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New small-molecule tubulin inhibitors*

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Abstract: The variety of biological agents directed toward the tubulin system exceeds those acting on DNA, making it an important target for cancer chemotherapy. However, the complicated chemical structures and restricted access to the natural resources, in combination with the development of drug resistance, limit the first generation of natural products. Considerable efforts in the search and synthesis of new synthetic compounds, such as small molecular tubulin inhibitors, gave access to novel potential/promising drugs. Among these substances, two series of novel, easily accessible indole classes were identified as tubulin-destabilizing agents. Owing to the synthetic nature, potent *in vitro* and *in vivo* antitumoral activity, and efficacy against multidrug-resistant (MDR) tumors, D-24851 and D-64131 have significant potential in cancer treatment.

INTRODUCTION

Malignant tumors represent one of the most common human diseases worldwide, and the subset of human cancer types that are amenable to curative treatment today is rather small. Although there is tremendous progress in understanding the molecular events that lead to malignancy, progress in the development of clinically innovative drugs that can cure humans is still slow. The world cancer drug market accounts for USD 14 billion in 2001 with Taxol®, BMS's drug for treating ovarian and breast cancers, being the biggest-selling, single anticancer agent. This success story brought the focus of oncology back to compounds that interfere with the cell cycle and to tubulin as a promising target, because specific agents inhibit the proliferation of tumor cell lines derived from various organs. Tubulin-containing structures are important for diverse cellular functions, including chromosome segregation during cell division, intracellular transport, development and maintenance of cell shape, cell motility, and possibly distribution of molecules on cell membranes. The drugs that interact with tubulin are heterogeneous in chemical structure [1]. However, a common characteristic of these agents is that while binding to tubulin, they cause its precipitation and sequestration to interrupt many important biologic functions that depend on the microtubular class of subcellular organelles.

One class of well-characterized and clinically used antimitotic drugs is of natural origin, namely, the taxanes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine, vinorelbine) and podophyllotoxins/colchicine (Fig. 1). These agents either inhibit the polymerization of tubulin (vinca alkaloids/cholchicine) or prevent the dissembly of microtubules (taxanes). Accordingly, three important binding domains have been identified.

More recently, epothilone A and B and their analogs raised interest due to high cytotoxicity and good stabilization of microtubules. These natural products were originally isolated from myxobacteria

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sorangium cellulosum [2]; now, numerous reports are published on total synthesis and structure variation. Their unique capability to inhibit taxol-resistant tumor cell lines and their good solubility are the biggest advantages as compared to taxanes. However, the complicated chemical structures and limited access to the natural resources, in combination with the development of drug resistance, limit the potential of these natural products in general. Other natural products or derived analogs (Fig. 2) are characterized by increased solubility or potency, but still are complicated in chemical structure. Another major drawback of taxanes and vinca alkaloids in clinical application is the development of neurotoxicity. The drugs interfere with the function of microtubules in axons, which mediate the neuronal vesicle transport.

New synthetic, small-molecule chemical entities that bind to tubulin, but are neither a substrate of transmembrane pumps nor interfere with the function of axonal microtubules, would strongly increase the therapeutic index in the treatment of malignancies.

A series of synthetic molecules that bind to tubulin are currently being evaluated in the preclinical or early clinical stage (Fig. 3). Among them, a molecule named D-24851 was identified that destabilizes microtubules in tumor cells and cell-free systems. *N*-(Pyridin-4-yl)-[1-(4-chlorbenzyl)-indol-3-yl]glyoxylic acid amide (D-24851) is a novel synthetic compound that was found in a cell-based screening assay to discover cytotoxic drugs.

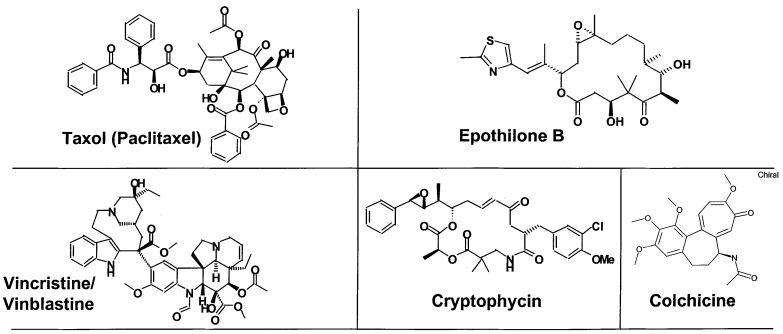

Fig. 1 Structure comparison of different tubulin binders.

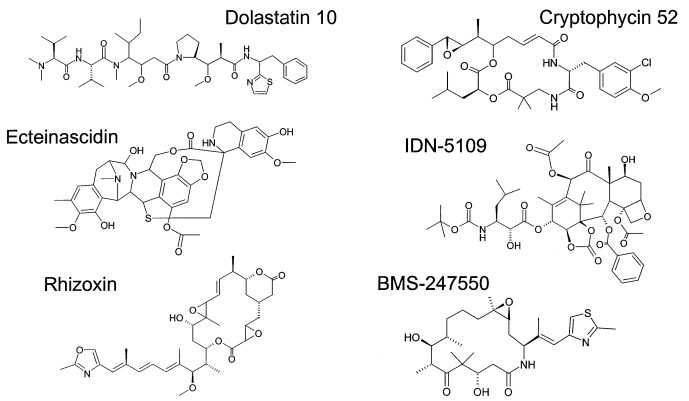

Fig. 2 Structure comparison of natural product-derived tubulin binders.

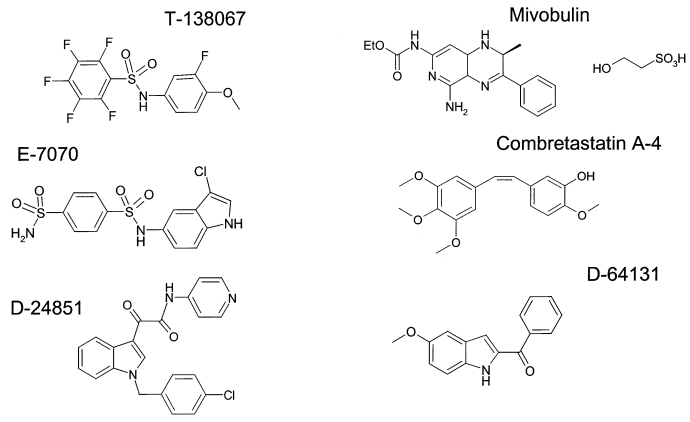

Fig. 3 Structure comparison of small molecule tubulin binders.

RESULTS AND DISCUSSION

D-24851 is a novel synthetic anticancer agent with significant antitumoral activity *in vitro* and *in vivo* (Table 1). It destabilizes microtubules in tumor cells, as well as in a cell-free system. The binding site of D-24851 does not overlap with the tubulin-binding sites of the well-characterized microtubule-destabilizing agents vincristine or colchicine. Futhermore, the molecule selectively blocks cell cycle progression at metaphase. *In vitro*, D-24851 exerts significant antitumoral activity against a variety of malignancies (e.g., prostate, brain, breast, pancreas, and colon). When compared with other microtubule-inhibiting compounds, D-24851 has a number of superior properties *in vivo*: (a) curative treatment of Yoshida AH13 rat sarcomas at almost nontoxic doses; (b) oral applicability; (c) lack of neurotoxicity at curative doses, which is a major drawback of taxanes and vinca alkaloids in the clinical use; and (d) efficacy toward MDR tumor cells. Therefore, D-24851 may have significant potential as a therapeutic agent in cancer therapy [3].

The mode of action of the molecule as a tubulin inhibitor was shown by indirect immunofluorescence microscopy using an antibody against α-tubulin and in a cell-free tubulin polymerization assay. D-24851 induced accumulation of cells with condensed nuclei and abnormal mitotic spindles. At higher concentrations, fragmentation of the spindle apparatus and degradation of microtubules were observed. The well-characterized vinca alkaloids and colchicine interact with different binding sites on tubulin and were known to destabilize microtubules. In fact, exposure of cells to vincristine also revealed fragmented mitotic spindles similar to those shown for D-24851. Paclitaxel, known as a microtubule-stabilizing agent, did not induce fragmentation of the spindle apparatus. This strongly suggests that D-24851 arrests cells at metaphase owing to modulating microtubule stability.

The destabilizing effect of D-24851 on microtubules was also seen in a cell-free assay using purified tubulin. Polymerization of tubulin was blocked by D-24851 in a concentration-dependent manner with an IC50 of approximately 0.3 μM, which may indicate a direct interaction of D-24851 with tubulin. The substoichiometric concentrations of the compound in relation to the tubulin concentration (10 μM) are sufficient to block tubulin polymerization, similar to vincristine or other vinca alkaloids.

D-24851 is a low-molecular-weight compound that shows no structural similarities to vinca alkaloids or colchicine and did not compete for the binding of radiolabeled vincristine or colchicine to tubulin. This suggests that D-24851 may bind to a novel binding site on tubulin that results in inhibition of tubulin polymerization. The IC50 value of vincristine for tubulin polymerization was ~10-fold lower than that of D-24851. The difference between both compounds in the inhibition of cell growth was

shown in a variety of different tumor cell lines. This suggests that D-24851 interferes with the function of tubulin, thereby inducing cell cycle arrest and consequently cell growth inhibition.

In vivo, D-24851 showed a remarkable antitumoral efficacy in the Yoshida AH13 rat sarcoma model. Oral application of D-24851 induced complete tumor regressions and resulted in curative treatment of the animals (Fig. 4). Of great importance is that, at curative doses of D-24851, no systemic toxicity in terms of body weight loss or hematological toxicities were observed *in vivo*. In contrast, vincristine or paclitaxel treatment at their maximal tolerated doses resulted only in a moderate inhibition of tumor growth, but in a significant toxicity in terms of body weight loss. These data demonstrate that D-24851 is more potent than vincristine or paclitaxel in the treatment of Yoshida AH13 tumors *in vivo*, which was also confirmed in other human tumor-bearing mice (e.g., MCF7, A549, or PC3). Furthermore, in the murine Renca (renal carcinoma) and in Renca lac Z. tumor model the effect of

Table 1 Antitumor activity of D-24851 *in vitro* and *in vivo*.

Tumor cell line	in vitro	in vivo
(tissue/species/ATCC no)	IC 50 [µM]	
SKOV3 (ovary / human/ HTB-77)	0.036	active
KB (cervix / human/CCL-17)	0.115	less active
HT 29 (colon / human/HTB-38)	0.072	-----
A549 (lung / human/CCL-185)	0.164	strong activity
PC-3 (prostate/ human/CRL-1435)	0.064	active
DU145 (prostate / human/ HTB-81)	0.148	less active
AsPC-1(pancreas/ human/CRL-1682)	0.285	-----
C6 (brain / rat/CCL-107)	0.200	-----
U 87 (brain / human/HTB-14)	0.077	strong activity
A 431 (vulvar squamous/ human)	-------	strong activity
MDA-MB 231 (mamma / human/HTB-26)	0.074	-----
MCF-7 (mamma / human)	-------	strong activity
AH13 (liver / rat)	-------	strong activity
DMBA (mamma / rat)	-------	strong activity
L1210 (leukemia / mouse)	0.053	strong activity

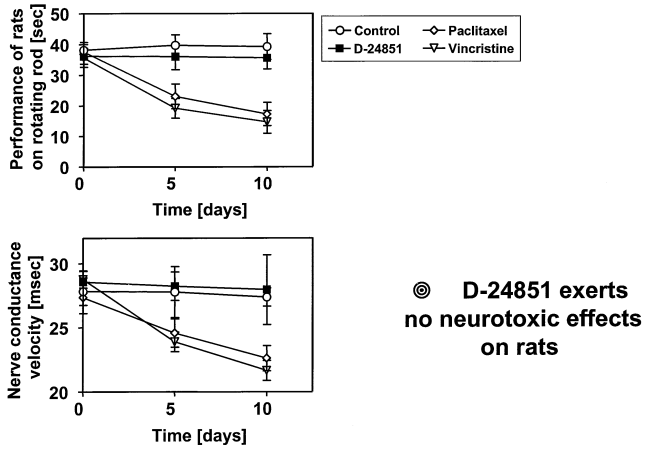

Fig. 5 Neurotoxicity.

D-24851 on the tumors of the kidney and the prevention of metastasis formation in the lung was studied [4]. A dose-dependent reduction of tumor weight in kidney and prevention of metastasis formation in the lung was observed in animals after oral treatment with D-24851 (15 and 17.5 mg/kg p.o.). Also, in the Renca lac Z. tumor model a reduction of the pulmonary metastasis could be observed in balb/c mice treated with D-24851.

In clinical studies, it has been demonstrated that cumulative doses of paclitaxel or vincristine doses are associated with development of neurotoxicity. The effect of these drugs on the nervous system of rats has also been shown previously. Intravenous administration of vinca alkaloids significantly impaired coordination and nerve conductance velocity (NCV) in the nerve tail. We also observed similar effects on rats after i.p. application of paxlitaxel or vincristine. On a molecular level, drug-impaired microtubule function in axons seems to be responsible for the neurotoxic effects. Microtubules were found to accumulate in axons after the administration of paclitaxel, whereas vinca alkaloids interfere with axonal transport, which induces spiralization of axonal microtubules. Although D-24851 also alters microtubule function, no neurotoxic effects on rats in terms of deficit in motor function or reduced NCV was seen at curative doses. One possible explanation for the lack of neurotoxicity of D-24851 could be that concentrations of D-24851 that are sufficient to block the cell cycle do not inhibit axonal vesicle transport. Alternatively, D-24851 may only interact with nonaxonal microtubules.

The use of cytotoxic agents is often accompanied by development of MDR tumor phenotype. A major determinant of MDR is the overexpression of drug efflux pumps, namely the p-gp170 and the multidrug resistance protein (MRP). The results suggest that D-24851 is a substrate neither of P-glycoprotein nor for MRP. Thus, D-24851 retains its cytotoxic activity toward MDR cells *in vitro* and *in vivo*. In contrast, paclitaxel and vincristine were shown to be actively transported by p-gp170 and, in part, by MRP. Of clinical importance is that D-24851 retains its antitumoral activity against cancer cell lines with resistance to cisplatin, the topoisomerase-I-inhibitor SN-38, and the thymidylate synthase inhibitors 5-FU and raltitrexed.

Additionally, a series of 2-arylindole derivatives was selected from screening as having potent antiproliferative activity. The lead compound D-64131, representing another class of small-molecule, indole-based compounds, was identified as a potent cytotoxic compound from *in vitro* screens and potent analogs with defined structure–activity relationships (SAR) were synthesized [5]. Studies revealed a cell cycle specific mode of action through inhibition of tubulin polymerization. D-64131 and analogs are acting on a cellular level as destabilizing tubulin inhibitors. In contrast to vincristine, doxorubicin or taxol, D-64131 is effective in cell lines with high gp170 p-glycoprotein expression. *In vivo* experiments in nude mice bearing human and rat xenograft tumors show efficacy at well-tolerated doses after p.o. administration. About 140 acylindol derivatives have been synthesized and tested for antiproliferative activity and inhibiton of tubulin polymerization. Besides D-64131, several further analogs have shown antitumoral efficacy and are available for extended preclinical development.

CONCLUSION

In summary, D-24851 is a novel tubulin-binding agent with significant antitumoral efficacy *in vitro* and *in vivo*. The lack of neurotoxicity and the potential in an oral formulation may provide an anticancer drug with a significant therapeutic index. Owing to its synthetic nature, its oral applicability, its potent *in vitro* and *in vivo* antitumoral activity, its efficacy against MDR tumors, and the lack of neurotoxicity, D-24851 may have significant potential for the treatment of various malignancies. Clinical Phase I trials with D-24851 will be initiated.

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