marked on resistant cells.

These results prompted us to think that our bivalent compounds interact with tubulin exploiting only the thiocolchicine, justifying in this way the partially retained bioactivity. However, the hydrophobic linker seems to disturb the interaction of pironetin with the target, as well as render our compounds better substrates for P-glycoprotein, as emerged considering that the activity decreases with the length of the linker. Furthermore, to confirm that the retained antiproliferative activity of the obtained compounds was actually related to their capacity to destabilize tubulin, tubulin polymerization assay was performed.

Table 1. Cytotoxicity data.

Compound	IC_{50} [nM][a]		R/S[b]
	A2780	***A2780AD***	
4	11.8±1	4300±600	364
9a	252±7	20500±500	81
9b	205±15	19000±6000	93
9c	196±0.2	46000±1500	235
Colchicine	13.6±2	663±23	49
N-deacetyl-10-thiocolchicine	7.8±1	30.9±0.6	4
Paclitaxel	0.4±0.08	1400±200	3500
Podophyllotoxin	8±1	10.1±0.7	1.2

[a] IC_{50} values determined in the parental ovarian carcinoma A2780 line and the MDR P-glycoprotein-overexpressing ovarian carcinoma A2780/AD10. IC_{50} values in nm were determined after two days' exposure to drugs using the MTT cell proliferation assay. Data are the mean ± SE of at least four independent experiments. [b] Ratio of IC_{50}(resistant cell line)/IC_{50}(parental cell line). Values are the calculated relative resistance of each mutant cell line obtained by dividing the IC_{50} value of the resistant line by the IC_{50} value of the parental line, A2780.

Podophyllotoxin and *N*-deacetyl-10-thiocolchicine were also tested, for comparative purposes. The results of the tubulin polymerization assay are reported in Figure 2. As can be deducted from the graphic, all the tested compounds show a strong destabilizing effect, indicating that the toxicity observed is exerted through inhibition of tubulin assembly. This is coherent with the assumption that the partial activity of our compounds depends from the interaction of the thiocolchicine moiety with tubulin. These results highlight a slightly difference in activity between compound **4** and compounds **9a-c**, differing in linker length. In particular, the plain inability of the obtained hybrid compounds to exploit the dual interaction with tubulin/microtubules can be inferred from the data. These observations prompted us to investigate the binding mode of these compounds through molecular modelling studies.

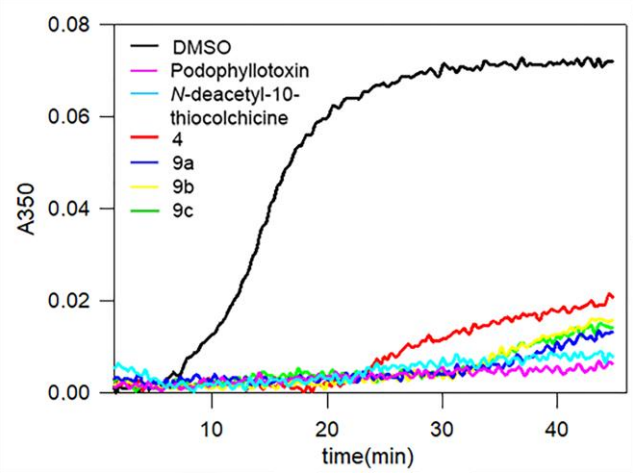


Figure 2. Time course of 25 μM tubulin polymerization at 37 ºC measured by 350 nm turbidimetry in the presence of DMSO vehicle (control) or 27.5 μM of ligand. The assay was performed as triplicate and the graph represent the mean of the three experiments.

Molecular dynamics simulations. Molecular Dynamics (MD) simulations are a useful tool to assess the conformational stability of a ligand within the binding site. MD simulations were performed on compounds **4**, **9a**, colchicine and *N*-deacetyl-10-thiocolchicine in complex with a tubulin α-β dimer. We chose the PDB 4O2B[17] (tubulin-colchicine complex) as the starting structure for MD simulations. This choice is justified by a higher resolution compared to any tubulin-thiocolchicine solved structure.[11] Moreover colchicine and thiocolchicine are very similar molecules and the conformation of the residues forming the binding site is largely superimposable. The experimental results suggested that ligands interact with tubulin through the *N*-deacetyl-10-thiocolchicine moiety. Accordingly, in the MD starting structures the *N*-deacetyl-10-thiocolchicine nucleus of each ligand was placed into the colchicine binding site while the pironetin moiety was placed between α-β subunits (see Supporting information, Figure S1). A 50ns MD simulation for each complex was performed and subsequently the root mean square deviation (RMSD) were computed with respect to the staring structure for each ligand after least square fit to protein C_α (Figure 3). From these data it resulted that the longer is the linker, the greater is the displacement of the ligands within the binding site.

On the other hand, colchicine and *N*-deacetyl-10-thiocolchicine show a low and stationary RMSD trend. In Figure S2 (Supporting information), the superimposition of the final structure of the MD of compound **4** with the crystallographic structure of tubulin-thiocolchinine complex can be compared (PDB ID: 5LP6). It is possible to infer that the presence of the linker has a displacing effect on the thiocolchinine nucleus, which can lead to a partial loss of its key hydrophobic interactions with the β-monomer.

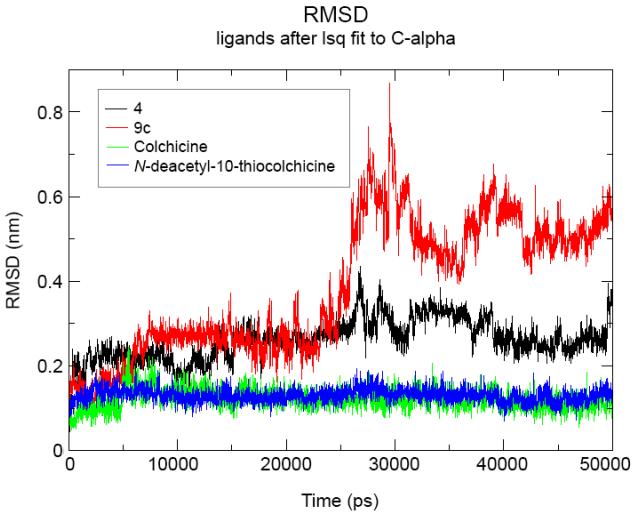


Figure 3. RMSD plot for the ligands. Colchicine and *N*-deacetyl-10-thiocolchicine show a low and stationary value of RMSD while the linker of compounds **4** and **9a** has a negative effect on the conformational stability of the molecules within the binding site.

Moreover, the root mean square fluctuations (RMSF) for ligands with heavy atoms was considered (Figure 4). Once again, it is evident that the presence of a long linker (compound **9a**, red line) leads to a higher mobility of the thiocolchicine moiety. This effect is less pronounced in the case of compound **4**, which has a shorter linker. Colchicine and *N*-deacetyl-10-thiocolchicine show the lowest RMSF values, suggesting that any of these linkers has a negative impact on the ligands binding mode.

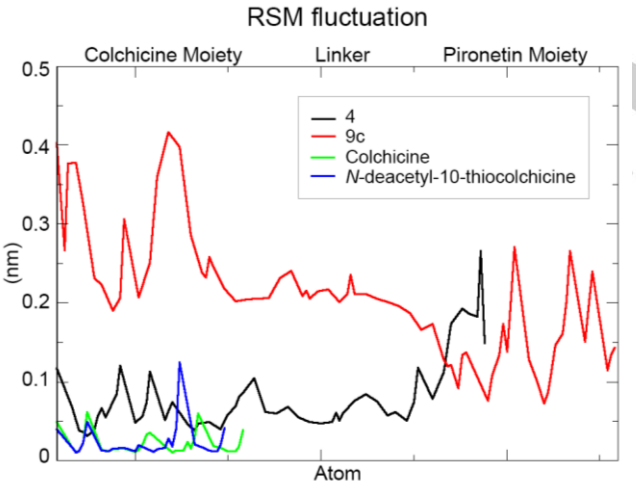


Figure 4. RMSF plot for the ligands. The fluctuations are higher for compound **9a**, which has the longest linker.

Conclusions

In conclusion, four different conjugate compounds have been efficiently synthetized by linking thiocolchicine nucleus with pironetin inspired derivatives. One of them presents 10 atom linker and three of them present 24 atom (C_{10}-N-C_2-N-C_{10}) linker. The detected reduced bioactivity and the apparent absence of interaction by the α-tubulin binder prompted us to employ an *in silico* approach to evaluate the binding mode of the compounds. The MD results suggest that a longer linker has a displacing effect on the thiocolchinine nucleus, probably resulting in a loss of activity. The observed diminished activity on resistant cells whispers that the lipophilic nature of the linker renders the described compounds, better substrates for p-Gp efflux pumps. The design and the synthesis of conjugate tubulin/microtubules targeting compounds by linking one alfa- with one beta-binder is feasible and handle. In contrast this study demonstrates that the aim to improve tubulin/microtubules targeting by an α,β–bivalent binder sounds unlikely due to the required dimension and the tricky relevance of the linker.

Experimental Section

Experimental details (chemistry, molecular dynamics simulations and Biology) are given in the Supporting Information.

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Keywords: bivalent compounds, thiocolchicine derivatives, tubulin binders.

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FULL PAPER

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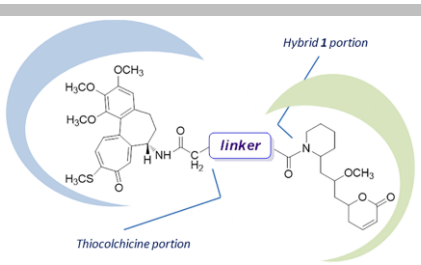

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