

# Vorinostat enhances the cytotoxic effects of the topoisomerase I inhibitor SN38 in glioblastoma cell lines

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**Abstract** Histone deacetylase (HDAC) inhibitors represent a promising class of anti-cancer agents that are actively being evaluated in the context of clinical trials in solid tumors, including glioblastoma. What makes these agents particularly attractive is their capacity to enhance the activity of commonly used cytotoxics in cancer therapy, including both chemotherapy and ionizing radiation. As recent investigations suggest HDAC inhibitors may potentiate the cytotoxicity of topoisomerase inhibitors, which continue to be a commonly used class of agents in the treatment of glioblastoma, we performed preclinical studies to determine if this combination may be a promising strategy in glioblastoma. The effects of the HDAC inhibitor vorinostat and SN38, which is the active metabolite of the topoisomerase I inhibitor CPT-11, was evaluated using the clonogenic assay. Various treatment schedules were tested to determine optimum drug sequencing. Induction of DNA double strand breaks (DSBs) with the combination of vorinostat and SN38 was evaluated using the neutral comet assay, and their subsequent repair was evaluated by  $\gamma$ H2AX foci kinetics using immunofluorescent cytochemistry. Vorinostat enhanced the cytotoxicity of SN38 in glioblastoma cell lines. Optimal treatment schedules involved maximal concurrent administration of agents. Pretreatment with either agent did not enhance cytotoxicity. Vorinostat potentiated SN38-induced DNA DSBs and attenuated their subsequent repair. These results indicate vorinostat enhances the cytotoxicity of SN38 in glioblastoma cell lines,

suggesting this combination may be a worthwhile strategy to test in the context of a clinical trial.

**Keywords** Glioblastoma · Vorinostat · Histone deacetylase inhibitor · Topoisomerase inhibitor · SN38

## Introduction

There are approximately 18,500 cases of newly diagnosed primary brain malignancies per year, with the most aggressive form, glioblastoma, being the most common. Although the incidence of glioblastoma is low when compared to such cancers as lung, prostate, and breast, because this disease is rapidly fatal and generally affects young and otherwise healthy individuals, they are among the most devastating malignancies in terms of average years of life lost [1].

Historically, achieving clinical gains in glioblastoma have been limited, however novel therapeutic strategies have emerged offering strong promise in this disease. In newly diagnosed glioblastoma, results published by the European Organization for Research and Treatment of Cancer (EORTC) [2] combining the alkylating agent temozolomide with radiation demonstrated a significant improvement in survival and now represents standard therapy in newly diagnosed glioblastoma. Despite representing progress, this approach still does not offer cure to a majority of patients, who develop disease recurrence within a year of definitive therapy.

The potential for clinical improvements in recurrent glioblastoma has also been recently identified. Although salvage regimens have been generally ineffective, with median progression free survival after recurrence being approximately 9 weeks [3], Vredenburgh et al. recently published Phase II data combining bevacizumab, a

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humanized monoclonal antibody that binds to and inhibits the activity of vascular endothelial growth factor (VEGF), with the topoisomerase I inhibitor CPT-11 in recurrent glioblastoma [4]. This study demonstrated striking improvements in progression free survival when compared to historic controls, leading to the adoption of this platform as a standard therapy for recurrent glioblastoma.

Although the addition of CPT-11 in this regimen has been a point of debate, as this combination has been adopted by many institutions, identifying novel agents with the capacity of enhancing its cytotoxic effects may lead to continued clinical gains in glioblastoma. Histone deacetylase (HDAC) inhibitors represent an emerging class of targeted anti-cancer therapy actively being investigated in glioblastoma [5] and has demonstrated the potential to enhance the activity of a variety of cytotoxics [6, 7], including both topoisomerase I [8, 9] and II [10–13] inhibitors. Proposed mechanisms for this favorable interaction has been attributed to the capacity of these agents to modulate chromatin architecture, leading to chromatin decondensation and increased topoisomerase inhibitor accessibility to DNA [11], as well as cell cycle phase distribution [8]. Based on the clinical interest of HDAC inhibitors in glioblastoma, coupled with their potential to enhance the cytotoxic effects of topoisomerase inhibitors, we performed *in vitro* studies in glioblastoma cell lines to determine if this combination may be a promising therapeutic strategy in this otherwise fatal disease.

## Materials and methods

### Cell lines and treatment

Human glioblastoma cell lines U87 and T98G were obtained from ATCC (Manassas, VA) and grown in RPMI 1640 (GIBCO, Carlsbad, CA) and Eagles minimum essential medium with Earle's salt and L-glutamine (Cellgro, Manassas, VA), respectively, and supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Carlsbad, CA) at 37°C under a humidified atmosphere with 5% CO<sub>2</sub>. SN38 was purchased from Fisher Scientific (Pittsburgh, PA), suspended in DMSO (Sigma, St. Louis, MO) at stock concentrations of 1 μM and stored at –20°C. Vorinostat was provided by Merck Research Laboratories (Whitehouse Station, NJ), suspended in DMSO at stock concentrations of 1 mM and stored at –70°C until use. Vehicle control was used in all experiments.

### Clonogenic assay

Exponentially growing cells were trypsinized to generate a single cell suspension and seeded into 6-well tissue culture

plates. After allowing cells time to adhere (6 h), cells were treated using the described experimental conditions. Plates were then washed, replaced with fresh medium, and incubated for colony formation for 10–14 days. Colonies were stained with crystal violet and the number of colonies containing at least 50 cells was determined, and surviving fractions were calculated. Irradiated feeder cells were used prior to U87 seeding to promote colony formation. A dose enhancement factor (DEF) was calculated to quantify differences between SN38 and SN38 + vorinostat cell survival curves. The DEF was defined as the SN38 dose alone/SN38 dose combined with vorinostat required for 50% decrease in survival [6, 14].

### Neutral comet assay

To measure DNA double strand breaks (DSBs), the neutral comet assay was performed with slight modifications using a commercially available kit according to the manufacturer recommendations (Trevigen, Gaithersburg, MD). In brief, monolayer cultures were treated as indicated and single cell suspensions were generated and washed with PBS. Cells were mixed with low melting agarose (1:10), and this mixture was placed onto the provided slides. Cell lysis was performed at 4°C for 1 h. Cells were then subjected to electrophoresis (1 V/cm) for 20 m at room temperature, fixed with 70% ETOH and DNA was stained with SYBR green 1 staining solution. Digital fluorescent images were obtained using the IP Labs software (Signal Analytics Corporation, Vienna, VA).

### Immunofluorescent analysis of γH2AX foci

Cells were seeded into Lab-Tek II standard tissue culture slides (Thermo Fisher, Rochester, NY) and treated as indicated. Cultures were then fixed with 4% paraformaldehyde, permeabilized with 0.2% NP40, and blocked with 5% skim milk. Slides were incubated in primary antibody of phospho-H2AX antibody (Upstate Biotechnology, Charlottesville, VA) at a concentration of 1:500 in BSA, secondary antibody of Alexa Fluor 488 goat anti-mouse IgG, and then mounted with anti-fade containing 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). Cells were analyzed on a Zeiss upright fluorescent microscope. The number of foci per cell was determined in 100 cells per condition.

### Statistical analysis

The statistical analysis was done for the described treatment conditions using a Student's *t* test. A probability level of a *P* value of <0.05 was considered significant.

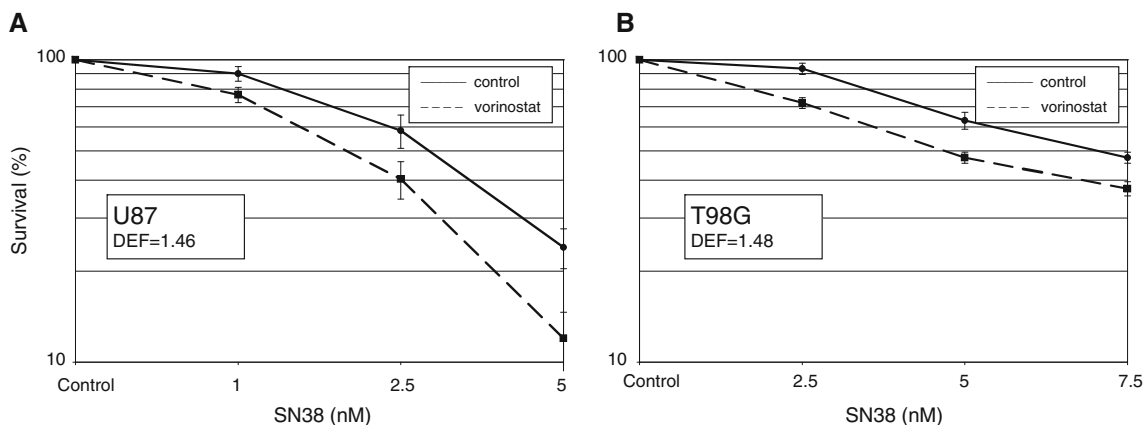
## Results

The aim of this study was to determine if the HDAC inhibitor vorinostat could enhance the cytotoxicity of the topoisomerase I inhibitor CPT-11 in glioblastoma cell lines. Towards this end, clinically relevant concentrations of vorinostat [15] was combined with graded concentrations of SN38, which is the active metabolite of CPT-11 [16], in culture medium of human glioma cell lines U87 and T98G. Cells were exposed to SN38 alone or combined with vorinostat for 48 h. Cultures were then replaced with fresh medium during the colony-forming period (10–14 days). Survival curves were generated after normalizing for the cell killing induced by vorinostat alone. As T98G cells were more resistant to both vorinostat and SN38, drug concentrations used in this cell line were slightly higher when compared to U87 (vorinostat: 2.5 versus 1.0  $\mu\text{M}$ ; SN38 2.5–7.5 versus 1.0–5.0 nM). Combining vorinostat with SN38 enhanced cytotoxicity, with a DEF at a surviving fraction of 0.5 of 1.46 and 1.48 in U87 and T98G cells, respectively (Fig. 1).

As sequencing between HDAC and topoisomerase inhibitor administration has been previously described as an important factor underlying their favorable interaction [8, 10], we sought to determine if different treatment schedules could influence response in our model system. Clonogenic survival studies were performed in U87 using fixed concentrations of vorinostat (1.0  $\mu\text{M}$ ) and SN38 (2.5 nM). Cells were exposed to either vehicle control, single agent alone for 48 h, or combined using five treatment schedules (Fig. 2). Treatment schedule A simulated the experimental conditions described in Fig. 1, with 48 h exposure of both agents together, followed by replacing with fresh medium. Treatment schedules B and C treated cells with both agents for 48 h, however only overlapped

their schedules for 24 h. Treatment schedules D and E exposed cells to either vorinostat or SN38 sequentially with no drug overlap. As shown in Fig. 2, sequential administration of vorinostat and SN38 (schedules D and E) demonstrated no statistically significant increase in cytotoxicity when compared to SN38 alone ( $P = 0.17$  and  $0.48$ , respectively). Both treatment schedules involving 24 h single agent pre-treatment, followed by 24 h drug overlap (schedules B and C) demonstrated a statistically significant increase in toxicity when compared to SN38 alone ( $P = 0.01$  and  $0.0007$ , respectively), although there was no difference between schedules B and C ( $P = 0.24$ ). As expected from results presented in Fig. 1, there was a statistically significant increase in cytotoxicity in schedule A (48 h overlap) when compared to SN38 alone ( $P = 0.0002$ ). When cytotoxicity of schedule A was compared to schedules B and C, there continued to be a statistically significant increase in cytotoxicity ( $P = 0.02$ ), reinforcing the potential for a mechanistic interaction between these agents.

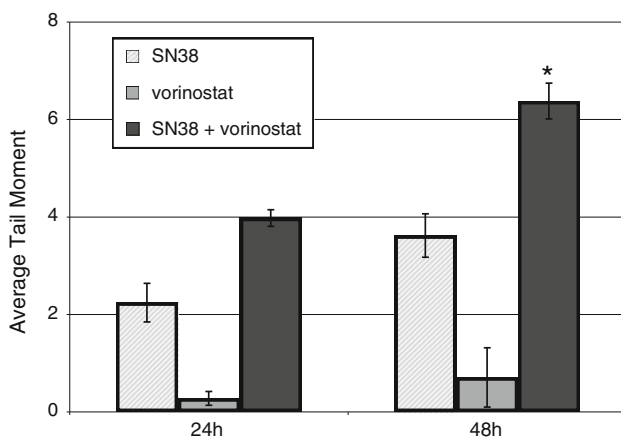
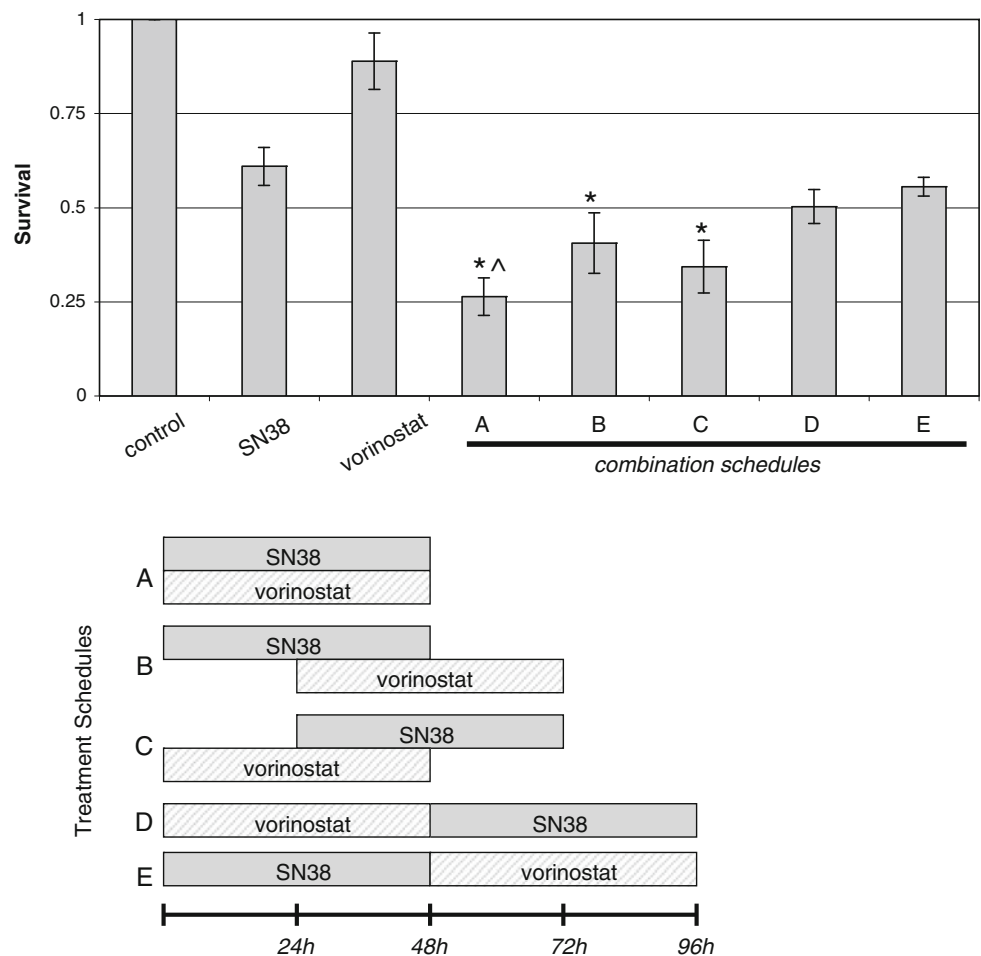
A key mechanism underlying topoisomerase I inhibitor activity involves its capacity to prevent religation of DNA strands by binding the topoisomerase I DNA complex, leading to DNA double-strand breaks (DSBs) and cell death [16]. Therefore, we sought to determine if the observed increase in cytotoxicity associated with the vorinostat and SN38 combination involved potentiation of DNA damage. DNA DSB induction was assessed using the neutral comet assay. U87 cells were exposed to SN38  $\pm$  vorinostat in a time-course manner up to 48 h using drug concentrations described in Fig. 2. As demonstrated in Fig. 3, SN38 alone demonstrated a time-course increase in DNA DSBs, as measured by the average tail moment. As expected, vorinostat alone did not demonstrate an increase in DNA DSBs, however combining vorinostat with SN-38



**Fig. 1** The effects of vorinostat on SN38-induced cytotoxicity. Glioblastoma cell lines were seeded in six-well tissue culture plates and exposed to SN38  $\pm$  vorinostat 6 h later. After 48 h drug exposure, culture plates were washed and replaced with fresh media.

Colonies were determined 10–14 days later, and survival curves were generated after normalizing for cell killing of vorinostat alone. DEF was calculated at a surviving fraction of 0.5. Points mean from three independent experiments; bars SD

**Fig. 2** The influence of sequencing in vorinostat and SN38 combinations. U87 cells were seeded in six-well tissue culture plates, allowed to adhere for 6 h, and exposed to SN38 alone (48 h), vorinostat alone (48 h), or to both agents combined (with cells being exposed to each agent for 48 h) using treatment schedules A–E. Culture plates were then replaced with fresh media, and colonies were determined 10–14 days later. Columns mean from three independent experiments, bars SD, \* statistically significant compared to SN38 alone (A versus SN38,  $P = 0.0002$ , B versus SN38,  $P = 0.01$ , C versus SN38,  $P = 0.007$ ; ^ statistically significant when compared to treatment schedules B and C ( $P = 0.02$ )

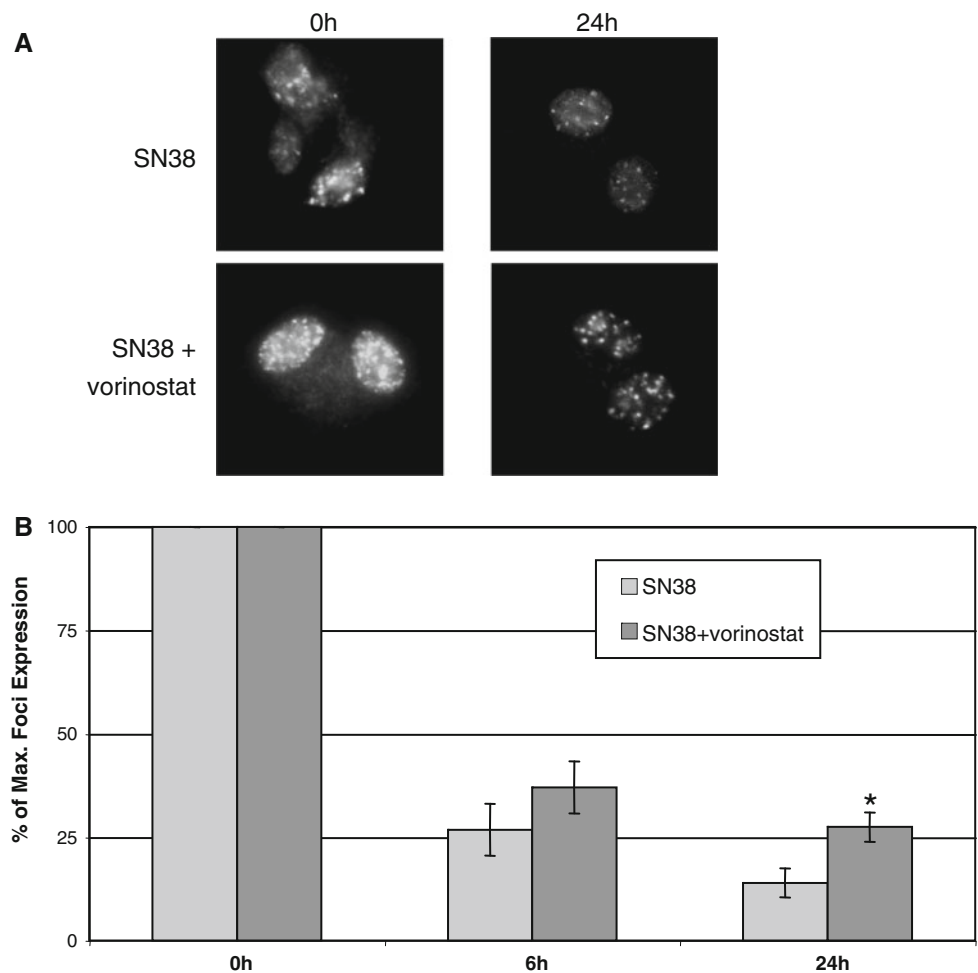


**Fig. 3** The effect of vorinostat on SN38-induced DNA DSBs. DNA DSBs were analyzed using the neutral comet assay. U87 cells were exposed to SN38, vorinostat, or the combination for 24 and 48 h and collected for analysis. Columns mean of three independent experiments, bars SD, \*  $P = 0.007$  (SN38 + vorinostat versus SN38)

demonstrated a potentiation of DNA damage when compared to SN38 alone, reaching statistical significance at 48 h ( $P = 0.007$ ).

It has been previously described that a key mechanism underlying the potential of HDAC inhibitors to enhance the response of cytotoxics is through abrogation of DNA DSB repair pathways [6, 7, 17–19]. To determine if vorinostat modulates DNA repair associated with SN38-induced damage, we evaluated phosphorylated histone H2AX kinetics. At sites of topoisomerase I inhibitor-induced DNA DSBs, the histone H2AX becomes rapidly phosphorylated ( $\gamma$ H2AX), forming readily visible foci and their subsequent dispersal is associated with DSB repair [6, 16, 20, 21]. U87 cells were exposed to SN38  $\pm$  vorinostat for 48 h and evaluated for  $\gamma$ H2AX foci. Immediately following 48 h drug exposure, there was a  $\sim 1.6$  fold increase in foci/cell with the SN38 and vorinostat combination when compared to SN38 alone, as demonstrated in representative micrographs (Fig. 4a). This is in accord with our neutral comet findings, demonstrating the capacity of vorinostat to potentiate SN38-induced DNA DSBs. To assess repair, cultures were then washed and replaced with fresh medium and evaluated for  $\gamma$ H2AX foci kinetics in a time-course manner [6, 18]. Results were normalized to the maximum number of foci/cell immediately after 48 h drug exposure

**Fig. 4** Vorinostat delays DNA repair kinetics. U87 cells were exposed to SN38 alone, vorinostat alone, or the combination for 48 h. Cells were then washed, replaced with fresh media, and analyzed for  $\gamma$ H2AX foci immediately after washing, and at stated times. A total of 100 nuclei per treatment were evaluated. The average number of foci/cell for cells treated with vorinostat alone, SN38 alone and in the SN38 + vorinostat combination after 48 h exposure was <1, 15 and 24, respectively. **a** Representative micrographs of  $\gamma$ H2AX foci in U87 cells following 48 h exposure of SN38 alone or vorinostat + SN38, and then after 24 h wash-off. **b** Data obtained in subsequent time points were normalized to these maximum levels. Columns mean of two independent experiments, bars SD, \*  $P = 0.03$  (SN38 + vorinostat versus SN38)



to SN38 alone and the SN38/vorinostat combination. As expected, following drug removal, there was rapid foci dispersal in both treatment arms suggesting repair. A modest decrease in foci dispersal was observed in the SN38 + vorinostat treatment arm when compared with cells treated with SN38 alone, which was statistically significant at 24 h (Fig. 4a, b;  $P = 0.03$ ). These results suggest that in addition to potentiating initial SN38-induced DNA DSBs, vorinostat also influences their subsequent repair.

**Discussion**

Glioblastoma continues to be a devastating malignancy in which therapeutic options are limited. Therefore, identifying novel combinatorial strategies in this malignancy is of critical importance. In this study, we identified the capacity of the HDAC inhibitor vorinostat to potentiate the cytotoxicity of SN38, the active metabolite of CPT-11, which continues to be a commonly used chemotherapy in glioblastoma. These findings are consistent with previous

preclinical investigations suggesting a favorable interaction between these two classes of anti-cancer agents in other tumor types, including breast cancer and melanoma [8, 9, 11], and clinical trials are currently underway testing this combination [9, 22]. Based on data presented, we are moving forth with early clinical trials testing the vorinostat and CPT-11 combination in glioblastoma.

Previous investigations in other cancer cell lines studying the combination of HDAC inhibitors with topoisomerase inhibitors identified drug sequencing to play a significant role in their favorable interaction. Marchion et al. showed that pretreatment with an HDAC inhibitor was required for the observed synergy with topoisomerase II inhibitors in breast cancer cell lines in vitro [10] and in vivo [12]. On the contrary, when combined with topoisomerase I inhibitors, Bevins et al. demonstrated increased efficacy when an HDAC inhibitor was administered post-treatment [8]. When combining vorinostat with the topoisomerase I inhibitor SN38 in glioblastoma cell lines, it appears that the most favorable scheduling is observed when interactions between the two agents are maximized. Combinations involving alternate sequencing of SN38 and

vorinostat as single agents (Schedule D and E, Fig. 2) demonstrated clearly inferior results when compared to other schedules, further supporting the mechanistic interaction between these classes of agents.

DNA damage caused by replication-mediated DSBs is likely a primary mechanism of tumor cell kill associated with topoisomerase I inhibitors [16]. Our findings demonstrate the potential of vorinostat to augment SN38-induced DSBs, likely playing a role in the observed increase in cytotoxicity. These findings may involve the potential of HDAC inhibitor to modulate chromatin architecture. This is supported by studies involving the HDAC inhibitor valproic acid, which demonstrated chromatin decondensation following HDAC inhibition led to enhanced sensitivity to DNA nucleases and increased DNA binding, suggesting an increase in access of macromolecules to DNA and enhanced cytotoxicity of topoisomerase II inhibitors [11].

In addition to potentiating SN38-induced DNA damage, our data also suggests vorinostat abrogates their subsequent repair. Our group and others have demonstrated the potential of HDAC inhibitors to modulate DNA repair following radiation response [6, 14, 18]. Although mechanisms underlying this interaction remain unclear, the potential of HDAC inhibitors to either down-modulate or acetylate repair proteins central to both homologous recombination and non-homologous end joining repair, may play a role [7, 17–19]. Although statistically significant, the influence of vorinostat on SN38-induced DSB repair appears modest when compared to repair associated with radiation damage. This difference may be associated with the apparent redundancy of repair pathways involved with the excision of the DNA topoisomerase I covalent complexes, indicating that several distinct signaling pathways may operate together during the repair of SN38-induced damage and compensate for individual pathways targeted by vorinostat [16].

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