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3 **Catalytic cleavage of disulfide bonds in small molecules and linkers**
4 **of antibody- drug conjugates**

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7 Donglu Zhang*, Aimee Fourie-O'Donohue, Peter S Dragovich, Thomas H Pillow, Jack D
8 Sadowsky, Katherine R Kozak, Robert T Cass, Liling Liu, Yuzhong Deng, Yichin Liu,
9 Cornelis ECA Hop, S Cyrus Khojasteh*

10

11 **Affiliations:**

12 Drug Metabolism & Pharmacokinetics (DZ, RTC, LL, YD, CECAH, SCK), Biochemical
13 and Cellular Pharmacology (AFO, KRK, YL), Discovery Chemistry (PSD, THP), Protein
14 Chemistry (JDS), Genentech, Inc., South San Francisco, CA 94080, USA.

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16

17 **Running title: Thioredoxin and glutaredoxin catalyze cleavage of disulfide bonds**
18 **of linkers of ADC**

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20 *Correspondence to: zhang.donglu@gene.com or khojasteh.cyrus@gene.com

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31 Key Words: ADC linker cleavage, Disulfide linker, Thioredoxin, glutaredoxin

32 Abbreviations: ADC, antibody drug conjugate; PBD, pyrrolo[2,1-c][1,4]benzodiazepine;
33 GRX, glutaredoxin, GSH, glutathione; TRX, thioredoxin.

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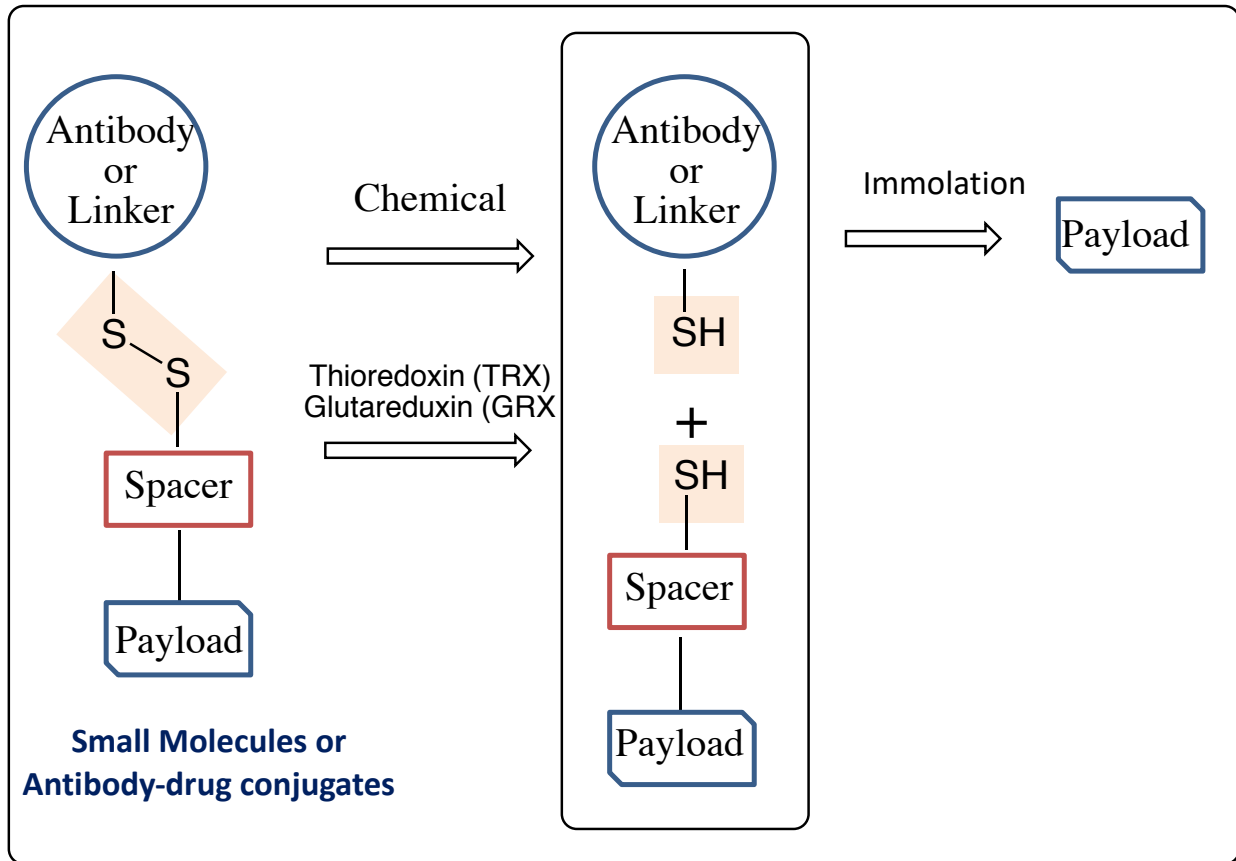
36 **Abstract**

37 Catalytic disulfide cleavage is an essential mechanism in cells for protein folding and
38 synthesis; however, the detailed enzymatic mechanism of disulfide bond cleavage in
39 xenobiotics is not well understood. This report describes an enzymatic mechanism of
40 disulfide bond cleavage in xenobiotic small molecules and antibody drug conjugate (ADC)
41 linkers. The chemically stable disulfide bonds in substituted disulfide-containing
42 pyrrolobenzodiazepine (PBD) monomer prodrugs in presence of glutathione or cysteine
43 were found to be unstable in incubations in whole blood of humans and rats. Thioredoxin
44 (TRX) and glutaredoxin (GRX) were the enzymes determined to be involved in this
45 reaction. For a diverse set of drug-linker conjugates, TRX generated cleaved products in
46 the presence of TRX-reductase and NADPH that are consistent with catalytic disulfide
47 cleavage and linker immolation. GRX was less rigorously studied mainly due to availability
48 and lower activity than TRX but its role in the catalytic cleavage was also confirmed for
49 this set of compounds. Collectively, these *in vitro* experiments demonstrate that TRX, as
50 well as GRX, can catalyze the cleavage of disulfide bonds in both small molecules and
51 ADC linkers.

52

53 **Visual Abstract**

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57 Introduction

58 The disulfide bond (C-S-S-C) is a common structural motif in proteins and has been
59 recently used in targeted drug-delivery approaches (i.e., prodrugs) (Chen and Hu 2009;
60 Vrudhula et al., 2002; Zhang et al., 2017a) that utilize high levels of the reducing agent
61 glutathione (GSH) to selectively release various cytotoxic agents in tumors (Gamcsik et
62 al., 2012; Hatem et al., 2017). Pillow et al. reported a self-immolating disulfide linker (β -
63 mercaptoethyl-carbamate, $-\text{SCH}_2\text{CH}_2\text{OCO}^-$) that can be directly attached to cysteine
64 thiols of antibodies where the cysteine residues are engineered into antibody light or
65 heavy chains (called THIOMABTM antibodies) (Pillow et al., 2017a; Pillow et al., 2017b;
66 Zhang et al., 2016). Cleavage of the disulfide linker was proposed to occur through GSH
67 or cysteine reductive cleavage of the cysteine–thiolate intermediate following conjugate
68 internalization and lysosomal proteolysis. In this mechanism, payload was released after
69 linker immolation following chemical cleavage of the disulfide bond (Pillow et al., 2017a;
70 Pillow et al., 2017b; Zhang et al., 2016). Disulfide bond linkers have also been used in
71 other antibody conjugates (Erickson et al., 2010; Kellogg et al., 2011), chemosensors
72 (Lee et al., 2013), and nanoparticles (Wang et al., 2014; Zhang et al., 2017b).

73 Pyrrolo[2,1-c][1,4]benzodiazepine (PBD monomer; **1**) and its dimer (PBD dimer; **2**) belong
74 to a class of DNA alkylators that covalently modify DNA minor grooves (Hartley, 2011).
75 Recently, several antibody drug conjugates (ADCs) using PBD analogs as drugs have
76 entered clinical trials (Jeffrey et al., 2013; Saunders et al., 2015). In the process of
77 developing the next generation of ADCs, we sought to design an ADC with a disulfide-
78 containing linker and the prodrug of a cytotoxic payload that could be selectively activated
79 by the high reducing potential present in many intratumor environments following targeted
80 antibody-mediated delivery (Figure 1) (Pei et al., 2018).

81 Disulfide bonds between cysteines are an integral part of protein structures and are
82 formed during protein synthesis, folding, and post-translational modifications. Thioredoxin
83 (TRX) and glutaredoxin (GRX) are cytosolic enzymes of 10-12 kDa in size that catalyze
84 cleavage of the disulfide bond formed between a cysteine residue and GSH, which is
85 initially formed to protect newly incorporated cysteine residues, or between cysteine
86 residues formed during posttranslational modifications (Azimi et al., 2011; Chen et al.,

87 2006; Hogg, 2003; Hogg, 2009). TRX can be located outside cells, in the cytoplasm, in
88 the nucleus, or in mitochondria with a cellular concentration of 2-12 μM and a plasma
89 concentration of up to 6 nM. TRX reductase and NADPH are required for TRX catalytic
90 activity (Holmgren and Bjornstedt, 1995; Mustacich and Powis, 2000). GRX concentration
91 in red blood cells can be 1 μM with an optimal pH 8 for catalytic activity. GRX activity also
92 requires a reductase and NADPH or GSH as a cofactor. In this study, the recombinant
93 enzymes TRX and GRX demonstrated catalytic activities for cleavage of disulfide bonds
94 in xenobiotics. The catalytic activities of disulfide cleavage in whole blood are consistent
95 with the activities of TRX and GRX, although low cofactor concentrations in blood may
96 limit their optimal catalytic activities toward xenobiotic disulfides.
97 During the process of testing for disulfide stability in buffer in the presence of GSH or
98 cysteine and whole blood, distinct stability profiles were observed for disulfides with few
99 substitutions at the adjacent carbons. These results suggested that a biological
100 mechanism exists to catalyze certain disulfide cleavages. Subsequently, we conducted
101 experiments to investigate the potential catalytic activity of TRX and GRX, two common
102 oxidoreductase enzymes that are present in whole blood (Pei et al., 2018; Bjornstedt et
103 al., 1995; Butera et al., 2014; Holmgren and Bjornstedt, 1995). Incubation of small
104 molecule disulfide compounds with TRX produced the expected products. Incubation of
105 these enzymes with the disulfide-linker ADC also produced the expected payload **2**. In
106 addition, incubation of an ADC containing both a disulfide prodrug and a disulfide linker
107 produced several products that were consistent with cleavage of either disulfide bond.
108 These results suggested that TRX and GRX can catalyze cleavage of disulfide bonds in
109 small molecules as well as in the linker of an ADC.

110

111 **Materials and Methods:**

112 Materials

113 Ammonium formate, formic acid, NADPH, human recombinant human TRX, rat liver TRX
114 reductase, and a proprietary TRX reductase inhibitor (catalog # T9199) were purchased
115 from Sigma-Aldrich (St. Louis, MO, USA). Human GRX I, rat recombinant TRX, and GRX
116 reductase were purchased from Creative Biomart (Shirley, NY). Compounds **1-10** and **12**

117 were made as described previously (Pei et al., 2018). Synthesis of compounds **11** and
118 **B8** are described in the Supplemental Information section. Human CD22 antibodies with
119 two engineered cysteine residues were generated as described previously (Ohri et al.,
120 2018; Bhakta et al., 2013; Junutula et al., 2016). ADC **13** was synthesized from the linker
121 drug **B8** as described previously (Zhang et al., 2016; Staben et al., 2016). The antibody
122 to drug ratio was 1.9 and 2.0 for ADCs **12** and **13**, respectively.

123 *In vitro* incubations in buffer or with enzymes

124 The compounds were incubated at 10 μ M with 0.03 or 0.2 mM cysteine, or 4 mM GSH,
125 in 100 mM Tris buffer pH 7.0 containing 5% methanol at 37°C. Aliquots were taken at 0,
126 1, 4, and 24 h and the samples were analyzed by LC-MS/MS.

127 Selected disulfide prodrugs **3**, **5**, and **10** at 10 μ M and ADCs **12** and **13** at 1.6 μ M (0.25
128 mg/mL) were separately incubated with human or rat recombinant TRX at 100 nM (1
129 μ g/mL), with TRX reductase at 20 nM and NADPH (5 mM), or with human recombinant
130 GRX at 100 nM (1.2 μ g/mL) and 80 μ M GSH, all in 100 μ L of Tris buffer (100 mM, pH 7.4)
131 for 1 or 2 h at 37°C in a water bath with shaking at 120 rpm. Control incubations without
132 NADPH or GSH were also included. In addition, the proprietary TRX reductase inhibitor
133 at 1 mM was used in some of incubations. Acetonitrile (0.2 mL) was added to quench the
134 reactions. After centrifugation, aliquots of 10 μ L were injected for LC/MS analysis using
135 the conditions described in next section.

136 LC/MS analysis for identification of small molecular catabolites

137 The samples from *in vitro* incubations of buffer, enzymes, or whole blood were injected
138 into a Sciex TripleTOF 5600 with a Hypersil Gold C18 column (100x2.1 mm, 1.9 μ M,
139 Thermo Scientific). The column was eluted at a flow rate of 0.4 mL/min with buffer A (0.1%
140 formic acid in 10 mM ammonium acetate) and buffer B (0.1% formic acid in 10 mM
141 ammonium acetate in 90% acetonitrile) with the following gradient profile: 5% B at 0-0.5
142 min, 5-25% B at 0.5-8 min, 25-75% B at 8-13 min, 75-95% B at 13-13.5 min, 95% B at
143 13.5-14.5 min, and 95-5% B at 14.5-15 min. All products were separated and
144 characterized by LC-MS/MS in a positive ESI ion mode. All analytes had the protonated
145 molecular ($[MH]^+$) as the major species with little source fragmentation. Full scan accurate

146 mass peak areas were used to estimate relative abundance of each species. The
147 disappearance of starting material, estimated on the basis of relative full scan peak areas,
148 was consistent with that estimated based on the relative abundance compared to time 0
149 h by MS or UV (200-350 nm). The disulfide cleavage versus time profiles were obtained
150 and percent parent remaining at individual time points was reported.

151 The identification of compounds was performed by LC/MS on a Triple TOF 5600 mass
152 spectrometer (AB Sciex) coupled with HPLC separation. The PBD-dimer (**2**) was
153 identified by a molecular ion at m/z 585.2730 (calculated at 585.2713; $C_{33}H_{37}N_4O_6$) and
154 by major fragments at m/z 504.2144 and 259.1096. Compound **11** was identified by a
155 molecular ion at m/z 781.2968 (calculated at 781.2941; $C_{39}H_{49}N_4O_9S_2$) and by a major
156 fragment at m/z 719.2991. Compound **16** was identified by a molecular ion at m/z
157 841.2889 (calculated at 841.2788; $C_{40}H_{49}N_4O_{12}S_2$) and by major fragments at m/z
158 823.2696, 705.2538, 608.2086, and 535.1915. Other compounds were identified by
159 comparison with synthetic materials.

160 Whole blood stability

161 Blood of human and rat (100 μ L of pool of mixed gender) was incubated with 10 μ M of
162 each compound (**1-10**) at 37°C for 0, 4, and 24 h ($n = 3$). Acetonitrile (300 μ L) was used
163 to quench the reaction. After vortexing and sonication for 5 min, the samples were
164 centrifuged for 10 min at 2000 xg . The supernatant (50 μ L) was mixed with 200 μ L of
165 water and 10 μ L was analyzed by LC-MS/MS. The GSH analysis was carried out with a
166 Shimadzu Nexera UPLC system coupled to a QTRAP 5500 AB Sciex in positive ion
167 mode. Mobile phase A was water with 0.1% formic acid, and mobile phase B was
168 acetonitrile with 0.1% formic acid. The chromatography was performed on a Thermo
169 HyperCarb column 50x2.1 mm, 3 μ m (Bellefonte PA, USA). Propranolol (100 nM) was
170 used as the internal standard. The calibration curve for quantitation of each compound
171 was constructed by plotting the compound to internal standard peak area ratio versus the
172 nominal concentration of the analyte with a weighted 1/x quadratic regression.

173 In preparation for the incubation of ADCs in whole blood, the vendor (Bioreclamation,
174 Westbury NY) shipped cold, whole blood overnight, and stability samples were created
175 immediately upon arrival. Initial dilutions of the ADC source material were made in buffer

176 (1X PBS, pH7.4, 0.5% BSA, 15 ppm ProClin) so that all molecules were at a concentration
177 of 1 mg/mL. This was followed by a 1:10 dilution (36 μ L of 1 mg/mL solution was diluted
178 in 324 μ L blood or buffer) to generate stability samples with a final concentration of 100
179 μ g/mL. Once mixed, 150 μ L of the whole blood/buffer stability samples was aliquoted into
180 two separate sets of tubes for the two different time points (0 and 24 h). The 0 h samples
181 were placed in a -80°C freezer and the 24 h samples were placed in a 37°C incubator
182 and shaken (~700 rpm). After 24 h, samples were removed from the incubator and stored
183 in a -80°C freezer until affinity-capture LC-MS was performed. The matrices used to
184 generate the samples were mouse (CB17 SCID), rat (Sprague-Dawley), and human.
185 Whole blood stability samples were analyzed by affinity-capture LC-MS with modifications
186 to the method described previously (Xu, et al., 2011). Briefly, streptavidin-coated (SA)
187 magnetic beads (Thermo Fisher Scientific, Waltham, MA) were washed 2x with HBS-EP
188 buffer (GE Healthcare, Sunnyvale, CA), then mixed with either biotinylated extracellular
189 domain of target (e.g., human erb2) or anti-idiotypic antibody for specific capture using a
190 KingFisher Flex (Thermo Fisher Scientific, Waltham, MA) and incubated for 2 h at room
191 temperature with gentle agitation. After the 2 h, the SA-bead/biotin-capture probe
192 complex was washed 2x with HBS-EP buffer, mixed with stability samples that were
193 diluted 1:16 with HBS-EP buffer and then incubated for 2 h at room temperature with
194 gentle agitation. After 2 h, the SA-bead/biotin-capture probe/sample complex was
195 washed 2x with HBS-EP buffer, followed by deglycosylation overnight with PNGase F
196 (New England BioLabs, Ipswich, MA). The SA-bead/biotin-capture probe/sample
197 complex was then washed 2x with HBS-EP buffer, followed by 2x washes of water
198 (Optima H₂O, Fisher Scientific, Pittsburgh, PA) and finally a 1x wash with 10%
199 acetonitrile. The beads were then placed in a solution of 30% acetonitrile and 0.1% formic
200 acid for elution and incubated for 30 min at room temperature with gentle agitation before
201 being collected. The eluted samples were then loaded onto an LC/MS (Synapt G2-S,
202 Waters, Milford, MA) for analysis.

203 ADC samples (10 μ L) were injected and loaded onto a PepSwift reversed phase
204 monolithic column (500 μ m \times 5 cm) (Thermo Fisher Scientific, Waltham, MA) maintained
205 at 65°C using a Waters Acquity UPLC system at a flow rate of 20 μ L/min with the following
206 gradient: 20% B (100% acetonitrile and 0.1% formic acid; A is 0.1% formic acid in water)

207 at 0-2 min, 35% B at 2.5 min, 65% B at 5 min, 95% B at 5.5 min, and 5% B at 6 min.
208 The column was directly coupled for online detection with Waters Synapt G2-S Q-TOF
209 mass spectrometry operated in positive ESI mode with an acquisition range of m/z 500
210 to 5000.

211 For stability data analysis, deconvolution of the raw spectrum within a selected ADC
212 elution time window was implemented with Waters BiopharmaLynx 1.3.3 software. Drug
213 loss or modifications were identified according to the corresponding mass shifts from the
214 starting ADC material. Peak labeling and the drug-to-antibody ratio (DAR) calculation
215 were performed with a custom Vortex script (Dotmatics, Bishops Stortford, United
216 Kingdom). Drug loss, cleavage, and formation of adducts were identified according to the
217 corresponding mass shifts from the starting ADC material. The relative abundance of
218 each ADC species in the analytical sample was represented by its MS signal intensity.
219 The relative ratios of ADC with different DARs were calculated by dividing the intensity of
220 the specific ADC species with the intensity of the total ADC species. DAR percent was
221 calculated as previously reported (Staben et al., 2016).

222

223 Results

224 The stability of disulfide bonds in substituted disulfide-containing PBD monomer prodrugs
225 (**3-10**) was tested in incubations with GSH and cysteine (Figure 1). Subsequent tests
226 showed that some stable compounds, which had been selected from a
227 glutathione/cysteine reduction assay, were unstable in incubations in whole blood of
228 humans and rats. Compounds **3-6**, all of which contain the disulfide prodrug functionality,
229 were relatively stable in incubations with 4 mM GSH or 30 μ M cysteine up to 24 h at 37°C
230 (Table 1). In incubations of human or rat whole blood, however, these compounds had a
231 low percent remaining of the starting material at the end of the incubation and were,
232 therefore, unstable under these conditions (Table 1 and Figure S1). In addition, more
233 enzymatic cleavage was observed in rat blood than in human blood. Interestingly,
234 disulfide compounds **7-10**, which have more substitutions on the carbon atoms next to
235 the disulfide bond, showed improved stability compared to the less substituted disulfide
236 compounds **3-6** in both GSH/cysteine reduction assays and whole blood incubations. The
237 product from these whole blood incubations was the expected PBD monomer **1**. Figure 2
238 (pathway A) shows that chemical reduction of the disulfide bond followed by immolation
239 of the β -mercaptoethyl-carbamate linker produced PBD monomer **1**. To better understand
240 the role of free thiols in the degradation of these disulfide compounds in blood, we also
241 determined GSH and cysteine concentrations in whole blood and in blood cells of rats
242 and humans (Table S1). GSH and cysteine concentrations in the plasma of rats and
243 humans were relatively low at single μ M ranges. On the other hand, GSH concentrations
244 in blood cells reached mM ranges, while cysteine concentrations were at low μ M ranges.
245 These results are consistent with literature values (Gamcsik et al., 2012; Hatem et al.,
246 2017; Johnson et al., 2008; Otani et al., 2011; Sato et al., 2005). In these *in vitro*
247 incubations, the concentrations of the reductants were higher than those found in whole
248 blood or blood cells of human or rat, yet they resulted in much less disulfide cleavage.

249 Figures 3A and 3C show product profiles of ADC **12** from 24 h incubations in whole blood
250 of human and rat. ADC **12**, which contains a disulfide linker, showed relatively good
251 stability with only low levels of deconjugation products (**P-1**) observed in both human and
252 rat blood samples after a 24 h incubation at 37°C.

253 A probe ADC molecule **13** was designed to contain both a disulfide prodrug functionality
254 and a disulfide linker on PBD dimer **2**. Compared to ADC **12**, which was relatively stable
255 in whole blood incubations (Figures 3A and 3C), the prodrug ADC **13** was not stable in
256 whole blood of human or rat and showed the formation of multiple products. These
257 products resulted from the loss of one or two prodrug functionalities (-196 Da), or the loss
258 of one or two linker drugs (-LD), or a combination of these degrading processes. Figures
259 3B, D, E, F show the degradation profiles of ADC **13**. Although the antibody-related
260 product profiles look similar between human and rat, the payload-related product profiles
261 are very different (Figure 4). In incubations with human blood, the intramolecular disulfide
262 **16** was the dominant product with payload **2** and prodrug **11** as minor products. In
263 contrast, incubation in rat blood generated all three compounds (**2**, **11**, **16**) as prominent
264 products. Rat blood presumably has a higher level of degradation activity than human
265 blood, leading to a greater extent of cleavage of **16** and a higher concentration of **2**.

266 Figure 3 shows the degradation pathway of ADC **13** in human and rat blood. In this
267 pathway, cleavage of the linker disulfide bond resulted in formation of intermediate **14**,
268 which can quickly immolate to form **11**. Disulfide cleavage in the prodrug functionality of
269 ADC **13** generated intermediate **15**, which can immolate to form conjugate **12** (which is
270 also labeled as P3 in the chromatograms). Intermediate **15** can also lead to formation of
271 **16** through intramolecular disulfide formation. Immolation of the less substituted β -
272 mercaptoethyl-carbamate linker in **15** might be slower than that of the β -
273 mercaptoisopropyl-carbamate linker in **14** (Zhang et al., 2018), which would allow for
274 sufficient time to form the intramolecular disulfide **16**. Cleavage of the prodrug disulfide
275 bond appeared to be operative in both human and rat blood. Further degradation of **11**,
276 **12**, or **16** released payload **2**. In this context, cleavage of the linker disulfide bond could
277 be a minor pathway in human blood but a major pathway in rat blood. ADC **13** primarily
278 underwent prodrug disulfide bond cleavage in both human and rat blood to form the
279 intramolecular disulfide **16**. The antibody-related product profile also indicated that
280 cleavage of the prodrug disulfide bond lead to loss of a 196 Da species to form P1-P4
281 (Figures 3B and D).

282 The disulfide stability data from the PBD monomer model compounds **3-6** and ADC **13**
283 suggest that there may be an enzymatic mechanism in whole blood that causes
284 instability of these otherwise stable disulfides. Figure 2 (pathway B) shows a proposed
285 mechanism for catalysis of disulfide cleavage through linker immolation, leading to the
286 release of PBD monomer **1** or PBD dimer **2**.

287 There is a good level (nM to μ M) of TRX and GRX in whole blood in human and animals
288 (Pei et al., 2018; Bjornstedt et al., 1995; Butera et al., 2014; Holgren et al., 1995), which
289 may have caused the instability of the disulfide compounds **3-6**. Experiments were,
290 therefore, conducted using recombinant TRX and GRX enzymes in incubations with these
291 disulfide compounds. The results showed that an appreciable level of PBD monomer **1**
292 was produced from compound **3** in the presence of TRX, TRX-reductase, and NADPH;
293 however, GRX did not form any PBD monomer **1** from **3** in the presence of GSH at a
294 concentration (80 μ M) that did not chemically cleave the disulfide bonds (Figure 5A).
295 Surprisingly, GRX formed a similarly low level of PBD monomer **1** from compounds **5** and
296 **10** (Table 2S), which have different disulfide structures from that in compound **3**. These
297 results suggest that GRX has different specificity and perhaps a narrower range of
298 substrate acceptance than does TRX for catalytic cleavage of disulfide bonds.

299 We next investigated whether the disulfide linker in ADC **12** is subject to catalytic disulfide
300 cleavage by TRX or GRX. Figure 5B (conditions a, b, and c) showed that incubations of
301 ADC **12** with both human and rat TRX produced PBD dimer **2** after 1-2 h incubations. In
302 comparison, a minimal level of PBD dimer **2** formed in incubations of human TRX without
303 NADPH or in the presence of a TRX-reductase inhibitor (Figure 5B, conditions g and h).
304 Incubations of ADC **12** with GRX produced a lower level of PBD dimer **2** (3-4 fold lower
305 than TRX incubations) (Figure 5B, conditions d, e, and f). No PBD dimer **2** was formed in
306 the control incubation in the presence of 80 μ M GSH cofactor without GRX. The TRX-
307 mediated cleavage of the linker disulfide bond, therefore, appeared to be time- and
308 NADPH-dependent and inhibited by a TRX reductase inhibitor (Figure 5B).

309 Figure 5C shows PBD-related product formation from incubation of ADC **13** with TRX or
310 GRX under various conditions. Payload **2** was the main product from incubations with
311 human and rat TRX (conditions a, b, and c), while prodrug **11** was a prominent metabolite

312 formed (conditions a, b, c, and d). This activity was not observed when TRX reductase
313 inhibitor was present in the incubation or no NADPH was used (conditions g and h). The
314 intramolecular disulfide **16** was a minor product of both TRX and GRX incubations. PBD
315 dimer **2** was identified following incubations with TRX in the presence of TRX-reductase
316 and NADPH or GRX at a concentration of GSH that did not cause any level of disulfide
317 linker cleavage (Figure 5C). Similar to whole blood incubations, cleavage of the disulfide
318 linker led to formation of proposed intermediate **14** that could quickly immolate to form
319 prodrug **11**. The disulfide in prodrug **11** could be further cleaved to form payload **2**.
320 Alternatively, disulfide cleavage in the prodrug functionality produced intermediate **15** that
321 underwent relatively slow immolation, leading to formation of intramolecular disulfide **16**.
322 Figure 2S shows the antibody-related product profiles of ADC **12** and **13** in the presence
323 of human or rat TRX as well as human GRX. The conjugate was more extensively
324 degraded in the incubation with TRX than in whole blood (Figures 3B and 3D), as
325 evidenced by an antibody product formed from complete linker cleavage that was not
326 observed in whole blood incubations. Either the prodrug disulfide bond or linker disulfide
327 bond in ADC **13** could be cleaved by TRX or GRX to form a mix of products (Figure 5C).
328 GRX showed a low level of catalytic activity for both types of disulfide bonds.

329 The linker disulfide bond in ADC **12** was also cleaved by TRX, but the extent of cleavage
330 was much less than that for ADC **13** as a significant amount of starting ADC **12** remained
331 in parallel incubations (Figure 2S, conditions b and d). Comparison of the antibody-related
332 product profiles of ADC **12** and ADC **13** in the presence of TRX clearly showed more
333 extensive degradation of ADC **13** than **12** (Figure 2S). Overall, the prodrug disulfide bond
334 is more susceptible to catalytic cleavage by enzymes than is the disulfide linker bond.

335

336 Discussion

337 Incubations of the disulfide-containing prodrugs **3**, **5**, and **10** with recombinant TRX and
338 GRX in the presence of cofactors showed that the catalytic activity of these enzymes is
339 required to cleave the disulfide bonds in these small molecules. Likewise, incubations of
340 ADC **12** showed the importance of the TRX and GRX enzymes in cleavage of the linker
341 disulfide bond. Incubation of ADC **13** further demonstrated that TRX and GRX can

342 catalyze cleavage of both prodrug and linker disulfide bonds from the same molecule.
343 These data clearly support catalytic disulfide cleavage activities of TRX and GRX. Both
344 antibody product- and PBD product-profiles were qualitatively similar between the
345 reactions of disulfide compounds with TRX or GRX enzymes in whole blood. Immobilization
346 following disulfide cleavage for the disulfide-containing compounds selected in these
347 studies facilitated product analysis and clean assessment of disulfide cleavage. The
348 disulfide linker cleavage in ADC **12** and **13** suggested that the disulfide bonds that connect
349 engineered-in cysteines and payloads are accessible to enzymes. The variable stabilities
350 of the ADC conjugates from the cysteines engineered at different locations on an antibody
351 may suggest different accessibilities of these linker disulfide bonds to TRX or GRX
352 enzymes (Ohri et al., 2018). Neither of these enzymes is expected to cleave inner
353 disulfide bonds such as inter-chain disulfides of an antibody.

354 Cellular disulfide cleavage has been implied in a number of previous reports of cell
355 incubations (Zhang et al., 2017b; Butera et al., 2014). TRX has been shown to catalyze
356 the allosteric disulfide bonds in proteins (Hogg, 2003; Hogg, 2009). To our knowledge,
357 there is no prior report of experimental data showing catalytic cleavage of disulfide bonds
358 in xenobiotics by a particular enzyme. Disulfide-containing drugs are rare, which may limit
359 investigations into catalytic disulfide cleavage. Romidepsin is a disulfide-containing
360 HDAC inhibitor prodrug, which acts as an anticancer agent to treat cutaneous T-cell
361 lymphoma (Amengual et al., 2018), and it binds to the thiol in the binding pocket of Zn-
362 dependent histone deacetylase upon disulfide cleavage. TRX and GRX could also be
363 involved in the metabolism of thiol-containing drugs such as albitiazolium (Caldarelli et
364 al., 2012).

365 Collectively, results support that TRX and GRX in whole blood may catalyze degradation
366 of disulfide-containing prodrugs and disulfide-linker ADC conjugates. Through careful
367 product characterization of disulfide-containing molecules, we demonstrated that TRX
368 and GRX catalyze the disulfide bond cleavage in xenobiotics; thus, representing a new
369 function of TRX and GRX.

370

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374

375 **Authorship Contributions.**

376 Participated in research design: Zhang, Khojasteh.

377 Conducted experiments: Zhang, Fourie-O'Donohue, Dragovich, Pillow, Sadowsky,
378 Kozak, Cass, Liu, Deng, Liu.

379 Contributed new reagents or analytic tools: Zhang, Dragovich, Pillow, Sadowsky.

380 Performed data analysis: Zhang, Liu, Deng, Liu, Khojasteh.

381 Wrote or contributed to the writing of the manuscript: Zhang, Fourie-O'Donohue,
382 Dragovich, Pillow, Sadowsky, Kozak, Cass, Liu, Deng, Liu, Hop, and Khojasteh.

383

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489

490 **Figure legends**

491 Figure 1. Chemical structures of the disulfide-containing prodrugs and ADC conjugates
492 in this study.

493 Figure 2. Chemical (A) and catalytic (B) disulfide cleavage mechanisms for disulfide-
494 containing prodrugs and disulfide linker-containing ADCs.

495 Figure 3. Degradation product LC-MS profiles of ADC **13** from human and rat blood
496 incubations.

497 Figure 4. Proposed payload-related product formation pathways of ADC **13** in incubations
498 in human and rat blood.

499 Figure 5. PBD-related product LC-MS profiles of disulfide **3** (A), ADC **12** (B), and ADC **13**
500 (C) in catalytic reactions by TRX and GRX.

501

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503
504 Table 1. Stabilities of disulfide-containing prodrugs in incubations with GSH, cysteine, or
505 human and rat whole blood.

506

507

Compound	% Disulfide remaining			
	GSH @4.0 mM ^a	Cysteine @30 μM ^a	Rat whole blood ^b	Human whole blood ^b
3	56	100	0.1	5
4	21	99	0.3	24
5	68	100	5	80
6	44	98	1	45
7	88	100	87	120 ^c
8	100	100	19	120 ^c
9	100	100	100	108 ^c
10	82	99	124 ^c	124 ^c

508 ^aDisulfide cleavage in the presence of the indicated concentration of GSH or cysteine at 24 h.
509 See Supporting Information for additional details.

510 ^bThe disulfide was incubated in whole blood, and aliquots were analyzed at 24 h. Procaine (10
511 μM) was used as positive control incubation with <3% remaining after 24 h.

512 ^c Higher than expected % remaining was reported. This is more likely due to the
513 bioanalytical variability.

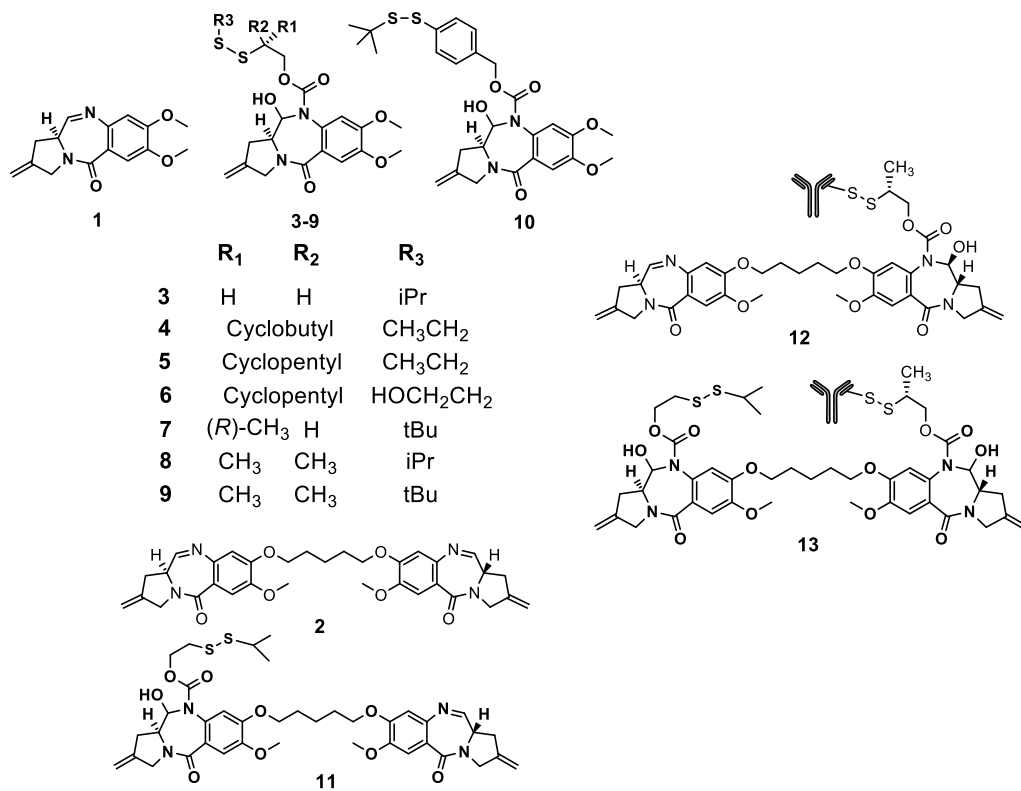
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517 Figure 1.

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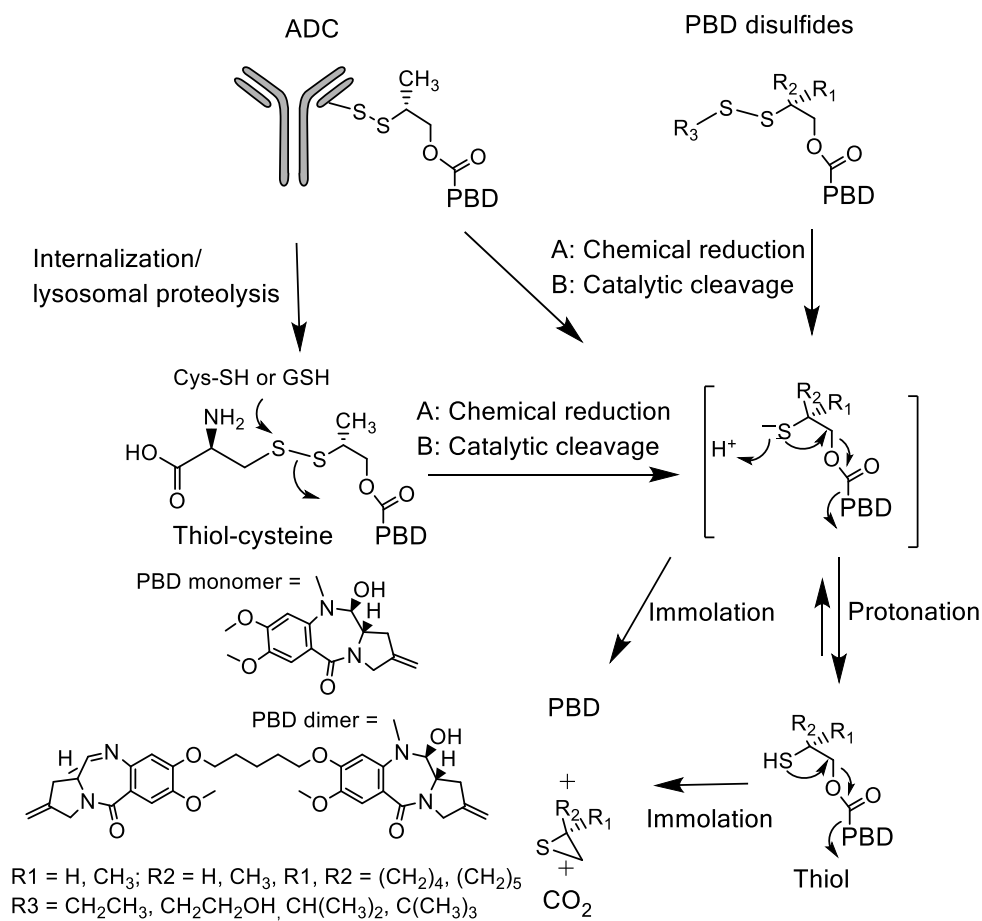
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522 Figure 2.

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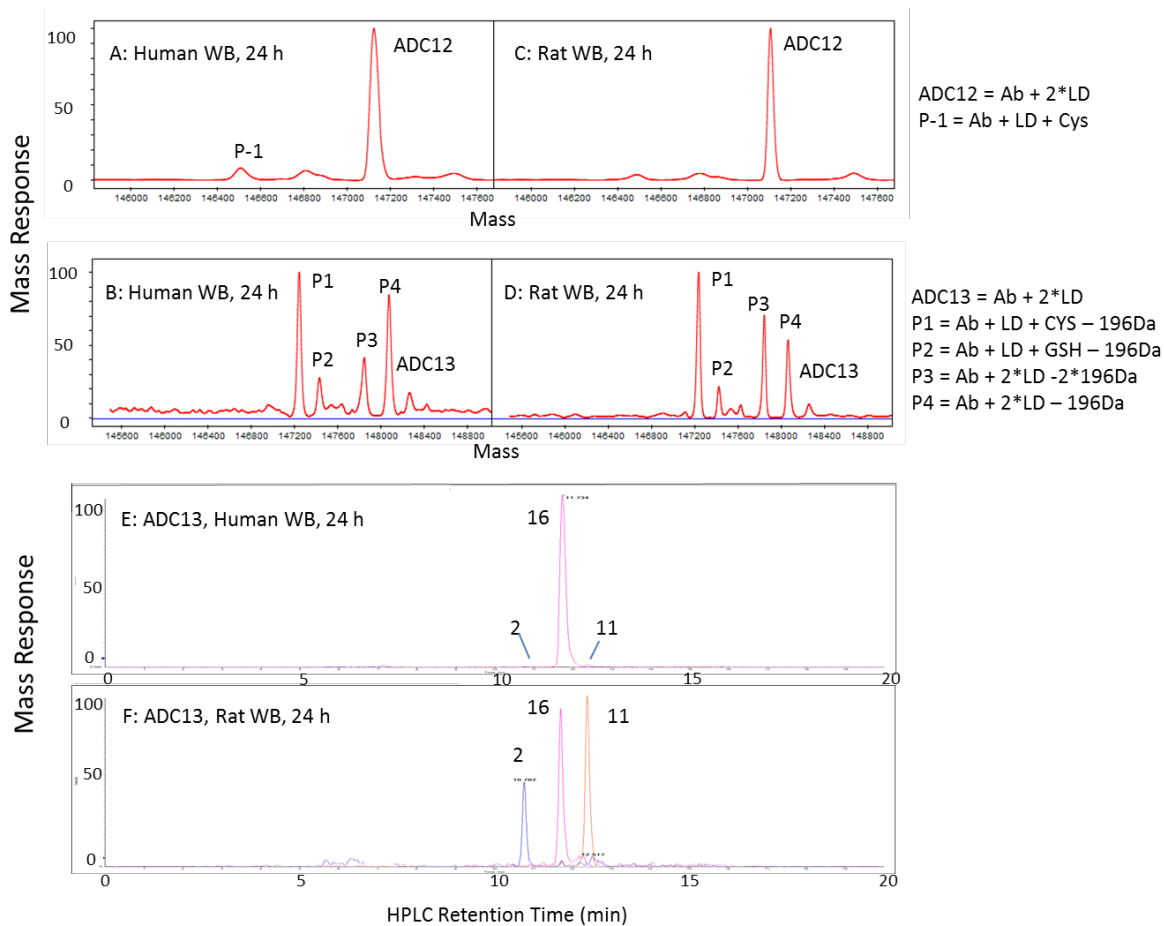
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527 Figure 3.



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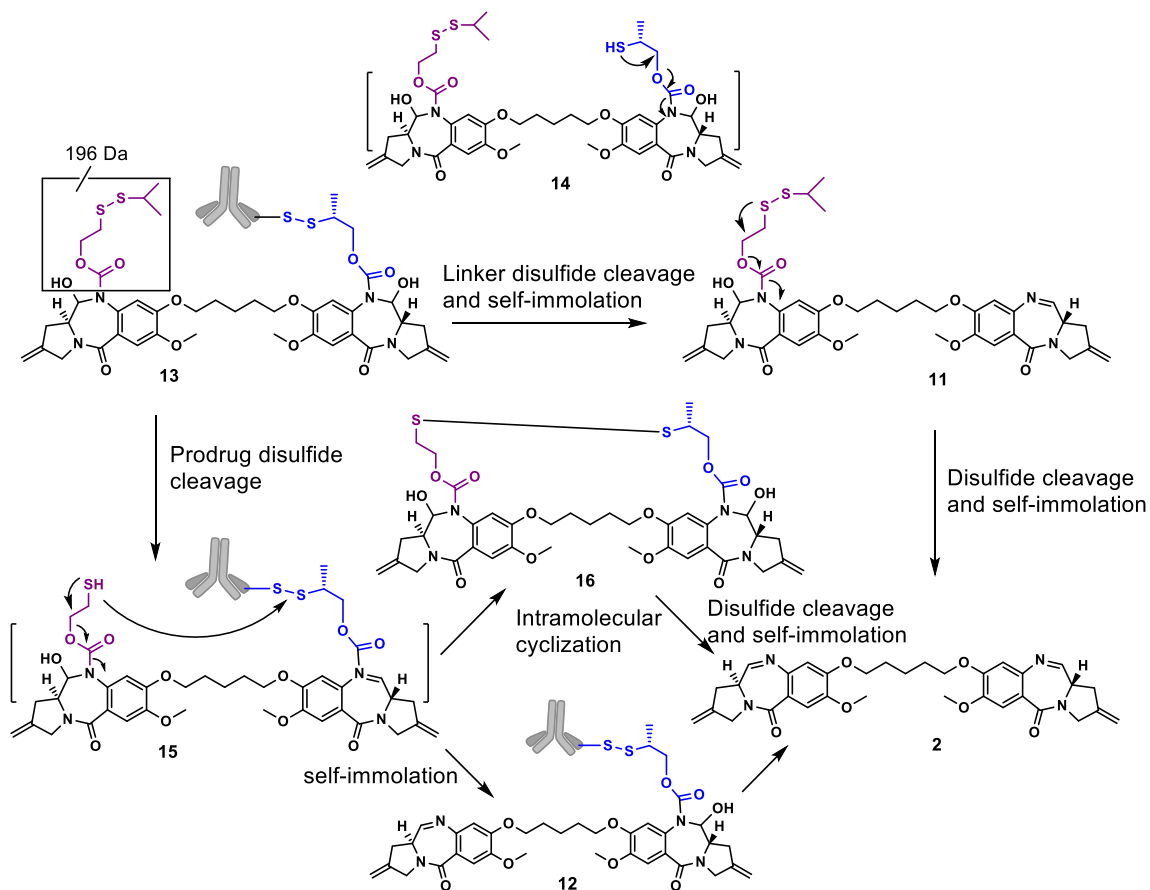
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532 Figure 4.

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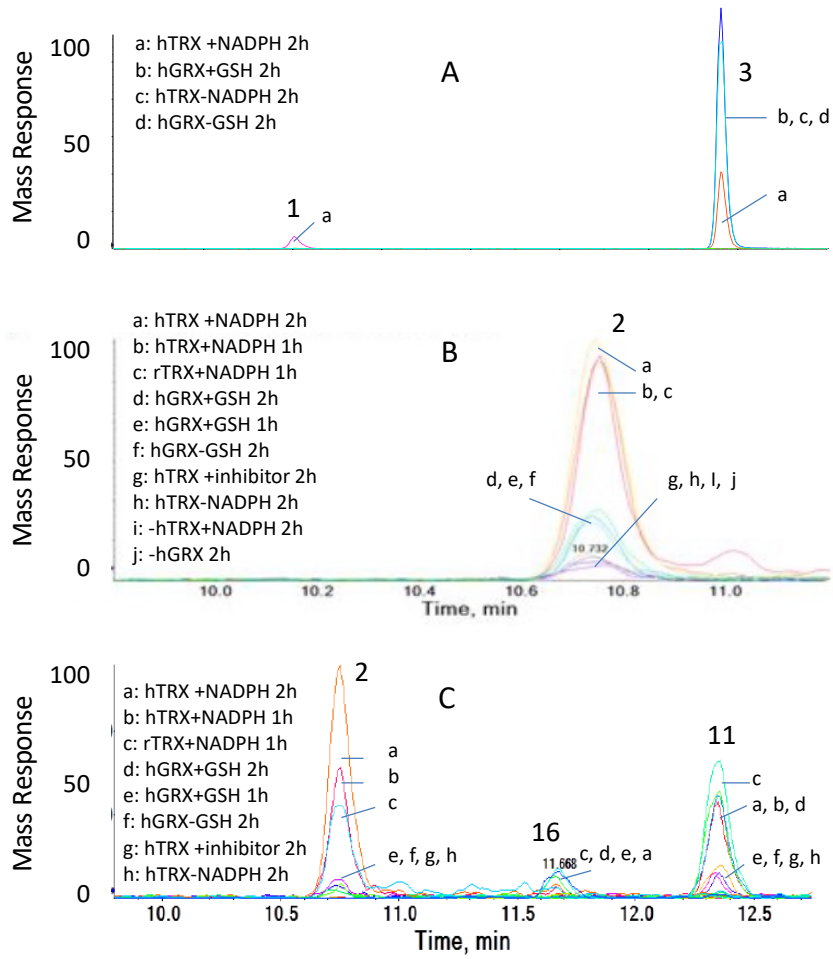


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536 Figure 5.

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