A-204197, a New Tubulin-binding Agent with Antimitotic Activity in Tumor Cell Lines Resistant to Known Microtubule Inhibitors

Stephen K. Tahir, Edward K.-H. Han, Bruce Credo, Hwan-Soo Jae, Jennifer A. Pietenpol, Caroline D. Scatena, Jinshyun R. Wu-Wong, David Frost, Hing Sham, Saul H. Rosenberg, and Shi-Chung Ng


ABSTRACT

Drug resistance is a prevalent problem in the treatment of neoplastic disease, and the effectiveness of many clinically useful drugs is limited by the fact that they are substrates for the efflux pump, P-glycoprotein. Because there is a need for new compounds that are effective in treating drug-resistant tumors, we tested A-204197 (4-[4-acetyl-4,5-dihydro-5-(3,4,5-trimethoxyphenyl)-1,3,4-oxadiazol-2-yl]-N,N-dimethylbenzenamine), a novel oxadiazoline derivative with antiproliferative properties, on cell lines that were either sensitive or resistant to known microtubule inhibitors. Cell lines that were resistant to paclitaxel, vinblastine, or colchicine were equally sensitive to A-204197 (proliferation IC₅₀s ranging from 36 to 48 nM) despite their expression levels of P-glycoprotein. The effect of A-204197 on cell growth was associated with cell cycle arrest in G₂-M, increased phosphorylation of select G₂-M checkpoint proteins, and apoptosis. In competition-binding assays, A-204197 competed with [³H]-labeled colchicine for binding to tubulin (Kᵢ = 0.75 μM); however, it did not compete with [³H]-labeled paclitaxel. A-204197 prevented tubulin polymerization in a dose-dependent manner (IC₅₀ = 4.5 μM) in vitro and depolymerized microtubules in a time-dependent manner in cultured cells. These findings indicate A-204197 is a promising new tubulin-binding compound with antimitotic activity that has potential for treating neoplastic diseases with greater efficacy than currently used antimitotic agents.

INTRODUCTION

Tubulin, the major protein component of microtubules, is the target of numerous antimitotic drugs (1–4). Known antimitotic agents generally fall into three distinct classes. The Vinca alkaloids, typified by vincristine, vinblastine, and vinorelbine, are well-characterized tubulin-binding drugs that interfere with the cell’s ability to properly form the mitotic spindle by preventing the normal polymerization of microtubules (4). They have importance in the treatment of leukemia, lymphomas, small cell lung cancer, and other malignancies (5). The taxanes such as paclitaxel (Taxol) and docetaxel (Taxotere) are newer antimitotic agents that stabilize microtubules and induce a net polymerization of microtubules (4). They are effective in the treatment of breast, lung, ovarian, head and neck, and bladder carcinomas (6). The third class, typified by colchicine, is comprised of a structurally diverse collection of small molecules that are related by the fact that all bind to a common site on tubulin known as the colchicine site and prevent the normal polymerization of microtubules (7). No representatives of this third class have yet been approved for use in cancer chemotherapy. Despite their differences, the consequence of disrupting tubulin and microtubule dynamics with these three classes of drugs appears to be the same: metaphase arrest in dividing cells and induction of apoptosis (8).

Although antimitotic compounds have been used clinically to treat patients with neoplastic disease, a major drawback is the loss of efficacy over time because of the development of resistance (9, 10). Drug resistance often develops through the expression of efflux pumps, such as P-gp and other MDR proteins (9–11). P-gp (p170) is a large integral membrane protein that binds many anticancer drugs and transports them from the cell. Resistance can be intrinsic or acquired, but in either case, tumors become refractory to a variety of structurally different compounds (9). Because many clinically used antimitotic compounds such as vinblastine and paclitaxel are substrates for P-gp, there is a need for new compounds that are effective in treating drug-resistant tumors that express P-gp.

Recently, as part of our efforts to identify clinically effective antitumor compounds with antiproliferative properties, we discovered A-204197, an oxadiazoline derivative (Fig. 1). In this study, we determined the mechanism of action of A-204197 and examined if its efficacy was affected by the P-gp status in select tumor cell lines.

MATERIALS AND METHODS

Chemicals. A-204197 was synthesized at Abbott Laboratories. Colchicine, vinblastine, and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO). [³H]Colchicine (specific activity = 61.4 Ci/mmol) and [³H]paclitaxel (specific activity = 6.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and Moravek Biochemicals (Brea, CA), respectively.

Cell Culture. HCT116 and HCT15 colon carcinoma cells (American Type Culture Collection, Rockville, MD) were cultured with DMEM and supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Inc., Grand Island, NY). The lung carcinoma cell line H460 was purchased from American Type Culture Collection. H460/T200 was derived from H460 cells by initially adding 10 nM paclitaxel to the H460 culture medium and thereafter doubling the paclitaxel concentration every 3–4 weeks up to 200 nM. H460 and H460/T200 cells were grown and maintained in RPMI 1640 plus 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Inc.). All cell lines were maintained in a humidified chamber at 37°C containing 5% CO₂.

SRB Assay. Cell proliferation assays were performed in 96-well microliter plates using the SRB colorimetric assay as described previously (12) after treatment with A-204197, paclitaxel, vinblastine, or colchicine (10⁻¹⁻¹⁻¹⁰⁻⁶ M) in duplicate for 48 h at 37°C.

Flow Cytometric Analysis. Adherent and floating cells were harvested, pelleted, and resuspended in 0.5-ml ice-cold staining solution (50 μg/ml PI, 40 units/ml RNase, 0.5% Triton X-100 in PBS). After 1 h at 4°C in the dark, the DNA content was analyzed using a Becton Dickinson FACSCalibur Flow Cytometer (San Jose, CA).

Caspase 3 Activation Assay. Caspase 3 activation by H460/T200 cells after treatment with various concentrations of A-204197 (10⁻¹⁻¹⁻¹⁻¹⁰⁻⁶ M) in

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2 S. K. T. and E. K.-H. H. contributed equally to this study.

3 The abbreviations used are: P-gp, P-glycoprotein; MDR, multidrug resistance; SRB, sulforhodamine B; H460, NCI-H460 lung carcinoma cell line; H460/T200, paclitaxel-resistant variant of H460 cells; SPA, scintillation proximity assay; PI, propidium iodide; A-204197, 4-[4-acetyl-4,5-dihydro-5-(3,4,5-trimethoxyphenyl)-1,3,4-oxadiazol-2-yl]-N,N-dimethylbenzenamine; AUC, area under the curve; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin).
A-204197, A NOVEL ANTIMITOTIC AGENT

RESULTS

A-204197 Inhibits the Proliferation of Human Tumor Cell Lines Independent of Their MDR Status. Because colchicine, vinblastine, and paclitaxel are substrates for P-gp, we compared them with A-204197 on the proliferation of three human cancer cells (H460, H460/T200, and HCT15). H460 non-small cell lung carcinoma cells have very low expression of P-gp; HCT15 colon adenocarcinoma cells have one of the highest levels of P-gp expression of cells from the National Cancer Institute tumor panel (18), and the expression of P-gp in H460/T200 cells, generated by selection in 200 nM paclitaxel, is ~10-fold greater than HCT15 cells. The IC50s of A-204197 on H460, HCT15, and H460/T200 proliferation using the SRB assay were similar after 48-h treatment (42, 36, and 48 nM respectively; Table 1). In contrast, the resistance of all three cell lines to paclitaxel, vinblastine, and colchicine increased in parallel with P-gp levels (Table 1). Thus, the two cells (HCT15 and H460/T200) having high P-gp expression levels were resistant to colchicine, vinblastine, and paclitaxel but remained sensitive to A-204197.

Cell Cycle Arrest in G2-M and Induction of Apoptosis after A-204197 Treatment. There was an accumulation of H460 cells with 4N DNA content and a concomitant decrease in cells with 2N DNA content after treatment with 0.1 μM paclitaxel, 0.5 μM A-204197, or 0.5 μM colchicine for 24 h (Fig. 2). In contrast, the cell cycle distribution of paclitaxel- and colchicine-treated H460/T200 cells looked similar to the untreated controls (Fig. 2), whereas A-204197 was equally effective in both cell lines. The accumulation of cells with 4N DNA content increased with time (Fig. 3A), followed by a decrease in cells with 4N DNA content and an increase in subdiploid cells at later time points (24 and 48 h), indicative of apoptotic cells. Consistent with an induction of apoptosis, we observed an increase in the ability of cell lysates to cleave the caspase 3 substrate, DEVD-AMC, when prepared from cells 24 h after A-204197 treatment (Fig. 3B). As expected, the effect of A-204197 on the cell cycle distribution of H460/T200 cells was similar to its effect on H460 cells despite the difference in P-gp expression.

A-204197 Induces Phosphorylation of G2-M Regulatory Proteins and Bcl-2. Slower migrating forms of the Cdc25C phosphatase and the antiapoptotic protein Bcl-2 were detected in H460 and HCT15 cells after 4 and 24 h treatment with 100 nM A-204197 (Fig. 4). In contrast, there was a shift to the faster migrating form of the cyclin-dependent kinase Cdc2, consistent with the hypophosphorylated, active form of the protein. The changes in Cdc25C, Bcl-2, and Cdc2 coincided with the appearance of phosphoepitopes recognized by MPM-2, an antibody that recognizes phosphorylated polypeptides found only in mitotic cells (17). Similar changes in the migration of Cdc25C, Bcl-2, and Cdc2 were observed in H460 cells treated with the antineoplastic agent paclitaxel for 24 h. The changes in protein migration in A-204197 and paclitaxel-treated H460 cells were attributable to phosphorylation, as the slower migrating forms of Cdc25C and Bcl-2 were lost when treated cellular extracts were exposed to phosphatase in vitro (data not shown and Ref. 17). In contrast to the H460 cells, the MDR HCT15 cells were insensitive to paclitaxel treatment, as there was no change in the phosphorylation of Cdc25C, Cdc2, or Bcl-2. Therefore, it appears that A-204197 arrests cells in mitosis, induces apoptosis, and is capable of inducing a mitotic arrest in cells that express P-gp that are insensitive to known microtubule inhibitors.

A-204197 Binds to the Colchicine Site of Tubulin and Prevents the Polymerization of Microtubules in Vitro. We determined if A-204197 interacted directly with tubulin by binding to either the

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<th>Compound</th>
<th>H460 (MDR-&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>HCT15 (MDR&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>H460/T200 (MDR&lt;sup&gt;a&lt;/sup&gt;)</th>
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<tr>
<td>A-204197</td>
<td>42.2 ± 6</td>
<td>36.4 ± 3</td>
<td>47.7 ± 4</td>
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<td>(14)</td>
<td>(18)</td>
<td>(7)</td>
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<td>Colchicine</td>
<td>49.4 ± 8</td>
<td>107 ± 9</td>
<td>194</td>
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<td>(n = 6)</td>
<td>(n = 8)</td>
<td>(n = 2)</td>
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<tr>
<td>Vinblastine</td>
<td>3.4 ± 0.4</td>
<td>43 ± 11</td>
<td>190 ± 51</td>
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<td></td>
<td>(n = 10)</td>
<td>(n = 9)</td>
<td>(n = 11)</td>
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<tr>
<td>Paclitaxel</td>
<td>7.4 ± 0.9</td>
<td>280 ± 29</td>
<td>530 ± 99</td>
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<td>(n = 31)</td>
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<sup>a</sup> Mean ± SE (number of determinations).
Fig. 2. Cell cycle distribution of H460 and H460/T200 cells after 0.1 μM paclitaxel, 0.5 μM colchicine, or 0.5 μM A-204197 treatment for 24 h. Cells were treated with the indicated compound, trypsinized, fixed, and stained with PI. The cell cycle distribution was measured by flow cytometry. Results are representative of two independent experiments.

Fig. 3. A, cell cycle progression of H460 and H460/T200 cells after 0.5 μM A-204197 treatment for 0–48 h. Cells were treated for the indicated times, then trypsinized, fixed, and stained with PI. The cell cycle distribution was measured by flow cytometry. Results are representative of two independent experiments. B, dose-dependent increase in caspase 3 activation (DEVD-AMC cleavage) in H460 cells after A-204197 treatment for 24 h. Each point represents the mean ± SE.
colchicine or paclitaxel-binding domains on tubulin using a competition-binding SPA. We found that A-204197 competitively inhibited $[^3H]$colchicine binding ($K_i = 0.75 \mu M$; Fig. 5A), whereas it did not compete with $[^3H]$paclitaxel (Fig. 5B). To test the effect of A-204197 on microtubules in vitro, we added purified, unpolymerized tubulin with various concentrations of A-204197 (0–30 $\mu M$) and measured polymerization at 37°C for 30 min (Fig. 5C). A-204197 reduced the amount of tubulin polymerized in a dose-dependent manner. The inhibitory concentration (IC$_{50}$) that reduced the amount of polymerized tubulin by 50% was 4.5 $\mu M$.

**A-204197 Depolymerizes Microtubules in Human Tumor Cell Lines.** The effect of A-204197 on the microtubule organization was first examined in HCT116 colon carcinoma cells. The normal microtubule distribution in untreated HCT116 cells is shown in Fig. 6A. Microtubule depolymerization was observed as early as 1 h as evident by breaks along the microtubules (data not shown). By 4 h, many cells with long microtubule fragments were visible after 0.5 $\mu M$ treatment (Fig. 6B). By 8 h, most cells had short microtubule fragments scattered throughout the cytoplasm (Fig. 6C), and there was a complete loss of microtubules with only a diffuse stain visible throughout the cytoplasm by 24 h (Fig. 6D).

We then examined the microtubule polymerization status in H460/T200 cells as compared with their parental cell line H460 after 24 h treatment with paclitaxel, colchicine, or A-204197. Consistent with the SRB proliferation data, we observed an effect on microtubules after paclitaxel or colchicine treatment in H460 but not in the drug-resistant H460/T200 cells (Fig. 7). In contrast, A-204197 was able to completely depolymerize the microtubules in both the H460 and H460/T200 cells (Fig. 7, D and H).

**DISCUSSION**

A-204197 in vitro was equally effective against all three cell lines (IC$_{50}$ = 36–48 $\mu M$) despite their P-gp MDR status. This feature was distinct from paclitaxel, vinblastine, and colchicine because HCT15 and H460/T200 cells were more resistant to these chemotherapeutics (IC$_{50}$ ranged from 3–530 $\mu M$) than H460 cells. Although A-204197 efficacy was not affected by the P-gp status in the cell lines tested, the
possibility exists that selective pressure will eventually lead to drug resistance. We have examined the effect of selective pressure of A-204197 on HCT15 cells and found that in contrast to paclitaxel-resistant cell lines that readily arose with drug selection, it was quite difficult to generate A-204197 resistant lines (data not shown). The one drug-resistant HCT15 line that resulted from long selection exhibited no alteration in P-gp expression, but it exhibited a novel resistant mechanism by overexpression of annexin IV, a small calcium and lipid-binding protein (19).

Many antimitotic drugs that interfere with the normal formation of the mitotic spindle, either by increasing microtubule stability or depolymerization, can cause cells to arrest at the prometaphase/metaphase-to-anaphase transition known as the mitotic checkpoint (20). Our results show that in addition to directly disrupting microtubules, treatment with A-204197 initiated a phosphorylation cascade resulting in the engagement of active Cdc2 kinase and phosphorylation of Cdc25C, Bcl-2, and MPM-2 epitopes. These changes in protein phosphorylation are consistent with cell cycle arrest in mitosis as shown previously (17).

Depending on the cell type, the mitotic block induced by other antimitotic compounds may persist for varying lengths of time; however, most cells will exit the cell cycle and undergo apoptosis (20, 21). Consistent with other antimitotic compounds, we observed a concomitant decrease in mitotic cells and an increase in a subdiploid population, suggesting that cells arrested in mitosis with A-204197 eventually became apoptotic.

The process by which antimicrotubule drugs induce apoptosis is poorly understood. It has been shown previously that apoptosis in cancer cells after high doses of paclitaxel could occur directly from a mitotic block (22, 23). Conversely, cells treated with low doses of paclitaxel formed multinucleated cells which either became apoptotic in wild-type p53-containing cells or underwent multiple rounds of DNA replication before undergoing apoptosis in p53-deficient cells (23). Other reports show that antimicrotubule drug-induced apoptosis may involve Bcl-2 and Raf-1 kinase phosphorylation (8, 24, 25). Chadebech et al. (24) have shown phosphorylation and degradation of Bcl-2 occurred in paclitaxel-treated cells arrested in mitosis. Basu and Haldar (25) demonstrated that the antimicrotubule agents colchicine, colcemid, and podophyllotoxin caused Bcl-2 phosphorylation and apoptosis in cancer cells. Yet another mechanism may involve both c-Jun N-terminal kinase-dependent pathways, because Wang et al. (26) have shown that paclitaxel activated the c-Jun N-terminal kinase/stress-activated protein kinase signal transduction pathway in a variety of human cell lines.

In summary, A-204197 is a new antimitotic agent that has properties distinct from colchicine, vinblastine, and paclitaxel. A-204197 and its derivatives may prove to be useful for the treatment of neoplastic diseases. Studies in vivo with A-204197 used either alone or in combination with other agents will further elucidate the effectiveness of A-204197 as a chemotherapeutic agent.

REFERENCES


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