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Histone deacetylase inhibitor (HDACI) mechanisms of action: emerging insights

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Abstract

Initially regarded as “epigenetic modifiers” acting predominantly through chromatin remodeling via histone acetylation, HDACIs, alternatively referred to as lysine deacetylase or simply deacetylase inhibitors, have since been recognized to exert multiple cytotoxic actions in cancer cells, often through acetylation of non-histone proteins. Some well-recognized mechanisms of HDACI lethality include, in addition to relaxation of DNA and de-repression of gene transcription, interference with chaperone protein function, free radical generation, induction of DNA damage, up-regulation of endogenous inhibitors of cell cycle progression, e.g., p21, and promotion of apoptosis. Intriguingly, this class of agents is relatively selective for transformed cells, at least in pre-clinical studies. In recent years, additional mechanisms of action of these agents have been uncovered. For example, HDACIs interfere with multiple DNA repair processes, as well as disrupt cell cycle checkpoints, critical to the maintenance of genomic integrity in the face of diverse genotoxic insults. Despite their pre-clinical potential, the clinical use of HDACIs remains restricted to certain subsets of T-cell lymphoma. Currently, it appears likely that the ultimate role of these agents will lie in rational combinations, only a few of which have been pursued in the clinic to date. This review focuses on relatively recently identified mechanisms of action of HDACIs, with particular emphasis on those that relate to the DNA damage response (DDR), and

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discuss synergistic strategies combining HDACIs with several novel targeted agents that disrupt the DDR or antagonize anti-apoptotic proteins that could have implications for the future use of HDACIs in patients with cancer.

Keywords

HDAC inhibitor; DNA damage response; DNA repair; cell cycle checkpoints; apoptosis; rational combinations

I. Introduction

In many respects, histone deacetylase inhibitors (HDACIs) represent prototypical "epigenetic" agents which act by modifying gene expression to restore the normal differentiation or death programs of transformed cells. In fact, the ability of HDACIs such as sodium butyrate to hyperacetylate histones and induce new gene expression in leukemia cells was discovered over thirty years ago (Reeves & Cserjesi, 1979). Over the ensuing decades, a large amount of data emerged concerning the mechanisms of action of these agents, culminating in the regulatory approval of two HDACIs, vorinostat (Zolinza®, Merck) and romidpesin (Istodax®, Celgene), for the treatment of patients with cutaneous and peripheral T-cell lymphoma (Watanabe, 2010). However, despite this large body of information, and intriguing evidence, both preclinical and clinical, suggesting a role for HDACIs in other malignancies (e.g., acute myeloid leukemia (AML), myelodysplastic syndromes (MDS) (Quintas-Cardama, Santos, & Garcia-Manero, 2011) and multiple myeloma (MM) (Richardson et al., 2013)), there is a general sense that HDACIs have not fully realized their potential as antineoplastic agents. One of the major barriers to this goal is continuing uncertainty about the mechanism of action by which these agents in fact trigger transformed cell death (Grant & Dai, 2012; Rosato & Grant, 2005). Complicating efforts to resolve this issue has been the emerging realization that HDACIs are truly pleiotropic agents which act through a wide variety of disparate and mutually interactive mechanisms. In this context, evidence that HDACIs modify gene expression, i.e., by altering chromatin structure, acetylating promoter regions, or disabling co-repressors, is undisputed. However, HDACIs target other nonhistone proteins which may, either directly or indirectly, influence cell fate, and it is highly likely that such actions intersect with those mediated by the canonical effects of HDACIs on gene expression (Grant & Dai, 2012; Rosato & Grant, 2005). Several excellent reviews of the mechanisms of action of this interesting group of agents in cancer cells have appeared in the literature (Bolden, Peart, & Johnstone, 2006; Dickinson, Johnstone, & Prince, 2010; Marks & Xu, 2009; Minucci & Pelicci, 2006; Schrump, 2009). The thrust of the present article is on recently identified mechanisms of HDACI lethality, particularly as they relate to the DNA damage response (DDR) network, paving the way for novel synergistic combination strategies with other targeted agents that exert complementary activities.

II. Mechanisms of HDACI lethality

DNA (chromatin) is wrapped around histone octamers to form nucleosomes, and these histones are reversibly modified in various ways in order to render DNA accessible to

transcription factors, the best characterized of which is acetylation (Grant & Dai, 2012; Rosato & Grant, 2005). Acetylation of histones is reciprocally regulated by histone acetyltransferases (HATs) and HDACs (Grant & Dai, 2012; Rosato & Grant, 2005). Acetylation of positively charged N-terminal lysine residues in the histone tails interferes with their binding to negatively charged DNA, allowing a more open, or relaxed chromatin configuration that favors gene transcription (Rosato & Grant, 2005). Conversely, deacetylation of histones favors compaction of chromatin, which is generally associated with gene silencing (Rosato & Grant, 2005), although some genes can be down-regulated by histone acetylation, depending on the cellular context (Peart et al., 2005). HDACs, which are frequently dysregulated in cancer, represent the products of 18 genes, which can be subdivided into 4 classes (Grant & Dai, 2012). Classes I (HDACs 1, 2, 3 and 8), II (HDACs 4, 5, 6, 7, 9 and 10) and IV (HDAC 11) are zinc-dependent enzymes, whereas the class III enzymes (sirtuins) are zinc-independent but nicotinamide adenine dinucleotide (NAD)-dependent (Rosato & Grant, 2005). The class IIb HDAC, HDAC6 is distinguished by its ability to deacetylate tubulin, which has important mechanistic implications related to disruption of aggresome formation and down-regulation of chaperone proteins by HDAC6 inhibitors (Bali, Pranpat, Bradner, Balasis, Fiskus, Guo, Rocha, Kumaraswamy, Boyapalle, Atadja, Seto, & Bhalla, 2005b; Hideshima et al., 2005). Clinically relevant HDACIs represent different chemical classes, e.g., hydroxamic acids (vorinostat, dacinostat, panobinostat, belinostat), benzamides (entinostat, mocetinostat) and cyclic tetrapeptides (romidepsin) (Bolden et al., 2006; Dickinson et al., 2010). Some of these (the hydroxamic acids) act as pan-HDACIs, whereas others predominantly target the class I (entinostat, mocetinostat) or class IIb (e.g., ACY-1215) enzymes (Bolden et al., 2006; Grant & Dai, 2012).

Key determinants of HDACI lethality (Figure 1) include:

- **i.** *down-regulation of anti-apoptotic proteins* such as caspase inhibitors (e.g., X-linked inhibitor of apoptosis (XIAP), survivin and cellular FLICE-like inhibitory protein (c-FLIP)) (Aron et al., 2003; C. S. Mitsiades et al., 2004; Rosato et al., 2006; Rosato et al., 2007; Sanda et al., 2007), Bcl-w (Sanda et al., 2007) and myeloid cell leukemia-1 (Mcl-1, through reversal of microRNA silencing) (Sampath et al., 2012),
- **ii.** *up-regulation of pro-apoptotic proteins* such as Bim, Bmf and Noxa (through acetylation of p53) (S. Chen, Dai, Pei, & Grant, 2009b; Dai, Chen, Kramer et al., 2008; Dai, Chen, Wang, Pei, Kramer et al., 2011; Inoue, Riley, Gant, Dyer, & Cohen, 2007; Tan et al., 2006; Terui et al., 2003; Xargay-Torrent et al., 2011; Zhao et al., 2005),
- **iii.** *activation of the death receptor pathway* (Glick et al., 1999; Insinga et al., 2005; Nebbioso et al., 2005),
- **iv.** *induction of Bid cleavage and activation* (Lindemann et al., 2007; N. Mitsiades et al., 2003; Ruefli et al., 2001), thus linking the intrinsic and extrinsic pathways of apoptosis,

v. *induction of the endogenous cyclin-dependent kinase (CDK) inhibitor p21* (H. Li & Wu, 2004; N. Mitsiades et al., 2003; Nawrocki et al., 2007; Richon, Sandhoff, Rifkind, & Marks, 2000; Sanda et al., 2007; H. Wang et al., 2012),

vi. *ROS generation* (Sanda et al., 2007) *and induction of DNA damage* (Dai, Rahmani, Dent, & Grant, 2005; Dasmahapatra et al., 2010; Dasmahapatra et al., 2011; Gaymes et al., 2006; Hu et al., 2010; Petruccelli et al., 2011; Rosato, Almenara, & Grant, 2003; Rosato et al., 2008; Rosato et al., 2010; Ruefli et al., 2001; Ungerstedt et al., 2005; Xu, Ngo, Perez, Dokmanovic, & Marks, 2006),

vii. *disruption of chaperone protein (in particular, heat shock protein 90 (Hsp90)) function (via acetylation)* (X. Yu et al., 2002), an effect that has been attributed to HDAC6 inhibition (Bali, Pranpat, Bradner, Balasis, Fiskus, Guo, Rocha, Kumaraswamy, Boyapalle, Atadja, Seto, & Bhalla, 2005b), leading to Hsp70-mediated proteasomal degradation of Hsp90 “client” oncoproteins (Nimmanapalli et al., 2003), and

viii. *inhibition of DNA repair* through acetylation of Ku70 (Cohen et al., 2004; Rosato et al., 2008; Subramanian, Opipari, Bian, Castle, & Kwok, 2005), down-regulation of the DNA repair proteins Ku86, BRCA1, Chk1, RAD50, RAD51 and MRE11 (meiotic recombination 11) (Adimoolam et al., 2007; J. H. Lee, Choy, Ngo, Foster, & Marks, 2010; Rosato et al., 2008), interference with the S-phase checkpoint through loss of HDAC3 function (Bhaskara et al., 2008), disruption of both the homologous (Adimoolam et al., 2007; Kachhap et al., 2010) and non-homologous end-joining (NHEJ) (Miller et al., 2010) processes of DNA repair and interference with HDAC-mediated coordination of ATR (ATM and Rad3-related) checkpoint function, double strand break (DSB) processing and autophagy (T. Robert et al., 2011; Shubassi, Robert, Vanoli, Minucci, & Foiani, 2012).

The pleiotropic actions of HDACIs also include (Figure 1):

i. *interference with the function of the co-repressor* (e.g., Bcl-6 in diffuse large B-cell lymphoma (DLBCL) (Cerchietti et al., 2010)) *and cofactor* (e.g., NCOR1/SMRT (nuclear receptor corepressor 1/silencing mediator of retinoic acid and thyroid hormone receptor)) *axis*, critical for maintaining chromatin structure and genomic stability (Bhaskara et al., 2008; Bhaskara et al., 2010)),

ii. *promotion of proteotoxic and endoplasmic reticulum (ER) stress via disruption of aggresome formation* via HDAC6 inhibition and acetylation of GRP78 (glucose regulated protein 78), a critical sensor of the ER stress response (Hideshima et al., 2005; Kawaguchi et al., 2003; Nawrocki et al., 2006; Nawrocki et al., 2007; Rao, Nalluri, Fiskus et al., 2010; Rao, Nalluri, Kolhe et al., 2010), as well as through inhibition of class I HDACs (Kahali, Sarcar, Prabhu, Seto, & Chinnaiyan, 2012),

iii. *disruption of cell cycle (especially mitotic spindle assembly) checkpoints* (Ishii, Kurasawa, Wong, & Yu-Lee, 2008; Magnaghi-Jaulin, Eot-Houllier, Fulcrand, & Jaulin, 2007; Stevens, Beamish, Warrener, & Gabrielli, 2008b; Warrener et al., 2003), dysregulation of which is frequent in neoplastic cells (Kastan & Bartek, 2004),

iv. *c-Jun N-terminal kinase (JNK) activation* (Dai et al., 2010; Dasmahapatra et al., 2010; Dasmahapatra et al., 2011; Vrana et al., 1999),

v. *signal transducer and activator of transcription (STAT) 5 and 3 inhibition* (Nguyen, Dai, Attkisson, Kramer, Jordan, Nguyen, Kolluri, Muschen, & Grant, 2011b; Rascle, Johnston, & Amati, 2003; Shao et al., 2010),

vi. *interference with proteasome function* (Fotheringham et al., 2009; C. S. Mitsiades et al., 2004),

vii. *anti-angiogenic effects* (Deroanne et al., 2002; Qian et al., 2004),

viii. generation of the pro-apoptotic lipid second messenger ceramide (Maggio et al., 2004) and

ix. *induction of autophagy*, possibly through acetylation of the autophagy signaling component Atg3 (Yi et al., 2012).

Autophagy induced by HDACIs can be cytoprotective (Carew et al., 2007; Gammoh et al., 2012), as can induction of p21 by these agents (Almenara, Rosato, & Grant, 2002), providing a basis for synergism with agents that block these phenomena (Almenara et al., 2002; Carew et al., 2007; Gammoh et al., 2012). The selective toxicity of HDACIs towards transformed cells may be explained in part by the ability of normal but not neoplastic cells to escape HDACI-induced oxidative injury by up-regulating thioredoxin (Ungerstedt et al., 2005), to repair HDACI-induced DNA damage (J. H. Lee et al., 2010) and by the activation of death receptor pathways in transformed cells (Insinga et al., 2005; Nebbioso et al., 2005).

III. The DDR signaling network, cell cycle checkpoints and HDACIs

The DDR represents a complex network of multiple signaling pathways involving cell cycle checkpoints, DNA repair, transcriptional programs, and apoptosis, through which cells maintain genomic integrity following various endogenous (metabolic) or environmental stresses (Dai & Grant, 2010). The cell cycle progresses in an orderly fashion and is monitored by safety mechanisms known as cell cycle checkpoints, which, on activation, function to halt cell division (A. N. Tse, Carvajal, & Schwartz, 2007). When DNA damage occurs, distinct, albeit overlapping and cooperating, checkpoint pathways are activated, which block S-phase entry (the G1/S-phase checkpoint), delay S-phase progression (the intra-S or S-phase checkpoint), or prevent mitotic entry (the G2/M-phase checkpoint). These events direct phase-specific DNA repair mechanisms through repair-specific gene transcription. If repair fails, checkpoints trigger apoptosis (Dai & Grant, 2010).

Components of the checkpoint mechanism include sensors, mediators, transducers and effectors, which work cooperatively in different phases of the cell cycle. Sensors recognize damage, and recruit the proximal transducers ATM (ataxia telangiectasia mutated) and ATR to lesions where they are initially activated. ATM and ATR, in turn, transduce signals to the distal transducers, checkpoint kinases 1 and 2 (Chk1 and Chk2). In general, ATM is activated by DSBs, resulting, for example, from ionizing radiation, whereas ATR is activated by DNA damage and DNA replication stress. The acetylation of ATM by the HAT Tip60 (Tat interactive protein 60) is a key step linking the detection of DNA damage and the

activation of ATM kinase activity (Sun, Xu, Roy, & Price, 2007). Classically, ATM has been thought to activate Chk2, and ATR primarily Chk1. However, more recent data have challenged the classic model of ATM and ATR being activated by distinct lesions and triggering independent downstream pathways, and coordinated cross-talk definitely exists between the two pathways. These concepts have been reviewed (Bucher & Britten, 2008; Dai & Grant, 2010; A. N. Tse et al., 2007) and are summarized in Figure 2.

The G1/S checkpoint is the first defense against genomic stress in cycling cells. In response to DNA damage, the G1 checkpoint prevents cells from entering the S-phase by inhibiting the initiation of DNA replication. At this checkpoint, Chk2 is activated by ATM to phosphorylate (and thereby inhibit) the cell division cycle (CDC)25A phosphatase, thus preventing activation of cyclin E(A)/CDK2 and temporarily halting the cell cycle. G1 arrest is sustained by ATM/Chk2-mediated phosphorylation of murine double minute homolog 2 (Mdm2) and p53, resulting in p53 stabilization and accumulation. p53 transcriptionally activates the endogenous CDK inhibitor p21, which in turn inhibits cyclin E(A)/CDK2 and preserves the association of the tumor suppressor Rb (retinoblastoma protein) with the transcription factor E2F (Bucher & Britten, 2008).

The intra-S or S-phase checkpoint is activated in response to structural DNA damage as well as stalled replication forks. Upon activation of this checkpoint, ATR/Chk1 and ATM/Chk2 phosphorylate CDC25A, resulting in enhanced proteolysis of the phosphatase and inhibition of its function through 14-3-3 σ binding. Cyclin E(A)/CDK2 is thereby inhibited, and progression through the S phase halted (A. N. Tse et al., 2007).

The G2/M checkpoint prevents mitotic entry of cells that have either incurred DNA damage during G2, or that have escaped the G1/S and intra-S checkpoints despite earlier genomic insults. The key downstream target of the G2/M checkpoint is the pro-mitotic cyclin B/CDK1 (cdc2) complex. During interphase, this complex is inactivated through phosphorylation by Myt1 and Wee1 (A. N. Tse et al., 2007). Chk1 may phosphorylate Wee1, mediating binding of Wee1 to 14-3-3 proteins, which in turn may stimulate the kinase activity of Wee1 against CDK1 (cdc2). Thus, both Chk1 and 14-3-3 proteins may act together as positive regulators of Wee1 (M. H. Lee & Yang, 2001). Activation of cyclin B/CDK1 (cdc2) requires dephosphorylation by the CDC25 phosphatases (A, B and C). Initiation of the G2/M checkpoint is mediated by ATR/Chk1, which phosphorylates (and thereby inhibits) CDC25A, -B and –C, whereas maintenance of this checkpoint requires p53 and its downstream effectors p21, 14-3-3 σ and growth arrest and DNA damage 45 (GADD45) (A. N. Tse et al., 2007).

Checkpoint dysfunction is common in human cancers and is considered a pathologic hallmark of neoplastic transformation (Kastan & Bartek, 2004). Conversely, agents used for cancer treatment, such as cytotoxic chemotherapy and ionizing radiation, also activate cell cycle checkpoints (A. N. Tse et al., 2007). Cancer cells are particularly dependent upon the S- and G2/M-phase checkpoints for repair of DNA damage due to pre-existing defects in G1/S checkpoint mechanisms, such as p53 and Rb mutations. Because the S-phase checkpoint facilitates slowing, rather than arrest, of the cell cycle, a cancer cell harboring DNA damage may progress through the S-phase checkpoint, only to halt at the G2/M

checkpoint. The latter is therefore a key guardian of the cancer cell genome, and its abrogation should lead to enhanced tumor cell death, sparing normal cells, which maintain an intact G1/S-phase checkpoint. G2/M checkpoint abrogation prevents cancer cells from repairing DNA damage, forcing them into a premature and lethal mitosis ("mitotic catastrophe") (Bucher & Britten, 2008). Hsp90 inhibitors down-regulate several key DDR proteins (e.g., ATR, Chk1 and Wee1) that are "clients" of the chaperone protein (Ha et al., 2011; Sugimoto et al., 2008; A. N. Tse, Sheikh, Alan, Chou, & Schwartz, 2009). Disruption of chaperone protein (Hsp90) function through acetylation of this protein, leading to proteasomal degradation of "client" oncoproteins (Nimmanapalli et al., 2003; X. Yu et al., 2002), is a widely recognized mechanism of action of HDACIs that has generally been attributed to HDAC6 inhibition (Bali, Pranpat, Bradner, Balasis, Fiskus, Guo, Rocha, Kumaraswamy, Boyapalle, Atadja, Seto, & Bhalla, 2005b), although similar effects have been observed with the class I selective HDACI entinostat (Nishioka et al., 2008). HDACIs have also been shown to down-regulate Chk1 and cause premature mitotic entry and apoptosis in lung cancer cells (Brazelle et al., 2010). Significantly, ATM forms a complex with HDAC1 and their mutual binding increases in response to ionizing radiation (G. D. Kim et al., 1999). HDAC4 has also been shown to be a component of the DDR (Basile, Mantovani, & Imbriano, 2006). HDAC4 shuttles from the cytoplasm into the nucleus following DNA damage independently of the activation of p53, colocalizes with p53 binding protein 1 (p53BP1) (Kao et al., 2003) and becomes associated with G2/M promoters through a p53-dependent mechanism (Basile et al., 2006), to epigenetically silence genes that control the G2/M transition. Silencing of HDAC4 via RNA interference abrogated DNA damage-induced G2 delay, and radiosensitized HeLa cells (Kao et al., 2003).

IV. Induction of DNA damage and inhibition of DNA repair by HDACIs

Although it has been reported that HDACIs can directly activate the DDR as a result of changes in chromatin structure without the requirement for DNA DSBs (Bakkenist & Kastan, 2003), ample evidence exists to support the notion that HDACIs directly induce DNA damage, resulting in DSBs (C. S. Chen et al., 2007; Dasmahapatra et al., 2010; Dasmahapatra et al., 2011; Gaymes et al., 2006; J. H. Lee et al., 2010; Miller et al., 2010; Namdar, Perez, Ngo, & Marks, 2010; Petruccelli et al., 2011; Rosato et al., 2008; Rosato et al., 2010). Furthermore, the chromatin remodeling and DNA damage signaling pathways are by no means mutually exclusive (Lukas, Lukas, & Bartek, 2011; Misteli & Soutoglou, 2009). Mechanistically, DNA damage and apoptosis induced by HDACIs is due, at least in part, to the generation of oxidative stress (Bhalla et al., 2009; Butler et al., 2002; Dai et al., 2005; Dasmahapatra et al., 2011; Petruccelli et al., 2011; Rahmani et al., 2005; Rosato et al., 2003; Rosato et al., 2006; Rosato et al., 2008; Rosato et al., 2010; Ruefli et al., 2001; Sanda et al., 2007; Ungerstedt et al., 2005; Xu et al., 2006). ROS generation by HDACIs has been attributed to down-regulation of antioxidant pathways (Butler et al., 2002; Ungerstedt et al., 2005). HDACIs have also been shown to down-regulate proteins that participate in base excision repair (BER) and nucleotide excision repair (NER), pathways for the repair of oxidative damage, and to trigger depolarization of the mitochondrial membrane (Rosato et al., 2008; Rosato et al., 2010), which can induce reactive oxygen species (ROS) generation. HDACI-induced nuclear factor kappa B (NFκB) activation(Dai, Rahmani, & Grant, 2003;

Dai et al., 2005; Dai et al., 2010; Dai, Chen, Wang, Pei, Funk et al., 2011; Mayo et al., 2003; Rosato et al., 2010; Roychowdhury et al., 2004) could also, in part, explain the production of ROS by these agents, through the induction of pro-inflammatory cytokines (J. J. Kim, Lee, Park, & Yoo, 2010). Not surprisingly, HDACIs sensitize tumor cells to DNA-damaging agents and ionizing radiation (IR)(Camphausen et al., 2004; Miller et al., 2010; Munshi et al., 2005; Munshi et al., 2006). DNA damage resulting from HDACI treatment has also been attributed to profound changes in chromatin structure, exposing portions of DNA normally protected from damage to DNA-damaging agents, inhibition of DNA repair pathways and/or modulation of expression of DDR and cellular survival genes (C. Robert & Rassool, 2012). At pharmacologic concentrations, vorinostat slows down replication forks in cancer cells, activates dormant replication origins and induces replication-mediated DNA damage, likely through inhibition of HDAC3 (Conti et al., 2010).

DSBs represent the most serious form of DNA damage in mammalian cells (reviewed in (Khanna & Jackson, 2001)). Histone H1.2 plays an important role in transmitting apoptotic signals from the nucleus to the mitochondria following DNA DSBs (Konishi et al., 2003). As noted above, ATM is a key mediator in the activation of cell cycle checkpoints by DSBs. ATM is recruited to DSB sites by the MRN (MRE11/NBS1/RAD50) complex (Falck, Coates, & Jackson, 2005), followed by its phosphorylation and conversion from an inactive dimer to an active monomer (Bakkenist & Kastan, 2003)(Bakkenist & Kastan, 2003; Bakkenist & Kastan, 2003; Falck et al., 2005). Upon activation, ATM phosphorylates MRN and downstream effectors to activate the G1/S, intra-S and G2/M checkpoints (reviewed in (O'Driscoll & Jeggo, 2006)). Homologous recombination (HR) repairs replication-associated DSBs and is a generally error-free pathway of DNA repair that is active in late S-phase and in G2, and utilizes the undamaged sister chromatid as the template for repair (C. Robert & Rassool, 2012). Critical steps in HR include CtBP interacting protein (CtIP)-mediated resection of the DSB in a 5′-3′ fashion (Huertas & Jackson, 2009; Sartori et al., 2007), followed by the recruitment and assembly of RAD51 into a nucleoprotein filament, which involves RAD52, x-ray repair cross-complementing proteins (XRCC) 2 and 3 and BRCA2 (N. Liu et al., 1998; Petalcorin, Sandall, Wigley, & Boulton, 2006; Sonoda et al., 2007). DSB repair by the NHEJ pathway is initiated by the Ku70/Ku86 heterodimer, which binds to ends of DNA (Walker, Corpina, & Goldberg, 2001) and activates DNA protein kinase (PK) (Calsou et al., 1999; Gottlieb & Jackson, 1993; Singleton, Torres-Arzayus, Rottinghaus, Taccioli, & Jeggo, 1999). The key step in NHEJ is the physical juxtaposition of DNA ends (C. Robert & Rassool, 2012). There is also an alternative DNA end-joining repair pathway that has been implicated in certain cancers (Nussenzweig & Nussenzweig, 2007).

HDACIs inhibit multiple processes of DNA repair (C. Robert & Rassool, 2012). First, these agents transcriptionally down-regulate a large number of DNA DSB repair proteins, e.g., RAD50, MRE11, Ku70, Ku80, Ku86, DNA PK, BRCA1, RAD51, Chk1, and Bloom syndrome (BLM) (Kachhap et al., 2010; J. H. Lee et al., 2010; Munshi et al., 2005; Munshi et al., 2006; Rosato et al., 2008). HDACIs additionally impair the recruitment of these proteins to repair foci and decrease levels of the transcription factor E2F1, which promotes gene expression of *RAD51*, *Chk1* and *BRCA1* (Kachhap et al., 2010). Another major mechanism of regulation of a number of proteins important in DNA repair (e.g., p53, Ku70,

flap structure-specific endonuclease 1 (FEN1), Werner syndrome (WRN), ATM, mediator of DNA damage checkpoint 1 (MDC1) and DNA PK) is through acetylation (Choudhary et al., 2009). HDACIs acetylate and thereby stabilize p53, leading to transcription of its target genes, including p21, triggering cell cycle arrest or apoptosis (J. Luo, Su, Chen, Shiloh, & Gu, 2000). The proteasomal degradation of p53 requires MDM2-HDAC1-mediated deacetylation (Ito et al., 2002). Ku70 acetylation by HDACIs reduces its binding to DNA and sensitizes prostate cancer cells to agents such as bleomycin, doxorubicin and etoposide, which induce DSBs (C. S. Chen et al., 2007). In addition, HDACIs disrupt the association of Ku70 with Bax by acetylating the former, releasing pro-apoptotic Bax and triggering apoptosis via the mitochondrial pathway (C. S. Chen et al., 2007). In colorectal cancer cells, acetylation of Ku70 by HDACIs disrupts the FLIP/Ku70 complex and triggers FLIP polyubiquitination and degradation by the proteasome, thus de-repressing the death receptor-mediated pathway (Kerr et al., 2012). Recently, HDAC1 and HDAC2 were shown to be rapidly recruited to sites of DSBs in osteosarcoma cells and to be responsible for deacetylation of the histone mark H3K56 (Miller et al., 2010). HDAC1- and 2-depleted cells were hypersensitive to DNA-damaging agents and showed sustained DNA-damage signaling and defective DSB repair, particularly by NHEJ (Miller et al., 2010). It was proposed that local condensation of chromatin as a consequence of histone deacetylation might be necessary for proper DSB repair through inhibition of transcription and prevention of Ku70 sliding off naked DNA (Miller et al., 2010). H3K56 acetylation has recently been shown to interfere with Ku localization at retrotransposons, leading to dissociation of condensin from retro-transposons, releasing condensin-mediated genomic associations during S-phase and upon DNA damage (A. Tanaka et al., 2012). ATR mediates the DDR of condensin-mediated genome organization (A. Tanaka et al., 2012). HDAC3 has also recently been shown to be essential for efficient DNA replication and repair, and for the maintenance of chromatin structure and genome stability (Bhaskara et al., 2008; Bhaskara et al., 2010). HDAC3 deacetylates histone marks H3K9, H3K14, H4K5 and H4K12 during late S-phase, and deletion of HDAC3 in cycling mouse embryonic fibroblasts (MEFs) led to a delay in cell-cycle progression, cell-cycle-dependent DNA damage, and apoptosis, which appeared to be associated with defective DNA DSB repair (Bhaskara et al., 2008; Bhaskara et al., 2010). HDAC9 and HDAC10 appear to participate in HR repair, although the precise mechanisms remain elusive (Kotian, Liyanarachchi, Zelent, & Parvin, 2011). The sirtuin SIRT6 participates in promoting DNA end resection, a crucial step in HR repair, the primary repair mechanism in S-phase (Branzei & Foiani, 2008), by deacetylating the DSB resection protein CtIP (Kaidi, Weinert, Choudhary, & Jackson, 2010). HDACIs promote acetylation of CtIP (Langerak, Mejia-Ramirez, Limbo, & Russell, 2011), triggering nuclear export and autophagic degradation (T. Robert et al., 2011). Adding further to the complexity of the mechanisms by which pharmacologic inhibition of HDACs might impact DNA repair processes in cancer cells is the fact that several HATs (e.g., HIV-1, TIP60, MOF) play critical roles in DNA repair (Sharma et al., 2010; Sun et al., 2009), acetylating histones and relaxing chromatin, thus improving accessibility of DNA repair proteins to DSB sites (Murr et al., 2006).

V. HDACIs and apoptotic pathways

Cancer cells invariably have abnormalities in one or more apoptotic pathways, determining a survival advantage for these cells over their normal counterparts (Hanahan & Weinberg, 2011). Furthermore, abnormalities in the apoptotic response also play a major role in the development of drug resistance by malignant cells (Davids & Letai, 2012). Most, if not all, anti-cancer drugs kill malignant cells through induction of apoptosis, and most tumor cells do retain their apoptotic machinery (Davids & Letai, 2012). Defects in apoptotic pathways thus represent an ideal target for therapeutic intervention.

At a molecular level, apoptosis is caused by the activation of caspases (Irvine, McMullin, & Ong, 2002; Schimmer, 2007; Testa & Riccioni, 2007). The *extrinsic*, or death receptor pathway is initiated by engagement of death receptors of the TNF (tumor necrosis factor) superfamily on the cell membrane by their respective ligands, such as Fas ligand (FasL), TNF or TRAIL (TNF-related apoptosis inducing ligand). c-FLIP is an endogenous inhibitor of this pathway. The *intrinsic*, or mitochondrial pathway of apoptosis is triggered by various cellular stresses, such as growth factor deprivation, DNA or microtubule damage, hypoxia, etc. and is the mechanism by which apoptosis is induced by cytotoxic chemotherapy. The seminal event in this pathway is mitochondrial outer membrane polarization (MOMP), which is controlled by the B-cell lymphoma 2 (Bcl-2) family of proteins and results from the formation of pores in the mitochondrial outer membrane (Davids & Letai, 2012). The inhibitors of apoptosis (IAPs) are cytoplasmic proteins that inhibit the action of caspases. The most important member of this family, XIAP, potently inhibits active caspases 9, 3 and 7, thus blocking apoptosis triggered by multiple caspase activation pathways (Datta et al., 2000).

The anti-apoptotic Bcl-2 family members are Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Bfl-1/A1 and Bcl-B. The “multi-domain” pro-apoptotic members are Bax, Bak and Bok (Labi, Grespi, Baumgartner, & Villunger, 2008). The other pro-apoptotic members, the so-called “BH3-only” proteins, include Bim, Bid, Bad, Bik, Hrk, Bmf, Puma and Noxa (Davids & Letai, 2012). The multi-domain proteins Bax and Bak (apoptosis “effectors”) are absolutely essential for the pro-apoptotic function of the BH3-only proteins (Labi et al., 2008).

Individual BH3-only proteins differ markedly in their ability to bind to different anti-apoptotic Bcl-2 relatives. For example, Bad and Bmf bind strongly with Bcl-2, Bcl-X_L, and Bcl-w, and Noxa binds only with high affinity to Mcl-1 and Bfl-1/A1. Thus, BH3-only proteins with different selective binding patterns (e.g., Bad plus Noxa) synergize in apoptosis induction, an important finding from co-transfection assays of great translational relevance (Kuroda & Taniwaki, 2009).

A link between the extrinsic and intrinsic pathways is provided by the cleavage of the proapoptotic Bcl-2 family “BH3-only” protein Bid by caspase 8 to produce a truncated molecule, tBid, that is able to directly activate the multi-domain apoptosis effectors Bax and Bak. Mitochondrial translocation of Bax (normally a cytosolic protein) and oligomerization of Bax and Bak lead to MOMP (Ott, Norberg, Zhivotovsky, & Orrenius, 2009).

HDACIs interact at multiple levels with both the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways of apoptosis, up-regulating pro-apoptotic and down-regulating anti-apoptotic proteins (Grant & Dai, 2012). The transcriptional signature of vorinostat in MM reveals down-regulation of caspase inhibitors (C. S. Mitsiades et al., 2004). HDACIs have been shown to down-regulate Bcl-xL, Mcl-1 and XIAP in both solid tumor (Gillespie, Borrow, Zhang, & Hersey, 2006) and malignant hematopoietic cells (Jona et al., 2011; Rosato et al., 2006; Rosato et al., 2007). Down-regulation of survivin and XIAP by HDACIs has been demonstrated in human glioma cells to be mediated through inhibition of cdc2 (CDK1) (E. H. Kim et al., 2005). Reversal of epigenetic silencing of critical microRNAs by HDACIs, leading to activation of the latter, may underlie the ability of HDACIs to down-regulate Mcl-1 (Saito et al., 2009; Sampath et al., 2012). In chronic lymphocytic leukemia (CLL) cells, romidepsin activates the extrinsic pathway of apoptosis via down-regulation of c-FLIP (Aron et al., 2003). E2F1-dependent up-regulation of the pro-apoptotic BH3-only protein Bim via induction of ASK1 (apoptosis signal-regulating kinase 1) is a major mediator of apoptosis in response to HDACIs (Tan et al., 2006; Zhao et al., 2005). Bim up-regulation by HDACIs plays a critical role in sensitizing human leukemia and myeloma cells to the BH3-mimetic ABT-737 (S. Chen, Dai, Pei, & Grant et al., 2009b). Reversal of epigenetic silencing of Bim by HDACIs may overcome resistance to glucocorticoids in pediatric acute lymphoblastic leukemia (ALL) (Bachmann et al., 2010) and to chemotherapy in BL (Richter-Larrea et al., 2010). Bim up-regulation by HDACIs is also implicated in synergistic interactions between this class of agents and proteasome inhibitors (PIs) (Dai, Chen, Kramer et al., 2008; Dai, Chen, Wang, Pei, Kramer et al., 2011). Recently, MAPK (mitogen activated protein kinase) pathway activation was identified as a key mechanism of resistance to romidepsin in cutaneous T-cell lymphoma (CTCL) cells, and the addition of MEK (MAPK kinase) inhibitors synergistically induced apoptosis in these resistant cells via restoration of Bim (Chakraborty et al., 2013). Besides Bim, a key role for induction of other pro-apoptotic BH3-only proteins such as Bmf and Noxa in HDACI-induced apoptosis and synergism with ABT-737 has been proposed (Inoue et al., 2007; Wiegmans et al., 2011). Up-regulation of Noxa by HDACIs may be due to acetylation of p53 (Terui et al., 2003). In mantle cell lymphoma (MCL) cells, vorinostat has been shown to hyperacetylate the promoter regions of *Bim*, *Bmf* and *Noxa*, thereby activating their transcription, and to synergize with ABT-263 (navitoclax) (C. Tse et al., 2008), the orally available analog of ABT-737 (Xargay-Torrent et al., 2011). HDACIs induce expression of TRAIL in human AML cells and activate the death receptor pathway of apoptosis (Insinga et al., 2005; Nebbioso et al., 2005). Synergism between HDACIs and TRAIL has been reported in preclinical studies in a number of hematologic (Al-Yacoub et al., 2012; Guo et al., 2004; Inoue et al., 2004) and solid organ malignancies (Bangert et al., 2012; Kauh et al., 2010; E. H. Kim et al., 2005), in some cases involving down-regulation of c-FLIP (Al-Yacoub et al., 2012; Bangert et al., 2012; Kauh et al., 2010). Finally, Bid cleavage and activation appears to be an important mechanism of HDACI lethality in malignant cells (Gillenwater, Zhong, & Lotan, 2007; Lindemann et al., 2007; Ruefli et al., 2001). In summary, HDACIs promote apoptosis induction via both the mitochondrial (intrinsic) and death receptor (extrinsic) pathways, down-regulating key anti-apoptotic proteins such as Mcl-1, Bcl-xL, XIAP and c-FLIP, up-regulating critical pro-apoptotic proteins, e.g., Bim,

Bmf and Noxa, and facilitating cross-talk between the two pathways through cleavage and activation of Bid.

VI. Rational combinations involving HDACIs and other novel targeted agents involving disruption of the DDR

Given the ability of HDACIs to induce DNA damage and the many ways in which these agents impair DNA repair mechanisms, it is not surprising that these drugs potentiate the effects of DNA-damaging chemotherapy (C. S. Chen et al., 2007; Rosato et al., 2008), in some cases in a sequence-dependent fashion (Marchion et al., 2004). Particularly in the case of topoisomerase II inhibitors, synergism may require pre-treatment with HDACIs (Marchion et al., 2004; Shiozawa et al., 2009). Some of these observations (Sanchez-Gonzalez et al., 2006; Shiozawa et al., 2009) have led to clinical trials combining HDACIs with genotoxic agents in AML and high-risk MDS (Garcia-Manero et al., 2012), and encouraging results from such single-institution studies have served as the basis of large, cooperative group-led phase III studies that are currently ongoing (e.g., NCT01802333). While such approaches undoubtedly hold promise and continue to be explored, the remainder of this review will focus on the potential for mechanism-based combinations of HDACIs involving novel, targeted molecules that disrupt the DDR or promote apoptosis via antagonism of anti-apoptotic protein function.

The frequency of DNA repair defects and cell cycle checkpoint dysfunction in cancer cells makes targeting the DDR a particularly attractive anti-cancer strategy, often in concert with agents capable of inducing DNA damage (Bouwman & Jonkers, 2012). For example, certain genetically defined subsets of AML, e.g., those harboring *fms*-like tyrosine kinase-internal tandem duplication (FLT3-ITD) mutations (approximately 25% of cases of AML) are characterized by defective NHEJ DNA repair pathways (Fan, Li, Small, & Rassool, 2010; L. Li et al., 2011). Similarly, certain leukemia-associated fusion proteins, such as promyelocytic leukemia zinc finger-retinoic acid receptor alpha (PLZF-RARα), promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) and runt related transcription factor 1-eight twenty one (RUNX1-ETO, AML1-ETO) confer a DNA repair-deficient phenotype, and expression of these fusion proteins increases sensitivity of AML cells to vorinostat-induced DNA damage and apoptosis (Petruccelli et al., 2013). Thus far, most efforts to develop G2/M checkpoint abrogators such as Chk1 inhibitors therapeutically have focused on combination strategies involving genotoxic agents, but clinical efficacy has been modest (Karp et al., 2012). Since HDACIs down-regulate Chk1 and Wee1, induce DNA damage and inhibit DNA repair pathways by multiple mechanisms (see above discussion), there is a strong theoretical rationale for combining these agents with G2/M checkpoint abrogators. Indeed, the novel Chk1 inhibitor MK-8776 synergizes with vorinostat in inducing apoptosis of AML cell lines and primary AML blasts, particularly the putative leukemia-initiating cell (LIC) population ($CD34^+CD38^-CD123^+$) while relatively sparing normal cord blood $CD34^+$ cells, accompanied by interference with the intra-S checkpoint, disruption of DNA replication, down-regulation of proteins involved in DNA replication (e.g., chromatin licensing and DNA replication factor 1 (CDT1)) and repair (e.g., CtIP and BRCA1) and sharp increases in DNA damage (Dai et al., 2013). The MK-8776/

vorinostat combination was effective against AML cells with intact or deficient p53 function, as well as those bearing the FLT3-ITD mutation (Dai et al., 2013). Similar results were obtained using the Wee1 inhibitor MK-1775 in combination with vorinostat and validated in a mouse xenograft model (Zhang, Y, et al. Proc Am Assoc Can Res (Abstr), **55:** 4556, 2014). The MK-1775/vorinostat combination was particularly active against FLT3-ITD AML, and demonstrated activity regardless of p53 mutational status (Zhang, Y, et al. Proc Am Assoc Can Res (Abstr), **55:** 4556, 2014). Indeed, genomic instability, with increased ROS production, DNA DSBs and defective repair may be a hallmark of myeloid malignancies (Sallmyr, Fan, & Rassool, 2008).

Disruption by HDACIs of mitotic spindle checkpoints in neoplastic cells (Qiu et al., 2000) and their down-regulation of oncogenic Hsp90 client proteins (Bali, Pranpat, Bradner, Balasis, Fiskus, Guo, Rocha, Kumaraswamy, Boyapalle, Atadja, Seto, & Bhalla, 2005a) such as Raf, Akt, FLT3 and Bcr-Abl (breakpoint cluster region-Abelson) underlie the ability of these agents to potentiate the actions of small-molecule inhibitors of aurora (Dai, Chen, Venditti et al., 2008; Fiskus et al., 2008; Nguyen, Dai, Attkisson, Kramer, Jordan, Nguyen, Kolluri, Muschen, & Grant, 2011a), FLT3 (Bali et al., 2004) and Bcr-Abl (Fiskus, Pranpat, Balasis et al., 2006; Fiskus, Pranpat, Bali et al., 2006; Nimmanapalli, Fuino, Stobaugh, Richon, & Bhalla, 2003; C. Yu, Rahmani, Almenara et al., 2003) kinases. Additionally, HDACIs induce “mitotic slippage” (Stevens, Beamish, Warrener, & Gabrielli, 2008a) and synergize with Janus-associated kinase (JAK)2 inhibitors (Novotny-Diermayr et al., 2012; Y. Wang et al., 2009). The aurora kinases, serine-threonine protein kinases that are overexpressed in a variety of cancer types, regulate various processes including chromosome alignment, segregation, centrosomal maturation, mitotic spindle formation, and cytokinesis during mitosis (Kelly et al., 2011). Their fundamental role in cell cycle regulation and aberrant expression in a broad range of malignancies has prompted the development of a number of small-molecule inhibitors (Kelly et al., 2011). HDACIs have been shown to enhance the lethality of dual aurora/Bcr-Abl tyrosine kinase inhibitors (TKIs) such as MK-0457 (VX-680) (Dai, Chen, Venditti et al., 2008; Fiskus et al., 2008) and KW-2449 (Nguyen, Dai, Attkisson, Kramer, Jordan, Nguyen, Kolluri, Muschen, & Grant, 2011a) against Bcr-Abl$^+$ cells, both sensitive and resistant to imatinib, including those bearing the T315 “gatekeeper” mutation. The latter two multi-targeted TKIs are no longer in clinical development. However, synergistic anti-leukemic interactions between AT9283, a small-molecule inhibitor of aurora, Bcr-Abl, FLT3 and Janus kinases (Dawson et al., 2010; Santo et al., 2011; R. Tanaka et al., 2010) and entinostat have been observed in Bcr-Abl$^+$ (CML and Philadelphia chromosome-positive ALL) cells, including those bearing T315I, as well as in AML cells, both FLT3-mutated and –wild type (Nguyen and Grant, unpublished observations).

Polo-like kinase 1 (PLK-1) is a conserved serine-threonine kinase involved in mitotic progression through interactions with cyclin B, the CDC25C phosphatase, and Wee1 (Archambault & Glover, 2009). It has been implicated in progression into M-phase, mitotic spindle formation, cytokinesis, and chromosome segregation (Lapenna & Giordano, 2009). PLK-1 plays an important role in the DDR (Smits et al., 2000), including recovery from the G2/M DNA damage checkpoint (Takaki, Trenz, Costanzo, & Petronczki, 2008). Moreover,

interactions between PLK-1 and multiple checkpoint proteins, including Chk1/2, p53, claspin, and FoxM1 (forkhead box protein M1), have been described (X. S. Liu, Song, & Liu, 2010). Co-treatment of imatinib-sensitive and –resistant Bcr-Abl$^+$ cells with the PLK-1 inhibitor BI2536 and vorinostat synergistically induced cell death with minimal toxicity towards normal cells (Dasmahapatra, Patel, Nguyen, Attkisson, & Grant, 2013). The combination triggered pronounced mitochondrial dysfunction, inhibition of phospho-BCR/ABL, caspase activation, PARP (poly ADP ribose polymerase) cleavage, ROS generation and DNA damage, and was dramatically effective in a xenograft model of Bcr-Abl$^+$ leukemia (Dasmahapatra et al., 2013). Although Bcr-Abl signals downstream to PLK-1 (Gleixner et al., 2010), enhanced Bcr-Abl pathway inhibition was not felt to be the sole mechanism for lethality of the BI2536/vorinostat regimen; rather, potentiation of DNA damage and disabling of the DDR appeared to predominate (Dasmahapatra et al., 2013). Potentiation by vorinostat of BI2536-mediated inhibition of PLK-1 phosphorylation and enhanced ROS generation were noted, and abrogation of G0/G1 arrest with striking accumulation of cells in G2/M phase was observed (Dasmahapatra et al., 2013). The strategy of combined PLK and HDAC inhibition also affords considerable promise in DLBCL and in AML, in which PLK-1 is highly expressed (L. Liu, Zhang, & Zou, 2007; Renner et al., 2009), at least in preclinical studies (Dasmahapatra and Grant, unpublished observations). Significantly, the novel PLK inhibitor volasertib was recently granted “breakthrough” status (Three more drugs judged “breakthroughs”.2013) by the US FDA (Food and Drug Administration) based on encouraging efficacy in AML (Maertens et al., 2012). Synergism between PLK inhibitors and HDACIs has also been demonstrated *in vitro* against prostate cancer cells (Wissing et al., 2013); however, there are no ongoing clinical trials investigating this combination. Based on striking synergism observed in preclinical studies (Dasmahapatra and Grant, unpublished observations), phase I trials combining volasertib with HDACIs are planned in patients with relapsed/refractory NHL and myeloid malignancies.

The combination of PIs with HDACIs remains an attractive one for several reasons, and has been extensively studied. A central theme underlying synergism between these classes of agents is the activation of the NF-κB survival pathway by HDACIs, and its inhibition by PIs, which prevent degradation of IκB (I kappa B), the negative regulator of NF-κB. The NF-κB pathway mediates a cytoprotective response strongly implicated in cancer development and progression (reviewed in (Karin, 2006)) that is activated by HDACIs and limits their lethality (Dai et al., 2005; Dai, Chen, Wang, Pei, Funk et al., 2011; Rosato et al., 2010). Deacetylation of the RelA subunit of NF-κB by HDAC3 promotes its association with IκBα, the physiologic inhibitor of NF-κB, thus acting as an intra-nuclear molecular switch that controls the duration of the NF-κB transcriptional response (L. Chen, Fischle, Verdin, & Greene, 2001). The activation of NF-κB by HDACIs has served as the basis of many synergistic strategies combining these agents with those that inhibit NF-κB by various mechanisms, such as the CDK inhibitor flavopiridol (alvocidib) (Gao, Dai, Rahmani, Dent, & Grant, 2004; Rosato et al., 2007), the IKK (I kappa B kinase) inhibitor parthenolide (Dai et al., 2010), the SIRT1 (sirtuin 1) agonist resveratrol (Yaseen et al., 2012) and PIs (Dai, Chen, Kramer et al., 2008; Dai, Chen, Wang, Pei, Kramer et al., 2011; Dasmahapatra et al., 2010; Dasmahapatra et al., 2011; Pei, Dai, & Grant, 2004; C. Yu, Rahmani, Conrad et al.,

2003). A number of these have been explored in clinical trials (Badros et al., 2009; B. Holkova et al., 2013; B. Holkova et al., 2011; B. Holkova et al., 2012; Kmieciak et al., 2013). Attention has also focused on the formation of aggresomes in response to proteasome inhibition, and the inhibition of aggresome function by HDACIs, at least by those exhibiting tubulin acetylase activity (Catley et al., 2006; Hideshima et al., 2005; Nawrocki et al., 2006), leading to enhanced proteotoxic stress and apoptosis; a related HDACI effect is the disruption of chaperone protein function, further adding to the cellular burden of mis-folded proteins and leading to ER stress and the unfolded protein response (reviewed in (Hideshima, Richardson, & Anderson, 2011)). However, PIs lead to ROS generation (Ling, Liebes, Zou, & Perez-Soler, 2003; Perez-Galan et al., 2006; C. Yu, Rahmani, Dent, & Grant, 2004) in multiple tumor types and consequently to DNA damage, and may also inhibit DNA repair (Vaziri et al., 2005). Specifically, bortezomib has been demonstrated to sensitize cells to apoptosis induced by DNA-damaging chemotherapy in a p53- and sequence-dependent manner involving down-regulation of 14-3-3 σ and survivin, both of which play critical roles in regulating G2/M progression and apoptosis (Vaziri et al., 2005). With respect to clinical development, the PI/HDACI combination strategy has advanced the furthest (phase III) in MM (Richardson, Schlossman et al., 2013), and multiple phase I and II trials are investigating this combination in a variety of hematologic malignancies and solid tumors (NCT00798720, NCT01276717, NCT00901147, NCT00667082). Promising results with the combination of panobinostat, bortezomib and dexamethasone in heavily pretreated patients with MM participating in the PANORAMA (Panobinostat or Placebo With Bortezomib and Dexamethasone in Patients With Relapsed Multiple Myeloma) trials, including those with bortezomib-refractory disease (Richardson, Schlossman et al., 2013), raise the possibility that panobinostat may gain regulatory approval for use in this patient population.

Although recognized for some time, synergism between HDACIs and BH3-mimetics provides a promising avenue of investigation that remains unexplored therapeutically. HDACIs up-regulate Bim, which is largely sequestered by Bcl-2 and Bcl-xL, and can be released from such binding by the *bona fide* BH3-mimetic ABT-737 (Oltersdorf et al., 2005), triggering Bax/Bak activation and MOMP, leading to apoptosis (S. Chen, Dai, Pei, & Grant et al., 2009b). Besides Bim, HDACIs release pro-apoptotic Bax from its binding with Ku70, and induce Bmf and Noxa. HDACIs also down-regulate Mcl-1 (Gillespie et al., 2006; Rosato et al., 2006; Rosato et al., 2007; Saito et al., 2009; Sampath et al., 2012), the primary determinant of resistance to ABT-737 (Konopleva et al., 2006; van Delft et al., 2006). Synergistic induction of apoptosis and autophagy has been observed in leukemia cell lines and primary AML cells exposed to the combination of HDACIs and the BH3-mimetic obatoclax (Wei et al., 2010). Both ABT-737 and obatoclax have also been shown to synergistically enhance entinostat-induced apoptosis in Hodgkin's lymphoma cell lines (Jona et al., 2011). In MCL cell lines and primary cells, vorinostat activated transcription (through promoter hyperacetylation) of *Bim*, *Bmf* and *Noxa*, and induced apoptosis with navitoclax in synergistic fashion (Xargay-Torrent et al., 2011). However, the development of both obatoclax and navitoclax has been discontinued due to the occurrence of infusional central nervous system toxicity and dose-dependent thrombocytopenia, respectively, despite promising efficacy of navitoclax in CLL (Roberts et al., 2012). ABT-199 (GDC-0199) is an orally available Bcl-2-selective BH3-mimetic developed by reverse engineering of

navitoclax that exhibits potent anti-tumor activity while sparing platelets (due to lack of Bcl-xL inhibition) (Souers et al., 2013). Although it has been suggested that Bak must be released from both Mcl-1 and Bcl-xL, but not Bcl-2, in order to induce apoptosis (Willis et al., 2005), this drug has been highly effective in a large number of tumor types preclinically (Pan et al., 2014; Touzeau et al., 2014; Vaillant et al., 2013; Vandenberg & Cory, 2013; Vogler, Dinsdale, Dyer, & Cohen, 2013) and in patients with CLL or small lymphocytic lymphoma (BCL-2 inhibitor yields high response in CLL and SLL.2014). Information is not currently available on the results of combining this agent with HDACIs. Similarly, published information is lacking on combination studies of HDACIs and XIAP antagonists (Smacmimetics), another rational combination based on the ability of HDACIs to down-regulate survivin and XIAP.

A potentially interesting combination is that of HDACIs with MLN4924 (Soucy et al., 2009), a first-in-class inhibitor of protein neddylation, a critical pathway of protein degradation located upstream of the proteasome (reviewed in (Rabut & Peter, 2008)). Targeting protein neddylation allows more selective interference with protein turnover than is possible with proteasome inhibition, potentially translating to a better therapeutic index for this drug compared with PIs (Nawrocki, Griffin, Kelly, & Carew, 2012; M. Wang et al., 2011). MLN4924 exhibits impressive activity in preclinical studies in DLBCL (Milhollen et al., 2010), AML (R. T. Swords et al., 2010a) and ovarian cancer (Jazaeri et al., 2013; Nawrocki et al., 2013), and has demonstrated single-agent activity in patients with AML (R. T. Swords et al., 2010b). Like PIs, MLN4924 potently inhibits the NF-κB pathway (Milhollen et al., 2010; R. T. Swords et al., 2010b), and like HDACIs, leads to ROS generation (R. T. Swords et al., 2010a). MLN4924 interferes with the turnover of the cullin-RING ligase substrate CDT1, a critical DNA replication licensing factor, thus inducing DNA re-replication, an irreversible cellular insult that results in apoptosis (Milhollen et al., 2011). This phenomenon appears to be most marked in S-phase (Lin, Milhollen, Smith, Narayanan, & Dutta, 2010). Besides down-regulating checkpoint proteins activated in response to DNA damage, HDACIs inhibit both HR repair and NHEJ (Koprinarova, Botev, & Russev, 2011). Finally, MLN4924 triggers a cytoprotective autophagic response (Z. Luo et al., 2012; Z. Luo, Pan, Jeong, Liu, & Jia, 2012) that may be counteracted by HDACIs (T. Robert et al., 2011; Shubassi et al., 2012).

While each of the combinatorial approaches involving HDACIs and other novel, targeted agents discussed above is supported by strong theoretical rationale and, in most cases, clear demonstration of synergism at the preclinical level, not all are equally likely to be developed clinically. For example, the multi-targeted TKIs MK0457 (VX-680) and KW-2449 are no longer in development, and the future development of AT9283 is unclear. Similarly, a number of Chk1/Chk2 inhibitors have been discontinued or deprioritized by the respective manufacturers (e.g., AZD7762, PF-00477736, XL844, MK-8776), although there are others in early phases of testing (e.g., LY2603618, LY2606368, GDC-0425, reviewed in (Thompson & Eastman, 2013)). However, the recent licensing by Merck of its Wee1 inhibitor MK-1775 to Astra Zeneca has raises the possibility of a clinical trial involving this agent (now AZD-1775) in combination with an HDACI. The combination of PIs with HDACIs has been extensively tested, both preclinically and clinically, and it is conceivable that the combination of panobinostat, bortezomib and dexamethasone will be granted

approval by the FDA for use in patients with relapsed/refractory MM. On theoretical grounds, the combination of MLN4924 and an HDACI appears particularly appealing, and AML might be the most logical choice for clinical investigation of this combination given the promising single agent activity of MLN4924 in this disease (R. T. Swords et al., 2010a; R. T. Swords et al., 2010b). Finally, the FDA's granting of “breakthrough” status to volasertib for AML (Three more drugs judged “breakthroughs”.2013) and the evidence of clinical activity of ABT-199 (GDC-0199) in patients with resistant lymphoid malignancies (BCL-2 inhibitor yields high response in CLL and SLL.2014) raise the possibility of regulatory approval for these agents, increasing the chances that combination trials with HDACIs will be pursued, given the compelling preclinical data with these classes of agents (S. Chen, Dai, Pei, & Grant, 2009a; Dasmahapatra et al., 2013; Wei et al., 2010; Wissing et al., 2013).

VII. Conclusions

It has become abundantly clear in recent years that HDACIs are unlikely to make a substantial impact in the clinic when used alone, and that the future of this very promising class of agents lies in rational combination therapy. A very large number of rational, HDACI-based combinations is possible, and new ones continue to emerge as the pleiotropic mechanisms of action of these drugs are progressively elucidated. In the therapeutic arena, the HDACI-based combinations that have advanced the farthest include those with cytotoxic chemotherapy (Garcia-Manero et al., 2012; Gojo et al., 2013), hypomethylating agents (Fandy et al., 2009; Juergens et al., 2011; Stathis et al., 2011)and PIs (Badros et al., 2009; Richardson, Schlossman et al., 2013).

However, although many trials have been completed (e.g., NCT00667082, NCT01105377, NCT00895934) or are ongoing (e.g., NCT01802333, NCT01305499, NCT00948064), none of these combinations has met with regulatory approval yet or been widely adopted in clinical practice. It follows from the above discussion that many other exciting avenues for rational combination exist, e.g., with agents that interfere with cell cycle checkpoints, mitosis, proteasome activity, protein neddylation and the function of anti-apoptotic proteins. Hopefully, the theoretical potential of these combinations and encouraging preclinical data will successfully be translated into novel therapies for patients with cancer in the near future.

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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APAF-1	apoptotic protease activating factor 1
ASK1	apoptosis signal-regulating kinase 1
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
Bcl-2	B-cell lymphoma-2
Bcl-xL	Bcl-extra long
Bcr-Abl	breakpoint cluster region-Abelson
Bad	Bcl-2 antagonist of cell death
BER	base excision repair
Bim	Bcl-2 interacting mediator of cell death
Bid	Bcl-2 interacting domain death agonist
Bik	Bcl-2 interacting killer
BLM	Bloom syndrome
Bmf	Bcl-2 modifying factor
CDC25	cell division cycle 25
CDK	cyclin-dependent kinase
CDT1	chromatin licensing and DNA replication factor 1
c-FLIP	cellular FLICE-like inhibitory protein
Chk	checkpoint kinase
CLL	chronic lymphocytic leukemia
CTCL	cutaneous T-cell lymphoma
CtIP	CtBP interacting protein

DNA	deoxyribonucleic acid
DDR	DNA damage response
DIABLO	direct IAP-binding protein with low pI
DISC	death-inducing signaling complex
DLBCL	diffuse large B-cell lymphoma
DSB	double strand break
ER	endoplasmic reticulum
ETO	eight twenty one
Fadd	Fas-associated protein with death domain
FasL	Fas ligand
FDA	Food and Drug Administration
FEN1	flap structure-specific endonuclease 1
FLT3	*fms*-like tyrosine kinase 3
FoxM1	forkhead box protein M1
GRP78	glucose regulated protein 78
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACI	histone deacetylase inhibitor
HR	homologous recombination
Hrk	harakiri
Hsp	heat shock protein
IAP	inhibitor of apoptosis
IκB	I kappa B
IKK	I kappa B kinase
ITD	internal tandem duplication
JAK	Janus-associated kinase
JNK	c-Jun N-terminal kinase
LIC	leukemia-initiating cell
MAPK	mitogen activated protein kinase
MCL	mantle cell lymphoma
Mcl-1	myeloid cell leukemia-1
MDC1	mediator of DNA damage checkpoint 1

Mdm2	murine double minute homolog 2
MDS	myelodysplastic syndromes
MEF	mouse embryonic fibroblast
MEK	mitogen activated protein kinase kinase
MM	multiple myeloma
MOMP	mitochondrial outer membrane polarization
MRE11	meiotic recombination 11
MRN	MRE11/NBS1/RAD50
NAD	nicotinamide adenine dinucleotide
NCOR1	nuclear receptor corepressor 1
NER	nucleotide excision repair
NF-κB	nuclear factor kappa B
NHEJ	nonhomologous end-joining
PANORAMA	Panobinostat or Placebo With Bortezomib and Dexamethasone in Patients With Relapsed Multiple Myeloma
PARP	poly ADP ribose polymerase
p53BP1	p53 binding protein 1
Ph	Philadelphia chromosome
PI	proteasome inhibitor
PK	protein kinase
PLK-1	polo-like kinase 1
PLZF	promyelocytic leukemia zinc finger
PML	promyelocytic leukemia
Puma	p53-upregulated modulator of apoptosis
RARα	retinoic acid receptor alpha
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RUNX1	runt related transcription factor 1
SIRT1	sirtuin 1
SMAC	second mitochondria-derived activator of caspases
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor

STAT	signal transducer and activator of transcription
Tip60	Tat interactive protein 60
TKI	tyrosine kinase inhibitor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
WRN	Werner syndrome
XIAP	X-linked inhibitor of apoptosis
XRCC	x-ray repair cross-complementing proteins

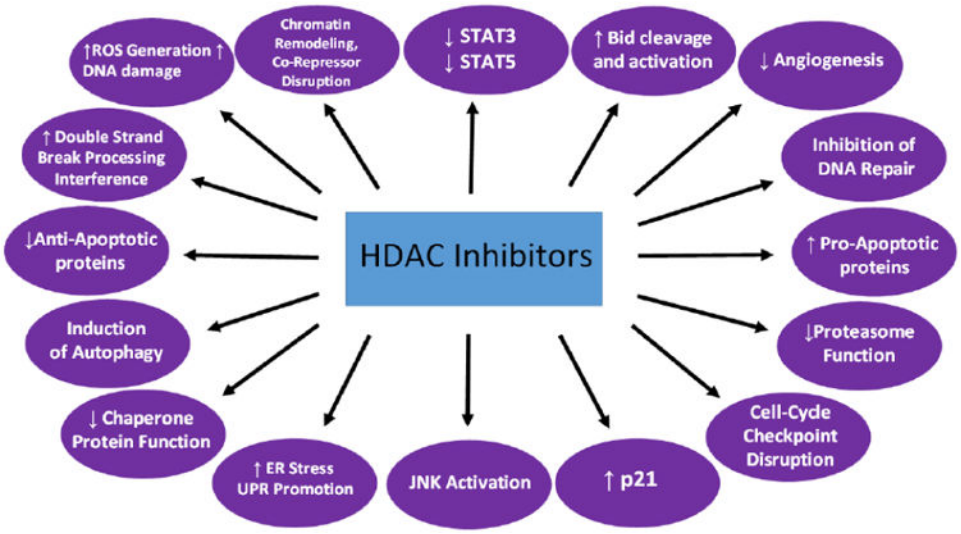

Figure 1.
Mechanisms of histone deacetylase inhibitor lethality.

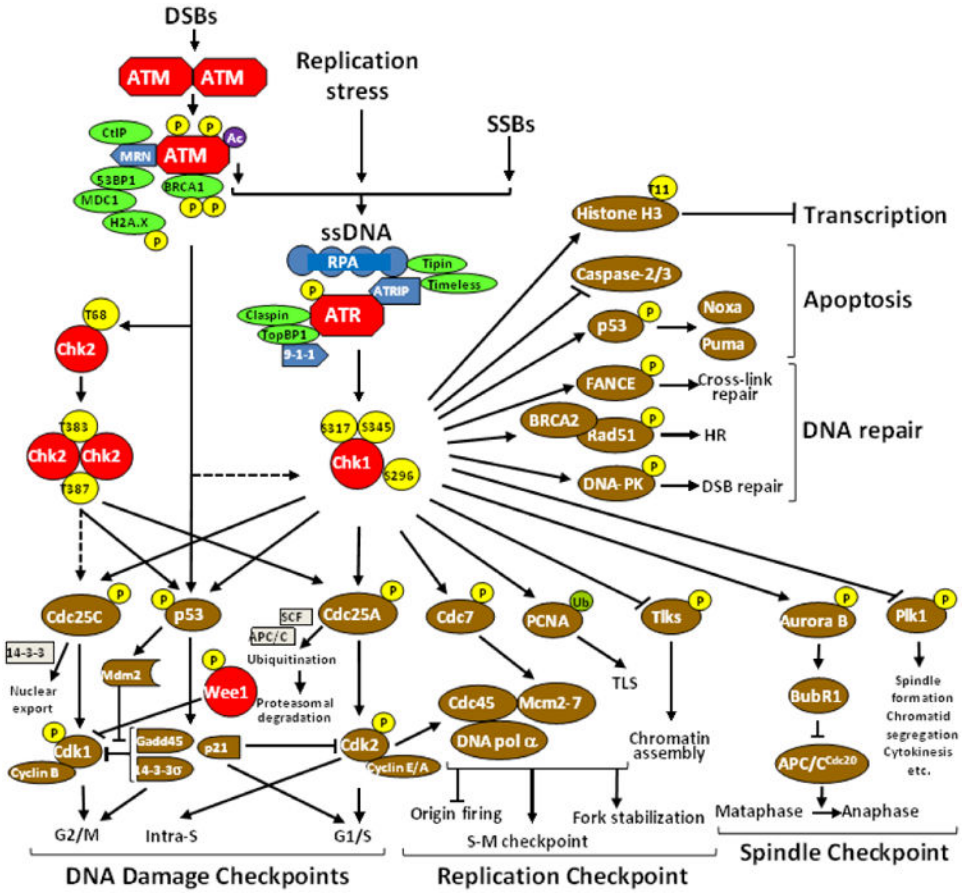

Figure 2.

The DDR signaling network. DNA damage (e.g., DSBs, SSBs, and stalled replication forks) generates ssDNA that initiates ATR-mediated Chk1 activation. In this context, the ATR/ATRIP (ATR interacting protein) complex is recruited to ssDNA lesions via binding of ATRIP with RPA that recognizes and coats ssDNA. In conjunction with recruited/activated sensors and mediators, ATR phosphorylates Chk1 at two canonical sites (Ser345 and S317), directly leading to its activation without the homodimerization and intramolecular transautophosphorylation that is required for Chk2 activation. Activated Chk1 then phosphorylates diverse downstream effectors, which in turn are involved in cell cycle checkpoints (i.e., intra-S-phase, G2/M-phase, and G1/S-phase checkpoints), the DNA replication checkpoint, and the mitotic spindle checkpoint, as well as DNA repair, apoptosis, and transcription. Modified, with permission, from (Dai & Grant, 2010).