Anti-Myeloma Effects of the Novel Anthracycline Derivative INNO-206
Eric Sanchez, Mingjie Li, Cathy Wang, Cydney M. Nichols, Jennifer Li, Haiming Chen, and James R. Berenson

Abstract

Purpose: Doxorubicin has shown efficacy especially in combination treatment for the treatment of multiple myeloma; however, its side effects limit its use. INNO-206 is an albumin-binding prodrug of doxorubicin, which is released from albumin under acidic conditions. Because INNO-206 has not been previously evaluated in any hematologic malignancy, we determined its anti–multiple myeloma effects.

Experimental Design: The anti–multiple myeloma effect of INNO-206 at different pH levels on multiple myeloma cell proliferation using multiple myeloma cell lines with the MTS assay and antiangiogenic activity using the chorioallantoic membrane/feather bud assay were determined. The anti–multiple myeloma effects and toxicity of INNO-206 were also compared with conventional doxorubicin and PEGylated liposomal doxorubicin (PLD) alone, and in combination with bortezomib, using our multiple myeloma xenograft models.

Results: INNO-206 inhibited blood vessel formation and reduced multiple myeloma cell growth in a pH-dependent fashion. INNO-206 alone produced marked anti–multiple myeloma effects in vivo at doses that doxorubicin was toxic, and the combination of INNO-206 plus bortezomib produced increased anti–multiple myeloma effects compared with either agent alone. In contrast, all mice receiving bortezomib with doxorubicin or PLD died.

Conclusions: These findings show that INNO-206 produces anti–multiple myeloma effects in vitro and in vivo. It also enhances the antitumor effects of bortezomib. These results suggest that INNO-206 may provide patients with multiple myeloma with an anthracycline that may be administered safely at higher doses compared with free doxorubicin, resulting in superior efficacy compared with the currently available anthracyclines to treat this B-cell malignancy.

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Introduction

Multiple myeloma is a malignancy of bone marrow–based plasma cells that comprises 1% of all malignancies in the United States (1) and is the second most common hematologic cancer (2). Doxorubicin is an active antineoplastic drug (3–4) but its clinical application is limited by its side effects (5–7). To increase the therapeutic potential of this agent while reducing its side effects, several delivery systems have been created, including the development of its PEGylated liposomal formulation (PLD; refs. 8–10). PLD has shown improved efficacy when combined with bortezomib compared with bortezomib alone for previously treated patients with multiple myeloma (11). We have showed using our multiple myeloma xenograft models that more frequent dosing of PLD at lower doses is more effective and better tolerated than higher doses administered less often (13). We have used this approach for treating patients with multiple myeloma (14, 15) by combining PLD with bortezomib and i.v. dexamethasone using a longer cycle with high response rates with improved tolerability. Doxorubicin has also been combined with bortezomib and dexamethasone with high response rates but was poorly tolerated (16).

Hypoxia in tumors has been shown to promote a lethal cancer phenotype through, in part, the induction of hypoxia-inducible factor (HIF)-1, which controls the expression of angiogenesis-related genes that contribute to tumor progression (17). Antiangiogenic therapy represents a promising approach for cancer treatment (18). Anthracyclines, including doxorubicin, reduce HIF-1 levels within tumor cells and inhibit tumor blood vessel development as we and others have shown (19, 20).

INNO-206 (CytRx Corporation) is an albumin-binding prodrug of doxorubicin that binds rapidly and selectively to untreated patients (12). This led to a high response rate but was associated with significant toxicity. We have showed using our multiple myeloma xenograft models that more frequent dosing of PLD at lower doses is more effective and better tolerated than higher doses administered less often (13). We have used this approach for treating patients with multiple myeloma (14, 15) by combining PLD with bortezomib and i.v. dexamethasone using a longer cycle with high response rates with improved tolerability. Doxorubicin has also been combined with bortezomib and dexamethasone with high response rates but was poorly tolerated (16).

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Anti–Multiple Myeloma Effects of INNO-206

Translational Relevance
Side effects of doxorubicin limit its clinical application. A delivery system that exploits albumin as a drug carrier was created to increase the therapeutic potential of anthracyclines. INNO-206 is an albumin-binding prodrug of doxorubicin that is released from albumin under acidic conditions, which occurs in the extracellular tissue of tumors. In vitro, we show the acid-dependent anti–multiple myeloma activity of INNO-206 and its superior cytotoxic effects when compared with doxorubicin. Using clinically achievable doses, INNO-206 produced significant anti–multiple myeloma effects in vivo, whereas free doxorubicin at the same or lower doses was toxic. Similar anti–multiple myeloma effects were obtained in a different model and enhanced anti–multiple myeloma effects were observed when INNO-206 was combined with bortezomib compared with either agent alone. INNO-206 should provide multiple myeloma patients with a new anthracycline that may be able to be administered at higher doses compared with free doxorubicin, resulting in superior anti–multiple myeloma efficacy and improved tolerability.

Materials and Methods
Reagents
INNO-206 (CytRx Corporation, Los Angeles) stock solutions (4.5 mg/mL) were prepared using 50% ethanol and 50% water and further diluted in sterile water. Bortezomib (Millennium Pharmaceuticals) was obtained at 1 mg/mL stock solution and diluted using 0.9% sodium chloride. Doxorubicin (Sigma-Aldrich) stock solution (2 mg/mL) was dissolved and diluted in phosphate-buffered saline. PLD stock solution (2 mg/mL) was diluted in sterile water. Evans blue (Sigma-Aldrich) was dissolved in sterile water and injected as a 2% solution. All drugs were administered i.v. in a volume of 100 μL.

Cell lines
The human multiple myeloma cell lines RPMI8226 and U266 were obtained from the American Type Culture Collection (Rockville), and the MM1S multiple myeloma cell line was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL, USA). The cell lines were maintained in RPMI-1640 (Omega Scientific) supplemented with 1% FBS, 2 mmol/L-glutamine, 100 I/U per mL penicillin, 100 μg/mL streptomycin, and essential amino acids in an atmosphere of 5% CO₂ at 37°C.

Cell viability assay
Cells were seeded at 1 × 10⁵ cells/100 μL/well in 96-well plates in RPMI-1640 media with FBS for 24 hours before treatment. Cells were cultured in the presence of medium, INNO-206 or doxorubicin for 48 hours. Next, cell viability was quantified using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Each well was treated with MTS for 1 to 4 hours, after which absorbance at 490 nm was recorded using a 96-well plate reader. The quantity of formazan product as measured is directly proportional to the number of living cells. Data graphed are means ± SEM using 3 replicates per data point.

Preparation of the feather buds
The FBs were prepared as previously described (20). Briefly, fertilized chick eggs (Charles River) were incubated horizontally for 8 days. Stage 33 chick embryonic dorsal skin with FBs was collected, cut into 2 × 2 mm sections, and placed on culture inserts in 6-well plates. The FBs were cultured with or without drugs for 48 hours. Images were analyzed using dissection microscopy to determine size, area, shape factor, and orientation of FBs.

CAM/FB coculture
For the CAM/FB coculture, fertilized chick eggs were incubated and windowed by day 8 (20). The FBs were transferred onto the CAM of an 8-day-old chick embryo. The eggs were sealed and incubated for an additional 4 days.

the cysteine-34 position of serum albumin after i.v administration and is released under acidic conditions (21). The bone marrow of patients with multiple myeloma has a strong osteolytic component (22–24) and osteoclasts accumulate around bone that is adjacent to myeloma cells (25). Osteoclasts dissolve bone mineral through secretion of hydrochloric acid (26–28), and pH measurements at the active osteoclast's ruffled border have shown pH levels of 3 to 4 (29). Osteoclasts remove the acidified products and liberate them into the extracellular space (30, 31) adjacent to multiple myeloma cells. These studies suggest that the pH in multiple myeloma bone marrow is acidic. The extracellular component of tumor tissues is also acidic (32–34). Importantly, the majority of chemotherapeutic drugs being weak bases are protonated extracellularly by tumor tissues, which reduces their cytotoxicity (33, 35). Compared with doxorubicin, the acid-dependent release of INNO-206 offers the opportunity to achieve higher levels of active doxorubicin near and within tumor cells.

Evaluation of INNO-206 in solid tumor xenograft models and murine renal cell carcinoma and orthotopic pancreatic carcinoma models has showed superior efficacy compared with free doxorubicin (21, 36). In addition, it exhibits a substantial increase in the maximum tolerated dose (MTD) in animals when compared with conventional doxorubicin (37). Clinically, INNO-206 showed a good safety profile in a phase 1 clinical trial and induced regressions of solid tumors known to be anthracycline-sensitive (38). However, INNO-206 has not been previously evaluated in any hematologic malignancy. Thus, we conducted our study to ascertain its antiangiogenic and anti–multiple myeloma effects in vitro and determine the tolerability and anti–multiple myeloma activity of this novel anthracycline alone and in combination with bortezomib using our human multiple myeloma xenograft models.

Cell viability assay
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CAM/FB coculture
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After this, FB development was determined using microscopy and photographs were taken. Expression of the endothelial gene Flk-1 was determined in the FB by using reverse transcription-PCR (RT-PCR). At least 3 independent experiments were carried out and each showed similar results.

RT-PCR and densitometry

Total RNA was isolated from each FB. RNA was resuspended in 0.1% diethyl pyrocarbonate-treated water, digested with DNase I (Sigma-Aldrich) to remove contaminating DNA and extracted with phenol/chloroform followed by ethanol precipitation. Total RNA (1 μg) was reverse-transcribed to cDNA and amplified using the ThermoScript System and a GeneAmp PCR System 9700 (Applied Biosystems) for 1 cycle at 94°C for 1 minute, and 1 cycle at 72°C for 1 minute. Primers: Flk-1 forward (5'-GATCATGCGTCAGACACC-3') and reverse primers (5'-GTCTTGACGCATGCTGCACC-3') under the same conditions as a loading control. Density histograms were created from JPEG images using the software ImageJ (version 1.38; http://rsb.info.nih.gov/ij/), an image analysis program created by the National Institutes of Health (NIH).

Xenograft models

Six- to 8-week-old male CB17 severe combined immunodeficient (SCID) mice were obtained from the Charles River Laboratories and maintained in a pathogen-free animal resources facility under sterile conditions. Animal studies were conducted according to protocols approved by the Institutional Animal Care and Use Committee. To establish the LAGx-1A tumor, a bone marrow biopsy was obtained from a female patient with multiple myeloma who had progressed on lenalidomide and bortezomib after this biopsy was obtained. The biopsy was implanted into the hind limb of a SCID mouse and passaged through succeeding generations (39). As assessed using flow cytometric analysis, this tumor showed marked expression of CD138. The LAGx-2 tumor was established from a bone marrow biopsy obtained from a male patient with multiple myeloma who secreted IgGκ. The patient had progressed on lenalidomide and melphalan and bortezomib after this biopsy was obtained. The biopsy was implanted into the hind limb of a SCID mouse and passaged through succeeding generations (39). As assessed using flow cytometric analysis, this tumor showed marked expression of CD138. The multiple myeloma tumors were excised, sectioned into 20 to 40 mm³ pieces, and implanted into the left superficial gluteal muscle. Seven days after tumor implantation, mice were randomized into treatment groups. Animals were euthanized when tumors reached 2.5 cm in diameter.

Treatment groups

For the LAGx-1A experiment, INNO-206 was administered to SCID mice at 10.8 mg/kg (doxorubicin equivalent dose of 8.0 mg/kg) once weekly. Mice were treated with conventional doxorubicin at 4.0 and 8.0 mg/kg once weekly. For the LAGx-2 experiment, INNO-206 was administered once weekly (W) at doses of 2.7 and 5.4 mg/kg, or on 3 consecutive days (W-F) weekly at doses of 0.9 and 1.8 mg/kg. Bortezomib was administered twice weekly (W, F) at a dose of 0.5 mg/kg. Doxorubicin was administered to SCID mice at 2, 4, and 8 mg/kg, and PLD was administered to SCID mice at 2 mg/kg once weekly. Each drug was administered i.v. in a volume of 100 μL.

Statistical analysis

Tumors were measured weekly using standard calipers and the formula for an ellipsoid volume was applied \[ \frac{4}{3} \pi \times (\text{width}/2)^2 \times (\text{length}/2) \]. Tumor growth and IgG curves were analyzed in terms of treatment group means and standard error (n = 10 mice/group). Statistical significance of differences observed in drug-treated mice versus control mice was determined using a Student t test. The minimal level of significance was \( P < 0.05 \). After injection of the Evans blue dye, density histograms were created from JPEG images using the software ImageJ (version 1.38; http://rsb.info.nih.gov/ij/), an image analysis program with many functions and was created by the NIH. Quantitative measurements of Evans blue staining were used to create density histograms, which measured the color blue in tumors after injection of the dye at 4 hours postdose and then subtracting endogenous blue from tumors injected at 0 hour.

Human IgG (hlgG) ELISA

The levels of hlgG secreted by LAGx-1A tumors (LAGx-2 tumors do not secrete paraprotein) were determined using an ELISA. Mice bearing multiple myeloma tumors were bled weekly via retro-orbital bleeding. Samples were spun at 10,000 rpm for 5 minutes and serum was collected. The hlgG ELISA kit (Bethyl Laboratories) was used according to the manufacturer’s specifications. Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a μQuant microplate spectrophotometer with KC junior software (Bio-Tek Instruments).

Figure 1. pH- and concentration-dependent inhibition of the viability of multiple myeloma cell lines after exposure to INNO-206 and doxorubicin. A, there is a pH- and concentration-dependent decrease in RPMI8226 cell viability after incubation with INNO-206 (black bars) or doxorubicin (white bars), but cells remained viable at all concentrations at pH 7. At pH 5, RPMI8226 cells showed reduced viability and were not viable at INNO-206 concentrations ≥0.54 μM/L. B, cell viability of MM1S cells after exposure to INNO-207 (black bars) and doxorubicin (white bars) at pH 7. In an acidic environment (pH 5), cell viability of MM1S cells was reduced after exposure to INNO-207 (black bars) compared with doxorubicin (white bars). C, U266 viability of INNO-206 and doxorubicin at pH 7 is shown. At pH 5, inhibition of U266 viability is decreased after exposure to INNO-206. D, with the addition of acid (pH 5), cell viability of MM1S cells was only slightly reduced compared with control. Data graphed are the mean ± SEM using 3 replicates.
Results

INNO-206 inhibits multiple myeloma cell line growth in vitro

Because INNO-206 shows that the highest level of releasing doxorubicin is at pH 5, we assessed the cytotoxicity of INNO-206 or doxorubicin in a concentration- and pH-dependent fashion in the 3 multiple myeloma cell lines RPMI8226, U266, and MM1S. First, drugs were prepared in pH 5 or 7 for 45 minutes before their addition to the cell culture. To compare equivalent concentrations of doxorubicin-bound INNO-206 to free doxorubicin, the INNO-206 concentrations were divided by 1.346 as this gives the amount of free doxorubicin contained within the INNO-206 compound. Cells were then exposed to increasing concentrations of INNO-206 from 0.27 to 2.16 μmol/L (free doxorubicin equivalent doses of 0.2–1.6 μmol/L) or doxorubicin (0.2–1.6 μmol/L) for 48 hours, and cell viability was determined with the MTS assay. A concentration- and pH-dependent decrease in viable RPMI8226 cells was observed after exposure to INNO-206 or doxorubicin (Fig. 1A). At pH 5, viable cells were essentially eliminated in cells cultured with INNO-206 at concentrations ≥0.54 μmol/L and doxorubicin was also effective but less so than INNO-206 (Fig. 1A). A similar concentration and pH-dependent inhibition of cell growth, as those observed earlier, was observed in the MM1S cell line after exposure to INNO-206 or doxorubicin (Fig. 1B). As the concentration was increased and pH was decreased, from pH 7 to 5, the percentage of viable MM1S cells within the INNO-206 group dramatically decreased, in contrast to what occurred with doxorubicin. In fact, the anti–multiple myeloma effects of doxorubicin at 0.4 and 0.8 μmol/L were less at pH 5 than 7. The diminishing anti–multiple myeloma effects of doxorubicin in an acidic environment were also observed in the U266 cell line (Fig. 1C), in contrast to INNO-206 where increased anti–multiple myeloma effects were observed at the lower pH. Because the data above was generated from drugs incubated at physiologic pH and at pH 5, the effect of an acidic pH alone on multiple myeloma cell lines was also tested. Exposure of multiple myeloma cells to pH 5 only resulted in a minimal reduction in viable cells compared to those cultured at pH 7. A representative example from all 3 cell lines tested is shown in Fig. 1D.

Antiangiogenic effect of INNO-206

We assessed the antangiogenic effects of INNO-206 at decreasing pH levels using our CAM/FB model (20). The FB was exposed to INNO-206 or doxorubicin for 2 days followed by attachment to the CAM for an additional 4 days. As controls, FBs that were not previously exposed to INNO-206 were attached to the CAM. Placode and dermal condensation of the FB occurred and the weight of the FB increased and feathers formed. In the presence of INNO-206, inhibition of FB development occurred in a concentration- and pH-dependent manner after 4 days of culture (Fig. 2A). Similar to our previous published results (20), doxorubicin inhibited feather development (Fig. 2B).

Next, we assessed endothelial gene expression in the FB tissue after attachment to the CAM. Specifically, Flk-1 transcript levels were assessed using RT-PCR, and RNA levels were significantly reduced in a concentration and pH-dependent fashion after incubation of the FB with INNO-206–containing medium compared with the FB without drug exposure after attachment to the CAM (Fig. 2C). Density histograms created from these RT-PCR images showed reduced Flk-1 levels after incubation of the FB with INNO-206 (Fig. 2D).

The in vivo anti–multiple myeloma effects and toxicity of INNO-206 versus conventional doxorubicin

Mice bearing the LAGk-1A tumor receiving INNO-206 once weekly via i.v. injection at 10.8 mg/kg (equivalent to 8.0 mg/kg of doxorubicin) showed significantly smaller tumor volumes and IgG levels on days 28 (tumor volumes: P = 0.0152; hIgG: P = 0.0019), 35 (tumor volumes: P = 0.0051; hIgG: P = 0.0006) and 42 (tumor volumes: P = 0.0036; hIgG: P = 0.0113) compared with vehicle–treated mice (Fig. 3A and B). This INNO-206 treatment regimen was well tolerated with 90% of mice surviving until the termination of the study (day 42). In contrast, doxorubicin administered once weekly at both 4.0 and 8.0 mg/kg via i.v. injection resulted in marked toxicity after the first treatment (day 7) and deaths began to occur by day 14 so that it was not possible to evaluate the effect of this drug’s effect on the animals’ tumor growth. By day 42, no mice were alive among mice receiving it at 4.0 or 8.0 mg/kg. These results show that more doxorubicin can be administered safely in its albumin-bound form (INNO-206) than free doxorubicin; and INNO-206 produces marked anti–multiple myeloma activity.

INNO-206 administered 3 times weekly at 1.8 mg/kg versus once weekly at 5.4 mg/kg

The anti–multiple myeloma effects of INNO-206 were also evaluated using another multiple myeloma xenograft (LAGk-2) model. In addition, different doses and schedules of INNO-206 were tested. LAGk-2–bearing mice treated with INNO-206 i.v. 3 times weekly at 1.8 mg/kg significantly reduced tumor volume compared with vehicle–treated mice on days 28, 35, 42, 49, and 56 (P = 0.0036, P = 0.0002, P = 0.0001, P = 0.0013, and P = 0.0013, respectively; Fig. 4). Mice receiving the once weekly INNO-206 injection at 5.4 mg/kg also showed significantly smaller tumor volumes than mice receiving vehicle on days 28, 35, 42, 49, and 56 (P = 0.0068, P = 0.0008, P = 0.0004, P = 0.023, and P = 0.0014, respectively; Fig. 4). There was a trend toward a greater reduction in tumor volume with weekly versus 3 times weekly dosing of the drug, but the difference between these 2 schedules was not significant.

INNO-206 administered 3 times weekly at 0.9 mg/kg versus once weekly at 2.7 mg/kg with or without bortezomib

On the basis of our single-agent experiments, we evaluated INNO-206 at 2.7 mg/kg (equivalent to 2 mg/kg of...
doxorubicin) once weekly and 0.9 mg/kg 3 times weekly alone and in combination with bortezomib. LAGk-2–bearing mice treated with INNO-206 i.v. 3 times weekly at 0.9 mg/kg showed significantly less tumor compared with vehicle-treated mice on days 28, 35, 42, 49, and 56 (\(P = 0.0122, P = 0.0023, P = 0.0008, P = 0.0320,\) and \(P = 0.0076,\) respectively; Fig. 5A). Mice receiving the once weekly INNO-206 injection at 2.7 mg/kg also showed significantly smaller tumor volumes than vehicle on days 28, 35, 42, 49, and 56 (\(P = 0.0337, P = 0.0091, P = 0.0253, P = 0.0526,\) and \(P = 0.0058,\) respectively; Fig. 5A).

Compared with vehicle alone, mice that received bortezomib at 0.5 mg/kg twice weekly showed a reduction in tumor volume on days 28, 35, 42, 49, and 56 (\(P = 0.0033, P = 0.0002, P < 0.0001, P = 0.0004,\) and \(P = 0.0003,\) respectively; Fig. 5B). Mice receiving the combination of INNO-206 (3 times weekly i.v. at 0.9 mg/kg) and bortezomib had smaller tumor volumes than mice in the vehicle control group on days 28, 35, 42, 49, and 56 (\(P = 0.0032,
Tumor volumes (mm³) vs. Days post tumor implantation

- Control (10/10 mice alive at day 42)
- INNO-206 10.8 mg/kg (9/10 mice alive at day 42)
- Doxorubicin 4 mg/kg (9/10 mice alive at day 42)
- Doxorubicin 8 mg/kg (9/10 mice alive at day 42)

**Figure 3.** Anti–multiple myeloma effects of INNO-206 in vivo using the LAGx-1A xenograft model. A, tumor volume is significantly less compared with control (vehicle-treated) mice, after treatment with INNO-206 at 10.8 mg/kg (at a doxorubicin equivalent dose of 8.0 mg/kg) once weekly via i.v. injection. Doxorubicin at 4.0 and 8.0 mg/kg, also administered once weekly via i.v. injection, resulted in significant toxicity and deaths. B, serum IgG levels were markedly reduced after INNO-206 treatment when compared with control. Data are presented as means ± SEM.

P = 0.0002, P = 0.0002, P = 0.0011, and P = 0.0008, respectively; Fig. 5B). The combination showed enhanced anti–multiple myeloma effects compared with the INNO-206 alone-treated mice (on days 35, 42, 49, and 56: P = 0.0053, P = 0.0009, P = 0.0133, and P = 0.0047, respectively) but it did not result in more antitumor effects than bortezomib alone. Overall, 60% (6/10) of mice survived this combination treatment without significant body weight loss (Fig. 5C).

Mice treated with once weekly INNO-206 at 2.7 mg/kg with bortezomib showed a more pronounced reduction in tumor volume on days 28, 35, 42, and 56 (P = 0.0018, P < 0.0001, P < 0.0001, and P = 0.0002, respectively; Fig. 5B) when compared with vehicle–treated mice. In fact, the tumor was not palpable in any mouse on day 49. Tumors in mice receiving this combination were consistently smaller throughout the initial treatment period (days 7–63) when compared with the single-agent groups, although this did not reach statistical significance given the small size of the tumors in the single agent groups. However, significant differences were observed on days 70, 77, and 84 among mice receiving once weekly INNO-206 (2.7 mg/kg) plus bortezomib, as these animals continued to show absence of tumor, whereas groups treated with bortezomib or INNO-206 alone showed regrowth of their tumors at these latter time points (compared with bortezomib alone on days 70, 77, and 84, P = 0.046, P = 0.0338, and P = 0.0372, respectively; compared with INNO-206 alone on days 70, 77, and 84, P = 0.0018, P = 0.0009, and P = 0.0009; Fig. 5B). No significant body weight loss was observed during the treatment in any group (Fig. 5C). Overall, 70% (7/10) and 90% (9/10) of mice survived the in the combination treatment and vehicle groups, respectively.

The anti–multiple myeloma effects of doxorubicin or PLD in combination with bortezomib were also evaluated at the same equivalent doxorubicin doses as those used in the INNO-206 plus bortezomib experiments. Mice receiving once weekly administration of 2 mg/kg of doxorubicin or PLD (doxorubicin equivalent dose of 2.7 mg/kg INNO-206) in combination with bortezomib (twice weekly at 0.5 mg/kg) did not die soon after treatment initiation (Fig. 5D). Specifically, 2 of 4 mice in each of the groups receiving doxorubicin alone, doxorubicin or PLD in combination with bortezomib died by day 21 post tumor implantation. By day 28, the remaining 2 mice from each of these 3 groups...
died (Fig. 5D). Furthermore, by day 35, all mice receiving PLD died and all mice receiving vehicle control and single-agent bortezomib were alive (data not shown).

Visualization of Evans blue dye–albumin complexes within 3 different multiple myeloma xenografts

To show albumin uptake within the multiple myeloma xenograft tumors, and because INNO-206 is doxorubicin bound to albumin, the Evans blue dye, a compound which irreversibly and rapidly binds to plasma albumin, was injected i.v. into tumor-bearing mice. Four hours postinjection, mice were sacrificed and tumors extracted. The exterior surfaces of the tumors were blue and cross-sections confirmed that the interior of the tumors were also blue; and, thus, these tissues contained Evans blue dye-albumin complexes (Fig. 6A: top row, Evans blue stained tumors; bottom row, tumors not stained with Evans blue). Using density histograms, quantitative measurements showing the uptake of albumin in these tumors were created by measuring the intensity of the color blue after Evans blue injection at 0 and 4 hours postdose (Fig. 6B). Cross-sections of tumors were also blue from mice bearing a different xenograft tumor (LAGκ-1A) sacrificed 4 hours postinjection, whereas tumors from mice sacrificed at time point zero post injection did not stain blue (data not shown).

Discussion

This report is the first to evaluate the antitumor effects of the novel albumin-binding doxorubicin prodrug INNO-206 in any hematologic malignancy. Our in vitro results, in 3 multiple myeloma cell lines using the same equivalent concentrations of doxorubicin present in INNO-206 and doxorubicin, showed that the anti–multiple myeloma effects of INNO-206 were more pronounced in an acidic environment whereas those of doxorubicin were diminished. These results are in contrast to those obtained in solid tumors, which showed that doxorubicin was approximately 10-fold more active than INNO-206 at the equivalent doxorubicin concentration.
The antiangiogenic effects we observed in vitro and in vivo support the acid-sensitive nature of the effects of INNO-206. It reduced angiogenesis as doxorubicin (pH 5) and was well tolerated, whereas doxorubicin was extremely toxic at both a similar (8.0 mg/kg) and even half (4.0 mg/kg) the equivalent of the doxorubicin dose administered with INNO-206 (10.8 mg/kg which is 8.0 mg/kg of free doxorubicin). Mice receiving INNO-206 at this dose showed a marked inhibition in tumor growth and IgG levels, and 9 of 10 mice were alive at day 42 posttumor implantation. In contrast, doxorubicin administered at 4.0 and 8.0 mg/kg, using the same schedule and route of injection as INNO-206, resulted in deaths in all animals by day 42. Other laboratories have shown that INNO-206 is superior to doxorubicin with respect to reducing cardiotoxicity and mitochondrial damage at equimolar as well as equitoxic doses in a rat model (42), and significantly lower levels of INNO-206 were observed in the heart, liver, and kidneys of mice when compared with similar studies assessing doxorubicin (21). The lack of deaths observed in our in vitro study with INNO-206 is consistent with a favorable shift in the lethal dose, which is 2- to 5-fold higher for INNO-206 compared with free doxorubicin (37, 43). INNO-206 was administered at twice the dose (INNO-206 at 10.8 mg/kg = doxorubicin equivalent dose of 8.0 mg/kg) as the low doxorubicin dose group (4.0 mg/kg), and it was well tolerated and resulted in significant anti–multiple myeloma activity whereas even the low dose of doxorubicin was toxic. These results are consistent with findings from a phase 1 trial of patients with advanced solid tumors showing an approximately 3-fold increase in the MTD of INNO-206 compared with the highest dose of doxorubicin that can be safely administered (38). Results from our LAGx-1A xenograft study provide further evidence that INNO-206 is able to be given safely at much higher doses than conventional doxorubicin. Preclinical xenograft studies conducted in other laboratories in solid cancers have similarly shown INNO-206 to be more efficacious than free doxorubicin and that more of this novel anthracycline can be administered than doxorubicin (36).

We and others have shown that the anti–multiple myeloma activity of doxorubicin is enhanced in the presence of bortezomib in vitro and in vivo (44). Clinically, one of the first effective combination chemotherapy treatments for relapsed/refractory multiple myeloma included doxorubicin with vincristine and oral dexamethasone, which rapidly gained widespread use in the frontline setting (3). Doxorubicin has also been combined with bortezomib and dexamethasone in the first line setting in patients with multiple myeloma with high response rates (16). Its PEGylated liposomal form, PLD, has showed improved pharmacokinetic properties and reduced toxicity compared with several recent reports showing the inhibition of blood vessel formation with doxorubicin and other anthracyclines (19, 20).

To establish whether our in vitro observations would translate into anti–multiple myeloma activity in vivo, we evaluated the anti–multiple myeloma effects of INNO-206 and compared it to equivalent and lower doses of free doxorubicin. Our multiple myeloma xenograft model LAGx-1A was sensitive to the effects of INNO-206 and it was well tolerated, whereas doxorubicin was extremely toxic at both a similar (8.0 mg/kg) and even half (4.0 mg/kg) the equivalent of the doxorubicin dose administered with INNO-206 (10.8 mg/kg which is 8.0 mg/kg of free doxorubicin). Mice receiving INNO-206 at this dose showed a marked inhibition in tumor growth and IgG levels, and 9 of 10 mice were alive at day 42 posttumor implantation. In contrast, doxorubicin administered at 4.0 and 8.0 mg/kg, using the same schedule and route of injection as INNO-206, resulted in deaths in all animals by day 42. Other laboratories have shown that INNO-206 is superior to doxorubicin with respect to reducing cardiotoxicity and mitochondrial damage at equimolar as well as equitoxic doses in a rat model (42), and significantly lower levels of INNO-206 were observed in the heart, liver, and kidneys of mice when compared with similar studies assessing doxorubicin (21). The lack of deaths observed in our in vitro study with INNO-206 is consistent with a favorable shift in the lethal dose, which is 2- to 5-fold higher for INNO-206 compared with free doxorubicin (37, 43). INNO-206 was administered at twice the dose (INNO-206 at 10.8 mg/kg = doxorubicin equivalent dose of 8.0 mg/kg) as the low doxorubicin dose group (4.0 mg/kg), and it was well tolerated and resulted in significant anti–multiple myeloma activity whereas even the low dose of doxorubicin was toxic. These results are consistent with findings from a phase 1 trial of patients with advanced solid tumors showing an approximately 3-fold increase in the MTD of INNO-206 compared with the highest dose of doxorubicin that can be safely administered (38). Results from our LAGx-1A xenograft study provide further evidence that INNO-206 is able to be given safely at much higher doses than conventional doxorubicin. Preclinical xenograft studies conducted in other laboratories in solid cancers have similarly shown INNO-206 to be more efficacious than free doxorubicin and that more of this novel anthracycline can be administered than doxorubicin (36).
doxorubicin (8) but it cannot be administered at higher doses than doxorubicin. Like doxorubicin, PLD has also been combined with bortezomib for previously treated patients with multiple myeloma (11) and both drugs with dexamethasone for previously untreated and relapsed or refractory patients with multiple myeloma (12, 14, 15). However, patients continue to show resistant disease and significant side effects with PLD-based therapies. Thus, we examined the effect of the combination of INNO-206 and bortezomib in another human multiple myeloma xenograft model. Although initially single agent and combination therapies showed similar anti–multiple myeloma effects, the combination of both agents proved superior with longer follow up. The toxicity profile, as judged by body weight loss and mortality, was similar between mice treated with single-agent and combination treatment. In addition, once weekly injection at a higher dose was superior in its anti–multiple myeloma effects compared with 3 times weekly (on consecutive days) at lower doses. In contrast, the toxicity of single-agent doxorubicin at 2 mg/kg, when administered once weekly, resulted in the deaths of all mice after only 3 injections, which is consistent with prior studies (45, 46). As expected, the addition of bortezomib to doxorubicin or PLD at the MTD dose (2 mg/kg), which is the equivalent of the doxorubicin dose used in the INNO-206 (2.7 mg/kg) plus bortezomib studies, was also not tolerated in these mice and all animals were dead by day 28.

Optimal scheduling of INNO-206 was also evaluated. Our previous results evaluating PLD showed more frequent daily administration at lower doses was more effective than higher doses given on a weekly schedule (13). Our clinical results with more frequent administration of lower doses of PLD when used in combination with bortezomib and dexamethasone have shown high response rates with reduced toxicity (14, 15). In contrast, weekly dosing of INNO-206 seems to be more effective than more frequent dosing of this anthracycline from our current study. It remains to be determined, whether based on these preclinical results that this drug will be more effective clinically when given at higher doses less frequently.

Notably, the doses of INNO-206 used in our xenograft studies (0.9–10.8 mg/kg/wk) are clinically achievable (2.7–32.4 mg/m²) and far below the well-tolerated dose of 200 mg/m² of INNO-206 (doxorubicin equivalent dose of 148.6 mg/m²) that was administered in the phase 1 clinical trial (38). This doxorubicin equivalent dose of INNO-206 is 2 to 2.5 times higher than the standard doxorubicin dose of 60 to 75 mg/m², which often results in many side effects in clinical practice.

Although this newer anthracycline INNO-206 binds to circulating albumin and patients with myeloma often have hypoalbuminemia (47), the exclusion criterion for the INNO-206 phase 1 trial (38) was defined as albumin concentrations <2 g/dL. The serum albumin levels in patients with multiple myeloma are rarely below this level; and, thus, very few patients would be excluded from receiving this compound based on a low serum albumin level. Furthermore, albumin is known to accumulate in solid tumors because of the EPR-effect (40, 41), resulting in higher concentrations of the albumin-bound-form of INNO-206 within the tumor tissues. However, the accumulation of albumin in multiple myeloma because of the EPR-effect has not been documented. Proof of concept of albumin accumulation in multiple myeloma was obtained in our preclinical models by injecting the Evans blue dye which binds rapidly and tightly to circulating albumin (48). Subcutaneously growing multiple myeloma tumors turned blue within a few hours postinjection, showing rapid tumor uptake of albumin. Furthermore, studies suggest that the interaction between multiple myeloma tumor cells and the high bone-resorptive activity of osteoclasts results in a localized acidic microenvironment in the bone marrow of patients with multiple myeloma (26–31). These factors are of clinical relevance because this should likely result in the release of even more doxorubicin from albumin-bound INNO-206 in the multiple myeloma bone marrow environment and/or intracellularly after cellular uptake because of the EPR-effect. The increasing role of albumin as a drug carrier in the clinical setting has been highlighted in a recent review, which outlines different drugs using this delivery system (48). Two such compounds have recently been approved for the treatment of metastatic breast cancer and diabetes mellitus, an albumin-bound paclitaxel nanoparticle (ABI-007) and a myristic acid derivative of insulin that binds to the fatty acid binding sites of albumin (NN304), respectively (48). In addition, a recent study showed enhanced anti–tumor activity in an ectopic multiple myeloma xenograft model after treatment with nanoparticle albumin-bound-tapamycin alone (ABI-009) and in combination with perifosine (NSC639966; ref. 49). The increased efficacy showed with these other types of albumin–bound pro-drugs combined with the promising preclinical in vitro and in vivo results from this study provide further support for initiating clinical trials with this novel anthracycline derivative INNO-206 for the treatment of multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Sanchez, M. Li, C.M. Nichols, J.R. Berenson

Writing, review, and/or revision of the manuscript: E. Sanchez, C.M. Nichols, H. Chen, J.R. Berenson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Li, C. Wang, C.M. Nichols, J. Li, H. Chen

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