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Short Communication

An *in Vivo* Model of Human Multidrug-Resistant Multiple Myeloma in SCID Mice

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We have established a reproducible* in vivo *model of human multiple myeloma in the severe combined immunodeficient (SCID) mouse using both the RPMI 8226 human myeloma cell line and the P-glycoprotein-expressing multidrug-resistant 8226/C1N subline. SCID mice 5 to 8 weeks of age were injected intraperitoneally with either 8226 drug-sensitive or P-glycoprotein-expressing multidrug-resistant myeloma cells (8226/C1N). Tumors were detected within 5 days after injection by the presence of human lambda light chain excretion in the mouse urine. Growth of the tumor was observed primarily in the abdominal cavity with spread to the abdominal organs. The anti-neoplastic agent doxorubicin was effective in treating the drug-sensitive 8226 human-SCID xenografts but had no effect on the multidrug-resistant 8226/C1N human-SCID xenografts. In the 8226-sensitive xenografts, treatment with doxorubicin resulted in a sharp decline in the concentration of human lambda light chain being excreted in the mouse urine. This correlated with an increased survival of the drug-treated animals. This mouse model offers an* in vivo *means of evaluating efficacy and toxicity of new therapeutic approaches, including development of chemosensitizers directed against P-glycoprotein in multidrug-resistant myelomas. (Am J Pathol 1993, 142:691–698)

Multiple myeloma is a plasma cell malignancy that is generally incurable in spite of a high initial response to chemotherapy.[1] Relapsing disease commonly heralds an increase in the incidence of drug resistance. Prior studies indicate that terminal, drug-resistant myeloma commonly expresses P-glycoprotein (PGP).[2] This protein is a cellular efflux pump encoded by the *MDR*-1 gene and is frequently responsible for the multiple-drug-resistant (MDR) phenotype.[3] Chemosensitizers, such as verapamil and cyclosporin A, can reverse PGP-mediated drug resistance in myeloma cells displaying the MDR phenotype *in vitro*[4] and in clinical trials.[2,5,6] Chemosensitizers, such as verapamil, have proven too toxic to administer at the concentrations required to be fully effective.[2,7] Thus, new chemosensitizers are sought that will be more potent and less toxic than the initial compounds. Development and testing of candidate compounds has been hampered by the lack of a suitable *in vivo* model.

This report describes an *in vivo* model that will be useful in the evaluation of more effective agents to combat drug resistance. We developed an MDR myeloma model in SCID mice, which are known to be permissive of B-cell human tumor xenografts. Our recent experience with long term engraftment of fresh human myeloma cells in the SCID mouse suggested this possibility.[8]

Whereas murine models of myeloma are known, i.e., pristane-induced, Balb/c peritoneal plasmacytomas[9,10] and spontaneous C57BL marrow myelomas,[11,12] these systems do not specifically model human myeloma associated with drug resistance. Mickisch et al[13] have developed a transgenic mouse model in which expression of the human *MDR*-1 gene in the bone marrow confers protection to nor-

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mal peripheral leukocytes from the myelosuppressive effects of chemotherapeutic agents. This model has been used to evaluate chemosensitizers indirectly by demonstrating increased myelosuppression when the chemosensitizer is combined with anti-neoplastic therapy.

The recent development of human xenografts in nude and SCID mice suggested a new experimental model.[14] Millar et al,[15] using the human myeloma cell line RPMI 8226, observed a low xenograft take rate in athymic nude mice. In contrast, recent studies by ourselves and others have found a high xenograft take rate in SCID mice with myeloma cell lines and fresh myeloma tissue.[8,16] Mice homozygous for the SCID mutation on chromosome 16 have a severe combined immune deficiency caused by the inability to rearrange their immunoglobulin and T-cell receptor genes correctly.[17–19] Because they lack both functional B and T cells, they are more severely immunocompromised than the nude mouse and, consequently, have a greater propensity for transplantation. The SCID model has been used to study the tumorigenicity of B-cell neoplasias such as Burkitt's lymphoma,[16] acute lymphocytic leukemia,[20] large cell lymphoma,[21] and myeloma.[8] In particular, the intraperitoneal model of human-SCID xenografts allows assessment of tumor dissemination in the context of a high xenograft rate.[8,16,21]

The purpose of our study was to determine the feasibility of using the RPMI 8226 human myeloma cell line and its MDR variants as a basis for a model of drug-resistant human multiple myeloma in the SCID mouse.

Materials and Methods

Animals

BALBc/C.17 mice homozygous for the SCID defect (scid/scid) were bred and maintained in a dedicated facility at the Arizona Health Sciences Center. The animals were housed in microisolator cages under specific pathogen-free conditions and were handled in a laminar flow hood. They were fed LM45 5%fat, autoclavable pellets (Tekland Premier, Madison, WI) and given autoclaved water supplemented with antibiotics. Mice were screened at regular intervals for the presence of bacteria, Sendai virus, mouse hepatitis virus, and Mycoplasma. All mice were evaluated for the presence of mouse IgG by ELISA assay, and only those animals with ≤1 mg/L of mouse Ig were used. Five- to eight-week-old male and female animals were used for all studies.

Cell Lines

The RPMI 8226 human multiple myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD). This cell line was originally derived from the peripheral blood of a 61-year-old male with multiple myeloma.[22] An MDR variant of this cell line, 8226/DOX40, was selected for resistance to doxorubicin.[23] A subcloned derivative of this cell line, 8226/C1N, was selected for lack of expression of the cellular adhesion molecule NCAM and was utilized in these studies (Hanneman et al, manuscript submitted). The 8226/C1N subline expresses PGP and displays the MDR phenotype just as the parent 8226/DOX40 line. The cells were grown in suspension culture in RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (100 units/ml), 1% (v/v) streptomycin (100 μg/ml), and 1% (v/v) L-glutamine (GIBCO Grand Island, NY; Bethesda Research Laboratories, Bethesda, MD). Cells were maintained at 37 C in a humidified 5% CO_2-95% ambient air atmosphere.

Generation of Human Multiple Myeloma in SCID Mice

Each animal received a total of 10 to 50 × 10^6 human 8226 cells or 10 × 10^6 8226/C1N cells injected intraperitoneally. Before injection, the cells were washed twice and resuspended in sterile phosphate-buffered saline, pH 7.4. In all instances, low passage number cells in logarithmic growth were used. The 8226 cell line produces and secretes λ light chain, and the development of human tumors in the recipient mice was monitored at 4- to 5-day intervals using a radial immunodiffusion assay (The Binding Site LTD, Birmingham, England) to measure the presence of human λ light chain in the urine. The sensitivity of this assay is ≤0.7 mg/L λ light chain. Up to 10 microliters of mouse urine was applied to the wells, and the assay plates were then incubated for 96 hours at room temperature. The diameter of the resulting precipitin ring was measured to the nearest 0.1 mm using a calibrating viewer (Kallestad Labs, Inc., Chaska, MN). Diameters of the sample wells were then compared to a linear calibration curve constructed using samples of known λ light chain concentration. Urinary creatinine was measured using an automated chemical analyzer (Paramax 720 ZX, Baxter Instruments, Los Angeles, CA). Autopsies were performed on the mice approximately thirty to forty days after cell inoculation to obtain tissues for histological examination and immunophenotyping.

Histological Evaluation

Mice were sacrificed by cervical dislocation, and complete "en block" autopsies were performed with gross and histological examination of all tissues. Some tissues were placed in 10% buffered formalin, paraffin-embedded, cut, and stained with hematoxylin and eosin; whereas additional tissue was snap frozen for phenotyping. Multiple sections of each organ were evaluated for the presence of human tumor.

Immunophenotyping

Tissue section phenotyping was performed on formalin-fixed or snap-frozen tissues using a standard three-stage immunohistochemistry method.[24] Snap-frozen tissues were permeabilized in 4 C acetone and assayed using relevant primary monoclonal antibodies (anti-λ, anti-κ, mouse anti-human; Becton-Dickinson, Mountain View, CA), followed by incubation with secondary biotin-avidin conjugates and horseradish peroxidase, labeled with diaminobenzidine tetrahydrochloride as the detection agent. PGP was detected using monoclonal antibodies JSB-1 (Applied Chemicals, Westbury, NY) and C-494 (Centocor, Malvern, PA) and using a biotin-avidin-detection method previously described.[24] An irrelevant isotype-matched primary antibody was substituted as a negative control.

Tumor Treatment with Doxorubicin

Recent evidence suggests that the SCID mouse may be unable to repair DNA double strand breaks and may therefore be more susceptible to the toxic effects of a drug such as doxorubicin.[25] To address this question, studies were performed to ascertain the maximal tolerated dose of doxorubicin in SCID mice. Mixed sex SCID mice weighing approximately 14 to 25 g were injected intraperitoneally with doxorubicin (Sigma Chemical Co., St. Louis, MO). The animals were injected with doses of doxorubicin ranging from 2.5 mg/kg to 75 mg/kg for single bolus administration and 0.1 mg/kg to 7 mg/kg in multiple dosing studies. Following administration of doxorubicin, the animals were monitored for weight loss and toxic deaths for at least two weeks. The maximal tolerated dose was defined as that dose of drug that did not result in the deaths of any animals within a group, whereas a higher dose resulted in the chemotherapy-related death of at least one animal. Once the maximal tolerated dose had been established, the efficacy of doxorubicin in SCID mice bearing the drug-sensitive 8226 parent cell line or the MDR 8226/C1N cell line was determined using a concentration below this value, to minimize drug-induced toxicities.

Results

In multiple experiments involving more than 100 animals, we determined the cell dosage necessary to establish a 100% incidence of tumor involvement. The drug-sensitive 8226 parent cell line required a dosage of 50×10^6 cells compared to a lesser dose of 10×10^6 cells for the 8226/C1N cell line to achieve a 100% xenograft take rate, as evidenced by overt plasmacytomas on gross and histological examination. Inoculums of 10 to 25×10^6 sensitive cells revealed no overt tumors at autopsy at 2 to 4 weeks; however, human plasma cell engraftment was evidenced by increasing λ light chain expression. Experiments testing the route of inoculation found the highest xenograft take rate with the intraperitoneal route (100% xenografts). In contrast, there were rare takes with the subcutaneous route (2.5%, 1 in 40 animals) and no intravenous takes (0%, 0 in 10 animals).

Inoculation with either cell line resulted in death in all animals when left untreated. The mean survival time for those animals injected with 10×10^6 8226/C1N was 42.3 ± 2.2 days ($n = 7$), 45.9 ± 11.2 days for animals receiving 25×10^6 8226 cells ($n = 15$), and 45.4 ± 7.3 days for those receiving 50×10^6 8226 cells ($n = 14$). Clinically, the animals showed weight loss and gradual fur loss.

The human-SCID myeloma xenografts of both drug-sensitive and drug-resistant 8226 cell lines appeared histologically as plasmacytomas (Figure 1, A and B). They were present predominantly on the peritoneum and in the perinephric fat, were invasive into the psoas muscle and diaphragm, and were metastatic to the liver, kidneys, pancreas, prostate, and testicles. Immunophenotyping revealed that all tumors were human λ light chain–positive and human κ light chain–negative (Figure 1c). The renal tubules in human-SCID 8226 myeloma xenografts showed striking human λ light chain in apical vesicles of the proximal renal tubules (Figure 1d). The human-SCID 8226/C1N xenografts showed strong surface PGP-expression as evidenced by reactivity with both JSB-1 and C-494, whereas the human-SCID 8226/S were negative (data not shown). The human-SCID xenografts were established rapidly and reproducibly under the conditions of intraperitoneal growth, with only rare involvement of the bone marrow (1 in 40 animals). Osteolytic lesions were not observed.

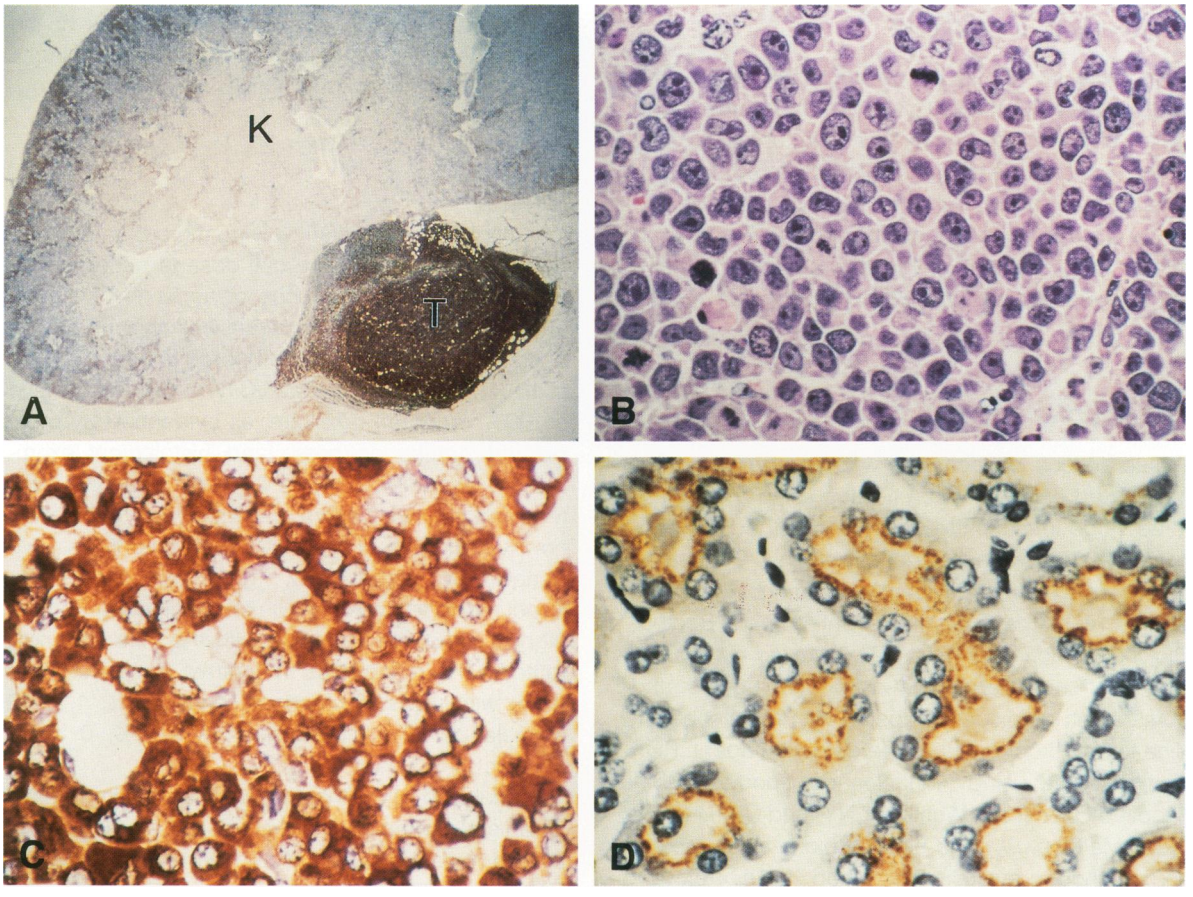

Figure 1. *Human λ Ig-bearing myeloma tumor:* **(A)** *microanatomic localization in perinephric fat (×10); K = kidney, T= tumor;* **(B)** *histological appearance of plasmacytoma (×250);* **(C)** *strong cytoplasmic λ Ig expression within the human-SCID myeloma xenograft (×400);* **(D)** *human λ light chain in SCID mouse renal tubules (×400).* **(A), (C),** *and* **(D)** *show Ig detection utilizing anti-λ immunoperoxidase method with methylene blue counterstain.* **(B)** *stained with hematoxylin and eosin.*

Measurement of urinary monoclonal human λ light chain excretion served as a sensitive measure of tumor xenograft take and tumor burden in these animals. As shown in Figure 2, excretion of human λ light chain was initially detected at day 5 and was observed to increase linearly over a period of at least 30 days in those mice receiving myeloma cells. Control animals injected with saline failed to demonstrate human light chain in the urine. λ light chain was detected in the urine although there were no palpable tumor masses in the animals. This profile of λ excretion has been reproduced in each subsequent experiment, now representing over 100 animals. The reproducibility of this data is observed in Figure 3A, which represents an experiment in which the λ light chain values from untreated SCID-human xenografts are nearly identical at equivalent points in time as those presented in the experiment shown in Figure 2.

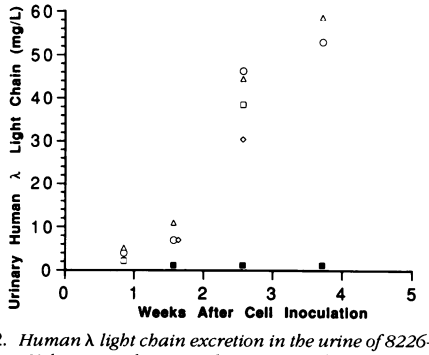

Figure 2. *Human λ light chain excretion in the urine of 8226-bearing SCID mice. Values were determined using an radial immunodiffusion assay. Open symbols represent individual animals injected with 8226/S cell line; closed symbol represents control animal not receiving tumor cells (n = 5).*

Control SCID mice receiving no tumor cells, displayed a mean urinary creatinine value of 55.8 ± 8.6 mg/dl (range: 40 to 65 mg/dl; $n = 6$) 30 days following a bolus saline injection. Animals injected with either 8226 myeloma cells or the drug-resistant 8226/C1N did not have a significant increase in urinary creatinine values compared to the control mice over a thirty-day period. In addition, treatment of SCID-human xenografts with doxorubicin had no effect on the urinary creatinine values.

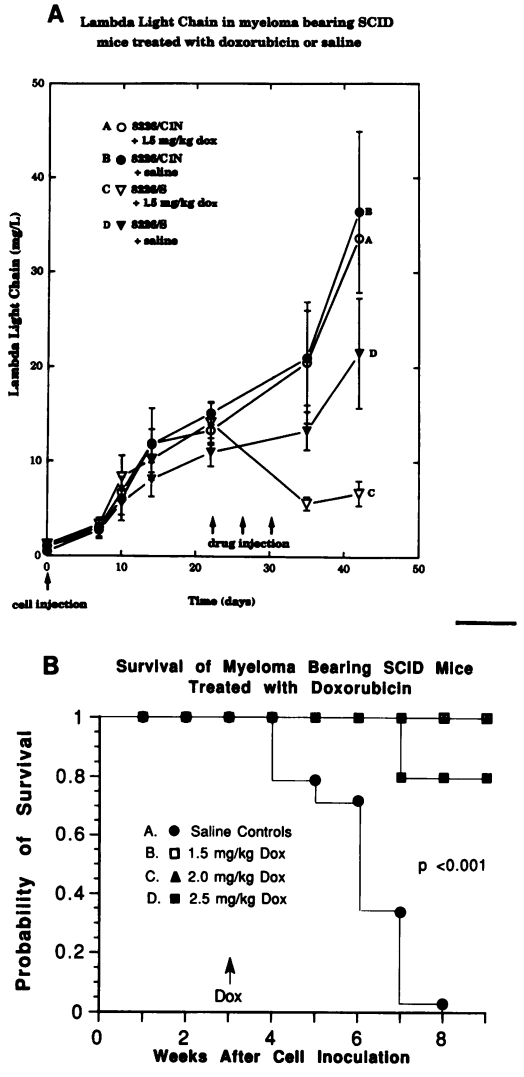

Figure 3. (A) *Human λ light chain excretion in the urine of 8226 and 8226/C1N tumor-bearing SCID mice before and after therapy with 1.5 mg/kg doxorubicin, administered on an every-4-day basis. Tumor cells were injected on day 0, and doxorubicin therapy initiated on day 21 (n = 5 in each group);* (B) *Kaplan-Meier curve showing the probability of survival of SCID mice bearing 8226 tumors. Cells were injected on day 0 and (•) saline or (□) 1.5 mg/kg, (▲) 2.0 mg/kg, or (■) 2.5 mg/kg bolus doxorubicin was initiated on day 21 after cell inoculation (n = 10 in each group).*

The maximum tolerated dose of doxorubicin on a multidose regimen was established as 2.0 mg/kg when administered intraperitoneally on days 1, 5, and 9 (Table 1). When tumor-bearing SCID mice were treated with 1.5 mg/kg of doxorubicin administered on days 21, 26, and 30 after injection of cells, there was a marked decrease in the level of human λ light chain in the urine of those animals bearing the drug-sensitive 8226 cells. In contrast, there was no change in the λ light chain excretion of those animals inoculated with the MDR variant 8226/C1N (Figure 3A).

In a separate experiment shown in Figure 3B, the reduction in the light chain values of those mice injected with the drug-sensitive 8226 tumors followed by doxorubicin translated into a significant ($P < 0.001$) increase in their survival time when compared to saline-treated controls. This experiment has been repeated three times with comparable results.

Discussion

The major findings of this study are: a) reproducible establishment of both a drug-sensitive and drug-resistant human multiple myeloma xenograft model in SCID mice; b) demonstration of differential treatment effects on drug-sensitive and drug-resistant SCID mouse myeloma with a prototypic drug, doxorubicin; c) establishment of human urinary immunoglobulin and animal survival as measurable endpoints in therapy experiments. These findings set the stage for subsequent *in vivo* studies to investigate new therapeutic approaches to MDR myeloma, including the evaluation of new chemosensitizers of PGP-mediated multidrug resistance.

The advantages of the SCID human myeloma xenograft model include: 1) a reliable tumor take rate; 2) ready demonstration by immunophenotyping of human versus murine origin of tumors; 3) quantification of tumor burden via urinary monoclonal light chain measurement; and 4) a reproducible, defined time frame of tumor growth and survival allowing for assessment of outcome in a speedy fashion.

Measurements of serum immunoglobulin have been demonstrated to correlate with the total tumor burden in patients with IgG myeloma.[26] The mea-

Table 1. *Maximal Tolerated Dose of Doxorubicin in SCID Mice*

Dose (mg/kg)	Deaths/ Total	Mean survival time (days)
Bolus administration		
75	2/2	<1
25	6/6	4
10	7/8	15.8
2.5	0/3	23*
1.0	0/3	23*
Multiple administration regimens		
Days 1, 3, 5		
2.5	4/5	19.6
1.0	0/5	45*
0.1	0/5	45*
Days 1, 5, 9		
7.0	5/5	8.4
5.0	5/5	14.4
3.0	5/5	9.8
2.5	1/5	23*
2.0	0/10	46*
1.5	0/10	46*

* Experiment terminated.

surement of human monoclonal light chain in the urine represents a novel and sensitive means of assessing human tumor burden in a mouse model. One of the advantages of our model is that tumor growth and cell kill can be assessed repeatedly by human monoclonal light chain assays without killing the animal and without relying solely on measurements like the percent of increased life span. Treatment can therefore be started at a specific tumor burden, thus permitting a direct comparison of the effectiveness of chemotherapeutic agents used at the same extent of disease in each animal. This approach would be preferable to the other models in which the tumor is treated at a predetermined time interval following the inoculation of a known number of tumor cells. In such systems, the actual tumor burden in individual animals may vary considerably, thus complicating direct comparisons. Our model permits the precise quantification of response and comparison of agents for mice with the same tumor burden. An additional advantage to the SCID-human xenograft model is that all animals will have documented tumor take by routine testing for human light chain urinary excretion before entry into therapeutic trials.

One obvious use of this model will be to test drugs that may be active against MDR myeloma. This model will serve to complement current *in vitro* assays, which are better suited to identify initially compounds effective in overcoming PGP-mediated drug resistance. Those compounds identified as promising on the basis of *in vitro* assays would then become candidates for *in vivo* evaluation of their ability to reverse PGP-mediated drug resistance. Not only will studies with the SCID *in vivo* model include pharmacokinetic and pharmacodynamic parameters, which are not easily studied in the *in vitro* setting, the model can also be used to identify the limiting organ-specific toxicities of new chemomodulators, both when administered alone and in combination with chemotherapeutic agents.

Renal failure is a common complication of multiple myeloma that can be significant at the time of clinical presentation. Although we are utilizing urinary light chain excretion as a measure of tumor burden, these measurements are made while the animal is still in relatively early stages of the disease. No renal damage was detected by urinary creatinine levels or by histological examination of kidneys at necropsy. As tumor burdens are allowed to grow untreated, however, one would expect to find an increase in the levels of urinary and serum creatinine as well as in other markers of nephrotoxicity. Future studies will include the use of serum indicators of renal function, such as serum creatinine, as well as to allow for a measure of creatinine clearance.

This model was developed to assess the efficacy and toxicities of new chemotherapeutic agents and chemosensitizers directed against the PGP-mediated MDR phenotype. Future uses of our model may also include studies of new agents and therapeutic strategies directed against non-PGP-mediated multidrug resistance in myeloma. This model may also serve as a more general model of multiple myeloma, providing a means to evaluate the effectiveness of various therapeutic strategies aimed at this disease and to elucidate the mechanisms by which myeloma cells may escape from chemosensitization. Studies are currently underway to develop an orthotopic model of human myeloma in the SCID mouse.

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