

Sea urchin embryo as a model organism for the rapid functional screening of tubulin modulators

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Identification of antimetabolic molecules that affect tubulin dynamics is a multistep procedure. It includes in vitro tubulin polymerization assay, studies of a cell cycle effect, and general cytotoxicity assessment. To simplify this lengthy screening protocol, we have introduced and validated an assay system based on the sea urchin embryos. The proposed two-step procedure involves the fertilized egg test for mitotic arrest and the behavioral assessment of a free-swimming blastula. In order to validate the assay, we have analyzed the effect of a panel of known antiproliferative agents on the sea urchin embryo. For all tubulin destabilizing drugs, we observed rapid spinning and lack of forward movement of an embryo. Both effects are likely to result from the in vivo microtubule disassembly caused by test molecules. Notably, the described assay yields rapid information on antiproliferative, antimetabolic, cytotoxic, and tubulin destabilizing activities of the molecules along with their solubility and permeability potential. Moreover, measured potencies of the test articles correlated well with the reported values in both in vitro and cell based assays.

INTRODUCTION

Tubulin is the major protein component of microtubules, a cytoskeletal organelle that plays an essential role in cell division, intracellular transport, cell motility, and shape maintenance (1). During mitosis, microtubules are required for mitotic spindle formation and chromosomal separation. Hence, targeting tubulin in rapidly dividing tumor cells is a sound strategy for cancer therapy (2). Approved drugs that affect microtubule dynamics include paclitaxel (TaxolTM) and vinblastine. Their use, however, is limited by poor therapeutic window, multiple drug resistance, low solubility, and general systemic toxicity. Hence, a considerable challenge to discover and develop novel small molecule inhibitors of tubulin dynamics exists for the pharmaceutical industry. Several small heterocycle-based compounds appeared recently and showed some unique characteristics; as a representative example, D-24851 demonstrated

anti-tumor effect in xenograft models (3). Indole-based D-24851 did not bind to any of the characterized binding sites on tubulin. Moreover, in a variety of tests, this phase II drug candidate did not show neurotoxicity, a common side-effect of antimetabolic agents.

Evaluation of antimetabolic agents displaying desirable efficacy and safety profiles similar to D24851 is rather lengthy. This multistep procedure includes studies of the in vitro effect on tubulin polymerization, cytotoxicity and cell cycle effects, and a panel of functional assays. In this paper, we report on a physiologically relevant one-pot procedure for the assessment of an antimetabolic activity of compound libraries. Simplicity of the experimental protocol, relevance of the model, and the potential for both medium-throughput and structure-activity relationship studies are the distinctive features of the developed screening protocol.

The sea urchin embryo has long been used as a model organism for the developmental biological studies (4–8).

A number of factors make this system suited for conducting a wide range of biological tests. These include straightforward artificial spawning, fertilization and rearing, rapid synchronous development, embryo optical transparency, and well understood embryogenesis. As a result, sea urchin embryos have been successfully used in studies of the effects of various antiproliferative agents (9–12).

Important to our objectives, there are two distinct processes directly connected to microtubule dynamics during early sea urchin embryogenesis. These are cleavage (i.e., a series of successive mitotic cycles occurring within approximately 30-min intervals) and ciliary swimming of hatched embryos within 9–12 h after fertilization. Both processes can be easily monitored and quantified, yielding the opportunity to screen compound libraries for the molecules affecting microtubule structure and function.

In our initial set of experiments, we investigated the effect of a potent

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tubulin-destabilizing drug D-24851 (3) on the embryonic development of sea urchin *Paracentrotus lividus* from the fertilized egg until the beginning of active feeding (mid-pluteus stage). Subsequently, we proposed a protocol for parallel screening of small molecules for tubulin destabilizing activity using the sea urchin embryos. The assay procedure was further validated by testing a series of diverse antiproliferative agents that affect different intracellular targets.

MATERIALS AND METHODS

Adult sea urchins *P. lividus* were collected from the Mediterranean Sea at the Cyprus coast and kept in an aerated seawater tank. Gametes were obtained by intracelomic injection of 0.5 M KCl. Eggs were washed with filtered sea water and fertilized by adding drops of diluted sperm. Embryos were cultured at room temperature under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered sea water up to the beginning of active feeding (mid-pluteus stage). The embryos were observed with a Biolam LOMO light microscope (LOMO PLC, St. Petersburg, Russia). Electronic images were obtained using digital camera Olympus C4000 digital camera with a microscope adaptor 10× (Optica M, St. Petersburg, Russia).

Test articles were obtained as follows. D-24851 [N-(pyridin-4-yl)-(1-[4-chlorbenzyl]-indol-3-yl)-glyoxyl-amid], SK2 [(2,3-di-hydro-benzo[1,4]dioxin-6-yl)-(5-[2-([pyridin-3-ylmethyl]-amino)-phenyl]-[1,3,4]oxadiazol-2-yl)-amine], SK3 [benzo(1,3) dioxol-5-yl-(5-[2-([pyridin-4-ylmethyl]-amino)-phenyl]-[1,3,4] oxadiazol-2-yl)-amine], and SK4 [(2,3-dihydro-benzo[1,4]dioxin-6-yl)-(5-[2-([pyridin-4-ylmethyl]-amino)-phenyl]-[1,3,4]oxadiazol-2-yl)-amine] were synthesized at Zelinsky Institute of Organic Chemistry (Moscow, Russia) as described elsewhere (3,13). Acrylamide, apigenin, 3'-azido-3'-deoxythymidine (azidothymidine), colchicine, cytochalasin D, dolastatin 15, 5-fluorouracyl, hydroxyurea, nocodazole, paclitaxel, and roscovitine were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), 4-phenylbutyric acid was

obtained from Acros Organics (Geel, Belgium), and 2-methoxyestradiol was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, UK). Anti-tumor medicines mitoxantrone (Mitoxantrone-Lans™; Lans Pharm, Russia), mitomycin C (Mitomycin-Vero™; Veropharm, Russia), and vinblastine (Vinblastin-Richter™; Gedeon Richter, Budapest, Hungary) were purchased at pharmacies as commercial preparative forms. Combretastatin A-4 disodium phosphate was a gift of OXiGENE (Waltham, MA, USA). Ruboxyl [14-(1-oxyl-2,2,6,6-tetramethylpiperidyl-4)-acetoxyrubomycin hydrochloride] was a gift of Dr. Vladimir A. Serezhenkov, Institute of Chemical Physics, Russian Academy of Sciences, Moscow, Russia. Structures of noncommercial compounds ruboxyl, SK2, SK3, and SK4 are presented in Figure 1.

Stock solutions of reagents were prepared as follows: acrylamide and hydroxyurea, 500 mM/filtered sea water; colchicine, 20 mM/filtered sea water; ruboxyl, 10 mM/filtered sea water; vinblastine, 5 mM/0.9% NaCl; combretastatin A-4, 5 mM aqueous solution; mitoxantrone, 3.8 mM aqueous solution; mitomycin C, 1.5 mM aqueous solution; cytochalasin D, 2 mM/95% ethanol; apigenin, azidothymidine, dolastatin 15, 2-methoxyestradiol, and paclitaxel, 5 mM/95% ethanol; 5-fluorouracyl and 4-phenylbutyric acid, 50 mM/95% ethanol; D-24851, 0.4 mM/95% ethanol prepared from 5–10 mM/dimethyl sulfoxide (DMSO); nocodazole, 0.25 mM/95% ethanol prepared from 5 mM/DMSO; SK2, SK3, and SK4, 0.05 mM/95% ethanol prepared from 5 mM/DMSO; roscovitine, 1 mM/95% ethanol prepared from 10 mM/DMSO. In all cases, the solvents at final concentrations caused no developmental changes.

For compound treatment, 5-mL aliquots of embryo suspension at required developmental stages were transferred to 6-well plates and incubated as a monolayer at a concentration up to 3000 embryos/mL. For D-24851, sea urchin embryos were exposed continuously to the compound both in 6-well plates and in 100-mL glass beakers with motor-driven plastic

paddles, at the following developmental stages: fertilized egg, early blastula, hatched blastula, gastrula, and early pluteus. All other test articles were added to fertilized eggs and to hatched blastulae. The development was observed up to mid-pluteus.

The antiproliferative activity was studied by exposing fertilized eggs (10–25 min after fertilization, 45–60 min before the first mitotic cycle completion) to two times the decreasing concentration of the compounds. The effects were quantitatively estimated as a threshold concentration resulting in cleavage alteration and embryo death before hatching or full mitotic arrest (vide infra).

RESULTS

In order to find detectable and quantifiable developmental changes caused by tubulin/microtubule binding chemicals, we studied the effect of D-24851 on several embryo stages: fertilized egg, early blastula, hatched blastula, early gastrula, and early

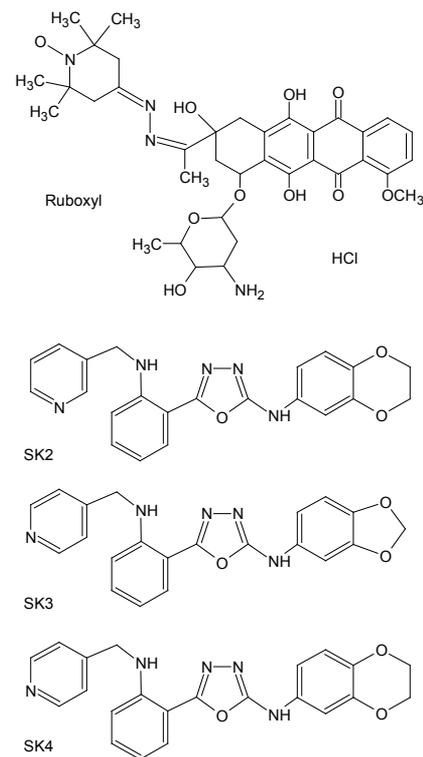


Figure 1. Structures of selected compounds tested on the sea urchin embryo.

pluteus. When applied to the fertilized eggs of sea urchin, 10–500 nM D-24851 altered cleavage and caused embryo death before hatching (Figure 2). The samples were observed at several time intervals, when intact embryos reached the following stages: 2 cells, 4 cells, 8 cells, 16 cells, early blastula, prehatching blastula, and hatched blastula. At concentrations of 5 nM and less, the compound did not produce any visible changes in embryo development. At 10 nM we observed cleavage delay (Figure 2E), followed by abnormal cleavage (Figure 2, F–H) and embryo death when intact embryos hatched. The extent of antimitotic effect was concentration-dependent and about 10-fold more pronounced in embryo suspension stirred by the paddle than incubated as monolayer in the plates. This outcome was explained by a low solubility of the compound and its tendency to form a fine suspension in a screening system. When control embryos reached early blastula stage, the arrested eggs acquired a specific tuberculous shape clearly different from that of intact eggs (Figure 2, K and L).

When added to early blastulae at the beginning of ciliogenesis, 50–100 nM D-24851 induced hatching delay of about 3 h, embryo immobilization, and developmental arrest at late gastrula stage. Embryo immobilization seemed to be the result of a specific inhibition of a ciliary microtubule assembly, rather than other toxic effects, since embryos were able to hatch, further forming primary mesenchyme cells, archenteron, and spicule rudiments.

Free-swimming embryos after hatching changed their swimming behavior markedly, when exposed to 100–500 nM D-24851. Namely, their forward movement gradually slowed down, viable embryos settled to the bottom of an incubation vessel, and subsequently started to spin counter-clockwise around the animal-vegetal axis when viewed from the vegetal pole (Figure 3D; video illustrations are available at www.chemblock.com). The effect progressed over the course of treatment and became clearly detectable after 10–13 h. Hatched blastulae (Figure 3A) were more sensitive than gastrulae (Figure 3C), probably due to the lesser extent of

ciliary machinery development. When applied to gastrula stage, D-24851 caused embryo spinning at concentrations of 200 nM and higher. Rapid spinning of embryos usually continued for 5–7 h. It then gradually slowed down followed by embryo disaggregation and death. When D-24851 was applied immediately after hatching, we observed gastrulation delay of 2–3 h and formation of embryos with low transparency, due to the increased

number of cells inside (Figure 3F) when intact embryos reached early pluteus stage (Figure 3E). In this case, embryos never developed past the gastrula stage with spicule rudiments. Neither morphological abnormalities of archenteron formation nor alteration of the primary mesenchyme cell ingression were observed. At early pluteus stage (Figure 3E), when embryos use ciliary band for locomotion instead of uniform surface

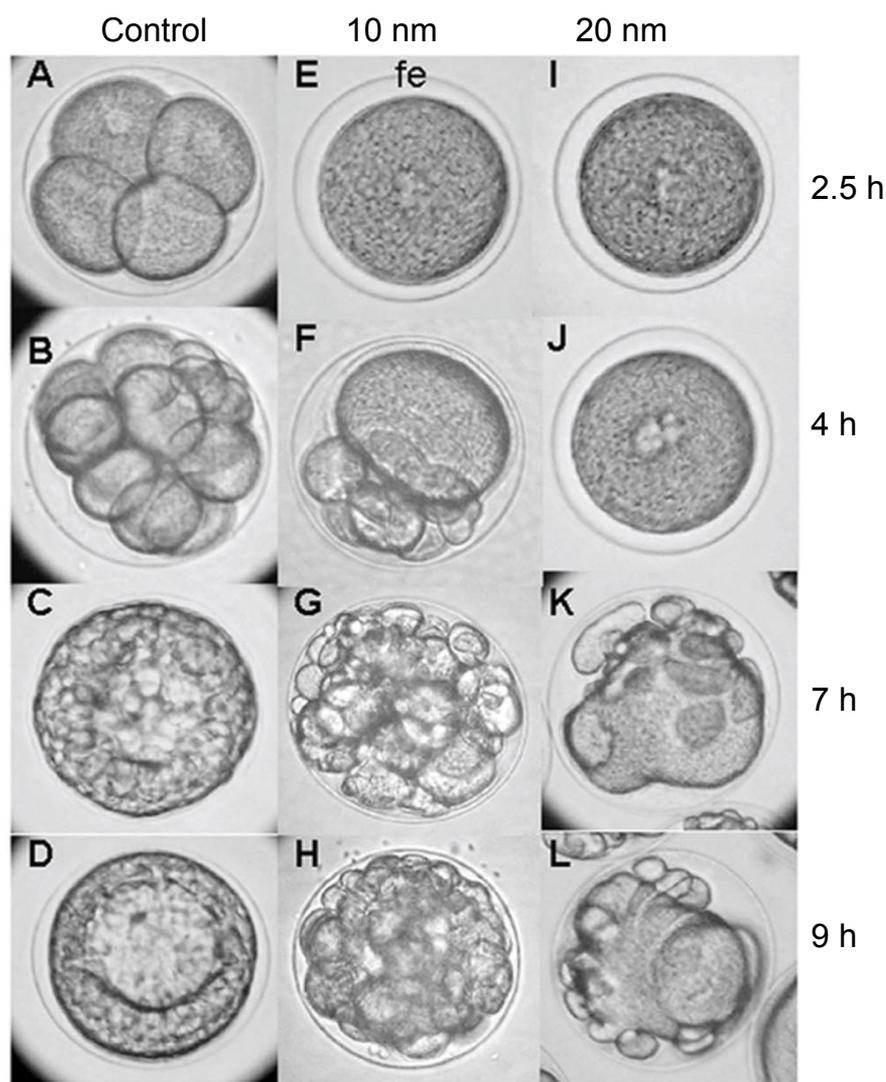


Figure 2. The effect of D-24851 on sea urchin fertilized egg development. Tests were performed in embryo suspension stirred by a paddle at 18°C. Time after fertilization: (A, E, and I) 2.5 h; (B, F, and J) 4 h; (C, G, and K) 7 h; (D, H, and L) 9 h. (A–D) Intact embryos. (A) 4-cell stage. (B) 16-cell stage. (C) Early blastula. (D) Mid-blastula just before hatching. Fertilized eggs (10–15 min after fertilization) were exposed continuously to D-24851, 10 nM (E–H) or 20 nM (I–L). Note cleavage alteration (F–H), cleavage arrest (E, I, and J), and formation of tuberculous eggs (K and L). For embryos cultivated as a monolayer in 6-well plates, the effects similar to those in panels E–H and I–L were observed at D-24851 concentrations of 50 and 100 nM, respectively. The average embryo diameter is 115 µm including the fertilization envelope (fe).

Table 1. The Effects of Antiproliferative Drugs on Sea Urchin Embryo Development

Compound	Threshold Concentrations (μM) ^a		Mechanism of Action (Reference)
	Cleavage Alterations	Embryo Spinning	
Tubulin/Microtubule Targeting Drugs			
Colchicine	50	50	Inhibits microtubule assembly through colchicine site (2,14).
Combretastatin A-4	0.005	1	Inhibits microtubule assembly through colchicine site (2).
Nocodazole	0.005	0.1	Inhibits microtubule assembly through colchicine site (2).
Vinblastine	0.2	10	Inhibits microtubule assembly through vinca-alkaloid domain (2).
Dolastatin 15	0.005	2	Inhibits microtubule assembly through vinca-alkaloid domain (15).
D-24851	0.01	0.1	Inhibits microtubule assembly through a new site (3).
SK2	0.001	0.005	Inhibits tubulin polymerization, causes mitotic arrest (13).
SK3	0.0005	0.005	Inhibits tubulin polymerization and tumor cell growth (13).
SK4	0.0005	0.005	Inhibits tubulin polymerization, causes mitotic arrest (13).
2-Methoxyestradiol	0.2	No ^b ($\leq 50 \mu\text{M}$)	Alters microtubule dynamics without disassembly through colchicine site (16,17).
Kinase Targeting Drug			
Roscovitine	1	No ($\leq 5 \mu\text{M}$)	Inhibits cyclin-dependent kinases cdc2, cdk2, and cdk5 (19).
Nonmicrotubule Cytoskeleton Targeting Drugs			
Acrylamide	5 mM	No ($\leq 20 \text{ mM}$)	Disrupts intermediate filaments (20).
Cytochalasin D	0.05	No ($\leq 10 \mu\text{M}$)	Disrupts F-actin microfilaments (21).
DNA Targeting Drugs			
Apigenin	50	No ($\leq 50 \mu\text{M}$)	Inhibits kinase and topoisomerase I. ^c
Azidothymidine	5	No ($\leq 50 \mu\text{M}$)	Interferes with telomerase reaction (22).
5-Fluorouracyl	20	No ($\leq 500 \mu\text{M}$)	Inhibits DNA synthesis due to thymidylate synthetase inactivation. ^c
Hydroxyurea	100	No ($\leq 10 \text{ mM}$)	Inhibits DNA synthesis due to ribonucleoside reductase inactivation. ^c
Mitomycin C	10	No ($\leq 200 \mu\text{M}$)	Causes DNA damage due to prooxidant action (10).
Mitoxantrone	1	No ($\leq 50 \mu\text{M}$)	DNA intercalating agent that inhibits DNA synthesis. ^c
Ruboxyl	10	No ($\leq 100 \mu\text{M}$)	DNA intercalating agent that inhibits DNA synthesis (23).
4-Phenylbutyric acid	500	No ($\leq 500 \mu\text{M}$)	Inhibits histone deacetylase, resulting in cell cycle gene expression modulation. ^c

^aThe repeated tests showed no differences in threshold concentration values.
^b2-Methoxyestradiol (10–50 μM) applied to hatched blastulae strongly inhibited forward swimming without induction of rapid spinning and caused developmental abnormalities similar to those of other tubulin destabilizers.
^cRetrieved from www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cancer_Research/Product_Lines/Antitumor_Agents.html#Anti-Proliferative_Agents.

ciliation, the sensitivity to D-24851 decreased, and changes of swimming pattern were induced only at drug concentrations of 400–500 nM.

We further examined the effects of several known tubulin destabilizing drugs, namely: colchicine, combretastatin A-4, dolastatin 15, 2-methoxyestradiol, nocodazole, vinblastine, SK2, SK3, and SK4. All tests were performed on both fertilized eggs and hatched blastulae. The results are summarized in Table 1.

It was found that all tubulin destabilizers altered cleavage and induced

the appearance of tuberculous eggs in case of a full mitotic arrest (Figure 4B). The range of tested concentrations was as follows: 10 μM –1 mM colchicine, 0.0005–5 μM combretastatin A-4, 0.002–2 μM dolastatin 15, 0.05–50 μM 2-methoxyestradiol, 0.002–2.5 μM nocodazole, 0.05–50 μM vinblastine, and 0.0002–0.05 μM SK2, SK3, and SK4. We have never observed, in one sample, normally developing embryos together with embryos displaying abnormalities. Therefore it was challenging to calculate the median inhibitory concentration (IC_{50}),

conventionally used for the quantitative estimation of inhibitory activities. Instead, in our assay, we used the threshold concentrations that resulted in cleavage abnormalities or arrest. At these concentrations, all tested tubulin destabilizers caused 100% cleavage alteration and embryo death before hatching, whereas at 2-fold lower concentrations, the compounds failed to produce any effect.

When applied after hatching, colchicine, combretastatin A-4, dolastatin 15, nocodazole, vinblastine, SK2, SK3, and SK4 changed

embryo morphology and swimming patterns similar to D-24851. 2-Methoxyestradiol (10–50 μM) caused the same morphological changes and markedly inhibited swimming, but failed to induce embryo spinning.

The degree of spinning effect was time-dependent. Spinning of embryos treated with 1–20 μM combretastatin A-4, 2 μM dolastatin 15, 0.1–0.5 μM nocodazole, 10–50 μM vinblastine, 0.005–0.01 μM SK2, 0.005–0.05 μM SK3, and 0.005–0.05 μM SK4 became visible in 0.5, 1, 1.5–0.25, 1.5, 4–1, 2, and 2 h respectively. This time dependence was most profound for colchicine, as a concentration of 1 mM resulted in embryo spinning after 1.5 h of treatment, whereas the same effect for 0.05 mM occurred after 11.5 h. In our assay, the threshold concentrations of tubulin destabilizers, which induced embryo spinning, were markedly higher than those that caused cleavage alterations (Table 1).

In contrast, microtubule stabilizer paclitaxel did not cause spinning, although it did strongly inhibited cleavage. The morphology of pacli-

taxel-treated eggs was noticeably different from that of the eggs treated with tubulin destabilizers (Figure 4C).

In our assay validation studies, we examined the effect of roscovitine, a potent selective inhibitor of cyclin-dependent kinases. It was found that roscovitine markedly delayed the first division of fertilized eggs in a concentration-dependent manner, followed by formation of abnormal embryos. After treatment of hatched blastulae, the compound inhibited both embryo development and swimming, but never induced embryo spinning (Table 1).

We also studied the effect of chemicals targeting nonmicrotubule cytoskeleton. Specifically, we looked at acrylamide, known to destroy intermediate filaments, and the potent cytostatic drug cytochalasin D. When applied to fertilized eggs of the sea urchin, acrylamide caused cleavage alterations, although the first 2–3 cell divisions occurred normally. Similarly, cytochalasin D inhibited cleavage up to a full mitotic arrest (Figure 4D). However, after treatment of hatched blastulae by acrylamide or cytochalasin D, we did not observe embryo spinning (Table 1).

We further examined the effect of DNA targeting agents in our assay system. Experiments showed that a panel of compounds known to inhibit DNA biosynthesis or damage DNA molecules altered cleavage after the 8-cell stage. When applied after hatching,

DNA targeting drugs did not change swimming behavior (Table 1), except for the lethal concentrations resulting in embryo immobilization and death.

DISCUSSION

There are well-defined developmental stages during sea urchin early embryogenesis that require a specific microtubule functions (5). At cleavage (Figure 2, A and B), microtubules compose the mitotic spindle responsible for chromosome orientation and segregation in M-phase of a cell cycle. At early blastula stage (Figure 2C), within several hours after fertilization, ectoderm cells produce microtubule-containing cilia. This process is accompanied by the increasing rate of tubulin biosynthesis for further assembly into microtubules (24). After hatching, the embryos start to swim actively due to the coordinated beating of cilia uniformly distributed over the embryo outer surface (Figure 3, A–C, hatched blastula and gastrula stages). Later, ectoderm ciliary bands appear around the mouth region, providing more complicated swimming behavior of pluteus larvae (25) (Figure 3E).

The detailed study of the effects of D-24851, a strong inhibitor of purified tubulin polymerization and tumor cell growth (3), revealed two quantifiable and easily detectable effects of antimitotic compound on the sea urchin embryo. These are (i) cleavage alterations, including tuberculous shape of arrested eggs and (ii) changes in swimming pattern after hatching. We reasoned that the latter one, especially rapid spinning around the animal-

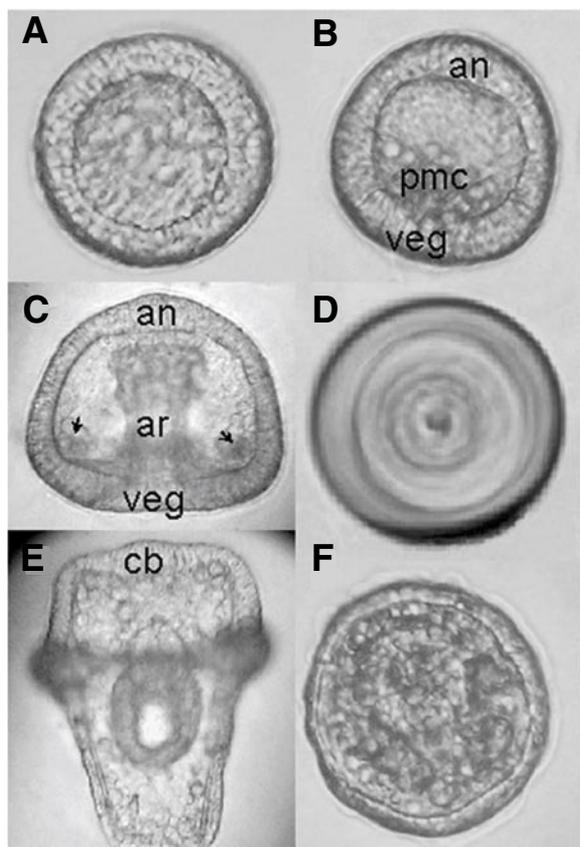


Figure 3. The effect of D-24851 on sea urchin embryo development after hatching. (A) Treatment of hatched blastulae 12 h after fertilization. Embryos were exposed continuously to 500 nM D-24851 at 18°C. (A–C) Hatched embryos swim with the animal pole (an) forward, slowly rotating counterclockwise around animal-vegetal axis, when viewed from the vegetal pole (veg). The embryo motility is provided by uniform ciliation. (B) Mesenchyme blastula with primary mesenchyme cells (pmc) inside blastocoel, 16 h. No visible differences could be observed between intact and treated embryos. (C) Intact late gastrula with fully developed archenteron (ar) and two symmetric spuculae rudiments (arrows), 24h. (D) Spinning counterclockwise blastula viewed from the vegetal pole, 19 h after fertilization, 7 h of treatment by D-24851. Video illustrations are available at www.chemblock.com. (E) Intact early pluteus, 33h. Swimming of the larva is provided by a ciliary band (cb) surrounding the mouth region. (F) Abnormal gastrula, 33 h after fertilization, 21 h of treatment by D-24851. The development of treated embryo is arrested at gastrula stage. For panels A, B, D, and F, the average embryo diameter is 115–120 μm . For panels C and E, the maximum embryo size is approximately 125 and 165 μm , respectively.

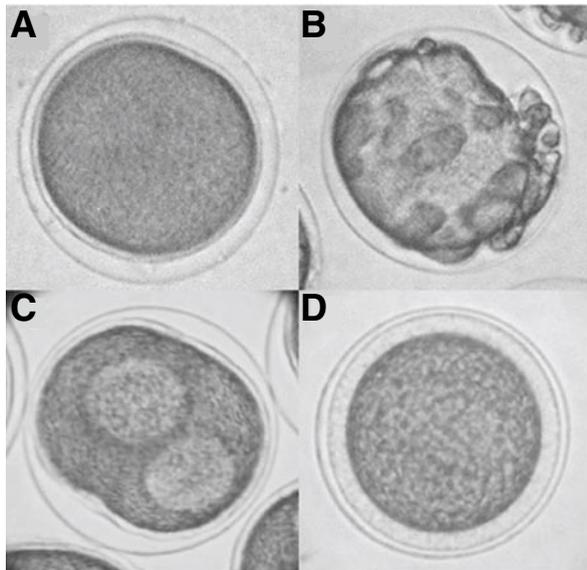


Figure 4. Sea urchin fertilized eggs treated by antimitotic drugs. (A) Intact fertilized egg. (B) Colchicine (0.2 mM), 11 h of treatment. (C) Paclitaxel (10 μ M), 3 h of treatment. The light spots inside a cell correspond to the mitotic apparatus. (D) Cytochalasin D (4 μ M), 5 h of treatment. Note the tuberculous cell shape in panel B, which is unique for tubulin destabilizing drugs. The same tuberculous cell shape was observed for other tubulin destabilizers: 0.01–5 μ M combretastatin A-4, 0.01–0.5 μ M D-24851, 0.05–0.1 μ M dolastatin 15, 10–50 μ M 2-methoxyestradiol, 0.01–2.5 μ M nocodazole, 1–50 μ M vinblastine, 0.002–0.05 μ M SK2, 0.001–0.05 μ M SK3, and 0.001–0.05 μ M SK4. The average egg diameter is 115 μ m.

vegetal axis, could be the result of a destabilizing effect of the compound on tubulin/microtubules. To support this assumption, we further examined the effects of nine known, chemically diverse tubulin destabilizing drugs (colchicine, combretastatin A-4, dolastatin 15, 2-methoxyestradiol, nocodazole, vinblastine, SK2, SK3, and SK4), along with the microtubule stabilizer paclitaxel, as well as a panel of antiproliferative agents targeting cyclin-dependent kinases (roscovitine), nonmicrotubule cytoskeleton (acrylamide and cytochalasin D), or DNA (apigenin, azidothymidine, 5-fluorouracyl, hydroxyurea, mitomycin C, mitoxantrone, 4-phenylbutyric acid, and ruboxyl). We found that tested tubulin destabilizers, independent of their chemical structure and tubulin binding site, induced cleavage alteration/arrest and embryo spinning. Rapid spinning of embryos exposed to high concentrations of colchicine and nocodazole has been previously reported for other sea urchin species, namely *Arbacia punctulata*,

Lytechinus pictus, and *Strongylocentrotus purpuratus* (26–28). The observed time-dependent degree of the spinning effect seemed to be a result of permeability and/or tubulin binding properties of a molecule. In all cases, tubulin destabilizing drugs induced embryo spinning at markedly higher concentrations than those that caused cleavage alterations (Table 2). This effect was most pronounced for dolastatin 15, which is known to be a weak tubulin binder (29). Interestingly, 2-methoxyestradiol, which inhibits tubulin polymerization (16) and blocks mitosis through alteration of microtubules dynamics without their disassembly (17), produced the same effects on *P. lividus* embryos as other tubulin destabilizers, except for

spinning. In addition, another tubulin binding compound paclitaxel, which stabilizes microtubules (2), did not cause spinning. Instead, it displayed strong cleavage inhibition activity. We reasoned that spinning of immobilized sea urchin embryos is a consequence of microtubule disassembly.

Cell cycle affecting molecules can interfere not only with mitotic spindle, but with cyclin-dependent kinases as well. In this connection, we studied the effect of roscovitine, a potent selective inhibitor of the cyclin-dependent kinases *cdc2/cyclin B*, *cdk2/cyclin A*, *cdk2/cyclin E*, and *cdk5/p35*, which is known to cause reversible mitotic arrest of sea urchin *Sphaerechinus granularis* eggs in late prophase (19). Similarly, we found that roscovitine induced abnormal cleavage, but failed to produce spinning effect. This is another argument in favor of the assumption that the spinning effect is a distinguishing feature of tubulin destabilizers.

In addition to microtubules, both actin-containing microfilaments and

intermediate filaments compose the cytoskeleton. These are also involved in cell division and ciliary movement. In the sea urchin embryo, intermediate filaments co-localize with the mitotic spindle and are believed to participate in various microtubule-dependent events (30). Ciliary basal apparatus of the blastula ectoderm cell is connected to cytoplasmic microtubules and cortical actin microfilaments, while intermediate filaments have been detected in cilia (30,31). It is likely that all these structures contribute to the stability of the sea urchin embryo cilium, although their specific role in regulating the ciliary beating still remains unclear. Therefore to validate our assay system, we examined acrylamide, a compound known to disrupt intermediate filaments without interfering with the actin-containing microfilaments and microtubules (20), and a potent cytostatic drug cytochalasin D that selectively degrades actin-containing microfilaments (21). Both compounds inhibited cleavage, however, and were unable to cause embryo spinning (Table 1). This suggests that among cytoskeleton targeting drugs, only tubulin destabilizers were able to induce changes of swimming behavior, such as the spinning of immobilized sea urchin embryos.

Our further studies showed, that a panel of DNA-targeting agents caused cleavage alterations after the 8-cell stage, which was previously reported as a characteristic feature of DNA synthesis inhibitors (7). When applied to free-swimming blastulae, DNA-targeting drugs did not induce any changes in swimming pattern, except for the lethal concentrations resulting in embryo immobilization and death. Similar effects of aphidicolin (an inhibitor of DNA polymerase α) (32), 5-azacytidine (an inhibitor of DNA methylation) (33), and DNA intercalator doxorubicin (11) have been observed on sea urchin *S. granularis*, *Psammechinus miliaris*, *A. punctulata*, and *P. lividus* embryos. These results further supported our observation that the embryo spinning phenomenon is unique to the tubulin destabilizing drugs.

We then attempted to address the question of the putative mechanism underlying the changes of sea urchin

Table 2. Efficacy of Tubulin Destabilizing Drugs Against Sea Urchin Embryos and Various Human Tumor Cell Lines

Compound	Threshold Concentration for <i>Paracentrotus lividus</i> Embryo (μM)			Cytotoxicity Against Human Tumor Cell Lines
	Cleavage Alteration	Cleavage Arrest	Embryo Spinning	Growth Inhibition Constant (IC_{50} , μM) (Reference)
SK4	0.0005	0.002	0.005	0.01 (A431 epidermoid carcinoma) (13) ^a
SK3	0.0005	0.002	0.005	15 (A431 epidermoid carcinoma) (13) ^a
SK2	0.001	0.005	0.005	0.1 (A431 epidermoid carcinoma) (13) ^a
Combretastatin A-4	0.005	0.01	1	0.0032 (mean panel of 60 tumor cell lines) (37)
Nocodazole	0.005	0.01	0.1	0.05 (U937 leukemia) (38); 0.14 (KB/HeLa) (39)
Dolastatin 15	0.005	0.05	2	0.003 (Burkitt lymphoma) (29)
D-24851	0.01	0.02	0.1	0.064 (PC-3 prostate); 0.115 (KB/HeLa) (3)
Vinblastine	0.2	1	10	0.23 (neuroglioma) (40)
2-Methoxyestradiol	0.2	10	—	0.21 (OVCAR-3); 0.32 (SF-539) (41)
Colchicine	50	100	50	0.002 (A549 lung) (42); 0.03 (KB/HeLa) (39)

^aConcentration (μM) required for cell cycle arrest in G2/M phase.
 IC_{50} , median inhibitory concentration.

embryo swimming behavior caused by tubulin destabilizers. As previously described (25,34), after hatching, sea urchin embryos swim with the animal pole forward, slowly rotating counter-clockwise around the animal-vegetal axis when viewed from the vegetal pole. Forces derived from ciliary effective strokes determine the direction of swimming and rotation. Adjacent rows of cilia produce metachronal waves that pass over the surface of the embryo and cause rotation. The pattern of metachronal waves is an inherent property of embryo morphology and determines the direction of rotation.

Inside the cell, a ciliary basal apparatus connected to cytoplasmic microtubules maintains the stability of cilium and effective stroke direction (31). Tubulin destabilizing drugs, such as colchicine and colcemid, have been found to disrupt cytoplasmic but not ciliary microtubules in cultured cells and sea urchin blastulae cells (26,35). The sea urchin embryos treated with tubulin destabilizers retained ciliary beating, but the effective stroke direction changed. Therefore, instead of forward swimming, embryos started to spin rapidly in the direction determined by metachronal waves. This is likely the result of a ciliary anchorage alteration due to disassembly of cytoplasmic microtubules.

Based on these data, we proposed a protocol for parallel screening of

chemical libraries for tubulin destabilizing activity using the sea urchin embryos. The details of compound treatment are the same as described in the Materials and Methods section. The assay sequence starts with a fertilized egg test for cleavage alteration/arrest, which is clearly detected at 3–6 h after fertilization. Molecules that cause cleavage alteration/arrest are selected for the subsequent treatment and assessment of free-swimming blastulae originating from the same embryo culture. Embryo spinning can be observed after 0.5–20 h of treatment, depending on the nature and concentration of the compound. Both spinning and lack of forward movement of an embryo are likely the result of the tubulin destabilizing activity caused by a molecule. The tuberculous egg shape seems to be an additional indicator of the tubulin destabilizing activity. Based on our experience, the assay is highly reproducible with regard to both effect and compound concentration (see Table 1). In our hands, we are able to reliably generate 90–100 data points per day using a standard optical microscope. We are in the process of evaluating several detection techniques, including light scattering, to increase throughput and automation possibilities.

Structural and functional similarities of tubulins from sea urchin embryos and vertebrates indicate that the results obtained from the sea urchin

embryo model could be extrapolated to the higher living systems. Several studies also suggest that tubulins originating from diverse animal sources contain highly conserved amino acid sequence (1). In addition, purified pig brain tubulin injected into the sand dollar *Clypeaster japonicus* eggs copolymerizes with native tubulin and incorporates into mitotic spindles during several cell cycles (36). Our tests of tubulin destabilizing drugs showed that the effective concentrations resulting in cleavage alteration/arrest and embryo death before hatching were close to the reported IC_{50} values for a panel of a human tumor cell lines (Table 2).

Notably, colchicine affected the sea urchin embryos at relatively high concentrations, when compared with its cytotoxicity against human tumor cell lines. This result could not be explained by the hydrophilic nature of the colchicine molecule, because other water-soluble drugs, vinblastine and combretastatin A4 disodium phosphate, altered sea urchin embryo cleavage at concentrations similar to those that caused inhibition of mammalian cell growth (Table 2). Another possibility might concern differences in the cellular permeability or the intracellular transport of the compound, a phenomenon previously described for some mammalian cell lines (43). In addition, during early sea urchin embryo

development, the cell cycle is markedly shorter than in mammalian cell culture. At the cleavage stages of *P. lividus*, cells divide every 35–40 min at optimum temperature. Colchicine binds to tubulin at a relatively slow rate (44) and therefore might not have enough time to suppress mitotic spindle formation in the sea urchin blastomeres.

In summary, we demonstrated that the sea urchin embryo is a relevant model for the assessment of antiproliferative, antimitotic, and cytotoxic effects of small molecules. This model allows for a rapid identification of tubulin destabilizing agents, although we cannot exclude a possible interaction of the tested molecules with microtubule-associated proteins, which may induce changes in the tubulin/microtubule dynamics. Currently, we are in the process of establishing an automated system for conducting functional screening of tubulin modulators using a light scattering technique.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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