Anti-Myeloma Effects of the Novel Anthracycline Derivative INNO-206

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Abstract

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

Experimental Design: The anti-MM effect of INNO-206 at different pH levels on MM cell proliferation using MM cell lines with the MTS assay and anti-angiogenic activity using the chorioallantoic membrane/feather bud assay were determined. The anti-MM effects and toxicity of INNO-206 were also compared to conventional doxorubicin and pegylated liposomal doxorubicin (PLD) alone, and in combination with bortezomib, using our MM xenograft models.
**Results:** INNO-206 inhibited blood vessel formation and reduced MM cell growth in a pH-dependent fashion. INNO-206 alone produced marked anti-MM effects *in vivo* at doses that doxorubicin was toxic, and the combination of INNO-206 plus bortezomib produced increased anti-MM effects compared to either agent alone. In contrast, all mice receiving bortezomib with doxorubicin or PLD died.

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

Side effects of doxorubicin limit its clinical application. A delivery system which 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 demonstrate the acid-dependent anti-MM activity of INNO-206 and its superior cytotoxic effects when compared to doxorubicin. Using clinically achievable doses, INNO-206 produced significant anti-MM effects in vivo whereas free doxorubicin at the same or lower doses was toxic. Similar anti-MM effects were obtained in a different model and enhanced anti-MM effects were observed when INNO-206 was combined with bortezomib compared to either agent alone. INNO-206 should provide MM patients with a new anthracycline that may be able to be administered at higher doses compared to free doxorubicin, resulting in superior anti-MM efficacy and improved tolerability.
Introduction

Multiple myeloma (MM) is a malignancy of bone marrow (BM)-based plasma cells that comprises 1% of all malignancies in the United States (1) and is the second most common hematological cancer (2). Doxorubicin is an active anti-neoplastic drug (3-4) but its clinical application is limited by its side effects (5-7). In order 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) (8-10). PLD has shown improved efficacy when combined with bortezomib compared to bortezomib alone for previously treated MM patients (11). This combination with oral dexamethasone has also been evaluated in untreated patients (12). This led to a high response rate but was associated with significant toxicity. We have demonstrated using our MM 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 MM patients (14, 15) by combining PLD with bortezomib and intravenous (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). Anti-angiogenic 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, Los Angeles, CA) is an albumin-binding prodrug of doxorubicin that binds rapidly and selectively to the cysteine-34 position of serum albumin after i.v administration and is released under acidic conditions (21). The BM of MM patients 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 MM cells. These studies suggest that the pH in MM BM 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 to 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 demonstrated superior efficacy compared to free doxorubicin (21, 36). Additionally, it exhibits a substantial increase in the maximum tolerated dose (MTD) in animals when compared to 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 hematological malignancy. Thus, we conducted our study to ascertain its anti-angiogenic and anti-MM effects in vitro and determine the tolerability and anti-MM
activity of this novel anthracycline alone and in combination with bortezomib using our human MM xenograft models.

**Materials and Methods**

**Reagents**

INNO-206 (CytRx Corporation, Los Angeles, CA, USA) stock solutions (5.4 mg/ml) were prepared using 50% ethanol and 50% water and further diluted in sterile water. Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) was obtained at 1 mg/ml stock solution and diluted using 0.9% sodium chloride. Doxorubicin (Sigma-Aldrich, St. Louis, MO, USA) 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, St. Louis, MO, USA) 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 MM cell lines RPMI8226 and U266 were obtained from the American Type Culture Collection (Rockville, MD, USA), and the MM1S MM cell line was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL, USA). The cell lines were maintained in RPMI 1640 (Omega Scientific, Tarzana, CA, USA) supplemented with 10% fetal bovine serum, 2 mmol/l 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 $1 \times 10^5$ cells/100 μL/well in 96-well plates in RPMI1640 media with FBS for 24 hours prior to 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, Madison, WI, USA). Each well was treated with MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) for 1-4 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, Wilmington, MA, USA) were incubated horizontally for 8 days. Stage 33 chick embryonic dorsal skin with FBs was collected, cut into 2x2 mm sections, and placed on culture inserts in 6-well culture dishes (Falcon). The FBs were cultured with or without drugs for 48 h. Images were analyzed using dissection microscopy to determine size, area, shape factor, and orientation of FBs.

CAM/FB co-culture

For the CAM/FB co-culture, 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. Following this, FB development was determined using microscopy and photographs were taken. Expression of the endothelial gene Flk-1 was determined in the FB using RT-PCR. At least three independent experiments were carried out and each showed similar results.

**RT-PCR and densitometry**

Total RNA was isolated from each FB. RNA was re-suspended in 0.1% diethyl pyrocarbonate-treated water, digested with DNase I (Sigma-Aldrich, St. Louis, MO, USA) to remove contaminating DNA and extracted with phenol/chloroform followed by ethanol precipitation. Total RNA (1 µg) was reversed-transcribed to cDNA and amplified using the ThermoScript System (Invitrogen, Carlsbad, CA, USA). PCR was performed using the ThermoScript System and a GeneAmp PCR System 9700 (Applied Biosystems. Foster City, CA, USA) for one cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and one cycle at 72°C for 5 min. Primers: Flk-1 (L) caaccagacggacagtggta, (R) acagactccctgcttttgct. GAPDH was amplified using forward (5′-AGCCACATCGCTCAGACACC-3′) and reverse primers (5′-GTACTCAGCGGCCAGCATCG-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/](http://rsb.info.nih.gov/ij/)), an image analysis program created by the National Institutes of Health (NIH).

**Xenograft models**
Six to eight-week old male CB17 severe combined immunodeficient (SCID) mice were obtained from the Charles River Laboratories (Wilmington, MA) 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 LAGκ-1A tumor, a BM biopsy was obtained from a female MM patient who had progressed on lenalidomide but responded to 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 LAGκ-2 tumor was established from a BM biopsy obtained from a male MM patient who secreted IgGκ. The patient had progressed on lenalidomide and methylprednisolone. Using immunofluorescence, this tumor also showed marked CD38 and CD138 expression. The MM tumors were excised, sectioned into 20 to 40 mm³ pieces, and implanted into the left superficial gluteal muscle. Seven days following tumor implantation, mice were randomized into treatment groups. Animals were euthanized when tumors reached 2.5 cm in diameter.

**Treatment groups**

For the LAGκ-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 LAGκ-2 experiment, INNO-206 was administered once weekly (W) at doses of 2.7 and 5.4 mg/kg, or on three 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 100µl.

**Statistical analysis**

Tumors were measured weekly using standard calipers and the formula for an ellipsoid volume was applied \((4/3 \pi \times [\text{width}/2]^2 \times [\text{length}/2])\). Tumor growth and IgG curves were analyzed in terms of treatment group means and standard error \((n = 10 \text{ mice/group})\). Statistical significance of differences observed in drug-treated mice versus control mice was determined using a Student’s t test. The minimal level of significance was \(P < 0.05\). Following 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/](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 following injection of the dye at 4 hrs post-dose and then subtracting endogenous blue from tumors injected at 0 hrs.

**Human IgG (hIgG) ELISA**

The levels of human immunoglobulin G (IgG) secreted by LAGκ-1A tumors (LAGκ-2 tumors do not secrete paraprotein) were determined using an enzyme-linked immunosorbent assay (ELISA). Mice bearing MM tumors were bled weekly via retro-orbital bleeding. Samples were spun at 10,000 rpm for 5 minutes and serum was collected. The human IgG ELISA kit (Bethyl Laboratories, Montgomery, TX) 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, Winooski, VT).

Results

INNO-206 inhibits MM cell line growth in vitro

Since INNO-206 shows 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 three MM cell lines RPMI8226, U266 and MM1S. First, drugs were prepared in pH 5 or 7 for 45 minutes prior to their addition to the cell culture. In order 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 µM (free doxorubicin equivalent doses of 0.2 to 1.6 µM) or doxorubicin (0.2 to 1.6 µM) for 48 hrs, and cell viability was determined with the MTS assay. A concentration- and pH-dependent decrease in viable RPMI8226 cells was observed following exposure to INNO-206 or doxorubicin (Figure 1A). At pH 5, viable cells were essentially eliminated in cells cultured with INNO-206 at concentrations ≥ 0.54 µM and doxorubicin was also effective but less so than INNO-206 (Figure 1A). A similar concentration and pH-dependent inhibition of cell growth, as those observed above, was observed in the MM1S cell line following exposure to INNO-206 or doxorubicin (Figure 1B). As the
concentration was increased and pH was decreased, from pH 7 to pH 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-MM effects of doxorubicin at 0.4 µM and 0.8 µM were less at pH 5 than pH 7. The diminishing anti-MM effects of doxorubicin in an acidic environment were also observed in the U266 cell line (Figure 1C), in contrast to INNO-206 where increased anti-MM 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 MM cell lines was also tested. Exposure of MM 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 three cell lines tested is shown in Figure 1D.

**Anti-angiogenic effect of INNO-206**

We assessed the anti-angiogenic 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 (Figure 2A). Similar to our previous published results (20), doxorubicin inhibited feather development (Figure 2B). Next, we assessed endothelial gene expression in the FB tissue following 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 following incubation of the FB with INNO-206-containing medium compared to the FB without drug exposure following attachment to the CAM (Figure 2C). Density histograms created from these RT-PCR images demonstrated reduced Flk-1 levels following incubation of the FB with INNO-206 (Figure 2D).

The *in vivo* anti-MM effects and toxicity of INNO-206 versus conventional doxorubicin

Mice bearing the LAGκ-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 to vehicle-treated mice (Figures 3A & 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 following 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 demonstrate that more doxorubicin can be administered safely in its albumin-bound form (INNO-206) than free doxorubicin; and INNO-206 produces marked anti-myeloma activity.
INNO-206 administered thrice weekly at 1.8 mg/kg versus once weekly at 5.4 mg/kg

The anti-MM effects of INNO-206 were also evaluated using another MM xenograft (LAGκ-2) model. Additionally, different doses and schedules of INNO-206 were tested. LAGκ-2-bearing mice treated with INNO-206 i.v. thrice weekly at 1.8 mg/kg significantly reduced tumor volume compared to 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; Figure 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; Figure 4). There was a trend toward a greater reduction in tumor volume with weekly versus thrice weekly dosing of the drug, but the difference between these two schedules was not significant.

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

Based on 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 thrice weekly alone and in combination with bortezomib. LAGκ-2-bearing mice treated with INNO-206 i.v. thrice weekly at 0.9 mg/kg showed significantly less tumor compared to vehicle-treated mice on days 28, 35, 42, 49, and 56 ($P = 0.0122$, $P = 0.0023$, $P = 0.0008$, $P = 0.023$, and $P = 0.0076$, respectively; Figure 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; Figure 5A).

Compared to vehicle alone, mice that received bortezomib at 0.5 mg/kg twice weekly demonstrated 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; Figure 5B). Mice receiving the combination of INNO-206 (thrice 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 ($P = 0.0032$, $P = 0.0002$, $P = 0.0002$, $P = 0.0011$, and $P = 0.0008$, respectively; Figure 5B). The combination showed enhanced anti-MM effects compared to 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 anti-tumor effects than bortezomib alone. Overall, 60% (6/10) of mice survived this combination treatment without significant body weight loss (Figure 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; Figure 5B) when compared to 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 to 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 to bortezomib alone on days 70, 77 and 84, \( P = 0.046 \), \( P = 0.0338 \), and \( P = 0.0372 \), respectively; compared to INNO-206 alone on days 70, 77 and 84, \( P = 0.0018 \), \( P = 0.0009 \), and \( P = 0.0009 \); Figure 5B). No significant body weight loss was observed during the treatment in any group (Figure 5C). Overall, 70\% (7/10) and 90\% (9/10) of mice survived the in the combination treatment and vehicle groups, respectively.

The anti-MM 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) died soon after treatment initiation (Figure 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 two mice from each of these three groups died (Figure 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 three different MM xenografts**

To demonstrate albumin uptake within the MM 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 post injection, 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 (Figure 6A: top row, Evans blue stained tumors: bottom row, tumors not
stained with Evans blue). Using density histograms, quantitative measurements
demonstrating the uptake of albumin in these tumors were created by measuring the
intensity of the color blue following Evans blue injection at 0 and 4 hrs post-dose (Figure
6B). Cross sections of tumors were also blue from mice bearing a different xenograft
tumor (LAGκ-1A) sacrificed 4 hours post injection, whereas tumors from mice sacrificed
at time point zero post injection did not stain blue (data not shown).

Discussion

The current report is the first to evaluate the anti-tumor effects of the novel
albumin-binding doxorubicin prodrug INNO-206 in any hematological malignancy. Our
in vitro results, in three MM cell lines using the same equivalent concentrations of
doxorubicin present in INNO-206 and doxorubicin, showed that the anti-MM 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 (21, 36). Enhanced uptake of
albumin in solid tumors is well documented and is termed the enhanced permeability and
retention of macromolecules (EPR) effect (41, 42). Interestingly, the cell lines used in
those experiments were derived from solid tumors (21, 36); and, thus, one would expect
that INNO-206 would be more active than doxorubicin in solid cancer cell lines due to
the uptake of albumin by those tumors. In contrast, our in vitro results in MM cell lines showed that INNO-206 was actually more active than free doxorubicin in an acidic environment (pH 5).

Our CAM/FB model (20) was used to evaluate the anti-angiogenic effects of INNO-206. It reduced angiogenesis as determined by Flk-1 gene expression and feather formation more so at pH 5 compared to pH 7. These studies further support the acid-sensitive nature of the effects of INNO-206, which is in agreement with the results observed from our in vitro pH-dependent studies and those of another laboratory (21). The anti-angiogenic effects that we observed with this novel anthracycline derivative are consistent with several recent reports demonstrating the inhibition of blood vessel formation with doxorubicin and other anthracyclines (19, 20).

To establish whether our in vitro observations would translate into anti-MM activity in vivo, we evaluated the anti-MM effects of INNO-206 and compared it to equivalent and lower doses of free doxorubicin. Our MM xenograft model LAGκ-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 post-tumor 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 (43), and significantly lower levels of INNO-206 were observed in the heart, liver and kidneys of mice when compared to similar studies assessing doxorubicin (21). The lack of deaths observed in our in vivo 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 to free doxorubicin (37, 44). 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-MM 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 demonstrating an approximately 3-fold increase in the MTD of INNO-206 compared to the highest dose of doxorubicin that can be safely administered (38). Results from our LAGκ-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 performed 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-MM activity of doxorubicin is enhanced in the presence of bortezomib in vitro and in vivo (45). Clinically, one of the first effective combination chemotherapy treatments for relapsed/refractory MM 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 MM patients with high response rates (16). Its pegylated liposomal form, PLD, has demonstrated improved pharmacokinetic properties
and reduced toxicity compared to doxorubicin (8) but it cannot be administered at higher
doses than doxorubicin. Like doxorubicin, PLD has also been combined with bortezomib
for previously treated MM patients (11) and both drugs with dexamethasone for
previously untreated and relapsed or refractory MM patients (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 MM xenograft model. Although initially single agent and combination
therapies showed similar anti-MM 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.
Additionally, once weekly injection at a higher dose was superior in its anti-MM effects
compared to thrice 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 (46, 47).
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 appears 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 mg/m²-32.4 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 myeloma patients often have hypoalbuminemia (48), the exclusion criterion for the INNO-206 phase 1 trial (38) was defined as albumin concentrations < 2 g/dL. The serum albumin levels in MM patients 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 due to the EPR-effect (41, 42), resulting in higher concentrations of the albumin-bound-form of INNO-206 within the tumor tissues. However, the accumulation of albumin in MM due to the EPR-effect has not been documented. Proof of concept of albumin accumulation in MM was obtained in our preclinical models by injecting the Evans blue dye which binds rapidly and tightly to circulating albumin (49). Subcutaneously growing MM tumors turned blue within a few hours post-injection, demonstrating rapid tumor uptake of albumin. Furthermore, studies suggest that the interaction between MM tumor cells and
the high bone-resorptive activity of osteoclasts results in a localized acidic microenvironment in the BM of MM patients (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 MM BM environment and/or intracellularly after cellular uptake due to 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 (49). 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 (49). Additionally, a recent study showed enhanced anti-tumor activity in an ectopic MM xenograft model following treatment with nanoparticle albumin-bound-rapamycin alone (ABI-009) and in combination with perifosine (NSC 639966) (50). The increased efficacy demonstrated 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 MM.

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References


FIGURE LEGENDS

Figure 1. pH- and concentration-dependent inhibition of the viability of MM cell lines following exposure to INNO-206 and doxorubicin. A, there is a pH and concentration-dependent decrease in RPMI 8226 cell viability following incubation with INNO-206 (black bars) or doxorubicin (white bars) but cells remained viable at all concentrations at pH 7. At pH 5, RPMI 8226 cells showed reduced viability and were not viable at INNO-206 concentrations ≥ 0.54 μM. B, cell viability of MM1S cells following 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 following exposure to INNO-207 (black bars) compared to 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 following exposure to INNO-206. D, with the addition of acid (pH 5), cell viability of MM1S cells was only slightly reduced compared to control. Data graphed are the mean ± SEM using 3 replicates.

Figure 2. Concentration and pH-dependent inhibition of FB development following treatment with INNO-206. Four groups of E8 embryonic skin tissue explants (FB) were cultured in dishes and exposed to increasing concentrations of INNO-206 for 48 hours at different pH 5 and 7. Figures are representative of samples run in triplicates. A, control FBs that were not exposed to INNO-206 formed feathers. In contrast, a reduction in feather development occurred in the presence of INNO-206 at pH 7. At pH 5, INNO-206 inhibited FB development much more markedly than at pH 7. B, doxorubicin also inhibited feather development compared to FB not exposed to drug. C, gene expression of the endothelial cell marker Flk-1 as determined using RT-PCR on FB was significantly reduced in a concentration and pH-dependent fashion following exposure of the FB to INNO-206 compared to the FB without drug exposure. D, density histograms demonstrate significantly reduced Flk-1 levels in a concentration- and pH-dependent fashion following exposure of the FB to INNO-206 compared to the FB without drug exposure.

Figure 3. Anti-MM effects of INNO-206 in vivo using the LAGκ-1A xenograft model. A, tumor volume is significantly less compared to control (vehicle-treated) mice, following 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 hIgG levels were markedly reduced following INNO-206 treatment when compared to control. Data are presented as means ± standard error of the mean.

Figure 4. Anti-MM effects of INNO-206 at 1.8 mg/kg thrice weekly or 5.4 mg/kg once weekly, using the LAGκ-2 xenograft model. Significant inhibition of tumor growth was observed in mice bearing human MM xenografts receiving these doses of INNO-206 once or thrice weekly when compared to controls.
Figure 5. Anti-MM effects of INNO-206 alone at 0.9 mg/kg thrice weekly or 2.7 mg/kg once weekly, bortezomib (Bort) alone or the drugs together in vivo using the LAGκ-2 xenograft model. A, a significant reduction in tumor growth was observed in mice bearing human LAGκ-2 tumors receiving INNO-206 (i.v.) at 2.7 mg/kg once weekly or thrice weekly at 0.9 mg/kg when compared to vehicle control. B, INNO-206 dosed once weekly at 2.7 mg/kg plus bortezomib combination therapy significantly inhibited tumor growth more than either agent alone. C, body weight was used to assess toxicity of treatment and minimal loss was observed in mice dosed with single agents or combination therapy (P = prior to initiation of treatment, E = end of study weight). D, mice did not tolerate doxorubicin or PLD (once weekly at 2 mg/kg, i.v. injection) in combination with bortezomib (twice weekly at 0.5 mg/kg via i.v. injection) and died due to toxicity. Data are presented as means ± standard error of the mean.

Figure 6. Albumin uptake in MM xenografts four hours following injection of a 2% Evans blue dye solution. A, intact (left) and cross section (right) of a LAGκ-2 MM tumor removed 4 hours (top row) and immediately (bottom row) following i.v. injection of Evans blue dye. B, density histograms of tumors obtained from these mice. Data is representative of three independent experiments.
Figure 2A

Dose Dependent Anti-Angiogenesis Effect of INNO-206 in the CAM/FB Model

pH 7

pH 5

Control  0.67 μM  2 μM  6.7 μM
Figure 2B

Anti-Angiogenesis Effect of Doxorubicin in the CAM/FB Model

Control

Doxorubicin 5 µM
Figure 2C

Endothelial Marker Flk-1 Gene Expression

<table>
<thead>
<tr>
<th>pH</th>
<th>Flk-1</th>
<th>GAPDH</th>
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<tbody>
<tr>
<td>pH 7</td>
<td></td>
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<tr>
<td>pH 6</td>
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<tr>
<td>pH 5</td>
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INNO-206 (μM) | 0.67 | 2 | 6.7 | - |
Doxorubicin (μM) | - | - | - | 5 |
Figure 3B

LAG3-1A

- 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 (0/10 mice alive at day 42)
- doxorubicin 8 mg/kg (0/10 mice alive at day 42)
Figure 4

LAGK-2

- Control
- INNO-206 1.8 mg/kg (3x/week)
- INNO-206 5.4 mg/kg (1x/week)

Tumor Volumes (mm³)

Days Post Tumor Implantation

Start Tx

0 10 20 30 40 50 60 70 80 90

0 500 1000 1500 2000 2500 3000
Figure 5C

Body Weights

- INNO-206 0.9 mg/kg (3x/week)
- INNO-206 2.7 mg/kg (1x/week)
- Bort 0.5 mg/kg (2x/week)
- INNO-206 0.9 mg/kg + Bort
- INNO-206 2.7 mg/kg + Bort