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

Jan Scicinski, Bryan Oronsky, Michael Taylor, Gang Luo, Timothy Musick, Joseph Marini,
Christopher M. Adams, William L. Fitch

RadioRx Inc., Mountain View, CA (JS,BO); Covance Laboratories Inc. Madison, WI (GL, TM, JM), NonClinical Safety Assessment, Mountain View, CA (MT), Dept of Anesthesiology (WLF) and Chemistry (CA), Stanford University, Stanford, CA
Metabolism and disposition of RRx-001

William L. Fitch, 178 Park Ave. Palo Alto, CA 94306

Phone: 6504501400

Email: bfitch@stanford.edu

Number of pages 31
Number of tables 3
Number of figures 3
Number of references 37
Number of words in abstract 246
Number of words in introduction 291
Number of words in discussion 1084

ABBREVIATIONS: RRx-001, Ethanone, 2-bromo-1-(3,3-dinitro-1-azetidinyl)-; DTT, dithiothreitol; Hb, hemoglobin; LC, liquid chromatography; LSC, liquid scintillation counting; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NO, nitric oxide; QWBA, quantitative whole body autoradiography; RBC, red blood cells
ABSTRACT

RRx-001, has shown promise as a novel cancer therapeutic agent. The disposition of RRx-001 was evaluated \textit{in vitro} and after intravenous (iv) administration to rats. At both 24 and 168 h after a single iv administration of $^{14}$C-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 kidney, with approximately 26% in the urine by the first 8 h and decreasing amounts in all subsequent collections to a total of 36.3% by 168 h. The partitioning of total radioactivity in red blood cells (RBC) and plasma was determined following \textit{in vitro} addition to human, rat, dog and monkey whole blood at 1 and 20 µM. In rat, at 30 min about 75% of the radioactivity is associated with RBC and 25% with plasma. In human, at 30 min about 25% of the radioactivity is associated with RBC and 75% with plasma. Analysis by LC/radiodetection/MS showed that $^{14}$C-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 hemoglobin was incubated with cold RRx-001 in buffer and a standard proteomics protocol 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 beta chain.
Introduction

RRx-001, a novel, non-explosive 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 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 cysteinyl residues in hemoglobin followed by the generation nitrogen oxides.

Several hypotheses have been proposed to account for the biological effects of RRx-001. 1)RRx-001 could alkylate glutathione (GSH) and other endogenous thiols, depleting the blood’s ability to modulate redox reactions. GSH depletion is well known in cancer chemotherapy (Ortega et al., 2011). 2)RRx-001 could increase circulating nitric oxide, a known radiosensitizer (Wardman et al., 2007). Nitric oxide can increase blood flow to tumors through cGMP mediated vasodilation. Nitric oxide could be increased due to RRx-001 either by a) covalently binding to Hb, altering the blood transport or Hb mediated production rate of nitric oxide(Hon et al., 2010) or b) being metabolized to forms of NOx, directly acting as a nitric oxide or nitrite prodrug (Scatena et al., 2010).

There has been a recent resurgence of interest in drugs that bind irreversibly and covalently to their targets (Singh et al., 2011). However the key to successful development outcomes for compounds that bind irreversibly is selectivity (Oronsky et al., 2012). This series of studies was undertaken to understand the selectivity of binding of RRx-001 as well as its disposition in preclinical species and in man.
Materials and Methods

Materials. The $^{14}$C-RRx-001 was synthesized by ViTrax Inc. (Placentia, CA), with the label in the carbonyl group. The specific radioactivity was 56.7 mCi/mmol, and the radiochemical purity was 97.6%. Nonradiolabeled RRx-001 was prepared by ATK Aerospace Systems, (Corrine, UT)(Straessler et al., 2012). RRx-001-GSH and RRx-001-Cys were synthesized by Scynexis (Research Triangle Park, NC). Dimethylacetamide, polyethylene glycol 400 and Hb A0, (ferrous stabilized human lyophilized powder) were purchased from Sigma Co. All other reagents and solvents used were commercially available and 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 No. RATBREC.137985M) was obtained from Bioreclamation LLC (Westbury, NY). Whole blood from dogs and monkeys was collected by venipuncture and then 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 using 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. All animals were acclimated to the experimental conditions 3 days prior to use. Food and water were supplied ad libitum throughout the acclimatization and experimental period. RRx-001 and $[^{14}$C]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 in individual, suspended, stainless steel wire-mesh cages.

**Rat Mass Balance** $[^{14}\text{C}]RRx\text{-}001$ (10 mg/kg, specific activity $21.2 \mu\text{Ci/mg}$) was administrated intravenously via the tail vain to 12 rats. Blood (approximately 1 ml) was collected from a jugular vein via syringe and needle and transferred into tubes containing Na$_2$EDTA anticoagulant at $0.08^a$, $0.25^b$, $0.5^c$, $1^d$, $2^a$, $4^b$, $8^c$, $24^d$, $48^a$, $72^b$ and $168^e$ h postdose from three animals (from subgroups designated $\text{abcde}$)/time point. Urine was collected in plastic containers surrounded by dry ice at 0-8 and 8-24 h and at 24-hour intervals though 168 h postdose. Feces was collected in glass containers surrounded by dry ice and transferred to plastic containers at 24-hour intervals though 168 h postdose. After the last collections, animals were sacrificed via exsanguination (cardiac puncture) under isoflurane anesthesia and blood, (approximately 2 to 10 ml) was collected into tubes containing Na$_2$EDTA. Samples were prepared for LSC analysis as previously described (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 hour at approximately $60^\circ\text{C}$, 0.1 M Na$_2$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 % of dose in blood at 168 h was calculated from the animal weights, dose administered, ng equivalent/g measured and 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 previously described (Solon et al., 2002).
Whole Blood/Plasma Ratio of $^{14}$C-RRx-001. The whole blood (1.5 ml) from rat, dog, monkey, and 3 individual male humans was fortified with $^{14}$C-RRx-001 at final concentrations of 1 and 20 µM. The fortified blood samples were incubated at 37°C for 30 min, and then centrifuged at 3,000 x g for 10 min to obtain plasma and RBC. Blood-to-plasma partitioning was calculated from radioactivity counts of whole blood and plasma.

Measurement of $^{14}$C-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 lysed by the addition of 3 ml ice-cold deionized water and set on ice for 5 min. Lysed 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 then centrifuged at 17,000g for 10 min. The hemolysate, in combination of the wash (supernatant), was treated with 10 ml of acidified acetonitrile (containing 0.1% hydrochloric acid) for 5 min on ice, and then centrifuged at 10,000 g 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 LSC for calculation of $^{14}$C-RRx-001 bound to Hb (pmol/mg). The total covalently bound to Hb (%) was calculated from $^{14}$C-RRx-001 bound to Hb (pmol/mg), literature reported Hb count (mg/ml of blood), and $^{14}$C-RRx-001 concentrations in blood.

LC/Radioflow/MS analysis of soluble RRx-001 blood metabolites $^{14}$C-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 minutes the incubation was terminated with an addition of ice-cold acetonitrile (2 volumes). The samples were centrifuged at 3,000 g for 10 min. A fraction of each supernatant (2 ml) was dried under a stream of nitrogen, and then reconstituted in 200 µL of 0.1% formic acid. LC (Method B) was performed using an Agilent (Santa Clara, CA, USA) 1200 column compartment, binary pump, WP autosampler and diode-array detector on a Phenomenex
Luna C18 100A, 250 x 4.6 mm 5μ column, 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 Thermo-Fisher LTQ Orbitrap XL mass spectrometer (San Jose, CA, USA) equipped with an ESI source with the following parameters: capillary temperature: 275°C; source voltage: 3.5V. Five scan events were utilized.

Scan 1 captures full scan (m/z 180–900) positive spectra with scans 2 and 3 capturing data dependent MS/MS and MS3 on the most intense ion from scan 1 and scans 4 and 5 capturing data dependent MS/MS and MS3 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 glutathione. The mixture was incubated at 50°C for 1 h and then analyzed by LC/MS. LC (Method A) was performed using an Agilent 1100 column compartment, capillary pump, autosampler and diode-array detector on a Varian (Palo Alto, CA, USA) Polaris 5m C18-A column, 2.1 x 250 mm, 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 was collected at 200 nm. Mass spectrometric analyses were carried out on a Thermo-Fisher LTQ ion trap mass spectrometer equipped with an ESI source operated in 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 A0 was dissolved at 100 mg/ml (0.53 mM) in pH 7.4 phosphate buffer. RRx-001 was dissolved in DMSO and then 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 and then
quenched with 1 volume of acetonitrile, vortexed and centrifuged to reisolate the Hb. A small fraction (< 500 µgram) of each reaction was aliquoted as a dried fraction. Each fraction was reconstituted in 8M urea, and 1% ProteaseMax surfactant (Promega, Madison, WI). Reduction was performed by adding 1.6 µl 500 mM DTT and incubating for 30 min at 55˚C. Alkylation was then performed in which 3.2 µl 1 M acrylamide was used and samples were left at room temperature for 30 min. Digestion was done with 1µg of trypsin diluted with 5µl 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 then centrifuged at 10000 g for 2 min. Using the 1.7ml spin top inserts, a stage tip was loaded and equilibrated with 40 µl of acetonitrile and spun at 2600 g for approximately 3 min. The stage tips were prepared for peptide binding by two wash steps of 40 µl 0.1% formic acid and spinning at 2600 g for 5-6 min. 75-80% of the tryptic digest supernatant was loaded to the spintube and spun at 2600 g for 10-12 min. The sample was washed twice by loading 40 µl of 0.1% formic acid to each stage tip and spun at 2600 g for 6-8 min. Then the peptides were eluted with two steps of 40 µl of 60% acetonitrile, 40% 0.1% formic acid and spinning at 2600 g for 3-5 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 columns, and run on an 80 min gradient from 2% mobile phase B to 35% mobile phase B using a Bruker-Michrom (Auburn,CA)Advance source 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 (v36) 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, N-terminal adducts were searched. In later
database searches the variable modification(s) of C₅H₆N₁O₂, C₅H₈N₁O₃ and C₃H₇N₂O₃ on Cys and His were done.
Results

Pharmacokinetics of $^{14}$C-RRx-001 Routes and rates of excretion were evaluated in rats. Following a single IV administration of $^{14}$C-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 116000 and 9420 ng equivalents $^{14}$C-RRx-001/g, respectively (Table 1). The volume of distribution of $^{14}$C-RRx-001 derived total radioactivity was 0.926 and 0.084 l/kg, respectively, based on plasma and blood concentration-time courses. Mean blood to 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 $^{14}$C-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 and plasma and urine from maximum levels at specified times postdose is shown in Figure 2. 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 postdose.

Chromatographic profiling of the radioactivity in the 0-8 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 glutathione 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 $^{14}$C-RRx-001. Radioactivity was quantifiable in all analyzed matrices except
bile and stomach contents at 24 h postdose. The matrix with the highest concentration of radioactivity at 24 h postdose was blood with 76500 ng equivalents $^{14}$C-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 (GI) tract, and eye lens. $^{14}$C-RRx-001 derived total radioactivity at 168 h in blood was 69600 ng equivalents $^{14}$C-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 glutathione in phosphate buffer at pH 7.4, the RRx-001 quickly and quantitatively disappeared with loss of bromide to give a glutathione 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 (Figure 1) and its structure was confirmed by accurate mass, MS/MS (Supplemental Figure S1), and NMR (data not shown). The MS/MS spectra of M1 and the other GSH adducts show classic glutathione adduct fragmentation (Wen and Fitch, 2009). Similar reaction of RRx-001 with cysteine yielded the Cys adduct (Figure 1, M2, (R)-2-amino-3-((2-(3,3-dinitroazetidin-1-yl)-2-oxoethyl)thio)propanoic acid), confirmed by comparison to a synthetic standard, accurate mass, MS/MS (Supplemental Figure S2) and NMR (data not shown). When M1 was treated with excess GSH a slow reaction was observed which 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 Figure S3). This reaction was accelerated in pH 8 ammonium bicarbonate buffer. 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 which appeared to have lost 1 oxygen to give an oxime structure (M+H=434). The proposed structure of M7 is supported by the MS/MS (Supplemental Figure S4). M6 is a product ((S)-2-amino-5-(((R)-1-((carboxymethyl)amino)-1-oxo-3-((2-oxo-2-(3-oxoazetidin-1-yl)ethyl)thio)propan-2-yl)amino)-5-oxopentanoic acid) having 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 Figure S5). These proposed structures and reactions are shown in Figure 1.

Blood/plasma Partitioning and Covalent Binding to Hb

The distribution of total 14C-RRx-001 in blood and plasma was determined following the addition of 14C-RRx-001 (1 and 20 µM) to blood from 3 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-to-plasma ratio was observed among the 3 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 69-79% in rat, 23-30% in dog, 22-29% in human, and 9.8-20% 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 HPLC using chromatographic method B. The representative radiochromatograms are shown in Figure 3. The
The retention time of $^{14}$C-RRx-001 would be approximately 15.4 min under the HPLC 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 $^{14}$C-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 by comparison to a synthetic standard. M2, the third largest peak in the 0 min human chromatogram, was identified as the Cys adduct by comparison to a synthetic standard. M2, the second largest peak in the 0 min human chromatograms, was identified as the GSH adduct of a mononitro analog by comparison of MS/MS to the earlier described M3. Smaller components of this mixture were identified from the MS data and designated as M4, M5, and M6 with supporting data in Table 3. M4 is the Cys adduct of the mononitro RRx-001 ((R)-2-amino-3-((2-(3-nitroazetidin-1-yl)-2-oxoethyl)thio)propanoic acid) expected to form via classic metabolism of M3. M5 is the Cys-Gly adduct of RRx-001 ((R)-2-(2-amino-3-((2-(3,3-dinitroazetidin-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, 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 as the top of Supplemental Figure S6. Among the ions present are the triply charged ion for beta chain peptide residues 83-95 at 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-β₉₃.

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 GTFATLSELHCDK 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 to 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 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 C₅H₆N₂O₃. 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 C₅H₅NO₂, C₅H₆N₂O₃, C₅H₆N₂O₄, C₅H₅NO₂, C₅H₇NO₃, C₅H₆N₂O₃ or C₅H₅N₃O₅. In addition the specific C₃H₅NO alkylated peptide from residue 1-8 of the beta chain was not detected in the control reaction; nor were the N-terminal peptide from the alpha chain or the peptide 100-127 of the alpha chain (that had previously been shown to be susceptible to methyl bromide or acrylamide alkylation) detectable. In Supplemental Table S2 the ions for the native peptide and the two new peptides
have been extracted and the chromatographic peaks integrated for the three incubation samples. Although it is difficult to directly compare quantitative results from single injection capillary LC/MS data, clearly the amount of native C₃H₅NO alkylated tryptic peptide is decreasing and RRx-001-related peptides are increasing in a dose-dependent manner.

The new peptides were further characterized by data-dependent MS/MS experiments. Supplemental Figure S7 shows the MS/MS of native tryptic peptide triply charged m/z 498.24 ion. The spectrum is dominated by y cleavage ions, y₃-y₉. These standard peptide collision induced fragment ions prove the structure of the peptide GTFATLSELHCDK and the presence of the C₃H₅NO alkylation on the Cys. Supplemental Figure S8 shows the assigned MS/MS spectrum for the m/z 511.57 ion. The spectrum shows remarkably comparable y₃-y₉ ions for this peptide with a C₅H₅NO₂ alkylation on the Cys. The spectrum of the hydrate ion m/z 517.5785 was very weak and only two minor ions could be assigned to y fragment species. The corresponding spectrum for the C₅H₆N₂O₃ peptide at m/z 521.9089 showed y₃-y₉ ions. All of the characterization data is included in Supplemental Table S3.

With the chemical reactivity of the dinitroazetidinyl group to excess thiols in mind, it was suspected that the standard proteomics protocol of high concentration DTT treatment had affected the loss of the nitro groups in the primary adduct. Attempts to perform proteomics without the DTT treatment were not successful.
Discussion

RRx-001 has novel activity in preclinical models of cancer and is currently under evaluation in a Phase 1 clinical study. RRx-001 is extremely labile in vivo. In vitro experiments demonstrated high reactivity with thiols. The reactions are shown in Figure 1. Rapid reaction with GSH or Cys gave M1 and M2. In whole blood M1 can undergo normal \( \gamma \)-glutamyl transpeptidase and peptidase M biotransformation to give M5 and M2. During bioanalytical method validation studies it was found that acivicin, a known inhibitor of \( \gamma \)-glutamyl transpeptidase (Zhang et al., 2005), partially stabilizes M1 in plasma. Presence of excess GSH leads to denitrification to M3. The formation of GSSG during the denitration of M1 to M3 implies the additional consumption of 2 equivalents of GSH (possibly through the intermediacy of an S-nitroglutathione (Balazy et al., 1998). A similar reaction on M2 could be the source of the M4 observed in whole blood. Small amounts of the ketone/hydrate M6 were observed in vitro and in whole blood; M7 is proposed as intermediate between M3 and M6. The reduction of M3 to M7 would consume additional GSH (Derbyshire et al., 2005). The hydrolysis of M7 to M6 would be expected to yield the released nitrogen as hydroxylamine; however this has not been demonstrated. M7 has not been observed in the ex vivo experiments but oximes and their tautomeric nitrosoalkanes are isoelectronic with \( \mathrm{O}_2 \) and bind strongly to heme iron (Sohl et al., 2004) so might be difficult to extract and detect.

Disappearance of parent drug is rapid and quantitative upon iv 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. Glutathione 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. Glutathione S-transferase is known to be active in erythrocytes (Lof et al., 2000) but its possible catalysis of this (already fast) reaction was not investigated. Liver glutathione S-transferase should not be exposed to RRx-001. Our studies of
blood/plasma ratios showed considerable plasma radioactivity levels after 30 min ex vivo incubation with whole blood. This radioactivity was not directly characterized but is likely due to adducts such as M1-M7 formed in erythocytes and then actively transported out to the plasma. Glutathione adducts are known to be actively transported by MRP1 and related transporters (Ortega et al., 2011) out of RBCs (Koeck 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 prior to transport out of the cell. Cancer cells typically have overexpressed MRPs 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 anti-cancer effects.

RRx-001 does deplete GSH from erythocytes 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 multi-factorial (Ortega et al., 2011). However, the relatively low effective dose of RRx-001 compared to 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 $^{14}$C-RRx-001 showed that the majority of the RBC-associated radioactivity was covalently linked to the Hb. For dog, monkey and man, the % associated with Hb varied from 10-30%. 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-β93 is the dominant site of
alkylation, analogous to reported alkylation by methyl bromide and acrylamide (Basile et al., 2008, Ferranti et al., 1996). Following the standard proteomic protocol of DTT reduction the observed peptide adducts had exclusively adducts with 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 unlabelled RRx-001 but the chromatogram was much more complex (data not shown). Confirmation of the specific hemoglobin adducts formed in man at low dose (and in the presence of endogenous thiol concentrations) will require the development of sensitive peptide MS/MS methods (Zhang et al., 2008) and was outside the scope of this study.

A most likely mode of action involves effects on circulating NOx levels. A direct effect from the conversion of RRx-001 to NOX is possible given the demonstrated loss of the nitro groups both on reaction with GSH and after alkylation to Hb. There are many enzymatic systems involved in the reduction of nitrite to nitric oxide (van Faassen et al., 2009) (Tiso et al., 2011) and the pharmacokinetics of NOX is complex (Hon et al., 2010), (Seth and Stamler, 2011, Stamler and Hess, 2010), (Schwab et al., 2010).

Less direct effects on hemoglobin’s normal modulation of NOx levels are also possible. We have shown that RRx-001 alkylates Cys-β93 and Cys-β93 is known to be critical to nitric oxide transfer (Allen et al., 2009). In addition, Cys-β93 forms mixed disulfides with glutathione and plays a pivotal role in the cooperative activities of hemoglobin, glutathione, and nitric oxide (Singel and Stamler, 2005, Bunn and Forget, 1986, Thomas et al., 2003). 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 hemoglobin has been shown to produce significantly more nitric oxide from nitrite under hypoxic conditions compared to native hemoglobin (unpublished observation). Further 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.
Acknowledgements

Thanks to C. Watson, R. Cunico, Bay Bioanalytical Laboratory, Inc. Hercules, CA, who first noted the appearance of M1, M3 and M7 in reactions of RRx-001 with GSH. Acknowledgment is given to Michael Shicker, Bonnie Jung, and Phil Manteufel from Covance for assistance with mass balance and QWBA studies.

Authorship contributions

Contributed in research design: Scicinski, Oronsky, Fitch, Taylor, Luo, Adams, Musick, Marini
Conducted experiments Fitch, Luo, Musick, Marini, Adams, Fitch
Contributed new reagents or analytic tools
Performed data analysis Fitch, Scicinski, Adams, Oronsky, Luo, Musick, Marina
Wrote or contributed to the writing of the manuscript Scicinski, Oronsky, Fitch, Luo, Taylor, Musick, Marini
References


This work was funded by RadioRx


Send reprint requests to William L. Fitch, 178 Park Ave. Palo Alto, CA 94306 Email: bfitch@stanford.edu
Legends for figures

Figure 1. Structure of RRx-001 and reactions with GSH

Figure 2. Concentrations of radioactivity in blood and plasma at specified times after a single iv administration of $^{14}$C-RRx-001 to male rats expressed as % of the concentration at 0.083 h. For urine the calculation is % of the administered dose collected in urine at each time point normalized to 100%

Figure 3 Radiochromatograms for soluble $^{14}$C-RRx-001 metabolites from rat (top) and male human (bottom) blood treated with 20 uM RRx-001 and immediately (left) or after 30 min (right) quenched with acetonitrile. The arrow shows the expected RT for RRx-001.
#### Tables

**Table 1**

Pharmacokinetic parameters for radioactivity in blood and plasma collected from male rats after a single 10 mg/kg iv administration of $^{14}$C-RRx-001

<table>
<thead>
<tr>
<th>Matrix</th>
<th>$t_{1/2}$ (h)</th>
<th>AUC$_{0-t}$ (ng eq·h/g)</th>
<th>AUC$_{0-\infty}$ (ng eq·h/g)</th>
<th>Vd (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>&gt;168</td>
<td>14900000</td>
<td>NC$^a$</td>
<td>0.084</td>
</tr>
<tr>
<td>Plasma</td>
<td>66.1</td>
<td>160000</td>
<td>200000</td>
<td>0.926</td>
</tr>
</tbody>
</table>

$^a$ Not calculated.
Table 2

Total $^{14}$C Blood to Plasma ratios and % bound to Hb in whole blood samples incubated with $^{14}$C-RRx-001. Each ratio is the mean of triplicate measurements with the standard deviation in parentheses. The human mean result is the average and standard deviation of the results for the triplicate individuals. Standard deviations of the % bound result were not calculated as individual hematocrits were not measured in the study.

<table>
<thead>
<tr>
<th>Conc.</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></td>
<td>Conc.</td>
<td>Mean(SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood-to-plasma ratio</td>
<td>1 uM</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>
</tr>
<tr>
<td></td>
<td>20 uM</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>% bound to Hb</td>
<td>1 uM</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 uM</td>
<td>79</td>
<td>30</td>
<td>20</td>
<td>21</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>
Table 3. Identified RRx-001 metabolites

<table>
<thead>
<tr>
<th>M</th>
<th>Name</th>
<th>Predicted M+H</th>
<th>RT (min) A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RRx-001</td>
<td>267.9564</td>
<td>12.8</td>
<td>15.1</td>
</tr>
<tr>
<td>M1</td>
<td>GSH adduct</td>
<td>495.1140</td>
<td>9.0</td>
<td>8.3</td>
</tr>
<tr>
<td>M2</td>
<td>Cysteine adduct</td>
<td>309.0499</td>
<td>7.8</td>
<td>7.6</td>
</tr>
<tr>
<td>M3</td>
<td>Mononitro GSH adduct</td>
<td>450.1289</td>
<td>6.9</td>
<td>5.9</td>
</tr>
<tr>
<td>M4</td>
<td>Mononitro Cys adduct</td>
<td>264.0649</td>
<td>4.7</td>
<td>4.3</td>
</tr>
<tr>
<td>M5</td>
<td>Cys-gly adduct</td>
<td>366.0714</td>
<td>8.0</td>
<td>6.2</td>
</tr>
<tr>
<td>M6</td>
<td>Dihydroxy GSH adduct (Ketone form)</td>
<td>437.1337</td>
<td>6.1</td>
<td>4.1</td>
</tr>
<tr>
<td>M7</td>
<td>Mononitroso GSH adduct</td>
<td>434.1340</td>
<td>6.5</td>
<td>Not observed in accurate mass experiments</td>
</tr>
</tbody>
</table>

All metabolite masses were accurate to 5 ppm.
Fig 2

![Graph showing the percentage of maximum vs. time (h) for blood, plasma, and urine.](image-url)