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Glabratephrin reverses doxorubicin resistance in triple negative breast cancer by inhibiting P-glycoprotein

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1 **Glabratephrin reverses doxorubicin resistance in triple negative breast cancer by inhibiting P-**
2 **glycoprotein**

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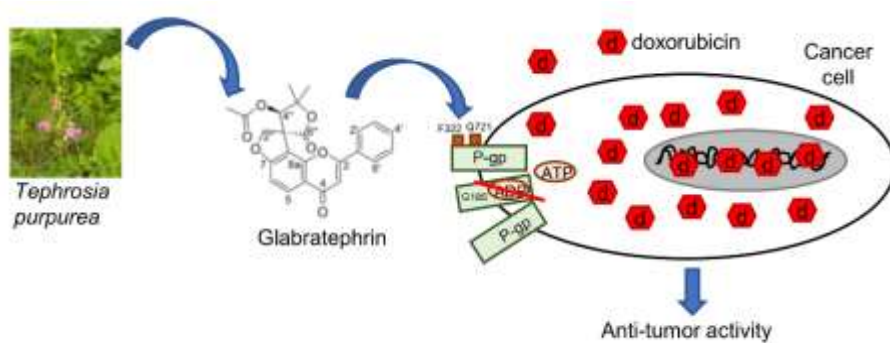
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23 **Highlights**

- 24 - Glabratephrin is a natural prenylated flavonoid from *Tephrosia purpurea*
- 25 - Glabratephrin reverses resistance to doxorubicin in triple negative breast cancer
- 26 - Glabratephrin binds P-glycoprotein and reduces its catalytic activity
- 27 - Glabratephrin is effective and safe in triple negative breast cancer xenografts

28

29 **Graphical abstract**



30

31

32 **Abstract**

33 Triple-negative breast cancer is one of the most aggressive breast cancer. The first therapeutic option
34 is chemotherapy, often based on anthracycline as doxorubicin. However, chemotherapy efficacy is
35 limited in by the presence of P-glycoprotein (Pgp), a membrane transporter protein that effluxes
36 doxorubicin, reducing its cellular accumulation and toxicity. Inhibiting Pgp activity with effective
37 and non-toxic products is still an open challenge.

38 In this work, we demonstrated that the natural product Glabratephrin (Glab), a prenylated flavonoid
39 from *Tephrosia purpurea* with a unique chemical structure, increased doxorubicin accumulation and
40 cytotoxicity in triple negative breast cancer cells with high levels of Pgp, characterized by both
41 acquired or intrinsic resistance to doxorubicin. Glab also reduced the growth of Pgp-expressing
42 tumors, without adding significant extra-toxicities to doxorubicin treatment. Interestingly, Glab did
43 not change the expression of Pgp, but it reduced the affinity for Pgp and the efflux of doxorubicin, as
44 suggested by the increased K_m and the reduced V_{max} . *In silico* molecular docking predicted that
45 Glab binds two residues (phenylalanine 322, glutamine 721) localized in the transmembrane domains
46 of Pgp, facing the extracellular environment. Moreover, site-directed mutagenesis identified glycine
47 185 as a critical residue mediating the reduced catalytic efficacy of Pgp elicited by Glab.

48 We propose Glab as an effective and safe compound able to reverse doxorubicin resistance mediated
49 by Pgp in triple negative breast cancers, opening the way to a new combinatorial approach that may
50 improve chemotherapy efficacy in the most refractory and aggressive breast cancer.

51
52 **Keywords:** Glabratephrin; P-glycoprotein; doxorubicin; triple negative breast cancer

53
54 **Chemical compounds:** doxorubicin (PubChem CID: 31703); Glabratephrin (PubChem CID:
55 12893624).

56 **Abbreviations:** MDR, multidrug resistance; ABCB1/Pgp: ATP binding cassette sub-family B1/P-
57 glycoprotein; Dox, doxorubicin; ABCC1/MRP1: ATP binding cassette sub-family C1/multidrug
58 resistance-associated protein 1 (MRP1); ABCG2/BCRP: ATP binding cassette sub-family G2/breast
59 cancer resistance protein; QSAR: quantitative structure-activity relationships; Glab: Glabratephrin;
60 FBS: fetal bovine serum; HIF-1 α : Hypoxia-inducible factor-1 α ; CI: Combination Index; LDH: lactate
61 dehydrogenase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AP: alkaline
62 phosphatase; CPK: creatine phosphokinase; cTnI/T: cardiac troponin I/T; Pi: phosphate; MOE:
63 Molecular Operating Environment; RMSD: root mean square deviation; ANOVA: analysis of
64 variance; Gln: glutamine; Phe: phenylalanine; Gly: glycine; Val: valine; Ser: serine; Asn; asparagine;
65 Ala: alanine; Thr: threonine; CS: collateral sensitivity; NBD: nucleotide binding domain; TMD:
66 transmembrane domain; DSPE-PEG: distearoyl-phosphatidylethanolamine-polyethylene glycol.

67

68 **1. Introduction**

69 Multi-drug resistance (MDR) is a major cause of chemotherapy failure. One of the main mechanisms
70 of chemoresistance is the high expression of adenosine triphosphate-binding cassette sub-family B1
71 (ABCB1)/P-glycoprotein (Pgp) that determines resistance to a broad spectrum of drugs used against
72 breast cancer [1]. Doxorubicin (Dox), a typical substrate of Pgp [2], is an anthracycline extensively
73 used to treat hematological and solid tumors, including triple negative breast cancer [3, 4]. Some of
74 the last generation of Pgp inhibitors, such as Tariquidar and Elacridar, have enhanced the efficacy of
75 anticancer drugs in preclinical studies, opening the perspective to their possible clinical use in
76 patients with refractory breast cancers. While no Phase I/II trials based on Elacridar were registered,
77 two trials using Tariquidar started (<https://clinicaltrials.gov/>) [5]. The phase-II NCT00048633 trial
78 tested the efficacy of Tariquidar in combination with anthracyclines or taxanes in patients with
79 advanced breast cancers, but it did not provide any conclusion about the clinical benefits derived from

80 this combination therapy. Similarly, no information about an increased efficacy of the combination of
81 Tariquidar and vinorelbine were reported in the NCT00001944 trial. These data dampened the
82 enthusiasm for setting larger phase III clinical trial with these two Pgp inhibitors. In addition, many
83 Pgp inhibitors showed high toxicity [6]: the first-generation inhibitors of Pgp, such as verapamil,
84 suffered of low therapeutic window and cardiotoxicity; the second-generation inhibitors displayed
85 lower cardiotoxicity but often produced unwanted drug-drug interactions when administered with
86 chemotherapeutic agents, limiting the efficacy of the latter [7]. Among the third-generation inhibitor,
87 Elacridar induced modest neutropenia, Tariquidar was not associated with grade III/IV toxicities [7],
88 but in both cases the lack of efficacy in terms of overall response rate limited the further clinical
89 development. Finding effective and safe Pgp inhibitors remains still an open challenge.

90 In this scenario, natural products have been considered more and extensively as potential alternatives
91 to synthetic molecules, although also natural compounds may suffer of the same drawbacks of
92 synthetic compounds, as non-specific binding with the targets and/or unfavorable pharmacokinetic
93 profiles [8]. The mechanisms at the basis of the reversion of chemoresistance by natural products are
94 pleiotropic and sometimes not univocal, also in consideration of the wide range of natural compounds
95 tested. Lipophilic terpenoids (e.g. monoterpenes, diterpenes, triterpenes, tetraterpenes), flavonoids,
96 steroids such as cardiac glycosides, prenyl and steroid derivatives of quinolone and indole have been
97 shown to inhibit Pgp, multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance
98 protein (BCRP) in a competitive manner [9], suggesting that these structures may fit the drug-binding
99 pockets present on the different ABC transporters and displace the substrates. For more hydrophilic
100 compounds with phenolic structure, it has been proposed that multiple H-bonds are formed with the
101 protein, leading to the transporter's destabilization [9] and to non-competitive inhibition. Quercetin,
102 catechins, morin and capsaicin, have been reported to inhibit Pgp more than other ABC transporters
103 [10-12]. Quantitative structure-activity relationships (QSAR) studies highlighted those specific

104 moieties, such as a double bond between positions 2 and 3, a methoxyl group in position 3 and a
105 hydroxyl group in position 5, that may confer this selectivity toward Pgp [12], leading to hypothesize
106 that these moieties bind specific sites on Pgp, altering the substrates affinity or the tridimensional
107 structure of the transporter, leading to a reduced catalytic efficacy. However, the effects of flavonoids
108 are often tumor type- and model-dependent. For instance, in Pgp-expressing colon cancer the activity
109 of quercetin, galangin and kaempferol on the efflux of adriamycin was unrelated to their structure,
110 and all the compounds are very weak competitors [13]. Moreover, within the same tumor the efflux
111 of adriamycin in crude membrane extracts of Pgp-expressing cells was significantly higher than the
112 efflux measured in cell cultures [13], leading to hypothesize that other factors – as flavonoids uptake,
113 change of Pgp structure in isolated membranes compared to living cells – impact on the final effect of
114 flavonoids on chemoresistance. Flavonoids and terpenoids have broader effects than simply
115 interacting with Pgp: for instance, they reduce the transcription of Pgp, inhibit the activity of
116 cytochrome p450 isoforms catabolizing chemotherapeutic drugs, improve the pharmacokinetic profile
117 of anti-cancer agents [12]. Overall, despite some contradictory findings, natural compounds with a
118 flavonoid structure may be noteworthy of further investigation about their efficacy and safety as
119 chemosensitizing agents, given their broad-spectrum effects on Pgp -expressing cells.

120 *Tephrosia purpurea*, a pantropical flowering plant of *Fabaceae* family widely used in traditional
121 medicine, was reported to contain rotenoids, isoflavones, flavanones, flavones, flavanols and
122 chalcones [14]. Prenylated flavonoids are the major isolated compounds from this plant. Among them
123 Glabratephrin (Glab) was repeatedly isolated as the major constituent of *T. purpurea* [15-18]. Glab,
124 and recently isoglabratephrin [15], are the only examples in nature of prenylated flavonoids with a 4-
125 hydroxy-2,7-dioxaspiro[4.4]nonan-1-one-3,3-dimethyl ring moiety. Glab has shown multiple
126 biological activities, ranging from anti-helminthic to anti-microbial, anti-fungal, anti-inflammatory,
127 anti-pyretic effects [15-18]. The ethanolic extract of *T. purpurea* has shown cytotoxic activity against

128 the nasopharyngeal KB cell line, where it inhibits cell proliferation and migration [18]. Also
129 Glabridin, a prenylated isoflavan with cyclic prenylation of ring A, is an anticancer agent [19] and a
130 potent inhibitor of Pgp expression and activity [20], but no studies about the chemosensitizing
131 properties of the rare spiro-prenylated compounds such as Glab and isoglabratephrin exist, despite
132 QSAR studies predict that the prenylated flavonoid structure as that present in Glab may interact with
133 Pgp [21]. Additionally, Glab was selected after a small-scale screening of 60 medicinal plant extracts
134 and compounds from the library of the Pharmacognosy Department in National Research Center,
135 Giza, Egypt, because it is extracted with a cost-effective isolation scheme that can fit industrial scale
136 up. This is of paramount importance for eventual medicinal and clinical applications.

137 The aim of this work was to set up a combinatorial treatment based on Glab and Dox, able to reverse
138 the resistance to this chemotherapeutic drug without unwanted systemic toxicity. We addressed this
139 issue by testing the efficacy of Glab in triple negative breast cancer cells expressing different amount
140 of Pgp, with an acquired or a constitutive resistance to Dox. In particular, we investigated the safety
141 and ability of Glab in restoring the efficacy of Dox, the first-line treatment for triple negative breast
142 cancer [22], in vitro and in vivo.

143 **2. Materials and Methods**

144 *2.1. Chemicals and Materials*

145 The lab supplies for cell cultures were obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ).
146 The electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). The protein content of
147 cell lysates was assessed with the BCA kit from Sigma Chemicals Co. (St. Louis, MO). Unless
148 specified otherwise, all reagents were purchased from Sigma Chemicals Co.

149 *2.2. Plant material*

150 The aerial parts of *T. purpurea* were collected in February 2010, Marfai Valley, Gabal Elba, Egypt
151 (22.2145 N, 36.3899 E, 450 m altitude). A voucher specimen has been deposited in the herbarium of

152 National Research Centre, Cairo, Egypt. The plant was identified by one of the authors (A. H. H. E).
153 The plant was air-dried in the dark, reduced to a 36 mesh-powder using a cutter mill and kept in
154 tightly sealed containers until the extraction.

155 2.3. Extraction and isolation of glabratephrin

156 Air-dried aerial plant powder (1200 g) was extracted with methylene dichloride (CH₂Cl₂)-methanol
157 (MeOH) (1:1, 3 L) by maceration at room temperature and repeated 20 min cycles of sonication (50%
158 amplitude; Hielscher UP200S, Ultrasound Sonicator, GmbH, Teltow, Germany). The solvent was
159 evaporated under vacuum at 40°C. The residue (70 g) was subjected to silica gel open column
160 chromatography (Silica gel 60 - 0.063-0.200 mm, 70-230 mesh -, Merck, Darmstadt, Germany) and
161 eluted with *n*-hexane, ethyl acetate (EtOAc), with increasing polarity gradient from 10% to 100%
162 EtOAc, then washed with MeOH. Similar fractions were pooled based on their separation by thin
163 layer chromatography (TLC), carried out on TLC Silica gel 60G F₂₅₄ 25 Glass plates (Merck), to yield
164 9 main fractions (FrA1-FrA9). FrA7 contained a major spot at R_f 0.5, emitting a blue fluorescence at
165 254 nm, and was subjected to silica gel column chromatography, eluted with *n*-hexane-EtOAc (2:1)
166 to yield 12 fractions (FrB1-FrB12). The 11th fraction, containing the major spot, was further purified
167 by Sephadex LH-20 column chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden),
168 eluted with CH₂Cl₂-methanol (MeOH) (1:1) to yield 94 mg of colorless crystals that were identified
169 as Glab (Figure 1), by ESIMS and ¹H-NMR analyses, whose spectrum (Supplementary Figure S1)
170 matched with the already published spectra of Glab [18]. The purity of the compound was 99%.

171 2.3. Cell lines

172 Human triple negative breast cancer MDA-MB-231 cells and murine mammary JC cells were
173 purchased from ATCC (Manassas, VA), and were cultured in RPMI-1640 medium with 10% fetal
174 bovine serum (FBS) and 1% penicillin-streptomycin (PS). MDA-MB-231/DX were generated by
175 culturing parental MDA-MB-231 cells in complete medium, adding Dox at increasing concentrations

176 every 5 passages (p0: 10 nM Dox; p5: 25 nM; p10: 50 nM; p15: 100 nM; p20: 250 nM; p25: 500 nM;
177 p30: 1000 nM). Human peripheral blood mononuclear cells (PBMC) from 3 different healthy donors
178 were obtained by the Blood Bank of AOU Città della Salute e della Scienza, Torino, Italy (#DG-
179 767/2015) and cultured in RPMI-1640 medium with 10% FBS, 1% PS. Human fibroblasts were a
180 kind gift of Prof. Francesco Novelli, Department of Molecular Biotechnology and Health Sciences,
181 University of Torino, Italy, and were maintained in DMEM medium with 10% FBS, 1% PS.

182 *2.4. Immunoblotting*

183 Cells were rinsed with ice-cold lysis buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton-X100),
184 supplemented with the protease inhibitor cocktail set III (80 µM aprotinin, 5 mM bestatin, 1.5 mM
185 leupeptin, 1 mM pepstatin; Calbiochem, San Diego, CA), 2 mM phenylmethylsulfonyl fluoride and 1
186 mM Na₃VO₄. Samples were sonicated (with 10 bursts of 1 s, amplitude 40%; Hielscher UP200S,
187 Ultrasound Sonicator) and centrifuged at 13,000 × g for 10 min at 4°C. 20 µg protein extracts were
188 subjected to SDS-PAGE and probed with the following antibodies: anti-Pgp (1:250, rabbit polyclonal
189 #sc-8313, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-MRP1 (1:500, mouse clone MRPm5,
190 Abcam, Cambridge, UK), anti-BCRP (1:500, mouse clone BXP-21, Santa Cruz Biotechnology Inc.),
191 anti-β-tubulin (1:1000, mouse clone D10, Santa Cruz Biotechnology Inc.), followed by a peroxidase-
192 conjugated secondary antibody (Bio-Rad Laboratories). The membranes were washed with Tris-
193 buffered saline-Tween 0.1% v/v solution, the proteins were detected by enhanced chemiluminescence
194 (Bio-Rad Laboratories). The relative quantitation of immunoblot was performed with the ImageJ
195 software (<https://imagej.nih.gov/ij/>). The band density of untreated MDA-MB-231 cells was
196 considered as 1 arbitrary unit.

197 *2.5. RT-PCR*

198 Total RNA was extracted and reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad
199 Laboratories). qRT-PCR was performed using the IQ™ SYBR Green Supermix (Bio-Rad

200 Laboratories). The PCR primer sequences, designed using the qPrimerDepot software
201 (<http://primerdepot.nci.nih.gov/>), were: *Pgp* (human): 5'-TGCTGGAGCGGTTCTACG-3', 5'-
202 ATAGGCAATGTTCTCAGCAATG-3'; *S14* (human): 5'-CGAGGCTGATGACCTGTTCT-3', 5'-
203 GCCCTCTCCACTCTCTCTT-3'. The relative gene expression levels were calculated using the
204 Gene Expression Quantitation software (Bio-Rad Laboratories).

205 2.6. Hypoxia Inducible Factor-1 α (HIF-1 α) activity

206 Nuclear proteins were extracted using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium),
207 and quantified. The activity of HIF-1 α was assessed on 10 μ g of nuclear proteins by the TransAM™
208 HIF Activity kit (Active Motif). The absorbance at 450 nm was measured with a Packard EL340
209 microplate reader (Bio-Tek Instruments, Winooski, VT). For each set of experiments, a blank was
210 prepared with bis-distilled water, and its absorbance was subtracted from that obtained in the
211 presence of nuclear extracts. Data were expressed as absorbance units/mg nuclear proteins.

212 2.7. Intracellular doxorubicin accumulation

213 Cells were washed twice with PBS 1X, detached by gentle scraping and centrifuged at 13,000 \times g for
214 5 minutes at 4°C. Cell pellets were re-suspended in 400 μ l of a 1:1 mixture of ethanol/0.3 N HCl and
215 sonicated (10-sec bursts). The protein content was assessed with the BCA kit. The amount of
216 intracellular doxorubicin was detected using a Synergy HT 96-well micro-plate reader (Bio-Tek
217 Instruments). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was
218 prepared in the absence of cells in each set of experiments; its fluorescence was subtracted from that
219 measured in each sample. Fluorescence was converted in nmoles doxorubicin/mg cell proteins using
220 a calibration curve.

221 2.8. Cell viability

222 Cell viability was evaluated by crystal violet staining, as reported previously [23]. To calculate the
223 IC₅₀, 1 \times 10⁴ cells were seeded in 96-well plates, treated with scalar concentrations (from 10⁻⁹ M to

224 10^{-2} M) of Glab for 72 h, and then stained with crystal violet solution. The quantitation of crystal
225 violet staining was performed by reading the absorbance at 540 nm with a Synergy HT 96-well
226 micro-plate reader (Bio-Tek Instruments). The mean absorbance of untreated cells was considered
227 100%; the absorbance units of the other experimental conditions were expressed as percentage of
228 viable cells vs. untreated cells. IC_{50} , i.e. the concentration of the compound that decreases cell
229 viability by 50%, was calculated with the GraphPad Prism (v 6.01) software. The Combination Index
230 (CI) was calculated by measuring the viability in cells incubated with scalar concentrations (from 10^{-10}
231 M to 10^{-5} M) of Dox and Glab, using the CalcuSyn software (www.biosoft.com/w/calculusyn.htm).

232 2.9. *In vivo tumor growth*

233 1×10^7 JC cells were mixed with 100 μ l Matrigel and orthotopically implanted in 6-week-old female
234 immunocompetent balb/C mice (Charles River Laboratories Italia, Calco), housed (5 per cage) under
235 12 h light/dark cycle, with food and drinking provided *ad libitum*. Tumor growth was measured daily
236 by caliper, according to the equation $(L \times W^2)/2$, where L=tumor length and W=tumor width. When
237 tumors reached the volume of 50 mm³, mice (n= 8/group) were randomized in the following groups
238 and treated on day 1, 7, 14 after randomization as reported: 1) vehicle group, treated with 200 μ l
239 saline solution intravenously (i.v.); 2) Glabratephrin group, treated with a 200 μ l water/10% DMSO
240 solution i.v., containing 5 μ M Glab; 3) doxorubicin group, treated with 5 mg/kg Dox, dissolved in
241 200 μ l water i.v.; 4) Glabratephrin + doxorubicin group, treated with 100 μ l of saline solution i.v.
242 containing 5 μ M Glab + 100 μ l water solution containing 5 mg/kg Dox. Tumor volumes were
243 monitored daily. Animals were euthanized at day 21 after randomization with zolazepam (0.2 ml/kg)
244 and xylazine (16 mg/kg). Tumors were excised, photographed, fixed in 4% v/v paraformaldehyde
245 overnight, and paraffin embedded. The paraffin sections were stained with haematoxylin-eosin or
246 immune-stained for Ki67 (1:100; rabbit #AB9260, Sigma-Aldrich, St. Louis, MO), as index of cell
247 proliferation, followed by a peroxidase-conjugated secondary antibody (1:100, Dako, Glostrup,

248 Denmark). Liver, kidneys and spleen were excised, fixed, and paraffin sections were examined after
249 haematoxylin-eosin staining. The sections were examined with a Leica DC100 microscope (Leica,
250 Weitzlar, Germany). Lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine
251 aminotransferase (ALT), alkaline phosphatase (AP), creatinine, creatine phosphokinase (CPK) and
252 CPK-MB, cardiac troponin I (cTnI) and T (cTnT) were measured on blood samples collected
253 immediately after euthanasia, using commercially available kits from Beckman Coulter Inc.
254 (Beckman Coulter, Miami, FL). To test the acute toxicity, 6-week-old female balb/C mice
255 (n=5/group) were treated with vehicle (200 µl saline solution i.v.) or with a 200 µl water/10% DMSO
256 solution containing 0.5, 1, 5, 10 µM Glab (i.v.) After 24 h the animals were sacrificed, the blood was
257 collected and the hematochemical parameters indicated above were assessed. In all studies,
258 researchers analyzing the results were unaware of the treatments received by animals. The Animal
259 care and experimental procedures were approved by the Bio-Ethical Committee of the Italian
260 Ministry of Health (#122/2015-PR).

261 *2.10. Rhodamine 123 efflux*

262 Cells were washed with PBS, detached and re-suspended in 1 ml of fresh medium containing 5%
263 FBS. The samples were maintained at 37°C for 20 min in the presence of 1 µg/ml Rhodamine 123.
264 After this incubation time, cells were washed and re-suspended in 0.5 ml of PBS. The intracellular
265 Rhodamine 123 content, which is inversely related to its efflux, was detected fluorimetrically using a
266 a Synergy HT 96-well micro-plate reader (Bio-Tek Instruments). Excitation and emission
267 wavelengths were 475 and 553 nm, respectively [24]. A blank was prepared in the absence of cells in
268 each set of experiments; its fluorescence was subtracted from that measured in each sample. The
269 results were expressed as nmoles/mg cell proteins.

270 *2.11. Doxorubicin efflux*

271 Cells were incubated for 10 min with increasing concentrations (0-50 μM) of Dox, with or without
272 Glab, then washed and analyzed for the intracellular concentration of Dox. A second series of dishes,
273 after the incubation under the same experimental conditions, were left for further 10 min at 37°C,
274 then washed and tested for the intracellular drug content. The difference of Dox concentration
275 between the two series during this time (dc/dt) was plotted versus the initial drugs concentration [24].
276 Values were fitted to Michaelis-Menten equation to calculate V_{max} and K_{m} , using the Enzfitter
277 software (Biosoft Corporation, Cambridge, United Kingdom).

278 *2.12. Pgp ATPase activity*

279 The Pgp ATPase activity was measured in membrane vesicles as described previously [24]. Cells
280 were washed with Ringer's solution (148.7 mM NaCl, 2.55 mM K_2HPO_4 , 0.45 mM KH_2PO_4 , 1.2 mM
281 MgSO_4 ; pH 7.4), lysed on ice with lysis buffer (10 mM HEPES/Tris, 5 mM EDTA, 5 mM EGTA, 2
282 mM dithiothreitol; pH 7.4) supplemented with 2 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin,
283 10 $\mu\text{g}/\text{ml}$ pepstatin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and subjected to nitrogen cavitation at 1200 psi for 20 min.
284 Samples were centrifuged at $300 \times g$ for 10 min in the pre-centrifugation buffer (10 mM Tris/HCl, 25
285 mM sucrose; pH 7.5), overlaid on a sucrose cushion (10 mM Tris/HCl, 35% w/v sucrose, 1 mM
286 EDTA; pH 7.5) and centrifuged at $14,000 \times g$ for 10 min. The interface was collected, diluted in the
287 centrifugation buffer (10 mM Tris/HCl, 250 mM sucrose; pH 7.5), centrifuged at $100,000 \times g$ for 45
288 min. The vesicle pellet was re-suspended in 0.5 ml centrifugation buffer and stored at -80°C until the
289 use, after the quantification of the protein content. 20 μg of total protein were incubated for 30 min at
290 37°C with 50 μl of the reaction mix (25 mM Tris/HCl, 3 mM ATP, 50 mM KCl, 2.5 mM MgSO_4 , 3
291 mM dithiothreitol, 0.5 mM EGTA, 2 mM ouabain, 3 mM NaN_3 ; pH 7.0). In each set of experiments,
292 a blank containing 0.5 mM Na_3VO_4 was included. The reaction was stopped by adding 0.2 ml ice-
293 cold stopping buffer (0.2% w/v ammonium molybdate, 1.3% v/v H_2SO_4 , 0.9% w/v SDS, 2.3% w/v
294 trichloroacetic acid, 1% w/v ascorbic acid). After 30 min incubation at room temperature, the

295 absorbance of the phosphate hydrolyzed from ATP was measured at 620 nm, using a Packard EL340
296 microplate reader (Bio-Tek Instruments). The absorbance was converted into nmoles hydrolyzed
297 phosphate (Pi)/min/mg proteins, according to the titration curve previously prepared.

298 *2.13. Docking studies*

299 The Molecular Operating Environment (MOE) software (Chemical Computing Group;
300 <https://www.chemcomp.com/Products.htm>) was used to model the binding of Glab with Pgp (Protein
301 Data Bank, PDB: 4M2S and 5KPI, as second crystal structure of mouse native Pgp showing 99%
302 homology with 4M2S, according to Clustalw-multiple sequence alignment) [25]. The binding sites
303 for QZ59-RRR, a cyclic-tris-(R)-valineselenazole, QZ59-SSS and verapamil, as well as the amino
304 acid residues involved in binding, identified by Aller et al [26], were used for docking these
305 compounds and Glab. Since 4M2S was co-crystalized with QZ59-RRR as a binding ligand, providing
306 a necessary tool for comparing the binding affinity of unknown compounds as Glab with the
307 reference compound, 4M2S was chosen for molecular docking study.

308 London ΔG scoring function was used for scoring. It was enhanced by using two different refinement
309 methods - the force-field and Grid-Min poses - to ensure that refined poses satisfy the specified
310 conformations. Rotatable bonds were allowed. The best 10 poses were retained and analyzed for the
311 binding poses best score. The database browser was used in MOE to compare the docking poses to
312 the ligand in the co-crystallized structure and to get the root mean square deviation (RMSD) of the
313 docking pose compared to the co-crystallized ligand position.

314 The molecule builder tool of MOE software was used to construct a tridimensional model of the
315 structures. Energy minimization was done through force-field MMFF94x optimization using a
316 gradient of 0.0001 for determining low-energy conformations with the most favorable (lowest
317 energy) geometry. The crystal structures of 4M2S receptor protein in complex with QZ59-RRR were
318 obtained from the PDB.

319 Hydrogen atoms and partial charges were added to the protein to assign ionization states and position
320 hydrogen atoms in the macromolecular structure. The binding free energy as well as hydrogen
321 bonding were used to rank the binding affinity of QZ59-RRR and Glab to Pgp. The evaluation of the
322 hydrogen bonds was done by measuring the hydrogen bond length, which did not exceed 3.5 Å. The
323 RMSD of the docking pose compared to the co-crystal ligand position was used in the ranking. The
324 mode of interaction of the native ligand within the crystal structure was used as a standard docked
325 model as well as for RMSD calculation.

326 *2.14. Overexpression of wild-type and mutated Pgp*

327 The pHa vector containing the full-length *mdr1* cDNA, encoding for Pgp, was purchased from
328 Addgene (Cambridge, MA) and subcloned into pCDNA3 vector. By sequencing the *mdr1* gene
329 present in the pCDNA3 vector, we verified that it contained the wild-type sequence of Pgp (data not
330 shown). pCDNA3 vector containing the wild-type *mdr1* cDNA, was subjected to PCR-based
331 mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA), following the manufacturer's
332 instructions to generate the mutated constructs Gly185Val, Ser400Asn, Gly412Ala, Ser893Ala,
333 Ser893Thr. The mutations were confirmed by DNA sequencing [27]. In transfection experiments, $5 \times$
334 10^4 cells were seeded in FBS-free medium and treated with 6 μ l of jetPEI transfection reagent
335 (Polyplus-transfection SA BIOPARC, Illkirch, France) and 3 μ g DNA empty-pCDNA3 (mock cells),
336 wild-type *mdr1*-pCDNA3 (wild-type Pgp) or mutated *mdr1*-pCDNA3 (mutated Pgp). After 6 h, cells
337 were washed and grown in complete medium for 48 h before the experiments indicated in the Results
338 section.

339 *2.15. Statistical analysis*

340 Results were analyzed by a one-way analysis of variance (ANOVA) and Tukey's test, using
341 GraphPad Prism software (v 6.01). $p < 0.05$ was considered significant. All data were expressed as
342 means \pm SD.

343

344 **3. Results**

345 *3.1. Doxorubicin accumulation in sensitive and resistant breast cancer cells*

346 From triple negative breast cancer MDA-MB-231 cells, we generated the MDA-MB-231/DX variant
347 by culturing parental cells in medium with increasing concentrations of doxorubicin, as detailed in the
348 Materials and Methods section, to obtain a cellular model of acquired resistance to Dox. Murine
349 mammary JC cells were used as a model of cells constitutively expressing Pgp and constitutively
350 resistant to Dox [28]. Starting from passage number 10, MDA-MB-231/DX subline increased Pgp
351 mRNA (Figure 2A) and protein (Figure 2B-C), reaching a plateau level at passage 20, corresponding
352 to culture conditions in a medium containing 250 nM Dox. Pgp increase was not accompanied by any
353 increase in MRP1 or BCRP (Figure 2B-C), two other transporters involved in Dox efflux [29]. The
354 increase in Pgp expression was accompanied by a progressive increase in the activity of HIF-1 α
355 (Figure 2D), a transcription factor activated by Dox [30] and an inducer of Pgp gene transcription
356 [31]. The same event was previously reported in the colon cancer HT29/DX subline, generated by
357 culturing parental cell in medium with increasing concentration of Dox [32], suggesting that it is a
358 process common to different cancer cell types during the acquisition of resistance.

359 For all the subsequent experiments, we used cells at passage 25, i.e. stably growing in medium
360 containing 500 nM Dox. As shown in Figure 3A-B, MDA-MB-231/DX cells had an intermediate
361 level of Pgp between parental MDA-MB-231 and JC cells. Consistently, MDA-MB-231/DX cells
362 retained significantly less Dox than parental MDA-MB-231 cells, and JC cells had the lowest
363 accumulation of the drug (Figure 3C). In the drug accumulation assay, we tested the concentration of
364 5 μ M Dox, i.e. the minimum concentration that discriminates sensitive from resistant cells in terms of
365 intracellular drug retention and cytotoxicity [32], and 25 μ M Dox, i.e. a concentration that is
366 cytotoxic for most cell lines, except for strongly Pgp-expressing cells as JC [33], and corresponds to

367 the highest concentration displaying good solubility in cell culture medium. Consistently with the
 368 intracellular retention data, in viability assays, 5 μ M Dox induced a significant reduction of MDA-
 369 MB-231 cells, that was less pronounced in MDA-MB-231/DX subline and absent in JC cells (Figure
 370 3D). Similarly, 25 μ M Dox, that was more accumulated within all cell lines except JC cells (Figure
 371 3C), produced a moderate decrease of cell viability in MDA-MB-231/DX cells but it had no effects in
 372 JC cells (Figure 3D). This different behavior can be explained by the different expression levels of
 373 Pgp between MDA-MB-231, MDA-MB-231/DX and JC cells. Based on these results, we considered
 374 MDA-MB-231, MDA-MB-231/DX and JC cells as Dox-sensitive, moderately Dox-resistant and
 375 strongly Dox-resistant cells, respectively.

376 *3.2. Glabratephrin increases doxorubicin-induced cytotoxicity in resistant cells at non-cytotoxic*
 377 *concentrations*

378 To investigate the effect of Glab, we first calculated its IC₅₀ in our models. While in Dox-sensitive
 379 cells, the IC₅₀ value was higher than 1 mM, in Dox-resistant cells it was reduced below 250 μ M
 380 (Table 1).

381 **Table 1. IC₅₀ (mM) of Glabratephrin in breast cancer cells**

Compound	IC ₅₀ (mM)		
	MDA-MB-231	MDA-MB-231/DX	JC
Glabratephrin	> 1	0.236 \pm 0.067 **	0.138 \pm 0.042 **

382 1×10^4 cells were seeded in quadruplicates in 96-well plates, treated with scalar concentrations (from
 383 10^{-9} M to 10^{-2} M) of Glab for 72 h. Cell viability was measured by crystal violet staining. IC₅₀, i.e. the
 384 concentration of Glab that decreased the cell viability by 50%, was calculated with the GraphPad
 385 Prism (v 6.01) software. Data are means \pm SD (n = 4). MDA-MB-231/DX and JC cells vs. MDA-
 386 MB-231 cells: ** p < 0.01.

387

388 The preferential sensitivity of Pgp-expressing cells prompted us to investigate if Glab may act as an
389 adjuvant of chemotherapeutic drugs substrates of Pgp, as Dox. In a preliminary screening, we
390 incubated the three cell lines with different concentrations of Glab (1 nM, 10 nM, 100 nM, 1 μ M, 10
391 μ M), with and without sub-toxic Dox concentrations (0.05, 0.5 and 5 μ M). Cell viability was
392 measured after 72 h. At all concentrations Glab alone reduced cell viability less than 20% (Figure 4A-
393 C). In Dox-sensitive MDA-MB-231 cells, Glab did not increase the cytotoxic effects of Dox (Figure
394 4A). In moderately Dox-resistant MDA-MB-231/DX cells, at all concentrations Dox reduced cell
395 viability no more than 20%, but Glab - associated with Dox - increased cell death starting from 10
396 nM concentration (Figure 4B). The cytotoxic effect was progressively stronger at increasing
397 concentrations of Glab and Dox. In highly Dox-resistant JC cells, Dox did not significantly reduce
398 cells viability, as expected. Interestingly, Glab rescued the cytotoxicity of Dox, starting from the
399 concentration of 100 nM; the number of viable cells was progressively reduced at the increasing of
400 Glab and Dox concentrations (Figure 4C). The effect of Glab and Dox was synergistic, as indicated
401 by the CI of 0.29721 in MDA-MB-231/DX cells (Figure 4D) and 0.10922 in JC cells (Figure 4E). To
402 evaluate the toxicity of the different combinations of Glab and Dox on non-transformed cells, we
403 measured the cell viability in human PBMC and fibroblasts. As reported in the new Supplementary
404 Figure S2A-B, the reduction in the viability of normal cells was comparable or lower than in breast
405 cancer cell lines.

406 *3.3. Glabratephrin reverses doxorubicin resistance in vivo without inducing systemic toxicities*

407 Prompted by the good anti-proliferative efficacy achieved against breast cancer cell lines and the
408 acceptable toxicity profile observed in non-transformed cells in vitro, we next evaluated the efficacy
409 of Glab in mice bearing JC tumors that were completely refractory to Dox (Figure 5A-B). In a
410 preliminary set of experiments to evaluate the acute toxicity, we determined that 5 μ M Glab was the
411 maximal dose of the compound that did not cause liver, kidney or heart toxicities, based on the

412 hematochemical parameters (Supplementary Table S1). This concentration was chosen for the
413 subsequent experiments of anti-tumor efficacy. While Glab alone did not have any effects, the
414 combination of Glab + Dox lowered the rate of tumor growth (Figure 5A), resulting in smaller tumor
415 masses (Figure 5B). The growth profile was characterized by an initial delay in the growth of tumor,
416 followed by a steady-state in the tumor volumes. This trend suggests that the effect of Glab + Dox
417 combination is cytostatic. In keeping with these results, the positivity for Ki67, an index of tumor
418 proliferation, was similar in animals treated with vehicle, Glab or Dox alone, but it was reduced in the
419 tumors from Glab + Dox group (Figure 5C), indicating a good antitumor efficacy of the combination.
420 We are aware that many Pgp inhibitors, particularly of first and second generation, were effective *in*
421 *vitro* but they failed in preclinical models for the high systemic toxicity [34]. Post-mortem
422 examination of liver, kidney and spleen did not show histological abnormalities in these organs for all
423 the treatment groups, including the animals treated with Glab and Dox combination (Figure 5D). At
424 the time of sacrifice, we also measured specific hematochemical parameters as indexes of possible
425 systemic toxicities in the treated animals (Table 2). According to these parameters, no signs of liver
426 toxicities - indicated by LDH, AST, ALT, AP - and kidney toxicities - indicated by creatinine - were
427 detected in Glab-treated animals, alone or in combination with Dox, in agreement with the lack of
428 histological abnormalities. In our experimental protocol, Dox was used at a the maximum tolerated
429 dose [35]. This regimen did not induce fatal events in the treated animals, but it elicited an
430 appreciable cardiac damage indicated by the increase of CPK and its cardiac specific isoform CPK-
431 MB, as well as cTnT. Glab did not affect cardiac parameters when used alone nor worsened the
432 damaged elicited by Dox when used in combination (Table 2), indicating that it did not exacerbate
433 Dox cardiotoxicity.

434 **Table 2. Hematochemical parameters of treated animals**

	Ctrl	Glab	Dox	Glab + Dox
LDH (U/l)	6578 ± 504	6892 ± 298	6791 ± 471	6792 ± 561
AST (U/l)	193 ± 39	145 ± 35	139 ± 41	127 ± 38
ALT (U/l)	34 ± 7	36 ± 5	38 ± 7	39 ± 10
AP (U/l)	113 ± 31	145 ± 23	139 ± 18	128 ± 27
Creatinine (mg/l)	0.071 ± 0.012	0.065 ± 0.008	0.061 ± 0.008	0.064 ± 0.009
CPK (U/l)	287 ± 77	281 ± 62	542 ± 45 *	591 ± 78 *
CPK-MB (ng/ml)	0.128 ± 0.062	0.110 ± 0.044	0.321 ± 0.076 *	0.296 ± 0.081 *
cTnI (pg/ml)	1.089 ± 0.034	1.028 ± 0.089	1.055 ± 0.041	1.032 ± 0.042
cTnT (pg/ml)	1.983 ± 0.301	1.872 ± 0.217	2.986 ± 0.104 *	3.117 ± 0.285 *

435 Balb/C mice (n=8 animals/group) were treated as described in Figure 5. Blood was collected
436 immediately after euthanasia and analyzed for lactate dehydrogenase (LDH), aspartate
437 aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatinine,
438 creatine phosphokinase (CPK) and CPK-MB, cardiac troponin I (cTnI) and T (cTnT). Data are
439 presented as means ± SD. * p < 0.05: vs Ctrl group.
440

441 *3.4. Glabratephrin increases intracellular doxorubicin accumulation and cytotoxicity in resistant*
442 *breast cancer cells by decreasing Pgp activity*

443 We next investigated whether Glab enhanced Dox cytotoxicity by increasing its intracellular retention
444 and/or reducing Dox efflux via Pgp. After 24 h incubation with 5 µM Dox and 100 nM Glab,
445 corresponding to the lowest concentration of Glab increasing the drug's cytotoxicity in both the
446 resistant cell lines analyzed, Dox was significantly more accumulated in the Pgp-expressing MDA-
447 MB-231/DX and JC cells, not in the Pgp-negative MDA-MB-231 cells (Figure 6A). Similarly, Glab

448 increased the retention of Rhodamine 123, another substrate of Pgp, in resistant cells, when co-
 449 incubated with the dye (Figure 6B). These data suggest that Glab may compete with Dox or
 450 Rhodamine 123 for their binding and efflux through Pgp.

451 To further explore this issue, we measured the kinetic parameters of Dox efflux from MDA-MB-
 452 231/DX and JC cells. As shown in Figure 6C and Table 3, MDA-MB-231/DX had a lower Vmax
 453 than JC cells, consistently with their lower expression of Pgp. In both cell lines, Glab reduced the
 454 Vmax of Dox efflux, suggesting that it decreased the maximal catalytic efficiency of Pgp. Our
 455 hypothesis was proved by the significant decrease of Pgp ATPase activity induced by Glab (Figure
 456 6D). By contrast, Glab did not change the amount of Pgp protein (Supplementary Figure S3),
 457 suggesting that the reduction of Vmax was caused by a decreased activity of the transporter, not by a
 458 different expression. In parallel, Glab increased the Km of Dox (Figure 6C; Table 3), indicating a
 459 reduction in Dox affinity for Pgp.

460 **Table 3. Kinetic parameters of Dox efflux**

Cells	Vmax ($\mu\text{moles}/\text{min}$)	Km (μM)
MDA-MB-231/DX - Glab	13.2 \pm 2.4	0.15 \pm 0.04
MDA-MB-231/DX + Glab	6.7 \pm 1.2 ***	0.27 \pm 0.05 **
JC - Glab	4.2 \pm 0.7	0.12 \pm 0.04
JC + Glab	1.9 \pm 0.9 *	0.24 \pm 0.04 **

461 Cells were incubated as reported in Figure 6C. Vmax and Km were calculated with the Enzfitter
 462 software. Data are presented as means \pm SD (n = 3). Glab-treated vs. untreated cells: * p < 0.05; ** p
 463 < 0.01; *** p < 0.001.

464

465 3.5. Molecular docking studies of Glabratephrin on Pgp

466 Glab was docked at QZ59-RRR, QZ59-SSS and verapamil binding sites [26]. The docking scores -
467 based on the binding affinity measured by RMSD values, on the predicted hydrogen bond and on the
468 binding free energy (S-score) - were used to evaluate binding affinity. The docking scores in the
469 binding site of QZ59-RRR were the only favorable for inhibitory activity of Glab. Applying the MOE
470 software, Pgp (PDB: 4M2S) structure was separated from the structure of the putative ligand. The
471 binding free energy as well as hydrogen bonding were used to rank the binding affinity of QZ59RRR
472 and Glab to Pgp. Docking accuracy was validated by re-docking the co-crystallized ligand QZ59-
473 RRR into the binding site of QZ59-RRR in Pgp (Figure 7A), with a RMSD of 1.31 Å and a binding
474 free energy of -10.07 kcal/mol (Table 4).

475 Glab docking calculation was carried out using the standard default variable in the MOE software.
476 Glab was docked into the same groove of the co-crystallized ligand QZ59-RRR (Figure 7B) and had
477 a comparable binding free energy (Table 4). The refined docking of Glab to Pgp is reported in Figure
478 7C.

479 **Table 4. Binding affinity evaluation using binding free energy**

Ligand	S- Score (Kcal/mol)	Number of H-bond involved	Aminoacids involved
QZ59-RRR	-10.07	2	Gln 721; Phe 979
Glabratephrin	-10.95	2	Gln 721; Phe 332

480 Gln: glutamine; Phe: phenylalanine

481 The binding mode of QZ59-RRR with Pgp (Supplementary Figure S4) was predicted to have one H-
482 bond donor with Gln721 at 3.2 Å distance and one arene interaction with Phe979 (Figure 8A). The
483 binding mode of Glab with Pgp was predicted to have one H-bond donor with Gln721 at 2.6 Å
484 distance and one arene interaction Phe332 (Figure 8B). These results suggest that both the carbonyl

485 group of the acetyl residue and the mono-substituted phenyl moiety of Glab are necessary to interact
486 with Pgp.

487 3.6. The Pgp domain centered around glycine 185 is necessary to mediate Glabratephrin activity

488 To identify a possible mechanism by which Glab, after binding Pgp, interferes with the catalytic
489 activity of the transporter, we over-expressed wild-type Pgp and five different mutants containing the
490 mutations mostly annotated in human tumors - Gly185Val, Ser400Asn, Gly412Ala, Ser893Ala,
491 Ser893Thr (ABCMDb/Database for Mutations in ABC proteins; <http://abcmutations.hegelab.org/>) in
492 MDA-MB-231 cells. As shown in Figure 9A, both wild-type and mutated Pgp were expressed at
493 comparable levels, higher than the levels of endogenous Pgp. Interestingly, Glab increased Dox
494 accumulation (Figure 9B) and ATPase activity (Figure 9C) in cells expressing wild-type Pgp and all
495 Pgp mutants, except in cells expressing Gly185Val-mutated protein (Figure 9B-C). These results
496 suggest that the domain containing Gly185 is important in determining the inhibitory effect of Glab
497 on Pgp activity.

498 4. Discussion

499 In this work, we reported for the first time the potential of Glab, a prenylated flavonoid extracted
500 from the medicinal plant *T. purpurea*, with a unique chemical structure, in reversing the resistance to
501 Dox mediated by Pgp in triple negative breast cancer cells.

502 A cytotoxic activity of Glab against oral cancer cells has been reported previously [14]. The IC₅₀
503 values measured in triple negative breast cancer cells, however, indicated that the compound reduced
504 cell viability only at high micromolar concentrations. Interestingly, IC₅₀ values were lower when the
505 levels of Pgp increased, either in cells with acquired or constitutive resistance to Dox. The peculiar
506 cytotoxicity of a compound in cells highly expressing Pgp is known as “collateral sensitivity” (CS)
507 [36] and is considered the most effective way to eradicate MDR. Thiosemicarbazones, 1,10-

508 phenanthrolines, as well as natural-products such as sesquiterpenic benzoquinones and flavonoids,
509 have been recently identified as potent inducers of CS [37]. According to the profile of IC_{50} , Glab is a
510 potential CS-inducer since it is slightly more effective as cytotoxic agent in cells with high levels of
511 Pgp, according to the IC_{50} . However, high micromolar concentrations, at which Glab resulted
512 moderately cytotoxic, are difficult to be reached in vivo. Hence, we excluded that Glab is a potent
513 anti-cancer agent when used alone.

514 On the other hand, Glab, in combination with Dox, exerted a synergistic toxicity against cells
515 expressing Pgp. Consistently, in xenografts of highly Pgp-expressing/Dox-resistant breast cancers
516 Glab rescued the anti-tumor efficacy of Dox. Besides exerting a strong reduction of tumor growth, the
517 combination of Glab and Dox did not show toxicity, as suggested by in vitro assays on non-
518 transformed cells, and *post-mortem* histological and hematochemical parameters of the treated
519 animals. Moreover, given the significant decrease of tumor volume elicited by this combination, we
520 propose Glab as an option to reduce the doses of Dox, preserving good efficacy against Pgp-
521 expressing tumors and reducing the dose-dependent cardiotoxicity that is the major side-effects of
522 anthracyclines.

523 Of note, the higher Pgp level was in triple negative breast cancer cells, the higher synergism between
524 Glab and Dox was observed. To explain this synergistic effect in Pgp-expressing cells, we
525 hypothesized that Glab may affect the expression or activity of Pgp, reducing the efflux of Dox. The
526 kinetic parameters of Dox efflux revealed a higher K_m and a lower V_{max} , together with a decreased
527 ATPase activity, in Pgp-expressing cells treated with Glab. By contrast, Glab did not change the
528 expression of Pgp. Upon binding, several substrates including Dox trigger the ATP hydrolysis and the
529 substrate transport [38]. Competitive Pgp inhibitors bind to the substrate binding sites and impair the
530 substrate binding coupled with the ATP hydrolysis; non-competitive inhibitors bind to the
531 transmembrane domains (TMD) of Pgp, to the intracellular linking domains or to the nucleotide

532 binding domains (NBD), impairing the catalytic cycle [38]. Our results pointed out that Glab
533 competes with Dox for its efflux through Pgp. This may be due to the reduced binding and/or reduced
534 release of Dox, indicated by the increased K_m , and to the uncoupling of substrate binding, ATP
535 hydrolysis and substrate efflux, indicated by the lower V_{max} .

536 To deepen our knowledge about the potential binding sites of Glab on Pgp, we performed molecular
537 docking studies and site-directed mutagenesis assays. According to the docking simulation, Glab is
538 predicted to bind Phe332 and Gln721, two residues mapping in the Pgp TMD 1 and 2, facing the
539 outer membrane leaflet [39]. They are outside the so-called hydrophobic pocket, i.e. the binding
540 pocket most likely involved in Dox transport [40], which is localized at the interface between Pgp and
541 inner membrane leaflet [41]. Therefore, it is unlikely that the increase in Dox K_m induced by Glab
542 was due to a decrease in the drug binding. We hypothesize that Glab may instead interfere with the
543 Dox release or with the substrate-triggered ATP hydrolysis [38]. The results of site-directed
544 mutagenesis indicated that Glab interferes with the domain centered around Gly185. This result is of
545 particular interest in a pharmacological perspective. Indeed, among the mutations analyzed, Ser400
546 and Gly412 mutations have no reported clinical significance; these amino acids are in exons 12 and
547 13 that are components of the NBD at the N-terminal side of Pgp [42]. Ser893 is localized between
548 the TMD 10 and 11 of Pgp; its mutation alters the efflux of lipophilic drugs, such as simvastatin [43],
549 ondasetron [44] and paclitaxel [45] but not Dox. Gly185 is in a large hydrophobic domain of Pgp
550 involved in drug release outside the cell [46]. Moreover, Gly185 dictates the conformation changes
551 induced by ATP hydrolysis and culminating in the extracellular release of the drugs: its mutation into
552 Val determines a more efficient coupling between these two processes [47]. Gly185Val mutation has
553 been involved in the resistance to lipophilic drugs such as colchicine, epipodophyllotoxins [48] and
554 Dox [24]. Moreover, this mutation impairs the effects of Pgp allosteric inhibitors, such as distearoyl-
555 phosphatidylethanolamine-polyethylene glycol (DSPE-PEG), which increases the K_m of Dox and

556 reduces the ATPase activity in wild-type Pgp, not in Gly185Val-mutated Pgp [24]. Of note, Glab
557 shows the same properties of DSPE-PEG, since it increased Dox Km and decreased Pgp ATPase
558 activity, but lose its efficacy in cells mutated at Gly185. We may hypothesize that the binding of Glab
559 on TMD 1 and 2 alters the conformation of Gly185-centered domain and disrupts the efficient
560 coupling between ATP hydrolysis and Dox efflux. In consequence of the decreased catalytic activity
561 of Pgp, the release of Dox towards the external side is less efficient, as suggested by the increased
562 Km of the drug and the reduced Vmax.

563 Dox is one of the first therapeutic option in triple negative breast cancer [22], but this type of breast
564 cancer is less responsive to Dox than other breast cancer types [49]. One of the main reasons for the
565 low success of Dox is the abundant presence of Pgp in triple negative breast cancer cells [50].
566 Increasing Dox efficacy in this setting is still an unmet need. Unluckily, most of the small molecules
567 designed as Pgp inhibitors [34] or selective killers of Pgp-expressing cells [36, 37] were effective *in*
568 *vitro* but not in preclinical models, because of low efficacy and/or undesired toxicities. Natural
569 products structurally analogue to Glab may offer a safe and effective alternative to inhibit Pgp.

570 In this work, we identified Glab as a potent Dox-sensitizer in Pgp-expressing triple negative breast
571 cancer cells, thanks to its inhibition of Pgp catalytic efficiency and Dox efflux. The increased
572 intracellular retention of Dox determines an increased cytotoxicity *in vitro* and a significant reduction
573 in tumor growth *in vivo*. Moreover, Glab did not display systemic toxicities according to the
574 hematochemical and histological parameters measured. These results may open the way to further
575 investigations of the efficacy of Glab in patient-derived triple negative breast cancer cells and
576 xenografts, to identify subset of patients – unresponsive to Dox because of high levels of Pgp and
577 characterized by poor prognosis – who may benefit from the use of Glab as a potentially adjuvant
578 agent.

579

580 **Disclosure of Potential Conflicts of Interest**

581 None

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741 **Figure Legends**

742 **Figure 1.** Extraction procedure and structure of Glabratephrin.

743

744 **Figure 2.** MDA-MB-231/DX subline generation. MDA-MB-231 cells were cultured in medium with
745 increasing concentration of Dox, as indicated in the Materials and methods section, generating the
746 Dox-resistant subline (MDA/DX). **A.** At time 0 (p0) and every 5 passages (p), Pgp mRNA was
747 measured by RT-PCR in triplicates. Parental MDA-MB-231 (MDA) cells were included as internal
748 control. Data are presented as means \pm SD (n=3). MDA-MB-231/DX cells vs. MDA-MB-231 cells:
749 ** p < 0.01; *** p < 0.001. **B.** Cells were lysed and subjected to immunoblotting for the indicated
750 proteins. Tubulin was used to check the equal protein loading. The figure is representative of 1 out of
751 3 experiments. **C.** Relative band density of Pgp, MRP1 and BCRP, performed with the ImageJ
752 software. Data are presented as means \pm SD (n=3). MDA-MB-231/DX cells vs. MDA-MB-231 cells:
753 ** p < 0.01; *** p < 0.001. **D.** HIF-1 α activity was measured by ELISA in duplicates. Data are
754 presented as means \pm SD (n=3). MDA-MB-231/DX cells vs. MDA-MB-231 cells: * p < 0.05; *** p <
755 0.001.

756

757 **Figure 3.** Doxorubicin accumulation and cytotoxicity in breast cancer cells with different degree of
758 resistance. **A.** MDA-MB-231 cells (MDA), MDA-MB-231/DX cells (MDA/DX) and JC cells were
759 lysed and subjected to immunoblotting for the indicated proteins. Tubulin was used to check the
760 equal protein loading. The figure is representative of 1 out of 3 experiments. **B.** Relative band density
761 of Pgp, MRP1 and BCRP, performed with the ImageJ software. Data are presented as means \pm SD
762 (n=3). MDA-MB-231/DX and JC cells vs. MDA-MB-231 cells: *** p < 0.001. **C.** Cells were
763 incubated 24 h with 5 or 25 μ M doxorubicin (Dox). The intracellular drug accumulation was

764 measured fluorimetrically in duplicates. Data are presented as means \pm SD (n = 3). MDA-MB-
765 231/DX cells vs. MDA-MB-231 cells: * p < 0.05; ** p < 0.01. **D.** Cells were grown 72 h in fresh
766 medium (Ctrl) or in medium with 5 or 25 μ M Dox. Cell viability was measured by crystal violet
767 staining, in quadruplicates. Data are presented as means \pm SD (n = 3). MDA-MB-231/DX cells vs.
768 MDA-MB-231 cells: * p < 0.05; Dox vs Ctrl cells: $^{\circ\circ}$ p < 0.01, $^{\circ\circ\circ}$ p < 0.001.

769

770 **Figure 4.** Effects of Glabratephrin on doxorubicin cytotoxicity in breast cancer cells with different
771 degree of resistance. **A-B-C.** MDA-MB-231 (MDA, panel **A**), MDA-MB-231/DX (MDA/DX, panel
772 **B**) and JC (panel **C**) cells were grown for 72 h in fresh medium (0) or in medium containing
773 Glabratephrin (Glab) at 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M, alone or in the presence of
774 doxorubicin (Dox; 0.05 μ M, 0.5 μ M, 5 μ M). Cell viability was measured by crystal violet staining in
775 quadruplicates. The heatmaps represent the mean percentage of viable cells in each condition, in a
776 colorimetric scale. The viability of untreated cells was considered 100% (n = 3 independent
777 experiments). For MDA-MB-231 cells: Dox 0.05 μ M vs. untreated cells: p < 0.01, Dox 0.5 and 5 μ M
778 vs. untreated cells: p < 0.01. For MDA-MB-231/DX cells: Dox 0.05, 0.5 and 5 μ M vs. untreated
779 cells: not significant; Dox + 10 nM Glab vs. Dox alone: p < 0.05; Dox + 100 nM/1 μ M/10 μ M Glab
780 vs. Dox alone: p < 0.001. For JC cells: Dox 0.05, 0.5 and 5 μ M vs. untreated cells: not significant;
781 Dox + 100 nM Glab vs. Dox alone: p < 0.05; Dox + 1 μ M/10 μ M Glab vs. Dox alone: p < 0.001. **D-**
782 **E.** MDA-MB-231/DX cells (MDA/DX, panel **D**) and JC cells (panel **E**) were grown for 72 h in fresh
783 medium or in medium containing Dox and Glab, either alone or in combination, in the range of
784 concentrations between 10^{-10} and 10^{-5} M. Cell viability was measured by crystal violet staining in
785 quadruplicates. The isobologram analyses were performed using the CalcuSyn software.

786

787 **Figure 5.** Effects of the combination of Glabratephrin and doxorubicin *in vivo*. JC cells were
788 orthotopically implanted into 6-week-old female balb/C mice. When tumor reached the volume of 50

789 mm³, mice (n= 8/group) were randomized and treated as reported in the following groups and treated
790 on day 1, 7, 14 after randomization as reported: 1) vehicle group (Ctrl), treated with 200 µl saline
791 solution intravenously (i.v.); 2) Glabratephrin group (Glab), treated with a 200 µl water/10% DMSO
792 solution i.v., containing 5 µM Glab; 3) doxorubicin group (Dox), treated with 5 mg/kg Dox, dissolved
793 in 200 µl water i.v.; 4) Glabratephrin + doxorubicin group (Glab+Dox), treated with 100 µl of saline
794 solution i.v. containing 5 µM Glab + 100 µl water solution containing 5 mg/kg Dox. **A.** Tumor
795 growth was monitored daily by caliper measurement. Data are presented as means ± SD. ***p<0.001:
796 Glab+Dox treatment vs all the other treatments (days 9-18). **B.** Photographs of representative tumors
797 of each group. **C.** Representative haematoxylin-eosin (HE) and Ki67 staining in each group of
798 treatments. For each experimental condition a minimum of 5 fields were examined. Ocular: 10X;
799 objective: 20X. **D.** Representative haematoxylin-eosin staining of liver, kidneys and spleen examined
800 *post-mortem* in each group of treatments. For each experimental condition a minimum of 5 fields
801 were examined. Ocular: 10X; objective: 10X (spleen), 20X (liver, kidneys).

802

803 **Figure 6.** Glabratephrin reduces Pgp activity. **A.** MDA-MB-231 cells (MDA), MDA-MB-231/DX
804 cells (MDA/DX) and JC cells were incubated 24 h with 5 µM doxorubicin (Dox), in the absence (-)
805 or presence (+) of 100 nM Glabratephrin (Glab). The intracellular drug accumulation was measured
806 fluorimetrically in duplicates. Data are presented as means ± SD (n = 3). Glab-treated vs. untreated
807 cells: * p < 0.05; *** p < 0.001. **B.** Cells were incubated 20 min with 1 µg/ml Rhodamine 123, in the
808 absence (-) or presence (+) of 100 nM Glab. The intracellular accumulation of Rhodamine 123 was
809 measured fluorimetrically in duplicates. Data are presented as means ± SD (n = 3). Glab-treated vs.
810 untreated cells: *** p < 0.001. **C.** Cells were incubated 10 min with increasing concentrations of Dox
811 (0.5-50 µM), in the absence (-) or presence (+) of 100 nM Glab. One series of dishes was analyzed
812 for the intracellular Dox concentration (c1); a second series was washed and let in the incubator for

813 additional 10 minutes, then analyzed for the intracellular Dox concentration (c2) as well. The dc/dt
814 value was considered indicative of Dox velocity of efflux. Data are presented as means \pm SD (n = 3).
815 **D.** Cells were grown 24 h in the absence (-) or presence (+) of 100 nM Glab. The Pgp ATPase
816 activity was measured spectrophotometrically in duplicates. Data are presented as means \pm SD (n = 3).
817 Glab-treated vs. untreated cells: * p < 0.05; ** p < 0.01.

818

819 **Figure 7.** Docking of QZ59-RRR or Glabratephrin on Pgp. **A.** Superposition of the co-crystallized
820 ligand QZ59-RRR (blue) and re-docked ligand QZ59-RRR (green) on Pgp (4M2S). **B.** Superposition
821 of the co-crystallized ligand QZ59-RRR (blue) and Glabratephrin (green) on Pgp (4M2S). **C.** Putative
822 binding mode of Glab with on Pgp (4M2S).

823

824 **Figure 8.** Modeling of the interactions between QZ59-RRR or Glabratephrin and Pgp. **A-B.** The
825 functional groups involved in the interaction between QZ59-RRR (panel **A**) or Glabratephrin (panel
826 **B**) and Pgp (4M2S) were displayed.

827

828 **Figure 9.** Glabratephrin loses its efficacy in Gly185Val mutated Pgp. MDA-MB-231 cells were
829 transfected with an empty vector (mock) or with expression vectors encoding for wild-type (wt) Pgp,
830 or Gly185Val, Ser400Asn, Gly412Ala, Ser893Ala, Ser893Thr-mutated Pgp. **A.** Cells were lysed and
831 subjected to immunoblotting for Pgp. Tubulin was used to check the equal protein loading. The figure
832 is representative of 1 out of 3 experiments. Numbers represent the relative band density of Pgp,
833 calculated with the ImageJ software. **B.** Cells were incubated 24 h in the absence (-) or presence (+)
834 of 100 nM Glabratephrin (Glab), with 5 μ M doxorubicin (Dox). The intracellular drug accumulation
835 was measured fluorimetrically in duplicates. Data are presented as means \pm SD (n = 3). MDA-MB-
836 231 cells overexpressing Pgp vs. mock cells: **** p < 0.001; Glab-treated cells vs corresponding
837 untreated cells: $^{\circ\circ\circ}$ p < 0.001. **C.** The Pgp ATPase activity was measured spectrophotometrically in

838 duplicates. Data are presented as means \pm SD (n = 3). MDA-MB-231 cells overexpressing Pgp vs.
839 mock cells: *** p < 0.001; Glab-treated cells vs corresponding untreated cells: °°° p < 0.001.

Glabratephrin reverses doxorubicin resistance in triple negative breast cancer by inhibiting P-glycoprotein

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Supplementary Table S1. Effects of different concentrations of Glabratephrin on hematochemical parameters of the treated animals

	Ctrl	Glab 0.5 μ M	Glab 1 μ M	Glab 5 μ M	Glab 10 μ M
LDH (U/l)	6089 \pm 609	6345 \pm 451	6434 \pm 509	6709 \pm 709	9234 \pm 789
AST (U/l)	236 \pm 67	198 \pm 48	221 \pm 67	187 \pm 59	509 \pm 98 *
ALT (U/l)	35 \pm 11	44 \pm 8	41 \pm 8	44 \pm 11	58 \pm 21*
AP (U/l)	109 \pm 47	128 \pm 46	117 \pm 43	104 \pm 34	171 \pm 54
Creatinine (mg/l)	0.087 \pm 0.013	0.096 \pm 0.011	0.076 \pm 0.012	0.085 \pm 0.013	0.134 \pm 0.012 *
CPK (U/l)	322 \pm 65	267 \pm 79	312 \pm 68	334 \pm 71	409 \pm 103
CPK-MB (ng/ml)	0.127 \pm 0.057	0.134 \pm 0.056	0.098 \pm 0.088	0.118 \pm 0.095	0.138 \pm 0.098
cTnI (pg/ml)	1.073 \pm 0.045	1.103 \pm 0.094	1.025 \pm 0.058	1.075 \pm 0.064	1.109 \pm 0.067
cTnT (pg/ml)	2.195 \pm 0.409	1.945 \pm 0.398	2.329 \pm 0.283	2.007 \pm 0.368	1.893 \pm 0.407

Balb/C mice (n=5 animals/group) were treated with vehicle (200 μ l saline solution i.v., Ctrl) or with a 200 μ l water/10% DMSO solution containing 0.5, 1, 5, 10 μ M Glabratephrin (Glab) i.v. After 24 h animals were sacrificed, blood was collected and analyzed for lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatinine, creatine phosphokinase (CPK) and CPK-MB, cardiac troponin I (cTnI) and T (cTnT). Data are presented as means \pm SD. * p < 0.05: vs Ctrl group.

Supplementary Figures

Supplementary Figure S1

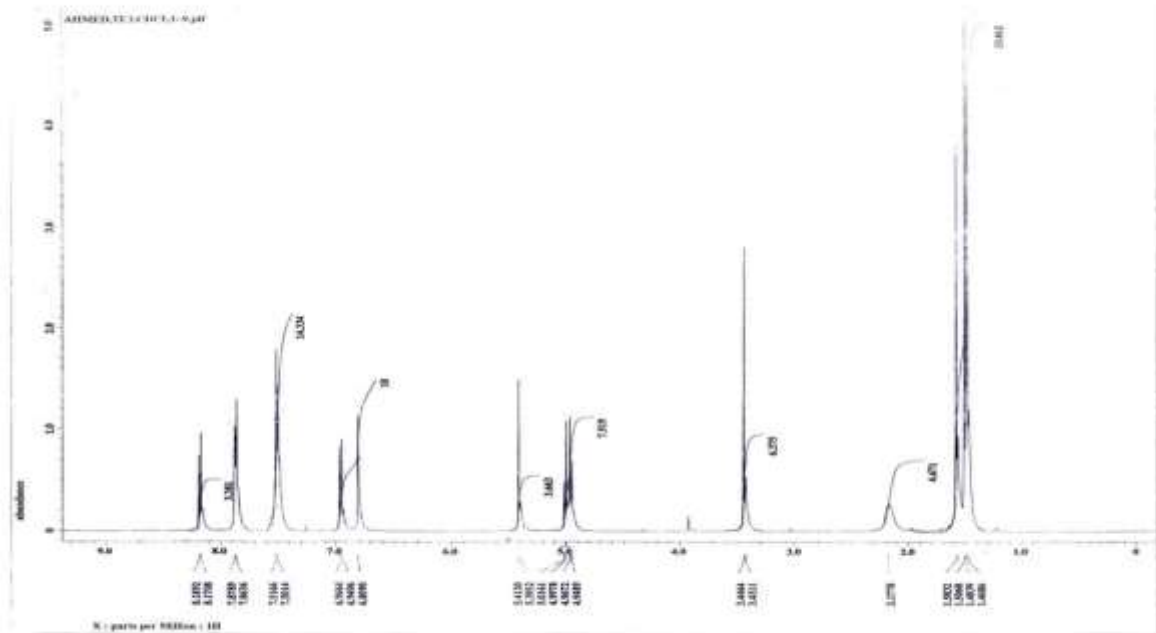


Figure S1. NMR spectrum of Glabratephrin (Pharmacognosy department in National Research Centre in Egypt).

Supplementary Figure S2

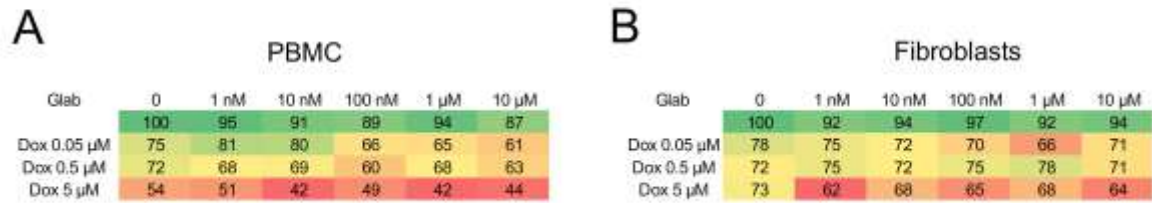


Figure S2. Effects of Glabratephrin and doxorubicin on non-transformed cells. Human peripheral blood mononuclear cells (PBMC, panel **A**) and human fibroblasts (panel **B**) were grown for 72 h in fresh medium (0) or in medium containing Glabratephrin (Glab) at 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M, alone or in the presence of doxorubicin (Dox, at 0.05 μ M, 0.5 μ M, 5 μ M). Cell viability was measured by crystal violet staining in quadruplicates. The heatmaps represent the mean percentage of viable cells in each condition, in a colorimetric scale. The viability of untreated cells was considered 100%, (n = 3 independent experiments). For PBMC: Dox 0.5 μ M vs. untreated cells: $p < 0.05$, Dox 5 μ M vs. untreated cells: $p < 0.01$; Dox 0.05 μ M + Glab 100 nM, 1 μ M, 10 μ M vs. untreated cells: $p < 0.05$; Dox 0.5 μ M + Glab 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M vs. untreated cells: $p < 0.01$; Dox 5 μ M + Glab 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M vs. untreated cells: $p < 0.001$. For fibroblasts: Dox 0.05 μ M + Glab 1 μ M: $p < 0.01$; Dox 5 μ M + Glab 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M vs. untreated cells: $p < 0.01$.

Supplementary Figure S3

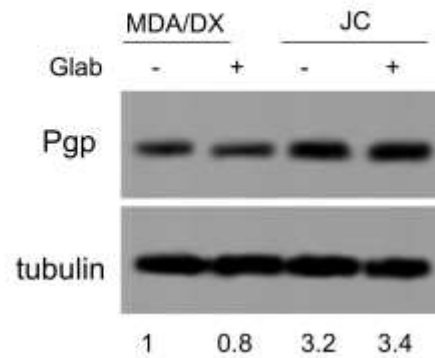


Figure S3. Expression of Pgp in cells incubated with Glabratephrin. MDA-MB-231/DX cells (MDA/DX) and JC cells were incubated in the absence (-) or in the presence of 100 nM Glabratephrin (Glab) for 24 h, then lysed and subjected to immunoblotting for Pgp. Tubulin was used to check the equal protein loading. The figure is representative of 1 out of 3 experiments. Numbers represent the relative band density of Pgp, calculated with the ImageJ software.

Supplementary Figure S4

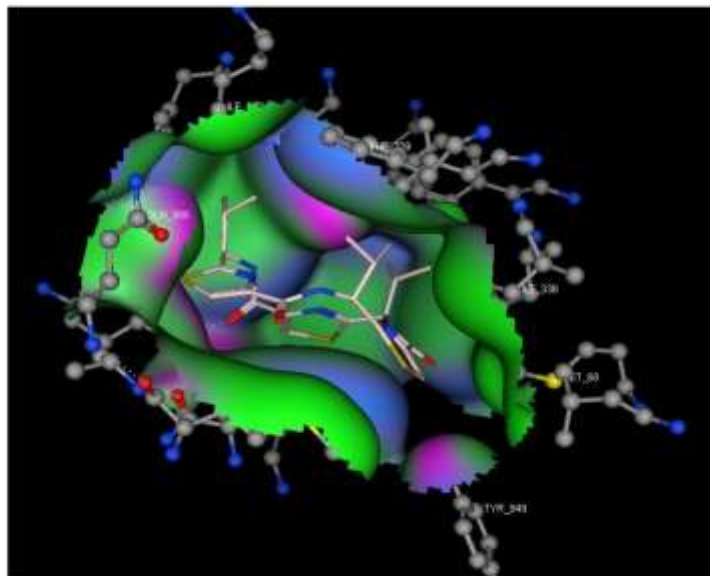


Figure S4. Docking simulation of QZ59-RRR to Pgp. Proposed model of interaction between QZ59-RRR and Pgp.