

Ursolic acid induces cell death and modulates autophagy through JNK pathway in apoptosis-resistant colorectal cancer cells

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Abbreviations: CRC, colorectal carcinoma; 5-FU, 5-fluorouracil; UA, ursolic acid; MSI, microsatellite instability; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; NAC, N-Acetyl-L-cysteine; STS, staurosporine; TUNEL, TdT mediated dUTP Nick End Labelling; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

1 **Abstract**

2 Colorectal carcinomas (CRC) with *P53* mutations have been shown to be
3 resistant to chemotherapy with 5-fluorouracil (5-FU), the most widely used
4 chemotherapeutic drug for CRC treatment. Autophagy is emerging as a promising
5 therapeutic target for drug resistant tumors. In the present study, we tested the effects of
6 ursolic acid (UA), a natural triterpenoid, on cell death mechanisms and its effects in
7 combination with 5-FU in the HCT15 p53 mutant apoptosis resistant CRC cell line. The
8 involvement of UA in autophagy and its *in vivo* efficacy were evaluated.

9 Our data shows that UA induces apoptosis independent of caspases in HCT15
10 cells, and enhances 5-FU effects associated with an activation of JNK. In this cell line,
11 where this compound has a more pronounced effect on the induction of cell death
12 compared to 5-FU, apoptosis corresponds only to a small percentage of the total cell
13 death induced by UA. UA also modulated autophagy by inducing the accumulation of
14 LC3 and p62 levels with involvement of JNK pathway, which indicates a contribution
15 of autophagy on JNK-dependent induction of cell death by UA. By using nude mice
16 xenografted with HCT15 cells, we verified that UA was also active *in vivo* decreasing
17 tumor growth rate.

18 In conclusion, this study shows UA's anticancer potential both *in vitro* and *in*
19 *vivo*. Induction of cell death and modulation of autophagy in CRC resistant cells was
20 shown to involve JNK signalling.

21

22 **Introduction**

23 Colorectal carcinoma (CRC) is the second leading cause of cancer related death
24 and 5-fluorouracil (5-FU) is the main chemotherapeutic agent used in the treatment of
25 this disease [1]. However, significant resistance to 5-FU has been reported and other
26 compounds are needed in order to increase treatment efficacy [2]. Resistance to 5-FU
27 (with reduced induction of apoptosis) has been associated with tumour cells that
28 harbour *P53* mutations [3-5]. Tumors presenting microsatellite instability (MSI) status,
29 which accounts for 15% of sporadic CRC, have also demonstrated *in vitro* resistance to
30 5-FU [6-8], suggesting little or no benefit from 5-FU treatment in MSI patients,
31 although clinical evidence is not always consistent [8]. These patients, in particular
32 those with MSI and p53 mutations, would clearly gain from new treatment modalities
33 for enhanced efficacy.

34 Apoptotic cell death is a fundamental cellular process that plays an important
35 role during development and tissue homeostasis and has also a profound effect on
36 cancer progression and response to treatment [9]. Apoptosis can be mediated by death
37 receptors (extrinsic pathway) or by the mitochondrial pathway (intrinsic pathway), both
38 involving the activation of caspases [9, 10]. Other alternative cell death mechanisms
39 independent of caspases have been proposed, such as modulation of autophagy [11].
40 Autophagy is considered a mechanism of cell survival with an important role in
41 preventing early phases of tumor development [12]. However, at late stages of tumor
42 development it may confer anticancer drug resistance [13, 14]. Thus, inhibition of
43 autophagy in resistant cancer cells can lead to cell death and it is currently considered
44 an alternative therapeutic approach [13].

45 The c-Jun N-terminal kinase (JNK), a stress-activated protein kinase of the
46 family of the mitogen activated protein kinase (MAPK), has been implicated in many

47 cellular events including apoptosis signalling [15, 16]. More recently, JNK was also
48 found to be a mediator of autophagy, contributing to autophagic cell death in some
49 types of cancer cells [17-22]. Activation of JNK can induce Beclin-1 expression [19],
50 mediate damage-regulated autophagy modulator (DRAM) [18, 22], as well as, mediate
51 p53 phosphorylation [17], effects that contribute to cell death.

52 Several phytochemicals have demonstrated the ability to modulate cancer cell
53 death through different signalling pathways [23, 24]. Activities, such as anti-
54 inflammatory and anticancer, have been attributed to ursolic acid (UA), a naturally
55 occurring triterpenoid found in fruits and herbs [25]. In a previous study [26], we
56 showed that UA has anticarcinogenic potential through inhibitory effects on PI3K
57 pathway in HCT15 MSI mutant p53 CRC cell line. The present study demonstrates that
58 UA induces cell death and modulates autophagy through JNK signaling. In addition,
59 UA enhances 5-FU-induced apoptosis in this resistant cell line where it demonstrated to
60 be even more efficient in inducing cell death than 5-FU alone. *In vivo* results using
61 xenografted nude mice showed that UA significantly decreased tumor growth while
62 increasing expression of autophagy markers (p62) and JNK, providing evidence for
63 UA's therapeutic potential against CRC.

64

65 **Material and methods**

66

67 **Reagents and antibodies**

68 Ursolic acid (UA), z-VAD-fmk (zVAD), staurosporine (STS), 5-Fluorouracil (5-
69 FU), SP600125 (SP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
70 (MTT) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis,

71 MO, USA). UA, zVAD, STS, 5-FU and SP were used as stock solutions dissolved in
72 dimethyl sulfoxide (DMSO).

73 Primary antibodies were purchased from the following sources: anti-phospho-
74 JNK (G-7), anti-JNK, anti-p53 and anti-MAPLC3 (clone 5F10) from Santa Cruz
75 Biotechnology, Inc. (Santa Cruz, CA, USA); anti-phospho-mTOR and anti-mTOR from
76 Cell Signaling (Danvers, MA, USA); anti-p62 (SQTM1) from Enzo Life Sciences
77 (Lorrach, Germany); anti-LC3 (clone 5F10) from NanoTools (Teningen, Germany); and
78 anti- β -actin from Sigma-Aldrich. Secondary antibodies HRP donkey anti-rabbit and
79 sheep anti-mouse were purchased from GE Healthcare (Bucks, UK).

80

81 **Cell line and culture conditions**

82 HCT15 and CO115 human colon carcinoma-derived cell lines were kindly
83 provided by Dr. Raquel Seruca (IPATIMUP, University of Porto, Portugal). Cell lines
84 were maintained at 37°C in a humidified 5% CO₂ atmosphere in RPMI-1640 medium
85 (Sigma-Aldrich) supplemented with 10mM HEPES, 0.1mM pyruvate, 1%
86 antibiotic/antimycotic solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS; EU
87 standard, Lonza, Verviers, Belgium). Cells were seeded onto six (2ml) and twelve (1ml)
88 well plates at a density of 0.75×10^5 cells/ml. Test compounds were added to culture
89 medium to the desired concentration ensuring that the DMSO concentration did not
90 exceed 0.5% (v/v); controls received vehicle only.

91

92 **Apoptosis analysis by TUNEL assay**

93 TUNEL (TdT mediated dUTP Nick End Labelling) assay was performed to
94 estimate the percentage of cells with DNA damage typical of apoptosis. After the
95 different treatments for 48h, cells were collected (both floating and attached cells), fixed
96 with 4% paraformaldehyde for 15min at room temperature and attached onto a

97 polylysine treated slide using a Shandon Cytospin. Centrifuged cells were then washed
98 in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2min on
99 ice. TUNEL assay was performed using a kit from Roche (Mannheim, Germany),
100 following the manufacturer's instructions. Cells were incubated with Hoechst for nuclei
101 staining. The percentage of apoptotic cells was calculated from the ratio between
102 TUNEL positive cells and total number of cells (nuclei staining with Hoechst), from a
103 count higher than 500 cells per slide under a fluorescent microscope. Results are
104 presented as mean \pm SEM of at least three independent experiments.

105

106 **Cell death analysis by PI staining**

107 After the different treatments (2h or 48h), cells were collected (both floating and
108 attached cells) and washed in ice cold PBS containing 5% (v/v) FBS. Cells were then
109 resuspended in ice cold PBS with propidium iodide (PI) added to a final concentration
110 of 0.5mg/ml. Cells were maintained on ice and protected from light. Twenty microliters
111 of the stained cell suspensions were placed on clean microscope slides and overlaid
112 carefully with coverslips. Immediately, cells were visualized on a fluorescent
113 microscope and photos taken from different fields. The percentage of dead cells (PI
114 positive) was calculated from the ratio between PI positive cells and total number of
115 cells (visualized under phase contrast), from a count higher than 500 cells per slide.
116 Results are presented as mean \pm SEM of at least three independent experiments.

117

118 **Western blot analysis**

119 Cells were subjected to different treatment combinations for 24h or 48h, and
120 total cell lysates were prepared to measure expression of different proteins. The cells
121 were washed with PBS 1X and lysed for 15min at 4°C with ice cold RIPA buffer (1%

122 NP-40 in 150mM NaCl, 50mM Tris (pH 7.5), 2mM EDTA), supplemented with 20mM
123 NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 20mM Na₂V₃O₄ and protease
124 inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified
125 using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
126 and BSA used as a protein standard. For western blot analysis, 20μg of protein were
127 resolved by SDS-polyacrylamide gel and electroblotted to a Hybond-P polyvinylidene
128 difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with
129 0.05% Tween-20) containing 5% (w/v) non-fat dry milk or 1% (w/v) BSA (bovine
130 serum albumin), washed in TPBS and incubated with primary antibody overnight. After
131 washing, membranes were incubated with secondary antibody conjugated with IgG
132 horseradish peroxidase for 1h and immunoreactive bands were detected using the
133 Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence
134 detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity
135 was quantified using the Quantity One software from Bio-Rad. β-actin was used as
136 loading control.

137

138 ***In vivo* experiment of UA treatment in mice xenografted with HCT15 cells**

139 Six to eight weeks-old female Balb/cA nude mice (Taconic BALBANU-F) were
140 kept in individually filtered ventilated housing, and acclimated before the experiment.
141 HCT15 cells (10⁶ cells in 100μl Hanks BSS) were injected subcutaneously into the right
142 flank of each animal and tumors were allowed to grow for one week. Mice were then
143 assigned to two groups of ten animals: Group 1 placebo and Group 2 UA. Animals
144 received orally once daily 0.1 ml of Nutella with or without UA (75mg/kg body weight)
145 for 14 days. Tumor growth was measured twice a week for two weeks or until tumor
146 volume was 1 cm³ whichever was attained first. Tumor size was calculated using the

147 formula: $V = \frac{3}{4} \pi (a/2)^2 b$, where a represents the smallest tumor diameter and b the
148 largest tumor diameter. No signs of toxicity were observed in animals. The experiment
149 was carried out at the Biocenter, University of Copenhagen, according to the regulation
150 of Danish national authorities for handling laboratory animals.

151

152 **Histological sections and immunohistochemistry**

153 At the end of the two weeks treatment period, tumors were excised, fixed in
154 formalin and paraffin-embedded. Five μm sections were cut, collected onto APS coated
155 slides, and dried at 37°C overnight. For immunohistochemistry analysis, slides were
156 deparaffinised, rehydrated and antigen retrieval was performed by placing slides in
157 0.05% citraconic anhydride solution, pH 7.3, for 30 min at 98°C [27] and, after drying at
158 37°C, 5 min incubation with 1% SDS in phosphate buffered saline (PBS). Sections were
159 then blocked with 5% normal goat serum in 0.05% tween-20/1% bovine serum
160 albumin/PBS and incubated with primary antibodies overnight at 4°C in humidity
161 chambers: rabbit ant-p62 (1:500), mouse anti-LC3 (5 $\mu\text{g}/\text{ml}$) and mouse anti-p-JNK
162 (1:100). After incubation, slides were washed with TPBS and incubated with secondary
163 antibodies (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568;
164 Invitrogen) for 1 h at 37°C. Slides were rinsed, nuclei were counter stained with DAPI
165 and mounted with 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 M Tris (pH 8.5).
166 Slides were observed in a fluorescent microscope and semi-quantitatively scored, and
167 photos taken in a confocal microscope.

168

169 **Statistical analysis**

170 Statistical analyses were done using t -test and two-away ANOVA, using
171 GraphPad Prism 4.0 software (San Diego, CA, USA). P -values ≤ 0.05 were considered

172 statistically significant. All results are presented as mean \pm SEM of at least 3
173 independent experiments. Images are representative of three independent experiments.

174

175 **Results**

176

177 **UA induces caspase-independent apoptosis in HCT15 and enhances 5-FU effect**

178 In a previous study, UA at 4 μ M was shown to decrease significantly cell
179 proliferation (by 50%), to inhibit PI3K/Akt pathway and to induce apoptosis as assessed
180 by TUNEL assay [26]. In the present study we aimed to clarify the mechanisms
181 involved in the cell death induced by UA that is only partly due to apoptosis induction.
182 As shown in Fig. 1A, the significant induction of TUNEL-positive cells by UA in
183 HCT15 cells was caspase independent, since the inclusion of the inhibitor of caspases z-
184 VAD (20 μ M) did not prevent the induction of apoptosis by UA after 48h of treatment.
185 The increase of TUNEL-positive cells by the classical inducer of apoptosis
186 staurosporine (STS, 0.250 μ M) was also independent of caspases in this apoptosis
187 resistant cell line. These results were corroborated by analysis of apoptosis markers by
188 western blotting (Fig. 1B), where UA did not induce the cleavage of caspase 9, caspase
189 3 or PARP-1. However, STS induced slightly the cleavage of PARP-1 and decreased
190 the levels of procaspase 3 and 9 (Fig. 1B). These results suggest that UA induces
191 apoptosis by a caspase-independent mechanism in HCT15 cells.

192 We had also shown previously that HCT15 cells are resistant to induction of cell
193 death by apoptosis by a common CRC chemotherapeutic drug 5-FU, probably due to
194 the p53 mutation and MSI status [28]. However, when we subjected HCT15 cells to the
195 combination of UA with 5-FU for 48h, at concentrations that were previously shown to
196 decrease cell growth by 50% [26, 28], a significant enhancement of apoptosis was

197 observed when compared with both compounds alone (Fig. 1C). Interestingly, this same
198 combination of 5-FU and UA when tested in normal human fibroblasts did not decrease
199 cell viability (Supplementary Fig. 1).

200

201 **UA induces JNK-dependent apoptosis in HCT15**

202 Since the stress-activated MAPK signalling pathways have been implicated in
203 cell death mechanisms [16], we further studied their involvement in UA-induced
204 apoptosis, as well as, the role of reactive oxygen species (ROS). As shown, in Fig. 2A,
205 co-incubation of UA with the antioxidant N-acetylcysteine (NAC) inhibited the % of
206 TUNEL-positive cells, suggesting an implication of oxidative stress as a contributor for
207 UA-induced apoptosis in HCT15 cells. Previously, we observed that UA did not change
208 MAPK/ERK pathway in HCT15 cells [26]. Here we studied the involvement of the
209 stress kinases p38 and JNK on apoptosis induced by UA in HCT15 cells. Using western
210 blot analysis, we observed that UA significantly induced phospho-JNK (active form)
211 expression (Fig. 2B). An increase of phospho-JNK expression was also observed for
212 STS and no effect was detected for 5-FU. UA did not change the expression of
213 phospho-p38 expression in HCT15 cells (data not shown).

214 To assess whether apoptosis induction by UA and UA plus FU were dependent
215 on JNK activation, incubations in the presence of 20 μ M SP600125 (SP), a JNK
216 inhibitor, were performed. As shown in Fig. 2C, SP inhibited TUNEL-positive cells
217 induced by UA, suggesting a dependence on JNK signalling for the UA-induced
218 apoptosis in HCT15 cells. SP also inhibited TUNEL-positive cells induced by STS
219 (Supplementary Fig. 2), an effect not observed with 5-FU (Fig. 2C). An almost
220 complete abrogation of TUNEL-positive cells induced by the combination of UA with

221 5-FU was observed in the presence of SP (Fig. 2C). These results indicate that
222 activation of JNK by UA is necessary for UA-induced apoptosis in HCT15 cells.

223

224 **UA induces cell death and modulates autophagy through JNK pathway in HCT15**

225 Although significant, apoptosis induced by UA in HCT15 only affects around
226 4% of total cell number, which does not reflect the extensive morphological changes
227 (and appearance of floating cells) induced by this compound, suggesting a much higher
228 percentage of cell death. Cell death was, therefore, subsequently measured using PI
229 staining, at 2h and 48h. As shown in Supplementary Fig. 3, UA produced a small
230 increase in cell death after 2h of incubation, indicating no acute necrotic effect.
231 However, after 48h, UA induced cell death in around 50% of cells, as shown by the
232 increase number of PI positive cells (Fig. 3A, Supplementary Fig. 3). On the other hand,
233 5-FU alone did not induce significant PI positive cell death and no cumulative effect
234 with UA was observed (Fig. 3A). These results indicate that UA induces cell death in
235 HCT15 cells more efficiently than 5-FU and also by mechanisms other than apoptosis.
236 We also tested whether JNK pathway was involved in the total cell death induced by
237 UA. As shown in Figure 3A, SP partially inhibited total cell death induced by UA, as
238 well as, the cell death induced by the combination of UA with 5-FU. These data suggest
239 a dependence on JNK signaling also for the total cell death induced by UA in HCT15
240 cells.

241 Recently, autophagy has been argued to be a potential target for induction of cell
242 death in chemoresistant cancer cells [13]. Therefore, we further investigated the
243 possible role of UA in autophagy and the involvement of JNK in this process. As shown
244 in Fig. 3B, UA induced an accumulation of both LC3-II (and to a lower extent also
245 LC3-I) and p62 levels in HCT15 cells after 48h of treatment, which were remarkably

246 prevented in the presence of SP, indicating a role of JNK activation in the accumulation
247 of these autophagic mediators. No effect on LC3-II protein expression was detected in
248 cells treated with 5-FU or SP alone (Fig. 3B). The LC3-II accumulated in cells treated
249 with UA plus 5-FU seems to be due to UA alone, an effect also inhibited in the presence
250 of SP. The possible role of UA in some upstream regulators of autophagy, such as p53
251 and mTOR, was also investigated. Figure 3B shows that UA decreased both p53 and
252 phospho-mTOR levels, as well as, the levels of p53 induced by 5-FU. These effects are
253 consistent with the potential of UA to modulate autophagy.

254 Interestingly, UA also modulates the same autophagic mediators in other CRC
255 cells, such as the MSI CO115 p53 wild-type cell line (Supplementary Fig. 4D).
256 Although in this cell line UA induced apoptosis dependent of caspases, total cell death
257 is higher (Supplementary Fig. 4A-C) indicating a possible role of autophagy on cell
258 death.

259

260 **UA decreases tumor growth in mice xenografted with HCT15 cells**

261 Because UA demonstrated to induce cell death more efficiently than 5-FU in
262 HCT15 cells, we evaluated *in vivo*, in mice xenografted with HCT15 cells, the effects of
263 UA and the possible implication of autophagy and JNK signaling on tumor growth. As
264 shown in Fig. 4A, UA significantly decreased tumor growth rate after 14 days of
265 treatment when compared to the control group. No significant effect on body weight
266 was observed between treatments (data not shown). Using immunohistochemistry
267 analysis of these tumors, a tendency for a higher expression of p62 and phospho-JNK in
268 UA group was observed as compared to controls (Fig. 4B, 4C). Colocalization of p62
269 and phospho-JNK was, however, only partial. No differences were observed on the
270 tumor expression of LC3 or the proliferation marker Ki67 between treatments (Fig. 4B,

271 4C). These results suggest the potential of UA in reducing tumor growth and the
272 possible involvement of JNK signalling and autophagy in the *in vivo* effects of UA.

273

274 Discussion

275 Several mechanisms of resistance have been reported that decrease 5-FU
276 efficacy, such as loss of p53 transactivation function [3-5]. Defects in apoptosis play a
277 central role in tumorigenesis and confer resistance to anticancer therapies [29].
278 Alternative strategies such as autophagy inhibition have been demonstrated to sensitize
279 tumor cells to anticancer drugs [13, 14]. In a previous study, we showed that UA
280 induces apoptosis in HCT15 mutant p53 MSI human CRC cell line [26]. This cell line is
281 also resistant to 5-FU [28], and here we showed that combination of this
282 chemotherapeutic agent with UA significantly enhanced apoptosis as compared with 5-
283 FU alone. This drug combination did not have any cytotoxicity in normal cells. The
284 induction of apoptosis in HCT15 cells by UA (with or without 5-FU) was shown to be
285 dependent on JNK pathway and independent of caspases. In the conditions used, the
286 apoptosis induced by UA is small and did not account for the total cell death (about
287 50%) observed, suggesting that other mechanisms must be involved.

288 Reactive oxygen species (ROS) have been shown to be involved in the
289 regulation of cell death and signalling through JNK pathway [30]. This pathway is
290 implicated in many cellular events related to cell death, such as apoptosis [15, 16] and
291 autophagy [17-22]. Our results showed that the antioxidant NAC partially inhibited
292 apoptosis induced by UA, suggesting the involvement of ROS on UA's effects. In
293 addition, UA activated JNK pathway, as shown by the increased levels of phospho-
294 JNK. Its inhibition with SP significantly decreased UA-induced cell death and the
295 increase of the autophagic mediators LC3 and p62. Therefore, both apoptosis and total

296 cell death induced by UA alone or UA in combination with 5-FU were shown to involve
297 JNK pathway, possibly in response to oxidative stress produced by UA. The importance
298 of JNK activation as one contributing mechanism to cell death induction in CRC has
299 previously been demonstrated for atorvastatin. This drug was shown to induce apoptosis
300 involving JNK activation and to synergistically interact with celecoxib, a selective
301 cyclooxygenase-2 inhibitor, in killing human CRC cells [31]. Also, UA has been shown
302 to induce JNK pathway in other cell lines [32-37] leading to cell death, however its
303 association with autophagy has never been reported.

304 Autophagy is activated under stress conditions, such as nutrient and/or growth
305 factor deprivation and, although it represents a mechanism of survival, it may assume a
306 cell death function in cancer cells when apoptosis is deregulated [11, 12]. Several
307 signalling proteins have been demonstrated to interfere with autophagy [13, 38]. In our
308 previous work, UA showed to decrease PI3K/Akt pathway [26]. Here, we observed that
309 UA also decreased the levels of phospho-mTOR, as well as, the levels of mutant p53.
310 Since an inhibition of mTOR is associated with an induction of autophagy [38, 39] and
311 the cytosolic mutant p53 has shown to inhibit autophagy [40, 41], our results suggest
312 that UA may induce autophagy by inhibiting the PI3K/Akt/mTOR signaling and
313 decreasing mutant p53.

314 On the other hand, the ability of UA to increase the levels of autophagic
315 mediators LC3 and p62 suggest that UA may be inhibiting autophagy. LC3-II is
316 associated with autophagosome membrane reflecting its abundance. Its increasing levels
317 have been interpreted as either the result of induction or inhibition of the autophagic
318 process [42]. However, the accumulation of both LC3-I and LC3-II after long periods of
319 incubation, as observed here, is taken as an indication of inhibition of autophagy [42].
320 In the case of p62 that is selectively incorporated into autophagosomes through binding

321 to LC3, its levels reflect its degradation by autophagy [42]. Therefore, an accumulation
322 of p62 represents an inhibition at later steps of the autophagic process. As a result, in
323 HCT15 cells, modulation of autophagy by UA seems to involve a dual effect: it may
324 facilitate initial stages but it inhibits autophagy at later steps. Nevertheless, UA seems to
325 be able to modulate autophagy independently of p53 status and of apoptosis cell
326 resistance, since UA had the same effects in other CRC cells, such as CO115 cell line.
327 Further experiments have to be performed to elucidate in detail the effects of UA on
328 autophagy and its implication on induction of cell death.

329 Since UA alone was more efficient in inducing cell death than 5-FU *in vitro*, the
330 *in vivo* potential of UA against CRC was evaluated in nude mice xenografted with
331 HCT15 cells. Interestingly, UA decreased tumor growth rate after 2 weeks of treatment,
332 without affecting body weight, and a tendency to increase the levels of p62 and
333 phospho-JNK in tumors was observed. These results suggest that the antitumor effect of
334 UA may involve the regulation of autophagy possibly by JNK signaling. The *in vivo*
335 potential of UA as an antitumorogenic agent has recently been suggested in other cancer
336 types [43, 44].

337 In conclusion, this study shows that UA enhances the apoptotic effect of 5-FU,
338 with an activation of JNK. UA induces cell death in CRC resistant cell line more
339 efficiently than 5-FU probably by inhibiting autophagy. The antitumor potential of UA
340 against CRC and the possible involvement of autophagy and JNK were observed *in*
341 *vivo*. The applicability of UA as a potential inhibitor of autophagy should be explored in
342 future studies and in strategies for treatment of CRC tumors resistant to conventional
343 chemotherapeutic drugs.

344

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Figure Legends

Figure 1 – Effect of both ursolic acid (UA) and 5-fluorouracil (FU), alone or in combination, on apoptosis in HCT15 cells. **(A)** Effect of UA 4 μ M (UA4) and staurosporine (STS) 0.25 μ M with or without a caspase inhibitor zVAD-FMK (zVAD) 20 μ M in the % of TUNEL-positive cells after 48h of treatment. **(B)** Effect of UA4 for 24h on the expression of protein markers of caspase-dependent apoptosis, as assessed by western blotting. **(C)** Effect of UA4 alone or in combination with 5-fluorouracil 100 μ M (FU100) for 48h in the % of TUNEL-positive cells. **(A, C)** Values are mean \pm SEM of at least 3 independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, when compared with control (CT); ++ $P \leq 0.01$, when compared with the respective compound alone; ## $P \leq 0.01$ and ### $P \leq 0.001$, when compared with FU or zVAD alone; NS, not significant differences observed between each other. In **B**, images are representative of at least 3 independent experiments with similar results. β -actin was used as loading control.

Figure 2 – Effect of JNK pathway on apoptosis induced by ursolic acid (UA) in HCT15 cells. **(A)** Effect of N-Acetyl-L-cysteine (NAC) 5mM in the % of TUNEL-positive cells induced by UA 4 μ M (UA4) after 48h of treatment. Values are mean \pm SEM of at least 3 independent experiments. *** $P \leq 0.001$, when compared to control (CT); ### $P \leq 0.001$, when compared with NAC alone; ++ $P \leq 0.01$, when compared with each other. **(B)** Effect of UA4, staurosporine (STS) 0.25 μ M and 5-fluorouracil 100 μ M (FU100) on phospho-JNK and total JNK levels, for 48h, using western blot. Images are representative of at least 3 independent experiments with similar results. β -actin was used as loading control. **(C)** Effect of UA 4 μ M, FU 100 μ M and SP600125 (SP) 20 μ M, a JNK inhibitor, alone or in combination, in the % of TUNEL-positive cells for 48h of treatment. Values are mean \pm SEM of at least 3 independent experiments. ** $P \leq 0.01$, *** $P \leq 0.001$, when compared with UA alone; θ $P \leq 0.05$ and $\theta\theta$ $P \leq 0.01$, when compared with SP alone; ### $P \leq 0.001$, when compared with FU alone. ++ $P \leq 0.01$ and +++ $P \leq 0.001$, when compared with each other; NS, not significant when compared with each other.

Figure 3 – Effect of both ursolic acid (UA) and 5-fluorouracil (FU), alone or in combination, on cell death and autophagic mediators in HCT15 cells. **(A)** Effect on cell death of UA 4 μ M, FU 100 μ M and SP600125 (SP) 20 μ M, alone or in combination, for 48h, as assessed by PI staining. Values are mean \pm SEM of at least 3 independent experiments. *** $P \leq 0.001$, when compared with UA alone; 000 $P \leq 0.001$, when compared with SP alone; ### $P \leq 0.001$, when compared with FU alone; ++ $P \leq 0.01$ and +++ $P \leq 0.001$, when compared with each other; NS, not significant when compared with each other. **(B)** Effect UA 4 μ M, FU 100 μ M and SP 20 μ M, alone or in combination, in the levels of LC3, p62, p53, phospho-mTOR and total mTOR, for 48h, using western blot. Images are representative of at least 3 independent experiments with similar results. β -actin was used as loading control.

Figure 4 – Effect of ursolic acid (UA) treatment *in vivo*. **(A)** Tumor progression of HCT15 cells xenografted in nude mice for 14 days, as expressed by relative tumor size. Mice were divided in two groups: control (placebo) and UA (75 mg/kg), each with 7 animals. The effect of time ($p < 0.001$) and treatment were observed ($p < 0.001$), as well as, the effect of the interaction ($p = 0.006$) shown in the figure as (++). **(B)** Representative confocal images of immunohistochemical analysis of the expression of p62 (green), ki67 (green), phospho-JNK (red) and LC3 (red) proteins in the tumors treated with UA in the *in vivo* experiment; bar: 20 μ m. **(C) Semi-quantification of the immunohistochemical sections of the expression of p62, phospho-JNK, LC3 and Ki67 proteins *in vivo*. Immunoreaction intensity was scored as 0 for negative staining, 1 for weak, 2 for intermediate and 3 for strong. Values are mean \pm SEM of 7 animals each.**

Figure 1
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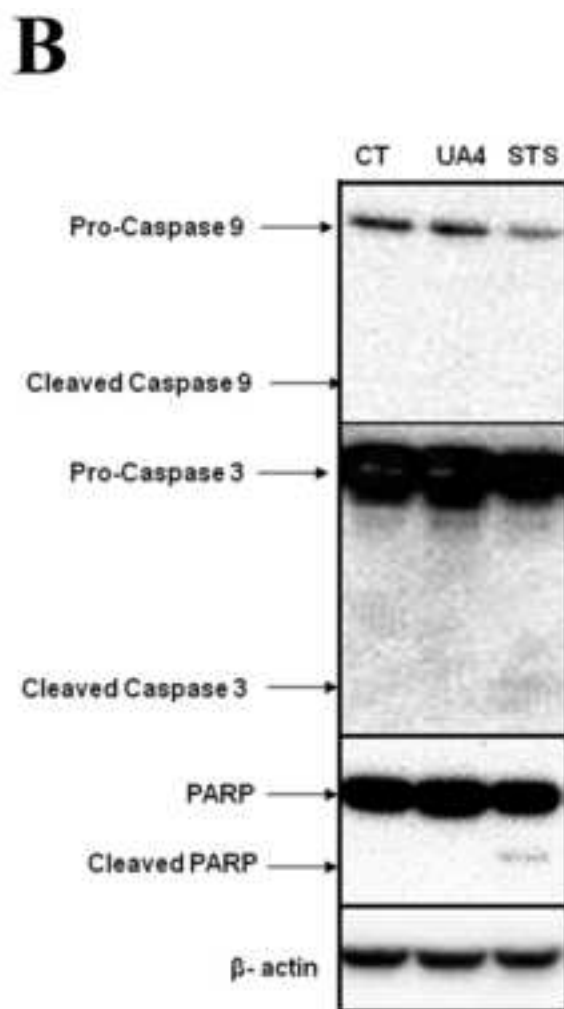
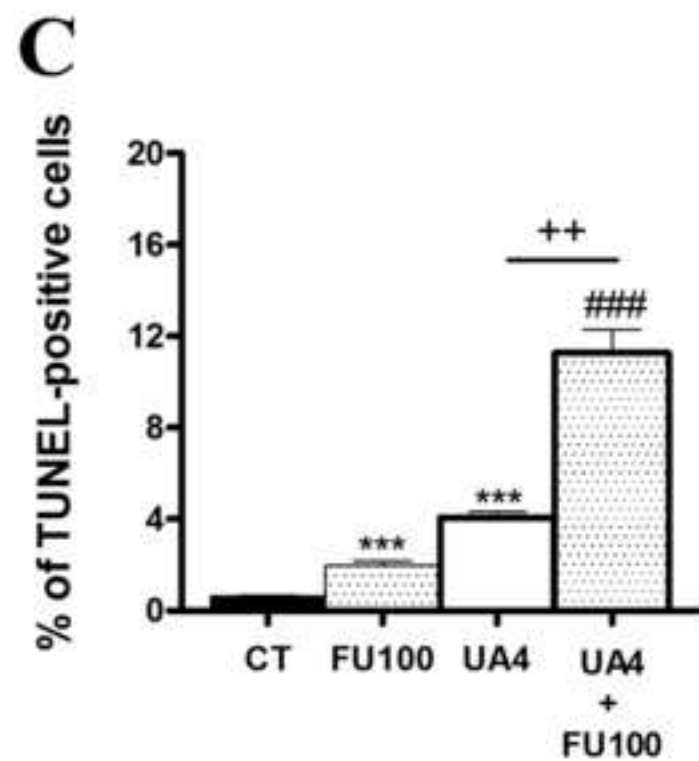
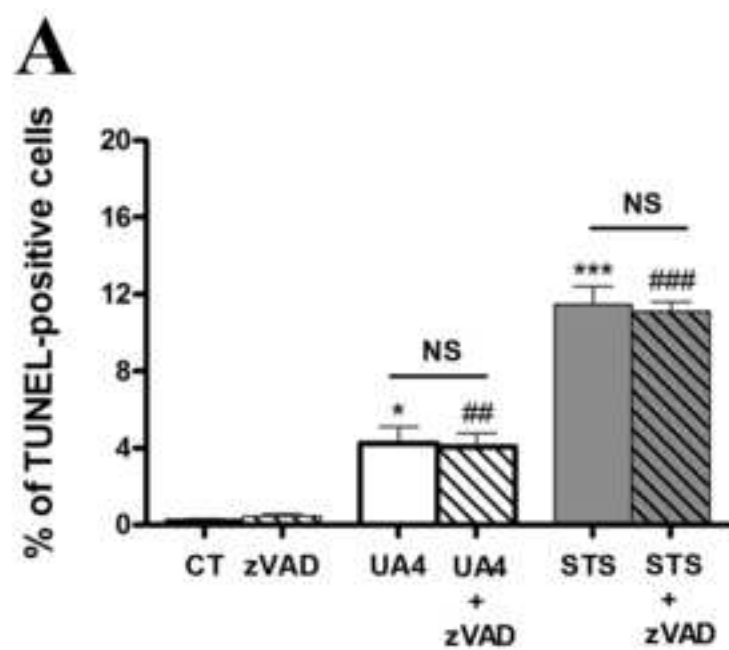


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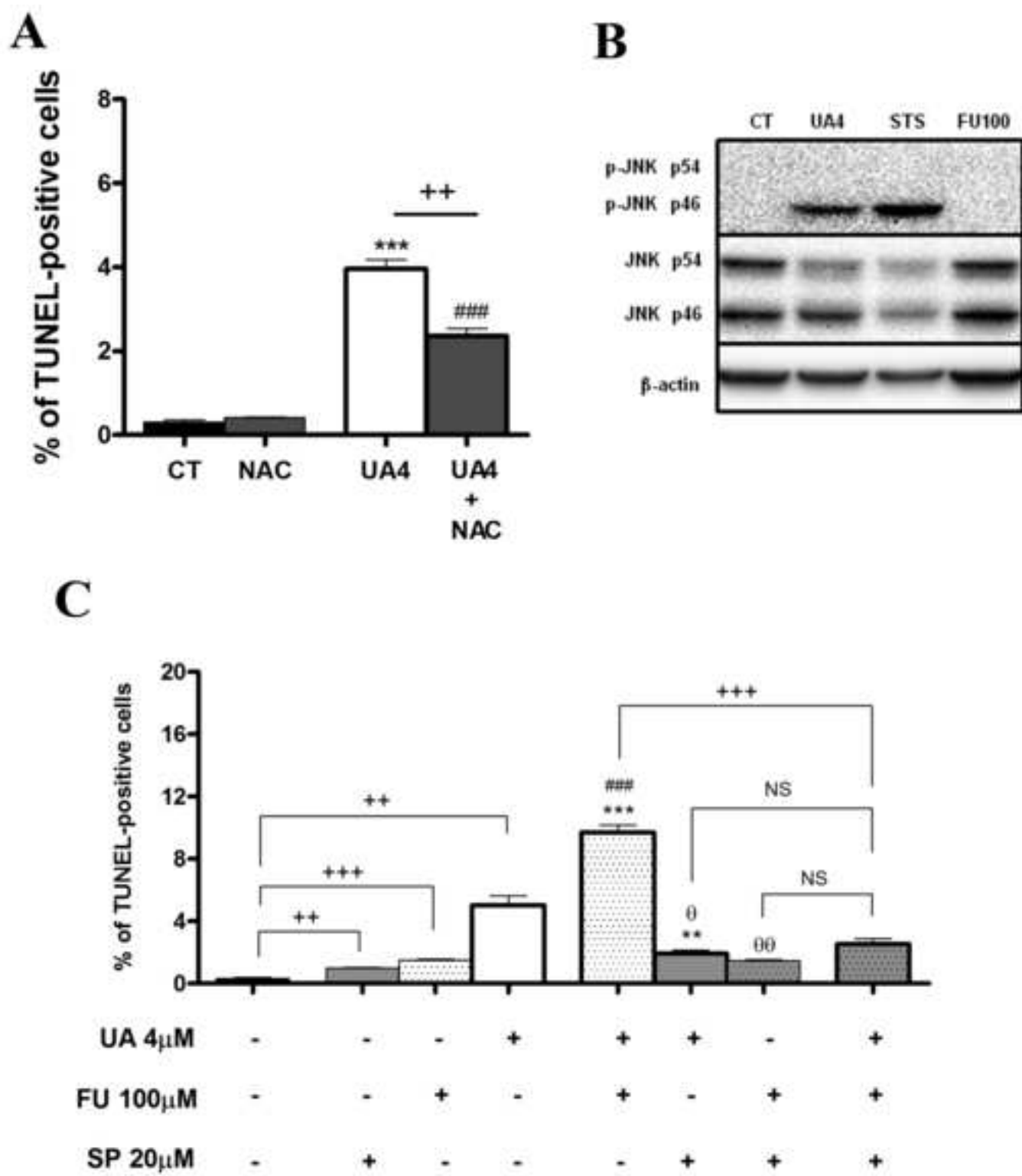


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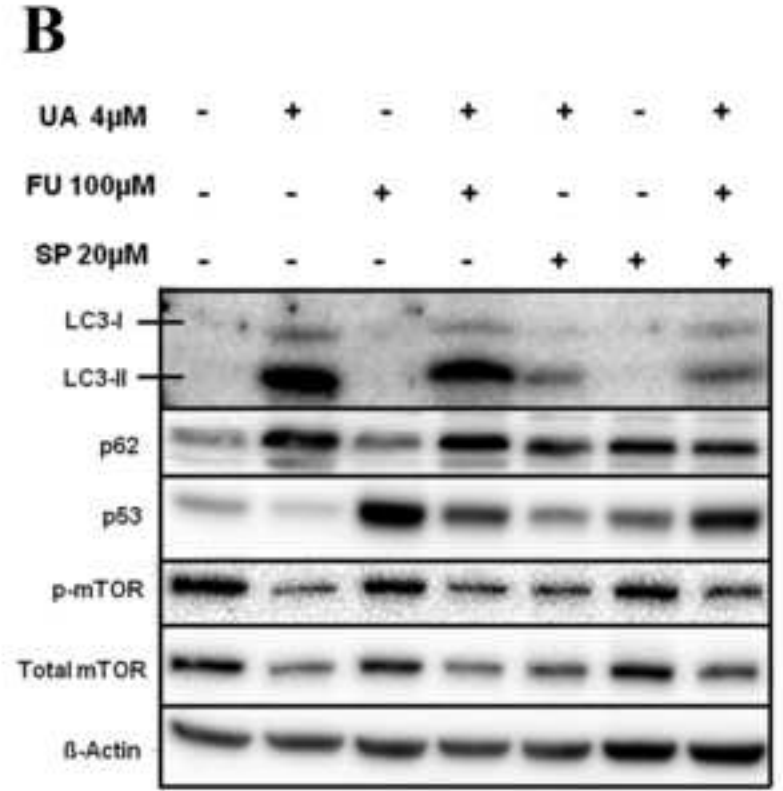
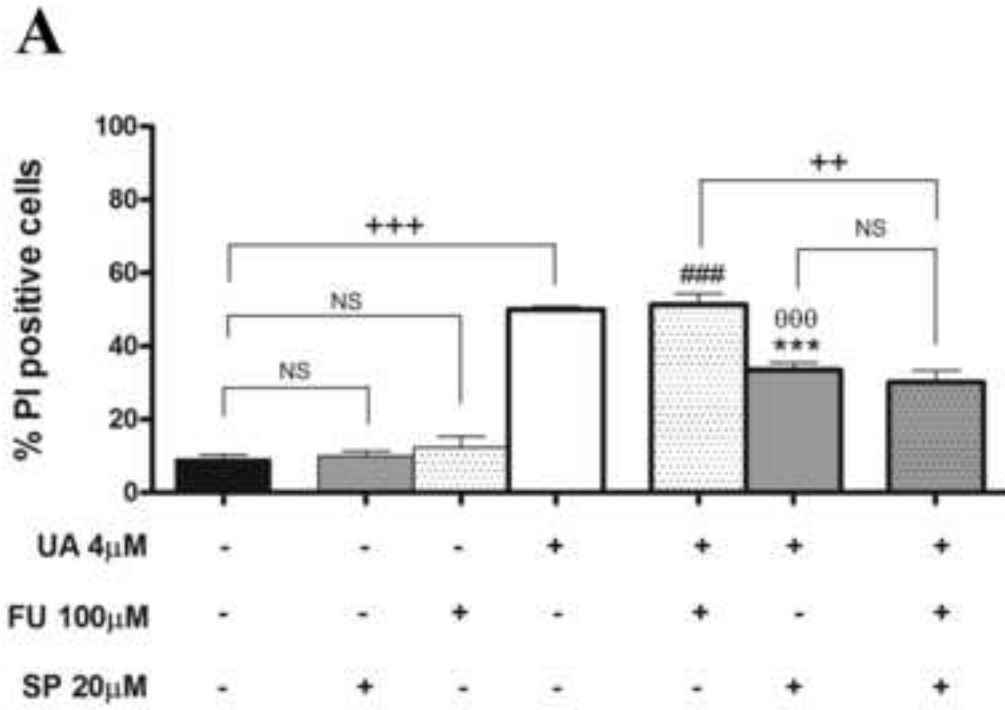
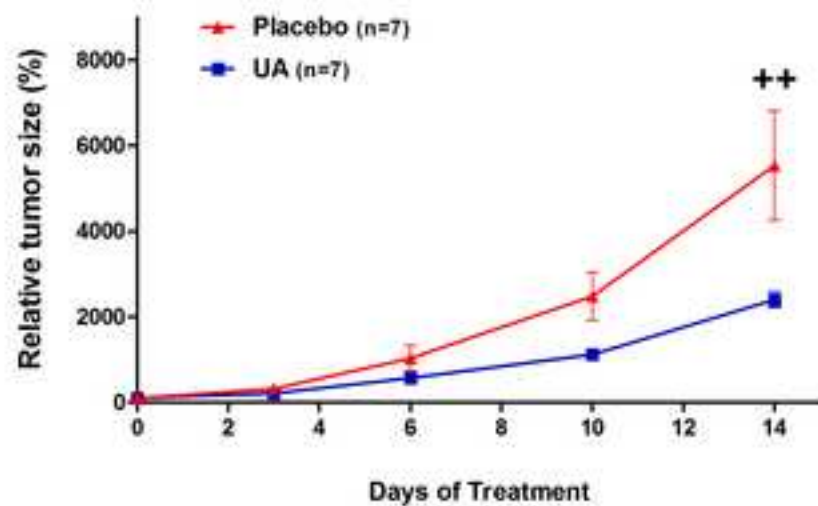
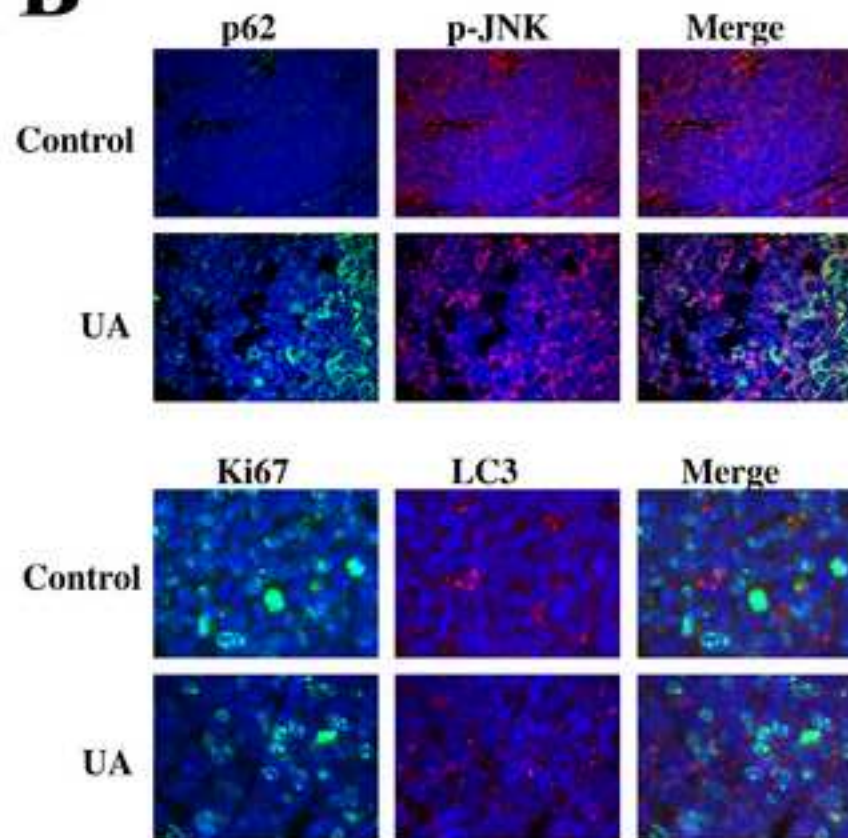


Figure 4
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A



B



C

