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Tools for the rational design of bivalent microtubule-targeting drugs

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ABSTRACT

Microtubule (MT) dynamic behaviour is an attractive drug target for chemotherapy, whose regulation by MT-stabilizing and destabilizing agents has been fruitfully applied in treating several types of cancers. MT-stabilizing agents are also emerging as potential remedies for neurodegenerative conditions, such as Alzheimer's and Parkinson's disease, although single-target drugs are not expected to fully cure these complex pathologies. Drug combination often displays enhanced efficacy with respect to monotherapies. In particular, MT-targeting bivalent compounds (MTBCs) represent a promising class of molecules; however, surprisingly, the majority of MTBCs reported so far exhibit equal if not less efficacy than their building monomers. In order to shed light on MTBCs poor performance, we characterised through a set of complementary approaches thiocolchicine (TH) and two bivalent TH-homodimers as prototype molecules. First, the binding affinities of these three molecules were assessed, then we obtained the crystallographic structure of a tubulin-TH complex. The binding affinities were interpreted in light of structural data and of molecular dynamics simulations. Finally, their effects on MT cytoskeleton and cell survival were validated on HeLa cells. The ensemble of these data provides chemical and structural considerations on how a successful rational design of MTBCs should be conceived.

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1. Introduction

Microtubules (MTs) are highly dynamic filaments that play important roles in several essential cellular processes, such as cell division or intracellular trafficking. MTs are hollow cylindrical assemblies of αβ-tubulin heterodimers, which can switch rapidly between phases of growth, pause, and shrinkage, a behaviour known as dynamic instability [1]. MT polymerization dynamics is tightly regulated through the binding of various MT-associated proteins but also through a wide range of naturally occurring and synthetic molecules [2]. Such dynamic behaviour is a very suitable drug target for chemotherapy. MT-targeting agents can be divided into two structurally distinct classes of drugs, MT-stabilizing agents and MT-destabilizing agents. Both MT-stabilizing and destabilizing agents have been successfully applied in the treatment of several types of cancer [3]. MT-stabilizing agents block MT depolymerisation targeting two different sites on β-tubulin, the taxoid or the laulimalide/peloruside binding site [4,5]. It is worth noting that the most recent MT-stabilizing agents are able to bind tubulin in a covalent manner, thus escaping the drug efflux pump at the basis of the resistance mechanism [6]. Recently, the anti-angiogenetic effect of this class of drugs renewed the interest in MT-stabilizing agents [7]. On the other side, MT-destabilizing agents hamper MT assembly and/or induce depolymerisation by binding to the vinca or the colchicine binding site [8]. Both such sites are localized at the interface between α- and β-subunits, whose reciprocal orientation is modified upon ligand binding [9,10]. This class of molecules also

Abbreviations: MT, Microtubules; MTBCs, MT-targeting bivalent compounds; MDA, microtubule depolymerizing agent; MSA, microtubule stabilizing agent; TH, thiocolchicine; THdim-S, short thiocolchicine homodimer; THdim-L, long thiocolchicine homodimer; TTL, tubulin tyrosine ligase; RB3, stathmin-like protein B3; T_2R, complex of two tubulin dimers and one RB3 molecule; K_d, dissociation constant; MD, molecular dynamics; GI_{50}, concentration for 50% of maximal inhibition of cell proliferation.

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includes vascular disrupting agents able to target pre-existing tumour vasculature [11], and, more recently, a new generation of antibody–conjugated MT-targeting agents that is improving the targeting of these drugs to tumors [12]. Interestingly, MT-targeting agents have also been shown to be neuroprotective in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [13,14]. In many neurodegenerative conditions, alterations in the stability of the MTs often precede impairment of axonal transport [15]. Therefore, MT-stabilizing agents may act as neuroprotective agents by preventing axonal damage [14].

In the last decades huge efforts have been made to develop MT-drugs targeting cancer and neurodegenerative diseases. However, it has become increasingly evident that single drugs cannot fully tackle very complex diseases. Among the approaches aimed to ameliorate their therapeutic index, multi-target therapies are a promising strategy. Combination of drugs often provides enhanced efficacy and reduced adaptive resistance compared to monotherapies [16]. In this context, bifunctional drugs, whose components bind to separate sites eliciting a multiple effect and increasing their pharmacological activity, represent a specific class of MT-targeting agents. In particular MT-targeting bivalent compounds (MTBCs) may have great pharmacological potential; however, most of such molecules display *in vitro* and in cell performances either similar or poorer as compared to the corresponding individual components [17–19].

In order to clarify the limitations of many available MTBCs and to draw a set of considerations on how to design future divalent molecules we have characterised, using a set of complementary approaches, thiocolchicine (TH) and two bivalent TH-homodimers, with a short (THdim-S) and a long (THdim-L) spacer, respectively (Fig. 1A, D, G). TH is a colchicine analogue in which the C-13 methoxy group is substituted with a thiomethyl moiety (Fig. 1A). This class of molecules exerts their biological effects by inhibiting tubulin assembly and suppressing MT formation [20]. TH is expected to bind at the colchicine site on tubulin, and previous studies indicate that the substitution of the oxygen to a sulphur atom actually increases the compound potency [21].

First, the binding affinities of TH and TH dimers were measured experimentally by fluorescence quenching. Then the crystallographic structure of tubulin in complex with TH was determined and the binding affinities of the two TH dimers were then correlated to the structural features of colchicine binding site through molecular dynamics simulations. Finally, cytotoxicity assessment and cytoskeleton organization were evaluated in HeLa cells. As a whole, the data presented clarify many aspects of MTBCs moderate performance *in vitro* and *in vivo* and, most importantly, provide a new set of tools for the rational design of effective MTBCs.

2. Materials and methods

A detailed description of this section is available as Supplementary Material.

2.1. Structure deposition

Atomic coordinates and structure factors for the T_2R–TTL in complex with TH have been deposited to the Protein Data Bank with accession code 5LP6.

3. Results

3.1. Binding affinity by fluorescence quenching

Typically, MTBCs consist of two active moieties bonded by a linker of various chemical nature. In order to evaluate the role of the linker present in MTBCs in determining the compound efficacy, two TH homodimers were used. While the chemistry of the two linkers is the same, their lengths are markedly different: 1 carbon atoms in the short homodimer (THdim-S) and 10 carbon atoms in the long (THdim-L) (Fig. 1D–G). Tubulin dimer contains eight tryptophan (Trp) residues spread over the two subunits and intrinsic Trp fluorescence has been previously used to determine the K_d of several colchicinoids [22]. The affinity of TH, THdim-S and THdim-L for tubulin was tested by titrating a fixed concentration of the protein with increasing ligand amounts. The fluorescence spectra of αβ-tubulin recorder in the presence of increasing concentrations of TH, THdim-S and THdim-L are shown in Fig. 1B, E, H. In all cases, the fluorescence intensity of the tubulin dimer decreases significantly with increasing ligand concentration as consequence of the complex formation. Notably, residual fluorescence emission maximum presents a red shift for TH and a blue shift for the dimers, indicative of a change in polarity of microenvironment surrounding Trp residues. The same effect was previously observed upon binding of both colchicinoids and colchicine site-binding agents [23]. The emission intensity at 335 nm was plotted over the concentration of the ligand for all compounds, as shown in Fig. 1C, F, I. Data were fitted using Equation (2) (see Supplementary Material), which describes a 1:1 protein-ligand stoichiometry and the values of K_d for the three compounds were calculated (Fig. 1C, F, I). The affinity measurements show that THdim-L does not display a better K_d for tubulin compared to TH (17.2 ± 2.5 μM, 19.9 ± 1.0 μM, respectively), while THdim-S displays an affinity even worse than TH towards the tubulin dimer (35.8 ± 4.5 μM). This observation indicates a fundamental role for the linker length in determining the binding affinity of the TH moieties at the colchicine-binding site. With the aim to scrutinize in depth the interaction between TH and tubulin's binding site the 3D structure of the tubulin-thiocolchicine complex was obtained by X-ray crystallography.

3.2. X-ray crystal structure of the tubulin-thiocolchicine complex

In order to assess the exact binding of TH to tubulin, we have determined the structure of the complex T_2R-TTL with (*S*)-TH, the active enantiomer of the ligand, at 2.9 Å resolution. The structure of the TH-complex could be superimposed with that obtained in the absence of the ligand (pdb code 4IIJ, r.m.s.d. of 0.47 Å over 2074 residues) [24], indicating that TH does not modify the overall conformation of tubulin. The overall electron density map is well defined for the two αβ-tubulin dimers and for RB3-SLD while the electron density for TTL is of lesser quality. Although each tubulin dimer possesses a TH binding site, unexpectedly only the tubulin dimer proximal to TTL displays a well-defined electron density map present at the colchicine site at the interface between α- and β-tubulin (Fig. S1). The orientation of TH in the binding site is well superimposable to that of colchicine in the T_2R complex previously reported (pdb code 1SA0) (Ravelli et al., 2004). TH lies in a pocket that is enclosed by strands L8 and L9, loop T7 and helices H7 and H8 of β-tubulin and the loop T5 of α-tubulin (Fig. 2A). The three rings of TH are deeply buried at the intra-dimer interface and form mainly hydrophobic contacts with several residues of β-tubulin. In addition weak electrostatic interactions are formed between the sulphur of ring C and the side chain and main chain of Asp350, and Val315 of β-tubulin. The binding of TH triggers the same conformational changes reported for the binding of colchicine: the T7 loop flips outwards and the loop T5 pulls back towards the α-tubulin upon ligand binding (Fig. 2A). The *N*-acetyl group (Fig. 1A) points towards a channel connecting the colchicine-binding site to the protein surface, plausibly the entry tunnel for TH. Such channel displays a mildly polar character and is made up by residues belonging to loops T5, T7 and strand L8. Taking advantage of the

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