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Rewriting the Epigenetic Code for Tumor Resensitization: A Review

Bryan Oronsky* , Neil Oronsky†, Jan Scicinski*, Gary Fanger*, Michelle Lybeck* and Tony Reid‡

*RadioRx Inc, Mountain View, CA, USA; †CFLS, LLC, San Jose, CA 95128, USA; ‡UCSD Moores Cancer Center, La Jolla, CA 92093, USA

Abstract

In cancer chemotherapy, one axiom, which has practically solidified into dogma, is that acquired resistance to antitumor agents or regimens, nearly inevitable in all patients with metastatic disease, remains unalterable and irreversible, rendering therapeutic rechallenge futile. However, the introduction of epigenetic therapies, including histone deacetylase inhibitors (HDACis) and DNA methyltransferase inhibitors (DNMTIs), provides oncologists, like computer programmers, with new techniques to "overwrite" the modifiable software pattern of gene expression in tumors and challenge the "one and done" treatment prescription. Taking the epigenetic code-as-software analogy a step further, if chemoresistance is the product of multiple nongenetic alterations, which develop and accumulate over time in response to treatment, then the possibility to hack or tweak the operating system and fall back on a "system restore" or "undo" feature, like the arrow icon in the Windows XP toolbar, reconfiguring the tumor to its baseline nonresistant state, holds tremendous promise for turning advanced, metastatic cancer from a fatal disease into a chronic, livable condition. This review aims 1) to explore the potential mechanisms by which a group of small molecule agents including HDACis (entinostat and vorinostat), DNMTIs (decitabine and 5-azacytidine), and redox modulators (RRx-001) may reprogram the tumor microenvironment from a refractory to a nonrefractory state, 2) highlight some recent findings, and 3) discuss whether the current "once burned forever spurned" paradigm in the treatment of metastatic disease should be revised to promote active resensitization attempts with formerly failed chemotherapies.

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Introduction

In response to changes in the environment, cancer adapts primarily by means of epigenetic modifications. The term epigenetics refers to the "source code" by which tumors are able to assimilate environmental events without changing the hardware, i.e., DNA. Therefore, epigenetic modifications are akin to rapid software updates that only involve alterations to gene expression or output rather than the genetic sequence itself. In contrast to the permanence of DNA mutations, the reversibility of epigenetic aberrations constitutes an attractive therapeutic target.

From an information technology perspective, it is possible to liken the tumor to malware designed specifically to damage or disrupt the source code of normal tissue through its pattern of gene expression.

The DNA of tumor cells is to computer hardware as epigenetics is to system software. While the DNA hardware is fixed and unchangeable, epigenetics, like software, is a form of code, and code is "hackable" or modifiable. Hence with epigenetic agents, gene expression in tumors is reprogrammable in the same way that computer code can be rewritten. Just as malicious code can be reengineered or neutralized, a feasible solution to the widespread problem of chemoresistance is to reprogram the tumor to restore sensitivity to previously tried therapies. Not surprisingly, this is perhaps easier said than done; however, it is becoming increasingly evident that chemoresistance is not necessarily written in stone; after all, the epigenome, by definition, is editable, like any software [1], and while the parts of the epigenome that code for chemoresistance are

Address all correspondence to: Bryan Oronsky, MD, PhD, RadioRx, 800 W El Camino Real, Suite 180, Mountain View, CA, USA. E-mail: boronsky@radiorx.com
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unknown, clues about the "why and how" have emerged from a commonality between the putative mechanisms of action of the agents described in this review.

The Dark Side of Epigenetics: Carcinogenesis and Resistance

While epigenetics is an exploitable anticancer mechanism, the plasticity of epigenetic changes, with subsequent molecular alterations that regulate the neoplastic phenotype, contributes to carcinogenesis, tumor promotion, chemoresistance, and radioresistance as much as or more than genetic variability [2]. In particular, the yin of epigenetic silencing of tumor suppressor genes is an important mechanism for carcinogenesis. For example, *MGMT* hypermethylation, plays a direct role in the accumulation of G-to-A mutations in the *KRAS* gene in colorectal tumors. This is the dark side of epigenetics: that it underlies and subserves the malignant phenotype. Conversely, since turnabout is fair play, the yang of epigenetic reactivation of these same silenced tumor suppressor genes is an invaluable anticancer strategy [3–9].

DNA Methyltransferase Inhibitors

Methyltransferases (MTases) transfer a methyl group to the C5 position of cytosine guanine dinucleotides (CpG). Overexpressed MTases lead to cytosine guanine dinucleotide hypermethylation around transcriptional start sites, which is associated with gene silencing and cancer [10]. MTases are an important player in many processes, and thus, their inhibition disrupts multiple signaling pathway nodes [11].

The prototype DNA methyltransferase inhibitors (DNMTIs) are nucleoside inhibitors 5-azacytidine (5-azaCdR) and 2′-deoxy-5-azacytidine (decitabine) that were upgraded from conventional cytotoxic therapies to the status of DNA demethylators with FDA approval for the treatment of hematological malignancies, myelodysplasia, and acute myeloid leukemia (AML), in 2004 and 2006, respectively [1].

Resensitization to previously failed therapies has been directly demonstrated with these agents most notably in ovarian cancer to restore platinum sensitivity in patients with platinum-resistant disease. Matei et al. administered low-dose decitabine before 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 of 10.2 months, with 9 patients (53%) free of progression at 6 months [12]; this is compared to the small percentage of short-lived objective responses (<10%) usually induced in this patient population [13]. Fu et al. 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 RR of 13.8% and a disease control rate (partial response plus stable disease) in 45% (13 of 29 evaluable patients) with platinum-resistant or refractory ovarian cancer [14]. Further confirmatory studies in this patient population are anticipated.

Juergens et al. conducted a combination phase I/II trial in extensively pretreated patients with recurrent metastatic non–small cell lung cancer with azacytidine and entinostat [see histone deacetylase inhibitors (HDACis) below], inhibitors of DNA methylation and histone deacetylation, respectively. Objective responses were observed [15], the therapy was well tolerated, and survival benefits (>1 year in approximately 20% of the patients and a median overall survival (OS) of 6.4 months) exceeded historical controls [1] (48% expected survival after 6 months). Interestingly, the authors attributed the long survival not to prolonged stable disease but to an "unusually robust response to subsequent cytotoxic therapies, with which the majority of patients were treated" [1], an observation that was also made in a phase 1 trial of RRx-001, as discussed below. The subsequent therapies in the non–small cell lung cancer trial included pemetrexed, docetaxel, erlotinib, anti–programmed cell death protein (PD-1) monoclonal antibodies, gemcitabine, irinotecan/bevacizumab, and cisplatin, suggesting that this combination of epigenetic inhibitors reverted the tumor microenvironment to a less resistant state, making it more widely susceptible to a variety of subsequent chemotherapeutic agents. SGI-110, a dinucleotide prodrug of decitabine and deoxyguanosine that protects the parent from deamination and thereby increases the systemic exposure, is currently in phase 2 for AML [16].

While the exact mechanisms of resensitization—and sensitization—remain unclear, the promiscuous demethylation activity of these DNMTIs, which hit multiple targets including the tumor suppressor gene *p53* that downmodulates glycolysis, may alter or reset the tumor biology and thwart chemoresistance [17–19].

Histone Deacetylase Inhibitors

HDACis inhibit the enzyme HDAC 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. Cancer is linked to histone hypoacetylation, due to overexpression of HDACs, and the anticancer effects of HDACis have been attributed to the restoration of the histone acetylation balance [20].

However, the developing story is more 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 [21]. Acetylation has broad regulatory functions on histones and nonhistone proteins. Substrates of nonhistone acetylation are multiple and include important cellular factors involved in cellular homeostasis such as p53, nuclear factor κB, and hypoxia-inducible factor 1α [22] that 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 are activated directly through deacetylation [23] and indirectly through ROS-induced DNA damage [24].

The HDACi, vorinostat, has been approved by the FDA for the treatment of cutaneous T-cell lymphoma, whereas entinostat has received breakthrough therapy status in estrogen receptor (ER)-positive breast cancer. However, their evaluation as combination chemotherapy in clinical trials in different tumor types hints obliquely at a resensitization potential [1]. Two of the trials in non–small lung cancer were not promising and the lack of activity may be related to dosing considerations; however in a phase II breast cancer trial of the aromatase inhibitor exemestane with the HDACi entinostat *versus* exemestane alone, the combination significantly improved overall survival by 8.3 months ($P = .04$), warranting additional testing to determine whether the improvement was due both to increased susceptibility of the tumor to the aromatase inhibitor and resensitization to subsequent therapies.

Romidepsin, a unique HDAC prodrug, which is converted intracellularly to a reduced form that binds to and inhibits class 1 HDACs, was

approved for the treatment of cutaneous T-cell lymphoma on the basis of studies that demonstrated an objective RR rather than overall survival, which unfortunately does not allow for an assessment of its resensitization potential.

These epigenetic therapies are representative of the current paradigm for rational drug discovery, which emphasizes structures that specifically target and antagonize the chromatin-modifying enzymes. An emerging diametrically opposed strategy involves structurally nonspecific drugs with no specific site of action that are promiscuous in their capacity to inhibit HDACs and DNA MTases. The redox-active RRx-001 and aliphatic acids such as valproic acid (VPA) exemplify this strategy.

RRx-001

With an iconoclastic pedigree from the aerospace industry and a chemical structure and mechanism of action that clearly differentiate it from the classic epigenetic agents, compelling preliminary clinical evidence suggests that the pan-epigenetic modulator, RRx-001, which inhibits DNA MTases and HDACs, resensitizes tumors to previously tried—and failed—therapies. In a multicenter phase 1 dose escalation study, RRx-001 demonstrated an acceptable safety profile at the maximal dose of 83 mg/m^2 and evidence of anticancer activity including one partial response and disease stabilization in five patients lasting >16 weeks. At 16.8 months, 50% of patients were still alive. Like the observation in the azacytidine and entinostat combination non–small cell lung cancer trial, the prolonged but nonsignificant overall survival significantly exceeded what was expected on the basis of the regorafenib CORRECT trial in which the median OS was 6.4 months. The increase in survival is attributed to robust clinical responses with subsequent post-progression treatments, including radiation, suggesting that the state of the tumors were changed epigenetically, rendering them hypersensitive to multiple cytotoxics.

In addition, the drug enhanced susceptibility to anticancer agents in five patients, four with colorectal cancer and one with non–small cell lung cancer that had previously demonstrated resistance. A case report that reviews the clinical course of two refractory colorectal patients with documented chemoresensitization after treatment with RRx-001 has been published [25].

RRx-001 allosterically modifies hemoglobin and maximally catalyzes the reduction of nitrite to bioavailable nitric oxide under hypoxia, which accumulates in poorly oxygenated tumors [26]. Nitric oxide rapidly combines with excess superoxide ($O_2\bullet^-$) in the tumor, outcompeting superoxide dismutase, to produce high levels of potent peroxynitrite (ONOO–), in the proverbial "Devil's Triangle" of oxidative stress [27]. In this way, RRx-001 channels its activity through redox and metabolic stress on the tumor, (refer to Figure 1), resulting in the oxidation of critical cysteine residues at catalytic sites of the enzymes DNA MTases 1 and 3a and HDACs, inhibiting their activity and resulting in global hypomethylation (RadioRx unpublished data). This inhibition of DNA MTases, in particular, results in the de-repression p53 and p21 expression, which are dramatically upregulated, presumably due to the demethylation of their regulatory regions, leading to cell cycle arrest and apoptosis [28].

These interesting preliminary clinical findings suggest that a more systematic "proof of concept" study is warranted to determine whether RRx-001 is a cause, contributor, or epiphenomenon to chemoresensitization. A randomized Phase 2 study in colorectal cancer has started, while multiple Phase 2 studies in breast, brain, liver, and bone with RRx-001 as a therapeutic resensitizer both as monotherapy and in combination are either in the planning stages or almost under way.

Valproic Acid

As a nonspecific inhibitor of multiple HDACs, the antiepileptic and mood stabilizer VPA [29] like the other aliphatics, butyric acid and phenylbutyric acid, reverses epigenetic silencing and induces an enhancement of gene expression. This epigenetic modulation of gene expression has led to anticancer activity [30] in a variety of *in vitro* and *in vivo* systems, with encouraging results in early clinical trials either alone or in combination with demethylating and/or cytotoxic agents in AML.

Like RRx-001, VPA induces oxidative stress, possibly through the generation of reactive intermediates [31], and since HDACs, as cysteine-dependent enzymes, are susceptible to ROS modulation and inhibition [32], the resultant altered gene expression patterns from their inactivation contribute to anticancer activity. In addition, like RRx-001, VPA-induced ROS formation is reversed by pretreatment with antioxidants like ascorbic acid [33].

Conclusions and Future Perspectives

A central tenet of treatment in oncology is that resistant tumors remain resistant, making reintroduction of the same therapy (drug rechallenge) a counterproductive strategy, capable of doing more harm than good, given the potential for toxicity without clinical benefit. Resensitization has been anecdotally reported in the literature after chemotherapy-free intervals ("drug holidays"), which provide empirical support to the notion that epigenetic reversibility may characterize the "natural history" of certain tumors [34]. Treatment with epigenetic agents may accentuate or accelerate this intrinsic reversibility, suggesting that acquired drug resistance is clinically circumventable with epigenetic modulation and that therefore rechallenge with failed therapies is a feasible anticancer strategy.

The central analogy in this review was to compare the DNA of the tumor cell to computer hardware and epigenetics to system software. The basic premise that software and epigenetics are each a form of code and that code, by design, is flexible and modifiable implies that the tumor can be circumvented and manipulated in the same way that a computer can be hacked. However, unlike software, which is static, the tumor is a biologic system that adapts in response to dynamic conditions; this is a disadvantage because it allows tumors to become resistant to treatment. It is also, paradoxically, an exploitable advantage because each adaptation puts an energy tax on the tumor in the form of adenosine triphosphate (ATP) expenditure (expend to defend), and energy is finite in accordance with the first law of thermodynamics [35].

Therefore, the assumption in oncology that tumor progression necessitates treatment succession, because the cancer will remain resistant to the initial therapy, should be revisited. The tumor operates on an energy deficit due to high rates of ATP turnover [36] especially under hypoxia to maintain survival. The cellular adaptations to chemotherapy including increased repair of DNA damage, enhanced drug inactivation [37], elevated intracellular levels of glutathione, overexpression of multiple drug resistance (MDR) [38], and other membrane efflux pumps that mediate resistance represent an additional drain on tumor ATP economy, resulting in a mismatch between ATP supply and ATP demand.

Resensitization demands a shift in perspective and treatment ethos: In the current paradigm of metastatic cancer, time is a one-way arrow

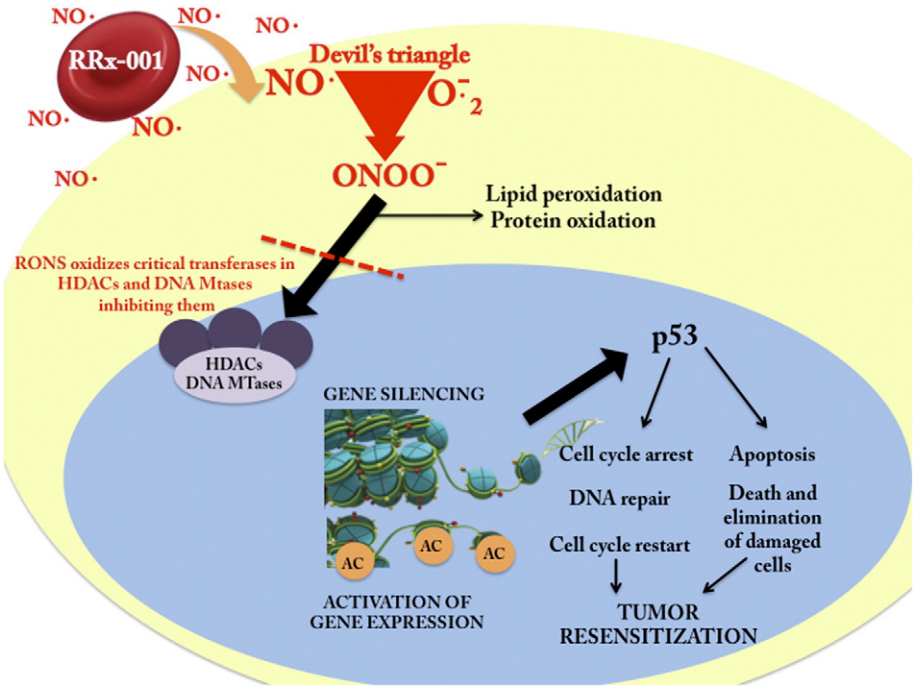

Figure 1. Basic mechanism of RRx-001–induced cytotoxicity. Higher levels of oxidative stress compared to normal tissues are a hallmark of tumors. RRx-001 delivers nitric oxide to tumors under hypoxia, which leads to the formation of ONOO–, transforming cellular stress from oxidative only to nitro-oxidative. ONOO– exerts its harmful effects on the tumor directly and indirectly. It oxidizes critical cysteine residues on the epigenetic regulators HDACs and DNA MTases, inhibiting them, which leads to p53 reactivation. Unless excess superoxide and RRx-001–derived NO production are terminated, this mechanism continues to propagate damage within the tumor cell in a vicious cycle. Moreover, ONOO– directly damages all macromolecules including lipids, proteins, and DNA. DNA damage induced by ONOO– activates a repair process, which eventually leads to ATP depletion and necrosis.

pointing inevitably toward therapeutic failure, which may justify aggressive chemotherapy protocols, often at the expense of quality of life considerations, to extend life.

Clearly then, resensitization has important diagnostic and therapeutic implications and needs to be examined on a more systematic, rather than on an anecdotally "one off" or case-by-case, basis.

Epigenetics stands at the intersection of nature *versus* nurture whereby epigenetic marks dynamically—and often reversibly—change or readjust in response to environmental factors. Cancer cells, challenged by an ever-changing environment, and in a constant state of flux, epigenetically "tinker" with genes, activating or inactivating them, in response to a variable environment. While the specific molecular mechanisms involved in resensitization or, perhaps more appropriately, "episensitization," which constitutes a reboot or restore to the original state, 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. Unlike targeted or molecular therapies, which aim to strictly regulate one pathway, one target, or one gene, epigenetic agents are "Swiss Army Knives" in the anticancer armamentarium, modifying the chromatin structure and thus influencing expression of multiple genes and a panoply of pathways including ribosomal proteins, oxidative phosphorylation, DNA/RNA polymerases, and Wnt/β-catenin signaling among others through inhibition of HDACs and DNA MTases [39]. Epigenetic modulators, like decitabine and the other epigenetic agents listed in Table 1, replace the specificity of molecularly targeted agents, designed to inhibit specific kinases, with the multiplicity of gene reactivation. As a therapeutic strategy, epigenetic modulation may seem, at present, like a relative shot in the dark, given the nonspecific nature of its gene-activating effects. However, since cancer cells build and require a growth-conducive microenvironment, which depends on silencing target genes, reactivation of these genes that, in aggregate, encompass a broad range of biologic functions may destabilize the tumor.

The identification of specific epigenetically modified drug-resistant genes, before therapy, with tumor biopsies, may help to direct and augment the efficacy of treatment. For example, the fact that methylation-dependent silencing of argininosuccinate synthetase (ASS1) [40], a rate-limiting enzyme involved in the biosynthesis of arginine, has been implicated in therapeutic resistance in several cancer types including renal cell carcinoma, hepatocellular carcinoma, malignant melanoma, glioblastoma multiforme, and platinum-resistant epithelial ovarian cancer suggests a role for demethylating agents in these ASS1 drug-resistant cancers [41].

Table 1. Epigenetic Therapies: Approved and under Development

Agent	Class	Approval Date	Approved Indication
Azacytidine	DNMTI	2004	Myelodysplastic syndrome
Decitabine	DNMTI	2006	AML
SGI-110	Second generation hypomethylating agent	Phase 2	AML
Vorinostat	HDACi	2006	Cutaneous T-cell lymphoma
Romidepsin	Class 1 HDACi	2009	Cutaneous T-cell lymphoma
Valproic acid	Nonspecific epigenetic agent	–	Off-label use
RRx-001	Pan-epigenetic agent	Phase 2	Multiple tumor solid tumor types

Nevertheless, despite their current nonspecific promiscuity, epigenetic agents may act on most or all tumor types, since aberrant methylation and deacetylation patterns are a hallmark of cancer cells. In particular, several of the anticancer agents described in this review activate and upregulate p53, which itself affects multiple targets [19]. Following genotoxic stress in response to traditional therapeutic strategies such as cisplatin, doxorubicin, 5-fluorouracil, fludarabine, mitoxantrone, etoposide, or X-ray radiation, p53 is upregulated; the capacity to maximally induce p53 is only limited by the systemic toxicity of these agents.

One strategy to promote episensitization might be to administer azacytidine and entinostat sequentially after progression on RRx-001 followed by therapies that have been previously tried and failed. Another strategy might be to combine several genotoxic and nongenotoxic therapies with p53 upregulating properties at lower and potentially less toxic doses. The success of this strategy could be measured with standard imaging procedures such as fluorodeoxyglucose (FDG) - positron emission tomography (PET).

RRx-001, HDACis, and DNMTIs all disrupt multiple signaling pathways and it is perhaps this *lack* of specificity that is responsible for their ability to resensitize cells to ineffective treatments [1]. The failure of so-called targeted agents to significantly increase overall survival and quality of life supports an evidence-based paradigm shift away from the systematic avoidance of previously tried therapies toward their potential reuse for resensitization.

With this resensitization paradigm shift, it would be theoretically possible to continue treatment instead of giving up after all conventional options have been exhausted, with reverted and reprogrammed tumors that are repeatedly susceptible to the same chemotherapies. Instead of a one-way arrow pointing inevitably in the direction of therapeutic failure, treatment would thereby alternate between resistance and resensitization, like a swinging pendulum. The desideratum is for patients to live out the rest of their lives with metastatic cancer in the form of a chronic condition, which is manageable and survivable, like diabetes, psoriasis, and human immunodeficiency virus (HIV), and not under the shadow of a progressively fatal disease.

Competing Interests

The authors declare that the drug RRx-001, mentioned in this review, is manufactured by RadioRx Inc, and the majority of the research and clinical trials associated with drug RRx-001 have been funded by RadioRx Inc. The authors declare that they have no other competing interests.

Author's Contributions

B.O. contributed with the majority of the writing of this manuscript. Remaining authors N.O., J.S., G.F., M.L., and T.R. contributed with additional writing and editing of the manuscript. All authors read and approved the final manuscript.

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