Structural Basis of Noscapine Activation for Tubulin Binding

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ABSTRACT: Noscapine is a natural alkaloid that is used as an antitussive medicine. However, it also acts as a weak anticancer agent in certain in vivo models through a mechanism that is largely unknown. Here, we performed structural studies and show that the cytotoxic agent 7A-O-demethoxy-amino-noscapine (7A-aminonoscapine) binds to the colchicine site of tubulin. We suggest that the 7A-methoxy group of noscapine prevents binding to tubulin due to a steric clash of the compound with the TS-loop of α-tubulin. We further propose that the anticancer activity of noscapine arises from a bioactive metabolite that binds to the colchicine site of tubulin to induce mitotic arrest through a microtubule cytoskeleton-based mechanism.

INTRODUCTION

Among systemic and wide spectrum anticancer chemotherapies, those directed against tubulin have shown to be extremely successful. Vinca drugs discovered in the 60’s were the first magic bullet against leukemia,1 while taxanes became in the 90’s the best choice for the treatment of solid tumors (breast, ovarian, prostatic, lung).2 More recently, high affiinity drugs targeting the vinblastine site on β-tubulin (e.g., auristatin) or the newly discovered maytansine site3 had shown excellent activity as antibody drug conjugates.4 Finally, newly developed chemotypes with improved properties targeting the vinca (eribulin)5 or the taxane (ixabepilone)6 sites have been approved as second-line treatments of metastatic cancers. However, no drug binding to the colchicine site has passed yet the trials required to become an approved anticancer drug, though this was the first druggable site described in tubulin.7 Colchicine itself and the colchicine site ligand mebendazole are currently the only drugs used for the treatment of gout8 and nematode infestation,9 respectively. Noscapine is a naturally occurring phthalideisoquinoline alkaloid obtained during opium harvesting.10 It is used in humans as an oral antitussive agent.11 The drug also displays a favorable toxicity profile and has been known for some time to act as a weak anticancer agent in certain in vivo models.12 However, its molecular mechanism of action in this context is unknown. Like other microtubule targeting agents, noscapine arrests cells in the G2/M phase of the cycle at elevated concentrations (2 μM), but it does not significantly alter microtubule formation in vitro.12,13 Accordingly, the cellular effect could be caused by small amounts of an active contaminant or by metabolic activation of a prodrug. Strikingly, 7A-O-demethylated noscapine derivatives (7A-hydroxy and 7A-amino derivatives) mirror the cellular phenotype of the parent molecule but induce a G2/M arrest at about 500 times increased potency.14 In a previous study, we suspected that the 7A-amino derivative of noscapine (7A-aminonoscapine) binds to the colchicine site of tubulin.13 Tubulin inhibition through the colchicine site is a substoichiometric process15 and involves blocking of the “curved to straight” conformational transition required for tubulin dimers to incorporate into the microtubule lattice.16

In this work, we performed an NMR-assisted docking process combining STD data and CORCEMA-ST calculations for the building of a 3D model that we further validated through the determination of the structure of the tubulin-7A-amino-noscapine complex by macromolecular crystallography. Our results enlighten the molecular mechanism of action of noscapine by suggesting that noscapine is a produg that requires an activation step via O-demethylation at its position 7A.

RESULTS

NMR-Directed Modeling of the Tubulin-7A-amino-noscapine Complex in Solution Indicates Binding to the Colchicine Site. STD-NMR is a useful tool to investigate the interaction of small ligands with macromolecules. Aiming at understanding the lack of activity of noscapine on microtubule

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assembly and at unveiling the binding mode of 7A-amino-
noscapine (Figure 1), we employed STD-NMR in combination
with computational modeling. We further used the NMR STD
and TR-NOESY data obtained previously to model binding
poses of 7A-aminonoscapine in the two tubulin dimers present
in the T2R-podophyllotoxin complex. In agreement with the observed drug competition with the
colchicine site ligand nocodazole, the best binding poses with
similar scores located to the colchicine sites of both tubulin
dimers in the T2R complex (β1-tubulin, 0.16; β2-tubulin, 0.18;
Figure 1B). In both cases, nearly identical calculated STD
profiles with rmsd values below 2 Å between them were revealed
(Figure 1C,D). The calculated profiles were fully compatible with those experimentally determined, indicating that protons
H9, H3A, and H4A of the ligand are closer to the protein surface and thus receive a larger saturation.

High-Resolution Crystal Structure of the T2R-TTL-7A-
aminonoscapine Complex. Despite noscapine showing a
weak colchicine-like activity, we were previously unable to
detect any direct binding of the compound to the colchicine site
using centrifugation- or spectroscopic-based assays. Thus, the
reason for the observed antitubulin activity of noscapine remains
unknown.

Both NMR-determined poses (i.e., in β1- and β2-tubulin of
the T2R complex) revealed 7A-aminonoscapine in the same
orientation, but its placement in the two binding sites is shifted
with respect to each other (Figure 1B), suggesting that the
method does not have enough accuracy to understand the
reason for noscapine’s inability to bind the colchicine site. To
investigate the structural reasons for the lack of activity of
noscapine, we performed soaking experiments with noscapine
and 7A-aminonoscapine using T2R-TTL crystals obtained from
a protein complex composed of two αβ-tubulin heterodimers,
the stathmin-like protein RB3 and tubulin tyrosine ligase.

The T2R-TTL system has successfully produced several
structures of ligands bound to tubulin using X-ray crystallog-

The structure of T,R-TTL-7A-aminonoscapine was solved at a 2.2 Å resolution, and we unequivocally found ligand density at the colchicine site (Figure 2A and Figure S2). Concomitantly, with the biochemical experiments, we could not identify any extra density from crystals soaked with the parental noscapine (not shown).

The ligand in the colchicine site occupies a similar space as colchicine (Figure 3A). Noticeably, the conformation of 7A-aminonoscapine using either NMR or crystallography is similar with an rmsd of 0.18 Å in β2-tubulin (Figure S1A), while the NMR-calculated pose is displaced from the actual crystallographic one in β1-tubulin (Figure S1B). The overall structure of tubulin in the T3R-TTL-7A-aminonoscapine complex superimposed well with that of the ligand free complex structure with an overall rmsd of 0.29 Å over 1996 Cα-atoms and a β-tubulin rmsd of 0.14 Å over 365 Cα-atoms, suggesting that the binding of 7A-aminonoscapine does not affect the conformation of tubulin.

The three-membered [1,3]dioxolo-isoquinoline ring of 7A-aminonoscapine is stacked between the side chains of βCys241 of helix βH7 and βLeu255 of helix βH8 (Figure 3B). The dioxolo moiety points into the pocket shaped by the side chains of βTyr202, βVal238, βLeu242, βLeu255, βIle318, and βIle378, and the oxygen O1 forms two water-mediated H-bonds to both the main chain carbonyl of βGly237 and the main chain amide of βCys241 of helix βH7. The N6-methyl group is equatorially oriented toward the nucleotide N-site of α-tubulin; however, it does not engage in electrostatic interactions with the nucleotide.

**Figure 2.** Overall tubulin-7A-aminonoscapine complex structure. (A) Ribbon representation of the tubulin-bound 7A-aminonoscapine on representation of the tubulin-bound 7A-aminonoscapine structure (PDB ID 6Y6D). The α- and β-tubulin chains are in dark and light gray, respectively. The ligand 7A-aminonoscapine (light green) and the nucleotides (orange) are in sphere and stick representation, respectively. The carbon atoms of the individual nucleotides are colored according to their chain assignments. Oxygen and nitrogen atoms are colored in red and blue, respectively. (B, C) Close-up view of the atomic interaction network observed between 7A-aminonoscapine (light green) and tubulin (gray) in two different orientations. Interacting residues of tubulin are shown in stick representation and are labeled. Oxygen and nitrogen atoms are colored in red and blue, respectively. Hydrogen bonds are depicted as black dashed lines. Secondary structural elements of tubulin are labeled in blue. For simplicity, only α-tubulin residues are indicated with an “α”.

**Figure 3.** (A) Superposition of the tubulin-7A-aminonoscapine (light green, PDB ID 6Y6D) and tubulin-colchicine (slate, PDB ID 4O2B) complex structures. The structures were superimposed onto the β1-tubulin of their respective T3R-TTL complexes. Interacting residues of tubulin discussed in the text are shown in stick representation and are labeled. Oxygen and nitrogen atoms are colored in red and blue, respectively. Hydrogen bonds are depicted as black dashed lines. Secondary structural elements of tubulin are labeled in blue. (B) Modeled noscapine molecule into the crystallographic determined the 7A-aminonoscapine binding site, highlighting how the 7A-methoxy group would clash into the αT5-loop. The clash is in agreement with the calculated NMR model and suggests why noscapine does not bind to tubulin.
The protonated N6 points toward the side chain of βCys241 and may engage in a weak interaction with the lone pairs of the sulphydryl group (4.9 Å distance). The 4-methoxy moiety forms a hydrophobic contact with the side chains of βLeu242 and βAla250. The benzofuran-1-one moiety is stacked between the side chains of βAsn258 and βLys352, and its 1A-carbonyl forms water-mediated H-bonds to the side chains of αAsn101 and βLys254 and to the main chain carbonyl of αSer178. The 7A-amino group points toward the tubulin intradimer interface and is in direct and water-mediated H-bond contact to both the main chain carbonyls of αThr179 and βAsn349. The 6A-methoxy moiety occupies the cavity shaped by the carboxy-terminal end of helix βH8, the amino-terminal end of strand βS9, and the αVal181 side chain on the αTS-loop.

The tubulin-colchicine and tubulin-7A-aminonoscapine structures superimpose very well (overall rmsd of 0.16 Å over 1882 Cα-atoms and a β-tubulin rmsd of 0.14 Å over 328 Cα-atoms; Figure 3A). The 7A-amino and the 6A-methoxy moieties of the benzofuran-1-one ring occupy almost the same space as the corresponding carbonyl and methoxy groups of the C-ring of colchicine, thereby establishing similar polar and hydrophobic contacts. Compared to both the colchicine-bound and the apotubulin state, the equatorial N6-methyl group of 7A-amino-colchicine, thereby establishing similar polar and hydrophobic contacts. The 7A-demethylated product of noscapine is a tubulin assembly inhibitor that is as potent as noscapine.23 The most reasonable explanation for those results is that derivatives containing such substitutes bind tubulin in a different way.

Our high-resolution crystal structure confirms that 7A-amino-noscapine targets the colchicine site of tubulin (Figure 2). This result corroborates that the inhibitory effects of noscapine derivatives on tubulin assembly into microtubules, and the subsequent cellular effects13,14 are exerted using the same molecular mechanism as colchicine. Based on our structural information, we can now explain the lack of noscapine’s activity in vitro and the activity improvement of some developed derivatives. The 7A-methoxy group in noscapine clashes with the αTS-loop of tubulin, precluding noscapine binding to the colchicine site (Figure 3B). The reduction of the noscapine lactone produced cyclic ether noscapinoids with increased activity,21 which could be explained by the loss of the H-bond network established by the 1A-carbonyl group with α-tubulin; it would also allow for a better accommodation of the 7A-methoxy group within the pocket. The best cellular toxicity activities were obtained with 9-halogenated derivatives22, which likely display increased contacts with strands S8–S9 in β-tubulin. Otherwise, N-substituted 6′-derivatives showed different effects on the inhibition of cell proliferation,15 which could be explained by the interaction of the different moieties with the βT7-loop. Finally, bulkier substituents at the 7A-position showed better activities than noscapine.23 The most reasonable explanation for those results is that derivatives containing such substituents bind tubulin in a different way.

An immediate question emerging from these observations is the following: from where does the colchicine-like activity observed for the parental noscapine come from? We hypothesize that the observed in vivo activity of noscapine is the result of its metabolic bioactivation. A prodrug is a molecule with a low or neglectable activity against a pharmacological target as compared with one of its metabolites.24 In humans, the metabolism of xenobiotics is mostly related with cytochrome P450 enzymes that, among other activities, perform the oxidation of a carbon group bound to oxygen or other heteratoms.25 This reaction leads to the release of the carbon and leaves an alcohol, amine, or thiol in the molecule,26 which is a common mechanism for the release of a drug from its prodrug.24 For instance, morphine or norcodeine are the consequence of O-demethylation and N-demethylation of codein, respectively.26 In fact, the oxidation mechanism is a common way of antitumoral prodrug activation (e.g., cyclophosphamide,27 dacarbazine,28 and tamoxifen29) where the hepatic CYP2D6 enzyme is mostly involved in the activation process, as exemplified by codeine and tamoxifen.24

The noscapine metabolism is well understood and includes an extensive “first pass” metabolism in rats, rabbits, and humans mainly by C–C cleavage, O-demethylation, and cleavage of the methylenedioxyl group.30–32 The metabolic map of noscapine has been extensively characterized by Fang et al.32 This study shows that the 7A-demethylated product is one of the major metabolites of noscapine in cells and in vivo after being processed by CYP3A5. The same metabolite was also identified in mice both unmodified and as its glucuronide after being processed by UGT1A1/3/9. The 7A-demethylated product of noscapine is a tubulin assembly inhibitor that is as potent as vinblastine, showing an IC₅₀ of 0.6 μM in A549 cells.33 Considering that the noscapine doses effective for tumor inhibition in mice are 120 mg/kg,34 while the dose of colchicine (when used as an antigout drug) is about 4000 times lower (i.e., 30 μg/kg35), it is reasonable to assume that only a small fraction of the metabolically processed 7A-demethylated noscapine derivative is required to justify the observed activity.

In conclusion, we propose that noscapine’s cytotoxicity is a consequence of the oxidation of its methoxyl group at position 7A, which leads to the formation of the active phenol. The resulting drug is then able to bind tubulin and block microtubule assembly in a colchicine-like manner.13

## DISCUSSION AND CONCLUSIONS

Our high-resolution crystal structure confirms that 7A-amino-noscapine targets the colchicine site of tubulin (Figure 2). This result corroborates that the inhibitory effects of noscapine derivatives on tubulin assembly into microtubules, and the subsequent cellular effects13,14 are exerted using the same molecular mechanism as colchicine. Based on our structural information, we can now explain the lack of noscapine’s activity in vitro and the activity improvement of some developed derivatives. The 7A-methoxy group in noscapine clashes with the αTS-loop of tubulin, precluding noscapine binding to the colchicine site (Figure 3B). The reduction of the noscapine lactone produced cyclic ether noscapinoids with increased activity,21 which could be explained by the loss of the H-bond network established by the 1A-carbonyl group with α-tubulin; it would also allow for a better accommodation of the 7A-methoxy group within the pocket. The best cellular toxicity activities were obtained with 9-halogenated derivatives22, which likely display increased contacts with strands S8–S9 in β-tubulin. Otherwise, N-substituted 6′-derivatives showed different effects on the inhibition of cell proliferation,15 which could be explained by the interaction of the different moieties with the βT7-loop. Finally, bulkier substituents at the 7A-position showed better activities than noscapine.23 The most reasonable explanation for those results is that derivatives containing such substituents bind tubulin in a different way.

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protein surface, which was considered rigid. In this work, a 25 Å parallelepiped build around β1- and β2-tubulin cavities was employed, and 20 poses were generated per cavity. The calculated STD spectrum of 7A-aminonoscapine bound to tubulin was compared for each pose using the CORCEMA-ST method with iterative rounds of model building and refinement. The final model was refined to 2.2 Å resolution with crystallographic R values of 18.4% (R_{merge}) and 22.4% (R_{free}). The model has good geometry with small root-mean-square deviations (rmsd) from ideal values for bond lengths and bond angles. The quality of the structures was assessed with MolProbity.

Data collection and refinement statistics of the crystallographic analysis (Table S1), comparison between crystal and NMR structures (Figure S1), and the map of tubulin-bound 7A-aminonoscapine (Figure S2) (PDF) are available. NMR models of the T_{2R-TTL}-7-demethoxy-amino noscapine complexes (PDB, PDF) are also provided. Molecular formula strings (CSV) are included.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00855.

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**Notes**

The authors declare no competing financial interest.

The PDB code for the crystallographic structure of the T_{2R-TTL}-7-demethoxy-amino noscapine complex is 6Y6D.

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**ABBREVIATIONS**

CORCEMA-ST, complete relaxation and conformational exchange matrix analysis of saturation transfer; STD, saturation transfer difference; TTL-T_R, bovine brain α/τ-tubulin, rat stathmin-like protein RB3, and chicken tubulin–tyrosine ligase complex; TR-NOESY, two-dimensional transferred nuclear Overhauser effect spectroscopy.


