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In vivo Evaluation of Indolyl Glyoxamides in the Phenotypic Sea Urchin Embryo Assay

Marina N. Semenova[1,*], Alex S. Kiselyov[2], Ilia Y. Titov[2], Mikhail M. Raihstat[2], Maxim Molodtsov[3,4], Ekaterina Grishchuk[5,6], Ilia Spiridonov[4,5] and Victor V. Semenov[2,7]

[1]*Institute of Developmental Biology, Russian Academy of Sciences, 26 Vavilov Str, 119334 Moscow, Russia*
[2]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47 Leninsky Prospect, 117913 Moscow, Russia*
[3]*Center for Theoretical Problems of Physico-Chemical Pharmacology, Russian Academy of Sciences, Kosigin Str 4, 119991 Moscow, Russia*
[4]*National Research Center for Hematology, Russian Academy of Medical Sciences, Novozykovsky proezd, 4a, 125167 Moscow, Russia*
[5]*MCD Biology Department, University of Colorado at Boulder, Boulder, CO 80309-0347, USA*
[6]*Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Baltiiskaya Str, 12, 125169 Moscow, Russia*
[7]*Chemical Block Ltd, 3 Kyriacou Matsi, 3723 Limassol, Cyprus*
**Corresponding author: Marina N. Semenova, ms@chemical-block.com*

We have devised a 'one-pot' phenotypic *in vivo* assay for the rapid evaluation of potential tubulin inhibitors using the sea urchin embryo model. An effect of a small molecule on two specific developmental stages of sea urchin embryo, namely: (i) fertilized egg test for antimitotic activity and (ii) behavioral monitoring of a free-swimming blastulae for changes in the embryo swimming pattern could be quantified by a threshold concentration resulting in respective abnormalities. Derivatives of the clinical candidate D-24851 featured good correlation between activity in tubulin polymerization assay and our *in vivo* data. Importantly, we demonstrated that in these series, the *N*-substitution of indole is non-essential to attain profound *in vitro* and cellular effects.

Key words: antimitotic agents, glyoxamide, phenotypic assay, sea urchin embryo, tubulin

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Tubulin remains one of better validated targets in oncology (1). Despite a great selection of compounds targeting tubulin, there is ongoing need for the small molecules that display (i) better therapeutic window (for example, tumor reduction versus neurotoxicity and cardiotoxicity), (ii) reduced non-specific toxicity, (iii) improved pharmacokinetics, (iv) lack of multi-drug resistance (MDR), and (v) synthetic feasibility. In addition, evaluation of antimitotic molecules affecting tubulin dynamics for *in vivo* studies is a convoluted, multistage process. Generally, early assessment of a new small molecule entity includes estimation of cytotoxic and antimitotic activities (G_2M arrest) followed by studies of *in vitro* tubulin polymerization effect of the molecule (2).

To introduce reliable *in vivo* read-out that allows the selection of potent mechanistically sound compounds targeting tubulin, we have devised phenotypic assay based on the sea urchin embryo (3). This simple organism model facilitates rapid 'one-pot' assessment of antiproliferative, antimitotic, and cytotoxic effects of small molecules on a living organism. The assay includes: (i) fertilized egg test for antimitotic activity displayed by cleavage alteration/arrest and (ii) behavioral monitoring of a free-swimming blastulae treated immediately after hatching (9–12 h after fertilization). Changes to the embryo swimming pattern, i.e. lack of forward movement, settlement to the bottom of the culture vessel, and rapid spinning around the animal–vegetal axis suggest a tubulin-destabilizing activity caused by a molecule (video illustrations are available at http://www.chemblock.com). Notably, this 'one-pot' assay yields data on both antiproliferative activity of the test molecule (e.g. D-24851) and on its tubulin polymerization effect (e.g. destabilizing versus stabilizing) (Figure 1).

Among new chemical entities introduced to the later-stage clinical trials, a compound D-24851 developed by Baxter (**1**, Table 1) attracted our attention. This molecule was reported to possess favorable functional activity [profound G_2M effect, lack of MDR, and *in vivo* neurotoxicity, promising *in vivo* efficacy (4,5)]. However, despite a number of studies describing structure–activity relationship for a series of D-24851 analogs (4–8), we felt that relatively little attention was paid to the respective unsubstituted analogs (NH indoles). In this study, we described both syntheses, *in vitro* and *in vivo* evaluation of these glyoxamide derivatives. Notably, we showed sound correlation between the data obtained in both assay systems suggesting that the sea urchin embryo is a promising surrogate *in vivo* model for evaluating antimitotic agents.

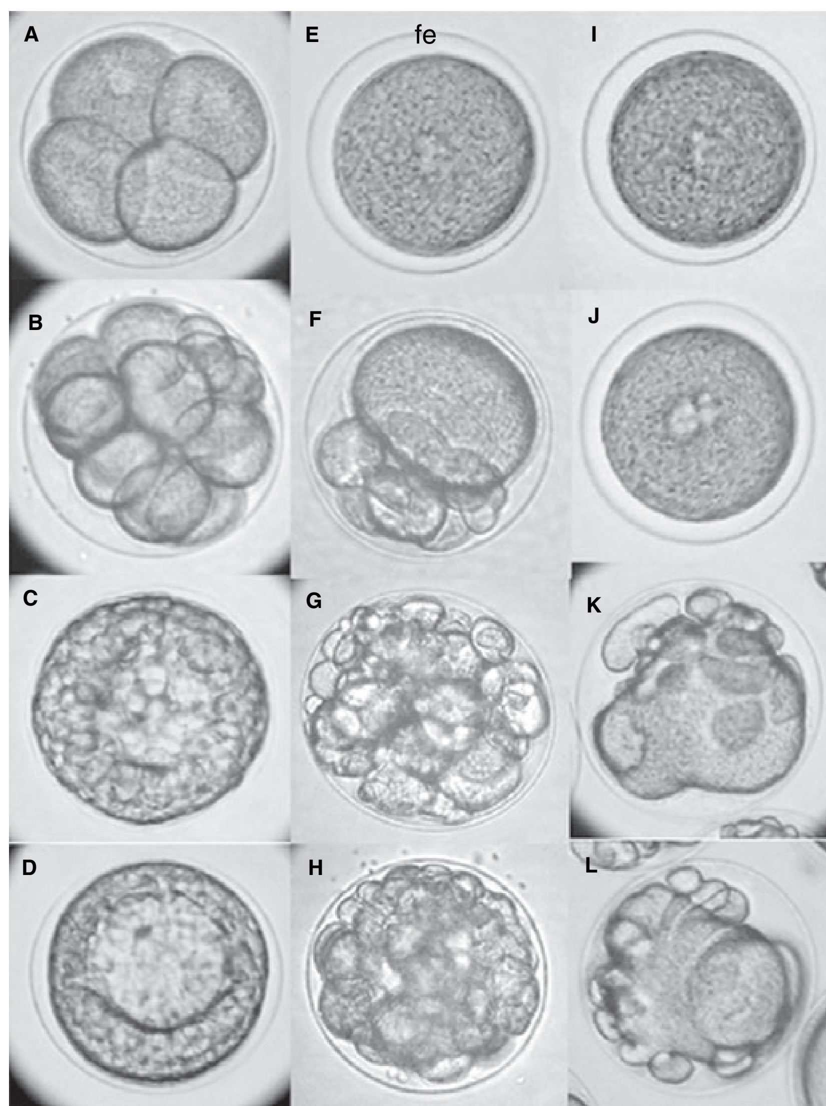


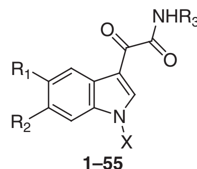
Figure 1: Typical effect of tubulin-destabilizing compound D-24851 **(1)** on sea urchin fertilized egg development. Time after fertilization (18 °C): (A, E, and I) – 2.5 h; (B, F, and J) – 4 h; (C, G, and K) – 7 h; (D, H, and L) – 9 h. (A–D) Intact embryos. (A) Four-cell stage. (B) 16-cell stage. (C) Early blastula. (D) Mid-blastula just before hatching. Fertilized eggs (10–15 min after fertilization) were exposed continuously to D-24851 at threshold (10 nM; E–H) or 2–10 times higher concentration (I–L). Note cleavage alteration (F–H), cleavage arrest (E, I, and J), and formation of tuberculous eggs (K, and L). The average embryo diameter is 115 μM including the fertilization envelope (fe). Microphotographs presented from **3**).

Results and Discussion

We have synthesized a series of indolyl glyoxamides **1–55** using protocol described earlier [Scheme 1 (4,7)]. In general, yields of the targeted molecules were in line with the reported values (80–95%). Purity of the materials for the biological testing exceeded 97% as evidenced by NMR, LC MS and combustion analyses.

For the sea urchin embryo assay, stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO) and/or in 95% EtOH at 5–10 mM concentrations. In our hands, dilution of stock solutions containing both DMSO and EtOH with sea water as required by the sea urchin embryo assay protocol, enhanced solubility of the test articles in the salt-containing media, as evidenced by the microscopic examination of both daughter and screening solutions. Further, we found that the maximal tolerated concentrations of DMSO and EtOH in the *in vivo* assay were 0.05% and 1%, respectively. Higher concentrations of both DMSO (≥0.1%) and EtOH (≥1%) caused non-specific alteration and retardation of the sea urchin embryo development independent of stage. The effects of molecules were quantified by a threshold concentration resulting in cleavage abnormalities and embryo death before hatching or full mitotic arrest (Table 1). Combretastatin A-4P (9), nocodazole (10),

Table 1: Effects of indolyl glyoxylamides **1–55** on sea urchin embryo development

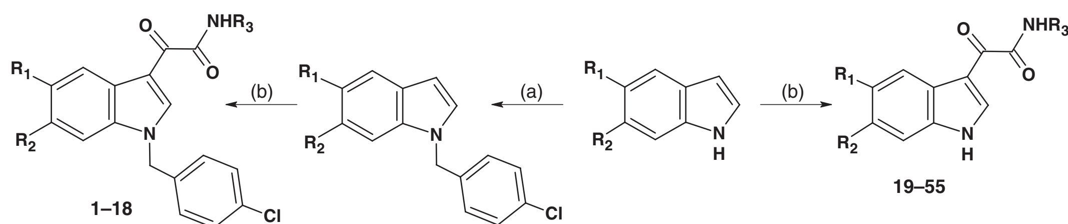


Compound numbers	R^1	R^2	R^3	X	Threshold concentration (uM)[a] Cleavage alteration	Cleavage arrest	Spinning	Destabilizing index (i)[b]
	Combretastatin A-4P				0.005	0.01	1	0.024[c]
	Vinblastine				0.2	1	5	0.0032[c]
	Nocodazole				0.005	0.01	0.1	0.05[c]
1	D-24851, H	H	4-Py	*p*Cl–$C_6H_4CH_2$	0.01	0.02	0.1	−0.9; 0.86[c]
2	H	H	*o*Cl-3-Py	*p*Cl–$C_6H_4CH_2$	>2.5	>2.5	>2.5	N/A
3	H	H	3,4-Methylenedioxyphenyl	*p*Cl–$C_6H_4CH_2$	0.05	0.1	1	−1.3
4	Br	H	3,4-Methylenedioxyphenyl	*p*Cl–$C_6H_4CH_2$	>4	>4	>5	N/A
5	H	H	3,4-Ethylenedioxyphenyl	*p*Cl–$C_6H_4CH_2$	0.1	2	0.05	−1.3
6	Br	H	3,4-Ethylenedioxyphenyl	*p*Cl–$C_6H_4CH_2$	>4	>4	>5	N/A
7	H	F	3,4-Ethylenedioxyphenyl	*p*Cl–$C_6H_4CH_2$	2	>4	0.05	N/A
8	H	H	2-(5-Me)-Isoxazolo	*p*Cl–$C_6H_4CH_2$	0.01	0.1	0.5	−0.9
9	H	H	*p*MeO–C_6H_4–	*p*Cl–$C_6H_4CH_2$	0.05	1	0.4	−1.2
10	H	H	*m*MeO–C_6H_4–	*p*Cl–$C_6H_4CH_2$	4	>5	5	N/A
11	H	H	*p*F_3CO–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>1	>1	>1	N/A
12	H	H	*p*F_3C–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>0.5	>0.5	>0.5	N/A
13	H	H	*m*F_3C–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>4	>4	>5	N/A
14	H	H	*p*Cl–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>5	>5	>5	N/A
15	H	H	*m*Cl–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>5	>5	>5	N/A
16	H	H	*p*F–C_6H_4–	*p*Cl–$C_6H_4CH_2$	4	>4	>5	−0.9
17	H	H	*m*F–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>4	>4	>5	N/A
18	H	H	*p*Br–C_6H_4–	*p*Cl–$C_6H_4CH_2$	>5	>5	>5	N/A
			(Fragments)					
19	H	H	4-Py	H	0.02	2	5	−0.7
20	H	H	4-Py–CH_2–	H	>4	>4	>5	N/A
21	H	H	3-Py	H	0.2	>4	>4	−0.1
22	Br	H	3-Py	H	>4	>4	>4	N/A
23	H	F	3-Py	H	1	>4	2	N/A
24	H	H	2-(5-Me)-Isoxazolo	H	2	>4	>5	N/A
25	H	F	2-(5-Me)-Isoxazolo	H	>4	>4	>5	N/A
26	**H**	**H**	*p*MeO–C_6H_4–	H	0.001	0.005	0.2–0.5	−0.7
27	H	H	*p*MeO–C_6H_4–CH_2–	H	>4	>4	>5	N/A
28	NO_2	H	*p*MeO–C_6H_4–	H	>4	>4	>5	N/A
29	Br	H	*p*MeO–C_6H_4–	H	>2	>2	>2	N/A
30	**F**	**H**	*p*MeO–C_6H_4–	H	0.002	0.01	>5	N/A
31	**H**	**F**	*p*MeO–C_6H_4–	H	0.001	0.005	1	N/A
32	H	H	*m*MeO–C_6H_4–	H	0.05	>4	>5	N/A
33	H	F	*m*MeO–C_6H_4–	H	0.2	>4	>5	N/A
34	H	H	*m*CF_3–C_6H_4–	H	>4	>4	>5	N/A
35	H	H	*p*Cl–C_6H_4–	H	>5	>5	>5	N/A
36	H	H	*p*F–C_6H_4–	H	0.02	>4	>5	N/A
37	H	H	*m*F–C_6H_4–	H	0.2	>5	>5	N/A
38	H	H	*m*Cl–*p*-F–C_6H_3–	H	0.1	>4	>5	N/A
39	H	H	3,4-diMeO–C_6H_3–	H	0.1	>4	>5	N/A
40	H	H	3,4,5-triMeO–C_6H_2–	H	0.1	1	>5	N/A
41	F	H	3,4,5-triMeO–C_6H_2–	H	1	4	>5	N/A
42	H	F	3,4,5-triMeO–C_6H_2–	H	0.1	1	>5	N/A
43	H	H	3,4-Methylenedioxyphenyl	H	0.002	0.005	0.05	-0.6
44	H	H	3,4-Methylenedioxyphenyl-CH_2-	H	>4	>4	>5	N/A
45	F	H	3,4-Methylenedioxyphenyl	H	0.005	0.02	0.2	N/A

Table 1: (*Continued*)

Compound numbers	R^1	R^2	R^3	X	Threshold concentration (uM)[a] Cleavage alteration	Cleavage arrest	Spinning	Destabilizing index (i)[b]
46	H	F	3,4-Methylenedioxyphenyl	H	0.002	0.01	0.5	N/A
47	H	H	3,4-Ethylenedioxyphenyl	H	0.002	0.005	0.02	−0.8
48	Br	H	3,4-Ethylenedioxyphenyl	H	>4	>4	>5	N/A
49	F	H	3,4-Ethylenedioxyphenyl	H	0.005	0.02	0.1	N/A
50	H	F	3,4-Ethylenedioxyphenyl	H	0.005	0.02	0.5	N/A
51	H	H	*p*Cl–C_6H_4–CH_2–	H	>4	>4	>4	N/A
52	H	H	*p*Cl–C_6H_4–	H	>4	>4	>5	N/A
53	H	H	*p*MeO_2C–C_6H_4–	H	0.1	>5	>5	−0.1
54	H	H	*p*MeC(O) –C_6H_4–	H	0.05	>5	>5	N/A
55	H	H	2-Thiazolo-	H	1	>4	>5	N/A

[a]Sea urchin embryo assay was conducted as described in ref/ (3); triplicate measurement showed no differences in threshold concentration values.
[b]95%SE = ±0.1.
[c]IC_{50} values for the *in vitro* polymerization of purified tubulin. From ref. (9) for combretastatin A-4P, ref. (10) for nocodazole and vinblastine, and ref. (4) for D-24851.



Scheme 1: General protocol for the synthesis of indolyl glyoxamides **1-55**: (a) *p*X-$C_6H_4CH_2Cl$ (X = Cl, F), *t*-BuOK, THF, 0 °C (90–95% yields); (b) oxalyl chloride, Et_2O, 0 °C; R_3NH_2, Et_3N, THF (80–95% yields).

vinblastine (10) and D-24851 (5) served as a benchmark reference compounds.

As evidenced from Table 1, *in vivo* sea urchin embryo assay yielded data that closely followed *in vitro* tubulin polymerization assay for the standards. For example, vinblastine, nocodazole, combretastatin A-4P, and D-24851 displayed sound effect on the sea urchin embryo (Table 1). Effective concentrations of the reference compounds resulting in embryo cleavage alteration/arrest were close to the reported IC_{50} values for a panel of human tumor cell lines (4–10). This was also in agreement with their *in vitro* activity in the tubulin polymerization assay (5,9,10).

In general, molecules containing mono- or bis-alkoxyaryl substituents (R^3) consistently displayed superior *in vivo* activity as well as tubulin-destabilizing properties (e.g. **3**, **5,** and **9**; Table 1). Notably, bromo substitution in the indole ring had an adverse effect on *in vivo* activity of the compound (compare **3** and **4**; **5** and **6**). Derivatives **2** and **11–18** were inactive in the sea urchin embryo test.

In the next series of experiments, we evaluated a number of NH-derivatives of D-24851 (**19–55,** Table 1) in our phenotypic assay as well as in a tubulin polymerization assays. Compound **19** was found to have reduced *in vivo* antitubulin activity compared to the parent D-24851. Molecules modified with *p*-methoxyphenyl anilines (**26**, **30,** and **31**), as well as cyclic bis-oxygenated species featuring 3,4-methylene- and 3,4-ethylenedioxyphenyl substituents (**43, 45–47, 49,** and **50**), displayed excellent *in vivo* and *in vitro* tubulin destabilizing activity (Table 1). Their effective concentrations causing cleavage alteration in the assay were <10 nM, matching *in vitro* tubulin polymerization inhibitory activities. Notably, bromo derivative **48** was inactive, whereas the respective fluoro species did not alter activity (**45, 46, 49,** and **50,** Table 1). 3,4-Dimethoxy- and 3,4,5-trimethoxyphenyl derivatives (**39–42**) displayed *in vivo* tubulin-independent antiproliferative activity in our phenotypic assay (Table 1).

Addition of methylene group between amino- and *p*-methoxyphenyl/3,4-methylenedioxyphenyl moiety (compare **19** and **20**; **26** and **27**; **43** and **44**; Table 1) ablated tubulin destabilizing effect. *Para*-substitution at R^3 (e.g. **9, 26,** and **31)** enhanced tubulin destabilizing activity. Respective *m*-analogs (**10**, **32,** and **33)** were less active or caused tubulin-unrelated cleavage alteration (Table 1). Compound **19** (4-Py) featured considerably better potency than **21** (3-Py). Similar pattern of inhibitory activity against human tumor cell lines for these indole derivatives has been described earlier (6). In this study, molecules **21** and **53,** which induced cleavage abnormalities, failed to cause both embryo spinning and inhibition of tubulin polymerization *in vitro*

(Table 1), suggesting the potential for the tubulin-independent nature of their antiproliferative effect.

Representative molecules **26**, **43,** and **47** have been profiled against a panel of kinases composed of enzymes implicated in various aspects of tubulin dynamics and cell cycle, specifically Cdk1, Cdk2, Auroras A/B, and Plk1. Other targets included tyrosine (EGFR, ErbB2, Flt1, VEGFR2, FGFR1, PDGFRβ, IGF1R, bcr-Abl, c-Met, and c-Kit) and serine/threonine kinases (PKA, Raf-1, JNK3, PI3α, and Src). Notably, the selected molecules did not show inhibition for the entire kinase panel (percent inhibition, PI > 30%) at 10 μM concentration. Interestingly, compound **54** displayed 50 nM activities in a cleavage alteration step. However, it was inactive in cleavage arrest/spinning follow-up. Of the panel kinases mentioned above, compound **54** only inhibited Auroras A and B (PI = 67% and 73%, respectively, at 10 μM) suggesting that the observed *in vivo* effect may in part be due to this kinase inhibition.

In conclusion, we have devised a streamlined phenotypic assay for the rapid evaluation of potential tubulin inhibitors. An effect of a small molecule on two specific developmental stages of sea urchin embryo, namely: (i) fertilized egg test for antimitotic activity and (ii) behavioral monitoring of a free-swimming blastulae for changes in the embryo swimming pattern could be quantified by a threshold concentration resulting in respective abnormalities. Derivatives of the clinical candidate D-24851 featured good correlation between activity in tubulin polymerization assay and *in vivo* data. Importantly, we demonstrated that in these series, the *N*-substitution of indole is nonessential to attain profound *in vitro* and cellular effects. For instance, respective 3,4-methylene- and 3,4-ethylenedioxyphenyl derivatives showed dramatic *in vivo* and *in vitro* effects across the assay panel. Based on our initial observations, the phenotypic sea urchin embryo assay is valid for the primary identification of specific antiproliferative agents affecting molecular targets other than tubulin.

Methods and Materials

Sea urchin embryo assay

Adult sea urchins *Paracentrotus lividus* were collected from Mediterranean Sea at Cyprus coast and kept in aerated seawater tank. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered sea water and fertilized by adding drops of a diluted sperm. Embryos were cultured at room temperature under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered sea water. The embryos were observed with light microscope Biolam LOMO [Microscopes.com (www.microscopes.com) Northbrook, IL, USA]. For compound treatment, 5 mL aliquots of embryo suspension were transferred to six-well plates and incubated as a monolayer at a concentration up to 3000 embryos/mL. The antiproliferative activity was assessed by exposing fertilized eggs (10–25 min after fertilization, 45–60 min before the first mitotic cycle completion) to twofold decreasing concentrations of a compound. Cleavage alteration and arrest were clearly detected at 3–6 h after fertilization (Figure 1). The effects were quantitatively estimated as a threshold concentration resulting in cleavage alteration and embryo death before hatching or full mitotic arrest. For tubulin-destabilizing activity, the compounds were tested on free-swimming blastulae just after hatching (9–12 h after fertilization), originated from the same embryo culture. Embryo spinning was observed after 0.5–20 h of treatment, depending on the nature and concentration of the compound. Both spinning and lack of forward movement were interpreted to be the result of the tubulin-destabilizing activity of a molecule.

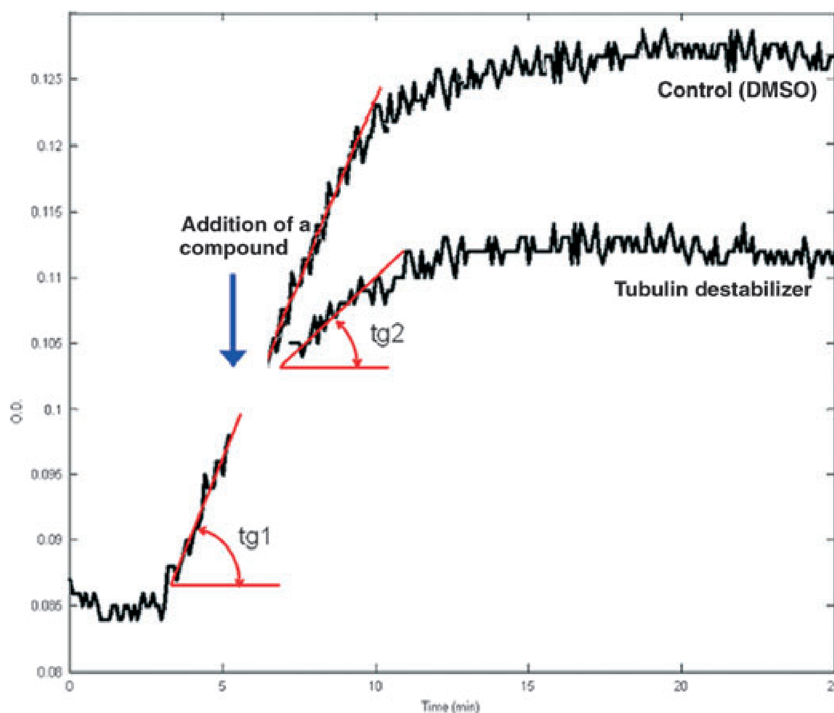


Figure 2: Turbidity curves expressing *in vitro* purified tubulin polymerization. tg1 and tg2 are tangents of linear regression slope angles before and after the addition of a compound, respectively. O.D. – optical density at 490 nm.

In vitro *tubulin polymerization assay*

In vitro tubulin polymerization was determined in a turbidity assay (11). Tubulin was prepared from calf brains by two cycles of polymerization/depolymerization (12), aliquoted and stored at −80 °C. The assay was conducted in 96-well plates in a microplate reader (ThermoMax, Molecular Devices Corp., Sunnyvale, CA, USA) at 37 °C in BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA) supplemented with 1 mM GTP (Sigma-Aldrich Corp., St Louis, MO, USA). Tubulin concentration was ca. 4 mg/mL [Bio-Rad assay (Hercules, CA, USA)]. Test compounds were initially dissolved in DMSO at 4 mg/mL (ca. 10 mM) and diluted with EtOH (1:20 by volume) to facilitate solvation and to reduce/prevent precipitation. Tubulin was allowed to polymerize for 5 min in the presence of GTP. Turbidity was recorded at 490 nm every 6 second until linear increase was insured. A compound solution (10 μM final concentration) was added to the resulting polymerizing tubulin under gentle mixing, reading resumed at 6-seconds intervals for additional 40 min. A typical destabilizing compound featured decrease in the activity slope of the polymerization curve (Figure 2). Linear regions of the graph were analyzed using MATLAB6.1 (MathWorks, Inc., Natick, MA, USA) software to calculate destabilizing index $i = (tg2 - tg1)/tg1$, expressing normalized difference between slopes of the curve before and after addition of the compound. The assay provides the direct estimation of inhibitory kinetics because a tested article is added to tubulin in the course of its polymerization.

Acknowledgment

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