Quantitative Analysis for Drug Binding and Fatty Acid Modification of Tubulin

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Objectives

Developing accurate and quantitative methods of analyzing reactivity of each cysteine in tubulin dimer to identify binding sites of anti-cancer drugs and to understand the polymerization mechanism of tubulin.

Introduction

α, β–Heterodimer of tubulin is a building block of microtubules, which are the major constituents of mitotic spindles.

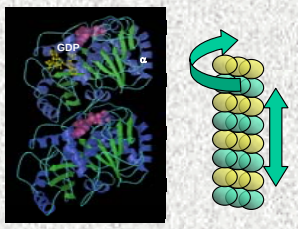

Fig. 1: Left: Structure of the heterodimer of tubulin (J. Löwe et al. *J Mol Biol.* 313: 1045-1057 (2001). Right: Linear microtubule formation.

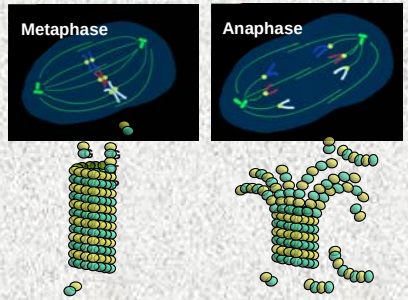

Fig. 2: Polymerization and depolymerization of tubulin in cell cycle.

Even though a variety of antimitotic agents targeting tubulin have been used widely, there are still big problems such as cytotoxicity and multidrug resistance. Several different binding sites of drugs to tubulin have been reported. The efficacy against multidrug resistant cell, and the cytotoxicity vary to agents. Because the different behaviors of drugs may be due to the different binding sites of drugs to tubulin, knowing the binding site is important to understand tubulin's behavior.
Tubulin's cysteines react very fast with sulfhydryl reagents without denaturation, and many papers reported that tubulin has two most reactive cysteines in dimer. Those cysteines might be related to tubulin's active site and/or polymerization mechanism, however it was unclear which cysteines were involved in.

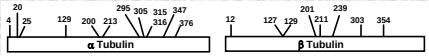

Fig. 3: The location of each cysteine in tubulin dimer.

Tubulin has 20 free cysteines in the dimer and they are potential binding sites of sulfhydryl reagent including anticancer drugs. Therefore, a method of identifying the most reactive cysteine is necessary.

Methods

1. Sample preparation

Reaction of sulfhydryl reagents with tubulin (various time and molar ratios).
Quenching and removal of excess molecules :
Acetone precipitation
Chloroform/Methanol precipitation (for fatty acid removal)
Resolubilization using high power sonication.
Reducing using TCEP.
Trypsin digestion.

2. MS analysis

HPLC/MS/MS for peptide identification.
HPLC-MS for quantitation.

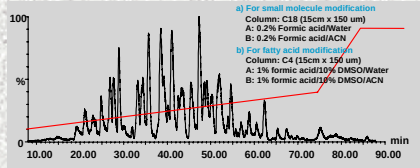

Fig. 4: Ion current chromatogram of trypsin digest of modified tubulin.

3. Data analysis

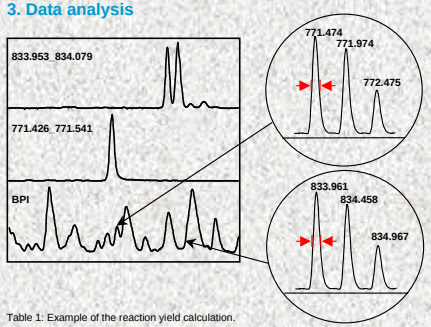

Table 1: Example of the reaction yield calculation.

Residue	Segment	Mw	m/z	Rt	Probe	Sum	Yield
347Cys	340–352	1540.740	771.378	48		277.3	
			277.3				
		1665.788	833.902	52	NEM		
			274.8			274.8	49.77%

Reaction yield was calculated by dividing the intensity of modified cysteine-containing peptide by overall intensity.

Results & Discussion

1. Binding site of anticancer drug, T138067 (Tularik Inc.)

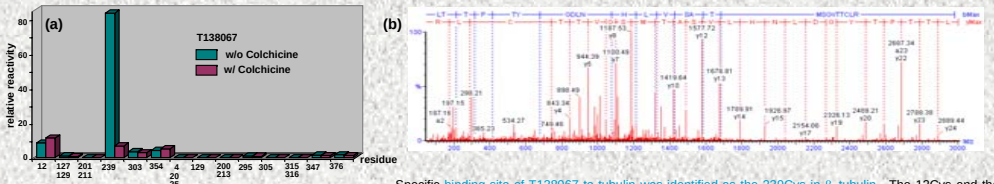

Fig. 5: (a) Reactivity of each cysteine toward T138067 w/ and w/o colchicine treatment; (b) The MS/MS fragmentation of the 239Cys-containing peptide (LTTPTYGDLNHLVSATMSGVTTCLR) modified by T138067.

Specific binding site of T138067 to tubulin was identified as the 239Cys in β–tubulin. The 12Cys and the 354Cys were minor binding sites. Treatment of colchicine before the reaction of T138067 reduced the reactivity of the 239Cys significantly since the binding of colchicine hampered the access of the T138067 to the 239Cys. This result shows that the binding site of colchicine is very close to the 239Cys rather than the 354Cys.

2. Reactivity changes toward mBrB (new method of identifying the binding sites of non-covalent anticancer drugs)

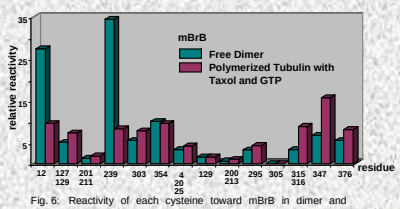

Fig. 6: Reactivity of each cysteine toward mBrB in dimer and polymerized form.

Table 2: Reactivity changes of each cysteine toward mBrB with various anticancer agents treatment.

residue \ agent	Taxol	Colch	GDP	Cryp	Vcr
12	-17.9	39.4	-20.7	-20.1	-20.4
127/129	2.2	1.0	0.4	-0.5	-0.4
201/211	0.6	0.1	0.1	0.0	-0.6
239	-26.1	-30.5	-2.8	-7.8	3.0
303	2.3	0.0	0.0	0.1	-0.6
354	-0.4	-2.3	-0.2	0.0	-2.7
4/20/25	0.9	0.2	0.3	0.7	-1.0
129	0.0	-0.3	-0.3	-0.4	-0.8
200/213	0.4	0.3	-0.1	0.3	0.0
295	1.1	-0.8	-0.3	-0.3	-0.9
305	0.0	0.0	0.0	0.0	0.0
315/316	5.4	1.4	-0.2	-0.5	-0.7
347	8.9	0.2	0.0	0.1	-1.6
376	2.7	1.4	0.9	0.3	0.1

The two most reactive cysteines toward mBrB were the 12Cys and the 239Cys in β–tubulin. The reactivity of these two cysteines was reduced after polymerization while other cysteines' reactivity changes were not significant. This result supports the protein breathing mechanism of tubulin. Only the 12Cys' reactivity decreased with treatment of GDP, cryptophycine (Cryp), vincristine (Vcr), which proves the binding sites of these three agents are near the 12Cys. After colchcine (Colch) treatment, the reactivity of the 239Cys decreased while 12Cys's reactivity increased. Since colchicine occupied the active site, mBrB could not access the 239Cys, yet binding of colchicine may affect on the vulnerability of the 12Cys by tertiary structure distortion.

3. Binding of NEM and its inhibition mechanism of (-) end elongation

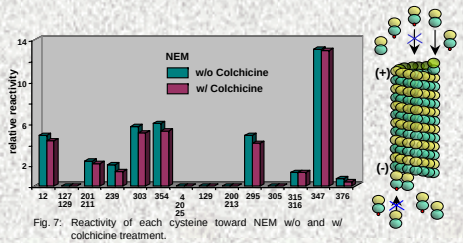

Fig. 7: Reactivity of each cysteine toward NEM w/o and w/ colchicine treatment.

The most reactive cysteine toward NEM was the 347Cys in α–tubulin, which is an essential part of inter-dimer interaction. This result explains the mechanism of (–) end elongation of NEM bound tubulin.

4. Binding sites of palmitoyl group

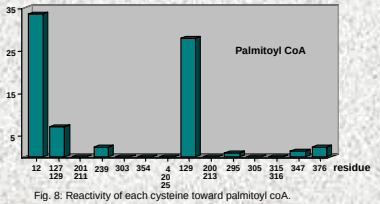

Fig. 8: Reactivity of each cysteine toward palmitoyl coA.

Conclusions

Direct quantitation using HPLC-MS has been applied to monitor the reactivity of each cysteine in tubulin dimers toward various sulfhydryl reagents. The 239Cys in β–tubulin was identified as the binding site of T138067 (anticancer drug, Tularik Inc.) with this method, and the colchicine binding site was also verified as the same site using competition analysis. A new method of identifying the binding sites of non-covalent binding agents has been developed. This method has proven as an useful tool of determining the various binding sites without additional modification of the agents.