**In vivo** synergy between topoisomerase II and histone deacetylase inhibitors: predictive correlates

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Abstract

Histone deacetylase inhibitors (HDACi) are a promising class of anticancer agents, yet the specific biological effects resulting in cell death are still poorly understood and clinically relevant markers of response are not adequately defined. The anticonvulsant valproic acid has recently emerged as an HDACi, and *in vitro* studies suggested that valproic acid may potentiate cytotoxic agents. We evaluated the pharmacologic and biological effects of valproic acid on histone acetylation, chromatin structure, and DNA damage induced by topoisomerase II inhibitors in mice bearing breast cancer tumors and developed an *ex vivo* methodology for response prediction using comet assays. The exposure of mice to valproic acid before exposure to epirubicin led to tumor regression when valproic acid was given for 48 hours at concentrations sufficient for histone hyperacetylation, down-regulation of heterochromatin maintenance proteins, and chromatin decondensation. Tumor response was accurately predicted by *ex vivo* comet moments. Valproic acid did not exacerbate epirubicin-related toxicity. Antitumor effects were not observed with valproic acid alone despite biologically active valproic acid concentrations. These findings suggest that exposure of tumor-bearing mice to valproic acid potentiated the antitumor effects of topoisomerase II inhibitors without enhancing toxicity. The HDACi-induced histone acetylation and modulation of heterochromatin correlated with potentiation of epirubicin-mediated DNA damage. However, these effects did not result in antitumor activity when using a HDACi alone and hence should not be considered a surrogate marker. *Ex vivo* comet assays may be useful as a predictive tool when tumor cells are limited and serial biopsies are difficult to obtain. [Mol Cancer Ther 2005;4(12):1993–2000]

Introduction

Histone acetylation is an important step in the regulation of transcription. Early trials with several histone deacetylase inhibitors (HDACi) have shown moderate activity in solid tumor malignancies when used as single agents (1–5). The mechanism by which HDACi control tumor growth is largely unknown.

HDACi not only regulate transcription but also may control chromatin dynamics and thereby influence access of macromolecules, such as DNA-damaging agents and transcription factors, to their target sites (6). Several investigators have proposed synergistic or additive interactions between HDACi and other antitumor agents (7–12). We have shown previously that the acetylation of histones by HDACi, such as suberoylanilide hydroxamic acid or valproic acid, leads to conformational changes of DNA and chromatin decondensation *in vitro*. The HDACi-induced chromatin decondensation was associated with a potentiation of DNA damage induced by topoisomerase II inhibitors in a sequence-specific manner (8, 9). Furthermore, we reported that the valproic acid–induced histone acetylation was obligatory but likely not sufficient for chromatin decondensation. Chromatin decondensation in the presence of HDACi was associated with down-regulation of proteins involved in the maintenance of heterochromatin, such as the structural maintenance of chromatin proteins 1 to 5, structural maintenance of chromatin–associated proteins, DNA methyltransferase 1, and heterochromatin protein 1 (HP-1; ref. 11). Data from cultured cells showed that the modulation of heterochromatin maintenance proteins was reversible on drug withdrawal and was obligatory for the potentiation of DNA-damaging agents. HDACi affect several biological functions, many of which may not be directly associated with their antitumor effects. We have reported previously a schedule-dependent synergistic interaction of valproic acid and topoisomerase II inhibitors. Here, we expanded these studies to define the relevance of *in vivo* biological effects of valproic acid for this synergistic interaction and propose a novel methodology to predict responses on limited tumor samples after exposure to HDACi.

Materials and Methods

Chemicals and Antibodies

Valproic acid was purchased from Sigma Chemical Co. (St. Louis, MO) and epirubicin was from Pfizer, Inc.
All other reagents were of analytic grade and purchased from standard suppliers. Acetyl histone H3 antibody and HP-1 antibody were purchased from Upstate Biotechnology (Chicago, IL).

**Cell Lines**

The cell lines used for this study were purchased from the American Type Culture Collection (Manassas, VA) and include the breast cancer cell lines SKBr-3, MCF-7, and MDA-361; the melanoma cell line A375-S2; and the ovarian cell line SKOV-3; and the colon cancer cell line KM12C. Cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies, Carlsbad, CA). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

**Apoptosis**

Topoisomerase II inhibitor cytotoxicity was evaluated in the presence of HDACi by apoptotic assays. Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation and evaluated with fluorescence microscopy using bis-benzimide staining. Briefly, harvested cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed with PBS. Cell nuclei were stained with 0.5 μg/mL bis-benzimide trihydrochloride (Hoechst 33258, Molecular Probes, Eugene, OR). Two hundred cells were counted for each experiment and evaluated for apoptotic scores [apoptotic nuclei] / (all nuclei) × 100. All experiments were repeated at least in triplicate.

**Animal Studies**

Four- to 6-week-old nude athymic female mice were obtained from the National Cancer Institute-Frederick Cancer Center. Experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol (R2462), and institutional guidelines for the proper and humane use of animals in research were followed. Estrogen pellets (60-day slow release, 0.72 μg) were placed under the dorsal skin 7 days before tumor inoculation for MCF-7 cell experiments. MCF-7 or MDA-361 breast cancer cells (5 × 10⁶) mixed 1:1 with Matrigel (Life Technologies) were injected on the right and left flanks. Once tumors reached 5 mm in largest diameter, mice (4–8 mice per cohort) were treated with saline alone, valproic acid only, epirubicin only, epirubicin followed by valproic acid, and valproic acid followed by epirubicin. Mice receiving valproic acid were injected with 500 mg/kg/d in 0.1 mL saline i.p. twice daily for five doses. Epirubicin (0 or 3 mg/kg in 0.1 mL saline) was injected either after the last dose or before the first dose of valproic acid. Tumor volume was calculated according to the formula: 

\[ V = 0.52 \times a \times b^2 \]

where \( a \) is largest superficial diameter and \( b \) is smallest superficial diameter. Once the tumors reached 15 mm in largest diameter, mice were euthanized. Tumor volumes and weights were assessed four times weekly. This experiment was repeated at least twice. For ex vivo experiments on isolated tumor cells, mice were treated i.p. with 0, 100, 250, 500, or 1,000 mg/kg/d valproic acid twice daily for five doses. Tumors were harvested 4 hours after the last dose of valproic acid and single cells were isolated by collagenase digestion (200 units/mL, Sigma). Statistical analysis was done using paired Student’s t test and multivariate analysis by ANOVA for comparisons within and among groups.

**Definition of the Maximally Tolerated Dose and Dose Justification**

A limited dose finding study was done at 0, 100, 250, 500, and 1,000 mg/kg/d in tumor-bearing mice. The 1,000 mg/kg/d dose was found to be too toxic. Concentrations of 500 mg/kg/d divided in two doses were tolerable but showed a relative weight gain retardation when compared with saline-treated mice. Reports from other investigators in this mouse model suggested excessive toxicities at 800 mg/kg/d (13, 14). The reported LD₅₀ for epirubicin in mice was 16 mg/kg (15). The epirubicin dose selected for the presented experiments was based on prior studies suggesting a feasibility of this dose when given weekly for at least 3 weeks; higher concentrations resulted in delayed deaths. Higher concentrations were feasible when given as a single injection.

**Histone Acetylation (Acetyl Histone H3) and HP-1**

Tumor cells isolated from mice injected with 0, 100, 250, or 500 mg/kg/d valproic acid for 48 hours were evaluated for histone H3 acetylation and HP-1 expression by immunofluorescence. Cells were fixed with 95% ethanol-5% acetic acid. The respected proteins were labeled with anti-acetylated histone H3 and HP-1 antibody and then exposed to fluorescent-labeled secondary antibodies (Alexa Fluor 546 and 488, respectively, Molecular Probes) as described previously (11). In brief, images of histone H3 and HP-1 fluorescence staining were acquired by confocal microscopy in separate channels (red and green) using fixed detector gain and amplification settings between experiments. Files were then converted to TIFF files and analyzed by Photoshop. The respected protein staining was measured in square pixel surface area and was reflected in the average number of square pixels from the nuclei of 50 cells per sample. For each measured nuclei, the background staining was measured in the immediate vicinity and subtracted from the average square pixel surface area. The use of a cytospin of cultured MCF-7 cells exposed to the respective experimental condition served as internal staining control and comparator. Levels for the fluorescence detection were set accordingly.

**Electron Microscopy**

Tumor samples (20–50 mm³) were fixed with 4% paraformaldehyde in PBS for 1 hour at 37°C. Samples were rinsed with PBS, dehydrated in increasing ethanol concentrations (50%, 70%, 80%, 90%, and 100%), and embedded in water-permeable LR White plastic. Cells were sectioned with a diamond knife at 90 nm thickness and stained for 2 minutes in 1% uranyl acetate and for 5 minutes with lead citrate and finally examined at 60 kV on a Philips 100M electron microscope at 2,700- and 6,300-fold magnification.

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¹ Munster et al., unpublished data.
Results

Topoisomerase II Inhibitors and HDACi Interactions
Are Schedule Dependent

In vitro studies indicated that exposure to HDACi sensitized cancer cells to topoisomerase II inhibitors (9, 11). Sensitization required concentrations and exposure times sufficient for the down-regulation of heterochromatin proteins. Furthermore, sensitization was seen at concentrations of either drug that was not associated with any antiproliferative or apoptotic effects, and a reversal of the drug sequence completely abrogated the valproic acid–induced potentiation of epirubicin (Fig. 1A). To assess whether dose and time dependence was a prerequisite of synergy between HDACi and topoisomerase II inhibitors in vivo, different schedules of this combination were tested in a breast cancer xenograft model. Mice (n = 8) were implanted with MCF-7 breast cancer tumors on both dorsal flanks. When tumors reached 5 mm in diameter, mice were randomly selected and treated with 500 mg/kg/d valproic acid given every 12 hours for 48 hours followed by a single injection of 3 mg/kg epirubicin or a single dose of epirubicin followed by 48 hours of valproic acid (Fig. 1B). Control arms included saline only, valproic acid only, and epirubicin only. Treatment cycles were repeated after 1 week. The definition and justification for dose selection were described in Materials and Methods. The selected doses for both drugs were the maximally tolerated doses served at concentrations of valproic acid and epirubicin that had minimal effects when used as single agents.

Animals were evaluated for toxicity thrice weekly. Although there were no treatment-related deaths, both valproic acid and epirubicin treatments were associated with toxicity. Although there were no treatment-related deaths, both valproic acid and epirubicin treatments were associated with toxicity.

Figure 1. Valproic acid potentiates epirubicin cytotoxicity in vitro and in vivo. A, schedule dependence of apoptosis observed in MCF-7 cells treated with increasing concentrations of valproic acid (0, 0.5, 1, 2, 3, or 5 mmol/L) for 48 h either immediately before (VPA: Epi; gray shading) or immediately after (VPA: Epi; no shading) a 4-h exposure to 0.5 μmol/L epirubicin or valproic acid alone (VPA: dark shading). The respective drugs were then replaced by non-drug-containing medium and apoptotic nuclei were assessed by nuclear fragmentation 24 h after drug removal. Bars, SE. B, tumor-bearing mice (4–8 mice per group) received i.p. injections of 500 mg/kg/d valproic acid given twice daily for five doses followed by one i.p. injection of epirubicin (3 mg/kg × 1) or one dose of epirubicin followed by five doses of valproic acid. Cycles were repeated beginning on days 8 and 15 (horizontal columns). The cohorts were as follows: saline (diamonds), valproic acid alone (circles), epirubicin alone (squares), valproic acid followed by epirubicin (open squares), or epirubicin followed by valproic acid (open triangles). Tumor growth was significantly inhibited (P < 0.001) in animals treated with valproic acid followed by epirubicin when compared with all other treatment groups. Bars, SE. C, percent average weight change (mean and 95% confidence intervals (95% C.I.) per treatment group after each treatment cycle.

Comet Assay

The alkaline comet assays were done and used according to the manufacturer’s recommendations (Trevigen, Gaithersburg, MD). Isolated tumor cells (5,000 per slide in duplicates) were imbedded in agarose at a final concentration of 1:10 and layered on glass slides. Slides were equilibrated to 4°C and submerged in cold Trevigen lysis buffer containing 1% DMSO for 30 minutes. Slides were then incubated for 1 hour at room temperature in alkaline electrophoresis solution (pH >13). Comet tails were generated by a 40-minute electrophoresis at 20 V, 4°C. Slides were then washed in distilled water and dried overnight. Slides were stained for 20 minutes with SYBR Green and the comet moment was quantified using the LAI Comet Analysis System. Statistical analysis was done using multivariate analysis by ANOVA. Experiments were repeated at least twice.

A complete disappearance of established tumors was not observed in any of these groups. The potentiation of epirubicin by pretreatment with valproic acid was observed at concentrations of valproic acid and epirubicin that had minimal effects when used as single agents.

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with a slowing of the expected weight gain compared with the control group (Fig. 1C). Mice in the treatment groups receiving combinations of valproic acid and epirubicin were not associated with increased toxicity as determined by limited toxicity assessment, including food intake, soiling, grooming behavior, locomotion, posture, activity, and responsiveness.

Effects of Valproic Acid on Histone Acetylation and Chromatin Structure

Histone modifications are an integral part in epigenetic control (16). The most important functions of histone modification include acetylation and methylation. The histone acetylation state is essential for the heterochromatin structure and dynamics. Hyperacetylation of core histones has been associated with pericentric heterochromatin. Furthermore, redistribution of HP-1 has been directly linked to histone acetylation (17, 18). Chromatin structure changes induced by HDACi have been reported by several investigators, including this group (8, 18–21). In particular, we have shown previously that prolonged exposure to valproic acid resulted in a depletion of several chromatin maintenance proteins. These included several structural maintenance of chromatin proteins, DNA methyltransferase 1, and HP-1. The reduced expression of these proteins was associated with chromatin decondensation \textit{in vitro} (11). As the expression of HP-1 has been linked to the acetylation state of the chromatin, we have not only measured the effects of valproic acid directly on histone H3 acetylation but also indirectly by evaluating its effects on HP-1. The expression levels of acetylated histone H3 and HP-1 were evaluated in MCF-7 tumor cells isolated from mice treated with 0, 100, 250, and 500 mg/kg/d valproic acid given over 48 hours. Tumor cells were isolated 4 hours after the last dose of valproic acid and the respective proteins were assayed by immunofluorescence using dual staining as described in detail in Materials and Methods (Fig. 2A). As discussed above, concentrations beyond 500 mg/kg/d were found to be too toxic. Statistically significant differences (\(P < 0.05\)) in histone H3 acetylation and HP-1 depletion were only seen in tumor cells from mice treated with 500 mg/kg/d (Fig. 2B and C).

To determine whether down-regulation of the heterochromatin maintenance proteins resulted in a structural change in the chromatin, the effects of valproic acid on MCF-7 tumors isolated from mice treated with 0 (left), 100 (middle), or 500 (right) mg/kg valproic acid for 48 hours were evaluated by electron microscopy (Fig. 3). Nuclei of mice treated with saline (0 mg/kg valproic acid) show a condensed pattern of evenly dispersed heterochromatin.
contrast, valproic acid treatment led to a dispersion of the heterochromatin suggestive of chromatin decondensation. Furthermore, valproic acid treatment resulted in an increase in the expression of cytoplasmic fat vacuoles (Fig. 3A, arrows), suggestive of HDACi-induced mammary differentiation as described by Munster et al. (22).

**Ex vivo Comet Moments Predict Valproic Acid–Induced Sensitization of Breast Cancer Tumors to Epirubicin**

The ability to predict early response remains a challenging quest in many clinical studies. Often, tumor tissues are not easily accessible and tumor cells are limited in numbers. Furthermore, in the majority of patients, serial biopsies for the sequential assessment of effects are not feasible. We have shown previously that the comet assay may be a useful tool to determine epirubicin-induced DNA damage in vitro in the presence of HDACi (9). The degree of the DNA damage is expressed in the comet moments and is quantified as a function of both the distance and the amount of DNA fragments that migrate in an electrical field. Here, we evaluated the relevance of biological valproic acid concentrations and the presence of the topoisomerase II targets in predicting DNA damage induced by epirubicin in cell lines that express topoisomerase IIα or IIβ but not those depleted in both isoforms (9). A survey of cell lines cultured in the presence or absence of valproic acid (2 mmol/L) for 48 hours before exposure to epirubicin for 4 hours showed potentiation in the epirubicin-induced apoptosis in all cells, except the topoisomerase II–depleted MDA-361 cells (Fig. 4A), even at higher epirubicin concentrations. The IC₅₀ for valproic acid in the MDA-361 was within the same range as other evaluated cell lines (data not shown). Furthermore, we have shown that, whereas HDACi did not potentiate the apoptosis induced by a topoisomerase II inhibitor, suberoylanilide hydroxamic acid and valproic acid potentiated the effects of the topoisomerase I inhibitor, topotecan, in this cell line (9). Here, the epirubicin-induced comet moments were evaluated as a function of valproic acid dose in MCF-7 cells, a sensitive model system. MCF-7 cells were isolated from tumor-bearing mice after a 48-hour exposure to increasing concentrations of valproic acid. Isolated cells were then exposed to epirubicin ex vivo for 1 hour. As shown in Fig. 4B, valproic acid was associated with a numerical dose-dependent increase in the DNA damage expressed in comet moments induced by epirubicin. However, multivariate analysis by ANOVA indicated a statistically significant increase in comet moments only at concentrations that were found to be required for histone acetylation and chromatin remodeling (500 mg/kg/d; P < 0.001). In
contrary, valproic acid treatment of mice bearing the topoisomerase II–depleted MDA-361 tumor cells showed no change in the epirubicin-induced DNA damage ($P = 0.12$) at any concentration of valproic acid or epirubicin (Fig. 4C; data not shown).

**Discussion**

Valproic acid is an anticonvulsant that has been in clinical use for decades. Its ability to inhibit histone deacetylase has only recently been described (23). We have previously reported a schedule-dependent synergistic interaction between HDACi and topoisomerase II inhibitors in vitro. Our preclinical studies suggested that preexposure to HDACi was associated with rapid histone acetylation. In contrast, the observed HDACi-induced modulation of chromatin maintenance proteins and chromatin decondensation required extended exposure to a HDACi. Preexposure of tumor cells to a HDACi was associated with increased interaction of topoisomerase II inhibitors with the DNA substrate and increased topoisomerase II inhibitor-mediated DNA damage in the presence of the topoisomerase II targets.

Here, we studied the relevance of the biological effects of the HDACi valproic acid on tumor growth alone and in combination with DNA-damaging agents in xenograft models and evaluated a potential role of the comet assay as a surrogate marker of response. Although valproic acid is less potent than many of the newer HDACi, the tolerability of high and cumulative doses of this drug may nonetheless render it an intriguing agent for further development, particularly in combination with cytotoxic agents (4, 24). The fact that valproic acid, because it is predominantly used as an antiseizure drug (25), easily crosses the blood-brain barrier may lend it a further advantage as it may facilitate the penetrance into sanctuary sites, such as the central nervous system. The well-established toxicity profile and long-term safety data may render valproic acid an ideal candidate for proof-of-principle studies that may be transferable to other HDACi in the same or different classes.

**In vitro** data from several breast cancer cell lines suggested that optimal effects are seen if the tumor cells are exposed to a HDACi for at least 48 hours. Longer preexposure did not further enhance synergy; however, shorter preexposure times or the reversal of the drug sequence abrogated synergistic effects (11). Similar findings were observed in the xenograft model. A 48-hour exposure of mice to valproic acid followed by a single injection of epirubicin resulted in tumor regression and a complete disappearance of 44% of the evaluable tumors. In contrast, treatment with epirubicin followed by valproic acid for 48 hours at the same concentrations did not result in any effects beyond those seen with each single agent alone (Fig. 1B). Despite the potentiation of the antitumor effects, the combination did not adversely affect the toxicity profiles in mice treated with drug combinations compared with those treated with valproic acid or epirubicin alone. Compared with the mice treated with saline, a relative weight gain deficit was observed in all treatment groups (Fig. 1C). The doses of valproic acid and epirubicin used were determined to be the maximally tolerated dose for this xenograft model as described in Materials and Methods. Although valproic acid plasma levels were not evaluated in this study, reported valproic acid levels in a comparable...
elsewhere suggested that, for the observed synergy, not II isoforms (9, 11). More extensive studies reported was not observed in cells depleted of both topoisomerase inhibitors to the DNA. However, this was
density, allowing an increase in binding of the top-
Topoisomerase II inhibitors was due to chromatin decon-
dentification of surrogates for responses is further
in the acetylation of histones in tumor cells (Fig. 2). Although histone acetylation marks biological activity of HDACi activity, we found that valproic acid alone did not result in significant antitumor effects, even at concentrations sufficient for histone acetylation and chromatin decondensation. Therefore, histone acetylation may be a pharmacologic marker of HDACi activity rather than a surrogate marker for antitumor effects. Similarly, microarray analysis of tumor samples from mice exposed to valproic acid showed a large proportion of genes to be affected in the absence of antitumor activity, which should prompt a careful evaluation of surrogate markers of response when designing clinical trials (data not shown).

The identification of surrogates for responses is further challenged by ethical constraints on exposing patients to additional risks, particularly when tumor tissues are not easily accessible or the number of tumor cells is limited. Furthermore, in the majority of patients, serial biopsies for the sequential assessment of drug effects are not feasible. We and others have shown previously that the comet assay may be a useful tool to determine early effects on DNA damage by topoisomerase II inhibitors (29, 30). In vitro DNA damage was enhanced in the presence of a HDACi (9). In this study, we evaluated the utility of the comet assay to determine the effects of valproic acid on epirubicin-induced DNA damage in tumor cells from mice treated with valproic acid. Tumor samples were obtained before and after valproic acid exposure and then exposed to epirubicin ex vivo. The comet assay may be done with a minimum of 5,000 cells; hence, it may provide a valuable surrogate system when tumor cells are limited. Furthermore, the ex vivo exposure to epirubicin allowed a comparison of two sets of data, each serving as its own reference, which may strengthen the statistical power. In concordance with the effects on histone H3 acety-
lation and HP-1 expression (Fig. 2), the latter serving as a quantitative marker of chromatin decondensation, a significant difference in the epirubicin-induced comet moments was only seen at concentrations required to affect the chromatin structure. Furthermore, valproic acid did not affect epirubicin-induced DNA damage in top-
Tumor treatment led to histone acetylation and chromatin decondensation but with only limited antitumor effects alone. This suggests that valproic acid may have more relevance in potentiating cytotoxic agents than as a single agent for the treatment of cancer. Furthermore, we have introduced a new application for the comet assay as a predictor of valproic acid–induced sensitization to DNA-damaging agents. This methodology may determine which patients that are treated with valproic acid may respond to antinecyline therapy.

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References


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