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Creating a “Pro-survival Phenotype” Through Histone Deacetylase Inhibition: Past, Present, and Future

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Abstract

Traumatic injuries and their sequela represent a major source of mortality in the United States and globally. Initial treatment for shock, traumatic brain injury, and polytrauma is limited to resuscitation fluids to replace lost volume. To date, there are no treatments with inherent pro-survival properties. Our lab has investigated the use of histone deacetylase inhibitors (HDACIs) as pharmacological agents to improve survival. This class of drugs acts through posttranslational protein modifications and is a direct regulator of chromatin structure and function, as well as the function of numerous cytoplasmic proteins. In models of hemorrhagic shock and polytrauma, administration of HDACIs offers a significant survival advantage, even in the absence of fluid resuscitation. Positive results have also been shown in two-hit models of hemorrhage and sepsis and in hemorrhagic shock combined with traumatic brain injury. Accumulating data generated by our group and others continue to support the use of HDACIs for creation of a pro-survival phenotype. With further research and clinical trials, HDACIs have the potential to be an integral tool in treatment of trauma, especially in the pre-hospital phase.

Keywords

Hemorrhage; shock; sepsis; epigenetic; survival; inflammation; genes; proteins; histone deacetylase inhibitor; traumatic brain injury

Background

In the United States, injuries are the leading cause of death in the younger population (aged 1 to 42 years) (1). In 2010, 23% of emergency department visits (>30 million) in the US were due to injuries (2), and resulted in nearly 180,000 deaths (1). Worldwide, injuries kill nearly 6 million people annually and cause harm to millions more, accounting for 10% of global mortality (3). Despite numerous advancements in trauma care, a recent multinational trial of more than 20,000 patients (4) once again demonstrated that majority of deaths still occur within the first few hours after injuries, with about half due to massive blood loss (5–8), making this the number one cause of *preventable* deaths. To improve outcomes we must develop more effective pre-hospital treatments. Resuscitation fluids simply replace the lost

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intra-vascular volume, but have no inherent pro-survival properties (9). In severely bleeding patients, early aggressive crystalloid resuscitation has not been shown to provide any survival benefits and may even worsen the outcomes (10). We now know that cellular damage suffered during shock can be further exaggerated by inappropriate resuscitation (11). Therefore, contemporary trauma care is moving rapidly away from aggressive crystalloid infusion towards “damage control resuscitation” that promotes minimal use of crystalloids, rapid hemorrhage control, and early administration of blood components (red cells, plasma and platelets) (12–15). However, use of blood products is not practical in the pre-hospital setting where most of the deaths occur, and especially in the battlefield. This has fueled an initiative to develop novel life-preserving strategies that could serve as a bridge to definitive care. Our team, through funding from the Department of Defense, and the National Institutes of Health, has pursued the development of pharmacological interventions that could alter the cellular response to injury and create a “pro-survival phenotype.

Epigenetic Modulations

A wide spectrum of responses is observed following trauma even when patients are matched for gender, age, risk factors, and injury severity. The markedly different phenotypes expressed by different people, as well as various tissues/cells within the same individual, support the presence of very precise systems that regulate gene transcription and protein functions. The rapidly expanding field of “epigenetics” focuses on mechanisms and phenomena that affect the phenotype of a cell or an organism without affecting the genotype (16, 17). Many types of epigenetic processes have been identified including acetylation, methylation, ubiquitylation, phosphorylation, and sumoylation. These processes act through modification of the secondary and tertiary structures of DNA (chromatin), making it more or less accessible for transcription. From a therapeutic standpoint, epigenetic modulation is exciting because the transcription of desirable genes and function of various proteins can be rapidly (and reversibly) modified by modulating these mechanisms. Current treatment for hemorrhagic shock (HS) focuses on maintaining sufficient tissue perfusion and vital organ function through administration of fluids and blood products and surgical control of hemorrhage; unfortunately, this resource intensive treatment is difficult to administer, especially in austere environments where advanced surgical interventions may not be immediately available. Additionally, this approach fails to address damage at the cellular level secondary to hypoperfusion (during hemorrhage) and reperfusion (during resuscitation). Focusing on the cellular pathophysiology of HS, our laboratory has explored the strategy of epigenetic modulation as a unique opportunity for pharmacological intervention, especially in a setting such as shock where early treatment could improve outcomes.

Our team has shown that ~7% of genes (and their downstream pathways) change their expression following hemorrhage and resuscitation and do so in an organ- and treatment-specific fashion (18, 19). One key change in hemorrhage is the alteration of histone acetylation patterns. Histone proteins, the chief components of chromatin, package and organize the DNA into structural units termed nucleosomes (20). Acetylation of lysine residues on histones is a well-established posttranslational modification and is a direct

regulator of chromatin structure and function (21). The enzymes histone acetylase (HAT) and histone deacetylase (HDAC) control the level of histone acetylation and act as critical gene transcriptional activators or silencers. Accumulating data have shown that lysine acetylation can also occur in non-histone proteins, indicating that HAT and HDAC regulate not only transcription but a variety of other cellular processes as well (22–24).

Histone Deacetylases and Their Inhibitors

In humans, at least eighteen different HDACs have been reported (25, 26). These are subdivided into three classical (Zn^{++} dependent) classes (I, II, and IV), as well as a separate class III that contains NAD^+ dependent sirtuins (Table 1). Members of class I are limited to the nucleus, whereas class II shuttle between the nucleus and cytoplasm and have tissue specific expression (25–27). Class III can be found in the nucleus (SIRT 1, 6 and 7), mitochondria (SIRT 3, 4 and 5), or the cytoplasm (SIRT2) (26, 28) while Class IV are mostly found in the cytoplasm. The different HDACs and their binding partners have distinct functions, unique cellular/organ distribution, and discrete physiological effects (25, 26, 29–31). This variability in organ distribution, disease specificity, and biological functions can be exploited when designing a targeted therapeutic intervention.

HDACI Treatment in Hemorrhagic Shock

Our initial studies evaluated the effects of hemorrhage and resuscitation on acetylation and HDAC/HAT activity in a rat model. Rats were subjected to volume-controlled hemorrhage and resuscitated with various fluids as well as HDAC inhibitors (HDACIs). Upon resuscitation, cardiac tissue was collected and evaluated for acetylation and activity of HDAC/HAT. Our results showed that hemorrhage and resuscitation resulted in an imbalance of HDAC/HAT activity (22). When resuscitation was limited to strictly crystalloid fluids there was a net de-acetylation of the nuclear fraction of proteins including histones and non-histone chromosomal proteins. The addition of HDAC inhibitors to resuscitative fluids induced histone hyperacetylation in the cardiac cells, even when administered after shock (22).

These findings led us to question whether the acetylation profile following HS and subsequent resuscitation was limited to cardiac tissue and other “high priority” organs generally targeted with physiologic responses of blood redistribution in the setting of shock. We therefore evaluated the effects of HS on additional organs, specifically lung and liver and discovered that the acetylation patterns were organ specific and highly dependent upon the fluids used for resuscitation. Furthermore, we discovered that these patterns could be rapidly and reversibly modulated through the use of HDACIs. On further evaluation with cDNA microarrays and RT-PCR we noted that the largest changes were in the expression of genes involved in metabolism, growth, proliferation, differentiation, and cellular signaling (23).

These studies demonstrated the feasibility of using HDACIs during resuscitation. However, neither survival nor organ damage was evaluated in these initial experiments. We therefore wanted to determine whether post-shock administration of the HDACIs valproic acid (VPA) and suberoyanilide hydroxamic acid (SAHA) could improve early survival in a lethal model

of hemorrhage (60% blood loss in rodents) without any conventional fluid resuscitation. In this model, unresuscitated shock was associated with 25% survival, whereas administration of VPA and SAHA (in the absence of fluid resuscitation) improved the survival to 75% and 83%, respectively ($p < 0.05$; vs. controls) (Figure 1) (32). In addition, administration of VPA resulted in a substantial increase in histone acetylation that peaked at 6 hours and diminished over time (32) (Figure 2). Similarly, in a swine model of 35% blood volume hemorrhage followed by a lethal ischemia-reperfusion injury, Causey et al have shown that VPA treatment after injury mitigated the adverse physiologic changes seen following major hemorrhage and was associated with decreased acidosis, coagulopathy, crystalloid requirement, and epinephrine during resuscitation. This was associated with increased levels of acetylated proteins in liver and lung tissues and increased expression of heat shock protein (HSP) 70 compared to controls (33). Furthermore, microarray and functional analysis identified four biologic processes that were altered in the vascular endothelium after ischemia involving apoptosis/cell death and angiogenesis/vascular development including reversal of the pathological induced over-expression of vascular endothelial growth factor (VEGF) (34). Interestingly, VPA has also been shown to reduce VEGF and hypoxia-inducible factor 1 α , production as well as loss of zonula occludens-1 (ZO-1) in an in-vitro model of hypoxia (35).

Though HDACIs are known to act through modulation of histones, and by directly acetylating key non-histone proteins, we have also some explored additional mechanisms to explain their beneficial effects. A detailed discussion is beyond the scope of this review, but testing in similar models has demonstrated hyperacetylation of beta-catenin fractions in the brain and the potential for increased protein stability, regulation of protein-protein interactions, and stimulation of DNA-binding and gene transcription (36). Additionally, we have evaluated the effects of HDACIs on the JNK pathway, which has been shown to play a major role in apoptosis in many cell death paradigms through down regulating anti-apoptotic bcl-w (B-cell leukemia/lymphoma-w) protein. We discovered that phospho-JNK expression was reduced and bcl-w expression was increased in the brains of hemorrhaged rats treated with VPA (37).

VPA Dosing and Toxicity

The positive benefits of VPA in hemorrhagic shock were challenged by Burns et al in a model of 60% hemorrhage in anesthetized swine. In this model, VPA was administered at 400 mg/kg in a volume of 1.33 ml/kg over 2 min or at 300 mg/kg in a volume of 2 ml/kg over 30 min. The response to VPA at both the 400 mg/kg and 300 mg/kg in this study appeared to be toxic with respiratory and cardiac arrest occurring shortly after infusion. Furthermore, case reports in the literature regarding acute overdoses of VPA often describe significant neurological impairment in addition to hypotension, tachycardia, respiratory depression necessitating intubation, and even cardiac arrest (38–41). There are also reports of significant non-lactate metabolic acidosis and other biochemical derangements (42, 43). In swine models, we initially noted similar effects when using high doses of VPA (300–400mg/kg) given rapidly (<30 min) [unpublished work]; however, when similar doses were administered over longer durations (30–60 min), they were well tolerated and exhibited the desired cytoprotective properties (44). In fact, 300mg/kg of VPA given over 3 hours was

effective in models of HS combined with traumatic brain injury (TBI) (45, 46). It is interesting to note that in a dose-escalation study using a rodent model of HS, high doses of VPA (>300 mg/kg) resulted in greater histone acetylation and activation of pro-survival protein signaling; however, doses as low as 250 mg/kg conferred the same survival advantage. In addition, treatment in a split-dose fashion (200 mg/kg followed 2 h later by another dose of 100 mg/kg) preserved cytoprotective effectiveness with the same survival advantage (47). To date, there is no similar dose-optimization study in large animal models.

It is important to note that there have been reports of coagulopathy, including acquired von Willebrand syndrome and decreased factor VII levels, platelet count, factor VIII, protein C, and fibrinogen secondary to VPA use in pediatric populations; however, such reports have been noted only in patients receiving long-term (> 4 weeks) VPA for treatment of neurological disorders (48–51). To our knowledge, there have been no published reports of acute coagulopathy secondary to single-dose or short-term VPA treatment. Data from our large animal HS models suggests no increased coagulopathy secondary to single high dose VPA treatment based on thromboelastography measurements [unpublished data].

HDACI Treatment in 2-Hit Models

Sepsis or septic shock is a leading cause of mortality in intensive care units. Patients that survive the acute episode of blood loss often exhibit a systemic inflammatory response syndrome (SIRS), and some develop nosocomial infections which can progress to septic shock (52). Prior work has shown that the HS-induced SIRS is very similar to sepsis at a molecular level (53). Treatment with pan-HDACIs has proven beneficial in sepsis, where a largely unopposed pro-inflammatory response causes an imbalance in acetylation of proteins and can lead to organ damage and death. In mice subjected to intraperitoneal injections of lipopolysaccharide (LPS) or cecal ligation and puncture (CLP) induced poly microbial sepsis, treatment with a pan-HDACI improved long-term survival and attenuated expression of pro-inflammatory mediators, including MyD88, tumor necrosis factor alpha (TNF- α), and interleukin (IL)-6 (54, 55). Importantly, these results were achieved in the absence of standard fluid resuscitation. Similar benefits were seen in a “two-hit” models, where hemorrhage was followed by a CLP insult in rodents (56). In that study, rats treated with VPA showed a significantly better long-term survival rate (86% vs. 14%, $p < 0.05$) (Figure 3a), suppressed inflammatory profile (Figure 3b), and lower severity of acute lung injury. To take these results further, we examined whether the combination of hypertonic saline (HTS) with VPA would allow us to achieve survival advantage at a lower dose of VPA. In the same “two-hit” rodent model described above, HTS + VPA treatment significantly improved survival (87.5%), compared with the other groups (14.3%; $p < 0.05$), while attenuating peritoneal myeloperoxidase (MPO) levels and TNF- α and IL-1 α levels in the serum, peritoneal cavity, and lung (57).

Many other researchers have reported positive findings with the use of HDACI in septic models. Recently, Takebe et al showed that induction of sepsis by CLP results in the appearance of apoptotic cells in lungs and spleens of mice. Treatment with CG200745, a broad-spectrum HDAC inhibitor, sharply reduced apoptosis in both lung and splenic tissues and prevented sepsis-associated increase in apoptotic markers such activated caspase 3 and

anti-cleaved poly (ADP-ribose) polymerase (58). Shang et al have also shown encouraging results using VPA in a rat model of lipopolysaccharide (LPS)-induced septic shock. In their model, VPA treated animals showed decreased serum markers of hepatic and renal dysfunction, and TNF- α , as well as decreased MPO activity and TNF- α in lung tissues (59). Similarly, in a model of LPS-induced acute lung Injury (ALI) in mice, Ji et al showed that VPA pretreatment attenuated the LPS-induced ALI with reduced histological injury scores and MPO activity in addition to decreased nuclear factor kappa B (NF- κ B) p65, nitric oxide, and inducible nitric oxide synthase in the lung tissues as well as TNF- α and IL-1 β in the bronchoalveolar lavage fluid (60).

Numerous mechanisms are likely to be involved in the beneficial effect of HDACIs. For example, using our two-hit model (hemorrhage and sepsis), we have shown that treatment with SAHA can protect against delayed consequences of severe sepsis including coagulopathy by improving fibrin cross-linkage, clot formation, and platelet function without significantly impacting clot initiation parameters (61). Another possible mechanism through which HDACIs act is through the Toll Like Receptor (TLR) signaling and associated downstream regulators. These play a crucial role in the innate immune system as the first line of defense against pathogens. TLR4 and TLR2 are the main sensors for pathogen-associated molecular pattern recognition from gram-negative and gram-positive bacteria, respectively. LPS binds to host TLR4, triggering an inflammatory reaction characterized by the release of a large number of inflammatory mediators including TNF- α and IL-6. Downstream TLR signaling occurs via two major pathways: myeloid differentiation factor 88 (MyD88)-dependent and independent pathways. The MyD88-dependent pathway further activates the mitogen activated protein kinase (MAPK) cascade and NF- κ B. An absence or deficiency of MyD88 strongly attenuates the hyper-inflammatory response and may explain the anti-inflammatory effects of HDACIs (54, 62). Accumulating evidence suggests that HDACIs may suppress MyD88 expression through acetylation of STAT-1 and NF- κ B (63). Other pathways, including those involving HSP 90 and hypoxia-inducible factor-1 α may also be involved (62, 64). Although the overwhelming majority of studies have shown that HDAC inhibitors have anti-inflammatory properties, some studies have reached a different conclusion. For example, Sugiura et al showed that VPA pre-treatment reduced survival and significantly augmented the proinflammatory response induced by LPS both *in vivo* and *in vitro* in a mouse model of lethal endotoxemia by inducing HMGB1 release (65). Differences in models, doses, and timing of treatment may be responsible for these contradictory results. Although the fundamental molecular and cellular signaling mechanisms require further investigation, treatment of sepsis with HDACIs represents a novel and promising therapeutic strategy.

Isoform Specific HDACI Treatment in Lethal Sepsis

In general, class I HDACs are ubiquitously distributed and play a role in cell survival and proliferation, whereas class II HDACs have tissue specific roles (66). All HDACs exist in multi-protein complexes which confer substrate selectivity and unique functions (based upon various binding partners) in addition to regulating inhibitor selectivity (31, 66). The isoform specific inhibitors target different types of HDAC and therefore can be used in various combinations to optimize results. HDAC6 belongs to class IIb HDAC based on

domain organization. It is a cytoplasmic microtubule-associated enzyme that associates with non-histone substrates, including HSP90, α -tubulin, and cortactin (67). HDAC6 overexpression results in tubulin deacetylation and increased cell motility (68, 69), whereas specific inhibition or downregulation of HDAC6 activity increases acetylation of α -tubulin and HSP90, leading to reduced cellular motility and degradation of HSP90 client proteins, such as Bcr-Abl, Raf-1, Akt, HER2/Neu, interleukin 1 receptor associated kinase 1, and hypoxia-inducible factor 1 α (70, 71).

To investigate the utility of isoform specific HDACIs and to gain a better insight into the underlying mechanisms responsible for the improved outcomes, we administered Tubastatin A, a specific inhibitor of HDAC6, to mice that were subjected to CLP or sham treatment. We found that Tubastatin A treatment increased the circulating monocytes and lymphocytes in the CLP animals and decreased the granulocyte percentage, thus decreasing the granulocyte-to-lymphocyte ratio (72). In a similar model, administration of Tubastatin A resulted in attenuation of stress hormone abnormalities compared to control animals with decreased corticosterone levels at 24 hours, and increased ACTH levels at 48 hours. In addition, Tubastatin A treated animals had significantly lower percentages of thymic atrophy, bone marrow depletion and atrophy, and cellular apoptosis in the splenic follicles (73)

SIRT1, a class II HDAC, is primarily a nuclear deacetylase that regulates protein activities that control various cellular processes, including cell proliferation, differentiation, apoptosis, metabolism, DNA damage, genome stability, and cell survival (74, 75). Using the CLP-sepsis model described above, we explored the effects of EX-527, a selective SIRT1 inhibitor, on survival, inflammatory cytokine production, coagulopathy and bone marrow atrophy during severe sepsis. Treatment with EX-527 significantly improved survival, and attenuated the levels of TNF- α and IL-6 (Figure 4) in blood and peritoneal fluid compared to the vehicle control. It also decreased TNF- α and IL-6 production by splenocytes in vitro. Selective inhibition of SIRT1 was associated with dramatic improvements in fibrin cross-linkage, platelet function and clot rigidity, but without a significant impact on the clot initiation parameters. Moreover, inhibition of SIRT1 significantly decreased the bone marrow atrophy.

Ongoing testing of various isoform-specific HDACI is in progress in different models of sepsis and hemorrhagic shock with promising results. In the future, these specific inhibitors may displace the pan-inhibitors as a potential treatment for sepsis and hemorrhagic shock.

HDACI Treatment in Traumatic Brain Injury and Hemorrhagic Shock

Given the promising results with HDACI treatment in HS we asked whether this treatment could be beneficial in TBI, a major burden of disease in the United States with a yearly incidence of 1.7 million people (76). In 2010 TBI was reported in 30,703 military members serving in combat zones, becoming the “signature injury” of the current military conflicts (77). In TBI, the extent of brain damage is determined by the severity of primary mechanical injury and the secondary injury caused by hypoxia and hypotension that contributes to cerebral edema, raised intracranial pressure, and ongoing cellular destruction (78).

Hypotension and hypoxemia are independently associated with significant increases in morbidity and mortality after TBI (79). The combination of TBI and hemorrhagic shock (HS) is common and especially lethal, with studies showing that HS can double the morbidity and mortality following TBI (80, 81). The type of fluid that is used for resuscitation is also important. Fluid resuscitation restores the lost intra-vascular volume and augments the systemic and cerebral circulations. Although isotonic crystalloids are commonly used because they maintain an appropriate osmotic environment, they do not have any specific cytoprotective properties. We have previously shown that administration of 6% hetastarch (Hextend [Hex]) after TBI decreases brain swelling without affecting size of the lesion, whereas early treatment with fresh frozen plasma (FFP) significantly decreased the extent of brain damage (82). As FFP is not available in the pre-hospital settings, we explored whether the efficacy of Hex could be improved with the addition of a neuroprotective HDACI. In in-vitro models, we have previously shown that treatment with VPA induces NF- κ B activation and reduces phospho-JNK activation leading to protection of neurons from hypoxia-induced apoptosis (83). Therefore, it was logical to test whether addition of VPA (300mg/kg) to hetastarch solution would yield synergistic effects. In a swine model of 40% blood loss with brain cortical impact and a 2 hour period of HS we demonstrated that the brain lesion size in the Hex + VPA group was significantly smaller than the animals that were treated with Hex alone (Figure 5). In addition, the number of CD11b-positive cells as well as MPO and IL-1 levels in the brains were significantly reduced by the VPA treatment (46), suggesting a decrease in post-traumatic inflammation. Building on our previous findings that fresh frozen plasma (FFP) is an even better resuscitation fluid for TBI than Hex (82), we conducted a study where the combination of FFP + VPA was administered in a similar model of TBI and HS. The addition of VPA to FFP decreased TBI lesion size by 25% and brain swelling by 40% (Figure 6). It is important to note that the decrease in brain injury in the VPA treated animals was not due to better resuscitation (improved hemodynamic status) as these animals actually had lower blood pressure and slower lactate clearance. Thus, the smaller lesion size most likely reflects the enhanced ability of the stressed cells to survive the insults, similar to what has been observed in a non-TBI polytrauma model (44).

Mechanisms Underlying VPA-induced Neuroprotection

To gain a better understanding of other possible mechanisms leading to enhanced neuroprotection with VPA treatment, we analyzed platelet function in swine undergoing TBI and HS. It has been shown that patients with TBI experience increased platelet activation but decreased function compared with non-TBI trauma patients (84). This platelet dysfunction is thought to be mediated by the so-called “exhausted platelet syndrome”, characterized by initial platelet hyperactivation and concomitant depletion of intracellular mediators, leading to platelet unresponsiveness to stimulation. In a swine model of combined TBI + HS, we observed that FFP + VPA treatment resulted in elevated platelet activation in both the brain and serum compared with FFP alone. Brain and serum P-selectin levels, a marker for platelet activation, were significantly higher in the FFP + VPA group compared with FFP alone. The addition of VPA also resulted in significantly greater serum and brain sCD40 ligand levels, another marker of active platelets (Figure 7). Furthermore, levels of brain

platelet endothelial cell adhesion molecule 1 (PECAM-1), a surface glycoprotein receptor shown to limit growth of platelet thrombi on collagen surfaces, were significantly lower in the FFP + VPA group compared with the FFP group (85). These results suggest that the previously observed neuroprotective effects of VPA may be due to preservation of platelet function as measured by increased platelet activation response after resuscitation.

Another mechanism responsible for VPA induced neuroprotection is pharmacologic modulation of cerebral metabolic derangements during TBI and HS. In a study that used cerebral microdialysis in pigs undergoing TBI and HS, we found that treatment with FFP + VPA infusion led to a decrease in cerebral lactate and pyruvate compared with the FFP alone group, implying superior glucose utilization for ATP production. We also noted a decrease in concentrations of glutamate in the brains of the FFP+ VPA animals, a known excitotoxic neurotransmitter implicated as the final common pathway in neuronal injury and death (86), and glycerol, a well-established marker of membrane-phospholipid damage after TBI (87, 88). These findings suggest an overall decrease in excitotoxicity and brain damage. Similarly, Morland et al showed that VPA administration leads to reduction of the extracellular accumulation of glutamate in a rat model of malonate toxicity (89). Finally, brain pyruvate dehydrogenase complex (PDH) activity, deficiency of which has been shown to lead to neurodegenerative disease (90), was greater in the mitochondrial fractions and lower in cytosolic fractions in the FFP+VPA group, indicating better mitochondrial membrane function and enhanced mitochondrial PDH retention (91).

To investigate the effects of VPA treatment on brain gene expression profiles, we performed a microarray analysis of cerebral RNA isolated from Hex and Hex + VPA treated animals 6 hours after HS and TBI. Gene expression profiles for 1668 probe sets mapping to 370 known genes were found to be differentially modulated by VPA treatment in swine, with an overall attenuation of genes encoding apoptosis, cell death, and necrosis. Pathway analysis showed a significant modulation of cell signaling, dendritic cell response, and complement systems in the VPA treated animals (92). Recent data by other investigators has further clarified the mechanisms that are involved in the HDACI-induced neuroprotection. Zhang et al showed that VPA treatment after TBI significantly attenuated brain edema, reduced contusion volume, and the rate of neuronal apoptosis. This was attributed to reduced capase-3 activity and activation of ERK and Akt signaling pathways (93). Dash et al showed that administration of VPA after TBI in a rat model was neuroprotective with reduction in blood-brain barrier (BBB) permeability as evidenced by decreased Evans Blue dye extravasation (94). In a model of transient focal cerebral ischemia, Wang et al (95) showed that post-ischemic VPA treatment decreased BBB disruption and brain edema and reduced activity of matrix metalloproteinase-9 (MMP-9), a known mediator of BBB disruption after stroke (96). Additionally, VPA treatment preserved tight junction proteins claudin-5 and ZO-1. Finally, Lee et al reported similar findings in a model of spinal cord injury where VPA treatment inhibited MMP-9 activity after injury, and attenuated blood-spinal cord barrier permeability and degradation of tight junction molecules such as occludin and ZO-1 (97). In addition, VPA upregulated anti-apoptotic proteins including phosphorylated Akt (pAkt), HSP 27, and HSP 70. For additional details, an excellent review by Chen et al (98) focusing on the emerging neuroprotective properties of VPA and underlying mechanisms is recommended.

HDACI Treatment in a Poly-trauma Swine Model

To determine the efficacy of HDACI treatment in complex polytrauma associated with HS, we designed a swine long-term survival study where Yorkshire swine underwent a protocol simulating different phases of trauma care: (1) Pre-hospital phase: Femur fracture, 60% hemorrhage, 30 min of shock, and infusion of saline (3× shed blood); and (2) Early hospital phase: high grade liver injury (simulating rupture of a previously contained hematoma) followed by liver packing (Operative Phase). The animals were randomized into 3 treatment groups (n = 6–8/group): no treatment (control), fresh whole blood (FWB), and intravenous VPA (400 mg/kg, given during the pre-hospital phase). Animals were monitored for 4 hours (Critical care Phase), with survival being the primary endpoint. VPA treatment significantly increase survival (86%) compared to control group (25%) ($p < 0.05$) (Figure 8). In addition, VPA treatment preserved the Akt survival pathway with increased pAkt, phospho-GSK-3b (Glycogen synthase kinase 3b), b-catenin and Bcl-2 protein levels compared to control group (all $p < 0.05$) (44). In a long-term survival study, we noticed no adverse effects following VPA treatment in a poly-trauma model (99).

Interestingly, in a model of 35% volume-controlled hemorrhage and aortic cross-clamping, Nelson et al showed that VPA administration was not associated with improved duration of survival compared with control animals. Animals receiving VPA showed shorter lengths of survival, higher crystalloid and vasopressor requirement, and worse hemodynamic and physiologic measurements throughout the resuscitative period (100). This is in contrast with earlier work from this group showing decreased resuscitative requirements and improved hemodynamic and physiologic measurements during a 6-hour resuscitative period (33). The authors attributed this negative effect of VPA to high dose of VPA used (400 mg/kg), which is known to cause increased cardiac suppression and instability and suggested that a lower dose (200–300 mg/kg) may reduce cardiac toxicity and improve the cell and tissue protective impacts of VPA.

Valproic Acid Safety and Tolerability

Besides its clinical use as an anticonvulsant and mood-stabilizing drug (101), VPA has beneficial effects in clinical depression (102), absence seizures (103, 104), tonic-clonic seizures, complex partial seizures (105), juvenile myoclonic epilepsy (106), migraine headaches, and schizophrenia. However, with its role as an HDACI, use of VPA in cancer therapy has been an area of intense research in the last decade. VPA has been shown to exert anti-tumor effects in a wide variety of cancers in preclinical studies, in vitro and in vivo, through modulation of multiple pathways including cell cycle arrest, angiogenesis, apoptosis, differentiation, and senescence (107). As we have shown above, VPA holds tremendous promise for use in pharmacological resuscitation as a strategy in hemorrhagic shock, polytrauma, and TBI that could alter the cellular response to injury and create a “pro-survival phenotype; however, the single doses used in our animal models (250–300 mg/kg intravenously) have not been used in clinical practice. Clinical studies evaluating higher doses of VPA administered intravenously in subjects with solid tumors and confirmed progressive, advanced stage malignant disease have shown that after 5 day treatment periods in a 21 day cycle, 60 mg/kg/day was defined as the maximal tolerated dose with doses of 90

to 120mg/kg/d resulting in neurocognitive impairment, with grade 3 or 4 neurological side effects (108). The dose of VPA that can promote survival while being safe in trauma patients is yet unknown and as mentioned above, high doses of VPA given over short infusions can lead to significant toxicity. However, our current preliminary large animal data reveal that lower doses (150mg/kg) of VPA are safe and equally cytoprotective (Accepted for presentation at the Western Trauma Association annual meeting 2015). Also, the fact that VPA dose of 60mg/kg/day in cancer patients can create a biological effect seems to suggest that the effective dose for trauma patients will be much lower than what has previously been used in the various animal studies. We received FDA approval in 2012 for a phase 1, single ascending dose, double blind, placebo controlled study to evaluate the safety and tolerability of valproic acid in healthy volunteers and trauma patients (ClinicalTrials.gov Identifier: NCT01951560). The objectives of this trial are to determine the safety, tolerability, pharmacokinetic, and pharmacodynamics profile of single ascending doses of valproic acid administered as intravenous infusion (IV) in doses ranging from 15 mg/kg to 250 mg/kg in healthy subjects. The two highest doses that are demonstrated to have an acceptable safety profile will be used in the second part of the trial in trauma subjects presenting with hemorrhagic shock. This study is currently ongoing and is expected to yield important information.

Conclusions

Conventional methods of treating massive blood loss have proven to be ineffective and at times can worsen the outcome. Keeping the injured alive is especially challenging in the pre-hospital setting/battlefield environment where resources are limited. Our research has shown that pharmacological treatment with HDACIs represents a novel and promising approach for not only treatment of shock, but sepsis, TBI, and polytrauma. These cytoprotective agents can be administered in austere settings to keep the injured alive, thus serving as a bridge to definitive care. How HDACIs exert their beneficial effect in such diverse lethal insults is a complex question but we are gaining a better understanding of the mechanisms underlying their action. With further research and clinical experimentation, the use of carefully selected pharmacological agents could become an integral component of trauma care in the future.

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Figure 1.

Kaplan-Meier plot showing percent survival per group over time. Percentages represent percent survival at primary endpoint, 3 hours. Sham: no hemorrhage, no resuscitation; NR: hemorrhage, no resuscitation; Saline: resuscitation with 0.9% saline 3× shed blood volume; VPA: valproic acid 300 mg/kg in 0.25 mL saline; SAHA: suberoyanilide hydroxamic acid 7.5 mg/kg in 0.25 mL saline; Vehicle: 0.25 mL 0.9% saline vehicle.

Figure 2.

Relative intensity of acetylated H3 K9 (AcH3K9)/actin was analyzed by densitometry. Data shown as mean $\pm$ standard error of mean (minimum of 3 animals at each time point).

Figure 3.

VPA treatment increases survival and suppresses the inflammatory profile in a two-hit model of shock followed by CLP. (A) Male Sprague-Dawley rats were subjected to sublethal hemorrhagic shock and then randomized into two groups (n = 7/group): VPA and vehicle control (VEH). Treatment with 300mg/kg VPA or normal saline was administered via the peritoneum. After 24 hours, all rats received CLP followed immediately by injection of the same dose of VPA or NS. Survival was monitored for 10 days. Treatment with VPA significantly improved survival (85.7% vs. 14.3%; $p < 0.05$). (B) Mice were harvested at 6 and 24 hours following CLP and peritoneal irrigation and serum were collected and analyzed for TNF- α and IL-6 levels by ELISA. VPA treatment significantly decreased the levels of peritoneal irrigation and serum TNF- α levels at 6 hours and IL-6 (means $\pm$ SEM).

Figure 4.

(A) EX-527 attenuates TNF- α levels markedly in blood in vivo. Blood samples were collected at 24 h after CLP and assayed for TNF- α levels by ELISA (means $\pm$ SEM, $n=6-7$ animals/group). (B) EX-527 attenuates IL-6 levels markedly in peritoneal fluid and blood in vivo. Peritoneal fluid and blood were collected at 48 h after CLP and assayed for IL-6 levels by ELISA (means $\pm$ SEM, $n = 6-7$ animals/group).

Figure 5.

Addition of VPA to Hex resuscitation reduces brain injury 6 hours after HS and TBI. (A) Representative brain slices stained with 2,3,5-Triphenyltetrazolium chloride. (B) Lesion size and brain swelling in different groups. Brain swelling is shown as percent increase compared with the contralateral hemisphere. **p* < 0.05 compared with isotonic sodium chloride solution treatment.

Figure 6.

Addition of VPA to FFP resuscitation reduces brain injury 6 hours after HS and TBI. (A) Representative brain slices stained with 2,3,5-triphenyltetrazolium chloride. (B) Lesion size and brain swelling in different groups. Brain swelling is shown as percent increase compared with the contralateral hemisphere. FFP, Fresh frozen plasma; NS, 0.9% normal saline; VPA, valproic acid. *p < 0.05 compared with NS. #p < 0.05 FFP versus FFP+VPA

Figure 7. Concentrations of serum CD40 ligand (CD40L) (A), and P-selectin (B), in FFP, and FFP+VPA groups. Data presented as group mean $\pm$ standard deviation. Asterisk (*) indicates p 0.05. P value represents differences in estimated marginal means based on the time points post-resuscitation (PR) and after 6 h of observation (6HOB) using a linear mixed model with Bonferroni post hoc testing.

Figure 8.

Kaplan Meier Survival Curves (swine poly-trauma model). In this highly lethal model, there was 75% mortality in the control group that received no further treatment. Resuscitation with FWB resulted in 100% survival. Animals that received the full dose of VPA survived until the end of experiment. There was no significant difference in survival between FWB and VPA treatment groups.

Table 1

Summary of different HDACs Classes and Their Inhibitors

HDAC Class	HDAC Isoform	Cellular Location	Isoform Specific Inhibitor	Non-specific Inhibitor	Effect
Class I Ubiquitous tissue distribution	HDAC1	Nuc	MS-275, FK-228	TSA SAHA Butyrate VPA	Pan-HDACIs (SAHA, VPA) improve survival in models of hemorrhage/trauma (32, 44, 109), sepsis (54, 55) and two-hit (56).
	HDAC2		FK-228, Apicidin		
	HDAC3		Apicidin		
	HDAC8				
	HDAC9				
Class IIa Tissue specific.	HDAC4	Nuc/Cyt	MC1568	TSA SAHA Butyrate VPA	No published data for specific HDAC class IIa inhibitors in hemorrhage, sepsis and two-hit.
	HDAC5				
	HDAC7				
	HDAC9				
	HDAC10				
Class IIb Tissue specific.	HDAC 6	Mainly Cyt	Tub A	TSA SAHA	Tub A alters the composition of circulating blood cells and improves survival in sepsis (72) and two-hit models (unpublished data). No published data in hemorrhage.
	HDAC10				
	SIRT1				
	SIRT2				
	SIRT3				
Class III	SIRT4	Nuc/Cyt	EX-527	Nicotinamide	EX-527 (110) and AGK2 (unpublished data) improve survival in sepsis. No published data in hemorrhage and two-hit models.
	SIRT5		AGK2		
	SIRT6		EX-527		
	SIRT7		Suramin		
	HDAC11				
Class IV	HDAC11	Nuc/Cyt		TSA, SAHA	No data for specific inhibitors

Cyt=Cytosol; HDAC=Histone deacetylase inhibitor; HDACIs=Histone deacetylase inhibitors; Mit= Mitochondria; Nuc=Nucleus; 2-hit= hemorrhagic shock followed by septic shock. SAHA= Suberoylanilide hydroxamic acid; TSA=Trichostatin A; Tub A=Tubastatin A; VPA=Valproic acid; MS-275= Entinostat or N-[[4-[(2-aminophenyl)amino]carbonyl]phenyl]methyl]-3-pyridinylmethyl ester; FK-228= Depsipeptide or Cyclo[(2Z)-2-amino-2-butenoyl-L-valyl-(3S,4E)-3-hydroxy-7-mercapto-4-heptenoyl-D-valyl-D-cysteinyl] cyclic (3-5) disulfide; MC-1568= 3-[5-(3-(3-Fluorophenyl))-3-oxopropen-1-yl]-1-methyl-1H-pyrol-2-yl]-N-hydroxy-2-propenamide; EX-527= 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide; AGK2= 2-cyano-3-[5-(2,5-dichlorophenyl)-2-furyl]-N-5-quinolinylacrylamide.