

Therapeutic anticancer efficacy of a synthetic diazonamide analog in the absence of overt toxicity

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Blocking cell division through the inhibition of mitosis is one of the most successful clinical strategies for the treatment of cancer. Taxanes and vinca alkaloids are in widespread use and have demonstrated substantive therapeutic efficacy. Both classes of compounds bind directly to tubulin, a structural component of the mitotic spindle. The ubiquitous utilization of tubulin in cell division in both cancerous and normal cells, however, tempers the broad spectrum of activity of currently used antimetabolites by significant toxicities in normal dividing tissue. Moreover, peripheral nerve cells that rely on microtubules to shuttle cargo along axonal processes are also damaged by tubulin-binding drugs. Thus, neutropenia and peripheral neuropathy are the most frequently cited dose-limiting toxicities of this class of chemotherapeutics. Here we report the preclinical assessment of AB-5, a structural and functional analog of the natural product diazonamide A. AB-5, like taxanes and vinca alkaloids, blocks cell division during mitosis. However, AB-5 works not by binding tubulin but rather through inhibition of a newly discovered role for ornithine- δ -aminotransferase in mitosis. We hereby report that, unlike other antimetabolites, AB-5 is extremely well tolerated by mice when administered under conditions where the drug cures xenografted tumors as effectively as taxanes and vinca alkaloids. AB-5-treated mice show no weight loss, no change in overall physical appearance, and no evidence of neutropenia. These observations raise the possibility that AB-5 may have clinical utility for cancer therapy under conditions largely devoid of chemotherapeutic toxicity and suggest that further preclinical evaluation of AB-5 is warranted.

antimetabolite agent | neutropenia | oncology | pharmacokinetics

The mitotic spindle is a molecular machine composed of polymerized tubulin (microtubules) and associated regulatory proteins acting in concert to appropriately segregate replicated chromosomes. Compounds that interfere with the mitotic spindle have proven highly effective against both hematological malignancies and solid tumors (1, 2). The two most widely used families of antimetabolite agents, taxanes and vinca alkaloids, block cell division by perturbation of microtubule dynamics (reviewed in ref. 3). The taxanes stabilize microtubules against depolymerization, resulting in mitotic cell cycle arrest and eventually cell death (4). By contrast, the vinca alkaloids inhibit the polymerization of tubulin into microtubules, causing G₂/M arrest of the cell cycle and subsequent cell death (4). Both families of compounds are known to exert their antimetabolite activities through direct binding to tubulin (5, 6). Despite the diverse array of spindle proteins that could be used as targets for novel chemotherapeutics, only tubulin-binding agents are currently in clinical use, although agents that interfere with the motor protein kinesin are entering clinical trials (3).

The ubiquitous expression and utilization of tubulin in cell division in both cancerous and normal cells limit the therapeutic utility of tubulin-binding drugs. Bone marrow suppression, in particular neutropenia, is a dose-limiting toxicity of both the taxanes and the vinca family alkaloids (7–9). Despite many efforts to improve these agents, including enumeration of new

classes of tubulin-binding drugs such as the epothilones, it is evident that neutropenia remains a substantive toxicological liability (3, 10). Given this largely unavoidable target-based toxicity, new efforts in the antimetabolite field have instead focused on improving dosing schedule, solubility, bioavailability, activity against resistant cell populations, and concomitant administration of hematopoietic growth factors or agents that ameliorate neurotoxic symptoms (11, 12). Thus, agents that inhibit mitosis with improved safety profiles, perhaps through cancer-specific modes of action, are needed.

Diazonamide A is a natural product originally isolated from the marine ascidian *Diazona angulata* (13). Evaluation of the compound by the Developmental Therapeutics Program at the National Cancer Institute, including analysis by the COMPARE algorithm of the resulting differential cytotoxicity pattern (14, 15), indicated diazonamide A might represent a tubulin-active agent, because its correlation coefficients of toxicity most closely paralleled antitubulin drugs (vinblastine, maytansine, paclitaxel, and vincristine). Cells treated with diazonamide A arrest at the G₂/M boundary and fail to form appropriately organized bipolar mitotic spindles (15, 16). Notably, however, studies by Cruz-Monserrate *et al.* (15) indicated that diazonamide A does not compete with vinblastine or colchicine for tubulin binding. In fact, our own studies have shown that neither biotinylated nor radiolabeled congeners of the natural product interact specifically with purified tubulin *in vitro*, raising the possibility that the compound does not act by binding directly to tubulin (17).

Earlier, we developed a successful laboratory synthesis of diazonamide A and identified its correct chemical structure (16, 18). Such efforts further enabled the preparation of derivatized forms of diazonamide A useful for biochemical studies of its mode of action. These experiments have shown conclusively that tubulin binding does not underpin the antimetabolite effects of this compound. Rather, diazonamide A interacts with a specifically proteolyzed form of ornithine δ -amino transferase (OAT) critically involved in a heretofore novel spindle assembly pathway controlling mitosis (17). Paradoxically, the OAT-mediated spindle assembly pathway appears not to be essential for normal cell division. Mice bearing an inactivating mutation in the gene encoding OAT develop to adulthood if supplemented with

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Conflict of interest statement: P.G.H. and X.W. are founders and shareholders of Joyant Pharmaceuticals, a company that has licensed rights to develop diazonamide A analogs for commercial use. N.S.W. serves as a paid consultant to Joyant Pharmaceuticals.

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Abbreviations: OAT, ornithine δ -amino transferase; GI50, growth inhibitory 50; q2–3dX6, Monday, Wednesday, and Friday dosing schedule for 2 weeks; qd \times 6, daily dosing schedule for 6 days.

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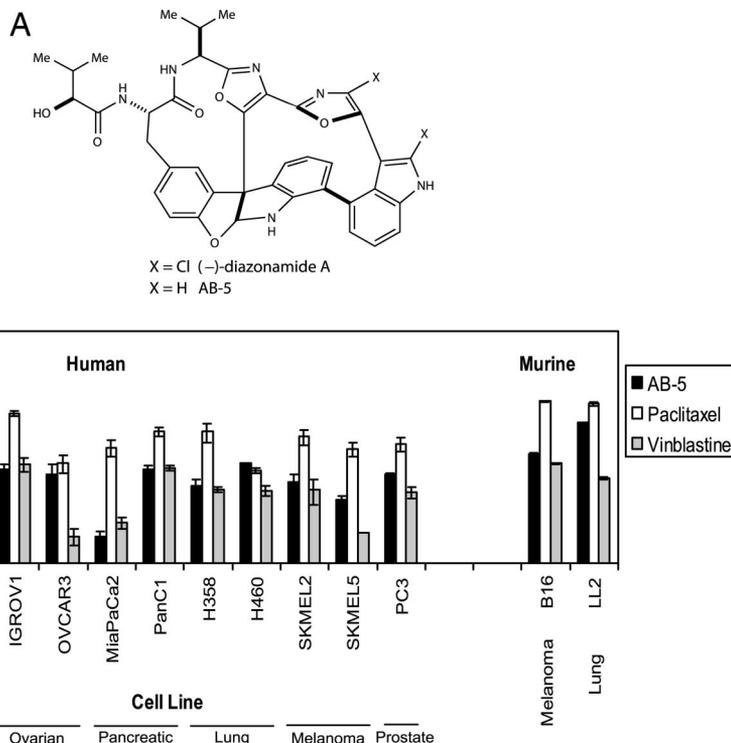


Fig. 1. Structures of native diazonamide A and a functionally equivalent synthetic analogue, AB-5, and *in vitro* activity of AB-5 against a panel of human and murine tumors. (A) Structures of diazonamide compounds: (–)-diazonamide A and AB-5. (B) Activity of AB-5 against a panel of human tumor lines was assessed by addition of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) or Cell Titer-Glo reagent after a 48-h incubation with compound. GI50 values were calculated as described in *Materials and Methods*. The mean \pm SEM for multiple replicate experiments is shown.

arginine, the metabolite produced by the mitochondrial biosynthetic pathway in which this enzyme is involved (19). Moreover, human patients carrying inactivating mutations in the OAT gene also develop to adulthood in the absence of symptoms that would be expected from deficits in mitotic cell division (20).

The OAT-mediated mechanism of action of diazonamide A in inhibiting cell division implied this compound might show a toxicity profile distinct from those exhibited by other tubulin-binding antimetabolites. Herein we describe rigorous studies of the pharmacokinetics, toxicity, and efficacy of AB-5, a synthetic variant of diazonamide A, in nude mice bearing xenografted human tumors. These data reveal substantive therapeutic efficacy of AB-5 in the absence of overt toxicity, suggesting this compound may have potential as an anticancer therapeutic.

Results and Discussion

AB-5 Activity *in Vitro*. Limited structure–activity relationship analysis revealed that a closely related analog of diazonamide A, designated AB-5 (Fig. 1A), exhibited activity indistinguishable from the parent compound in standard cell viability assays. Because AB-5 could be made in fewer steps and in higher yield than diazonamide A, all experiments performed here were carried out by using AB-5. An *in vitro* screen of AB-5 activity across a broad spectrum of tumors revealed nanomolar growth inhibitory 50 (GI50) values against both human and murine tumors similar to the activities of two widely used antimetabolites, vinblastine sulfate and paclitaxel (Fig. 1B). These observations correlate closely with previous studies of native diazonamide A conducted by the Developmental Therapeutics Program at the National Cancer Institute described above.

Analysis of AB-5 Pharmacokinetics and Metabolism. To assess whether AB-5 might have therapeutic utility, *in vivo* studies were initiated to evaluate its stability, metabolism, half-life, and

volume of distribution upon i.v. administration into mice. The compound was formulated in a 1:1 mixture of cremaphor:ethanol (10%) and diluted in 5% dextrose (90%) before *in vivo* administration. With this formulation, 20 mg/kg could be easily administered to mice as a single-bolus i.v. injection with no observable acute toxicity. A simple noncompartmental analysis of plasma levels of AB-5 in mice after a bolus i.v. injection of 20 mg/kg revealed a low clearance rate and an intermediate terminal half-life that predicted efficacious levels of compound should persist on a daily or every-other-day schedule of dosing (Fig. 2A). Plasma levels at 24 h after injection were \approx 15 ng/ml (21 nM), at or above the GI50 value for all tumors examined to date, and measurements of AB-5 levels in s.c. xenografted tumors were 10-fold above the average *in vitro* GI50 values 48 h after injection (data not shown).

The low clearance values for AB-5 suggested that metabolism of this compound might be limited. Analysis of AB-5 metabolism in an *in vitro* murine S9 assay demonstrated the generation of a single metabolite with a molecular weight change (+16) and shift in chromatographic retention time consistent with site selective monohydroxylation (Fig. 2B and C). When the assay was repeated by using human S9 fractions, an identical profile was obtained (Fig. 2D), indicating that metabolism of this compound in mouse and human may be similar. The putative hydroxylated metabolite of AB-5 was purified and tested for antimetabolic activity on HCT116 and PC3 tumor cells. Data from PC3 cells are shown in Fig. 2E. Within limits of quantitation, the metabolite displayed antimetabolic activity nearly indistinguishable from the parental AB-5 compound.

Efficacy of AB-5 Against Human Tumor Xenografts. Based on the aforementioned pharmacokinetic studies, a Monday, Wednesday, and Friday dosing schedule for 2 weeks (q2–3dX6) using 20 mg/kg given as either an i.v. or i.p. bolus was chosen for analysis

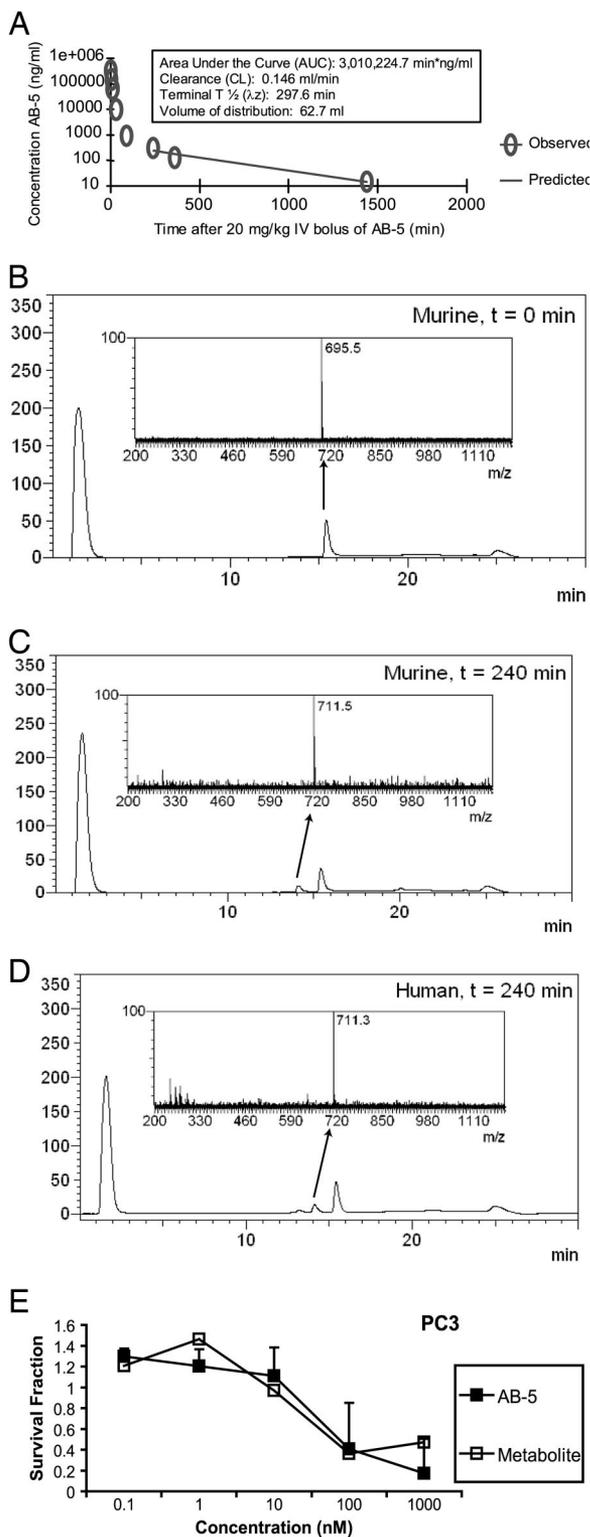


Fig. 2. Analysis of pharmacokinetics and metabolism of AB-5. (A) Pharmacokinetic analysis of AB-5 was conducted after a single i.v. bolus dose of 20 mg/kg AB-5. Mice were bled by cardiac puncture at the indicated times, and plasma was isolated. AB-5 was isolated, and levels were analyzed as described in *Materials and Methods*. Each data point represents the average of five mice. (B–D) Metabolism of AB-5 was assessed by incubation of 10 μ M AB-5 with 1 mg of mouse CD-1 or pooled human S9 protein (In Vitro Technologies) at 37°C. Cofactors necessary for phase I and II metabolism were added as described in *Materials and Methods*. Protein was precipitated by the addition of one volume of methanol. Samples were analyzed on a Shimadzu LCMS-2010 instrument. The HPLC absorbance chromatogram is shown with the mass of

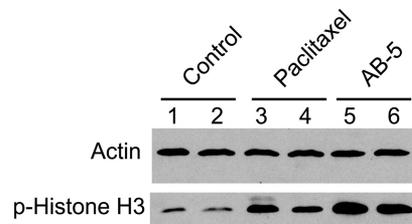


Fig. 3. Assessment of phospho-histone H3 in xenografted tumors. Nude mice were injected at an s.c. site with 10^7 PC3 cells in the left flank. Eight days later, the mice were randomized into three groups of two mice each and treated with vehicle control (lanes 1 and 2), 20 mg/kg paclitaxel (lanes 3 and 4), and 20 mg/kg AB-5 (lanes 5 and 6). The mice were killed 12 h later, and extracts from the tumors were immunoblotted with antiphosphorylated histone H3 and antiactin antibodies.

of AB-5 efficacy against s.c. xenografts. This dose and schedule were well tolerated for up to six doses, with no significant weight loss. Paclitaxel, another antimetabolic that is also administered by using cremaphor:ethanol as an excipient, was chosen as a positive control for most subsequent efficacy and toxicity experiments. In some experiments, vinblastine sulfate was also evaluated. Nude mice, injected s.c. with 7.5×10^6 to 10^7 tumor cells, were randomized into treatment groups when tumors reached 100–300 mm³ in size. Mice were given 20 mg/kg paclitaxel (21), 20 mg/kg AB-5, or 0.45–0.7 mg/kg vinblastine q2–3dX6, and tumor volume was monitored over time. Data from three tumors, HCT116 (colon), PC3 (prostate), and MDA-MB-435 (breast), are shown in [supporting information \(SI\) Table 1](#). In most experiments, significant regression in tumor volume was observed in all animals for paclitaxel and AB-5, with cures, defined as animals that were tumor-free >100 days after the final dose, noted in 40% of the animals dosed with AB-5 and 55% of animals dosed with paclitaxel on the q2–3dX6 schedule administered i.v. At doses of vinblastine administered, tumor regression was apparent but not as significant as that noted for either paclitaxel or AB-5.

Effects of AB-5 *in Vivo* on Histone H3 Phosphorylation. In the accompanying paper (17), Wang *et al.* show clearly that AB-5 acts *in vitro* as an antimetabolic, blocking cells in G₂/M. To confirm that the antitumor activity of AB-5 *in vivo* proceeds by a similar mechanism, PC3 tumors from mice treated for 12 h with vehicle, 20 mg/kg paclitaxel, or 20 mg/kg AB-5 were analyzed by Western blotting for the status of the mitotic marker phosphorylated histone H3 (Fig. 3). Not surprisingly, tumors treated with both paclitaxel and AB-5 showed significant induction in the level of histone H3 phosphorylation, suggesting that AB-5 acts *in vivo* as an antimetabolic, just as it does *in vitro*.

Analysis of AB-5 Toxicity. Although AB-5 functions as an antimetabolic, both *in vitro* and *in vivo*, Wang *et al.* (17) have shown that it does so through a unique mode of action involving a heretofore unrecognized role for OAT in cell division (17). Mice deficient in OAT

the indicated peak shown in *Inset*. (B) AB-5 incubated with murine S9 protein for 0 min elutes as a single peak with the indicated *M*, 695.5. (C) AB-5 incubated with murine S9 protein for 240 min yields a novel compound with *M*, 711.5 and a shorter retention time in addition to the original parental compound. (D) AB-5 incubated with human S9 protein for 240 min yields a similar more rapidly eluting compound also with *M*, 711.3. (E) The AB-5 metabolite was generated by incubation of AB-5 with human S9 protein for 4 h and then fractionated by HPLC on a C18 column. The amount of metabolite isolated was determined semiquantitatively by comparison with an AB-5 standard curve. The metabolite and AB-5 parental compound isolated in the same experiment were used in a 48-h cell viability experiment with PC3 prostate cells, as described in Fig. 1.

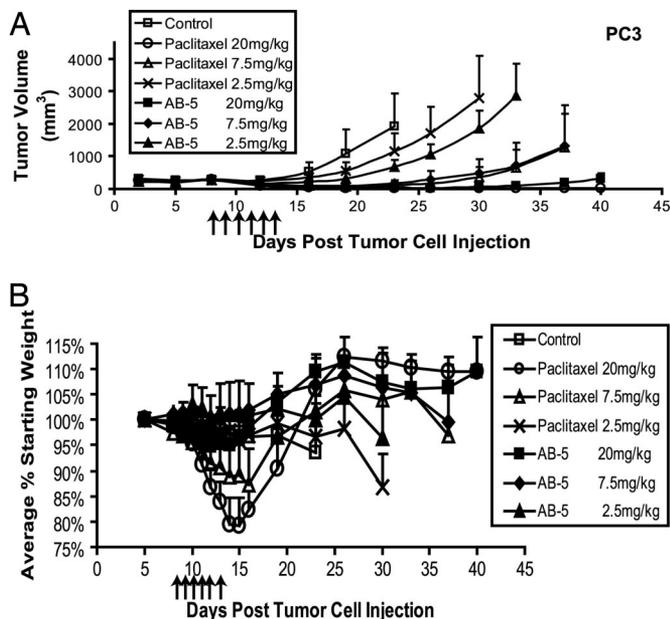


Fig. 4. Comparison of AB-5 and paclitaxel efficacy. (A) Nude mice were injected at an s.c. site with 10^7 PC3 cells in the left flank. When tumors reached ≈ 250 mm³ in size, the mice were randomized into treatment groups, and therapy was initiated at the indicated doses qd \times 6, as described in *Materials and Methods*. Arrows indicate the days on which compound was administered. Tumor volume was calculated according to the formula (length \times width²)/2. (B) Animals were weighed and observed daily.

are viable (19), suggesting that this molecule may be redundant for normal cell division, which in turn raises the possibility that AB-5 may have a safety profile distinct from that of tubulin-binding antimetabolites. Because both paclitaxel and AB-5 were well tolerated based on animal weights and showed similar efficacy when given at 20 mg/kg i.v. q2–3d \times 6, a daily dosing schedule was used to evaluate toxicities potentially induced by greater compound exposure over a shorter period. In addition, several doses of both compounds were evaluated to more thoroughly compare activity in relationship to toxicity. Groups of six to seven nude mice were given paclitaxel or AB-5 at 20, 7.5, and 2.5 mg/kg for 6 consecutive days. Efficacy was comparable at the lower doses (7.5 and 2.5 mg/kg) of both compounds (Fig. 4A). At 20 mg/kg, there were more complete responses with paclitaxel (2/5) than with AB-5 (1/6). However, significantly greater toxicity was noted. Two days after cessation of therapy, mice treated with 20 mg/kg paclitaxel had lost >20% of their starting body weight, and one mouse was killed because of significant morbidity (Fig. 4B). Even mice treated with 7.5 mg/kg of paclitaxel lost an average of 13% of starting body weight. Conversely, mice treated at all dose levels of AB-5 showed minimal fluctuation in weight ($\leq 5\%$).

To more fully evaluate the difference in toxicity between AB-5 and paclitaxel, in particular any effects on neutropenia, the most common clinical side effect of paclitaxel and related antimetabolites, groups of four to five mice were treated with 20 mg/kg AB-5, paclitaxel, or vehicle control at a daily dosing schedule for 6 days (qd \times 6). Animal weight was monitored daily, and by day eight, the paclitaxel mice had lost on average 20% of their body mass (Fig. 5A). In addition to weight loss, paclitaxel-treated mice showed signs of reduced activity, abnormal gait, and a dermatological condition characterized by severe flaking of their skin (SI Movie 1). AB-5-treated mice, by contrast, showed neither weight loss nor any changes in general behavior or appearance (Fig. 5A and SI Movie 1). All animals were killed on day eight for examination of blood and bone marrow cells. Paclitaxel-treated mice showed a significant decrease in both the percent-

age and absolute number of neutrophils in both peripheral blood and bone marrow (Fig. 5B and C). Strikingly, percentages and absolute numbers of neutrophils in AB-5-treated mice were indistinguishable from vehicle-treated control mice.

The experiment was repeated in animals xenografted with HCT116 colon tumor cells. An additional antimetabolite agent having demonstrated clinical success, vinblastine sulfate, was included in this second trial of both toxicity and efficacy. With regard to efficacy, the three compounds were indistinguishable in their ability to halt growth of the HCT116 tumor and induce its regression (Fig. 5D). Treatment of mice with both paclitaxel and vinblastine sulfate at this dose and schedule resulted in significant weight loss and neutropenia in both peripheral blood (data not shown) and bone marrow (Fig. 5E). By contrast, AB-5-treated mice lost no weight, showed no overt signs of toxicity, and gave no indication of neutropenia. Despite the apparent lack of toxicity of AB-5 against normal murine tissues in these experiments, *in vitro* data have shown that AB-5 is as active against murine tumors as paclitaxel and vinblastine (Fig. 1b and N.S.W., unpublished observations). These data provide confirmatory evidence that AB-5 is as efficacious as paclitaxel and vinblastine sulfate under conditions that avoid overt toxicity.

Conclusions

AB-5 is a small-molecule antimetabolite having therapeutic activity comparable to clinically used antimetabolites in the absence of overt toxicity. More rigorous studies in additional species will be required to fully investigate the other toxicities AB-5 might cause when administered at a therapeutically effective dose. However, the complete absence of weight loss and generally healthy appearance of treated mice may be taken to indicate that the compound is well tolerated by other organ systems. The favorable toxicity profile, relative stability, low clearance, similar metabolism in mice and humans, and strong efficacy both *in vitro* and *in vivo* against a broad panel of human tumors warrant further preclinical development of AB-5 as an anticancer agent.

Materials and Methods

Reagents. Cell lines used in this study were obtained from the Developmental Therapeutics Branch of the National Cancer Institute or from the American Type Culture Collection. Paclitaxel was purchased from Polymed Therapeutics (Houston, TX). Diazonamide A; its synthetic variant, AB-5; and the internal standard, AB-9, used for quantitation by liquid chromatography/MS were synthesized on site. Detailed spectroscopic data for AB-5 and AB-9 are provided in *SI Text*. Vinblastine sulfate, antiactin antibody (A2066), and all other chemicals were purchased from Sigma (St. Louis, MO). The antiphosphorylated histone H3 antibody (sc-12927R) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used for flow cytometry were purchased from BD Pharmingen (San Diego, CA).

In Vitro Cell Viability Assays. Cells were plated at optimally determined densities, and compounds were added in 10-fold dilutions the next day. Forty-eight hours later, cell viability was assessed by addition of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] (Sigma) or CellTiter-Glo Reagent (Promega, Madison, WI). GI50 values were calculated according to the formula $100 \times (T - T_0) / (C - T_0) = 50$, where T is optical density of test well after 48 h exposure to compound, T_0 is optical density at time 0, and C is control optical density.

Pharmacokinetic Analysis. NCR nu/nu mice, purchased from the National Cancer Institute (Frederick, MD), were injected with 20 mg/kg AB-5 i.v. as a bolus in 0.2 ml of 10% cremaphor/ethanol diluted with 5% dextrose. Animals were given an inhalation overdose of CO₂, and blood was harvested by cardiac puncture with a sodium citrate-coated needle and syringe containing 50 μ l

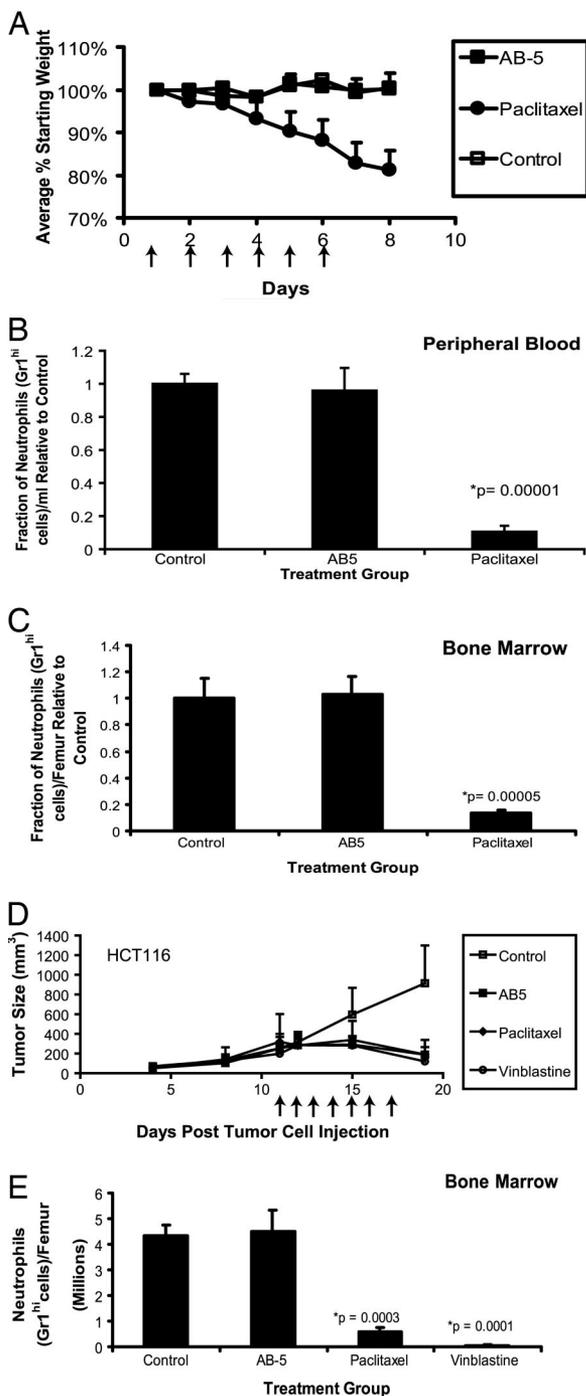


Fig. 5. Assessment of efficacy and neutropenia in mice treated with paclitaxel and vinblastine vs. AB-5. (A–C) Non-tumor-bearing mice were injected with either paclitaxel or AB-5 at 20 mg/kg i.v. qd \times 6, as described in *Materials and Methods*. Animals were observed and weighed daily. On day eight, the mice were anesthetized and bled by the tail vein into Li-heparin-coated Eppendorf tubes. Neutrophil counts were obtained as described in *Materials and Methods*. (A) Animal weights were obtained daily at approximately the same time each day. (B) The absolute neutrophil count in blood was obtained by multiplying the percentage of Gr1^{hi} cells \times number of white cells per milliliter of blood. (C) The absolute neutrophil count in bone marrow was obtained by multiplying the percentage of Gr1^{hi} cells \times number of white cells per femur. Each data point represents the average of five animals \pm SEM. (D and E) Nude mice were injected with 10^7 HCT116 colon tumor cells at an s.c. site. When tumors reached 200–300 mm³ in volume on day 12, therapy was initiated at the indicated doses qd \times 6. Mice were injected with either paclitaxel or AB-5 at 20 mg/kg i.v. qd \times 6 in 10–20% cremaphor/ethanol diluted with a solution of 5% dextrose, cremaphor/ethanol vehicle in 5%

of sodium citrate. Plasma was isolated by centrifugation of the blood for 10 min at 8,000 \times g and frozen at -80°C until analysis. One hundred microliters of plasma was spiked with 1 μg of an internal standard, AB-9. Proteins were precipitated by acetonitrile, and the supernatant was run over a Waters (Milford, MA) OASIS HLB solid-phase extraction column. Compound was eluted from the column with 2% acetic acid in methanol. Samples were run on a Shimadzu (Columbia, MD) LCMS-2010 instrument in negative single ion monitoring mode using a Capcell PAK C18 MG S3 column (Shiseido, Tokyo, Japan) for chromatography. The compound was eluted with a stepwise gradient from 40% to 100% methanol in water. Samples were quantitated by calculation of the area under the curve for the parent ion mass for AB-5 and AB-9, allowing internally controlled determination of the ratio of AB-5 to AB-9. A standard curve was prepared by using blank murine plasma (Biomed, Foster City, CA) spiked with various concentrations of AB-5 and 1 μg of AB-9. Pharmacokinetic parameters for plasma were determined by using a noncompartmental analysis tool on the WinNonlin software package (Pharsight, Mountain View, CA).

In Vitro Metabolism Studies. A 10 μM concentration of AB-5 was incubated in 1 ml of 0.1 M Tris/0.5% NaHCO₃/3 mm of MgCl₂ solution with 1 mg of mouse CD-1 S9 protein (In Vitro Technologies, Baltimore, MD) at 37°C. Cofactors necessary for phase I and II metabolism were added as recommended by In Vitro Technologies. Protein was precipitated by the addition of one volume of methanol. Samples were analyzed on a Shimadzu LCMS-2010 instrument, as described above, except runs were performed in scan mode.

Xenograft Studies. NCR nu/nu mice were injected s.c. with 7.5–10 \times 10⁶ tumor cells in the left flank. Five to 10 days later when tumors reached \approx 100–300 mm³ in size, mice were randomized into treatment groups of four to seven mice per group. AB-5 and paclitaxel were first diluted in a 1:1 mixture of cremaphor:ethanol and then added at final dilutions of 10% and 20%, respectively, to a 5% solution of dextrose. These compounds were administered i.v. or i.p., as indicated, in a total volume of 0.2 ml. Mice were weighed and tumors measured using vernier calipers two to three times per week. Tumor volume was calculated according to the formula (length \times width²)/2.

All animal work was approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center.

Assessment of Histone H3 Phosphorylation. Eight days after implantation of 10^7 PC3 cells s.c. in the left flank, nu/nu mice were injected i.v. with 20 mg/kg AB-5 or 20 mg/kg paclitaxel, as described above. Mice were killed 12 h later, and tumors were snap-frozen. Tumors were later minced and homogenized in RIPA buffer (50 mM Tris/300 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS) containing 1 mM NaVO₃, 10 mM β -glycerophosphate, 50 mM NaF, 0.1 mg/ml PMSF, and 1 miniprotease inhibitor tablet (Roche, Indianapolis, IN) per 10 ml. One hundred micrograms of protein was blotted with antiphosphorylated histone H3 and antiactin antibodies.

dextrose only (Control), or 2 mg/kg vinblastine sulfate in 5% dextrose. Animals were weighed and observed daily. On day 19, the mice were anesthetized and bled, and an analysis of neutropenia and efficacy was conducted as described in *Materials and Methods*. Each data point represents the average of four animals \pm SEM. The data were analyzed by a one-way ANOVA followed by Dunnett's test to evaluate differences between each treated group and the control group (STATISTICA). Significant *P* values are reported for each figure.

Assessment of Blood and Bone Marrow Neutrophil Numbers. NCR nu/nu mice were first injected with 10^7 HCT116 cells s.c. in the left flank 12 days before the onset of therapy or were treated without prior tumor cell inoculation. Animals were then injected i.v. with 20 mg/kg AB-5 or 20 mg/kg paclitaxel formulated as described above or 2 mg/kg vinblastine sulfate sodium salt in 5% dextrose qd \times 6. The mice were weighed and observed daily. On day eight after initiation of therapy, the mice were anesthetized and bled by the tail vein into Li-heparin-coated microcentrifuge tubes (Brinkman Instruments, Westbury, NY). After death, a single femur was removed from each animal, and bone marrow was isolated by crushing the bone with a mortar and pestle containing cold PBS/0.5% BSA/5 mM EDTA and filtering out residual bone spicules and debris through a 100- μ m filter. For the total leukocyte count in blood, an aliquot of whole blood was directly diluted with trypan blue and 4% acetic acid. Erythrocytes in bone marrow were first lysed by incubation in a Tris-ammonium chloride lysis solution before white cells were counted, as above. Before staining, whole blood was lysed with ammonium chloride. Both bone marrow and blood samples were then stained with an antibody to the myeloid differentiation antigen Gr1 (clone RB6-8C5; Pharmingen) followed by streptavidin APC (Pharmingen) and analyzed by flow cytometry using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) and

CellQuest software (Becton Dickinson). The absolute neutrophil count in blood was obtained by multiplying the percentage of Gr1^{hi} cells \times number of white cells per milliliter of blood. The Gr1 mAb stains two populations. Gr1^{hi} cells represent granulocytes (neutrophils and eosinophils), whereas Gr1^{lo} cells represent monocytes (22). Backgating of the Gr1^{hi} population onto the light-scatter profile indicated that this population falls within the granulocyte region of the light-scatter profile. The absolute neutrophil count in bone marrow was obtained by multiplying the percentage of Gr1^{hi} cells \times the number of white cells per femur.

Statistical Analysis. The data were analyzed by a one-way ANOVA followed by Dunnett's test to evaluate differences between each treated group and the control group (STATISTICA; StatSoft, Tulsa, OK). Significant *P* values are reported for each figure.

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