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Preclinical Studies of the Novel Vascular Disrupting Agent MN-029

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Abstract. *Background: Vascular disrupting agents (VDAs) are designed to cause a rapid and selective shutdown of the established tumor vasculature, which leads to secondary ischemic tumor cell death. Materials and Methods: We examined the efficacy of a novel VDA, MN-029, in the rodent KHT sarcoma model. Results: A significant reduction in the functional vessel number was observed after intraperitoneal injection of MN-029 at a dose of 100 mg/kg. Histological evaluation showed extensive necrosis (~90%) by 24 h. MN-029 treatment to the tumor-bearing mice also resulted in a dose-dependent tumor cell killing. When used in combination with radiation or cisplatin chemotherapy, a 100 mg/kg dose of MN-029 significantly enhanced tumor killing compared to that seen with radiation or cisplatin alone. Conclusion: The results demonstrated that MN-029 could cause rapid vascular shutdown in solid tumors, dose-dependent secondary tumor cell killing, and effective enhancement of the antitumor effects of radiation and cisplatin chemotherapy.*

The interest in novel therapies aimed against tumor vasculature arises from the dependence of solid tumors on a functional blood vessel network for survival, expansion and dissemination (13). It has long been observed that tumor vessels differ greatly from vessels in normal tissues (38). They are not only primitive in nature, but also morphologically and functionally abnormal. The structural defects and ineffective perfusion capacity of tumor microvessels lead to chaotic and heterogeneous blood flow, which inevitably fails to meet the metabolic demands of the tumor tissue (38). The ability to target tumor vessels represents an exciting new development in the expanding modalities of anticancer treatments. Two key approaches to targeting the tumor blood vessel network have been developed (11, 13, 28). One aims to inhibit the angiogenic process in tumors (antiangiogenic approach) (13), the other seeks to preferentially destroy the existing tumor vasculature (vascular disrupting approach) (3, 10, 33). Though both selectively target the tumor vasculature, distinctions between antiangiogenic and vascular disrupting therapies should be made. The two approaches differ in their mode of action, as well as in their therapeutic applications (28).

Vascular disrupting agents (VDAs) exert their selective action through exploiting structural, phenotypic and functional differences between tumor and normal endothelium (36). Destruction of the endothelium results in secondary tumor cell death from lack of oxygen and nutrients, due to the rapid and extensive vascular shutdown. Treatment with these agents ultimately leads to widespread central necrosis in solid tumors (26). The class of small molecule VDAs, which includes DMXAA, CA4DP, AVE8062A, ZD6126 and OXi4503, has now been demonstrated to posses antitumor efficacy in a wide variety of preclinical tumor models, including transplanted and spontaneous rodent tumors, orthotopically-transplanted tumors and human tumor xenografts (1, 4, 7, 16-21, 23, 25).

Encouraged by the results from preclinical investigations, VDAs have entered clinical trials. In addition, the development and evaluation of second generation compounds is actively being pursued. The current studies examined the antivascular and antitumor efficacies of the microtubule-destabilizing agent MN-029, a novel benzimidazole carbamate, in the rodent KHT sarcoma model.

Materials and Methods

Animal and tumor model. KHT sarcoma cells were injected *i.m.* ($2x10^5$ cells in a volume of 0.01 ml) into the hind limbs of 6- to 8-week-old female C3H/HeJ mice (Frederick Cancer Research Facility, MD, USA). Once the tumors had reached a size equivalent to a weight of ~0.5 g, the mice were randomly assigned to groups that were untreated or treated with MN-029, cisplatin, or radiation administered alone or in combination.

Drug treatment. MN-029 (MediciNova, Inc., San Diego, CA, USA) was prepared at 6 mg/ml in 20% hydroxypropyl-β-cyclodextrin. Cisplatin (Bristol-Myers Squibb Co., Princeton, NJ, USA) was dissolved in 0.9% saline and injected *i.p.* in a volume of 0.01 ml/g body weight.

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Irradiation. Tumors of non-anesthetized mice were irradiated locally using a 6MV Clinac 600c linear accelerator (Varian Oncology Systems, Palo Alto, CA, USA) operating at a dose rate of 4 Gy/min. The mice were confined to plastic jigs designed so that the tumor-bearing legs extended through openings on the sides, thus allowing the tumors to be irradiated locally.

Hoechst-33342 studies of patent tumor blood vessels. Hoechst-33342 (bisBenzimide, Sigma, Saint Louis, MO, USA) solution was prepared in 0.9% sterile saline immediately before use. KHT sarcoma-bearing mice were either untreated or treated with 100 mg/kg MN-029. Hoechst-33342 was then administered at 40 mg/kg *i.v.* (volume 5 ml/kg) at various times after MN-029 injection (34). One minute after Hoechst-33342 injection, the mice were killed, and the tumors were resected and immediately immersed in liquid nitrogen for subsequent frozen sectioning. For each tumor sample, 10-μm cryostat sections were cut at three different levels between one pole and the equatorial plane. The sections were studied under UV illumination using a fluorescent microscope. Blood vessel outlines were identified by the surrounding halo of fluorescent Hoechst-33342 labeled cells. Vessel counts were performed using a Chalkley point array for random sample analysis (6). Briefly, each section was viewed at 10x objective magnification. A 25-point Chalkley grid was positioned randomly over the field of view. Any points falling within halos of fluorescent cells were scored positive, and a minimum of 6 sections per tumor were examined. Data from 3-5 tumors were pooled and presented (32).

Morphological and morphometric analysis. The tumors were dissected from the hind limb 24 h after the MN-029 treatment and fixed in 10% neutral buffered formalin. Standard hematoxylin-eosin (H&E) staining was carried out on the mid-cross sections of the formalin-fixed tumor specimens. The sections were viewed and captured using a Zeiss Zxiophot 2 microscope (Carl Zeiss Jena, GmbH, Jena Germany) with Sony DXC970 color camera (Sony Corporation, Tokyo, Japan). Entire tumor sections were reconstructed by tiled field mapping and the total tumor area, as well as the area of tumor necrosis, was determined using a MCID5.5 image analysis program (Imaging Research Inc., Ontario, Canada). Areas of tumor necrosis were identified from the hyperchromatic, pleomorphic, mitotically-active viable tumor cells. The tumor necrotic fraction was then determined by the ratio of necrotic area *versus* total tumor area (32) with the aid of the Image J software program (National Health Institute, Bethesda, MD, USA).

Clonogenic cell survival. Clonogenic cell survival in treated or untreated tumors was determined using an *in vivo* to *in vitro* cell survival assay, as previously described (32). Briefly, 24 h after treatment, KHT sarcomas were excised and single cell suspensions were prepared using a combined mechanical and enzymatic dissociation procedure. The cells were counted and various dilutions were prepared. KHT cells were mixed with 0.2% agar containing α-minimum essential medium supplemented with 10% fetal bovine serum and plated into 24-well plates. Two weeks later, the resulting colonies were counted with the aid of a dissecting microscope. The tumor surviving fractions were calculated by multiplying the determined fraction of surviving cells by the ratio of cells recovered in treated and untreated tumors.

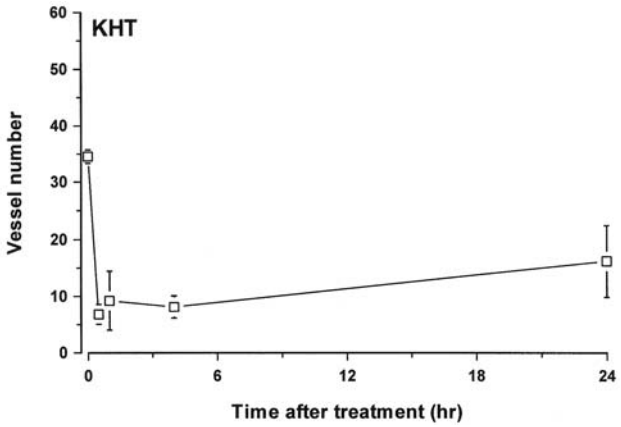

Figure 1. *Patent blood vessels identified in KHT sarcomas at various times after treating mice with a 100 mg/kg dose of MN-029. At each time-point, mice were injected with Hoechst 33342 (40 mg/kg) and tumors were removed 1 min later. Vessel numbers were determined using a Chalkley point array for random sample analysis. Data are the mean±SE of 3-5 tumors.*

Results

To evaluate the effect of MN-029 on the vasculature of KHT sarcomas, functional vascular volumes were determined with the use of the perivascular stain Hoechst-33342. The results showed that administration of MN-029 led to a rapid vascular shutdown, which became readily apparent within 30 min after exposure to a 100 mg/kg dose (Figure 1). Indeed, functional vessels were visible only at the periphery of the tumor, indicating that this dose of the VDA led to an almost complete vascular shutdown in these tumors.

Histological analysis of KHT sarcomas performed 24 h post MN-029 treatment demonstrated widespread central tumor necrosis, consistent with the findings of the Hoechst-33342 studies. Untreated KHT tumors, which frequently entrapped normal skeletal muscle, showed little evidence of tumor necrosis (Figures 2A and 2B). In contrast, extensive central tumor necrosis was apparent 24 h after treatment with a 100 mg/kg dose of MN-029 (Figures 2C and 2D). In the treated groups, viable tumor cells were observed only at the periphery of the tumor adjacent to the surrounding normal tissue. The extent of tumor necrosis was clearly treatment dose-dependent (Figure 3). For example, 24 h post VDA treatment, a 25 mg/kg dose of MN-029 resulted in ~20% tumor necrosis, whereas a 100 mg/kg dose resulted in ~90% tumor necrosis. Tumor cell death, secondary to the induction of ischemia, also was found to be MN-029 dose-dependent (Figure 4).

The response of KHT sarcomas to MN-029 treatment combined with conventional anticancer therapies was also investigated. Based on preliminary results and prior experience

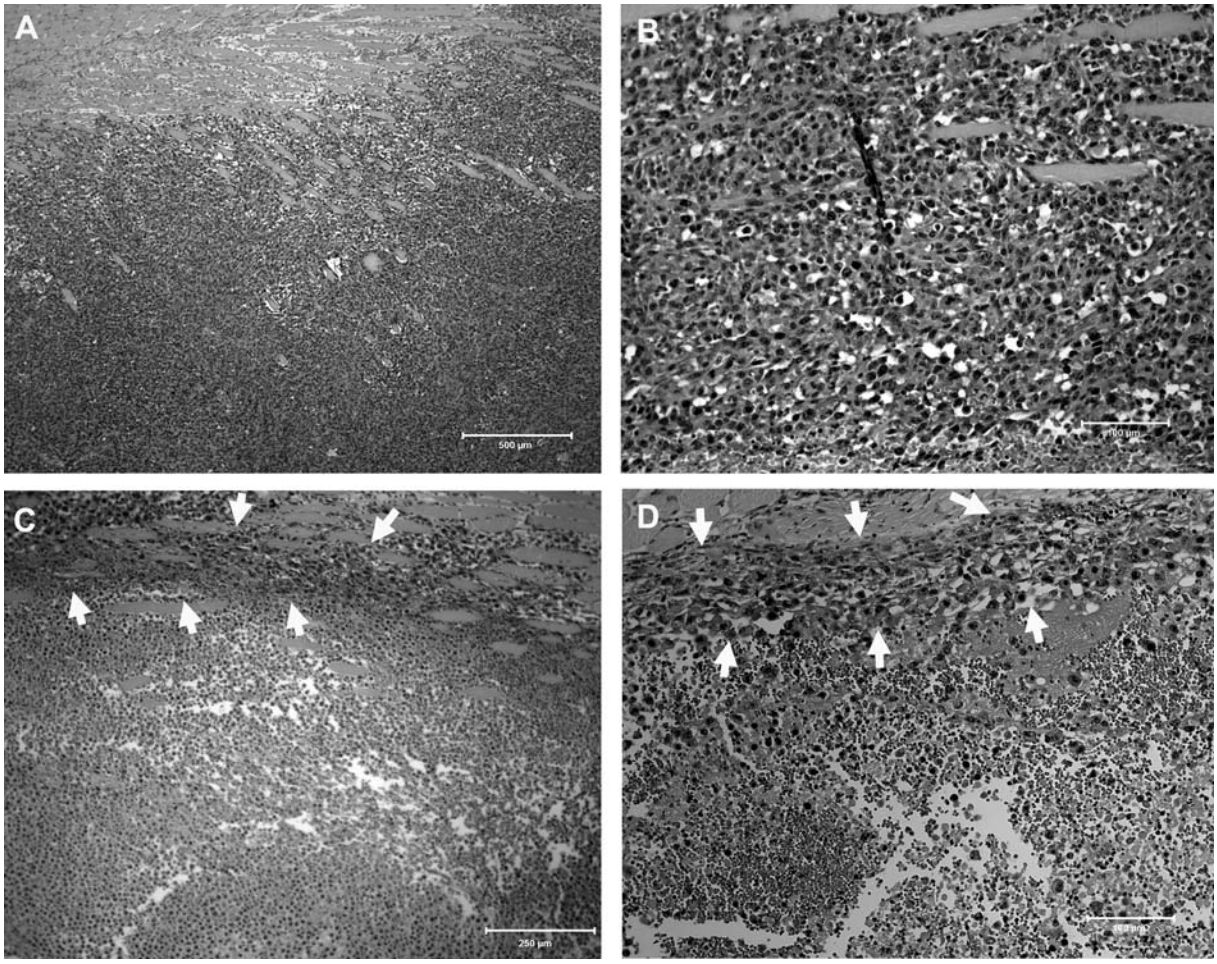

Figure 2. *Histological assessments of KHT sarcomas in mice treated with MN-029. (A, B) Low (5x) and high (20x) magnification of H&E-stained untreated tumors show little necrosis. (C, D) KHT tumors assessed 24 h after MN-029 treatment (100 mg/kg) show extensive areas of necrosis when viewed at low or high magnifications. Arrows indicate rim of viable tumor tissue.*

with other VDAs (31, 32), MN-029 was administered 1 h post chemotherapy/radiotherapy in these studies. Clonogenic cell survival assessed in KHT sarcomas 24 h after treatment was used as the tumor response end-point. The results showed that the inclusion of MN-029 in the treatment strategy reduced tumor cell survival ~20-fold below that achieved with cisplatin treatment alone (Figure 5). Similarly, when KHT sarcoma-bearing mice were irradiated with a range of doses (0 – 25 Gy) prior to the administration of a 100 mg/kg dose of MN-029, tumor cell survival was found to be 20- to 50-fold lower in animals treated with the combination than mice treated with radiation alone (Figure 6).

Discussion

The concept of using drugs to directly damage tumor vasculature was advocated by Denekamp (9) nearly 20 years

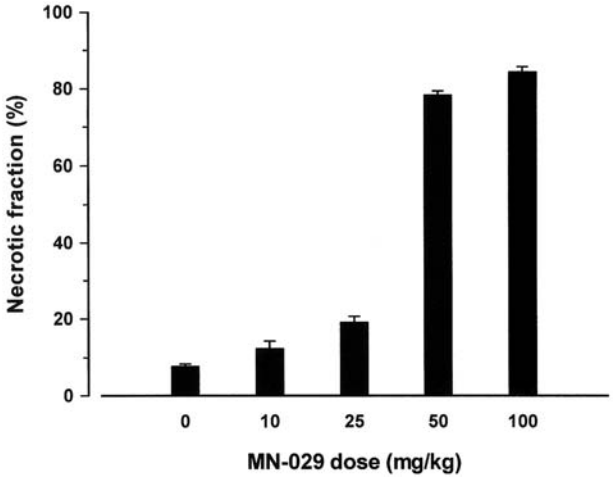

Figure 3. *Percent necrosis determined in KHT sarcomas 24 h after treating mice with graded doses of MN-029. Data are the mean±SE of 3-5 tumors.*

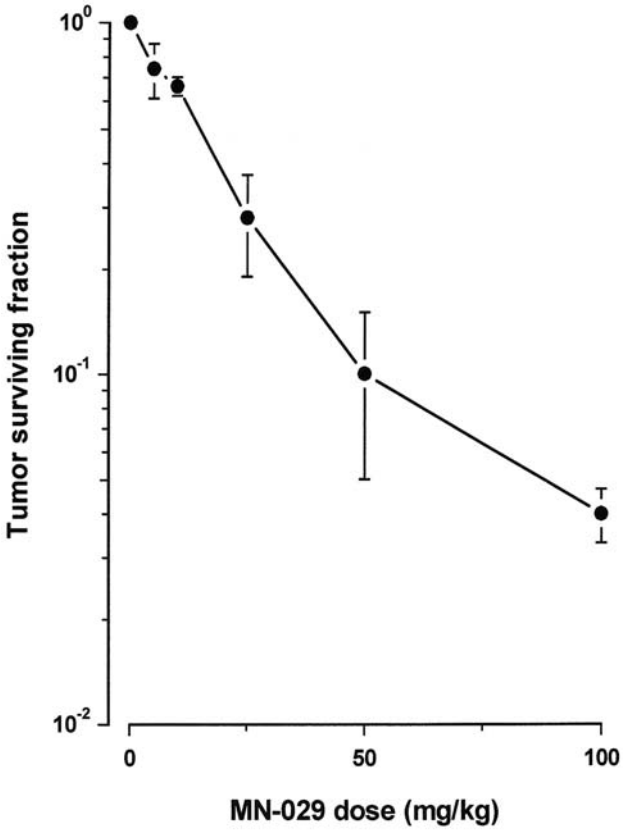

Figure 4. *Clonogenic cell survival in KHT sarcomas treated with a range of doses of MN-029. Data were determined 24 h after treatment and are the mean±SE of 5-25 tumors.*

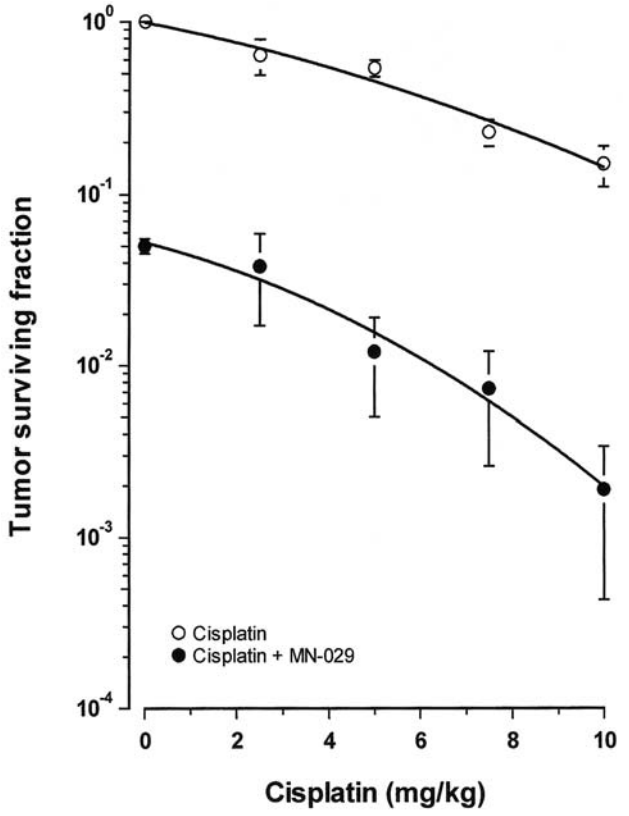

Figure 5. *Clonogenic cell survival in KHT sarcomas treated with either cisplatin alone (open circles) or a combination of cisplatin plus MN-029 (solid circles). MN-029 was administrated 1 h post chemotherapy. Data are mean±SE of 3-6 experiments.*

ago. It has reemerged recently as a potential viable cancer therapy, primarily because of the development of agents showing selective targeting of growing tumor endothelium (1, 4, 7, 16-21, 23, 25). Indeed both ligand-directed VDAs and small molecule VDAs have been shown to exploit differences between tumor and normal tissue endothelia to induce selective occlusion of tumor vessels (3, 29). Both types of agents have demonstrated potent antivascular and antitumor efficacy in a wide variety of preclinical tumor models (1, 4, 7, 16-21, 23, 25, 29). While lead agents are now undergoing clinical evaluation (5, 12, 27, 35), the pursuit and development of structurally or mechanistically new VDAs is actively ongoing.

MN-029 binds reversibly at the colchicine binding site. Its mechanism of action is based on the reversible inhibition of tubulin polymerization, resulting in the disruption of the cell cytoskeleton. The present investigations evaluated the *in vivo* vascular effects and antitumor efficacy of this agent in the KHT sarcoma.

Treatment with MN-029 was found to result in a rapid onset of the disruption of the tumor vasculature. When fluorescent microscopy was used to visualize Hoechst-33342 perivascular staining, a dramatic reduction of patent tumor vessels was noted in KHT sarcoma sections within 30 min post MN-029 treatment (Figure 1). Such a dramatic shutdown of tumor vasculature is a typical response of tumor vasculature to VDA treatment, having been previously observed with other agents in this (21, 24, 32) and other solid tumor models (3, 7, 14, 17, 37). The suppression of functional vessels by MN-029, which lasted for ~24 h, also was consistent with the histological findings of widespread tumor and dose-dependent necrosis evident 24 h after treatment (Figures 2 and 3).

The central necrosis coupled with a thin rim of viable tumor cells at the periphery of the tumor observed after treatment with a 100 mg/kg dose of MN-029 is a characteristic feature of tumors treated with VDAs (2, 7, 8, 14, 16, 17, 21, 32).

These residual areas of tumor tissue can act as a source of tumor regrowth, thus, in general, it is highly unlikely that VDA treatment alone will eradicate the entire tumor mass. Still, the destruction of large tumor areas, particularly in the

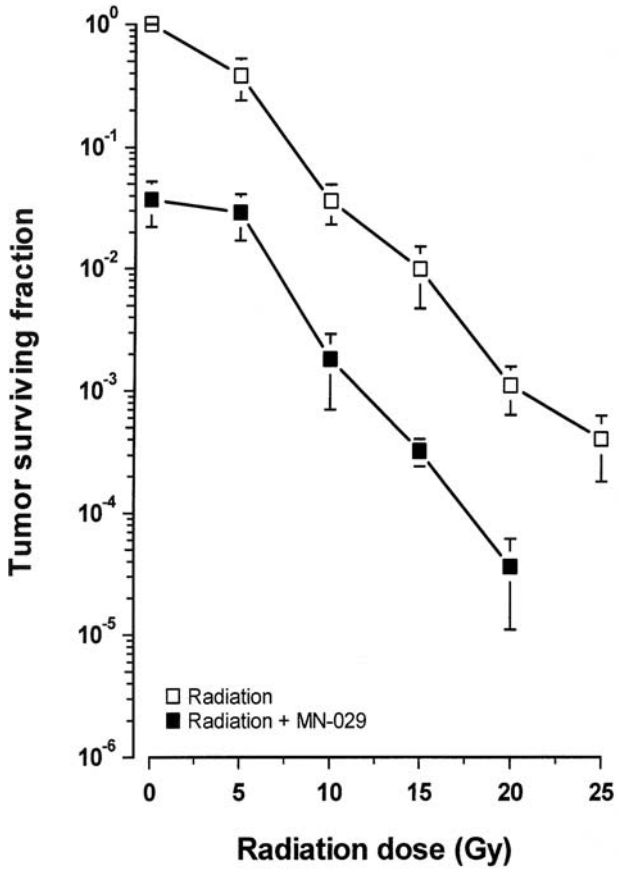

Figure 6. *Clonogenic cell survival in KHT sarcomas treated with either radiation alone (open squares) or a combination of radiation plus MN-029 (solid squares). MN-029 was administrated 1 h post radiotherapy. Data are mean±SE of 3-6 experiments.*

central and typically most radiation and chemotherapy treatment-resistant regions of the tumors, is highly desirable. The strategy of combining VDAs with conventional cytotoxic modalities therefore represents a treatment strategy that holds significant therapeutic potential and indeed has been successfully applied preclinically with a variety of VDAs (22, 24, 31, 32). This was also the case in the present studies which incorporated MN-029 treatment into conventional radiation and chemotherapy treatments. The results showed marked gains in treatment efficacy when MN-029 was combined with cisplatin or radiotherapy (Figures 5 and 6).

In conclusion, MN-029 was found to demonstrate striking antivascular effects in tumors, leading to the induction of necrosis and a consequential rapid loss of clonogenic neoplastic cells. This VDA also was successfully incorporated into conventional cisplatin or radiation therapy treatments. MN-029 proved to be a potent VDA with similar efficacies as other agents in this class, so that future clinical evaluation is warranted.

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