Preclinical Evaluation of the Metabolism and Disposition of RRx-001, a Novel Investigative Anticancer Agent

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ABSTRACT:

RRx-001 has shown promise as a novel cancer therapeutic agent. The disposition of RRx-001 was evaluated in vitro and after intravenous administration to rats. At both 24 and 168 h after a single intravenous administration of [14C]-RRx-001 (10 mg/kg), the majority of radiolabel was in the blood. The recovery of label in excreta was quite low, but the major route of radiolabel excretion was via the urine. In human, rat, dog, and monkey whole blood at 1 and 20 min, approximately 75% of the radioactivity is associated with RBCs and 25% with plasma. In human, at 30 min, approximately 25% of the radioactivity is associated with RBCs and 75% with plasma. Analysis by liquid chromatography/radiodetection/mass spectrometry showed that [14C]-RRx-001 reacted rapidly with whole blood to give four major soluble metabolites: the GSH and Cys adducts of RRx-001 (M1 and M2) and the corresponding mononitro GSH and Cys adducts (M3 and M4). Human Hb was incubated with cold RRx-001 in buffer, and a standard proteomics protocol was used to separate and identify the tryptic peptides. Standard peptide collision-induced fragment ions supported the structure of the peptide GTFATLSELHCDK with the alkylation on the Cys-93 locus of the Hb β chain.

Introduction

RRx-001, a novel, nonexplosive molecule modified from a class of solid rocket propellants, has shown promise as a novel cancer therapeutic agent in a number of cell lines and tumor models (Bednarski et al., 2009; Ning et al., 2012). In mouse models, RRx-001 administered intravenously as a single agent was equipotent to cisplatin while targeted intravenously as a single agent was equipotent to cisplatin while better tolerated. RRx-001 also showed activity as a radiosensitizer in both in vitro and in vivo models (Ning et al., 2012). The activity of RRx-001 is thought to be associated with a nucleophilic substitution by circulating thiol compounds and covalent binding of RRx-001 to cysteiny1 residues in Hb, followed by the generation of nitrogen oxides.

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Abbreviations: RRx-001, ethanone, 2-bromo-1-(3,3-dinitro-1-azetidinyl); DTT, dithiothreitol; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NO, nitric oxide; QWBA, quantitative whole-body autoradiography; RBC, red blood cells.
search Triangle Park, NC). Dimethylacetamide, polyethylene glycol 400, and Hb A\textsubscript{2} (ferrous stabilized human lypohlized powder) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents used were of either of analytical or high-performance liquid chromatography grade.

Human blood was obtained, but not pooled, from volunteers who had not had any known medication during the previous 7 days. Sodium heparin was used as the anticoagulant. Whole blood from rats (lot number RATBREC.137985SM) was obtained from Bioreclamation LLC (Westbury, NY). Whole blood from rhesus monkeys was collected by venipuncture and pooled from animals held at Covance. The blood was used within 24 h of receipt and was stored at approximately 5°C when not in use. The hematocrit of the blood from each species was determined before use with a CritSpin (StatSpin; Iris Sample Processing, Westwood, MA).

**Animal Studies.** Animal studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Covance. Male Wistar Hanover rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals were acclimated to the experimental conditions 3 days before use. Food and water were supplied ad libitum throughout the acclimatization and experimental period. RRx-001 and [14C]RRx-001 were dissolved in 1 part dimethylacetamide, 2 parts polyethylene glycol 400, and 33 parts normal saline. All animals were housed in individual, suspended, stainless steel, wire-mesh cages during acclimation. During the test period, animals were housed as appropriate for sample collection: beginning at least the day before dose administration and throughout the test period, animals designated for collection of excreta and expired air were housed in glass metabolism, and animals designated for pharmacokinetic and QWBA analyses were housed as appropriate for sample collection: beginning at least the day before dose administration and throughout the test period, animals designated for pharmacokinetic and QWBA analyses were housed in individual, suspended, stainless steel, wire-mesh cages.

**Rat Mass Balance.** [14C]RRx-001 (10 mg/kg; specific activity, 21.2 μCi/ mg) was administered intramuscularly via the tail vein to 12 rats. Blood (approximately 1 ml) was collected from a jugular vein via syringe and needle and transferred into tubes containing Na\textsubscript{2}EDTA anticoagulant at 0.083%, 0.25%, 0%, 0.5%, 1%, 2%, 4%, 8%, 24%, 48%, 72%, and 168 h after dose from three animals (from subgroups designated in superscript letters a, b, c, d, and e) per time point. Urine was collected in plastic containers surrounded by dry ice at 0 to 8 and 24 h and at 24-h intervals through 168 h after dose. Feces was collected in glass containers surrounded by dry ice and transferred to plastic containers at 24-h intervals through 168 h after dose. After the last collections, animals were sacrificed via exsanguination (cardiac puncture) under isoflurane anesthesia and blood (approximately 2–10 ml) was collected into tubes containing Na\textsubscript{2}EDTA. Samples were prepared for liquid scintillation counting analysis as described previously (Musick et al., 2008). Blood was mixed by inverting several times, and duplicate weighed aliquots were taken. A sufficient amount of commercial solubilizing agent was added to digest each sample. Samples were incubated for at least 1 h at approximately 60°C. 0.1 M Na\textsubscript{2}EDTA was added to reduce foaming, and 30% hydrogen peroxide was added to remove the color. The samples were allowed to sit at least overnight to allow any foaming to dissipate. The percentage of dose in blood at 168 h was calculated from the animal weights, from the dose administered and the measured blood concentration and under the assumption that the amount of blood in a rat is 7% of its body weight (Lee and Blaufox, 1985). Rat QWBA was conducted as described previously (Solon et al., 2002).

**Whole Blood/Plasma Ratio of [14C]-RRx-001.** The whole blood (1.5 ml) from rat, dog, monkey, and three individual male humans was fortified with [14C]-RRx-001 at final concentrations of 1 and 20 μM. The fortified blood samples were incubated at 37°C for 30 min and centrifuged at 3000g for 10 min to obtain plasma and RBCs. Blood-to-plasma partitioning was determined from radioactivity counts of whole blood and plasma.

**Measurement of [14C]-RRx-001 Incorporation into Hb.** The RBC pellets were washed twice with 2 ml of ice-cold saline and recentrifuged. The washed RBC pellets were lyed by the addition of 3 ml of ice-cold deionized water and set on ice for 5 min. Lyed RBC samples were centrifuged at 13,000g for 10 min to separate cell debris (pellet) and hemolysate (supernatant). Cell debris pellets were washed with 4 ml of ice-cold deionized water and centrifuged at 17,000g for 10 min. The hemolysate, in combination of the wash (supernatant), was treated with 10 ml of acidic acetonitrile (containing 0.1% hydrochloric acid) for 5 min on ice and centrifuged at 10,000g for 10 min. The obtained Hb pellets were washed with acetonitrile and dried to completeness under a stream of nitrogen at 30°C and in an oven at 60°C for 15 min. Dried and weighed Hb samples were dissolved in solution, and the radioactivity was measured by liquid scintillation counting for calculation of [14C]-RRx-001 bound to Hb (picomoles per milligram). The total covalently bound to Hb (in percentages) was calculated from [14C]-RRx-001 bound to Hb (picomoles per milligram), the Hb count (milligram per milliliter of blood) reported in literature, and the [14C]-RRx-001 concentrations in blood.

**LC/Radioflow/MS Analysis of Soluble RRx-001 Blood Metabolites.** [14C]-RRx-001 (20 μM) was incubated with rat and human blood from two individuals (one from each gender) as described above. After 0 and 30 min, the incubation was terminated with the addition of ice-cold acetonitrile (2 volumes). The samples were centrifuged at 3000g for 10 min. A fraction of each supernatant (2 ml) was dried under a stream of nitrogen and reconstituted in 200 μl of 0.1% formic acid. LC (method B) was performed using an Agilent Technologies (Santa Clara, CA) 1200 column compartment, binary pump, well plate autosampler, and diode-array detector on a Luna C18 100A column (250 × 4.6 mm, 5 μ; Phenomenex, Torrance, CA), in a column heater at 50°C. The flow rate was 1.0 ml/min with a gradient from 5% B (A is 0.1% formic acid in water; B is 0.1% formic acid in acetonitrile) to 95% B in 20 min followed by 2 min at 95% B and back to 5% B at 22.1 min. Radioactivity profiles were determined using a model 3 Betaram radiodetector (Lablogics Systems Inc., Brandon, FL). MS analyses were carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source with the following parameters: capillary temperature, 275°C; source voltage, 3.5 V. Five scan events were used. Scan 1 captures full-scan (m/z 180–900) positive spectra, with scans 2 and 3 capturing data-dependent MS/MS and MS\textsuperscript{3} on the most intense ion from scan 1 and scans 4 and 5 capturing data-dependent MS/MS and MS\textsuperscript{3} on the second most intense ion from scan 1.

**Reaction of RRx-001 with Thiols.** RRx-001, 0.2 mM in phosphate-buffered saline, pH 7.4, was mixed with 1.8 mM reduced GSH. The mixture was incubated at 50°C for 1 h and analyzed by LC/MS. LC (method A) was performed using an Agilent Technologies 1100 column compartment, capillary pump, autosampler, and diode-array detector on a Polaris 5 m C18-A column (2.1–250 mm; Varian, Inc., Palo Alto, CA), in a column heater at 50°C. The flow rate was 0.3 ml/min with a gradient from 0% B (A is 0.1% formic acid in water; B is 0.1% formic acid in acetonitrile) to 50% B in 10 min followed by 2 min at 100% B. UV data were collected at 200 nm. Mass spectrometric analyses were carried out on an LTQ ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source operated in the positive mode. The heated capillary temperature in the source was held at 250°C. Data-dependent MS/MS was conducted with 35% relative collision energy, an isolation width of 2 m/z units, an activation q of 0.25, and an activation time of 30 ms.

**Reaction of RRx-001 with Commercial Hb.** Hb A\textsubscript{2} was dissolved at 100 mg/ml (0.53 mM) in phosphate buffer, pH 7.4. RRx-001 was dissolved in dimethyl sulfoxide and added to the Hb solution to give concentrations of 0, 0.53, and 5.5 mM. Each reaction was incubated at 37°C for 18 h, quenched with 1 volume of acetonitrile, vortexed, and centrifuged to resolute the Hb. A small fraction (≤500 μg) of each reaction was aliquoted as a dried fraction. Each fraction was reconstituted in 8 M urea and 1% ProteaseMax surfactant (Promega, Madison, WI). Reduction was performed by adding 1.6 μL of 500 mM DTT and incubating for 30 min at 55°C. Alkylation was then performed in which 3.2 μl of 1 M acrylamide was used, and samples were left at room temperature for 30 min. Digestion was done with 1 μg of trypsin diluted with 5 μl of 50 mM ammonium bicarbonate. Samples were placed in 37°C for overnight digestion. The tryptic digest was acidified by adding 5 μl of 50% formic acid and centrifuged at 10,000g for 2 min. Using the 1.7 ml spin top inserts, a stage tip was loaded and equilibrated with 40 μl of acetonitrile and spun at 2600g for approximately 3 min. The stage tips were prepared for peptide binding by two wash steps of 40 μl of 0.1% formic acid and spinning at 2600g for 5 to 6 min. Seventy-five percent to 80% of the tryptic digest supernatant was loaded to the spinolute and spun at 2600g for 10 to 12 min. The sample was washed twice by loading 40 μl of 0.1% formic acid to each stage tip and spun at 2600g for 6 to 8 min. Then, the peptides were eluted with two steps of 40 μl of 60% acetonitrile and 40% of 0.1% formic acid and spun at 2600g for 3 to 5 min. Samples were evaporated to dryness in a SpeedVac.
LC/MS/MS for Identification of Alkylation Sites. The dried peptides were reconstituted in 2% acetonitrile in 0.2% aqueous formic acid, loaded onto an in-house packed nano LC C18 reversed-phase column, and run on an 80-min gradient from 2% mobile phase B to 35% mobile phase B using an Advance source (Bruker-Michrom, Auburn, CA) at a flow rate of 600 nl/min. The peptides were infused into the LTQ Orbitrap Velos set in data-dependant acquisition mode with fragmentation using the ion trap. For the database searches, all .RAW files were converted to a mzXML format and searched against the human ipi database (version 36) using 20 ppm mass tolerance for the precursor ions. Initially, the variable modifications of Met oxidation, Lys acetylation, Cys propionamide, and the assumed RRx-001 Cys, His, and N-terminal adducts were searched. In later database searches, the variable modification(s) of C5H6N1O2, C5H8N1O3, and C5H7N2O3 on Cys and His were done.

Results
Pharmacokinetics of 14C-RRx-001. Routes and rates of excretion were evaluated in rats. After a single intravenous administration of 14C-RRx-001 (10 mg/kg) to male Wistar Hanover rats, the concentrations of radioactivity in blood and plasma at the first collection time point (0.083 h) were 116,000 and 9420 ng equivalents 14C-RRx-001/g, respectively (Table 1). The volume of distribution of 14C-RRx-001-derived total radioactivity was 0.926 and 0.084 l/kg, respectively, based on plasma and blood concentration-time courses. Mean blood/plasma concentration ratios were 12.4 at 0.083 h and continually increased throughout the study to a mean value of 222 at 168 h. These results indicated that most 14C-RRx-001-derived radioactivity partitioned into rat erythrocytes.

Approximately 26% of the total radioactivity was excreted via urine in the first sampling time (0–8 h) with decreasing but measurable amounts in all subsequent collections to a total of 36.3% at 168 h. Excretion to feces was minor with 1.5% of the dose on day 1, 0.3% on day 2, and 0.06% on day 7. Insignificant amounts of radioactivity were associated with expired air or cage washes. The decrease in radioactivity in blood, plasma, and urine from maximum levels at specified times after dose is shown in Fig. 1. Clearly, the blood-associated radioactivity has a much longer lifetime than the plasma-associated radioactivity. A mean of 61.80% of the administered radioactivity was calculated to be contained in the blood at 168 h after dose. Chromatographic profiling of the radioactivity in the 0- to 8-h urine collection was performed (data not shown); however, the presence of large amounts of polyethylene glycol in the formulation obscured mass spectra from this sample. But the major peak was tentatively identified as the GSH adduct (M1) of RRx-001 by concordance of retention time to that of an authentic reference standard. The other peaks were not identified.

Tissue Distribution of Radioactivity. QWBA was also performed in rat at 24 and 168 h after administration of 14C-RRx-001. Radioactivity was quantifiable in all analyzed matrices except bile and stomach contents at 24 h after dose. The matrix with the highest concentration of radioactivity at 24 h after dose was blood with 76,500 ng equivalents 14C-RRx-001. By 168 h, the concentrations of radioactivity declined in most analyzed tissues; radioactivity was still quantifiable in all analyzed matrices except bile, contents of various components of the gastrointestinal tract, and eye lens. 14C-RRx-001-derived total radioactivity at 168 h in blood was 69,600 ng equivalents 14C-RRx-001/g, much higher than that in any other matrix determined. The concentrations of radioactivity in tissues are presented in Supplemental Table S1 as mean tissue/blood concentration ratios. All other tissues were <0.1 tissue/blood at both time points.

In Vitro Reactivity. Preliminary experiments demonstrated that RRx-001 was stable for an extended period as formulated and dissolved in water or pH 7.4 buffer (data not shown). Upon reaction with 1 equivalent of reduced GSH in phosphate buffer at pH 7.4, RRx-001 quickly and quantitatively disappeared with loss of bromide to give a GSH adduct [(S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-3-((2-(3,3-dinitroazetidin-1-yl)-2-oxoethyl)thio)-1-oxopropan-2-yl)amino)-5-oxopentanoic acid] as observed using LC/MS and chromatographic method A. The GSH adduct of RRx-001 was designated as M1 (Fig. 2), and its structure was confirmed by accurate mass, MS/MS (Supplemental Fig. S1), and NMR (data not shown). The MS/MS spectra of

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{SG}
\end{align*}
\]

M6

\[
\begin{align*}
& \text{HO} \\
& \text{N=N} \\
& \text{N=O}
\end{align*}
\]

M7

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{SG}
\end{align*}
\]

M3

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{Br}
\end{align*}
\]

M1

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{SG}
\end{align*}
\]

M2

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{Cys-gly}
\end{align*}
\]

M5

\[
\begin{align*}
& \text{O=O} \\
& \text{N=N} \\
& \text{Cys}
\end{align*}
\]

M4

FIG. 1. Structure of RRx-001 and reactions with GSH.
M1 and the other GSH adducts show classic GSH adduct fragmentation (Wen and Fitch, 2009). Similar reaction of RRx-001 with cysteine yielded the Cys adduct [Fig. 2; M2, (R)-2-amino-3-((2-(3,3-dinitroazetidin-1-yl)-2-oxoethyl)thio)propanoic acid], confirmed by comparison with a synthetic standard, accurate mass, MS/MS (Supplemental Fig. S2), and NMR (data not shown). When M1 was treated with excess GSH, a slow reaction was observed that led to oxidation of GSH to GSSG and reductive denitration of M1 to yield M3 [(S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-3-((2-(3-nitroazetidin-1-yl)-2-oxoethyl)thio)-1-oxopropan-2-yl)amino)-5-oxopentanoic acid]. M3 was also confirmed by accurate mass and MS/MS (Supplemental Fig. S3). This reaction was accelerated in ammonium bicarbonate buffer, pH 8. In the final reaction mixture, small amounts of two other products were observed. M7 was a product [(S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-3-((2-(3-hydroxyimino)azetidin-1-yl)-2-oxoethyl)thio)-1-oxopropan-2-yl)amino]-5-oxopentanoic acid] of further reduction of M3 that appeared to have lost one oxygen to give an oxime structure (M + H = 434). The proposed structure of M7 is supported by the MS/MS (Supplemental Fig. S4). M6 is a product [(S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-1-oxo-3-((2-oxo-2-((3-nitroazetidin-1-yl)ethyl)thio)propan-2-yl)amino]-5-oxopentanoic acid) that lost both nitro groups to give a ketone (M + H = 419). In the full-scan mass spectrum of M6, the presence of coeluting ketone and hydrate (M + H = 437) forms of M6 are evident. 3-Azetidinones are expected to prefer the hydrate form (Ozone et al., 2002) but readily lose water during electrospray ionization. The proposed structure of M6 is supported by accurate mass and MS/MS (Supplemental Fig. S5). These proposed structures and reactions are shown in Fig. 2.

**Blood/Plasma Partitioning and Covalent Binding to Hb.** The distribution of total 14C-RRx-001 in blood and plasma was determined after the addition of 14C-RRx-001 (1 and 20 μM) to blood from three individual human donors, rat, dog, and monkey (Table 2). The blood/plasma ratio of 14C-RRx-001 was much higher in rats than in the other species tested. Considerable individual variability of blood/plasma ratio was observed among the three individual human samples. And more importantly, 14C-RRx-001 was found to covalently bind to Hb in a species-dependent manner. The covalent binding to Hb was 69 to 79% in rat, 22 to 30% in dog, 22 to 29% in human, and 9.8 to 20% in monkey (Table 2).

**Identification of Soluble Small-Molecule Products of RRx-001 Reaction.** The soluble metabolites from human and rat whole blood treated with 14C RRx-001 were isolated by acetonitrile precipitation after incubation for 0 and 30 min and analyzed by high-performance liquid chromatography using chromatographic method B. The representative radiochromatograms are shown in Fig. 3. The retention time of 14C-RRx-001 would be approximately 15.4 min under the high-performance liquid chromatography elution conditions; clearly, in the shortest practical sampling time, no parent drug was detected in either species. The total radioactivity of soluble metabolites was higher from human blood than that from rat, consistent with the observation that higher covalent binding of 14C-RRx-001 was found to rat Hb than to human Hb. The metabolites labeled M1 and M2 appeared to decrease over time in both species. M1, the largest peak in the 0-min chromatograms, was identified as the GSH adduct compared with a synthetic standard. M2, the third-largest peak in the 0-min human chromatogram, was identified as the Cys adduct compared with a synthetic standard. M2, the second-largest peak in the 0-min human chromatograms, was identified as the GSH adduct compared with a synthetic standard. M3 was also confirmed by accurate mass and MS/MS (Supplemental Fig. S4). M6 is a product [(S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-1-oxo-3-((2-oxo-2-((3-nitroazetidin-1-yl)ethyl)thio)propan-2-yl)amino)]-5-oxopentanoic acid] expected to form via classic metabolism of M3. M5 is the Cys-Gly adduct of RRx-001 [(R)-2-amino-3-((2-(3-nitroazetidin-1-yl)-2-oxoethyl)thio)propanamido)acetic acid] expected to be the metabolic intermediate between M1 and M2. M6 is the ketone/hydrate form of the GSH adduct. M7, the oxime product, has not yet been observed in reactions with blood.

**Identification of Human Hb Alkylation Sites.** Commercial human Hb at 0.53 mM in buffer was reacted with RRx-001 at concentrations of 0, 0.53, and 5.5 mM. The precipitated protein was reduced, and the reduced protein was subjected to tryptic digestion and analyzed by liquid chromatography–tandem mass spectrometry. The reaction of human Hb with RRx-001 was found to covalently bind to Hb in a species-dependent manner. The covalent binding to Hb was 69 to 79% in rat, 22 to 30% in dog, 22 to 29% in human, and 9.8 to 20% in monkey (Table 2).

### TABLE 2

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Rat</th>
<th>Dog</th>
<th>Monkey</th>
<th>Human 1</th>
<th>Human 2</th>
<th>Human 3</th>
<th>Human Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood/plasma ratio</td>
<td>1 μM</td>
<td>2.1 (0.11)</td>
<td>0.83 (0.011)</td>
<td>0.82 (0.009)</td>
<td>1.2 (0.083)</td>
<td>0.76 (0.008)</td>
<td>1.0 (0.039)</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>4.0 (0.10)</td>
<td>1.2 (0.093)</td>
<td>1.5 (0.27)</td>
<td>1.2 (0.019)</td>
<td>0.76 (0.003)</td>
<td>1.0 (0.041)</td>
</tr>
<tr>
<td>Percentage bound to Hb</td>
<td>1 μM</td>
<td>69</td>
<td>23</td>
<td>9.8</td>
<td>24</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>79</td>
<td>30</td>
<td>20</td>
<td>21</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>
treated with acrylamide (to protect residual cysteines), and proteolyzed with trypsin to yield smaller peptide fragments. LC/MS/MS was used to separate and identify the tryptic peptides. Base peak chromatograms from these samples showed that most major tryptic peptides were unchanged by RRx-001 treatment. Among the smaller peaks, altered peptides were sought through automated software search and manual inspection. The small peak at 26.1 min gave the full-scan spectrum shown at the top of Supplemental Figure S6. Among the ions present are the triply charged ion for \[m/z=498.2431\] (predicted monoisotopic \[m/z=498.2420\]) and the corresponding doubly charged ion at \[m/z=746.8595\] (predicted monoisotopic \[m/z=746.8591\]). The sequence for this peptide is GTFATLSELHCDK and for the native peptide includes the C3H5NO alkylation from acrylamide on Cys-93.

The bottom panel of Supplemental Figure S6 shows the corresponding region of the high-dose treated chromatogram. Many peptide ions remain unchanged, but the peaks for the native C3H5NO alkylated peptide are greatly reduced, and new triply charged ion packets at monoisotopic \[m/z=511.5749\] and 517.5785 are present. Along with the corresponding doubly charged ions at \[m/z=766.8581\] and 775.8632, these \[m/z\] values translate to two peptides with sequence GTFTATLSELHCDK, but now altered with additional C5H5NO2 or C5H7NO3 groups. The \[m/z=511.57\] and 517.58 ions in the chromatograms from low- and high-dose incubations have indistinguishable retention times at 26.5 min, slightly longer in all cases compared with the retention of the native alkylated peptide (26.3 min). The calculated masses for the alkylating groups, C5H5NO2 or C5H7NO3, differ by one water molecule and are proposed to represent

### TABLE 3

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Name</th>
<th>Predicted M + H</th>
<th>Retention Time</th>
</tr>
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<tr>
<td></td>
<td>RRx-001</td>
<td>267,9564</td>
<td>12.8</td>
</tr>
<tr>
<td>M1</td>
<td>GSH adduct</td>
<td>495,1140</td>
<td>9.0</td>
</tr>
<tr>
<td>M2</td>
<td>Cysteine adduct</td>
<td>309,0499</td>
<td>7.8</td>
</tr>
<tr>
<td>M3</td>
<td>Mononitro GSH adduct</td>
<td>450,1289</td>
<td>6.9</td>
</tr>
<tr>
<td>M4</td>
<td>Mononitro Cys adduct</td>
<td>264,0649</td>
<td>4.7</td>
</tr>
<tr>
<td>M5</td>
<td>Cys-gly adduct</td>
<td>366,0714</td>
<td>8.0</td>
</tr>
<tr>
<td>M6</td>
<td>Dihydroxy GSH adduct (ketone form)</td>
<td>437,1337</td>
<td>6.1</td>
</tr>
<tr>
<td>M7</td>
<td>Mononitroso GSH adduct</td>
<td>434,1340</td>
<td>6.5</td>
</tr>
</tbody>
</table>

All metabolite masses were accurate to 5 ppm.
an in-source fragmentation and thus only one solution structure (corresponding to the Hb form of M6). A second altered peptide was detected by targeted examination of an extracted ion chromatogram calculated for a peptide alkylated in an analogous way to M3 with C₂H₄N₂O₂. This triply charged ion m/z 521.9089 eluted at 29.2 min-in much less abundance than the 511.5749 and 517.5785 triply charged peptide ions. The expected N-(3,3-dinitroazetidinyl)acetyl derivative (analogous to M1) would have a triply charged ion at m/z 536.9040 but was not detected. No other drug-related peptides could be detected using searches for peptides modified with C₂H₄NO₂, C₂H₅NO₃, C₂H₆NO₃, or C₂H₇NO₃. This triply charged ion isoelectronic with O₂ and bind strongly to heme iron (Sohl et al., 2004), so they might be difficult to extract and detect.

Disappearance of the parent drug is rapid and quantitative upon intravenous administration to blood. Based on the rapidity of reaction, no intact drug is likely to reach a peripheral site of action; the activities must be mediated through the metabolites of RRx-001. GSH is the major circulating small-molecule thiol compound, present primarily in RBCs; plasma has limited free GSH. Accordingly, we found that the RRx-001 GSH adduct M1 was the major small-molecule metabolite in both rat and human blood. GSH S-transferase is known to be active in erythrocytes (Loft et al., 2000), but its possible catalysis of this (already fast) reaction was not investigated. Liver GSH S-transferase should not be exposed to RRx-001. Our studies of blood/plasma ratios showed considerable plasma radioactivity levels after a 30-min ex vivo incubation with whole blood. This radioactivity was not directly characterized but is likely due to adducts such as M1 to M7 formed in erythrocytes and actively transported out to the plasma. GSH adducts are known to be actively transported by MRP1 and related transporters (Ortega et al., 2011) out of RBCs (Köck et al., 2007).

Cysteine is known to have a much lower blood concentration (Lang et al., 2001). The Cys adduct M2 was also detected in human blood, but at lower levels, and appeared to have limited stability. As the denitrated metabolites M3 and M6 were observed in vitro in the presence of high GSH concentration, these may be formed inside RBCs before transport out of the cell. Cancer cells typically have overexpressed multidrug resistance-associated protein, and any M1 or other amino acids that permeated into the cell would be expected to be transported out rapidly, suggesting that intracellular concentrations of these amino acids would be very low. Therefore, it is unlikely that these metabolites in themselves are the cause of peripheral anticancer effects.

RRx-001 does deplete GSH from erythrocytes and thus potentially could affect cancer cell GSH levels. The extent to which RRx-001 gives rise to M3 and or M4 (or the equivalent Hb forms), a total of 3 equivalents of thiol have been consumed. The effect of GSH in cancer cell death is multifactorial (Ortega et al., 2011). However, the relatively low effective dose of RRx-001 compared with free thiol concentration (~2–5 mmol/kg, in humans) (Wardman, 2007) suggests that GSH depletion is only one of the multifactorial mechanisms contributing to the observed activity.

Isolation of Hb from rat blood treated with 14C-RRx-001 showed that the majority of the RBC-associated radioactivity was covalently linked to the Hb. For dog, monkey, and man, the percentage associated with Hb varied from 10 to 30%. The very high covalent binding in rat blood can be accounted for by the unusually high nucleophilic reactivity of the Cys-125β found in rat globin versus other vertebrate species (Neis et al., 1984; Miranda, 2000). Comparison of tryptic peptides between untreated and RRx-001-treated human Hb showed that Cys-993 is the dominant site of alklylation, analogous to reported alklylation by methyl bromide and acrylamide (Ferranti et al., 1996; Basile et al., 2008). Following the standard proteomic protocol of DTT reduction, the observed peptide adducts had exclusive adducts with the ketoazetidinyl and mononitroazetidinyl groups. Similar evidence for the Hb forms corresponding to M6 and M3 was also observed in a comparable standard protocol LC/MS/MS analysis of the tryptic digest of Hb isolated from human blood incubated with unlabeled RRx-001, but the chromatogram was much more complex (data not shown). Confirmation of the specific Hb adducts formed in man at low dose (and in the presence of endogenous thiol concentra-
Less direct effects on Hb’s normal modulation of NOx levels are also possible. We have shown that RRx-001 alkylates Cys-β93 and Cys-γ93 is known to be critical to nitric oxide transfer (Allen et al., 2009). In addition, Cys-β93 forms mixed disulfides with GSH and plays a pivotal role in the cooperative activities of Hb, GSH, and nitric oxide (Bunn and Forget, 1986; Thomas et al., 2003; Singel and Stamler, 2005). Recent studies have implicated that Cys-β93 modification or mutation can increase nitric oxide production from nitrite (Shiva et al., 2011), and RRx-001 functionalized Hb has been shown to produce significantly more nitric oxide from nitrite under hypoxic conditions compared with native Hb (F. Kuypers, M. Fens, and J. Scicinski, unpublished observation). Additional studies to define the mode of action of RRx-001 with models of functional Hb and/or analytical speciation of the precise metabolic fate of the RRx-001 nitro groups are in progress.

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Authorship Contributions

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