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Clinical Cancer Research

Molecular Pathways

Molecular Pathways: Old Drugs Define New Pathways: Non-Histone Acetylation at the Crossroads of the DNA Damage Response and Autophagy

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Abstract

Histone deacetylases (HDAC) modulate acetylation and the function of histone and non-histone proteins. HDAC inhibitors have been developed to block the aberrant action of HDACs in cancer, and several are in clinical use (vorinostat, romidepsin, and valproic acid). Detailed understanding of their action is lacking, however, and their clinical activity is limited in most cases. Recently, HDACs have been involved in the control of the DNA damage response (DDR) at several levels and in directly regulating the acetylation of a number of DDR proteins (including CtIP and Exo1). Mechanistically, acetylation leads to the degradation of double-strand break repair enzymes through autophagy, providing a novel, direct link between DDR and autophagy. These observations, obtained in yeast cells, should now be translated to mammalian model systems and cancer cells to reveal whether this acetylation link is maintained in mammals, and if and how it is deregulated in cancer. In addition to HDACs, DDR and autophagy have been addressed pharmacologically, suggesting that the acetylation link, if involved in cancer, can be exploited for the design of new anticancer treatments. *Clin Cancer Res; 18(9); 2436–42. ©2012 AACR.*

Background

HDAC inhibitors and the confusing concept of epigenetic therapy

Chromatin alterations play a fundamental role in cancer onset and progression. Unlike genetic mutations, epigenetic alterations are reversible, and the quest for epigenetic therapies has resulted in the development of several compounds targeting epigenetic enzymes. To date, 4 drugs have been approved by the U.S. Food and Drug Administration (FDA) for cancer treatment (1).

Histone deacetylases (HDAC) are among the best-characterized epigenetic targets (2). Two HDAC inhibitors, vorinostat and romidepsin, are now approved by the FDA for the treatment of cutaneous T-cell lymphoma, and another old drug, valproic acid (VPA), used in the treatment of epilepsy, has been recognized to act as a weak HDAC inhibitor (1, 2). Clinically, however, the use of HDAC inhibitors as a monotherapy led to much more modest antitumor activity compared with the preclinical results, suggesting that combination with other agents may be required to achieve relevant clinical effects (3, 4). Treatment with HDAC inhibitors is not devoid of side effects. Toxicity has been ascribed to the lack of selectivity in most existing HDAC inhibitors, which aspecifically target the 11 human HDACs. Although romidepsin displays some selectivity, it does not have decreased side effects (5).

Thus, epigenetic therapy, at least in the case of HDACs, is in trouble. One of the key explanations for this partial failure is the fact that HDAC inhibitors not only modulate gene expression by blocking the removal of acetyl lysine from histones (resulting in reactivation of aberrantly suppressed genes) but also block the action of HDACs on several other substrates; indeed, the acetylation of non-histone proteins has recently been shown to contribute to the regulation of most cellular functions and has been suggested to influence the susceptibility of the proteins to other posttranslational modifications (6). Focusing on the transcriptional, that is epigenetic, effects of HDAC inhibitors may be strategically wrong, and their non-epigenetic effects may be as relevant or more relevant than the epigenetic ones; HDAC inhibitor–based (epigenetic) therapy therefore needs to be revisited.

HDAC inhibitors and the DNA damage response

The term "DNA damage response" (DDR) refers to the sophisticated cellular networks that cells have evolved to sense, recognize, and repair, in a cell-cycle–dependent manner, different types of DNA damage, caused by endogenous and exogenous stressors. The phosphoinositide 3-kinases ATM and ATR are master regulators of DDR in

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mammals. ATM is activated by double-strand breaks (DSB), whereas ATR senses replication protein A (RPA)–coated single-stranded DNA (ssDNA). Both sensors relay the signal to CHK1 and CHK2, 2 protein kinases that activate downstream substrates and orchestrate the cellular events leading to cell-cycle arrest and DNA repair. If the repair is effective, DDR is inactivated, enabling the recovery of the cell functions. If the damage cannot be repaired, chronic DDR activation promotes cell death (7).

DSBs are among the most harmful lesions that cells can experience. Their presence can trigger genome rearrangements and the loss of genetic information at the break site. To repair DSBs, cells use 2 major pathways: nonhomologous end joining (NHEJ) and homologous recombination. NHEJ operates throughout the cell cycle; it is initiated by the loading of the Ku70/Ku80 complex onto the free DNA ends and requires minimal processing of the DNA ends. Homologous recombination is limited to S and G_2 phases of the cell cycle and requires extensive DNA-end resection by the MRN complex in conjunction with auxiliary factors, including CtIP and Exo1, to create stretches of ssDNA that are first coated by RPA, which then is exchanged with RAD51 to complete the homologous recombination process (8).

Deficiency in 1 or more components of the DDR cascade leads to genome instability and cancer predisposition (9). Inherited cancers exemplify this concept, because they frequently arise from mutations of genes involved in DDR (e.g., BRCA1/2 in breast and ovarian cancers). Defects in DDR, however, weaken the ability of cancer cells to repair therapy-induced DNA lesions. Indeed, cancer cells are more sensitive to DNA-damaging agents than most normal cells, and several anticancer drugs work by generating DNA damage. To an extreme, DDR defects can be further enhanced by drugs inhibiting the DDR pathways still functioning in cancer cells; this synthetic lethality approach has been validated preclinically and clinically through the use of PARP inhibitors to weaken DDR in cancer cells carrying BRCA1/2 mutations to levels that are not compatible with survival (10).

HDACs modulate the DDR cascade at different levels (Fig. 1A). Interestingly, treatment with HDAC inhibitors does not generate DSBs *per se*, but activates DDR (11). One potential explanation for this observation is that chromatin relaxation following inhibition of HDACs may expose otherwise tightly packed portions of DNA to damage from endogenous and exogenous stressors, such as reactive oxygen species (ROS), ionizing radiation (IR), and cytotoxic drugs. In support of this hypothesis, elevated levels of ROS have been observed after treatment with HDAC inhibitors, and ROS-induced DNA damage has been shown to be one of the mechanisms through which HDAC inhibitors trigger cell death (12). Increased accessibility to DNA-damaging agents, however, is unlikely to be the only mechanism responsible for the effects of treatment with HDAC inhibitors. HDACs have also been shown to regulate the transcription rate and the protein levels of several components of the DDR cascade (13–24).

In view of these results, it is not surprising that HDAC inhibitors cooperate with DNA-damaging agents in inducing cancer cell death. Since the early observations, almost 3 decades ago, that the HDAC inhibitor sodium butyrate increases the radiosensitivity of human colon carcinoma cell lines, a large number of studies have reported a synergistic action of nontoxic concentrations of HDAC inhibitors with IR or a variety of drugs that directly or indirectly cause DSBs, such as platinum analogues, topoisomerase inhibitors, and DNA intercalators (4, 12, 25–27). These studies have been done *in vitro*, using cell lines, or *in vivo*, in murine xenografts of those same cell lines (4, 12, 26, 27).

The combination of DNA-damaging agents with HDAC inhibitors induces a higher number and prolonged duration of phosphorylated histone H2AX (γH2AX) nuclear foci, which are classical markers of DSBs (12, 17, 18, 21, 23, 26, 28–30). Vorinostat has been shown to act as a radiosensitizer of lung cancer and melanoma cells through a strong inhibitory effect on the NHEJ pathway (22, 31). In prostate and glioma cancer cells, radiosensitization by vorinostat is achieved by attenuating the expression of homologous recombination DNA repair genes (16, 18). Inhibition of the expression of radiation-induced DNA-repair proteins (RAD50, DNA-PK, Rad51, Ku80, and Ku86) contributes to vorinostat-mediated radiosensitization of pancreatic, osteosarcoma, rhabomyosarcoma, and neuroblastoma cells (14, 17, 21).

Almost invariably, the synergism or cooperating effect is, as expected, due to increased cell death, apparently through apoptosis (13, 14, 16, 17, 21–23, 29, 31). A limitation of these studies, in most cases, is that they have focused on the description of the cellular events and have investigated, to a very limited extent, the molecular mechanisms underlying the phenomenon. Thus, we have ample information on the ability of HDAC inhibitors to sensitize tumor cells to DNA-damaging treatments, but these results are not necessarily helpful in directing clinical strategies.

Autophagy in anticancer therapy

Autophagy represents an evolutionarily conserved, self-digestive process that prevents the toxic accumulation of damaged cellular organelles and proteins. These components, once sequestered into double-membrane autophagic vesicles, the autophagosomes, are delivered to the lysosomes and finally degraded by lysosomal enzymes. In this way, the resulting catabolites can be recycled for the cellular metabolic needs (32). The major regulator of autophagy is a kinase, mTOR, which inhibits autophagy (33). Downstream of mTOR, proteins encoded by the family of autophagy-related genes (ATG) are essential for the execution of autophagy (32).

Apart from its constitutive catabolic function, autophagy is rapidly upregulated in response to stress stimuli that increase the cellular requirement for energy production, such as starvation and growth factor withdrawal or damage mitigation, occurring after oxidative damage due to aging

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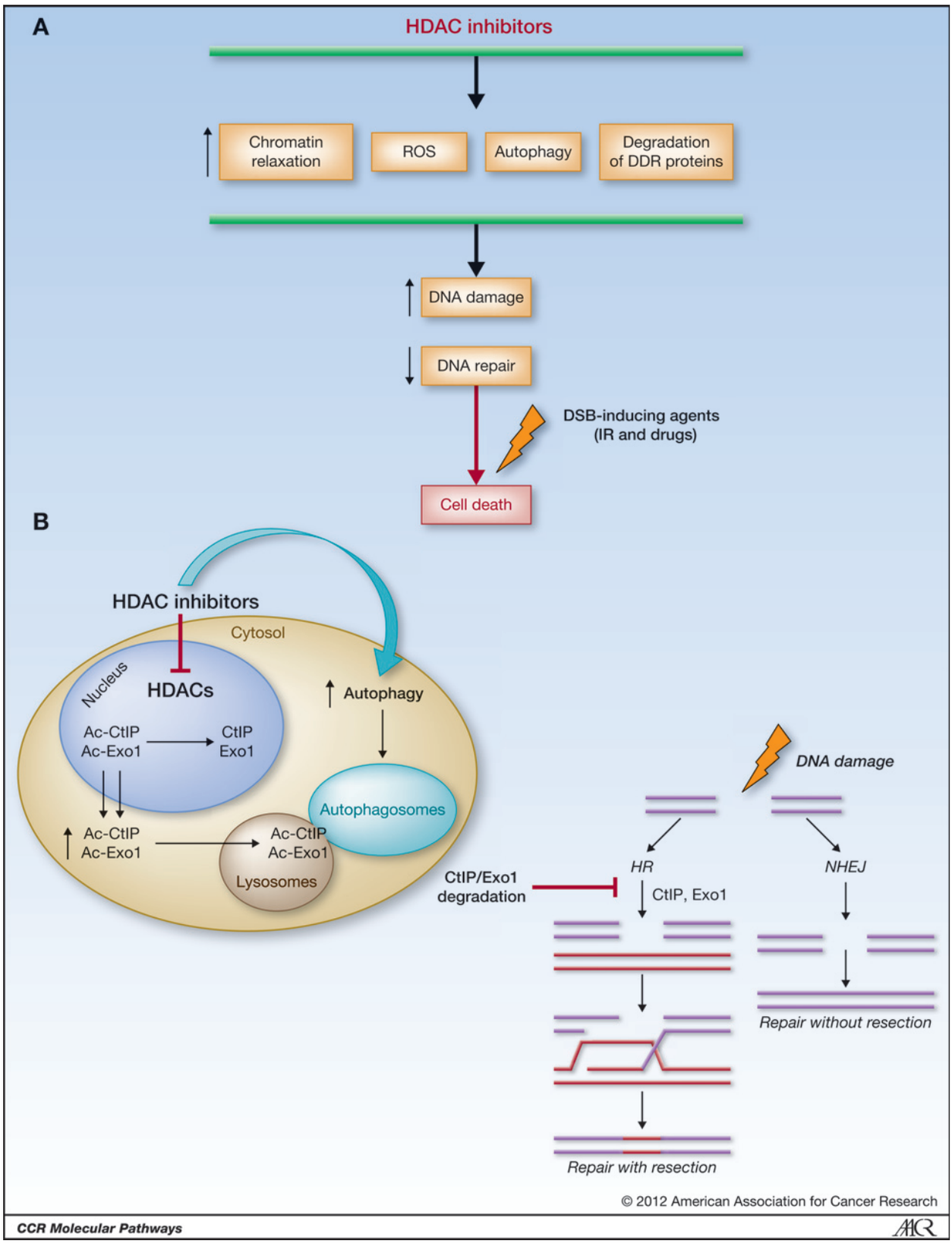

Figure 1. A, a new stranglehold on cancer cells. HDAC inhibition, through several mechanisms, leads to increased DNA damage and impaired DNA repair. Further DNA damage (from IR or drugs) results in a level of DNA damage that is not compatible with cancer cell survival. B, the acetylation link between DDR and autophagy. Acetylation (controlled by HDAC activity) affects the stability of several DDR proteins (CtIP, Sae2 in yeast, and Exo1). Treatment with HDAC inhibitors results in hyperacetylation of CtIP and Exo1, leading to their degradation through the autophagic pathway. Reduced levels of CtIP and Exo1, crucial factors for DNA-end resection, affect the homologous recombination (HR) pathway, concluding in impairment of DSB repair.

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and hypoxia (34). Activating autophagy, cells generate energy and metabolites by digesting their own organelles and macromolecules. Sustained autophagy, beyond a crucial point needed for survival, results in autophagy-associated cell death (35).

Genetic studies have claimed that autophagy can have a tumor-suppressive role, and loss of autophagy regulators has been described in several human cancers (36). It has been shown that many anticancer treatments in clinical use today, as well as various therapies that are under investigation, induce autophagy in tumor cells (37). These data and the indication that autophagy is an alternative mechanism of programmed cell death have heightened the interest in manipulating autophagy to improve cancer treatment, particularly in apoptosis-defective contexts (38). Several clinical trials modulating autophagy with FDA-approved drugs are already active (36, 39). However, contradictory results have led to disagreement about how to influence the process advantageously. Indeed, the observation that tumor cells may show an "autophagy addiction" has led to the proposal that inhibition of autophagy may impair their survival (40–42). On the other hand, autophagy may be required by cancer cells to survive and better defend themselves from several anticancer drugs, and in this case, inducers of autophagy may lead to tumor cell death (34). Intriguingly, a few reports suggest that HDAC inhibitors, including VPA, induce autophagy in mammalian cells (43, 44).

The acetylation of DNA damage response proteins links the DNA damage response with autophagy

Several DDR proteins undergo reversible acetylation that affects their activity, thus suggesting that this may represent an additional layer of regulation of DDR by treatment with HDAC inhibitors (6, 31, 45).

A combination of pharmacologic and genetic studies in yeast cells has recently provided an acetylation link between 2 previously unrelated processes: DDR and autophagy (46). VPA treatment alone had no effect on the cells, but upon exposure to different DNA-damaging agents, VPA-treated cells were not able to engage a proper DDR response and could not correctly repair DSBs. DSB resection rates were slower in VPA-treated cells due to a severe reduction in the levels of 2 nucleases essential for the resection reaction, Sae2 (CtIP in mammals) and Exo1. Two specific yeast HDACs, Hda1 and Rpd3, were required, because the VPA phenotype was recapitulated in *rpd3 hda1* double mutants. Consistent with a previous study in human cells (47), Sae2 was found acetylated in VPA-treated yeast cells, while Exo1 acetylation, found in mammalian cells, was not observed in these conditions in yeast (6). The missing link between Sae2 acetylation and its degradation is provided by autophagy. VPA stimulated autophagy in yeast, as in mammalian cells (43, 46). Genetic and chemical inactivation of autophagy stabilized Sae2 in VPA-treated and HDAC-deleted cells, whereas induction of autophagy by rapamycin (a clinically approved mTOR inhibitor) resulted in the destabilization of Sae2. Because the action of Rpd3 and Hda1 is opposed to that of Gcn5 (a histone and protein acetylase that works in the acetylation–deacetylation network) in *gcn5* mutants, VPA and rapamycin-mediated destabilization of Sae2 was attenuated (46). These results led to a model in which acetylation plays a regulatory role in coordinating autophagy and DSB repair (Fig. 1B).

Clinical–Translational Advances

Exploiting the acetylation link between DNA damage response and autophagy: current status and new challenges

Currently, the results of a few phase I/II clinical trials using combinational strategies have been reported, with encouraging, though not definitive, results (3, 4).

In an attempt to verify the radiosensitizing properties of HDAC inhibitors, vorinostat has been employed in a phase I clinical radiotherapy trial to treat advanced gastrointestinal cancers. The initial results confirmed the safety of the combination (300 mg of vorinostat once daily in conjunction with 30 Gy of radiation over 2 weeks), but the high variability in the tumor volume regression reported (26% mean reduction, 23% SD) cannot be considered as a validation of the approach (48). Other clinical studies are ongoing in a variety of cancers including brain, lung, and pancreatic tumors (49).

Similarly, initial clinical studies testing the combination of HDAC inhibitors with DNA-damaging drugs have yielded conflicting results. Notwithstanding the preclinical examples of synergistic cancer cell killing, a phase I clinical trial in patients with advanced solid tumor malignancies treated with vorinostat and doxorubicin resulted in a very limited clinical benefit (50). The *in vitro* evidence and xenograft observations that VPA potentiates epirubicin-induced cell death without exacerbating toxicity (51) have been translated into a phase I clinical study in solid tumor malignancies, in which objective responses were seen in 22% of the patients and 39% had stable disease (52). In a limited phase II dose-expansion trial in patients with metastatic breast cancer, 9 of 14 evaluable patients had an objective response and 1 patient had a complete clinical response (50). A phase II clinical trial is ongoing to further delineate the efficacy of the combination (49).

VPA pretreatment has been shown to increase the cytotoxicity of the topoisomerase inhibitor karenitecin in melanoma cells and in animal xenografts (53). The VPA–karenitecin combination was clinically evaluated in patients with stage IV melanoma with encouraging results: 47% of the patients had stable disease with median progression-free survival of 10.3 weeks versus 34% with stable disease with median progression-free survival of 7.9 weeks in patients treated with single-agent karenitecin (53).

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In this framework of increasing interest in the potential use of HDAC inhibitors with DNA-damaging drugs, the observation that triggering unprogrammed autophagy-mediated turnover of key DDR proteins, either by inhibiting HDACs and/or mTOR, would contribute to the sensitivity to DNA-damaging agents by dampening DDR has several implications (Fig. 1B; ref. 46).

At a general level, it further emphasizes that the study of HDAC inhibitors, and their use in cancer therapy, must definitively come to terms with the fact that considering epigenetic mechanisms alone is not going to provide in most (if not all) cases a satisfactory understanding of the mode of action of these compounds and, therefore, is not going to result in successful therapies.

The proposed model may then provide new insights on the molecular mechanisms behind the reported sensitization to the DNA-damaging agents of HDAC inhibitors and mTOR inhibitors (4, 12, 26, 36, 54); this is particularly important, considering the controversial role attributed to autophagy induction in the cancer therapeutic response. It must be stressed, however, that our understanding of the acetylation link between DDR and autophagy is much less advanced in mammalian cells. CtIP deacetylation by SIRT6 was shown to positively regulate DSB repair in humans, but in this case, its effects on protein stability were not analyzed (47). It would be interesting to see if HDAC inhibitor–induced hyperacetylation and/or autophagy has a role in the downregulation of the several DDR proteins associated with the increased cell sensitivity to DNA-damaging agents upon treatment with HDAC inhibitors (13, 14, 16–18, 21–24).

Several preclinical studies have reported that the combination treatment of rapamycin or other inducers of autophagy and HDAC inhibitors has a greater antitumor activity compared with either agent alone (55–59). In some cases, the antitumor effect has been linked to the downregulation of key proteins, including HIF-1α in prostate and renal carcinoma cells, c-myc in primary diffuse large B-cell lymphoma (DLBCL) cells, and survivin in renal cancer cells (55, 56, 58). Induction of autophagy has not been studied in these reports, but it is interesting to note that these proteins are posttranslationally modified by acetylation (60–62). Very provocative in this context is the indication that mTOR is modified by acetylation (6) and that vorinostat-induced autophagic cell death in endometrial stromal sarcoma cells has been associated with a strong decrease in mTOR protein expression (63). In acute myelogenous leukemia, inhibition of mTOR signaling has been proposed to overcome HDAC inhibitor resistance (64) and, conversely in DLBCL cells, inhibition of HDACs to rescue mTOR inhibitor resistance (55). Even more recently, HDAC1 has been directly shown to regulate metabolism in mammalian cells through deacetylation of the AMPK kinase, thus leading to its interaction and activation by the upstream LKB1 kinase (65). In this way, HDAC1 can act as a direct energy sensor in the cells, hinting at complex regulatory links among metabolism, DDR, and autophagy.

Seven phase I/II clinical trials are currently ongoing mainly to evaluate the safety and tolerability and the recommended doses for the combination of autophagy inducers (sirolimus, temsirolimus, and everolimus) with HDAC inhibitors (vorinostat and panobinostat) in multiple myeloma, Hodgkin and non-Hodgkin lymphoma, and advanced solid cancers. including kidney and prostate cancers and nasopharyngeal carcinoma (49).

Perspectives

Finally, we would like to point out how these studies highlight the importance of 2 powerful approaches to the development of anticancer therapies:

- The study of old drugs remains a valuable source of pharmacologic innovation (66). Though they are not the most potent drugs available against the studied targets, VPA and rapamycin are approved drugs in clinical practice, and as such, once more details are available in mammalian cancer cells, it could be possible to quickly establish a clinical proof of concept.
- Yeast maintains its validity as a cell model system to explore pathways underlying the sensitivity and/or resistance to drugs and other therapies (67). Although genome-wide RNA interference cell screenings can now be done in mammalian cells (and even *in vivo*; ref. 68), these studies are enormously more time consuming and costly than screenings in yeast.

For these reasons, our 2 groups (with different sets of expertise in mammalian and yeast model systems) have decided to start a joint effort ("target from yeast to mammals," or TYM) to do extensive genome-wide screenings in yeast to identify genes and/or pathways involved in the response to established or new drugs, followed by the validation of those genes or pathways in diversified mammalian model systems. Although it is not exhaustive, we believe that this strategy has the potential to rapidly unravel unexpected links and quickly point out novel therapeutic avenues to cancer therapy.

Disclosure of Potential Conflicts of Interest

S. Minucci is a shareholder of Genextra, a holding company in the field of biotechnology. One of the scientific programs of Genextra is the development of novel HDAC inhibitors. None of the molecules studied by Genextra are discussed in this work. The other authors declare no conflicts of interest.

Authors' Contributions

Conception and design: O. A. Botrugno, F. Vanoli, M. Foiani, S. Minucci
Writing, review, and/or revision of the manuscript: O. A. Botrugno, F. Vanoli, M. Foiani, S. Minucci
Development of methodology: T. Robert
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Robert

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Robert
Study supervision: S. Minucci

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