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 Ziyang 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.

1. Introduction

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

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

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

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

2. Materials and methods

2.1 Reagents

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

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

2.2 Cell culture and cell viability

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

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

2.3 RNA sequencing and data analysis

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

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

Gene expression differences between samples treated by Vinigrol and their corresponding control samples were analyzed by the Bioconductor package DESeq2⁴⁰. Fold change value 2.0 and p-value smaller than 0.01 were used as thresholds to distinguish dysregulated genes. Those genes are considered as gene signatures to perform CMap analysis. In this work, the version of the reference database is CMap build02. To input gene signature produced by RNA-seq technique, 22283 Affymetrix IDs in CMap build02 were converted to 13714 gene symbol IDs. A local CMap analysis system was set up to perform analysis. Additionally, both Gene Ontology and KEGG enrichment analysis were performed to deduce the potential biological functions by a Bioconductor package, ClusterProfiler⁴¹. Genes with at least one read in treatment or control samples were considered as the enrichment analysis background.

2.4 EDU Cell cycle assay

MCF7 cells were treated with Vinigrol or DMSO for 22h, followed by treatment of 10μM 5-ethynyl-2-deoxyuridine (EdU). Three hours after the EdU treatment, cells were harvested and analyzed for EdU signaling using the BeyoClick™ EdU Cell 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

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

2.7 ER tracker staining

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

2.8 Establishment of ATF4/DDIT3 repression and activation cell lines

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

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: TACACGCCTAACTAGTCGTTC)
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

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

2.11 Determination of XBP1 splicing by RT-PCR

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

3. Results

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

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

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

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

3.2 Vinigrol induces PERK-mediated ER stress in MCF7

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

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

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

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

repression of ATF4 and DDIT3 using CRISPR/dCas9 inhibition (CRISPRi) system¹⁶. The knockdown efficiency of target genes was verified by qRT-PCR (Fig. 3A), which demonstrated that mRNA expression levels of ATF4 and DDIT3 were significantly repressed in MCF7 cells comparing with the dummy guide RNA (gRNA)-infected negative control (NC) MCF7 cells. Since ATF4 and DDIT3 are key intermediate regulator on PERK arm of UPR signaling, we also checked the mRNA and protein expression of other upstream and downstream regulators HSPA5/BiP, eIF2 α and GADD45A as did on MCF7 wildtype. Upon Vinigrol treatment, repression of either ATF4 or DDIT3 could significantly alleviate the drug-induced upregulation scale of HSPA5/BiP and GADD445A mRNA level, albeit not fully rescued the increase (Fig. 3B). Such alleviation was also observed on the protein level. Comparing to NC-MCF7 wildtype cells, the upregulation of both HSPA5/BiP and p-eIF2 α were almost completely abolished in ATF4 or DDIT3 CRISPRi repression cells (Fig. 3C). These results indicate an important feedback loop of ATF4 and DDIT3 to retro-regulate their upstream UPR mediators such as HSPA5/BiP and eIF2 α , beyond transducing stress signals to their downstream targets. Interestingly, repression of ATF4 largely refrained DDIT3 from being stimulated by Vinigrol-induced ER stress at both mRNA and protein level (Fig. 3B-C). On the other hand, DDIT3 repression barely influenced the ATF4 transcription and translation (Fig. 3B-C), even though the gRNA targeting on DDIT3 provided higher knockdown efficiency than ATF4 gRNA (Fig. 3A-B). These data further confirmed DDIT3 as the transcriptional target of ATF4, but not vice versa. Consequently, ATF4 functions on top of DDIT3 to promote the PERK signaling. Both transcriptional factors, however, play essential roles in retaining the Vinigrol-induced ER stress responses.

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

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

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

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

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

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

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

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 readily 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

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

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

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

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

660

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

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

677

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

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679

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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 50μM Vinigrol treatments, analyzed by flow cytometry. Data are represented as mean
699 ± 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 μ M Vinigrol treatment for 24 hrs,
707 quantified by qRT-PCR. (C) (Left) Protein levels of ER stress-related genes after 50
708 μ M 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 μ M 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 IRE1α 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 μM 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 ± 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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Supporting information

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

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