Evaluation of D-Methionine as a Novel Oral Radiation Protector for Prevention of Mucositis

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Abstract
Purpose: Oral mucositis is a common acute morbidity associated with radiation and/or chemotherapy treatment for cancer. D-Methionine (D-Met), the dextrro-isomer of the common amino acid L-methionine, has been documented to protect normal tissues from a diverse array of oxidative insults.
Experimental Design: We evaluated if D-Met could selectively prevent radiation-induced oral mucositis using in vitro cell culture models as well as an in vivo model of radiation injury to the oral mucosa in C3H mice. Results: Unlike free-radical scavengers, which protected both normal and transformed tumor cells in vitro from radiation-induced cell death, treatment with D-Met in culture protected nontransformed primary human cells from radiation-induced cell death (protective factor between 1.2 and 1.6; P < 0.05) whereas it did not confer a similar protection on transformed tumor cells. D-Met treatment also provided significant protection to normal human fibroblasts, but not to tumor cell lines, from radiation-induced loss of clonogenicity (protection factor, 1.6 ± 0.15). D-Met treatment did not alter DNA damage (as measured by histone phosphorylation) following irradiation but seemed to selectively mitigate the loss of mitochondrial membrane potential in nontransformed cells, whereas it did not provide a similar protection to tumor cells. Tumor control of implanted xenografts treated with radiation or concurrent cisplatin and radiation was not altered by D-Met treatment. Pharmacokinetics following administration of a liquid suspension of D-Met in rats showed 68% bioavailability relative to i.v. administration. Finally, in a murine model of mucositis, a dose-dependent increase in protection was observed with the protective factor increasing from 1.6 to 2.6 over a range of oral D-Met doses between 200 and 500 mg/kg (P < 0.0003).
Conclusions: D-Met protected normal tissues, but not tumor cells, in culture from radiation-induced cell death; it also protected normal cells from radiation-induced mucosal injury in a murine model but did not alter tumor response to therapy. Further studies on the use of D-Met to protect from oral mucositis are warranted.

The combination of chemotherapy and radiation therapy has a common role in the treatment of squamous cell cancer of the head and neck either definitively or in the postoperative setting (1). However, using this treatment, severe oral mucositis, which is a painful, debilitating, and dose-limiting side effect of radiation and/or chemotherapy, is common (2). Pain, loss of taste, dysphagia, and ulceration of the oral mucosa are common and almost universal complaints in patients receiving external radiation for head and neck cancer, which add to the cost, morbidity, and mortality of treatment (3, 4).

With the improved ability to manage other cytotoxicity-related side effects, oral mucositis has become one of the major dose-limiting toxicities of chemotherapy and radiation therapy (5, 6). Mucositis is considered to be an inevitable but transient side effect of therapeutic head and neck radiation (7–9), afflicting at least 50% of all patients receiving radiation therapy for head and neck cancer. The incidence and severity of oral mucositis are strongly related to dose, fraction size, radiation portals, fractionation, and type of ionizing irradiation (10, 11). In addition, severe and persistent acute mucositis may also contribute to consequential late toxicity (10).

The development of an effective pharmacologic treatment for the prevention of oral mucositis has been elusive (for review, see ref. 12). There are more than 50 published studies documenting investigations aimed at the prevention or reduction of oral mucositis. The range of medications used is...
extensive, including topical antimicrobials (13–15), narrow-stimulating cytokines (16–20), keratinocyte growth factor (21–23), inflammatory modifiers (24–26), palliative rinses (27), glutamine supplements (28), amifostine (29, 30), cryotherapy (31), and laser treatment (32). To date, keratinocyte growth factor has shown the most promise, with significant protection from oral mucositis observed when administered along with a combined total body radiation and chemotherapy regimen before bone marrow transplant (23). Unfortunately, none of these agents has been clearly shown to be effective in preventing oral mucositis from radiation therapy for head and neck cancer although trials are still planned or ongoing for a number of them.

D-Methionine (D-Met) is the dextro-isomer of the essential amino acid L-methionine (L-Met). Biochemically, methionine acts as a synthetic substrate and may enhance the intracellular production of the key antioxidant reduced glutathione (GSH; ref. 33). D-Met also selectively increases mitochondrial GSH, an effect that can prevent oxidative stress–induced apoptosis (34, 35). D-Met may also have secondary effects on other endogenous cellular antioxidants including superoxide dismutase and catalase (33, 34, 36) or may be altering the activity of the ion transport mechanisms within the cell (37). Further, D-Met delivered topically, i.v., i.p., or orally has been studied as a protector from ototoxicity following cisplatinum-, amino-glycoside-, or noise-induced injury in a number of animal models (for review, see ref. 36).

Clinically, D-Met has adequate bioavailability and no reported side effects in human studies (38–40). The D-isomer has a longer half-life and enhanced bioavailability as compared with L-Met, and it has been suggested that D-Met itself is not toxic unless it is converted to the L-isomer (41). In addition, 60% to 70% of D-Met is excreted without conversion to the L-isomer (42–46). D/L-Met is approved for use therapeutically L-isomer (42–46). D/L-Met is approved for use therapeutically (41–45). D/Met delivered topically, i.v., i.p., or orally has been studied as a protector from ototoxicity following cisplatinum-, amino-glycoside-, or noise-induced injury in a number of animal models (for review, see ref. 36).

Therefore, given the mounting evidence that D-Met can protect from reactive oxygen species–mediated injury to normal tissues and its long-term safe oral use in humans, we investigated, using preclinical models, whether D-Met treatment could be used as a radiation protector from oral mucositis following radiation therapy.

### Materials and Methods

**Cell culture.** The following cell lines were used: primary human keratinocytes (HeKn; Cascade Biologics), primary human fibroblasts and primary human umbilical vein endothelial cells (Clonetics), human salivary gland cells (HSG; kindly provided by Dr. Bruce I. Baum, NIH, Bethesda, MD), murine squamous cell carcinoma cells (SCC VII), human squamous cell carcinoma cell lines (UMSCC1, UMSCC6, and UMSCC11B, all obtained from T.E. Carey, The University of Michigan, Ann Arbor, MI), Chinese hamster lung fibroblasts (V79), human colon carcinoma cell lines (LoVo and HT29), and a human lung carcinoma cell line (A549; all obtained from American Type Culture Collection). HeKn cells were cultured in serum-free Epilife medium supplemented with antibiotics and human keratinocyte growth factors at 37°C in 5% CO2. Human umbilical vein endothelial cells were grown in endothelial cell growth medium EGM-2 supplemented with growth factors. Both HeKn and human umbilical vein endothelial cells were used between passages 2 and 4. The SCC VII, HSG, V79, UMSCC6, UMSCC1, and UMSCC11B cell lines were maintained in DMEM, and HT29, A549 and Lovo cells in RPMI 1640 containing 10% heat inactivated FCS, 1% nonessential amino acids, 1% l-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO2.

**Radiation protectors.** N-Acetyl-cysteine (NAC), glutathione, D-Met, and L-Met were all obtained from Sigma-Aldrich; each was formulated as a 1,000X stock solution in PBS prepared fresh daily and cells were treated at the indicated concentrations. For in vivo studies, the D-Met solution was filter sterilized through a 0.2-µm filter before either i.p. or i.v. injection. For oral administration, a high-concentration (200 mg/mL) suspension of D-Met (MRx-1024) was obtained from MRx Therapeutics, Inc.

**Radiation treatment.** Cells or animals were irradiated using the University of Michigan Experimental Radiation Core at room temperature using a Pantak DXT300 orthovoltage unit at a dose rate of ~3 Gy/min. Dosimetry was carried out using an ionization chamber directly traceable to the National Institute of Standards and Technology calibration.

**Trypan blue assay.** Eighteen to twenty-four hours before radiation, cells were seeded in 6-cm dishes to achieve 20% to 30% confluence at the time of radiation. Cells were irradiated alone, with a 1-h pretreatment with the indicated radiation protector or with the drug applied within the first 15 min after irradiation. To assess cell viability at the indicated time points, floating cells were removed and retained and the plates were then trypsinized. Both adherent and floating cells were subjected to centrifugation (1,000 × g, 3 min) and resuspended in 50-µL PBS containing trypan blue (0.4% solution; Sigma). Cell viability was manually counted with a hemocytometer and is reflected as the number of cells excluding trypan blue as a percentage of total numbers of cells counted. A minimum of 200 cells were counted from each sample and the results were based on three independent experiments. Results are plotted as average viable cells (±SE) with differences between groups determined via a two-tailed Student’s t test. Radiation-protective factors for each of the drugs were determined at day 5 after radiation as the ratio of cell viability with drug treatment and radiation compared with radiation alone.

**Clonogenic cell survival assay.** Clonogenic assays were done using standard techniques with cells subcultured at clonal density immediately after irradiation (48). Cell survival curves were fitted using the linear quadratic equation, and the mean inactivation dose was calculated according to the method of Fertl et al. (49). The radiation protection factor (PF) was calculated by dividing the mean inactivation dose for D-Met–treated cells by that obtained for control treated cells. A PF > 1 indicates radiation protection.

**H2A.X staining.** V79 cells were plated 18 to 24 h before treatment with PBS, D-Met (1 mg/mL), NAC (5 mmol/L), or glutathione (10 mmol/L) for 1 h before irradiation. Cells were treated with 3-Cy ionizing radiation and, within 10 min, cell extracts were prepared on ice as previously described (50), fractionated by SDS-PAGE on a 12% Bis-Tris gel (Invitrogen), and electrobolted onto polyvinylidene difluoride membranes. Membranes were then probed with a monoclonal anti-phospho-histone H2A.X (Ser139), clone JBW301 (Upstate/Millipore), or with an antibody against α-actin (Thermo-Fischer) followed by the appropriate horseradish peroxidase–conjugated secondary antibody (Thermo-Fischer) before visualization by enhanced chemiluminescence (Thermo-Fischer). Bands were quantified using NIH Image and data expressed as relative expression compared with actin control.

**Mitochondrial transmembrane potential (ψm).** MitoLight (Chemicon Int.) was used to detect mitochondrial membrane potential (ψm) in HSG, HeKn, LoVo, and HT29 cells. Following treatment, the cells were stained with MitoLight (according to the manufacturer’s instructions) for 15 min at 37°C in the dark, washed with 1 × PBS, and viewed at 2, 4, and 6 h postirradiation by fluorescence microscopy. The percentage of cells with retained mitochondrial membrane potential (scored as cells having >10 red/orange staining foci per cell) was counted as compared with nonviable cells. At least 300 cells were counted for each
condition, and experiments were done in triplicate. The numbers plotted are the averages ± SE.

**Acute lip reaction assay.** Animals were handled according to the established procedures of the University of Michigan Laboratory Animals Maintenance Manual. The experiments were carried out using 7- to 8-wk-old female C3H mice (Charles River Laboratories). The animals were kept, six per cage, in conventional housing. Access to food and water was not restricted. Mice undergoing lip irradiation were fed soft food. Mice were treated with mouth radiation alone or in combination with D-Met treatment as indicated. Radiation was delivered in five consecutive daily fractions (days 1-5 with fractions of 6 Gy). Mice were anesthetized by i.p. injection of a mixture consisting of 9 mg/kg xylazine (Bayer Co.) and 56 mg/kg ketamine (Fort Dodge Laboratories, Inc.). Six mice were placed supine on a specially made plastic and foam mold and the entire body was covered by a 6 half-value layer lead shield, except the anterior portion of the snout. The mouths of all six mice were irradiated in a single field. Before utilization, thermoluminescent dosimetry was used to ensure that the dose delivered to each area within the setup was uniform (±2%). Mice were randomly allocated to each of the six radiation slots. The following series of conditions were assessed in two separate experiments with six mice in each group, and the control and radiated groups duplicated for each experiment: (a) control (no treatment); (b) radiation (6 Gy daily for 5 d); (c) D-Met i.v. (300 mg/kg daily) + radiation; (d) D-Met i.p. (300 mg/kg daily) + radiation; and (e) D-Met oral (200, 300, or 500 mg/kg daily).

Lip toxicity was scored daily according to a standard scoring system (ref. 51; see Supplementary Table S2) from 0 to 7 by two independent observers who were blinded as to which treatment the mice received. Scores from each observer were averaged as were the scores across each group. Data were plotted as the mean ± SE of the erythema score daily for 14 d starting with the first day of treatment. Differences between treated groups relative to control or i.v. administration of the drug were determined using Student’s t test (two-tailed) using Microsoft Excel (Microsoft), with P < 0.05 considered as significant. Radiation protection was reported as a protective factor (PF) for peak erythema score [PF = (peak score for radiated animals) / (peak score for control animals)] or as a PF for the area under the curve (AUC) of erythema protection was reported as a protective factor (PF) for peak erythema score {PF = [(peak score for radiated animals) - (peak score for control animals)] / [(peak score for treated group) - (peak score for control animals)]} or as a PF for the area under the curve (AUC) of erythema score versus time. Kinetic analysis and AUCs were calculated using PK Functions for Microsoft Excel, a series of Add-in functions for Excel spreadsheets, designed and written by Joel I. Usansky, Atul Desai, and Diane Tang-Liu (Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA).

**Tumor models.** HT29 and A549 cell lines were used for tumor xenograft models in CD1 nu/nu mice (Charles River Laboratories) whereas C3H mice were used for SCCVII xenografts. Tumors were generated as previously described (52) by s.c. injection of 2 × 10⁶ viable cells in the right flank of each mouse. Tumors were measured with calipers in two dimensions every 2 to 3 d and treatment initiated once tumors had reached a volume of 100 mL. Tumor volumes were calculated as π/6(length × width²). Data are expressed as the average tumor volume (±SE) relative to volume at the start of treatment. Radiation therapy was delivered using daily fractions of between 2 and 6 Gy (as indicated for each experiment) with 6 half-value layers of lead placed over the animal with a cut-out over the tumor. Animals were anesthetized daily with ketamine/xylazine for radiation therapy.

SCCVII tumor–bearing C3H mice were treated for 7 d with one of the following five treatments: (a) PBS, (b) D-Met (300 mg/kg i.p.); (b) radiation (6 Gy for 7 daily fractions); (c) D-Met administered i.p. 1 h before irradiation; or (d) D-Met administered i.p. 1 h postirradiation. In a separate experiment, SCCVII tumor–bearing C3H mice were treated for 5 d with (a) PBS, (b) radiation (4 Gy for 5 daily fractions) with cisplatin (2.4 mg/kg daily i.p. 2 h before radiation), or (c) radiation + cisplatin + D-Met (300 mg/kg daily orally) administered 1 h before radiation.

HT29 and A549 tumor–bearing animals were treated with (a) PBS, (b) radiation (2 Gy for 10 fractions over 12 d), or (c) radiation + D-Met (300 mg/kg i.p. daily 1 h before radiation).

**Pharmacokinetics.** The i.v. and oral pharmacokinetic profiles of a single dose of D-Met (150 mg/kg i.v., 150 mg/kg orally, or 300 mg/kg orally) were determined using male Sprague-Dawley rats. Blood samples were collected at predetermined times and the plasma was analyzed for D-Met using a high-performance liquid chromatography assay as described below. On the day of treatment, D-Met was administered i.v. to groups (n = 3/group) of rats as a bolus injection in the tail vein or by oral gavage. Blood samples (~0.5 mL/sample) were collected from three animals per time point by tail artery puncture into 500-μL lavender top heparinized tubes. Collection times for i.v. administration were before administration and 0.03, 0.25, 0.5, 1, 2, 4, and 6 h postadministration. The collection times for oral administration were the same, except the first time point (0.03 h) was not used.

The blood was centrifuged immediately and the resulting plasma stored in Eppendorf tubes frozen at -80°C until analysis. Plasma samples were derivatized with 100 μL of 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (Marley’s reagent, Sigma-Aldrich), 40 μL of 1 mol/L sodium bicarbonate, and 1 mL of acetone, and incubated at 50°C for 4 h. Following incubation, 20 μL of 2 mol/L hydrochloric acid were added and the mixtures were centrifuged, following which an aliquot of the supernatant was transferred to a clean test tube and evaporated under nitrogen. The residue was reconstituted in 250 μL of 50 mmol/L Tris buffer (pH 7.0) and derivatized D-Met was detected by UV at 340 nm. Standard concentrations used were from 1 to 200 μg/mL. Pharmacokinetic data were analyzed as indicated above.

**Results**

**Selective protection of normal cells in culture.** Given the previous evidence suggesting that D-Met could provide protection in animal models against hearing loss resulting from cisplatinum-, aminoglycoside-, or noise-induced injury (36, 37, 53–55), we investigated if D-Met could also protect normal cells from radiation-induced cell death. Primary human cell cultures or transformed tumor cell lines were incubated with D-Met or representative antioxidants, which have previously been studied as radiation protectors (NAC or GSH), with each drug administered either 1 h before radiation or within 15 minutes after a single 10-Gy fraction. Control treated primary keratinocytes (Fig. 1A) maintained 94.5 ± 0.5% viability for up to 5 days, which was markedly reduced by radiation treatment (57.5 ± 1.0% viability; P < 0.03, compared with control). However, this loss of viability was attenuated by either pretreatment (73.8 ± 2.4%; P < 0.04, compared with radiated) or posttreatment (73.0 ± 3.1%; P < 0.01, compared with radiated) with D-Met (1 mg/mL). Overall, this led to a radiation-protective factor (PF), either before or after treatment with D-Met, of 1.3 ± 0.08. Treatment of primary human keratinocytes with either NAC (2 mmol/L; Fig. 1B) or GSH (5 mmol/L; Fig. 1C) before or after radiation treatment in an identical treatment paradigm resulted in a similar degree of radiation protection with PF of 1.4 ± 0.10 for NAC (P < 0.02, compared with irradiated) and 1.2 ± 0.05 for glutathione (P < 0.01, compared with irradiated). Similar radiation
protection was also observed for D-Met using other nontransformed human cells (human umbilical vein endothelial cells, PF 1.2 ± 0.05; fibroblasts, PF 1.3 ± 0.03; or HSG cells, PF 1.7 ± 0.15; data not shown).

Because D-Met provided radiation protection in nontransformed cells, we next evaluated the protective ability of D-Met and other radiation protectors in tumor cell lines. A transformed human squamous cell tumor cell line (UMSCC6) was treated with radiation with or without D-Met, NAC, or GSH. Five days following ionizing radiation, UMSCC6 cell viability decreased to 49.0 ± 3.0%, which was reduced compared with control cells, which were 96.0 ± 0.6% viable at this time point (P < 0.003, irradiated compared with control). Cells treated with D-Met and radiation (Fig. 1D) had viability that was indistinguishable from that of cells that received only radiation whether or not D-Met was present at the time of irradiation (viability, 45.0 ± 3.6%; P > 0.2, compared with radiated). In each case, D-Met had a PF of 0.98 ± 0.04, which was indistinguishable from unity. Although D-Met did not protect UMSCC6 cells from radiation-induced loss of viability, both NAC and GSH were protective, with PF of 1.2 ± 0.07 (P < 0.03, compared with irradiated; Fig. 1E) and 1.3 ± 0.04 (P < 0.02, compared with radiated; Fig. 1F), respectively.

A similar lack of radiation protection by D-Met for tumor cells was observed in a panel of transformed tumor cell lines that were all tested under similar conditions, including SCCVII (Supplementary Fig. S1A), UMSCC1 (Supplementary Fig. S1B), UMSCC11b (Supplementary Fig. S1C), HT29 (Supplementary Fig. S1D), LoVo (data not shown), and A549 (data not shown). In each case, there was no difference in cell viability with D-Met treatment compared with radiation-treated cells (P > 0.100 in all cases). However, treatment with NAC or GSH provided significant radiation protection in each of the cell lines tested (data not shown).
Colony formation assay. The standard assay used to assess radiation-mediated cell death is the colony formation assay, which measures not only short-term response to a radiation treatment (i.e., apoptosis) but also long-term reproductive viability. Colony formation assays could not be reproducibly done for normal human keratinocytes due to difficulties with colony growth at clonal density. Therefore, primary human fibroblasts were instead assessed, and pretreatment with D-Met (1 mg/mL) for 1 hour before radiation treatment resulted in a significant increase in cell survival, with a protective factor (PF) of $1.6 \pm 0.15$ (Fig. 2A). In contrast, a series of tumor cells were also assessed, and in each case there was no apparent radiation protection afforded by D-Met treatment using SCCVII (PF = $0.94 \pm 0.10$; Fig. 2B), UMSCC1 (PF = $1.1 \pm 0.15$; Fig. 2C), or UMSCC6 (PF = $0.97 \pm 0.07$; Fig. 2D) cells. The lack of D-Met–mediated protection of tumor cells by colony formation assay supports the selective radiation protection observed in trypan blue assays.

Assessment of DNA damage response to ionizing radiation. Having established the ability of D-Met to confer protection to nontransformed human cells in culture, but not to a panel of human tumor cell lines, we next investigated the mechanism of radiation protection that might explain the differences observed between D-Met, NAC, and GSH. Following treatment with ionizing radiation, the predominant lethal effect is due to the generation of DNA double-strand breaks, and the phosphorylation of histone H2AX can be used as a surrogate for this event. V79 cells (Chinese hamster lung fibroblasts), which have low endogenous phosphorylation of histone H2AX, were assessed to determine if there was an alteration of this phosphorylation following ionizing radiation. As can be seen in Fig. 3A, by Western blot analysis, there was low basal phosphorylation of histone H2AX before irradiation; however, 3-Gy ionizing radiation caused an increase in H2AX phosphorylation status that was not altered by pretreatment with D-Met. In contrast, in cells pretreated with either NAC or GSH, there was a partial suppression of the radiation-associated increase in H2AX phosphorylation.

Figure 3A represents a single representative example, whereas in Fig. 3B gel densitometry was used to quantify the change in H2AX phosphorylation ($n = 3$ experiments). Following irradiation, there was an increase in phosphorylation in all groups that was statistically elevated compared with control ($P < 0.01$ for all groups). However, in cells treated with D-Met before irradiation, there was no difference in the relative induction of H2AX phosphorylation compared with cells that only received irradiation (16.6 \pm 3.6-fold for X-ray therapy alone versus 16.0 \pm 2.4-fold for X-ray therapy + D-Met; $P > 0.5$). In contrast, following pretreatment with NAC or GSH, there was a dampening of the relative induction of H2AX phosphorylation compared with irradiated cells [5.9 \pm 1.4-fold for NAC ($P < 0.03$) and 8.3 \pm 0.7-fold for GSH ($P < 0.01$)].
Mitochondrial membrane potential. Because D-Met did not alter the initial DNA damage event, we next investigated downstream signals that are associated with radiation-induced cell death. Previously, we showed that in both solid tumor and leukemic tumor cell lines, following ionizing radiation there is a loss of mitochondrial membrane potential ($\psi_m$) before radiation-mediated cell death (56). To assess the effect of D-Met treatment on this process, nontransformed cells (keratinocytes and HSG cells) or transformed tumor cells (LoVo and HT29) were irradiated with 10 Gy and then mitochondrial function was evaluated. Untreated keratinocytes and LoVo cells maintained an intact mitochondrial membrane potential throughout the course of the experiment, visualized as red/orange fluorescing cells (Fig. 4A and B, left columns). In addition, in both cell lines, following irradiation there was a significant reduction in the number of cells that maintained functional mitochondria (resulting in loss of red/orange staining and instead almost uniform green staining Fig. 4A and B, middle columns). Finally, when pretreated with D-Met (1 mg/mL) for 1 hour before irradiation, there was a significant protection of mitochondrial function in keratinocytes (Fig. 4A, right column), which was not observed in LoVo cells (Fig. 4B, right column). Quantitative scoring of keratinocytes (Fig. 4C) revealed only 28.9 ± 1.7% of cells with intact $\psi_m$ 6 hours after ionizing radiation as compared with 97 ± 1.0% in control cells ($P < 0.001$), whereas in D-Met– and radiation-treated cells, 66 ± 1.7% of cells still had functional mitochondria at this time point ($P < 0.001$, compared with radiation only–treated cells). In contrast, following ionizing radiation, there was an almost complete loss of $\psi_m$ in both LoVo and HT29 cells by 6 hours and there was no significant protection of $\psi_m$ in either LoVo
pretreatment with D-Met was evaluated. A, SCCVII tumor-bearing mice treated with mock irradiation (○), D-Met alone (■), or fractionated irradiation alone (6 Gy × 7 fractions; □); pretreated with D-Met i.p. daily before irradiation (▲); or posttreated with D-Met i.p. daily after irradiation (▲). B, animals bearing HT29 tumors were treated in an analogous fashion although only daily pretreatment with D-Met was evaluated. C, animals bearing A549 tumors. For HT29 and A549 tumors, 10 daily fractions of 2 Gy were used. D, SCCVII tumors were treated with control (○), daily cisplatin and radiation (4 Gy × 5 fractions with cisplatin 2.4 mg/kg daily; △), or daily cisplatin, radiation, and oral D-Met (●). Points, mean relative tumor volume of five to seven animals per group; bars, SE.

We next assessed if D-Met would interfere with chemoradiation therapy–mediated tumor control (Fig. 5D). SCCVII flank tumors were again established in mice and treated with PBS alone, cisplatin + radiation, or cisplatin + radiation + D-Met. As seen previously for SCCVII tumors, the control treated animals exhibited rapid tumor growth, reaching an average volume six to seven times the starting volume within 14 days after the start of the experiment. The combination of radiation and chemotherapy caused a substantial regression of tumors that reached a nadir that was 70% the starting volume 8 days after the start of treatment, after which the tumors slowly regrew (P < 0.004, compared with control). Finally, daily pretreatment with D-Met seemed to have no effect either on tumor regression following concurrent chemoradiotherapy or on the eventual regrowth of tumors following the completion of treatment (P > 0.10, both for absolute nadir reached and for final tumor volume).

**Pharmacokinetics of D-Met in rats.** An ideal radiation protector would be orally bioavailable and not require daily i.v. or s.c. injection. Therefore, a high-concentration suspension formulation of D-Met was used to test the pharmacokinetics of oral D-Met administered as compared with i.v. administration. Following an i.v. dose of D-Met (150 mg/kg), plasma concentrations of the compound peaked at the first time point (470 μg/mL) and then declined in a multieponential fashion with a terminal half-life of 0.9 hours (Supplementary Table S1; Fig. 6). After an oral dose (150 or 300 mg/kg), D-Met was rapidly absorbed, reaching a peak plasma concentration 30 minutes after administration of 71 and 175 μg/mL, respectively (Supplementary Table S1; Fig. 6); the terminal half-life did not differ by route of administration. The AUC0-inf following oral administration of 300 mg/kg D-Met was 356.1 (μg h/mL),
which corresponds to a bioavailability of 68% compared with i.v. administration. In addition, following oral administration, both peak concentration and AUC were proportional to dose.

Protection from radiation-induced damage to normal mucosa.

Given its potential role as a protector from normal tissue injury, we finally investigated if D-Met could also protect from radiation-induced oral mucositis in a mouse model (51, 57, 58). Irradiation of mice with 6 Gy daily for 5 days resulted in mucositis (reddening, swelling, desquamation of the lips) compared with control animals, as scored using a previously reported standardized scale (Fig. 7A; Supplementary Table S2). A peak mucositis score of 3.5 ± 0.25 on a scale from 0 to 7 was observed 12 days after the start of radiation treatment and was elevated compared with control animals (peak value for control of 0.4 ± 0.2; *P* < 0.001). Pretreatment of animals with 300 mg/kg D-Met by i.v., i.p., or oral administration did not alter the timing for the onset of mucositis but did decrease both the peak value (all with peak <1.5 units compared with 3.5 in irradiated animals) and the area under the toxicity versus time curve (AUC) compared with animals treated with radiation alone (*P* < 0.03, for each group compared with irradiated). To evaluate the dose-dependent nature of protection using oral D-Met, the model was also evaluated with daily oral administration of D-Met at 200 and 500 mg/kg (Fig. 7B). Qualitatively, the higher-dose treatment resulted in increased protection when compared with the lower dose; however, to assess these differences more quantitatively, radiation-protective factors were calculated for each treatment group (Fig. 7C). At the uniform dose of 300 mg/kg, protective factors ranged from 2.0 for oral administration to 3.3 for i.v. administration. Although there was no statistically significant difference in radiation PF between i.v., i.p., or oral administration when delivered at a dose of 300 mg/kg, there was a trend toward enhanced protection (i.v. > i.p. > oral) that did not achieve statistical significance (*P* > 0.05, i.p and oral treatments compared with i.v. treatment). However, the ratio of radiation protection for i.v. administration compared with oral administration of 61% was similar to the 68% bioavailability previously identified for oral administration. Finally, there was a dose-dependent increase in radiation protection observed when the three different oral doses were evaluated (200, 300, or 500 mg/kg) with PF for the AUC of erythema score of 1.64, 2.08, and 2.63, respectively (*P* < 0.0003, χ² test).

Conclusions

Oral mucositis is a significant problem following treatment with ionizing radiation, and the data presented here suggest that D-Met may provide protection from radiation-induced mucosal injury. Interestingly, although D-Met has been...
suggested to be a weak free-radical scavenger (59), it does not contain a free sulfhydryl group and as such may be acting in a different fashion than classic free radical scavengers (such as GSH or NAC, which both contain free sulfhydryl groups). This possibility is supported by the data presented herein that D-Met did not cause any reduction in the initial radiation-induced DNA damage whereas both NAC and GSH resulted in reductions in early H2AX phosphorylation, consistent with their mechanism of action as free radical scavengers. The lack of significant direct inhibition of DNA damage is also supported by the fact that when animals were pretreated with D-Met and p53 activation was monitored, using a validated transgenic bioluminescent model of radiation-induced p53 activity (50), there was no decrement in bioluminescence compared with animals treated with radiation alone. In contrast, amifostine, which does act as a free radical scavenger, did reduce p53 activation as measured by bioluminescence, which is supportive of an equivalent activation of p53 following ionizing radiation with or without D-Met treatment.7 The fact that D-Met may be acting in a mechanism independent of free radical scavenging may be particularly relevant for a recent large randomized trial that suggested that single or combination use of two different free radical scavengers (vitamin E and β-carotene) administered concurrently with radiation treatment for head and neck cancer resulted in a modest decrease in acute toxicity (60); however, this was at the expense of increased tumor recurrence (60) with a decrease in both cause-specific and overall survival (61) and an increase in second primary tumors of the head and neck (62).

Interestingly, in contrast to free radical scavengers, D-Met seems to provide selective protection to nontransformed human cells, but not to transformed murine and human tumor cell lines. The mechanism of this selective difference in radiation protection remains an open area of investigation; however, it does seem that D-Met may alter mitochondrial events in nontransformed cells following ionizing radiation, maintaining in radiation protection from the mitochondrial membrane potential, a phenomenon that does not seem to occur in tumor cell lines.

The events within the cell that mediate this difference also remain to be determined. However, we did observe that although D-Met does not alter the immediate generation of free radicals following ionizing radiation in either normal or transformed cells, it does seem to block a later burst of reactive oxygen species (56) in nontransformed human cells ~1 hour after radiation, an alteration that was not effected in transformed tumor cells (data not shown). This alteration in reactive oxygen species production from the mitochondria is related to increases in mitochondrial glutathione or subsequent alterations in other antioxidant pathways following methionine treatment, as has previously been suggested (33–35, 54). Interestingly, D-Met also altered the oxidative response to ionizing radiation therapy in zebra fish, which correlated both with increased survival and a decrease in neurotoxic injury (63).

Finally, the utility of D-Met as a radiation protector in vivo as compared with either L-Met or the racemic mixture is worth exploring. In culture, D-Met and L-Met provide a similar degree of protection from loss of cell viability to keratinocytes or fibroblasts following ionizing radiation (data not shown). However, it has previously been documented in both rodents and man that L-Met has a shorter half-life than D-Met, whereas D-Met is only partially metabolized to L-Met with the remainder secreted in unchanged form in the urine (46, 64). Evaluation of L-Met in the oral mucositis model described here provided statistically significant protection as compared with radiation alone (data not shown). However, consistent with previous pharmacokinetic evaluations, there was a 2-fold decrease in protection for orally administered L-Met in vivo when compared with D-Met. Therefore, the advantage observed for D-Met as compared with L-Met in terms of mucosal protection in vivo is likely not mechanistic but instead related to decreased clearance.

In conclusion, D-Met may represent a safe and orally available compound that can protect oral mucosa from radiation-induced cytotoxicity in a dose-dependent manner while potentially not providing any significant tumor protection. Further studies on the mechanism underlying the apparent ability of D-Met to selectively protect nontransformed cells from radiation-induced cell death are needed. In addition, evaluation of the pharmacokinetics and efficacy of D-Met to protect against radiation or chemoradiation-induced mucositis in patients undergoing treatment for squamous cell cancer of the head and neck may be warranted.

References
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