The Inhibitor of Histone Deacetylases Sodium Butyrate Enhances the Cytotoxicity of Mitomycin C

Anastas Gospodinov, Stanislava Popova, Ivelina Vassileva, and Boyka Anachkova

Abstract

The use of histone deacetylase inhibitors has been proposed as a promising approach to increase the cell killing effect of DNA damage–inducing drugs in chemotherapy. However, the molecular mechanism of their action remains understudied. In the present article, we have assessed the effect of the histone deacetylase inhibitor sodium butyrate on the DNA damage response induced by the crosslinking agent mitomycin C. Sodium butyrate increased mitomycin C cytotoxicity, but did not impair the repair pathways required to remove mitomycin C-induced lesions as neither the rate of nucleotide excision repair nor the homologous recombination repair rate were diminished. Sodium butyrate treatment abrogated the S-phase cell-cycle checkpoint in mitomycin C–treated cells and induced the G2–M checkpoint. However, sodium butyrate treatment alone resulted in accumulation of reactive oxygen species, double-strand breaks in DNA, and apoptosis. These results imply that the accumulation of reactive oxygen species–mediated increase in DNA lesion burden may be the major mechanism by which sodium butyrate enhances the cytotoxicity of mitomycin C.

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Introduction

Histone deacetylase inhibitors (HDACi) are seen as promising epigenetic drugs that are used in mono and combination cancer therapy, but it is still unclear what mechanisms cause their clinical effects (1). The initial rationale of using HDACi in anticancer therapy was to induce global transition to euchromatic state and thus express heterochromatin-silenced genes (2). HDACs, however, have broad spectrum of targets that include histones as well as nonhistone proteins, and their inhibition results in similarly broad range of effects — from complex transcription-associated effects to roles on protein stability and protein–protein interactions (3). HDACi affect cell-cycle distribution and activate cell-cycle checkpoints. Thus, cells treated with sodium butyrate displayed G1 arrest due to disruption of the Rb-mediated signaling and suppressed expression of cyclins required for S-phase entry (4). In addition to the G1 block, HDACi reduced the expression of cyclin B1, a key cyclin for G2–M transition and resulted in G2 block (5). In some cases, the HDACi–induced growth arrest has been shown to be irreversible and tightly linked to the induction of p21 (6, 7).

In relation to the DNA damage response, different studies reported effects of the HDACi on DNA repair pathways and induction of apoptosis that could potentiate the DNA damaging agents used in cancer therapy. Treatment with HDACi resulted in defects in double-strand break (DSB) repair by both homologous recombination (HR) repair (8) and nonhomologous end joining (NHEJ; refs. 8, 9). The effect on NHEJ, which is the principal DSB repair pathway in mammals, was attributed to downregulation of Ku70 and Ku86 (10) or to the disruption of critical deacetylation events at the break sites (9). Another study found that sodium butyrate attenuated nucleotide excision repair (NER) and enhanced cell-killing effect of psoralen-induced lesions (11). HDACi induce cell death directly via various other mechanisms such as hyperacetylation of promoters of TRAIL, DR5, FAS, and FASL death receptor pathway members (12, 13) or production of proapoptotic reactive oxygen species (ROS) and oxidative injury (14–17).

An important way by which HDACi increase the cytotoxic effect of DNA damaging agents is by canceling the cell-cycle checkpoints, which allows entry of cells with damaged DNA in S-phase or mitosis (18, 19) and greatly facilitates their demise. Recently, it has been reported that sodium butyrate could abrogate the G1–S checkpoint activated by cisplatin, force cells in S-phase with damaged DNA, and thus, promote cell killing (20). On the other hand, sodium butyrate was not able to abrogate the G2 phase checkpoint induced in gamma-irradiated HeLa cells (8) indicating that different cell-cycle arrest points have different sensitivities to HDACi modulation.

The aim of the present investigation was to study the effect of HDAC inhibition on the cytotoxicity of an agent

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that induces DNA interstrand crosslinks and block cells in S-phase. We attempted a systematic examination of the effect of the HDACi sodium butyrate on mitomycin C (MMC)-induced DNA damage response (Fig. 1A). Sodium butyrate is the sodium salt of butyric acid, which is a 4 carbon normal fatty acid and is a natural metabolite in many organisms including bacteria populating the gastrointestinal tract. MMC is used as a chemotherapeutic agent to treat upper gastrointestinal, breast and bladder tumors. MMC is metabolized by reductive enzymes to generate reactive DNA alkylation species and when activated, it alkylates guanine at the N2-position to form DNA monoadducts and DNA intra- and interstrand crosslink adducts (21). MMC-induced lesions activate the S-phase DNA damage checkpoint (22) and are repaired by NER (23) and HR (24).

Our observations indicated that sodium butyrate increased MMC cytotoxicity. Treatment with sodium butyrate did not impair the repair pathways required to remove MMC-induced lesions as neither the rate of NER, nor HR repair rate were diminished. Sodium butyrate was able to abrogate the MMC-induced S-phase checkpoint and cells arrested in G2, which could in part explain the enhanced cytotoxicity of cells treated with both agents. Sodium butyrate treatment induced time-dependent accumulation of ROS, DNA damage, and massive apoptosis that could be inhibited by a ROS scavenger. We conclude that the mechanism by which sodium butyrate enhances the cytotoxicity of mitomycin C is complex and a major factor of this enhancement is the ROS-mediated overall increase in the DNA lesion burden induced by MMC.

Materials and Methods

Cell lines, culture conditions, transfection, and cell treatment

HeLa and HCT-116 cells were obtained from the American Type Culture Collection and were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), and penicillin/streptomycin and pyruvate. The male mouse ES cell line containing direct repeat GFP (DR-GFP) construct integrated in the hprt locus (clone 18-1/10) were obtained from the laboratory of Dr. Zdenko Herceg (International Agency for Research on Cancer, Lyon, France) in 2011. The cell line has been described previously (25) and was not authenticated in our laboratory. ES cells were initially grown on “feeders” and routinely on gelatin in DMEM supplemented with 20% FCS, leukemia inhibitory factor, penicillin/streptomycin, glutamine, pyruvate, and nonessential amino acids in 5% CO2. Transfection of plasmids was carried out using Lipofectamine 2000, following the manufacturer’s recommendations. To inhibit HDACs, cells were treated with either 5 mmol/L sodium butyrate or 5 mmol/L vorinostat (suberoylanilide hydroxamic acid) added to the culture medium and incubated for the indicated times.

Host cell reactivation assay, homologous recombination assay, and cell-cycle analysis

Host cell reactivation assay, using UV-irradiated plasmids, was carried out as described earlier (26). Briefly, plasmid DNA was dissolved in Tris-EDTA buffer (10 mmol/L Tris–HCl, pH 8, and 1 mmol/L EDTA) poured in Petri dishes to form 1 to 2 mm thick layer and UV irradiated for 2 and 3 minutes with a UV lamp (emission maximum 254 nm and exposure rate of 8.3 J.m–2.s–1). Damaged and control undamaged pEGFP-C1 were introduced into cells using Lipofectamine 2000 transfection reagent.
For the HR assay, the I-SceI expression vector pCBASce34 was transfected into ES cells using Lipofectamine 2000. Plasmid pCAGGS (without the I-SceI gene) and GFP-expressing plasmid pEGFP-C1 were used for mock and transfection efficiency controls, respectively. Flow cytometry analysis was conducted 48 hours after transfection using a FACScalibur apparatus with Cellquest software (Becton Dickinson). For analysis of GFP expression, cells were harvested by trypsinization and analyzed by a FACScalibur apparatus with Cellquest software (Becton Dickinson).

To analyze cell-cycle profiles, cells were harvested by trypsinization and fixed in 70% ethanol. Before analysis, cells were resuspended in PBS, treated with RNase A (20 µg/mL) and stained with propidium iodide (20 µg/mL), and analysis conducted by a FACScalibur apparatus with Cellquest software (Becton Dickinson).

To measure bromodeoxyuridine (BrdUrd) incorporation, cells were cultured in medium containing 15 µmol/L BrdUrd for 45 minutes, harvested, washed in PBS, fixed in 70% ice-cold ethanol, and stored at −20°C. To denature DNA, fixed cells were resuspended in solution containing 2N HCl and 0.5% Triton X-100 and incubated for 30 minutes at room temperature. To neutralize the acid, cells were resuspended in 0.1 mol/L Na2B4O7 and then blocked in 2% bovine serum albumin (BSA) in PBS – 0.1% Tween-20. Cells were incubated with mouse anti-BrdUrd antibody (Becton Dickinson cat# 347580) diluted at a ratio of 1:50, rinsed, and incubated with an anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody. RNA was digested with 20 µg/mL RNase A and stained with propidium iodide (5 µg/mL).

Western blotting and immunofluorescence microscopy

Protein lysates were resolved on SDS-PAGE (6% or 12.5%, as appropriate) and blotted on nitrocellulose membrane (Bio-Rad). The primary antibodies used were mouse anti-RAD51 antibody (Abcam #14B4), mouse anti-phospho-H2AX antibody (1:1000; Abcam #18311), rabbit anti-phospho-ATM (1:1000; Abcam #81290), acetyl-H4 (Lys5/8/12/16) antibody (Upstate, cat# 17-229), and mouse anti-actin (1:10,000; Sigma). Proteins were visualized using Li-cor Odyssey IR imaging system with appropriate IRDye-labeled secondary antibodies (Li-cor).

For immunofluorescence, cells were grown on coverslips, washed in PBS, fixed with methanol for 5 minutes at −20°C, washed with PBS, and blocked in 3% BSA in PBS for 1 hour. Staining was done using mouse anti-RAD51 antibody (Abcam #14B4) diluted 1:100 overnight at 4°C. Secondary FITC-conjugated anti-mouse IgG (Sigma) were used at 1:200 dilution for 1 hour at room temperature, and after 3 times for 5-minute washes with PBS, slides were mounted using ProLong Gold mounting medium (Invitrogen).

ROS assay

After experimental manipulation, floating and attached cells were harvested and pooled, washed once in PBS, and incubated in PBS with 10 µmol/L 2',7'-dichlorofluorescein diacetate (H2DCFDA) for 15 minutes at room temperature. Samples were then washed 3 times (5 minutes each), resuspended in the same buffer, and after 30 minutes, incubation in the dark at room temperature was analyzed by fluorescence-activated cell sorting (FACS). For fluorescent imaging, cells were grown on cover slips, treated and processed as above, and examined under a fluorescent microscope.

Results

Sodium butyrate enhances cytotoxicity of mitomycin C

We first checked the effect of sodium butyrate on histone H4 acetylation using an acetyl-H4-specific antibody. Western blot analysis of total extracts from cells treated for 8, 16, and 24 hours with 5 mmol/L sodium butyrate or left untreated as a control indicated that acetylation strongly increased in a time-dependent manner (Fig. 1B). To assess the effect of sodium butyrate on mitomycin C (MMC) cytotoxicity, we conducted clonogenic survival assay. HCT-116 cells were pretreated with 5 mmol/L sodium butyrate for 6 hours to induce histone hyperacetylation before addition of MMC for additional 18 hours without removal of sodium butyrate from the medium. Following treatment, cells were washed, plated at a density of 500 cells/cm², and left to grow in fresh medium. Colony formation was assessed 9 days later. The result showed that 5 mmol/L sodium butyrate alone caused about 20% reduction in the viability of the cells, 50 mmol/L MMC caused 50% reduction, whereas the combined effect of the 2 agents was stronger than that of either one alone and reached over 90% reduction in viability (Fig. 1C). Similar results were obtained with HeLa cells (data not shown).

Sodium butyrate does not impair nucleotide excision repair rates

NER is a major pathway that deals with MMC-induced lesions as it removes DNA adducts and intranucleoside strand links and participates in the repair of interstrand crosslinks (ICL) caused by the drug. To assess efficiency of NER, we used the host cell reactivation assay of in vitro UV-damaged plasmids. UV induces cyclobutane pyrimidine dimers and 6-4 photoproducts that are repaired only by NER. pEGFP-C1 was irradiated with 1 or 1.5 kJ/m² UV to obtain 0.8 to 1 lesion per kbp (27, 28) and transfected in HeLa cells. After transfection, cells were cultured for 6 hours in fresh medium and for 24 hours in the presence or absence of sodium butyrate and expression efficiency of GFP assessed using flow cytometry analysis (Fig. 2A and B). In HeLa cells, there were insignificant fluctuations in the repair efficiency between sodium butyrate-treated and -untreated cells (Fig. 2C). Similar outcome was observed when the experiment was carried out with the cell line HCT-116 (not shown). These results suggest that treatment with 5 mmol/L sodium butyrate did not...
significantly change NER capacity as measured by the host cell reactivation assay.

Effect of sodium butyrate on homologous recombination repair rates

Homologous recombination repair is essential for ICL repair. To measure the effect of sodium butyrate on HR repair rate, we took advantage of the DR-GFP construct integrated in the hprt locus of mouse ES cells (25, 29; Fig. 3A). Expression of I-SceI endonuclease induces a single DSB in an out-of-frame GFP reporter, which if repaired by HR with the tandem GFP fragment, restores a functional GFP gene and its expression can be detected by flow cytometry analysis. To evaluate the effect of sodium butyrate on HR repair, ES cells were transfected with I-SceI expression vector and treated with 5 mmol/L sodium butyrate or left untreated as control. After 48 hours of treatment, cells were collected and the size of the GFP population was measured by flow cytometry analysis (Fig. 3B Top). In parallel, cells were transfected with pEGFP-C1 plasmid to assess possible transcriptional effect of sodium butyrate on GFP expression, as GFP expression in that plasmid is driven by the same (CMV) promoter as that of the I-SceI–containing plasmid (Fig. 3B Bottom). After normalization of the GFP-positive cells transfected with the I-SceI restrictase expression plasmid to the expression efficiency of the control, it was seen that sodium butyrate treatment did not result in HR repair rate defect, but instead stimulated HR repair (with about 40%; Fig. 3C). That could be attributed to higher accessibility of both the restrictase and the HR repair machinery to DNA under the conditions of histone hyperacetylation.

To further confirm these results, we checked Rad51 recruitment into nuclear foci after MMC treatment under the conditions of HDAC inhibition. Rad51 functions as a helical nucleoprotein filament loaded on the single-stranded DNA 3' overhangs that carries out the search for and exchange with the homologous duplex and thus, constitutes the core of the HR repair reaction (30). Treatment of HeLa cells with 5 mmol/L MMC for 18 hours followed by immunofluorescent staining resulted in clearly visible Rad51 foci in both sodium butyrate–treated and –untreated cells (Fig. 3D). There were no apparent defects in Rad51 foci formation in sodium butyrate–treated cells, confirming the result obtained using the DR-GFP reporter. Taken together, the data about the rates of NER and HR repair in sodium butyrate-treated cells indicate that sodium butyrate did not compromise the repair of ICLs.
Effect of sodium butyrate on cell-cycle distribution, DNA damage signaling, and apoptosis

To check whether sodium butyrate affected DNA damage signaling in the MMC-treated cells, we analyzed their cell-cycle distribution. HeLa cells were treated with 5 mmol/L sodium butyrate for 6 hours before the addition of 5 μmol/L MMC and incubated for additional 18 hours. Following treatment, cells were analyzed by FACS. The obtained results indicated that MMC treatment alone caused S-phase accumulation, consistent with earlier reports on the effect of MMC (22). Treatment with sodium butyrate blocked cells in G1 and G2, causing depletion of S-phase cells, which has also been described (31–33). In cells treated with both agents, S-phase cells were diminished, whereas the G2 fraction was increased over 5-fold, compared with those treated with MMC only. This suggests that upon HDAC inhibition, the S-phase checkpoint was abrogated and cells accumulated in G2, which implied activation of the G2–M checkpoint (Fig. 4A and B).

To further confirm these results, we checked BrdUrd incorporation in HeLa cells that were either treated for 6 hours with 2 μmol/L MMC or with 2 μmol/L MMC and 5 mmol/L sodium butyrate simultaneously (Fig. 4C). Staining with an anti-BrdUrd antibody followed by FACS analysis indicated that in MMC-treated cells, DNA synthesis was strongly inhibited compared with the untreated control, and the fraction of cells that were BrdUrd positive was diminished more than 3.5-fold. Treatment with both agents resulted in an increase in the population of replicating cells compared with the cells treated only
with MMC to reach a value that was slightly lower than that of the control. These data clearly showed that sodium butyrate impaired activation of the S-phase checkpoint in cells treated with MMC.

To test the G2–M DNA damage signaling in a more direct way, we checked the abundance of the phosphorylated active form of ATM checkpoint kinase by Western blot analysis. HeLa cells were treated with 5 mmol/L sodium butyrate for 24 and 48 hours or were left untreated and 5 mmol/L MMC was added during the last 18 hours of treatment (Fig. 4D). In parallel, cells were treated with sodium butyrate only for 24 and 48 hours and left untreated as controls.

Figure 4. Cell-cycle distribution and DNA damage signaling in cells treated with sodium butyrate, MMC, and a combination of the drugs. A, HeLa cells were treated with 5 mmol/L sodium butyrate for 24 hours, 5 μmol/L MMC for 18 hours, a combination of the drugs (MMC added during the last 18 hours of the 24-hour sodium butyrate treatment), or left untreated as a control. After propidium iodide staining, cells were analyzed by FACS analysis. B, cell-cycle distribution of cells treated as in A. Light gray bars, percentage of G1 cells; dark gray bars, S-phase cells; gray bars, G2 cells; dotted bar, sub-G1 fraction. Data are medium of two 2 independent experiments ± s.d.m. C, scatter plots showing BrdUrd incorporation and DNA content (stained with propidium iodide) in control cells, cells treated for 6 hours with 5 μmol/L mitomycin C or a combination of 5 μmol/L mitomycin C and 5 mmol/L sodium butyrate. Indicated are the percentages of replicating cells. D, phosphorylated ATM in cells treated with sodium butyrate for the indicated times and with 5 μmol/L mitomycin C for the last 18 hours of sodium butyrate treatment or with MMC only. E, levels of phosphorylated ATM in cells treated with sodium butyrate for the indicated time periods. F, HeLa cells were treated with 5 mmol/L of sodium butyrate for 24 hours, 5 μmol/L MMC for 18 hours, and a combination of the drugs (MMC added during the last 18 hours of sodium butyrate treatment) or left untreated as control. Total proteins extracts were analyzed by Western blot analysis for the accumulation of the p85 PARP apoptotic fragment. G, HCT-116 cells were treated and analyzed as in F.
untreated as controls (Fig. 4E). Western blot analyses indicated that cells treated with sodium butyrate displayed increased amounts of phosphorylated ATM, irrespective of whether they were or were not treated with MMC. These results are consistent with the FACS data and indicate a shift to G2–M ATM-dependent checkpoint activation in cells treated with MMC and sodium butyrate.

As treatment with sodium butyrate induced DNA damage signaling and appearance of sub-G1 cells (Fig. 4A and B), we next checked whether the reduction in viability in sodium butyrate and MMC-treated cells was a result of apoptosis. To this end, we looked for the accumulation of the p85 fragment of PARP that is a marker characteristic for ongoing apoptosis (34). The result indicated that the treatment of HeLa and HCT-116 cells with either of the drugs induced apoptosis, sodium butyrate being the more potent proapoptotic agent. At 24 hours of treatment, the combined effect of sodium butyrate and MMC was stronger than that of either of the drugs alone (Fig. 4F and G).

To extend these observations we analyzed the phosphorylation of the histone variant H2AX, which is the principle chromatin substrate of ATM and is widely used as an indicator for the formation of DSBs. Cells treated with 5 mmol/L of sodium butyrate for 24 and 48 hours, with or without addition of MMC exhibited increased amount of γ-H2AX (Fig. 5A and B), strongly suggesting accumulation of DSBs in the cells treated with the HDACi. H2AX is phosphorylated in broad chromatin regions surrounding the DSBs that can be visualized as nuclear foci by immunofluorescent microscopy, which allows analysis of DSB formation at the cellular level. The comparison between the pattern of γ-H2AX foci formation in MMC- and SB-treated cells showed that these formed as early as 8 hours after treatment with either of the agents (Fig. 5C). However, the density of foci formation in sodium butyrate-treated cells was lower than in MMC-treated cells. The appearance of γ-H2AX foci in sodium butyrate-treated cells argues that sodium butyrate induces DSB formation.

**Induction of apoptosis in HDACi-treated cells is due to DSB formation by ROS**

It has been reported that sodium butyrate may cause significant deregulation of the activities of enzymes that cope with ROS inside the cell (35). To test the possibility that the sodium butyrate-induced DSBs are ROS-dependent, we treated HeLa cells with sodium butyrate for 8 and 24 hours and then stained the cells with the cell-permeant dye 2′,7′-dichlorodihydrofluorescein diacetate, which is a chemically reduced form of fluorescein and is widely used as an indicator for ROS. FACS analysis and fluorescent microscopy indicated that after 8 hours in sodium butyrate, cells were already showing increased staining. At 24 hours of treatment, the median value of the fluorescence has increased nearly 2-fold and much more brightly stained cells were observed under the microscope (Fig. 6A and B). Similar results were obtained using the HCT-116 cell line. To verify that increased intracellular concentration of ROS led to apoptosis, we used dithiothreitol (DTT), a substance known to be an effective scavenger of ROS and checked apoptosis induction in HeLa cells treated with sodium butyrate, DTT, or both agents simultaneously. FACS analysis indicated that while apoptotic sub-G1 population was readily observed in sodium butyrate-treated cells, it was absent when the cells were treated with a combination of sodium butyrate and DTT (Fig. 6C). This result strongly suggested that apoptosis in sodium butyrate-treated cells depended on the generation of ROS. To further these observations, we checked accumulation of the p85 PARP fragment as a marker of apoptotic induction. As expected, the p85 PARP fragment, while abundant in sodium butyrate-treated cells, was absent when DTT was added (Fig. 6D). Taken together, our results indicate that sodium butyrate enhances the cell killing effect of the crosslinking agent MMC mostly as a result of increased ROS accumulation in the cells and induction of DSBs and apoptosis.

In addition, we used another structurally unrelated to sodium butyrate HDACi vorinostat (Supplementary Fig. S1A) to test whether the observed effects were specific to sodium butyrate or common to different classes of HDACi. Treatment with 5 μmol/L vorinostat resulted in histone hyperacetylation, appearance of DSBs as judged by accumulation of phosphorylated H2AX, and an increase of p85 PARP, indicative of apoptosis induction (Supplementary Fig. S1B). These changes were concomitant with increase of ROS production (Supplementary
Fig. S1C) that was observed in cells following staining with 2′,7′-dichlorodihydrofluorescein diacetate, indicating that both sodium butyrate and vorinostat induced similar effects.

Discussion

Here, we have shown that the combined effect of the HDACi sodium butyrate and the DNA crosslinking agent MMC on reduction of cell viability was stronger than that of either one of the agents alone. If sodium butyrate affected the efficiency of repair of MMC-induced DNA lesions, it could have significant effect on MMC cytotoxicity. However, we did not detect differences in NER capacity between sodium butyrate-treated and -untreated cells. A previous report found reduced NER rate after sodium butyrate treatment (11). The discrepancy could be due to differences in the experimental design (plasmid host cell reactivation vs. cell-based assay) or the cell types used. Regarding the repair by HR, sodium butyrate treatment resulted in a higher HR repair rate (Fig. 3B). This is in
agreement with previous data that histone acetylation at lesion sites was needed for efficient HR repair (25, 36, 37). The result from the reporter assay (Fig. 3C) was strengthened by the fact that Rad51 foci formation after induction of ICLs by MMC was also not impaired in cells treated with sodium butyrate (Fig. 3D). Taken together, the data about the rates of NER and HR repair indicated that the increased cytotoxicity of ICL lesions in sodium butyrate-treated cells did not result from changes in their repair capacity.

Another possible cause for increased sensitivity of sodium butyrate-treated cells to MMC could be the abrogation of DNA damage–induced checkpoint signaling. The distribution of the cells in the cell cycle under conditions of hyperacetylation of chromatin showed that the MMC-induced ATR-dependent block of the cells in S-phase was abrogated. This was confirmed by the increased rate of BrdUrd incorporation in MMC-damaged cells that were treated with sodium butyrate. The observation is in line with a recent report that HDAC inhibition in yeast specifically counteractivated activation of the yeast ATR ortholog, Mec1 (38). However, here we observed a shift in cell-cycle signaling from S-phase ATR-dependent block of the cells to ATM-dependent blockage in G2–M phase of the cell cycle. That is why we do not think that perturbations of cell-cycle signaling could be the reason for the sensitization of MMC-treated cells by sodium butyrate. In search for another reason for the increased apoptosis rate, we treated cells with sodium butyrate only and found phosphorylation of H2AX, indicating the presence of DSBs.

These results raised the question about the cause of DSBS production. Because there were data about HDACi-mediated ROS generation (15), we also tested this possibility. The results showed that sodium butyrate treatment resulted in a time-dependent accumulation of ROS. The apoptotic effect caused by sodium butyrate was largely abolished in the presence of the ROS scavenger DTT (Fig. 6C and D). This suggested that apoptosis induction under our conditions was abrogated. This was confirmed by the increased rate of BrdUrd incorporation in MMC-damaged cells that were treated with sodium butyrate. The observation is in line with a recent report that HDAC inhibition in yeast specifically counteracted activation of the yeast ATR ortholog, Mec1 (38). However, here we observed a shift in cell-cycle signaling from S-phase ATR-dependent block of the cells to ATM-dependent blockage in G2–M phase of the cell cycle. That is why we do not think that perturbations of cell-cycle signaling could be the reason for the sensitization of MMC-treated cells by sodium butyrate. In search for another reason for the increased apoptosis rate, we treated cells with sodium butyrate only and found phosphorylation of H2AX, indicating the presence of DSBS.

One explanation for the production of ROS by sodium butyrate could be that the high concentration of sodium butyrate would enhance the synthesis of medium and long chain fatty acids and the increased lipid concentration may induce ROS. On the other side, a recent proteomic study of HDACi-treated cells found altered expression levels of enzymes associated with energy metabolism, antioxidative stress, and cellular redox control, which suggests an excessive production of ROS under conditions of hyperacetylation of chromatin (43). In addition, HDAC inhibition has led to increased transcription of the TRAIL gene that resulted in accelerated caspase activation and promoted mitochondrial damage (44). To choose between the hypotheses of elevated lipid source and transcriptional perturbation leading to ROS production, we repeated some of the experiments with vorinostat. Vorinostat is an HDAC inhibitor that is structurally unrelated to sodium butyrate, but also inhibits class I and class II HDACs. The application of vorinostat resulted in phosphorylation of H2AX and p85 PARP accumulation that was concomitant with ROS production in the treated cells. These results favor the latter hypothesis and indicate that ROS-mediated apoptosis is a common effect of HDAC inhibition. The results also suggest that the data obtained with sodium butyrate as a model compound could be extended to clinically applicable HDACi.

HDACi are an emerging new class of agents that could enhance the effectiveness of cancer therapy. It has been reported that different HDACi sensitize tumor cells towards the killing effect of many anticancer agents (11, 45–47) and this effect has been attributed to interference with DNA repair pathways (8, 11, 48) or abrogation of the cell-cycle checkpoints (18–20). Our results suggest that the accumulation of intracellular ROS-mediated increase in the lesion burden may be the major mechanism by which sodium butyrate enhances the cytotoxicity of MMC-treated cells. The results also explain the selectivity of HDACi to tumor cells, as normal, not transformed human fibroblasts treated with HDACi accumulate thioredoxin, a natural ROS scavenger, making the normal cells less sensitive to HDACi (49, 50).

Taken together, our results underscore the importance of understanding the mechanistic effect of each modulator of the DNA damage response to use the best regimen of combination with genotoxic drugs to manipulate the clinically desired outcome of treatment of tumor cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B. Anachkova
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gospodinov, S. Popova, I. Vassileva
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Writing, review, and/or revision of the manuscript: A. Gospodinov, B. Anachkova
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Vassileva
Study supervision: B. Anachkova

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