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Discovery of new targets of phenolic acids in Danshen using label-free cell phenotypic assay

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26 **Abstract**

27 Traditional Chinese medicines (TCMs) have been used in clinic for thousands of
28 years. Their reliable therapeutic efficacies are closely related to their multi-target
29 mechanisms of action (MOAs). Discovery of these targets is important for
30 understanding their clinical features. Danshen is the dried root of *Salvia miltiorrhiza*,
31 a traditional Chinese medicine (TCM) used for the treatment of cardiovascular and
32 cerebrovascular diseases. Although its clinical features are well recognized, targets of
33 its active constituents are poorly understood. Here, label-free cell phenotypic assay
34 was used to investigate the potential targets of phenolic acids in Danshen.
35 Pharmacological profiling of 10 known phenolic acids in Danshen using HT-29 and
36 A431 cells revealed that lithospermic acid, salvianolic acid A, salvianolic acid B,
37 salvianolic acid C and Danshensu all displayed agonistic activity at the GPR35;
38 however, salvianolic acid A and salvianolic acid C at high doses were also active to
39 alter intracellular Ca^{2+} via another unknown target. Since GPR35 has been implicated
40 in inflammation and cardiovascular diseases, the discovery of GPR35 as one target of
41 phenolic acids in Danshen was useful for elucidating their mechanisms of action in
42 the treatment of these diseases. This study also highlights the potential of label-free
43 cell phenotypic assay for discovering multiple targets of TCM.

44

45 **Key words:** label-free cell phenotypic assay, multiple targets, phenolic acids,
46 Danshen

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48 ## 1. Introduction

49 Traditional Chinese medicines (TCMs) are attracting considerable attention owing
50 to their reliable clinical efficacy, thus being important resources for lead compounds
51 or drugs.[1, 2] Most of them provide therapeutic effect by interacting with multiple
52 targets. For instance, paclitaxel (anticancer), reserpine (antihypertensive), artemisinin
53 (antimalarial) and rapamycin (immunosuppression) act on at least two targets.
54 Studying these mechanisms of action (MOAs) is important for understanding their
55 clinical features. Target-based approaches that screen compounds against a single
56 target protein are not effective for discovering multiple targets of compounds.[3] While
57 phenotype-based approaches can provide an unbiased way to find active compounds,
58 which are useful for identifying multiple targets or pathways.[4]

59 As one of new generation phenotype-based assay techniques, label-free cell
60 phenotypic assay has become an attractive approach for determining target
61 engagement of drugs.[5-7] This assay uses label-free resonant waveguide grating (RWG)
62 biosensor to convert drug-induced dynamic redistribution of cellular constituents in
63 cells into an integrated and kinetic response, termed dynamic mass redistribution
64 (DMR).[14] The DMR is recorded as a shift in resonant wavelength in picometer (pm)
65 and represents a cell phenotypic response covering a wide range of
66 targets/pathways.[6-9] Furthermore, due to its non-invasiveness, DMR assay can be
67 performed in multiple formats and permits intervention with probe molecules, thus
68 enabling mechanistic elucidation of drug pharmacology.[10-12]

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69 Danshen, the dried root of *Salvia miltiorrhiza*, is a traditional Chinese medicine
70 (TCM) widely used in China for the treatment of cardiovascular and cerebrovascular
71 diseases.[13, 14] The Danshen Dripping Pill is currently in phase III clinical trial with a
72 great hope to be the first Food and Drug Administration (FDA) approved TCM.
73 Danshen extract contains two main types of ingredients, water-soluble phenolic acids
74 and lipophilic diterpenoid quinines. Until now, more than 100 compounds have been
75 isolated,[15] which contain about 30 phenolic acid compounds. According to the number
76 of caffeic acid units, these phenolic acids can be classified into five subgroups:
77 monomers, dimmers, trimmers, tetramers and others.[14] These compounds occur in the
78 Danshen extract with various concentrations. Based on fingerprinting analysis of the
79 Danshen extract,[16] caffeic acid, danshensu, rosmarinic acid and salvianolic acid B
80 exist in all the studied species; salvianolic acid B and rosmarinic acid are relatively
81 rich in the extract. In terms of Danshen preparations,[17] the tablets and capsules
82 contain both water-soluble phenolic acids and lipophilic diterpenoid quinines, while
83 the dropping pills and injections contain mainly phenolic acids. Among these phenolic
84 acids, the contents of danshensu, protocatechuic aldehyde and salvianolic acid A were
85 higher than those in other solid samples. Salvianolic acid B is the predominant
86 compound in the crude extract, but decreases in the dropping pills and injections.
87 Given that a water decoction and an injection agent are the common forms of
88 Danshen in clinic, the water-soluble phenolic acids are believed to be responsible for
89 its biological activities. These water-soluble components have been shown to have
90 activities as antioxidant, anti-ischemia reperfusion, anti-hypertension, antiplatelet

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91 aggregation, anti-inflammation and anti-fibrosis.[18] Determining target engagement
92 represents an important step towards understanding the pharmacological activities of
93 the water-soluble phenolic acids in Danshen, thus the clinical features of Danshen
94 products.[19] Recently, *in silico* analysis and experimental validation have demonstrated
95 that salvianolic acid A exhibits anti-inflammatory effect via the induction of heme
96 oxygenase 1 through p38 MAPK pathway in lipopolysaccharide (LPS)-stimulated
97 RAW264.7 cells.[20] Salvianolic acid B may inhibit angiotensin converting enzyme
98 (ACE) and thus attenuate angiotensin I-induced vasoconstriction, which might
99 partially account for its anti-hypertension effect.[21] Salvianolic acid B may also
100 prevent cardiac remodeling by inhibiting matrix metalloproteinase-9.[22] Furthermore,
101 salvianolic acid A, salvianolic acid B and caffeic acid all can inhibit the
102 protein-protein interactions via the SH2 domains of the Src-family kinases, Src and
103 Lck.[23] These findings suggest that these phenolic acids may interact with multiple
104 targets rather than single target. Therefore, further discovery of new targets of
105 phenolic acids in Danshen is of importance to understand and elucidate their clinical
106 features.

107 Here we use label-free cell phenotypic assay to discover new targets of phenolic
108 acids from Danshen with a hope to elucidate its *in vivo* therapeutic effects.

109

110 **2. Experimental**

111 **2.1. Materials**

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112 Lithospermic acid, salvianolic acid A, salvianolic acid B, salvianolic acid C,
113 danshensu, caffeic acid, isoferulic acid, carnosol and protocatechuic aldehyde were
114 purchased from Shanghai Yuanye Bio-technology Co., Ltd. (China). Salvianolic acid
115 D was gifted from Prof. Jingbo Zhu (Dalian Polytechnic University). Their strucures
116 were shown in **Fig. 1**. Acetylcholine chloride, bradykinin, cyclopiazonic acid,
117 histamine, kynurenic acid, thapsigargin, zaprinast and SPB05142 (CID2745687) were
118 obtained from Sigma Chemical Co. (St Louis, MO, USA). ML145 was obtained from
119 Tocris Bioscience Co. (St. Louis, MO, USA). Rabbit polyclonal β-actin antibody,
120 rabbit polyclonal extracellular-signal regulated kinase 1/2 antibody (anti-ERK1/2) and
121 rabbit polyclonal anti-GPR35 were obtained from Proteintech (Wuhan, China). Rabbit
122 polyclonal anti-phosphorylated extracellular-signalregulated kinase 1/2
123 (Thr202/Tyr20) was from Bioworld Technology, co. Ltd (Naijing, China). Epic®
124 384-well biosensor cell culture compatible microplates were obtained from Corning
125 Incorporated (Corning, NY, USA). Human colorectal adenocarcinoma HT-29 and
126 human epidermoid carcinoma A431 cell lines were obtained from Cell Bank of
127 Shanghai Institute of Cell Biology, Chinese Academy of Sciences.
128 Acetylcholine chloride, cyclopiazonic acid, danshensu, lithospermic acid,
129 salvianolic acid A, salvianolic acid B, salvianolic acid C and thapsigargin were
130 stocked in 100 mM, while all other compounds were stocked in 10 mM. Except for
131 acetylcholine which was prepared in water, all others compounds were dissolved in
132 100% dimethyl sulfoxide (DMSO). All compounds were diluted freshly by the assay

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133 buffer (1x Hank's balanced salt solution (HBSS) buffer, 10 mM Hepes, pH 7.2) to the
134 assayed concentrations.
135
136 **2.2. Cell culture**
137 HT-29 cells were cultured using McCoy's 5A Medium (#DY0324S0414L, Sango
138 Biotech, Shanghai, China) with 10% fetal bovine serum (#10099141, Gibco, Life
139 Technologies), 50 μg/mL penicillin and 100 μg/mL streptomycin in a humidified
140 37^oC/5% CO_2 incubator. A431 cells were cultured in Dulbecco's Modified Eagle's
141 Medium (DMEM) (#SH30022.01B, Thermo Scientific HyClone) supplemented with
142 10% fetal bovine serum, 50 μg/mL penicillin and 100 μg/mL streptomycin at 37^oC
143 under air/5% CO_2.
144
145 **2.3. Dynamic mass redistribution assay**
146 All DMR assays were performed using an Epic® BT system (Corning, NY, USA).
147 Cells were directly seeded in Epic 384-well biosensor microplates with a seeding
148 density of 32,000 and 25,000 cells per well for HT-29 and A431 cells, respectively.
149 After cultured for ~20 h, these cells formed a monolayer in the cell culture medium
150 with a confluency of ~95%. A431 cells were further starved for 24 h using serum-free
151 medium. After culture, cells were manually washed using the assay buffer and
152 maintained with 30 μL of the assay buffer for 1 h in the Epic system.
153 For profiling compounds in HT-29 cells, a 2-min baseline was first established,
154 followed by adding compounds and monitoring the compound-induced DMR signals
155 for 1 h. Then a 2-min baseline was re-established, followed by adding 1 μM zaprinast
156 or 16 μM acetylcholine and monitoring DMR signals for another 1 h. Similarly, for
157 profiling compounds in A431 cells, a 2-min baseline was first established, followed

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158 by compound addition and DMR recording for 1 h. A 2-min baseline was then
159 re-established, followed by adding 16 nM bradykinin or 1 μM histamine and
160 monitoring DMR signals for 1 h.

161 For DMR agonist assay, after establishment of a 2-min baseline, phenolic acids at
162 varied doses were added individually and the DMR signals were recorded for 1 h. For
163 DMR desensitization assay, cells were pretreated with these compounds for 1 h,
164 followed by recording a 2-min baseline, adding 1 μM zaprinast and monitoring DMR
165 signals for 1h. For DMR antagonist assay, HT-29 cells were pretreated with an
166 antagonist, SPB05142 for 5 min or ML145 for 10 min, following by recording a
167 2-min baseline, adding a ligand at its indicated concentration (EC_{20}, EC_{50} or EC_{80})
168 and monitoring DMR signals for 1 h.

169 To examine compounds for altering intracellular Ca^{2+}, a two-step assay was also
170 used. Different doses of salvianolic acid A, salvianolic acid C, thapsigargin and
171 cyclopiazonic acid were first assayed, followed by recording the DMR signal induced
172 by cyclopiazonic acid at 16 μM for 1 h.

173

174 **2.4. Western blot**

175 After cells were treated with compounds for 15 min, whole cell lysates were
176 harvested using lysis buffer (KeyGEN BioTECH, Naijing, China). For each group, 20
177 μg total protein was electrophoresed through a 12% (w/v) acrylamide gel and
178 electroblotted onto polyvinylidene difluoride (PVDF) membranes. Antibodies were
179 diluted as follows: β-actin (1:300), ERK1/2 (1:300), p-ERK1/2 $^{Thr202/Tyr204}$ (1:1000)
180 and GPR35 (1:500). Detection was performed by GTVTM Ⅲ Detection
181 System/Mo&Rb (GeneTech, Shanghai, China).

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183 **2.5. Data analysis**

184 All DMR data were acquired using Epic Imager software (Corning, NY, USA) and
185 processed by Imager Beta 3.7 (Corning), Microsoft Excel 2010 and GraphPad Prism
186 6.02 (GraphPad Sofware Inc., San Diego, CA, USA). All DMR signals were
187 background corrected. All EC_{50} or IC_{50} values described were calculated based on the
188 maximal amplitude of DMR signal within 40 min post-stimulation. All DMR data
189 were from two independent measurements, each in duplicate (n = 4).

190

191 **3. Results and discussion**

192

193 **3.1. Profiling of phenolic acids from Danshen**

194 We first profiled the activity of 10 phenolic acids in Danshen using RWG
195 biosensor-enabled label-free cell phenotypic assay in HT-29 and A431 cells. The
196 concentration of each compound for profiling was 10 μM. These phenolic acids are
197 known to present in Danshen (**Fig. 1**). A two-step DMR assay was performed. The
198 first step was DMR agonist assay to characterize all compounds for their agonist
199 activities in HT-29 and A431 cells. The second step was DMR desensitization assay to
200 examine the ability of each compound to desensitize the cellular response induced by
201 a panel of agonist probe molecules, each at its saturating dose. Since HT-29 cells
202 endogenously express GPR35[24] and muscarinic M3 receptor,[12, 25] zaprinast[26] and
203 acetylcholine[12] were chosen as their agonist probes, respectively. Similarly, A431
204 endogenously express bradykinin B_2 receptor[25,26] and histamine H_1 receptor[27],
205 bradykinin and histamine were chosen as agonist probes, respectively. Based on the
206 EC_{100} reported in literature, the doses of probe agonists examined were set to be 1 μM,
207 16 μM, 16 nM and 1 μM for zaprinast, acetylcholine, bradykinin and histamine,

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208 respectively. Additionally, the expression level of GPR35 in HT-29 and A431 cells
209 was examined using western blot and DMR assay (**Fig. S1**). Results indicated that the
210 expression level of GPR35 in HT-29 cells was much higher than that in A431 cells.

211 Results showed that in HT-29 cells salvianolic acid A and salvianolic acid C
212 triggered a large DMR, while lithospermic acid, salvianolic acid B and danshensu led
213 to a relatively small DMR, and all other five compounds resulted in negligible DMR
214 (**Fig. 2a**). Interestingly, the five active compounds, including salvianolic acid A,
215 salvianolic acid B, salvianolic acid C, lithospermic acid and danshensu, all attenuated
216 the zaprinast-induced signal (**Fig. 2b**). Furthermore, the ability of salvianolic acid A
217 and salvianolic acid C to attenuate the zaprinast-induced signal was greater than that
218 of lithospermic acid, salvianolic acid B and danshensu. However, all compounds had
219 no or little effect on the acetylcholine-induced signal (**Fig. 2c**). Combining the
220 agonistic activity with the desensitization pattern suggests that lithospermic acid,
221 salvianolic acid A, salvianolic acid B, salvianolic acid C and danshensu may have
222 agonistic activity at the GPR35.

223 Different label-free pharmacological patterns were observed in A431 cells. The
224 DMR responses at 30 min post-stimulation showed that salvianolic acid A and
225 salvianolic acid C triggered relatively strong DMR signal, while lithospermic acid and
226 salvianolic acid B led to small DMR signal, and other compounds were inactive (**Fig.
227 2d**). Furthermore, both salvianolic acid A and salvianolic acid C attenuated the
228 bradykinin- and histamine-induced DMR signals (**Fig. 2e** and **2f**, respectively). Given
229 that in A431 cells there is little GPR35 expressed (**Fig. S1**), histamine activates the H_1
230 receptor triggering G_q-signaling,[5] and bradykinin activates B_2 receptor triggering both
231 G_s- and G_q-pathways[27], these results suggest that salvianolic acid A and salvianolic
232 acid C may alter intracellular Ca^{2+} via another unknown target.

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233

234 **3.2. Activity of phenolic acids at the GPR35**

235 We next examined the potential agonistic activity of lithospermic acid, salvianolic
236 acid A, salvianolic acid B, salvianolic acid C and danshensu at the GPR35 using three
237 different DMR assays. DMR agonist assay was employed to determine their agonist
238 activities. DMR desensitization assay and DMR antagonist assay were used to
239 examine the specificity of these five compounds to activate GPR35.

240 First, DMR agonist assay showed that lithospermic acid, salvianolic acid A,
241 salvianolic acid B, salvianolic acid C and danshensu with the concentration range of
242 0.03 ~ 250 μM all triggered dose-dependent positive DMR signals, but with different
243 amplitudes (**Fig. 3**). According to DMR characteristics, these five compounds can be
244 divided into two groups. The first group consists of salvianolic acid A and salvianolic
245 acid C, both triggering a quite large DMR with almost identical characteristics (**Fig.
246 3a**). The second group consists of lithospermic acid, salvianolic acid B and danshensu,
247 all triggering a moderate DMR (**Fig. 3b**). Except for salvianolic acid B and danshensu,
248 whose DMR signals seem not reach saturation at the maximal concentration of 250
249 μM examined, the dose responses of salvianolic acid A, salvianolic acid C and
250 lithospermic acid were best fitted with a single-phase non-linear regression (**Fig. 3c**
251 and **3d**). The EC_{50} values of these compounds are showed in **Table 1**. Furthermore,
252 the maximal DMR of lithospermic acid, salvianolic acid B and danshensu all shared
253 similarity to those of zaprinast and kynurenic acid (**Fig. 4**). Zaprinast[26] and kynurenic
254 acid[28] are two known GPR35 agonists and often used as probe agonists for GPR35.
255 Of note, salvianolic acid A or salvianolic acid C at 16 μM gave rise to a DMR also
256 similar to that of zaprinast, but at high doses triggered a DMR that is much greater

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257 than the maximal DMR of zaprinast, suggesting that both compounds may also
258 activate another unknown target or pathway, besides GPR35.
259 Second, DMR desensitization assay was performed against the DMR of 1 μM
260 zaprinast. Results showed that these five compounds all dose-dependently
261 desensitized the DMR of zaprinast, leading to an apparent IC_{50} of 3.48 ± 0.38 μM,
262 72.74 ± 22.96 μM, 2.70 ± 0.31 μM, 109 ± 23 μM and 30.42 ± 2.25 μM (n = 4 for all)
263 for salvianolic acid A, salvianolic acid B, salvianolic acid C, lithospermic acid and
264 danshensu, respectively (**Fig. 5**; **Table 1**). These results suggest that these compounds
265 activate GPR35.
266 Third, DMR antagonist assay was performed to confirm the specificity of these
267 compounds to activate GPR35. Known GPR35 antagonists SPB05142[29, 30] and
268 ML145[31, 32] were used to pretreat HT-29 cells at different doses, followed by detecting
269 DMR signal induced by each compound at its respective dose close to EC_{80}, EC_{50} or
270 EC_{20}. Results showed that SPB05142 displayed different inhibitory effects on the
271 DMR of different compounds at different doses. When the dose of each compound
272 was assayed at its EC_{80}, SPB05142 dose-dependently and completely blocked the
273 DMR of 125 μM lithospermic acid or 100 μM danshensu, leading to an IC_{50} of 2.29 ±
274 0.52 μM and 2.98 ± 1.35 μM (n = 4), respectively (**Fig. 6a**). However, SPB05142
275 only partially blocked the DMR of 100 μM salvianolic acid B with an IC_{50} of 0.71 ±
276 0.42 μM (n = 4) (**Fig. 6a**), but had little effect on the DMR of 125 μM salvianolic acid
277 A, or 100 μM salvianolic acid C (**Fig. 6b**). On the other hand, SPB05142 weakly
278 attenuated the DMR of salvianolic acid A and salvianolic acid C at their EC_{50} (**Fig.
279 6c**), but dose-dependently inhibited their DMR at their respective EC_{20} (15 μM and 4
280 μM, respectively) with an IC_{50} of 2.52 ± 0.48 μM and 1.57 ± 0.64 μM (n = 4),

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281 respectively (**Fig. 6d**). Almost identical inhibition pattern was observed using ML145
282 as an antagonist (**Fig. S2, Table 1**).

283 We further examined the activity of salvianolic acid A, salvianolic acid C,
284 salvianolic acid B, lithospermic acid and danshensu using ERK phosphorylation assay.
285 The activation and signaling of many GPCRs induce ERK phosphorylation, including
286 GPR35.[33] As in **Fig. 7**, it shows that salvianolic acid A, salvianolic acid C, salvianolic
287 acid B, lithospermic acid and danshensu lead to ERK phosphorylation (**Fig. 7a)**. As
288 controls, the known GPR35 agonist zaprinast also triggers ERK phosphorylation (**Fig.**
289 **7b**). Moreover, the GPR35 antagonist ML145 attenuated ERK phosphorylation
290 induced by these compounds (**Fig. 7c**). These results suggest that salvianolic acid A,
291 salvianolic acid C, salvianolic acid B, lithospermic acid and danshensu resulted in the
292 phosphorylation of ERK via the activation of GPR35.

293 Together, these results suggest that all five compounds display agonistic activity at
294 the GPR35. Lithospermic acid and danshensu are mostly specific to activate GPR35
295 in HT-29 cells. However, salvianolic acid B may also activate another unknown target,
296 and both salvianolic acid A and salvianolic acid C at high doses may activate multiple
297 targets/pathways, triggering a quite large DMR.

298

299 **3.3. Deconvolution of possible target(s), besides GPR35, of salvianolic acid A and**
300 **salvianolic acid C**

301 Both salvianolic acid A and salvianolic acid C appear to display polypharmacology.
302 This was evidenced by that both triggered a larger DMR consisting of contributions
303 from GPR35 as well as other unknown pathway(s) in HT-29 cells. Furthermore, both
304 compounds also triggered a large DMR in A431 cells and attenuated the bradykinin-
305 and histamine-induced DMR signals, probably due to heterologous desensitization

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306 mechanism. The activation of G_q-coupled receptors is known to sequentially activate
307 trimeric G proteins and phospholipase C (PLC), the latter of which then hydrolyzes
308 the membrane lipid phosphatidylinositol bisphosphate, yielding diacylglycerol (DAG)
309 and inositol triphosphate (IP3). IP3 binds to and opens a calcium channel in the
310 endoplasmic reticulum, causing calcium mobilization. On the other hand, Danshen is
311 known to cause vasorelaxant actions due to the inhibition of Ca^{2+} flux.[34, 35] Therefore,
312 we hypothesized that salvianolic acid A and salvianolic acid C may alter intracellular
313 Ca^{2+} via an unknown target. To validate this hypothesis, we used thapsigargin and
314 cyclopiazonic acid as the probe molecules. Thapsigargin is a non-competitive
315 inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA),[36] while
316 cyclopiazonic acid is a specific inhibitor of SERCA.[37] Both inhibitors can raise
317 intracellular calcium concentration by blocking the ability of the cell to pump calcium
318 into the sarcoplasmic and endoplasmic reticula.[35]

319 DMR agonist assay showed that salvianolic acid A and C triggered robust DMR
320 with similar characteristics in A431 cells (**Fig. 8a** and **b**), and salvianolic acid C
321 exhibited slightly higher potency than salvianolic acid A (**Fig. 8c**). On the other hand,
322 thapsigargin and cyclopiazonic acid triggered comparable DMR in A431, whose
323 characteristics were different from those of salvianolic acid A and C (comparing **Fig.
324 8d** and **e** with **Fig. 8a** and **b**). As expected,[35] thapsigargin exhibited higher potency
325 than cyclopiazonic acid (**Fig. 8f**). The IC_{50} values to trigger DMR were found to be
326 122.46 ± 11.75 μM, 60.75 ± 3.01 μM, 0.30 ± 0.02 μM and 2.08 ± 0.09 μM (n = 4) for
327 salvianolic acid A, salvianolic acid C, thapsigargin and cyclopiazonic acid,
328 respectively.

329 DMR desensitization assay showed that all four compounds caused dose-dependent
330 desensitization of cells responding to the second stimulation with 16 μM

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331 cyclopiazolic acid (**Fig. 9**). The IC_{50} values to desensitize the cyclopiazolic acid DMR
332 were found to be 71.43 ± 9.53 μM, 26.06 ± 1.70 μM, 0.023 ± 0.002 μM and 5.12 ±
333 0.50 μM (n = 4 for all) for salvianolic acid A, salvianolic acid C, thapsigargin and
334 cyclopiazonic acid, respectively. These results suggest that salvianolic acid A and
335 salvianolic acid C alter intracellular Ca^{2+} via an unknown target and share similar
336 mechanism to those of thapsigargin and cyclopiazonic acid. Further elucidation of the
337 exact target intervened by salvianolic acid A and salvianolic acid C is warranted and
338 currently under investigation.

339 **4. Conclusion**

340 Danshen water extract has clinical benefits for treating cardiovascular and
341 cerebrovascular diseases and contains several phenolic acids. Here we used label-free
342 cell phenotypic assay to investigate the possible targets of these phenolic acids from
343 Danshen. We found that lithospermic acid, salvianolic acid A, salvianolic acid B,
344 salvianolic acid C and danshensu all displayed agonistic activity at the GPR35, but
345 with different potency. We also found that salvianolic acid A and salvianolic acid C
346 also alter intracellular Ca^{2+} via a mechanism that is similar to two known SERCA
347 inhibitors. The multi-target activity of these phenolic acids may be related to the
348 clinical features of Danshen products. Specifically, GPR35 has been implicated in
349 inflammation, hypertension, coronary artery disease and cancer. Thus, the agonistic
350 activity of multiple phenolic acids in Danshen may be useful for elucidating the
351 mechanism of Danshen products in the treatment of cardiovascular diseases.

352

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415 **Table 1** The active phenolic acids and their pharmacological characteristics in HT-29
416 cells.

Compounds	EC_{50} (μM)	IC_{50}^{*} (μM)	IC_{50}^{**} (μM)	IC_{50}^{***} (μM)
Lithospermic acid	37.63 ± 13.83	109.04 ± 23.79	2.29 ± 0.52	0.47 ± 0.07
Salvianolic acid A	37.69 ± 3.77	3.48 ± 0.38	2.52 ± 0.48	0.15 ± 0.10
Salvianolic acid B	weak	72.74 ± 22.96	0.71 ± 0.42	1.48 ± 0.22
Salvianolic acid C	18.83 ± 1.11	2.70 ± 0.31	1.57 ± 0.64	0.24 ± 0.03
Danshensu	weak	30.42 ± 2.25	2.98 ± 1.35	0.25 ± 0.02

417 * IC_{50} to desensitize the DMR of 1 μM zaprinast in HT-29 cells.
418 ** IC_{50} of SPB05142 to block the DMR of a phenolic acid compound at a dose of 125 μM, 100 μM,
419 100 μM, 15 μM and 4 μM for lithospermic acid, salvianolic acid B, danshensu, salvianolic acid A and
420 salvianolic acid C, respectively. The HT-29 cells were pre-treated with the antagonist for 5 min.
421 *** IC_{50} of ML145 to block the DMR of compounds at the same doses as above. The HT-29 cells were
422 pre-treated with the antagonist for 10 min.
423
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425 **Figure legends**

426 **Fig. 1.** Chemical structures of 10 known phenolic acids in Danshen.

427

428 **Fig. 2** A label-free cell phenotypic profiling technique to identify potential
429 polypharmacology of phenolic acid compounds in HT-29 cells (a-c) and A431 cells
430 (d-f). (a) The DMR amplitudes at 30 min post-stimulation of 10 μM compounds as a
431 function of compounds in HT-29 cells. (b) The DMR response of 1 μM zaprinast after
432 the cells were pre-stimulated with each compound for 1 h. (c) The DMR response of
433 16 μM acetylcholine after the cells were pre-stimulated with each compound for 1 h.
434 (d) The DMR amplitudes of 10 μM compounds as a function of compounds in A431
435 cells. (e) The DMR response of 16nM bradykinin after the cells were pre-stimulated
436 with each compound for 1 h. (f) The DMR response of 1 μM histamine after the cells
437 were pre-stimulated with each compound for 1 h. The compounds are lithospermic
438 acid (1), salvianolic acid A (2), salvianolic acid B (3), salvianolic acid C (4),
439 salvianolic acid D (5), danshensu (6), caffeic acid (7), isoferulic acid (8), carnosol (9),
440 protocatechuic aldehyde (10) and control (11). All data represents mean ± s.d. from 2
441 independent measurements, each in duplicate (n = 4).

442

443 **Fig. 3** DMR dose responses of compounds in HT-29 cells. (a and b) Real time DMR
444 signals of salvianolic acid A (a) and danshensu (b); (c and d) DMR amplitudes as a
445 function of their doses: salvianolic acid A and salvianolic acid C (c), salvianolic acid

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446 B, lithospermic acid and danshensu (d). All data represents mean ± s.d. from 2
447 independent measurements, each in duplicate (n = 4).
448

449 **Fig. 4** DMR dose responses of probes in HT-29 cells. (a, b) Real time DMR signals of
450 zaprinast (a) and kynurenic acid (b); (c) DMR amplitudes as a function of their doses.
451 All data represents mean ± s.d. from 2 independent measurements, each in duplicate
452 (n = 4).
453

454 **Fig. 5** The DMR amplitudes of 1 μM zaprinast as a function of compounds doses in
455 HT-29 cells after pre-stimulation with these compounds for 1 h. (a) Salvianolic acid A
456 and salvianolic acid C; (b) salvianolic acid B, lithospermic acid and danshensu. All
457 data represents mean ± s.d. from 2 independent measurements, each in duplicate (n =
458 4).
459

460 **Fig. 6** The DMR amplitudes of compounds as a function of SPB05142 doses in HT-29
461 cells after pre-treated with the antagonist for 5 min. (a) Lithospermic acid, salvianolic
462 acid B and danshensu at their EC_{80} of 125 μM, 100 μM and 100 μM, respectively; (b)
463 salvianolic acid A and salvianolic acid C at their EC_{80} of 125 μM and 100 μM,
464 respectively; (c) salvianolic acid A and salvianolic acid C at their EC_{50} of 35 μM and
465 20 μM, respectively; (d) salvianolic acid A and salvianolic acid C at their EC_{20} of 15
466 μM and 4 μM, respectively. All data represents mean ± s.d. from 2 independent
467 measurements, each in duplicate (n = 4).

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468 **Fig. 7** ERK phosphorylation assay of active phenolic acids. (a) Western blot of
469 p-ERK1/2 after treated with salvianolic acid A (SAA), salvianolic acid C (SAC),
470 salvianolic acid B (SAB), lithospermic acid (LA) and danshensu (DSS) at the
471 concentration of 20 μM, 10 μM, 50 μM, 50 μM and 50 μM, respectively. (b) Western
472 blot of p-ERK1/2 after treated with the known GPR35 agonist zaprinast (Zap) at 1 μM
473 and western blot of p-ERK1/2 after treated with compounds that have the same
474 concentration as (a) in the presence of ML145 (25 μM). "Con" represented the blank
475 control.

476

477 **Fig. 8** Real time DMR signals of salvianolic acid A (a), salvianolic acid C (b),
478 thapsigargin (d) and cyclopiazonic acid (e) at different doses in A431 cells and their
479 DMR amplitudes as a function of their doses (c and f). All data represents mean ± s.d.
480 from 2 independent measurements, each in duplicate (n = 4).

481

482 **Fig. 9** The DMR amplitudes of 16 μM cyclopiazonic acid as a function of compounds
483 doses in A431 cells after pre-stimulation with these compounds for 1 h. (a)
484 Salvianolic acid A and salvianolic acid C; (b) thapsigargin and cyclopiazonic acid. All
485 data represents mean ± s.d. from 2 independent measurements, each in duplicate (n =
486 4).

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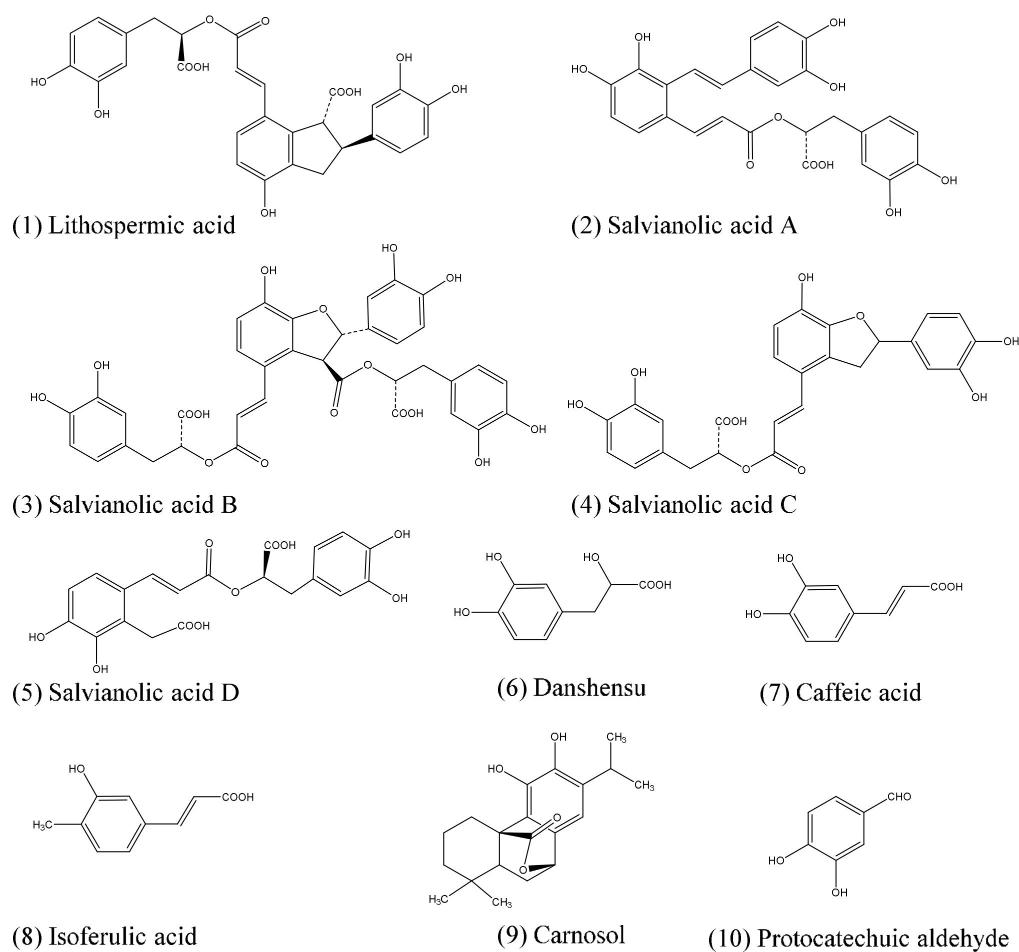
489 **Fig. 1**



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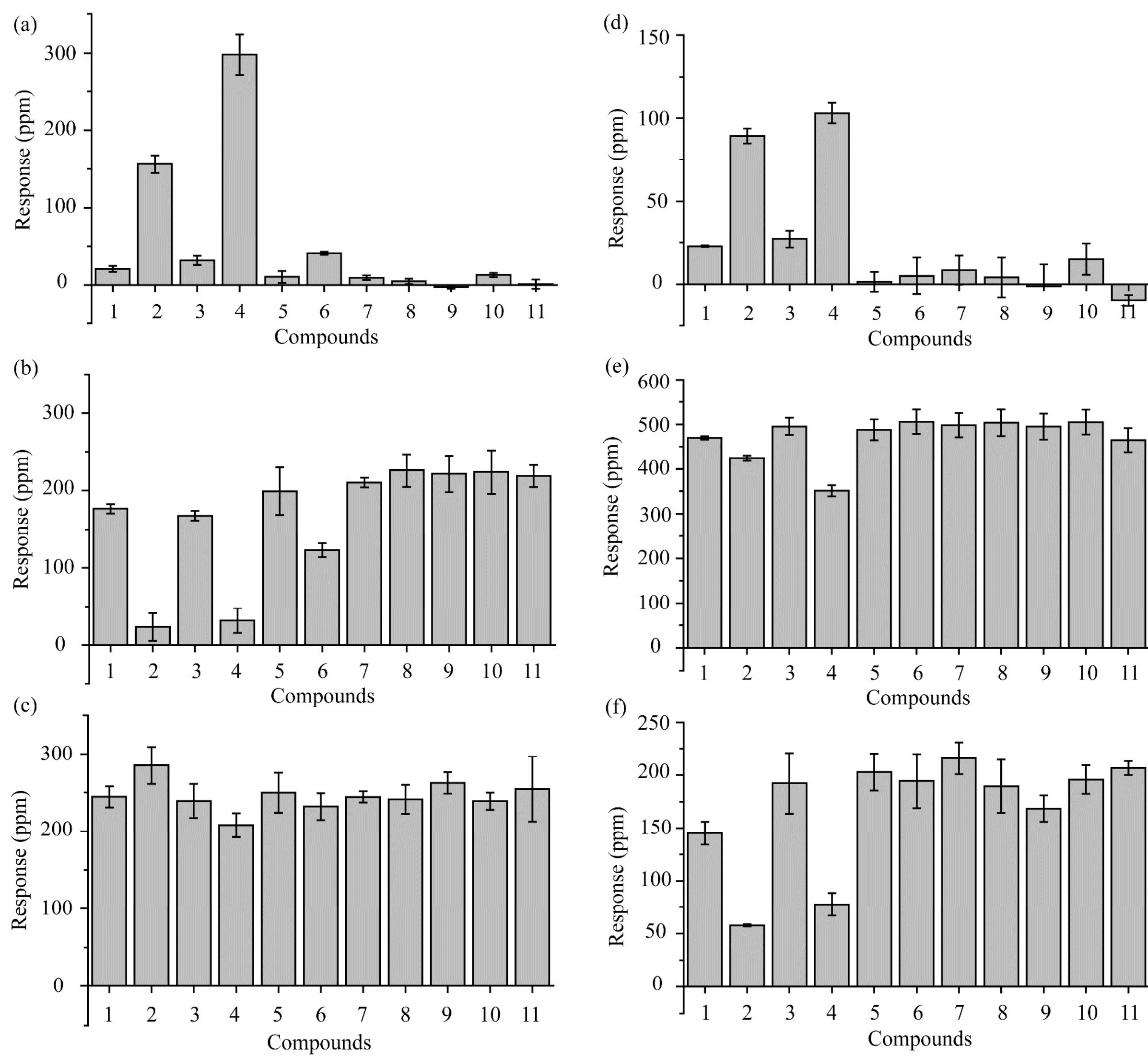
500 **Fig. 2**



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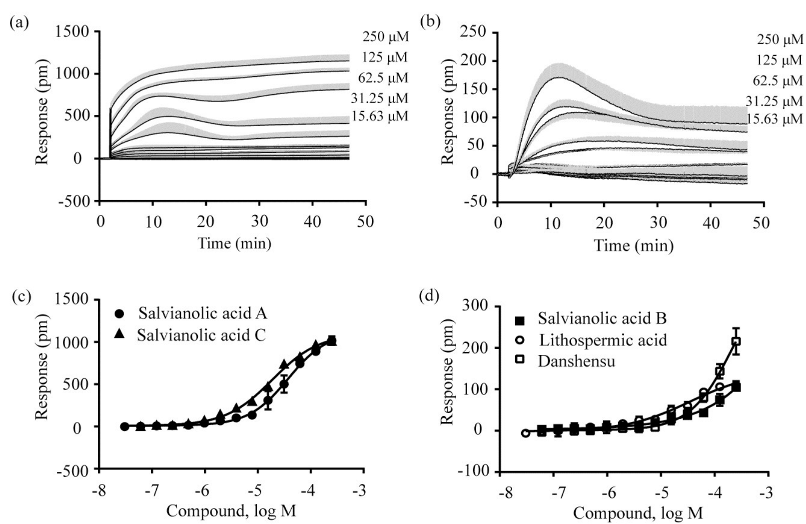
510 **Fig. 3**



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527 **Fig. 4**

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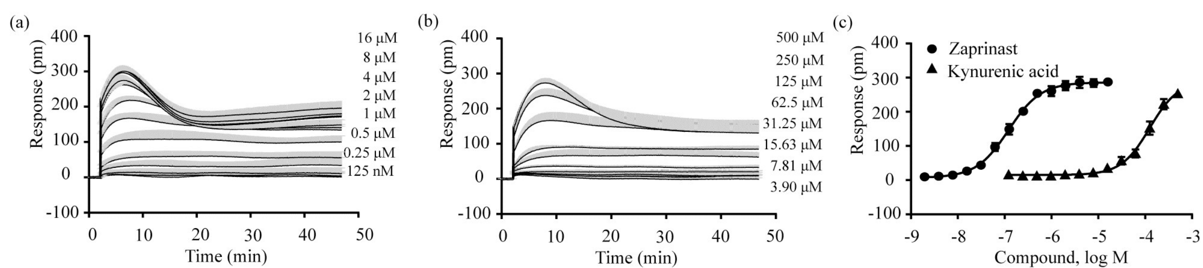

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531 **Fig. 5**

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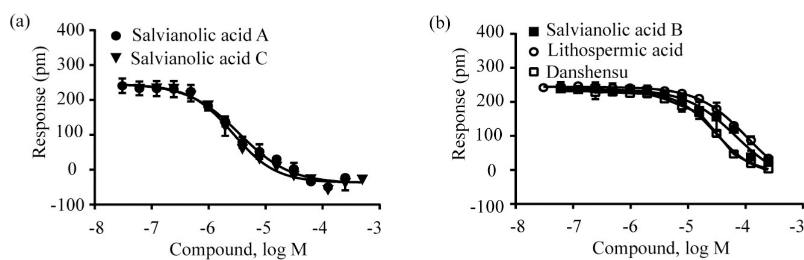

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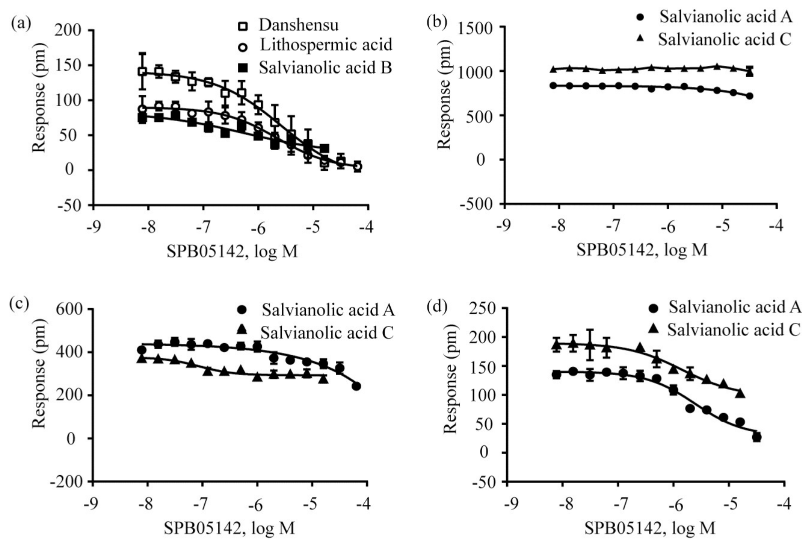
553 **Fig. 6**



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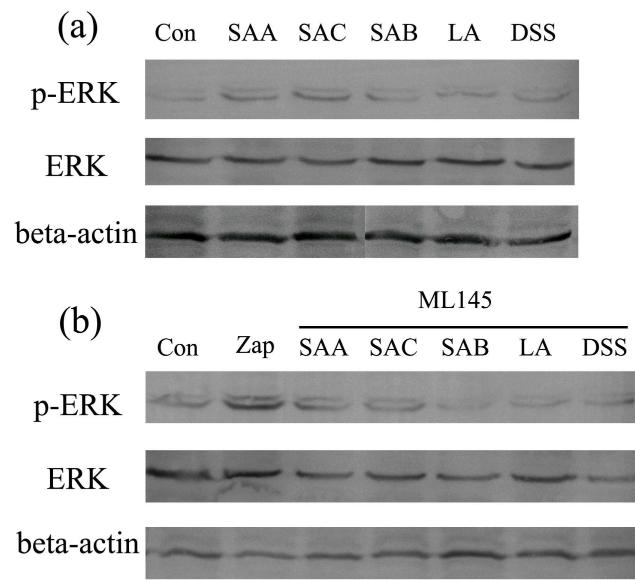
571 **Fig. 7**



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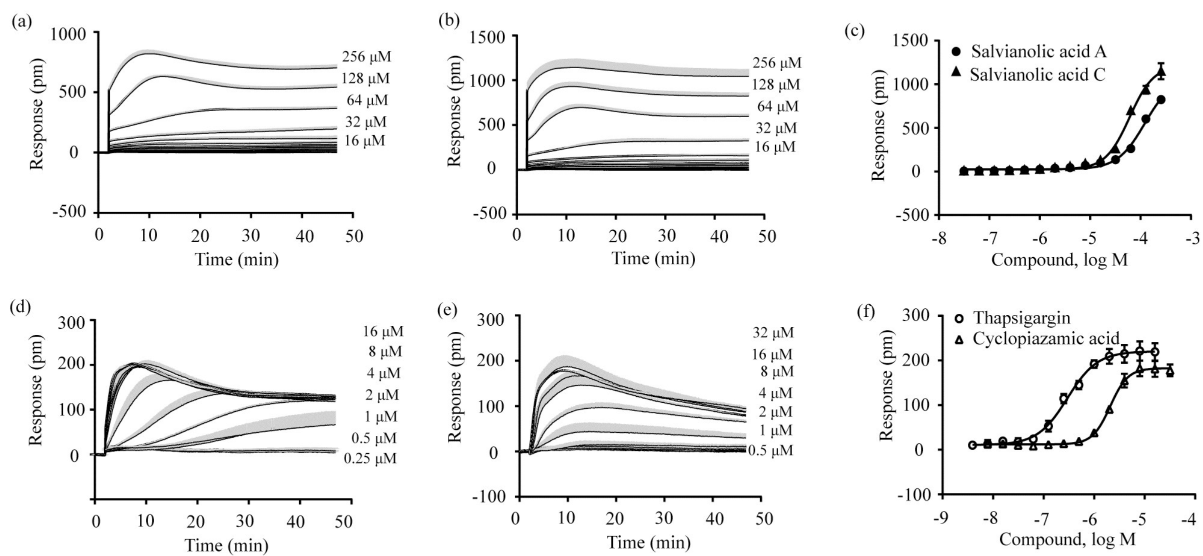
575 **Fig. 8**



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578 **Fig. 9**

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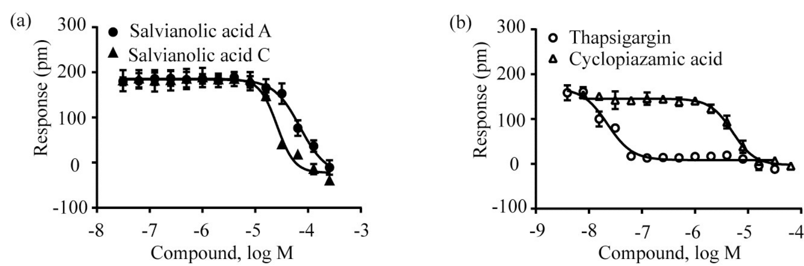

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