Apoptosis
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SHORT COMMUNICATION

Proteasome inhibitors trigger mutations via activation of caspases and CAD, but mutagenesis provoked by the HDAC inhibitors vorinostat and romidepsin is caspase/CAD-independent

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Abstract
Genotoxic anti-cancer therapies such as chemotherapy and radiotherapy can contribute to an increase in second malignancies in cancer survivors due to their oncogenic effects on non-cancerous cells. Inhibition of histone deacetylase (HDAC) proteins or the proteasome differ from chemotherapy in that they eliminate cancer cells by regulating gene expression or cellular protein equilibrium, respectively. As members of these drug classes have been approved for clinical use in recent times, we investigated whether these two drug classes exhibit similar mutagenic capabilities as chemotherapy. The HDAC inhibitors vorinostat/SAHA and romidepsin/FK288 were found to induce DNA damage, and mis-repair of this damage manifested into mutations in clonogenically viable surviving cells. DNA damage and mutations were also detected in cells treated with the proteasome inhibitor bortezomib. Exposure to both drug classes stimulated caspase activation consistent with apoptotic cell death. Inhibition of caspases protected cells from bortezomib-induced acute (but not clonogenic) death and mutagenesis, implying caspases were required for the mutagenic action of bortezomib. This was also observed for second generation proteasome inhibitors. Cells deficient in caspase-activated DNase (CAD) also failed to acquire DNA damage or mutations following treatment with bortezomib. Surprisingly, vorinostat and romidepsin maintained an equivalent level of killing and mutagenic ability regardless of caspase or CAD activity. Our findings indicate that both drug classes harbour mutagenic potential in vitro. If recapitulated in vivo, the mutagenicity of these agents may influence the treatment of cancer patients who are more susceptible to oncogenic mutations due to dysfunctional DNA repair pathways.

Keywords Genotoxicity · HDAC inhibitors · Proteasome inhibitors · Second malignant neoplasms · Bortezomib · Vorinostat

Introduction

Clinically used chemotherapies often function by directly damaging DNA and subsequently triggering apoptosis to kill cancer cells. While this has proven effective in curing some patients of various types of cancers [1], the broad activity of these drugs may influence the emergence of resistant clones in relapsed patients and possibly cause defects in the DNA of non-cancerous cells resulting in mutations in otherwise healthy cells [2]. If these mutations occur in genes that are responsible for regulating proliferation or cell death pathways the cell can become cancerous [3]. About one-fifth of cancer survivors develop a second neoplasm later in life and a proportion of this may be attributed to the oncogenic effects of DNA damaging therapies [4].

Research has been undertaken over the years to hopefully cure cancers that would otherwise be less responsive to classical chemotherapy and radiotherapy. Many novel treatments activate cell death pathways independent of damaged DNA [5, 6], and may therefore provide an additional advantage: not causing mutations that can lead to second cancers. Histone deacetylase (HDAC) inhibitors and proteasome inhibitors target altered gene expression and protein equilibrium, respectively, to eliminate cancerous cells [7, 8].

Inhibition of HDAC proteins promote the acetylation of core histone proteins, which modulates chromatin structure to regulate transcription often enhancing gene expression.

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Non-histone protein substrates involved in DNA repair, cell cycle progression or cytoskeletal organisation can also be targets of HDAC inhibitors [9]. This can lead to a multitude of cell signalling events such as apoptosis, cell differentiation or cell cycle arrest [10]. HDACs are grouped into four classes based on homology to yeast analogues and localisation: class I, II, III and IV, and a number of inhibitors have been developed with differing specificities to HDAC proteins [11]. Both the pan HDAC inhibitor vorinostat (SAHA) and the class I/II targeting inhibitor romidepsin (FK288) have been approved for therapeutic use in cutaneous or peripheral T-cell lymphoma patients [7]. HDAC inhibitors also show promise in combination with other therapies including chemotherapy and radiotherapy, as enhanced histone acetylation exposes chromatin to better enable accessibility for these DNA targeting treatments [12].

Originally developed as a potential therapy for cachexia, proteasome inhibitors have been repurposed as a treatment for haematological cancers [8]. Proteasome inhibitors target the 20S proteasome triggering apoptosis in cancerous cells through proteotoxic stress by causing an accumulation of ubiquitinated proteins [8]. Bortezomib (Velcade) was the first in the class of proteasome inhibitors to be approved, and is presently used to treat multiple myeloma and mantle cell lymphoma [8, 13]. Second generation proteasome inhibitors have also been developed; two of which, ixazomib and carfilzomib, are also approved for the treatment of multiple myeloma [14]. These second generation proteasome inhibitors exhibit very similar mechanisms of action to bortezomib but differ in their binding to the chymotrypic-like sites of the 20S proteasome. Bortezomib and ixazomib are boronic acid reversible inhibitors whereas carfilzomib is an epoxyketone irreversible inhibitor [14].

The DNA damaging nature of HDAC inhibitors like vorinostat and romidepsin has been widely reported [15–20] particularly in the context of cooperating with chemotherapy or radiotherapy in order to exacerbate the DNA damage response and increase apoptotic signalling in cancer cells [21]. There are fewer reports describing the ability of proteasome inhibition to cause DNA damage [22, 23]. However, the ability of these drugs to harbour mutagenic potential, that is a cell that survives drug treatment but mis-repairs the damaged DNA, or elevate the risk of cured patients to second malignancies, is yet to be defined.

This study therefore aimed to elucidate whether HDAC or proteasome inhibitors cause mutations in surviving cells, which may therefore influence the risk of treated patients to second malignancies. The hypoxanthine–guanine phosphoribosyltransferase (HPRT) gene mutation assay was used as this assay capitalises on the ability of HPRT competent cells to metabolise the purine analogue 6-thioguanine (6-TG) into a lethal product, therefore cells bearing drug-induced HPRT loss-of-function mutations are able to grow in the presence of 6-TG [24]. DNA damage was also quantitated by staining for cells bearing phosphorylated H2AX protein. Our data reveal that DNA damage could be detected upon exposure to HDAC or proteasome inhibitors, and that these agents indeed provoke mutations. Caspases and CAD were responsible for mutagenesis caused by bortezomib but surprisingly did not contribute to the mutations provoked following HDAC inhibition despite caspases being active.

Results

HDAC and proteasome inhibitors are mutagenic

To investigate whether drugs that inhibit HDAC proteins or the proteasome cause mutations we employed the HPRT mutation assay to quantitate mutations in surviving TK6 lymphoblastoid (Fig. 1a) and LN18 glioblastoma (Fig. 1b) cells. Cisplatin, a clinically used chemotherapy drug that generates DNA crosslinks and has been implicated in the development of second malignancies [25], was included as a mutagenic control. Cisplatin caused a reduction in the clonogenic survival of both TK6 and LN18 cells and this was accompanied by an increase in the number of 6-TG-resistant (6-TG^R) cells implying mutations at the HPRT locus.

Vorinostat and romidepsin also provoked a dose dependent reduction in clonogenic potential in both cell types. LN18 cells were 1000-fold less sensitive to romidepsin when compared to TK6 cells. This may reflect the elevated levels of class I HDAC proteins present in high-grade glioblastoma cells (like LN18 cells); requiring micromolar concentrations of class I-targeting HDAC inhibitors for toxicity [26]. Nanomolar concentrations of romidepsin were previously reported to kill other lymphoid cells [27, 28] which is consistent with our observations in TK6 cells. Vorinostat triggered an increase in the frequency of HPRT mutations at doses as low as tenfold less than the peak plasma concentration (Cmax) achieved in patients. Romidepsin treatment also led to an increased number of 6-TG^R cells indicating that specific targeting of class I/II HDAC proteins was also mutagenic. Concentrations of romidepsin that were 6000-fold lower than patient Cmax provoked mutations in TK6 cells.

Both TK6 and LN18 cells were sensitive to bortezomib-induced clonogenic death at similar concentrations. HPRT mutations were also observed in surviving cells at concentrations that were at least 60-fold lower than Cmax achieved in patients. In TK6 cells, bortezomib was similar to cisplatin as exposure was only mutagenic at doses that were highly toxic, but the mutagenicity of vorinostat or romidepsin peaked at doses that enabled clonogenic survival of about 30–50% of cells. These assays demonstrate that HDAC inhibition by vorinostat or romidepsin, or inhibition of the proteasome

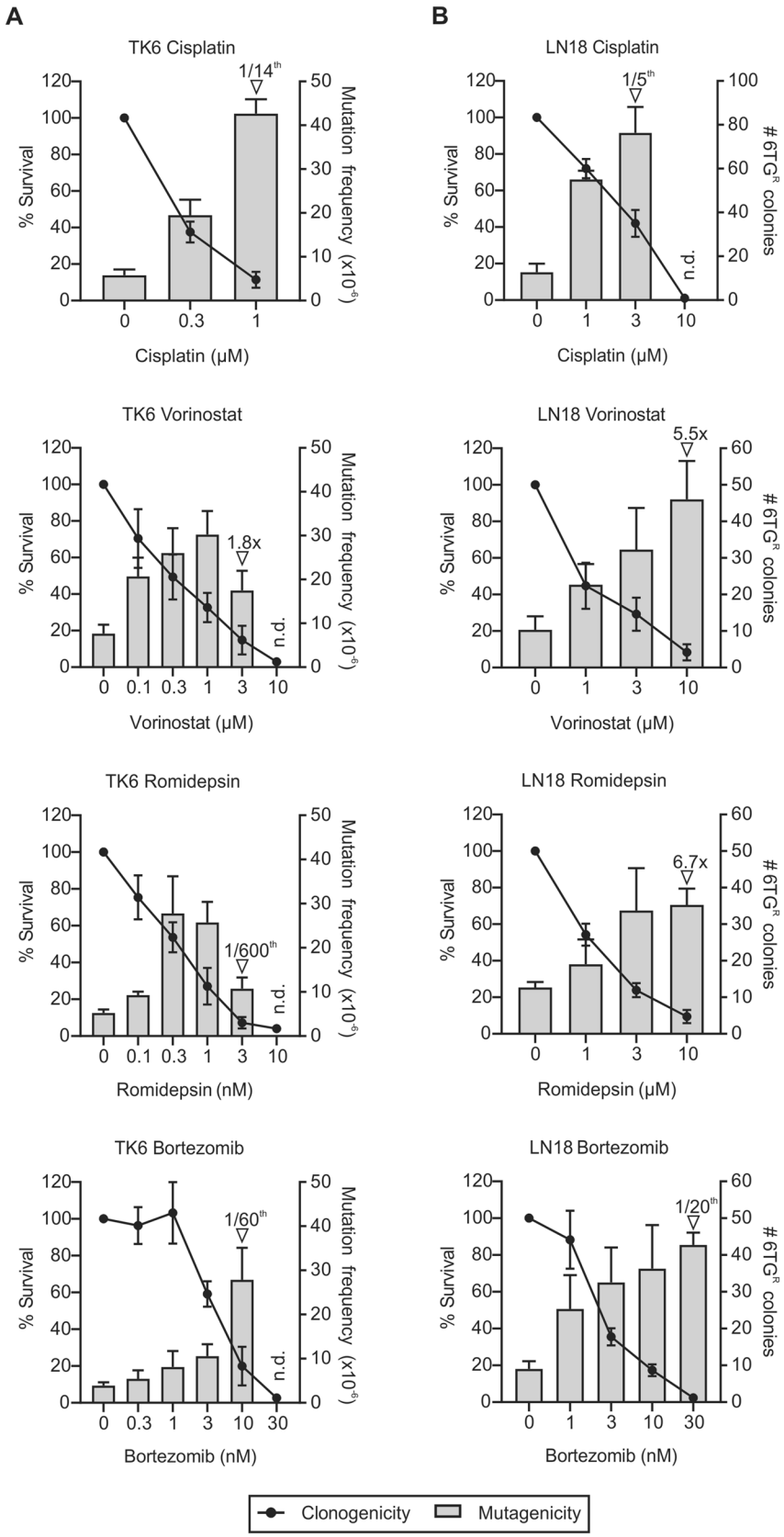
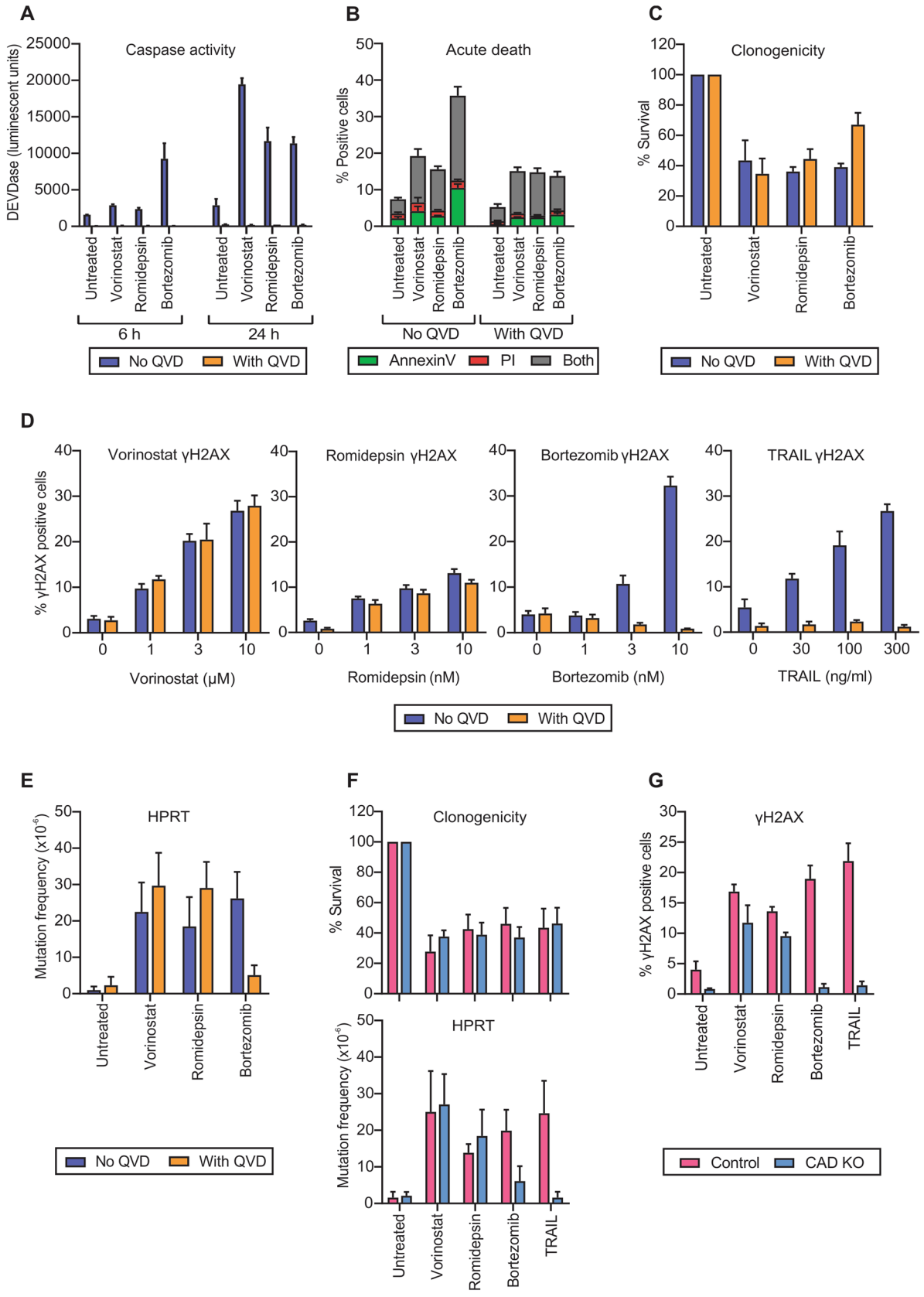


Fig. 1 Treatment of TK6 or LN18 cells with HDAC or proteasome inhibitors increases HPRT mutation frequencies. **a** TK6 or **b** LN18 cells were incubated with indicated doses of cisplatin, vorinostat, romidepsin or bortezomib for 24 h. Following treatment cells were harvested and clonogenicity assays performed to determine the degree of clonogenic survival (lines). Surviving cells were then grown in 6-TG containing media to estimate the frequency of HPRT mutations (columns). Triangles indicate the published peak plasma concentrations of the drugs in patients in relation to the highest dose tested for mutagenicity. *n.d.* not done. Error bars indicate standard error of the mean from at least three independent experiments


A
Caspase activity
DEVDase (luminescent units)
25000
20000
15000
10000
5000
0
Untreated
Vorinostat
Romidepsin
Bortezomib
6 h
24 h
No QVD
With QVD
B
Acute death
% Positive cells
50
40
30
20
10
0
No QVD
With QVD
AnnexinV
PI
Both
C
Clonogenicity
% Survival
120
100
80
60
40
20
0
D
Vorinostat γH2AX
Romidepsin γH2AX
Bortezomib γH2AX
TRAIL γH2AX
% γH2AX positive cells
Vorinostat (μM)
Romidepsin (nM)
Bortezomib (nM)
TRAIL (ng/ml)
E
HPRT
Mutation frequency (x10⁻⁶)
F
Clonogenicity
% Survival
HPRT
Mutation frequency (x10⁻⁶)
G
γH2AX
% γH2AX positive cells
TRAIL
Control
CAD KO

◂**Fig. 2** Mutagenesis triggered by bortezomib but not vorinostat or romidepsin is dependent on caspases and CAD. TK6 cells were incubated with no inhibitor or 10 μM Q-VD-OPh (QVD) for 1 h then subjected to 10 μM vorinostat, 10 nM romidepsin or 30 nM bortezomib for a further 6 or 24 h. **a** Caspase activity was measured at 6 or 24 h using the Caspase-3/7-Glo reagent, **b** acute cell death and **c** clonogenic survival was determined following 24 h treatment. **d** Non- or QVD-pre-treated cells were treated with indicated doses of drugs for 6 h and the number of γH2AX positive cells was quantitated by flow cytometry. **e** Cells were treated the same as panels **a**–**c** then surviving cells grown in 6-TG containing media to estimate the frequency of HPRT mutations. Cas9/CRISPR control or CAD-deficient TK6 cells were treated with 10 μM vorinostat, 10 nM romidepsin, 30 nM bortezomib or 300 ng/ml TRAIL. **f** Clonogenic survival and the frequency of HPRT mutations were determined after 24 h treatment in drug, while **g** the proportion of γH2AX positive cells after 6 h incubation in drug was determined. Error bars indicate standard error of the mean from at least three independent experiments

by bortezomib can provoke mutations in surviving cells that remain clonogenically viable.

Mutagenesis by proteasome inhibition is caspase- and CAD-dependent

Our previous characterisation of the mechanisms of mutagenesis by the death ligand TRAIL and the microtubule targeting drug vincristine revealed that mutations arose due to mis-repair of CAD-mediated DNA double strand breaks (DSBs) following sub-lethal caspase signalling [29]. HDAC and proteasome inhibitors were reported to induce caspase activation via intrinsic apoptotic signalling [30] so we hypothesised that these drugs too may provoke some mutations via caspase and CAD signalling pathways. Bortezomib induced DEVDase activation after 6 h while it took longer for caspase activation to ensue following vorinostat or romidepsin exposure (Fig. 2a). Pre-incubation with the pan caspase inhibitor Q-VD-OPh (QVD) abolished DEVDase activity (Fig. 2a) and protected more than half of bortezomib-treated cells from acute death whereas caspase inhibition did not dramatically alter the small number of annexinV and/or PI positive cells following vorinostat or romidepsin treatment (Fig. 2b). While QVD ensured the cell membranes of most cells remained intact after 24 h treatment with bortezomib (Fig. 2b) it only maintained the clonogenic potential of a subset of those cells (Fig. 2c). This was most likely due to impaired mitochondrial function affecting clonogenicity. Caspase inhibition did not alter the level of clonogenic death provoked by vorinostat or romidepsin (Fig. 2c). To assess whether these drugs provoke DNA damage via sublethal caspase activity we quantitated the proportion of cells bearing phosphorylated H2AX protein (γH2AX) in the presence or absence of QVD (Fig. 2d). TRAIL treatment provoked a dose dependent increase in γH2AX positive cells and, as expected, this was abolished when caspases were inhibited. An increase in cells harbouring γH2AX was observed following exposure to bortezomib and, like TRAIL, pre-treatment with QVD blocked this damage. Vorinostat and romidepsin also promoted H2AX phosphorylation in a dose dependent manner, but the addition of QVD did not affect this (Fig. 2d).

To test whether the lack of DNA damage observed when caspases were inactive following bortezomib treatment altered the frequency of mutations, surviving cells that had been drug-treated in the presence or absence of QVD were grown in 6-TG to quantify HPRT mutations. Background 6-TG^R levels were observed in QVD pre-treated cells that had been treated with bortezomib, compared to a tenfold higher frequency in bortezomib-treated cells bearing active caspases (Fig. 2e). Cells treated with either vorinostat or romidepsin still acquired mutations despite caspase inhibition with QVD (Fig. 2e) although the magnitude of this in the presence of QVD appeared slightly higher than in its absence.

The caspase-mediated mutagenesis of TRAIL was also CAD dependent [29], therefore we suspected that the caspase-dependent mutagenesis observed for bortezomib was also due to the mis-repair of DSBs generated by CAD. All drugs impaired clonogenic survival to similar levels in both control and CAD KO cells but HPRT mutations failed to manifest in CAD-deficient cells treated with bortezomib or TRAIL (Fig. 2f). Fewer CAD KO cells contained γH2AX than control cells regardless of treatment (Fig. 2g). Vorinostat or romidepsin treatment provoked H2AX phosphorylation in a similar proportion of control and CAD KO cells, however exposure to bortezomib or TRAIL only stimulated H2AX phosphorylation in cells expressing CAD (Fig. 2g). This indicates that bortezomib, like TRAIL, provokes mutations via the mis-repair of DNA damage that is created by the action of CAD.

To explore whether the caspase-dependent mutagenesis observed in bortezomib-treated cells is a general feature of proteasome inhibition, we tested the ability of a panel of second-generation proteasome inhibitors with different affinities for the proteasome to provoke DNA damage. LLVYase activity was used to measure proteasome activity as this indicates chymotrypsin-like proteasome function [31]. All inhibitors caused a near complete loss of LLVYase activity, consistent with their proteasome inhibition mechanism of action (Fig. 3a). QVD did not affect this, implying that caspase activation occurred as a consequence of impaired proteasomal function. Of the panel of proteasome inhibitors, TK6 cells were most sensitive to bortezomib and delanzomib while oprozomib was least toxic (Fig. 3b). QVD was able to protect at least 70% of cells from acute death regardless of drug, implying that all proteasome inhibitors caused caspase-dependent death. All proteasome inhibitors provoked a dose dependent increase in H2AX phosphorylation, correlating with

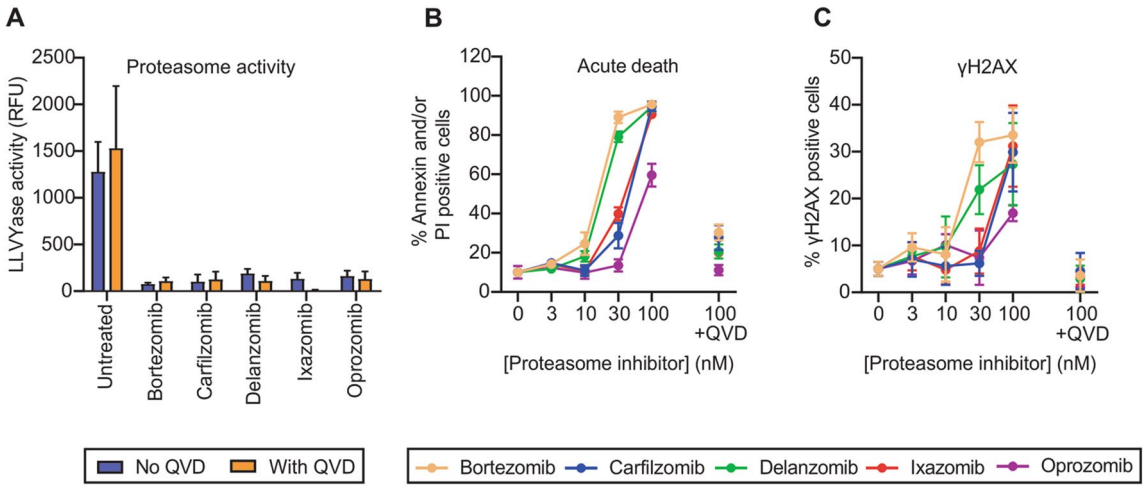


Fig. 3 Second generation proteasome inhibitors provoke caspase-dependent DNA damage. **a** TK6 cells were incubated with no inhibitor or 10 μM QVD for 1 h then subjected to 100 nM of each proteasome inhibitor for a further 3 h. Cells were lysed and proteasome activity measured using the Suc-LLVY-AMC fluorogenic substrate. Cells were incubated with the indicated doses of each proteasome inhibitor for 24 h plus 1 h pretreatment with 10 μM QVD for the highest dose. **b** Acute cell death was measured by annexinV and/or PI staining or **c** the proportion of γH2AX positive cells determined

the impact on survival (Fig. 3c). As with the observations for bortezomib, caspase inhibition by QVD abolished the DNA damage associated with all other proteasome inhibitors.

Vorinostat-induced DNA damage occurs upstream of intrinsic mitochondrial signalling

Our data imply that caspases do not contribute to the mutations provoked by HDAC inhibitors. DNA damage caused by HDAC inhibition has been reported to occur due to reactive oxygen species (ROS), some of which can originate from the mitochondria after damage [32]. Bcl-2 was over-expressed in TK6 cells (Fig. 4a) to address whether damage to the mitochondria by vorinostat correlates with damage to the DNA. Cells over-expressing Bcl-2 were protected from acute death caused by vorinostat or the chemotherapeutic doxorubicin (Fig. 4b) and exhibited low DEVDase levels after treatment when compared to the MBP control (Fig. 4c). This indicates that cytochrome *c* release from the mitochondria and subsequent apoptosome formation had not occurred in these cells to enable executioner caspase activation. Interestingly, an increased proportion of γH2AX positive cells was still detected in Bcl-2 over-expressing cells treated with vorinostat (Fig. 4d) implying that H2AX phosphorylation occurred with little mitochondrial damage. As expected, doxorubicin caused DNA damage regardless of the status of the mitochondria.

Discussion

Research over the past few decades has revealed that the initiation of cell death as a result of the DNA damage induced by classical chemotherapy and radiotherapy can be less effective in relapsed patients, possibly due to the acquired or inherent chemoresistance of a selected subpopulation of cells within an initially heterogeneous tumour population [33], but may also result in serious long term side effects such as second malignancies in cured patients [1]. Novel anti-cancer agents that activate cell death pathways without the need for the cell to recognise and respond to a genotoxic insult may avoid these mutagenic consequences.

This study revealed that HDAC inhibition by vorinostat or romidepsin, or proteasome inhibition by bortezomib provoked mutations in clonogenically competent surviving cells in two different cell types. The mutagenic potential of these drugs was supported in assays that detected phosphorylated H2AX proteins to measure DNA damage. The remodelling of chromatin by HDAC inhibitors has been reported previously to provoke DNA damage [34]. While studies highlighted synergy between HDAC inhibitors with other DNA damaging therapies to enhance the anti-tumour effect [12] our results suggest that the HDAC inhibitors vorinostat and romidepsin can alter the genomes of surviving cells that fail to correctly repair their damaged DNA. Indeed, cells treated with the pan HDAC inhibitor trichostatin A reportedly exhibited chromosomal loss

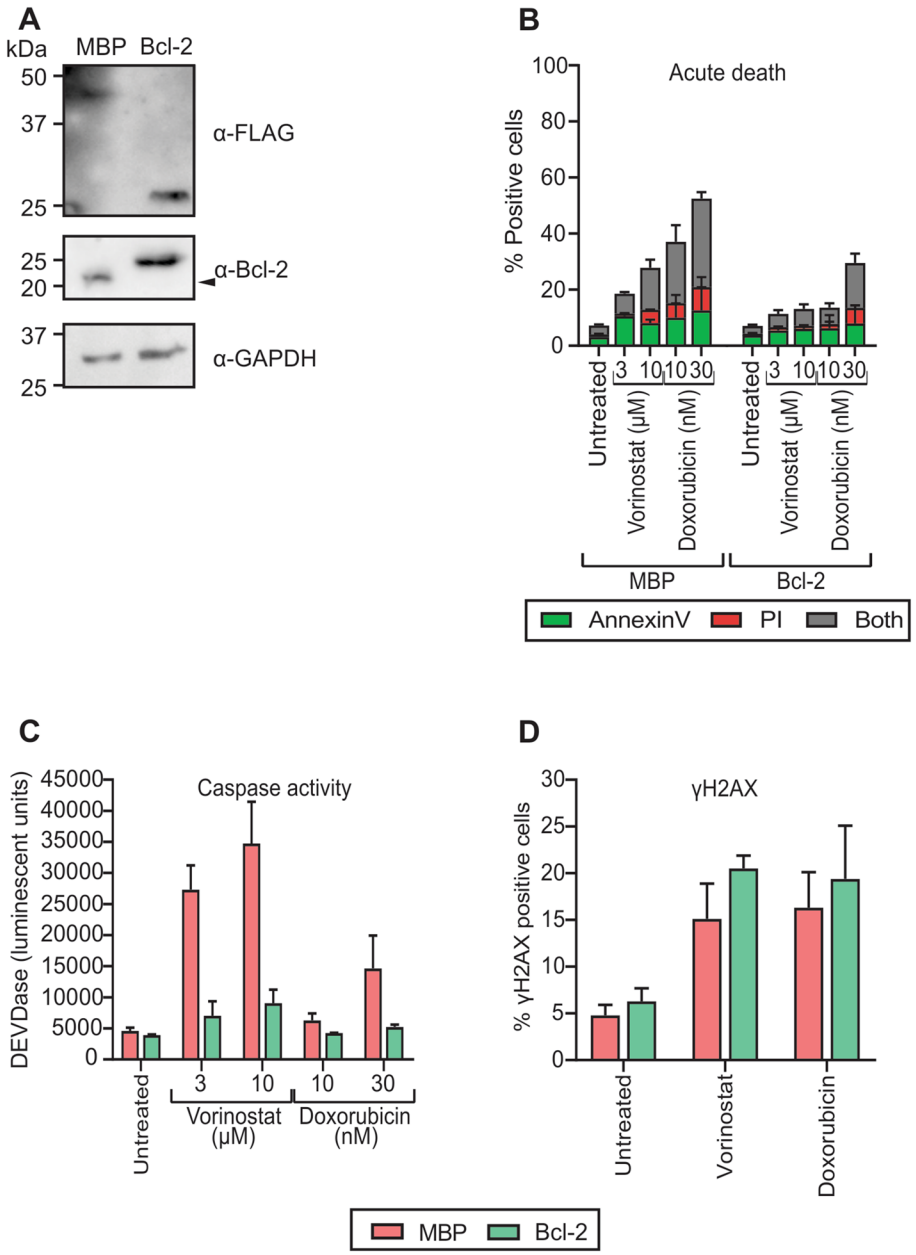


Fig. 4 Over expression of Bcl-2 does not affect vorinostat-induced DNA damage. TK6 cells were stably transfected with FLAG-tagged MBP or Bcl-2 expression plasmids. **a** Expression was confirmed by immunoblotting using anti-FLAG, -Bcl-2 or -GAPDH antibodies (to indicate loading). Cells were left untreated or incubated with indicated doses of vorinostat or doxorubicin. **b** Acute cell death was measured after 24 h by annexinV and/or PI staining, **c** while caspase activity (measured by the Caspase-3/7-Glo reagent), or **d** the proportion of γH2AX positive cells, upon 10 μM vorinostat or 30 nM doxorubicin exposure, was determined after 6 h incubation in drug. Error bars indicate standard error of the mean from three independent experiments

and abnormalities but analysis of surviving cells was not investigated in that study [20]. To our knowledge this is the first study to report the ability of either HDAC or proteasome inhibitors to provoke mutations in surviving cells that maintain clonogenic potential.

Both drug classes tested in this study could induce apoptotic death due to high caspase activity as DEVDase levels increased upon treatment. Pharmacological caspase inhibition blocked the DNA damage and mutations associated with bortezomib treatment, but the mutation frequencies and degree of DNA damage were unaffected by caspase inhibition in vorinostat- or romidepsin-treated cells. Additionally, cells lacking expression of the nuclease CAD failed to acquire HPRT mutations or display evidence of H2AX phosphorylation upon bortezomib exposure, a similar observation to that in TRAIL-treated cells. Hence, CAD did not account for mutagenicity associated with vorinostat or romidepsin. The ability of bortezomib to generate caspase-dependent DNA damage was also shared by other second generation proteasome inhibitors implying that the process of caspase activation following proteasome inhibition can be mutagenic. We therefore propose a model of mutagenesis for proteasome inhibition as a result of sublethal caspase signalling leading to the activation of CAD, which generates DSBs that are mis-repaired.

In contrast, HDAC inhibition by vorinostat or romidepsin created mutations via a mechanism not requiring caspases or CAD. ROS has been implicated in HDAC inhibitor-mediated toxicity and genotoxicity [19, 32, 35, 36]. The ability of HDAC inhibitors to unwind chromatin to facilitate the accessibility of transcription factors and enhance gene expression may also expose DNA to damage by intracellular factors like ROS. Although we did not assess ROS production in this study, our data indicate that caspases do not play a role in vorinostat- or romidepsin-induced death or mutagenesis implying that any DNA damage as a result of potential ROS formation is independent of caspases. Furthermore, DNA damage provoked by vorinostat was detected in cells over-expressing Bcl-2 which presumably contained intact mitochondria as treatment did not induce caspase activation. It is therefore unlikely that vorinostat caused DNA damage as a result of ROS that originated from the mitochondria.

We observed a similar mutagenic profile for vorinostat and romidepsin despite their different specificities for HDAC proteins. The mutagenic mechanism of these drugs presumably must involve inhibition of class I and/or II HDACs that both drugs target. Class I and II HDACs regulate expression of high fidelity homologous recombination (HR) DNA repair proteins, such as ATM, RAD51 and BRCA1. Inhibition of these HDACs by various inhibitors (including vorinostat) reduced cellular capacity for HR repair [37–39]. Given that defective HR or ATM function enhanced the mutagenesis of chemotherapies [40], downregulation of these factors by HDAC inhibition may also contribute to its mutagenic potential. Vorinostat-induced DNA damage was also dependent on the cell's replication status as γH2AX proteins co-localized with replication factories, and replication forks travelled slower upon vorinostat exposure [41]. The repair of DSBs facilitated by ATM often occurs within heterochromatin but DNA repair was ATM-independent and slower when DSBs were located in loosely packed chromatin [42]. It is possible that DNA damage occurring in regions of highly accessible chromatin, such as when HDAC proteins are inhibited, is rapidly repaired, probably by low fidelity repair machineries, thereby increasing the opportunity for mis-repair.

A number of HDAC inhibitors as well as proteasome inhibitors are approved for clinical use and newer drugs with similar mechanisms are being evaluated for therapeutic use. Further studies are needed to determine if the mutations observed in this study utilising in vitro assays translate to in vivo contexts, to probe whether these drugs possess oncogenic properties.

Methods

Cell lines and reagents

The TK6 lymphoblastoid [43] and LN18 glioblastoma [44] cell lines were purchased from ATCC (Manassas, VA, USA). TK6 cells were grown in RPMI-1640 containing HEPES buffer (Invitrogen; Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (Invitrogen). LN18 cells were cultured in Dulbecco's modified Eagle medium with high glucose (Invitrogen) supplemented with 10% heat inactivated FBS. All cells were grown at 37 °C in air supplemented with 5% CO_2. TK6 Cas9-CRISPR control, CAD KO 1.0 and FLAG-MBP cells were described previously [29].

Drugs used in this study were vorinostat (Selleck Chemicals; Houston, TX, USA), romidepsin (Selleck Chemicals), cisplatin (Sigma; Castle Hill, NSW, Australia), doxorubicin (Sigma), soluble TRAIL (Peprotech; Rocky Hill, NJ, USA), bortezomib (Selleck Chemicals), carfilzomib (Selleck Chemicals), delanzomib (Selleck Chemicals), ixazomib (Selleck Chemicals), oprozomib (Selleck Chemicals) and 6-thioguanine (6-TG; Sigma). These antibodies were used: rabbit anti-H2AX (Ser 139) clone 20E3 (Cell Signaling Technology; Danvers, MA, USA), mouse anti-FLAG (M2) (Sigma), mouse anti-Bcl-2 (Abcam; Cambridge, UK), mouse anti-GAPDH (Merck Millipore; Mellerica, MA, USA), goat anti-rabbit-FITC (Merck Millipore), donkey anti-rabbit-HRP (GE Healthcare Life Sciences; NJ, USA), rabbit anti-mouse-HRP (Sigma).

Cell survival assays

Acute cell death assays [45] and clonogenic survival assays [46] were conducted as described.

HPRT assay

HPRT assays for TK6 and LN18 cells were conducted using a previously published method [46] except that 10^5 LN18 cells were seeded in 10 cm plates in media containing 50 μM 6-TG.

γH2AX detection by flow cytometry

Detection and quantitation of cells bearing γH2AX protein was assayed as conducted previously [46], except that a 1:200 dilution of each antibody was used.

Stable transfection

One million TK6 cells were transfected with pEF-FLAG-Bcl-2 plasmid [47] using the Nucleofector SF solution using the DN-100 program with a Nucleofector device (Lonza) as previously described [40].

Immunoblotting

Immunoblotting was carried out according to a previously published protocol [46].

Caspase and proteasome activity assays

To measure caspase activity, 10^4 cells were seeded in 96-well white plates in media alone or media containing 10 µM Q-VD-OPh (R&D Systems; Minneapolis, NM, USA) and incubated for 1 h, then drug was added and incubated for 6 or 24 h. Caspase-3/-7 Glo solution (Promega; Fitichburg, WI, USA) was mixed into each well and incubated for 30 min at room temperature. Luminescence was recorded using a Spectromax M5 (Molecular Devices; CA, USA).

To measure proteasome activity [31], untreated and treated cells were mechanically homogenised on ice in a hypotonic lysis buffer (50 mM HEPES, pH 8.0) by sonication using a microson ultrasonic cell disruptor (Misonix) on power five for 5 s, then centrifuged at 13,000×*g* for 5 min at 4° and mixed 1:1 with stabilisation solution (40 mM HEPES, 1 mM EDTA, 20% glycerol, pH 8.0). Reactions were prepared in clear 96 well plates containing activity buffer (0.5 mM ATP, 1 mM DTT, 0.5 mg/mL BSA), 100 µM Suc-LLVY-AMC (Enzo life sciences; NY, USA) and 10 µg of protein that had been quantified using a micro BCA kit. Fluorescence was measured using a Spectramax M5 (Molecular Devices) and slope of the curve interpolated using GraphPad Prism.

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Compliance with ethical standard

Conflict of interests The authors declare that they have no conflict of interest.

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