

Episensitization: Therapeutic Tumor Resensitization by Epigenetic Agents: A Review and Reassessment

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Abstract: Resistance to chemotherapy, biological and targeted therapies is an important clinical problem. Resistance can arise and/or be selected for multiple mechanisms of action. Unfortunately, acquired resistance to antitumor agents or regimens is nearly inevitable in all patients with metastatic disease. Until recently, it was believed that this resistance was unalterable and irreversible, rendering retreatment with the same or similar drugs futile in most cases. However, the introduction of epigenetic therapies, including HDAC inhibitors and DNA methyltransferase inhibitors (DNMTIs), has provided oncologists with new strategies to potentially overcome this resistance. For example, if chemoresistance is the product of multiple non-genetic alterations, which develop and accumulate over time in response to treatment, then the ability to epigenetically modify the tumor to reconfigure it back to its baseline non-resistant state, holds tremendous promise for the treatment of advanced, metastatic cancer. This minireview aims (1) to explore the potential mechanisms by which a group of small molecule agents including HDACs (entinostat and vorinostat), DNA hypomethylating agents such as the DNMTIs (decitabine (DEC), 5-azacytidine (5-AZA)) and redox modulators (RRx-001) may reprogram the tumors from a refractory to non-refractory state, (2) highlight some recent findings in this area, and (3) discuss the therapeutic potential of resensitization approaches with formerly failed chemotherapies.

Keywords: Epigenetics, HDAC inhibitors, reactive oxygen species, resensitization.

INTRODUCTION

In contrast to the permanence of DNA mutations, the reversibility of epigenetic aberrations constitutes an attractive therapeutic target. Therefore, an innovative treatment approach to the widespread problem of chemoresistance is to reprogram the tumor to restore sensitivity to therapies that were initially effective, but subsequently failed secondary to the development of resistance. While conceptually challenging, it is becoming increasingly evident that chemoresistance may be reversible in some situations. Here we discuss some promising approaches to this problem and the putative mechanisms of action of the agents that may be useful for this purpose.

DNA METHYLTRANSFERASE INHIBITORS (DNMTIs)

Methyltransferases (MTases) transfer a methyl group to the C5 position of cytosine guanine dinucleotides (CpG). Of the three active DNA MTases identified (DNMT1, DNMT3A and DNMT3B), DNMT1 is the most abundant enzyme of this family found in mammals. DNA Methyl transferases require S-adenosylmethionine as the methyl group donor and result in the formation S-adenosylhomocysteine as a product of the reaction (Fig 1). Overexpressed MTases lead to CpG hypermethylation around transcriptional start sites which are associated with gene silencing and cancer [1]. MTases are important players in many processes and thus their inhibition disrupts multiple signaling pathway nodes [2]. The prototype DNMTIs are nucleoside inhibitors 5-azacytidine (5-azaCdR) and 2'-deoxy-5-azacytidine (decitabine). These cytidine analogues are incorporated into DNA and RNA during replication and transcription, inhibiting the action of MTases; this results in hypomethylation and activation of silenced genes that are important for control of pathways critical to proliferation and differentiation.

These agents, known as antimetabolites, (Fig. 2) were initially used as conventional cytotoxic therapies, but now, particularly at lower doses, are also recognized as DNA demethylators. FDA approval

for the treatment of myelodysplasia and acute myelogenous leukemia (AML), was granted for these epigenetic modifiers in 2004 and 2006, respectively [3].

Resensitization to previously failed therapies has been directly demonstrated with these agents, most notably in ovarian cancer, to restore sensitivity in patients with platinum-resistant disease. Matei *et al.*, [4] administered low-dose decitabine prior to carboplatin in 17 patients with heavily-pretreated and platinum-resistant ovarian cancer in a Phase 2 clinical trial, resulting in a 35% objective response rate (RR) and progression-free survival (PFS) of 10.2 months, with nine patients (53%) free of progression at 6 months. This was encouraging, given the relatively low rate of objective responses (<10%), usually of short duration, observed in this patient population. In addition, Li *et al.*, [5] reported a phase I/II study of 5-azacytidine and carboplatin that demonstrated durable responses (median duration of therapy 7.5 months) with an overall response rate of 13.8% and a disease control rate (partial response plus stable disease) in 45% (13/29 evaluable patients) with platinum-resistant or refractory ovarian cancer. Further confirmatory studies in this patient population are anticipated.

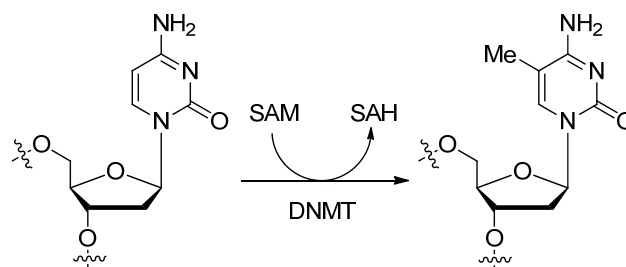


Fig. (1). DNA cytosine-5 methylation, catalyzed by DNMT. SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine.

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Juergens *et al.*, conducted a combination phase I/II trial in extensively pretreated patients with recurrent metastatic non-small

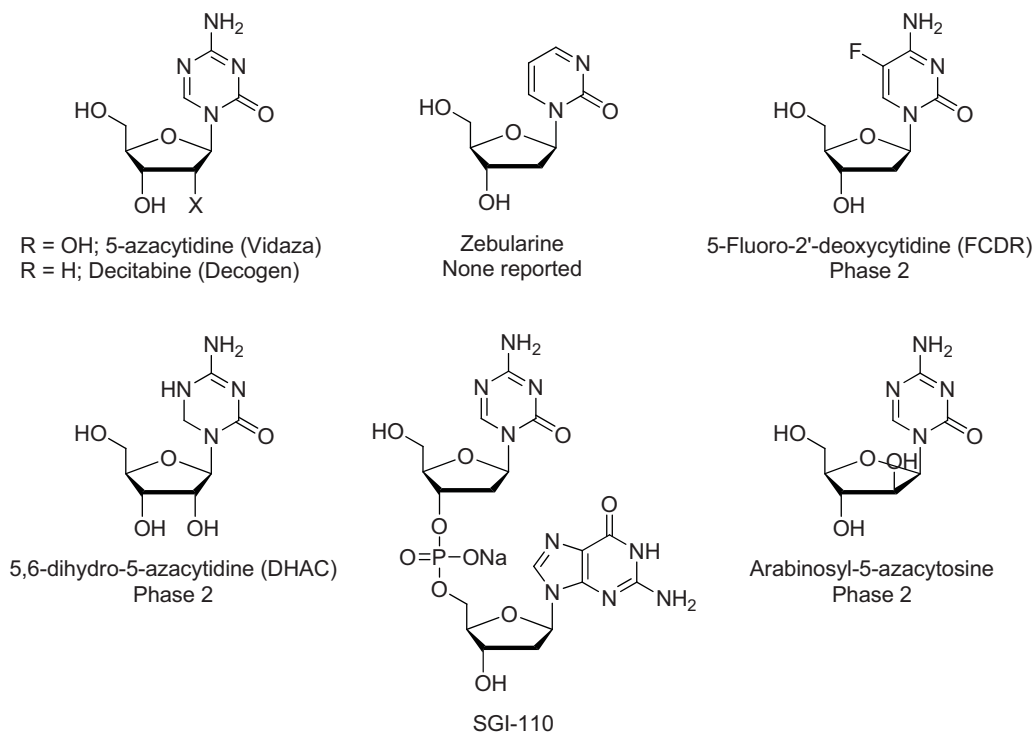


Fig. (2). Nucleoside DNMT inhibitors.

cell lung cancer with azacytidine and entinostat (see HDACi below), inhibitors of DNA methylation and histone deacetylation, respectively. Objective responses were observed, [6] and survival benefits (>1 year in approximately 20% of the patients and a median OS of 6.4 months) exceeded historical controls [3]. Interestingly, the authors did not attribute the long survival to prolonged stable disease, but rather to an “*unusually robust response to subsequent cytotoxic therapies, with which the majority of patients were treated*” [3]. A similar observation was also made in the Phase 1 trial of RRx-001, discussed below. Subsequent therapies in the NSCLC trial included perimetrexed, docetaxel, erlotinib, an anti PD-1 monoclonal antibody, gemcitabine, irinotecan/bevacizumab, and cisplatin, suggesting that this combination of epigenetic inhibitors reverted the tumor to a less resistant phenotype, making it more widely susceptible to a variety of subsequent therapeutic agents.

The non-nucleoside DNMTIs constitute a distinct class of inhibitors that act predominately by binding the active site of MTases, without the requirement of incorporation into DNA or RNA (Fig. 3). This class of inhibitors is currently an area of active research, with researchers anticipating similar epigenetic activity to the nucleosides [7, 8], though with the promise of lower toxicity. Although preclinical studies have shown reactivation of tumor suppressor genes [9], none of these compounds have been investigated clinically. While the exact mechanisms of resensitization—and sensitization—is very poorly understood, the promiscuous demethylation activity of these DNA Methyltransferase Inhibitors, which hit multiple targets including the tumor suppressor gene p53 [10], and can affect metabolic pathways (e.g. shift the tumor cells away from glycolysis) [11, 12], may alter or reset the tumor and thereby affect the sensitivity of the tumor to a variety of subsequent or concomitant therapies.

HISTONE DEACTYLATING INHIBITORS (HDACi)

The histone deacetylating inhibitors (HDACs) inhibit the enzyme histone deacetylase responsible for gene silencing through hypoacetylation of histones. Histone deacetylation increases the

electrostatic attraction between the positive charges of the histones and negative charges of the DNA, which ensures tight binding and renders promoter regions inaccessible to polymerases for gene transcription (Fig. 4).

Cancer is linked to histone hypoacetylation, due to over-expression of HDACs, and the anti-cancer effects of HDAC inhibitors have been attributed to the restoration of the histone acetylation balance [13, 14]. However, this process is complex, involving at least six human HDAC enzymes, a broad spectrum of protein classes, multiple mechanisms that include induction of reactive oxygen species (ROS) and pleiotropic biologic effects for which the putative target is unknown or uncertain [15]. Acetylation has broad regulatory functions on histones and non-histone proteins. Substrates of nonhistone acetylation are multiple and include important cellular factors involved in cellular homeostasis such as p53, nuclear factor- κ B (NF- κ B), and hypoxia-inducible factor (HIF-1 α) [16] which overlap with the DNA methylation inhibitors described above and RRx-001 described below. In particular the effect on p53 is highlighted for this review: the p53 tumor suppressor protein and glycolytic regulator is activated directly through deacetylation [17] and indirectly through ROS-induced DNA damage [18]. HDAC inhibitors fall into a number of broad classes. These include the short chain fatty acids and the hydroxamic acids (Fig. 5) and the benzamides and cyclic tetrapeptides (Fig. 6).

Two HDAC inhibitors, vorinostat and entinostat, have been approved by the FDA for the treatment of leukemia. They have also been studied in solid tumors, as a component of combination therapy, with results suggesting the potential for a resensitization effect. While there was no significant activity in two non-small lung cancer trials, [19, 20] perhaps due, in part, to dosing considerations, a randomized phase II breast cancer trial of the aromatase inhibitor exemestane with the HDACi entinostat versus exemestane alone demonstrated significantly improved overall survival. [21] These encouraging results merit additional testing to better characterize

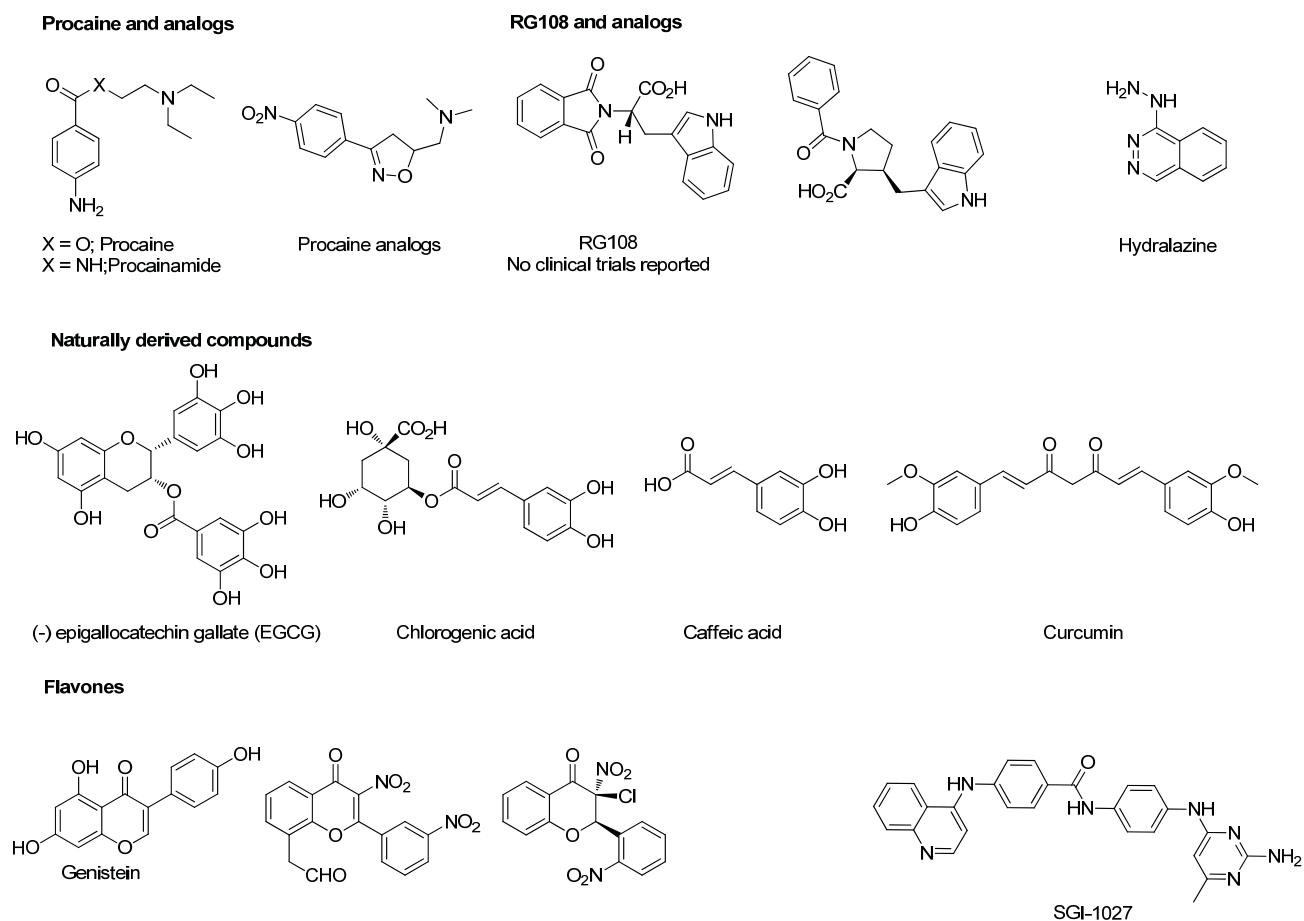


Fig. (3). Non-nucleoside DNMT inhibitors.

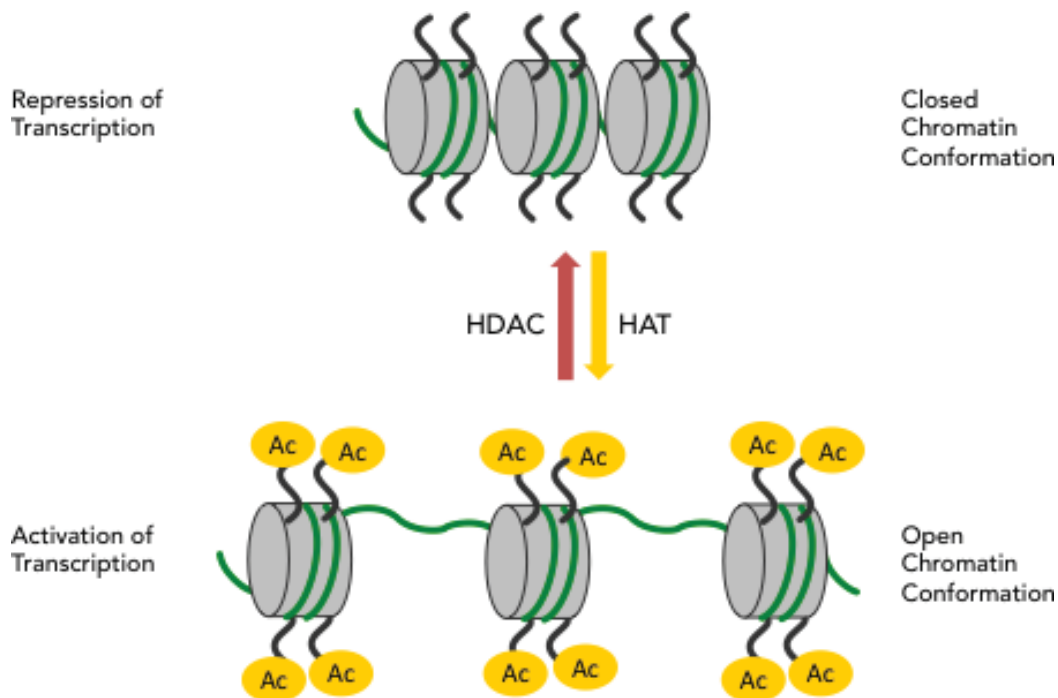
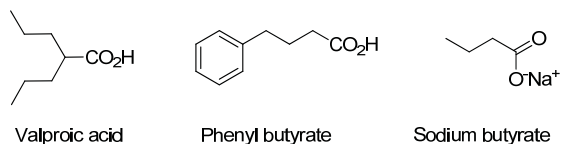
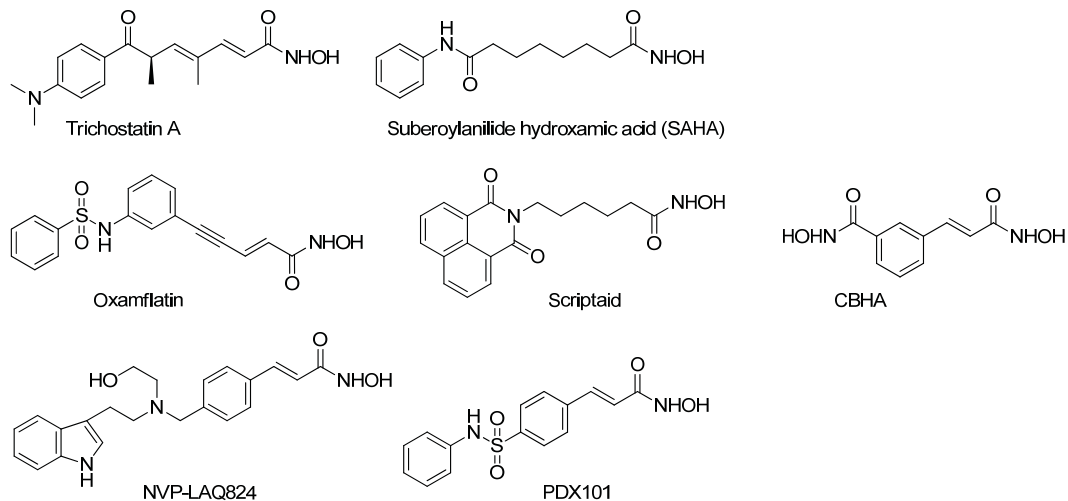
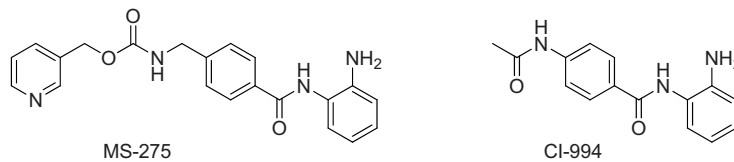
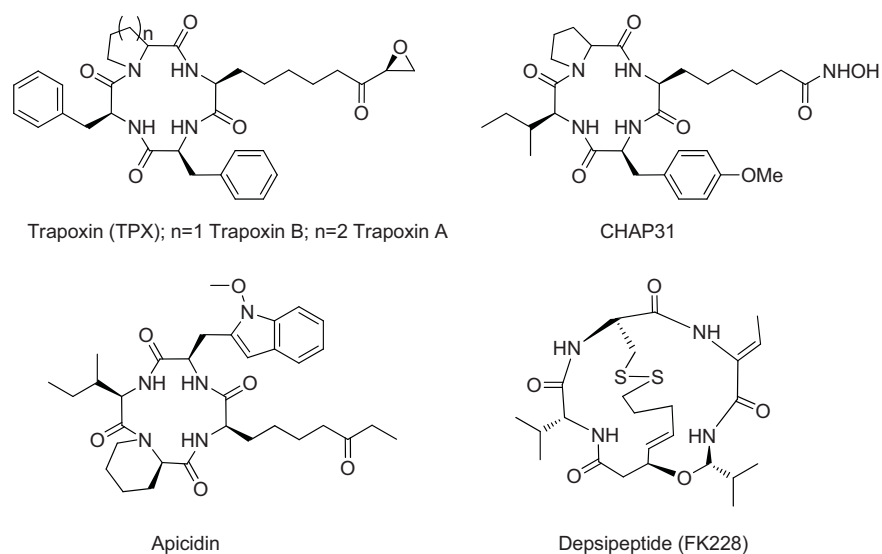


Fig. (4). Histone acetylation and repression or activation of transcription.

Short Chain Fatty Acids**Hydroxamic acids****Fig. (5).** HDAC Inhibitors: Short chain fatty acids and hydroxamic acids.**Benzamides****Cyclic tetrapeptides****Fig. (6).** HDAC Inhibitors: benzamides and cyclic tetrapeptides.

this survival benefit, and elucidate the underlying mechanism of the improved efficacy in the combination therapy arm.

RRx-001

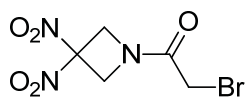
RRx-001 is a novel therapeutic, originating from the aerospace industry, with a chemical structure and mechanism of action that is clearly differentiated from classic cytotoxic and epigenetic agents (Fig. 7). As a redox active agent, RRx-001 inhibits tumor promotion and leads to apoptosis through the induction of oxidative and nitrosative stress in tumors that are already overburdened with free radicals leading to modulation and/or inhibition of multiple targets including HDACs and DNMTs. RRx-001 has shown promise as a novel cancer therapeutic agent in a number of cell lines and tumor models, in the absence of unacceptable off-target effects and systemic toxicity.

In a multi-center Phase 1 dose escalation study, RRx-001 demonstrated an acceptable safety profile at the maximal dose of 83 mg/m² and evidence of anti-cancer activity, including one partial response and disease stabilization in nine patients lasting at least 16 weeks. At 16.8 months, 50% of patients were still alive. Similar to the observation in the azacytidine and entinostat combination NSCLC trial, there was prolonged overall survival that greatly exceeded that which was expected on the basis of the regorafenib CORRECT trial [22], in which the median OS was 6.4 months. This increased survival is attributed, in part, to robust clinical responses with subsequent post-progression therapies, suggesting that tumors and/or the tumor microenvironment was modified leading to resensitization of tumors to previously failed therapies. It is believed that RRx-001-mediated epigenetic modification of the tumors may have played a role in this apparent resensitization.

RRx-001-enhanced susceptibility to chemotherapy was observed in at least 5 patients, 4 with colorectal cancer and 1 with NSCLC, with drugs to which the patients were previously resistant. A case report was recently published describing the resensitization of two colorectal cancer patients to FOLFIRI, which both patients had previously failed [23]. RRx-001 has multiple mechanisms of action, including induction of redox and metabolic stress on the tumor, which can disrupt multiple cellular pathways *via* sulfhydryl oxidation, Nrf2 activation and p53 upregulation, PARP cleavage, HIF-1 alpha and inhibition of G6PD, a central substrate of the Pentose Phosphate Pathway (RadioRx, unpublished data). Based on the potential for episensitization a number of Phase 2 studies in multiple tumor types including colorectal, breast, brain and liver will be initiated.

EPIGENETIC EFFECTS AND OXIDATIVE STRESS

The influence of ROS on epigenetic events has been studied extensively, particularly in the field of aging [24] and age-related illnesses, supporting a direct and indirect causal relationship between oxidative stress and epigenetic changes. Indirectly, oxidative stress leads to glutathione depletion and impairment of the one carbon cycle: DNA methylation and histone methylation depend upon the availability of methyl groups from S-adenosylmethionine (SAM). An increase in cysteine synthesis for GSH regeneration leads to a depletion of methionine, resulting in decreased synthesis of SAM, and an overall defect in methylation reactions. Oxidative stress also directly inactivates HDACs [25]. HDAC inhibition results in histone acetylation and a more open or



RRx-001

Fig. (7). RRx-001.

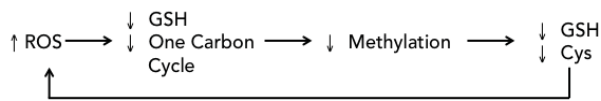


Fig. (8). ROS epigenetic cycle.

permissive chromatin conformation, rendering DNA more susceptible to ROS damage [26]. Indeed, HDAC inhibitors are associated with ROS generation [27, 28], resulting in DNA damage induction and repair, as measured through increased gamma-H2AX expression [29], thereby fueling a self-propelling loop of ROS-mediated cytotoxicity followed by repair and resolution processes (Fig. 8).

HDAC inhibitors increase the activity of DNA synthesis inhibitors such as fludarabine. The widely used anti epileptic and mood stabilizer, valproic acid (VPA), a small, branched fatty acid, has been investigated as an HDAC inhibitor both preclinically and clinically [30]. Preclinically, it enhanced fludarabine-induced apoptosis *via* mitochondria-mediated apoptosis, which in turn was associated with ROS generation, intracellular ATP depletion and reduced levels of ATM, an important protein in DNA damage response [31]. In addition, VPA increased the cytotoxicity of the aurora kinase inhibitor, VE465, as evidenced by higher levels of cleaved PARP. Ovarian cells were particularly sensitive [32].

Clinically, in two studies in patients with high-risk acute myeloid leukemia or myelodysplastic syndrome, the combination of VPA and a demethylating agent demonstrated higher OS and CR rates compared to the combination of VPA and chemotherapy, such as all-trans retinoic acid ATRA [33, 34]. Other studies in have similarly reported improvements in survival associated with epigenetic changes [35-37]. As an inhibitor of HDACs, which collectively deacetylate a variety of non-histone proteins, VPA has pleiotropic effects that are linked to increased levels of reactive oxygen species [38]. Leukemia is particularly sensitive to oxidative damage [39]. These results suggest that the chemosensitizing effect of VPA is associated with oxidative stress and induction of p53, with a resulting inhibition of glycolysis and improved therapeutic efficacy.

We hypothesize that RRx-001 has epigenetic modifying effects because it significantly affects the intracellular redox status of tumor cells [40]; the enzymes responsible for histone methylation and DNA methylation rely on critical redox intermediates derived from metabolic pathways, potentially leading to changes in gene expression. Furthermore, RRx-001 induces double-stranded DNA breaks secondary to the generation of ROS and RNS, and affects a variety of signaling pathways, which can impact the activity of p53 and p21, and deregulate tumor metabolism and cellular energetics. In this way, oxidative stress, through the alteration of DNA-methylation and histone modifications, may contribute to epigenetic changes. This is currently an area of active laboratory and clinical investigation, with several phase 2 trials planned with RRx-001 both as a single agent and in combination with chemotherapy and radiation therapy.

CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to modulate gene expression of tumors by either epigenetic modification or other mechanisms of action in order to resensitize them to previously failed therapies presents an entirely new paradigm for the treatment of cancer. As discussed here, data, in some cases preliminary, suggest that drugs such as HDACs, DNMTs and RRx-001 may have the ability to resensitize or 'episensitize' tumors to formerly failed chemotherapy drugs/regimens and that this episensitization is dependent upon the disruption of the expression or activity of an array of cellular targets, some of which may affect cellular energetics. These targets include p53, PARP, NFκB, and HIF-1α.

While the specific molecular mechanisms involved in epigenetic silencing are admittedly unclear, multiplicity may be more important than specificity i.e. the simultaneous inhibition of multiple pharmacologic targets that are crucial to cellular metabolism and energy status. In particular, the anti-cancer agents described in this review all activate and upregulate p53, which can have numerous effects, including its function as a gatekeeper at the crossroads of metabolism in cancer cells, with potential secondary effects on multiple other targets as well as the tumor glycolytic rate [12].

Since, RRx-001, HDAC inhibitors and DNA methyltransferase inhibitors all disrupt multiple signaling pathways, perhaps this lack of specificity (i.e. affecting multiple pathways and cellular functions) contributes to their ability to resensitize tumor cells to treatments [3] to which they have become resistant. This is an active area of investigation, the results of which may provide promising new therapeutic strategies for enhancing the responsiveness of patients' tumors to subsequent therapies following progression of disease. This new paradigm has near term translational applicability, with the potential to impact the natural history of a variety of refractory and metastatic cancers.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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