

Running title: VD₃ reduces cisplatin sensitivity

Transcriptomic analysis of vitamin D3 (cholecalciferol)-mediated attenuation of cisplatin sensitivity in gastric cancer cells *in vitro*

Ke Chen*, Yu Pan

Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

*Correspondence: chenke68@zju.edu.cn

Received March 10, 2026 / Accepted July 20, 2026

Cisplatin remains a cornerstone of chemotherapy for gastric cancer, but acquired resistance frequently limits its therapeutic efficacy. Vitamin D3/cholecalciferol (VD3) is widely used as a nutritional supplement; however, its influence on cisplatin responsiveness in gastric cancer cells remains unclear. In this study, gastric cancer cell lines were treated with cisplatin in the presence or absence of VD3, and cell viability, apoptosis, reactive oxygen species (ROS) accumulation, DNA damage-associated signaling, and transcriptomic changes were evaluated. VD3 significantly attenuated cisplatin-induced cytotoxicity in AGS, HGC-27, and MKN-45 cells. Co-treatment with VD3 reduced cisplatin-induced apoptosis, ROS accumulation, PARP cleavage, and γ H2AX activation. Transcriptomic analysis showed that VD3 markedly reduced the number of cisplatin-responsive differentially expressed genes and altered gene programs associated with extracellular matrix organization, steroid metabolism, DNA damage response, and ABC transporter-related pathways. These findings suggest that VD3 exposure can attenuate cisplatin-induced cellular responses in gastric cancer cells and modulate transcriptional programs associated with cisplatin treatment. Given that the study was performed under *in vitro* conditions, further investigations are required to determine the underlying mechanisms and the potential clinical relevance of these observations.

Key words: gastric cancer; cholecalciferol; cisplatin resistance; transcriptome sequencing; apoptosis

Gastric cancer is the fifth most common cancer worldwide and the third leading cause of cancer-related death [1, 2]. Because early-stage gastric cancer often lacks specific symptoms and reliable diagnostic biomarkers, many patients are diagnosed only after the disease has progressed to an advanced stage, contributing to its high mortality rate [3]. In 2018, gastric cancer accounted for

approximately 784,000 deaths globally [1]. Surgical resection, combined with chemotherapy and radiotherapy, remains the cornerstone of treatment. Cisplatin (DDP) is a first-line chemotherapeutic agent for gastric cancer, particularly in advanced disease; however, acquired resistance frequently develops and is associated with poor clinical outcomes [4].

DDP exerts its antitumor effects primarily by forming DNA cross-links that trigger DNA damage and apoptosis. However, prolonged exposure can activate DNA repair mechanisms and other adaptive responses in cancer cells, thereby reducing treatment efficacy. The mechanisms underlying DDP resistance in gastric cancer are complex and multifactorial. In addition to enhanced DNA repair, reduced intracellular drug accumulation, altered drug targets, and dysregulation of cell survival and death pathways have all been implicated in resistance development [5]. Several signaling pathways, including PI3K/AKT, NF- κ B, MAPK, and STAT3, also contribute to the regulation of DDP responsiveness [6-9]. In gastric cancer, resistance is further influenced by marked molecular and phenotypic heterogeneity. Gastric cancer cells may acquire resistance through increased drug efflux, glutathione-dependent detoxification, impaired apoptosis, epithelial-mesenchymal transition, cancer stemness, autophagy, metabolic reprogramming, and tumor microenvironment-derived survival signals [10]. Hypoxia/HIF-1 α signaling has also been closely linked to chemoresistance by promoting stemness, epithelial-mesenchymal transition, metabolic adaptation, anti-apoptotic signaling, and the expression of drug efflux transporters [11]. Together, these mechanisms contribute to heterogeneous responses to DDP and highlight the need to identify additional factors that modulate DDP sensitivity. A better understanding of these regulatory mechanisms may facilitate the development of more effective therapeutic strategies for overcoming DDP resistance in gastric cancer.

Vitamin D₃/cholecalciferol (VD₃) is a secosteroid obtained from dietary sources or synthesized endogenously in the skin. Cutaneous vitamin D₃ synthesis is not a direct conversion of cholesterol into vitamin D₃. Rather, 7-dehydrocholesterol, an intermediate of the postsqualene cholesterol biosynthetic pathway, serves as the immediate precursor. Upon exposure to ultraviolet B radiation, 7-dehydrocholesterol is photochemically converted into pre-vitamin D₃, which subsequently undergoes temperature-dependent thermal isomerization to form vitamin D₃ [12]. This photochemical process also generates other cholesterol-derived secosteroidal photoproducts,

72 including lumisterol and tachysterol, indicating that cutaneous vitamin D biology extends beyond
73 vitamin D₃ formation alone [12]. After synthesis in the skin or dietary intake, vitamin D₃ is
74 metabolized to 25-hydroxyvitamin D₃ and subsequently to 1,25-dihydroxyvitamin D₃, the
75 hormonally active vitamin D metabolite. Although the kidney is the principal endocrine source of
76 circulating 1,25-dihydroxyvitamin D₃, vitamin D activation is not confined to renal tissue.
77 CYP27B1 is expressed in a variety of extra-renal tissues, enabling local production of active
78 vitamin D metabolites and supporting intracrine, autocrine, and paracrine vitamin D signaling [13].
79 This local vitamin D metabolic and signaling axis is particularly relevant to cancer biology because
80 both normal and malignant tissues may express CYP27B1, CYP24A1, and the vitamin D receptor
81 (VDR), thereby influencing tissue-specific responses to vitamin D [14]. In addition to the classical
82 CYP2R1/CYP27B1/CYP24A1 pathway, vitamin D₃ can undergo CYP11A1-initiated hydroxylation
83 to generate non-classical vitamin D₃ hydroxyderivatives. Lumisterol can likewise be converted into
84 biologically active metabolites [15]. These CYP11A1-derived vitamin D and lumisterol metabolites
85 have been associated with the regulation of oxidative stress, DNA damage responses, inflammation,
86 and cellular proliferation [15]. In cancer models, both classical and CYP11A1-derived vitamin D₃
87 hydroxyderivatives have demonstrated antitumor activity, partly through inhibition of GLI1 and
88 β -catenin signaling pathways [16]. Furthermore, vitamin D signaling may interact with circadian
89 regulatory networks through nuclear receptors such as RORs, REV-ERBs, and AhR, highlighting
90 the complexity of vitamin D biology beyond its traditional endocrine functions [17].
91 Over the past several decades, the diverse physiological and pathological functions of vitamin D₃
92 and its metabolites have become increasingly recognized. Vitamin D signaling has been implicated
93 in a wide range of conditions, including metabolic syndrome, viral infection, cardiovascular disease,
94 and cancer [18-22]. Accumulating preclinical and clinical evidence suggests that vitamin D
95 supplementation may reduce the risk of several malignancies and influence cancer incidence,
96 progression, and mortality [23-26]. For example, epidemiological studies have reported that higher
97 dietary intake of vitamin D₃ and calcium is associated with a reduced risk of certain cancers [27-29].
98 In addition, long-term administration of selected vitamin D analogs following conventional
99 chemotherapy has been reported to decrease colorectal cancer recurrence [30]. Antitumor effects of
100 vitamin D₃ have also been observed in colorectal, breast, pancreatic, and head and neck cancers

101 [31-35]. Despite these findings, the role of vitamin D₃ in modulating responses to anticancer
102 chemotherapy, particularly DDP-based treatment, remains incompletely understood.
103 Therefore, the present study investigated whether vitamin D₃/cholecalciferol exposure modulates
104 DDP sensitivity in gastric cancer cells and explored the associated cellular and transcriptomic
105 alterations. By examining the effects of vitamin D₃ on DDP-induced cytotoxicity, apoptosis,
106 oxidative stress, DNA damage-associated signaling, and gene expression profiles, we sought to
107 provide new insights into factors that may influence DDP responsiveness in gastric cancer. The
108 findings may contribute to a better understanding of the biological interactions between vitamin D₃
109 and DDP and provide a basis for future studies evaluating their combined use in cancer therapy.

111 **Materials and methods**

112 **Reagents and treatment.** Vitamin D₃/cholecalciferol (VD₃; #S4063) and DDP (#S1166) were
113 purchased from Selleck Chemicals (Houston, TX, USA). VD₃ was dissolved in dimethyl sulfoxide
114 (DMSO) to prepare a stock solution and stored at -20 °C, protected from light. Cells treated with an
115 equivalent concentration of DMSO served as the vehicle control.

116 **Cell culture.** The human gastric cancer cell lines AGS (Homo sapiens, female, stomach; RRID:
117 CVCL_0139), HGC-27 (Homo sapiens, female, stomach; RRID: CVCL_1279), and MKN-45
118 (Homo sapiens, male, stomach; RRID: CVCL_0434) were obtained from Procell Life Science &
119 Technology Co., Ltd. (Wuhan, China). All cell lines were authenticated by short tandem repeat
120 (STR) profiling and confirmed to be free of mycoplasma contamination. AGS cells were cultured in
121 F-12K medium, whereas HGC-27 and MKN-45 cells were maintained in Roswell Park Memorial
122 Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher
123 Scientific, Waltham, MA, USA). All cells were incubated at 37 °C in a humidified atmosphere
124 containing 5% CO₂.

125 **Cytotoxicity assay.** Gastric cancer cells in the logarithmic growth phase were seeded into 96-well
126 plates at a density of 1×10^4 cells/well and allowed to attach for 24 h at 37 °C in a humidified 5%
127 CO₂ incubator. The culture medium was then replaced with fresh medium containing the indicated
128 concentrations of the test compounds. Each treatment was performed in triplicate wells. After 24 h
129 of treatment, the medium was removed and Cell Counting Kit-8 (CCK-8) reagent was added

130 according to the manufacturer's instructions. The plates were incubated for an additional 1 h, and
131 the optical density (OD) at 450 nm was measured using a Synergy H1/Synergy 2 microplate reader
132 (BioTek Instruments, Winooski, VT, USA). Cell viability was calculated relative to the
133 corresponding vehicle control and used to evaluate treatment-induced cytotoxicity.

134 **Quantitative real-time PCR (qRT-PCR).** Total RNA was extracted from HGC-27 cells using
135 TRIzol reagent according to the manufacturer's instructions. RNA concentration and purity were
136 determined spectrophotometrically. Complementary DNA (cDNA) was synthesized using a reverse
137 transcription kit. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR
138 Master Mix on a real-time PCR detection system. Relative mRNA expression levels were calculated
139 using the $2^{-\Delta\Delta C_t}$ method, with GAPDH serving as the internal control. To validate the RNA-seq
140 results, the expression levels of CYP24A1, HMGCR, GDF15, CDKN1A, and SPIDR were
141 analyzed by qRT-PCR. The primer sequences used in this study are listed in Supplementary Table
142 S1.

143 **Flow cytometry.** Cells were collected and washed twice with pre-cooled phosphate-buffered saline
144 (PBS) to remove residual drug. After centrifugation at $1,500 \times g$ for 5 min, the supernatant was
145 discarded and the cell pellet was resuspended in 195 μ l of Annexin V-FITC binding buffer from the
146 Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China).
147 Subsequently, 5 μ l of Annexin V-FITC and 10 μ l of propidium iodide (PI) staining solution were
148 added. The samples were gently mixed and incubated for 15 min at room temperature in the dark.
149 Apoptosis was then analyzed using a CyFlow flow cytometer (Sysmex Partec GmbH, Görlitz,
150 Germany).

151 **Western blotting.** Cells were harvested and washed two to three times with $1\times$ PBS, followed by
152 centrifugation at $1,000 \times g$ for 5 min. After removal of the supernatant, the cell pellets were lysed in
153 an appropriate volume of lysis buffer and incubated on ice for 10-15 min. The lysates were then
154 centrifuged at $13,000 \times g$ for 10 min at 4°C , and the supernatants containing total cellular proteins
155 were collected for analysis.

156 Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit
157 according to the manufacturer's instructions. Equal amounts of protein were separated by sodium
158 dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto

polyvinylidene fluoride (PVDF) membranes (FFP36; Beyotime Biotechnology, Shanghai, China). The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline containing Tween 20) for 1 h at room temperature and then incubated overnight at 4 °C with the appropriate primary antibodies, including anti-cleaved PARP antibody (Cell Signaling Technology, Danvers, MA, USA), anti-H2AX antibody (Cell Signaling Technology), and anti- α -tubulin antibody (Beyotime Biotechnology). After three washes with TBST (10 min each), the membranes were incubated with the corresponding secondary antibodies for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection reagent (Fude Biological Technology Co., Ltd., Hangzhou, China) and imaged with a ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Reactive oxygen species assay. Sterile coverslips were prepared by soaking in 75% ethanol for 24 h and were subsequently washed three times with sterile PBS before being placed into 6-well plates. Gastric cancer cells in the logarithmic growth phase were seeded onto the coverslips at a density of 5×10^5 cells/well and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was then replaced with fresh medium containing the indicated concentrations of the test compounds, and the cells were incubated for an additional 24 h. Intracellular reactive oxygen species (ROS) levels were assessed using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The DCFH-DA working solution was prepared by diluting the stock solution 1:1,000 in serum-free medium to a final concentration of 10 μ M. After treatment, the culture medium was removed and the DCFH-DA solution was added to each well. Cells were incubated for 20 min at 37 °C in a 5% CO₂ incubator and then washed three to five times with serum-free medium to remove excess probe. Intracellular ROS generation was visualized using a fluorescence microscope with excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence intensity was used as an indicator of intracellular ROS levels.

Transcriptome sequencing data analysis. Total RNA samples were submitted to Jingguang Gene Technology Co., Ltd. (China) for Illumina high-throughput sequencing. Gene expression levels were quantified using the transcripts per million (TPM) method. Differential expression analysis was performed in R using the limma package after data normalization. Genes with $p < 0.05$ and $|\log_{2}FC| > 1$ were considered differentially expressed genes (DEGs) and were selected for

subsequent analyses. Functional enrichment analyses of DEGs were conducted using the clusterProfiler package to identify significantly enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The results were visualized using the ggplot2, pheatmap, and GOpot packages. Gene Set Enrichment Analysis (GSEA) was performed using GSEA software version 4.1.0 to evaluate the enrichment of predefined gene sets.

Protein-protein interaction (PPI) networks were constructed using the STRING database (<https://string-db.org/>). The interaction data were subsequently imported into Cytoscape (<https://www.cytoscape.org/>) for network visualization and analysis, with upregulated and downregulated genes displayed in different colors. Subnetwork analysis was performed using the MCODE plugin with the following parameters: degree cutoff ≥ 2 , node score cutoff ≥ 0.2 , k-core ≥ 2 , and maximum depth=100. Subnetworks with MCODE scores > 6 were selected for further investigation.

Genes identified within the selected subnetworks were subjected to GO and KEGG annotation analyses using the ClueGO plugin to characterize their associated biological functions and signaling pathways. Finally, hub genes within the PPI network were identified using the cytoHubba plugin based on topological network algorithms, enabling the prediction of key regulatory genes potentially involved in the observed transcriptomic changes.

Data analysis. Statistical analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data are presented as the mean $\pm$ standard deviation (SD) from at least three independent experiments unless otherwise specified. Differences among multiple groups were evaluated using one-way analysis of variance (ANOVA). A two-sided p-value < 0.05 was considered statistically significant.

Results

VD₃ decreased the sensitivity of gastric cancer cells to DDP and inhibited DDP-induced apoptosis. Before evaluating the effect of VD₃ on DDP response, we first examined the effects of different VD₃ concentrations on gastric cancer cell viability. Based on these preliminary results, VD₃ concentrations that did not show obvious cytotoxicity, namely 40 and 60 μ M, were selected for combination treatment with DDP (Supplementary Figure S1). We then used the CCK-8 assay to

217 assess the cytotoxic effects of DDP in AGS, HGC-27, and MKN-45 cells. As shown in Figure 1A,
218 increasing DDP concentrations progressively reduced cell viability in the absence of VD₃. However,
219 VD₃ co-treatment significantly increased cell viability compared with DDP treatment alone.
220 Moreover, the protective effect became more evident with increasing VD₃ concentrations. These
221 results indicate that DDP sensitivity differs among gastric cancer cell lines and that VD₃ reduces
222 DDP sensitivity in these cells.

223 We next evaluated apoptosis after 24 h of treatment by flow cytometry. DDP significantly induced
224 apoptosis in gastric cancer cells, whereas VD₃ alone had no obvious effect on the apoptotic rate.
225 Compared with DDP treatment alone, co-treatment with VD₃ significantly reduced apoptosis
226 (Figure 1B). Because apoptotic cells typically show chromatin condensation and nuclear
227 fragmentation, Hoechst staining was also performed to assess nuclear morphological changes. As
228 shown in Supplementary Figure S2, approximately one-third of AGS cells treated with DDP alone
229 exhibited condensed or fragmented nuclei, whereas these apoptotic features were markedly reduced
230 after VD₃ co-treatment. These findings further support that VD₃ attenuates DDP-induced apoptosis
231 in gastric cancer cells.

232 **VD₃ inhibits DDP-induced apoptosis by reducing PARP1 cleavage and γ H2AX accumulation.**

233 To further investigate the role of VD₃ in modulating DDP-induced apoptosis, we examined PARP1
234 cleavage, a well-established marker of apoptotic cell death, by western blotting. As shown in Figure
235 2, cleaved PARP1 was undetectable in both the control and VD₃-treated groups. In contrast, DDP
236 treatment markedly increased PARP1 cleavage in all three gastric cancer cell lines. Co-treatment
237 with VD₃ substantially reduced the abundance of cleaved PARP1, particularly in HGC-27 and AGS
238 cells. These findings are consistent with the flow cytometric and Hoechst staining results, further
239 supporting the inhibitory effect of VD₃ on DDP-induced apoptosis.

240 Because DDP exerts its cytotoxic effects primarily through DNA damage, we next assessed γ H2AX,
241 a widely used marker of DNA damage response activation. DDP treatment markedly increased
242 γ H2AX levels in gastric cancer cells, whereas co-treatment with VD₃ attenuated DDP-induced
243 γ H2AX accumulation (Figure 2). These results indicate that VD₃ reduces the activation of DNA
244 damage-associated signaling induced by DDP. However, it should be noted that reduced γ H2AX
245 accumulation alone does not directly demonstrate enhanced DNA repair capacity.

VD₃ reduces DDP-induced oxidative stress. Previous studies have shown that DDP can promote intracellular ROS accumulation, leading to mitochondrial dysfunction, DNA damage, and apoptosis. Given the reported antioxidant properties of VD₃, we investigated whether VD₃ modulates the oxidative stress response induced by DDP. As shown in Figure 3, DDP treatment for 24 h significantly increased intracellular ROS levels in gastric cancer cells. In contrast, co-treatment with VD₃ markedly reduced DDP-induced ROS accumulation. These findings suggest that VD₃ attenuates oxidative stress induced by DDP, which may contribute to the reduced apoptosis and γ H2AX activation observed in gastric cancer cells.

The combination of VD₃ reduced the number of DDP-induced differentially expressed genes. To explore the mechanisms by which VD₃ modulates DDP sensitivity, transcriptomic analyses were performed in HGC-27 cells treated with vehicle control, DDP, VD₃, or DDP plus VD₃. Compared with the control group, DDP treatment resulted in 499 significantly upregulated genes and 514 significantly downregulated genes (Figure 4A). In contrast, VD₃ treatment alone induced relatively limited transcriptional changes, with 14 genes significantly upregulated and 36 genes significantly downregulated (Figure 4B). Cells treated with DDP plus VD₃ exhibited 274 upregulated and 198 downregulated genes relative to the control group (Figure 4C). Furthermore, comparison of the DDP plus VD₃ group with the DDP-alone group identified 35 significantly upregulated genes and 24 significantly downregulated genes (Figure 4D).

To further evaluate the impact of VD₃ on DDP-induced transcriptional responses, overlap analysis was performed between the differentially expressed genes identified in the DDP group and those identified in the DDP plus VD₃ group. Among the 1,013 genes differentially expressed following DDP treatment, 629 were no longer significantly altered after co-treatment with VD₃, whereas 88 additional genes became differentially expressed in the presence of VD₃ (Figure 4E). These findings suggest that VD₃ substantially modifies the transcriptional response to DDP and reduces the overall number of DDP-responsive differentially expressed genes.

VD₃ modulates DDP-responsive transcriptional programs associated with lipid metabolism. To gain further insight into the biological significance of the transcriptomic changes, we examined the 30 most differentially expressed genes across the treatment groups. As shown in Supplementary Figure S3A, DDP treatment markedly altered the expression of genes involved in cell growth,

cell-cycle regulation, and DNA damage responses, including growth differentiation factor 15 (GDF15), cyclin-dependent kinase inhibitor 1A (CDKN1A), and scaffold protein involved in DNA repair (SPIDR). In contrast, VD₃ treatment primarily affected genes associated with lipid metabolism and vitamin D catabolism, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and cytochrome P450 family 24 subfamily A member 1 (CYP24A1) (Supplementary Figure S3B). Following co-treatment with DDP and VD₃, GDF15 and CDKN1A remained significantly altered relative to the control group, whereas SPIDR was no longer differentially expressed. In addition, genes such as ASNS and CTSL showed notable expression changes in the co-treatment group (Supplementary Figure S3C), suggesting that these genes may be involved in the cellular response to combined DDP and VD₃ treatment. Comparison of the DDP plus VD₃ group with the DDP-alone group further revealed differential expression of genes related to lipid metabolism and vitamin D catabolism, including OLR1 and CYP24A1 (Supplementary Figure S3D).

To validate the RNA-seq findings, selected genes were analyzed by qRT-PCR in HGC-27 cells treated with vehicle control, VD₃, DDP, or DDP plus VD₃. The validated genes included *CYP24A1*, *HMGCR*, *GDF15*, *CDKN1A*, and *SPIDR*, which are associated with vitamin D metabolism, steroid metabolism, DDP-induced stress responses, and DNA damage-associated signaling. The qRT-PCR results showed expression patterns that were generally consistent with those obtained from RNA-seq analysis, supporting the reliability of the transcriptomic data (Supplementary Figure S4).

GO-BP and KEGG enrichment analyses of differentially expressed genes. To further investigate the biological processes associated with the modulation of DDP responses by VD₃, GO biological process (GO-BP) enrichment analyses were performed on the identified differentially expressed genes. As shown in Figure 5A, DDP treatment significantly suppressed genes involved in extracellular matrix (ECM) organization while enriching genes associated with the negative regulation of protein phosphorylation. Comparison of the VD₃-treated and control groups revealed significant enrichment of processes related to steroid metabolism, lipid catabolism, and acetyl-CoA metabolism following VD₃ treatment (Figure 5B). In cells treated with DDP plus VD₃, the downregulation of ECM-associated genes induced by DDP was partially attenuated, resulting in a modest increase in the expression of ECM-related genes compared with DDP treatment alone

304 (Figure 5C). Furthermore, comparison of the DDP plus VD₃ group with the DDP-alone group
305 showed enrichment of vitamin metabolic processes and suppression of steroid biosynthetic
306 processes in the co-treatment group (Figure 5D).

307 To further characterize the affected signaling pathways, KEGG enrichment analyses were
308 conducted for the differentially expressed genes identified in each comparison. As shown in Figure
309 6A, DDP treatment significantly affected several pathways, including the NF- κ B signaling pathway,
310 ECM–receptor interaction, and TNF signaling pathway. In contrast, VD₃ treatment primarily
311 influenced the steroid biosynthesis pathway (Figure 6B). In the DDP plus VD₃ group, steroid
312 biosynthesis remained one of the most prominently altered pathways, whereas ECM–receptor
313 interaction was no longer significantly enriched (Figure 6C). When the DDP plus VD₃ group was
314 directly compared with the DDP-alone group, relatively few pathway-level differences were
315 observed, with steroid biosynthesis representing the principal altered pathway (Figure 6D).

316 **GSEA analysis of signaling pathway alterations associated with VD₃ treatment.** Because
317 signaling pathway activity can be influenced by coordinated changes in multiple genes, analyses
318 based solely on differentially expressed genes may not fully capture pathway-level alterations.
319 Therefore, GSEA was performed using the complete transcriptomic dataset to provide a more
320 comprehensive assessment of pathway enrichment. As relatively few differentially expressed genes
321 were identified between the DDP plus VD₃ group and the DDP-alone group, KEGG enrichment
322 analysis detected only a limited number of significantly enriched pathways. In contrast, GSEA
323 revealed broader transcriptomic changes associated with VD₃ treatment. Compared with DDP
324 treatment alone, co-treatment with VD₃ was associated with enrichment of gene sets related to ABC
325 transporters and ECM–receptor interaction, whereas gene sets involved in steroid biosynthesis were
326 negatively enriched (Figures 7A-7C). These findings suggest that VD₃ modulates transcriptional
327 programs associated with transporter activity, extracellular matrix signaling, and steroid metabolism
328 in DDP-treated gastric cancer cells.

329 **Protein interaction network analysis identifies candidate pathways and hub genes associated**
330 **with VD₃ treatment.** To further characterize the molecular networks associated with VD₃ treatment,
331 a PPI network was constructed using the differentially expressed genes identified between the DDP
332 plus VD₃ group and the DDP-alone group. Analysis using the STRING database generated a

333 network comprising 17 nodes and 13 edges, including 10 upregulated and 7 downregulated genes
334 (Figure 8A). The genes within this network were subsequently analyzed using the ClueGO plugin
335 for GO-BP and KEGG enrichment analyses. GO-BP analysis indicated enrichment of biological
336 processes related to the negative regulation of lipid storage and the regulation of steroid
337 biosynthetic processes (Figure 8B). Consistent with these findings, KEGG analysis identified
338 steroid biosynthesis as the principal enriched pathway associated with VD₃ treatment (Figure 8C).
339 To identify key regulatory genes within the network, hub gene analysis was performed using the
340 CytoHubba plugin. TNF and HSPG2 were identified as the highest-ranking hub genes and may
341 represent important components of the transcriptional network associated with VD₃ treatment in
342 DDP-exposed gastric cancer cells (Figure 8D).

343 **Protein-protein interaction network analysis of genes no longer differentially expressed after**
344 **VD₃ co-treatment.** To further investigate the transcriptional changes attenuated by VD₃, we
345 performed PPI network analysis on genes that were differentially expressed following DDP
346 treatment but were no longer significantly altered after co-treatment with VD₃. The resulting PPI
347 network comprised 427 nodes and 875 edges (Supplementary Figure S5A). These genes were
348 subsequently analyzed using the MCODE plugin to identify highly interconnected subnetworks,
349 yielding two core subnetworks with MCODE scores greater than 6 (Supplementary Figures S5B,
350 S5C).

351 Functional annotation of genes within these subnetworks was performed using the ClueGO plugin.
352 GO-BP analysis revealed significant enrichment of biological processes related to
353 collagen-activated tyrosine kinase receptor signaling, whereas KEGG analysis identified ECM–
354 receptor interaction as a major enriched pathway (Supplementary Figures S5D, S5E). These results
355 suggest that genes whose DDP-induced expression changes were attenuated by VD₃ are primarily
356 associated with tyrosine kinase receptor signaling and ECM-related pathways. These findings
357 indicate that VD₃ may modulate transcriptional programs involved in these pathways in
358 DDP-treated gastric cancer cells.

359

360 Discussion

DDP exerts its antitumor activity through multiple mechanisms, most notably by forming DNA adducts through interactions with purine bases, thereby inducing DNA damage, activating intracellular signaling pathways, and ultimately triggering apoptosis. However, treatment-related toxicity and acquired drug resistance remain major obstacles that limit its clinical efficacy. Therefore, elucidating the molecular mechanisms underlying DDP resistance is essential for improving therapeutic outcomes. Reduced intracellular drug accumulation, detoxification by glutathione and metallothioneins, and enhanced DNA damage repair have all been implicated in the development of DDP resistance [36]. In the present study, VD₃ attenuated DDP-induced cytotoxicity in gastric cancer cell lines under *in vitro* conditions, suggesting that VD₃ exposure may reduce DDP sensitivity in certain cellular contexts. Consistent with this observation, apoptosis assays demonstrated that the reduced cytotoxic response was associated, at least in part, with decreased DDP-induced apoptosis. DDP is known to induce DNA double-strand breaks (DSBs), which initiate a cascade of signaling events that ultimately lead to apoptotic cell death [37, 38]. Previous studies have reported a strong positive correlation between DSB formation and γ H2AX accumulation, supporting the use of γ H2AX as a marker of DNA damage response activation [39]. In agreement with these findings, our western blot analyses showed that DDP treatment markedly increased γ H2AX levels in gastric cancer cells, indicating activation of DNA damage-associated signaling pathways. Because DDP-induced DNA lesions activate the cellular DNA damage response, γ H2AX is widely used as an indicator of this process, particularly following DSB formation. Nevertheless, γ H2AX should be interpreted cautiously because it reflects activation of DNA damage-associated signaling rather than DNA repair capacity itself [40]. In our study, co-treatment with VD₃ reduced DDP-induced γ H2AX accumulation, suggesting attenuation of DNA damage-associated signaling. However, this finding should not be interpreted as evidence of enhanced DNA repair. γ H2AX levels can be influenced by multiple factors, including the extent of DNA damage, ROS-associated stress, apoptotic cell fraction, cell-cycle distribution, and upstream DNA damage response signaling. Therefore, although our results indicate that VD₃ attenuates DDP-induced γ H2AX activation, additional functional studies are required to determine whether VD₃ directly affects DNA repair processes or influences other mechanisms involved in the cellular response to DDP.

ROS are continuously generated as byproducts of mitochondrial oxidative metabolism under physiological conditions. Maintenance of ROS homeostasis is essential for normal cellular survival and signal transduction [41]. However, excessive ROS accumulation can disrupt mitochondrial function, activate mitochondrial apoptotic pathways, and induce oxidative damage to cellular macromolecules, including DNA [42-45]. Previous studies have shown that DDP promotes intracellular ROS generation, which may further exacerbate DNA damage and contribute to apoptosis through both oxidative stress-dependent and mitochondrial pathways [46]. In the present study, we evaluated the effects of DDP and VD₃ on oxidative stress in gastric cancer cells. Consistent with previous reports, DDP treatment markedly increased intracellular ROS levels. In contrast, co-treatment with VD₃ significantly attenuated DDP-induced ROS accumulation. These findings suggest that VD₃ mitigates the oxidative stress response triggered by DDP. Given the established links between ROS accumulation, DNA damage signaling, and apoptosis, the reduction in ROS levels may partially contribute to the decreased γ H2AX accumulation, reduced apoptosis, and attenuated cytotoxicity observed following VD₃ co-treatment. However, the precise mechanisms underlying these effects remain to be elucidated.

Our experimental findings demonstrated that VD₃ attenuated the cytotoxic effects of DDP in gastric cancer cells under *in vitro* conditions. To gain further insight into the molecular basis of this phenomenon, we performed transcriptomic analyses of cells treated with DDP, VD₃, DDP plus VD₃, or vehicle control. DDP treatment induced extensive transcriptional alterations, affecting genes involved in several pathways, including NF- κ B signaling, ECM–receptor interaction, and TNF signaling. In contrast, VD₃ treatment alone primarily affected genes associated with steroid metabolism. Given that VD₃ is a steroid-derived molecule, modulation of steroid metabolic pathways is consistent with its known biological activity. Notably, co-treatment with VD₃ markedly reduced the number of DDP-responsive differentially expressed genes, suggesting that VD₃ modifies the transcriptional response to DDP. Functional enrichment analyses indicated that many of the genes whose DDP-induced expression changes were attenuated by VD₃ were associated with DNA damage response-related processes and ECM-receptor interaction pathways. These observations suggest that VD₃ may influence transcriptional programs affected by DDP, particularly those related to DNA damage-associated signaling and extracellular matrix regulation; however,

419 direct functional validation is required to establish causal relationships. To further support the
420 transcriptomic findings, representative genes identified by RNA-seq, including *CYP24A1*, *HMGCR*,
421 *GDF15*, *CDKN1A*, and *SPIDR*, were evaluated by qRT-PCR. The expression patterns obtained by
422 qRT-PCR were generally consistent with the RNA-seq results, supporting the reliability of the
423 transcriptomic analysis.

424 Direct comparison between the DDP plus VD_3 group and the DDP-alone group further revealed
425 transcriptomic changes associated with steroid metabolic processes, ECM–receptor interaction, and
426 ABC transporter-related pathways. GSEA additionally identified enrichment of gene sets related to
427 ABC transporters and ECM–receptor interaction, together with negative enrichment of steroid
428 biosynthesis-related genes following VD_3 co-treatment. Because ABC transporters are
429 well-recognized mediators of drug efflux and chemoresistance, these findings raise the possibility
430 that altered transporter-related transcriptional programs may contribute to the reduced DDP
431 sensitivity observed in VD_3 -treated cells. However, the current data are based primarily on
432 transcriptomic analyses and do not establish functional changes in transporter activity. Finally, PPI
433 network analysis identified *TNF* and *HSPG2* as candidate hub genes within the VD_3 -associated
434 transcriptional network. Future studies are required to determine whether these genes, or other
435 candidate regulators identified in this study, functionally contribute to the modulation of DDP
436 responses by VD_3 .

437 Several limitations of this study should be acknowledged. First, the experiments were conducted
438 primarily in established gastric cancer cell lines and evaluated short-term drug responses under *in*
439 *vitro* conditions. Consequently, the findings require validation in animal models, patient-derived
440 systems, and clinical samples. Moreover, *in vitro* models cannot fully recapitulate the
441 pharmacokinetics of DDP, systemic vitamin D metabolism, the tumor microenvironment, immune
442 regulation, or patient-specific vitamin D status. Second, the compound examined in this study was
443 VD_3 /cholecalciferol, a precursor form of vitamin D, rather than the hormonally active metabolite
444 1,25-dihydroxyvitamin D_3 . Intracellular conversion of cholecalciferol to 25-hydroxyvitamin D_3 ,
445 1,25-dihydroxyvitamin D_3 , or non-classical vitamin D-derived metabolites was not directly
446 evaluated. Previous studies have shown that CYP11A1-derived vitamin D and lumisterol
447 metabolites participate in the regulation of oxidative stress, DNA damage responses, inflammation,

and cell proliferation, and that both classical and CYP11A1-derived vitamin D hydroxyderivatives exhibit anticancer activity in basal and squamous cell carcinoma models [15, 16]. Therefore, whether these metabolites contribute to the observed changes in ROS accumulation, γ H2AX activation, apoptosis, and DDP sensitivity remain to be determined. Third, DNA repair activity was not directly assessed. Although γ H2AX was used as a marker of DDP-induced DNA damage response activation, reduced γ H2AX accumulation cannot distinguish between decreased DNA damage generation, altered repair kinetics, reduced apoptosis, changes in cell-cycle distribution, or modulation of upstream DNA damage response signaling pathways. Accordingly, the present data do not establish a direct effect of VD₃ on DNA repair capacity. Finally, although selected RNA-seq findings were validated by qRT-PCR, the transcriptomic analyses remain largely correlative. Additional mechanistic studies are required to identify the direct molecular mediators responsible for the VD₃-associated modulation of DDP responses. Therefore, the results of this study should be interpreted with caution and should not be directly extrapolated to clinical recommendations regarding vitamin D supplementation during DDP-based chemotherapy.

In summary, the present study demonstrates that VD₃ attenuates DDP-induced cytotoxicity, apoptosis, ROS accumulation, and γ H2AX activation in gastric cancer cells under *in vitro* conditions. Transcriptomic analyses further suggest that VD₃ modulates gene expression programs associated with steroid metabolism, ECM-related pathways, ABC transporter-associated pathways, and DNA damage response-related processes. Collectively, these findings provide preliminary evidence that VD₃ exposure can influence cellular responses to DDP in gastric cancer cells. However, the specific vitamin D-derived metabolites involved, the underlying molecular mechanisms, and the clinical relevance of these observations remain to be elucidated through further mechanistic, preclinical, and clinical studies.

Acknowledgements: This study was supported by the Zhejiang Province Basic Public Welfare Research Project (LGF22H030010 to KC).

References

477 [1] BRAY F, FERLAY J, SOERJOMATARAM I, SIEGEL RL, TORRE LA et al. Global cancer
478 statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers
479 in 185 countries. *CA Cancer J Clin* 2018; 68: 394-424. <https://doi.org/10.3322/caac.21492>

480 [2] CHEN W, ZHENG R, BAADE PD, ZHANG S, ZENG H et al. Cancer statistics in China,
481 2015. *CA Cancer J Clin* 2016; 66: 115-132. <https://doi.org/10.3322/caac.21338>

482 [3] LIU H, WU W, LIU Y, ZHANG C, ZHOU Z. Predictive value of cystic fibrosis
483 transmembrane conductance regulator (CFTR) in the diagnosis of gastric cancer. *Clin Invest*
484 *Med*. 2014; 37: E226-232. <https://doi.org/10.25011/cim.v37i4.21728>

485 [4] ZHAO TT, XU H, XU HM, WANG ZN, XU YY et al. The efficacy and safety of targeted
486 therapy with or without chemotherapy in advanced gastric cancer treatment: a network
487 meta-analysis of well-designed randomized controlled trials. *Gastric Cancer* 2018; 21:
488 361-371. <https://doi.org/10.1007/s10120-018-0813-2>

489 [5] MARIN JJ, AL-ABDULLA R, LOZANO E, BRIZ O, BUJANDA L et al. Mechanisms of
490 Resistance to Chemotherapy in Gastric Cancer. *Anticancer Agents Med Chem* 2016; 16:
491 318-334. <https://doi.org/10.2174/1871520615666150803125121>

492 [6] JULIACHS M, MUNOZ C, MOUTINHO CA, VIDAL A, CONDOM E et al. The
493 PDGFRbeta-AKT pathway contributes to CDDP-acquired resistance in testicular germ cell
494 tumors. *Clin Cancer Res* 2014; 20: 658-667.
495 <https://doi.org/10.1158/1078-0432.CCR-13-1131>

496 [7] BENEDETTI G, FREDRIKSSON L, HERPERS B, MEERMAN J, VAN DE WATER B et
497 al. TNF-alpha-mediated NF-kappaB survival signaling impairment by cisplatin enhances
498 JNK activation allowing synergistic apoptosis of renal proximal tubular cells. *Biochem*
499 *Pharmacol* 2013; 85: 274-286. <https://doi.org/10.1016/j.bcp.2012.10.012>

500 [8] LIN CC, YEH HH, HUANG WL, YAN JJ, LAI WW et al. Metformin enhances cisplatin
501 cytotoxicity by suppressing signal transducer and activator of transcription-3 activity
502 independently of the liver kinase B1-AMP-activated protein kinase pathway. *Am J Respir*
503 *Cell Mol Biol* 2013; 49: 241-250. <https://doi.org/10.1165/rcmb.2012-0244OC>

504 [9] GUEGAN JP, EZAN F, THERET N, LANGOUET S, BAFFET G. MAPK signaling in
505 cisplatin-induced death: predominant role of ERK1 over ERK2 in human hepatocellular
506 carcinoma cells. *Carcinogenesis* 2013; 34: 38-47. <https://doi.org/10.1093/carcin/bgs317>

507 [10] LI Z, SHU X, LIU X, LI Q, HU Y et al. Cellular and Molecular Mechanisms of
508 Chemoresistance for Gastric Cancer. *Int J Gen Med* 2024; 17: 3779-3788.
509 <https://doi.org/10.2147/IJGM.S473749>

510 [11] OZCAN G. The hypoxia-inducible factor-1alpha in stemness and resistance to
511 chemotherapy in gastric cancer: Future directions for therapeutic targeting. *Front Cell Dev*
512 *Biol* 2023; 11: 1082057. <https://doi.org/10.3389/fcell.2023.1082057>

513 [12] SLOMINSKI AT, TUCKEY RC, SLOMINSKI RM, HOLICK MF, JETTEN AM. On the
514 role of cholesterol, vitamin D, lumisterol, and tachysterol signaling in psoriasis. *J Invest*
515 *Dermatol* 2026; 146: 887-889. <https://doi.org/10.1016/j.jid.2026.01.005>

516 [13] ADAMS JS, HEWISON M. Extrarenal expression of the 25-hydroxyvitamin
517 D-1-hydroxylase. *Arch Biochem Biophys* 2012; 523: 95-102.
518 <https://doi.org/10.1016/j.abb.2012.02.016>

- 519 [14] TOWNSEND K, BANWELL CM, GUY M, COLSTON KW, MANSI JL et al. Autocrine
520 metabolism of vitamin D in normal and malignant breast tissue. *Clin Cancer Res* 2005; 11:
521 3579-3586. <https://doi.org/10.1158/1078-0432.CCR-04-2359>
- 522 [15] SLOMINSKI AT, KIM TK, JANJETOVIC Z, SLOMINSKI RM, LI W et al. Biological
523 Effects of CYP11A1-Derived Vitamin D and Lumisterol Metabolites in the Skin. *J Invest*
524 *Dermatol* 2024; 144: 2145-2161. <https://doi.org/10.1016/j.jid.2024.04.022>
- 525 [16] SLOMINSKI AT, BROZYNA AA, KIM TK, ELSAYED MM, JANJETOVIC Z et al.
526 CYP11A1- derived vitamin D hydroxyderivatives as candidates for therapy of basal and
527 squamous cell carcinomas. *Int J Oncol* 2022; 61: 96. <https://doi.org/10.3892/ijo.2022.5386>
- 528 [17] SLOMINSKI AT, SLOMINSKI RM, KAMAL M, HOLICK MF, TUCKEY RC et al. Is
529 Vitamin D Signaling Regulated by and Does It Regulate Circadian Rhythms? *FASEB J*
530 2025; 39: e71321. <https://doi.org/10.1096/fj.202503003R>
- 531 [18] DAKSHINAMURTI K. Vitamins and their derivatives in the prevention and treatment of
532 metabolic syndrome diseases (diabetes). *Can J Physiol Pharmacol* 2015; 93: 355-362.
533 <https://doi.org/10.1139/cjpp-2014-0479>
- 534 [19] BRUSTAD N, STOKHOLM J, BONNELYKKE K, CHAWES BL. Vitamin D-FUT2
535 interaction and risk of lower respiratory tract infections in childhood. *Allergy* 2025; 80:
536 582-584. <https://doi.org/10.1111/all.16266>
- 537 [20] GIAMPAZOLIAS E, PEREIRA DA COSTA M, LAM KC, LIM KHJ, CARDOSO A et al.
538 Vitamin D regulates microbiome-dependent cancer immunity. *Science* 2024; 384: 428-437.
539 <https://doi.org/10.1126/science.adh7954>
- 540 [21] DE LA GUIA-GALIPIENSO F, MARTINEZ-FERRAN M, VALLECILLO N, LAVIE CJ,
541 SANCHIS-GOMAR F et al. Vitamin D and cardiovascular health. *Clin Nutr* 2021; 40:
542 2946-2957. <https://doi.org/10.1016/j.clnu.2020.12.025>
- 543 [22] MANSON JE, COOK NR, LEE IM, CHRISTEN W, BASSUK SS et al. Vitamin D
544 Supplements and Prevention of Cancer and Cardiovascular Disease. *N Engl J Med* 2019;
545 380: 33-44. <https://doi.org/10.1056/NEJMoa1809944>
- 546 [23] KUZNIA S, ZHU A, AKUTSU T, BURING JE, CAMARGO CA JR et al. Efficacy of
547 vitamin D(3) supplementation on cancer mortality: Systematic review and individual patient
548 data meta-analysis of randomised controlled trials. *Ageing Res Rev* 2023; 87: 101923.
549 <https://doi.org/10.1016/j.arr.2023.101923>
- 550 [24] BERNHARDT SM, BORGES VF, SCHEDIN P. Vitamin D as a Potential Preventive Agent
551 For Young Women's Breast Cancer. *Cancer Prev Res (Phila)* 2021; 14: 825-838.
552 <https://doi.org/10.1158/1940-6207.CAPR-21-0114>
- 553 [25] SKRAJNOWSKA D, BOBROWSKA-KORCZAK B. Potential Molecular Mechanisms of
554 the Anti-cancer Activity of Vitamin D. *Anticancer Res* 2019; 39: 3353-3363.
555 <https://doi.org/10.21873/anticancer.13478>
- 556 [26] SUNIL KUMAR BV, SINGH S, VERMA R. Anticancer potential of dietary vitamin D and
557 ascorbic acid: A review. *Crit Rev Food Sci Nutr* 2017; 57: 2623-2635.
558 <https://doi.org/10.1080/10408398.2015.1064086>
- 559 [27] TOUVIER M, CHAN DS, LAU R, AUNE D, VIEIRA R et al. Meta-analyses of vitamin D
560 intake, 25-hydroxyvitamin D status, vitamin D receptor polymorphisms, and colorectal
561 cancer risk. *Cancer Epidemiol Biomarkers Prev* 2011; 20: 1003-1016.
562 <https://doi.org/10.1158/1055-9965.EPI-10-1141>

- [28] SLATTERY ML, WOLFF RK, HERRICK JS, CAAN BJ, SAMOWITZ W. Calcium, vitamin D, VDR genotypes, and epigenetic and genetic changes in rectal tumors. *Nutr Cancer* 2010; 62: 436-442. <https://doi.org/10.1080/01635580903441204>
- [29] CHO E, SMITH-WARNER SA, SPIEGELMAN D, BEESON WL, VAN DEN BRANDT PA et al. Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst* 2004; 96: 1015-1022. <https://doi.org/10.1093/jnci/djh185>
- [30] KOTLARZ A, PRZYBYSZEWSKA M, SWOBODA P, MILOSZEWSKA J, GRYGOROWICZ MA et al. Differential interference of vitamin D analogs PRI-1906, PRI-2191, and PRI-2205 with the renewal of human colon cancer cells refractory to treatment with 5-fluorouracil. *Tumour Biol* 2016; 37: 4699-4709. <https://doi.org/10.1007/s13277-015-4311-4>
- [31] SANTOS JM, HUSSAIN F. VD(3) mitigates breast cancer aggressiveness by targeting V-H(+)-ATPase. *J Nutr Biochem* 2019; 70: 185-193. <https://doi.org/10.1016/j.jnutbio.2019.05.005>
- [32] LIU Y, WANG X, SUN X, LU S, LIU S. Vitamin intake and pancreatic cancer risk reduction: A meta-analysis of observational studies. *Medicine (Baltimore)* 2018; 97: e0114. <https://doi.org/10.1097/MD.00000000000010114>
- [33] GILZAD-KOHAN H, SANI S, BOROUJERDI M. Calcitriol Reverses Induced Expression of Efflux Proteins and Potentiates Cytotoxic Activity of Gemcitabine in Capan-2 Pancreatic Cancer Cells. *J Pharm Pharm Sci* 2017; 20: 295-304. <https://doi.org/10.18433/J37W7R>
- [34] BOSTICK RM. Effects of supplemental vitamin D and calcium on normal colon tissue and circulating biomarkers of risk for colorectal neoplasms. *J Steroid Biochem Mol Biol* 2015; 148: 86-95. <https://doi.org/10.1016/j.jsbmb.2015.01.010>
- [35] BOCHEN F, BALENSIEFER B, KORNER S, BITTENBRING JT, NEUMANN F et al. Vitamin D deficiency in head and neck cancer patients - prevalence, prognostic value and impact on immune function. *Oncoimmunology* 2018; 7: e1476817. <https://doi.org/10.1080/2162402X.2018.1476817>
- [36] GHOSH S. Cisplatin: The first metal based anticancer drug. *Bioorg Chem* 2019; 88: 102925. <https://doi.org/10.1016/j.bioorg.2019.102925>
- [37] SANCHO-MARTINEZ SM, PRIETO-GARCIA L, PRIETO M, LOPEZ-NOVOA JM, LOPEZ-HERNANDEZ FJ. Subcellular targets of cisplatin cytotoxicity: an integrated view. *Pharmacol Ther* 2012; 136: 35-55. <https://doi.org/10.1016/j.pharmthera.2012.07.003>
- [38] BASU A, KRISHNAMURTHY S. Cellular responses to Cisplatin-induced DNA damage. *J Nucleic Acids* 2010; 2010: 201367. <https://doi.org/10.4061/2010/201367>
- [39] ROTHKAMM K, LOBRICH M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A* 2003; 100: 5057-5062. <https://doi.org/10.1073/pnas.0830918100>
- [40] VALENTE D, GENTILESCHI MP, GUERRISI A, BRUZZANITI V, MORRONE A et al. Factors to Consider for the Correct Use of gammaH2AX in the Evaluation of DNA Double-Strand Breaks Damage Caused by Ionizing Radiation. *Cancers (Basel)* 2022; 14: 6204. <https://doi.org/10.3390/cancers14246204>
- [41] EBRAHIMI SO, REIISI S, SHAREEF S. miRNAs, oxidative stress, and cancer: A comprehensive and updated review. *J Cell Physiol* 2020; 235: 8812-8825. <https://doi.org/10.1002/jcp.29724>

607 [42] MA L, WANG H, WANG C, SU J, XIE Q et al. Failure of Elevating Calcium Induces
608 Oxidative Stress Tolerance and Imparts Cisplatin Resistance in Ovarian Cancer Cells. *Aging*
609 *Dis* 2016; 7: 254-266. <https://doi.org/10.14336/AD.2016.0118>

610 [43] LIN L, ZHENG J, ZHU W, JIA N. Nephroprotective effect of gelsemine against
611 cisplatin-induced toxicity is mediated via attenuation of oxidative stress. *Cell Biochem*
612 *Biophys* 2015; 71: 535-541. <https://doi.org/10.1007/s12013-014-0231-y>

613 [44] IKNER A, SHIOZAKI K. Yeast signaling pathways in the oxidative stress response. *Mutat*
614 *Res* 2005; 569: 13-27. <https://doi.org/10.1016/j.mrfmmm.2004.09.006>

615 [45] BARZILAI A, ROTMAN G, SHILOH Y. ATM deficiency and oxidative stress: a new
616 dimension of defective response to DNA damage. *DNA Repair (Amst)* 2002; 1: 3-25.
617 [https://doi.org/10.1016/s1568-7864\(01\)00007-6](https://doi.org/10.1016/s1568-7864(01)00007-6)

618 [46] GALLUZZI L, SENOVILLA L, VITALE I, MICHELS J, MARTINS I et al. Molecular
619 mechanisms of cisplatin resistance. *Oncogene* 2012; 31: 1869-1883.
620 <https://doi.org/10.1038/onc.2011.384>

621

622

Figure Legends

Figure 1. VD₃ attenuates DDP-induced cytotoxicity and apoptosis in gastric cancer cells. A) HGC-27, AGS, and MKN-45 cells were treated with the indicated concentrations of DDP in the presence or absence of VD₃ for 24 h, and cell viability was assessed using the CCK-8 assay. Cell viability was normalized to the corresponding vehicle control group. B) Apoptosis in HGC-27, AGS, and MKN-45 cells following treatment with vehicle control, VD₃, DDP, or DDP plus VD₃ for 24 h was analyzed by Annexin V-FITC/PI flow cytometry. Quantitative data are presented as the mean±SD of three independent experiments. Statistical significance was determined by one-way ANOVA. **p < 0.01 vs. the control group; #p < 0.05 and ##p < 0.01 vs. the DDP-treated group

Figure 2. VD₃ reduces DDP-induced PARP1 cleavage and γH2AX accumulation. A-C) Western blot analysis of PARP1, cleaved PARP1, and γH2AX in HGC-27 (A), AGS (B), and MKN-45 (C) cells following treatment with vehicle control, VD₃, DDP, or DDP plus VD₃ for 24 h. α-Tubulin was used as the loading control. Representative immunoblots and corresponding quantitative analyses are shown. Data are presented as the mean±SD of three independent experiments. Statistical significance was determined by one-way ANOVA. ***p < 0.001 vs. the control group; ###p < 0.001 vs. the DDP-treated group.

Figure 3. VD₃ attenuates DDP-induced oxidative stress in gastric cancer cells. A-C) Intracellular ROS levels in HGC-27 (A), AGS (B), and MKN-45 (C) cells following treatment with vehicle control, VD₃, DDP, or DDP plus VD₃ for 24 h were assessed using the DCFH-DA fluorescent probe. Representative fluorescence images and corresponding quantitative analyses are shown. Fluorescence intensity was normalized to the vehicle control group. Data are presented as the mean±SD of three independent experiments. Statistical significance was determined by one-way ANOVA. **p < 0.01 vs. the control group; #p < 0.05 and ##p < 0.01 vs. the DDP-treated group

Figure 4. Transcriptomic changes among the control, DDP, VD₃, and DDP plus VD₃ groups in HGC-27 cells. RNA-seq was performed in HGC-27 cells treated with vehicle control, VD₃, DDP, or

DDP plus VD₃ for 24 h. Differentially expressed genes were identified using the criteria of $|\log_2FC| > 1$ and $p < 0.05$. A-D) Volcano plots showing differentially expressed genes in the Control vs. DDP (A), Control vs. VD₃ (B), Control vs. DDP plus VD₃ (C), and DDP vs. DDP plus VD₃ (D) comparisons. Red dots represent upregulated genes, blue dots represent downregulated genes, and gray dots represent genes that were not differentially expressed. E) Venn diagram showing the overlap of differentially expressed genes among the indicated comparison groups.

Figure 5. GO enrichment analysis of differentially expressed genes. Gene Ontology (GO) biological process enrichment analysis was performed using differentially expressed genes from the indicated comparison groups: Control vs. DDP (A), Control vs. VD₃ (B), Control vs. DDP plus VD₃ (C), and DDP vs. DDP plus VD₃ (D).

Figure 6. KEGG pathway enrichment analysis of differentially expressed genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using differentially expressed genes from the indicated comparison groups: Control vs. DDP (A), Control vs. VD₃ (B), Control vs. DDP plus VD₃ (C), and DDP vs. DDP plus VD₃ (D).

Figure 7. GSEA of pathway-level changes associated with VD₃ treatment in DDP-treated HGC-27 cells. GSEA was performed using RNA-seq data from HGC-27 cells treated with DDP plus VD₃ compared with cells treated with DDP alone. Representative enriched pathways include ABC transporter-related pathways (A), ECM-receptor interaction (B), and steroid biosynthesis (C).

Figure 8. Protein-protein interaction network analysis of candidate genes associated with VD₃-mediated modulation of DDP response. A) Protein-protein interaction (PPI) network constructed using differentially expressed genes identified in the DDP plus VD₃ versus DDP comparison. B) GO functional annotation analysis of genes included in the PPI network. C) KEGG pathway annotation analysis of genes included in the PPI network. D) Hub gene ranking based on network topology analysis using the cytoHubba plugin in Cytoscape. Candidate hub genes were identified according to their network scores.

[Download full resolution image](#)

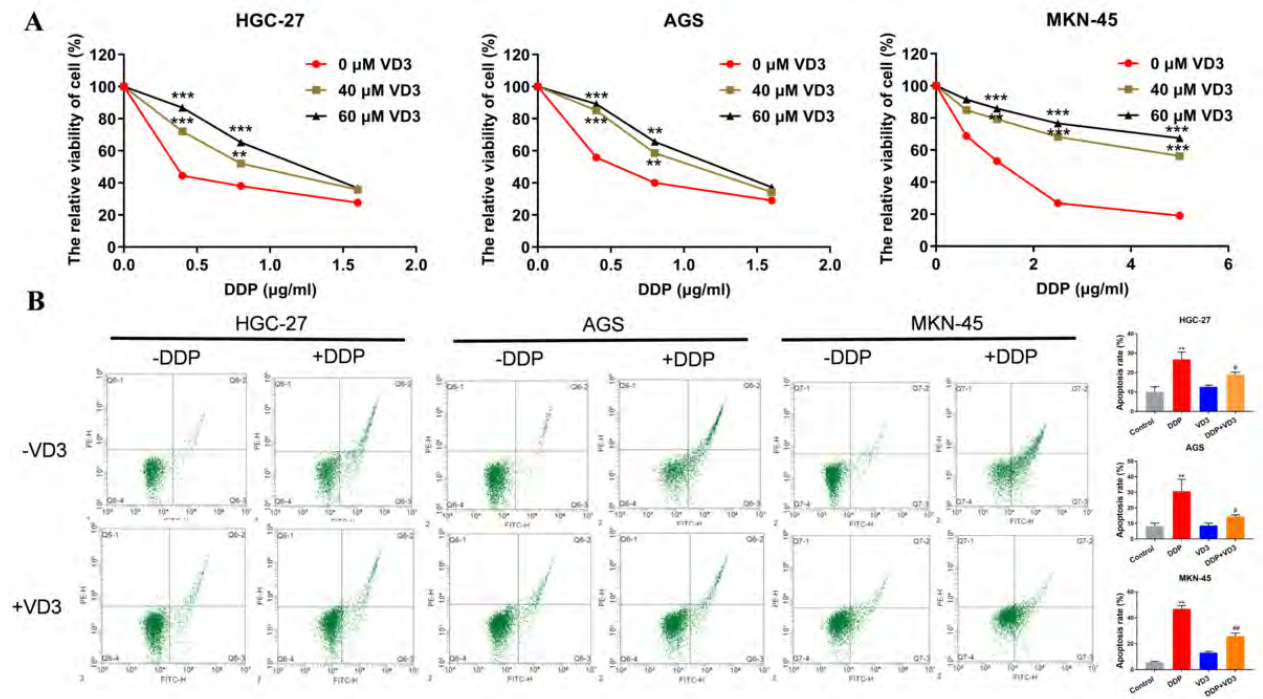


Fig. 2 [Download full resolution image](#)

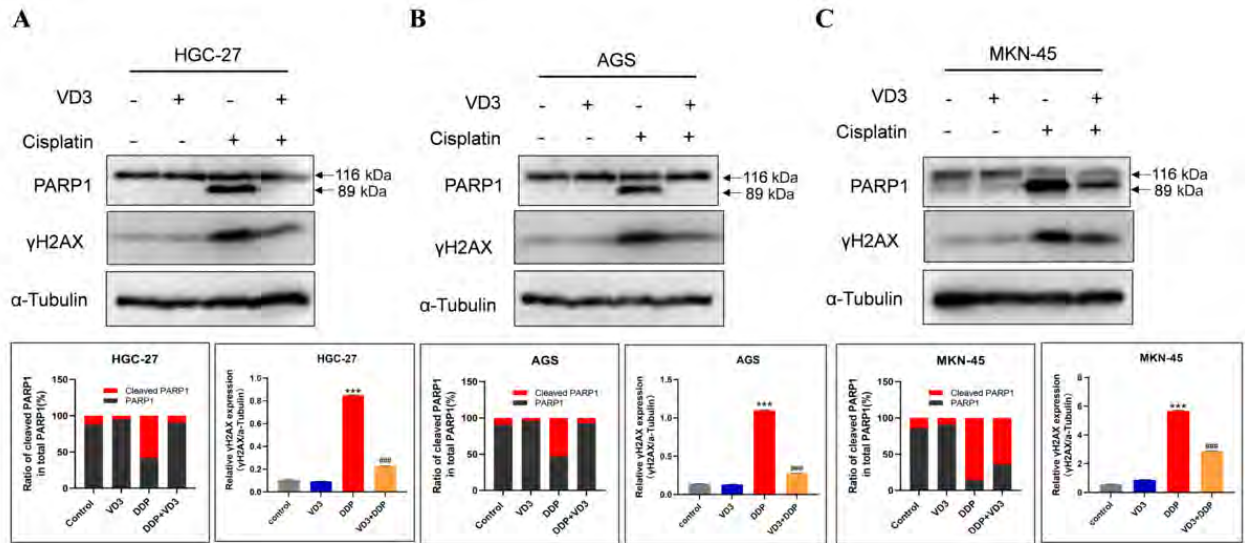


Fig. 3 [Download full resolution image](#)

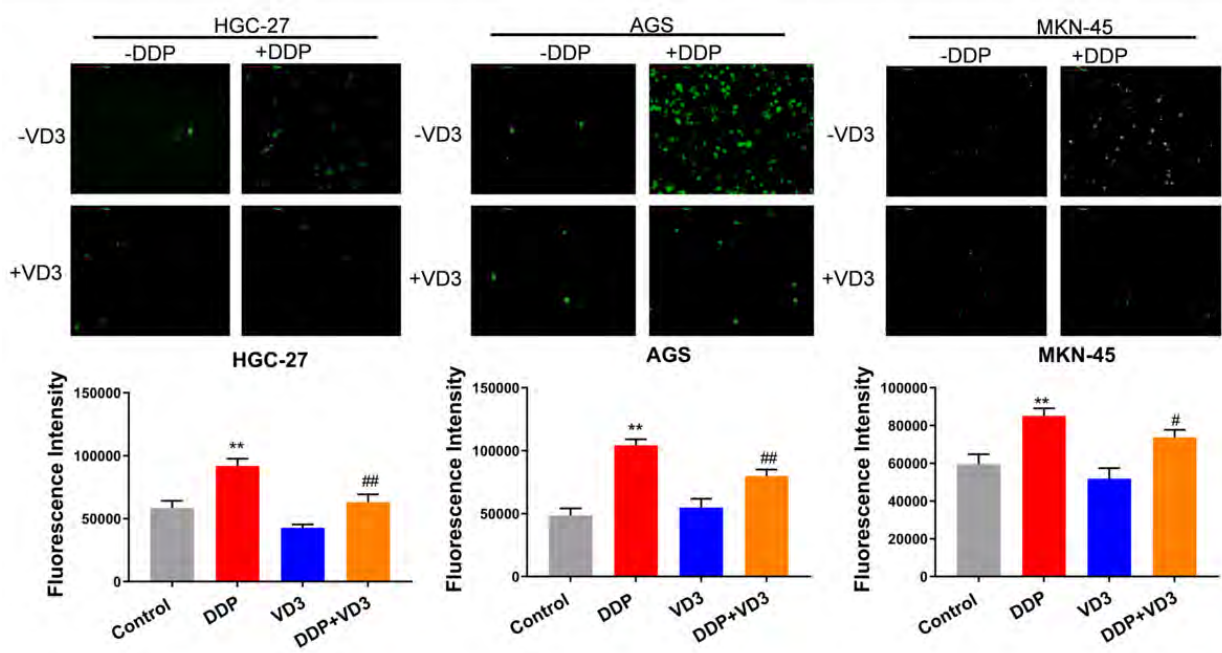


Fig. 4 [Download full resolution image](#)

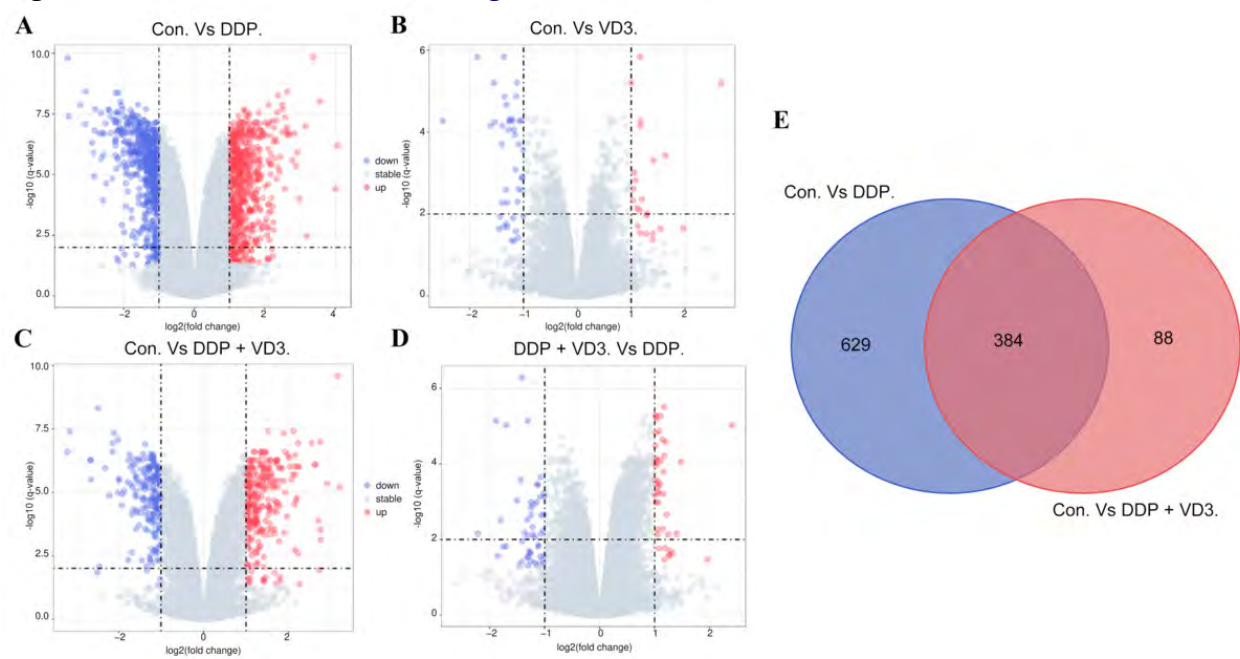


Fig. 5 [Download full resolution image](#)

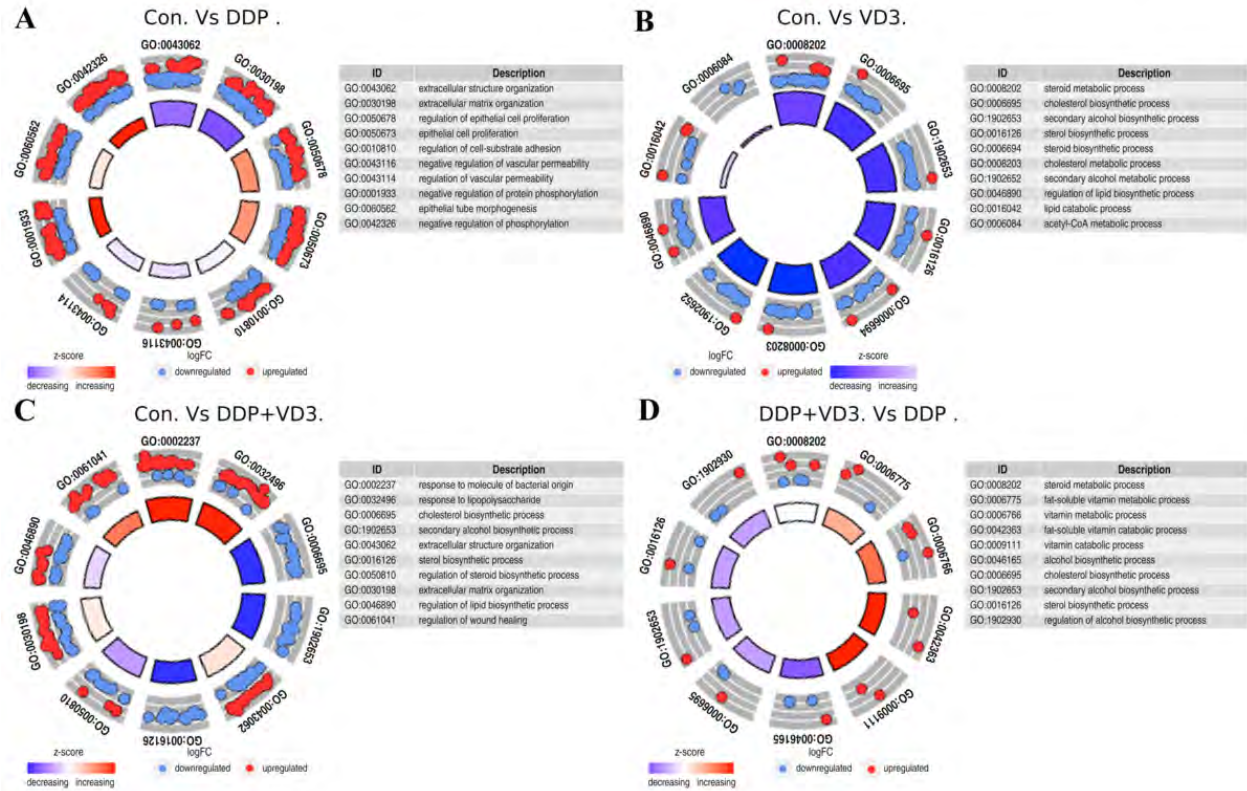


Fig. 6 [Download full resolution image](#)

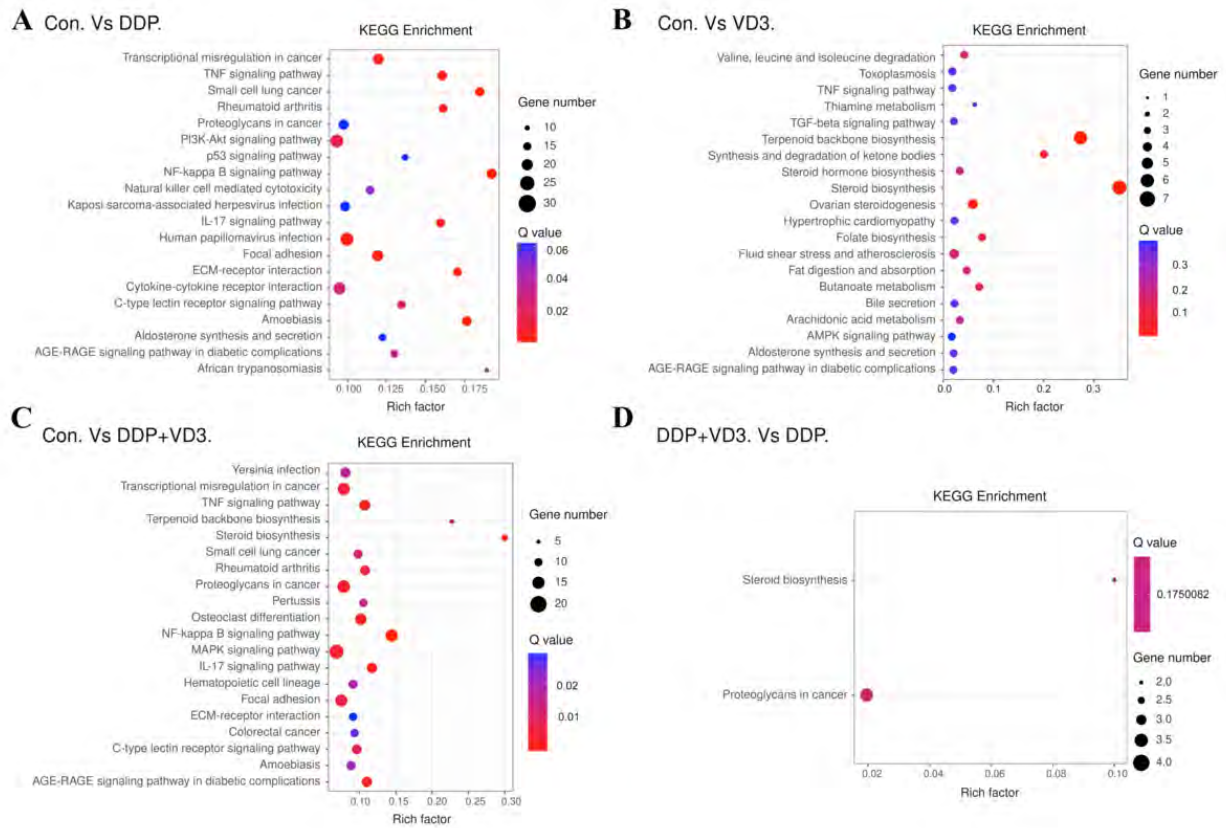
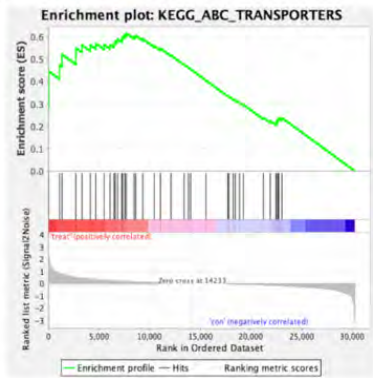
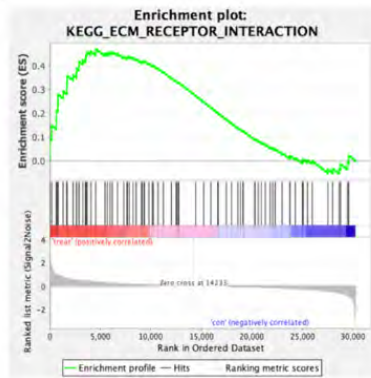


Fig. 7 [Download full resolution image](#)

A



B



C

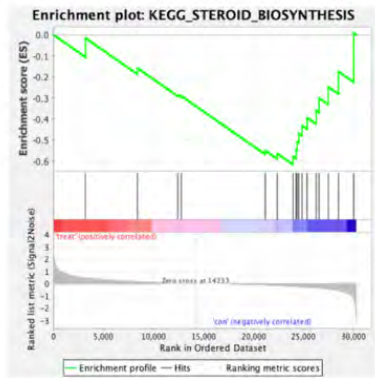


Fig. 8 [Download full resolution image](#)

