DR5 disulfide bonding as a sensor and effector of protein folding stress

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Abstract

New agents are needed that selectively kill cancer cells without harming normal tissues. The TRAIL ligand and its receptors, DR5 and DR4, exhibit cancer-selective toxicity, but TRAIL analogs or agonistic antibodies targeting these receptors have not received FDA approval for cancer therapy. Small molecules for activating DR5 or DR4 independently of protein ligands may bypass some of the pharmacological limitations of these protein drugs. Previously described Disulfide bond Disrupting Agents (DDAs) activate DR5 by altering its disulfide bonding through inhibition of the Protein Disulfide Isomerases (PDIs) ERp44, AGR2, and PDIA1. Work presented here extends these findings by showing that disruption of single DR5 disulfide bonds causes high-level DR5 expression, disulfide-mediated clustering, and activation of Caspase 8-Caspase 3 mediated pro-apoptotic signaling. Recognition of the extracellular domain of DR5 by various antibodies is strongly influenced by the pattern of DR5 disulfide bonding, which has important implications for the use of agonistic DR5 antibodies for cancer therapy. Disulfide-defective DR5 mutants do not activate the ER stress response or stimulate autophagy, indicating that these DDA-mediated responses are separable from DR5 activation and pro-apoptotic signaling. Importantly, other ER stressors, including Thapsigargin and Tunicamycin also alter DR5 disulfide bonding in various cancer cell lines and in some instances, DR5 mis-disulfide bonding is potentiated by overriding the Integrated Stress Response (ISR) with inhibitors of the PERK kinase or the ISR inhibitor ISRIB. These observations indicate that the pattern of DR5 disulfide bonding functions as a sensor of ER stress and serves as an effector of proteotoxic stress by driving extrinsic apoptosis independently of extracellular ligands.

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44 **Introduction**
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46 Cancer remains one of the most lethal diseases, making the identification of safer and more effective therapies

47 urgent. Identification of cancer drug targets that are essential for malignant cells, but not normal cells, is key.

48 Targeting proteins involved in the folding and maturation of oncoproteins, but not "house-keeping" proteins,

49 holds great promise. Protein Disulfide Isomerases (PDIs) comprise a family of 22 human enzymes that play

50 essential roles in the folding of secreted and membrane proteins [1]. Previous work showed that PDIs may be

51 favorable targets for anticancer agents [2-6]. However, much of this work focused on canonical PDIs with

52 CXXC active site motifs and little is known about non-canonical PDIs that possess CXXS active site trapping

53 motifs that lack the second, resolving cysteine. Previous work indicated that bicyclic thiosulfonate compounds

54 termed Disulfide bond Disrupting Agents (DDA) bind to the PDIs PDIA1, ERp44, AGR2, and AGR3 through

55 their active site Cys residues [7]. DDAs block client binding to PDIA1 and ERp44 and prevent disulfide-

56 mediated AGR2 dimerization. Further, mutation of the active site Cys residues of ERp44 and AGR2 ablate

57 binding to biotinylated DDAs. Collectively, these results suggest that DDAs inhibit the catalytic activity of

58 PDIA1, ERp44, AGR2, and AGR3 by covalently modifying their active site Cys residues.

59 Importantly, DDAs show significant activity against breast tumors and metastatic lesions in animal

60 models without affecting surrounding stromal cells or normal tissues [8, 9]. Tumor cell death occurred through

61 apoptosis, and DDA-mediated apoptosis was associated with downregulation of the HER-family oncoproteins

62 EGFR, HER2, and HER3 and upregulation and activation of DR5, a receptor for the pro-apoptotic ligand

63 TRAIL. However, significant questions remain regarding DDA modes of anticancer action, determinants of

64 cancer responsiveness to DDAs, and the features controlling DDA safety and metabolic stability. The work

65 presented here was designed to address these questions. The results reveal that DR5 plays a central role in

66 the cancer-selective, pro-apoptotic effects of the DDAs, that DR5 levels and signaling activity through the

67 Caspase 8-Caspase 3 axis are controlled by the state of DR5 disulfide bonding, and that multiple inducers of

68 endoplasmic reticulum protein folding stress alter DR5 disulfide bonding. These observations suggest that DR5

69 functions as both a sensor and effector of proper disulfide bond formation in proteostasis.

3

70   **Results**

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72   *DDA-triggered selective ER retention (sERr) is associated with elevated DR5 levels and signaling*: The DDAs

73   used herein are presented in Fig. 1A. We proposed that DDAs exhibit rapidly reversible covalent bonding to

74   protein thiols by disulfide bond formation, with the exception of the target PDIs that form stable disulfide bonds

75   with DDAs [10]. In further support of this premise, we incubated T47D cell extracts with the biotinylated DDA

76   probe Bio-Pyr-DTDO alone or combined with a 100-fold excess of the unlabeled DDA competitors shown in

77   Fig. 1B. Bands recognized by Bio-Pyr-DTDO were identified as PDIA1, ERp44, and AGR2 by mass

78   spectrometry and immunoblot as reported previously [7]. Endogenous biotinylated proteins are observed in the

79   absence of Bio-Pyr-DTDO treatment (asterisks). As expected, Bio-Pyr-DTDO binding was blocked by the more

80   reactive, less selective DDAs DTDO, D5DO, D7DO, and RBF3. In contrast, the less reactive, more selective

81   DDA tcyDTDO did not affect Bio-Pyr-DTDO binding, nor did the thiol-reactive deubiquitinase inhibitor b-AP15

82   [11]. The thiol-reactive compound *N*-ethylmaleimide prevented Bio-Pyr-DTDO binding to DDA targets. These

83   observations support the selectivity of bicyclic DDAs against a subset of PDIs.

84   Also consistent with previous work, the bicyclic DDAs dFtcyDTDO and dMtcyDTDO increased the

85   levels of DR5, and immunoblot analysis under non-reducing conditions showed an electrophoretic mobility shift

86   of monomeric DR5 and an increase in disulfide bonded oligomeric forms of DR5 (Fig. 1C). A previous study

87   from the Tirosh laboratory showed that under Endoplasmic Reticulum (ER) stress conditions, trafficking of

88   some mis-disulfide bonded transmembrane receptor tyrosine kinases became arrested in the ER through the

89   formation of large disulfide bonded complexes involving ERp44 [12]. This mechanism was termed selective ER

90   retention, or sERr. Since DDAs induce ER stress and inhibit ERp44 client binding [7], we examined whether

91   DDA treatment activates or inhibits sERr. Increasing dFtcyDTDO blocked maturation of MET, an established

92   marker of sERr and also prevented maturation of PCSK9 through its auto-cleavage (Fig. 1D). This was

93   associated with increased expression of the ER stress marker XBP1s. Analysis of the same samples under

94   non-reducing conditions showed that increasing dFtcyDTDO concentrations increased EGFR oligomerization,

95   and elevated levels of monomeric and oligomeric DR5. dFtcyDTDO had a modest effect on PDIA1 client

96   binding. In contrast, dFtcyDTDO blocked the formation of lower molecular mass ERp44 disulfide bonded

4

97 complexes with clients (red arrows), while very high mass ERp44 complexes (green arrow) were elevated. This

98 observation suggests that DDAs caused sERr. However, the observation that high levels of mis-disulfide

99 bonded monomeric DR5 accumulated suggests that DR5 may evade sERr.

100   The DDA dMtcyDTDO also induced sERr as indicated by near complete blockade of MET maturation

101 (Fig. 2A). This effect was not altered by signaling inhibitors of mTORC1 (rapamycin), Akt (MK2206), EGFR

102 (Gefitinib), or EGFR and HER2 (Lapatinib). The PERK kinase suppresses protein synthesis under ER stress

103 conditions in part through phosphorylation of eIF2α [12], and previous work [12] showed that sERr is strongly

104 potentiated by PERK inhibition. In MDA-MB-468 cells, Thapsigargin induced a partial block of MET processing

105 that was strongly potentiated by the PERK inhibitor GSK2606414 (hereafter called PERKi) as expected. Since

106 DDAs activate sERr, and sERr is potentiated by PERKi, cell viability studies were performed to examine the

107 effect of DDA/PERKi combination treatment. While PERKi alone had little effect on cell viability, PERKi strongly

108 potentiated dFtcyDTDO cytotoxicity in MDA-MB-468 breast cancer cells (2B, left panel) and WM793 melanoma

109 cells (2B, right panel). sERr was initially investigated primarily in HepG2 hepatoma cells [12], so we compared

110 the combinatorial effects of dMtcyDTDO and PERKi in HepG2 and MDA-MB-468 cells. In both lines PERKi

111 alone had no effect on MET or PCSK9 processing (Fig. 2C). However, PERKi increased the levels of

112 unprocessed MET in both lines and elevated levels of unprocessed PCSK9 in HepG2 cells. This is consistent

113 with PERKi permitting continued synthesis of nascent MET and PCSK9 under ER stress conditions.

114 Combinatorial DDA/PERKi treatment was associated with increased Caspase 8 cleavage, which could explain

115 the enhanced toxicity to cancer cells. PERKi did not strongly potentiate DDA upregulation of DR5, but

116 potentiated Caspase 8 cleavage/activation (Fig. 2D). DR5 knockout partially blunted Caspase 8 cleavage. The

117 compound ISRIB [13] negates the integrated stress response (ISR) by overcoming the effects of

118 eIF2α phosphorylation [14]. When combined with dMtcyDTDO, ISRIB and PERKi produced similar

119 enhancements in the levels of unprocessed MET and PCSK9 (Fig. 2E). This is consistent with both agents

120 overcoming ISR triggered by DDA treatment. Combinatorial DDA/PERKi activation of Caspase 8 in MDA-MB-

121 468 cells was not altered by forced CDCP1 expression, which disrupts cell-cell adhesion and confers

122 suspension growth [15], (Supplemental Fig. S1A), and PERKi actions were not mimicked by inhibition of

123 eEF2K [6] that controls translation initiation (Supplemental Fig. S1B). Together, these observations suggest

124 that PERKi enhances DDA toxicity to cancer cells by potentiating DR5 pro-apoptotic signaling rather than

125 upregulating DR5 expression.

126

127 *Multiple ER stress inducers alter DR5 disulfide bonding:* Since ERp44 is a DDA target, we examined if ER

128 stress alters DR5 disulfide bonding in the absence of ERp44, or in HepG2 ERp44 knockout cells in which wild

129 type or catalytically null (C29S) versions of ERp44 were reintroduced. The most notable effect was that the ER

130 stressor Thapsigargin and PERKi had little effect alone, but irrespective of the presence or absence of ERp44,

131 Thapsigargin + PERKi decreased DR5 electrophoretic mobility and increased its levels (Fig. 3A). This

132 suggests that while DDAs are sufficient to alter DR5 disulfide bonding alone, other ER stressors may perturb

133 DR5 disulfide bonding, particularly if combined with agents that override the ISR. Consistent with this,

134 treatment of MDA-MB-468 cells with dFtcyDTDO altered DR5 levels and disulfide bonding, while Tunicamycin

135 increased DR5 levels without altering its mobility, and PERKi alone had no discernable effect (Fig. 3B).

136 Tunicamycin + PERKi induced a partial shift in DR5 mobility, similar to that seen with dFtcyDTDO treatment,

137 and this shift was associated with higher Caspase 8 cleavage and more numerous Caspase 3 cleavage

138 products. Unlike DR5, DR4 is *N*-glycosylated. DR4 levels were not changed under any of the conditions, but

139 DR4 mobility was increased by Tunicamycin, presumably due to its deglycosylation.

140 Analysis of the effects of other ER stressors on MDA-MB-468 cells indicated that while PERKi alone

141 had little effect on Caspase 8 cleavage, PERKi potentiated induction of Caspase 8 cleavage by Thapsigargin,

142 Tunicamycin, Cyclosporine A, and Dithiothreitol (Fig. 3C). Increased Caspase 8 cleavage correlated with DR5

143 oligomerization, and in some cases, reduced mobility of monomeric DR5. Immunoblot of MET under reducing

144 conditions showed that dFtcyDTDO and Thapsigargin induced sERr. Tunicamycin + PERKi induction of sERr

145 is difficult to assess given the potentially offsetting effects of deglycosylation and lack of MET proteolytic

146 processing. PERKi caused greater accumulation of unprocessed MET in combination with Thapsigargin than

147 with dFtcyDTDO. Consistent with this observation, protein synthesis assays showed that PERKi increased

148 protein synthesis in the presence of Tunicamycin, but further decreased protein synthesis in the presence of

149 dFtcyDTDO (Fig. 3D). Analysis of the effects of ER stressors on HepG2 cells showed little effect of PERKi or

150 dFtcyDTDO on DR5 levels, while PERKi caused DR5 mobility shifts when combined with Thapsigargin or

6

151   Tunicamycin (Fig. 3E). Thapsigargin + PERKi caused sERr as assessed by MET processing. Levels of the

152   PERK downstream effector ATF4 increased in response to Thapsigargin, Tunicamycin, and Cyclosporine A. In

153   each case ATF4 upregulation was partially reversed by PERKi.

154   We next compared ER stress responses observed in PERK knockout and control HepG2 cells. In

155   control cells Thapsigargin increased monomeric and oligomeric DR5 levels and PERKi-cotreatment caused

156   upshifting of the long DR5 isoform and increased DR5 oligomerization (Fig. 3F). As expected, PERKi had no

157   discernable effect on the levels or electrophoretic mobility of DR5 in the PERK knockout cells. We consistently

158   observed a smaller band recognized by the PERK antibody in the presence of PERKi + ER stressors. This

159   likely results from Caspase cleavage of PERK since it is decreased by Caspase inhibitor Q-VD-OPH. Due to

160   differences in the responses of MDA-MB-468 and HepG2 cells to the various ER stressors, we examined ER

161   stress-induced changes in DR5 electrophoretic mobility under non-reducing conditions in neuroblastoma (SH-

162   SY5Y), cervical carcinoma (A431), and human mammary epithelial (HMEC) cells. In SH-SY5Y cells,

163   dFtcyDTDO induced an upward DR5 shift. dFtcyDTDO + PERKi did not further slow DR5 mobility, but

164   increased DR5 oligomerization (Fig. 3G). DR5 mobility and oligomerization were most strongly affected by

165   PERKi combined with Thapsigargin or Tunicamycin in the A431 cells. HMECs exhibited the smallest effect of

166   any of the ER stressors on monomeric DR5 levels, but exhibited a low level of DR5 oligomerization when ER

167   stressors were combined with PERKi. Similar analyses in WM793 melanoma cells showed that dFtcyDTDO

168   alone induced DR5 shifts under non-reducing conditions that were not further potentiated by PERKi or ISRIB

169   (Fig. 3H). Thapsigargin, but not Tunicamycin, induced partial DR5 shifts that were accentuated by PERKi.

170   Together, the findings in Figs. 1 and 2 reveal that changes in DR5 disulfide bonding caused by ER stressors

171   differ among various cancer and non-transformed cell lines and that in some cases ER stress alone is

172   sufficient to alter monomeric DR5 mobility and induce its oligomerization, while in other cases, these effects

173   are potentiated by PERKi.

174

175   *EGFR overexpression elevates DDA-induced accumulation of mis-disulfide bonded, monomeric DR5:* Given

176   previous observations that DDAs downregulate HER-family proteins [16], and that EGFR overexpression

177   sensitizes cells to DDA cytotoxic effects [17], we examined if DDAs differentially perturb DR5 disulfide bonding

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178 and levels in various cancer lines versus non-transformed cells. The DDAs dMtcyDTDO and dFtcyDTDO did

179 not increase DR5 levels in non-transformed MCF10A mammary epithelial cells, HaCaT human keratinocytes,

180 or the T47D luminal breast cancer cell line (Fig. 4A). In contrast, the DDAs induced robust increases in DR5

181 expression in the MDA-MB-468 and HCC1937 triple-negative breast cancer cell lines. Similarly, while

182 dFtcyDTDO, dFtcyDTDO + PERKi, and Tunicamycin + PERKi reduced DR5 mobility in MDA-MB-468 cells

183 (Fig. 4B), only Tunicamycin + PERKi increased DR5 levels and decreased its mobility in HMECs. Since MDA-

184 MB-468 cells express high EGFR levels [18], we examined if EGFR overexpression is sufficient to confer

185 sensitivity of DR5 to DDA-induced changes in disulfide bonding in MCF10A cells. dFtcyDTDO decreased DR5

186 mobility in the EGFR overexpressing cells, but not the vector control cells (Fig. 4C). ER stressor Cyclosporine

187 A did not induce this effect. Analysis of the disulfide bonding status of the DDA targets AGR2, ERp44, and

188 PDIA1 showed that EGFR overexpression increased levels of disulfide-bonded oligomers of these PDIs as

189 observed previously [7]. AGR2 is secreted by some cells [19-22], so we examined if dFtcyDTDO or

190 Cyclosporine A caused AGR2 secretion. As expected [23], Cyclosporine A caused Cyclophilin B secretion, but

191 AGR2 secretion was not observed under these conditions.

192   Since we previously observed that as with EGFR, MYC overexpression sensitizes cells to DDA-driven

193 apoptosis [7], we examined the effects of MYC on DR5 disulfide bonding. dFtcyDTDO shifted DR5 mobility in

194 MYC overexpressing cells, albeit not to the extent observed with EGFR overexpression (Fig. 4D). GRP78

195 immunoblot indicated that dFtcyDTDO induced a stronger ER stress response in the EGFR and MYC

196 overexpressing cells as compared with the vector control. We previously showed that DDAs selectively

197 upregulate DR5 in a subset of cancer cells and oncogene transformed epithelial lines [7, 8]. The results in Fig.

198 4 show that this DR5 upregulation by ER stressors is associated with changes in the disulfide bonding of the

199 monomeric forms of DR5, and in some cases is associated with disulfide-mediated DR5 oligomerization.

200

201 *DR5 levels and oligomerization states are altered by perturbation of DR5 auto-inhibitory domain disulfides:* A

202 recent study showed that the disulfide bond-rich extracellular domain of DR5 serves to prevent receptor

203 oligomerization and pro-apoptotic signaling in the absence of its ligand TRAIL [24]. A more recent article

204 narrowed down the DR5 autoinhibitory domain to a positive patch involving residues R154, K155, and R157

8

205   [25]. Based on a crystal structure [26], these basic residues share a common orientation due to two disulfide

206   bonds, C156-C170 and C139-153 (Fig. 5A). We hypothesized that loss of these two disulfide bonds may

207   disrupt the auto-inhibitory domain, culminating in DR5 clustering and activation of Caspase 8-Caspase 3-

208   mediated apoptosis independently of TRAIL or DDA treatment. This hypothesis was tested by doxycycline-

209   inducible expression of wild type and disulfide-defective DR5 point mutants. High-level inducible expression of

210   the long form of wild type DR5 required induction by doxycycline combined with DDA treatment as described

211   previously [8], while mutation of one or both of the Cys residues of the C160-C178 disulfide bond conferred

212   high level DR5 expression in the absence of DDA treatment, as did the C81S mutation (Fig. 5B). Interestingly,

213   DDA treatment still caused an upward shift in these mutants under reducing conditions suggesting that DDAs

214   may disrupt multiple DR5 disulfide bonds. The DR5 disulfide-defective mutants, but not wild type DR5, also

215   caused formation of high molecular weight DR4 oligomers in the absence of DDA treatment suggesting that

216   endogenous DR4 may co-aggregate with ectopic, mis-disulfide bonded DR5 oligomers.

217   We next examined if Caspase activation limits expression of DR5[C153S], DR4, or the murine TRAIL

218   receptor (mDR5) by inhibiting Caspases with Q-VD-OPH [27]. Q-VD-OPH increased the inducible expression

219   of all three receptors and prevented formation of the p18 fragment of Caspase 8, but not the p41/p43 fragment

220   (Fig. 5C). This likely indicates that Q-VD-OPH does not inhibit receptor-driven Caspase 8 autocleavage, but

221   inhibits the previously described [28] Caspase 3 cleavage of Caspase 8. We further examined the relationship

222   between DR5 and DR4 oligomerization and Caspase activation using C-terminal DR5 or DR4 deletion

223   constructs since such mutants were previously shown incapable of coupling to Caspase 8 activation [29].

224   Doxycycline and doxycycline + dMtcyDTDO produced similar effects on the levels of the wild type and mutant

225   DR5 and DR4, although the mutants were incapable of activating the Caspase 8-Caspase 3 cascade (Fig. 5D).

226   Since DDAs cause ER stress, we examined if ER stress is independent of DR5-mediated Caspase activation.

227   dMtcyDTDO upregulated ER stress markers, decreased AKT phosphorylation, and increased disulfide-

228   mediated EGFR oligomerization irrespective of Caspase activation (Fig. 5E).

229   DR5 mutants lacking the disulfide bonds that form the positive patch exhibit high expression and

230   oligomerization in the absence of DDA treatment, unlike wild type DR5 (Fig. 5F). Upregulation of DR5 by

231   disruption of positive patch Cys residues was observed with two antibodies that recognize the C-terminal

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232 (#8074) or N-terminal (sc-166624) regions of DR5, although the latter antibody exhibited a strong binding

233 preference for oligomeric DR5 isoforms over monomeric DR5 (Fig. 5G). The PhosphoSite database

234 (Phosphosite.org) lists K245 as a major site of DR5 ubiquitination. Mutation of this site to Arg modestly

235 increased receptor levels in the doxycycline and doxycycline + DDA treated samples, but did not mimic the

236 ability of the C-S mutations to exhibit high level expression in the absence of DDA treatment. In summary, the

237 results in Fig. 5 indicate that individual mutation of several different DR5 disulfides, including the positive patch

238 disulfides, is sufficient for high level expression of DR5 and activation of Caspases independent of DDA

239 treatment or ER stress. Further, stabilization of mis-disulfide bonded DR5 does not require Caspase activation,

240 but, activation of Caspase 8 by mis-disulfide bonded DR5 requires its C-terminus that is necessary for DISC

241 formation [29].

242

243 *DDAs activate autophagy and autophagy inhibitors potentiate DDA-induced DR5 accumulation:* Proteasomal

244 degradation (ERAD) and autophagy are important modes for the disposal of misfolded proteins. However, the

245 fate of mis-disulfide bonded, aggregated proteins in the secretory pathway is underexplored. Since DDAs

246 induce ER stress [17], and ER stress frequently activates autophagy [30], we examined if DDAs stimulate

247 autophagy. dFtcyDTDO treatment induced an upward DR5 shift in a concentration-dependent manner. GPR78

248 expression and autophagy marker LC3 lipidation were both increased to maximal levels at the lowest

249 dFtcyDTDO concentration tested (Fig. 6A). Treatment with the autophagy/lysosome inhibitor Bafilomycin A1

250 increased levels of monomeric and oligomeric DR5 isoforms in the absence of DDA treatment (Fig. 6B).

251 Similar studies employing the autophagy/lysosome inhibitor Chloroquine showed increased DR5 levels and

252 accumulation of oligomeric EGFR compared with vehicle treatment. Combining dFtcyDTDO with Chloroquine

253 or PERKi increased DR5 levels and Caspase 8 cleavage over that observed with dFtcyDTDO alone (Fig. 6C).

254 Cell viability assays of cells treated as in Fig. 6C showed that combining dFtcyDTDO with either PERKi or

255 Chloroquine reduced viability more than dFtcyDTDO, and combining the three agents decreased viability the

256 most (Fig. 6D). An inhibitor of the autophagy PI3-kinase VPS34 (VPS34i, [31]) increased DR5 expression to a

257 similar extent as Bafilomycin, and combining dFtcyDTDO with either VPS34i or Bafilomycin increased

258 expression of monomeric DR5 more than each individual treatment (Fig. 6E). Of these treatments, only

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259   dFtcyDTDO or the dFtcyDTDO-containing treatments upshifted monomeric DR5. The strong accumulation of

260   upshifted monomeric DR5 caused by autophagy inhibitors suggests that autophagy plays a role in degrading

261   mis-disulfide bonded DR5.

262

263   *DDAs upregulate DR5 paralog DcR2:* The pro-apoptotic TRAIL receptors DR4 and DR5 share conserved

264   disulfide-rich domains with the TRAIL decoy receptors DcR1 and DcR2 that bind TRAIL, but cannot activate

265   Caspases. Specifically, the DR5 autoinhibitory domain is largely conserved with DR4, DcR1, and DcR2 (Fig.

266   7A). We considered that DDAs might stabilize DcR1 or DcR2 in a similar manner as DR5. Since we previously

267   found that the prolyl isomerase inhibitor Cyclosporine A potentiated DDA cytotoxic effects [9], we examined

268   Cyclosporine A effects on the levels of decoy receptors. We did not detect DcR1 in the cell lines examined, but

269   observed upregulation of DcR2 after dFtcyDTDO treatment (Fig. 7B). We also observed that Cyclosporine A,

270   but not FK606, which inhibits a different family of prolyl isomerases, decreased DDA upregulation of DcR2, but

271   not DR5. dFtcyDTDO increased DcR2 levels more at low concentrations than high concentrations in some

272   experiments, but irrespective of the pattern of DcR2 upregulation by dFtcyDTDO, it was blocked by co-

273   treatment with Cyclosporine A (Fig. 7B-D). Quantitation of band intensities revealed that Cyclosporine A

274   treatment potentiated the effects of low (310 nM) dFtcyDTDO concentration on DR5 oligomerization (Fig. 7E,

275   left panel) and decreased DcR2 upregulation by dFtcyDTDO (Fig. 7E, right panel).

276

277   *DR5 bonding status influences antibody recognition, but not trafficking to the cell surface:* Since sERr prevents

278   some transmembrane receptors from reaching the cell surface [12] and DR5 was shown to be activated in the

279   Golgi by binding to aggregated proteins [32], we examined DR4 and DR5 cell surface labeling by flow

280   cytometry. Using the Clone DJR2-4 (7-8) antibody for flow cytometry analyses, Doxycycline induction of the

281   wild type, C153S, and C156S mutants showed DR5 increased cell surface labeling, however co-treatment with

282   dFtcyDTDO decreased apparent DR5 surface localization (Fig. 8A, upper panel). DR4 flow cytometry studies

283   showed that doxycycline increased DR4 surface levels and this was not altered by dFtcyDTDO co-treatment.

284   Since recognition of DR5 by the flow cytometry antibody could be hindered by changes in DR5 disulfide

285   bonding, we examined levels of DR5 and its disulfide bonding mutants using three different commercially

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286 available antibodies (Fig. 8B). The #8074 antibody directed against the cytoplasmic, C-terminal portion of DR5

287 recognized all of the DR5 proteins, including the monomeric and oligomeric forms of DR5. As shown here (Fig.

288 5) and elsewhere [8], wild type DR5 was only maximally expressed in cells induced with doxycycline and

289 treated with DDAs. The sc166624 DR5 antibody directed against the N-terminal cysteine-rich region

290 preferentially recognized the oligomeric forms of DR. In contrast, Clone DJR2-4 (7-8) directed toward the N-

291 terminal cysteine-rich portion of DR5 did not recognize the C119S/C137S DR5 mutant, but bound the C153S,

292 C156S, C160S, and C160S/C178S DR5 mutants. However, dFtcyDTDO treatment ablated DR5 recognition by

293 this antibody. These observations suggest that binding of Clone DJR2-4 (7-8) antibody is sensitive to DR5

294 disulfide bonding, which is altered by DDA treatment. Since this antibody is commonly used for DR5 labeling in

295 flow cytometry studies and multiple ER stressors, including DDAs, alter DR5 disulfide bonding, lack of signal

296 with this antibody may be indicative of changes in DR5 disulfide bonding rather than DR5 downregulation or

297 internalization.

298 Previous work showed that DDA treatment increased surface localization of DR5 as measured by biotin

299 labeling [7]. The same approach was used to examine the localization of the DR5 mutants to the cell surface.

300 The results showed that the C119S/C137S, C153S, and C156S mutants trafficked to the cell surface,

301 particularly in the context of DDA treatment (Fig. 8C). Under these conditions, expression of DR5 disulfide

302 bonding mutants did not elicit an ER stress response as indicated by the markers GRP78, XBP1s, and PERK

303 activation (phosphorylation). However, dFtcyDTDO activated all these indicators of ER stress. A similar cell

304 surface biotinylation experiment showed trafficking of wild type and mutant DR5 to the cell surface. Surface

305 localization of the C119S/C137S and C153S DR5 mutants occurred in both the presence or absence of

306 dFtcyDTDO or the ER stress inducer Cyclosporine A (Fig. 8D). Expression of mis-disulfide bonded DR5

307 mutants did not upregulate the ER stress or autophagy markers GRP78, or LC3, respectively, but DDA

308 treatment upregulated both markers. Together, these results indicate that under conditions where DR5

309 disulfide bonding is perturbed by either mutagenesis or DDA treatment, DR5 traffics to the cell surface. This is

310 consistent with previous work showing that DDA treatment increases cancer cell sensitivity to the DR4/5 ligand

311 TRAIL [8].

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12

313   *DDA safety and identification of a metabolically stable DDA analog:* Previous work with the DDAs RBF3 [16]

314   and tcyDTDO [8] did not reveal evidence of toxicity under conditions in which they induce the death of primary

315   and metastatic breast cancer cells in mouse models. We also examined the metabolism of the DDA tcyDTDO

316   by liver microsomes [9], but did not present analyses of the stability of dMtcyDTDO and dFtcyDTDO toward

317   metabolism in liver and intestinal microsomes. Recent work demonstrated the activity of both dMtcyDTDO and

318   dFtcyDTDO in mouse models of breast cancer [33], but did not examine the effects of these compounds on

319   normal tissues such as the liver or hematopoietic cells. Examination of breast tumor tissue from mice treated

320   with vehicle or 10 mg/kg dMtcyDTDO showed extensive death of tumor tissue in the dMtcyDTDO-treated, but

321   not the vehicle-treated mice (Fig. 9A, upper panels). Liver tissues from vehicle or dMtcyDTDO-treated mice

322   were indistinguishable (Fig. 9A, lower panels). Analysis of complete blood cell counts from tumor-bearing mice

323   treated with vehicle or 10 mg/ml dMtcyDTDO were in the normal range for healthy mice (Supplemental Table:

324   S1). The apparent decrease in platelets across the samples was likely due to partial clotting prior to analysis.

325   Stability studies in human liver microsomes supplemented with NADPH showed that FtcyDTDO was

326   metabolically stable ($t_{1/2}$ > 60 min), while tcyDTDO, and dMtcyDTDO metabolized by phase I enzymes with

327   half-lives of 11.9, and 47.9 min, respectively. Similar studies employing human intestinal microsomes showed

328   that the half-life of all three DDA compounds exceeded 60 min. Although more thorough analysis of these

329   DDAs is needed, studies performed to date indicate that dFtcyDTDO has a more favorable metabolic stability

330   profile than tcyDTDO or dMtcyDTDO.

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Discussion

Previous work showed that the DDA compounds induce ER stress, which is associated with high-level DR5 expression and disulfide-mediated oligomerization [8, 17, 34]. Further, mutational disruption of a subset of DR5 disulfide bonds was demonstrated to stabilize DR5 and trigger disulfide-mediated DR5 oligomerization. However, the relationship between the DDA-induced ER stress response and DDA effects on DR5 were unexplored. The results presented here show that individual mutational disruption of a total of five of the seven DR5 disulfide bonds each causes the same DR5 stabilization, including the disulfides within the previously described auto-inhibitory domain [24, 25]. The observation that DDAs still induce a mobility shift of monomeric disulfide point mutants of DR5 suggests that while loss of individual disulfide bonds is sufficient to stabilize DR5 and promote pro-apoptotic signaling, DDAs disrupt multiple DR5 disulfide bonds.

Numerous studies have demonstrated transcriptional regulation of DR5 through a PERK-ATF4-CHOP-DR5 pathway [35, 36]. In this model, PERK inhibition is predicted to block DR5 upregulation by ER stress. Our previous studies in breast cancer lines found little effect of knocking out or overexpressing CHOP on DR5 levels, suggesting that other DR5 regulatory mechanisms exist [8]. We found here that PERK inhibition potentiated DR5 pro-apoptotic signaling, but only modestly increased DR5 levels. A report showed that DR5 is activated by binding to misfolded proteins in the Golgi [32], so PERKi might elevate DR5 oligomerization through this mechanism. Alternatively, overriding ISR may activate DR5 by increasing protein folding flux under ER stress conditions and surpass the ability of the DDA targets ERp44, PDIA1, and AGR2 to catalyze disulfide bond formation or their functions in protein folding checkpoints. A recent report showed that breast cancer metastases exhibit elevated ER stress and are responsive to a new, more selective PERK inhibitor [37]. This along with our previous study showing DDA activity against breast cancer metastases [8] provides a rationale for DDA/PERKi combination therapy for the treatment of metastatic breast cancer. Likewise, the observation that Cyclosporine A blocks DDA-induced upregulation of DCR2 further supports the previous contention [9] that DDA/Cyclosporine co-treatment may exhibit enhanced efficacy against breast malignancies.

Overexpression of the EGFR or MYC oncoproteins was shown to sensitize cells to DDA cytotoxic effects, but the underlying mechanisms were not investigated [8]. Results presented here show that EGFR or MYC overexpression permits DDA perturbation of DR5 disulfide bonding that is not observed in vector control

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non-transformed MCF10A mammary epithelial cells. This may partially explain the ability of DDAs to mimic the cancer-specific cytotoxic effects of the DR5/4 ligand TRAIL. Interestingly, expression of disulfide bonding mutants of DR5 does not trigger an ER stress response or activate autophagy. Further, mis-disulfide bonded DR5 traffics to the cell surface, consistent with the previous observation that DDAs synergize with TRAIL to kill cancer cells [8]. The present results extend previous work on the relationship between ER stress and DR5 activation by showing that in addition to DDAs, other ER stressors, including Tunicamycin and Thapsigargin, can alter DR5 disulfide bonding in a manner that is potentiated in some cases by PERKi co-treatment. A subject of ongoing investigation is why ER stressors that alter DR5 disulfide bonding do not have the same effect on its paralog DR4. This may relate to the different N- and O-glycosylation patterns observed for these receptors [38-41], or regulation of DR4 and DR5 stabilities by different E3 ubiquitin ligases [42-44]. Together, our work [7, 8, 17] and that of others [32], suggest that DR5 has evolved as a direct sensor and effector of ER stress/protein misfolding and that ER stress can activate DR5 through transcriptional mechanisms and at the protein level through altered DR5 disulfide bonding and DR5 binding to misfolded proteins. Importantly, DR5 exhibits a TRAIL-independent gain of function under these conditions that inactivate a wide variety of other transmembrane oncoproteins, including EGFR and MET.

DDA studies have been performed largely in breast cancer cell lines, therefore it will be critical to determine the importance of these DDA-driven effects in non-transformed cells and across other tumor types. This DDA cancer selectivity is further supported by the Broad Institute's Dependency Map (DepMap; depmap.org/portal/). Of the 22 human disulfide isomerases, only four, ERp44, AGR2, AGR3, and TMX1, are considered as "strong dependencies" in the DepMap, and our studies have shown DDAs to inhibit three of these, ERp44, AGR2, and AGR3. It is possible that the client proteins of ERp44, AGR2, and AGR3 vary with tumor type. As an example, out of 17,347 genes in the DepMap CRISPR screen, the colon cancer lines C80, COLO205, LS513, and SNUC4 rank AGR2 as the first, fourth, third, and fifth most important gene, respectively. Interestingly, ERN2 encodes the IRE1α homolog IRE1β whose expression is restricted to Goblet cells. The DepMap lists IRE1β as the top predictor of AGR2 dependence. Two recent reports show that AGR2 functions as an inhibitor of IRE1β that overcomes the cytotoxic effects of this enzyme [45], and that the active site Cys81 of AGR2 is required for IRE1β inhibition [46, 47]. It will be important to determine if DDAs exhibit

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387   anticancer activity against tumor lines in which AGR2 is required to prevent IRE1β-mediated cancer cell death.

388   This is an area of active investigation by our team.

389   More generally, DDAs may serve as important tools for investigating disulfide bonding quality control in

390   the secretory pathway. Significant interest has recently focused on DDAs and similar compounds for their

391   ability to function in transport across cell membranes in thiol-mediated uptake [48] and as redox-sensitive

392   probes [49] indicating the value of six membered cyclic dichalcogenides for diverse biological applications. As

393   an inhibitor of ERp44, DDAs may override two key protein disulfide bonding checkpoints, the retrograde Golgi-

394   ER recycling of secretory proteins discovered by Sitia and Colleagues [50-55], and the selective ER retention

395   mechanism of some receptor tyrosine kinases discovered by Tirosh and Colleagues [12]. Based on these new

396   mechanistic insights and molecular and pharmacological tools, the stage is set to investigate the molecular and

397   biological functions of the non-canonical PDIs, and strongly selective cancer dependencies, ERp44, AGR2,

398   and AGR3.

399

Materials and Methods

Cell culture, preparation of cell extracts, and immunoblot analysis

MCF10A cells were cultured in a humidified incubator set at 37°C containing 5% CO_2 as described previously [56]. All other cell lines were grown in Dulbecco's Modified Eagle's Medium (GE Healthcare Life Sciences, Logan, UT) with 10% fetal bovine serum (10% FBS–DMEM). A431, AsPC1, HaCaT, HCC1937, HepG2, HMEC, MCF10A, MDA-MB-468, SH-SY5Y, and T47D were purchased from American Type Culture Collection (ATCC) (Manassas, VA). SUM149pt was purchased from Applied Biological Materials, Inc. (Richmond, BC, Canada). ERp44 and PERK knockout HepG2 cell lines were described previously [12]. WM793 cells were kindly provided by Dr. W. Douglas Cress, Moffitt Cancer Center. Generation of the MCF10A/Vector, MCF10A/EGFR and MCF10A/MYC cell lines is described in previous work [8]. Derivation of the HCI-012/LVM2/LR10 cell line is described in previous work [8, 9]. Generation of DR5 knock out MDA-MB-468 cells and MDA-MB-468 cells stably expressing DR5 using the Tet-ON system is previously described [8]. Generation of the MDA-MB-468 cells stably expressing CDCP1 or vector control is described in previous work [15].

Cell lysates were prepared as described in a previous publication [57]. Immunoblot analysis was performed employing the following antibodies purchased from Cell Signaling Technology (Beverly, MA) [Akt, #4691; P-Akt[T308], #13038; ATF4, #11815; CDCP1, #13794; Cleaved Caspase 3, #9664; Cleaved Caspase 8, #9496; Cyclophilin B, #43603; DCR2, #8049; DR4, #42533; DR5, #8074; eE2F, #2332; P-eE2F[T56], #2331; GRP78, #3177; HER3, #4754; LC3, #3868; MET, #3127; PARP, #9532; PCSK9, #55728; PDIA1, #3501; PERK, #5683; and XBP1s, #12782], from Santa Cruz Biotechnology (Santa Cruz, CA) [Actin, sc-47778; AGR2/3, sc-376653; AGR3, sc-390940; c-Myc (9E10), sc-40; EGFR, sc-373746; ERp44, sc-393687; PDIA6, sc-365260; and pY99, sc-7020], and from Rockland Immunochemicals, Inc. (Limerick, PA), Streptavidin-Alkaline Phosphatase conjugated (SA-AP), S000-05.

Quantitative Analysis of Immunoblot Results

Protein levels in immunoblots were quantified using Adobe Photoshop (Berkeley, CA) and ImageJ (NIH, Bethesda, MD), as previously described [58], followed by normalization to Actin as a loading control.

429

Materials

431 Reagents were purchased from the following companies: Tunicamycin and Chloroquine: Sigma-Aldrich (St.

432 Louis, MO); Thapsigargin: AdipoGen (San Diego, CA); Lapatinib: Selleck Chemicals (Houston, TX);

433 Doxycycline: Enzo Life Science (Farmingdale, NY); TORIN1, and dithiothreitol (DTT): TOCRIS Bioscience

434 (Minneapolis, MN); Cyclosporine A (CsA): Biorbyt (Duran, NC); Rapamycin and PERK Inhibitor I

435 (GSK2656157): Calbiochem (Burlington, Massachusetts); Bafilomycin A1, Gefitinib, ISRIB, MK2206, and Q-

436 VD-OPH: Cayman Chemical (Ann Arbor, MI); *N*-ethylmaleimide (NEM): Thermo Fisher Scientific (Grand

437 Island, NY); eEF2K inhibitor (A-484954) and VPS34 inhibitor Vps34-IN-1: MedChemExpress (Monmouth

438 Junction, NY); FK506: InvivoGen (San Diego, CA); b-AP15: MedKoo Biosciences (Chapel Hill, NC).

439

Tumor studies and histochemical analysis

441 012/LVM2/LR10 xenograft tumor studies were carried out in adult female NOD-SCID-γ (NSG) mice, as

442 described in a previous publication [9]. After the development of palpable tumors (approximately 4 mm^3), mice

443 were randomly assigned to two treatment groups: DMSO (Vehicle) and 10 mg/kg dMtcyDTDO). Mice were

444 treated every weekday for twenty days by intraperitoneal injection, administering 50 µL per injection. At the end

445 of the twenty-day period, tissue samples were collected and fixed in 4% paraformaldehyde/Phosphate-

446 Buffered Saline (PBS), followed by paraffin-embedding, sectioning and staining with hematoxylin and eosin

447 (H&E) by the University of Florida Molecular Pathology Core (https://molecular.pathology.ufl.edu/).

448 Prior to endpoint, peripheral blood was collected by facial vein puncture into EDTA-treated tubes; complete

449 blood cell counts (CBCs) were obtained using an Element HT5 fully automated hematology analyzer (Heska,

450 Loveland, CO).

451

Disulfide Bond-mediated Oligomerization

453

454 Disulfide bond-mediated oligomerization under non-reducing conditions was analyzed as described in previous

455 work [7].

456

Vector Construction

In order to construct the Tet-DR4 expression vector, DR4 (Addgene plasmid #61382) was amplified using the

following primers: 5′-TTTTATCGATCACCATGGCGCCCGTCGCCGTCTGG-3′ and

5′-TTTTGGATCCTCACTCCAAGGACACGGCAG-3′ and cloned into the pRetroX-TetOne-Puro vector with a

modified cloning site that incorporates Not I, Bcl I, and Cla I sites 5′ to the BamH I site (Clontech, Mountain

View, CA, USA).

The initial mutations of C81S, C119S and C160S in DR5 were performed in pcDNA3 with QuikChange

mutagenesis and the following primers, respectively: 5′-

CCAGCCCCTCAGAGGGATTGAGTCCACCTGGACACCATATC-3- and 5′-

GATATGGTGTCCAGGTGGACTCAATCCCTCTGAGGGGCTGG-3′, 5′-

GCTTGCGCTGCACCAGGAGTGATTCAGGTGAAGTGG-3′ and 5′-

CCACTTCACCTGAATCACTCCTGGTGCAGCGCAAGC

-3′ and 5′- CGGAAGTGCCGCACAGGGAGTCCCAGAGGGATGGTCAAGG -3′ and 5′-

CCTTGACCATCCCTCTGGGACTCCCTGTGCGGCACTTCCG

-3′. Mutations were verified by sequencing. The following primers were used to add a 5′-EcoRI and a

3′-BamHI site to C81S, C119S and C160S DR5 by PCR amplification: 5′-

TTTTGAATTCCACCATGGAACAACGGGGACAGAAC-3′ and 5′-

TTTTGGATCCTTAATGATGATGATGATGATGGGACATGGCAGAGTCTGC-3′. The C119S and

C160S DR5 mutants were subsequently cloned into the EcoRI and BamHI sites of pRetroX-TetOne-

Puro vector (Clontech, Mountain View, CA, USA). Mutation of the DR5 C94 was produced in the DR5

C81S construct to produce the DR5 C81S/C94S using QuikChange mutagenesis and the following

primers: 5′-CATATCTCAGAAGACGGTAGAGATAGCATCTCCTGCAAATATGGACAGG-3′ and 5′-

CCTGTCCATATTTGCAGGAGATGCTATCTCTACCGTCTTCTGAGATATG-3′. Mutation of the DR5

C137S was introduced into the DR5 C119S construct to produce the DR5 C119S/C137S using

QuikChange mutagenesis and the following primers: 5′-

19

482   CCACGACCAGAAACACAGTGAGTCAGTGCGAAGAAGGCACCTTC -3′ and 5′-

483   GAAGGTGCCTTCTTCGCACTGACTCACTGTGTTTCTGGTCGTGG -3′. The C178S mutation of

484   DR5 was introduced into the DR5 C160S construct to produce the DR5 C160S/C178S using

485   QuikChange mutagenesis and the following primers: 5-

486   CACCCTGGAGTGACATCGAAAGTGTCCACAAAGAATCAGGTAC -3′ and 5′-

487   GTACCTGATTCTTTGTGGACACTTTCGATGTCACTCCAGGGTG-3′. The C153S and C156S

488   mutations of DR5 were produced using QuikChange mutagenesis and the following primers,

489   respectively: 5′-GAAGAAGATTCTCCTGAGATGAGCCGGAAGTGCCGCACAGGG-3′ and 5′-

490   CCCTGTGCGGCACTTCCGGCTCATCTCAGGAGAATCTTCTTC-3′ and 5′-

491   CTCCTGAGATGTGCCGGAAGAGCCGCACAGGGTGTCCCAGAGGG-3′ and 5′-

492   CCCTCTGGGACACCCTGTGCGGCTCTTCCGGCACATCTCAGGAG-3′. The K245R DR5 mutation

493   was produced by QuikChange Mutagenesis and the following primers: 5′-

494   GTCCTTCCTTACCTGCGAGGCATCTGCTCAGGT-3′ and 5′-

495   ACCTGAGCAGATGCCTCGCAGGTAAGGAAGGAC-3′. Tet-DR5 [ΔC41] was produced by amplifying

496   DR5 by PCR, adding 5′-EcoRI and 3′-BamHI sites using the following primers: 5′-

497   TTTTGAATTCCACCATGGAACAACGGGGACAGAAC-3′ and 5′-

498   TTTTGGATCCTTACTTGTCGTCATCGTCTTTGTAGTCGACAGAGGCATCTCGCCCGG-3′ followed

499   by cloning into the EcoRI and BamHI sites of the pRetroX-TetOne-Puro vector. The Tet-DR4[ΔC43]

500   was produced by amplifying DR4 by PCR, adding 5′-ClaI and 3′-BamHI sites using the following

501   primers: 5′-TTTTATCGATCACCATGGCGCCCGTCGCCGTCTGG-3′ and 5′-

502   TTTTGGATCCTTACTTGTCGTCATCGTCTTTGTAGTCGATCGAGGCGTTCCGTCCAGTTTTG-3′

503   followed by cloning into the ClaI and BamHI sites of the pRetroX-TetOne-Puro vector.

504   In order to clone ERp44, total RNA from T47D cells was extracted with TRIzol Reagent

505   (Invitrogen, Waltham, MA USA) according to the manufacturer's protocol. Total cellular RNA was

506   reverse transcribed to synthesize first-strand cDNA using the PCR conditions listed: 25 °C for 10 min,

507 42 °C for 30 min, and 95 °C for 5 min. DNA encoding ERp44 was subsequently amplified using the

508 following primers: 5′- TTTTGGATCCCACCATGCATCCTGCCGTCTTCC-3′ and 5′-

509 TTTTCTCGAGTTAAAGCTCATCTCGATCCCTC-3′. The PCR fragment encoding ERp44 was cloned

510 into the 5′ BamHI and 3′ XhoI sites of the pMXs-IRES-Blasticidin retroviral vector (RTV-016) (Cell

511 Biolabs, Inc., San Diego, CA USA). The following primers were used to produce the C29S mutation of

512 ERp44 using QuikChange mutagenesis: 5′-

513 CTTTAGTAAATTTTTATGCTGACTGGAGTCGTTTCAGTCAGATGTTG-3′ and 5′-

514 CAACATCTGACTGAAACGACTCCAGTCAGCATAAAAATTTACTAAAG-3.′ All mutations were

515 verified by sequencing.

516

517 **MTT Cell Viability Assays**

518 In order to evaluate cell viability, cells were plated at 7,500/well in 96-well plates and incubated at

519 37°C for 24h. Cells were subsequently treated with various compounds for 72 h at 37°C. Following

520 removal of the cell media, cells were incubated with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

521 diphenyltetrazolium bromide) (Biomatik, Wilmington, DE, United States) in PBS at 37°C for 1 h. The

522 MTT solution was subsequently removed and the MTT formazan product was dissolved in 100 µl of

523 DMSO, followed by measurement of MTT formazan absorbance (570 and 690 nm) in a plate reader.

524

525 **Protein Synthesis Assays**

526 Leucine incorporation into proteins was assayed using ^{3}H-Leucine (cat. # NET460001MC) obtained

527 from Perkin Elmer (Waltham, MA), as described in a previous publication [33].

528

529 **Chemical Synthesis of DDAs**

530 The DDAs presented in Fig. 1A. were prepared based on existing literature procedures from our team and others.

531 RBF3, D5DO, and D7DO were obtained according to the methods described by Field and colleagues [59, 60].

532 DTDO was synthesized as we described previously [16], as well as tcyDTDO [9], dMtcyDTDO and dFtcyDTDO

533 [61], and Bio-Pyr-DTDO [7].

534

535 **Metabolic stability using rat and human liver and intestinal microsomes**

536 To understand the rate of metabolism of compounds across species the *in vitro* metabolic stability of

537 each compound was performed using liver and intestinal microsomes from rats and humans in

538 triplicate. Verapamil was used as a positive control to check the activity of the microsomes. The

539 incubation mixtures consisted of liver or intestinal microsomes (1 mg /ml protein for liver microsomes

540 and 0.5 mg/ml protein for intestinal microsomes), substrate (10 μM), and NADPH (1 mM) in a total

541 volume of 0.2 ml potassium phosphate buffer (50 mM, pH 7.4). Reactions were initiated with the

542 addition of NADPH and kept in an incubator shaker at 37°C. Aliquots of 20 μl were collected at 0, 5,

543 10, 15, 30, 45, and 60 min and mixed with 100 μl of acetonitrile with formic acid (0.1% v/v) containing

544 phenacetin (50 ng/ml; internal standard) for the termination of the reaction. The samples were then

545 vortex mixed and filtered through a 0.45 μm PTFE Solvinert membrane filtration plate under

546 centrifugation at a speed of 2000 ×g for 5 min at 4°C. The filtrates were subjected to UPLC-MS/MS

547 analysis.

548 The intrinsic clearance of the compounds was calculated using a half-life employing the 'substrate

549 depletion' approach. The apparent half-life was calculated from the pseudo-first-order rate constants

550 obtained by linear regression of log (concentration) and time plots. The *in vitro* intrinsic clearance for

551 compound was estimated using the formula:

552 $$t_{1/2} = ln(2)/k$$

553 $$Cl_{int} \ (\mu L/min/mg \ protein) = \frac{0.693}{t_{1/2}} \ X \ \frac{V_{\text{incubation in } \mu L}}{\text{Protein Concentration in mg}}$$

554 Where k is the slope of the line obtained by plotting the natural logarithmic of the percentage of

555 parent remaining versus time and V is the volume of incubation.

556   The *in vitro* intrinsic clearance from rat and human liver microsomes were scaled to whole-organ

557   (hepatic) *in vivo* intrinsic CL (CL$_{int, H}$) using the scaling factors available in the literature using equation

558   [62]:

559    $$Cl_{\text{int, H}} \text{ (mL/min*kg)} = \frac{0.693}{t1/2 \text{ (min)}} \text{ X } \frac{\text{Volume of incubation (mL)}}{\text{Protein in incubation (mg)}} \text{ X } \frac{\text{Liver weight } (g)}{\text{Body weight (kg)}} \text{ X SF}$$

560   The scaling factor used for the rat was 45 (45 mg microsomal protein/g liver) and liver weight (g) per

561   kg body weight was 40 g/kg while for human scaling factors was 29 (29 mg microsomal protein/g

562   liver) and liver weight (g) per kg body weight was 24 g/kg [63, 64].

563

564   **LC-MS/MS analysis:**

565   UPLC-MS/MS analysis was carried out using a Waters Acquity Class I Plus UPLC coupled

566   with a Waters Xevo TQ-S Micro triple quadrupole mass spectrometer. The chromatographic

567   separation was achieved using Acquity UPLC CSH C18 column (2.1 mm x 50 mm, 1.7 μm) using the

568   mobile phase consisting of 0.1% formic acid (A) – methanol (B) with a gradient program of 80 % A

569   held for 0.5 min, then decreased A to 65% reaching 1.0 min and further decreased to 40 % A by 2.5

570   min and held at 40 % until 3.0 min, then sharply decreased back to the initial conditions by 3.1 min

571   and maintained until 3.5 min. The column and autosampler temperatures were kept at 50 °C and 4

572   °C, respectively. The mobile phase was delivered at a flow rate of 0.35 mL/min and the injection

573   volume was set to 2 μL. The MassLnyx software version 4.2 was used for instrument control and

574   TargetLynx for data analysis. The mass spectrometer was operated in positive ion mode and

575   detection of the ions was performed in the multiple reaction monitoring (MRM) mode. The monitored

576   ion transitions (*m/z*) and instrument conditions can be seen in Table 1. Each compound was

577   monitored using two precursor-to-daughter ion transition pairs, one as a quantifier and another as a

578   qualifier to get better selectivity for each compound. The ion spray voltage was set at 3000 V, the

579   desolvation temperature was 400 °C, the desolvation gas flow was 850 L/h, and the cone gas flow

580   was 50 L/h.

23

581 Table 1. Mass parameters for tcyDTDO, dMtcyDTDO, dFtcyDTDO, and internal standard (IS)

Compound	Parent (*m/z*)	Daughter (*m/z*)	Cone (V)	Collision (V)	Type
tcyDTDO	207.16	81.03	46	24	Qualifier
tcyDTDO	207.16	109.02	46	14	Quantifier
dFtcyDTDO	243.10	105.00	44	20	Qualifier
dFtcyDTDO	243.10	125.10	44	16	Quantifier
dMtcyDTDO	267.10	139.10	34	11	Qualifier
dMtcyDTDO	267.10	235.00	34	4	Quantifier
Phenacetin	180.11	110.02	34	20	IS

582

583 **Flow Cytometry Analysis**

584 Cells were lifted from plates using cell scrapers and washed in ice cold PBS. Single cell suspensions were

585 prepared, counted, and diluted to 1×10^6 cells/100 μL. Subsequently, cells were stained for DR4 (DJR1-APC,

586 Cat: 307208, Biolegend) and DR5 (DJR2-4-PE, Cat:307406, Biolegend) markers for 30 min at 4 °C. Cells were

587 then washed twice in ice-cold PBS and stained with viability dye (violet fluorescent reactive dye, Cat:L34955,

588 Invitrogen). FACS Buffer (1% FBS, 0.5 mM EDTA in PBS (400 μL)) was subsequently added. Cells were not

589 fixed or permeabilized. Stained samples were analyzed using single-color compensation and FMO controls on

590 a Sony SP6800 spectral analyzer and quantified using FlowJo V10.8.1 (BD Biosciences). Cells were gated in

591 the following sequence: SSC-A x FSC-A, FSC-H x FSC-A, SSC-H x SSC-A, and Live Cells, to determine Mean

592 Fluorescence Intensity (MFI) of DR5 or DR4.

593

594 **Statistical Analysis**

595 Statistical analysis of protein levels detected by immunoblot, MTT viability assays and protein synthesis assays

596 were performed as described in a previous publication [33].

597
598

24

599 **Figure Legends**
600
601 **Fig. 1:** DDA compounds that selectively inhibit AGR2, PDIA1, and ERp44 block the maturation of select

602 transmembrane and secreted proteins, but strongly upregulate DR5. A. Chemical structures of representative

603 DDAs used in the manuscript. B. Demonstration of the selectivity of biotinylated DDA binding to the target

604 proteins PDIA1, ERp44, and AGR2. Extracts from T47D cells were incubated with the indicated competitors for

605 2h and then incubated for 1 h with biotinylated-DDA, followed by sample analysis by gel electrophoresis and

606 blotting with Streptavidin-Alkaline Phosphatase detection. C. Non-reducing immunoblot analysis of the effect of

607 24 h treatment of the indicated cells with the specified DDAs at 2.5 μM each. M represents monomeric DR5

608 isoforms and O represents disulfide-bonded DR5 oligomeric complexes. S and L refer to the short and long

609 forms of DR5 and S′ and L′ refer to the same DR5 isoforms with altered electrophoretic mobility caused by

610 DDA treatment. Actin serves as a loading control. D. Left panel, reducing immunoblot analysis of MDA-MB-

611 468 cells treated with increasing dFtcyDTDO concentrations showing higher expression of XBP1s and

612 decreased levels of the mature forms and increased relative levels of the pro- forms of MET and PCSK9. Right

613 panel, non-reducing immunoblot analysis using the indicated antibodies. Red arrows represent oligomeric

614 ERp44 isoforms lost upon dFtcyDTDO treatment and the green arrow represents high molecular mass ERp44

615 isoforms elevated by dFtcyDTDO treatment. O and M represent the Oligomeric and Monomeric protein

616 isoforms in panels C and D.

617

618 **Fig. 2:** PERK inhibition amplifies the pro-apoptotic effects of DDAs on cancer cell lines. A. Reducing

619 immunoblot analysis of MDA-MB-468 cells treated for 24 h with the indicated combinations of dMtcyDTDO (2.5

620 μM), Rapamycin (100 nM), TORIN1 (100 nM), MK2206 (5 μM), Gefitinib (10 μM), Lapatinib (10 μM),

621 Thapsigargin (400 nM), and PERKi (1μM). Red arrows denote pro- or mature protein isoforms. B. MTT cell

622 viability assays of MDA-MB-468 cells (left panel) or WM793 cells (right panel) treated for 72 h as indicated.

623 Data are plotted as the average (N = 6), with error bars representing standard deviation. C. Reducing

624 immunoblot analysis of HepG2 or MDA-MB-468 cells treated for 24 h as indicated with 2.5 μM dMtcyDTDO or

625 1 μM PERKi. Red arrows denote pro- or mature protein isoforms. D. Reducing immunoblot analysis of the

626 indicated cell lines treated as specified for 24 h with dMtcyDTDO (2.5 μM) or PERKi (1 μM). E. Reducing

25

627 immunoblot analysis of MDA-MB-468 cells or SUM149pt cells treated for 24 h as indicated with dMtcyDTDO

628 (2.5 μM), ISRIB (200 nM), or PERKi (1μM).

629

630 **Fig. 3:** A variety of ER stressors alter DR5 disulfide bonding. A, upper panel. ERp44-deficient HepG2 cells into

631 which vector, wild type or catalytically null ERp44 were reintroduced were treated as indicated for 24 h and

632 analyzed by non-reducing immunoblot. A, lower panel. Expanded region of the DR5 immunoblot showing

633 altered DR5 disulfide bonding in the Thapsigargin/PERKi combination treatment. B. Non-reducing immunoblot

634 of MDA-MB-468 cells treated as indicated for 24 h. C. MDA-MB-468 cells were treated as indicated for 24 h

635 and subjected to non-reducing (DR5, Cleaved Caspase 3, DR4, Actin) or reducing (MET) immunoblot analysis.

636 D. Protein synthesis assays of cells pre-treated for 24 h as indicated before protein synthesis was measured

637 by ^{3}H-Leucine incorporation over a 2 h pulse. Data are plotted as the average (N = 6), with error bars

638 representing standard deviation. E. HepG2 cells were treated as indicated for 24 h and subjected to non-

639 reducing (DR5, Cleaved Caspase 3, DR4, Actin) or reducing (MET) immunoblot analysis. F. Control and PERK

640 knockout HepG2 cells were treated for 24 h as indicated and subjected to non-reducing immunoblot analysis.

641 G. Non-reducing immunoblot analysis of the indicated cell lines treated as specified for 24 h. Note the DR5

642 oligomerization in A431 cells when Thapsigargin, Tunicamycin, or dFtcyDTDO are combined with PERK

643 inhibition. H. Non-reducing immunoblot analysis of WM793 cells treated as specified for 24 h. Unless otherwise

644 specified, the following concentrations of compounds were used in A-H above: dFtcyDTDO (2.5 μM),

645 Thapsigargin (400 nM), Tunicamycin (500 ng/ml), Cyclosporine A (10 μM), Dithiothreitol (DTT; (2.5 mM)),

646 ISRIB (200 nM), or PERKi (1μM). O and M represent Oligomeric and Monomeric protein isoforms in panels A,

647 C, E, G, and H.

648

649 **Fig. 4:** DDA upregulation of DR5 occurs in breast cancer cells or mammary epithelial cells overexpressing

650 MYC or EGFR. A. The indicated cell lines were treated for 24 h with 2.5 μM dMtcyDTDO or dFtcyDTDO and

651 analyzed by immunoblot under reducing conditions. B. MDA-MB-468 cells or Human Mammary Epithelial Cells

652 (HMEC) were treated for 24 h as indicated and subjected to non-reducing immunoblot. D may represent PERK

653 degradation products produced by Caspases. C. MCF10A cells engineered to overexpress EGFR or the

654 corresponding vector control line were treated as indicated for 24 h with 2.5 μM dFtcyDTDO, 10 μM

655 Cyclosporine A, or vehicle. The medium was collected and concentrated for analysis of secreted proteins and

656 the cell extracts were analyzed for internal proteins. Immunoblot analysis was performed under non-reducing

657 conditions. O and M represent Oligomeric and Monomeric protein isoforms in panels B and C. D. MCF10A

658 cells engineered to overexpress EGFR or MYC were treated for 24 h with 2.5 μM dFtcyDTDO or vehicle and

659 cells were analyzed by non-reducing immunoblot. Bands shown represent monomeric protein isoforms.

660

661 **Fig. 5:** Genetic disruption of multiple DR5 disulfide bonds induces its stabilization and pro-apoptotic signaling.

662 A. Structural model of DR5 showing its disulfide bonds, and the positive patch autoinhibitory domain described

663 in the literature. B. Non-reducing immunoblot analysis of MDA-MB-468 cells engineered with doxycycline-

664 inducible expression of wild type (WT) DR5 or the indicated Cys to Ser disulfide bond mutants. Cells were

665 treated as indicated for 24 h with 1 μg/ml doxycycline and 2.5 μM dMtcyDTDO. The red arrow denotes DR4

666 oligomers that coincide with DR5 oligomerization. C. Reducing immunoblot analysis of the indicated MDA-MB-

667 468 stable cell lines. Cells were treated for 24 h as specified with 1 μg/ml doxycycline or doxycycline + 10 μM

668 Q-VD-OPH. The catalog numbers of DR5 and DR4 antibodies are shown. D. Non-reducing immunoblot

669 analysis of the indicated MDA-MB-468 cell lines with doxycycline-inducible expression of wild type DR4 and

670 DR5, and DR4 and DR5 C-terminal deletion constructs defective in apoptotic signaling. Cells were treated for

671 24 h as specified with 1 μg/ml doxycycline or doxycycline + 2.5 μM dMtcyDTDO. E. Non-reducing immunoblot

672 analysis of the indicated MDA-MB-468 cell lines with doxycycline-inducible expression of wild type and

673 apoptosis-defective DR5. Cells were treated for 24 h as specified with 1 μg/ml doxycycline or doxycycline + 2.5

674 μM dMtcyDTDO. F. Non-reducing immunoblot analysis of the indicated MDA-MB-468 doxycycline-inducible

675 stable cell lines. Cells were treated for 24 h as indicated. G. Non-reducing immunoblot analysis of the indicated

676 MDA-MB-468 cell lines with doxycycline-inducible expression of wild type and apoptosis-defective DR5. Cells

677 were treated for 24 h as specified with 1 μg/ml doxycycline or doxycycline + 2.5 μM dMtcyDTDO. O and M

678 represent Oligomeric and Monomeric protein isoforms in panels B and D-G.

679

680 **Fig. 6:** DDAs activate autophagy and inhibitors of autophagy/lysosomal degradation upregulate DR5. A. Non-

681 reducing immunoblot analysis of MDA-MB-468 cells treated as indicated for 24 h. B. Non-reducing immunoblot

682 analysis of vector control or DR5 knockout MDA-MB-468 cells treated with 2.5 μM dFtcyDTDO or 1 μM

683 Bafilomycin A1 for 24 h. C. Non-reducing immunoblot analysis of MDA-MB-468 cells treated for 24 h as

684 indicated with 1 μM PERKi (P), 15 μM Chloroquine (C), or 2.5 μM dFtcyDTDO (dF). D. MTT Cell viability assay

685 of MDA-MB-468 cells treated as indicated for 72 h. Data are plotted as the average (N = 6), with error bars

686 representing standard deviation. E. Non-reducing immunoblot analysis of MDA-MB-468 cells treated for 24 h

687 as indicated with 2.5 μM dFtcyDTDO (F) or 1 μM Bafilomycin A1 (BFA1), VPS34 inhibitor (VPS34i), 400 nM

688 Thapsigargin, or 500 ng/ml Tunicamycin. O and M represent Oligomeric and Monomeric protein isoforms in

689 panels A, B, C, and E.

690

691 **Fig. 7:** DDAs upregulate TRAIL Decoy Receptor 2, an effect overridden by Cyclosporine A. A. Sequence

692 alignment of the putative autoinhibitory motifs of DR4, DR5, DCR1, and DCR2. B. Non-reducing immunoblot

693 analysis of MDA-MB-468 cells treated for 24 h with the indicated concentrations of dFtcyDTDO, combined with

694 DMSO vehicle, 5 μM Cyclosporine A or 100 nM FK506. C. Non-reducing immunoblot analysis of MDA-MB-468

695 cells treated for 24 h with the indicated concentrations of dFtcyDTDO, combined with DMSO vehicle or 5 μM

696 Cyclosporine A. D. Non-reducing immunoblot analysis of MDA-MB-468 cells treated for 24 h with the indicated

697 concentrations of dFtcyDTDO, combined with DMSO vehicle or 5 μM Cyclosporine A. Data are plotted as the

698 average (N = 3), with error bars representing standard error. Asterisks denote $p < 0.05$ compared to control

699 using Student's unpaired t-test. E. Densitometry analysis of the relative levels of total, monomeric, and

700 oligomeric forms of DR5 (left panel) or total levels of Cyclophilin B or DCR2 (right panel) from panels 7B-D. O

701 and M represent Oligomeric and Monomeric protein isoforms in panels B-D.

702

703 **Fig. 8:** Effects of altered disulfide bonding on DR5 cell surface localization and antibody recognition. A. Flow

704 cytometry analysis of the indicated doxycycline-inducible MDA-MB-468 stable cell lines with an antibody to

705 DR5 (Clone DJR2-4 (7-8)) (top panel) or DR4 (bottom panel). Prior to analysis, cells were treated for 24 h as

706 indicated with 10 μM Q-VD-OPH, 1 μg/ml doxycycline, or 2.5 μM dFtcyDTDO. Dots represent the average

28

707 values from three independent biological replicates performed in triplicate. * Represents $p < 0.05$, ****

708 represents $p < 0.0001$, and ns represents not significant ($p > 0.05$). B. Non-reducing immunoblot analysis of

709 the indicated MDA-MB-468 stable cell lines treated for 24 h as specified. Note the alternate staining patterns

710 observed with different DR5 antibodies. O and M represent Oligomeric and Monomeric protein isoforms. C.

711 The indicated MDA-MB-468 stable cell lines were treated for 24 h as indicated with 1 μg/ml doxycycline and

712 2.5 μM dFtcyDTDO and subjected to cell surface protein biotin labeling. Cell surface proteins (External; Ext.)

713 were affinity purified with Streptavidin-agarose, and the unlabeled flow-through (Internal; Int.) proteins were

714 also collected. Both fractions were analyzed by non-reducing immunoblot using the indicated antibodies. D.

715 Cell surface protein labeling experiment as in panel C except that cell lines were treated with the indicated

716 combinations of 1 μg/ml doxycycline, 2.5 μM dFtcyDTDO, and 10 μM Cyclosporine A.

717

718 **Fig. 9:** Metabolic stability of select DDAs and lack of DDA effects on liver morphology. A. Hematoxylin and

719 Eosin stained breast tumor (upper panels) and liver tissue samples (lower panels) from mice bearing

720 012/LVM2/LR10 tumors after treatment with vehicle (peanut oil) or 10 mg/kg dMtcyDTDO by oral gavage for 20

721 days. B. Stability of tcyDTDO, dMtcyDTDO, or dFtcyDTDO metabolism in rat or human liver microsomes in the

722 presence or absence of 1 mM NADPH. Verapamil serves as a positive control. C. Stability of tcyDTDO,

723 dMtcyDTDO metabolism, or dFtcyDTDO in rat or human intestinal microsomes in the presence or absence of 1

724 mM NADPH. Verapamil serves as a positive control. Data points are plotted as the average (N = 3), with error

725 bars representing standard deviation.

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963

964 **Author Contributions**
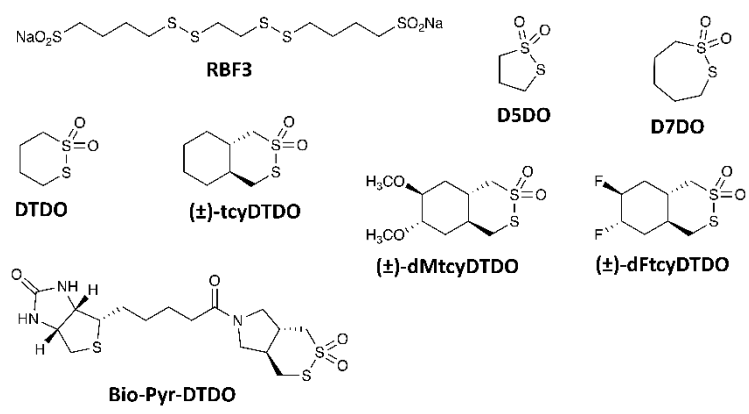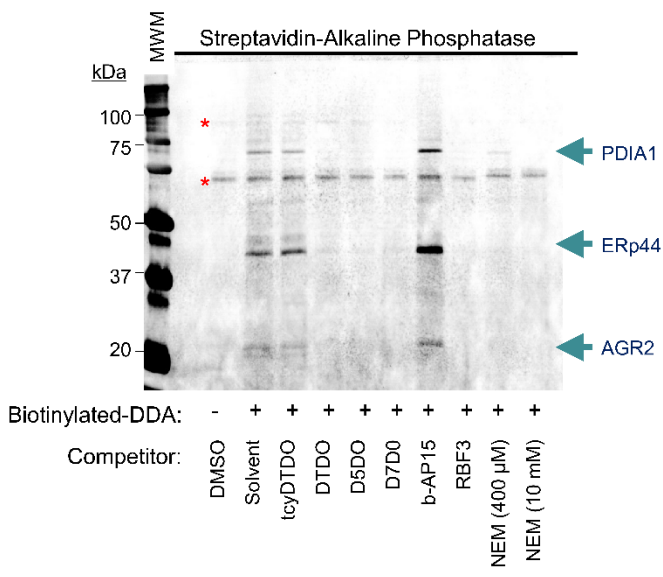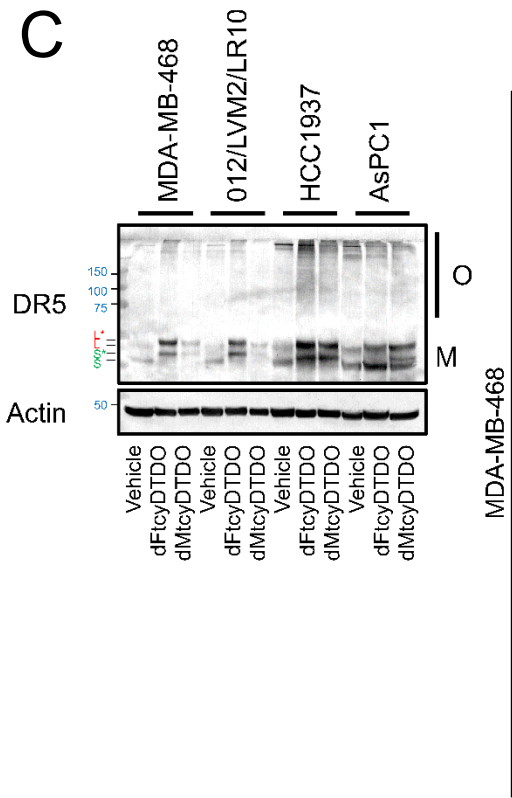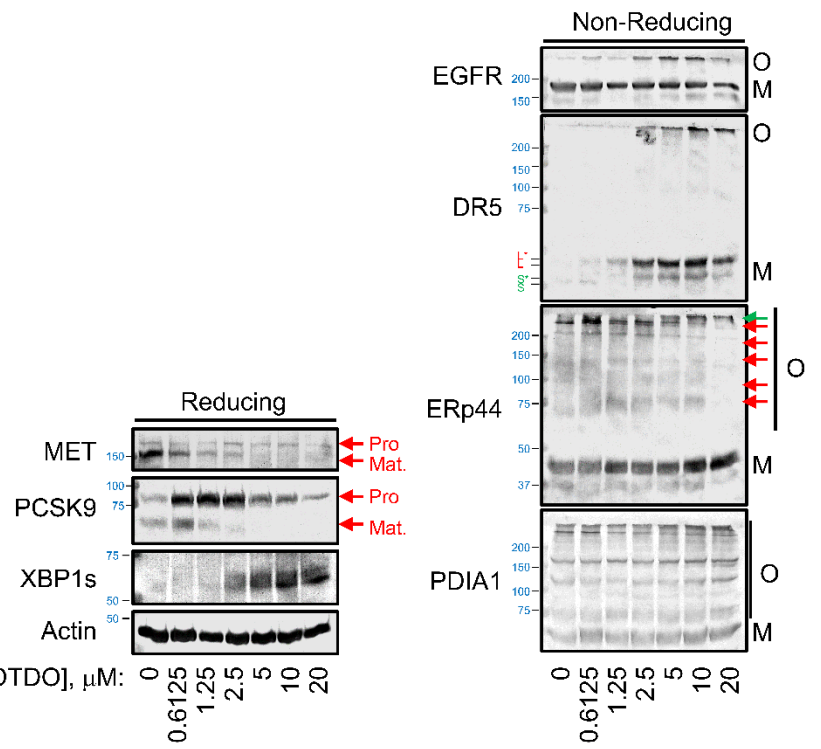
965 Performed experiments: MS, ZD, SE, GT, GA, MW, HS, BF, SK, BL
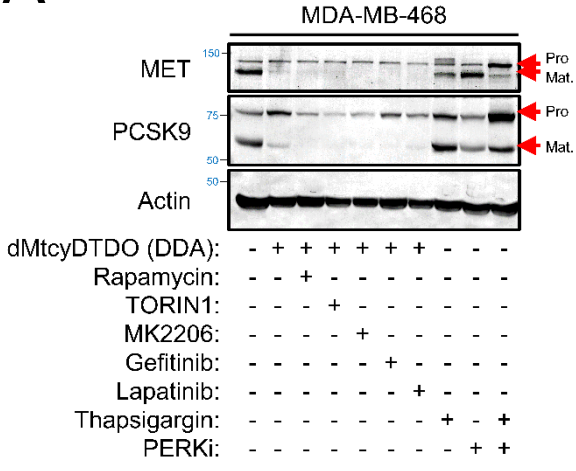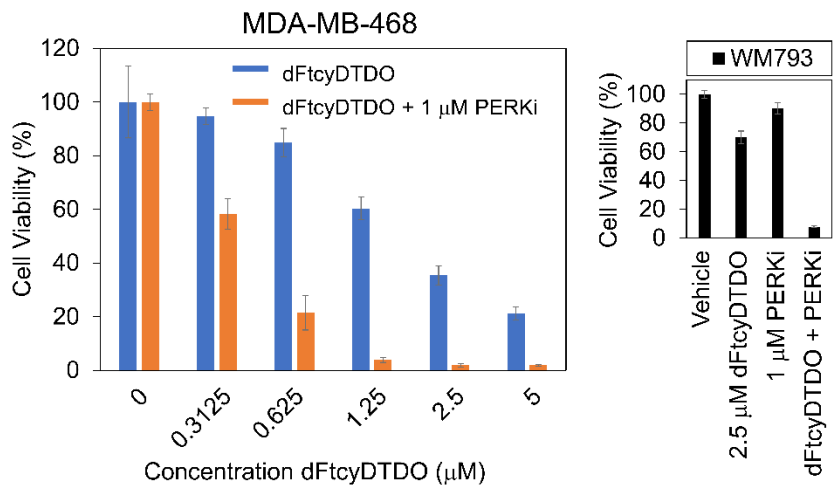
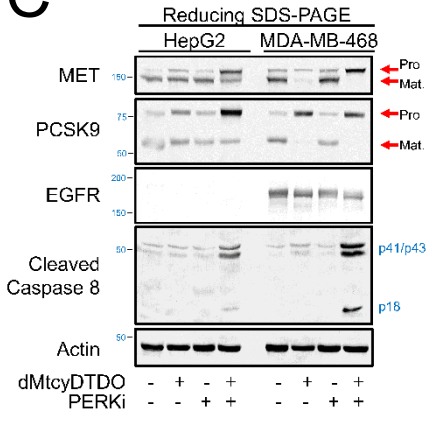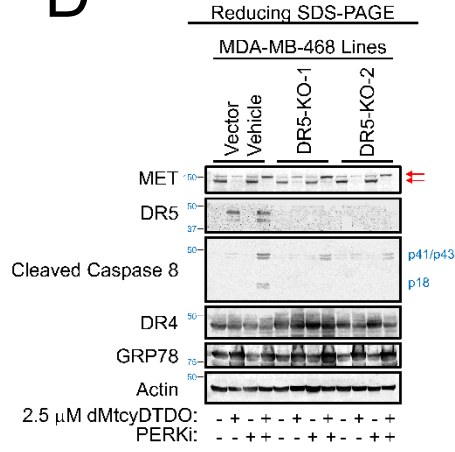966 Directed research: ML, AS, OG, JH, BT, RC, BL

967 Provided key reagents and expertise: JH, BT

968 Wrote and edited manuscript: ML, ZD, GT, CWC, AS, OG, RC, BL

969

970

Law et al., Fig. 1

A

MDA-MB-468

MET — Pro / Mat.

PCSK9 — Pro / Mat.

Actin

dMtcyDTDO (DDA):	-	+	+	+	+	+	+	-	-	-
Rapamycin:	-	-	+	-	-	-	-	-	-	-
TORIN1:	-	-	-	+	-	-	-	-	-	-
MK2206:	-	-	-	-	+	-	-	-	-	-
Gefitinib:	-	-	-	-	-	+	-	-	-	-
Lapatinib:	-	-	-	-	-	-	+	-	-	-
Thapsigargin:	-	-	-	-	-	-	-	+	-	+
PERKi:	-	-	-	-	-	-	-	-	+	+

B

MDA-MB-468

Cell Viability (%) vs Concentration dFtcyDTDO (μM)
Legend: dFtcyDTDO (blue), dFtcyDTDO + 1 μM PERKi (orange)
Concentrations: 0, 0.3125, 0.625, 1.25, 2.5, 5

WM793

Cell Viability (%)
Vehicle, 2.5 μM dFtcyDTDO, 1 μM PERKi, dFtcyDTDO + PERKi

C

Reducing SDS-PAGE

HepG2   MDA-MB-468

MET — Pro / Mat.

PCSK9 — Pro / Mat.

EGFR

Cleaved Caspase 8 — p41/p43 / p18

Actin

| dMtcyDTDO | - | + | - | + | - | + | - | + |
| PERKi | - | - | + | + | - | - | + | + |

D

Reducing SDS-PAGE

MDA-MB-468 Lines

Vector Vehicle   DR5-KO-1   DR5-KO-2

MET

DR5

Cleaved Caspase 8 — p41/p43 / p18

DR4

GRP78

Actin

| 2.5 μM dMtcyDTDO: | - | + | - | + | - | + | - | + | - | + | - | + |
| PERKi: | - | - | + | + | - | - | + | + | - | - | + | + |

E

Reducing SDS-PAGE

MDA-MB-468   SUM149pt

MET

PCSK9

DR5

Cleaved Casp. 8 — p41/p43 / p18

sXBP1

Actin

dMtcyDTDO:	-	+	-	-	+	+	-	+	-	-	+	+
ISRIB:	-	-	+	-	+	-	-	-	+	-	+	-
PERK Inh.:	-	-	-	+	-	+	-	-	-	+	-	+

Law et al., Fig. 2

Law et al., Fig. 3

Law et al., Fig. 4

Law et al., Fig. 5

Law et al., Fig. 6

Law et al., Fig. 7

Law et al., Fig. 8

A Vehicle | 10 mg/kg dMtcyDTDO

Breast Tumor

Liver

B Human and Rat Liver Microsome Metabolism

tcyDTDO | dMtcyDTDO | dFtcyDTDO

RLM_NADPH, HLM_NADPH, RLM_No NADPH, HLM_No NADPH, Verapamil_RLM, Verapamil_HLM

Parameter	tcyDTDO		dMtcyDTDO		dFtcyDTDO	
	RLM	HLM	RLM	HLM	RLM	HLM
$T_{1/2}$ (min)	> 60 min	11.9±0.2	22.4±1.2	47.9±1.3	> 60 min	> 60 min
Intrinsic clearance, CL_{int} (µL/min/mg protein)		58.3±1.2	31±1.7	14.5±0.4	-	-
Whole liver clearance, $CL_{int,in\ vivo}$ (mL/min*kg)		40.6±0.8	55.8±3.1	10.1±0.3	-	-
Hepatic clearance, CL_h (mL/min*kg)		13.8±0.1	27.7±0.8	6.8±0.1	-	-
Extraction Ratio		0.66±0	0.5±0.01	0.32±0.01	-	-
Clearance Category	Low	Moderate	Moderate	Moderate	Low	Low

C Human and Rat Intestinal Microsome Metabolism

tcyDTDO | dMtcyDTDO | dFtcyDTDO

RIM_NADPH, HIM_NADPH, RIM_No NADPH, HIM_No NADPH, Verapamil_RIM, Verapamil_HIM

Parameter	tcyDTDO		dMtcyDTDO		dFtcyDTDO	
	RIM	HIM	RIM	HIM	RLM	HLM
$T_{1/2}$ (min)	> 60 min	> 60 min	53.2±6.4	> 60 min	24±4.1	> 60 min
Intrinsic clearance, CL_{int} (µL/min/mg protein)	-	-	26.3±3	-	59±10.5	-

Law et al., Fig. 9