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## A high-throughput model for screening anti-tumor agents capable of promoting polymerization of tubulin *in vitro*<sup>1</sup>

Wei HU, Hui DONG, Yue-zhong LI<sup>2</sup>, Xi-tao HU, Guan-jun HAN, Yin-bo QU

*State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Ji-nan 250100, China*

**KEY WORDS** antitumor drug screening assays; tubulin polymerization promotor; microtubules

### ABSTRACT

**AIM:** To establish a high-throughput model for screening anti-tumor agents capable of promoting the polymerization of tubulin *in vitro*. **METHODS:** Tubulin was prepared in different purity for two screening steps. The first step was a high-throughput screening (HTS) for a set of 1500 samples using the GTP-containing tubulin and the end-reading method. The second step was performed on 119 hits from the first screening by a kinetic assay with GTP-lacking tubulin. **RESULTS:** The HTS for 1500 samples was accomplished in less than 3 h. From the screening, 108 samples were identified with >20 % promotion activity at 10 mg/L. Five of 108 were further confirmed by the kinetic assay using the purified tubulin subsequently. Three of the hit compounds were Etoposide A or its analogs, the other two compounds had new structures with a common pharmacophore for cytotoxic natural products that stabilize microtubules. In an MTT test, the five selected samples from the screening showed a minimal IC<sub>50</sub> at 0.28±0.06 nmol/L to Hela cells. **CONCLUSION:** The two-step screening method is a high-throughput, cost-effective, and efficient approach to identify microtubule-stabilizing agents.

### INTRODUCTION

Discovery compounds that exhibit specific features, such as binding, inhibiting or catalytic properties, is a central task in drug development and medical applications. The advances in genomics, bioinformatics, combinatorial chemistry, cell-based assays, and high-throughput screening (HTS) have led

to a new concept of drug discovery<sup>[1]</sup>. A large number of hypothetical targets are incorporated into molecular- or cell-based assays and exposed to many compounds representing numerous variations on a few chemical themes or, more recently, less variations on a greater number of themes in HTS configurations. The HTS method is now widely used, and will certainly accelerate the generation of leading compounds for further development as a useful therapeutic agents.

Microtubule is an important component of eukaryotic cytoskeleton that is essential for separation of the duplicated chromosome pairs during mitosis. It also has many other significant functions in cells, such as intracellular transportation, maintenance of cell shape, cellular locomotion, and transmission of signals between cell surface receptors and nuclear effectors<sup>[2]</sup>. The antimitotic drug Taxol<sup>®</sup> (paclitaxel), elucidated in 1971 from

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<sup>2</sup> Correspondence to Prof Yue-zhong LI.

Phn & Fax 86-531-8564-288. E-mail lilab@sdu.edu.cn

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the Pacific yew tree *Taxus brevifolia*<sup>[3]</sup>, has undergone extensive clinical development as a result of its efficiency in the treatment of refractory ovarian cancer and its potential value for the treatment of breast, lung, and other cancers<sup>[4]</sup>. The primary target of paclitaxel is the tubulin/microtubule system. Paclitaxel promotes hyperstabilization of microtubule, which is resistant to depolymerization conditions, such as calcium and cold *in vitro*<sup>[5]</sup>, and arrests cells in mitosis, eventually leading to cell death<sup>[6]</sup>. That is to say, paclitaxel can enhance microtubule polymerization so as to be called microtubule-stabilizing agents in contrast to vinblastine, colchicine and other antimetabolic compounds, which inhibit microtubule polymerization both *in vitro* and in cells<sup>[7]</sup>. The success of paclitaxel in therapies of human tumors as well as disadvantages lead to renewed interests in searching and developing novel compounds that mimic the activity of paclitaxel<sup>[8]</sup>.

The methods for screening microtubule-stabilizing agents usually employ the purified tubulin and its polymerization-depolymerization reaction *in vitro*<sup>[9]</sup>, which is lamed in HTS mainly because of the efficient preparation of tubulin, the expense for purification of tubulin, the maintenance of purified tubulin activity, and high-throughput detection of polymerization of microtubule *in vitro*<sup>[10,11]</sup>. Furthermore, the samples used in screening are often crude and contain various components, which might greatly affect the reaction of polymerization of microtubules *in vitro* and lead to false results. In this paper, we described an economic HTS method for screening microtubule-stabilizing agents from natural products.

## MATERIALS AND METHODS

**Materials and instruments** GTP was purchased from Amresco Co, DEAE-Sephadex and Sephadex G-50 were the products of Pharmacia Co. Paclitaxel (Taxol<sup>®</sup>) was purchased from Sigma Co with 97 % purity (HPLC). Other reagents and solvents used in the experiments were of analytic grade. Hitachi 55P-72 centrifuge was employed in the experiments.

Samples used in the screening assay were mainly microbial metabolites, which were either crude, separated or purified by HPLC or other separation methods.

Eight-channel Eppendorf pipette was used for liquid handling. SPECTRAMax 190 96-well reader (Sigma Co) was used for colorimetric detection. The 96-well plates used for the screening were the products of Sigma

Co.

**Preparation of tubulin** The method<sup>[12,13]</sup> was modified for purification and preparation of tubulins from pig brain tissue. All operations were at 2 °C unless indicated otherwise. Fresh pig brains were homogenized with the solution containing 1 mol/L monosodium glutamate and 0.1 mmol/L GTP. The homogenate was centrifuged at 100 000×g for 1 h. The supernatant was mixed for 1 h with 0.1 mmol/L GTP and DEAE-Sephadex at the ratio of 4:1 (v:v), and the resin had been equilibrated with 0.8 mol/L glutamate. After the resin had settled for about 15 min, approximately 60 %-70 % of the fluid was decanted, and the remaining fluid was freed by vacuum aspiration. The moist resin was mixed with a solution containing 0.8 mol/L glutamate and 0.1 mmol/L GTP and then vacuum aspirated. This washing step was repeated twice. The washed resin was mixed with a solution containing 1.0 mol/L NaCl, 1.0 mol/L glutamate, and 0.1 mmol/L GTP. After 20 min, the tubulin-rich eluate was collected by vacuum aspiration. The eluate from five repeats was collected as DEAE-tubulin, which was then purified by heat-polymerization and cold-depolymerization. The DEAE-tubulin was mixed with 1 mmol/L GTP, warmed to 37 °C, incubated for 45 min, and centrifuged for 1 h at 100 000×g at 37 °C to obtain warm polymerized microtubule sediment. The sediment was suspended in a solution containing 1 mol/L glutamate and 0.1 mmol/L GTP iced at 0 °C for 30 min, centrifuged at 100 000×g at 4 °C for 40 min to obtain cold supernatant (purified tubulin). The purified tubulin was then used in HTS assay, and stored in liquid nitrogen in 4 mL aliquots. The GDP/GTP-free tubulin was prepared on Sephadex G-50 with 1 mol/L glutamate. This preparation was also stored frozen in liquid nitrogen and employed in the kinetic tubulin-polymerization assay.

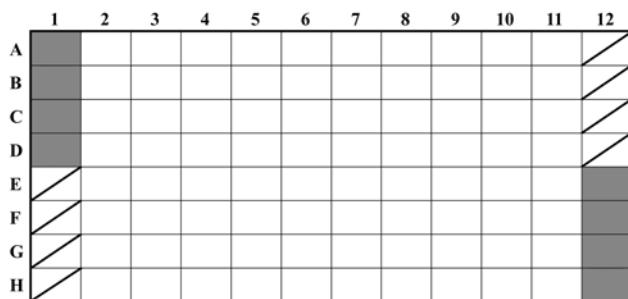
The purity of the tubulin sample was determined by SDS-PAGE as described by Laemmli and Favre using 10 % separating gel<sup>[14]</sup>, and protein concentrations were determined with the BCA kit (Pierce Co) and bovine serum albumin was used as standard.

Transmission electron microscopy was performed on the thin sections of polymerized microtubules from the purified tubulin or DEAE-tubulin *in vitro*, which were fixed in a solution as described by Phyllis *et al*<sup>[15]</sup>.

**Polymerization assay** Tubulin polymerization was monitored by turbidimetry with measurement of the change in absorbance at 350 nm in a SPECTRAMax 190 96-well reader. Unless indicated otherwise, sample

size was 0.1 mL each well and tubulin concentration was 1 g/L, and other components are described in the individual experiments.

**Sample preparation** Individual compounds were dissolved at 1 g/L concentration in 4 % Me<sub>2</sub>SO solution containing 1 mol/L glutamate. The sample solution was diluted 10-fold by adding 10 μL to 90 μL of the dissolving solution in A2-H11 wells on 96-well polystyrene plates as mother plates with the layout format<sup>[16]</sup> shown in Fig 1. All mother plates were duplicated to daughter plates for screening by transferring 10 μL of the solution using automatic eight-channel pipette. All the above operations were performed on ice.



**Fig 1.** Layout of samples (open blocks), blanks (lineated blocks), and controls (filled blocks) on the 96-well plate. Rows A to H and columns 1 to 12 are shown.

**First high-throughput screening (HTS) assay (End-point assay)** On the daughter plates with the same layout as the mother plates (Fig 1), 10 μL 4 % Me<sub>2</sub>SO solvent were distributed in eight blank wells (A12-D12, E1-1), and controls were in other eight wells (E12-H12, A1-D1) using known active compound paclitaxel in Me<sub>2</sub>SO (10 μL, 0.1 g/L). At 2 °C, an assay mixture (90 μL) containing 4 % Me<sub>2</sub>SO, 0.1 mmol/L GTP, 0.1 mmol/L CaCl<sub>2</sub> and 1 g/L tubulin, was added to all wells with 8-channel pipette. The plate was transferred to the reader for immediate reading at 350 nm and 2 °C, and UV absorption was recorded (Fig 2). Then all the plates were moved to an incubator at 37 °C for 20 min. The plate was transferred to the plate reader for reading at 350 nm and 37 °C again, together the UV absorption of each well (Fig 2). The percent end-point promote coefficient ( $P_e$ ) was calculated by the formula below.

In the formula,  $A_x^{37}$  and  $A_x^2$  equal the average absorbance of control wells or compound wells at 37 °C and 2 °C, respectively.  $A_0^{37}$  and  $A_0^2$  equal the average absorbance of blank wells at 37 °C and 2 °C, respectively.

$$P_e = \frac{\Delta A_x - \Delta A_0}{\Delta A_0} \times 100 \%$$

$$\Delta A_x = A_x^{37} - A_x^2 \quad \Delta A_0 = A_0^{37} - A_0^2$$

**Second confirmation screening assay (kinetic assay)** After the first HTS step, some active compounds were screened with the kinetic confirmation assay. In this step, the layout of samples was as the same as that in the end-point assay. But the assay mixture changed to the solution containing 4 % Me<sub>2</sub>SO, 0.1 mmol/L CaCl<sub>2</sub>, and 1 g/L tubulin without GTP. The plate was transferred from 2 °C to the plate reader for immediate reading at 350 nm and 37 °C, UV absorption change was monitored for 25 min, and the slope of absorption change from 6 to 16 min was recorded (Fig 3).

The average of the slopes for the 8 blanks was used as 0 % activity and the average of the slopes for the 8 controls was used as 100 % activity. The slope from sample wells was divided by the average, given percent kinetic promote coefficient ( $P_k$ ) to the value of 100 % in the presence of screening compound (Fig 3).

**MTT assay** In order to evaluate the anti-tumor activity of the compounds screened by the two-step screening assays, a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed with Hela cell line as described by James *et al*<sup>[17]</sup>, and the IC<sub>50</sub> of the compounds was determined.

## RESULTS

**The tubulin used in the assay** We purified tubulin with a two-step method. The first step was to elute pig brain tubulin from DEAE-Sephadex with 1 mol/L NaCl and 0.8 mol/L glutamine-Na (DEAE-tubulin), and then it was used in one cycle of polymerization in the presence of NaCl and glutamine, and depolymerization in the presence of glutamine. It was found that the loss of tubulin in the cycle was much reduced (only about 20 %-40 % of the total tubulin) compared to the polymerization-depolymerization cycles without elution step, which might be caused by removal of other proteins in the first step. With this two-step method, purified tubulin was obtained (Fig 4A). The microtubules from DEAE-tubulin or purified tubulin had a similar shape in the absence of glutamine (Fig 4B). The tubulin could be polymerized in the presence of paclitaxel *in vitro* (Fig 5).

GTP-induced polymerization of purified tubulin at 37 °C was under 350 nm in the presence of glutamate,

**A**

0.12	0.10	0.23	0.07	0.10	0.12	0.12	0.09	0.12	0.11	0.11	0.12
0.10	0.12	0.13	0.11	0.14	0.14	0.14	0.38	0.07	0.18	0.09	0.07
0.09	0.70	0.17	0.19	0.08	0.11	0.90	0.11	0.11	0.14	0.10	0.05
0.11	0.10	1.26	0.06	0.11	0.13	0.12	0.18	0.05	0.19	0.08	0.07
0.07	0.11	0.09	0.14	0.12	0.07	0.11	0.07	0.11	0.09	0.08	0.10
0.10	0.12	0.49	0.05	0.11	0.18	0.10	0.09	0.09	0.13	0.10	0.09
0.08	0.12	0.13	0.13	0.12	0.16	0.13	0.13	0.11	0.12	0.11	0.09
0.07	0.11	0.11	0.07	0.08	0.12	0.10	0.11	0.38	0.06	0.12	0.09

**B**

0.67	0.52	0.62	0.48	0.47	0.51	0.50	0.48	0.51	0.52	0.53	0.50
0.59	0.52	0.52	0.51	0.53	0.52	0.53	0.83	0.42	0.54	0.47	0.42
0.63	1.09	0.56	0.56	0.72	0.50	1.30	0.50	0.51	0.57	0.52	0.47
0.62	0.49	1.35	0.50	0.51	0.50	0.52	0.55	0.49	0.54	0.48	0.39
0.52	0.50	0.52	0.56	0.50	0.50	0.50	0.40	0.50	0.49	0.49	0.60
0.49	0.50	0.84	0.59	0.52	0.55	0.49	0.50	0.50	0.52	0.48	0.58
0.50	0.54	0.54	0.52	0.51	0.30	0.52	0.54	0.53	0.69	0.50	0.63
0.43	0.50	0.51	0.46	0.50	0.53	0.48	0.50	0.76	0.47	0.50	0.65

**C**

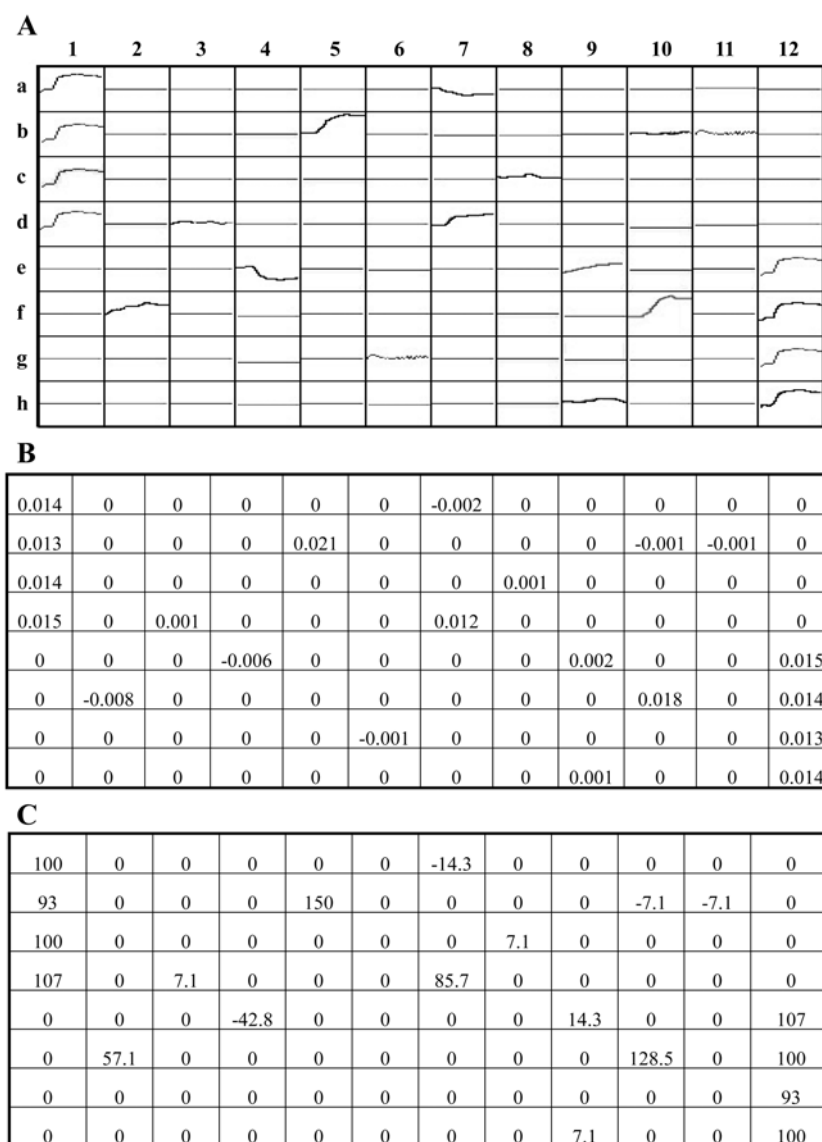
42.5	8.8	1.0	6.2	-4.2	1.0	-1.5	1.0	1.0	6.2	8.8	-1.5
26.9	3.6	1.0	3.6	1.0	-1.5	1.0	16.6	-9.3	-6.8	-1.6	-9.3
39.9	1.0	1.0	-4.2	65.8	1.0	3.6	1.0	3.6	11.4	8.9	8.8
32.1	1.0	-76.7	14.0	3.6	-4.2	3.6	-4.2	13.9	-9.3	3.6	-17.1
16.6	1.0	11.4	8.8	-1.5	11.4	3.6	-14.5	3.6	3.6	6.2	29.5
1.0	-1.5	-9.3	39.9	6.2	-4.2	1.0	6.2	6.2	1.0	-1.6	26.9
8.8	8.8	6.2	1.0	1.0	-63.7	1.0	6.2	8.8	47.7	1.0	39.9
-6.7	1.0	3.6	1.0	8.8	6.2	-1.6	1.0	-1.5	6.2	-1.5	45.1

**Fig 2. Typical data conversion from end-point reading to percent promote coefficient ( $P_e$ ). A: Raw data showing the absorbance at 350 nm 2 °C in each of the 96 wells (layout of samples, blanks, and controls as shown in Fig 1). B: Raw data showing the absorbance at 350 nm 37 °C in each of the 96 wells. C: Converted percent end-point promote coefficient ( $P_e$ ).**

and the kinetics of polymerization presented a typical “S” curve (Fig 5, curve 1). At 37 °C, the purified tubulin would not polymerize to microtubule without GTP (the nucleotides were removed from the tubulin by chromatography, Fig 5, curve 2). Paclitaxel could remarkably promote the assembly of purified tubulin in the presence of GTP, and made the reaction occur with high percent of total polymerization (Fig 5, curve 3). Even without GTP, assembly could occur with paclitaxel, and the kinetic of polymerization also presented typical ‘S’ curve (Fig 5, curve 4). Therefore, on the two-step of screening, promoting activity of compounds could be shown by end-point value or kinetic

curve.

Since Me<sub>2</sub>SO solvent was used in dissolving compounds for the screening assay, we investigated the effects of Me<sub>2</sub>SO on the assembly of purified tubulin. As Me<sub>2</sub>SO concentration increases, the purified tubulin was disproportionately assembled into microtubules even in the presence of MAPs as described by Himes *et al*<sup>[18]</sup>. The reaction of tubulin showed abnormal kinetic curve at 10 % or higher concentrations of Me<sub>2</sub>SO, and normal kinetic curve at concentrations of Me<sub>2</sub>SO below 5 %. However, the compounds, especially paclitaxel, did not dissolve well at Me<sub>2</sub>SO concentrations less than 3 %. The result suggested that presence of 4 % Me<sub>2</sub>SO



**Fig 3. Typical data conversion from kinetic reading to percent promote coefficient ( $P_k$ ). A: Raw data showing the absorbance at 350 nm at 37 °C over a period of 25 min in each of the 96-wells (layout of samples, blanks, and controls as shown in Fig 1). B: Slopes for the absorption change in each well. C: Converted percent kinetic promote coefficient ( $P_k$ ).**

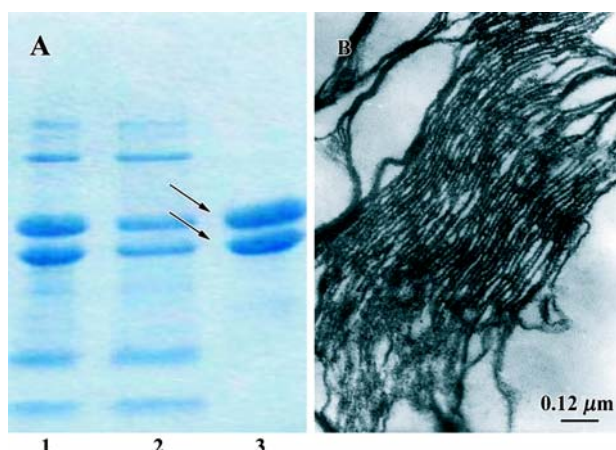
in the screening assay did not markedly affect the assembly of purified tubulin.

After initial experiments mentioned above, we defined the assay condition as final concentrations at 1 g/L tubulin, 0.1 mmol/L GTP and 0.1 mmol/L  $\text{CaCl}_2$ , and 10 mg/L screening compounds in a total volume of 0.1 mL of 4 %  $\text{Me}_2\text{SO}$ . The blank was 4 %  $\text{Me}_2\text{SO}$  solvent with 1g/L tubulin, 0.1 mmol/L GTP and 0.1 mmol/L  $\text{CaCl}_2$ , and paclitaxel in 4 %  $\text{Me}_2\text{SO}$  at final concentration of 10 mg/L were used as the control.

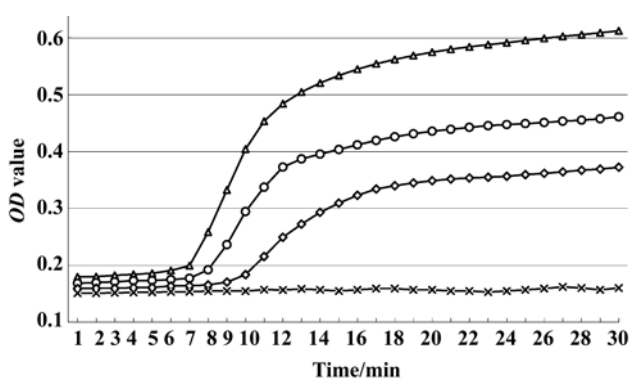
**Screening of 1500 samples** With first HTS assay (end-point assay), a total of 1500 samples were placed onto thirty eight 96-well plates with one repeat.

The contents for each well of blanks, controls and tests distributing on the 96-well plates were added separately and sequentially before the end-point reading of UV absorption at 350 nm at 2 °C. The absorbance of samples in each well was read after the incubation at 37 °C for 20 min. The addition of assay mixture took less than 5 min for each plate and was carried out simultaneously while incubating at 37 °C, so that the total assay time for each plate was limited by incubation.

Samples with no effect on assembly of purified tubulin showed  $\Delta A_x$  of UV absorption change was similar as the blanks ( $\Delta A_0$ ). A fraction of samples (23 out of the total 1500 samples) showed irregular  $\Delta A_x$  due to



**Fig 4. A:** SDS-PAGE analysis of tubulin isolated with DEAE-Sephadex and one time polymerization-depolymerization cycle (1, DEAE-Tubulin; 2, warm supernate; 3, purified tubulin). **B:** Electron micrographs of purified tubulin.



**Fig 5. Polymerization of purified tubulin at different contents *in vitro*.** Glutamate-induced polymerization of purified tubulin at 37 °C and 350 nm in the presence of GTP (○, curve 1); without GTP (×, curve 2); in the presence of GTP and paclitaxel (△, curve 3); in the presence of paclitaxel without GTP (◇, curve 4).

precipitation and the data were discarded. However, deep color or slight cloudy appearance that affected the endpoint reading did not seem to affect the kinetic reading. Thus, these samples were transferred to the second kinetic reading screening directly as uncertainty samples.

As expected, most samples showed no or weak activities. There were 437 samples showed 5 %-10 %  $P_e$  values, 264 samples showed 10 %-20 %  $P_e$  values, 101 samples showed 20 %-30 %  $P_e$  values, 7 samples showed greater than 30 %  $P_e$  values, and the rest showed less than 5 %  $P_e$  values at the assay concentration of 10 mg/L. We took 108 samples with higher than 20 %  $P_e$  values as hits for further screening, and the hit rate was 7.2 % of the total 1500 samples. Very

few samples (11 of 1500) showed abnormal  $P_e$  values less than -20 %, and they were also transferred to the secondary assay.

As mentioned above, 108 hits and 11 uncertainty samples were transferred to the kinetic screening, placed in duplicates onto four 96-well plates and assay mixture was added separately and sequentially. The reaction mixtures were kinetically monitored with the UV absorption at 350 nm and 37 °C for 25 min. The total assay time for each plate was limited by the plate reading. In this screening system, there was no GTP in the solution, negative samples showed no change of the slope of UV absorption, while samples with positive activities changed the slope at various degrees, reflecting their promoting effects on the assembly of tubulin *in vitro* (Fig 5, curve 4).

Most of the hits showed promotion activities on the assembly of tubulin. There were two samples displayed 80 %-100 % promotion activity, four samples exhibited greater than 100 % activity. And all the uncertain samples showed no activity. We took the six samples with higher than 80 % promotion as hits for further characterization, and the hit rate was 0.4 % of the total 1500 samples.

Paclitaxel was used at different concentrations (0.01, 0.1, 1, 10, 100, and 1000 μmol/L) for assay quality control. It showed consistent promotion activity on the assembly of tubulin with the expected potency (>1 μmol/L) reflected by the pooled data from the 38 plates.

**Characterization of hit compounds** Crude samples were purified on Shimadzu LC-6A preparative HPLC by Shimadzu C18 ODS preparative column, and the final hits with promotion activities higher than 80 % were further investigated at six concentrations (0.05, 0.5, 1, 1.5, 5, and 15 μmol/L). Among those, five compounds showed activities higher than 0.5 μmol/L, leading to a confirming hit rate of 83 %. One compound showed precipitation and was considered false positive. After performing MTT test for each active compound, the most active compound obtained from this screening showed an  $IC_{50}$  of  $0.28 \pm 0.06$  nmol/L in HeLa cells. There were total of four compounds with  $IC_{50}$  lower than 5 nmol/L.

As far as the active compound structures were concerned, three of them were Epothilone A or its analogues<sup>[19]</sup>, which were isolated from fermentation products of Myxobacteria. Two compounds were new structures with a common pharmacophore for cyto-

toxic natural products that stabilize microtubules as described by Ojima *et al*<sup>[20]</sup>. These compounds are under further structure determined, structure-activity analysis (SAR) and structural modification for developing potent and selective anti-tumor agents.

## DISCUSSION

The screening model described in the present paper has integrated several critical factors for a successful HTS program: target identification or selection; reagent preparation (tubulins purification); compound management and high-throughput library screening<sup>[21]</sup>.

Although paclitaxel plays an important role in the treatment of certain human tumors, its toxicities and aqueous insolubility are continuing to be a problem. Another major concern in the clinical treatment of human tumors with paclitaxel is the acquisition of drug resistance, which often leads to chemotherapeutic failure<sup>[22]</sup>. In addition, P-glycoprotein expression type of multidrug-resistant (MDR) tumor cells resist paclitaxel<sup>[23]</sup>. Great attention has been paid to find novel compounds with the similar or better activity as paclitaxel, and the tubulins are thus becoming the molecular target amenable to small molecule drug discovery.

Usually, there are two approaches to obtain tubulins. One employs the characteristics of tubulin, ie polymerizing into microtubule at a higher temperature and microtubule depolymerizing into  $\alpha$ - and  $\beta$ -tubulin dimers at lower temperature *in vitro*. After several cycles of polymerization-depolymerization, the tubulins are purified. The other method is using ion exchange resin (eg DEAE-Sephadex) to separate the tubulins from other cellular components. The former method can get purified tubulins, but with very low efficiency. For instance, from 10 pig brains (approximately 900-1000 g), we can only obtain 10-50 mg purified tubulin after three cycles, depending on the freshness of the brain. After the first cycle, the tubulin only occupies small portion of the sample. Further cycles will lose most tubulin due to removal of unwanted protein. The latter method, in contrast, can recover most of the tubulin from the DEAE-resin (DEAE-tubulin). However, there are still many other proteins left in the product, accounting for about 30 %-40 % of the total protein. The assay of polymerization activity *in vitro* confirmed that, except for the first cycle-tubulin, the rest displayed very stable polymerization behavior. So, we developed a two-step purification method for screening purposes. The loss

of tubulin in the cycle was much reduced and the tubulin in different purities could be obtained for different purposes. Considering associated expenses and screening scale, the DEAE-tubulin could be used in screening of a large number of samples. Active samples could be retested in the second step with purified tubulin without GTP.

A critical prerequisite for lead compound discovery screening is the compound screening library itself. Common HTS library typically consists of both synthesized and natural compounds. Considering only six types of compounds that mimic the effects of paclitaxel on tumors have been found from numerous sources<sup>[10]</sup>, we focused not only on increasing library size, but also on sample quality, such as analytical purity, molecular weight and structure diversity. For our library, we balanced sheer numbers of samples with a reasonable number of representative replicate analogues. The 1500 samples screened were mainly isolated from bacterial fermentation products, especially different *Sorangium cellulosum* strains. In order to ensure all screening compounds be present in the assay plate, they were generally dissolved in an accommodated H<sub>2</sub>O/Me<sub>2</sub>SO mixture, in which the Me<sub>2</sub>SO concentration was under the limitation tolerated by tubulins. The best concentration of library compound within the assay plate is also a matter of debate. Screening at high concentrations often leads to more false positive signals. So, we chose a relatively high concentration (10 mg/L) in the primary HTS screening, and six concentrations for hits to evaluate their actual promoting potency. In all cases, the goal is to economically and efficiently compile a screening compound library from which HTS leads can be rapidly discovered.

We carried the screening of 1500 compounds during 2 h 50 min, and finished the kinetic reading screening assay in 2 h 30 min for 4 plates with a total of 119 samples. Assay miniaturization is an alternative to compound compression as a means to increase HTS capacity. In the simplest terms, miniaturization corresponds to higher screening plate density and smaller screening volumes. It will allow for more assays to be performed in parallel in a conventional space, and the screening capacity could be extended dramatically. As the compound number decreases significantly, the veracity of screening becomes the key factor. Therefore, purified tubulin without GTP and kinetic detection were used. Of the samples tested, the assay system proved to be a high-throughput, cost-effective and efficient

model for screening anti-tumor agents capable of promoting the polymerization of tubulin *in vitro*.

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## REFERENCES

- 1 Jurgen D. Drug discovery: a historical perspective. *Science* 2000; 287: 1960-4.
- 2 Dustin P. Microtubules. 2nd ed. Berlin: Springer-Verlag; 1984. p 482.
- 3 Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents: VI. the isolation and structure of paclitaxel, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* 1971; 93: 2325-7.
- 4 Rowinsky EK, Donehower RC. The clinical pharmacology and use of antimicrotubule agents in cancer chemotherapeutics. *Pharmacol Ther* 1991; 52: 35-84.
- 5 Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by paclitaxel. *Nature* 1979; 277: 665-7.
- 6 Schiff PB, Horwitz SB. Paclitaxel stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1980; 77: 1561-5.
- 7 Parness J, Horwitz SB. Taxol binds to polymerized tubulin *in vitro*. *J Cell Biol* 1981; 91: 479-87.
- 8 He L, Orr GA, Horwitz SB. Novel molecules that interact with microtubules and have functional activity similar to Taxol. *Drug Discov Today* 2001; 6: 1153-64.
- 9 Bollag DM, McQueney PA, Zhu J, Hensens O, Koupal L, Liesch J, *et al*. Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. *Cancer Res* 1995; 55: 2325-33.
- 10 Li YZ, Hu W, Zhou L. Natural anti-tumor compounds with activity of promoting microtubule assembly. *Acta Pharm Sin* 2001; 36: 15-60.
- 11 He LF, O' Geotge A, Horwitz SB. Novel molecules that interact with microtubules and have functional activity similar to Taxol<sup>TM</sup>. *Drug Disc Today* 2001; 6: 1153-64.
- 12 Ernest H, Chii ML. Glutamate-induced polymerization of tubulin: characteristics of the reaction and application to the large-scale purification of tubulin. *Arch Biochem Biophys* 1981; 209: 29-40.
- 13 Ernest H, Chii ML. Separation of active tubulin and microtubule-associated proteins by ultracentrifugation and isolation of a component causing the formation of microtubule bundles. *Biochemistry* 1984; 23: 4173-84.
- 14 Laemmli UK, Favre M. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J Mol Biol* 1973; 80: 575-99.
- 15 Phyllis GW, Anthony AD, Michael CL, Ernest H. Induction of polymerization of purified tubulin by sulfonate buffers. *Eur J Biochem* 1981; 120: 129-36.
- 16 Ye QZ, Nan FJ, Hu LY, Qian Z, Qian J. High-throughput screening for human collagenase 1 inhibitors. *Acta Pharmacol Sin* 2000; 21: 1021-26.
- 17 James C, William GD, Gazdar AF, John DM, James BM. Evaluation of tetrazolium-based semiautomated colorimetric assessment of chemosensitivity testing. *Cancer Res* 1987; 37: 936-42.
- 18 Himes RH, Burton PR, Gaito JM. Dimethyl sulfoxide-induced self-assembly of tubulin lacking associated proteins. *J Biol Chem* 1977; 252: 6222-8.
- 19 Hardt IH, Steinmetz H, Gerth K, Sasse F, Reichenbach H, Hofle G. New natural epothilones from *Sorangium cellulosum*, strains So ce90/B2 and So ce90/D13: isolation, structure elucidation, and SAR studies. *J Nat Prod* 2001; 64: 847-56.
- 20 Ojima I, Chakravaty S, Inoue T, Lin S, He L, Horwitz SB, *et al*. A common pharmacophore for cytotoxic natural products that stabilize microtubules. *Proc Natl Acad Sci USA* 1999; 96: 4256-61.
- 21 James AL, Ian CT, William GS, David GO, James K, Edward JH, *et al*. HTS in the new millennium: the role of pharmacology and flexibility. *J Pharmacol Toxicol Methods* 2000; 44: 273-89.
- 22 Gottesman M, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 1993; 62: 385-427.
- 23 Chou TC, Zhang XG, Balog A, Su DS, Meng D, Savin K, *et al*. Desoxyepothilone B: an efficacious microtubule-targeted antitumor agent with a promising *in vivo* profile relative to epothilone B. *Proc Natl Acad Sci USA* 1998; 95: 9642-7.