Molecular radiobiology

Radiosensitisation of bladder cancer cells by panobinostat is modulated by Ku80 expression

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ABSTRACT

Background and purpose: In muscle-invasive bladder cancer there is an urgent need to identify relatively non-toxic radiosensitising agents for use in elderly patients. Histone deacetylase inhibitors radiosensitise tumour cells but not normal cells *in vitro* and variously downregulate DNA damage signalling, homologous recombination (HR) and non-homologous end-joining (NHEJ) repair proteins. We investigated panobinostat (PAN) as a potential radiosensitiser in bladder cancer cells.

Materials and methods: Clonogenic assays were performed in RT112 bladder cancer cells, and RT112 cells stably knocked down for RAD51 or Ku80 by shRNAi. Resolution of γH2AX foci was determined by immunofluorescence confocal microscopy, cell cycle progression by FACS analysis and protein expression by western blotting.

Results: PAN had a greater radiosensitising effect in Ku80KD than RT112 or RAD51KD cells; enhancement ratios 1.35 for Ku80KD at 10 nM (IC_{20} for Ku80KD) and 1.31 for RT112 and RAD51KD at 25 nM (IC_{40} for both). PAN downregulated MRE11, NBS1 and RAD51, but not Ku70 and Ku80, increased γH2AX foci formation in a dose-dependent manner and delayed γH2AX foci repair after ionising radiation.

Conclusions: PAN acts as a radiosensitiser in bladder cancer cell lines, and appears to target HR rather than NHEJ. As muscle-invasive bladder tumours have reduced Ku-DNA binding, PAN could be particularly useful as a radiosensitiser in bladder cancer.

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Bladder cancer is the 9th commonest cancer worldwide, with over 330,000 people diagnosed and over 130,000 dying from the disease annually [1]. Muscle-invasive bladder cancer can be treated by neo-adjuvant chemotherapy, followed by either surgical removal of the bladder (cystectomy) or radical radiotherapy (RT) with or without concurrent chemotherapy (CRT), with similar results [2]. Older patients tolerate surgery and chemotherapy poorly. As the overall population ages, we are witnessing increasing numbers of patients with bladder cancer who are unable to tolerate toxic treatments. There is therefore an urgent need to test novel, less toxic agents in combination with ionising radiation, as potential clinical radiosensitisers in this disease.

Double strand breaks (DSB) are the lethal DNA lesions caused by ionising radiation. Cells have two major mechanisms to repair DSB: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ predominates in G1/early S phase but is error-prone; Ku70, Ku80 and DNA-PK are its key initiators [3]. HR is active in late S/G2 phase, as it requires a sister chromatid to act as a repair template, and is error free; the MRE11/RAD50/NBS1 complex and RAD51 are key components [4,5].

Histone deacetylase inhibitors (HDACi) have known tumour growth suppressive effects *in vitro* and *in vivo*, and these agents act by opening up chromatin thus promoting gene expression, and also by effects on non-histone proteins, including transcription factors, transcription co-regulators, signalling mediators and DNA repair enzymes (reviewed in [6]). High levels of HDACs have been found in bladder tumours, so HDACi's may be useful in this disease. SAHA and MS275 were well tolerated in phase I studies in bladder cancer [6]. Whilst trichostatin A and belinostat inhibit the growth of bladder cancer cells in nanomolar and micromolar concentrations, respectively [7,8], we are not aware of similar studies using panobinostat (PAN), a potent HDACi.

Several HDACi's radiosensitise a range of tumour cell types and variously downregulate MRN complex or core NHEJ or HR proteins in tumour but not normal cells, whilst compromising DNA repair post-ionising radiation (reviewed in [9]). In addition, HDAC

inhibitors have shown no evidence of toxicity in normal cells and the HDACi H6CAHA even caused an increase in post-irradiation surviving fraction in normal prostate cells [10–12]. Their potential clinical use as radiosensitisers has been reported in anaplastic thyroid carcinoma, glioma, and gastrointestinal carcinoma (reviewed in [9]). However, to date there are no published clinical trials of radiotherapy combined with an HDACi in bladder cancer.

Muscle-invasive bladder tumours have defective NHEJ repair [13,14], so HDACi's would be particularly useful if they targeted the HR pathway, resulting in 'synthetic lethality', and hence an increased therapeutic ratio, in combination with IR [15].

We therefore investigated PAN as a potential radiosensitiser in muscle-invasive bladder cancer. PAN radiosensitised RT112 bladder cancer cells and stably-transfected Ku80 knockdown cells, and appeared to act through the HR pathway, and increased γH2AX foci formation and delayed resolution after ionising radiation.

Materials and methods

Reagents

Panobinostat (LBH589; PAN) was purchased from Selleck Chemicals and dissolved in dimethyl sulfoxide (DMSO; Sigma) to a stock concentration of 1 mmol/L. Single-use aliquots were stored at −80 °C.

Cell culture conditions

TP53 wild-type RT112 bladder transitional cell carcinoma cells were grown in RPMI-1640 (Sigma) supplemented with 10% v/v foetal bovine serum (FBS; Sigma), 2 mmol/L L-glutamine (Sigma). Stably transfected knock-down (KD) 795J (RAD51KD), C13 (Ku80KD) and pSil8 (non-silencing control (NSC)) cells were created from RT112 cells as previously described [15], and cultured in RPMI-1640 medium, 10% v/v FBS, 2 mmol/L L-glutamine (Sigma), 0.04 μg/ml G-418 solution (Roche). All cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37 °C and exponentially growing cells were used in all experiments.

Chemosensitivity clonogenic assay

Cells were plated at 700–1000 cells per plate in 10 cm dishes (Greiner Bio-One) for 24 h at appropriate drug concentrations. The next day plates were washed twice with RPMI-1640 medium and 10 ml of fresh medium was added to each plate. Fourteen days later each plate was stained with 0.25% Brilliant blue R (Sigma–Aldrich): 40% methanol: 7% acetic acid for 30 min. Colonies containing more than 50 cells were counted automatically using a Colcounter (Oxford Optronix).

Radiosensitisation clonogenic assay

Cells (7.5×10^5) were plated in 10 cm dishes, and the following day, treated either with DMSO or PAN at appropriate concentrations. After a further 24 h incubation, cells were trypsinised and appropriate numbers plated in 10 cm dishes and irradiated at a dose-rate of 1.7 Gy/min using a caesium-137 source (GSR D1, Gamma-Service Medical GmbH). Cells were then incubated at 37 °C for 14 days, before staining and counting as above. The surviving fraction and radiation survival curves were determined and plotted in GraphPad Prism using the linear-quadratic model as previously described [15].

Western blotting

Western blotting was performed as previously described [15], using the following antibodies: mouse monoclonal anti-Ku80

(Neomarkers), anti-Ku70 (Abcam), anti-Mre11 (Abcam), anti-H3K18 (Cell Signalling) and anti-β-actin (Abcam), and rabbit monoclonal anti-NBS1 (Abcam) and anti-RAD51 (Proteintech Europe).

Cell cycle fluorescence-activated cell sorting (FACS) analysis

Cell-cycle analysis was performed as previously described [15].

Immunofluorescence

Cells (2.5×10^5 per dish) were plated onto sterile glass coverslips, and the following day treated with PAN or DMSO. Twenty-four hours later cells were either fixed or irradiated to 5 Gy prior to fixation, at the time points indicated. Cells were fixed by incubation for 20 min in 4% paraformaldehyde (Thermo Scientific)/0.1% Triton X-100 (Sigma) at room temperature, followed by three PBS rinses, incubation in 0.5% Triton/PBS for 15 min, then three further PBS rinses. Coverslips were then blocked for 30 min at room temperature in 5% bovine serum albumin (VWR International Ltd.) before incubation in mouse monoclonal anti-γH2AX antibody (Millipore) overnight at 4 °C. Following three PBS washes, coverslips were incubated in secondary anti-rabbit (Alexa 488, Invitrogen) and anti-mouse (Alexa 568, Invitrogen) antibodies for 1 h before rinsing in PBS three times. Coverslips were mounted onto slides with Fluoromount G (Sigma) containing 0.1 μg/ml DAPI (Sigma), dried and scanned on a confocal microscope (Zeiss LSM 780).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism software. Clonogenic assays were performed in duplicate at least three times, with the results expressed as mean and standard deviation (SD). The sensitiser enhancement ratio (SER) was calculated at the 0.1 survival fraction (10% survival). In the immunofluorescence experiments, slides were prepared twice for each condition and at least 70 cells counted per slide.

Results

Panobinostat kills bladder cancer cells in the nanomolar range, causes G2/M cell cycle arrest, and causes γH2AX foci increase

First, we determined the inhibitory effect of panobinostat (PAN) on growth of RT112, RAD51KD, Ku80KD and NSC cells by clonogenic assay. After 24 h treatment, IC_{50} values for PAN were 27 nM in RT112, 25 nM in RAD51KD, 19 nM in Ku80KD and 27 nM in NSC cells; it was therefore more toxic in Ku knockdown cells (Fig. 1A and Supplementary Fig. 1A).

We also studied PAN's effect on γH2AX foci formation in RT112 cells (Fig. 1B and Supplementary Fig. 2). We detected a significant increase in the number of γH2AX foci per cell ($P = 0.022$ at 25 nm and $P = 0.002$ at 50 nM).

We then tested the effects of increasing concentrations of PAN on cell cycle progression (Fig. 1C and Supplementary Fig. 3). PAN caused G2/M phase arrest in a concentration-dependent manner (statistically significant at 25, 50 and 100 nM PAN, $P = 0.01$, $P = 0.02$ and $P = 0.02$, respectively).

Panobinostat downregulates MRE11, NBS1 and RAD51 proteins

Next, we determined the effects of PAN on key DNA damage signalling and repair proteins by western blotting. PAN downregulated protein levels of MRE11 and NBS1 in a dose-dependent manner (Fig. 1D). RAD51 was also downregulated, but PAN did
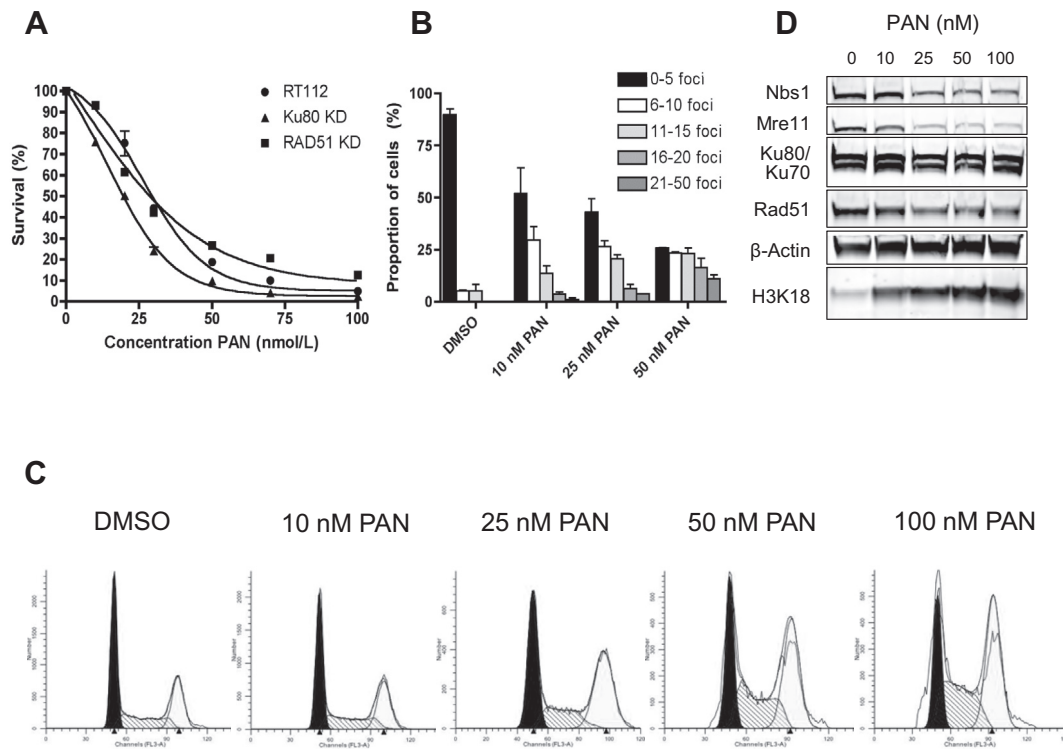
Fig. 1. (a) Clonogenic assays following 24 h treatment of RT112, RAD51KD and Ku80KD cells with increasing PAN concentrations. All experiments were performed at least three times. Error bars represent the mean and standard deviation (SD). (b) Quantification of γH2AX foci in DMSO- or PAN-treated cells, 24 h after treatment (see Supplementary Fig. 2 for confocal images). (c) Cell-cycle phase distributions in cells treated with increasing concentrations of PAN for 24 h. (d) Western blots from RT112 cells incubated with PAN for 24 h, showing downregulation of MRE11, NBS1 and RAD51 but not Ku70 or Ku80, with acetylation of H3K18 from 10 nM or greater PAN.

not affect Ku70 or Ku80 levels. Acetylation of histone H3K18 confirmed PAN's action at 10 nM and above.

Panobinostat radiosensitises bladder cancer cells, particularly Ku-deficient

Cells were treated at 10 nM and 25 nM PAN concentrations, approximately equivalent to RT112's IC_{10} and IC_{40}. PAN significantly sensitised Ku80KD cells at 10 nM (SER = 1.35, $P = 0.04$) with less marked effects in parental RT112 cells (SER = 1.16, $P = 0.05$) and RAD51KD cells (SER = 1.22, $P = 0.04$). At 25 nM PAN (IC_{40}), the SER for both RT112 and RAD51KD cells was 1.31 ($P = 0.01$ and $P = 0.03$, respectively), but this was higher for Ku80KD cells at a Ku80KD IC_{20} equivalent dose (1.56, $P = 0.05$; Fig. 2A and B).

Panobinostat delays γH2AX foci resolution after 5 Gy irradiation

We examined γH2AX foci resolution at 4, 24 and 48 h post 5 Gy irradiation in PAN and DMSO treated samples (Fig. 2C and D). We detected a significant delay in γH2AX foci resolution in PAN treated samples at 24 and 48 h time points ($P = 0.003$ and $P = 0.03$, respectively).

Effects of panobinostat on RAD51 and Ku70/Ku80

RAD51, Ku70/80 and histone H3K18 expression were measured by Western blot after 25 nM PAN ± 5 Gy (Fig. 3). RAD51 and Ku80 levels were reduced as expected in the respective knockdown cells. PAN reduced RAD51 levels in all cell lines and attenuated the RAD51 upregulation seen after 5 Gy. Ku70 and Ku80 protein levels were not markedly affected by PAN treatment.

Discussion

To our knowledge, this is the first reported study using PAN in bladder cancer cell lines and the first to show its radiosensitising effects in bladder cancer.

We found PAN to have potent cytotoxic effects in the RT112 bladder cancer cell line and its Ku80 and RAD51 stably transfected knockdown counterparts, with downregulation of RAD51, MRE11 and NBS1 but not Ku70 or Ku80 in RT112 cells. Strikingly, PAN was more toxic in the Ku80KD cell line than RT112 or RAD51KD cells. We tested PAN's effects on cell cycle progression in RT112 cells and, at doses of 25 nM and above, found a significant accumulation of cells in G2/M phase, where HR is the predominant DSB repair mechanism, but this was not seen at 10 nM.

Our RAD51 KD cell lines showed a similar radiosensitivity profile by clonogenic assay to the parental RT112 cell line, whilst the Ku80 KD cell line was more radiosensitive, with surviving fractions after 6 Gy of IR of 27%, 28% and 18%, respectively. HR appears to be less important than NHEJ for repairing IR-induced breaks in higher eukaryotes, especially in G1 phase, although HR contributes in the late S/G2 phase [16]. We found PAN to be an efficient radiosensitiser in the RT112 bladder cancer cells used in this report and also two other high grade bladder cancer cell lines (Supplementary Fig. 1). Here, PAN was a particularly efficient radiosensitiser in Ku80KD cells. PAN had an equal efficacy at Ku80KD IC_{20} (10 nM) and RT112 IC_{40} levels (25 nM), with SERs of 1.35 and 1.31, respectively. These data suggest that PAN's greater radiosensitising effect in KuKD cells is not due solely to the increased toxicity of the drug in these cells. Neither is it likely to be due to cell cycle effects at the lower doses (data not shown). Our results were similar to those we previously found for the tyrosine kinase inhibitor imatinib, which targets RAD51 [15]. However, our PAN results were more striking

A

B

C

D

No IR   4h post 5 Gy   24h post 5 Gy   48h post 5 Gy

DMSO

PAN 25 nM
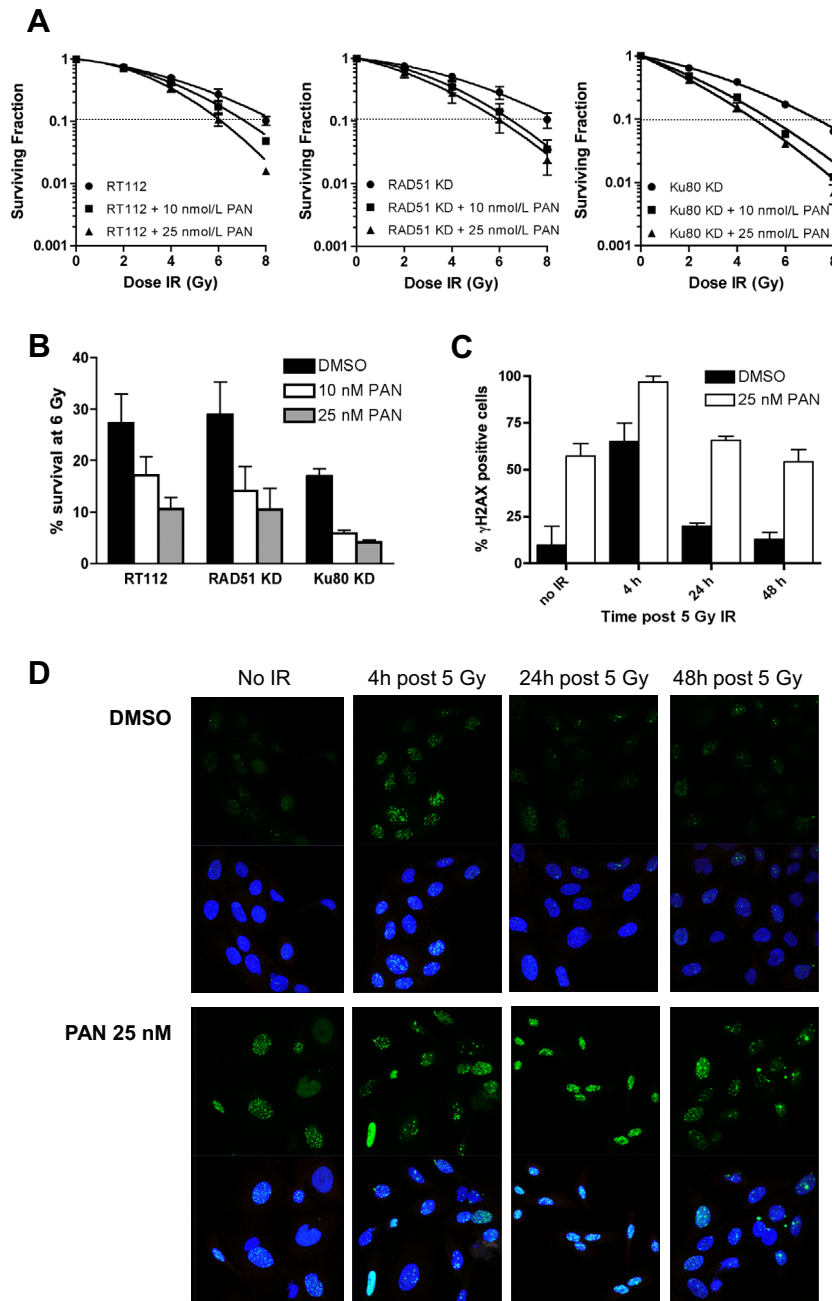
Fig. 2. (a) Clonogenic assays in RT112, RAD51KD and Ku80KD cells treated with increasing ionising radiation doses and 10 nM or 25 nM PAN; (b) Percentages of cells surviving after 6 Gy, quantified from (a); (c) quantification of γH2AX foci from (d); (d) Confocal images of γH2AX foci (top panels) and DAPI stained cells (bottom panels) for cells treated with and without 5 Gy IR and 25 nM PAN.

than with imatinib, and this may reflect the additional effects of PAN on MRE11 and NBS1. Downregulation of the MRN complex by siRNA or NBS1 or MRE11 mutation is known to result in radio-sensitisation [17,18] and the additional impact of PAN on these proteins higher in the DNA damage signalling pathway may account for our findings.

We measured γH2AX as a marker of DNA damage and repair and found that PAN upregulated the number of γH2AX foci seen at 24 h. Studies using other HDACi's also show similar effects [19]. When cells were irradiated with 5 Gy after incubation with 25 nM PAN, we detected a significant delay in γH2AX foci resolution, indicating supressed repair of DNA DSB in PAN treated cells. Geng et al. [20] showed similar effects for PAN with 3 Gy irradiation in lung cancer cells. We are, however, aware of the limitations

of the γH2AX assay as a measure of DSB repair, particularly in non-G0/G1 cells, since γH2AX formation can occur at single-stranded DNA regions during replication or repair [21]. Also background, endogenous 'cryptogenic' foci can limit the ability to detect residual IR-induced γH2AX foci [22].

In using only the γH2AX assay to measure DNA damage and repair, we have not comprehensively examined the effects of PAN on the NHEJ and HR pathways. To do this requires assays such as the NHEJ assay suitable for use on small scale extracts from cell lines [23] and the widely reported HR I-SceI-based reporter assay [24].

We have found previously that Ku80 expression by western blot and immunohistochemistry is variable and does not correlate with bladder cancer grade and stage [13]. However, muscle-invasive bladder tumours display reduced Ku-DNA binding (as previously
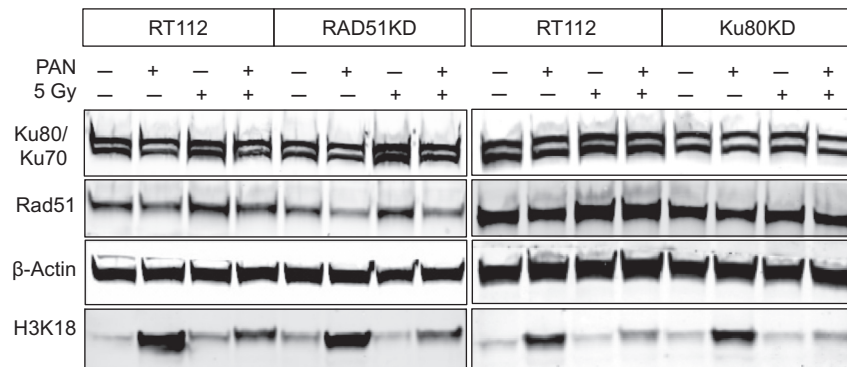
Fig. 3. Western blot analyses of RT112, RAD51KD and Ku80KD cells. Cells were incubated with or without 25 nM PAN for 24 h, and then harvested and irradiated to 5 Gy or left untreated. Cells were lysed 4 h later. Experiments were performed at least twice.

seen by Pucci et al. [25]), thus rendering them functionally NHEJ-defective. Therefore agents targeting the HR pathway could result in 'synthetic lethality', whereby normal cells, with intact NHEJ, can still repair IR-induced DNA damage via NHEJ despite HR being targeted, whilst tumour cells defective in NHEJ are less able to repair the damage. In terms of biomarker development for the prediction of response to panobinostat, it would be interesting to test whether low Ku-expressing tumours respond better to panobinostat, although the reduced Ku-DNA binding phenotype may override this effect.

Whilst others have variously shown effects of HDACi's on the MRN complex, RAD51 and Ku70/80, we believe we are the first to study all three aspects together [9]. PAN is a potent HDACi and the plasma levels used in this study can be reached safely in patients [26]. PAN is therefore a promising agent for investigation as an efficient future therapy in combination with radiation in bladder cancer.

Conflicts of interest

None to declare.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.06.021.

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