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Histone deacetylase inhibitors decrease intra-abdominal adhesions with one intraoperative dose by reducing peritoneal fibrin deposition pathways

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Abstract

Background—We previously demonstrated that postoperative peritoneal injury and inflammation contribute to adhesiogenesis. Recent evidence suggests that in addition to their role of interfering with the acetylation status of nuclear histone proteins, histone deacetylase inhibitors (HDACIs) including valproic acid (VPA) can target nonhistone proteins to resolve inflammation and modulate immune cells. We hypothesized that HDACIs could reduce adhesions.

Methods—Seventy-two rats underwent laparotomy with creation of 6 peritoneal ischemic buttons to induce adhesions. A single intraperitoneal (IP) dose of 50 mg/kg VPA was administered intraoperatively, whereas controls received vehicle. To evaluate the timing, 25 rats underwent ischemic button creation with either an intraoperative or a delayed IP dose of VPA at 1, 3, or 6 hours postoperatively. On postoperative day 7, adhesions were quantified. To investigate mechanisms, ischemic buttons were created in 24 rats and either VPA or saline was administered in 1 intraoperative dose. At 3 or 24 hours later, peritoneal fluid was collected and fibrinolytic activity measured. Alternatively, button tissue was collected 30 minutes postoperatively to measure tissue factor, fibrinogen, and vascular endothelial growth factor (VEGF) by real-time polymerase chain reaction or Western blot.

Results—A single intraoperative dose of VPA reduced adhesions by 50% relative to controls ($P < .001$). Delayed dosing did not reduce adhesions. In operated animals, peritoneal fibrinolytic activity was not different between groups. Tissue factor mRNA was downregulated by 50% ($P = .02$) and protein by 34% ($P < .01$) in animals administered VPA versus saline. VPA decreased fibrinogen protein by 56% and VEGF protein by 25% compared with saline ($P = .03$).

Conclusion—These findings suggest that VPA rapidly reduces the extravasation of key adhesiogenic substrates into the peritoneum. A single, intraoperative intervention provides an ideal dosing strategy and indicates an exciting new role for HDACIs in adhesion prevention.

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POSTOPERATIVE INTRA-ABDOMINAL ADHESIONS are a vexing problem. Although adhesions are a long-recognized complication of abdominal operations, years of research has yet to identify an effective means for their universal prevention. Indeed, the incidence of postoperative adhesions is still as high as 94% in patients undergoing abdominopelvic operations, even with meticulous operative technique.^{1,2} Because adhesions can cause serious long-term sequelae, including small bowel obstruction, chronic pelvic pain, and infertility in women, they are associated with costs as high as \$5 billion annually in the United States.¹⁻³ Currently, the only US Food and Drug Administration-approved adhesion prevention strategies are bioresorbable physical barriers such as Seprafilm (Genzyme Corp., Cambridge, Mass), which reduce adhesions by preventing apposition of injured tissues.^{4,5} Although effective where placed,⁶ there are significant limitations to the use of physical barriers, including difficulties with handling and application^{7,8} and their ineffectiveness elsewhere in the peritoneum.⁹ There is also clinical evidence that physical barriers fail to prevent small bowel obstruction, despite their localized efficacy in adhesion reduction.¹⁰⁻¹² Although many pharmacologic interventions are also capable of reducing adhesions in preclinical animal models, subsequent translational studies have been slow. In fact, a recent study examining a 20-year span of the National Hospital Discharge Survey showed that there has been no significant change in overall rates of adhesion-related complications, suggesting that no progress has been made in adhesion prevention and novel strategies are needed to reduce morbidity.¹³

Multiple pathways are involved in adhesiogenesis, and many of these are also fundamental to normal wound healing.¹⁴ Surgery causes tissue injury and ischemia, inducing an acute inflammatory response in which a fibrinous exudate is extravasated into the peritoneum at the site of injury. The subsequent downregulation of the peritoneal fibrinolytic system accounts, in part, for nascent adhesion formation.^{15,16} If the suppression of peritoneal fibrinolysis persists, these fibrinous matrices can develop into permanent adhesions. Hence, early resolution of the fibrinous exudate by the proteolytic enzyme plasmin is critical to adhesion prevention.^{14,17} Plasmin is converted in the peritoneal cavity from inactive plasminogen by tissue-type plasminogen activator (tPA), which accounts for the majority of the fibrinolytic capacity of the peritoneum.¹⁸ Peritoneal fibrinolytic activity is compromised postoperatively; however, it can be augmented pharmacologically thereby decreasing adhesion formation.^{16,19-22} We have previously demonstrated in our ischemic button model that several classes of compounds including a neurokinin-1 receptor antagonist,¹⁶ statins,²⁰ and *N*-acetyl-L-cysteine¹⁹ all reduce adhesions by 50–60%. Despite differing pathways, these compounds increase peritoneal fibrinolytic activity in the immediate postoperative period. Because it seems that targeting the peritoneal fibrinolytic system cannot completely eliminate adhesion formation, maximum efficacy may only be achieved when alternative pathways are defined.

Histone deacetylase inhibitors (HDACIs) are classes of compounds that alter transcription by changing the acetylation status of histones; however, many can deacetylate nonhistone proteins as well, allowing for epigenetic regulatory effects.²³ Recently, HDACIs have shown efficacy in the treatment of inflammatory conditions, cancer, and hemorrhagic shock.²⁴⁻²⁷ Valproic acid (VPA) is a broad and prototypical class I HDACI and although it has been US Food and Drug Administration–approved for the treatment of epilepsy and bipolar disorders

for many decades, newer indications have been described. For example, VPA has a strong antitumor effect in multiple solid cancers, for which it is in clinical trials, and it induces an anti-inflammatory and prosurvival phenotype in animal models of hemorrhagic shock,^{28,29} ischemia reperfusion injury,³⁰ traumatic brain injury,³¹ and septic shock.³² Additionally, VPA has been shown to modulate substrates in the fibrin formation pathway, including tissue factor and fibrinogen,^{33,34} both key participants early in adhesion formation. Perhaps of more importance, although vascular endothelial growth factor (VEGF) is best known for its role in angiogenesis, its function in increasing vascular permeability may play a key part in adhesion formation by initiating the exudation of fibrin precursors into the peritoneum.^{35,36} Given their promising antiproliferative and anti-inflammatory properties, we hypothesized that HDACIs could reduce postoperative adhesions.

MATERIALS AND METHODS

Materials

VPA (USP grade) was obtained from (Sigma Aldrich, St. Louis, Mo). Suberoylanilide hydroxamic acid (SAHA) and MS-275 were obtained from Cayman Chemical (Ann Arbor, Mich).

Animals

Male Wistar rats (weighing 200–225 g; Charles River Laboratories, Wilmington, Mass) were housed at constant room temperature and under 12-hour light/12-hour dark cycles and allowed access to food and water ad libitum. The Institutional Animal Care and Use Committee at the Boston University School of Medicine approved this study, and all procedures described were performed in accordance with recommendations outlined in the Guide for the Care and Use of Laboratory Animals: Eighth Edition (NRC 2011) published by the National Academies of Science.

Adhesion operation

Intraperitoneal (IP) ischemic buttons were created as described previously.¹⁶ In brief, after a midline laparotomy, 6 ischemic buttons (3 on each side of midline wound) were created by grasping and ligating 5 mm of peritoneum with 4-0 silk suture. This method was recently shown to be the most consistent and reproducible preclinical model for intra-abdominal adhesion formation compared with parietal peritoneum excision, parietal peritoneum abrasion, and cecal abrasion.³⁷ Any test compound or vehicle control was administered IP just before the wound was closed in 2 layers. In some experiments, as described herein, preoperative or postoperative test compound was administered IP via transabdominal injection. Rats survived until postoperative day 7, at which time they were humanely killed, their abdomens explored, and adhesion formation was quantified by scoring the percentage of buttons with attached fibrinous adhesions in each rat. For example, an animal that had adhesions attached to 4 of its 6 ischemic buttons received a score of 66%.

Dose–response and efficacy studies

To determine the optimal dose and dosing regimen of VPA, 72 male Wistar rats underwent laparotomy with ischemic button creation as described. Rats received IP administration of

either 1 mL of 0.9% saline (control, $n = 24$) or 1 mL VPA dissolved in saline at 200 mg/kg ($n = 6$), 100 mg/kg ($n = 12$), 50 mg/kg ($n = 12$), or 25 mg/kg ($n = 6$) at 24 hours preoperatively, intraoperatively, and 24 hours postoperatively. An additional group received a single intraoperative dose of VPA at 50 mg/kg ($n = 12$). On postoperative day 7, animals were humanely killed and adhesions were quantified.

Timing of therapeutic efficacy studies

To evaluate the window of therapeutic efficacy for intervention with the optimal dose as determined, an additional 25 rats underwent ischemic button creation with either an intraoperative or a delayed IP dose of 50 mg/kg VPA at 1, 3, or 6 hours postoperatively. On postoperative day 7, adhesions were quantified as percent of ischemic buttons with adhesions.

Efficacy studies of other HDACIs

Other HDACIs, with similar effects, include SAHA and MS-275. To determine whether other HDACIs exhibit anti-adhesion properties similar to VPA, 42 male rats underwent the adhesion inducing operation. A single IP dose of 50 mg/kg VPA, 50 mg/kg SAHA, or 10 mg/kg MS-275 was administered intraoperatively. VPA was dissolved in 1 mL 0.9% saline, and SAHA and MS-275 were dissolved in 1 mL 50% DMSO. Control animals received 1 mL of the appropriate vehicle alone.

Effects of VPA on colonic wound healing

To assess whether VPA affects normal wound healing, 12 male Wistar rats underwent laparotomy with a standardized colonic anastomosis as described previously.²⁰ In brief, after laparotomy and complete transection of the proximal colon, an end-to-end colonic anastomosis was created using a single running layer of 7-0 polypropylene suture. Animals received either 1 intraoperative dose of VPA at 50 mg/kg ($n = 6$) or 0.9% saline ($n = 6$). Animals were humanely killed at 7 days postoperatively and, after removing a 4-cm segment containing the anastomosis, the segment underwent measurements of burst pressure as described previously.²⁰ In addition, the dermal closure site was inspected daily and the fascia at sacrifice for any signs of dehiscence.

Peritoneal fluid collection and fibrinolytic assay

To evaluate the effects of VPA on peritoneal fibrinolytic activity, rats underwent laparotomy with ischemic button creation with intraoperative administration of 50 mg/kg VPA or saline as described. At 3 or 24 hours later, the peritoneum was lavaged with 600 μL of phosphate-buffered saline containing 1 international unit (IU)/mL of heparin before mixing it with an equal volume of 0.2 mol/L sodium acetate (pH 3.9). Fluid was immediately flash frozen in liquid nitrogen and stored at -80°C until use. Peritoneal fluid was collected from nonoperated animals for comparison. A tPA activity bioassay was performed as described previously.³⁸ In brief, fluid samples were thawed and acidified with 0.2 volumes 0.375 N hydrochloric acid and then diluted 10-fold with distilled water. The fibrinolytic activity of tPA in each sample was assayed in duplicate by adding 50 μL of the diluted sample to each well of a 96-well microtiter plate containing 50 μL tPA stimulator (0.6 mg/mL cyanogen

bromide–digested fibrinogen; American Diagnostica, Stamford, Conn). Next, 150 μ L of assay buffer containing 16.7 mg/mL human plasminogen (Athens Research and Technologies, Athens, Ga), 667 mmol/L S-2251 substrate (American Diagnostica), and 20 mmol/L Tris (pH 8.3) was added to each well and mixed gently. Cleavage of the S-2251 substrate by tPA-activated plasmin produces a yellow color that absorbs at 405 and 490 nm (calibration blank). The change in absorbance was measured at 37°C over the course of 10 hours using a spectrophotometric microplate reader (SpectraMax 250; Molecular Devices, Sunnyvale, Calif). The fibrinolytic activity of tPA in each sample was determined by extrapolation from a human tPA (Calbiochem, San Diego, Calif) standard curve.

Peritoneal tissue collection, RNA isolation, and quantitative real-time polymerase chain reaction

Rats underwent laparotomy with ischemic buttons with intraoperative administration of either 50 mg/kg VPA or saline as described. At 30 minutes postoperatively, adhesive tissue was collected by resecting each peritoneal ischemic button with any attached adhesions and a 0.5-cm rim of surrounding peritoneal tissue. For comparison, peritoneal tissue was also collected from nonoperated rats. Aliquots of tissues for mRNA and protein were immediately flash frozen in liquid nitrogen and stored at –80°C until use.

Total RNA was isolated from the peritoneal tissue using the SV Total RNA Isolation System (Promega, Madison, Wisc) and cDNA was prepared as described previously³⁸ with the GeneAmp PCRSystem2400 (Applied Biosystems, Foster City, Calif). Real-time polymerase chain reaction was carried out with the StepOne Plus System (Applied Biosystems) using primer sets from Applied Biosystems to amplify the mRNA of tissue factor, fibrinogen, VEGF-A, VEGF-R1, and VEGF-R2. Levels of mRNA expression were normalized to β -actin, a constitutively expressed gene that did not vary among treatment groups. Data were expressed as a percent of nonoperative controls.

Western blot analysis for protein in adhesion tissue

Rats underwent laparotomy with ischemic buttons and intraoperative administration of either 50 mg/kg VPA or saline. Thirty minutes later, peritoneal adhesive tissue was collected and stored as described. The frozen tissue was then ground with a mortar and pestle in liquid nitrogen, and suspended in 500 μ L RIPA buffer (50 mmol/L Tris; pH 7.4; 1% NP-40; 0.25% deoxycholate; 150 mmol/L NaCl; 1 mmol/L EDTA) containing a mammalian protease inhibitor cocktail used at 100 $\times$ (Sigma-Aldrich). Lysates were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, Penn) for 10 seconds on ice. After 1 hour incubation on ice, the homogenate was centrifuged at 850 $\times g$ for 15 minutes at 4°C, and the supernatant (cytosolic fraction) was recovered for Western blot analysis. Total protein content was determined using Bio-Rad Protein assay (Bio-Rad, Hercules, Calif). Cytosolic extracts (20 μ g/sample) were separated on 4–12% Bis-Tris mini-gels (Invitrogen, Grand Island, NY) and transferred to Hybond enhanced chemiluminescent membranes (Amersham Pharmacia Biotechnology, Piscataway, NJ). The membranes were blocked for 1 hour at room temperature in blotto (1 $\times$ 25 mmol/L Tris HCl; pH 7.4; 2.5 mmol/L KCl; 125 mmol/L NaCl; 0.1% Tween 20; and 5% nonfat dry milk [Tris-buffered saline (TBS)]) and incubated with a polyclonal antibody for either tissue factor (Abcam, ab104513 Cambridge, Mass),

fibrinogen (Abcam ab74057), or VEGF (Santa Cruz Biotechnology sc53462, Dallas, Tex), diluted 1:5,000 in blotto, for 1 hour at room temperature. Membranes were then subjected to vigorous washes, 4×15 minutes in TBS/0.1% Tween 20, and incubated for 1 hour at room temperature in secondary antibody, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Thermo Scientific Pierce, Rockford, Ill) diluted 1:10,000 in blotto. Membranes were then vigorously washed again, 4×15 minutes in TBS/0.1% Tween 20 at room temperature. Bands were detected by chemiluminescence with the Pierce enhanced chemiluminescent Western Blotting Substrate (ThermoFisher Scientific, Waltham, Mass) according to the manufacturer's instructions. Western blots were scanned and quantified using Scion Image software (NIH Image, Bethesda, Md). Mean values were determined and expressed as a percentage of nonoperated controls.

Statistical analysis

Data were analyzed either with 1-way analysis of variance or the Student *t*-test where appropriate using analytical software (Sigma-Stat; SPSS Inc, Chicago, Ill). In all analyses, before parametric statistical analyses, if the analyses of the data failed either normality or equivariance testing, $\log_{10}$ transformation was performed. If the data still failed these criteria, the nonparametric Dunn test of analysis of variance by ranks followed by the appropriate nonparametric post hoc test was used. When significant effects were detected, the differences between specific multiple means were determined by post hoc analysis with the Student–Newman–Keuls test. All data are expressed as mean values $\pm$ standard error of the mean.

RESULTS

VPA reduces postoperative adhesion formation with a single intraoperative dose, but is not effective with delayed dosing

When administered in a 3-dose IP regimen (24 hours preoperatively, intraoperatively, and 24 hours postoperatively), dosing studies showed that both 100 and 50 mg/kg were equally effective, reducing adhesions by 37.6% and 40.5%, respectively, ($P < .001$) compared with controls (Fig 1, A). Three-dose regimens of 25 and 200 mg/kg administered IP were not effective in reducing postoperative adhesions. To determine if preoperative and postoperative dosing was necessary, we trialed a single intraoperative IP dose and showed that 50 mg/kg optimally reduced postoperative adhesion formation by 50.4% compared with control animals ($P < .001$) administered only saline (Fig 1, A). Based on these results, the remainder of the mechanistic studies were carried out using the single, intraoperative 50-mg/kg dose administered IP.

To determine the optimal therapeutic window of administration and if delayed dosing was effective, we conducted a delayed dosing trial and showed that only intraoperative administration was effective ($P < .001$) and subsequent doses at 1, 3, or 6 hours postoperatively did not reduce adhesions (Fig 1, B).

SAHA and MS-275 reduce postoperative adhesion formation with similar efficacy to VPA

To determine whether the efficacy of VPA was unique to this class of HDACIs, we tested 2 other HDACIs using the same intraoperative timing of administration. We showed that SAHA administered IP as a single intraoperative dose of 50 mg/kg reduced postoperative adhesions by 48% ($P < .001$) and MS-275 administered IP as a single intraoperative dose at 10 mg/kg reduced postoperative adhesions by 45% ($P < .001$) compared with their respective vehicle controls (Fig 1, C).

VPA does not compromise normal intestinal wound healing

When considering any new pharmacologic agent administered IP during surgery for any indication, it is imperative to determine that peritoneal wound healing is not affected. Using a very rigorous colonic anastomosis model in which a complete colon transection was repaired with an end-to-end, hand-sewn anastomosis, we found that administration of 50 mg/kg of VPA at surgery had no negative effects on colonic anastomotic strength when compared with animals administered saline only. Average colonic burst pressure was 264 ± 15.8 mmHg for animals administered VPA and 225 ± 18.7 mmHg for animals administered saline, which were very similar to values we previously reported with other compounds.^{20,39} In addition, there were no indications of dehiscence at the dermal closure site, which was inspected daily or the fascia at killing, even at the 200-mg/kg dose.

VPA does not regulate peritoneal fibrinolytic activity

Our previous studies with NK-1R antagonists,¹⁶ statins,²⁰ and *N*-acetyl-L-cysteine¹⁹ showed that these compounds reduced adhesion formation, in part, by modulating peritoneal fibrinolytic activity. We therefore tested whether VPA reduced adhesion formation by a similar mechanism of action. Using the same dose and timing regimen as above, we showed that peritoneal fibrinolytic activity at 3 and 24 hours postoperatively was not significantly different between animals administered VPA and saline (Fig 2).

VPA downregulates tissue factor mRNA expression and protein levels in peritoneal adhesive tissue

Because the fibrinous exudate and the subsequent formation of the peritoneal fibrin matrix are fundamental elements of adhesion formation and VPA did not affect peritoneal fibrinolytic activity, we sought to examine alternative mechanisms by which VPA reduced adhesion formation. This involved examining coagulation pathway factors associated with adhesion formation upstream of the fibrinous exudate; because the administration of VPA at 1 hour postoperatively was ineffective in reducing adhesions, we chose the 30-minute postoperative time point. At 30 minutes postoperatively, we showed that the administration of VPA significantly downregulated ($P = .02$) the mRNA expression of tissue factor by $>50\%$ (Fig 3, A). Correspondingly, tissue factor protein levels were significantly reduced ($P < .01$) by 33.6% in animals administered VPA compared with controls (Fig 3, B).

VPA downregulates fibrinogen protein levels in peritoneal adhesive tissue

Perhaps one of the most significant events of adhesion formation is the plasmin-mediated conversion of fibrinogen to fibrin, the scaffold upon which fibrinous adhesions form and

eventually mature. We showed that 30 minutes after a single intraoperative IP dose of VPA, fibrinogen protein levels were reduced by 56% ($P = .03$) compared with controls (Fig 4).

VPA downregulates VEGF-A protein in peritoneal adhesive tissue

Because VEGF plays a key role in the regulation of vascular and mesothelial permeability, it is more than likely a key factor in regulating the extravasation of adhesiogenic clotting factors such as fibrin from the plasma compartment to the peritoneum. VEGF mRNA expression was not different in animals administered VPA versus those given saline (Fig 5, A). However, we showed that, 30 minutes after a single intraoperative dose of VPA, VEGF-A protein levels were reduced by >25% ($P = .045$) compared with animals administered saline alone (Fig 5, B).

VPA downregulates VEGF-R2 mRNA in peritoneal adhesive tissue

VEGF-R1 mRNA expression was not different in animals administered VPA compared with those given saline (Fig 6, A). However, we showed that 30 minutes after a single intraoperative dose of VPA, VEGF-R2 mRNA levels in peritoneal adhesion tissue were reduced by nearly 50% ($P = .001$) compared with animals administered saline alone (Fig 6, B).

DISCUSSION

The formidable problem of postoperative intra-abdominal adhesions remains unsolved, not only because of our incomplete understanding of early adhesiogenic events, but also because available preventative measures are limited and lacking in operative practicality. This study significantly increases our understanding of the very early events that precipitate intra-abdominal adhesion formation. With the data presented herein, we have demonstrated a novel role for HDACIs, particularly VPA, in reducing adhesion formation with a single IP dose administered just before closing the midline incision. The fact that IP dosing is only effective up to 1 hour postoperatively indicates that key adhesiogenic events occur rapidly after peritoneal injury. Additionally, these data show that the peritoneal fibrinolytic system is not affected by VPA, thereby excluding this potential mechanism of adhesion reduction in the pathway of VPA's efficacy. In addition, we have proposed a mechanism of action that involves the reduction of adhesiogenic substrates extravasating into the peritoneum after surgically induced peritoneal injury.

Previous work from our laboratory implicated peritoneal fibrinolysis as a key element of adhesion prevention. We showed that numerous agents such as a neurokinin-1 receptor antagonist,¹⁶ statins,²⁰ and *N*-acetyl-L-cysteine,¹⁹ reduce adhesions primarily by upregulating the peritoneal fibrinolytic system. We were somewhat surprised to discover that VPA does not reduce adhesion formation via this classic pathway. Instead, it seems that VPA decreases peritoneal fibrinogen and tissue factor protein levels, thereby limiting the availability of fibrin substrates to be cleaved into fibrin. Because peritoneal fibrin formation is an important inciting event in adhesiogenesis, decreasing its production via the action of VPA could reduce mature adhesions. Furthermore, we have shown that VPA reduces VEGF-A protein levels in peritoneal ischemic button tissue. Because VEGF is a potent mediator of

vascular permeability,⁴⁰ it follows that VPA's ability to decrease VEGF may limit exudation of fibrin precursors from the vasculature into the peritoneal space after tissue injury. Interestingly, we also show that although VPA does not effect the mRNA levels of the VEGF receptor VEGF-R1, VPA does significantly reduce the VEGF-R2 mRNA expression. This observation has 2 implications germane to adhesion formation. The binding of VEGF to the VEGF-R1 receptor mediates the upregulation of tPA,⁴¹ so the reduction of VEGF protein and the VEGF-R1 receptor by VPA blunts the upregulation of tPA. Additionally, the binding of VEGF to the VEGF-R2 receptor plays a key role in the breakdown of cell junctions,⁴¹ thus facilitating vascular permeability. The downregulation of the VEGF-R2 receptor by VPA mitigates this effect and prevents vascular leakage. So, although previously studied compounds may act by increasing peritoneal fibrinolysis and thereby degrading fibrin, which has already begun to form the basis for adhesions, HDACIs may instead limit the availability of fibrin substrates in the peritoneum and therefore prevent the initiation of adhesiogenesis. This represents a novel and not as yet described mechanism of adhesion prevention.

VPA has been widely prescribed for seizure disorders since its approval nearly 40 years ago and is well tolerated in doses as high as 3,000 mg/d. Side effects include mild thrombocytopenia and prolonged bleeding times; otherwise, it has an excellent safety profile. Indeed, the proposed mechanism of action for VPA has been described in the context of other disease states in experimental rodent models which have used doses up to 600 mg/d or 12 times higher than the 50 mg/d used in the present study. In models such as hemorrhagic and septic shock, VPA has been shown to induce an anti-inflammatory phenotype, thought to be related to its ability to modulate acetylation of nonhistone proteins.^{27,28,31,32,42} VPA therapy has been associated with decreased levels of circulating fibrinogen in human subjects,^{43,44} and although the resulting coagulopathy is thought to be an untoward systemic side effect of VPA administration, the same effect may be beneficial to adhesion prevention when its action occurs locally within the peritoneum. HDACIs are also known to suppress tissue factor expression in endothelial cells where it mediates thrombosis.³³ Mesothelial cells do express tissue factor,⁴⁵ and the reduction of tissue factor mRNA and protein by VPA reduces in adhesive peritoneal tissue may be derived from mesothelial cells. VPA also mitigates endothelial cell dysfunction in shock models by modulating expression of VEGF,³⁶ as it did in our study. Therefore, all of the mechanisms we propose in the current study have also been observed and postulated to be of therapeutic utility in a number of clinical and research scenarios not related to adhesion formation.

Importantly, we have shown that a very low dose of VPA is effective in reducing adhesions when given as 1 intraoperative IP dose in solution. For practical reasons relating to operating room logistics and surgeon preferences, adhesion prevention strategies must be easy to use in any operative setting; they must also be effective. Herein, we have shown that a single peritoneal lavage of a drug-containing saline solution at the conclusion of an operation is very effective and would be an ideal delivery time. The fact that the administration of VPA must occur within 1 hour of the operation certainly indicates that key events surrounding the leakage of vascular clotting components, especially fibrinogen, into the peritoneal space is a very early adhesiogenic event. The VPA-induced reduction in VEGF reduces the extravasation of fibrinogen within 30 minutes. Interestingly, the short temporal dependence

of VPA's administration has been recently observed in a rat ischemia–reperfusion model showing that the therapeutic window of efficacy after a single dose of VPA was between 0 and 90 minutes.³⁰ Other pharmacologic interventions have demonstrated efficacy, but only when given in multiple IP doses, which is impractical in actual surgical practice.¹⁹ Although we have not tested the adhesion reduction capacity of oral administration of HDACIs, experience has shown us that very few agents have oral efficacy for adhesion reduction,²⁰ and that IP dosing seems to maximize effectiveness.

Anastomotic wound healing was not affected by IP administration of VPA, nor was fascial or skin wound healing. Because events in adhesiogenesis share common pathways with normal wound healing, pharmacologic agents that decrease adhesions may compromise anastomotic healing. For instance, methylene blue decreases adhesions, but also weakens anastomotic wound healing, which negates its clinical utility.⁴⁶ Similarly, the use of tPA to decrease postoperative adhesions has adverse complications, such as bleeding and poor wound healing.^{47,48} Therefore, any potential intervention must be capable of inhibiting adhesions without deleterious effects on normal healing processes, and VPA meets these requirements.

HDACIs including VPA have also recently been shown to initiate a pro-resolving cascade that includes the reduction of inflammatory cytokine production by macrophages, the promotion of neutrophil apoptosis, and increased macrophage phagocytosis.⁴⁹ Further investigation revealed an association between HDACIs and the expression of annexin A1, an anti-inflammatory membrane-bound protein that has been shown to have a particular impact on the processes that regulate the resolution of acute inflammation.⁵⁰ These studies further propose nongenomic modulation of annexin A1 in immune cells as a novel mechanism of action for HDACIs, which presumably underlies their reported efficacy in other inflammatory pathologies including perhaps the formation of adhesions.⁴⁹

We have demonstrated that HDACIs, with particular focus on VPA, are capable of reducing postoperative adhesions when administered in a single intraoperative IP dose, without untoward effects on intestinal wound healing. Such a dosing scheme represents an ideal delivery strategy that lacks the constraints and limitations of physical barriers, and may be applied in almost any open or minimally invasive surgical setting. A caveat of these studies, of course, is the fact that these findings are in a rodent model of adhesion formation and it remains unknown whether or not these results will translate to humans. However, the cellular targets of HDACIs underlie the important mechanisms in adhesiogenesis and may help to better define the molecular events that contribute to adhesion formation and significant morbidity for patients. With a more robust understanding of adhesion biology and with the discovery of easily applied and effective agents for reducing adhesions, we are hopeful that a solution to this feared surgical problem may be near.

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Fig 1.

Efficacy data showing percent adhesion formation with the intraperitoneal (IP) administration of histone deacetylase inhibitors versus vehicle controls (*Con*). Adhesions were quantified on postoperative day 7. (A) IP valproic acid (*VPA*) dosing trials, demonstrating 3-dose regimens (*pre-op*, preoperatively; *intra-op*, intraoperatively; *post-op*, postoperatively) of VPA at 200 ($n = 5$), 100 ($n = 12$), 50 ($n = 12$), or 25 ($n = 6$) mg/kg, compared with a single intraoperative dosing regimen of 50 mg/kg ($n = 16$), which was most effective at reducing postoperative adhesions compared with saline *Con* ($n = 18$). (B) IP

VPA 50 mg/kg ($n = 5$ per group) timing trials, demonstrating the single intraoperative regimen as the only effective dosing strategy, compared with delayed dosing regimens in which VPA was administered at 1, 3, or 6 hours postoperatively and was not effective. (C) Comparison of different HDAIs including VPA (50 mg/kg; $n = 16$), suberoylanilide hydroxamic acid (*SAHA*; 50 mg/kg; $n = 6$), and MS-275 (10 mg/kg; $n = 6$) compared with controls (Con; $n = 18$). Data are expressed as mean values $\pm$ standard error of the mean. $*P < .001$ compared with controls.

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Fig 2.

Fibrinolytic activity in peritoneal fluid at 3 and 24 hours showing that intraoperative administration of valproic acid (VPA) does not change peritoneal fibrinolytic activity. *Non-op Con*, Nonoperated animals administered intraperitoneal (IP) saline ($n = 12$); *Non-op VPA*, nonoperated animals given IP VPA ($n = 12$); *Op Con*, operated animals given intraoperative IP saline ($n = 18$); *Op VPA*, operated animals given intraoperative IP VPA ($n = 18$). Data are expressed as mean values $\pm$ standard error of the mean.

Fig 3.

Tissue factor mRNA and protein expression in peritoneal adhesion tissue, as percent of nonoperated control, at 30 minutes postoperatively. (A) Intraperitoneal (IP) valproic acid (VPA; $n = 4$) decreased tissue factor mRNA expression compared with operated (*Op*) controls (*Con*; $n = 5$). (B) IP VPA ($n = 5$) decreased tissue factor protein expression compared with operated controls ($n = 5$). Data are normalized to actin, expressed as the mean values $\pm$ standard error of the mean, and presented as a percentage of nonoperative (*Non-op*) control levels. * $P < .02$ compared with saline controls.

Fig 4.

Fibrinogen protein in peritoneal adhesion tissue at 30 minutes postoperatively.

Intraperitoneal valproic acid (*VPA*; $n = 6$) decreased fibrinogen protein expression compared with operated (*op*) controls (*Con*, $n = 6$). Data are normalized to actin, expressed as the mean values $\pm$ standard error of the mean and presented as a percentage of nonoperative (*non-op*) control levels. * $P = .03$ compared with saline controls.

Fig 5.

Vascular endothelial growth factor (VEGF)-A mRNA and protein expression in peritoneal adhesion tissue, as percent of nonoperated (*non-op*) control (*Con*), at 30 minutes postoperatively. (A) Intraperitoneal (IP) valproic acid (*VPA*; $n = 6$) did not change VEGF-A mRNA expression compared with operated controls ($n = 6$). (B) IP VPA ($n = 6$) decreased VEGF-A protein expression compared with operated controls ($n = 6$). Data are normalized to actin, expressed as the mean values $\pm$ standard error of the mean, and presented as a percentage of nonoperative control levels. * $P < .05$ compared with saline control.

Fig 6.

Vascular endothelial growth factor (VEGF) receptor 1 and 2 mRNA expression in peritoneal adhesion tissue, as percent of nonoperated (*non-op*) control (*con*), at 30 minutes postoperatively. (A) Intraperitoneal (IP) valproic acid (*VPA*; $n = 6$) did not change VEGF-receptor 1 (VEGF-R1) mRNA expression compared with operated controls ($n = 6$). (B) IP VPA decreased VEGF-R2 mRNA expression compared with operated controls. Data are normalized to actin, expressed as the mean values $\pm$ standard error of the mean, and presented as a percentage of nonoperative control levels. * $P < .05$ compared with saline controls.