

# Discovery of RRx-001, a Myc and CD47 Downregulating Small Molecule with Tumor Targeted Cytotoxicity and Healthy Tissue Cytoprotective Properties in Clinical Development

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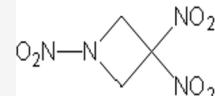
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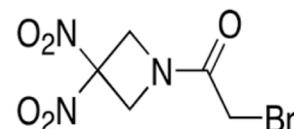
Supporting Information

**ABSTRACT:** After extensive screening of aerospace compounds in an effort to source a novel anticancer agent, RRx-001, a first-in-class dinitroazetidine small molecule, was selected for advancement into preclinical and clinical development. RRx-001 is a minimally toxic small molecule with a distinct chemical structure and mechanism of action. The paradox of RRx-001 is that it mediates both antitumor cytotoxicity and normal tissue protection. The question of exactly how RRx-001 does this, and by means of what mechanism(s), depending on the route of delivery, intravenous or intratumoral, are explored. RRx-001 is currently in phase 2 and 3 clinical trials for the treatment of multiple solid tumor malignancies and as a supportive care drug.

## TNAZ



## RRx-001



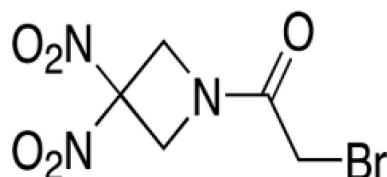
Chemical Formula:  $C_5H_6BrN_3O_5$

Molecular Weight: 268.02

Molecular structure of RRx-001  
(1-bromoacetyl- 3,3 dinitroazetidine)

## INTRODUCTION

RRx-001 is a first-in-class aerospace-based<sup>1</sup> therapeutic,<sup>2</sup> with a chemical structure shown in Figure 1, containing an acyl



Chemical Formula:  $C_5H_6BrN_3O_5$   
Molecular Weight: 268.02

Figure 1. Structure of RRx-001

bromide and a geminal dinitroazetidine group, in global phase 3 clinical trials<sup>3</sup> that have demonstrated both selective antitumor activity in several cancer types including small cell lung cancer (SCLC),<sup>4</sup> high-grade neuroendocrine carcinomas (HGNEC), metastatic colorectal cancer (CRC), ovarian cancer, brain metastases, and glioblastoma (GBM) as a single agent, as a radiochemosensitizer, and as an immunosensitizer.<sup>5</sup> Further, in clinical trials, RRx-001 has demonstrated protection of the bone marrow, kidneys, and gastrointestinal (GI) tract from chemo- and radiation therapy and will additionally be entering phase 3 trials as a supportive care drug. To date, over 300 patients have been treated in multiple phase 1, 2, and 3 trials with RRx-001 in

the absence of any dose-limiting toxicities and no maximally tolerated dose (MTD) has been reached. The most common intravenous toxicity or side effect in phase 1 was a local, temporary burning sensation and venous inflammation at the site of administration.<sup>6</sup>

The discovery of RRx-001 was the product of a unique “hands on” partnership between ATK, an American aerospace and defense company, academia, venture capital, and biotech that involved two parts serendipity and intuition, one part *in vivo* observation, and one part novel combinatorial chemistry in which a nitro group on the melt cast explosive, trinitroazetidine (TNAZ), was replaced with an acyl bromide to render the subsequent compound, RRx-001, less impact sensitive, and more reactive with thiols. The decision to search the previously unmined aerospace industry for potentially bioactive chemical compounds was made in opposition to the so-called streetlight effect which plagues the pharmaceutical industry and which refers to an old joke about a drunk searching for his keys under a streetlight, even though he lost them in the park, because “the light is better here”.<sup>7</sup> Accordingly, the tendency, which was not followed with RRx-001, is to disproportionately focus on well-

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Table 1. Compounds for Investigation of Structure–Activity Relationships (SARs)

Structure	MW	mg equivalent per 10 mg ABDNAZ	Abbreviation	Rationale
	268.02		RRx-001 (ABDNAZ)	Control
	189.13	7.1	ADNAZ	Tested activity of gem dinitro in absence of alkylating center but with a very similar compound
	178.03	6.6	Des-nitro ABDNAZ (ABAZ)	Tested contribution of alkylating center in absence of gem dinitro group.
	327.13	12.2	DNAZ-TMAB	Tested importance of water solubility and cell permeability
	308.27	11.5	ABDNAZ-Cys	Tested activity of metabolite formed in serum, transport and water solubility
	223.57	8.3	CIADNAZ	RRx-001 is rapidly alkylated. This compound tested, in parallel, whether the alkylation ability of ABDNAZ is optimum
	315.02	11.8	IADNAZ	RRx-001 is rapidly alkylated. This compound tested, in parallel, whether the alkylation ability of ABDNAZ is optimum
	319 (average)	6.0*	A/F (1:1) mixture of BDNPA and BDNPF	Tested importance of azetidine while retaining dinitro groups

\* The mg equivalent of A/F was adjusted to compensate for the presence of 2 dinitro groups

Table 2. 4× Tumor Growth Delay of RRx-001 and Analogs

no. of mice	4× TGT (day)	TGD (day)	P value ( <i>t</i> test)							
			CTL	ABDNAZ	ADNAZ	ABAZ	TMAB	Cys	CIADNAZ	IADNAZ
control	7	2.8 ± 0.6								
ABDNAZ	7	4.8 ± 1.1	2.0 ± 1.1	0.002						
ADNAZ	7	3.6 ± 0.6	0.8 ± 0.6	004	002					
ABAZ	7	3.8 ± 0.6	1.0 ± 0.6	0.008	006	0.5				
DNAZ-TMAB	7	3.6 ± 0.6	0.8 ± 0.6	0.03	0.02	1.0	0.4			
ABDNAZ-cys	7	3.9 ± 0.7	1.1 ± 0.7	001	0.08	0.4	0.8	0.4		
CIADNAZ	7	3.4 ± 0.5	0.6 ± 0.5	0.08	0.01	0.6	0.2	0.6	0.2	
IADNAZ	7	3.9 ± 0.6	1.1 ± 0.6	0.006	0.08	0.3	0.8	0.3	1.0	0.1
A/F	7	3.3 ± 0.4	0.5 ± 0.4	0.1	0.01	0.3	0.1	0.3	0.1	0.06

known therapeutic targets and chemical structures, i.e., those under the streetlight that are easiest to study and not on lesser known (or unknown) therapeutic targets and chemical structures, i.e., those beyond the streetlight that are hardest to study but nevertheless potentially as or more important.

While target-based screening is the current predominant paradigm in drug discovery, RRx-001 along with other energetic compounds were assessed by *in vitro* and *in vivo* phenotypic screens on the premise that their chemical structures, unprecedented (and untested) in the annals of pharmaceutical development, were not necessarily related to or predictive of bioactivity.

In contrast to the target-based screening, phenotypic screens involve a form of serendipity, because the mechanism of activity of the drug candidate in question is unknown and, therefore, difficult or impossible to predict in advance. The rationale to evaluate energetic compounds in particular for therapeutic activity is that the toxicological impacts of postdetonation residues on flora and fauna (and even humans in some cases) are

already known and, therefore, potentially predictive of *in vivo* safety, a cost-intensive preclinical hurdle that many drug development candidates fail to clear, even with evidence of significant anticancer activity.

Of all the energetic compounds that were tested, RRx-001 induced the most potent antiproliferative activity *in vitro* and inhibited the growth of multiple tumor types *in vivo* both under hypoxia and normoxia with minimal systemic toxicity.<sup>8</sup> Finally, the structure activity relationship (SAR) of RRx-001 was studied, which demonstrated conclusively that replacement or substitution of any part of RRx-001 decreased the anticancer activity both *in vivo* and *in vitro*. These compounds are shown in Table 1.

A syngeneic SCC VII tumor model in C3H mice was used to test the efficacy of RRx-001 (ABDNAZ) and its analogues with cisplatin (CDDP) as a positive control. All the compounds, except CDDP, were injected intraperitoneally (ip) at an equimolar dose to 12 mg/kg RRx-001 (ABDNAZ), every other day for three times total. CDDP was dosed at 50%

equimolar dose to 12 mg/kg RRx-001. Results showed that RRx-001 was more toxic to tumors than its derivatives ( $P = 0.01$ – $0.08$ ) but not more tumor-toxic than cisplatin. There was no statistically significant difference in terms of tumor growth delay time among the other compounds (Table 2) (Figures 2 and 3).

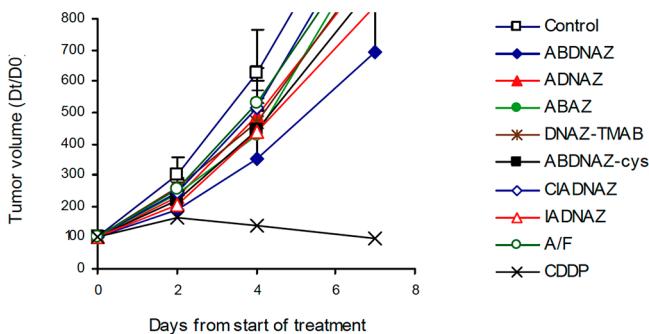


Figure 2. SCCVII tumor growth curves

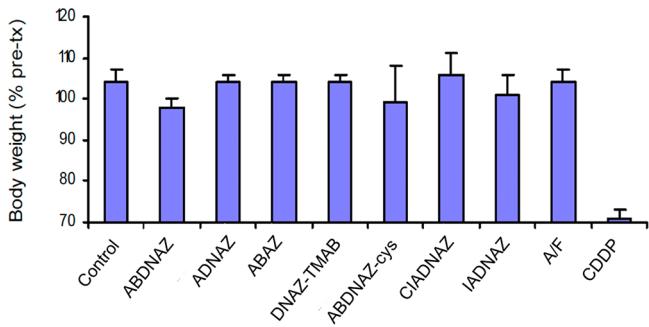


Figure 3. Body weight of tumor-bearing mice on day 7.

While cisplatin at a 6.7 mg/kg dose given every other day  $\times$  3 (50% of equimolar dose of 12 mg/kg RRx-001) demonstrated the most potent tumor inhibitory growth, it was very toxic and resulted in 100% animal deaths (7/7) on day 8.

RRx-001 is a nongenotoxic alkylating agent, meaning that it forms covalent adducts with thiol sulfurs but not amino nitrogens in proteins and nucleic acids; hence, while RRx-001 depletes cellular stores of cysteine, thioredoxin, and glutathione (GSH), which indirectly leads to nucleic acid oxidation,<sup>9</sup> it does not directly interact with DNA or RNA. The biological target of RRx-001 is thiols, which leads to

- (1) **Direct damage** when RRx-001 is injected intratumorally or given by hepatic artery infusion (HAI) from the denaturation of proteins and the modification of kinases, phosphatases, cell cycle regulators, fatty acids, cell membranes, etc., as well as the potential for immune cell activation due to antigen and danger signal release from dying tumor cells/
- (2) **Indirect damage** when RRx-001 is administered intravenously through reactive oxygen species/reactive nitrogen species (ROS/RNS) generation, formation of inflammatory foam cell macrophages and vascular normalization, which reprograms the tumor microenvironment and potentiates synergistic interactions with chemotherapy, targeted therapy, radiation, and immunotherapy/
- (3) **Normal tissue protection** from the cytotoxic effects of radiation and chemotherapy through the compensatory

stimulation and upregulation of endogenous defense mechanisms (Figure 4).

The objective of this review is to highlight the mechanisms by which RRx-001 mediates its cytotoxic and chemoradioprotective activities, depending on the method of administration, i.e., intravenous, intratumoral or by hepatic artery infusion.

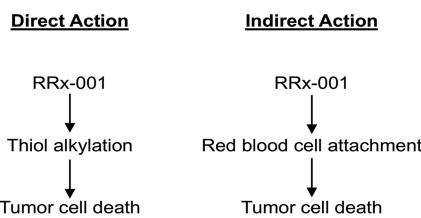


Figure 4. RRx-001 may act directly or indirectly on tumors, depending on how it is administered.

## CHEMISTRY

The synthesis of RRx-001 is provided in Scheme 1. The synthetic process of RRx-001 is highly hazardous and should not be attempted by unauthorized parties. Multikilogram GMP quantities of high purity RRx-001 API have been synthesized from a two-step sequence of oxidative nitration of 1-*tert*-butyl-3-hydroxymethyl-3-nitroazetidine

HMNAZ, 1, followed by acylative dealkylation and functionalization with bromoacetyl bromide. Intermediate *tert*-butyl-3,3-dinitroazetidine (TBDNAZ, 2) is achieved. The final compound, RRx-001 (3), is isolated by extraction and purified by recrystallization from ethyl acetate/hexane. Serious explosive hazards are mitigated with the use of water extraction in place of filtration.

## RESULTS

**Intravenously Administered RRx-001.** As a nonpolar electrophile, RRx-001 rapidly traverses the membrane of the red blood cell (RBC) and reacts selectively with nucleophilic sulfhydryl groups abundant in the RBC such as reduced glutathione and the highly conserved  $\beta$ -globin chain cysteine 93 residue.<sup>10,11</sup> These reactions, which proceed by nucleophilic substitution of the  $\alpha$ -bromine with a sulfur atom from a thiol group, result in stable thioether bonds, as shown in Figure 5.

The rapid and irreversible binding of RRx-001 to hemoglobin has several immediate and downstream consequences:

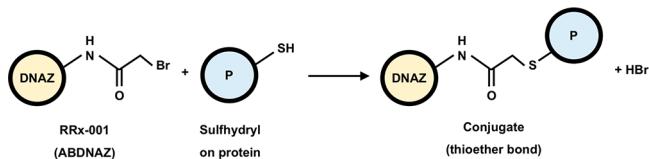
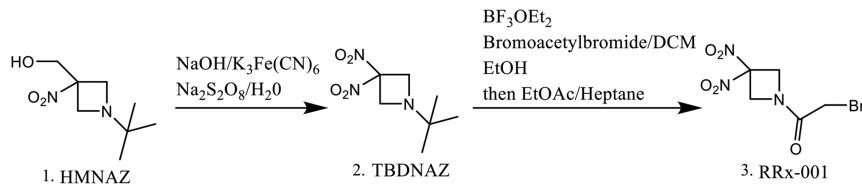
**Immediate Consequence 1.** Because RRx-001 binds to only a small subpopulation of red blood cells (RBCs), there is no anemia and no hematotoxicity.<sup>12</sup>

**Immediate Consequence 2.** Systemic toxicity is minimal,<sup>13</sup> and no drug–drug interactions or induction/inhibition of cytochrome P450 enzymes occur because RRx-001 is immediately sequestered postinjection in red blood cells, which selectively adhere to hypoxic tumor vasculature.

**Immediate Consequence 3.** Nitric oxide (NO)<sup>14,15</sup> is displaced from its binding site on  $\beta$ -cysteine 93, which leads to localized infusional discomfort and venous inflammation with direct IV administration, side effects that are ameliorated by a 10 min ex vivo incubation with an aliquot (12 mLs) of whole blood, which is the current method of delivery.<sup>16</sup>

**Immediate and Downstream Consequence 4.** Modification of Cys  $\beta$ 93 with RRx-001 increases the oxygen affinity of the RBC, which facilitates O<sub>2</sub> delivery to more hypoxic areas of the tumor.<sup>17</sup>

## Scheme 1. RRx-001 Synthetic Scheme



**Figure 5.** RRx-001 reaction scheme for chemical covalent conjugation of the bromoacetyl “warhead” to a sulfhydryl (in this case  $\beta$ -cysteine93) on hemoglobin (Hb), which is enhanced by the inductive effect (arrows) of the geminal dinitro groups on the azetidine ring. DNAZ, dinitroazetidine.

**Immediate Consequence 5.** Hemoglobin undergoes denaturative precipitation, which results in the formation of microvesicles (MVs) to remove the damaged hemoglobin (Hb).<sup>18,19</sup> These MVs, which are shed from the RBCs, deposit iron, nitric oxide, heme, and lipid (because the RBC membrane is comprised mainly of cholesterol and phospholipids) in healthy tissues, inducing oxidative stress and the upregulation of compensatory pathways to mitigate it. This adaptive upregulation is basis for the chemoradioprotective effects of RRx-001, to be discussed in more detail in the mechanism section below.

**Immediate and Downstream Consequence 6.** RRx-001-bound red blood cells are more rigid and increase expression of several tumor vascular adhesion molecules such as  $\alpha 4\beta 1$  integrin (VLA-4), CD36 (thrombospondin receptor), Lu/BCAM (CD239), ICAM-4, and phosphatidylserine (PS), facilitating selective adhesion to the tumor endothelium and subsequent phagocytosis by tumor associated macrophages (TAMs).<sup>20</sup> This phagocytosis is followed by subsequent degradation and release of the contents of the RBC in the tumor parenchyma; the released contents of the RBC include oxidized lipids, heme, NO,<sup>21</sup> iron, and RRx-001 metabolites, which account for the benign safety profile of RRx-001, because all the cytotoxic “action” takes place inside rather than outside of the tumor.

**Cytotoxic Mechanisms. Vascular Normalization.** Vascular normalization, a term coined by Jain,<sup>22</sup> refers to “pruning” rather than destruction of the morphologically aberrant, chaotic, and leaky blood tumor vessels, to improve influx of anticancer agents and oxygen. Clinical and preclinical evidence demonstrates that RRx-001 administration at lower doses decreases permeability, interstitial fluid pressure and vessel tortuosity, enhances pericyte coverage, and improves perfusion, which lead to improved entry of chemotherapy and its accumulation deep within the tumor.

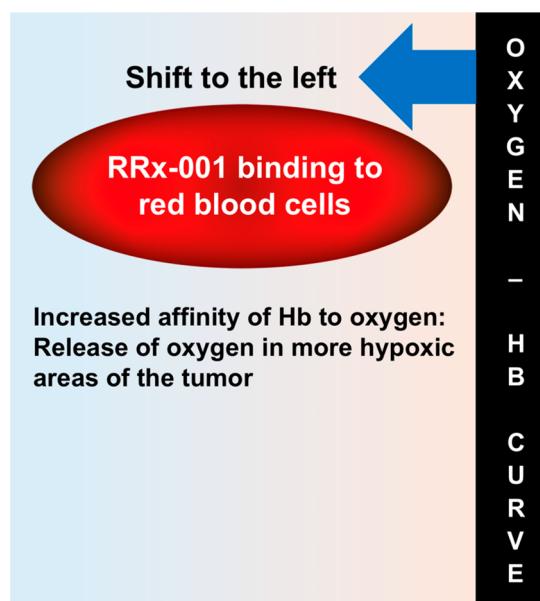
The molecular mechanisms by which lower dose RRx-001 drives vascular normalization effects<sup>23–25</sup> and makes chemotherapy and radiation<sup>26</sup> potentially more effective as a result are 3-fold:

- (1) **Increased Hb oxygen affinity:** RRx-001 increases the oxygen affinity of hemoglobin, leading to unloading of O<sub>2</sub> in more hypoxic areas, which lowers expressions of hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) and vascular endothelial growth factor (VEGF).<sup>27</sup>

(2) **M2-to-M1 macrophage polarization:**<sup>28</sup> RRx-001-induced tumor associated macrophage polarization from protumor M2 to antitumor M1 increases vessel normalization through decreased secretion of pro-angiogenic factors, such as VEGF,<sup>29</sup> adrenomedullin (ADM), platelet derived growth factor (PDGF), and matrix metalloproteinases (MMPs).<sup>30</sup>

(3) **Reverse Robin Hood syndrome (RRHS):** RRHS, a term that has been used to describe paradoxical changes in cerebral hemodynamics after a stroke,<sup>31</sup> refers in this context to RRx-001 RBC-induced occlusion of hypoxic tumor vessels which “steal” blood flow away from the occluded, oxygen-poor vessels to nonoccluded, less oxygen-poor ones.<sup>32</sup> This redistribution of blood flow leads to a regression or pruning of the dysfunctional vasculature with a more homogeneous drug and oxygen distribution as a result.<sup>33</sup>

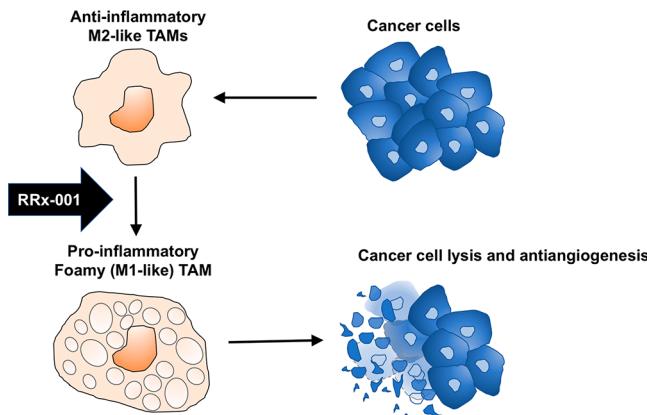
- (a) Left shift of hemoglobin (Hb)-oxygen curve leads to unloading of O<sub>2</sub> in more hypoxic areas<sup>34</sup> and a consequent decrease of HIF-1 $\alpha$  and VEGF (Figure 6)



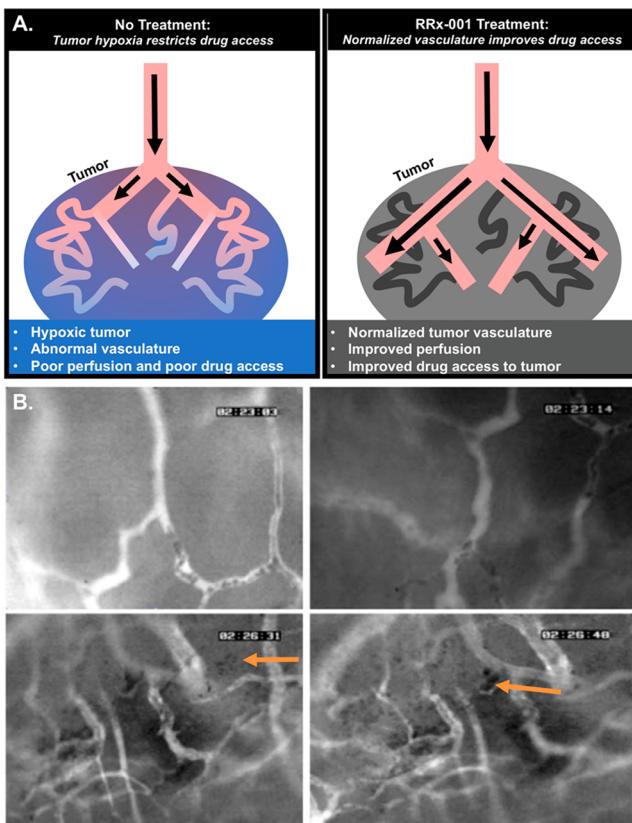
**Figure 6.** RRx-001-induced vascular normalization mechanisms.

(b) TAMs are skewed toward a pro-angiogenic phenotype, which express high levels of VEGF;<sup>35</sup> however, RRx-001-mediated metabolite, iron, and heme delivery to the tumor microenvironment elicits pro-inflammatory macrophages with antiangiogenic properties<sup>36</sup> (Figure 7).

(c) The sticky and more rigid RRx-001-bound red blood cells that lodge in the hypoxic tumor vasculature provoke the reverse Robin Hood-like steal of blood flow from the oxygen-poor to more oxygen-rich regions of the tumor<sup>37</sup> (Figure 8).



**Figure 7.** RRx-001-mediated metabolite, iron, and heme delivery to the tumor microenvironment causes tumor-associated macrophage repolarization from an M2 (anti-inflammatory) phenotype to M1 phenotype, eliciting pro-inflammatory macrophages with antiangiogenic properties.



**Figure 8.** (A) Rigid RRx-001-bound red blood cells lodge in the abnormal hypoxic tumor vasculature and provoke a reverse “Robin Hood-like” steal of blood flow from the oxygen-poor to more oxygen-rich regions of tumors. (B) Logjamming of the RRx-001 RBCs in the tumor vasculature and their internalization by TAMs. Orange arrows indicate red blood cells aggregating in the tumor.

**M1 Foam Cell Transformation, Macrophage Polarization, and Stimulation of Phagocytosis.** Macrophages are broadly categorized as pro-inflammatory M1 or anti-inflammatory M2 types. M2-like tumor-associated macrophages (TAMs), which are hallmark by the release of IL-4 or IL-10, IL-13, VEGF, and TGF- $\beta$  and the expression of the immune checkpoints, PD-1 and CD47, correlate with poor prognoses

due to the promotion of immunosuppression, angiogenesis, and chemoresistance; by contrast, the presence of M1 macrophages, which secrete pro-inflammatory cytokines such as TNF- $\alpha$ , IL-23, IL-1 $\beta$ , and IL-12, in the tumor microenvironment (TME), is indicative of better outcomes.<sup>38</sup> Therefore, one anticancer strategy, given the inherent plasticity of macrophages and their ability to switch between phenotypes depending on the composition of the microenvironmental milieu is to target the tumor-promoting M2-like TAMs and repolarize or reeducate them to tumor-inhibiting M1-like macrophages.<sup>39</sup>

The covalent encapsulation of RRx-001 in red blood cells<sup>40</sup> efficiently vectors it and its metabolites to the hypoxic tumor vasculature, where multicellular adhesions between the RRx-001-bound, oxidatively stressed RBCs, white blood cells (WBCs), platelets, and endothelial cells culminate in vaso-occlusion, hemolysis, and erythrophagocytosis by tumor associated macrophages (TAMs) because oxidized and/or hemolyzed RBCs are targets for TAMs, which are thought to exit and reenter the tumor, shown in Figure 8B. Hence, RRx-001 might be properly called an “erythrophagoimmunotherapeutic”.<sup>41</sup>

Reactive oxygen species generated by hemoglobin denaturation, RRx-001 metabolites and NO release oxidize lipoproteins such as LDL and lipoprotein(a) within the RBC membrane, facilitating erythrophagocytosis by tumor associated macrophages, with their subsequent conversion into lipid-laden foam cells of the M1 phenotype, as seen in a serial patient biopsy in Figure 9.

In the tumor, these M1-like foam cells initiate an inflammatory response through the secretion of various pro-inflammatory mediators such as IFN- $\gamma$ , COX-2, iNOS, TNF- $\alpha$ , IL-6, IL-1, and MCP-1 and reactive oxygen and nitrogen species, and through their lysis, which leads to the formation of a necrotic core<sup>42</sup> that is seen in almost all RRx-001-treated patients,<sup>43</sup> as shown in Figure 10.

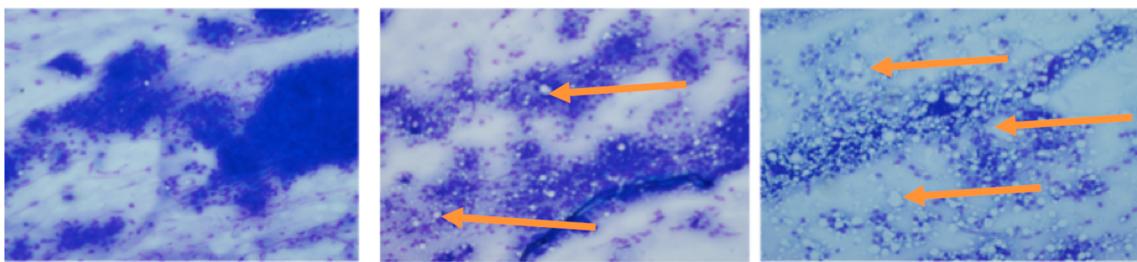
Recent data has demonstrated that these RRx-001-induced foam cells overexpress PPAR- $\gamma$ , which, in turn, inhibits the transcription factor, MYC.<sup>44</sup> Finally, the inhibition of MYC reduces expression of CD47, the “don’t eat me” signal, which functions as an immune checkpoint and drives phagocytic, antitumor activity.<sup>45</sup>

A graphic summary of the intravenous mechanism of action is shown in Figure 11.

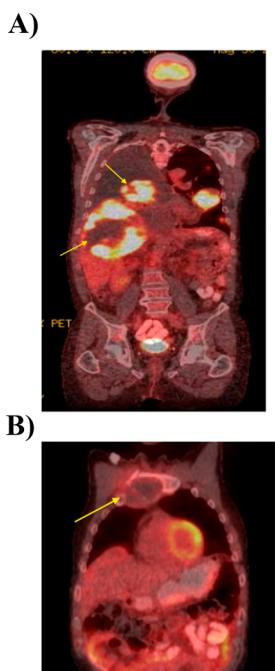
In support of its immunotherapeutic activity, IV RRx-001 was combined with 3 mg/kg nivolumab given every 2 weeks in a single arm, open-label phase 1 pilot study (NCT02518958) called PRIMETIME. Twelve patients with traditionally non-checkpoint inhibitor-responsive tumor types received  $\geq 1$  dose of RRx-001 and nivolumab. The combination was safe and well-tolerated with preliminary evidence of anticancer activity based on an objective response rate at 12 weeks of 25%

**Protective Mechanisms.** In addition to its cytotoxic activity through vascular normalization, tumor associated macrophage (TAM) polarization and CD47 downregulation,<sup>46</sup> RRx-001 also mediates chemoradioprotective effects preclinically and clinically. Preclinically, RRx-001 increases survival and ameliorates renal, bone marrow, cardiac, and GI toxicity in response to sublethal and lethal doses of radiation and chemotherapy. Clinically RRx-001 has demonstrated potential reduction of GI,<sup>47</sup> bone marrow, and renal toxicities.<sup>48</sup>

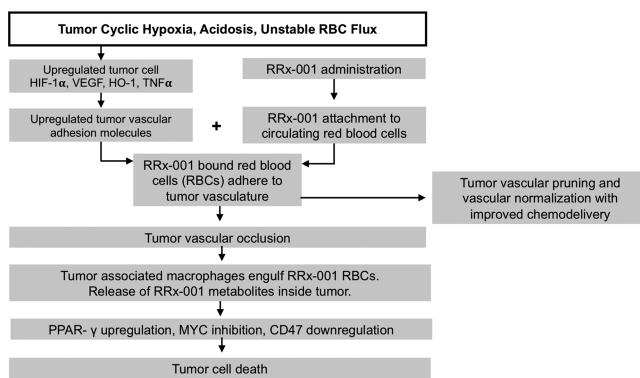
A discussion of normal tissue protection in oncology inevitably invites comparisons with the aminothiol, Amifostine, approved as a chemo- and radioprotectant but not used in the



**Figure 9.** Foam cells in a biopsy of an RRx-001-treated patient due to accelerated erythrophagocytic activity from RRx-001 metabolites. The three windows are from left to right, pretherapy, after 6 weeks of RRx-001 and after 12 weeks of RRx-001. The orange arrows indicate the macrophages that are overloaded with lipids.



**Figure 10.** Necrotic cores of RRx-001-treated tumors. (A) (top) PET-CT scan of patient with pancreatic cancer showing extensive central necrosis in liver and lung lesions. Necrosis was confirmed in the liver lesion both in the rim and the center. (B) (bottom) PET-CT scan of patient with metastatic sternal lesion (arrowed). Sternal metastasis is largely necrotic and not very FDG avid, post RRx-001.



**Figure 11.** Red blood cell-based antitumor mechanism summary.

clinic in part due to the possibility, which has never been definitively excluded, that Amifostine also protects tumors.<sup>49</sup> Because of this potential nonspecificity/nonselectivity, the

burden of proof is on any subsequent chemoprotectant to verify a lack of tumor protection.

In the case of RRx-001, multiple preclinical experiments<sup>50</sup> and clinical experience<sup>51–54</sup> in several different tumor types including head and neck cancer (H&N), small cell lung cancer (SCLC), high-grade neuroendocrine carcinoma,<sup>55</sup> glioblastoma (GBM), melanoma brain metastases,<sup>56</sup> colorectal cancer, and ovarian cancer provide evidence in support of RRx-001's antineoplastic activity both as a single agent and in combination with radiation and chemotherapy.

The differential RRx-001 red cell-based response between normal and tumor tissue is likely a function of the expression, "the dose makes the poison". Whereas RRx-001 bound RBCs adhere to the tumor vasculature prior to internalization by TAMs, which deliver high-levels of iron, oxidized lipids (oxLDLs) and RRx-001 metabolites directly to the tumor, normal tissues only receive "microdoses" of the same iron, oxidized lipids (oxLDLs), and RRx-001 metabolites from shed RBC microvesicles (MVs). This MV deposition leads to an upregulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2),<sup>57</sup> p53, and peroxisome proliferator-activated receptor γ (PPARγ) pathways, which protect against oxidative damage.

In this way, RRx-001 acts as an indirect cytoprotectant through the induction of a state of compensatory gene expression for DNA repair and anti-inflammation at low doses that alleviates the burden of anticancer agent-mediated side effects, while high doses delivered specifically to the tumor result in cytotoxicity.<sup>58</sup> This biphasic bell-shaped stress response with RRx-001, in which beneficial effects are counterintuitively induced at low doses while high doses result in toxicity,<sup>59</sup> is comparable to the dose response relationships, which exist with other more familiar biologic stressors such as exercise (e.g., regular exercise vs overtraining), drinking (e.g., moderation vs alcoholism), and eating (e.g., caloric restriction vs starvation).

The specific use of RRx-001 as a chemoradioprotectant will be put to the test in a phase 3 trial to protect against oral mucositis in first line H&N cancer.

**IV Pharmacokinetics.** Because RRx-001 is extremely rapidly metabolized, the glutathione metabolite (RRx-001-GSH) was chosen as a surrogate for exposure to RRx-001. In the phase I First-in-Man PK GCP study, RRx-001-GSH was quantified in plasma predose, during infusion, at the end of the infusion and time points after completion of the infusion.

Terminal half-life, which did not vary greatly across dose levels or between sampling days was 27.6 min. The calculated average  $AUC_{inf}$  and, to a lesser extent,  $C_{max}$  for both day 1 and day 22/50 for each cohort, was dose dependent. However, because no correlation between the PK parameters of RRx-001-GSH and

the activity and toxicity of RRx-001 is evident, RRx-001-GSH levels only serve to reflect systemic exposure to RRx-001.

**Intratumorally Administered RRx-001.** As mentioned previously, RRx-001 is an electrophile, which preferentially binds to sulfur containing ligands<sup>60</sup> rather than the hydroxy and amino groups present in nucleic acids.<sup>61</sup> Cellular thiols are attractive anticancer targets because (1) the most abundant thiol-containing peptides, reduced glutathione ( $\gamma$ -Glu-Cys-Gly; GSH), and thioredoxin (Trx), are significantly upregulated in cancers, which is thought to contribute to the development of tumor cell chemo- and radioresistance and cell protection and (2) so-called hyperreactive cysteines<sup>62</sup> with enhanced nucleophilicity are found in diverse enzymatic classes such as deubiquitinases, oxidoreductases, kinases, and in previously “undruggable” oncoproteins.

Preclinically intratumoral (IT) administration and hepatic artery infusion (HAI) of RRx-001 leads to highly selective tumor cell killing via (a) caspase-3 activation, (b) PARP cleavage, (c) cell cycle arrest, (d) superoxide/oxyradical ion generation, (e) inhibition of proteasome-associated deubiquitinases, (f) protein denaturation, (g) mitochondrial membrane depolarization, (h) endoplasmic reticulum (ER) stress, and (i) activation of type-I interferons (IFN-I).<sup>63</sup> Moreover, in the event that RRx-001 “escapes” and enters the systemic circulation, as with IV infusion, RRx-001 binds to hemoglobin on circulating red blood cells and free thiols like reduced glutathione (GSH). Consequently, toxicity from IT administration is anticipated to be minimal.

RRx-001 also has the potential to induce immunogenic cell death with the release of damage-associated molecular patterns (DAMPs) from dying cancer cells, such as extracellular ATP, calreticulin (CRT), and high mobility group box 1 protein (HMGB1) through plasma membrane disruption due to alkylation of exofacial thiols.<sup>64</sup>

The cytotoxic antiproliferative effects of RRx-001 (ABDNAZ) were studied on a panel of 12 cancer cell lines in vitro with a CCK-8 cell counting kit. The IC<sub>50</sub> values (concentration required for 50% inhibition of cell growth) ranged from 1.8 to 6.0  $\mu$ mol/L (Table 3). Comparison with cisplatin (CDDP) showed that RRx-001 (ABDNAZ) had similar activity to CDDP against four human cancer cell lines and SCC VII cells, with IC<sub>50</sub>

**Table 3. IC<sub>50</sub> of RRx-001 (ABDNAZ) against Cancer Cell Lines in Vitro**

IC <sub>50</sub> of ABDNAZ against cancer cell lines in vitro			
cell line	cell type	IC <sub>50</sub> (pmol/L)	SD
SCC VII <sup>a</sup>	SCC	1.8	0.3
22B	oral SCC	2.3	0.5
PANC-1	pancreatic carcinoma	2.3	0.7
M21	melanoma	2.6	0.5
U87	glioblastoma	2.7	0.6
RKO	colon carcinoma	3.0	0.4
HT29	colorectal adenocarcinoma	3.4	0.3
SNB75	glioblastoma	3.8	1.4
MCF-7	breast adenocarcinoma	4.0	0.5
A498	renal cell carcinoma	4.9	1.3
IMR32	brain neuroblastoma	5.1	
A549	nonsmall cell lung carcinoma	6.0	1.8

<sup>a</sup>NOTE: The SCC VII is a murine cancer cell line. All others are of human origin.

values of  $2.6 \pm 1.6 \mu\text{mol/L}$  and  $4.4 \pm 2.2 \mu\text{mol/L}$  for RRx-001 (ABDNAZ) and CDDP ( $P > 0.05$ ), respectively.<sup>65</sup>

## DISCUSSION AND CONCLUSIONS

The focus of this review is on the mechanistic effects of the minimally toxic phase III immunotherapeutic anticancer agent or “erythrophagoimmunotherapeutic”, RRx-001, which has been used as an intravenous chemosensitizer, immunosensitizer,<sup>66</sup> and a radiosensitizer in clinical trials in multiple tumor types including small cell lung cancer (SCLC), high-grade neuroendocrine carcinomas (HGNEC), metastatic colorectal cancer (CRC), brain metastases and glioblastoma (GBM), and will be trialed as a single agent in leukemia and myelodysplastic syndrome (MDS) as well as hepatocellular carcinoma (HCC) via hepatic artery infusion. In addition to radiochemosensitization and single agent activity, RRx-001 also acts as a vasculoprotector and chemoprotector<sup>67</sup> in nontransformed cells.

RRx-001-related cytoprotection is a function of microvesicle deposition from oxidatively stressed RBCs. This RBC-induced microvesiculation, which delivers microdoses of heme, hemoglobin, iron, nitric oxide, and RRx-001 metabolites to normal tissues, activates multiple conserved protective enzymatic and signaling systems<sup>68</sup> that increase adaptability and stress tolerance.<sup>69</sup> Akin to vaccine-induced immunity, RRx-001-exposed patients are, therefore, theoretically “inoculated” against larger future exposures of chemotherapy and/or radiation.

The anticancer mechanisms of RRx-001 differ depending on whether the route of administration is intravenous or intratumoral. When administered intratumorally or by intrahepatic artery infusion under balloon occlusion, RRx-001 covalently alkylates and modifies the cysteine thiols of cancer-associated proteins, which results in potent cytotoxicity and the potential for immunogenic cell death (ICD). Intravenously, the main mechanisms of action are vascular normalization, repolarization of TAMs, and downregulation of the innate antiphagocytic checkpoint, CD-47.<sup>70</sup>

Ironically, although RRx-001 is described as a novel, first-in-class agent, its mechanism of induced red blood cell dysfunction during intravenous administration is one of the most ancient by analogy with the hemoglobin-based mechanism of defense used by crocodiles, present since the Jurassic period from approximately 200 million years ago.<sup>71,72</sup> Despite the presence of hazardous microbial concentrations in their swampy habitats, crocodilian morbidity and mortality from bacterial infection (and cancer) are uncommonly rare;<sup>73,74</sup> antimicrobial and anticancer protection is attributed in part to hemocidins, proteolyzed ROS-producing residues of hemoglobin with antimicrobial activity that are liberated from the red blood cells of crocodiles (and other reptiles).<sup>75</sup> These hemoglobin-derived fragments from crocodiles potentially serve as primordial archetypes for the cytotoxic and protective activity, respectively, of the tumor-endothelial-bound RBCs and Hb-laden microvesicles released from RRx-001-bound red blood cells.

The long evolutionary history of the crocodile suggests that an important function of the RBC, and hemoglobin, may be to produce cytotoxic effects in pathogenic states such as cancer and counteractive effects in normal tissues that render them more resistant to future stress.

## ■ EXPERIMENTAL SECTION

**Synthetic Chemistry.** General Procedures. Step 1. Synthesis of TBDNAZ. Deionized water, sodium hydroxide, and HMNAZ (1 equiv) were added to a glass reaction vessel and the resulting solution was cooled to 5 °C at which time a premade solution of potassium hexanocyanoferrate (1 equiv)/sodium nitrite (4 equiv) in deionized water was charged while maintaining the solution temperature at 5 °C. The solution was agitated at 5 °C for NLT 0.5 h, at which time sodium persulfate (2 equiv) was charged while maintaining the solution temperature at NMT 20 °C. The solution was cooled to 5 °C and agitated for NLT 2 h and was then allowed to warm to RT over NLT 1 h. After reaction completion was confirmed by IPC, dichloromethane was charged, and the resulting biphasic mixture was agitated for NLT 0.25 h, allowed to settle, and then separated. The aqueous layer was extracted a second time with dichloromethane, and the organic layers were combined and sodium sulfate was charged to dry the solution. The sodium sulfate was removed by filtration, and the dichloromethane was removed via rotary evaporation under reduced pressure to a set volume to give a solution of *t*-butyl-DNAZ of approximately 15 wt %, which was used directly in the following step.

Step 2. Synthesis of RRx-001. To the 15 wt % solution of *t*-butyl-DNAZ in a glass vessel at 20 °C, was charged boron trifluoride etherate (0.1 equiv) followed by the slow addition of bromoacetyl bromide (1 equiv) over NLT 1 h while maintaining the reaction mixture temperature at NMT 25 °C. The reaction mixture was then agitated at 25 °C for NLT 12 h until reaction completion was confirmed by IPC testing. To the reaction mixture was charged dichloromethane and deionized water, and the resulting biphasic mixture was allowed to stir at 20–25 °C for NLT 2 h and the layers were then separated. To the organic layer was charged deionized water, and after agitation for NLT 0.5 h the layers were separated and the dichloromethane extracts were combined and sodium sulfate was added to dry the solution. After filtration of the sodium sulfate, ethanol was charged and the solvent was removed using rotary evaporation under reduced pressure to a set volume of solvent remained. The resulting slurry was filtered and washed with ethanol to give a wet cake of crude RRx-001, which was then charged to a clean glass reaction vessel to which ethyl acetate was charged. The mixture was stirred at room temperature for NLT 1 h until complete dissolution was achieved, at which time heptane was charged over NLT 0.5 h. The resulting slurry was filtered and the cake was washed with heptane and the material was dried in vacuo to a constant weight giving white crystalline RRx-001 in 32% overall yield (from HMNAZ) in >99% purity by HPLC.

**RRx-001 and Cisplatin Experiment Animal Data.** For the study illustrated in Table 2 and Figures 2 and 3, all animal experiments were conducted accordingly to the Guide for the Care and Use of Laboratory Animals (U.S. National Research Council, 2010). Mice (C3H, male, aged 7–8 weeks and weighing 22–25 g) sourced from Charles River were inoculated subcutaneously in the back with  $5 \times 10^5$  SCC VII tumor cells suspended in 0.05 mL of Hank's solution. Ten days post injection, mice were randomized into nine groups (tumor size range 50–300 mm<sup>3</sup>). On treatment day 0, no statistically different size in tumors was observed among groups. Tumors were measured (length and width) before treatment and after (3× per week) until tumor volumes reached 4 times the day 0 volume. The formula used to calculate tumor volume in mm<sup>3</sup>: (tumor volume =  $\pi/6 \times$  length × width<sup>2</sup>). Final data are expressed as % of pretreatment volume (day 0 measurement) as a function of days from start of treatment. Exponential regression analysis was used to calculate the quadrupling tumor growth time (4× TGT, in days). Tumor growth delay (TGD) time was defined as the difference between the treated tumors' 4× TGT compared to the same for control tumors (not treated). The 4× TGT and TGD times were determined for each animal, after which an average was calculated for each group. Body weight measurements were conducted 3× per week.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

Molecular strings and HPLC uploaded as supplementary files. The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00599>.

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### Notes

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## ■ ABBREVIATIONS USED

ABDNAZ, RRx-001; ADM, adrenomedullin; CDDP, cisplatin; CRC, colorectal cancer; GBM, glioblastoma; GI, gastrointestinal; GSH, glutathione; H&N, head and neck cancer; HAI, hepatic artery infusion; Hb, hemoglobin; HCC,

hepatocellular carcinoma; HGNEC, high-grade neuroendocrine carcinomas; HIF-1 $\alpha$ , hypoxia-inducible factor 1 alpha; HMNAZ, 1-*tert*-butyl-3-hydroxymethyl-3-nitroazetidine; MMPs, matrix metalloproteinases; MTD, maximally tolerated dose; MVs, microvesicles; NO, nitric oxide; oxLDLs, oxidized lipids; PDGF, platelet derived growth factor; PPAR $\gamma$ , peroxisome proliferator-activated receptor-gamma; PS, phosphatidylserine; RBC, red blood cell; RRHS, reverse Robin Hood syndrome; SCLC, small cell lung cancer; TAMs, tumor associated macrophages; TBDNAZ, *tert*-butyl-3,3-dinitroazetidine; TME, tumor microenvironment; TNAZ, trinitroazetidine; VEGF, vascular endothelial growth factor; WBCs, white blood cells

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