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Preparation of Fluorescent Tubulin Binders

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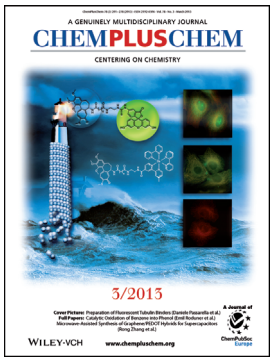
Thiocolchicine, taxol and cephalomannine have been used as building blocks for the preparation of four different fluorescent compounds designed to image the tubulin/microtubule network in cells. Thiocolchicine gave the best results and, in particular, the compound derived from conjugation with fluorescein minimally inhibits tubulin polymerization, is cell permeable and binds microtubules. Thus, it meets some of the demanding requirements for a new fluorescent dye that can secure a direct assay to evidence the tubulin/microtubules network in cells.

Introduction

Imaging of tissues or intracellular structures is an important goal in biomedicine.[1] Radioactive molecular imaging,[2] magnetic resonance imaging,[3] and optical imaging[4] have been recognized as powerful techniques in this field. Easy manipulation and high sensitivity make optical imaging the most widely used technique. In light of our interests concerning the biology of tubulins and their implication in cancer and neurodegenerative diseases[5] we need fluorescent dyes specific for tubulin which provide a useful tool for in-cellular and in vivo imaging studies. Among the current approaches to investigate tubulin and microtubule organization in cells, transfection of GFP-constructs and microinjection of fluorescent tubulin are widely used being efficient in tracking cytoskeleton dynamic processes. With the aim of obtaining new fluorescent dyes that can secure a direct assay to evidence the tubulin/microtubules network both in cells and in vivo and to overcome the invasive techniques of transfection or microinjection as well as the efficient, but time-consuming, immunofluorescence staining (Figure 1), we designed a small library of modified tubulin binders directly conjugated with fluorescent probe molecules.[6]



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Based on our experience,[7] the formation of conjugate compounds by modification of known tubulin binders modulates their effects on tubulin/microtubules dynamics. For this reason, we planned to use as tubulin binders thiocolchicine, paclitaxel and cephalomannine, which are representative destabilizing and stabilizing agents. We made use of fluorescein isothiocyanate (FITC) and an osmium–terpyridine complex previously reported by us[8] and others[9] for the introduction of fluorescent moieties (Figure 2).

Results and Discussion

Fluorescein presents a number of attractive features: a) its excitation wavelength (488 nm) does not interfere with the absorbances of cellular proteins, b) it has been extensively used in the preparation of fluorescent sensors,[10] c) it is easily accessible and d) it does not require sophisticated microscopic instruments.

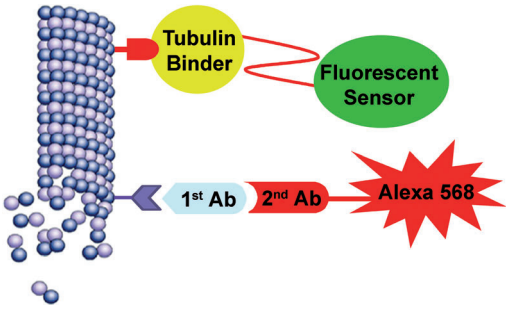

Figure 1. Comparison of new modified tubulin binders directly conjugated with fluorescent probes with time consuming immunofluorescence staining.

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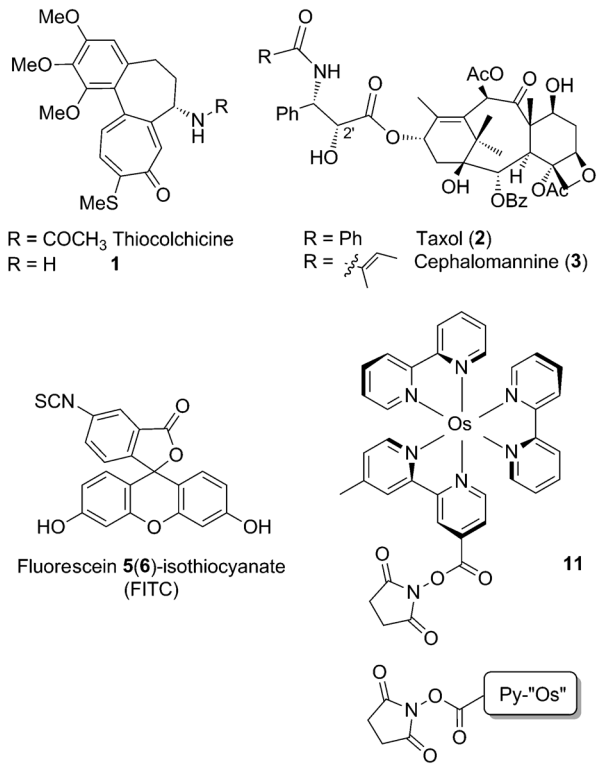


Figure 2. Structures of the building blocks used in this study. Bz = benzoyl.

To prepare our fluorescent probes, deacetylthiocolchicine was treated with suberic acid to give the monoamide derivative **4** that, by subsequent condensation with mono-*tert*-butoxycarbonylaminoethylamine in the presence of DIPEA, secured compound **5** (Scheme 1). Removal of the Boc protecting group with TFA afforded the amine derivative **6** that we used as a key building block in the generation of two different fluorescent compounds. First, we treated the thiocolchicine derivative **6** with FITC. The reaction was carried out in the dark using DMF as solvent and afforded the desired fluorescent product **7** in 80% yield. The fluorescent-tagged product was purified by HPLC and characterized by MALDI TOF MS (*m/z* 960) and ^{1}H NMR analysis. The purity was assessed by HPLC (>95%).

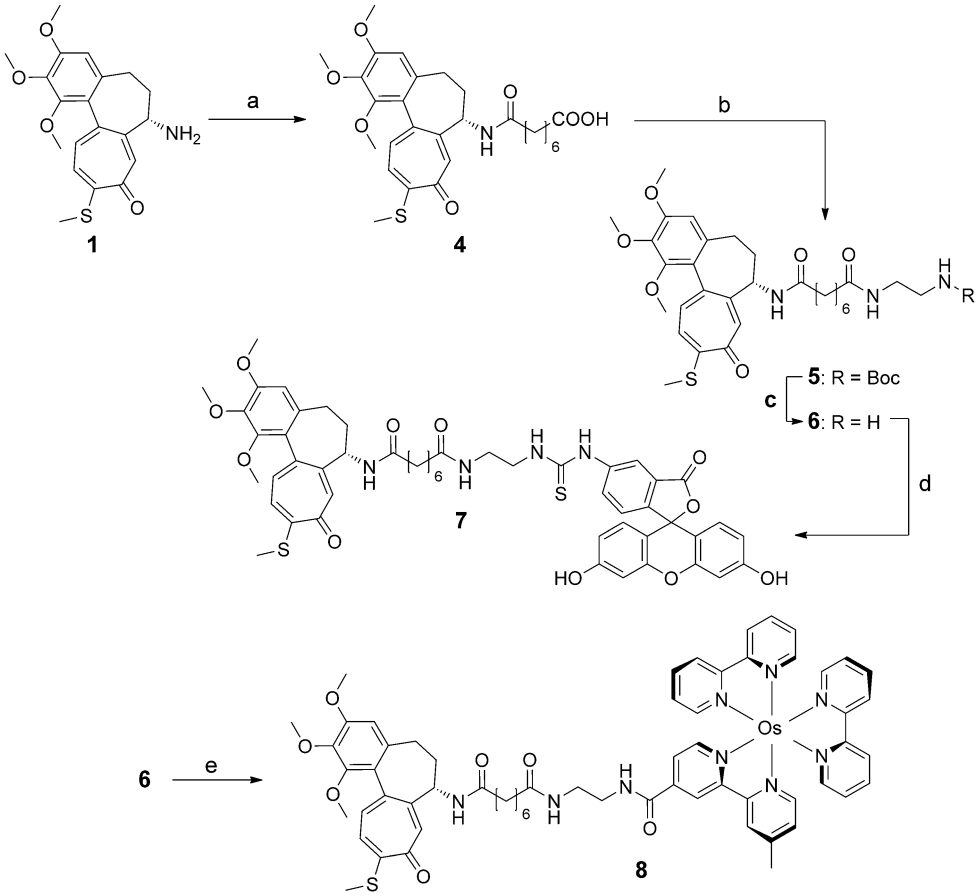
The second fluorescent probe prepared was an osmium–terpyridine complex. There has been a recent surge of interest in the application of luminescent transition-metal complexes as probes and tags in biological imaging and assay. The field has so far been dominated by the use of rhenium(I), ruthenium(II) and iridium(III) complexes.[11] In all these cases, intense luminescence comes from relatively long-lived triplet charge-transfer states with relatively large Stokes shifts that facilitate separation of the excitation and emission wavelengths. Despite the many similarities between the coordination chemistry of ruthenium and osmium, osmium(II) complexes have remained largely unexplored.

This is particularly surprising given that the absorption maxima and luminescence from ^{3}MLCT states of osmium(II) complexes tend to be red-shifted compared to their analogous ruthenium(II) complexes. As such, osmium(II) complexes offer the possibility of excitation towards the red edge of the visible spectrum while observing the emission in the near IR, minimising problems with autofluorescence from biological samples. Osmium(II) bipyridyl complexes have been used as FRET acceptors in probes of nucleotide structure[12] and as an antenna chromophore to sensitise lanthanide luminescence in d–f hybrid probes.[8,13] However, the use of osmium species as a luminescent tag on molecules and assemblies of biological interest remains unexplored. Therefore, the second compound that we synthesized contained, as the fluorescent component, an osmium complex (**11**; Figure 2) that we prepared as reported in Scheme 2. The preparation proceeded using 2-2′-bipyridine and osmium trichloride as starting materials that were combined in degassed DMF in the presence of lithium chloride. The third bipyridine system was introduced by reaction with 4′-methyl-2,2′-bipyridine-4-carboxylic acid. The carboxylic group was activated by reaction with *N*-hydroxsuccinimmide. As previously mentioned, the Os complexes were selected for the long wavelength absorption, which allows excitation with wavelengths up to 700 nm. At these wavelengths, tissue absorbance and autofluorescence are minimal, and excitation can be accomplished with LEDs or laser diodes. The reaction of **6** with **11** gave the desired compound **8** after 48 hours (24 h at RT and 24 h at 60 °C).

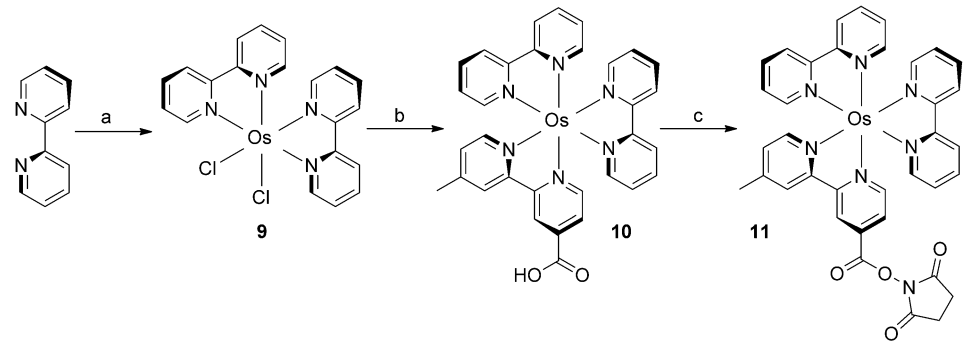
As for the previous product, the purification of the thiocolchicine derivative labelled with the Os fluorophore was performed by preparative HPLC and the desired compound was characterized by MALDI TOF analysis (*m/z* 1271.4) and its purity by HPLC.

The preparation of the taxol and cephalomannine derivatives proceeded by protection of the hydroxy group at position 2′ and the subsequent introduction of the linker at position 7 (Scheme 3). In the case of taxol, the TES group was preferred for the protection of the hydroxy group whereas the cephalomannine was transformed into the corresponding TBDMS derivative. The reaction with 4-nitrophenyl chloroformate and subsequently with N-Boc ethylendiamine secured compounds **14** and **15**, respectively. Treatment with TFA resulted in the simultaneous deprotection of the hydroxy group and the primary amino group, in order to achieve the final reaction with FITC. Unfortunately, compounds **16** and **17** were unstable and difficult to manipulate resulting in low yields in both cases (Scheme 3).

Compounds 7 and 8 have the potential to be used as tools in biological studies depending on their ability to interact with and affect the microtubule system in vitro and in cells. To get an insight into their biological activity, we initially investigated their effects on tubulin polymerization in vitro. Bovine tubulin (purified from brain) was mixed with a standard solution of each compound, in the presence of guanosine triphosphate (GTP), and incubated at 37 °C to allow microtubule assembly. After 30 minutes, the polymerized and the unpolymerized fractions were collected by centrifugation, separated by SDS-PAGE and quantified by densitometry, as previously described.[7d] We report in Figure 3 the ratios of polymerized/unpolymerized tubulin obtained in the presence of the different compounds. As expected, thiocolchicine strongly inhibits tubulin polymerization. The estimated EC_{50} value of the polymerized/unpoly-



Scheme 1. Reagents and conditions: a) $HOOC(CH_2)_6COOH$, BOPCl, TEA; b) $H_2N(CH_2)_2NHBoc$, DIPEA, HATU, CH_2Cl_2, RT; c) TFA, CH_2Cl_2, 0 °C; d) FITC, DMF, RT. e) **11**, DMF. Boc = *tert*-butoxycarbonyl, BOPCl = bis(2-oxo-3-oxazolidinyl)-phosphinic chloride, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TEA = triethylamine.



Scheme 2. Reagents and conditions: a) LiCl, 2,2′-bipyridine, $OsCl_3$, then Et_3N, DMF, reflux; b) 4′-methyl-2,2′-bipyridine-4-carboxylic acid, 70% EtOH, reflux; c) *N*-hydroxysuccinimide, DCC, MeCN, RT. DCC = 1,3-dicyclohexylcarbodiimide.

merized tubulin mixture is of (4.01 ± 1.33) μM whereas the concentration of thiocolchicine required to induce 50% reduction of tubulin polymerization is around 10 μM. On the contrary, we observed that **7** minimally—but not significantly—inhibits microtubule assembly, whereas **8** was completely ineffective.

To investigate if the synthesized compounds are cell permeable and bind tubulin in cells, we incubated human lung carcinoma cell line A549 with the thiocolchicine derivatives according to different experimental protocols. First, we performed an in vivo staining: we loaded cells with the fluorescent compounds, fixed them and carried out immunostaining with a monoclonal anti-α-tubulin antibody to reveal the microtubule network. As reported in Figure 4, control cells display a widespread network of long microtubules, other than the typical accumulation of microtubules, at one side of the nucleus, in the region called the microtubule organizing centre (MTOC). Interestingly, the fluorescent signal corresponding to Compound **7** almost completely overlaps with α-tubulin staining, as demonstrated by the merge pictures. This result suggests that **7** is cell permeable and maintains the ability to bind microtubules in cells. On the contrary, osmium complex **8** was completely undetectable in cells (data not shown) suggesting that this thiocolchicine derivative is not cell permeable and/or has lost the ability to bind microtubules. Second, we assessed the ability of the compounds to bind microtubules in fixed cells. A549 cells were incubated with compounds **7** and **8** after being fixed and immunostained with monoclonal anti-α-tubulin antibody. Figure 4 shows that **7** does not maintain its ability to stain the microtubule network in fixed cells, but displays an unspecific binding to the perinuclear region. As found in the in vivo set of experiments, **8** (TC-Osmium) was completely undetectable (data not shown) confirming its lack of binding to microtubules. The differences observed in the binding of compound **7** to microtubules between live and fixed cells could be explained by the mechanism of action of colchicine.

Indeed, free colchicine itself probably does not bind directly to microtubule ends but it first binds to soluble tubulin, induces slow conformational changes in the tubulin and, ultimately, forms a poorly reversible final-state tubulin–colchicine com-

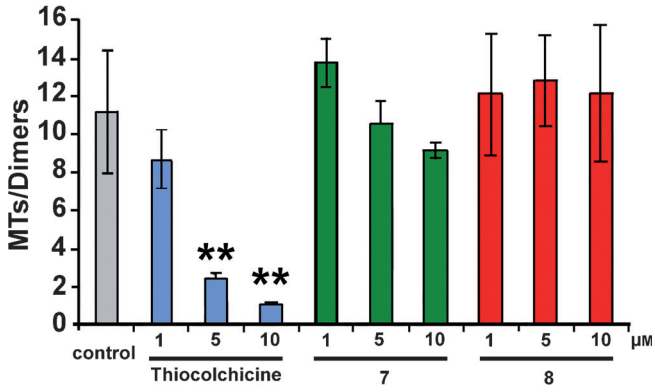


Figure 3. Effects of compounds **7** and **8** on tubulin polymerisation. Thiocolchicine has been used as reference compound.

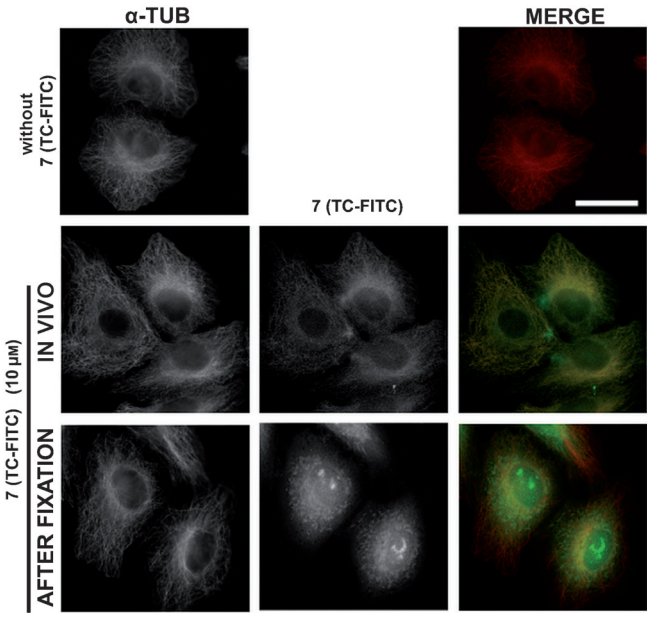
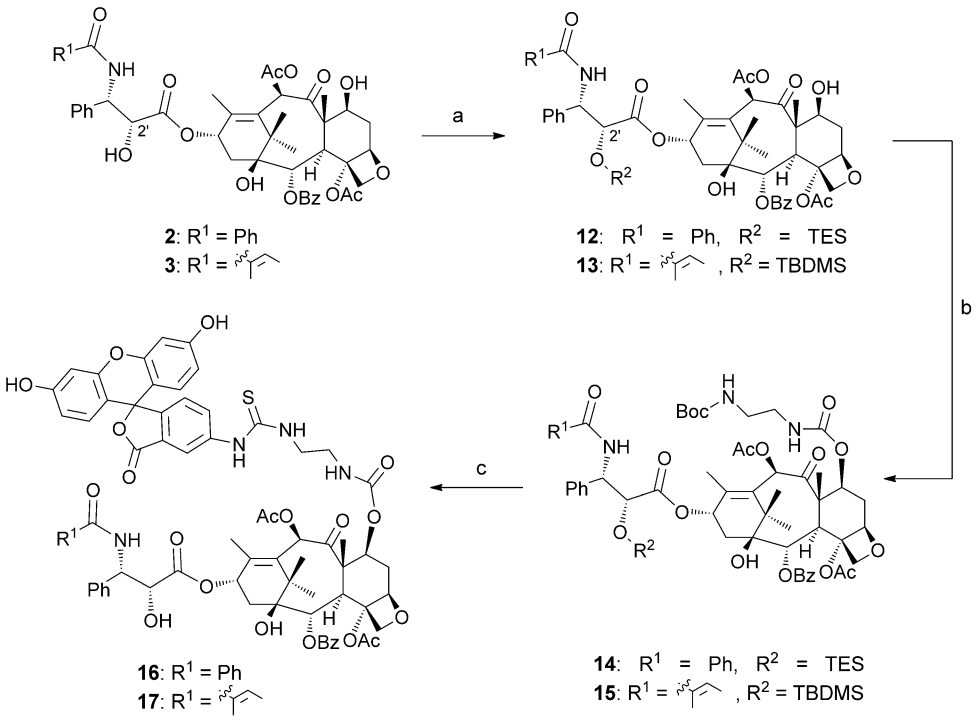


Figure 4. Microtubule network in A549 cells as revealed by immunostaining with antibody anti α-tubulin (α-TUB) in cells not loaded (without **7**) or loaded with 10 μM **7** in vivo or incubated with **7** after fixation. Scale bar, 20 μm.

plex, which then copolymerizes into the microtubule ends.[14] Therefore, if the microtubules/tubulin dimers cannot exchange, as the case of fixed cells reported in Figure 4, compound **7** cannot bind to microtubule. Thus, our results demonstrated the highly specificity of compound **7** for microtubules, when they are free to polymerize/depolymerize, and suggest that its use could be complementary to that of known microtubule stabilizer binder including fluorescent epothilone.[15]

Conclusion

In this study, we describe the preparation of four different fluorescent compounds using thiocolchicine, taxol and cephalomannine as building blocks. Thiocolchicine was modified to give a fluorescein derivative **7** and an osmium derivative **8**. Taxol and cephalomannine were modified to give the corresponding fluorescein derivatives by functionalization of position 7. Unfortunately, the taxane derivatives were unstable. Compound **7** minimally inhibits tubulin polymerization, is cell permeable and binds to microtubules microtubules in a cellular context requiring a dynamic exchange between free and polymerized tubulin. This is to say that it meets some of the demanding requirements for a new fluorescent dye that can secure a direct assay to evidence the tubulin/microtubule network in cells.



Scheme 3. Reagents and conditions: a) R^2Cl, imidazole, CH_2Cl_2; b) 4-Nitrophenyl chloroformate, DMAP, CH_2Cl_2; $H_2N(CH_2)_2NHBoc$, DMAP, DMF; c) Trifluoroacetic acid, CH_2Cl_2, 0 °C; FITC, DIPEA, DMF, RT. DMAP = 4-dimethylaminopyridine, TBDMS = *tert*-butyldiphenylsilyl, TES = triethylsilyl.

Experimental Section

Tubulin purification and assembly assay

Tubulin was purified from bovine brain purchased from a local slaughterhouse, conserved before use in ice-cold PBS and used as soon as possible. According to Castoldi and Popov (2003), pure tubulin was obtained by two cycles of polymerization-depoly-

merization in a high-molarity PIPES buffer (1 M K-PIPES, pH 6.9, 2 mM EGTA and 1 mM $MgCl_2$), and protein concentration was determined by the MicroBCA assay kit (Pierce). The test the effects of the thiocolchicine derivatives, the compounds were dissolved in dimethyl sulfoxide (DMSO), added in different concentrations (1–10 μM) to a reaction mixtures (20 μM tubulin, 10% glycerol, 1 mM GTP in BRB80 buffer) and incubated for 30 minutes at 37 °C. As control conditions were used either unmodified thiocolchicine or DMSO alone. At the end of polymerization, unpolymerized and polymerized fractions of tubulin were separated by centrifugation at 16500x*g* for 30 minutes at 25 °C. The collected microtubules were resuspended in SDS-PAGE sample buffer (2% w/v SDS, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 0.001% w/v bromophenol blue, and 62.5 mM Tris, pH 6.8) and the unpolymerized tubulin was diluted 3:1 with 4X SDS-PAGE sample buffer. Equal proportions of each fraction were resolved by a 7.5% SDS-gel and stained with Coomassie blue. Densitometric analyses of stained gels were performed by using ImageJ software (National Institute of Health), and data were elaborated using STATISTICA (StatSoft Inc., Tulsa, OK). Significant differences were assessed by one-way ANOVA with Tukey HSD post hoc test. Experiments were done in triplicate and data are expressed as means ± SEM.

Cell culture and fluorescence microscopy

Human lung carcinoma cell line A549 (CCL-185; American Type Culture Collection, Rockville, MD, USA) was grown in minimal essential medium with Earle's (E-MEM), supplemented with 10% fetal bovine serum (Hyclone Europe, Oud-Beijerland, Holland), 2 mM L-glutamine, 100 U mL^{-1} penicillin, and non-essential amino acids. Cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2. To test the ability of thiocolchicine derivatives to be incorporated into the cells and bind microtubules A549 cells (50000 cell mL^{-1}) were seeded on glass coverslips and grown in control medium. After 24 h, cells were either incubated 1 hour with 10 μM thiocolchicine derivatives and then fixed or vice versa, that is, cells were fixed and then incubated 1 hour with the fluorescent compounds. At the end of the treatments, cells were fixed and permeabilized for 10 minutes with methanol at −20 °C, washed with PBS, and blocked in PBS + 5% bovine serum albumin (BSA) for 15 minutes at room temperature. To localize tubulin, the cells were incubated with monoclonal anti-α-tubulin antibody (clone B-5–1–2, Sigma–Aldrich) 1:500 in PBS for 1 h at 37 °C. We used goat anti-mouse Alexa Fluor 594 (Molecular Probes) 1:1000 in PBS + 1% BSA for 45 minutes at 37 °C as secondary antibodies. Nuclei staining was performed by incubation with DAPI (0.25 μg mL^{-1} in PBS) for 15 minutes at room temperature. The coverslips were mounted in Mowiol (Calbiochem)-DABCO (Sigma–Aldrich) and examined with a Zeiss Axiovert 200 microscope equipped with a 63x Neofluor lens (Zeiss, Oberkochen, Germany) and confocal laser scan microscope imaging system (TCS SP2 AOBS, Leica Microsystems, Heidelberg, Germany) equipped with an Ar/Ar-Kr 488 nm, 561 nm and 405 nm diode lasers.

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Keywords: chephalomannines · fluorescence · fluorescent binders · osmium · thiocolchicines · tubulins

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