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ORIGINAL CONTRIBUTION

Urolithin A induces prostate cancer cell death in p53-dependent and in p53-independent manner

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Abstract

Purpose Pomegranate and walnuts are widely consumed dietary sources and contain several bioactive compounds, including the ellagitannins (ETs). ETs are polyphenols that are metabolized in the gut microbiota to urolithin A (UA). p53 is a tumor suppressor that lost its activity through MDM2 activation in about half cancers. The purpose of this study was to investigate the influence of UA on the p53-MDM2 interaction pathway in prostate cancer cell lines.

Methods Three human prostate cancer cell lines were used that harbor different p53 genotypes; LNCaP ($p53^{+/+}$), 22RV1($p53^{-/+}$) and PC3 ($p53^{-/-}$). Cell viability was determined by CellTiter-Glo Luminescent assay. Apoptosis was confirmed by measuring annexin V by flow cytometry. The expression of p53, its target proteins, and apoptotic markers were measured by western blotting. Real-time qPCR was used to measure the gene expression of p21, a main target gene of p53. Co-immunoprecipitation–immunoblotting was used to assess the inhibition of interactions between p53 and MDM2 and to assess the effect of UA on MDM2-mediated p53 polyubiquitination.

Results We found UA inhibited CaP cells' viability and induced apoptosis. For 22RV1 and LNCaP, we found UA increased p53 protein expression and its main target protein, p21, and MDM2, forming an autoregulatory feedback loop. In addition, UA increased the p53 proapoptotic proteins PUMA and NOXA. Moreover, UA inhibited the interaction between p53 and MDM2 and inhibited MDM2-mediated p53 polyubiquitination. UA downregulated MDM2 and XIAP protein expression in PC3 cells and upregulated p21 and p14ARF in a p53-independent manner.

Conclusion The influencing of UA on p53-MDM2 pathway may partly contribute to its anticancer effect.

Keywords Urolithin A · p53 · MDM2 · Prostate cancer · Polyphenols

Introduction

Carcinoma of the Prostate (CaP) is the most common cancer in men worldwide and is the second leading cause of cancer-related death in men in the United States [1]. When CaP is localized within its primary sites, treatments involved prostatectomy and 68% of patient become CaP-free for up to 10 years. For more advanced CaP, the main form of treatment involved androgen-ablation such as surgical or chemical castration. However, significant numbers of patients relapse CaP resulting in the emergence of androgen-independent CaP (AIPC) [2, 3]. Although docetaxel-based therapy is mostly used for AIPC, it still confers low survival rates for those patients which limited the treatment options for advanced CaP [4]. Therefore, it is important to target CaP independently on chemo and hormonal therapy. Several mutations in critical cellular pathways are acquired during CaP progression. One of the most common mutations is acquired in 50% of all cancers is the tumor suppressor gene TP53 including loss or gain of its functions [5, 6]. The transcription factor p53 is considered a guardian or caretaker of the genome due to its tumor-suppressor activity. p53 can control expression of genes involved in cell cycle arrest, apoptosis and DNA repairs [7]. The main target gene for p53 is p21 which mediates cell cycle arrest from G1 to the S phase. Moreover, BCL-2 family proteins PUMA and NOXA are mainly expressed by p53 and mediated p53-dependent apoptosis [8, 9]. p53 activation is regulated by its post-translation modifications (PTM) such as phosphorylation, acetylation, sumoylation [10–12]. Previous reports have shown that p53 phosphorylation and acetylation

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enhance the expression of its target genes [11, 13, 14], while other reports have shown p53 ubiquitination and sumoylation are associated with p53 nuclear export and inhibition of p53 transcriptional activity [15–17]. In addition to the regulations by PTM, p53 is negatively regulated by MDM2. MDM2 is an E3 ligase that mediates suppression of p53 transcriptional activity accompanied by p53 ubiquitination and degradation in the proteasome [18]. Moreover, MDM2 itself is a target gene for p53, therefore making an autoregulatory feedback loop in which p53 expresses its own inhibitor [19] Although p53 retains its wild-type form in about 50% of carcinoma cells, its activity is diminished by MDM2. Therefore, MDM2 becomes a novel target for cancer therapy in cancer cells harbor wild-type p53. Epidemiological studies suggest that consumption of a selected variety of fruit and vegetables rich in polyphenolic compounds is effective against several cancers including CaP cells and in vivo xenograft models [20–22]. Polyphenols that are derived from natural fruit have anticancer activity by acting on several mechanisms such as cell cycle arrest [23], induction of apoptosis [24], and inhibition of angiogenesis [25]. Pomegranate and walnuts are a widely consumed fruits worldwide. Previous studies have shown that pomegranates and walnuts have anticancer effects [26–28]. The effect of pomegranate and walnut on cancers is attributed to their polyphenolic compounds, particularly ellagitannins (ETs). The ETs hydrolyse in the stomach to EA. EA then metabolizes in the gut microbiota to the main bioavailable metabolite, urolithin A (UA) [29]. Previous studies have shown that UA is detected in the human prostate gland after the consumption of pomegranate juice and walnuts [30]. These studies have also shown that UA exerts its anticancer effects against CaP via different mechanisms. For example, UA downregulates androgen receptor and prostate-specific antigen (PSA) expression in human LNCaP cells [31]. Another study shows that UA induces cell cycle arrest and apoptosis in PC3 and DU-140 cell lines [32]. Although these studies confirm the apoptotic effects of UA in suppression of CaP, the molecular mechanisms of p53 regulation by UA and the anticancer effects of UA in CaP via targeting of p53-MDM2 pathway are not fully characterized. In the current study, we have found that UA activates p53 and its main transcriptional proteins p21, PUMA and NOXA by disrupting the interaction between p53 and MDM2. In addition, UA inhibits MDM2-mediated polyubiquitination of p53. In addition, UA inhibits PC3 cells (p53 null) by downregulating MDM2 and activating p21 in a p53-independent manner. We have confirmed the apoptotic effect of UA in three human CaP cell lines, 22RV1 and LNCaP and PC3.

Materials and methods

Cell cultures

Three human prostate cancer LNCaP ($p53^{+/+}$), 22RV1 ($p53^{-/+}$) and PC3 ($p53^{-/-}$) cells were purchased from American Type Tissue Culture Collection (Rockville, MD, USA). Mouse embryonic fibroblast (MEF) cells that possess double knockouts of p53 and MDM2 were obtained from Professor Guillermina Lozano (MD Anderson Cancer Center, University of Texas, USA). Wild-type MEF cells were obtained from ATCC. All cell lines were grown in a 37 °C incubator with 5% CO_2 according to the American Type Culture Collection protocols.

Reagents and antibodies

UA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-cleaved PARP, anti-cleaved caspase 3 (Asp175), anti-PMDM2-ser166, anti-PUMA (D30C10), anti-NOXA (D8L7U), anti-p53 ser15, anti-p53 ser 20, anti-GAPDH (14C10), and anti-β-actin (8H10D10) antibodies were purchased from Cell signaling biotechnology, Inc. (Danvers, MA, USA). Anti-MDM2 antibody (SMP14) was purchased from BD Biosciences (San Jose, CA, USA). Anti-p53 (DO-1), anti-p21 (F-5) and anti-ubiquitin (P4D1) antibodies were purchased from Santa Cruz technology (Santa Cruz, CA, USA).

Cell viability assay

All cells were incubated in 96-well plates overnight at a concentration of 120,000 cells/ml. Cells were then treated with UA or control (0.09% DMSO) and cell viability was measured by CellTiter-Glo Luminescent assay (Promega) according to the manufacturer's protocol.

Immunoprecipitation

The p53 was immunocaptured and purified using p53 immunocapture kit according to the manufacturer's protocol (Abcam). Briefly, cells were harvested for 48 h before treatment to be confluent for at least 70% confluency. Cells were then treated with 40 µM EA or UA for 24 h. Cells were then washed three times with cold PBS and scrapped using lauryl maltoside extraction buffer provided in the kit. Cells then transferred to chilled Eppendorf tubes and kept on ice for 30 min and then centrifuged for 20 min at 17×*g* speed. The supernatant was then transferred to new chilled Eppendorf tubes and then subjected to BCA assay. 1 mg of cell lysate was used and incubated with p53 antibody coated with

agarose beads in cold room for 16 h. The beads containing the p53 then washed three times with washing buffer. The p53 then was eluted from the beads using SDS elution buffer and then subjected to western blot analysis by loading equal volume of each sample on SDS-PAGE.

Immunoblotting

Immunoblotting was conducted as described previously [33]. All cells lines were incubated in 100 mm dishes in serum-free media containing either UA, or the appropriate controls. The dishes were then scraped, and the lysate was collected in a microcentrifuge tube and placed on ice for 30 min. The lysate was then passed through 21-gauge needle to break up the cell aggregates. Cell lysate was centrifuged at 14,000×*g* for 10 min and was quantified by BCA reagent (Thermo Fisher Scientific, Inc., Rockford, IL, USA). An equal concentration of each sample was loaded onto SDS-PAGE for separation. The gel then was transferred to nitrocellulose membrane (Bio-rad) using the semi-dry transfer cell TRANS-BLOTSD (Bio-Rad Laboratories, Hercules, CA) with transfer buffer (containing 230 mM glycine, 25 mM Tris, 0.7 mM SDS, 20% methanol). The membrane was then blocked using Odyssey-blocking buffer (LI-COR Biosciences) for 1 h at room temperature. Membranes were incubated with the indicated primary antibodies overnight and the appropriate secondary antibody for 1 h. Protein bands were visualized using the LI-COR system.

Ubiquitination assay

LNCaP cells were treated with 40 μM UA for 24 h. Afterward, 20 μM of proteasome inhibitor (MG132) was added to the culture medium for 6 h prior to harvesting. Endogenous p53 was immunocaptured as described above and then immunoblotted with the ubiquitin antibody to detect p53 polyubiquitination.

Quantitative RT-PCR

CaP cells were treated with 40 and 80 μM UA for 24 h. Total RNA was extracted and purified from the cell lines with miRNeasy Mini Kit (Qiagen) according to the manufacture guidelines. The cDNA was generated from the total RNA with using iScript cDNA Synthesis Kit (Biorad). The quantitative real time PCR was performed with a Bio-Rad real time thermal cycler using 57 °C as annealing temperature. Specific primers were used for human p21 F (CTGAGACTC TCAGGGTCGAA); p21 R (CGGCGTTTGGAGTGGTAG AA); human MDM2 F (TGGCGTGCCAAGCTTCTCTGT); MDM2 R (ACCTGAGTCCGATGATTCCTGCT); human GAPDH F (CAGCCTCAAGATCATCAGCA); and GAPDH R (GTCTTCTGGGTGGCAGTGAT).

Results

Urolithin A induces apoptosis in 22RV1 and LNCaP cells

To confirm the previous finding that UA induces cell death in LNCaP and to test the apoptosis in 22RV1, both cell lines were treated with various concentrations of UA or vehicle for 24 and 48 h and cell viability was measured using Celltiter Glo assay. There was no significant inhibition in cell proliferation at all concentrations tested (Fig. 1a) for 24 h. However, reduction in cell viability was confirmed after 48 h treatment of UA. To validate that UA induces apoptosis, cells were treated with 40 and 80 μM for 24 h and cleaved PARP was measured. Cleaved PARP was increased at 40 and 80 μM, confirming apoptosis in 24 h treatment in all cells used (Fig. 1b).

UA increased p53 protein expression and its target genes

Based on the result of apoptosis, we examined the effect of UA on p53 expression in 22RV1 and LNCaP cells. Our results show that UA increased the endogenous p53 at 40 and 80 μM in 24 h in 22RV1 and slightly increased in LNCaP (Fig. 2). Following the observed induction of p53 protein expression in 22RV1 and LNCaP, we sought to investigate whether this p53 protein induction was a result UA-induced p53 phosphorylation. Indeed, we found that UA induced p53 phosphorylation at Ser15 and Ser20 in both 22RV1 and LNCaP (Fig. 2).

Given that we identified the increased in p53 protein expression in response to UA treatment, we investigated whether UA causes the induction of p53 target genes, MDM2 and p21. As expected, we found p21 and MDM2 are induced after UA treatment for 24 h in both 22RV1 and LNCaP cells at both the protein and mRNA level (Fig. 3a, b). These findings indicate that UA increases p53 protein expression and its target genes. Since phosphorylated MDM2 at ser166 enables it to enter the nucleus where it binds and inhibits p53 [34], we investigated the effect of UA to pMDM2 at Ser166. We found that UA downregulated pMDM2 at Ser166 in LNCaP but not in 22RV1 (Fig. 3a, b).

To determine whether the proapoptotic effects of UA on 22RV1 and LNCaP cells were p53-dependent, we

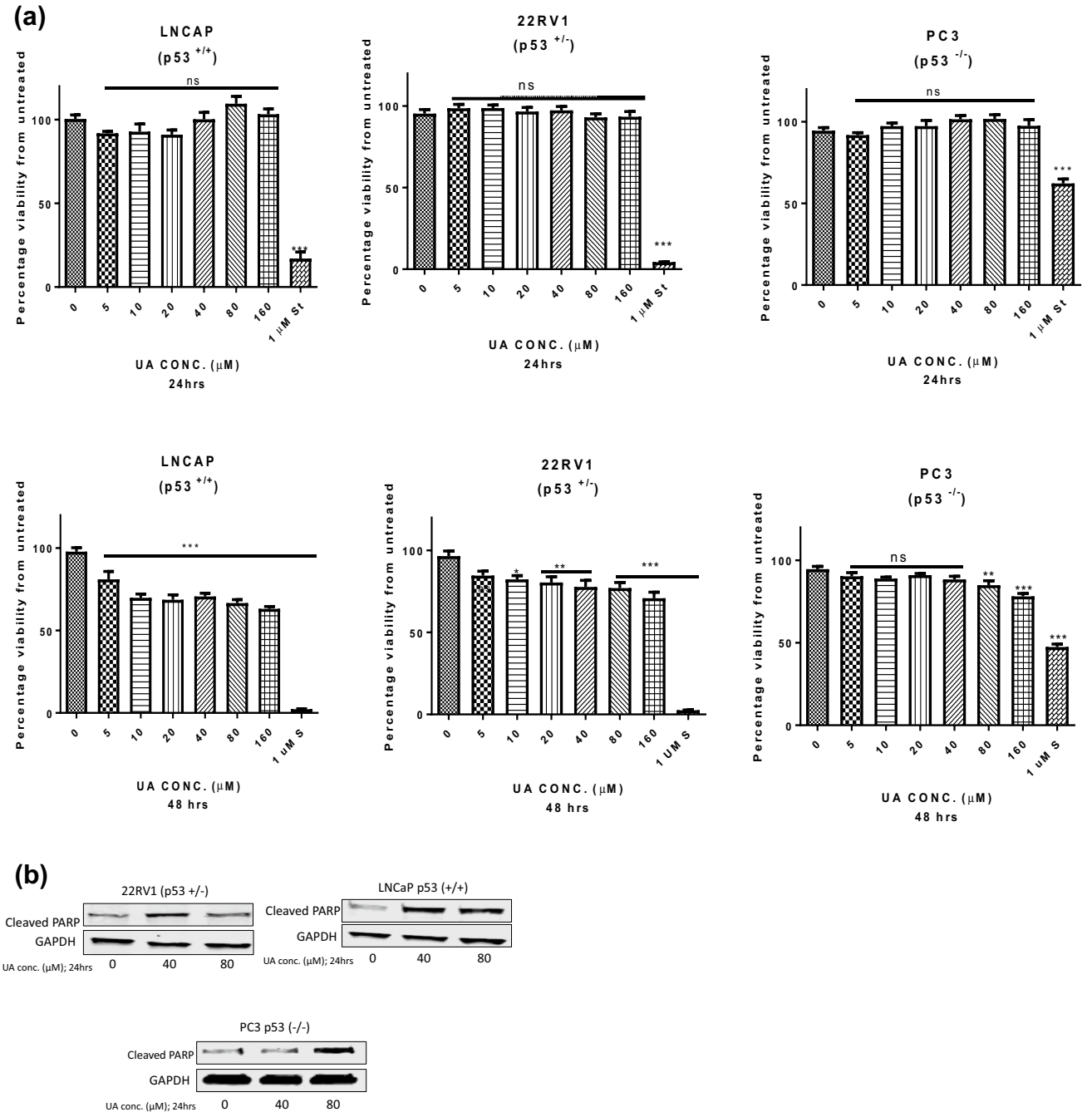


Fig. 1 **a** Cell titer Glo assays were performed to determine cell viability upon incubation with increasing concentrations of UA in 22RV1, LNCaP, and PC3 cells for 24 and 48 h. 1 μM of staurosporine (ST) was used as a positive control. One-way ANOVA of three measurements in triplicates (mean ± SEM) was performed. **b** Determination of apoptotic marker cleaved PARP protein levels by western blot

examined the effect of UA on both PUMA and NOXA in 22RV1 and LNCaP cells. The expressions of both these proapoptotic proteins were increased by UA in CaP cells and was p53-dependent (Fig. 4a, b).

UA inhibits the interaction of p53 and MDM2

Given the results shown above, UA increased p53 accumulation and its target gene and caused MDM2 autoregulatory

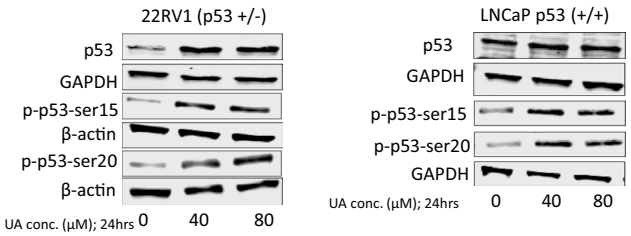


Fig. 2 UA induces p53 accumulation and increases p53 Ser15 and Ser20 phosphorylation. 22RV1 and LNCaP cells were seeded in 100 mm dishes and treated with 40 and 80 μM concentrations of UA for 24 h. The cells were then harvested, and protein levels were determined by western blot using the indicated antibodies. The blots are representative of three independent experiments

feedback loop, we investigated the effect of UA on the physical interaction between p53 and MDM in the LNCaP cell line harboring a wild type of p53. Following treatment of LNCaP with 40 μM UA for 24 h, co-immunoprecipitation was performed using the p53 immunocapture kit (described in the method section) to detect the interaction between p53 and MDM2. As shown in Fig. 5, endogenous p53 was immunocaptured in both vehicle control and treated sample. The interaction between p53 and MDM2 was markedly decreased by UA treatment (Fig. 5). This result indicates UA inhibits the interaction between p53 and MDM2.

UA inhibits MDM2-mediated p53 ubiquitination

Based on the finding that UA promotes the stability of p53 and inhibits p53 interactions with MDM2, we further investigated the effects of UA on the ubiquitination of endogenous p53. LNCaP cells were treated with 40 μM for 24 h. Endogenous p53 was immunoprecipitated as indicated in the method section. The p53 polyubiquitination was markedly decreased by the 40 μM UA for 24 h treatment (Fig. 6). This result suggests that UA inhibits p53 ubiquitination that is mediated by MDM2.

UA suppress CaP cells in p53-independent manner

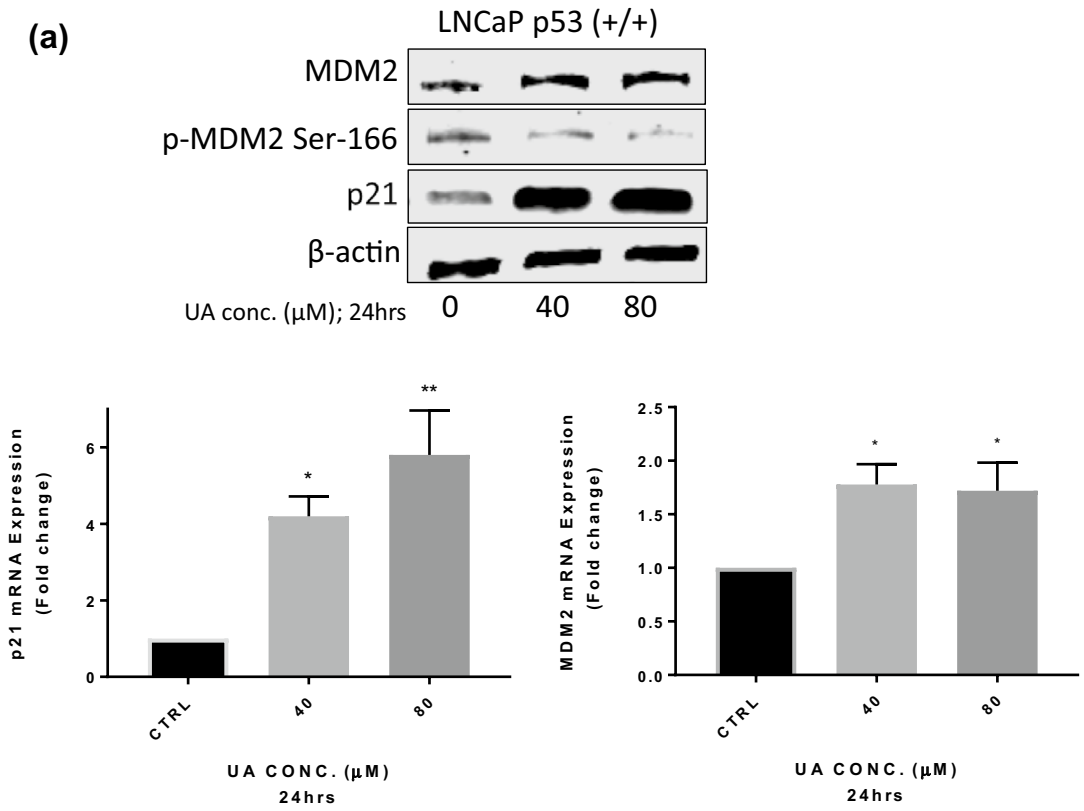
To see the effect of UA in the absence of p53, PC3 cell line was used because it harbors p53 knockout. In contrast to LNCaP and 22RV1 cell, UA did not induce MDM2 protein expression in PC3, but it downregulated MDM2 protein expression at 40 and 80 μM (Fig. 7a). Interestingly, the MDM2 gene expression was not changed after UA treatment (Fig. 7b). We therefore sought to investigate the effect of UA on p14ARF protein expression. p14ARF is a tumor suppressor that is known to antagonize the MDM2 ligase activity [35]. The protein expression of p14ARF was increased after UA treatment, explaining the downregulation of MDM2 (Fig. 7a). Furthermore, we looked for p21 in PC3 CaP cells. Interestingly, we found UA increased p21 at 40 and 80 μM at both the mRNA and protein level, independent of p53 (Fig. 7a, b). To further address the apoptotic effect of UA in p53-independent manner, we investigated the effect of UA on anti-apoptotic protein, XIAP that is induced by MDM2 [18]. UA showed downregulation of XIAP protein expression and elevation of cleaved caspase 3 (Fig. 7c). These results confirm that UA induces apoptosis by downregulating MDM2 and XIAP in p53-independent manner.

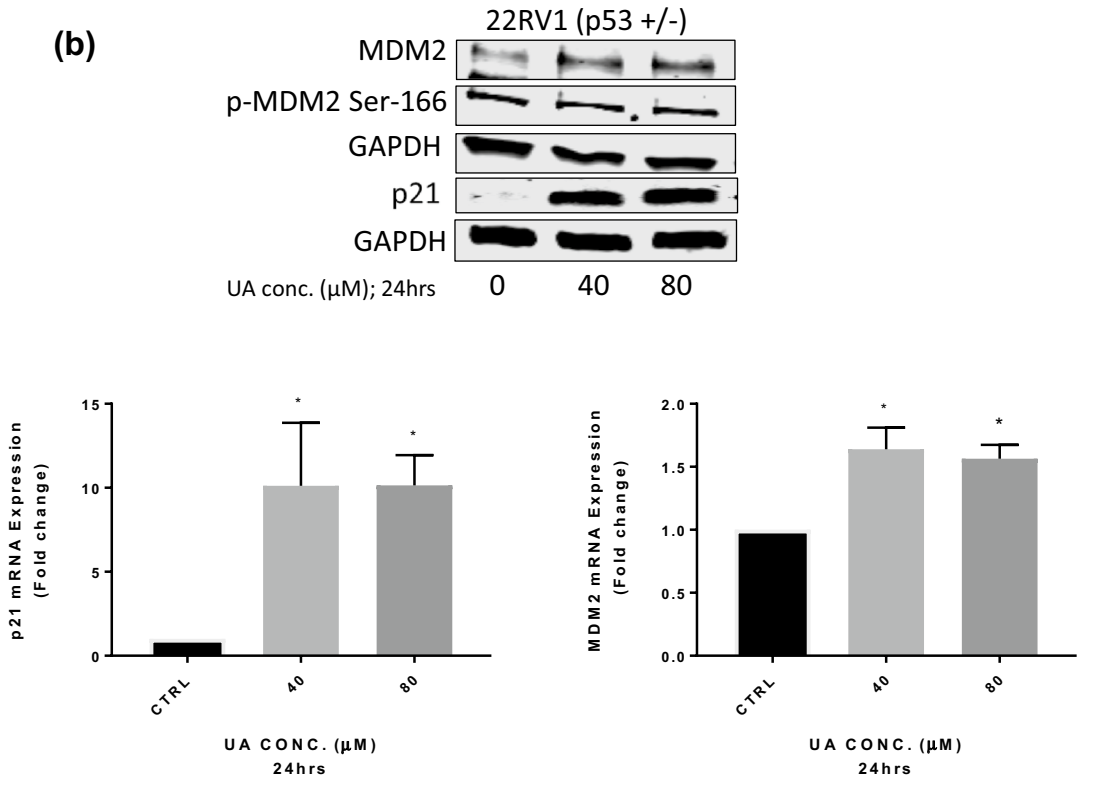
UA induced responses are independent of p53 and MDM2

To clarify the effect of p53 and MDM2 status on the ability of UA to induce apoptosis, cell viability assay was performed on MEF cell line ($p53^{-/-}$, $MDM2^{-/-}$) and on wild-type MEF (Fig. 8b). Although UA did not show significant inhibition in MEF's ($p53^{-/-}$, $MDM2^{-/-}$) proliferation, treatment of 40 and 80 μM UA for 24 h increased the cleaved caspase 3 protein expression, indicating apoptosis in MEF cells independently on p53 and MDM2 (Fig. 8b). Furthermore, we tested the effect of UA on p21 in MEF ($p53^{-/-}$, $MDM2^{-/-}$). Interestingly, in contrast to PC3 cells, p21 protein expression was not detected after 24 h of UA treatment as compared to wild-type MEF cells (Fig. 8c).

Discussion

Urolithin A is a major metabolite of ETs which are abundant in pomegranate, berries and walnut. UA has been extensively studied because of its anticancer effects. Although several reports have shown that UA exerts its anticancer activity on several cancer cell lines, the underlying mechanism of the influence of UA on p53-MDM2 pathway is not yet fully understood. Previous studies showed that UA-induced apoptosis in PC3 and DU-145 that are p53 null and mutated, respectively [32]. Another study showed UA inhibits transcription of PSA and androgen receptor mRNA in LNCaP cells [31]. Although this UA action is important in the early stages of CaP that are dependent on androgen receptors, advanced CaP becomes androgen-independent. Other studies have shown that UA induces apoptosis and induces p21 mRNA in the LNCaP cell line that retained wild-type p53 [36]. Although p21 is a major target gene of p53, no data show the effect of UA on p53 and its main negative regulator MDM2. We first looked at the effect of UA on CaP cells' viability. Although previous studies showed that UA inhibits cell viability in CaP cells in 24 h, in the current study, we did not find significant effect on CaP viability after UA treatment for 24 h. However, we found the CaP cells' viability declined after 48 h treatment. Moreover, we confirmed that UA induces apoptosis by inducing cleaved PARP


(a)
LNCaP p53 (+/+)
MDM2
p-MDM2 Ser-166
p21
β-actin
UA conc. (μM); 24hrs
0
40
80
p21 mRNA Expression (Fold change)
MDM2 mRNA Expression (Fold change)
CTRL
40
80
UA CONC. (μM)
24hrs


(b)
22RV1 (p53 +/-)
MDM2
p-MDM2 Ser-166
GAPDH
p21
GAPDH
UA conc. (μM); 24hrs
0
40
80
p21 mRNA Expression (Fold change)
MDM2 mRNA Expression (Fold change)
CTRL
40
80
UA CONC. (μM)
24hrs

◂**Fig. 3** **a** (Upper panel) UA upregulates p53 target genes, MDM2 and p21. LNCaP cells were seeded in 100 mm dishes and treated with 40 and 80 μM concentrations of UA for 24 h. The cells were then harvested, and protein levels were determined by western blot using the indicated antibodies. The blots are representative of three independent experiments. (Lower panel) p21and MDM2 mRNA levels were determined using quantitative real time-qPCR. Bars represent p21 and MDM2 mRNA in LNCaP cells treated with control (0.09% DMSO) or with 40 and 80 μM UA for 24 h. Data are expressed in fold changes compared to the vehicle control and normalized using GAPDH as internal control. **b** similar experiments were performed using 22RV1 cell line. The qPCR data are (mean ± SEM) of three independent experiments. * and ** $p < 0.05$

in three human CaP cell lines (22RV1 and LNCaP and PC3). We assumed this discrepancy between the viability assay and apoptosis in 24 h is because apoptosis is an early event of cell death, while cell viability declines at late event of apoptosis. Therefore, even that the cells still viable in 24 h, but apoptosis was confirmed after 24 h treatment. This was further confirmed when we saw CaP viability declined after 48 h of UA treatment. To further investigate the effect of UA on the upstream signaling pathway of cleaved PARP, the effect of UA on p53-MDM2 pathway was examined in 22RV1 and LNCaP cells. The MDM2 is negatively regulated p53 at the protein level. Therefore, we looked on protein expression of p53 after UA treatment. Western blot data indicate that UA stimulated p53 protein expression and that of its target proteins, p21, MDM2, PUMA and NOXA. This suggests that UA induces cell cycle arrest by elevating p21 gene and protein expression and induces apoptosis in 22RV1 and LNCaP cells in a p53-dependent manner by inducing PUMA and NOXA protein expression. p53 protein induction, stabilization, and localization are mainly regulated by post-translational modifications, such as phosphorylation, acetylation and ubiquitination [10, 11, 17]. The accumulation of p53 demonstrates that p53 phosphorylation at Ser15 is in response to DNA damage and phosphorylation at Ser20 weakens the interaction between p53 and MDM2 [37, 38]. In the current study, we demonstrated that the increased p53 protein expression by UA in 22RV1 and LNCaP cells was accompanied by the phosphorylation of p53 at Ser15 and Ser20, indicating that UA induces its action by causing DNA damage. When p53 is phosphorylated at Ser15, p53 is stabilized inside the nucleus where this phosphorylation enables p53 to bind to the promoter region of p21 to increase the level of p21 expression [39]. This confirms that UA induces cell cycle arrest by increasing the p53 main target protein, p21. As shown in Fig. 2, the total p53 protein level was increased with UA treatment in 22RV1, but slightly increased in LNCaP. We assumed that UA act differently in 22RV1 and LNCaP cells on p53 protein expression. The 22RV1 cell is derived from primary tumor of the prostate [40]. Moreover, 22RV1 cells have a heterozygous missense mutation in the tetramerization domain of the p53 (amino acids 323–363) [41]. On the other hand, the LNCaP cells is metastatic cells that are derived from lymph node and have two wild-type alleles of the p53 gene [41]. Therefore, the total p53 protein expression induced by UA in 22RV1 was different from that in LNCaP cells. The tumor suppressor p53 induces apoptosis mainly via expression of the proapoptotic proteins PUMA and NOXA [8]. In the current study, the increased level of p53 by UA was accompanied with increased levels of PUMA and NOXA. It has been shown that the proapoptotic proteins PUMA and NOXA are able to bind mitochondrial anti-apoptotic proteins such as BCL-2, BCL-XL, BCL-W, MCL1 and BCL-2-related protein A1 (BCL-2A1) [42]. Moreover, UA has been shown to inhibit Bcl-2 in LNCaP cells [31]. Therefore, we conclude that UA induces apoptosis via p53-dependent mechanisms by inducing PUMA and NOXA proteins expression. One of p53's downstream target gene, MDM2, is an E3 ligase enzyme that is responsible for p53 polyubiquitination and degradation in the proteasome [43]. Therefore, increased levels of p53 may cause increased levels of its main negative regulator, MDM2, forming an autoregulatory feedback loop. In the current study, we found that UA treatment markedly increased p53 expression and its negative regulator, MDM2. Since MDM2 upregulation by p53 will mediate p53 polyubiquitination in the cytoplasm, we further investigated the effect of UA on p53 ubiquitination. Although UA increases the level of MDM2, MDM2-mediated p53 polyubiquitination was markedly decreased by UA. Moreover, the co-immunoprecipitation results showed that UA inhibited the interaction between p53 and MDM2.

The apoptotic effect of UA in PC3 that has null p53 indicates the effect of UA in PC3 cells is p53-independent. We observed that UA did not increase MDM2 in PC3 cells but rather it downregulated it. Since PC3 cell line has no p53, no autoregulatory feedback loop can occur with MDM2. Our data showed that UA did not show a significant effect on MDM2 at the transcription level, thus we speculate that MDM2 protein downregulation might result from a direct interaction of UA with MDM2, or UA may affect other regulators of MDM2. p14ARF is known to antagonize MDM2 interactions with proteins and inhibits its ligase activity and also known to inhibit the G1 and G2 in the cell cycle [35]. Previously, it has been demonstrated that the polyphenolic compound, apigenin, downregulated MDM2 in 22RV1 cells by increasing p14ARF protein expression [44]. We found UA increased p14ARF in PC3 cells, which may explain the downregulation of MDM2 protein. Moreover, our data show that p21 mRNA and protein expressions were increased with UA treatment in PC3 cells, independently of p53. These data confirmed the evidence that p21 can be expressed in the absence of p53 [45]. Previous study showed that MDM2 knockdown by antisense therapy enhanced p21 expression in PC3 cells

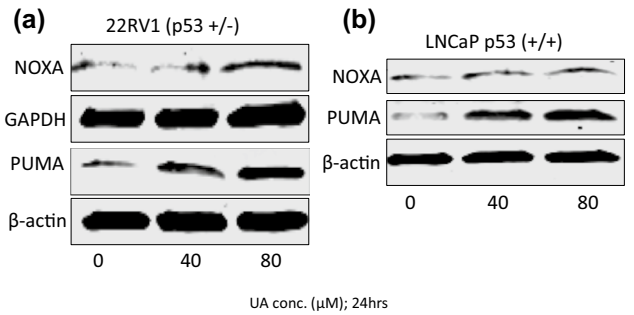


Fig. 4 **a** UA upregulates p53 main proapoptotic proteins, PUMA and NOXA. LNCaP cells were seeded in 100 mm dishes and treated with 40 and 80 μM concentrations of UA for 24 h. The cells were then harvested, and protein levels were determined by western blot using the indicated antibodies. **b** Similar experiments were performed using 22RV1 cell line. The blots are representative of three independent experiments

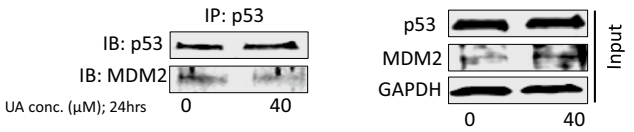


Fig. 5 UA inhibits the interaction of p53 and MDM2. LNCaP cells were treated with 40 μM UA for 24 h. Cell lysate was used for IP using p53 immunocapture kit as described in the method section. Western blot analysis was performed with the indicated antibodies following SDS-PAGE to detect the interaction of p53 and MDM2. The same cell lysate was used to determine the expression of p53 and MDM2 by western blot analysis. The blots are representative of three independent experiments

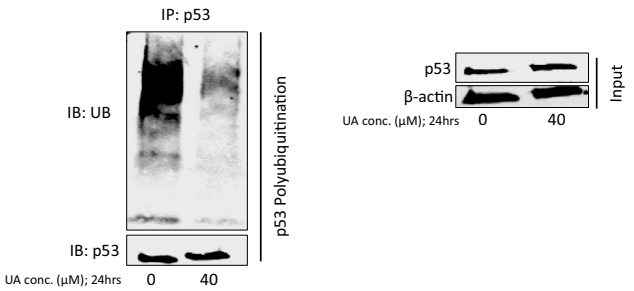


Fig. 6 UA inhibits MDM2-mediated p53 ubiquitination. LNCaP cells were treated with 40 μM UA for 24 h and then subjected to 6 h treatment with 20 μM MG132 before cell harvesting. The cell lysate was subjected to p53 antibody coated with agarose beads to capture the endogenous p53 as described in the method section. The lysate then subjected to western blot analysis to determine p53 polyubiquitination using ubiquitin antibody. The blots are representative of three independent experiments

and increased cell sensitivity to apoptosis [46]. Although p21 act as cyclin-dependent kinase inhibitor, there are also evidences that p21 can also induce apoptosis [47]. In another study, it has been found that p21 can be negatively regulated by MDM2 in PC3 cells independent of p53 [48]. We therefore assumed that the increased level of p21 by UA was by the downregulation of MDM2 protein by UA. Our data suggest that UA induces cell cycle arrest by inducing both p21 and p14ARF in p53-independent manner. Another MDM2 function independently of p53 is the regulation of inhibitors of apoptosis proteins (IAPs) [18] Among IAPs, XIAP protein inhibits specifically caspase 9, 3, and 7, resulting in apoptosis inhibition. Overexpression of MDM2 interacts with XIAP mRNA, increasing its translation level leading to resistance to cancer treatment and poor prognosis [49] Our data showed that UA downregulated MDM2 and XIAP proteins expression and increased cleaved caspase 3. We concluded that UA induced apoptosis by downregulating MDM2 in PC3 cells in p53-independent manner. These data suggest that UA is a potential therapy for advanced CaP independently of hormonal therapy.

In addition to the three CaP cell lines that have different genotype for p53, we also used a MEF cell line that has a double knockout for p53 and MDM2. Our data show that UA induces apoptosis in MEF ($p53^{-/-}$, $MDM2^{-/-}$) by increasing cleaved caspase 3 protein expression. Thus, our results suggest that the induction of p53 protein expression and its target genes may partly contribute to the anticancer activity of UA. We further looked on the protein expression of p21 on MEF ($p53^{-/-}$, $MDM2^{-/-}$) cells. The protein expression of p21 was below detection limit in MEF ($p53^{-/-}$, $MDM2^{-/-}$) as compared to wild-type MEF, suggesting the requirement of p53 in p21 expression in these cells. These data were in agreement with previous study in which p21 was not detected in MEF ($p53^{-/-}$, $MDM2^{-/-}$) cells [50].

In conclusion, our study identifies MDM2 as a potential target of the natural compound Urolithin A for cancer therapy by inducing protein expression of p53 and by inhibiting MDM2-mediated p53 ubiquitination and degradation in cells that harbor wild-type p53. Moreover, UA suppress CaP independent of p53 by downregulating MDM2 and XIAP proteins and increasing cleaved caspase 3. Since p53 accumulation in normal cells is the problem of most MDM2 inhibitors [8], further research is needed to address the effect of UA on p53 expression in normal cells.

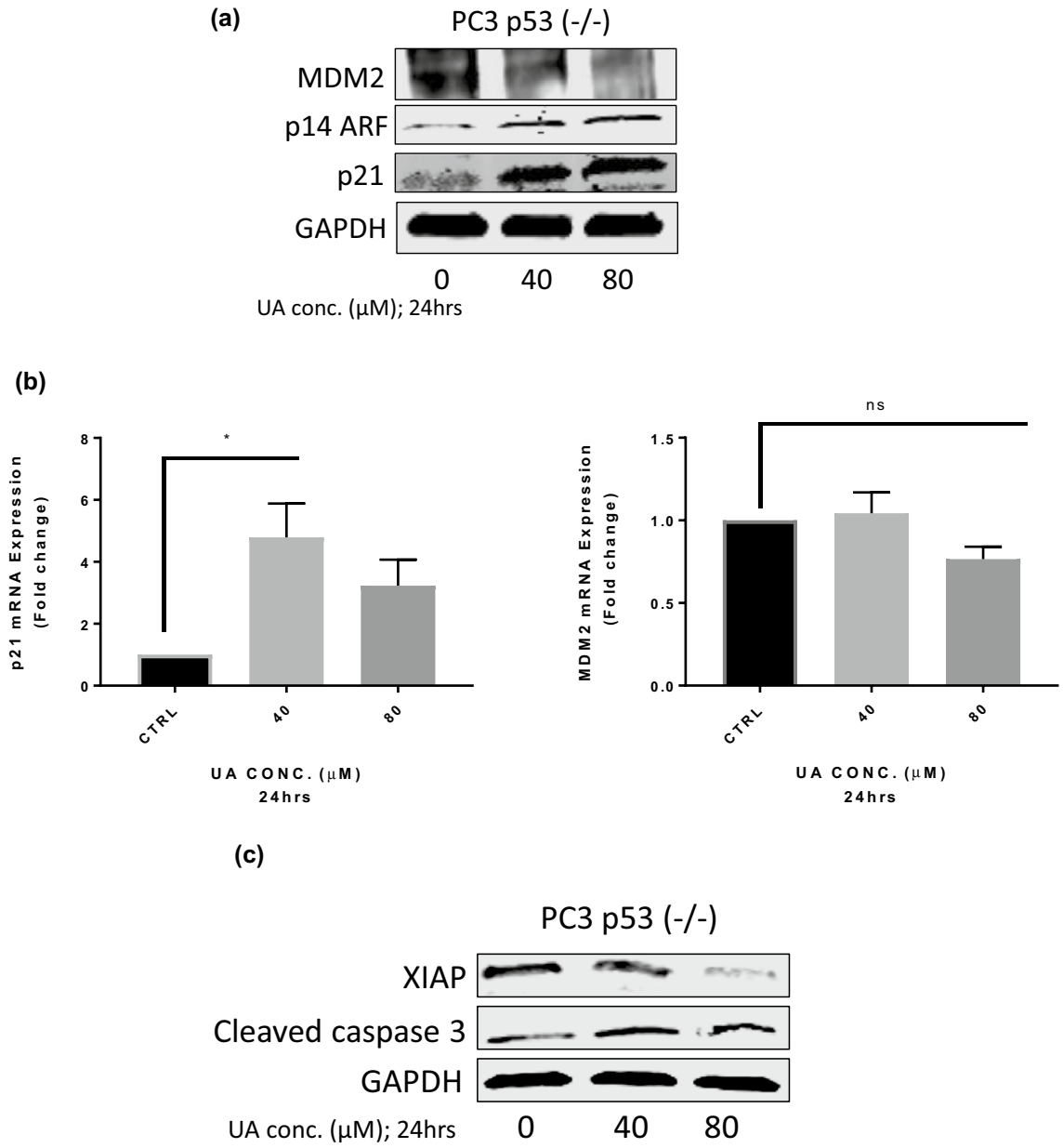


Fig. 7 UA suppresses CaP cells in p53 independent manner. PC3 cells were treated with 40 and 80 μM of UA for 24 h. **a** The protein expression of MDM2, p14ARF and p21 were detected by western blot analysis. **b** p21and MDM2 mRNA levels were determined using quantitative Real time-qPCR. Bars represent p21 and MDM2 mRNA in LNCaP cells treated with control (0.09% DMSO) or with 40 and 80 μM UA for 24 h. Data are expressed in fold changes compared to the vehicle control and normalized using GAPDH as internal control. **c** protein expression of XIAP and cleaved caspase 3 were detected by conducting western blot

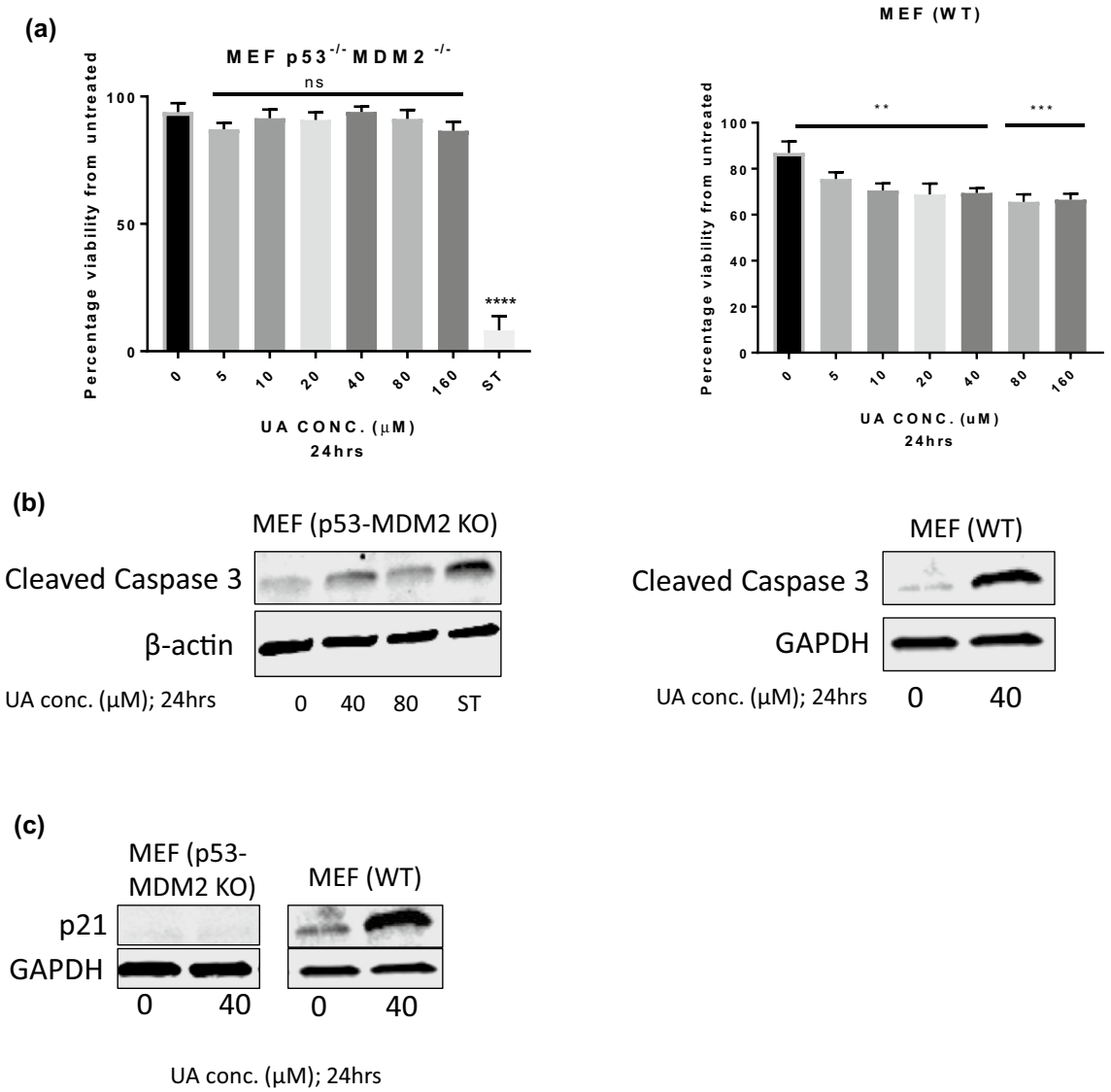


Fig. 8 UA effect are p53-MDM2-independent. **a** Cell titer Glo assays were performed to determine cell viability upon incubation with increasing concentrations of UA on MEF ($p53^{-/-}$, $MDM2^{-/-}$) and wild-type MEF cells. 1 µM of staurosporine (ST) was used as a positive control. One-way ANOVA of 3 measurements in triplicates (mean ± SEM) was performed. **b** Determination of apoptotic marker cleaved caspase 3 protein levels by western blot. **c** UA showed no detectable effect on p21 expression in MEF ($p53^{-/-}$, $MDM2^{-/-}$) as compared to wild-type MEF

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest related to this manuscript.

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