NM-3, an Isocoumarin, Increases the Antitumor Effects of Radiotherapy without Toxicity


ABSTRACT

We examined the effects of a new antiangiogenic isocoumarin, NM-3, as a radiation modifier in vitro and in vivo. The present studies demonstrate that NM-3 is cytotoxic to human umbilical vein endothelial cells (HUVECs) but not to Lewis lung carcinoma (LLC) cells nor Seg-1, esophageal adenocarcinoma cells, in clonogenic survival assays. When HUVEC cultures are treated with NM-3 combined with ionizing radiation (IR), additive cytotoxicity is observed. In addition, the combination of NM-3 and IR inhibits HUVEC migration to a greater extent than either treatment alone. The effects of treatment with NM-3 and IR were also evaluated in tumor model systems. C57BL/6 female mice bearing LLC tumors were given injections for 4 consecutive days with NM-3 (25 mg/kg/day) and treated with IR (20 Gy) for 2 consecutive days. Combined treatment with NM-3 and IR significantly reduced mean tumor volume compared with either treatment alone. An increase in local tumor control was also observed in LLC tumors in mice receiving NM-3/IR therapy. When athymic nude mice bearing Seg-1 tumor xenografts were treated with NM-3 and IR, significant tumor regression was observed after combined treatment (NM-3 and IR) compared with IR alone. Importantly, no increase in systemic or local tissue toxicity was observed after combined treatment (NM-3 and IR) when compared with IR alone. The bioavailability and nontoxic profile of NM-3 suggests that the efficacy of this agent should be tested in clinical radiotherapy.

INTRODUCTION

Tumor angiogenesis is essential for tumor progression and the formation of metastases. In the adult, ~0.01% of endothelial cells undergo cell division. By contrast, the fraction of proliferating endothelial cells in tumors is proposed to be 50-fold higher (1, 2). These differences between proliferating endothelial cells in tumor tissue versus normal tissue can be exploited through the use of angiogenesis inhibitors. Furthermore, a single tumor vessel may supply as many as 104 tumor cells, thereby amplifying the antitumor effects of antiangiogenic compounds (1, 2). Importantly, tumor endothelium is derived from normal host cells and, in contrast to tumor cells, is genetically stable, which suggests that tumor endothelium is unlikely to develop resistance to cytotoxic agents (3). Recent investigations have focused on the development of antiangiogenic agents, particularly those that can be administered p.o. and for prolonged periods, as alternatives to standard cytotoxic anticancer therapies.

Investigations of antiangiogenic compounds have been conducted in preclinical and clinical trials. Strategies to inhibit angiogenesis have included the use of neutralizing antibodies to angiogenic proteins, integrin molecules, and growth factor receptors. (4–6) Also, kinase inhibitors, natural products such as TNP-470, and antibiotic-derivatives such as minocycline have been used (7, 8). Angiostatin and endostatin, enzymatic degradation products of plasminogen and type XVIII collagen, respectively, are reported to induce tumor regression and prolong tumor dormancy in murine model systems (9, 10). However, in most reports, angiostatin and endostatin are tumoristatic, not tumoricidal, and tumor regrowth ensues after cessation of treatment. Tumor cures have been limited when most angiogenesis inhibitors are used as a sole method of treatment.

Radiotherapy is an important standard cancer treatment for local and regional disease. Sixty percent of all cancer patients receive radiotherapy during the course of their disease. The importance of improving radiotherapy is further highlighted by the fact that approximately 20–30% of cancer deaths are attributable to local/regional failures and 50–60% of cancer patients die with local tumor as a component of treatment failure (11). Recent advances in radiotherapy are attributed to improved methods of physical delivery, which allow for dose escalation and combination of radiotherapy with commonly used antineoplastic agents (12, 13). Combining radiotherapy and cytotoxic agents has improved local control and cure rates in a variety of human cancers but at the cost of significant acute normal tissue toxicity (14–19). Nonetheless, these results verify that local control can translate into an increase in cures.

Maucci et al. (20) have recently demonstrated that combined treatment with angiostatin enhances the effects of IR4 on tumor regression. The combination of endostatin and IR has resulted in similar findings (Hanna, et al., in press). Moreover, Gorski et al. (21) reported that the use of vascular endothelial growth factor antibody and IR produces greater than additive antitumor effects when compared with either treatment alone. These findings collectively indicate that the combination of radiotherapy and angiogenic blockade enhances the therapeutic ratio of IR by targeting both tumor cells and tumor vessels. Importantly, the therapeutic gains of the combined treatment modalities are achieved without increased toxicity when compared with treatment with radiotherapy alone.

Recently, an isocoumarin derivative, 2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid (NM-3), was synthesized from cytogenin, a compound isolated from the culture filtrate of Streptovorticillum eurocidium (22, 23). Antiangiogenic effects after p.o. administration of NM-3 have been demonstrated in the mouse dorsal air sac assay system. Although modest antitumor effects were reported when NM-3 was used as the sole therapeutic agent (22), recent investigations suggest that antiangiogenic compounds effectively enhance the antitumor effects of IR (20, 21). The low-toxicity profile of NM-3, its oral bioavailability, and the ease of production,
suggest that NM-3 might be a clinically useful antiangiogenic agent for combination studies with IR.

Here, we report that when NM-3 is combined with radiotherapy, an increase in local tumor control is observed without an increase in normal tissue toxicity. We also show that NM-3 is selectively cytotoxic to endothelial cells but not to tumor cells. We propose a new paradigm for enhancement of radiocurability whereby standard risk/benefit analysis, in terms of calculating the therapeutic index, is not applicable because the radio-enhancing agent has little or no antitumor effect when administered alone but enhances the antitumor effect of IR without increasing normal tissue effects.

MATERIALS AND METHODS

Cell Culture. LLC cells, provided by Dr. Judah Folkman (Harvard Medical School, Boston, MA), were maintained in DMEM (75%) and F-12 (25%; Life Technologies, Inc., Grand Island, NY) medium, with 10% heat-inactivated fetal bovine serum (Intergen, Purchase NY) and 1% penicillin/streptomycin (Life Technologies, Inc.). Human esophageal adenocarcinoma cells (Seg-1), kindly provided by Dr. David Beer (University of Michigan Medical School, Ann Arbor, MI), were maintained in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells of a human epidermoid carcinoma, SQ-20B, were maintained in DMEM:F-12 (3:1), 20% fetal bovine serum, and 0.4 µg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO). HUVECs and HAECs were obtained from Clonetics Corporation (San Diego, CA) and maintained in complete EGM-2 medium per manufacturer’s instructions.

Preparation and Administration of NM-3. NM-3 was obtained from T. Nakashima (Central Research Lab., Mercian Corp., Fujisawa, Japan) and ILEX Oncology. For in vitro experiments, NM-3 was prepared at a stock concentration of 1 mg/ml in PBS and filter sterilized. Serial dilutions were prepared in sterile PBS as needed.

For in vivo studies, 1.25 mg/ml NM-3 was dissolved in PBS and filter sterilized. Each animal received 400 µl (equivalent to 25 mg/kg) by i.p. injection. Control mice received an equal volume of PBS. In one study, mice bearing Seg-1 xenografts were treated with 100 mg/kg/day NM-3 (suspending in 40% polyethylene glycol-400 in PBS).

Matrigel Assay. The human glioblastoma cell line (U373 MG) was incubated overnight with the hypoxia-mimetic compound cobalt chloride (250 µM; Refs. 24–26), harvested, and mixed with ice-cold Matrigel (Collaborative Biomedical Products) at a concentration of 2 x 10^5 cells/ml. Athymic nude NCRNU female mice (Taconic Farms) were anesthetized and injected s.c. in the right hind limb of athymic nude female mice (Frederick Cancer Research Institute, Frederick, MD). Seg-1 cells (3 x 10^6 cells in 100 µl of serum-free medium) and SQ-20B cells (5 x 10^6 cells in 100 µl of PBS) were injected into the right hind limb of athymic nude female mice (Frederick Cancer Research Institute, Frederick, MD). LLC, Seg-1, and SQ-20B tumors were grown to a volume of 297.3 ± 13.3 mm^3 (n = 43), 564.2 ± 22.2 mm^3 (n = 31), or 681.8 ± 17.2 mm^3 (n = 45), respectively, before the mice were divided into experimental groups. Tumor volume was determined by direct measurement with calipers as described previously (28).

Mice bearing LLC tumors were given injections with 25 mg/kg/day of NM-3 as a single i.p. injection 4 h prior to IR treatment. These mice received a total dose of 100 mg/kg NM-3, with treatment beginning two days prior to IR treatment. Seg-1 tumor-bearing animals were treated with 100 mg/kg NM-3 i.p. for 4 consecutive days. In Seg-1 tumor experiments, both NM-3 and IR were started on the same day (day 0), with NM-3 given 3–4 h prior to IR. Mice bearing SQ-20B tumors received 50 mg/kg/day NM-3 (in two doses) for 8 days. In an additional experiment to assess local tumor control, C57BL/6 mice bearing LLC tumors (mean initial volume, 151.1 ± 4.6 mm^3; n = 29) received 5 days of NM-3 at 50 mg/kg/day (split into two doses). In all of the experiments, control and IR animals were given i.p. injections with an equivalent volume PBS. Tumors were irradiated using a General Electric Maxitron X-ray generator operating at 150 kV, 30 mA, using a 1 mm aluminum filter at a dose rate of 188 cGy/min. Fractionation schemes were based on tumor-doubling times and tumor volume. LLC tumors were treated with a total dose of 40 Gy (20 Gy on 2 consecutive days); Seg-1 tumors were treated with 20 Gy (four 5-Gy fractions); and SQ-20B tumors were treated with 65 Gy (5 Gy for 2 days, 15 Gy for 2 days with 3 days between, a single dose of 10 Gy and 5 Gy for 3 days). To assess local control in LLC, a total dose of 66 Gy was given (12 Gy for 3 days, then 15 Gy for 2 days). Mice were shielded with lead except for the tumor-bearing hind limb. The care and treatment of the mice were in accordance with institutional guidelines.

Tumor Histology. Animals were euthanized and tumors excised at specified time points throughout the experiments. Tumor samples were preserved in paraffin or OCT Embedding Compound (VWR Scientific, Chicago, IL). Four-µm sections were cut from OCT blocks and mounted on poly-L-lysine coated slides. Briefly, sections were fixed in 1% paraformaldehyde (methanol-free; Polysciences, Warrington, PA). After blocking with goat serum and quenching endogenous peroxidase activity, slides were incubated at 4°C overnight with rabbit-anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, CA) diluted to 0.1 µg/ml and were then incubated for 25 min at 37°C with rabbit-anti-rat IgG biotinylated secondary antibody (2 µg/ml; Vector, Burlingame, CA). Appropriate negative controls were performed with rat IgG2a isotype control (PharMingen), CD31 monoclonal antibody alone, and secondary antibody alone. ABC Vectastain Standard Elite was applied and the tissues were developed with the Vector DAB Kit. Each slide was counterstained with a solution (0.03%) of light green SF yellowish (Fisher, Hanover Park, IL). Ten high-power fields (×400) from each tumor section were examined using a Nikon Microphot-FX microscope equipped with a Sony digital camera. Vessels were counted using Macintosh Image Pro-Plus imaging software. Additional tumor tissue was placed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 6-µm thickness, stained with H&E, and examined by light microscopy at ×400.

Statistical Methods. Significance was determined using one-way ANOVA, Student’s t test, and Fisher’s exact test using Jandel Scientific software.

NM-3 INCREASES ANTITUMOR EFFECTS OF RADIOTHERAPY
RESULTS

NM-3 Is Antiangiogenic in a Matrigel Assay. Matrigel plugs from mice receiving daily injections of PBS vehicle showed a robust angiogenic response. (Fig. 1, upper panel) An extensive network of vessels was observed both within the Matrigel matrix as well as in the stromal interface between the skin and the Matrigel. In contrast, Matrigel plugs from mice receiving twice-daily injections of NM-3 (100 mg/kg/day) showed a dramatic reduction in neovascularization. (Fig. 1, lower panel) The few vessels present showed minimal vascular branching, which were predominantly in the stromal interface.

NM-3 Is Selectively Cytotoxic to Endothelial Cells. The lowest dose of NM-3 (10 ng/ml) was cytotoxic to HUVECs but not to either LLC or Seg-1 cells (P = 0.06; Fig. 2A). The selectivity of NM-3 cytotoxicity for endothelial cells, but not tumor cells, was consistent at all concentrations tested (100, 1000, and 10,000 ng/ml; P < 0.05). NM-3 was also selectively cytotoxic to HAECs in a dose-dependent fashion (data not shown). When HUVECs were exposed to NM-3 prior to IR, additive killing was observed at 500, 700, and 900 cGy (Fig. 2B). No enhancement of IR-induced cytotoxicity was observed when LLC cells were exposed to NM-3 and 100–900 cGy IR (Fig. 2C). Similar results were observed for Seg-1 cells (data not shown). Taken together, these data support a selective additive interaction between NM-3 and IR in endothelial cells. Notably, DAPI staining of HUVEC cultures revealed that NM-3-induced endothelial cell death was not mediated by apoptosis (data not shown).

NM-3 and IR Inhibit Endothelial Cell Migration. We observed that NM-3 at a concentration of 100 ng/ml had no detectable effect on HUVEC migration when compared with UTC (UTC = 54.25 ± 2.11 versus NM-3 alone = 51.83 ± 2.17 cells/10 hpfs). By contrast, IR inhibited HUVEC migration (41.25 ± 1.51 cells/10 hpfs, or 76% of control.) In addition, the combination of NM-3 and IR inhibited migration to a greater extent than either agent alone (27.5 ± 4.2, or 51% of control; P < 0.001; Fig. 3).

NM-3 and IR Inhibit Primary Tumor Growth. The effects of NM-3 and IR on tumor growth were assessed in LLC, Seg-1, and SQ-20B tumors. By day 7, LLC tumors (initial volume 297.3 ± 13.3 mm³; n = 43) in the control group and the NM-3 group grew to
8.02 ± 1.24 and 6.19 ± 0.44 times original volume, respectively. Animals in these treatment groups were sacrificed on day 7 because of tumor burden. LLC tumors in the IR (20 ± 20 Gy) group initially grew and then regressed to 1.06 ± 0.08 times original volume (day 7). Regression was followed by regrowth to 2.98 ± 0.83 times original volume at day 15 when the experiment was terminated. Combined treatment with NM-3 and IR significantly reduced mean tumor volume when compared with IR alone at day 3 (P < 0.001), 4 (P = 0.013), 5 (P < 0.001), 9 (P = 0.016), 11 (P = 0.032), 13 (P = 0.015), and 15 (P = 0.041; Fig. 4A).

Seg-1 tumors (initial volume, 564.4 ± 22.2 mm³; n = 31) in the control group, and the NM-3-treated group grew to 6.30 ± 0.42 and 6.74 ± 0.62 times original volume by day 15, at which time the animals were sacrificed because of tumor burden. Tumors in the IR (four doses of 5 Gy) group initially regressed (0.64 ± 0.03 times original volume, day 7) and then regrew (1.83 ± 0.11, day 15). Combined treatment with NM-3 and IR significantly reduced mean tumor volume at days 15 (P = 0.037), 17 (P = 0.019), 19 (P = 0.039), 21 (P = 0.014), and 23 (P = 0.004) when compared with IR treatment alone (Fig. 4B). The combined treatment group demonstrated a growth delay of 14 days compared with the control group and 3 days compared with IR-alone group.

In experiments conducted using SQ-20B tumors (initial tumor volume = 681.8 ± 17.2 mm³; n = 45), control tumors grew to 5.2 ± 0.94 times original volume by day 20. A similar pattern of tumor growth was observed in the NM-3-alone treatment group (4.3 ± 1.03, day 20). Animals in these two groups were subsequently sacrificed because of tumor burden. Tumors in the IR treatment group (65 Gy) doubled in size by day 6 and regressed to 50% of original volume at day 23. By day 38, IR-treated tumors regrew to twice original volume. Animals treated with NM-3 and IR doubled in size by day 6 and then regressed to 77% of original volume at day 23. Unlike the tumors treated with IR alone, tumors receiving combined therapy with NM-3 and IR failed to reach original volume by day 38 (P = 0.006; t test; data not shown).

Experiments were next conducted to confirm that growth delay translates to local control in a multifractional experiment. C57BL/6 mice bearing LLC tumors (initial volume, 151.1 ± 4.6 mm³; n = 29) were treated with 50 mg/kg/day of NM-3 and 66 Gy over 5 consecutive days. Tumors in the control group grew to 11.83 ± 1.1 times original volume by day 7. Tumors in the IR and NM-3/IR groups grew initially, but began regressing on day 7 (IR = 1.36 ± 0.12 times original volume and NM-3/IR = 1.08 ± 0.08 times original volume; Fig. 4C). On day 21 after initiation of treatment, no measurable tumor was present in 5 of 12 mice in the IR-alone group. However, when animals were treated with NM-3 plus IR, 9 of 11 appeared to be tumor-free (P = 0.089 by Fisher’s exact test). The day 21 time point was chosen in these studies because, after this time, mice in both treatment groups began dying of lung metastases.
We report that a new isocoumarin, NM-3, increases the antitumor effects of radiotherapy. In this context, selective antitumor effects are achieved with NM-3 and IR without adverse normal tissue effects. These findings indicate that standard risk/benefit analyses may not be relevant to the investigation of treatment with NM-3 and IR.

The present results describe a new paradigm in which a nontoxic agent potentiates the effects of IR. In this context, selective antitumor effects are achieved with NM-3 and IR without adverse normal tissue effects. This favorable therapeutic index is attributable to the selective effects of NM-3 on the tumor vasculature.

The mechanism by which NM-3 exerts antitumor effects is not known at this time. However, our in vitro data suggest that NM-3 alters several stages of the angiogenic process including endothelial cell survival, migration, and tube formation. Studies are under way in our laboratory to elucidate the pathways underlying interaction between NM-3 and IR.

An important focus of current clinical radiotherapy is selective targeting of tumors. This approach relies on physical techniques to achieve high doses of radiation to the tumor. Examples are intensity-modulated radiotherapy and stereotactic radiotherapy. Improved tumor control depends on radiation dose escalation. However, the application of dose escalation is limited to relatively small tumors or larger tumors in which normal tissues in the field can tolerate the high radiation doses.

Another current clinical strategy to enhance radiocurability is the use of chemical modifiers. One class of chemical modifiers is proposed to directly radiosensitize hypoxic tumor cells (29). However, the available hypoxic tumor cell sensitizers possess significant toxicity and have yet to yield positive clinical results. A third strategy of radiosensitization is the use of standard cytotoxins as chemical modifiers of the radiation response. These agents are proposed to directly sensitize tumor cells to IR. Clinical protocols combine IR treatment with cisplatinum, mitomycin C, 5-fluorouracil, hydroxyurea, or combinations of these agents. Although these drugs are likely to be additive with radiation in most clinical circumstances, they have improved cures in head and neck cancer, lung cancer, carcinoma of the cervix, and anal cancer (14–19). Clinical results from these combined modality trials reinforce the concept that increased local control increases overall cures. However, these combination regimens have considerable acute tissue toxicities, which lead to patient deaths, morbidity, and increased cost of cancer care when compared with treatment with radiotherapy alone.

The present results describe a new paradigm in which a nontoxic agent potentiates the effects of IR. In this context, selective antitumor effects are achieved with NM-3 and IR without adverse normal tissue effects. These findings indicate that standard risk/benefit analyses may not be relevant to the investigation of treatment with NM-3 and IR.
NM-3 increases antitumor effects of radiotherapy

IR because NM-3 has no known normal tissue toxicity and little antitumor effect alone but enhances the antitumor effects of IR. The combination of NM-3 with radiotherapy improves both tumor growth delay and radiocurability. In vitro assays of angiogenesis support mechanisms of NM-3/IR interaction through increased endothelial cell killing and decreased migration. These data are supported by reports of protein fragment angiogenesis inhibitors that potentiate radiotherapy and strengthen the concept of targeting both the tumor vasculature and tumor cells with IR.

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