

Running title: V9302 inhibits non-small cell lung cancer

V9302, an inhibitor of glutamine transport, suppresses proliferation and migration, and induces apoptosis in non-small cell lung cancer cells through ROS-mediated mTOR/p70S6K pathway

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This study aimed to investigate the effects and underlying mechanisms of V9302, an inhibitor of glutamine transport, on non-small cell lung cancer (NSCLC) cells. Proliferation was assessed using the cell counting kit-8, colony formation, and EdU assays. Mitochondrial membrane potential was evaluated through JC-1 staining. Cell cycle distribution, apoptosis, and reactive oxygen species (ROS) levels were analyzed by flow cytometry, while migration was assessed using wound healing and Transwell assays. Western blotting was performed to determine protein expression levels. The antitumor efficacy of V9302 *in vivo* was evaluated using a xenograft mouse model with PC-9 cells. The results demonstrated that V9302 inhibited cell proliferation and induced G1-phase arrest in human lung adenocarcinoma PC-9 and A549 cells. Western blotting showed that V9302 significantly inhibited the ASCT2 protein expression in both PC-9 and A549 cells. Additionally, V9302 promoted apoptosis through a mitochondrial-dependent pathway, as evidenced by elevated levels of cleaved PARP, cleaved Caspase 3, cleaved Caspase 9, and Bax. V9302 also suppressed cell migration by downregulating N-cadherin and vimentin expression. Notably, V9302 triggered significant ROS accumulation and inhibited mTOR/p70S6K pathway activation, an effect that was partially restored by N-acetylcysteine, a ROS scavenger. Pretreatment with mTOR activator MHY1485 mitigated the inhibitory effects of V9302 on cell proliferation and migration, as well as its induction of apoptosis. Furthermore, V9302 inhibited tumor growth and induced apoptosis in a xenograft mouse model, without inducing detectable visceral toxicity. In conclusion, these findings demonstrate that V9302 reduces cell proliferation and migration, and causes apoptosis through the ROS-mediated mTOR/p70S6K pathway in NSCLC cells. These findings provide a novel theoretical foundation for advancing both academic and clinical research on NSCLC treatment.

Key words: non-small cell lung cancer; V9302; apoptosis; reactive oxygen species; mTOR/p70S6K pathway

46

47 Non-small cell lung cancer (NSCLC) is the predominant subtype of lung cancer, accounting for
48 nearly 85% of all lung cancer cases [1]. As a highly aggressive tumor, NSCLC is often diagnosed at
49 late stages, accompanied by metastases, which limits therapeutic options and leads to poor
50 prognoses [2]. Despite significant advances in cancer research and therapeutic innovations, the
51 5-year relative survival rate for advanced NSCLC remains limited [3]. Therefore, the identification
52 of novel biomarkers and therapeutic targets, as well as the elucidation of their underlying molecular
53 mechanisms, is vital for improving NSCLC treatment.

54 Recent studies emphasize the critical role of glutamine in cancer cell metabolism, particularly in
55 energy production, biosynthesis, and the maintenance of cellular homeostasis [4]. Cancer cells
56 exhibit a greater dependence on Gln for survival and proliferation compared to normal cells. To
57 meet their increased demand for Gln, cancer cells upregulate membrane transporters that facilitate
58 Gln import, thereby enhancing the uptake of this critical amino acid [5], these transporters include
59 the alanine-serine-cysteine transporter 2 (ASCT2), SLC6A14 (solute carrier family 6 member 14),
60 and SLC7A6 (solute carrier family 7 member 6) [6]. ASCT2, the primary Gln transporter, is
61 significantly upregulated in various tumor types, and studies have shown that inhibition of ASCT2
62 activity yields promising antitumor efficacy [7]. V9302, a selective ASCT2 inhibitor, has been
63 demonstrated to reduce cell growth and proliferation, while promoting apoptosis and oxidative
64 accumulation in cancer cells [8]. Nevertheless, the precise function and underlying mechanisms of
65 V9302 in NSCLC remain inadequately understood. Therefore, elucidating the molecular pathways
66 modulated by V9302 is essential for evaluating its therapeutic potential in NSCLC. Studies indicate
67 that physiological levels of reactive oxygen species (ROS) facilitate tumor progression by
68 enhancing proliferation and migration, while supraphysiological ROS accumulation leads to
69 oxidative damage, resulting in growth arrest and apoptosis through the activation of stress response
70 pathways [9, 10]. In addition, ROS activates multiple signaling pathways that regulate cellular
71 stress responses [11]. Notably, the mTOR/p70S6K pathway is frequently hyperactivated in tumors
72 and has emerged as a potential drug target in various malignancies [12-14].

73 This study aims to characterize the effects of V9302 on proliferation, migration and apoptosis in
74 NSCLC cells, and to investigate the role of ROS and the mTOR/p70S6K pathway in these
75 processes, with the goal of providing new theoretical insights and experimental evidence for the
76 clinical management of NSCLC.

78 **Materials and methods**

79 **Cell culture and treatment.** PC-9 and A549 cells, human lung adenocarcinoma cell lines obtained
80 from Wuhan Punosai Biotechnology (Wuhan, China), were cultured in RPMI-1640 medium (Gibco,
81 USA) supplemented with 10% fetal bovine serum (FBS, Excell Bio, Shanghai, China) and 1%
82 penicillin-streptomycin (New Cell & Molecular Biotech, Suzhou, China) at 37 °C under standard
83 conditions. Chemical compound included V9302 (#GC34852, Glpbio, Shanghai, China),
84 N-acetylcysteine (NAC) (#HY-B0215, MedChemExpress, Shanghai, China), and MHY1485
85 (#HY-B0795, MedChemExpress, Shanghai, China).

86 **Cell viability analysis.** After seeding in 96-well plates (5,000 cells/well), the cells were treated
87 with V9302. Following a 24 h incubation, the culture medium was carefully removed and replaced
88 with cell counting kit-8 (#K1018, CCK-8, APExBIO, USA) reagent. After incubation at 37 °C for
89 30 minutes in the dark, optical density (OD) at 450 nm was measured using a microplate reader
90 (Synergy, USA). The half-maximal inhibitory concentration (IC₅₀) was calculated using GraphPad
91 Prism 8 software based on the OD values.

92 **Measurement of Gln and GSH levels.** Total Gln and glutathione (GSH) levels were measured
93 using the Gln assay kit (BC5305, Solarbio, Beijing, China) and GSH assay kit (#BC1175, Solarbio,
94 Beijing, China), respectively. After treatment with V9302, PC-9 and A549 cells were lysed and
95 analyzed according to the supplier's protocol. The levels of Gln and GSH were determined by
96 spectrophotometry (Thermo Fisher Scientific, USA) at 450 nm and 412 nm, respectively.

97 **EdU incorporation assay.** To detect cell proliferation using an EdU cell proliferation kit (#C0071S,
98 Beyotime, Shanghai, China), cells were plated in confocal dishes (20,000 cells/well) and then
99 treated with the indicated conditions for 24 h. After EdU labelling (10 µM, 2 h, 37 °C), the cells
100 were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 10
101 min, and then incubated with the click reaction mixture containing Alexa Fluor azide for 30 min at
102 ambient temperature protected from light. Nuclei were stained with 1 µg/ml DAPI for 5 min.
103 Fluorescence images were captured using an Axio Observer.Z1 microscope (Zeiss, Germany), and
104 quantification was performed using Image J software.

105 **Colony formation assay.** After seeding PC-9 and A549 cells (500 cells/well in 6-well plates) and
106 allowing for 24 h of attachment, the cells were treated with V9302 for 10 days. Following treatment,

107 the cells were washed twice with PBS, fixed with 4% formaldehyde for 20 min, stained with 2%
108 crystal violet for 10 min and then photographed. Colony quantification was performed using Image
109 J software across three independent experiments.

110 **Cell cycle analysis.** The cell cycle was detected using a cell cycle detection kit (#BB-4104, Bestbio,
111 Shanghai, China). Cells were inoculated in 6-well plates (500,000 cells/well) and cultured with
112 V9302 for 24 h. After trypsinization and PBS washing, the cells were fixed with 70% ethanol at
113 4 °C overnight. For cell cycle analysis, the fixed cells were co-stained with RNase A and PI for 30
114 min at 37 °C in the dark [15]. A minimum of 10,000 events/sample were collected using a
115 CytoFLEX flow cytometer (Beckman, USA), and the experiment was independently repeated three
116 times. Data analysis was conducted using FlowJo v10.8.1 software as previously described.

117 **Apoptosis assay.** Apoptosis was detected using a cell cycle detection kit (#BB-4101, Bestbio,
118 Shanghai, China). Cells were inoculated in 6-well plates (200,000 cells/well), treated with the
119 indicated conditions for 24 h, and harvested by trypsinization. After a 15 min incubation in the dark
120 with Annexin V-FITC and PI, apoptosis was assessed by analyzing 10,000 events/sample using a
121 CytoFLEX flow cytometer (Beckman, USA) [16]. The experiment was performed in three
122 independent replicates, and data analysis was conducted using FlowJo v10.8.1 software.

123 **Mitochondrial membrane potential (MMP) assay.** MMP was assessed using a MMP assay kit
124 (#C2006, Beyotime, Shanghai, China) following the manufacturer's protocol. Cells were seeded in
125 6-well plates (100,000 cells/well) and treated with V9302 for 24 h. JC-1 staining (10 µM) was
126 carried out at 37 °C for 20 min under dark conditions, followed by fluorescence imaging using an
127 Axio Observer.Z1 fluorescence microscope (Zeiss, Germany). The JC-1 red/green fluorescence
128 intensity ratio, which reflects changes in MMP, was analyzed using Image J software.

129 **Intracellular ROS level measurement.** Intracellular ROS levels were detected using a ROS assay
130 kit (#S0033S, Beyotime, Shanghai, China). After a 24 h exposure to the indicated treatment in
131 6-well plates (200,000 cells/well), cells were processed with DCFH-DA (Beyotime, Shanghai,
132 China,) (37 °C, 30 min), rinsed with serum-free medium to remove excess dye, harvested by
133 trypsinization, and promptly analyzed using a flow cytometer (Beckman, USA). The levels of ROS
134 were quantified using FlowJo v10.8.1 software.

135 **Wound healing assay.** Following seeding (500,000 cells/well in 6-well plates) and 24 h of
136 attachment, a uniform scratch was created at 80-90% confluence using a 200 µl pipette tip. After

137 three PBS washes, the cells were treated with V9302 for 24 h in medium comprising 2% FBS.
138 Wound healing was documented using a microscope (Olympus, Japan) immediately (0 h) and 24 h
139 post-scratch, and the wound closure was quantified using Image J software.

140 **Transwell migration assay.** After resuspension in serum-free medium, cells were seeded into the
141 upper chamber (20,000 cells/well), with 10% FBS medium placed in the lower chamber as a
142 chemoattractant. Following a 24 h incubation with the indicated treatment, the membranes were
143 fixed with 4% paraformaldehyde for 15 min, and non-migrated cells were removed by swabbing.
144 Migrated cells were stained with 0.1% crystal violet for 20 min, washed with PBS, and imaged
145 under a microscope (Olympus, Japan) at 200× magnification. Cell numbers were quantified in five
146 random fields per well using Image J software.

147 **Western blotting analysis.** After a 24 h incubation with the indicated treatment, cells were lysed
148 using RIPA (#P0013C, Beyotime, Shanghai, China) lysis solution. Protein concentrations were
149 determined using a bicinchoninic acid (BCA) kit (#ST2222-5g, Beyotime, Shanghai, China).
150 Protein (20-30 µg) was separated by SDS-PAGE and electrotransferred to a PVDF membrane.
151 Following blocking with 5% skim milk in TBST, the membrane was incubated sequentially with
152 primary antibodies (4 °C, overnight) and corresponding HRP-labelled secondary antibodies (room
153 temperature, 1 h), followed by visualization using an Enhanced Chemiluminescent (ECL) substrate
154 (#P10100, NCM Biotech, Suzhou, China). Images were captured using a gel imaging system
155 (Sevier, China). Quantitative analysis was performed with Image J software, with GAPDH or
156 β-Tubulin as the loading reference [17]. Antibodies included anti-PARP (#T40050, Abmart,
157 Shanghai, China), anti-ASCT2 (#5345), anti-Bax (#5023), anti-Bcl-2 (#4223), anti-Caspase-3
158 (#14220), anti-Caspase-9 (#9502), anti-cleaved Caspase-3 (#9661), anti-cleaved Caspase-9 (#7237),
159 anti-β-Tubulin (#2146), anti-mTOR (#2972), anti-phospho-mTOR (#2971), anti-p70S6K (#9202),
160 anti-phospho-p70S6K (#9234) (Cell Signaling Technology), anti-GAPDH (#60004-1-Ig),
161 anti-E-cadherin (#20874-1-AP), anti-N-cadherin (#22018-1-AP), and anti-vimentin (#60330-1-Ig)
162 (Proteintech).

163 **Xenograft mouse model.** BALB/c nude mice (SPF grade, female, 4-6 weeks old, weight 18-20 g)
164 were obtained from Hangzhou Ziyuan Laboratory Animal Technology Co. Ltd. The experimental
165 protocol was approved by the Ethics Committee of Bengbu Medical University (Approval No.
166 2025A819). PC-9 cells (5,000,000 cells suspended in 200 µl Matrigel/PBS) were subcutaneously

167 implanted into the right flank of the mice. When tumors reached an average volume of
168 approximately 100 mm³, the animals were randomly assigned to a control group and a V9302
169 treatment group, with six mice/group. V9302 was administered via intraperitoneal injection at a
170 dose of 50 mg/kg, prepared in a vehicle composed of 5% DMSO, 40% PEG400, and 55% saline.
171 Mice in the control group received an equal volume of vehicle alone. Treatments were performed
172 once daily for 21 consecutive days. Tumor dimensions and body weight were measured every three
173 days by an investigator blinded to group allocation, and tumor volume was calculated using the
174 formula: $V = (\text{length} \times \text{width}^2)/2$. On day 22, mice were humanely euthanized for tumor excision,
175 weighing, and sample collection for subsequent analysis.

176 **Immunohistochemistry (IHC).** Tissue sections were exposed to 3% H₂O₂ for endogenous
177 peroxidase activity quenching and subsequently blocked with serum. The sections were then
178 incubated with primary antibodies against Ki67, cleaved Caspase-3 and E-cadherin at 4 °C
179 overnight. After binding with secondary antibodies and 3,3'-Diaminobenzidine (DAB) staining, the
180 sections were imaged using a light microscope (Olympus, Japan).

181 **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** Apoptosis in
182 tumor tissues was detected using a TUNEL apoptosis detection kit (#HY-K1091, MedChemExpress,
183 Shanghai, China) according to the manufacturer's protocol. Briefly, paraffin-embedded tissue
184 sections (4 μm) were deparaffinized, rehydrated, and treated with proteinase K. After blocking
185 endogenous peroxidase activity, sections were incubated with biotinylated Nucl. Mix and rTdT at
186 37 °C for 1 h, followed by streptavidin-HRP. Staining was visualized with DAB, and sections were
187 counterstained with methyl green. Images were captured using an optical microscope [18]
188 (Olympus, Japan).

189 **Hematoxylin-eosin (H&E) staining.** After treatment with xylene for transparency, the specimen
190 was paraffin-embedded and sectioned into piece (thickness 4 μm). As described previously [19], the
191 sections were stained with HE and then visualized through a microscope (Olympus, Japan).

192 **Statistical analysis.** The quantitative results, presented as mean±SD from triplicate assays, were
193 analyzed using GraphPad Prism 8.0 and SPSS 27 software. Statistical comparisons among groups
194 were performed using one-way ANOVA followed by the LSD test, with $p < 0.05$ considered to
195 indicate a statistically significant difference.

196

197 **Results**

198 **V9302 inhibits the proliferation of NSCLC cells.** To assess the cytotoxic effects of V9302 in
199 PC-9 and A549 cells, the cells were exposed to varying concentrations of V9302 for 24 h. The
200 CCK-8 assay demonstrated a concentration-dependent reduction in cell viability in both cell lines
201 (Figure 1B), with IC₅₀ values of 13.41±0.45 μM and 20.02±0.73 μM, respectively. To investigate
202 the impact of V9302 on Gln metabolism in A549 and PC-9 cells, we employed Gln assay kit and
203 GSH assay kit to measure intracellular levels of Gln and GSH. The results demonstrated that V9302
204 significantly reduced the intracellular content of both Gln and GSH (Figures 1C, 1D). Western
205 blotting revealed that V9302 significantly inhibited the expression of ASCT2 protein in both PC-9
206 and A549 cells (Figures 1E, 1F). Additionally, V9302 treatment decreased the relative proportion of
207 EdU-positive cells and colony formation, as shown by EdU and colony formation assays (Figures
208 1G, 1H). The cell cycle plays a crucial role in regulating cell proliferation. Flow cytometry findings
209 demonstrated that V9302 triggered G₁-phase arrest in both PC-9 and A549 cells (Figure 1I). These
210 findings suggest that V9302 influences Gln metabolism and inhibits cell proliferation in NSCLC
211 cells.

212 **V9302 induces apoptosis in NSCLC cells.** To reveal the effect of V9302 on apoptosis in both cell
213 lines, we performed Annexin V-FITC/PI staining and observed a significant increase in apoptosis
214 rates in the treated cells (Figure 2A). To determine whether V9302-induced apoptosis involved the
215 mitochondrial pathway, we assessed changes in MMP using the JC-1 probe. V9302 treatment
216 significantly reduced the red/green fluorescence intensity ratio, indicating MMP dissipation (Figure
217 2B). The decrease in the Bcl-2/Bax ratio, along with the cleavage of Caspase-3, Caspase-9, and
218 PARP, plays a critical role in mitochondrial pathway-mediated apoptosis. Western blot analysis
219 demonstrated that V9302 upregulated Bax expression and the cleavage of PARP, Caspase-3, and
220 Caspase-9, while downregulated Bcl-2, PARP, and Caspase-3 expression in the cells, resulting in
221 decreased Bcl-2/Bax ratio (Figure 2C). Collectively, these findings suggest that V9302 induces
222 apoptosis via the mitochondrial pathway in NSCLC cells.

223 **V9302 restrains the migration of NSCLC cells.** To test the anti-migratory activities of V9302, we
224 conducted a wound healing assay in both cell lines. V9302 significantly reduced the percentage of
225 wound closure in both cell lines (Figures 3A, 3B). Consistently, Transwell migration assays
226 demonstrated that V9302 treatment markedly inhibited cell migration (Figure 3C, 3D). During

227 tumor cell migration, E-cadherin expression decreases, disrupting epithelial homeostasis, while
228 N-cadherin and vimentin expression increases, which mediates dynamic adhesion and enhances cell
229 motility. Western blot analysis revealed that V9302 reduced the levels of vimentin and N-cadherin
230 while increasing the expression of E-cadherin in both cell lines (Figure 3E). These findings confirm
231 that V9302 inhibits the migration of NSCLC cells.

232 **V9302 inhibits mTOR/p70S6K pathway through ROS generation in NSCLC cells.** Intracellular
233 ROS production is closely linked to Gln metabolism. Flow cytometry analysis showed that V9302
234 exposure induced marked intracellular ROS accumulation in both PC-9 and A549 cells. (Figure 4A).
235 Furthermore, previous studies have demonstrated that ROS modulates multiple signaling cascades,
236 with elevated ROS levels inhibiting the function of the mTOR/p70S6K pathway. Western blot
237 analysis confirmed that V9302 significantly decreased the levels of p-mTOR and p-p70S6K, while
238 exerting no marked effect on the total levels of mTOR and p70S6K in the cells (Figure 4B).
239 Pre-treatment with the ROS scavenger NAC, partially reversed V9302-induced suppression of
240 p-mTOR and p-p70S6K in both cell lines (Figure 4C, 4D). These findings suggest that V9302
241 inhibits the activation of the mTOR/p70S6K pathway through a ROS-dependent mechanism.

242 **V9302 modulates proliferation, migration and apoptosis through mTOR/p70S6K pathway in**
243 **NSCLC cells.** To further elucidate the function of mTOR/p70S6K pathway in V9302-mediated
244 suppression of NSCLC cell proliferation, we treated A549 and PC-9 cells with the mTOR activator
245 MHY1485. EdU incorporation and CCK-8 assays revealed that MHY1485 partially restored
246 V9302-induced inhibition of proliferation in both cell lines (Figures 5A-5C). Meanwhile,
247 MHY1485 attenuated V9302-induced apoptosis (Figure 5D), as evidenced by decreased cleavage of
248 PARP, Caspase-3, and Caspase-9 (Figure 5E). We then examined whether MHY1485 could reverse
249 V9302-mediated inhibition of migration. Transwell assays showed that MHY1485 significantly
250 alleviated V9302-induced suppression of migration (Figure 6A). Furthermore, MHY1485 partially
251 restored E-cadherin level and increased the expression of vimentin and N-cadherin levels (Figure
252 6B). Collectively, these results demonstrate that V9302 modulates cell migration, proliferation, and
253 apoptosis through the mTOR/p70S6K signaling pathway in NSCLC cells.

254 **V9302 represses the growth of xenografted NSCLC cells *in vivo*.** To assess the antitumor activity
255 of V9302 *in vivo*, a xenograft model using PC-9 cells was established in nude mice. V9302
256 treatment significantly inhibited tumor growth, with both tumor size and weight being dramatically

257 reduced compared to the control group (Figures 7A, 7C, 7D), while no significant differences in
258 body weight were observed between the V9302 and control groups (Figure 7B). To assess potential
259 hepatotoxicity and nephrotoxicity, we measured corresponding serum indicators such as alanine
260 aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), blood urea nitrogen
261 (BUN) by ELISA. No significant differences in these indicators were observed between the V9302
262 and control groups (Figure 7E). Histopathological analysis of important organs (liver, spleen,
263 kidney, lung, and heart) using HE staining showed no significant toxicity in V9302-treated mice
264 compared to the control group (Figure 7F). TUNEL staining revealed a significantly higher
265 apoptosis index in the V9302-treated group compared to the control group (Figures 7G, 7H).
266 Furthermore, IHC analysis demonstrated a marked reduction in Ki67 expression, along with
267 significant upregulation of E-cadherin and cleaved Caspase-3 levels (Figures 7I, 7J). Taken together,
268 these findings suggest that V9302 exerts anti-tumor effects in NSCLC *in vivo*.

269

270 Discussion

271 Recent studies have highlighted the reprogramming of Gln transporters as a critical mechanism in
272 cancer therapy, given that Gln uptake and utilization are essential for the survival and proliferation
273 of cancer cells [7]. Among these transporters, ASCT2 plays a central role in regulating intracellular
274 Gln levels [7]. ASCT2 is significantly upregulated in multiple cancers, including colorectum,
275 prostate, and breast cancers, and its expression is strongly correlated with cancer cell proliferation,
276 invasion, and metastasis [20-23]. Therefore, inhibition of ASCT2 has demonstrated strong
277 anti-tumor effects. ASCT2 inhibitors, such as benzels erine and L- γ -glutamyl-p-nitroanilide, have
278 been developed and are currently undergoing clinical evaluation [24, 25]. However, subsequent
279 studies have demonstrated that these compounds are not suitable for specifically targeting Gln
280 addition via ASCT2 in tumor cells, due to their low binding affinity, lack of specificity, and the
281 need for high effective doses [21, 25-27]. Notably, a novel ASCT2 inhibitor, V9302, has shown
282 significant anti-tumor activity across a range of cancers, including glioma and renal cell carcinoma,
283 by effectively inhibiting tumor growth [28-30].

284 V9302 is the first specific and effective small-molecule inhibitor of ASCT2, which inhibits tumor
285 growth by suppressing ASCT2-mediated Gln uptake, thereby affecting intracellular Gln metabolism,
286 inhibiting cell proliferation, and inducing apoptosis [8]. Our findings demonstrate that V9302 exerts

287 potent anti-tumor effects in NSCLC through dual mechanisms: metabolic disruption and
288 proliferation suppression. By competitively inhibiting Gln uptake, V9302 not only depletes
289 intracellular GSH pools and disrupts energy metabolism but also significantly reduces tumor growth
290 and Ki67 expression *in vivo*. These findings underscore the dependence of NSCLC on Gln for both
291 antioxidant defense (GSH synthesis) and bioenergetics, suggesting that inhibition of Gln
292 transporters may overcome the limitations of targeting downstream enzymes alone.

293 Furthermore, studies have demonstrated that V9302 disrupts the biosynthesis of key proteins during
294 the G₁/S transition, leading to G₁-phase arrest and subsequent suppression of cell proliferation [31,
295 32]. This form of sustained growth arrest is a hallmark of cellular senescence, a state that acts as a
296 critical barrier to tumor initiation by engaging tumor suppressor pathways such as p53/p21 [33]. In
297 this study, cell cycle analysis revealed a significant accumulation in the G₁-phase, indicating that the
298 G₁-phase arrest induced by V9302 resulted in reduced proliferation of NSCLC cells. This finding
299 provides a mechanistic explanation for the compound's anti-proliferative effects, as Gln depletion
300 likely disrupts the biosynthesis of key proteins required for the G₁/S transition. Additionally,
301 previous studies have demonstrated that V9302 caused PARP cleavage in breast cancer cells,
302 promoting apoptosis [34], further suggesting its pro-apoptotic potential. Apoptosis is predominantly
303 mediated through the mitochondrial pathway, which is regulated by mitochondrial membrane
304 integrity and the ratio of Bcl-2 family proteins. Mitochondrial damage has emerged as a crucial
305 mechanism for tumor suppression, as it disrupts cellular homeostasis and triggers cell death [35].
306 The increased Bax/Bcl-2 ratio destabilizes the mitochondrial outer membrane, leading to the
307 breakdown of MMP and cytochrome C efflux, which subsequently triggers the activation of
308 Caspase-9 and Caspase-3, culminating in PARP cleavage, a hallmark of apoptotic cell death [36-38].
309 In this study, V9302 upregulated Bax expression while downregulating Bcl-2, resulting in a
310 reduction in MMP, followed by the cleavage of PARP, Caspase-3, and Caspase-9. Furthermore,
311 V9302 increased cleaved Caspase-3 levels and enhanced apoptosis index of tissue cells *in vivo*.
312 These findings not only confirm V9302's pro-apoptotic effects across various cancer types but also
313 mechanistically link its metabolic inhibition (Gln uptake blockade) to the activation of intrinsic
314 apoptosis, suggesting a coordinated dual action that amplifies its therapeutic potential against
315 NSCLC.

316 Beyond its antiproliferative effects, V9302 has been shown to modulate cancer cell migration [39].

317 Tumor cell migration is a complex process facilitated by dynamic remodeling of the cytoskeleton,
318 extracellular matrix degradation, changes in adhesion, and epithelial-mesenchymal transition,
319 ultimately leading to the acquisition of motility, invasion and metastasis [40]. These processes are
320 characterized by distinct molecular changes, including reduced E-cadherin expression, which
321 disrupts cell adhesion, the disassembly of intercellular connections, and the initial dissociation of
322 epithelial cells. Meanwhile, the levels of vimentin and N-cadherin increases, enhancing matrix
323 degradation and promoting metastatic potential [41]. In this study, V9302 inhibited cell migration
324 by upregulating E-cadherin expression and downregulating vimentin and N-cadherin levels in both
325 cell lines.

326 As is well known, the oxidative system and its gene regulatory factors play crucial roles in various
327 types of tumors. Disruption or loss of their normal functions can lead to alterations in cell
328 metabolism, proliferation, and apoptosis [42]. ROS production is triggered by various cellular stress
329 conditions, such as impaired mitochondrial function and DNA damage [43]. In the present study,
330 V9302-mediated inhibition of Gln transport significantly impaired Gln metabolism and depleted
331 intracellular GSH levels. The depletion of GSH compromises cellular antioxidant capacity,
332 resulting in ROS accumulation, oxidative stress, and consequent redox imbalance within the cells
333 [39]. Therefore, we performed further validation and found that V9302 significantly increased the
334 intracellular ROS levels in PC-9 and A549 cells, suggesting that the anti-tumor effect of V9302 on
335 NSCLC may be associated with the disruption of intracellular redox homeostasis through ROS
336 accumulation. ROS are known to regulate cell death by modulating multiple signaling pathways,
337 such as the mTOR pathway. Within the complex landscape of redox biology, ROS regulate mTOR
338 signaling through multiple upstream mechanisms. Specifically, H_2O_2 can trigger AMPK activation
339 to modulate mTORC1 via regulatory-associated protein of mTOR (Raptor) phosphorylation,
340 although this effect exhibits notable cell-type specificity. Additionally, oxidative stress may directly
341 engage the PI3K/Akt pathway to stimulate mTOR activity. A critical node for redox input is the
342 tuberous sclerosis complex (TSC); ROS can activate TSC2 to suppress mTORC1 and, in response
343 to oxidative stress, dynamically recruit the TSC complex to peroxisomes to induce autophagy. At
344 the transcriptional level, the master redox regulator NRF2 directly modulates mTOR expression
345 [44-46]. As a critical regulator of cellular homeostasis, mTOR, a serine/threonine protein kinase,
346 governs protein synthesis, cell growth, proliferation, and apoptotic death [47]. Furthermore,

347 p70S6K, a key downstream factor in the mTOR pathway, plays a major role in regulating
348 translation [48]. The mTOR-mediated phosphorylation of p70S6K to form p-p70S6K promotes
349 tumor progression by enhancing proliferation, invasion, metastasis, and altered metabolism [49].
350 Extensive research has demonstrated that mTOR/p70S6K is hyperactivated in various malignancies,
351 including breast, prostate, and cervix cancers, positioning it as a promising therapeutic target [48,
352 50, 51]. Our results demonstrate that V9302 substantially downregulate the expression of p-p70S6K
353 and p-mTOR in both cell lines, indicating the potential of V9302 in the treatment of NSCLC. To
354 investigate whether ROS mediated the effect of V9302 on mTOR/p70S6K, we found that NAC
355 pretreatment abrogated V9302-induced suppression of mTOR/p70S6K signaling. These findings
356 demonstrate that V9302 inhibits mTOR/p70S6K signaling through ROS-dependent mechanisms, as
357 evidenced by the NAC-mediated reversal of this effect. This provides mechanistic insight into
358 V9302's antitumor activity, highlighting oxidative stress as a critical mediator of its metabolic
359 interference in NSCLC.

360 As a key regulator of tumor progression, mTOR/p70S6K pathway modulates cell proliferation,
361 metastasis, and apoptosis [52, 53]. This pathway promotes cell cycle progression and inhibits
362 mitochondrial-dependent apoptosis by regulating the levels of cyclin-dependent kinases, the
363 Bcl-2/Bax ratio, and other proteins through phosphorylation [54, 55]. Furthermore, the
364 mTOR/p70S6K pathway contributes to tumor metastasis by regulating epithelial-mesenchymal
365 transition [56]. Notably, MHY1485, an agonist of mTOR activation, partially rescues drug-induced
366 inhibition of tumor cells [57]. Our findings demonstrate that V9302 effectively targets this critical
367 pathway, with MHY1485-mediated mTOR activation significantly attenuating V9302's anti-tumor
368 effects on cell migration, proliferation, and apoptosis. This pharmacological rescue experiment
369 provides compelling evidence that mTOR/p70S6K inhibition represents a primary mechanism
370 underlying V9302's therapeutic activity. These results position mTOR/p70S6K inhibition as a
371 promising strategy for concurrently targeting multiple hallmarks of cancer, warranting further
372 investigation into its clinical applications, particularly in combination with existing targeted
373 therapies.

374 In summary, V9302 inhibits proliferation and migration; while enhancing apoptosis in NSCLC cells,
375 an effect associated with the inhibition of mTOR and p70S6K phosphorylation through ROS
376 accumulation. (Figure 8). This study provides an experimental foundation and novel insights into

the potential use of V9302 for the treatment of NSCLC.

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Figure Legends

Figure 1. The suppressive effects of V9302 on the proliferation and growth in PC-9 and A549 cells. A) The chemical structure of V9302. B) The inhibitory rates of PC-9 and A549 cells at different concentrations of V9302 were evaluated at 24 h. C) Gln levels in PC-9 and A549 cells after V9302 treatment were measured using Gln assay kit. D) GSH levels were quantified with GSH assay kit. E,

578 F) Western blotting analysis for the protein expression of ASCT2 with GAPDH as loading control.
579 The relative protein level was quantitated by Image J analysis software. G) The proliferation of the
580 cells was assessed using EdU assay. H) Colony formation assay was carried out to analyze the cell
581 proliferation. (I) Cell cycle distribution of PC-9 and A549 cells was measured with PI staining after
582 V9302 treatment for 24 h (n=3, *p < 0.05 vs. 0 μ M group).

583
584 **Figure 2.** V9302 administration induced apoptosis in PC-9 and A549 cells. A) Annexin V-FITC/PI
585 staining was utilized to evaluate apoptosis in PC-9 and A549 cells after V9302 treatment for 24 h. B)
586 JC-1 fluorescence staining was used to observe the changes of MMP in the cells. C) Western
587 blotting analysis for the protein expression of Bax, Bcl-2, cleaved Caspase-3, cleaved Caspase-9,
588 and cleaved PARP, with β -Tubulin as loading control. The relative protein level was quantitated by
589 Image J analysis software (n=3, *p < 0.05 vs. 0 μ M group).

590
591 **Figure 3.** V9302 restrained migration in PC-9 and A549 cells. A, B) Wound-healing assay for PC-9
592 and A549 cells after exposure to V9302. Migration images were captured at 0 h and 24 h after
593 scratching. C, D) Transwell assay for migration in PC-9 and A549 cells after V9302 treatment for
594 24 h. E) Western blotting analysis for protein expression of E-cadherin, N-cadherin and vimentin,
595 with GAPDH as loading control. The relative protein level was quantitated by Image J analysis
596 software (n=3, *p < 0.05 vs. 0 μ M group).

597
598 **Figure 4.** V9302 significantly increased intracellular ROS levels in PC-9 and A549 cells, and ROS
599 accumulation played a pivotal role in mediating the antitumor effects. A) ROS levels in cells treated
600 with varying concentrations of V9302 were assessed by flow cytometry. B) The protein expression
601 of mTOR/p70S6K signaling pathway-related molecules in PC-9 and A549 cells was determined by
602 Western blotting, with GAPDH as the internal control. C) Following NAC (2 mM) pretreatment,
603 ROS levels were evaluated by flow cytometry. D) After NAC (2 mM) pretreatment, the protein
604 expression of mTOR/p70S6K signaling pathway-related molecules was examined by Western
605 blotting (n=3, *p < 0.05 vs. 0 μ M group, [#]p < 0.05 vs. Control group).

606
607 **Figure 5.** mTOR activation attenuated the effects of V9302 on proliferation and apoptosis in PC-9

608 and A549 cells. A) CCK8 was applied to determine the viability of cells pretreated with MHY1485.
609 B, C) Proliferation was assessed by EdU assay in cells pretreated with MHY1485 (2 μ M). D)
610 Apoptosis rates were measured via flow cytometry following MHY1485 (2 μ M) pretreatment. E)
611 The expression levels of Bcl-2, Bax, Caspase-3, Caspase-9, and PARP were evaluated by Western
612 blotting after MHY1485 (2 μ M) pretreatment, with β -Tubulin as the internal reference (n=3, *p <
613 0.05, **p < 0.01 vs. Control group).

614
615 **Figure 6.** mTOR activation attenuated V9302-mediated inhibition of migration in PC-9 and A549
616 cells. A) Transwell migration assay was performed to evaluate the migratory capacity of cells
617 following MHY1485 (2 μ M) pretreatment. B) Protein expression levels of E-cadherin, N-cadherin,
618 and vimentin were analyzed by Western blotting in MHY1485-pretreated (2 μ M) cells, with
619 GAPDH as the internal reference (n=3, *p < 0.05, **p < 0.01 vs. Control group).

620
621 **Figure 7.** V9302 demonstrated *in vivo* anti-neoplastic effects. A) Macroscopic view of the tumor
622 after treatment for 2 weeks in the two groups. D) body weight, C) tumor volume, and B) The tumor
623 weight of mice processed with V9302. E) ELISA was employed to determine changes in the
624 indicators of renal function (BUN and CRE) and liver function (AST and ALT). F) HE staining
625 graphs of the liver, kidney, heart, spleen, and lung; scale bar=50 μ m. G, H) TUNEL assay was
626 employed to analyze the apoptosis of tumor cells; scale bar=50 μ m. I, J) Representative IHC graphs
627 of Ki67, cleaved Caspase-3 and E-cadherin; scale bar=50 μ m (n=6, *p < 0.05, **p < 0.01 vs.
628 Control group).

629
630 **Figure 8.** Schematic diagram of the mechanism of V9302 inhibits NSCLC via
631 ROS-mTOR/p70S6K pathway. V9302 downregulates ASCT2 expression to restrict glutamine
632 uptake, leading to an accumulation of intracellular ROS and subsequent inhibition of the
633 mTOR/p70S6K pathway. This cascade effectively suppresses NSCLC cell proliferation and
634 migration, while simultaneously inducing apoptosis.

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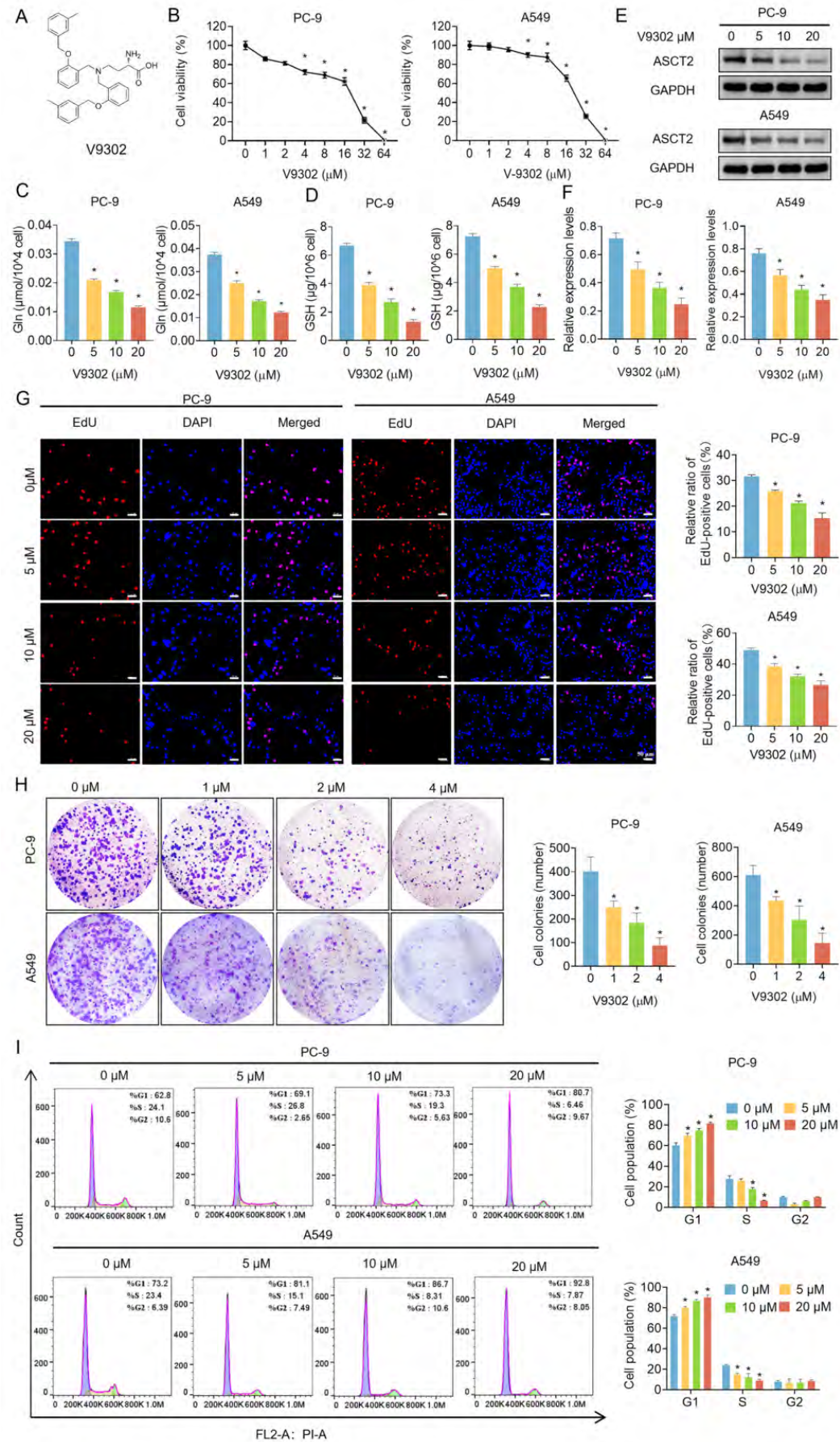


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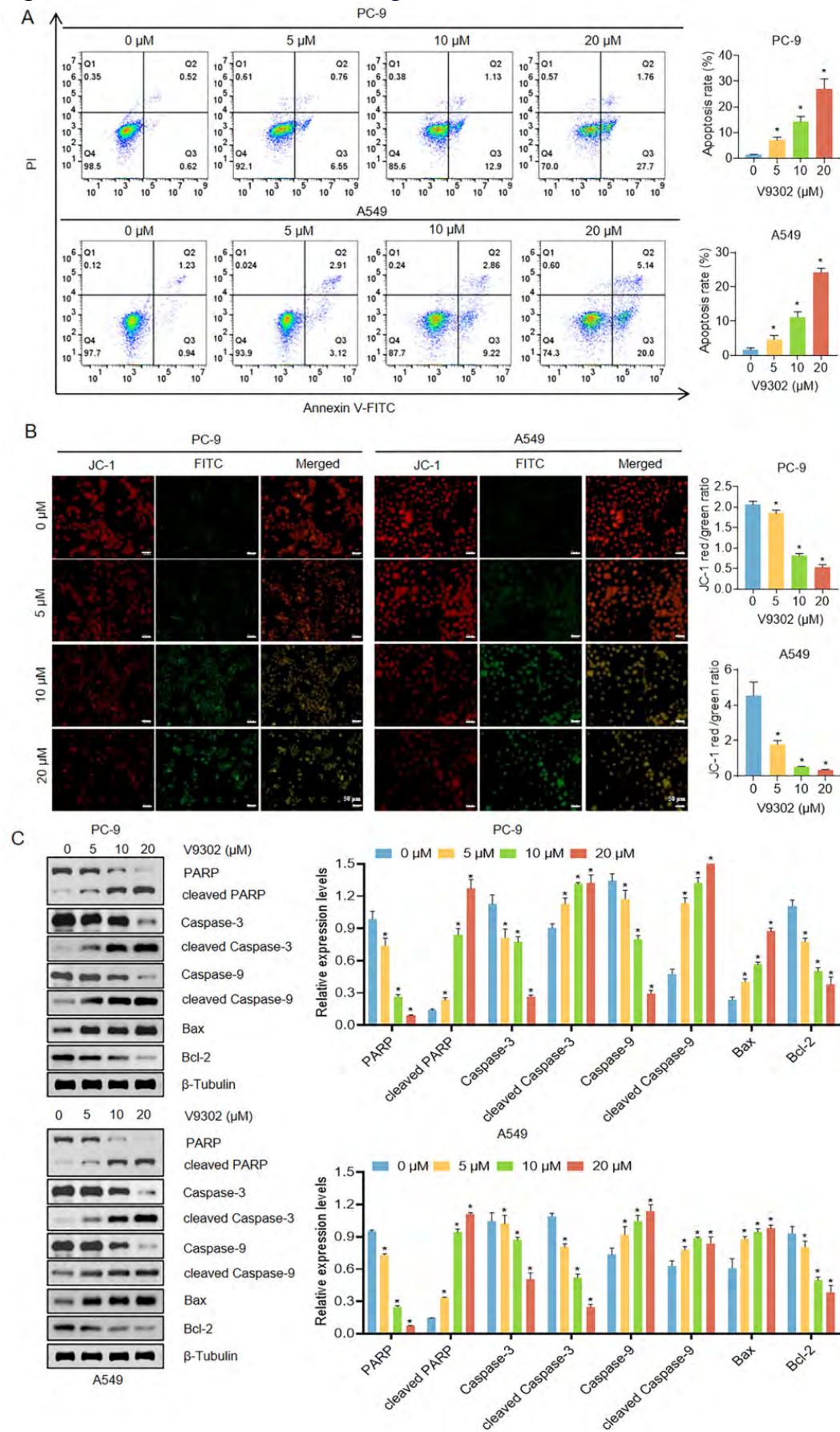


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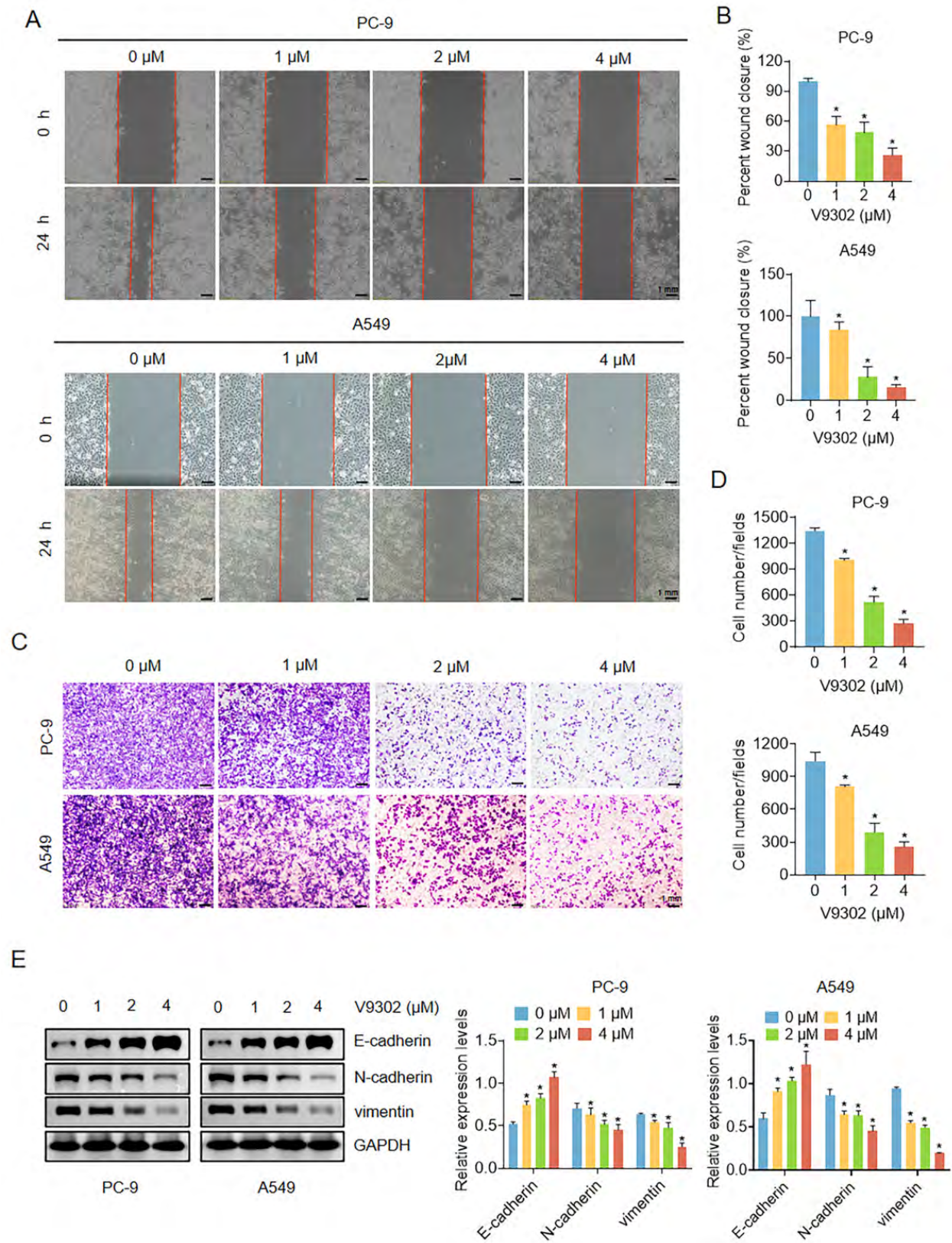


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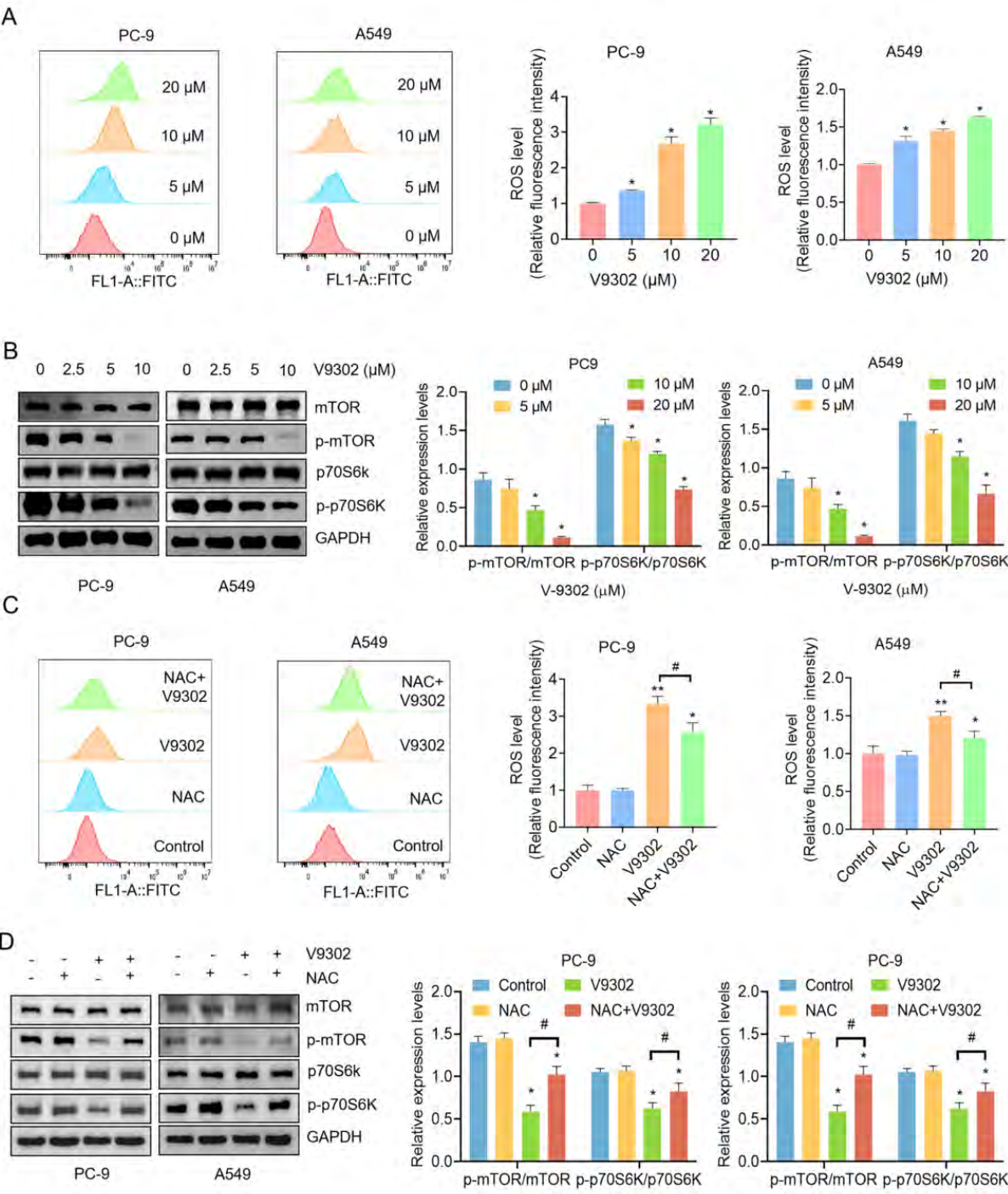


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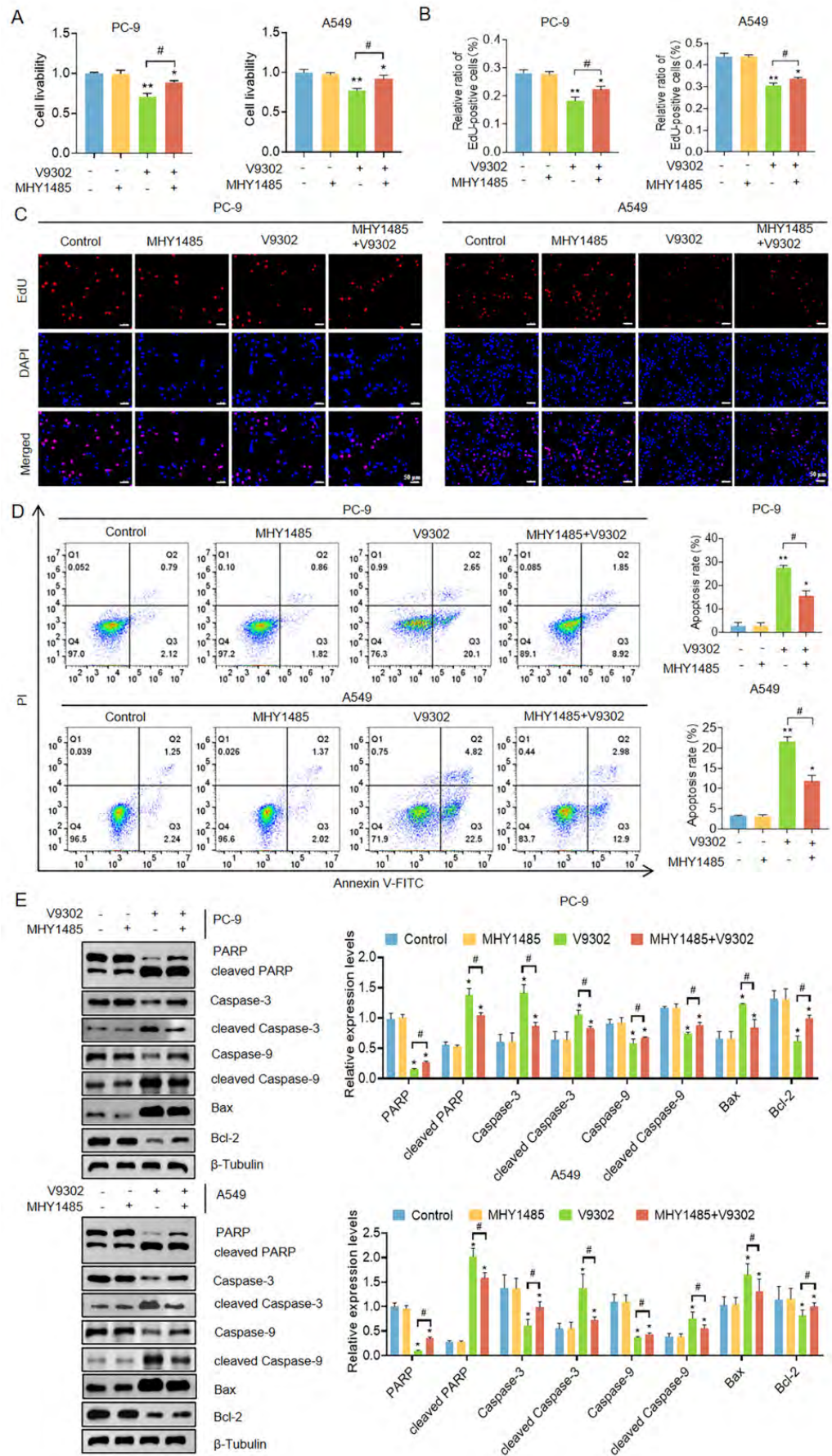


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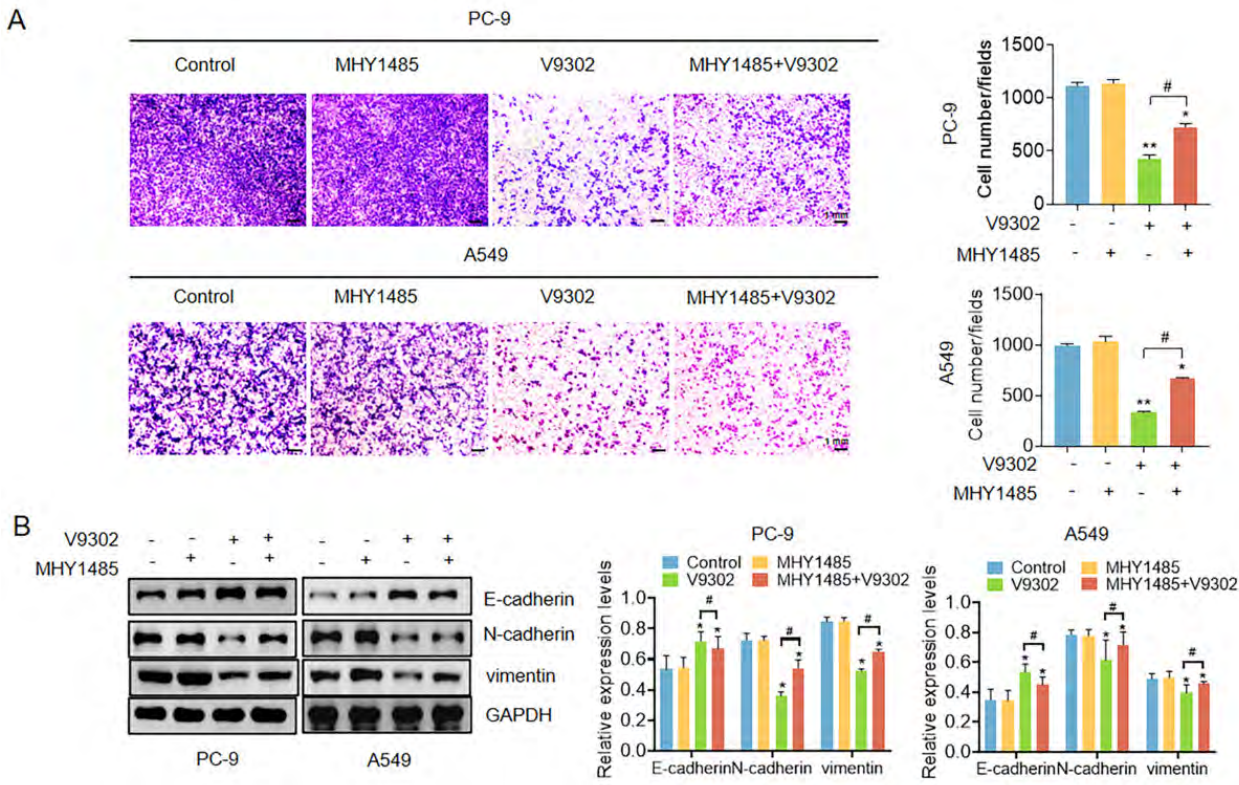


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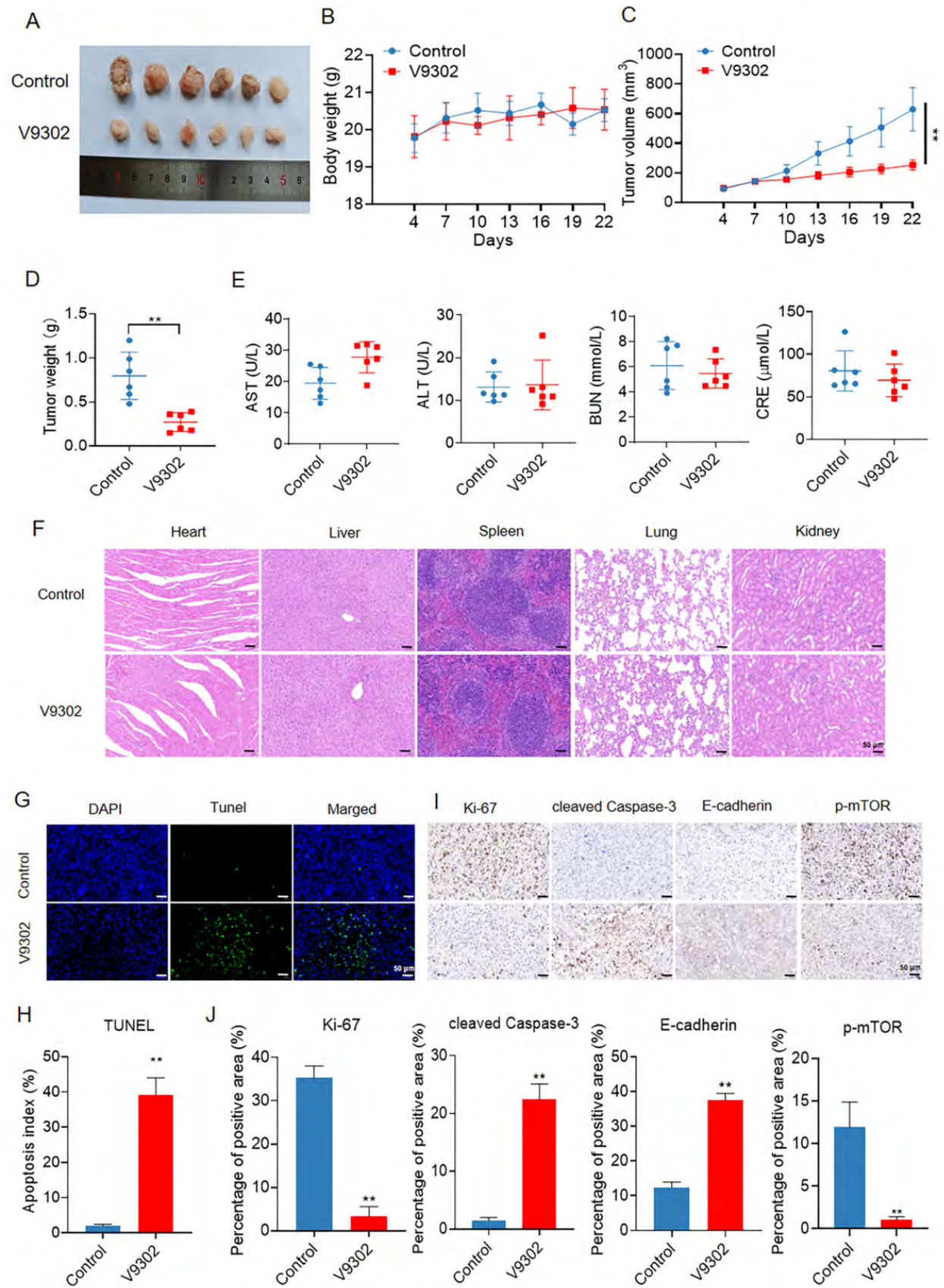


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