

Radiosensitization *In Vivo* by Histone Deacetylase Inhibition with No Increase in Early Normal Tissue Radiation Toxicity



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Abstract

As the population ages, more elderly patients require radiotherapy-based treatment for their pelvic malignancies, including muscle-invasive bladder cancer, as they are unfit for major surgery. Therefore, there is an urgent need to find radiosensitizing agents minimally toxic to normal tissues, including bowel and bladder, for such patients. We developed methods to determine normal tissue toxicity severity in intestine and bladder *in vivo*, using novel radiotherapy techniques on a small animal radiation research platform (SARRP). The effects of panobinostat on *in vivo* tumor growth delay were evaluated using subcutaneous xenografts in athymic nude mice. Panobinostat concentration levels in xenografts, plasma, and normal tissues were measured in CD1-nude mice. CD1-nude mice were treated with drug/irradiation combinations to assess acute normal tissue effects in small intestine using the intestinal crypt assay, and later effects in small and large

intestine at 11 weeks by stool assessment and at 12 weeks by histologic examination. *In vitro* effects of panobinostat were assessed by qPCR and of panobinostat, TMP195, and mocetinstat by clonogenic assay, and Western blot analysis. Panobinostat resulted in growth delay in RT112 bladder cancer xenografts but did not significantly increase acute (3.75 days) or 12 weeks' normal tissue radiation toxicity. Radiosensitization by panobinostat was effective in hypoxic bladder cancer cells and associated with class I HDAC inhibition, and protein downregulation of HDAC2 and MRE11. Pan-HDAC inhibition is a promising strategy for radiosensitization, but more selective agents may be more useful radiosensitizers clinically, resulting in fewer systemic side effects. *Mol Cancer Ther*; 17(2): 381–92. ©2017 AACR.

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Introduction

Muscle-invasive bladder cancer (MIBC) is a disease of an increasingly elderly population (1); Gray and colleagues (2) highlighted the underuse of aggressive therapy in over 80 year olds in the United States, and in the United Kingdom, other cause mortality accounts for only 34% of deaths in 80+-year-old MIBC patients (3). MIBC can be treated with radiotherapy or cystectomy with similar outcomes (1, 4, 5). Although radiotherapy is well tolerated by elderly patients, concurrent chemoradiation (CRT) is more effective (6–8). However, CRT causes systemic toxicities and

more severe bowel and bladder toxicity than radiotherapy alone; these side effects may be difficult for elderly patients to tolerate. As the population ages, new radiosensitizers, which are less toxic to normal tissues, and hence more suitable for CRT in elderly patients, are urgently needed.

Over the past decade, histone deacetylases (HDAC) have emerged as important cancer therapeutic targets. HDAC inhibitors are promising radiosensitizers in preclinical studies, and although in clinical studies combined treatment appears well tolerated acutely (9), data are lacking (especially for HDAC inhibitors in combination with radiotherapy) for longer term, particularly bowel, toxicities (10). HDAC expression is aberrant in multiple cancer types, including bladder cancer (11, 12). HDACs are classified as class I (HDAC1, 2, 3, and 8), IIa (HDAC4, 5, 7, and 9), IIb (HDAC6 and 10), III (sirtuins), and IV (HDAC11). Conventional HDAC inhibitors target only the Zn²⁺-dependent class I, II, and IV enzymes.

The pan-HDAC inhibitor panobinostat is one of the most potent HDAC inhibitors (13) and was recently reported in phase I trials in combination with radiotherapy in glioma and lung cancer. We previously found panobinostat to be an efficient radiosensitizer in the nanomolar range *in vitro* associated with downregulation of the DNA damage signaling proteins MRE11 and NBS1 and the homologous recombination (HR) protein RAD51 (14).

Here, we tested the hypothesis that panobinostat can spare normal tissues while being an effective tumor radiosensitizer

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in vivo. Panobinostat proved to be an efficient radiosensitizer in a bladder cancer xenograft model. However, in contrast to the known radiation modifier gemcitabine, clinically relevant doses of panobinostat did not increase the severity of acute normal tissue toxicity bowel surrounding mouse bladder irradiated using a small animal radiation research platform (SARRP). We also developed a model that can be used to investigate later normal tissue effects in large intestine and bladder, which could also be used to investigate other pelvic malignancies preclinically. Furthermore, we demonstrated that radiosensitization and effects on the MRE11–RAD50–NBS1 (MRN) complex were primarily class I mediated, using class-selective HDAC inhibitors and transient HDAC1 or 2 knockdown.

Materials and Methods

All animal work was done in accordance with UK Home Office Guidelines and institutional guidelines, approved by the University of Oxford Animal Welfare and Ethical Review Body (AWERB), under University of Oxford project licences PPL 30/2922 and 30/3172. Group sizes were chosen to detect large effect sizes.

Cell lines

RT112 cells were a gift from Margaret Knowles (University of Leeds, Leeds, United Kingdom; obtained 2009) and were authenticated by extensive genomic analysis (15) and used within 10 passages. CAL29 and T24 cell lines were purchased from DSMZ (in 2011) and used within 15 passages. A stably transfected Ku80 knockdown cell line (KuKD) was created from RT112 (in 2010) as described previously, and used up to passage 10 (16). RT112, T24, and KuKD, but not Cal29, cell lines were validated by short tandem repeat analysis in 2015 (DNA Diagnostics Centre). No traces of mycoplasma/bacteria were found in any of the cell lines by DAPI staining in 2016. All four cell lines were tested for mycoplasma in June 2017 by TOKU-E PCR Mycoplasma Detection Kit and found to be negative. Culture conditions are described in Supplementary Material.

Drugs and drug treatments

Panobinostat (for *in vitro* studies) and mocetinostat (17) were purchased from Selleck Chemicals. Gemcitabine was purchased from Accord Healthcare Ltd. TMP195 (18) was a generous gift from GlaxoSmithKline and, for *in vivo* experiments, panobinostat was a generous gift from Novartis.

Xenograft model for growth delay study

Cells were prepared in phenol red-free Matrigel (BD Biosciences) and RPMI medium 1:1, and 100 μ L (2×10^6 cells) injected into the flank of 6-week-old female athymic nude mice (Harlan Laboratories). Tumors were measured every second day with calipers. Xenograft volumes were calculated using ($a \times b \times c \times \pi/6$), and xenografts were allowed to reach 100 mm³ before randomizing mice into four groups and treating with: mock treatment [4% DMSO, 4% β -cyclodextrin in distilled water intraperitoneally; $n = 5$ RT112, $n = 4$ Ku knockdown (KuKD)], panobinostat (Novartis Pharmaceuticals, diluted in 4% DMSO, 4% β -cyclodextrin in water; $n = 7$ RT112, $n = 6$ KuKD) to a dose of 10 mg/kg, days 1, 3, and 5 intraperitoneally, daily irradiation [IR, 20 Gy in 5 fractions, 300 kV, using a Gulmay-320 cabinet irradiator (15); $n = 6$] or panobinostat + IR ($n = 6$).

Panobinostat concentration levels in plasma, xenografts, bladder, and intestines

Panobinostat concentrations were measured in CD1-nude mice bearing 100 mm³ RT112 xenografts as described in Supplementary Methods.

Normal tissue response models

Drug treatment alone. Six- to 8-week-old CD1-nude mice (Charles River Laboratories) were treated with mock treatment or panobinostat (10 mg/kg, intraperitoneally) for 2, 6, or 24 hours ($n = 1$ /group). A further 4 mice were treated similarly with mock treatment or gemcitabine (100 mg/kg, diluted in sterile water intraperitoneally). Bromodeoxyuridine (BrdUrd, 60 mg/kg, diluted in PBS) was injected intraperitoneally 30 minutes prior to sacrifice. The effects of drug on apoptosis and replication were assessed by Swiss roll as outlined in Supplementary Methods.

Drug/irradiation combinations. To assess the acute effects on surrounding normal tissues of adding a radiosensitizing drug to irradiation *in vivo*, 5- to 7-week-old CD1-nude mice were treated with mock treatment, panobinostat (10 mg/kg intraperitoneally) or gemcitabine (100 mg/kg intraperitoneally). Approximately 6 hours later, mice were treated supine with 10, 12, or 14 Gy to the lower abdomen, including lower small intestine, in a SARRP (Xstrahl) with 220 kV X-rays, 13 mA, half-value layer 0.794 mm Cu, using a 178-degree arc treatment with a 14-mm circular collimator. Mice were sacrificed at 3.75 days and tissue processed as described in Supplementary Methods.

For assessment of late normal tissue effects, mice were treated vertically, head down in the SARRP with 5 Gy daily over 5 days, using a 356-degree arc treatment and 10-mm collimator, with the isocenter positioned at the posterior caudal bladder wall, to avoid the small intestine, with some mice receiving panobinostat 10 mg/kg on days 1, 3, and 5. Eleven weeks later, mice were isolated for 24 hours and feces collected, weighed, counted, and measured. Following sacrifice at 12 weeks, the intestines were formalin fixed and paraffin embedded and examined histologically by a veterinary pathologist. Lesions were scored semiquantitatively on a scale of 0 to 5 (0: no lesion present; 5: entire tissue affected; ref. 19).

Crypt assay

H&E-stained Swiss roll slides were analyzed in a crypt assay modified for use in mice receiving partial abdominal irradiation, using Swiss rolls, as outlined in Supplementary Methods.

The percentage of surviving crypts was calculated as:

$$\frac{\text{Number of regenerating crypts per mm}}{\text{Number of control crypts per mm}} \times 100$$

Fluorescence slides of BrdUrd and cleaved caspase-3 were scanned using an Aperio FL digital pathology scanner (Leica). Twenty-five crypts from 4 samples under different time points were analyzed to determine how many cells in the crypt were BrdUrd or cleaved caspase-3 positive.

Clonogenic assays

Clonogenic assays were performed as described previously (15). Clonogenic survival assays under hypoxic conditions were performed as described by Pires and colleagues using an H34 Hypoxystation (Don Whitley Scientific; ref. 20). Briefly, cells were

seeded in 5-cm dishes, treated with DMSO or 25 nmol/L panobinostat 2 hours later, and incubated at 2% O₂ before IR. Cells were irradiated 24 hours later, and the medium replaced 15 minutes post-IR. Dishes were incubated for a further 12 days before staining and counting.

qPCR

Real-time qPCR was performed by $\Delta\Delta C_t$ method (see Supplementary Material for further details).

Western blots

Western blot samples were prepared as described previously (15, 16) with antibodies detailed in Supplementary Table S1 and details in Supplementary Material.

siRNA transfection

Cells (4×10^5) were plated 24 hours prior to treatment with 120 nmol/L siRNA (Invitrogen) or nonsilencing control (NSC). siRNA was combined with Oligofectamine (Invitrogen) and added to serum-free medium for 4 hours before adding medium containing 10% v/v FBS. Twenty-four hours later, cells were harvested for Western blot analysis or trypsinized and replated for clonogenic assay.

HR assays

The I-Sce1 assay was performed as in ref. 21 and a PCR-based method as per manufacturer's instructions (see Supplementary Material for further details).

Statistical analysis

GraphPad Prism 6.0 was used for all statistical analyses, with results represented as mean and SEM. A two-way ANOVA with Bonferroni multiple comparison was used to analyze clonogenic survival curves. A one-way ANOVA with Bonferroni correction or Tukey multiple comparison was used to compare more than two samples; a two-tailed unpaired Student *t* test was used to compare two samples. The Kaplan–Meier method was used to present time to tumor trebling with log-rank (Mantel–Cox) used to test significance.

Results

Panobinostat increases growth delay in irradiated bladder cancer cell xenografts

To test panobinostat as a radiosensitizer *in vivo*, RT112 cells were injected into the flank of athymic nude mice, and mice were treated once xenografts reached 100 mm³ (Fig. 1A). The Kaplan–Meier curve for time to treble tumor volume was significantly prolonged in the panobinostat + IR group compared with IR ($P = 0.007$, Fig. 1B; Supplementary Fig. S1A). The loss in body weight from baseline for mice treated with panobinostat + IR was significantly greater than that for IR alone ($P = 0.03$), but this was short lived (Supplementary Fig. S1B).

There was no significant difference in the observed transient body weight reduction between IR and panobinostat + IR in xenografts derived from 30% KuKD bladder cancer cells (Supplementary Fig. S1C and S1D). These grew significantly more slowly than the parental RT112 xenografts ($P < 0.001$) but displayed a striking sensitivity to radiation, and with panobinostat + IR, there was only a 1.3-fold increase in growth in 55 days (Supplementary Fig. S1E).

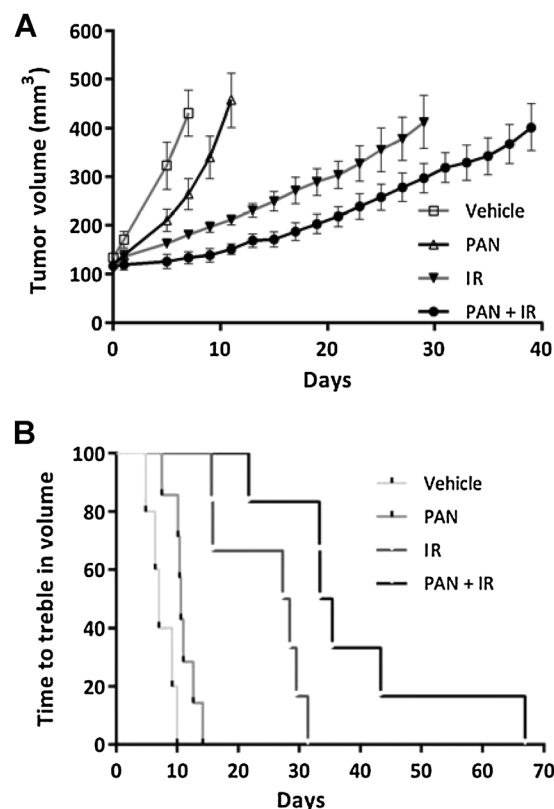


Figure 1.

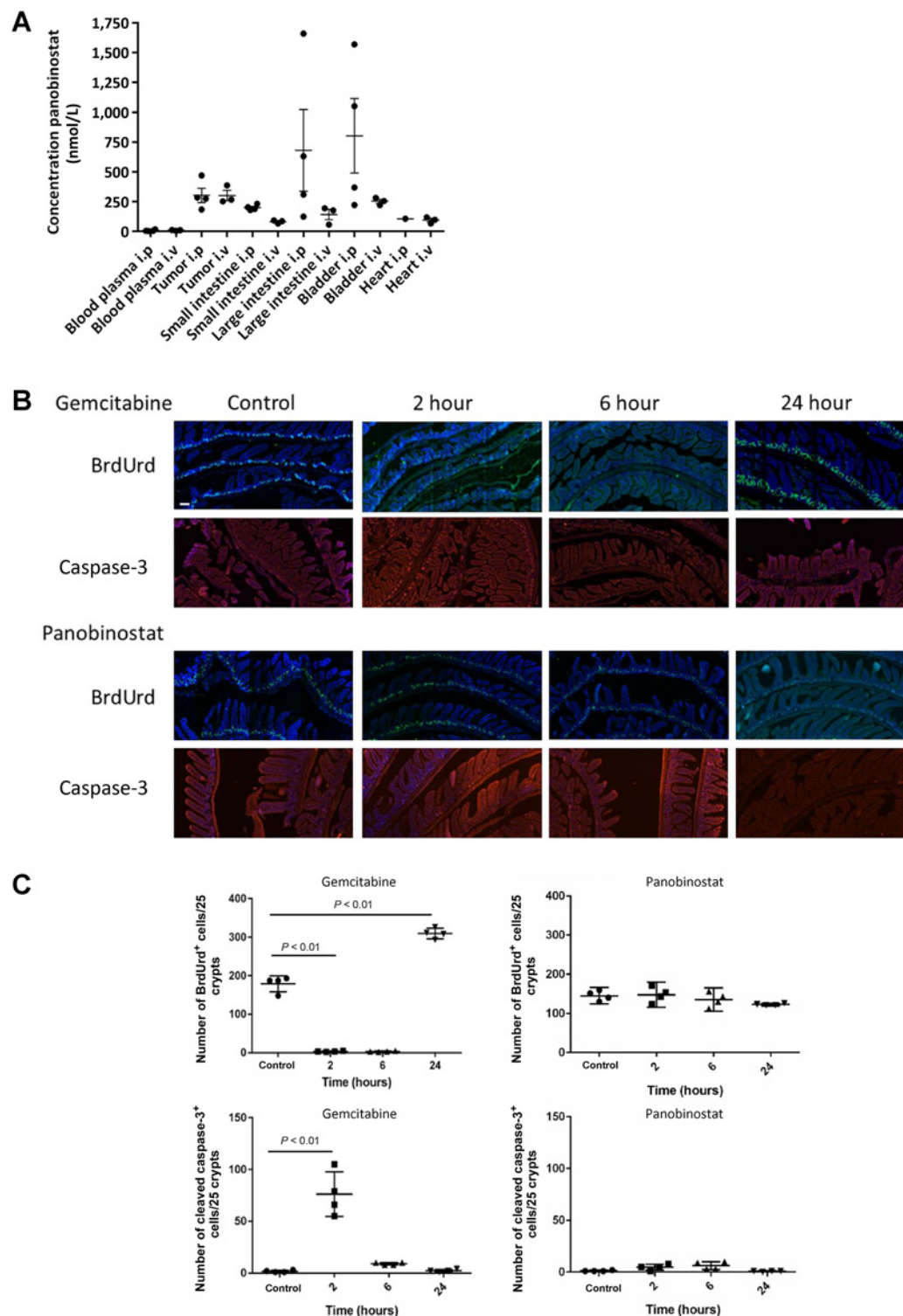
Panobinostat (PAN) causes increased growth delay in irradiated bladder cancer cell xenografts. **A**, Treatment of RT112 xenografts with vehicle, panobinostat, IR (5×4 Gy), or panobinostat + IR ($n = 5$ vehicle, $n = 7$ panobinostat, $n = 6$ IR, $n = 6$ panobinostat + IR). **B**, Kaplan–Meier survival curve of mice with RT112 xenografts showing plots of time to treble tumor volume.

Panobinostat preferentially accumulates in xenografts relative to plasma

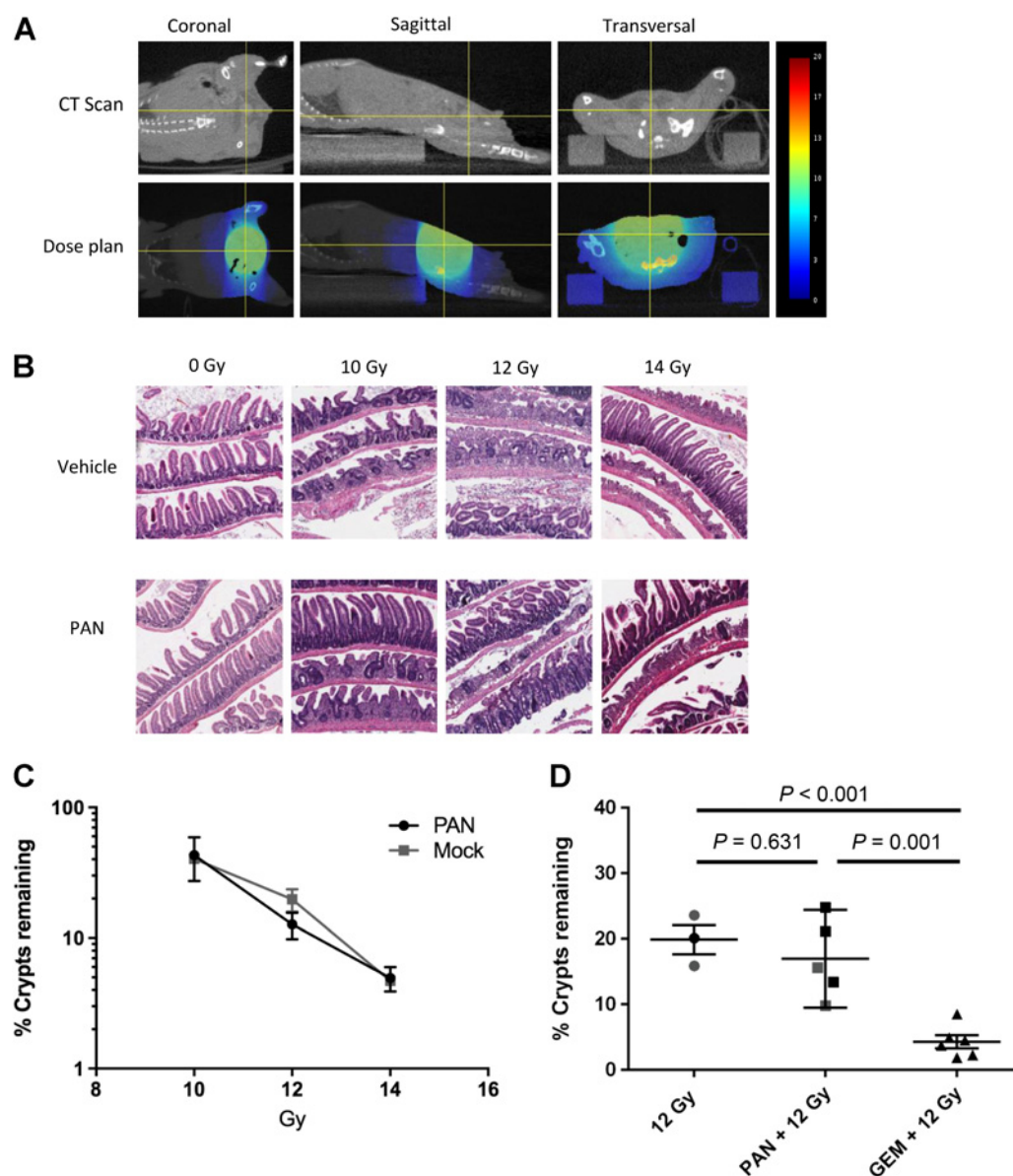
Mice bearing RT112 xenografts of approximately 100 mm³ were treated with intraperitoneal ($n = 4$) or intravenous ($n = 3$) panobinostat (with vehicle alone control, $n = 2$) and tissue and blood collected 6 hours later. Intraperitoneal concentrations were similar to intravenous in plasma, tumor, and heart (Fig. 2A; Supplementary Table S2). However, small intestinal panobinostat concentration was significantly lower in the intravenous arm ($P = 0.001$). The intravenous concentrations in large intestine and bladder were not significantly different from the intraperitoneal concentrations due to a large range of values in the intraperitoneal group ($P = 0.24$ and $P = 0.20$). Therefore, panobinostat preferentially accumulated in xenografts relative to plasma, but intraperitoneal administration resulted in high local concentrations of drug (above median tumor concentrations) in bladder and large intestine in most samples.

Panobinostat has no effect on apoptosis or cell proliferation within 24 hours of treatment

Small intestine from mice treated with gemcitabine or panobinostat and culled at 2, 6, and 24 hours showed

**Figure 2.**

Panobinostat accumulation in normal tissues and methodology to assess acute toxicity. **A**, Xenograft-bearing mice were treated with panobinostat 10 mg/kg intraperitoneally ($n = 4$) or intravenously ($n = 3$), or mock treated ($n = 2$). Xenograft, bladder, 4 to 5 cm of small and large intestine, heart, and blood were collected 6 hours later. **B**, Mice were treated with panobinostat or gemcitabine and 30 minutes prior to sacrifice at 2, 4, or 24 hours injected with BrdUrd ($n = 1$ /group). Top, areas of fluorescent BrdUrd expression (green); bottom, images show cleaved caspase-3 (red). The nucleus was stained by DAPI in the BrdUrd images. The 24-hour mouse of panobinostat treatment was a repeat, as the first mouse had to be sacrificed within 22 hours after drug administration on welfare grounds (see Discussion). Examination of limited tissues postmortem did not determine whether this was due to panobinostat. **C**, Quantification of number of BrdUrd-positive cells per 25 crypts and number of cleaved caspase-3-positive cells per 25 crypts following treatments in **B**. Four areas were scored per condition ($n = 1$ for each time point).

**Figure 3.**

Methodology to assess acute toxicity using the small animal radiation research platform. **A**, Supine SARRP setup showing dosimetry for 14-mm arc treatment. **B**, H&E-stained sections of small intestinal Swiss rolls demonstrating lost and regenerating crypts after 10, 12, and 14 Gy radiation. **C**, Small intestinal crypt assay survival for panobinostat and IR, $n = 2$ per group. Data were normalized to mean crypts per mm of two mock and one panobinostat (PAN) only for each of three small intestine segments. **D**, Effect on small intestinal crypt survival of 12 Gy IR ($n = 3$), panobinostat + 12 Gy ($n = 5$), and gemcitabine (GEM) + 12 Gy ($n = 6$; including data from **C** marked in gray) and from 4 independent studies. Three mice from 12 Gy IR and one mouse from panobinostat + 12 Gy were excluded because the slide did not meet the criteria: either no damage or crypt loss < 3 mm. New data were normalized to mean crypts per mm of two mock, one panobinostat only, and one gemcitabine only for each of three small intestine segments.

strikingly different effects following gemcitabine compared with panobinostat in terms of apoptosis and cellular proliferation (Fig. 2B and C). While gemcitabine treatment reduced cellular proliferation as measured by BrdUrd expression, panobinostat treatment had no effect on proliferation, and while gemcitabine resulted in an increase in cellular apoptosis at 2 hours as shown by cleaved caspase-3 staining, panobinostat did not.

Panobinostat does not increase acute intestinal toxicity following ionizing radiation

The effects of adding intraperitoneal panobinostat and gemcitabine on acute radiation intestinal toxicity were tested in CD1-nude mice using our modified crypt assay, with irradiation to the lower abdomen only (Fig. 3A) (22). No loss of small intestinal crypts was observed in mice treated with vehicle or drug alone (number of crypts per mm for mock = 18.6 ($n = 2$), panobinostat

=19.6 ($n = 1$), gemcitabine =18.0 ($n = 1$), Fig. 3B). There was no significant difference in crypt loss between panobinostat+IR and IR alone at 10 Gy ($P = 0.87$), 12 Gy ($P = 0.28$), or 14 Gy ($P = 0.84$, $n = 2$ per group, Fig. 3B and C). Further analysis was made with mice treated with 12 Gy alone, panobinostat +12 Gy or gemcitabine +12 Gy (Fig. 3D, $n = 3$ for 12 Gy, $n = 5$ for panobinostat +12 Gy, $n = 6$ for gemcitabine +12 Gy). There was no significant difference in crypt loss between 12 Gy and panobinostat +12 Gy ($P = 0.63$). However, gemcitabine +12 Gy showed a significant crypt loss (4.3% crypts remained in gemcitabine +12 Gy; 12 Gy vs. gemcitabine +12 Gy, $P < 0.001$, panobinostat +12 Gy vs. gemcitabine +12 Gy, $P = 0.001$) similar to that seen for 14 Gy alone (4.7%). In large bowel Swiss rolls, there was no difference in IR alone or panobinostat + IR in terms of crypt regeneration and inflammation, as assessed by a veterinary pathologist ($n = 2$ per group, Supplementary Table S3A).

Panobinostat does not increase radiation intestinal and bladder toxicity at 12 weeks

Mouse small intestine is exquisitely sensitive to radiation at the doses proposed; irradiation of even small segments of bowel can be fatal. We therefore developed a novel method to assess potential late effects of panobinostat + IR compared with IR alone. Mice were treated vertically, head-down in the SARRP, with gravity assisting the removal of small intestine from the treatment volume. Preliminary experiments using freshly euthanized mice, irradiated then immediately subjected to autopsy, reassured us that the entire small intestine would be at a safe distance from the field edge (Supplementary Fig. S2). The 10-mm field was centered on the caudal posterior bladder wall, to include lower large bowel in the field, avoiding the small intestine (Fig. 4A). Mice were treated with five fractions of 5 Gy (total 25 Gy) over 5 days. The loss of body weight from baseline of mice treated with panobinostat + IR was significantly greater compared with IR alone ($P = 0.03$) and mock-treated mice ($P < 0.001$), but weights had returned to baseline within 9 days (panobinostat + IR) and 6 days (IR) posttreatment (Fig. 4B).

There was no significant difference in mean weight of dried feces or mean number of fecal pellets (23) across the groups at 11 weeks (Fig. 4C–E). In all groups, over 85% of pellets were >4.5 mm in length. On histologic examination of the large intestines at 12 weeks, cage 4 mouse 4, treated with panobinostat + IR, had histologic appearances consistent with a moderate diffuse ulcerative colitis. Although *Helicobacter* was a possible cause, we were unable to rule out the possible involvement of panobinostat or radiation in its development. Otherwise, only one mouse per treatment group had mild (grade 2 on a 0–5 semiquantitative scale) changes, including inflammation and crypt hyperplasia (Supplementary Table S3B).

Panobinostat radiosensitizes hypoxic bladder cancer cell lines

Our use of subcutaneous human bladder cancer cell xenografts meant that we could not directly investigate the effects of panobinostat on the tumor microenvironment *in vivo*. However, bladder tumors are known to have hypoxic regions that respond less well to radiotherapy than well oxygenated areas. Moreover, as hypoxic conditions that mimic the tumor microenvironment have significant effects on the chromatin (24), it is important to test HDAC inhibition in this context. On clonogenic assay, surviving fractions at 6 Gy (SF_6) for normoxic and hypoxic

(2% O_2) RT112 cells were 0.27 and 0.35, respectively ($P = 0.37$), with 25 nmol/L panobinostat radiosensitizing normoxic RT112 cells ($SF_6 = 0.10$; ref. 14) and significantly radiosensitizing hypoxic RT112 cells ($SF_6 = 0.18$, $P = 0.04$). The oxygen enhancement ratio for panobinostat + IR at 10% survival was 1.22, and for IR alone was 1.12 (Supplementary Fig. S3). These data imply that panobinostat could overcome resistance to radiotherapy in the hypoxic regions of bladder cancer xenografts.

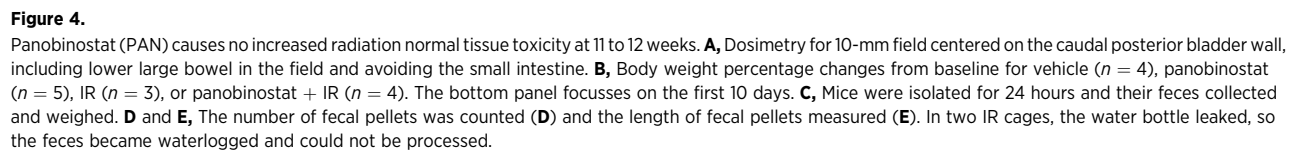
Radiosensitization and expression of proteins are mediated via class I HDAC effects

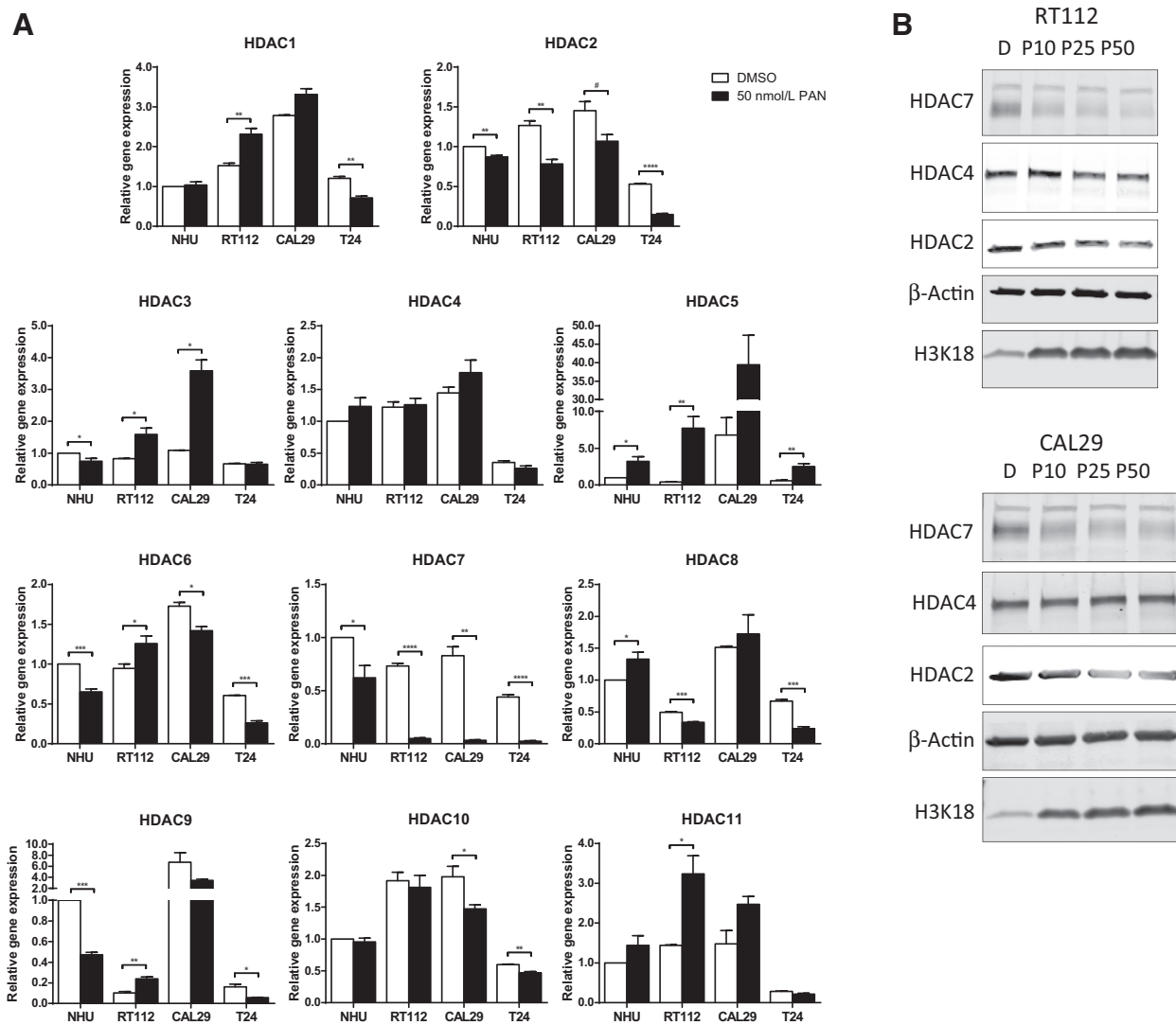
As panobinostat is a nonselective HDAC inhibitor, we hypothesized that its radiosensitizing action could be mediated via an individual HDAC or HDAC class rather than its pan-HDAC effects. We established the baseline mRNA expression of 11 individual HDAC genes, normalized to *GAPDH* expression, in a normal human urothelial (NHU) cell line and RT112, CAL29, and T24 bladder cancer cell lines (Supplementary Fig. S4A). mRNA levels of *HDAC1* and *HDAC2* were the highest across the cell lines, with approximately 5-fold higher expression than other HDACs. mRNA expression levels of untreated (DMSO) and panobinostat-treated samples were compared using a clinically achievable dose of 50 nmol/L (Fig. 5A; ref. 25). *HDAC2* (class I HDAC) was downregulated by panobinostat in all treated cells. *HDAC7* (class IIa) was markedly downregulated in tumor cells up to 22-fold compared with untreated cells; in marked contrast, in NHU cells, downregulation was only 1.5-fold. At the protein level, panobinostat caused downregulation of *HDAC2* and *HDAC7* protein in RT112 and CAL29 cells (Fig. 5B; Supplementary Fig. S4B).

The class I-selective agent mocetinostat and class IIa-selective agent TMP195 were then studied in RT112 and T24 bladder cancer cell lines by clonogenic assay. After 24-hour treatment, mocetinostat was effective in the micromolar range in bladder cancer cells (Supplementary Fig. S4C) and radiosensitized both cell lines (Fig. 6A), although to a lesser degree than panobinostat, with sensitizer enhancement ratios all less than 1.25. Similarly to panobinostat, mocetinostat downregulated MRE11 (and to a lesser extent NBS1 and RAD51) protein levels in a concentration-dependent manner (Fig. 6B). In contrast to mocetinostat, the class IIa-selective inhibitor TMP195 was nontoxic to RT112 cells, did not downregulate the MRN proteins or RAD51 (Supplementary Fig. S4D; Fig. 6B), and did not radiosensitize cells in clonogenic assay (Fig. 6C). Furthermore, TMP195 did not increase acetylation of histone H3 at residue 18 (Fig. 6B), in contrast to panobinostat and mocetinostat (Supplementary Fig. S4E; Fig. 6B). Mocetinostat downregulated *HDAC2* and *HDAC8* (class I) protein expression (Fig. 6D).

Transient knockdown of *HDAC1* or *HDAC2* protein levels in RT112 resulted in downregulation of levels of MRE11 and NBS1 ($n = 2$, Fig. 6E; Supplementary Fig. S4F). Furthermore, transient knockdown of *HDAC1* or 2, but not *HDAC7*, resulted in radiosensitization (Fig. 6F). Overall, these results imply that the radiosensitizing effects of panobinostat are primarily mediated via its class I-selective effects, in particular through its effects on *HDAC1* and 2.

Using both I-SceI-based and PCR-based HR assays, we demonstrated that panobinostat treatment resulted in reduced HR activity, and this was also seen for class I inhibitor mocetinostat (Fig. 6G–I) but not the class IIa inhibitor TMP195 (Supplementary Fig. S4G).



**Figure 5.**

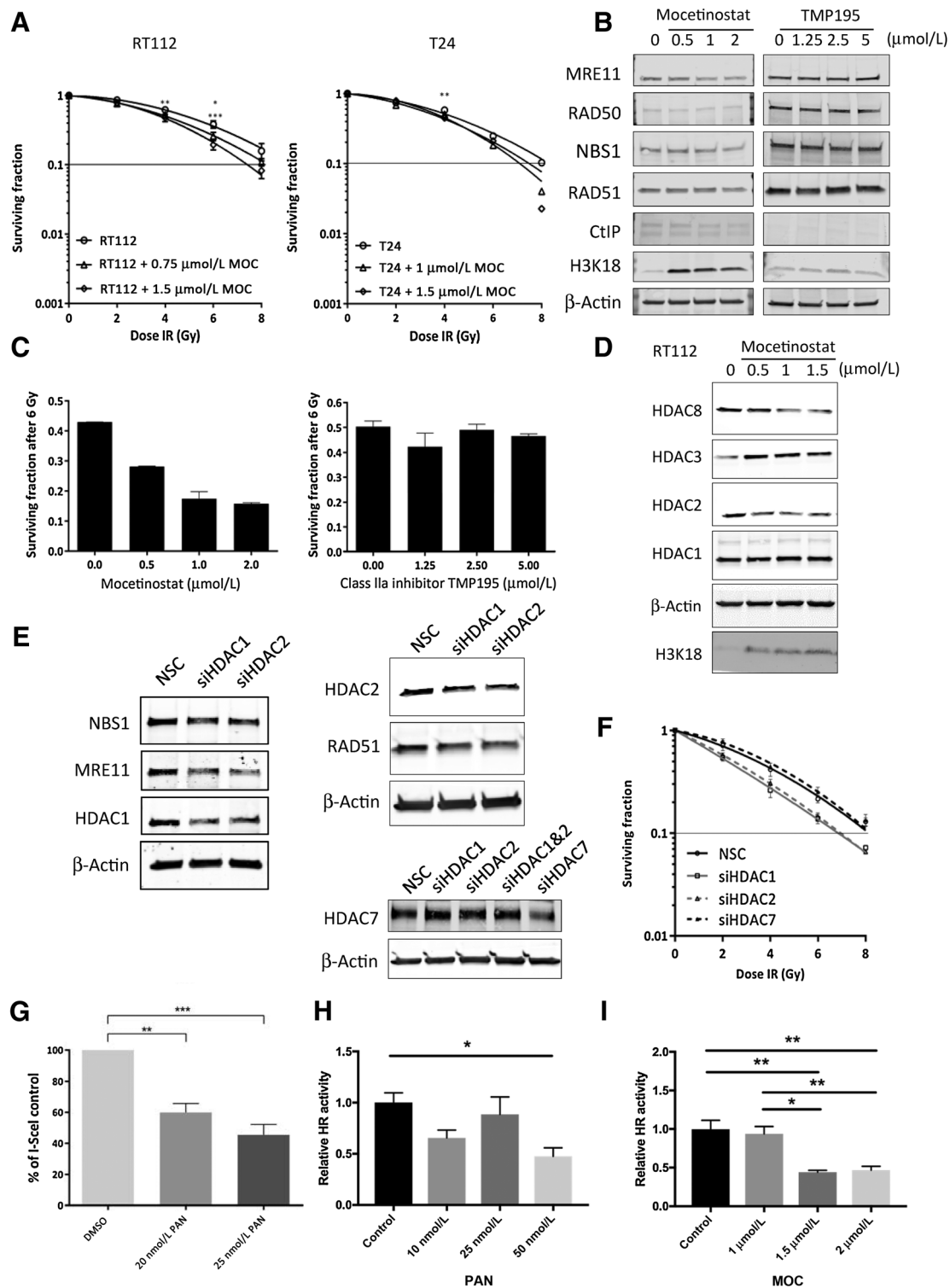
HDAC expression in response to panobinostat in a panel of bladder cell lines. **A**, Samples were treated with DMSO alone or with 50 nmol/L panobinostat (PAN) for 24 hours and mRNA extracted. Expression was normalized to *GAPDH* from untreated NHU cells. **B**, Protein expression of HDAC2, HDAC4, and HDAC7 in RT112 and CAL29 cells, after 24-hour 10, 25, and 50 nmol/L panobinostat incubation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Discussion

There is an urgent need to find nontoxic radiosensitizing agents to treat elderly bladder cancer patients. In most patients, radiotherapy treatment to the bladder causes tiredness and increased urinary and bowel frequency, and long-term some experience bladder shrinkage, increased bowel frequency, and rectal bleeding. Currently used radiosensitizing agents tend to exacerbate these side effects (6–8). HDAC inhibitors are promising as radiosensitizers as they have minimal effects on normal cells *in vitro* (26, 27) while targeting tumor cells (28–32), with even the suggestion of normal cell sparing with H6CAHA *in vitro* (26).

To our knowledge, this study is the first to address the mechanism and interrelation of the effect of HDAC inhibitors in both tumors and early normal tissue damage, although recently,

Kalanxhi and colleagues (33) found that mice treated with suberoylanilide hydroxamic acid (SAHA) without radiation showed a significant decrease in body weight but no apoptosis 3 hours after the last of five daily doses of drug. We developed a modified crypt assay, which could be used to study acute effects of local abdominal treatments rather than whole abdominal irradiation. We showed that panobinostat did not add to the acute intestinal toxicity caused by 10 to 14 Gy IR. On the other hand, another common radiation modifier, gemcitabine, demonstrated crypt damage as early as 2 hours after administration based on our BrdUrd and caspase-3 staining. Furthermore, gemcitabine significantly radiosensitized the small intestine, such that gemcitabine + 12 Gy gave the equivalent crypt loss to 14 Gy alone. Our modified assay method could be useful to others investigating pelvic malignancies, which require assessment of bowel toxicity

**Figure 6.**

Effects of class I HDAC inhibitors on bladder cancer cells. **A**, Clonogenic assay of RT112 and T24 cells incubated with mocetinostat (MOC) for 24 hours ($n = 3$). Ten percent cell survival represented by horizontal lines. **B**, Effects of mocetinostat or TMP195 on MRN complex and selected HR proteins in RT112 cells ($n = 2$). **C**, Radiosensitizing effects of mocetinostat and TMP195 at 6 Gy in RT112 cells ($n = 3$). **D**, Effects of mocetinostat on protein expression of class I HDACs in RT112 cells ($n = 2$). **E**, Effects of transient knockdown of *HDAC1* and *HDAC2* genes on MRE11, NBS1, and RAD51 protein expression ($n = 2$) and effects on protein levels of HDAC2 and HDAC7 following knockdown by siRNA, in RT112 cells. **F**, Clonogenic assay after transient knockdown of HDACs 1, 2, and 7 in RT112 cells. **G**, I-SceI assay for panobinostat (PAN; $n = 3$). **H** and **I**, HR assays for panobinostat ($n = 4$) and mocetinostat ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

where only part of the abdomen is irradiated. However, a limitation of our current method is that in 2 mice, the small intestine appeared to receive no irradiation, and in another 2 mice, less than 3 mm had been irradiated, perhaps due to a large cecum and the bladder receiving most of the dose. Since these experiments were undertaken, we have modified the set up so that the same sized field is moved slightly superior to encompass more bowel while still treating at least the top of the bladder. The transient weight loss seen after treatment was less marked in non-tumor-bearing animals, suggesting that in xenograft studies, weight loss may be due to systemic effects and/or effects associated with tumor kill, possibly cytokine-mediated, rather than reflecting acute intestinal toxicity.

We also developed a novel SARRP method to look at late effects in bowel and bladder. The SARRP permits focused delivery of ionizing radiation, to a relatively homogeneous dose, simulating treatments delivered to human patients. Mice were treated vertically, which allowed gravity to remove the small intestine from the treatment field (34), and our pretreatment cone-beam CT scans gave us confidence that the intestine was outside the field for each treatment. This method will also allow us to irradiate orthotopic bladder tumors, produced by the method of Jager and colleagues (35). A recent report described a method of treating C3H mice using a SARRP to model chronic radiation cystitis, but this involved radiation to the bladder alone without surrounding bowel (36). Our animal license prevented study of animals beyond 12 weeks, but true late effects would require more than 90 days to elapse, so we cannot exclude the possibility that such effects could have emerged at a later time point. Twenty-five Gy in 5 fractions was chosen as this dose schedule is at the bottom of the sigmoid dose-response curve for late effects (23), and so if panobinostat were to increase toxicity, this should be observable at a 30-week time point. One mouse developed ulcerative colitis of unknown etiology, although panobinostat and/or IR could not be ruled out as a possible contributing factor. Therefore, more mice would need to be treated out to 30 weeks to establish any lack of long-term toxicity from panobinostat as a radiosensitizer. Of note, one mouse out of 26 mice receiving panobinostat had to be sacrificed within 22 hours after drug administration showing clinical signs of shaking and reluctance to move. The only significant change in the limited tissues (liver, kidney, heart, thymus, and intestine) examined from this animal were minimal lymphoid atrophy, depleted hepatocyte glycogen, and diffuse bilateral renal tubular dilatation. It is unclear whether these findings are related to panobinostat administration.

Panobinostat at 10 mg/kg days 1, 3, and 5 was sufficient to increase growth delay with 20 Gy in 5 fractions of IR in xenografts. The striking effect of panobinostat in KuKD xenografts supports our hypothesis that, due to synthetic lethality, panobinostat may be useful in nonhomologous end joining-defective bladder cancer (37). Panobinostat accumulated preferentially in xenografts compared with plasma as previously seen by Atadja and colleagues (13). Our plasma concentrations are clinically achievable (25). We found that intraperitoneal administration resulted in higher concentrations of panobinostat in small and large intestine and bladder compared to intravenous administration. However, this local "bathing" of the bowel and bladder with drug following intraperitoneal injection neither increased its toxic effects in this area nor exacerbated radiation side effects. Furthermore, whereas gemcitabine reduced BrdUrd and increased cleaved caspase-3 levels, panobinostat did not.

Our subcutaneous xenograft human bladder cancer cell model is limited compared with orthotopic syngeneic models, as it cannot assess tumor invasiveness or the immune response, nor is it an ideal model with which to examine the tumor microenvironment. Bladder cancers are known to have a hypoxic fraction of around 10%, with high levels of hypoxia associated with lower survival, and a survival advantage was found in the BCON phase III trial for carbogen breathing and nicotinamide with radiotherapy versus radiotherapy alone (7). Panobinostat reduces resistance to cisplatin in hypoxic cells (38) and SAHA radiosensitizes hypoxic cells (39). Our similar findings for panobinostat imply it may be useful in hypoxic tumors.

We initially hypothesized that the striking differential effect on HDAC7 expression seen in NHUs and tumor cells could explain the differential effects of panobinostat in combination with IR on tumor and normal tissue seen *in vivo*. However, HDAC1 and 2 were much more highly expressed than the remaining HDACs, and we found radiosensitization and inhibition of MRE11 protein expression via class I (HDAC1 to some extent and 2 with a greater effect) but not IIa (HDAC7) inhibition. HDAC1 and HDAC2 have been shown to form a heterodimer with different localization and functions (40) when the dimer is disrupted, which account for the dual effect of knockdown of either protein. Furthermore, radiosensitization was seen following HDAC1 and HDAC2 but not HDAC7 siRNA knockdown. Our HR assay results also confirmed this result, as mocetinostat decreased the HR activity while TMP195 failed to show any effect. Therefore, class I-selective inhibitors might be as effective as panobinostat with fewer systemic side effects from off-target effects on other HDACs. Although mocetinostat is only effective in the micromolar range, the HDAC1 and 2-selective inhibitor romidepsin (41) showed promise at nanomolar concentrations with radiotherapy for cutaneous T-cell lymphoma (42).

Although a phase I clinical trial of panobinostat as a radiosensitizer could rapidly be commenced in bladder cancer, as panobinostat is already in clinical use, potent class I-selective HDAC inhibitors should be studied preclinically, as they may have fewer systemic side effects, and such studies might lead to clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Radiosensitization *In Vivo* by Histone Deacetylase Inhibition with No Increase in Early Normal Tissue Radiation Toxicity

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