

1 **Title page**

2 **Original article**

3 **Diterpenoid Vinigrol activates ATF4/DDIT3-mediated PERK/eIF2 α**
4 **arm of unfolded protein response to drive breast cancer cell death**

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28 **Author contributions**

29 Yuhui Hu and Xinsheng Yao conceived this collaborative project. Hao Gao and
30 Chuanxi Wang provided naturally purified Vinigrol, and Yuhui Hu supervised the
31 work reported in this study. Wencheng Wei performed the RT-qPCR, western blot,
32 FACS, cell viability experiment (with help from Ziying Gao), data analysis and
33 drafted the first version of manuscript. Wencheng Wei, Yunfei Li and Sanxing Gao
34 constructed the gRNA plasmids, Yunfei Li and Sanxing Gao generated the CRISPR
35 cell lines. Yanxiang Jiang constructed the RNA-seq libraries, Hao Wang and Yan Zhao
36 performed the RNA-seq related analyses. Yuhui Hu interpreted the data and wrote the
37 manuscript with input from all authors.

38 **Declaration of Interests**

39 A patent covering the anti-cancer effect of Vinigrol has been submitted by the
40 Southern University and Science and Technology.

41 Authors declare no conflict of interest.

42

43 **Manuscript**

44 **Original article**

45 **Diterpenoid Vinigrol activates ATF4/DDIT3-mediated PERK/eIF2 α**
46 **arm of unfolded protein response to drive breast cancer cell death**

47 **Abstract** : Vinigrol is a natural diterpenoid with unprecedented chemical structure,
48 driving great efforts into its total synthesis and the chemical analogs in the past
49 decades. Despite its pharmacological efficacies reported on anti-hypertension and
50 anti-clot, comprehensive functional investigations on Vinigrol and the underlying
51 molecular mechanisms are entirely missing. In this study, we carried out a complete
52 functional prediction of Vinigrol using a transcriptome-based strategy, Connectivity
53 Map, and identified “anti-cancer” as the most prominent biofunction ahead of
54 anti-hypertension and anti-depression/psychosis. A broad cytotoxicity was
55 subsequently confirmed on multiple cancer types. Further mechanistic investigation
56 on MCF7 cells revealed that its anti-cancer effect is mainly through activating
57 PERK/eIF2 α arm of unfolded protein response (UPR) and subsequent upregulation of
58 p53/p21 to halt the cell cycle. The other two branches of UPR, IRE1 α and ATF6, are
59 functionally irrelevant to Vinigrol-induced cell death. CRISPR/Cas9-based gene
60 activation, repression, and knockout systems identified essential contribution of
61 ATF4/DDIT3 not ATF6 to the death process. This study unraveled a broad anti-cancer
62 function of Vinigrol and its underlying targets and regulatory mechanisms, and also
63 paved the way for further inspection on the structure-efficacy relationship of the
64 whole compound family, making them a novel cluster of chemical hits for cancer
65 therapy.

66 **Keywords:** Vinigrol; Unfolded Protein Response; Anticancer; ATF4; DDIT3;
67 PERK/eIF2 α ; Connectivity Map; CRISPR gene editing.

68 **Running Title:**

69 Vinigrol activates ATF4/DDIT3-mediated PERK UPR for breast cancer cell death.

70 **1. Introduction**

71 Vinigrol is a diterpenoid with a brand new chemical structure originally purified from
72 the fungal strain *Virgaria nigra* in 1987¹. It is the only terpenoid consisting of two
73 cis-fused ring systems and an eight-membered ring bridge, and thus persisted as a
74 formidable challenge for chemical synthesis¹⁻⁶ (Fig. 1A). Large efforts from chemists
75 in the past half century have led to a success in its total synthesis²⁻³, which in turn
76 raised questions for biologists on the potential medical applications of Vinigrol and its
77 synthetic analogues. Unfortunately, the existing knowledge on Vinigrol's bioactivities
78 was solely based on one rudimentary study from early years, offering only the
79 pharmacological observations on anti-hypertension and anti-platelet aggregation
80 effects^{7,8}. Neither the underlying mechanisms nor the molecular targets of Vinigrol
81 were investigated. Thus, a comprehensive and unbiased exploration on the functions
82 and regulatory mechanisms of Vinigrol is pivotal to transforming the chemical success
83 into the medical uses.

84 Understanding the biological functions of a given compound without target
85 information, such as a natural product like Vinigrol, is much more difficult and
86 time-consuming, compared to the drug hits originated from the target-driven
87 screening strategy. The functional assessments often require prior knowledge from the
88 bioactivity of their similar compounds. However, this cannot be achieved for a
89 compound with unprecedented backbone structure such as Vinigrol. The fast advance
90 in functional genomics in the past two decades, particularly the
91 transcriptome-profiling technologies, powered with bioinformatic tools, together
92 opened the chance to gain the first unbiased functional prediction of a given
93 compound. This was firstly introduced by the concept named Connectivity Map
94 (CMap), a chemical transcriptome approach brought by Broad Institute in 2006⁹. The
95 CMap project developed a database containing large transcriptional expression
96 profiles responding to chemical and genetic perturbagens. The latest version of CMap
97 is called L1000 consisting of >3 million gene expression profiles and >1 million
98 replicate-collapsed gene signatures¹⁰, thus creating multiple dimensional connections
99 between genes, chemicals, diseases, and biological status. In the context of

100 pharmacology, CMap is a powerful tool in exploring potential activities of compounds
101 based on the idea that gene expression changes could be used as the universal
102 language to connect the compound without functional annotation to those with known
103 functions. This can be achieved by calculating a “Connectivity Score”, cScore, with
104 an algorithm reflecting the similarities between the inquiry compound to the reference
105 chemicals in CMap database in terms of their gene expression profiles. CMap has
106 been powering both biologists and pharmacists to successfully predict the functions
107 and mechanisms of actions (MOA) of their compounds of interest ¹¹⁻¹³.

108 Gene target identification of a compound without functional annotation is
109 another big challenge in pharmaceutical and pharmacological study. The new
110 generation of gene editing technology, CRISPR (Clustered Regularly Interspaced
111 Short Palindromic Repeats), has revolutionized the entire life sciences within only a
112 few years ¹⁴. Together with CRISPR-associated nuclease (Cas9), which is directed to
113 its target DNA sequence by a short RNA fragment named guide RNA (gRNA), the
114 CRISPR/Cas9 complex can cut its DNA target from virtually any genome.
115 Consequently, a DNA indel can form at the chosen genomic site, resulting in a
116 frameshift of the mRNA transcript and subsequent nonfunctional protein. This system
117 is referred as CRISPR knockout (CRISPRko). Additionally, scientists engineered a
118 enzymatically inactive ("dead") versions of Cas9 (dCas9) to eliminate CRISPR's
119 nuclease activity, while preserving its ability to target desirable sequences. Together
120 with various transcriptional regulators fused to dCas9, it is possible to turn almost any
121 gene on or off or adjust its level of transcription ¹⁵⁻¹⁷. The dCas9-based
122 transcriptional inhibition and activation systems are commonly referred to as
123 CRISPRi and CRISPRa, respectively. These techniques have a wide range of
124 applications, including for example disease elimination, creation of hardier plants,
125 fight against pathogens and more. In the field of pharmaceutical research, CRISPR has
126 demonstrated an unprecedented power to quantitatively pinpoint both sensitive and
127 resistant gene targets of a drug out of the whole genome gRNA screening and thus
128 unraveled its MOA ¹⁸⁻²⁰. When applied with a single gene gRNA, CRISPR emerges as
129 a gold standard to assert a functional gene target of a given compound/drug, through

130 inspecting the activity alterations upon gene manipulation²¹. CRISPR has brought us
131 into a new era of chemical-genetics for targets-oriented drug discovery.

132 Endoplasmic reticulum (ER) is a eukaryotic cellular organelle essential for the
133 production, processing, and transport of proteins and lipids. ER dysfunction or stress
134 leads to accretion of misfolded proteins at ER and subsequent initiation of an
135 evolutionarily conserved signaling response, namely unfolded protein response (UPR).
136 Under several physiological and pathological stresses, cells use UPR to sense the
137 pressure and transduce the stress signal through several distinct pathways to
138 downstream transcriptional and post-transcriptional regulations^{22,23}. Consequently,
139 UPR can exert cytoprotective effects by restoring ER function and cellular
140 homeostasis. However, excessive ER stress can also activate intrinsic cellular
141 pathways, leading to cell death^{24,25}. In vertebrates, activation of UPR involves the
142 stimulation of three major signal transducers locating on the ER membrane, i.e.,
143 protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 alpha (IRE1 α),
144 and activating transcription factor 6 (ATF6) (Fig. 2A)^{23,25,26}.

145 PERK is a signal-sensitive type I transmembrane protein, which forms a
146 homodimer and trans-autophosphorylate when exposed to ER stress²⁷. The most
147 crucial PERK phosphorylation substrate found so far is eukaryotic translation
148 initiation factor 2 (eIF2 α). Upon UPR activation, PERK phosphorylates serine 51 of
149 eIF2 α , which subsequently leads to a pause in overall protein synthesis in eukaryotic
150 cells²⁷ but selectively allows translation of activating transcription factor 4 (ATF4),
151 whose target genes include DNA Damage-Inducible Transcript 3 (DDIT3). Under a
152 sustained ER stress response, ATF4 and DDIT3 contribute to the induction of cell
153 apoptosis and autophagy²⁸. On another path, IRE1 α , a type 1 ER transmembrane
154 serine/threonine kinase also containing an endoribonuclease (RNase) domain, initiates
155 the most conserved UPR signaling branch²⁵. Under ER stress, IRE1 α dimerizes and
156 autophosphorylates to elicit its RNase activity^{29,30}, thereby leading to a catalytic
157 excision of 26-nt intron within the mRNA encoding the transcription factor
158 X-box-binding protein 1 (XBP1)³¹⁻³³. This stress-stimulated splicing event generates
159 a stable and frameshifted translation product known as XBP1s, the active form of the

160 transcription factor that controls the expression of downstream UPR effector genes³⁴.
161 In the third branch of UPR signaling, full-length ATF6 translocates from the ER to the
162 Golgi apparatus, where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P)
163 to release its C-terminal fragment ATF6f^{35,36}. As an active transcription factor, ATF6
164 fragment can enter the nucleus and upregulate the expression levels of target genes
165 including specific components in the ER-related protein degradation system and
166 XBP-1^{25,31}. In a recent report, both ATF6 and XBP1 are postulated to inhibit
167 colorectal cancer cell proliferation and stemness through activating PERK signaling³⁷.
168 Overall, the three arms of UPR act in parallel and may also combinatorically to
169 maintain ER proteostasis and sustain cell function under ER stress. However, whether
170 all the three branches are equally important to regulate the stress-induced cell death is
171 far from clear.

172 In this study, we firstly sought to unbiasedly predict the complete bioactivities of
173 Vinigrol using CMap and confirmed its broad anti-cancer effect on various cancer cell
174 lines. Mechanistically, ER stress turned out as the most affected pathway by Vinigrol.
175 Among the three UPR signaling branches, we demonstrated PERK/eIF2 α axis but not
176 IRE1 α and ATF6 as the essential pro-death pathway. In this process, ATF4/DDIT3
177 function as the essential mediators targeted by Vinigrol, as cross-validated by
178 CRISPR/mediated gene repression and activation. Together, our study provided the
179 first mechanistic evidence for the anti-cancer effect of Vinigrol, bringing the whole
180 compound family as novel anti-cancer drug hits.

181 **2. Materials and methods**

182 *2.1 Reagents*

183 Vinigrol (purity 99%) was purified from Fungus was provided by group of Xinsheng
184 Yao and Hao Gao at Jinan University (Guangzhou, China). CellTiter-Glo
185 Luminescent Cell viability (CTG) assay (G7573) was from Promega (Wisconsin,
186 USA). 5x protein loading buffer (P0283), BeyoClick™ EdU-488 assay (C0071),
187 BeyoClick™ EdU-594 assay (C0078), and ER tracker red (C1041) were from
188 Beyotime (Jiangsu, China). Anti-HSPA5(WL03157), anti-ATF4 (WL02330), and

189 Enhanced chemiluminescent plus reagent kit (WLA006C) were from Wanleibio Co.,
190 Ltd. (Liaoning, China). Anti-DDIT3 (15204-1-AP) was from Proteintech (Wuhan,
191 China). Anti- β -tubulin (HC101-02) was from Transgenbiotech (Beijing, China).
192 Anti-p21(#2947), anti-CyclinD1 (#2926), anti-CDK2 (#2546), anti-eIF2 α (#5324),
193 anti-p-eIF2 α (phospho Ser51) (#3597) were from Cell Signaling Technology (Danvers,
194 USA). Anti-p-CDK2 (phospho Thr-14) (ab68265) was from Abcam (Cambridge, UK).
195 Anti-p53(SC-126) was from Santa Cruz (Dallas, USA). Trizol reagent (#R401-01)
196 was from Vazyme (Jiangsu, China). 1640 medium (C11875500BT), FBS (#10270106),
197 and Penicillin-Streptomycin (15140-122) was from Gibco (New York, USA).

198 *2.2 Cell culture and cell viability*

199 Human breast cancer cells MCF7 were from ATCC. It was maintained in 1640
200 medium with 10% FBS in an incubator with 5% CO₂. Culture media were
201 supplemented with penicillin and streptomycin. The culture medium was changed
202 every two days. MCF7 cells at 70% confluence were used for the next experiment.

203 CTG assay was used to measure cell viability. 10,000 cells per well were seeded
204 in a 96-well plate with 0.1 ml 1640 medium. After 72 hours of drug exposure, 50 μ l
205 CTG buffer was added to each well to measure cellular ATP levels. The 96-well plates
206 were shaken 3 min to get cell lysis and then centrifuge at 1000g, 3 min. Luminescent
207 signals were stabilized for 10 min at 25 $^{\circ}$ C and recorded. Each well collection was
208 about 100ms.

209 *2.3 RNA sequencing and data analysis*

210 Total RNA was extracted from the MCF7 cells by Trizol reagent. Next-generation
211 sequencing of mRNA-derived cDNA libraries was performed on the Illumina HiSeq
212 X Ten platform at Novogene (Tianjin, China). Control and Vinigrol (50 μ M)
213 treatment group were each sequenced with two replicates. At least 9 million reads per
214 sample were generated. All sequencing data were aligned to the GRCh38 genome
215 using the HISAT2 package³⁸. Unique aligned reads were retained to obtain reads
216 count matrices with feature Counts³⁹. The Gene Transfer Format (GTF) file used for

217 quantitative analysis is Ensembl release 90. The Lexogen QuantSeq 3'mRNA-seq
218 library kit (Lexogen, Vienna, Austria) was used to prepare the RNA-seq library in this
219 study. To avoid false increase of gene expression by internal poly-A on the transcripts,
220 we modified the GTF file before generating reads count matrices. The modification is
221 subjected to the last base position of the last exon of each gene in the reference
222 genome. The original sequence of the gene is trimmed to the flanking region of this
223 base. The region of shortened gene body contains 1000nt from this base on five prime
224 directions and 500nt on three prime directions. It is worth noting that the extension on
225 five prime only includes the coding sequence region. In addition, if the length of the
226 original transcript is shorter than 1000nt, the gene is not trimmed. In another direction,
227 if the interval between two genes is smaller than 500nt, the extension on three prime
228 directions is equal to the interval.

229 Gene expression differences between samples treated by Vinigrol and their
230 corresponding control samples were analyzed by the Bioconductor package DESeq2
231 ⁴⁰. Fold change value 2.0 and p-value smaller than 0.01 were used as thresholds to
232 distinguish dysregulated genes. Those genes are considered as gene signatures to
233 perform CMap analysis. In this work, the version of the reference database is CMap
234 build02. To input gene signature produced by RNA-seq technique, 22283 Affymetrix
235 IDs in CMap build02 were converted to 13714 gene symbol IDs. A local CMap
236 analysis system was set up to perform analysis. Additionally, both Gene Ontology and
237 KEGG enrichment analysis were performed to deduce the potential biological
238 functions by a Bioconductor package, ClusterProfiler⁴¹. Genes with at least one read
239 in treatment or control samples were considered as the enrichment analysis
240 background.

241 *2.4 EDU Cell cycle assay*

242 MCF7 cells were treated with Vinigrol or DMSO for 22h, followed by treatment of
243 10 μ M 5-ethynyl-2-deoxyuridine (EdU). Three hours after the EdU treatment, cells
244 were harvested and analyzed for EdU signaling using the BeyoClick™ EdU Cell
245 Proliferation Kit with Alexa Fluor 488 according to the manufacturer instructions.

246 EdU reaction buffer (CuSO₄, Alexa Fluor 594 azide or 488 azides, and additive buffer)
247 was added to the cells for Click-iT reaction and then incubated for 30 min at 25 °C in
248 the dark. Cells were then washed with 3% BSA/DPBS. After click reaction, cells were
249 analyzed on a flow cytometry instrument.

250 *2.5 Western blot analysis*

251 Proteins were extracted by RIPA lysis buffer (150mM NaCl, 50mM Tris-HCl, pH 7.4,
252 1% Triton, 1mM EDTA, 1% sodium deoxycholate, and protease inhibitor mixture)
253 treatment for 15 min in MCF7 cells. Supernatants were collected and centrifuged at
254 13,000 rpm for 10min at 4 °C. BCA assays were used to quantify protein contents.
255 Protein samples were then diluted with 5x loading buffer and incubated at 96 °C for 10
256 min in a heating block. Protein samples were separated by 10% SDS-PAGE gel and
257 then electrically transferred to 0.45µm PVDF membranes (Millipore, IPVH00010).
258 Membranes were then blocked with 5% non-fat milk in TBST for one hour at room
259 temperature. Proteins were detected by incubation with indicated primary antibodies
260 overnight at 4 °C followed by incubation with HRP-conjugated secondary antibodies
261 for one hour at 25 °C. Membranes were subsequently incubated with ECL reagent for
262 1-2 min and exposed at BIO-RAD ChemiDoc TM XRS+ system (California, USA).
263 All blots were stripped and reblotted with anti-β-tubulin or anti-β-actin as loading
264 controls. All signals were obtained in the linear range for each antibody, quantified
265 using ImageJ pro-plus, and normalized to β-tubulin. The antibodies and the dilutions
266 for western blots used in these studies are as follows: eIF2α (1:2000), p-eIF2α S51
267 (1:500), HSPA5 (1:2000), and ATF4 (1:1000). DDIT3(1:500), p53(1:300), p21
268 (1:1000), CDK2(1:1000), p-CDK2 Thr14(1:2000), CyclinD1(1:500), β-tubulin
269 (1:5000).

270 *2.6 Quantitative real-time PCR*

271 Total RNA (500 ng) was extracted from the MCF7 cells by Trizol Reagent. PCRs
272 were performed to detect ER stress-related genes. cDNA was synthesized with the
273 HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, R211-01). qPCRs were

274 performed with Hieff qPCR SYBR Green Master Mix (Yeasen, #11201ES08).
275 qRT-PCR conditions were as follows: 95 °C for 5 min, 40 cycles of amplification at
276 95 °C for 15 s and 60 °C for 1 min, analyzed on the BIO-RAD real-time PCR system.
277 The normalized expression of the assayed genes relative to LMNA was computed for
278 all samples. Primers used to detect the assayed genes are shown in Table S1.

279 *2.7 ER tracker staining*

280 Cells were grown overnight on coverslips in 24-well plates. At 24h after treatment
281 with Vinigrol, cells were incubated with ER-Tracker red for 15 min at 37°C. Stained
282 cells were then washed twice with PBS and fixed with 4% formaldehyde for 30
283 minutes at 25 °C, followed by hoechst33342 staining and visualized using confocal
284 microscopy (Nikon A1R), using an emission wavelength of 594 nm.

285 *2.8 Establishment of ATF4/DDIT3 repression and activation cell lines*

286 To generate the MCF7 cell line stably expressed dCas9-KRAB-MeCP2,
287 approximately 500,000 cells in 6-well plates were transfected with 2000ng
288 dCas9-KRAB-MeCP2-containing PiggyBac expression plasmids (Addgene plasmid
289 #110821) and 400ng of transposase vector PB200PA-1 using PEI. The medium was
290 changed after 24 h. Two days later, cells were treated with 5 µg/ml blasticidin (Yeasen,
291 #60218ES10). Cells were passaged in blasticidin medium for more than two weeks to
292 select dCas9 repressor integrant-containing cells. Single clones were obtained with
293 FACS and the highest inhibition clones were chosen for qRT-PCR verification. We
294 established plasmids contains gRNA ATF4 (F: GCATGGCGTGAGTACCGGGG, R:
295 CCCCGGTACTCACGCCATGC), gRNA DDIT3 (F:
296 GGACCGTCCGAGAGAGGAAT, R: ATTCCTCTCTCGGACGGTCC), and gRNA
297 negative control (NC) (F: GACGACTAGTTAGGCGTGTA, R:
298 TACACGCCTAACTAGTCGTC). After virus packaging, selected single clones were
299 transfected with gRNA vectors and further selected by 1 µg/ml puromycin (MCE,
300 HY-15695). Inhibition efficiency was verified with qRT-PCR.

301 For CRISPR acitivation (CRISPRa) system, MCF7 cells were transfected with a

302 lentiviral vector expressing dCas9-SunTag10x_v4-P2ABFP-NLS, BFP-positive cells
303 were sorted with FACS. These cells were subsequently transfected with a lentiviral
304 vector expressing scFv-GCN4-GFP-VP64, and GFP-positive cells were sorted with
305 FACS. Single colonies with strong transcriptional activation were sorted for CRISPRa
306 with FACS. Plasmids contain gRNA ATF4 (F: GTAAACGGTTGGGGCGTCAA, R:
307 TTGACGCCCCAACCGTTTAC), gRNA DDIT3 (F:
308 GCCCTAGCGAGAGGGAGCGA, R: TCGCTCCCTCTCGCTAGGGC), and gRNA
309 NC (F: GAACGACTAGTTAGGCGTGTA, R: TACACGCCTAACTAGTCGTTTC)
310 were established and their activation efficiency was verified with qRT-PCR.

311 *2.9 Internally controlled growth assay*

312 MCF7 CRISPRi cells with stable repression of ATF4, DDIT3, and NC conjugated
313 with RFP reporter and cells with NC gRNA but no RFP-tag were constructed.
314 RFP-positive cells were mixed with RFP-negative cells and were co-cultured for three
315 days. Flow cytometry was used to analyze the proportional change of RFP-positive
316 cells after repeated Vinigrol treatment. In this internally controlled growth assay ¹⁸,
317 changes in the proportion of RFP-positive cells indicated proliferation speed variation
318 between RFP-positive cells and RFP-negative cells. Proportion of RFP-positive cells
319 was normalized with the proportion of RFP-negative cells compared with the
320 normalized proportion at the original time point. The ratio for each time point was
321 normalized to the same percentage for DMSO control cells, defined as relative
322 enrichment. Therefore, a relative enrichment >1 indicates that RFP positive cells
323 confer protection against treatment, while enrichment <1 indicates sensitization.

324 *2.10 Establishment of ATF6 knockout cell lines*

325 The Cas9 stable-expressing MCF7 cells (MCF7-Cas9) were established by lentiviral
326 transfection. Cas9 coding sequence were integrated into the genome of cells
327 (Addgene#52962) and selected with 1 µg/mL blasticidin for nine days. Knockout
328 efficiency was tested using positive control gRNA and indels were confirmed by PCR
329 and DNA sequencing. Knockout cell lines were established by overexpressing gRNA

330 lent guide-Puro (Addgene #52963). After 48h of viral transfection, cells were selected
331 with 1 μ g/ml puromycin. Single cells were seeded in individual wells of a 96-well
332 plate and cultured for two weeks. At least 24 individual cell colonies were marked and
333 transferred into a 24-well plate until a sufficient number of cells were obtained to
334 extract genomic DNA. Indels and frameshifts were confirmed by PCR and DNA
335 sequencing. Finally, target gene expression levels were measured with western blot.
336 The ATF6 gRNA sequences are listed as follows: TTAGCCCGGGACTCTTTCAC.

337 *2.11 Determination of XBP1 splicing by RT-PCR*

338 The XBP-1 mRNA splicing in MCF7 cells was analyzed by PCR⁴². The primers'
339 sequences for PCR amplification of human XBP-1 are
340 5'-GAATGAAGTGAGGCCAGTGG-3' and 5'-GGGGCTTGGTATATATGTGG-3'.
341 PCR products were subjected to a 3 % agarose gel with Star Green DNA dye (GenStar,
342 Cat. E107-01) by electrophoresis.

343 **3. Results**

344 *3.1 Vinigrol shows anticancer potency via DNA damage and cell cycle arrest*

345 We firstly applied CMap analysis, a chemical transcriptome-based bioinformatic
346 approach, to explore possible biological activities of Vinigrol. 3' RNA-seq was
347 performed to obtain the gene expression profiles of Vinigrol-treated MCF7 cells and
348 the differentially expressed genes (DEGs) were identified (see Method). Overall,
349 more than 700 and 330 genes were significantly up- and down-regulated, respectively
350 (Fig. 1B). The DEGs was used as the “gene-signature” to calculate the cScores in
351 comparison to the 6100 transcriptome profiles retrieved from CMap build02 database,
352 which is produced from the cancer cells treated with 1309 compounds/drugs with
353 known biofunctions. In total, 6100 cScores were obtained, normalized and ranked
354 from +1 to -1 (Table S4), reflecting the highest expression similarity (cScore at +1)
355 and dissimilarity (cScore at -1), respectively. Among all the 6100 profiles, those with
356 top positive cScores are enriched for the approved anti-cancer drugs and cytotoxic
357 compounds, suggesting anticancer as the most prominent biofunction of Vinigrol

358 (Fig.1C). Next, we verified the toxicity of Vinigrol on human breast cancer cell line
359 MCF7 (Fig.1D) and 8 other cancer cell lines and identified its broad-spectrum
360 anticancer activity with IC50 at 20-60 μ M (ATP measurement with CTG assay) (Table
361 S2). Toxicity of Vinigrol on noncancerous cells was significantly slighter compared to
362 cancer cells (Table S2). Phenotypically, Vinigrol treatment within only 24 hrs could
363 induce severe cytoplasmic vacuolization around nucleus in MCF7 cells at middle to
364 high doses before complete shrinkage and death (Fig. 1E).

365 We continued the mechanistic studies of Vinigrol on MCF7 cell lines. Firstly,
366 using EdU/hoechst33342 DNA labeling assay followed by FACS flow cytometry
367 quantification, we observed that the proportion of EdU-positive cells, i.e. cells at S
368 phase, were largely reduced whereas the percentage of cells at G2/M phase
369 significantly increased, indicating a cell cycle arrest at G2/M phase (Fig. 1F-1G). This
370 effect of Vinigrol was in a dose-dependent manner, cumulating a complete loss of
371 DNA replication at more than 75 μ M of Vinigrol. To dig out the arrest regulators, we
372 further checked the Cyclin/Cyclin-dependent kinase (CDK) cell cycle checkpoints
373 and p53/p21 DNA damage checkpoints (Fig. 1H). Western blot results showed that
374 Vinigrol upregulated p53 and p21, indicating a fast activation of cellular DNA
375 damage response (DDR). Simultaneously, CDK2 was inactivated as demonstrated by
376 a dramatic increase of phosphorylation on its inhibitory site Thr-14. Accordingly, the
377 cell cycle regulator cyclin D1 was severely downregulated. Altogether, it
378 demonstrated that Vinigrol could induce DNA damage and activation of classic
379 p53/p21 DDR pathway, which subsequently inactivated CDK2/Cyclin D1 checkpoints,
380 blocked the replication process, and prevent the cells from moving forward to division.
381 Notably, such effect came out fast and intensified in a time- and dose-dependent
382 manner.

383 *3.2 Vinigrol induces PERK-mediated ER stress in MCF7*

384 In light of the Vinigrol's anticancer effect, we performed gene enrichment analysis to
385 explore its potential molecular mechanisms. The up- and down-regulated genes upon
386 Vinigrol treatment were subjected to gene ontology (GO) analysis, respectively, using

387 all expressed genes as background (Fig. 2B). GO enrichment analysis suggested that
388 many of the upregulated genes are involved in PERK-mediated ER stress (Fig.
389 2B-2D). Strong and extended ER stress can result in cell dysfunction and even cell
390 death^{24,25}. Quantitative Reverse Transcription PCR (qRT-PCR) measurements
391 revealed continuous rises of mRNA level of the key PERK-mediated ER stress genes,
392 including stress initiator HSPA5/Bip, intermediary ATF4, and downstream death
393 effector DDIT3 and GADD45A (growth arrest and DNA damage inducible 45 alpha),
394 along with increase of drug doses and treatment duration (Fig. 2E). Consistent with
395 the mRNA changing dynamics, protein levels of hallmark molecules including
396 HSPA5/Bip, p-eIF2 α , eIF2 α , ATF4, and DDIT3, also sustainably increased (Fig. 2F,
397 Fig.S1). These data showed that Vinigrol can induce a fast activation of ER-stress
398 sensor HSPA5 and PERK arm of UPR, which subsequently phosphorylates eIF2 α ,
399 leading to induction of downstream transcriptional regulator ATF4 and pro-death
400 effector DDIT3. Notably, the ER stress activation by Vinigrol was in a time- and
401 dose-dependent manner, whose dynamics was in accordance with the changes of
402 p53/p21 DNA damage responders and cell cycle checkpoints (Fig. 1G), suggesting
403 additive role of ER stress in regulating the cell death. This was further confirmed by
404 reduced cell viability through combined treatment of PERK-specific inhibitor
405 GSK2606414 with Vinigrol (Fig. S2). When we labeled the ER of MCF7 cells with
406 fluorescent dye ER Tracker Red, we observed that the ER gradually lost its structure,
407 enlarged and vacuolized after Vinigrol treatment. Vacuolization in the ER was also
408 exacerbated with higher doses of Vinigrol (Fig. 2G). ER dilation and cytoplasm
409 vacuolization are key morphological features of ER stress. Altogether, these results
410 showed that Vinigrol could induce cell deaths by activating PERK-mediated ER stress
411 pathway.

412 *3.3 Vinigrol induced ER stress can be alleviated by DDIT3 and ATF4 knockdown*

413 PERK-mediated ER stress is related to increased abundance of ATF4, a transcriptional
414 factor that directly activates DDIT3 transcription²⁸. To determine whether Vinigrol
415 induces cell death is via the ATF4-DDIT3 axis, we developed cell lines with stable

416 repression of ATF4 and DDIT3 using CRISPR/dCas9 inhibition (CRISPRi) system ¹⁶.
417 The knockdown efficiency of target genes was verified by qRT-PCR (Fig. 3A), which
418 demonstrated that mRNA expression levels of ATF4 and DDIT3 were significantly
419 repressed in MCF7 cells comparing with the dummy guide RNA (gRNA)-infected
420 negative control (NC) MCF7 cells. Since ATF4 and DDIT3 are key intermediate
421 regulator on PERK arm of UPR signaling, we also checked the mRNA and protein
422 expression of other upstream and downstream regulators HSPA5/BiP, eIF2 α and
423 GADD45A as did on MCF7 wildtype. Upon Vinigrol treatment, repression of either
424 ATF4 or DDIT3 could significantly alleviate the drug-induced upregulation scale of
425 HSPA5/Bip and GADD445A mRNA level, albeit not fully rescued the increase (Fig.
426 3B). Such alleviation was also observed on the protein level. Comparing to NC-MCF7
427 wildtype cells, the upregulation of both HSPA5/Bip and p-eIF2 α were almost
428 completely abolished in ATF4 or DDIT3 CRISPRi repression cells (Fig. 3C). These
429 results indicate an important feedback loop of ATF4 and DDIT3 to retro-regulate their
430 upstream UPR mediators such as HSPA5/Bip and eIF2 α , beyond transducing stress
431 signals to their downstream targets. Interestingly, repression of ATF4 largely refrained
432 DDIT3 from being stimulated by Vinigrol-induced ER stress at both mRNA and
433 protein level (Fig. 3B-C). On the other hand, DDIT3 repression barely influenced the
434 ATF4 transcription and translation (Fig. 3B-C), even though the gRNA targeting on
435 DDIT3 provided higher knockdown efficiency than ATF4 gRNA (Fig. 3A-B). These
436 data further confirmed DDIT3 as the transcriptional target of ATF4, but not vice versa.
437 Consequently, ATF4 functions on top of DDIT3 to promote the PERK signaling. Both
438 transcriptional factors, however, play essential roles in retaining the Vinigrol-induced
439 ER stress responses.

440 *3.4 Vinigrol-induced cell death can be alleviated by ATF4/DDIT3 knockdown*

441 Stress induced UPR can contribute to cellular homeostasis and recovery, and cell
442 death. We therefore investigated the concrete role of ATF4/DDIT3 in
443 Vinigrol-induced death process. Cell viability measurement (CTG assay) revealed a
444 clear rescue of ATF4 or DDIT3 knockdown on cell growth when treated with Vinigrol

445 at the concentrations near IC50 (Fig. 4A). The growth protection by knocking down
446 ATF4 and DDIT3 were further supported by EDU/hoechst33342 cell cycle analysis.
447 Comparing to the NC cells with dummy gRNA, repression of the genes retained the
448 DNA replication of the cells, as demonstrated by increased percentage of S-phase
449 cells (Fig. 4B-C). Notably, such increase was only observed upon Vinigrol treatment,
450 not in vehicle treatment controls, which excluded the gene knockdown, by itself, from
451 protecting the DNA replication. Accordingly, when we checked the protein level of
452 p21, a key cell cycle negative regulator and DNA damage checkpoint, repression of
453 ATF4 and DDIT3 specifically diminished the drug-induced upregulation of p21 (Fig.
454 4D), and thus promoted the cell cycle process.

455 To further investigate the effects of ATF4 and DDIT3 knockdown on Vinigrol
456 sensitivity in an extended time frame, we applied an internally controlled growth
457 assay (see 2.9 in Materials and methods). Briefly, in this experiment, the red
458 fluorescent protein (RFP) was introduced to label the ATF4 or DDIT3 gRNA infected
459 cells, which were co-cultured, respectively, with the non-fluorescent dummy gRNA
460 cells (NC) followed by three runs of Vinigrol treatment and withdraw (Fig. 4E). The
461 proportion of RFP to NC cells was quantified after each run of drug treatment by flow
462 cytometry to indicate the relative growth rates of CRISPRi gene edited cells compared
463 to non-edited cells. Such RFP/NC proportion was further normalized by the same
464 co-culture setup but with only DMSO control treatment, in order to exclude the gene
465 effect on the cell growth that is irrelevant to drug effects. Overall, RFP positive cells
466 displayed relative enrichment greater than 1 and keep stepping up with repeated drug
467 treatment, which indicated that repression of ATF4 or DDIT3 confers growth
468 protection against Vinigrol treatment (Fig. 4F). Together, these findings clearly
469 indicate ATF4 and DDIT3 as two essential pro-death mediators that are induced by
470 Vinigrol and directly contribute to its anti-cancer effect.

471 *3.5 Vinigrol induced cell death can be exacerbated by ATF4/DDIT3 overexpression*

472 After establishing the necessity of ATF4/DDIT3 axis in Vinigrol induced cell deaths
473 through CRISPRi gene editing system, we wondered if activation of this pathway

474 would exacerbate Vinigrol induced cell death. To this end, we constructed MCF7 cells
475 stably overexpressing ATF4 or DDIT3 transcripts using
476 CRISPR/dCas9-SunTag-VP64 gene engineering system, namely CRISPR activation
477 (CRISPRa) system^{17,43} coupled with the gRNAs specifically targeting ATF4 and
478 DDIT3, respectively. The overexpression of both genes was efficient as quantified by
479 using qRT-PCR (Fig. 5A). In contrast to the knockdown results, ATF4 or DDIT3
480 overexpression significantly exacerbated the Vinigrol-induced ER stress as
481 demonstrated by the enlarged upregulation magnitudes of HSPA5/Bip, GADD45A,
482 and p-eIF2a at mRNA level (Fig. 5B) and protein level (Fig. 5C). As expected,
483 activation of ATF4 promoted DDIT3 transcription and led to protein level increase in
484 both Vinigrol and control treatments (Fig. 5B-C). Consequently, CTG cell viability
485 measurement and EdU cell cycle analysis showed that cell death induced by Vinigrol
486 was significantly aggravated after ATF4 or DDIT3 overexpression (Fig. 5D-5F).
487 Specifically, the fraction of S-phase cells was further reduced when overexpressing
488 ATF4 or DDIT3 upon Vinigrol treatment. Notably, without the drug treatment,
489 activation of either gene could significantly promote the cells into S-phase (Fig.
490 5E-G), indicating strong protective effects on the cell growth. When such basal gene
491 effects were normalized out to obtain the Vinigrol-induced gene effects (by comparing
492 the reduction fold between Vinigrol/DMSO groups), we found that the DNA
493 replication stress induced by Vinigrol was deteriorated with ATF4 or DDIT3
494 overexpression (Fig. 5F). This was further supported by the enhanced upregulation
495 scale of p21 in CRISPRa engineered cells (Fig. 5G). In the internally controlled
496 growth assay (Fig. 4E), in contrast to the growth rescue from ATF4 and DDIT3
497 knockdown, ATF4 and DDIT3 overexpression cells were almost depleted after
498 repeated Vinigrol treatment (Fig. 5H). Altogether, our results from both CRISPR
499 inhibition and activation systems provide solid evidence to pinpoint ATF4/DDIT3 as
500 the functional targets of Vinigrol, whose expression levels dictate the cell sensitivity
501 and resistance to the anti-cancer effect of Vinigrol.

502 *3.6 PERK, but not ATF6 or IRE1 α , directly contribute to Vinigrol induced cell death*

503 Among the three ER stress pathways, we next inspected the role of ATF6 and IRE1 α
504 pathways in Vinigrol induced cell death. From the RNA-seq analysis, most of ER
505 stress related genes were significantly upregulated upon Vinigrol treatment (Fig.
506 6A-6B). However, ATF6 and IRE1 α pathways were not enriched by GO and KEGG
507 analysis. ATF6 is an essential messenger of ER stress signaling pathway. Previous
508 studies indicated that it is involved in sustaining cell viability³⁷. ATF6 gene
509 expression level increased in a dose- and time-dependent manner after Vinigrol
510 treatment (Fig. 6C). Consistently, ATF6 protein level significantly increased at 6 hrs
511 and quickly decreased at 24 hrs (Fig. 6D). Furthermore, we used CRISPR/Cas9 to
512 generate single clones of MCF7 cells carrying homozygous deletion of ATF6 and
513 treated them with Vinigrol. Unexpectedly, we found that loss of ATF6 did not affect
514 Vinigrol sensitivity compared with controls in CTG cell viability assay (Fig. 6E-F),
515 which suggested that ATF6 is dispensable for Vinigrol-triggered cell death, and the
516 fast increase of ATF6 expression level is likely a temporary response of cells to
517 Vinigrol treatment.

518 IRE1 α /XBP1 pathway plays important roles in UPR. Under extended stress,
519 IRE1 α activation can cause cell death by activating the MAPK pathway^{44,45}. Vinigrol
520 treatment did not induce XBP-1 splicing; only slightly spliced-XBP-1 was detected at
521 100 μ M Vinigrol treatment for 24 hrs. Brefeldin A (BFA), a ER stress inducer was
522 known to activate IRE1 α to splice XBP-1⁴⁶. As a positive control in our study, BFA
523 rapidly led to a fast splicing of XBP-1 at low concentrations in short time (Fig. 6G),
524 which in turn suggested that the IRE1 α pathway was not activated in Vinigrol induced
525 cell deaths. To summarize, our data indicated that Vinigrol functions through
526 PERK-mediated ER stress instead of ATF6 and IRE1 α pathways to cause cell death in
527 MCF7 cells.

528 **4. Discussion**

529 The fundamental goal of this study is to decipher the bioactivity and functional
530 mechanism of Vinigrol, a natural diterpenoid with unprecedented chemical structure.
531 The success of its full synthesis achieved in the past decade not only create a series of

532 compound analogues, but also shed lights on their potential medical use through
533 elaborated pharmacological research and drug development. In the context of
534 molecular biology, we are also intrigued by a more fundamental question: how the
535 structure uniqueness of Vinigrol interferes with the cellular environment of a
536 mammalian cell.

537 In light of the inventions in transcriptome analytic techniques, i.e., Microarray as
538 the 1st generation of invention and then RNA-seq afterwards, we applied CMap for
539 the first functional assessment of Vinigrol. Up to now, three versions of CMap
540 database have been updated with the number of total expression profiles increasing
541 from a hundred (Build01), 6100 (Build02), and above a million (L1000). Notably,
542 only the first two versions provide the experimentally quantified whole transcriptome
543 datasets that are performed on Affymatrix Microarray. In L1000, on the other hand,
544 the expression profiles of 12291 genes were computationally imputed from the actual
545 quantification of only 978 genes, which would reduce the calculation accuracy of
546 cScore¹⁰. To achieve more precise functional predictions, we decided to compare our
547 RNA-seq data of Vinigrol to Build02, whose 1309 perturbagens consist of most of
548 approved drugs and drug hits with annotated actions and molecular targets.

549 Among the 20 best-matched drugs (Table S3), apart from anti-cancer effect, our
550 results unraveled several other potential biofunctions of Vinigrol, such as
551 anti-hypertension (similar to, for example, adrenergic receptor antagonist drug
552 phenoxybenzamine and suloctidil), anti-depression and antipsychosis (similar to
553 protriptyline, thioridazine, prochlorperazine, perphenazine). Interestingly, the
554 anti-hypertensive activity of Vinigrol was reported along with its discovery in 1988,
555 the only pharmacological study of Vinigrol ever published till now⁷. In this early
556 study, Vinigrol was orally given to conscious spontaneously hypertensive rats and its
557 anti-hypertensive activity was observed, yet the underlying mechanism and molecular
558 targets were completely missing. Experimentally approved anti-hypertension and
559 anti-cancer (this study) effect of Vinigrol demonstrate the unbiasedness and reliability
560 in functional prediction using chemical transcriptome-based approach. Undoubtedly,
561 the predicted effects of Vinigrol on neuronal and psychotic diseases such as

562 depression, AD, and schizophrenia are in high medical needs and definitely worth of
563 further exploration. However, to find the correct targets perturbation and suitable
564 disease models persists as the leading challenge to scientists, and our CMap results
565 and RNA-seq oriented gene and pathway analyses provide valuable
566 multi-dimensional information to guide their experimental design.

567 In our gene set enrichment analyses, ER stress/UPR relevant terms stood out as
568 the pathways that are mostly likely targeted by Vinigrol (Fig. 6A). However, all the
569 three UPR branches, PERK/eIF2 α , ATF6, and IRE1 α are firstly known to be essential
570 in restoring the cellular homeostasis and, when under excessive and prolonged
571 activation, also result in stress-induced inflammation and apoptosis^{24,25,27,28,45,46}. To
572 fully understand the role of ER stress in the anti-cancer effect of Vinigrol, it is
573 necessary to address the question whether the three pathways equally attributes to the
574 stress adaptation of the cell or mediates the cell death in response to Vinigrol. We
575 applied multiple cellular, biochemical, and molecular assays, together with
576 CRISPR/Cas9 mediated gene knockout, repression, and activation systems, to
577 specifically investigate the key mediators regulating each branch. The results clearly
578 showed that Vinigrol-induced cell death largely attributes to the activation of
579 PERK/eIF2 α arm instead of IRE1 α /XBP1 and ATF6 branches. Despite the IRE1 α
580 transcript slightly increases, it fails to cleave the XBP1 RNA and keep the pathway
581 silent. It was reported that proteasome inhibitors can induce ER stress but prevent
582 IRE1 α from splicing XBP-1⁴⁷. In our CMap analysis, the expression profiles of
583 Vinigrol treatment matched those of celastrol (Table S3), a proteasome inhibitor that
584 prevents XBP-1 splicing. Vinigrol's proteasome inhibition activity may be the cause
585 of inactivated XBP-1 during Vinigrol treatment. The activation of ATF6 expression
586 was dramatic shortly after Vinigrol treatment, suggesting it also plays a role. However,
587 not until we achieved its homozygous deletion by using CRISPR/Cas9 knockout
588 system could we affirm its irrelevance to the cell growth (Fig. 6F). Thus, we postulate
589 the activation of ATF6 as a downstream stress response rather than a functional
590 mediator of Vinigrol's anti-cancer effect.

591 PERK/eIF2 α is probably the most profoundly studied branch among the three,
592 involving the translational control by eIF2 α that is phosphorylated by PERK, and
593 downstream overexpression of two transcriptional factor ATF4 and DDIT3. Therefore,
594 we carefully inspected the changing dynamics of ATF4 and DDIT3 together with their
595 upstream mediator HSPA5/Bip, eIF2 α and several downstream effectors, and revealed
596 that they are all gradually upregulated by Vinigrol in a dose- and time-dependent
597 manner. Taking advantages of CRISPRi and CRISPRa, we cross-validated the
598 essential regulatory roles of ATF4/DDIT3 in promoting the death process stimulated
599 by Vinigrol. Specifically, knockdown of each gene restrains all the studied genes from
600 being over-activated by Vinigrol and thus alleviated the death, whereas CRISPRa
601 exerts the opposite effects. It is worth mentioning that the influential scales of gene
602 overexpression and knockdown sometimes are not at the same magnitude in terms of
603 their functional consequences, even if they are opposite. This is largely depending on
604 the basal expression of a gene. Specifically, in the MCF7 cells growing in normal
605 condition, the basal expression of DDIT3 is barely detected, therefore its further
606 knockdown renders relatively minor influence compared to its overexpression on the
607 cell growth (Fig. 4A, 5D). Thus, it is always encouraged to apply both repression and
608 activation in gene-editing experiments to cross-validate the results. Interestingly, even
609 without Vinigrol treatment, the CRISPRa enhanced basal expression of DDIT3 elicits
610 dramatic increase of S-phase cells (Fig. 5E-F). Previous reports described more of
611 pro-death effect of DDIT3 under ER stress through inducing the expression of
612 pro-apoptosis gene targets such as death receptor 5 (DR5) and Growth arrest and
613 DNA damage-inducible protein 34 (GADD34)^{25,48}. Here, we clearly demonstrated a
614 favorable effect of slight upregulation of DDIT3 to promote the DNA replication of
615 MCF7 tumor cells. However, its excessive and prolonged activation stimulated by
616 chemicals such as Vinigrol, halts the cell cycle and facilitates the death process that is
617 mediated by the best understood regulatory axis p53/p21. Altogether, our study
618 unveiled a broad anti-cancer function of Vinigrol with good potential as a clinical
619 medication. Nevertheless, we strongly recommend monitoring the expression of key

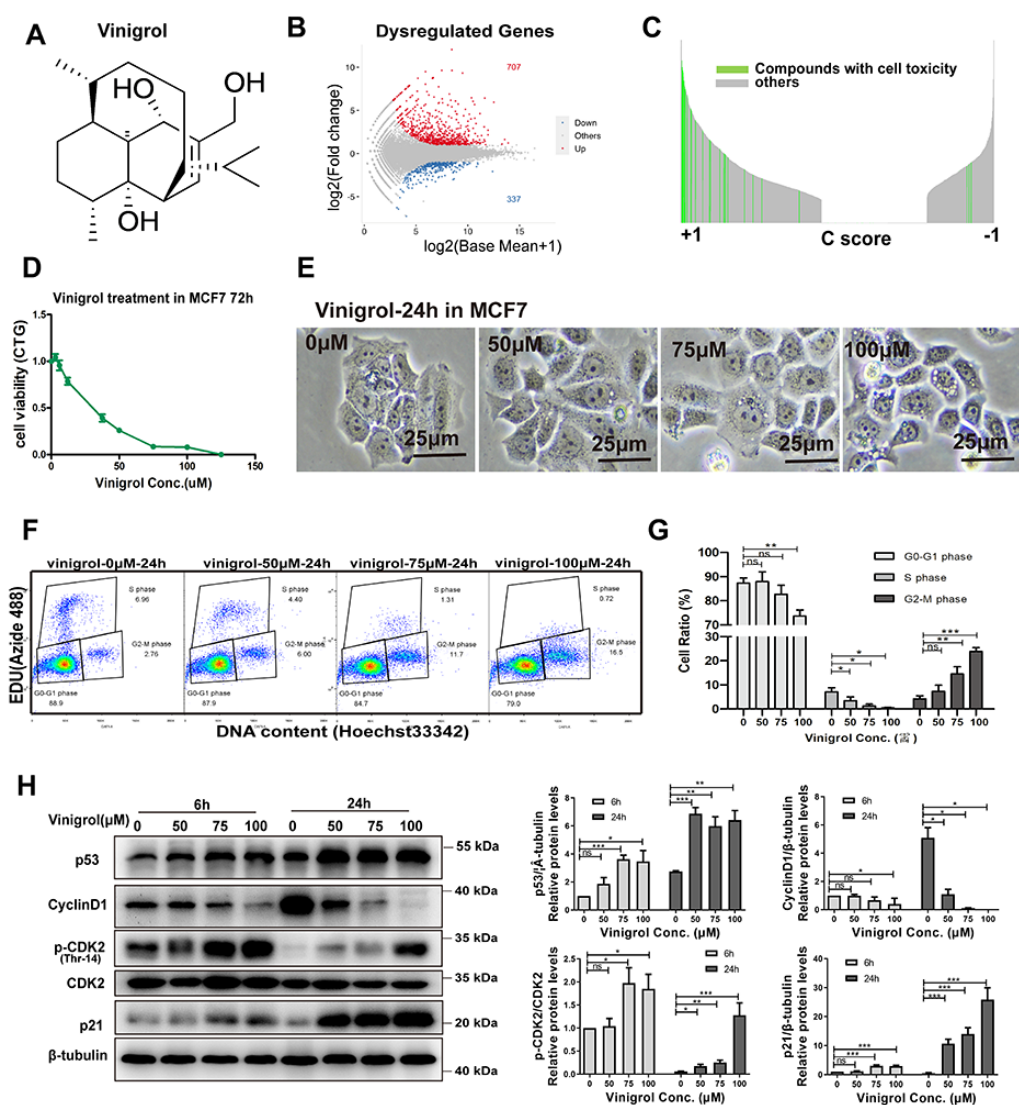
620 regulators such as ATF4 and DDIT3 before and during chemotherapeutic process to
621 guarantee a sufficient activation.

622 **5. Conclusion**

623 Vinigrol, a natural diterpenoid with unprecedented chemical structure, has gained a
624 huge endeavor in the past decade for its chemical synthesis. The success of chemists
625 on its full synthesis created a series of compound analogs with similar structures that
626 await functional and mechanistic investigation.

627 In this study, we firstly applied CMap to unbiasedly predict the complete
628 bioactivities of Vinigrol. Anti-cancer appear to be the most prominent biofunction
629 followed by anti-hypertension and anti-depression/psychosis. A broad cell toxicity on
630 various cancer cell lines was further confirmed. Mechanistically, ER stress turns out
631 as the most affected pathway by Vinigrol. Among the three UPR braches, we
632 demonstrate PERK/eIF2 α axis but not IRE1 α and ATF6 as the essential pro-death
633 pathway, which subsequently activates the cell cycle negative regulator and DNA
634 damage checkpoints p51/p21. In this process, ATF4/DDIT3 function as the essential
635 mediators targeted by Vinigrol, as cross-validated by CRISPRi/a genetic engineering.
636 In conclusion, we provided the first evidence for the anti-cancer effect of Vinigrol and
637 the underlying mechanisms. With the recent achievement of Vinigrol's chemical
638 synthesis, our results also paved the way for further comparisons and elucidation of
639 the structure-effect relationship of the whole compound family, making them a novel
640 chemical cluster with potential for cancer therapy. Additionally, this study serves as a
641 valuable resource on the genes and pathways affected by Vinigrol, as well as a
642 complete list of potential bioactivities to facilitate other pharmacological validations.
643

644 **Figures and Figure captions**



645

646 **Figure 1.** Vinigrol shows anticancer potency via DNA damage and cell cycle arrest.

647 (A) Chemical structure of Vinigrol. (B) MA plot figure analysis by 3'RNA-seq. (C)

648 Ranking of 6100 cScores of Vinigrol with the compounds in CMap Build02. The

649 length of each line indicates the absolute value of cScore. Green lines represent

650 toxicity compounds and grey lines represent non-toxicity compounds. (D) Cell

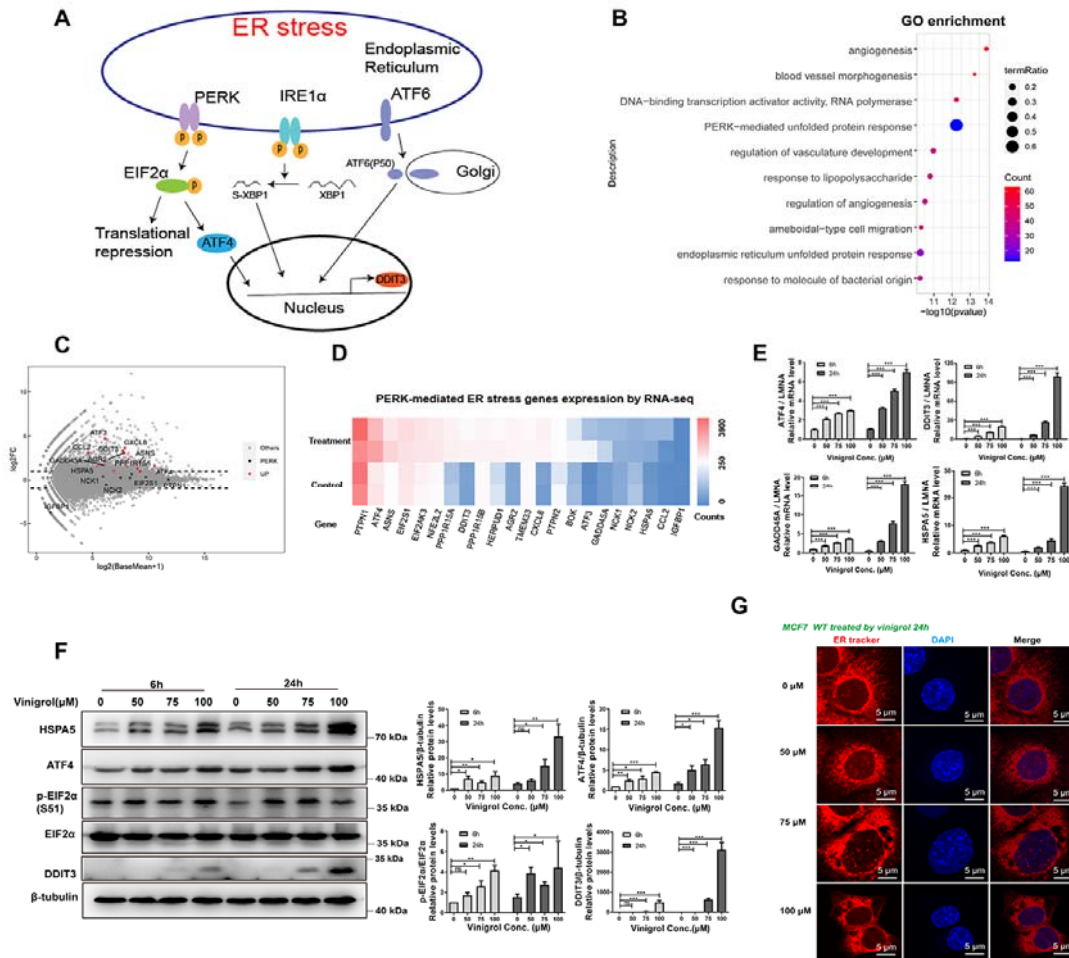
651 viability after Vinigrol treatment 72 hrs measured by CTG assay. (E) Cell morphology

652 after Vinigrol treatment 24 hrs. (F) Flow cytometry analysis of MCF7 cells treated 24

653 hrs for different Vinigrol doses, stained with EdU and Hoeschst, (G) Proportion of

654 cells at different cell cycles after Vinigrol treatment 24 hrs at varied doses. (H) (Left)

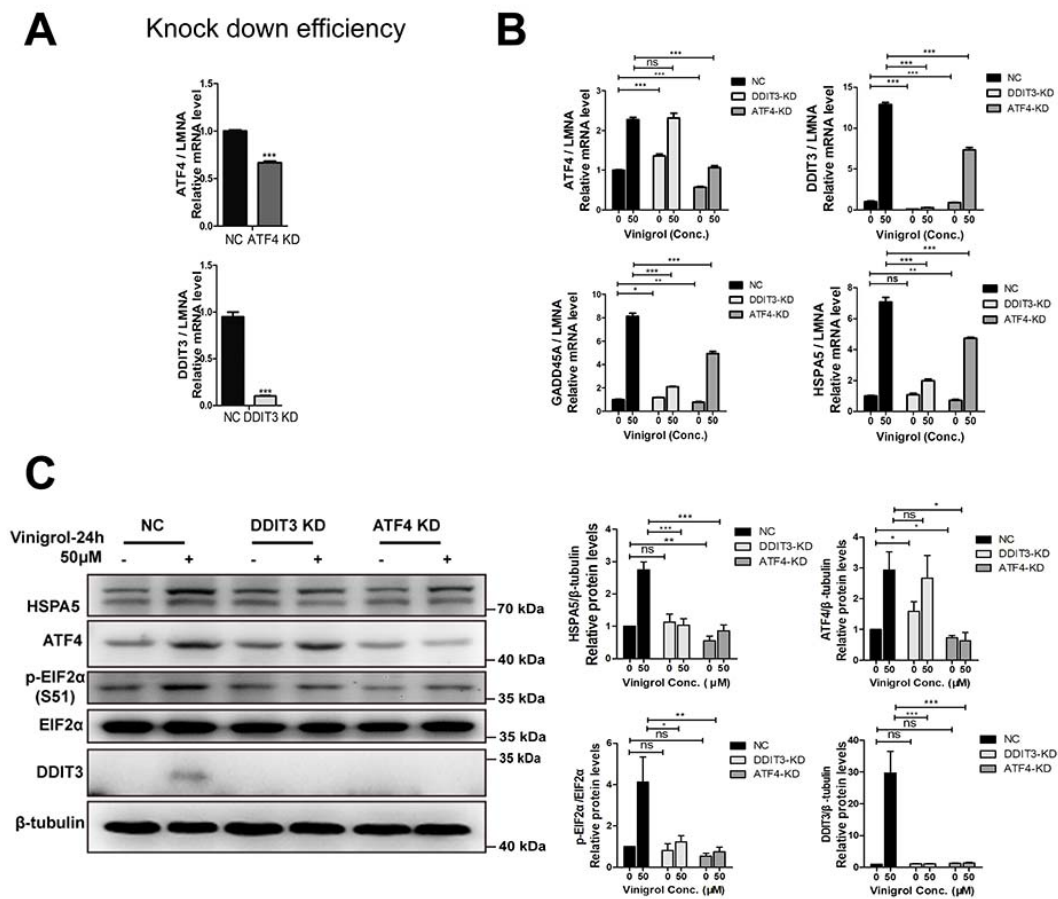
655 Western blot analysis of cell cycle checkpoint-related proteins after varied doses of
 656 Vinigrol treatment for 6 hrs and 24 hrs. (Right) semi-quantification of protein levels in
 657 Western blots. Data are represented as mean \pm SEM (n = 3), statistical significance
 658 was analyzed by two tail T-test. * represent $p < 0.05$, ** represent $p < 0.01$, ***
 659 represent $p < 0.001$, ns represent not significant.



660

661 **Figure 2.** Vinigrol induces PERK mediated ER stress in MCF7. (A) 3 branches of ER
 662 stress pathway. (B) Gene Ontology analysis indicates that genes upregulated by
 663 Vinigrol are significantly enriched in the term “PERK-mediated unfold protein
 664 response”. (C) MA-plot for gene expression comparison between Vinigrol (50 μ M, 6
 665 hrs) treated for and control MCF7 cells. Red and Black dots denote genes on
 666 PERK-mediated ER stress signaling pathway with significant expression changes (Red)
 667 and no changes (Black). (D) Heatmap representing normalized read counts of genes

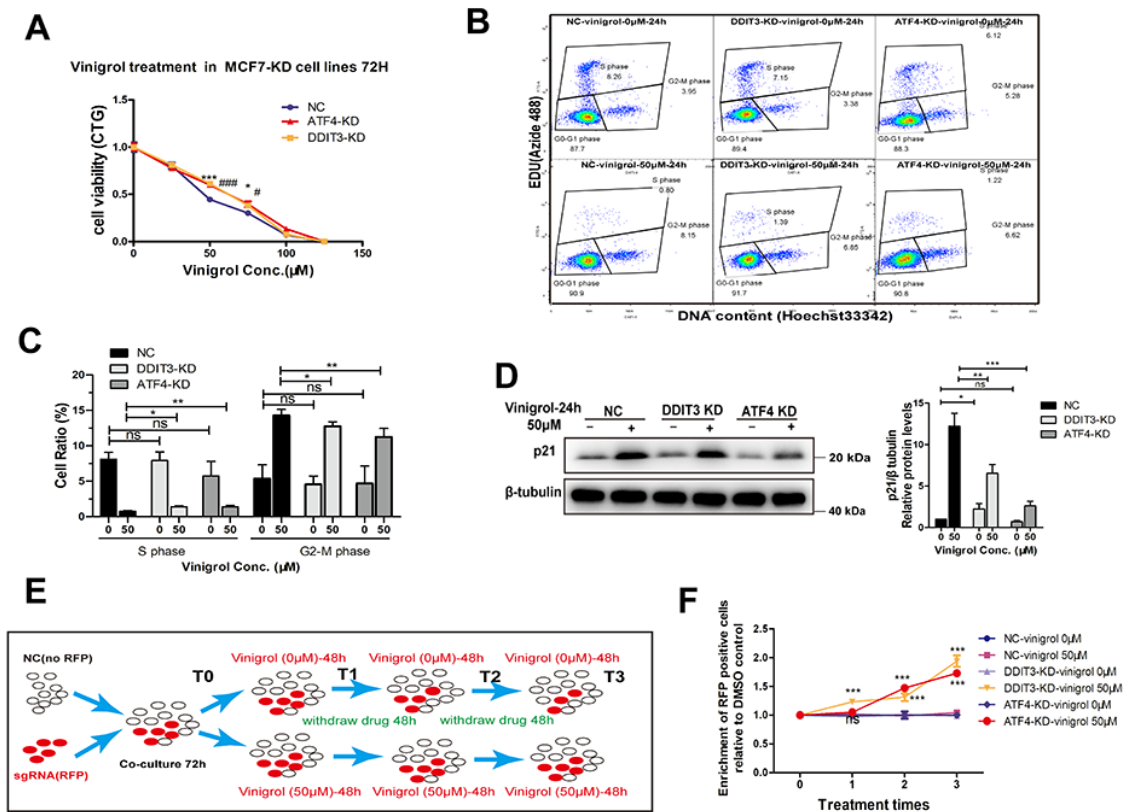
668 on PERK-mediated ER stress signaling pathway from RNA-seq data. (E) mRNA
 669 expression levels of ER stress-related genes with different doses of Vinigrol treatment
 670 for different time, quantified by qRT-PCR. (F) (Left) Protein levels of ER
 671 stress-related genes with different doses of Vinigrol treatment for different time,
 672 quantified by western blot. (Right) Semi-quantification of protein levels. (G)
 673 Visualization of ER and nuclei with ER Tracker and DAPI in MCF7 cells after
 674 different doses of Vinigrol treatment 24 hrs. Data are represented as mean \pm SEM (n =
 675 3), statistical significance was analyzed by T-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns
 676 represent not significant.



677

678 **Figure 3.** ER stress-induced by Vinigrol was alleviated after ATF4/DDIT3
 679 knockdown. (A) Knockdown efficiency of ATF4 and DDIT3 in MCF7 by
 680 CRISPR/dcas9 inhibition system, *compared with NC, *** $p < 0.001$. (B) mRNA

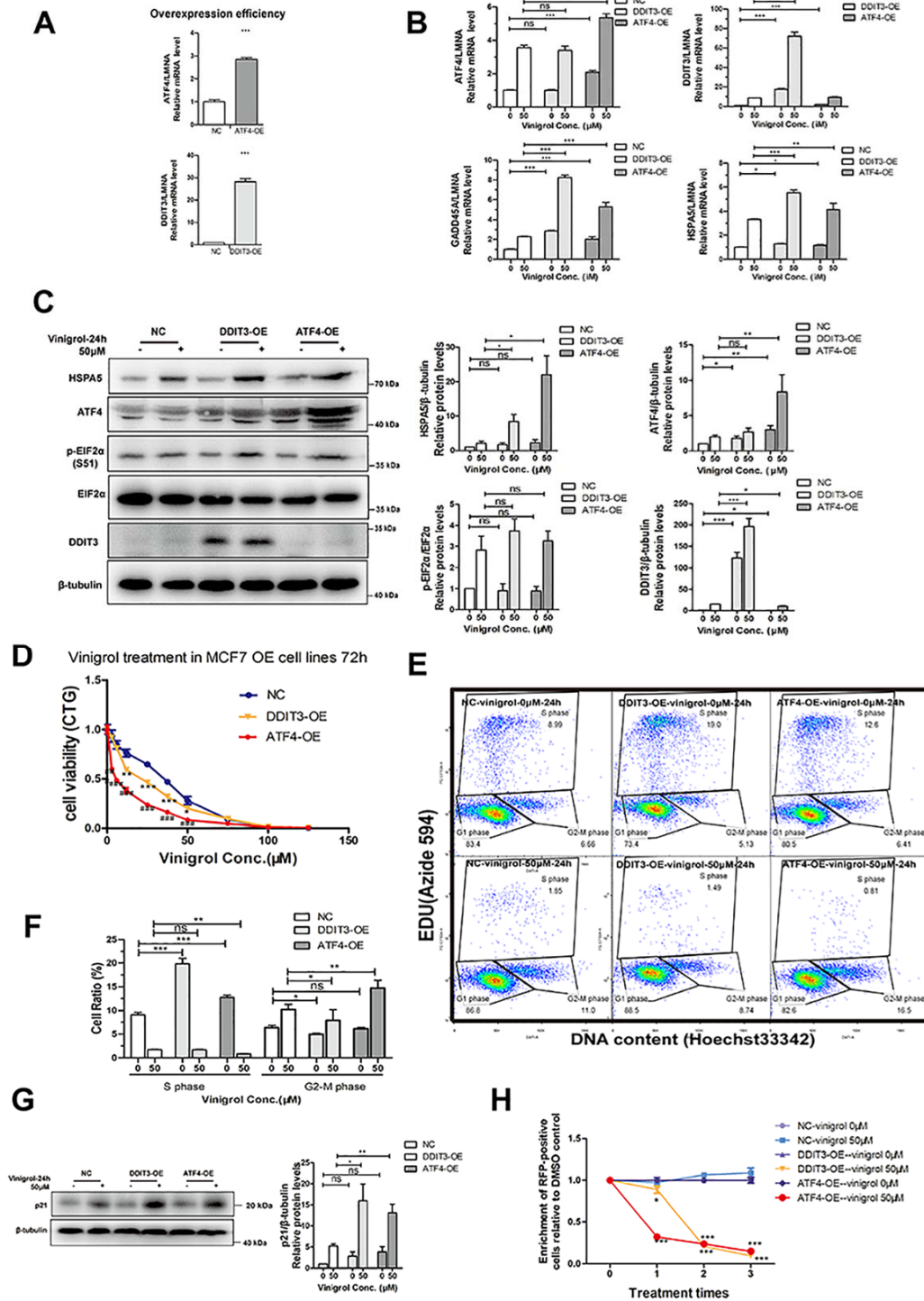
681 expression levels of ER stress-related genes after 24 hrs of Vinigrol (50 μ M) treatment,
 682 quantified by qRT-PCR. (C) (Left) Protein levels of ER stress-related protein after
 683 Vinigrol (50 μ M) treatment for 24 hrs, detected by Western blot. (Right)
 684 Semi-quantification of protein levels. Data are represented as mean \pm SEM (n = 3),
 685 statistical significance (p<0.05) was analyzed by T-test. *p<0.05, **p<0.01,
 686 ***p<0.001, ns represent not significant.
 687



688

689 **Figure 4.** Cell death induced by Vinigrol was alleviated after ATF4/DDIT3
 690 knockdown. (A) Cell viability quantified by CTG assay after Vinigrol treatment for 72
 691 hrs. ATF4-KD group compared with the NC group, # p<0.05, ## p<0.01, ### p<0.001;
 692 DDIT3-KD group compared with the NC group, * p<0.05, ** p<0.01, *** p<0.001;
 693 ns: not significant. (B) EDU/Hoechst33342 cell cycle analysis after DDIT3 and ATF4
 694 knockdown, analyzed by BD FACS. (C) Quantification of cells in different stages of
 695 cell cycle. (D) Protein level of p21 after 50 μ M Vinigrol treatment for 24 hrs, detected
 696 by western blot. (E) Workflow of internally controlled growth assay. (F) Enrichment

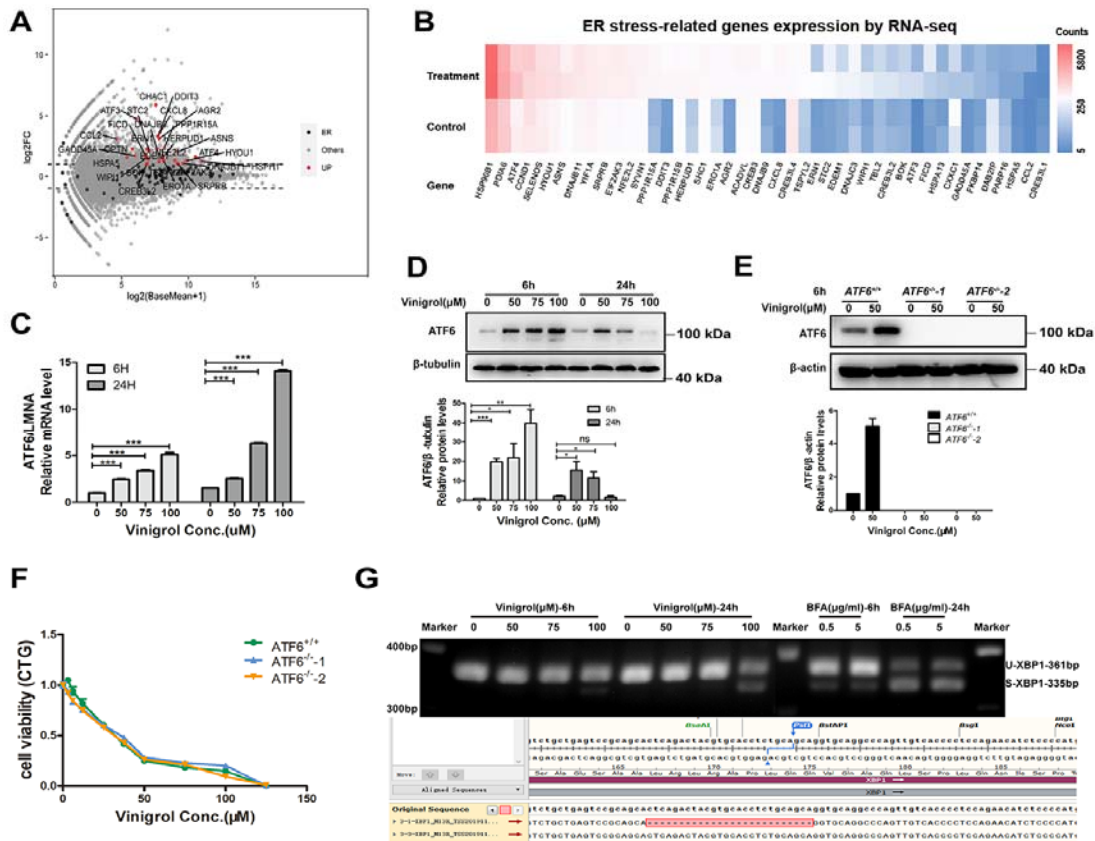
697 of RFP positive cells relative to DMSO-treated control cells across three repeated
698 50 μ M Vinigrol treatments, analyzed by flow cytometry. Data are represented as mean
699 \pm SEM (n = 3), statistical significance was analyzed by T-test, * p<0.05, ** p<0.01,
700 *** p<0.001, ns represent not significant.



701

702 **Figure 5.** Cell death induced by Vinigrol was exacerbated by overexpression of
 703 ATF4/DDIT3. Cell death induced by Vinigrol was exacerbated by overexpression of
 704 ATF4/DDIT3. (A) Overexpression (OE) efficiency of ATF4 and DDIT3 by

705 CRISPR/dcas9 activation system, *compared to NC, *** $p < 0.001$. (B) mRNA
706 expression levels of ER stress-related genes after 50 μM Vinigrol treatment for 24 hrs,
707 quantified by qRT-PCR. (C) (Left) Protein levels of ER stress-related genes after 50
708 μM Vinigrol treatment for 24 hrs, detected by western blot. (Right)
709 Semi-quantification of protein levels. (D) Cell viability after Vinigrol treatment for 72
710 hrs, quantified by CTG assay. ATF4-OE group compared with NC group, # $p < 0.05$, ##
711 $p < 0.01$, ### $p < 0.001$; DDIT3-OE group compared with NC group, * $p < 0.05$, **
712 $p < 0.01$, *** $p < 0.001$; ns: not significant. (E) EDU/Hoechst33342 cell cycle analysis
713 after ATF4 and DDIT3 overexpression, analyzed by BD FACS. (F) Quantification of
714 cells in different cell cycle stages. (G) Protein level of p21 after 50 μM Vinigrol
715 treatment 24 hrs, detected by western blot. (H) Enrichment of RFP positive MCF7
716 cells relative to DMSO-treated control cells in internally controlled growth assay.
717 Data are represented as mean \pm SEM ($n = 3$), statistical significance was analyzed by
718 T-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns represent not significant.



719

720 **Figure 6.** Effect of Vinigrol on ATF6 and IRE1 α pathways in MCF7. (A) Expression
721 levels of ER stress-related genes shown in MA plot in RNA-seq analysis. Red and
722 Black dots denote genes on ER stress signaling pathway with significant expression
723 changes (Red) and no changes (Black). (B) Expression levels (normalized RNA-seq
724 read counts) of ER stress-related genes shown in heatmap. (C) mRNA level of ATF6
725 after varied doses of Vinigrol treatment for different time, quantified by qRT-PCR. (D)
726 Protein levels of ATF6 expression level after Vinigrol treatment in MCF7 cell,
727 detected by western blot. (E) Protein levels of ATF6 after 50 μ M Vinigrol treatment
728 for 6 hrs in ATF6^{-/-} MCF7 cell lines, quantified by western blot. (F) Cell viability after
729 Vinigrol treatment in ATF6^{-/-} MCF7 cell lines, quantified by CTG assay. (G) Splicing
730 of XBP1 by IRE1 quantified by RT-PCR, with BFA as a positive control. Sanger
731 sequencing results of the two PCR amplicons revealed 26-nt deletion of s-XBP1. Data
732 are represented as mean \pm SEM (n = 3), statistical significance was analyzed by T-test.
733 * p < 0.05, ** p < 0.01, *** p < 0.001, ns represent not significant.

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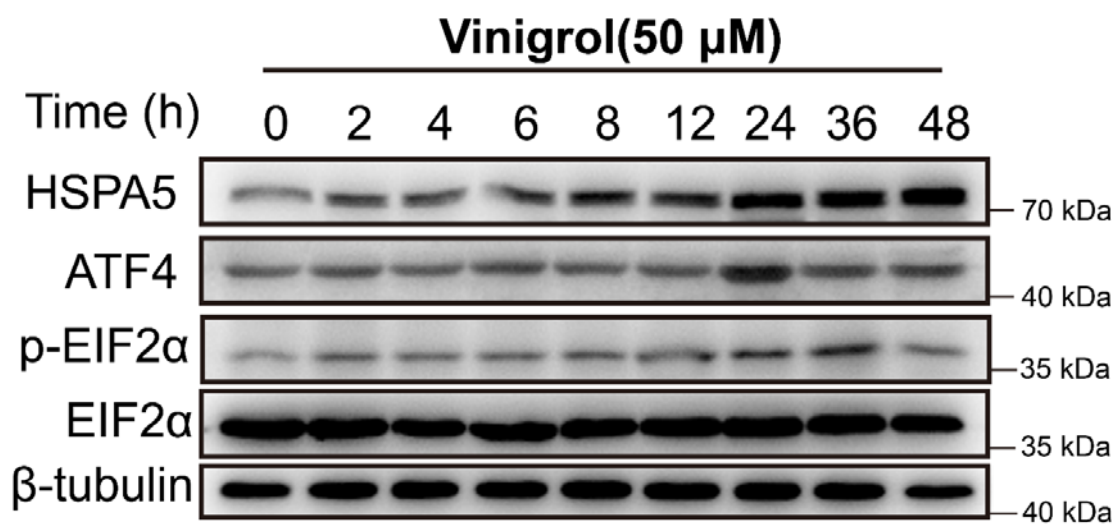
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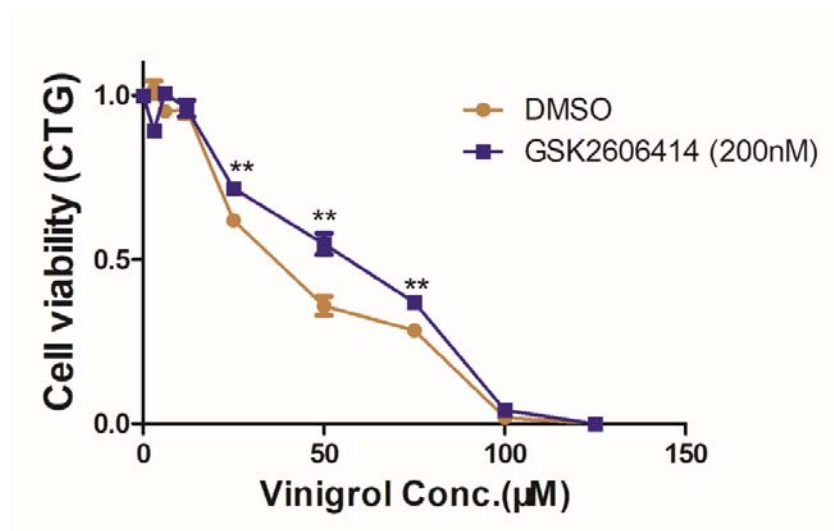
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861 **Supporting information**



863 **Figure S1.** The ER stress-related protein expression level of MCF7 cells after Vinigrol (50 μ M)
864 treatment at different time.



866 **Figure S2.** Cell viability of MCF7 cells treated with Vinigrol with and without
867 GSK2606414 (200nM) for 72 hrs.
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