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PRECLINICAL STUDIES

Urolithins impair cell proliferation, arrest the cell cycle and induce apoptosis in UMUC3 bladder cancer cells

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Summary Ellagitannins have been gaining attention as potential anticancer molecules. However, the low bioavailability of ellagitannins and their extensive metabolization in the gastrointestinal tract into ellagic acid and urolithins suggest that the health benefits of consuming ellagitannins rely on the direct effects of their metabolites. Recently, chemopreventive and chemotherapeutic activities were ascribed to urolithins. Nonetheless, there is still a need to screen and evaluate the selectivity of these molecules and to elucidate their cellular mechanisms of action. Therefore, this work focused on the antiproliferative effects of urolithins A, B and C and ellagic acid on different human tumor cell lines. The evaluation of cell viability and the determination of the half-maximal inhibitory concentrations indicated that the sensitivity to the studied urolithins varied markedly between the different cell lines, with the bladder cancer cells (UMUC3) being the most susceptible. In UMUC3 cells, urolithin A was the most active molecule, promoting cell cycle arrest at the G2/M checkpoint, increasing apoptotic cell death and inhibiting PI3K/Akt and MAPK signaling. Overall, the present study emphasizes the chemopreventive/chemotherapeutic potential of urolithins, highlighting the stronger effects of urolithin A and its potential to target transitional bladder cancer cells.

Keywords Apoptosis · Cell cycle · Ellagitannins · Intracellular signaling pathways · UMUC3 · Urolithins

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Introduction

The discovery of efficient and safe anticancer molecules from natural products is still a current demand in medical research. Among such compounds, ellagitannins emerge as promising leads. Ellagitannins, which are found in fruits and nuts such as pomegranates, strawberries and walnuts, are a subclass of hydrolysable tannins that contain one or more hexahydroxydiphenic acid units esterified to a polyol (usually α-D-glucopyranose) [1]. The therapeutic significance of this group of polyphenols as anticancer, antioxidant and anti-inflammatory agents has been demonstrated in vitro and in vivo [2, 3].

It has been established that dietary ellagitannins are partially hydrolyzed in the upper gastrointestinal tract into ellagic acid, which is poorly absorbed [4, 5], and metabolized by gut microbiota into urolithins [6, 7]. Urolithins are a subfamily of metabolites generated by the opening and decarboxylation of ellagic acid lactone rings, followed by a sequential dehydroxylation that promotes the production of a series of hydroxylated dibenzopyranones derivatives [8, 9]. As an important note, urolithins or its conjugates (mainly glucuronides) have been found in plasma at micromolar concentrations [7, 10] and in human tissues, including the prostate gland [11] and

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colon [12]. The concentration of urolithins in plasma, urine and feces varies considerably between individuals, which is probably related to different gut microbiota compositions [7, 9, 13, 14]. Despite this variability, urolithin A (3,8-dihydroxy-6H-dibenzopyran-6-one) and its monohydroxylated analog, the urolithin B, are the main urolithins reported in different mammals, including humans [6, 7, 15]. Furthermore, urolithins remain in the body for long periods of time due to enterohepatic recirculation and are found in the urine for up to 4 days after consumption of ellagitannin-containing foods [7, 16]. Considering this evidence and the very low bioavailability of ellagitannins, urolithins have been proposed to be responsible for the biological activity of ellagitannins and the systemic health effects related to ellagitannin consumption.

Corroborating these observations, several studies highlight the antioxidant capacity [17], estrogenic and/or anti-estrogenic activities [18] and anti-inflammatory properties [9] of urolithins. In addition, in vitro chemopreventive and chemotherapeutic effects were recently ascribed to urolithins and their derivatives [19–22], but many of the cellular and molecular mechanisms implicated in these activities remain to be determined. To gain new insights into the putative role of urolithins as novel drugs for cancer therapy, we investigated the antiproliferative effects of the most abundantly produced urolithins in humans, urolithins A and B, and their trihydroxylated precursor, urolithin C, in different tumor cell lines (Fig. 1). The role of each compound in the mechanisms associated with cell proliferation, specifically the cell cycle and cell death, as well as their effects on the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs) intracellular signaling pathways, were determined. Additionally, this work aimed to establish a structure-activity relationship, which may constitute valuable information in the design of urolithin derivatives that present potent antiproliferative activities.

Materials and methods

Chemicals

DMEM, RPMI-1640, resazurin sodium salt, trypan blue, penicillin, streptomycin, β-tubulin antibody and ellagic acid were obtained from Sigma-Aldrich Química (St. Louis, MO, USA) and fetal bovine serum was obtained from Gibco (Paisley, UK). Propidium iodide (PI). RNase solution and an annexin V-fluorescein isothiocyanate (FITC)/PI double staining kit were purchased from Immunostep (Salamanca, Spain). Protease and phosphatase inhibitor cocktails were obtained from Roche (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA) and polyacrylamide was obtained from BioRad (Hercules, CA, USA). Alkaline phosphatase-conjugated secondary antibodies and enhanced chemifluorescence substrate (ECF) were purchased from GE Healthcare (Chalfont St. Giles, UK). Phospho (p-) extracellular signal-regulated protein kinases 1 and 2 (Erk1/2; Thr202/Tyr204), stress activated protein kinase/c-Jun amino terminal kinase (SAPK/JNK; Thr183/Tyr185), p38 MAPK (Thr180/Tyr182) and Akt (Ser473) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Urolithins A (3,8-dyhydroxy-urolithin; 98.3% purity), B (3-hydroxy-urolithin; 99.4% purity) and C (3,8,9-dyhydroxy-urolithin; 98.3% purity) (Fig. 1) were acquired from Dalton Pharma Services (Toronto, Canada). Urolithins were diluted in DMSO and stored at −20 °C, and ellagic acid was diluted in 1 M NaOH, as described by the supplier.

Cell culture

HepG2 (human hepatocellular carcinoma; ATCC® HB-8065™) cells were cultured in DMEM with 1 g/L of glucose; A549 (human lung adenocarcinoma epithelial cells; ATCC® CCL-185™), MCF-7 (human breast carcinoma; ATCC® HTB-22™) and BJ (human skin fibroblasts derived from normal foreskin; ATCC® CRL-2522™) cells were cultured in DMEM with 5 g/L of glucose; and UMUC3 (human bladder transitional carcinoma; ATCC® CRL-1749™) were cultured in RPMI-1640 medium with L-Glutamine. All media contained 10% (*v*/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C and 5% CO_2 in a humidifier incubator.

Assessment of cell viability

Cell viability/metabolic activity was determined using the resazurin assay [23]. Resazurin (blue) is reduced into resorufin (pink) by metabolically active cells; therefore, the magnitude of dye reduction is correlated with the number of viable cells.

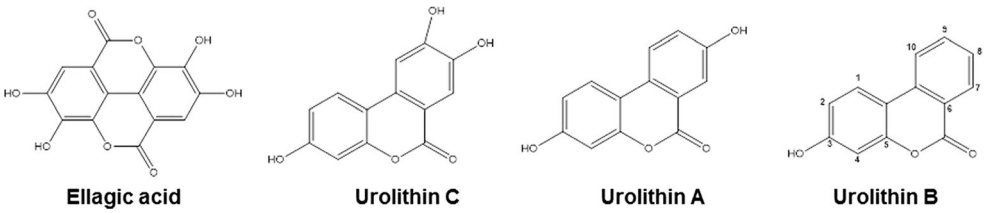

Fig. 1 Chemical structures of ellagic acid and urolithins

The cells were plated in 48-well plates for 12 h, the medium was carefully removed, and the cells were treated with serial concentrations of urolithins A, B and C (from 0.25 to 200 μM) and ellagic acid (from 0.5 to 75 μM) for 48 h. Similarly, sequential concentrations of DMSO and NaOH were used as controls. Resazurin (50 μM) was added to the cells 1 h before fluorescence recording. Compared with the other cell lines, BJ cells have a lower metabolic activity; thus, the incubation of BJ cells with resazurin was performed for 4 h. Absorbance at 570 nm was determined using a standard spectrophotometer (SLT, Austria), with a reference wavelength of 620 nm. Treated cells were compared with their respective controls and the IC_{50} values, which represent the concentration required to inhibit 50% of the cell viability, were determined by nonlinear regression.

Cell cycle and cell death analysis

UMUC3 cells were plated in 12-well plates and analyzed after 24 and 48 h of treatment with urolithins A (23.92 μM), B (41.8 μM) and C (16.28 μM). To investigate the cell cycle distribution, the cells were trypsinized, washed twice with PBS and fixed overnight with ice-cold 70% aqueous ethanol. The cells were then incubated with PI in RNase solution for 15 min at room temperature and analyzed by flow cytometry.

Apoptosis was evaluated using an annexin V-fluorescein FITC/PI double staining kit as described by the supplier. Briefly, UMUC3 cells were harvested by trypsinization, washed with PBS, incubated with annexin V and PI and analyzed by flow cytometry.

The results were obtained on a Becton Dickinson FACSCalibur cytometer using the Cellquest software (BD Biosciences). PI histogram modeling was performed using ModFit LT software (Verity Software House). To obtain accurate instrument settings, the flow cytometer was calibrated with fluorescent standard microbeads (CaliBRITE Beads; BD Biosciences).

Trypan blue exclusion assay

After 48 h of each treatment, the cells were trypsinized and suspended in cell culture medium. A sample of this suspension was diluted with the same volume of trypan blue dye and the cells were immediately counted using a Neubauer's hemocytometer. Control cells were incubated with the same volume of DMSO available in urolithin-treated cells.

Preparation of UMUC3 cell extracts

UMUC3 cells were cultured in 24-well plates for 12 h and further incubated with urolithins A, B and C at different amounts of time according to the proteins studied. Whole-cell lysates were obtained with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 2 mM ethylenediaminetetraacetic acid) supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails tablets. The cells were scraped and centrifuged at 12,000 rpm for 10 min at 4 °C to remove cell debris. The supernatant was collected and stored at −80 °C until use.

Western blot analysis

The protein concentration of cell lysates was determined using the bicinchoninic acid protein assay. The samples were denatured in sample buffer (0.125 mM Tris pH 6.8, 2% (*w*/*v*) sodium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol and bromophenol blue) and heated for 5 min at 95 °C. Briefly, equal amounts of proteins were separated by 4–10% (*v*/v) SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with the primary antibodies for phosphorylated proteins Erk1/2, SAPK/JNK and p38 MAPKs and Akt (1:1000) and with anti-rabbit or anti-mouse alkaline phosphatase-linked IgG secondary antibodies for 1 h at room temperature. The bands were visualized using the ECF substrate and the TyphoonTM FLA 9000 imaging system (GE Healthcare). The band densitometry was quantified using the Image Quant 6.0 software (Molecular Dynamics, Amersham Biosciences). The membranes were reprobed for β-tubulin antibody (1:20,000) to demonstrate that similar amounts of protein were loaded in the gels.

Statistical analysis

The data were expressed as the means ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by *Dunnett's post-hoc test*. The statistical analysis was performed using Prism 5.0 Software (GraphPad Software). Differences were considered significant at $p < 0.05$.

Results and discussion

Effect of urolithins and ellagic acid on cell viability

In this study, we aimed to identify the most potent antiproliferative ellagitannin-derived metabolites, evaluate the sensitivity of several tumor cell lines to urolithins A, B and C, and to investigate the selective effect of these urolithins in cancer cells. The resazurin reduction colorimetric assay was used to assess the viability of cells treated with urolithins and ellagic acid. Human tumor cell lines (UMUC3, A549, HepG2 and MCF-7) and non-tumor cells (BJ) were treated with increasing concentrations of those compounds for 48 h. For urolithins, the data points of the dose-response curves were fitted with a

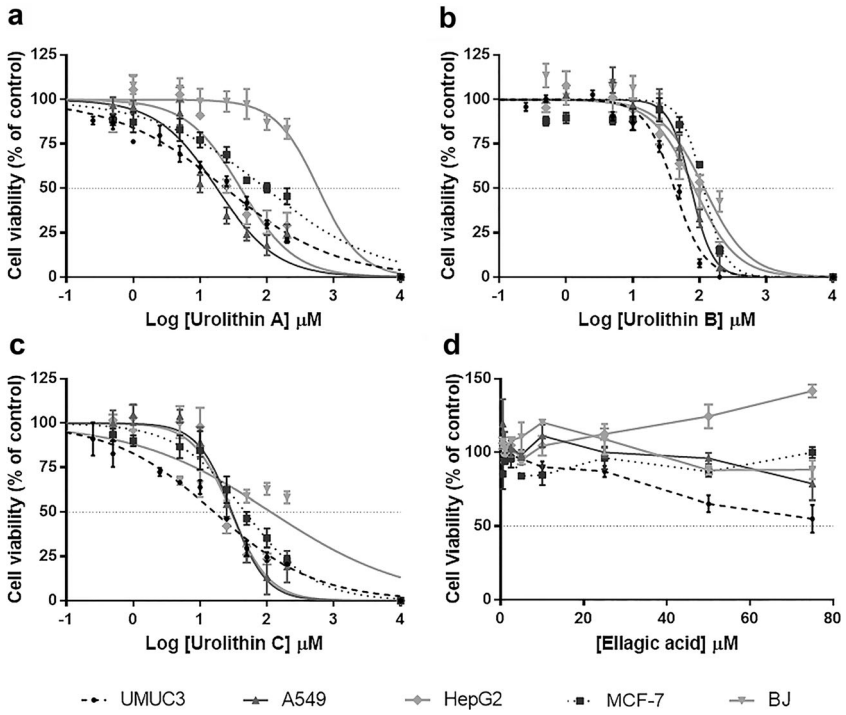

Fig. 2 Dose-response effect of urolithins and ellagic acid on cell viability of different cell lines. Cells were treated with different concentrations of urolithin A (**a**), urolithin B (**b**), urolithin C (**c**) and ellagic acid (**d**) for 48 h. The data points correspond to the means ± SEM of at least three independent assays. For urolithins, the dose-response curves were fitted to a sigmoidal function to calculate the mean IC_{50} values

sigmoidal function to calculate the IC_{50} values, which were further used in subsequent studies. The data showed that urolithins A, B and C reduced cell viability in a dose dependent manner (Fig. 2). Overall, urolithins A and C were the most active samples, presenting with the lowest IC_{50} values among all tested tumor cell lines (Table 1). The effect of urolithins was also dependent on the cell type. A549 and UMUC3 were highly sensitive to the cytotoxic effect of the urolithins, with IC_{50} values above 20 μM for urolithin A (17.81 μM) and C (16.28 μM), respectively. Notably, the highest IC_{50} values were observed in the non-tumor BJ cells, and urolithin A showed approximately 5 times less toxicity (IC_{50} = 586 μM) on normal fibroblasts compared with urolithins B (IC_{50} = 117.8 μM) and C (IC_{50} = 114.9 μM). Altogether, these results suggest that urolithins, and particularly urolithin A, exhibited a preferential cytotoxicity against human tumor cell lines. This selectivity is critical for the development of new effective therapeutic agents with reduced adverse effects in non-tumor cells.

In all cell lines, none of the tested concentrations of ellagic acid decrease cell viability by 50% or more. Consequently, a characteristic dose-response sigmoidal curve was not obtained, and the IC_{50} could not be determined using sigmoidal curve fitting. The highest concentration of ellagic acid (75 μM) assayed was greater than most of the IC_{50} values of urolithins A and C in tumor cells and exerted marginal effects on the viability of almost all cells lines, suggesting that ellagic acid had no significant cytotoxic activity on these cells.

To our knowledge, this is the first report to analyze the effects of urolithins in A549, UMUC3 and BJ cell lines. Our results are in agreement with those obtained in HepG2 cells by Kallio et al. [24], who demonstrated that urolithins A and B, but not ellagic acid, decreased liver cell viability and that urolithin A was more active than urolithin B. Moreover, our data showed that urolithin C had the lowest IC_{50} value in HepG2 cells and was thus the most active molecule in this cell line. Additionally, among the tested tumor cell lines, the MCF-7 cells were the most resistant to treatment with

Table 1 IC_{50} values and confidence intervals (CI) of urolithins A, B and C in different cell lines

	Urolithin A (μM)		Urolithin B (μM)		Urolithin C (μM)	
	IC_{50}	95% CI	IC_{50}	95% CI	IC_{50}	95% CI
UMUC3	23.92	[19.64–29.14]	41.8	[37.74–46.29]	16.28	[12.55–21.11]
MCF-7	95.56	[69.62–131.2]	114.9	[101.0–130.6]	47.25	[36.99–60.35]
A549	17.81	[13.63–23.42]	74.83	[65.97–84.89]	30.09	[24.21–37.39]
HepG2	40.53	[30.73–53.46]	83.72	[67.87–103.3]	29.29	[22.98–37.32]
BJ	586	[215.8–1591]	117.8	[89.86–154.4]	114.9	[63.51–208.0]

urolithins. However, the urolithins were still able to decrease the viability of MCF-7 cells; again, urolithin C presented with the lowest IC_{50}. Accordingly, previous studies in MCF-7 cells indicated that urolithins A and B can bind estrogen receptors, thereby exhibiting estrogenic and anti-estrogenic activities [18], and can inhibit aromatase activity [25]; these events may be critical for breast cancer chemoprevention.

Overall, we considered the UMUC3 cell line to be the most susceptible to these molecules since the IC_{50} values for all tested urolithins in these cells were lower than 50 μM (urolithin A - 23.92 μM; urolithin B - 41.8 μM; urolithin C − 16.28 μM). Based on these data, the UMUC3 cells were used in subsequent experiments. Furthermore, since ellagic acid was the least active molecule and its IC_{50} could not be calculated, we did not perform additional experiments with this compound.

Effect of urolithins on the cell cycle

Defects in cell cycle checkpoints are associated with an increase of genetic instability and uncontrolled cell proliferation, which could lead to the onset of carcinogenesis. Several transitional bladder carcinoma cell lines, including UMUC3 cells, have defective cell cycle checkpoints [26]. Therefore, targeting the cell cycle machinery may lead to the inhibition of cell proliferation and induction of apoptotic cell death, thus presenting potential value in identifying novel therapies to circumvent disease progression. Hence, the effects of urolithins on the cell cycle were evaluated in UMUC3 cells. The cells were treated for 24 or 48 h with the predetermined concentrations of urolithins A, B and C, which corresponded to the IC_{50} values. The cells were then analyzed for the distribution of G0/G1, S and G2/M phases by flow cytometry. At both time points, urolithin A clearly decreased the number of cells at the G0/G1 stage. However, its effect was more pronounced after 24 h since there was a 30.0% reduction in the percentage of cells in G0/G1 compared to control cells, whereas after 48 h of incubation, the percentage of cells in G0/G1 decreased by 21.6% relative to that of the control. This decrease in the percentage of cells in G0/G1 was accompanied by a significant rise in the percentage of cells in G2/M. In fact, in cells incubated for 24 h with urolithin A, the percentage of cells in G2/M increased by 26.1% relative to that of the control cells. However, in cells incubated with urolithin A for 48 h, the percentage of cells in G2/M increased by 13.1% relative to the control. These results seem to indicate that urolithin A induces a cell cycle arrest in G2/M, a phenomenon that is more evident after 24 h of incubation (Fig. 3). Accordingly, cell cycle arrest in the G2/M phase was also previously reported in colon and prostate cell lines treated with urolithin A [20, 27–29]. Several key proteins are involved in controlling the G2/M checkpoint. A study performed by Vicinanza et al. [20] demonstrated that urolithin A changed the cell cycle distribution, increased cdc 2 kinase phosphorylation at tyrosin-15 and induced an accumulation of cyclin B1. Those results suggested the inactivation of the cyclin B1/cdc2 kinase complex, a known regulator of the G2/M transition.

Our data also showed that urolithin C decreased the percentage of cells in the G0/G1 phase, though this effect was less pronounced than that of urolithin A. In fact, after 24 h of incubation with urolithin C, the percentage of cells in G0/G1 decreased by 10% compared to the control cells (Fig. 3a). However, this reduction was not sustained after 48 h of incubation with urolithin C (Fig. 3b). Therefore, our data showed that unlike urolithin A, urolithin C does not induce a cell cycle arrest.

The results showed that urolithin B also reduced the percentage of cells in G0/G1 but was less effective than urolithin A, and its effect was more pronounced at 48 h of incubation (decreased by 18.1% compared to the control). In addition, our data indicated that urolithin B was the only urolithin that significantly increased the percentage of cells in the S phase (the percentage of cells increased by 18.7% compared to the control cells) (Fig. 3b). These results are in agreement with those previously observed in colon cell lines [28]. Overall, we observed that the different urolithins elicit a dissimilar cellular response, suggesting that each urolithin triggers a specific signaling transduction profile.

Apoptosis/necrosis modulation by urolithins

Apoptosis induction is still considered a chemopreventive and chemotherapeutic strategy in cancer. To identify the type of cell death, specifically apoptosis and/or necrosis, UMUC3 cells were incubated with urolithins A, B or C, for 24 (Fig. 4a) and 48 h (Fig. 4b), and then stained with annexin V-FITC/PI. The data showed a statistically significant increase in the proportion of early apoptotic, late apoptotic and necrotic cells after 48 h of treatment with urolithin A (Fig. 4b). In contrast, urolithins B and C induced neither apoptosis nor necrosis (Fig. 4). This selective profile attributed to urolithin A was not observed in T24 cells, a cell line derived from a transitional cell carcinoma, in which both urolithin A and B induced apoptotic cell death [19]. These differences are probably due to genetic and molecular differences between the studied cell lines.

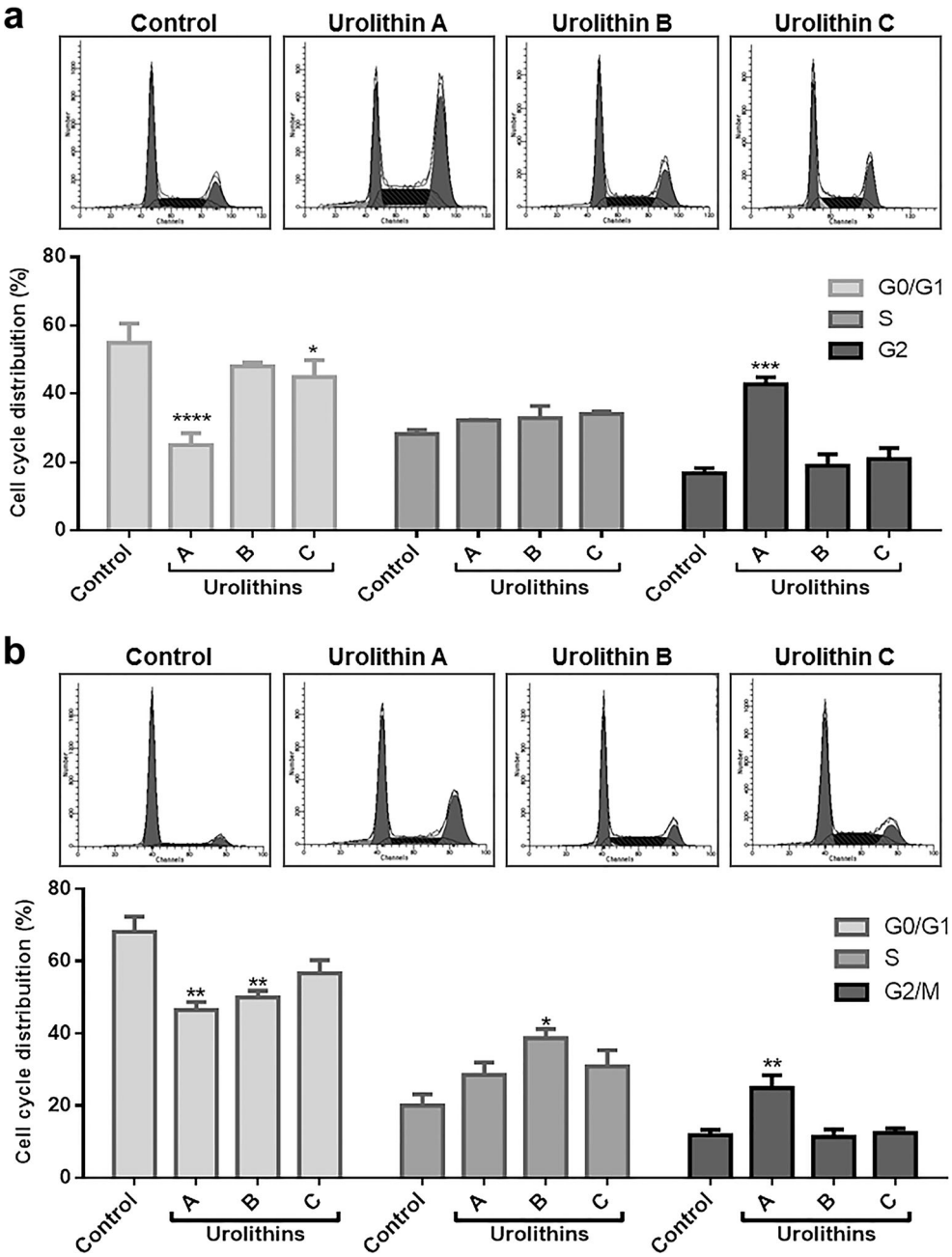

Fig. 3 Effects of urolithins on cell cycle progression in UMUC3 cells. Cells were incubated with urolithins A, B and C for 24 (**a**) and 48 (**b**) h and then the cell cycle progression was analyzed by flow cytometry with PI. The data are expressed as the percentage of PI-positive cells and are the means ± SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the control

Determination of UMUC3 cell viability using the trypan blue exclusion assay after incubation with urolithins a, B and C

As previously described, the resazurin assay was used to evaluate the IC_{50} values for cell viability. The absence of significant effects on apoptotic/necrotic cell death and the magnitude of the effects of urolithins B and C on the cell cycle distribution led us to use the trypan blue exclusion assay, which measures the integrity of the plasma membrane, to determine cell viability [30]. The cells were treated for 48 h with the concentrations of urolithins that corresponded to the IC_{50} values determined using the resazurin assay.

The results showed a slight, though statistically significant, decrease in cell viability after incubation with urolithins B (20%) and C (22%). For urolithin A, the results were consistent with those obtained using the resazurin assay, with a 48% decrease in cell viability (Fig. 5). The resazurin-based assay has been broadly used as an indicator of metabolically active mitochondria. However, this assay might over/under estimate the number of viable cells. Considering these results, we concluded that the cells treated with urolithins B and C are probably metabolic defective; thus, for the time periods studied, the results do not directly reflect the number of cells. Therefore, this study reinforces the concept that more than one viability test may need to be applied in order to evaluate

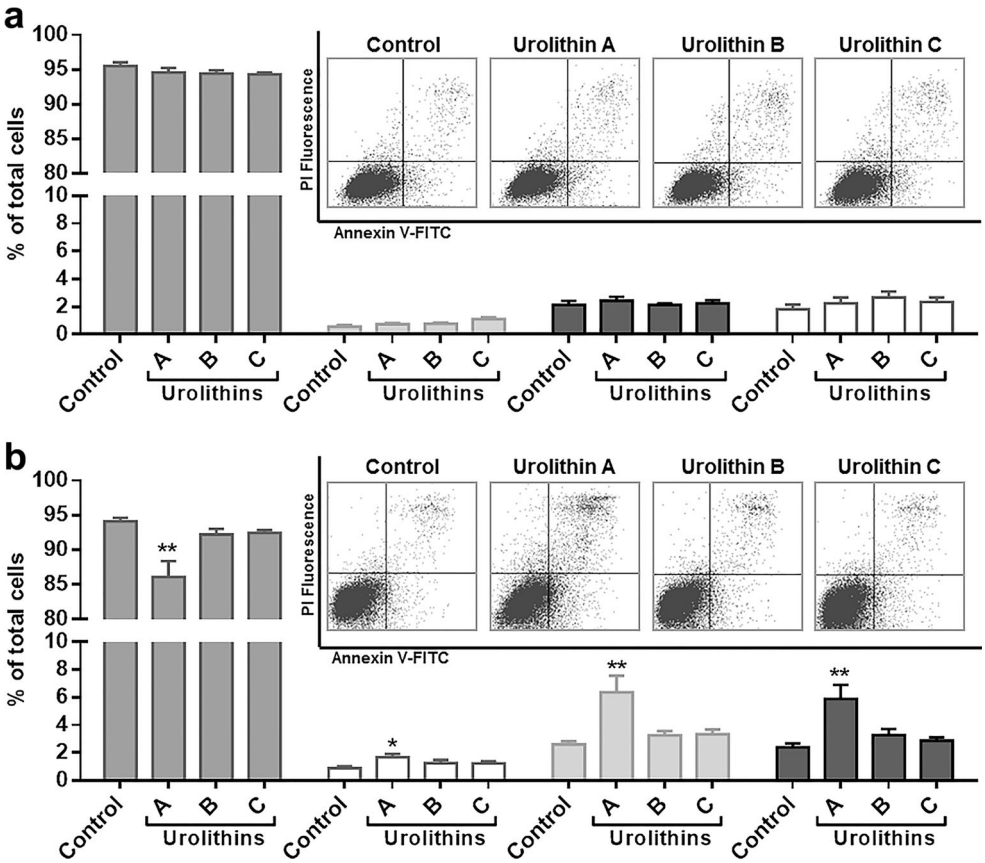

Fig. 4 Detection of apoptosis/necrosis after treatment with urolithins. Cells were incubated with urolithins A, B and C for 24 (**a**) and 48 (**b**) h. Annexin V-FITC/PI labelling was detected by FACSCalibur flow cytometer. The populations of early apoptotic cells (annexin V-positive/PI-negative), late apoptotic cells (annexin V-positive/PI-positive), necrotic cells (annexin V-negative/PI-positive) and viable cells (annexin V-negative/PI-negative) were evaluated as the percentage of total cells and are given as the means ± SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ when compared to the control

different parameters and to exclude false positive/negative results [31]. Importantly, we may also conclude that urolithin A is the most active molecule in UMUC3 cells, strongly suggesting its exploitation in in vivo models of transitional bladder cancer.

Effect of urolithins on the intracellular signaling pathways PI3K/Akt and MAPKs

We also evaluated the effects of the urolithins on the activation status of the PI3K/Akt and MAPKs signaling pathways, which are implicated in cell survival and proliferation. This analysis was performed by measuring the levels of phosphorylated kinases by western blot at different time points. The PI3K/Akt pathway promotes cellular survival by allowing defective cells to overcome cell cycle checkpoints and suppress apoptosis [32]. Indeed, the aberrant activation of this pathway is characteristic of a wide range of cancers, including bladder cancer [33]. Accordingly, pharmacological inhibition of the PI3K/Akt pathway to reduce the invasive capacity of the UMUC3 cell line has been addressed [34]. Based on those findings, we evaluated the effect of urolithins A, B and C on the phosphorylation status of Akt in bladder cancer UMUC3 cells (Fig. 6a-c). Our data showed that urolithin A decreased the levels of p-Akt at early time points (Fig. 6a), suggesting the potential of urolithin A as a chemopreventive/chemotherapeutic agent.

ERKs regulate several cellular processes, including proliferation, survival and prevention of apoptosis, whereas JNKs and p38 MAPKs usually positively regulate apoptosis and cell differentiation [35]. The majority of bladder cancers are highly dependent on ERK activation [36]. Interestingly, our results demonstrated that urolithin A decreased the phosphorylation of ERK 1/2 (Fig. 6d) and SAPK/JNK (Fig. 6 g) after incubation for short time periods. We may therefore suggest that the antiproliferative effects of urolithin A could be

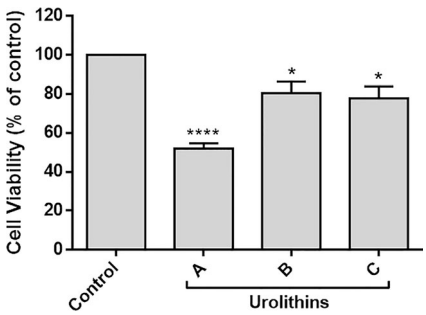

Fig. 5 Trypan blue exclusion test of cell viability. Cells were incubated with urolithins A, B and C for 48 h and then assayed for cell viability. The data are presented as the means ± SEM of at least 3 independent experiments and are expressed as percentages of the control. * $p < 0.05$, **** $p < 0.0001$ when compared to the control

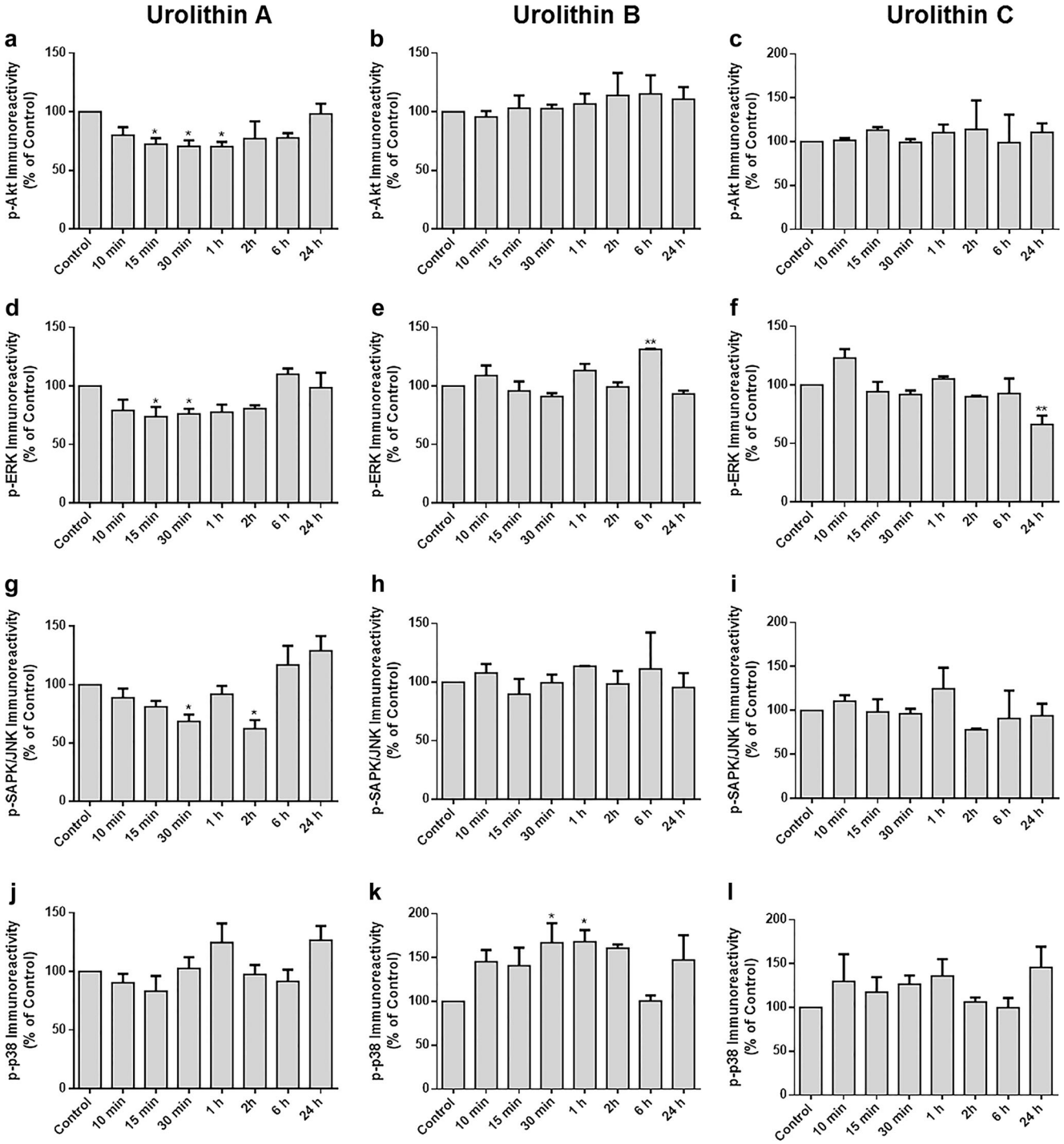

Fig. 6 Effect of urolithins on PI3K/AKT and MAPKs signaling pathways. UMUC3 cells were incubated with urolithins A, B and C for different amounts of time. The levels of Akt, ERK1/2, SAPK/JNK and p38 phosphorylated proteins were evaluated by western blot. Representative immunoblots are presented in Fig. 7. The data are presented as the means ± SEM of at least 3 independent experiments and are expressed as percentages of the control. *$p < 0.05$, **$p < 0.01$ when compared to the control

related to ERK 1/2 inhibition. However, the concomitant and intermittent decrease of SAPK/JNK phosphorylation could negatively regulate apoptosis. In fact, the effect of urolithin A in apoptotic cell death was only detected 48 h after the cells were treated. Studies using specific MAPK inhibitors should be addressed to further disclose the mechanisms by which urolithin A promotes apoptosis. Furthermore, cells treated with urolithins B and C demonstrated sporadic differences in ERK 1/2 and p38 phosphorylation. For example, urolithin C

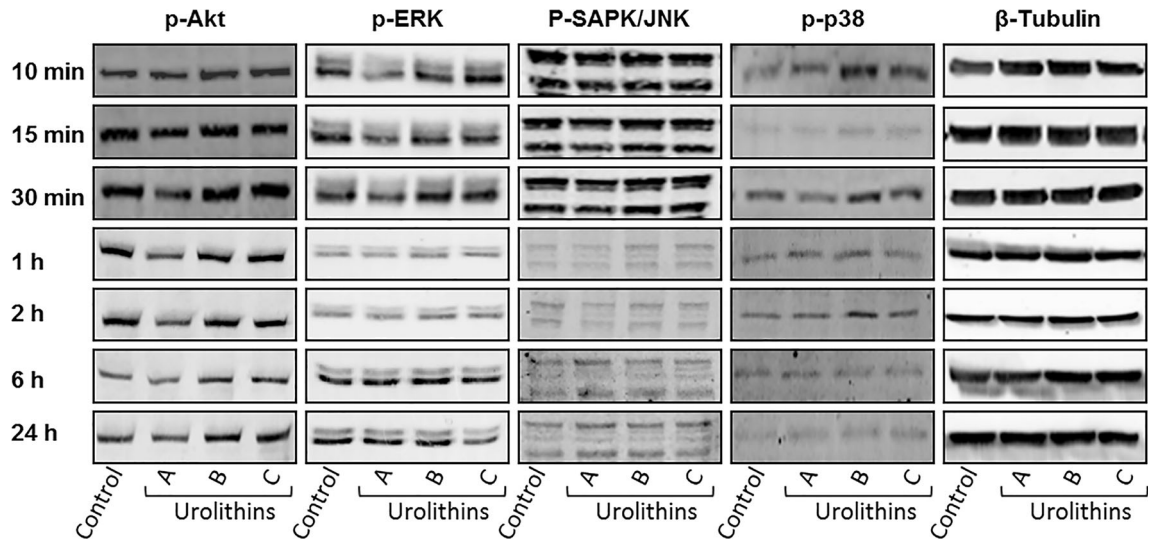

Fig. 7 Representative immunoblots of the data from Fig. 6

decreased the expression of p-ERK 1/2 after 24 h of treatment (Fig. 6f), whereas urolithin B increased the expression of p-ERK 1/2 at 6 h (Fig. 6e). Urolithin B also increased the expression of p-p38 at different time points (Fig. 6k).

Clear differences were observed in the activity profiles of each urolithin in terms of their effects on proliferation, the cell cycle, apoptosis and cell signaling pathways. Overall, urolithin A exhibited a preferential cytotoxicity against human tumor cell lines, promoting cell cycle arrest at the G2/M checkpoint after 24 h and 48 h of treatment, increasing apoptotic cell death and inhibiting Akt and ERK phosphorylation in UMUC3 cells. These differences appear to be related to the number/position of hydroxyl groups (Fig. 1). Indeed, the antiproliferative activity of urolithin A on cancer cell lines was notably more significant than that of urolithin B, reinforcing the hypothesis that the presence of a hydroxyl group at position 8 in urolithin A is partially responsible for the increase in biological activity. Conversely, the additional hydroxyl group in urolithin C at position 9 seems to have the opposite effect. Despite the similarities in the ranges of IC_{50} values of urolithins C and A against cancer cell lines, the tested concentrations of urolithin C did not modulate apoptotic/necrotic cell death or the cell cycle, events that are generally implicated in carcinogenesis. Our data are in accordance with those of other studies, which also demonstrated that urolithin A has a remarkable antiproliferative activity in cancer cells. In fact, comparatively to urolithins B or C, urolithin A showed the highest antiproliferative effect in Caco-2 and SW480 cells, HT-29 colon cancer cells; this activity was, at least in part, mediated by cell cycle arrest at the S and G2/M phases [28, 37]. Moreover, in vitro studies have demonstrated that urolithin A modulates several pathways implicated in cancer signaling and inflammation [9, 38, 39] highlighting the potential therapeutic value of urolithin A.

Even though urolithins A and B had been identified in human plasma and urine after ingestion of foods containing ellagitannins, the more abundant metabolites found in these samples were their glucuronide conjugates [40]. Although glucuronidation in other cell lines led to decreases in the biological activities of urolithins [28], it would be interesting to evaluate the antiproliferative effects of these metabolites in bladder cancer cells. Furthermore, and since the metabolites of different ellagitannins are present in biological samples, the effects of the combination of those molecules should also be addressed.

Conclusions

The increasing occurrence of human malignancies and the side effects associated with conventional therapies highlight the importance of discovering alternative drugs. In particular, the treatment of metastatic bladder cancer still remains one of the main challenges in urologic oncology, and the resistance of this carcinoma to a wide range of chemotherapeutic agents is the main reason for treatment failure.

In recent years, the therapeutic potential of dietary ellagitannins has been investigated. However, it has also been demonstrated that the bioavailability of ellagitannins is very low. By contrast, their metabolites, the urolithins, have relatively higher bioavailability. This knowledge aroused the interest of the scientific community, as these compounds have been proposed to be responsible for the anticancer activity and systemic health effects associated with the consumption of ellagitannins. Herein, we showed that urolithins A, B and C have antiproliferative effects in several cancer cell lines. In particular, urolithin A induced cell cycle arrest, increased apoptosis and modulated the intracellular PI3K/Akt and MAPK signaling pathways in UMUC3 cells. Of utmost importance,

we have shown the selective effects of urolithins in tumor cells and have established a structure-activity relationship. These findings constitute valuable information for the future design of urolithin derivatives. Overall, this paper generated important insights into the therapeutic value of urolithins, strongly supporting and encouraging further work on urolithins as potential chemopreventive/chemotherapeutic drugs for transitional bladder cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval This article does not contain any data from studies involving human participants or animals.

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