2-Aroylindoles, a Novel Class of Potent, Orally Active Small Molecule Tubulin Inhibitors


ASTA Medica Oncology, D-60314 Frankfurt/Main [T. B., T. R., M. S.]; Tumor Biology Center, D-79106 Freiburg [H. H. F., A. M. B.]; Department of Internal Medicine/German Cancer Center, University of Essen Medical School, 45112 Essen [U. V.]; and Faculty of Chemistry and Pharmacy, Institute of Pharmacy, University Regensburg, D-93040 Regensburg [S. M., H. P.; J. H., M. F.; H. H.]; Germany.

ABSTRACT

2-Aroylindoles with 5-methoxy-1H-2-indolyl-phenylmethanone (D-64131) as the lead structure were discovered as a new class of synthetic, small molecule tubulin inhibitors. By competitively binding with [3H]colchicine to αβ-tubulin and inhibiting microtubule formation, cycling cells were arrested in the G2-M phase of the cell division cycle. The proliferation of tumor cells from 12 of 14 different organs and tissues was inhibited with mean IC50 of 62 nM and 24 nM by D-64131 and D-68144, respectively, comparable with the potency of paclitaxel with mean IC50 of 10 nM. By measuring the cytotoxicity in a human colon carcinoma cell line, SMTIs derived from screening compound libraries or derivatization of natural compounds have been described recently (9-17). To our current knowledge, natural and synthetic compounds bind to distinct sites within β-tubulin and, by destabilization or stabilization of microtubules, cause mitotic catastrophe (3).

Tubulin binding molecules interfere with the dynamic instability of microtubules and thereby arrest mitotic cells in the M-phase of the cell division cycle, finally leading to apoptotic cell death. Tubulin binding molecules from nature are colchicine and the Vinca alkaloids vinblastine and vincristine, rhazoxin, maytansine, combretastatin A4, epothilone, and paclitaxel (3). SMTIs derived from screening compound libraries or derivatization of natural compounds have been described recently (9-17). To our current knowledge, natural and synthetic compounds bind to distinct sites within β-tubulin and, by destabilization or stabilization of microtubules, cause mitotic catastrophe (3).

The tubulin inhibitors used for human cancer therapy have severe drawbacks, i.e., high toxicity (especially neurotoxicity), marginal oral bioavailability and poor solubility, complex synthesis or isolation procedures, and most importantly, the development of drug resistance, which is a common phenomenon. New simple and synthetic compounds with oral bioavailability and high therapeutic index for first- and second-line therapy are therefore highly attractive.

By high throughput screening for antiproliferative agents, 2-aryloindoles were identified as a novel class of antimitotic compounds (18). In this report, we show that D-64131 as well as potent analogues are antimitotic by binding to β-tubulin, thereby destabilizing microtubules and arresting mitotic cells in the M-phase. D-64131 and analogues displayed broad antitumoral activity against cycling cells only, which is independent from the MDR/MDR phenotype or p53 status. Finally, in nude mice D-64131 proved to be well tolerated and highly active against the human amelanotic melanoma MEXF 989 xenograft.

INTRODUCTION

The mitotic spindle is a complex dynamic assembly of microtubules, motor proteins (kinases and dynein families), MAPs, and catastrophe factors (1). This self-organizing machine uses energy from nucleotide hydrolysis to segregate sister chromatids accurately into daughter cells (2). Compounds from nature targeting the mitotic spindle apparatus are potent cytotoxic drugs. Taxol (paclitaxel), the semisynthetic analogue Taxotere (docetaxel), and the Vinca alkaloids vincristine, vinblastine, and vinorelbine have significant antitumoral activity toward, breast, ovarian, and non-small cell lung cancer, among others. The discovery of new natural and semisynthetic compounds being cytotoxic by interference with tubulin has attracted much attention within the last years, and microtubules have become a compelling target for anticancer drug discovery (3, 4).

Microtubules are hollow tubes consisting of α- and β-tubulin heterodimers that polymerize parallel to a cylindrical axis (5). Mitotic microtubules are very dynamic structures, switching between growing and shortening states, a process known as dynamic instability and driven primarily by the catastrophe rate (1, 6). MAPs bind to and stabilize microtubules by reducing the catastrophe rate or increasing the polymerization rate (7, 8).

MATERIALS AND METHODS

Chemicals and Biologicals. General chemicals, 4,6-diamidino-2-phenylindole, DAPI, were purchased from Sigma (Munich, Germany). Muristerone A was from Invitrogen (Groningen, the Netherlands). [α-32P]GTP and streptavidin-coated yttrium SPA beads were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). [3H]colchicine was from NEN (Boston, MA). Biotinylated MAP-free bovine brain tubulin was from Cytoskeleton (Denver, CO). Tubulin for the polymerization assay was purified from bovine brain according to Vallee (19). 2-Aryloindolines were synthesized at the Institute of Pharmacy, University Regensburg (18).

Tumor Cell Lines. The human tumor cell lines A431 (valva epidermoid carcinoma/CRL 1555), HeLa/KB (cervical carcinoma, CCL-17), U373 (astrocytoma, HTB-17), U87 (glioblastoma, HTB-14), SKOV3 (ovarian adeno-carcinoma/CRL 1555), HeLa/KB (cervical carcinoma, CCL-17), U373 (astrocytoma, HTB-17), U87 (glioblastoma, HTB-14), SKOV3 (ovarian adeno-car...
cinoma, HTB-77), HT29 (colon adenocarcinoma, HTB-38), MDA-MB 231 (breast adenocarcinoma, HTB-26), HeLa (endometrial adenocarcinoma, HTB112), A549 (lung cancer, CCL 185), PC3 (prostate adenocarcinoma, CRL-1435), AsPC-1 (pancreatic adenocarcinoma, CRL1682), Cal27 (squamous cell carcinoma of the tongue, CRL2095), Saos-2 (osteogenic sarcoma, HTB-85), T24 (bladder transitional cell carcinoma, HTB-4) were from ATCC (Manassas, VA). The human colon adenocarcinoma cell line RKO and the human colon adenocarcinoma cell line RKO21 were described recently (20). The murine cell line L1210 (leukemia/CCL 219) was obtained from ATCC. The HT29, HT29-R1, HT29-R24, the raltitrexed-resistant HT29/ICID, A2780/Dx5 and MCF-7/adr, the MRP-expressing HT1080/DR4, the 5-fluorouracil-resistant HT29/R1 and HT29-R24, the raltitrexed-resistant HT29/ICID, and the SN-38 resistant HCT-8/SN38 cell lines have been described (22–26).

Cytotoxicity Assays. The test compounds were dissolved in DMSO at final concentrations of 10 mM and stored at −20 °C. The XTT assay (27) was used as described to determine proliferation by quantification of cellular metabolic activity. The various tumor cell lines were cultivated in microtiter plates (2–15 × 10^4 cells/100 μl/well) and incubated with different concentrations of cytotoxic agents for 48 h. Alternatively, drug sensitivity was assessed with the sulforhodamine B assay as described recently (9).

Cytotoxidity in the RKO exo p21 Cell Model. Expression of p21^{wat} was induced by treatment with 3 μm muristerone A for 24 h, arresting RKO colon carcinoma cells in the G1 and G2 phases of the cell division cycle (20). RKO cells with/without p21^{wat} expression (2 × 10^5 cells/well induced; 6 × 10^5 cells/well not induced) were treated with the test compound for 48 h at 37 °C. Cellular metabolic activity/proliferation was determined by the XTT assay as described above.

Indirect Immunofluorescence Microscopy. Detection of α-tubulin in HeLa/KB cells by immunofluorescence was done as described (9). Briefly, cells were incubated with test compound for 24 h, washed with PBS, and fixed in 50% v/v methanol:50% v/v acetic acid for 15 min at −20 °C. After drying at room temperature and saturation with 2% v/v BSA, 10% v/v FCS, in PBS for 30 min, staining was performed with an anti-α-tubulin antibody (clone B-5-1-2; Sigma) and a Cy3-conjugated rabbit antimouse antibody (315-165-003; Dianova). Nuclear staining was performed with 1 μg/ml 4,6-diamidino-2-phenylindole as described (9).

Flow Cytometry. HeLa/KB cells in subconfluent, proliferating culture were exposed to the cytotoxic agents for 24 h at 37 °C, detached with trypsin, and finally collected by centrifugation. After washing, fixation, and staining, cells were analyzed by FACs as described (9). Data points were connected, and the respective IC_{50} calculated using a nonlinear regression program (GraphPad Prism).

Human Tumor Xenografts. D-64131 was freshly dissolved in DMSO and subsequently diluted with PBS containing 0.05% v/v Tween 80 to obtain a final DMSO concentration of 1%. Oudbred nude mice, 6–8 weeks of age, of NMRI genetic background were used for all experiments. For the experiments, the human amelancric melanoma MEXF 989 tumor xenograft model (28) was chosen based on in vitro selectivity of D-64131 toward melanoma (data not shown) and engrafted from tumors in serial passage growing s.c. in nude mice. Fragments of ~25 mg were implanted s.c. in both flanks of the animals. Details of the animal experiments and data analysis were described by Mahboobi et al. (18). The MEXF 989 tumor-bearing nude mice were treated p.o. with doses of 200 and 400 mg/kg/day on days 1–5, 8–9, and 15–18. In two independent experiments, the drug doses and treatment schedule used were determined as being well tolerated in non-tumor-bearing nude mice before initiation of tumor experiments.

Tubulin Polymerization Assay. The assay was basically performed according to Bossale et al. (29). Tubulin heterodimers (0.8 mM/ml; 80 μg assay), isolated from bovine brain by cycles of polymerization and depolymerization, were incubated with test compounds (4 μg/ml in the initial screening, different concentrations in the final IC_{50} determination scheme) in PEM (100 mM PIPES, 1 mM EGTA, and 1 mM MgCl_{2}) buffer (pH 6.6) containing 1 mM GTP in a total volume of 100 μl at 37 °C for 1 h. Samples (75 μl) were then transferred to a 96-well Millipore Multiscreen Durapore hydrophilic 0.22-μm pore size filtration plate. Recovered microtubules on the filters were stained with 50 μl of Amido Black solution [0.1% w/v napthol blue black (Sigma), 45% v/v methanol, and 10% v/v acetic acid] for 2 min. Vacuum was applied, and unbound dye was removed by two additions of 200 μl of destain solution (90% v/v methanol, 2% v/v acetic acid). The microtubule bound dye was eluted by incubation with 200 μl of elution solution (25 mM NaOH, 0.05 mM EDTA, and 50% v/v ethanol) for 20 min. Next, 160 μl of elution solution was transferred to a 96-well plate, and the absorbance was measured at 600 nm using the Wallac Victor Multilabel counter (Perkin-Elmer/Wallac, Freiburg, Germany).

Tubulin Binding Assay. The tubulin binding assay was performed according to Tahit et al. (30) using biotin-labeled tubulin, streptavidin-coated yttrium SPA beads, and [3H]colchicine (1 μCi/ml; specific activity, 76.5 Ci/mmol). Briefly, the binding mixture includes 0.08 μM [3H]colchicine, 1 mM GTP, and 0.5 μg of biotin-tubulin in G-PERM buffer, pH 6.9 (80 mM PIPES, 1 mM MgCl_{2}, 1 mM EGTA, and 5% glycerol) in 100-μl final volume. The test compound and [3H]colchicine were added before tubulin. After incubation at 37 °C for 2 h, 20 μl of SPA beads (80 μg in P-GEM buffer) were added. After further incubation for 30 min under agitation at room temperature, the SPA beads were allowed to settle down for 45 min, and scintillation counting was done on a MicroBeta Trilux counting device (Perkin-Elmer Wallac).

Tubulin GTPase Assay. The tubulin GTPase assay was performed with slight modifications according to Roychowdhury et al. (31). Highly purified, lyophilized, MAP-free bovine brain tubulin was reconstituted in PERM buffer (pH 6.6) and stored in aliquots at −80 °C. The reaction mixture used for the GTPase assay contained 1 mM/ml tubulin, 1 mM MgCl_{2}, 100 μM α^{32}PGTP (specific activity, 3000 Ci/mmol), and 1 mM monosodium glutamate in P-Perm buffer. Alternatively, 4.5% (v/v) glycerol was used for induction of tubulin polymerization. Test compounds, dissolved in 10% v/v DMSO, were added before addition of glutamate and α^{32}PGTP (final concentration, 1% DMSO). Tubulin polymerization was started by incubation at 37 °C for 1 h and terminated by addition of SDS at 1% final concentration. GTP hydrolysis was measured by thin-layer chromatography of the reaction mixture using polyethyleneimine cellulose plates. The chromatograms were developed with 0.35 M NH_{4}CO_{3}. Chromatography plates were exposed to X-ray films and quantified by phosphorimager analysis using a Fuji BAS-1800II device.

RESULTS

2-Aroylindoles Are Potent Antiproliferative, Tubulin Binding Agents. Within a robotic screening program, 2-aroylindoles were initially identified as potent, antiproliferative agents. About 160 analogues were synthesized, and D-64131 was identified as a lead structure. Structural variations were introduced in the indole and phenyl ring for analysis of structure-activity relationships. In contrast to 2-benzoyl-5-methylindole derivatives, many 5-methoxyindole derivatives exhibited high cytotoxicity, and single or multiple substitutions within the phenyl ring (e.g., OCH_{3}, F, NO_{2}, and NH_{2}) were compatible with high cytotoxicity (Fig. 1 and Table 1; Ref. 18). No clear effect of electron-donating and withdrawing groups was noted and, in comparison with the natural compounds combretastatin a and colchicine, multiple methoxy substitutions were no prerequisite for high biological activity, as exemplified by D-64131. This compound as well as D-68143, D-68144, D-68150, D-68172, D-70316, and D-81187, as analogues with highest antiproliferative activity against HeLa/KB cervical carcinoma and U373 glioblastoma cell lines, were selected for further characterization in vitro (Fig. 1 and Table 1). In mode-of-action studies, D-64131 was tested against different targets within the cell division cycle, and tubulin was found as a putative molecular target. Further studies with selected analogues subsequently showed that polymerization of bovine brain tubulin as well as binding of [3H]colchicine to biotinylated αβ-tubulin were dose-dependently inhibited by D-64131 and other analogues (Fig. 2). The IC_{50} from these biochemical assays correlated well with the antiproliferative activity (Table 1). Thus, we conclude that the antiproliferative activity of 2-aroylindoles is based on binding to tubulin, interfering with the
A NEW CLASS OF MICROTUBULE INHIBITORS FOR CANCER THERAPY

2-Aroylindoles Are Cell Cycle Specific, Arresting Tumor Cells in G2-M. The antiproliferative effect of 2-aroylindoles was evaluated in two independent cellular models, i.e., by flow cytometric analysis of cells in the G1, S, or G2-M phase of the cell division cycle and by determination of the cytotoxicity toward RKO colon carcinoma cells with ectopic inducible expression of p21WAF1. As shown in Fig. 4A and summarized in Table 2, HeLa/KB cervical carcinoma cells are dose-dependently arrested in G2-M before induction of apoptotic cell death, as evidenced by a significant number of cells in sub-G1. The RKO p21WAF1 model was chosen to prove that the cytotoxicity depends actively on cycling cells. Inducible expression of p21WAF1 renders RKO cells completely resistant toward compounds such as paclitaxel that rely on the progression through a specific phase of the cell cycle (20). As exemplified for D-64131 and D-68144 in Fig. 4B and summarized in Table 2, cytotoxicity is only evident on cycling RKO cells without p21WAF1 expression. No significant cytotoxicity was detected toward RKO cells that were cell cycle arrested in G1 and G2 through inducible expression of p21WAF1. We conclude that 2-aroylindoles arrest cells in the M-phase of the cell cycle and display cytotoxicity only toward cycling cells.

Antiproliferative Activity of D-64131 and D-68144 toward Different Tumor Cell Lines. The in vitro activity spectrum toward cell lines representing different tumor entities was evaluated with

<table>
<thead>
<tr>
<th>Compound</th>
<th>D-64131</th>
<th>D-68143</th>
<th>D-68144</th>
<th>D-68148</th>
<th>D-68150</th>
<th>D-68172</th>
<th>D-70316</th>
<th>D-81187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxicity IC50 [µM]</td>
<td>0.074</td>
<td>0.106</td>
<td>0.027</td>
<td>0.082</td>
<td>0.250</td>
<td>0.019</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>Inhibition of polymerization IC50 [µM]</td>
<td>0.53</td>
<td>1.29</td>
<td>0.53</td>
<td>0.99</td>
<td>5.60</td>
<td>0.81</td>
<td>0.39</td>
<td>0.14</td>
</tr>
<tr>
<td>Colchicine binding</td>
<td>0.51</td>
<td>0.30</td>
<td>0.28</td>
<td>0.27</td>
<td>2.50</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* IC50, 0.29 µM at 26% maximal inhibition.

function of the mitotic spindle apparatus. This conclusion was further supported by confocal microscopic analysis of mitotic spindles in HeLa/KB cervical carcinoma cells after removal of unassembled tubulin. Many cells display abnormal microtubule structures, such as fragmented mitotic spindles or multiple spindle poles after treatment with D-64131 or vincristine (data not shown). Abnormal microtubule structures are also detectable in cells treated with the tubulin-stabilizing drug paclitaxel. Nuclear blebbing and abnormal nuclear structures typical for apoptotic cells are detectable in many cells treated with D-64131, Taxol, or vincristine (data not shown).

Effect of D-64131 on β-Tubulin GTPase Activity. The dynamic instability of microtubules is driven by β-tubulin-dependent hydrolysis of GTP (6). Synthetic or natural compounds like paclitaxel, vincristine, or colchicine either stimulate or repress the GTPase of β-tubulin associated with polymerization (32, 33). As shown in Fig. 3A, only highly purified tubulin is devoid of any β-tubulin-independent GTPase activity and was used for all additional experiments. Tubulin polymerization and β-tubulin GTPase were activated by raising temperature to 37°C and addition of 1 mM glutamate or 4.5% glycerin (Fig. 3A and data not shown). In case of glycerin-dependent polymerization, the GTPase is activated by paclitaxel and colchicine, with D-64131 and vincristine having no significant effect (Fig. 3B). For glutamate-dependent polymerization, the GTPase is activated by colchicine and vincristine in concentrations ≤1 µM (Fig. 3C). Paclitaxel or vincristine at higher concentrations of >1 µM are inhibitory, whereas D-64131 again showed only marginal influence (Fig. 3C). On the basis of these data, it appears that, unlike the natural tubulin inhibitors, D-64131 did not significantly effect the polymerization dependent GTPase of β-tubulin.

B

A

Fig. 2. Inhibition of tubulin polymerization and [3H]colchicine binding. The inhibition of polymerization of bovine brain tubulin by selected 2-aroylindole analogues is shown in A, whereas inhibition of [3H]colchicine binding to biotinylated tubulin is shown in B. Mean IC50 of independent experiments are summarized in Table 1.
D-64131 and D-68144, paclitaxel, vincristine, and colchicine were included as reference compounds. As summarized in Table 3, D-64131, D-68144, and colchicine were cytotoxic against 12 of 14 tumor types, whereas paclitaxel and vincristine were cytotoxic against 13 of 14 tumor types. The HeLaA endometrial carcinoma cell line was resistant toward all tubulin inhibitors as tested. Cytotoxicity was independent from the p53 status; cells with mutant (e.g., HT29, PC-3, MDA-MB431, and Saos-2) and wild-type p53 (e.g., A549 and RKO) were equally targeted. The mean cytotoxicity of D-64144 with 24 nm was identical to that of colchicine but 2.4- and 3.8-fold weaker compared with that of paclitaxel and vincristine, respectively. These results demonstrate that 2-arylidines have broad cytotoxicity toward cancer cells of different origin and an antitumor efficacy comparable with that of paclitaxel.

**D-64131 and D-68144 Are Cytotoxic toward MDR/MRP Tumor Cell Lines.** One major mechanism of acquired drug resistance is the overexpression of efflux pumps, *i.e.*, the p-gp170/MDR and MRP (34). We compared the antitumoral activity of D-64131 and D-68144 to that of vincristine, paclitaxel, and colchicine in the murine L1210 VCR cell line. The L1210 VCR murine leukemia cell line was selected for resistance toward vincristine, overexpressing the p-gp170/MDR protein (9). As summarized in Table 4, D-64131 and D-68144 are equally potent toward the parental L1210 and L1210 VCR cell lines.

### Table 2: Cytotoxicity in the RKO p21<sup>−/−</sup> model and G<sub>2</sub>-M cell cycle arrest

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Antiproliferative activity IC&lt;sub&gt;50&lt;/sub&gt; [nM]</th>
<th>G&lt;sub&gt;2&lt;/sub&gt;-M cell cycle arrest IC&lt;sub&gt;50&lt;/sub&gt; [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>&gt;10</td>
<td>0.006</td>
</tr>
<tr>
<td>Vincristine</td>
<td>&gt;10</td>
<td>0.001</td>
</tr>
<tr>
<td>D-64131</td>
<td>&gt;10</td>
<td>0.042</td>
</tr>
<tr>
<td>D-68144</td>
<td>&gt;10</td>
<td>0.082</td>
</tr>
<tr>
<td>D-68144</td>
<td>&gt;10</td>
<td>0.018</td>
</tr>
<tr>
<td>D-68150</td>
<td>&gt;10</td>
<td>0.050</td>
</tr>
<tr>
<td>D-68172</td>
<td>&gt;10</td>
<td>0.246</td>
</tr>
<tr>
<td>D-70316</td>
<td>&gt;10</td>
<td>0.019</td>
</tr>
<tr>
<td>D-81187</td>
<td>&gt;10</td>
<td>0.037</td>
</tr>
</tbody>
</table>

ND, not done.

### Table 3: Cytotoxicity profile toward tumor cell lines from different tissues/organisms

<table>
<thead>
<tr>
<th>Tumor cell line (organ/tissue)</th>
<th>Growth inhibition/IC&lt;sub&gt;50&lt;/sub&gt; [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-64131</td>
<td>D-68144</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>Vincristine</td>
<td></td>
</tr>
<tr>
<td>MDA MB231 (breast)</td>
<td>62</td>
</tr>
<tr>
<td>HeLaA (endometrium)</td>
<td>1000</td>
</tr>
<tr>
<td>A431 (vulva)</td>
<td>48</td>
</tr>
<tr>
<td>SKOV3 (ovary)</td>
<td>24</td>
</tr>
<tr>
<td>HeLaA/KB (cervix)</td>
<td>48</td>
</tr>
<tr>
<td>HT 29 (colon)</td>
<td>37</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>63</td>
</tr>
<tr>
<td>PC-3 (prostate)</td>
<td>56</td>
</tr>
<tr>
<td>AsPC-1 (pancreas)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ca27 (tongue)</td>
<td>65</td>
</tr>
<tr>
<td>U 87 (brain)</td>
<td>88</td>
</tr>
<tr>
<td>Saos-2 (bone)</td>
<td>42</td>
</tr>
<tr>
<td>Renca (kidney)</td>
<td>144</td>
</tr>
<tr>
<td>T24 (bladder)</td>
<td>63</td>
</tr>
<tr>
<td>Mean</td>
<td>62</td>
</tr>
</tbody>
</table>

As expected, the resistance factor (ratio IC<sub>50</sub> L1210 VCR/IC<sub>50</sub> L1210) for the reference compounds ranged from 70 to 109. In addition, the antitumoral efficacy of D-64131 in comparison to paclitaxel and vincristine was evaluated in resistance mediated by the MRP and in human tumor cells with resistance to cisplatin, the topoisomerase I inhibitor SN-38 (7-ethyl-10-hydroxycamptothecin) and the thymidylate synthase inhibitors 5-fluorouracil and raltitrexed. Taken together, D-64131 retained unaltered cytotoxic efficacy toward all resistant sublines as tested (Table 4). In summary, the cytotoxicity of 2-arylidines, as represented by D-64131 and D-68144, is not altered by various resistance phenotypes of high clinical relevance.

### Table 4: Antitumoral efficacy toward tumor cell lines with different resistance phenotypes

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Antiproliferative activity IC&lt;sub&gt;50&lt;/sub&gt; [nM]</th>
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<tbody>
<tr>
<td>D-64131</td>
<td>D-68144</td>
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<tr>
<td>Vincristine</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>Colchicine</td>
<td></td>
</tr>
<tr>
<td>MDR1</td>
<td>L1210</td>
</tr>
<tr>
<td></td>
<td>0.102</td>
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<tr>
<td></td>
<td>L1210/VCR</td>
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<tr>
<td></td>
<td>0.077 (0.7)</td>
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<tr>
<td></td>
<td>0.045 (1.7)</td>
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<tr>
<td></td>
<td>1.46 (70)</td>
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<tr>
<td></td>
<td>153 (109)</td>
</tr>
<tr>
<td></td>
<td>5.48 (107)</td>
</tr>
<tr>
<td>MDR1</td>
<td>MCF-7</td>
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<tr>
<td></td>
<td>0.04</td>
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<td></td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>0.001</td>
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<tr>
<td></td>
<td>0.003</td>
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<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>MDR1</td>
<td>A2780</td>
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<td>0.024</td>
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<td>0.0006</td>
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<td>0.005</td>
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<tr>
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<td>ND</td>
</tr>
<tr>
<td>MDR1</td>
<td>A2780/Dx5</td>
</tr>
<tr>
<td></td>
<td>0.02 (0.8)</td>
</tr>
<tr>
<td></td>
<td>0.024 (40)</td>
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<tr>
<td></td>
<td>0.145 (29)</td>
</tr>
<tr>
<td>MRP</td>
<td>HT1080</td>
</tr>
<tr>
<td></td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>MRP</td>
<td>HT1080/DR4</td>
</tr>
<tr>
<td></td>
<td>0.02 (0.7)</td>
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<tr>
<td></td>
<td>0.018 (23)</td>
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<tr>
<td></td>
<td>0.005 (1.7)</td>
</tr>
<tr>
<td>MRP</td>
<td>HT29</td>
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<td>0.055</td>
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<td>0.003</td>
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<tr>
<td>MRP</td>
<td>HT29/R1</td>
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<td>0.006 (0.9)</td>
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<td>HT29/DR24</td>
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<tr>
<td>MRP</td>
<td>H29/ICD</td>
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<td>0.093 (1.6)</td>
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<td>MRP</td>
<td>Topoisomerase 1</td>
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<td>0.02 (1.5)</td>
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<td>0.133 (4)</td>
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<sup>a</sup> ND, not done.

### DISCUSSION

D-64131 was discovered by random screening for antiproliferative drugs, exhibiting high cytotoxicity toward human cancer cells. Mode-of-action studies revealed that D-64131 inhibited tubulin polymerization. On the basis of this finding, D-64131 was selected as a lead structure, and more than 160 analogues with the 2-arylidine core...
structure were synthesized (18). These compounds represent a new chemical entity of a synthetic SMTI. From a structural point of view, 2-arylidoles have the indole ring as a scaffold and resemble D-24851 [N-(pyridin-4-yl)-(1-(4-chlorbenzyl)-indol-3-yl)-glyoxylamid], heterocombretastatins, diaryl-indoles, or 3-formyl-2-phenylldol (9, 13–15), although substituents as well as the overall structures differ significantly. Other synthetic SMTIs include A-105972 (10), the carbamate derivatives CI-980 and 1069C85 (11, 12).

![Figure 3](image)

**Fig. 3.** Effect on the β-tubulin GTPase. The GTPase activity of MAP-containing and highly purified MAP-free bovine brain tubulin is shown in A. The amount of GDP and GTP was determined under various experimental conditions as shown and described in “Materials and Methods.” PEM, PIPES/EGTA/MgCl2, buffer; Glut, monosodium glutamate. MAP-free tubulin was subsequently used for the determination of the effect of colchicine, vincristine, paclitaxel, or D-64131 on the polymerization-dependent β-tubulin GTPase. Polymerization of tubulin was induced by addition of 4.5% (v/v) glycerin (B) or 1 mM glutamate (C) and incubation at 37°C for 2 h. The GTPase activity is expressed as the quotient of GDP/GTP concentrations as quantified by phosphorimager analysis (background activity at 0°C is shown as a dotted line).

![Figure 4](image)

**Fig. 4.** Cell cycle specificity. The cell cycle specificity of selected compounds was determined by flow cytometry in A. The percentage of HeLa/KB cervical carcinoma cells in G2-M phase of the cell division cycle was quantified, and dose-response curves (left panel) as well as selected FACS histograms (right panel) are shown. In B, the cell cycle-dependent cytotoxicity of D-64131 and D-68144 was evaluated in the RKO p21/Waf1 cell model. RKO colon carcinoma cells were pretreated with 30 μM muristerone A to induce p21waf1, leading to cell cycle arrest in G1 and G2. Cell proliferation/metabolic activity was determined 48 h after treatment with compounds.
A NEW CLASS OF MICROTUBULE INHIBITORS FOR CANCER THERAPY

Dose [mg/kg] | Schedule | Route | Opt. T/C | GD 200% | BWC [%]
--- | --- | --- | --- | --- | ---
400 | d1-5,8,9-15-18 | p.o. | 15/28 | 23.4 | -4
200 | d1-5,8,9-15-18 | p.o. | 29/35 | 8.6 | -4

In this regard, the fluorescence of D-64131 with inhibition of [3H]colchicine binding. Because bovine brain tubulin is distinct to that of colchicinoids, but additional and/or the conformational change induced by binding of 2-aroylindoles is possible (35). Another explanation might be that the binding site of taxanes, vincristine and vinblastine are destabilizing tubulin and advanced breast cancer (39). Taxanes, although widely used in

Microtubule inhibitors are potent antimitotic drugs, arresting dividing cells in M-phase and inducing programmed cell death. These characteristics were also evident for 2-aroylindoles. As determined by flow cytometry, HeLa/KB cervical carcinoma cells were dose-dependently arrested in M-phase with 4N chromosomes before cell death became evident by cells with <2N chromosomes (cell population in sub-G1) and a massive reduction of attached, vital cells. The superior specificity of 2-aroylindoles was shown in three independent experimental settings, i.e., the RKO p21\textsuperscript{waf1} cell model, a biochemical kinetic screen and, finally, signal transduction to the c-fos promoter. In the RKO p21\textsuperscript{waf1} cell model, the colon adenocarcinoma cells are arrested in the G1 and G2 phase of the cell division cycle by cedysone-inducible ectopic expression of the cdk inhibitor p21\textsuperscript{waf1} (20). Because tubulin-targeting compounds only hit mitotic cells, RKO cells are protected by p21\textsuperscript{waf1} expression. As shown in Fig. 4B and Table 2, 2-aroylindoles are only cytotoxic to cycling RKO cells without p21\textsuperscript{waf1} expression. Because D-64131 is an analogue of the platelet-derived growth factor-receptor kinase inhibitor 5-methoxy-bis (1H-2-indol)-1-methanone (37), specificity was evaluated against serine/threonine and tyrosine-specific kinases, i.e., platelet-derived growth factor receptor β, Jak2, epidermal growth factor receptor/HER1, KDR/vascular endothelial growth factor-R2, protein kinase B/akt1, cyclin-dependent kinase 1/cyclin B, cyclin-dependent kinase K2/cyclin E, and protein kinase C\textgamma. In concentrations up to 10 \muM, all 2-aroylindoles as included in this study did not exhibit any inhibitory activity (data not shown). In the final assay for specificity, D-64131 did not affect the serum-dependent transcriptional activation of the immediate-early gene c-fos (data not shown), thus excluding inhibitory of kinases like raf1, mitogen-activated protein kinase kinase, or p42/44 mitogen-activated protein kinase involved in signaling to the c-fos promoter (38). We conclude that D-64131 and analogues are specific for tubulin, exhibiting cytotoxicity only against cycling cells.

Paclitaxel and Taxotere are stabilizing tubulin inhibitors used clinically for the treatment of breast, ovarian, and non-small cell lung cancer, whereas vincristine and vinblastine are destabilizing tubulin inhibitors indicated for acute lymphatic leukemia, Morbus Hodgkin, and advanced breast cancer (39). Taxanes, although widely used in cancer therapy (40), have severe drawbacks: (a) natural/semisynthetic compounds with complex structure; (b) low solubility and very limited oral bioavailability as single agents; (c) dose-limiting toxicity such as neutropenia, peripheral neuropathy, and most likely vehicle (Cremaphor EL)-related hypersensitivity reactions; and (d) development of drug resistance and tumor metastases in the brain. Clearly, there is a high medical need for tubulin inhibitors with a new mode of action and drug profile. D-64131 and analogues are very promising, displaying properties of high clinical relevance: (a) strong and broad antiproliferative activity toward tumor cells derived from different tissues/organs (Table 3); (b) no substrate of the MDR/MDR efflux pump and no cross resistance toward tumor cells displaying various resistance phenotypes (Table 4); and (c) oral bioavailability and efficacy at well-tolerated doses (Fig. 5).

At present, the best described mechanism of resistance to tubulin-binding agents is the MDR phenotype mediated by the P-glycoprotein 170 efflux pump (34). Because D-64131 or D-68144 are neither substrates of the MDR/MDR efflux pump nor is there any cross-resistance, they have the potential for treatment of taxane-resistant patients. Another resistant phenotype relates to β-tubulin structure and/or function such as described for 1A9 human ovarian carcinoma.
cells and paclitaxel with mutations in the class I/M40 β-tubulin isoform (41). Because 2-aryloindoles are structurally and mechanistically different to natural tubulin inhibitors, it is highly likely that they are active toward these cancer genotypes as well.

In animal studies in nude mice, D-64131 was well tolerated after oral dosing up to 400 mg/kg with no signs of toxicity at efficacious doses in the MEXF 989 melanoma xenograft tumor model. As such, D-64131 is superior to the synthetic SMTIs T138067 or A-105972, which were only effective after i.p. dosage (10, 17). Published clinical Phase II data for the carbamate CB980 are disappointing (42), but new chemical entities such as D-24851 are headed for the clinic (9, 43). 2-Aroyloindoles with D-64131 as a prototypic representative are new candidates for Phase I clinical trials, which are scheduled after completion of ongoing preclinical development studies.

ACKNOWLEDGMENTS

The technical assistance of A. Masch, E. Thoennes, S. Falk, and R. Deckenbach is gratefully acknowledged. We also thank Maria Hörter, GBF, Braunschweig for FACS analysis; J. Braunger and F. Flach, Byk Gulden GmbH, Konstanz for confocal microscopy; and U. Riemer for preparing the figures.

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2-Aroylindoles, a Novel Class of Potent, Orally Active Small Molecule Tubulin Inhibitors

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