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Tetrahydrobenzimidazole TMQ0153 triggers apoptosis, autophagy and necroptosis crosstalk in chronic myeloid leukemia

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26 **Abstract**

27 By comparing imatinib-sensitive and -resistant CML cell models, we investigated the
28 molecular mechanisms by which tetrahydrobenzimidazole derivative TMQ0153 triggered
29 caspase-dependent apoptosis at low concentrations accompanied by loss of mitochondrial
30 membrane potential (MMP) and increase of cytosolic free Ca²⁺ levels. Interestingly, at higher
31 concentrations, TMQ0153 induced necroptotic cell death with accumulation of ROS, both
32 preventable by *N*-acetyl-L-cysteine (NAC) pretreatment. At necroptosis-inducing
33 concentrations, we observed increased ROS and decreased ATP and GSH levels, concomitant
34 with protective autophagy induction. Inhibitors such as bafilomycin A1 (baf-A1) and siRNA
35 against beclin 1 abrogated autophagy, sensitized CML cells against TMQ0153 and enhanced
36 necroptotic cell death. Importantly, TMQ0153-induced necrosis led to cell surface exposure of
37 calreticulin (CRT) and ERp57 as well as the release of extracellular ATP and high mobility
38 group box (HMGB1) demonstrating the immunogenic potential of TMQ0153. We validated
39 the anti-cancer potential of TMQ0153 by *in vivo* inhibition of K562 xenografts in zebrafish.
40 Taken together, our findings provide evidence that cellular stress and redox modulation by
41 TMQ0153 concentration-dependently leads to different cell death modalities including
42 controlled necrosis in CML cell models.

43

44 **Keywords:** Tetrahydrobenzimidazole; Metabolic stress; ROS; Cell death; Chronic myeloid
45 leukemia

46

47 **Introduction**

48 Imatinib kills leukemic cells essentially *via* apoptosis but triggers primary or
49 secondary resistance in approximately 20~30% of patients ¹. Second-generation tyrosine
50 kinase inhibitors (TKIs) such as dasatinib and nilotinib re-activate apoptotic cell death
51 induction ^{2, 3} in patients with imatinib resistance, however, *de novo* resistance against these
52 TKIs were also reported ⁴.

53 Pharmacological agents that target BCR-ABL-independent molecular targets in CML
54 by initiating non-apoptotic cell death may overcome both BCR-ABL- and apoptosis-related
55 resistance mechanisms by targeting unrelated vulnerabilities of CML cells specifically related
56 to oxidative and metabolic stress metabolism. Transformation of leukemic cells by BCR-ABL
57 is associated with metabolic alterations and increased reactive oxygen species (ROS)
58 generation ⁵. In addition, ROS levels are tightly regulated in normal hematopoiesis but
59 become chronically elevated in CML ⁶. Targeting the altered metabolism and accumulation of

60 ROS in CML cells could be of therapeutic interest as exacerbation of intracellular ROS levels
61 constitute one of the main mechanisms of most chemo- and radio-therapeutic agents,
62 eventually killing cells whether by apoptosis or programmed necrosis, depending on the cell's
63 metabolic status ⁷.

64 Controlled necrosis pathways cause a disequilibrium of the redox metabolome
65 leading to depletion of ATP and glutathione (GSH) eventually triggering an energetic
66 catastrophe. The crosstalk between the redox metabolism, autophagy and necroptosis offers
67 an interesting therapeutic target in CML. Under oxidative and metabolic stress, autophagy
68 becomes a cellular homeostasis mechanism that aims to reestablish the cellular energy
69 balance, among others ⁸ allowing cell survival under stress conditions. Moreover, autophagy
70 cross-talks with apoptosis at the level of caspase-8 degradation as well as with non-apoptotic
71 or necrotic programmed cell death mechanisms ⁹.

72 We previously reported the synthesis of various tetrahydrobenzimidazole derivatives
73 and investigated their cytotoxic potential against hematopoietic cancer cell lines compared to
74 peripheral blood mononuclear cells (PBMCs) from healthy donors ¹⁰. Among these
75 derivatives, the quinone TMQ0153 exhibited significant differential cytotoxicity against
76 cancer cells. The usefulness of such quinones as inducers of non-canonical cell death in CML
77 remains to be investigated.

78

79 **Material and methods**

80 **Compounds**

81 Tetrahydrobenzimidazole (TMQ) 0153 was synthesized from p-benzoquinone as previously
82 described ¹⁰. 2'-deoxy-5-azacytidine (5-aza; A3656), 3-methyl adenine (3-MA; M9281),
83 bafilomycin (baf-A1; #B1793), N-acetyl-L-cysteine (NAC; LAA21), thapsigargin (TSG;
84 T9033), PP242 (P0037), shikonin (SHK; S7576) and necrostatin-1 (Nec-1; N9037) were
85 purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroquine (CQ; NZ-51031-K200)
86 was purchased from Enzo Life Science (ENZ-51031-0050). Tiron (SC-253669), Trolox (SC-
87 200810), buthionine sulfoximine (BSO) (SC-200824) were obtained from Santa-Cruz
88 Biotechnology (CA, USA). Hydrogen peroxide (H₂O₂) was purchased from Junsei Chemical
89 (23150-0350) (Tokyo, Japan).

90

91 **Cell culture**

92 Chronic myeloid leukemia cell lines K562, KBM-5 and MEG01 were cultured in RPMI 1640
93 medium (Lonza, Walkersville, MD, USA) supplemented with 10 % (v/v) fetal calf serum
94 (FCS; Biowest, Riverside, MO, USA) and 1 % (v/v) antibiotic–antimycotics (Lonza,
95 Walkersville, MD, USA) at 37 °C and 5 % of CO₂. KBM-5 cells were kindly donated by Dr.
96 Bharat B. Aggarwal. Imatinib-resistant KBM5-T315I cells (KBM5R) cells were obtained by
97 sequentially increasing the concentration of imatinib from 0.25, to 1 µM imatinib in IMDM
98 media supplemented with 10 % (v/v) fetal calf serum and 1 % (v/v) antibiotic–antimycotics
99 ¹¹. Imatinib-resistant K562 (K562R) cells were a gift of the Catholic University of Seoul and
100 cultured in RPMI 1640 medium with 25 mM HEPES (Lonza) supplemented with 10 % (v/v)
101 FCS and 1 % (v/v) antibiotic-antimycotics. Both resistant cell types were cultured with 1 µM
102 of imatinib and washed three times before each experiment. Lung carcinoma A549 and breast
103 adenocarcinoma MCF7 cells were obtained from the American Type Culture Collection
104 (ATCC, Manassas, USA) were cultured in RPMI 1640 medium (Lonza, Walkersville, MD,
105 USA) supplemented with 10 % (v/v) fetal calf serum (FCS; Biowest, Riverside, MO, USA)
106 and 1 % (v/v) antibiotic–antimycotics (Lonza, Walkersville, MD, USA). Normal B
107 lymphocyte RPMI-1788 from the Korean cell line Bank (KCLB, Seoul, South Korea) were
108 cultured in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10 %
109 (v/v) fetal calf serum (FCS; Biowest, Riverside, MO, USA) and 1 % (v/v) antibiotic–
110 antimycotics (Lonza, Walkersville, MD, USA) at 37 °C and 5 % of CO₂. All cells were
111 cultured according to standard procedures.

112

113 **Cell viability and cell death assessment**

114 A Trypan blue exclusion assay (Lonza) was used to assess cell viability and IC₅₀ values were
115 also calculated on data obtained from Trypan blue assay. The mode of cell death was
116 determined and quantified after determination of the nuclear morphology was evaluated under
117 fluorescence microscopy (Nikon Eclipse Ti-U, Nikon Instruments Korea, South Korea) after
118 cell staining with 1 µg/mL Hoechst 33342 (Sigma-Aldrich,) and 1 µg/mL propidium iodide
119 staining (Sigma-Aldrich). Caspase 3/7 activity was assessed by Caspase-Glo 3/7 Assay
120 (Promega, Madison, WI, USA), and intracellular ATP levels were measured using the
121 CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

122

123 **Colony formation Assays**

124 Colony formation assays were performed as previously published ¹².

125

126 **Protein extraction and western blotting**

127 Whole cell extracts were prepared using M-PER[®] (ThermoFisher, R7007, Waltham, MA,
128 USA) supplemented by 1x protease inhibitor cocktail (Complete EDTA-free; Roche, Basal,
129 Switzerland) according to manufacturer's instructions. Western blots were performed using
130 the following primary antibodies: anti-caspase 7 (9494S), anti-caspase 9 (9502S), anti-caspase
131 8 (9746), anti-PARP-1 (9542), anti-Mcl-1 (4572S), anti-LC3B (2775), anti-p62 (5114), anti-
132 Beclin 1 (3738) and anti-RIP3 (#13526) from Cell Signaling (Danvers, MA, USA); anti-
133 caspase 3 (sc-56053), anti-PARP-1 (C2-10; sc-53643) from Santa Cruz Biotechnology (CA,
134 USA); anti-Bcl-xL (610212), anti-RIP1 (610458) from BD Pharmingen (San Jose, CA, USA);
135 anti-beta actin (5441) from Sigma Aldrich. Bands were quantified using Image Quant TL (GE
136 Healthcare, Pittsburgh, PA, USA).

137

138 **Morphology analysis**

139 For Giemsa staining, cells were spun onto a glass slide for 5 minutes at 800 g using a cytopad
140 with caps (ELITech Biomedical Systems, USA). Cells were then fixed and stained with the
141 Diff-Quik staining kit (Dade Behring S.A., USA) according to the manufacturer's protocol
142 and pictures were taken under a microscope (Nikon Eclipse Ti-U, Nikon Instruments Korea,
143 South Korea). A total of 50 cells were counted in one area, and three independent areas were
144 counted for each set of three independent experiments.

145

146 **Transmission electron microscopy**

147 For transmission electron microscopy (TEM), 5×10^6 cells were pelleted and fixed in 2.5 %
148 glutaraldehyde (Electron Microscopy Sciences, USA) diluted in 0.1 M sodium cacodylate
149 buffer, pH 7.2 (Electron Microscopy Sciences, USA) overnight. Cells were then rinsed with
150 sodium cacodylate buffer twice and post-fixed in 2 % osmium tetroxide for 2 h at room
151 temperature. Samples were washed with distilled water and then stained with 0.5 % uranyl
152 acetate at 4 °C for overnight. After 24 h, samples were dehydrated through a graded series of
153 ethanol solutions to water followed by propylene oxide, and then infiltrated in 1:1 propylene
154 oxide/Spurr's resin. Samples were kept overnight embedded in Spurr's resin, mounted in
155 molds and left to polymerize in an oven at 56 °C for 48 h. Ultrathin sections (70–90 nm) were
156 obtained with ultramicrotome, EM UC7 (Leica, Germany). Sections were stained with uranyl
157 acetate and lead citrate and subsequently examined with a JEM1010 transmission electron
158 microscope (JEOL, Japan).

159

160 **Analyses of autophagic vesicles**

161 For fluorescence microscopy analysis, 3×10^6 cells were stained with Cyto-ID® Green dye
162 and Hoechst 33342, according to manufacturer's instructions (Enzo Life Science). Cells were
163 observed by confocal microscopy (Leica TCS SP8, Germany).

164

165 **Measurement of cytosolic calcium levels**

166 Experiments were based on published procedures with modifications¹³. Cells were stained
167 with 500 nM Fluo-3-AM (Thermo Fisher, R7007, Waltham, MA, USA) for 25 min at 37 °C.
168 After 15 min at room temperature, cytosolic Ca²⁺ levels were assessed by flow cytometry
169 (FACS Calibur, Becton Dickinson, San Jose, CA, USA) and data were recorded statistically
170 (10,000 events/sample) using the CellQuest Pro software (BD, Biosciences). Data were
171 analyzed using the Flow-Jo 8.8.7 software (Tree Star, Inc., Ashland, OR, USA) and results
172 were expressed as mean fluorescence intensity (MFI).

173

174 **Determination of the oxygen consumption rate**

175 The oxygen consumption rate (OCR) was measured using a Seahorse XFp cell mito stress
176 Assay (#103010-100, Agilent, USA) ran on a Seahorse XFp analyzer (Agilent, Yongsan-gu,
177 Seoul) according to manufacturer's instructions. Briefly, cells were seeded at 200,000 cells per
178 well and treated with TMQ0153 for 4 h in 175 µL medium. Before measurements, plates were
179 equilibrated in a CO₂-free incubator at 37 °C for 1 h. Analysis were performed using 1.5 µM
180 oligomycin, 0.5 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1
181 µM rotenone/antimycin A as indicated. Data were analyzed using the Seahorse XF Cell Mito
182 stress rest report generator software (Agilent).

183

184 **Transfections**

185 Cells were transfected with 4.5 µL HiPerFect Transfection reagent (HPF; Qiagen,
186 Germantown, MD, USA,) and 5 nM small interfering RNAs (siRNAs; Qiagen) targeting the
187 human beclin 1 (BCN-1) gene [NM003766; SiBEC-1_1: Hs_BECN1_1 (SI00055573) and 10
188 nM SiBEC-1_2: Hs_BECN1_2 (SI00055580)] or non-targeting (AllStars Negative Control
189 siRNA) as described elsewhere¹⁴. 24 h post-transfection, medium was replaced, and cells
190 were treated as indicated on figures.

191

192 **Analysis of ROS, mitochondrial membrane potential, mitochondrial and lysosomal**
193 **membrane mass**

194 ROS levels were probed by using 10 μ M of 2,7-dichlorodihydrofluorescein diacetate
195 (H_2DCF -DA; Life Technologies, Carlsbad, USA) and analyzed by flow cytometry as
196 previously described¹⁵. To monitor lysosomal mass, mitochondrial membrane potential and
197 mitochondrial mass, cells were incubated at 37 °C for 30 min with 20 nM LysoTracker Red
198 DND-99, 50 nM MitoTracker Red CMXRos (all from Molecular Probes, Invitrogen, Grand
199 Island, NY, USA), respectively, and then analyzed by flow cytometry. Data were recorded
200 statistically (10,000 events/sample) using the CellQuest Pro software. Data were analyzed
201 using the FlowJo 8.8.7 software and results were expressed as mean fluorescence intensity
202 (MFI).

203

204 **Glutathione measurements**

205 Reduced (GSH) and oxidized (GSSG) glutathione measurements were performed using the
206 GSH/GSSG-Glo™ Assay kit (Promega, Madison, WI, USA) following the manufacturer's
207 instructions. The luminescence signal was acquired using with a microplate luminometer
208 Centro LB 960 and data were recorded using the MikroWin 2000 software package (Berthold
209 Technologies, Bad Wildbad, Germany).

210

211 **Quantification of HMGB1 release**

212 Quantification of HMGB1 release in cell culture supernatants was assessed by enzyme-linked
213 immunosorbent assay kit from Shino-Test-Corporation (Jinbocho, Chiyoda-ku, Tokyo, Japan)
214 according to the manufacturer's instructions. Absorbance data were collected using with a
215 SpectraMax i3x microplate reader and data were recorded using the SoftMax Pro 7.0 software
216 package (Sunnyvale, California, USA).

217

218 **Measurement of extracellular ATP content**

219 Extracellular ATP levels in the supernatant were assessed by the ENLITEN® ATP Assay
220 system bioluminescence detection kit (Promega, Madison, WI, USA) following the
221 manufacturer's protocol. Luminescence signal was acquired with a microplate luminometer
222 Centro LB 960 and data were recorded using the MikroWin 2000 software package (Berthold
223 Technologies, Bad Wildbad, Germany).

224

225 **Analysis of calreticulin and ERp57 exposure**

226 Cells were collected, washed twice with 1x PBS and fixed in 0.25 % paraformaldehyde in 1x
227 PBS at 4 °C. After 5 min incubation, cells were washed twice in cold 1x PBS and incubated for

228 30 min at RT with anti-calreticulin (CRT; Ab2907, Abcam, Cambridge, UK) or ERp57
229 (Ab10287, Abcam) primary antibody diluted (1:50) in cold blocking buffer (2 % FCS in 1x
230 PBS) and then incubated for 30 min with an Alexa488-conjugated monoclonal secondary
231 antibody (A11034) diluted (1:50) in blocking buffer. Isotype-matched Alexa488-conjugated
232 IgG antibodies were used as a control. Samples were then analyzed by flow cytometry. Data
233 were recorded statistically (10,000 events/sample) using the CellQuest Pro software and
234 analyzed using the Flow-Jo 8.8.7 software Results were expressed as mean fluorescence
235 intensity (MFI). Samples were also analyzed by fluorescence microscopy (Nikon Eclipse Ti-
236 U, Nikon Instruments Korea, South Korea).

237

238 **Zebrafish toxicity assays and cancer cell xenografts**

239 Cancer xenograft assays were performed as previously published ¹². Briefly, 200 K562 cells
240 were stained for 2 h by 4 μ M of Cell tracker CM-Dil dye (Invitrogen, Grand Island, NY,
241 USA), then treated with TMQ0153 at indicated concentrations for 8 h and injected as
242 described.

243

244 **In silico drug-likeness properties**

245 In silico drug-likeness properties according to Lipinski's 'rule-of-five' and other parameters
246 for drug-likeness and oral bioavailability were evaluated by using the SCFBio website
247 (www.scfbio-iitd.res.in/).

248

249 **Bioinformatics analysis**

250 The Microarray Innovations in Leukemia (MILE) dataset ^{16,17} (GSE13159) was downloaded
251 from the Gene Expression Omnibus repository ¹⁶ and normalized using the Robust Multichip
252 Average (RMA) algorithm from the affy R package (version 1.62.0) ¹⁸. Boxplot was
253 generated using the *ggboxplot* function of the *ggpubr* R package (version 0.2.2) in R 3.6.0 ¹⁹
254 and RStudio ²⁰.

255

256 **Statistical analysis**

257 Data are expressed as the mean \pm S.D. and significance was estimated by using one-way or
258 two-way ANOVA tests using Prism 8 software, GraphPad Software (La Jolla, CA, USA).
259 Statistical significances were evaluated at *p*-values below 0.05 and represented by the
260 following legend: **p* \leq 0.05, ***p* \leq 0.01, ****p* \leq 0.001; posthoc analyses Dunnett; Sidak;
261 Tukey). All histograms represent the mean \pm SD of at least three independent experiments.

262

263 **Results**

264 **Cellular uptake and drug-likeness potential of TMQ0153 in human leukemia cells**

265 We first assessed the cellular uptake of auto-fluorescent TMQ0153 by K562 cells
266 using flow cytometry after 8 h, 24 h, 48 h and 72 h of treatment with concentrations up to 50
267 μ M. Auto-fluorescence was detected in all three channels (FL1, FL2 and FL3) up to 72 h
268 (**Suppl. Fig. 1A**) and TMQ0153 (50 μ M) was localized by confocal microscopy in the
269 cytoplasm after 24 h of treatment (**Suppl. Fig. 1B**) validating the time- and dose-dependent
270 uptake of TMQ153. Moreover, TMQ153 follows Lipinski's 'rule of five' (**Table I**),
271 confirming its potential drug-likeness properties.

272

273 **TMQ0153 inhibits cell viability, proliferation and colony formation capacity of drug-** 274 **sensitive and -resistant cancer cells**

275 We then validated the effect of TMQ0153 (**Fig. 1A**) on imatinib-sensitive (**Fig. 1B, C**)
276 and -resistant K562 (K562R) KBM5, imatinib-resistant KBM5 (KBM5R) and MEG01 cells
277 (**Suppl. Fig. 2A-D**). TMQ0153 decreased colony formation capacity of various imatinib-
278 sensitive and resistant CML cell types (**Fig. 1D and Suppl. Fig 2E**). To extend our findings
279 to an *in vivo* situation, zebrafish K562 xenograft formation was dose-dependently reduced by
280 TMQ153, compared to controls (**Suppl. Fig 3**). To assess for acute toxic side effects, no
281 morphological alterations or toxicity of developing zebrafish larvae were observed at
282 concentrations up to 50 μ M, confirming the safety of this compound. (**Fig. 1E**). We
283 generalized our findings by using solid tumor cell lines lung A549, prostate PC3 and breast
284 MCF7 cancer cells where TMQ153 also induced cell death (**Table II**).

285

286 **TMQ0153 induces concentration-dependent differential cell death modalities**

287 We then investigated the cell death mechanism induced by TMQ0153. As shown in
288 **Fig. 2A and Suppl. Fig. 4A**, apoptotic cell death was induced at concentrations up to 20 μ M
289 after 24 h, rescued by z-VAD pretreatment. Non-apoptotic, z-VAD-insensitive cell death was
290 dose-dependently induced at concentrations over 30 μ M after 8, 24, 48 and 72 h (**Fig. 2A and**
291 **Suppl. Fig. 4A-B**) confirming a concentration-dependent induction of caspase-dependent and
292 -independent cell death modalities in both K562 and K562R cells. As shown in **Fig. 2B**, after
293 24 h of treatment with TMQ0153, procaspase-9, -8 and -3 were significantly activated leading
294 to PARP-1 cleavage. TMQ0153 treatment led also to a decrease of the anti-apoptotic proteins
295 myeloid cell leukemia (Mcl)-1 and B-cell lymphoma-extra large (Bcl-xL) in a dose-dependent

296 manner (**Fig. 2C**). We confirmed our findings by measuring an increased caspase 3/7 activity
297 following a treatment with up to 20 μ M TMQ0153 for 24 h, which was abrogated in presence
298 of z-VAD (**Fig. 2D**).

299 **TMQ0153 activates necrostatin-1 sensitive necroptotic cell death**

300 We then investigated the mechanisms involved in TMQ0153-mediated non-apoptotic
301 cell death at higher concentrations (*i.e.* from 30 μ M) after 8 and 24 h in K562 cells. We pre-
302 treated K562 cells with the RIP1 inhibitor Nec-1 to assess for necroptosis induction.
303 TMQ0153 induced over 50 % of non-apoptotic, PI positive cells after 24 h of treatment with
304 50 μ M. Nec-1 pretreatment significantly prevented non-apoptotic cell death induction
305 between 30-50 μ M after 24 h, compared to shikonin, used as a positive control (**Fig. 3A**).
306 These results indicated that TMQ0153 induced cell death in K562 cells *via* the necroptotic
307 cell death pathway.

308 Necroptosis is known to be accompanied by a modulation of intracellular ATP,
309 increased expression levels of RIP1 and the appearance of a non-apoptotic cleavage fragment
310 of PARP-1 between 50 and 75 kDa²¹. Our results showed a significant decrease of
311 intracellular ATP levels after 4, 8, 16 and 24 h at 30 μ M TMQ0153 compared to 20 μ M (**Fig.**
312 **3B**). Western blot results confirmed that TMQ0153-treated cells presented increased RIP1
313 expression levels and this expression was inhibited by a Nec-1 pretreatment. Also, the
314 differentially cleaved necrotic PARP1 fragment revealed by the C2-10 antibody, supported
315 that TMQ0153 induced necrotic cell death at 30 μ M after 24 h of treatment (**Fig. 3C**).
316 Besides, we observed a concomitant accumulation of cytosolic Ca²⁺ levels at 30 and 50 μ M
317 TMQ0153 (**Fig. 3D**). Hematopoietic cell lines largely lack RIP3 expression and this loss of
318 RIP3 may reduce sensitivity against cytotoxic agents²².

319 We then investigated the basal expression levels of RIP3 by western blot analysis in
320 different cancer cell types with known RIP3 expression levels. As expected, RIP3 was
321 silenced in K562, A549, PC3 and MCF7 cells (**Suppl. Fig. 5A**). We then treated K562 cells
322 with the DNA hypomethylating agent 5-aza-2'-deoxycytidine (5-aza) at 1 μ M for 6 days to
323 re-express RIP3. As shown in **Suppl. Fig 5B**, 5-aza treatments increased RIP3 protein
324 expression levels. We then co-treated these cells with TMQ0153 to investigate potential
325 sensitization. Results showed that TMQ0153 treatment of 5-aza-pretreated K562 cells led to
326 significantly enhanced cell death levels (54 %) compared to TMQ0153 treatment alone
327 (21 %). Overall, our results indicated that the cytotoxicity of TMQ0153 in K562 cells is
328 mediated by necroptosis at higher concentrations. In addition, a pretreatment with DNA

329 hypomethylating agents like 5-aza may further potentialize the effect of TMQ0153 in CML
330 cells devoid of RIP3.

331

332 **TMQ0153 induces an early onset of autophagy in K562 cells followed by controlled** 333 **necrosis**

334 As we observed extensive vacuole formation (**Suppl. Fig. 6A**) in treated K562 cells
335 prior to cell death induction, we hypothesized that TMQ0153 was triggering an initial
336 intracellular stress reaction, potentially *via* autophagy. An initial morphological analysis of
337 TMQ0153-treated K562 cells by confocal microscopy and Diff-Quik staining allowed
338 quantifying early vesicle formation (**Suppl. Fig. 6B**). Treatment of K562 cells with
339 TMQ0153 at necroptosis-inducing concentrations (30 μ M) for 2, 4, 6 and 8 h showed the
340 onset of vesicle formation by confocal microscopy after CYTO-ID staining further
341 ascertaining autophagic activity (**Fig. 4A**). Western blot analysis demonstrated that TMQ0153
342 time-dependently induced the conversion of LC3-I to LC3-II at 30 μ M after 2, 4, 6 and 8 h,
343 prior to necroptosis induction. In agreement with these results, sequestosome-1
344 (SQSTM1)/p62 (**Fig. 4B**) was degraded. Moreover, in the presence of the lysosome inhibitor
345 baf-A1, we observed an enhanced accumulation of LC3-II and p62 levels in TMQ0153-
346 treated cells after 2 and 4 h confirming an active autophagic flux (**Fig 4C**). Results were
347 confirmed by transmission electron microscopy (TEM) confirming extensive vacuolization
348 (**Fig. 4D**).

349 To investigate the effect of TMQ0153 on cellular metabolism, we used a Seahorse
350 XF Analyzer. Our results showed a significant decrease of the oxygen consumption rate
351 (OCR) upon 4 h of treatment with 30 μ M TMQ0153 (**Fig. 5A**), which precedes necroptosis
352 induction. As these results indicated that TMQ0153 disrupted mitochondrial bioenergetics, we
353 further detected mitochondria with damaged morphologies by TEM (4h, 30 μ M) in line with
354 the observed metabolic alterations.

355 Considering the antagonizing effect of autophagy and apoptosis, we then investigated
356 the effect of autophagy induction on caspase-8 expression levels. Our results showed a
357 progressive reduction of pro-caspase-8 levels during autophagy induction between 2 and 8 h
358 with 30 μ M TMQ0153 (**Fig. 5B**). Moreover, inhibition of autophagy by 3-methyladenine (3-
359 MA) prevented reduction of pro-caspase-8 levels (**Fig. 5C**). These results are in line with
360 previous observations, where the onset of autophagy is antagonizing apoptotic cell death and,
361 as a result, favoring caspase-independent cell death modalities.

362

363 **Autophagic inhibitors augment necroptosis induced by TMQ0153 in K562 cells**

364 To investigate the relationship between TMQ0153 induced autophagy and
365 necroptosis, K562 cells were treated with TMQ0153 in the presence of autophagic inhibitors.
366 Nuclear morphology analyses showed that the inhibition of TMQ0153-induced autophagy by
367 baf-A1 significantly enhanced necroptosis in K562 cells compared to cells treated with
368 TMQ0153 alone (**Fig. 6A**). In addition, baf-A1-, 3-MA- and CQ-mediated inhibition of
369 autophagy led to necroptotic cell death in line with a switch from apoptotic to necrotic cell
370 demise and concomitant with the reduction of procaspase-8 levels (**Fig. 5B**).

371 To avoid non-specific effects of chemical inhibitors, we then also investigated the
372 effect of TMQ0153-induced autophagy in beclin 1-siRNA-transfected cells. Silencing beclin
373 1 enhanced necroptosis (**Fig. 6B**) and decreased autophagy (**Fig. 6C**). In agreement with our
374 hypothesis, TMQ0153-treated beclin 1 knockdown cells showed a reduction of caspase3/7
375 activity levels. Moreover, enhanced levels of necrotic PARP-1 cleavage (50-75 kD) were
376 detected when using the C2-10 anti-PARP antibody (**Fig. 6D, E**).

377

378 **CML patient cells are characterized by increased expression levels of genes involved in**
379 **oxidative stress**

380 We used a patient cohort regrouping 74 healthy donors and leukemia patients including 76
381 patients that were diagnosed with CML^{16,17} to assess the gene expression level of oxidative
382 stress-related genes. In particular, we were interested in checking the gene expression level of
383 the NADPH oxidases (NOX) family constituted of 7 members (NOX1-NOX5, DUOX1,
384 DUOX2), as they are largely responsible for the production of ROS²³. Our results show that
385 the expression level of NOX regulator cytochrome b-245 heavy chain (CYBB) was more
386 elevated in CML patients compared to healthy donors (**Suppl. Fig. 7**) leading to an increased
387 NOX activity and thus an increase in ROS production²⁴. Based on these observations, we
388 hypothesized that the resulting increased ROS levels in CML patient cells could be
389 therapeutically targeted by exacerbation of intracellular ROS levels by TMQ153 leading to a
390 pro-oxidant treatment approach.

391

392 **TMQ0153 depolarizes mitochondrial membrane potential and triggers necroptotic cell**
393 **death through ROS formation**

394 Accumulation of intracellular ROS is known to depolarize the mitochondrial
395 membrane potential (MMP). First, we performed a morphological analysis of mitochondria
396 by TEM after treatment of K562 with TMQ0153 (30 μ M). Our results showed that 30 μ M

397 TMQ0153 induced mitochondrial morphological changes such as enlarged and swollen
398 mitochondria compared to control after 8 h (**Fig. 7A and Supplementary Fig 8A-B**). Next,
399 we measured the MMP in K562 cells after 24h of treatment with TMQ0153. Results
400 demonstrated that TMQ0153 increased the proportion of cells with low MMP in a dose-
401 dependent manner (**Fig. 7B**). In addition, TMQ0153-mediated reduced cell viability and
402 MMP loss were totally prevented by the ROS scavenger NAC (**Fig. 7C**).

403 Next, we investigated the implication of ROS in the generation of necroptotic cell
404 death²⁵. To identify the nature and roles of intracellular ROS induced by TMQ0153, K562
405 cells were pretreated with and without various antioxidants: NAC, Trolox and Tiron.
406 TMQ0153 treatment triggered ROS production. However, apoptotic concentrations of
407 TMQ0153 (20 μ M) did not induce significant levels of ROS compared to a necrosis-inducing
408 concentration (30 μ M) after 4 and 8 h whereas pretreatment with NAC significantly abrogated
409 ROS formation at 30 μ M (**Fig. 8A and Suppl. Fig. 9A**) in both K562 and K562R cells.
410 Vitamin E derivative Trolox that reduces the levels of lipid peroxidation when the oxidation
411 was initiated inside the plasma membrane²⁶ or mitochondrially-localized Tiron²⁷ did not
412 significantly reduce ROS levels (**Suppl. Fig. 9B**).

413 Since the balance of intracellular reduced and oxidized glutathione (GSH) levels
414 reflects the redox state of the cells, we evaluated total GSH levels and the GSH/GSSG ratio
415 after 4 and 24 h of treatment. Results revealed that both total GSH levels and GSH/GSSG
416 ratio were significantly reduced upon 30 μ M TMQ0153 treatment (**Fig. 8B**). After GSH
417 depletion, redox and cellular stress are considered to be potent inducers of mitochondrial
418 dysfunction through ROS accumulation²⁸. Therefore, we examined the effect of Nec-1 on
419 intracellular ROS accumulation by TMQ0153 and found that Nec-1 pretreatment decreased
420 cellular ROS levels in TMQ0153-treated K562 cells after 4 and 8 h (**Fig. 8A**). This finding
421 could suggest that Nec-1 protects against TMQ0153-induced necroptosis by suppressing the
422 intracellular burden of ROS formation after necroptosis induction. Finally, ROS-induced
423 LMP is increasing in K562 cells after TMQ0153-induced necroptotic cell death in a time-
424 dependent manner at 30 μ M as demonstrated by fluorescence microscopy and FACS (**Fig.**
425 **8C**).

426

427 **TMQ0153-treated K562 cells release immunogenic cell death markers**

428 Our results showed a decrease in intracellular ATP levels and increased
429 mitochondrial ROS and cytoplasmic Ca²⁺ levels eventually concomitant with necroptosis
430 induction. Since these changes were described to contribute to the immunogenicity of the

431 dying cells, we also assessed the release of HMGB1 and demonstrated that TMQ0153
432 triggered accumulation HMGB1 in the supernatant in a dose- and time-dependent manner in
433 K562 cells (**Fig. 9A**). In addition, 24 h of treatment with TMQ0153 induced extracellular
434 ATP secretion into the supernatant of K562 cells at 30 and 50 μ M (**Fig. 9B**). Furthermore, the
435 potential immunogenic signal from dying cells also includes proteins that are exposed at the
436 surface of stressed or dying cells. Results showed that TMQ0153-treated cells increased
437 significantly the ectopic expression of calreticulin (Ecto-CRT) (**Fig. 9C and Suppl. Fig10A**)
438 and Ecto-ERp57 (**Fig. 9D and Suppl. Fig10B**). These results indicate that TMQ0153-induced
439 necroptosis could further augment immunogenicity of dying K562 cells.

440

441 **Discussion**

442 CML still has high morbidity and mortality among the leukemia patients²⁹. Even
443 though the development of TKIs is an effective treatment against CML, severe side effects
444 and mutations of BCR-ABL are considered as one of main reasons for drug resistance³⁰. For
445 this reason, novel therapies that target the molecular or metabolic characteristics of CML are
446 highly required.

447 In the present study, we attempted to address the interplay between apoptosis,
448 autophagy and necroptosis in CML cell models, by using an experimental pro-oxidant
449 therapeutic approach with the cytotoxic synthetic hydroquinone derivative TMQ0153 aiming
450 to disrupt oxidative and metabolic stress homeostasis. As a result, we observed a ROS-and
451 concentration-dependent induction of protective autophagy eventually leading to Nec-1-
452 sensitive necroptosis, whereas low TMQ0153 concentrations do not trigger any significant
453 increase of ROS levels and led to caspase-dependent apoptosis.

454 Quinone derivatives such as 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin)
455 induce apoptosis or necrosis in AML by producing ROS and decreasing Mcl-1 and Bcl-2 anti-
456 apoptotic proteins³¹. However, the effect of quinone-induced ROS on the apoptotic
457 machinery remains largely unknown. In our study, 20 μ M TMQ0153 induced apoptosis in
458 K562 cells along with caspase 8, 9 and 3 cleavage, that are both prevented in presence of z-
459 VAD. In addition, we found that apoptosis was associated with an early downregulation of
460 anti-apoptotic protein Mcl-1 and Bcl-xL after TMQ0153 treatment.

461 We showed that TMQ0153 induces cell death in K562 cells *via* a RIP1-dependent
462 necroptotic cell death pathway. 2,3,5-tris-hydroquinone was reported to induce ROS
463 production and increase intracellular Ca^{2+} levels that contribute to PARP-1-mediated necrosis
464 in HK-2 cells³². β -lapachone is reduced to β -lapachone hydroquinone, which induces

465 programmed necrosis through the ROS production and a RIP1-dependent cell death pathway
466 in human hepatocellular carcinoma³³. Indeed, our results showed that as a pro-oxidant
467 TMQ0153 induced necroptosis through downstream mediators including RIP1 leading to
468 mitochondrial dysfunction as a response to energy depletion. Previous research investigated
469 that cells lacking RIP3 expression were resistant to typical programmed necrotic stimuli but
470 became sensitive when RIP3 is re-expressed²². Our results supported these results as we
471 observed a sensitization against TMQ0153 by the re-expression of RIP3 after treatment with
472 the DNA demethylating agent 5-azacitidine²².

473 Autophagy is triggered as a pro-survival strategy in human cancer cells treated with
474 mammalian target of rapamycin (mTOR) inhibitor rapamycin³⁴, sarco/endoplasmic reticulum
475 (ER) Ca²⁺-ATPase (SERCA) inhibitor stemphol¹³ or the naphthoquinone shikonin³⁵. Indeed,
476 the inhibition of shikonin-induced autophagy increased necroptosis as well as PARP-1-
477 mediated cell death in A549³⁵. Our results support the idea that autophagy protects against
478 necroptosis in TMQ0153-treated K562 as we confirmed that autophagy was detected at early
479 time points before the induction of necroptotic cell death with 30 μM TMQ0153 most likely
480 due to an early cellular stress response in K562 cells.

481 In cancer cells, metabolic stress may arise from insufficient energy or oxygen supply.
482 Autophagy can be induced as an alternative source of energy and metabolites³⁶. Our results
483 showed that K562 cells treated with autophagy inhibitors such as baf-A1 enhanced
484 necroptotic cell death concomitantly with a non-apoptotic degradation of caspase-8, thus
485 preventing canonical apoptosis induction. These results were confirmed by a profile of PARP-
486 1 degradation typical of necrosis/necroptosis.

487 In beclin 1-silenced K562 cells, TMQ0153 induced the appearance of necrotic PARP-
488 1 band and enhanced necroptotic cell population suggesting that autophagy serves a protective
489 role. In addition, we observed that TMQ0153 induced autophagic responses in K562 cells as
490 evidenced by LC3-positive autophagy like vacuoles, and the increased conversion of LC3-I to
491 LC3-II. We also found that pretreatment with baf-A1, which blocks autophagosomal
492 degradation, increased the formation of LC3-II in K562 cells after treatment with TMQ0153.

493 We provided evidence that TMQ0153 induced the dysfunction of mitochondria as
494 shown by TEM and OCR analysis, as well as oxidative stress *via* increasing ROS levels and
495 triggering accumulation of cytoplasmic Ca²⁺ levels. GSH is an important antioxidant in
496 cellular metabolism and the reduction of GSH levels was described to induce autophagy,
497 apoptotic or necrotic/necroptotic cell death^{37,38}. We observed that intracellular GSH levels
498 decreased during the onset of autophagy and necroptosis induced by TMQ0153. This means

499 that TMQ0153, as a pro-oxidant compound, amplifies ROS stress in K562 cells prior to
500 necroptosis induction. Necroptotic cell death potentially further exacerbates ROS generation.

501 Weak release of lysosomal enzymes leads to apoptosis, whereas a massive release of
502 lysosomal enzymes results in necrosis ³⁹. In our study, TMQ0153 induced increased ROS and
503 LMP at early time points but are decreased later when necroptosis is induced. Wiedmer et al.
504 showed that autophagy is upregulated for the clearance of damaged lysosomes, leading to cell
505 recovery, thus playing a pro-survival role ⁴⁰. Hence, lysosomal instability triggered by
506 TMQ0153 contributes to final steps of necroptosis. Besides LMP, mitochondrial dysfunction
507 is another executioner of necroptosis ²⁵. Our results showed that TMQ0153 treatment
508 generated mitochondrial alterations and dysfunction associated with a loss of MMP.

509 Necroptosis triggers cell membrane rupture and the release of cellular cytoplasmic
510 contents into the extracellular spaces such as HMGB1 and ATP ¹¹. TMQ0153 treatment
511 significantly released HMGB1 and increased extracellular ATP levels. Immunogenic cell
512 death (ICD) is a form of chemotherapy-induced tumor cell death, which is mediated by
513 damage associated molecular patterns (DAMPs) that triggers effective antitumor immune
514 responses ⁴¹. ICD-inducers are able to mediate endoplasmic reticulum (ER) stress resulting in
515 the cell surface presentation of calreticulin and ERp57 ^{11,42}. Here, we showed that TMQ0153
516 treatment led to CRT and ERp57 surface expression. These results provided evidence that
517 TMQ0153 released ICD markers as an essential feature of this chemotherapeutic compound.

518 In summary (**Fig. 10**), TMQ0153 possesses a potent pro-oxidant capacity effective
519 against imatinib-sensitive and -resistant CML cells underlining the interest of TMQ0153 as an
520 experimental pre-clinical therapeutic agent accompanied by release of ICD markers.

521

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531

532 **Conflict of interest statement**

533 The authors declare no conflict of interest.

534

535 _____

536 **Tables**

537

538 **Table I: *In silico* prediction for the drug-likeness of TMQ0153 compared to**
539 **hydroquinone based on Lipinski's 'rule of five'⁴³.**

Drug-likeness parameter	Values		
	Theoretical	TMQ0153	Hydroquinone
MW (kDa)*	$180 \leq x \leq 500$	276.5	110
Hydrogen bond donors	≤ 5	1	2
Hydrogen bond acceptors	≤ 10	4	2
LogP (lipophilicity)	≤ 5	2.16	1.10
Molar refractivity	$40 \leq x \leq 130$	74.5	29.8

540 *MW: molecular weight

541

542

543 **Table II: Effect of TMQ0153 on human cancer cell viability.**

Cancer model		IC ₅₀ (μM)*			
Cancer type	Cell line	8 h	24 h	48 h	72 h
Blood	K562	> 50	35.4 ± 0.6	28.7 ± 0.7	26.1 ± 0.7
	K562R	> 50	> 50	> 50	43.4 ± 1.8
	KBM5	> 50	> 50	20.0 ± 2.3	14.5 ± 0.3
	KBM5R	> 50	> 50	20.7 ± 2.7	15.1 ± 0.8
	MEG01	> 50	> 50	45.3 ± 2.7	28.8 ± 1.7
Lung	A549	> 50	> 50	> 50	> 50
Prostate	PC3	> 50	> 50	> 50	49.0 ± 0.9
Breast	MCF7	> 50	> 50	> 50	46.7 ± 2.4

544 *IC₅₀ values were calculated on data obtained from Trypan blue assays and represent the mean ± S.D. of
 545 three independent experiments.

546 R: Imatinib-resistant.

547

548

549 **Figure Legends**

550

551 **Fig. 1. Effect of TMQ0153 on chronic myeloid leukemia cell viability.** (A) Chemical
552 structure of TMQ0153. (B) Time- and (C) dose-dependent effect of TMQ0153 on K562 cell
553 proliferation (left panel) and viability (right panel). (D) Inhibitory effect of increasing
554 concentrations of TMQ0153 on the colony forming capacities of the indicated CML cell lines.
555 Upper panel: pictures representative of three independent experiments. Lower panel:
556 quantification of the colony numbers. (A-D) All data represent mean (\pm S.D.) of three
557 independent experiments. Statistical significance was assessed as * $p < 0.05$, ** $p < 0.01$, *** p
558 < 0.001 compared to untreated cells. Two-way ANOVA (Cell viability); post hoc: Sidak's
559 test. One-way ANOVA (Colony formation); post hoc: Sidak's test. (E) Acute toxicity assay
560 on zebrafish embryos after 24 h of treatment with increasing concentrations of TMQ0153.
561 Pictures are representative of 10 fishes used for each condition (left panel) and the
562 corresponding quantification of viable embryos (right panel). Ethanol (EtOH, 70 %) was used
563 as a positive control for toxicity.

564

565 **Fig. 2. TMQ0153 triggered a concentration-dependent induction of caspase-dependent**
566 **and independent non-apoptotic cell death in K562 cells.** (A-D) K562 cells were treated
567 with various concentrations of TMQ0153 in presence or absence of the pan caspase inhibitor
568 carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD; 50 μ M). (A)
569 After 8, 24, 48 and 72 h of treatment the type of cell death triggered by TMQ0153 was
570 characterized by fluorescence microscopy after Hoechst/propidium iodide (PI) staining.
571 Pictures representative of three independent experiments (top panel). (B) Analysis of caspase
572 and poly [ADP-ribose] polymerase (PARP)-1 cleavage by western blot after 24 h of
573 treatment. (C) Effect of 24 h of treatment on Mcl-1 and Bcl-xL protein expression levels (top
574 panels) and the corresponding quantifications (middle and lower panels). (B-C) β -actin was
575 used as loading control. (D) Quantification of caspase- 3/7 activity levels after 24 h of
576 treatment. Etoposide (Eto; 100 μ M, 24 h) was used as a positive control for apoptosis
577 induction. All pictures are representative of three independent experiments and graphs
578 represent the mean (\pm S.D.) of three independent experiments. Statistical significance was
579 assessed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells. Two-way
580 ANOVA (microscopy analysis); post hoc: Sidak's test. One-way ANOVA (caspase-3/7
581 assay); post hoc: Tukey's test. One-way ANOVA (western blot); post hoc: Dunnett's test.

582

583 **Fig. 3. TMQ0153 induced a necrostatin-1-sensitive type of cell death in K562 cells.** (A)
584 K562 cells were incubated in presence or absence of 60 μ M necrostatin (Nec)-1 for 1 h before
585 a treatment with the indicated concentrations of TMQ0153. Shikonin (SHK; 5 μ M) was used
586 as a positive control for necrosis induction. (A) Nuclear morphology analyses by fluorescence
587 microscopy following Hoechst/ propidium iodide (PI) staining after 8 and 24 h of treatment.
588 Pictures representative of three independent experiments (left panels) and the corresponding
589 quantification (right panels). (B) Measurement of intracellular ATP levels at the indicated
590 concentrations and time points. (C) Receptor-interacting protein kinase (RIP)1 protein levels
591 and PARP-1 cleavage were determined by western blotting using C2-10 antibody. Shikonin
592 (SHK; 5 μ M, 24 h) and necrostatin (Nec)-1 (60 μ M, 1 h) were used as a positive control and
593 inhibitor for RIP1. Etoposide (Eto; 100 μ M, 24 h) and ethanol (EtOH; 10 %, 2 h) were used
594 as positive controls for apoptotic and necrotic PARP-1 cleavage, respectively. (D) Cytosolic
595 Ca^{2+} levels were measured using Fluo-3-AM. Thapsigargin (TSG; 300 nM, 24 h) was used as
596 a positive control for intracellular Ca^{2+} accumulation. β -actin was used as loading control. All
597 pictures are representative of three independent experiments and all graphs represent the mean
598 (\pm S.D.) of three independent experiments. Statistical significance was assessed as * p < 0.05,
599 ** p < 0.01, *** p < 0.001 for the indicated comparisons. Two-way ANOVA (microscopy
600 analysis, cell viability); post hoc: Sidak's test. Two-way ANOVA (cell titer glo assay); post
601 hoc; Tukey's test. One-way ANOVA (intracellular Ca^{2+} assay); post hoc; Dunnett's test.

602

603 **Fig. 4. TMQ0153 stimulated autophagy prior to necroptosis.** (A) Confocal UV
604 microscopy analysis after staining with Cyto-ID (left panel) and the corresponding
605 quantification of fluorescence intensity (right panel). (B) Western blot detection of LC3 and
606 p62 protein levels (left panel) and the corresponding densitometric analysis (right panel). (C)
607 Similar analysis in cells pretreated with 40 nM bafilomycin A1 (baf-A1) for 1 h (upper panel)
608 and the corresponding densitometric analysis (lower panel). 10 μ M PP242 for 4 h was used as
609 a positive control for autophagy induction. (D) Transmission electron microscopy at 12.000x
610 and 25.000x magnification: arrows indicate autophagolysosomes. Statistical significance was
611 assessed as * p < 0.05, ** p < 0.01, *** p < 0.001 compared to untreated cells unless otherwise
612 specified. One-way ANOVA (autophagosome assay); post hoc; Dunnett's test, One-way
613 ANOVA (western blot); post hoc; Tukey's test. Two-way ANOVA (mito stress test); post
614 hoc; Sidak's test.

615

616 **Fig. 5. Effect of TMQ0153 on cellular metabolism.** (A) Oxygen consumption rate (OCR)
617 was measured by Seahorse XFp analyzer. (B) Caspase 8 analysis by western blot. Etoposide
618 (Eto; 100 μ M, 24 h) was used as a positive control for apoptotic caspase cleavage. (C)
619 Caspase 8 analysis by western blot in the presence or absence of autophagy inhibitors: 40 nM
620 baf-A1, 10 mM, 3-methyladenine (3-MA) and 75 μ M chloroquine (CQ). In western blot
621 analyses, β -actin was used as a loading control. All pictures are representative of three
622 independent experiments and data represent mean (\pm S.D.) of three independent experiments.
623 Statistical significance was assessed as * p < 0.05, ** p < 0.01, *** p < 0.001 compared to
624 untreated cells unless otherwise specified. One-way ANOVA (western blot); post hoc;
625 Tukey's test. Two-way ANOVA (mito stress test); post hoc; Sidak's test.

626

627 **Fig. 6. Inhibition of autophagy increases TMQ0153-induced necroptosis.** (A) Effect of
628 autophagy inhibitors [40 nM baf-A1, 10 mM, 3-methyladenine (3-MA) and 75 μ M
629 chloroquine (CQ)] on 30 μ M TMQ0153-induced K562 cell death assessed by nuclear
630 morphology analysis after 8 h of treatment. (B-E) K562 cells were transfected with specific
631 small interferin (si)RNAs against beclin 1 [(SiBCN-1), 5 nM SiBCN-1_1 and 10 nM SiBCN-
632 1_2] for 24 h. (B) Upper panel: effect of siRNA on beclin 1 protein expression level. Lower
633 panel: transfected cells were treated with 30 μ M of TMQ0153 and a nuclear morphology
634 analysis was carried out after 8 h of treatment. (C-E) Effect of siRNA on TMQ0153-induced
635 autophagy quantified by flow cytometry after Cyto-ID staining (C); on caspase-3/7 activity
636 (D), and PARP-1 cleavage using C2-10 antibody (E). Etoposide (Eto; 100 μ M, 24 h) and
637 EtOH (10 %, 2 h) were used as positive controls for apoptotic and necrotic PARP-1 cleavage,
638 respectively. In western blot analyses, β -actin was used as a loading control. All pictures are
639 representative of three independent experiments and data represent mean (\pm S.D.) of three
640 independent experiments Statistical significance was assessed as * p < 0.05, ** p < 0.01, *** p
641 < 0.001, **** p < 0.0001 for the indicated comparisons. Two-way ANOVA (nuclear
642 morphology analysis); post hoc; Sidak's test. One-way ANOVA (Cyto-ID assay); post hoc;
643 Dunnett's test. One-way ANOVA (caspase 3/7 assay); post hoc; Tukey's test.

644

645 **Fig. 7. TMQ0153 induced mitochondrial dysfunction and MMP in K562 cells.** (A) Cells
646 were treated with TMQ0153 for 8 h and mitochondrial morphology was assessed by TEM at
647 12.000x and 25.000x magnification. Single arrows and double arrows indicate respectively
648 dilated and giant mitochondria, asterisks indicate autophagophores. (B) Mitochondrial
649 membrane potential (MMP) analysis in cells treated with increasing concentrations of

650 TMQ0153 for 24 h. The fraction of low MMP presenting cells is depicted. (C) Cells were pre-
651 incubated for 1 h in presence or absence of 50 mM N-acetyl-L-cysteine (NAC) followed by a
652 treatment with the indicated concentrations of TMQ0153. After 24 h of treatment, MMP
653 (upper panel) cell viability (bottom panel) were assessed by trypan blue assay and flow
654 cytometry, respectively. All pictures are representative of three independent experiments and
655 data represent the mean (\pm S.D.) of three independent experiments. Statistical significance
656 was assessed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells unless
657 otherwise specified. One-way ANOVA (mitochondrial membrane); post hoc; Dunnett's test.
658 One-way ANOVA (cell viability); post hoc; Tukey's test.

659

660 **Fig. 8. TMQ0153 triggers necroptotic cell death through decreased GSH levels,**
661 **involvement of LMP and ROS formation.** (A) Cells were pre-incubated for 1 h in presence
662 or absence of 50 mM NAC or 60 μ M necrostatin (Nec)-1. After 4 and 8 h of treatment with
663 TMQ0153 at 20 and 30 μ M, reactive oxygen species (ROS) levels were measured by flow
664 cytometry following dichlorofluorescein diacetate (H₂DCFDA) staining. H₂O₂ was used as a
665 positive control for ROS induction. (B) Quantification of total GSH levels (left panel) and
666 GSH (glutathione)/glutathione disulfide (GSSG) ratio (right panel). 50 μ M Buthionine
667 sulfoximine (BSO) was used as a positive control for the inhibition of GSH synthesis. (C)
668 Cells were stained with Hoechst and LysoTracker Red and analyzed by fluorescence
669 microscopy. LysoTracker Red fluorescence intensity was quantified using Image J 1.8.0
670 software (upper panel). LysoTracker Red intensity was quantified by FACS (bottom panel).
671 Chloroquine (CQ; 75 μ M, 4 h), PP242 (PP, 10 μ M, 4h) and baf-A1 (40 nM, 4 h) were used as
672 a positive and negative controls for autophagy inhibition and induction, respectively. All
673 pictures are representative of three independent experiments and data represent the mean (\pm
674 S.D.) of three independent experiments. Statistical significance was assessed as * $p < 0.05$,
675 ** $p < 0.01$, *** $p < 0.001$ compared to untreated cells unless otherwise specified. One-way
676 ANOVA (LMP); post hoc; Dunnett's test. One-way ANOVA (ROS, GSH assay); post hoc;
677 Tukey's test.

678

679 **Fig. 9. TMQ0153 treatments released immunogenic cell death markers from K562 cells.**
680 (A) Supernatants from TMQ0153-treated cells were assessed for high mobility group box
681 (HMGB)1 levels. Cells were treated for 24 h with the indicated concentrations of TMQ0153,
682 followed by the analysis of (B) extracellular ATP release, (C) calreticulin exposure by
683 fluorescence microscopy after 24 h of TMQ0153 treatment at indicated doses, (D) ectopic

684 ERp57 expression by fluorescence microscopy after 24 h of TMQ0153 treatment at indicated
685 doses. Oxaliplatin (Oxa, 30 μ M, 24 h) and shikonin (SHK; 5 μ M, 24 h) were used as positive
686 controls for immunogenic cell death induction. All pictures are representative of three
687 independent experiments and data represent the mean (\pm S.D.) of three independent
688 experiments. Statistical significance was assessed as * p < 0.05, ** p < 0.01, *** p < 0.001
689 compared to untreated cells. One-way ANOVA (HMGB1 assay); post hoc; Tukey's test. One-
690 way ANOVA (extracellular ATP assay, calreticulin expression, ERp57 expression); post hoc;
691 Dunnett's test.

692

693 **Fig. 10. Overall mechanism of action of TMQ0153 in CML cells.** (A) TMQ0153 regulates
694 apoptosis, necrosis/necroptosis and autophagy *via* a pro-oxidant cellular stress response in
695 CML. The cell death modalities triggered by TMQ0153 are controlled by various factors
696 including energy/ATP availability, the amplification of damages caused by ROS or cellular
697 stress. Depending on the severity of damage and amount of stress encountered by the cells,
698 low doses of TMQ0153 triggered apoptosis, whereas higher concentrations induced
699 necroptosis. TMQ0153-induced autophagy contributes to apoptosis inhibition in favor of a
700 RIP1-dependent necroptotic cell death.

701

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Figure 1

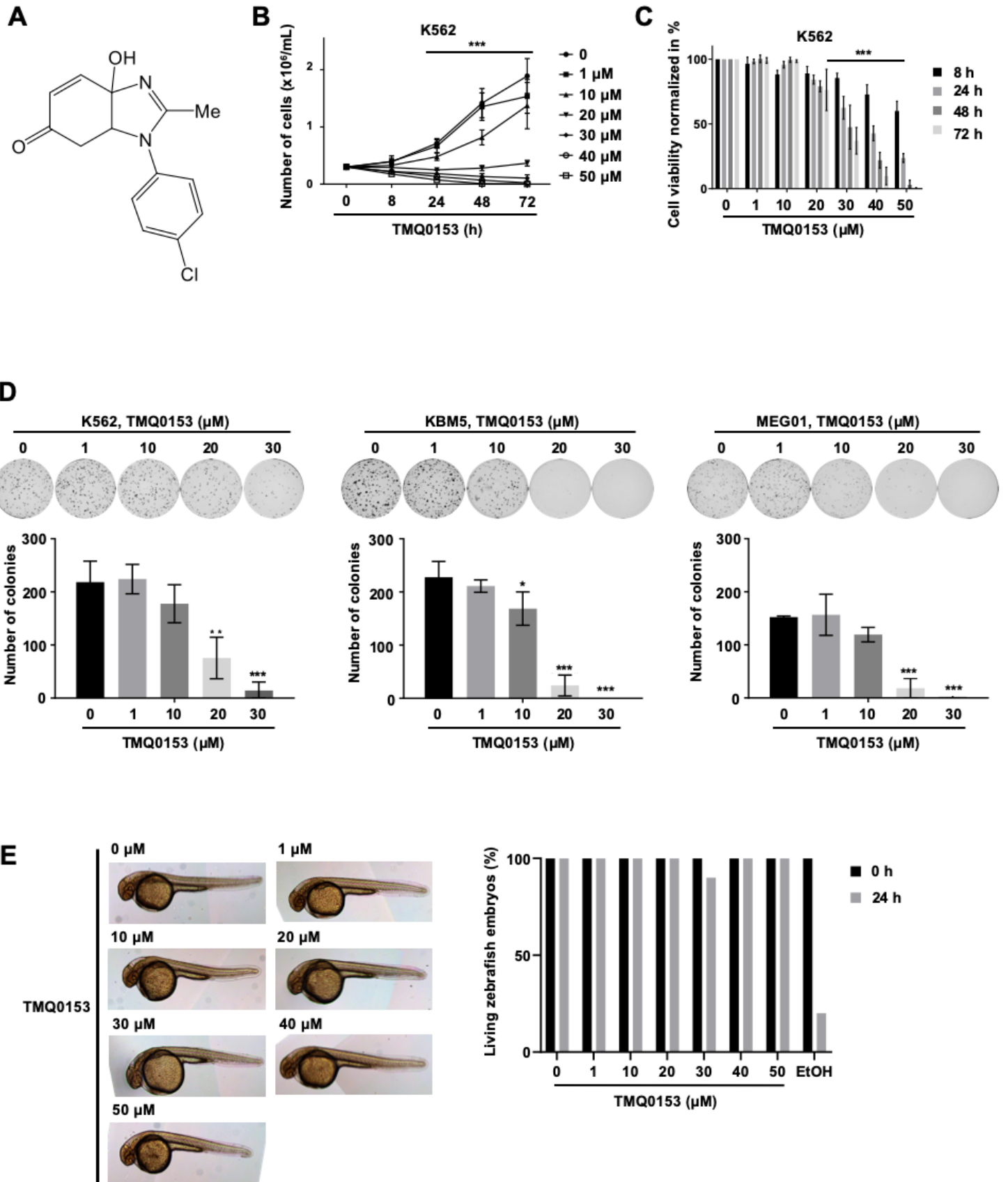


Figure 2

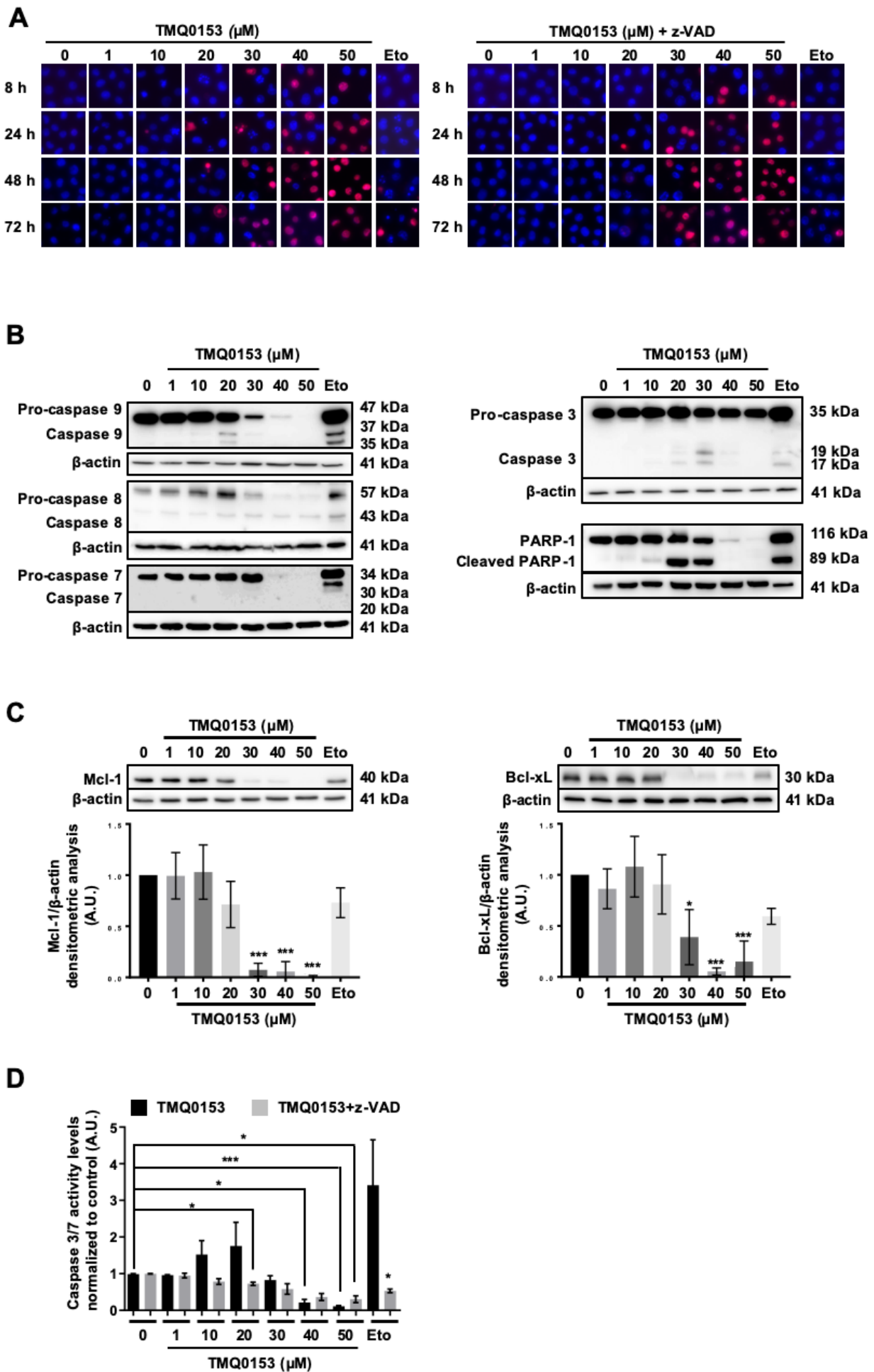


Figure 3

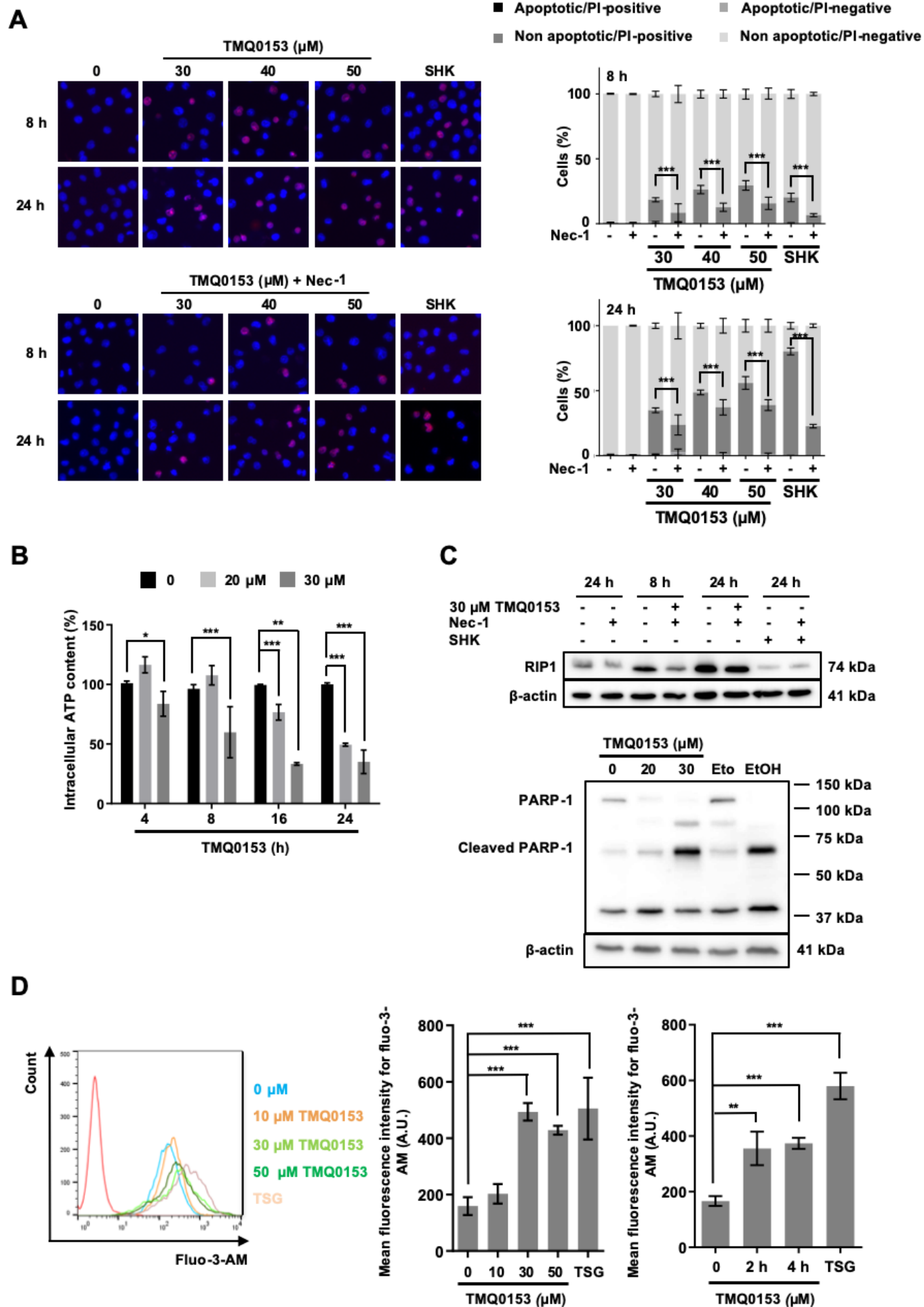


Figure 4

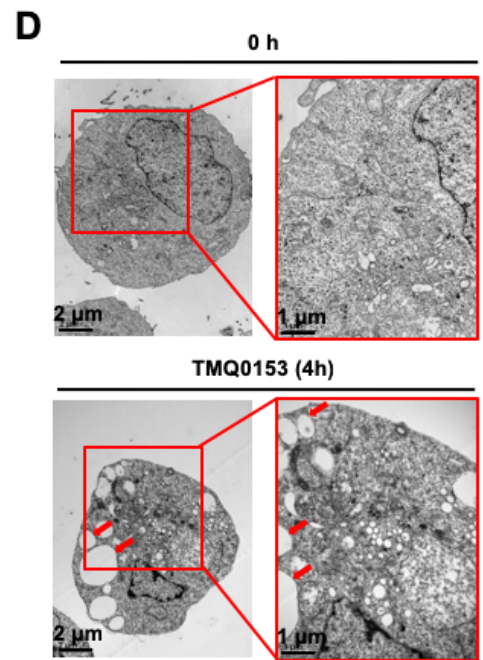
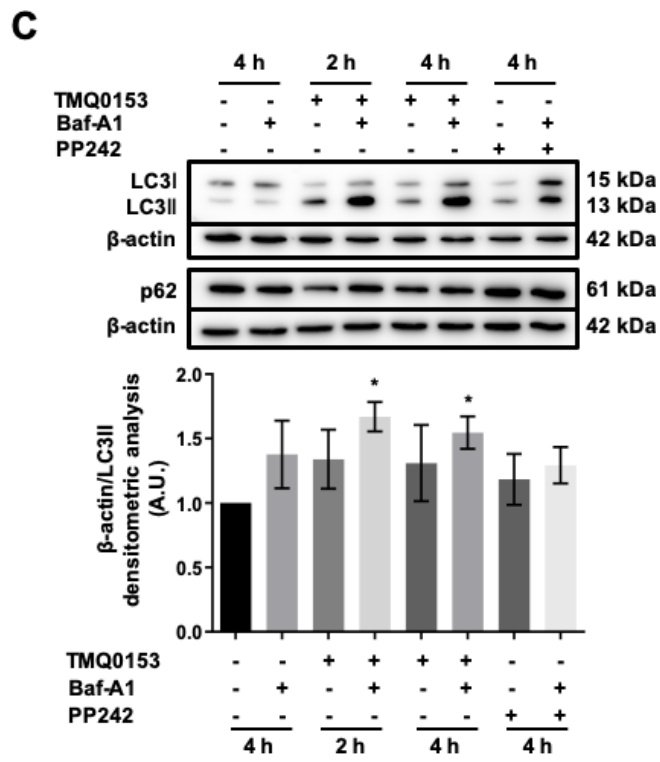
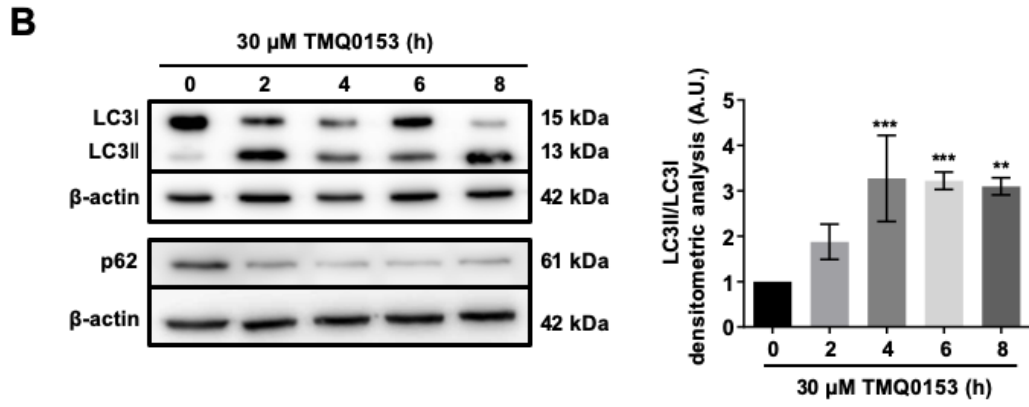
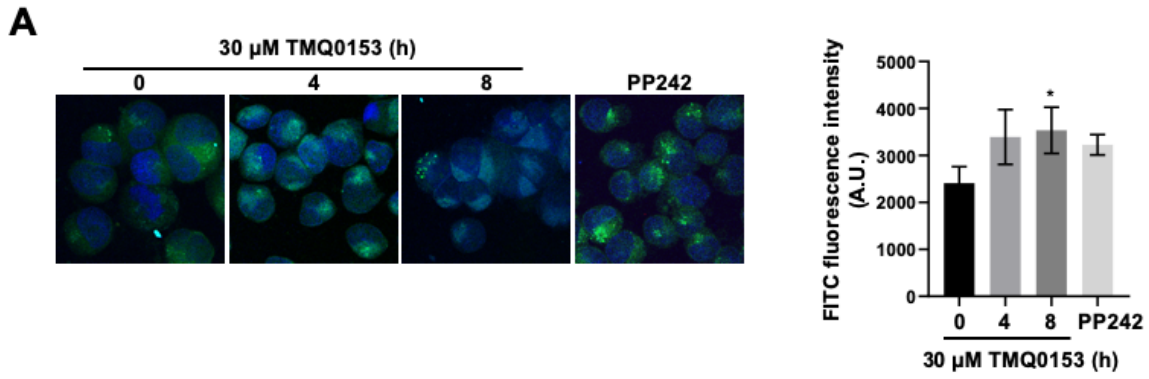
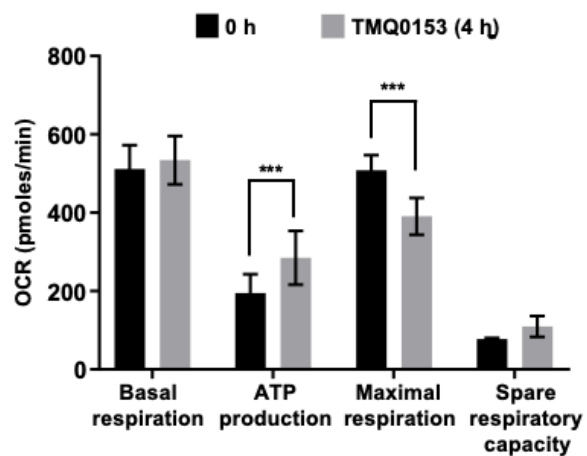
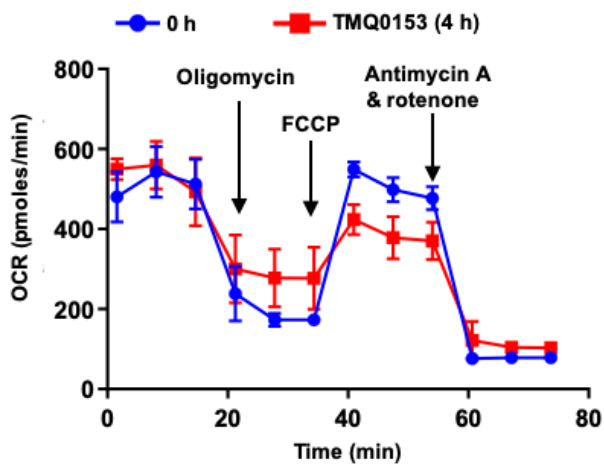
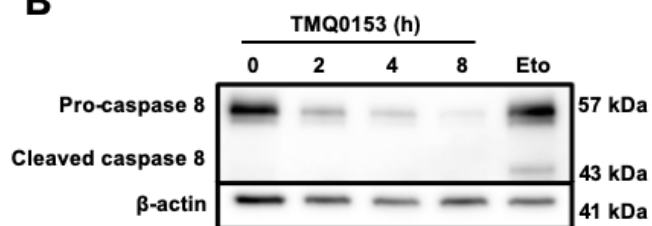


Figure 5

A



B



C

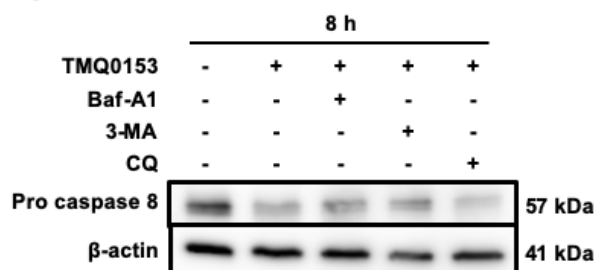


Figure 6

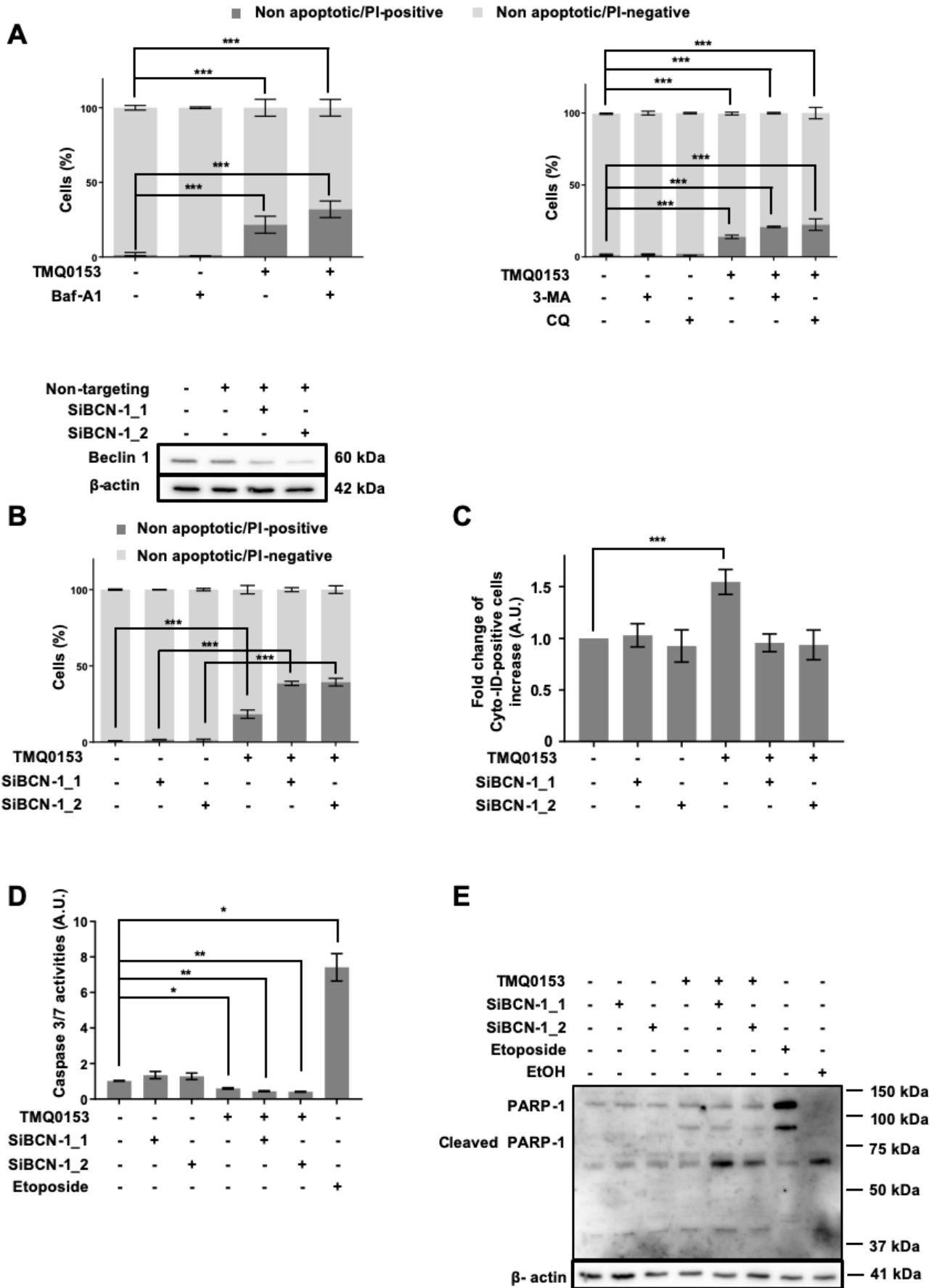
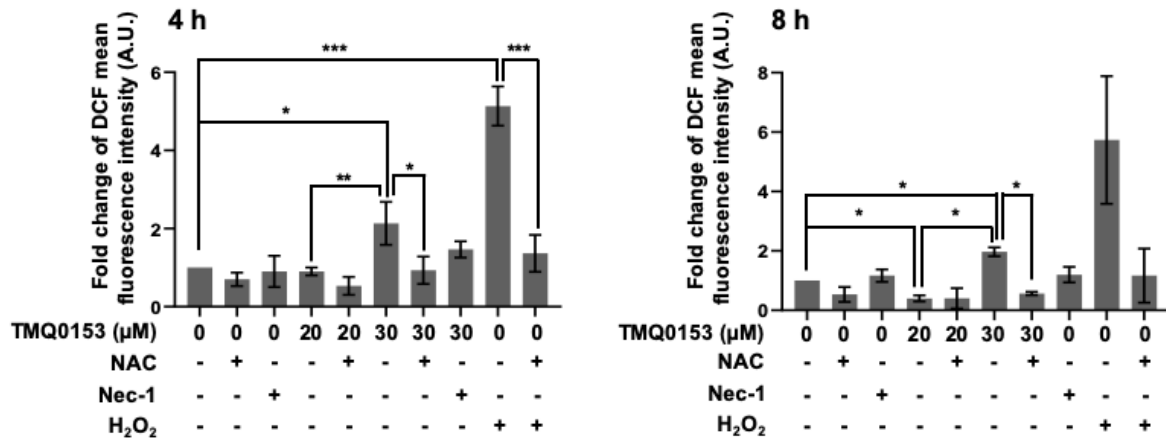
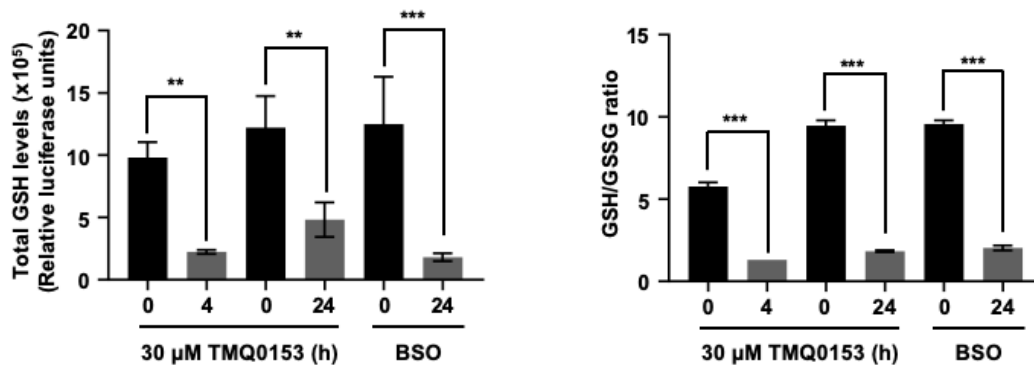


Figure 8

A



B



C

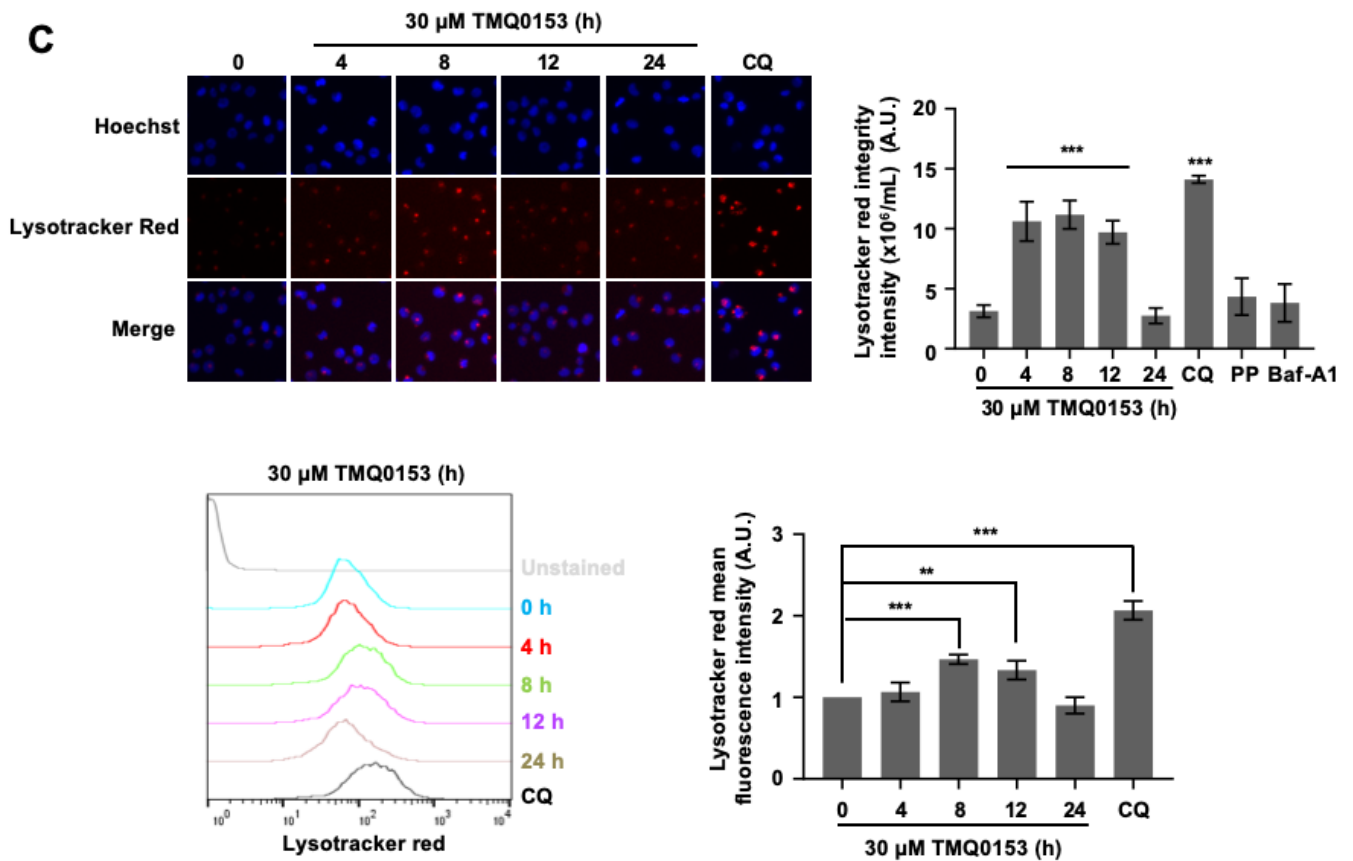


Figure 9

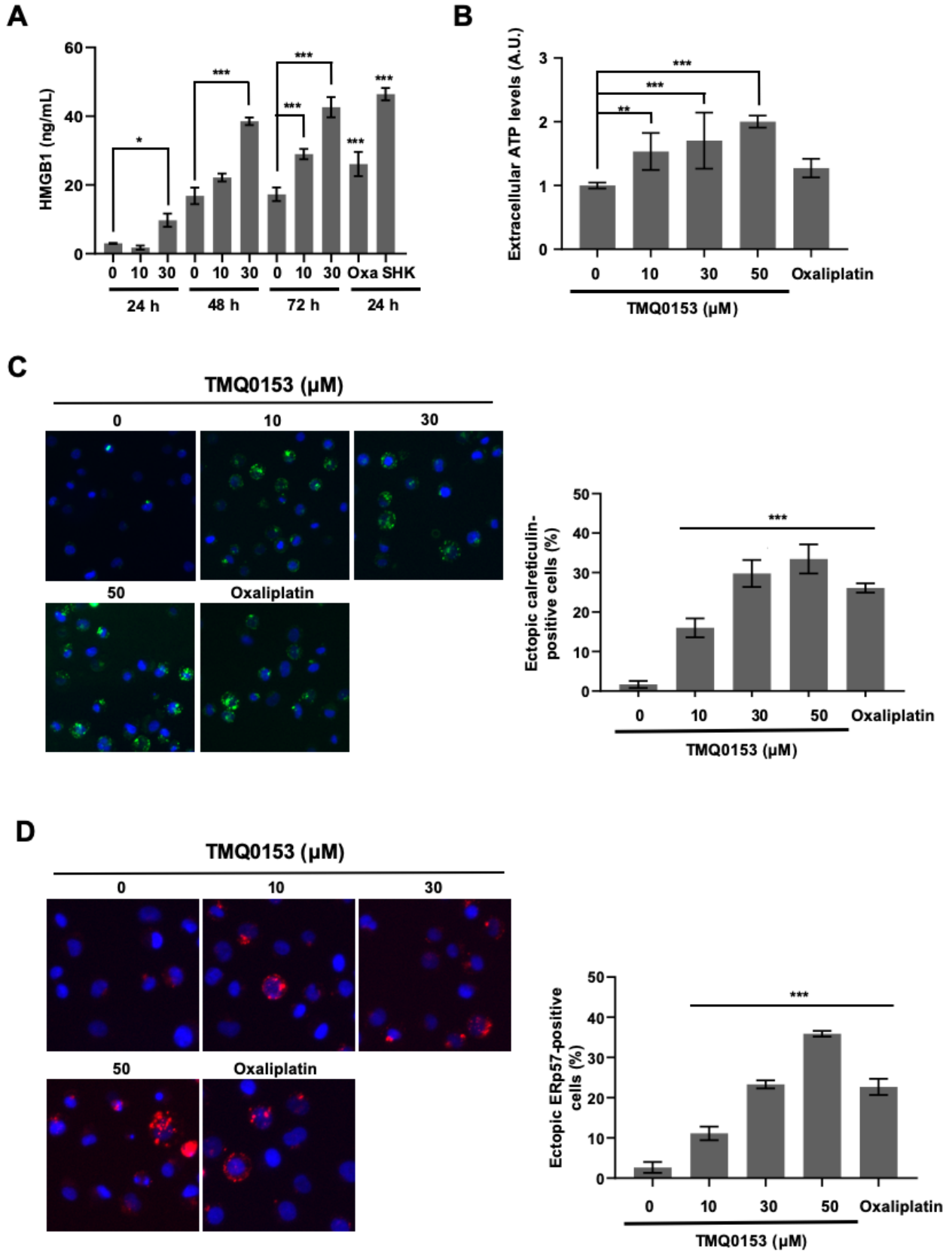


Figure 10

