

Review

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Review

Multifaceted Properties of Usnic Acid in Disrupting Cancer Hallmarks

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Abstract: Cancer, a complex group of diseases marked by uncontrolled cell growth and invasive behavior, is characterized by distinct hallmarks acquired during tumor development. These hallmarks, first proposed by Douglas Hanahan and Robert Weinberg in 2000, provide a framework for understanding cancer's complexity. Targeting them is a key strategy in cancer therapy. It includes inhibiting abnormal signaling, reactivating growth suppressors, preventing invasion and metastasis, inhibiting angiogenesis, limiting replicative immortality, modulating the immune system, inducing apoptosis, addressing genome instability, and regulating cellular energetics. Usnic acid (UA) is a natural compound found in lichens that has been explored as a cytotoxic agent against cancer cells of different origins. Although the exact mechanisms remain incompletely understood, UA presents a promising compound for therapeutic intervention. Understanding its impact on cancer hallmarks provides valuable insights into the potential of UA in developing targeted and multifaceted cancer therapies. This article explores UA activity in the context of disrupting hallmarks in cancer cells of different origins based on articles that emphasize the molecular mechanisms of this activity.

Keywords: Usnic Acid (UA) ; antiproliferative activity; cancer treatment; cancer hallmarks; lichens

1. Introduction

Cancer is a complex group of diseases characterized by uncontrolled cell growth and the ability of cells to invade surrounding tissues. The hallmarks of cancer are a set of fundamental characteristics acquired by cancer cells during the development of the disease. Initially proposed by Douglas Hanahan and Robert Weinberg in 2000, these hallmarks provide an organizing principle for understanding the complexity of cancer. The original six hallmarks included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [1]. Subsequently, two emerging hallmarks were added: reprogramming of energy metabolism and evading immune destruction. These hallmarks were underpinned by two enabling characteristics: genome instability and inflammation [2]. The concept of the hallmarks of cancer has been instrumental in rationalizing the diverse and complex nature of cancer, providing a framework for research and potential therapeutic interventions [3].

Targeting the hallmark of sustained proliferative signaling, therapies aim to inhibit the abnormal growth signals that drive cancer cells to divide uncontrollably. Effective cancer therapy often involves strategies to disrupt the evasion of growth suppressors by cancer cells. Inhibiting invasion and metastasis is a key focus of cancer therapies, aiming to prevent the spread of cancer cells to distant tissues and organs. Therapeutic interventions targeting angiogenesis, a hallmark that involves the formation of new blood vessels, seek to deprive tumors of their blood supply and impede their growth. Addressing the hallmark of replicative immortality, therapies aim to limit the ability of cancer cells to divide and escape the natural cellular aging process continuously. Immune system modulation is a crucial aspect of cancer therapy, focusing on enhancing the body's ability to recognize and eliminate cancer cells, countering the hallmark of immune evasion. Therapies targeting resistance to cell death mechanisms are designed to induce apoptosis or other kinds of death in cancer



cells. Combating the hallmark of genome instability and mutation involves therapies to enhance DNA damage repair and prevent the accumulation of genetic alterations that drive cancer progression. Therapeutic approaches targeting the ability of cancer cells to sustain chronic inflammation aim to disrupt the tumor-promoting microenvironment and hinder cancer progression [2,3].

Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione, UA) is one of the most investigated bioactive compounds found in lichens. It occurs in nature as (−) and (+) isomers as well as a racemic mixture [4]. These enantiomers may exhibit different biological activities and interactions due to their distinct spatial arrangements, making them important considerations in pharmaceutical and biological applications [5,6]. UA has been studied for its various biological activities such as antibacterial, antiviral, antimycotic, antiprotozoal, anti-inflammatory, analgesic, neuroprotective and anti-cancer (reviewed in [5,7–9]). The anti-cancer activity of (−)-UA was reported in 1975 when Kupchan and Kopperman isolated this compound from *Cladonia* species and treated mice harboring Lewis lung carcinoma with (−)-UA at a dose range of 20–200 mg/kg and that extended the life of animals even to 52% over the untreated control group [10].

This article aims to summarize the mechanisms of UA activity in the context of targeting cancer hallmarks in various in vitro models and shed some light on evidence of anticancer UA effects in vivo.

2. UA Inhibits Cell Proliferation and Induces Apoptosis of Cancer Cells

Cancer cells' ability to proliferate and survive harsh conditions is connected with such hallmarks as sustained proliferative signaling, which is associated with the activity of oncogenes, evading cell cycle suppressive and proapoptotic signals and replicative immortality. UA has been shown to affect these features in cancer cells.

The main pathways signaling cells' survival and proliferation encompass growth factors and their receptors, Hedgehog and Wnt signaling, to name a few. It was reported that (+)-UA reduced transcriptional activity of β-catenin/LEF and c-jun/AP-1, the final effectors of Wnt and MAPK pathways, respectively. Thus, expression levels of c-myc, CD44, and cyclin D1, proteins crucial for cancer cell survival and proliferation, were reduced in A549, H1650, H1975 and H460 non-small-cell lung cancer cells treated with (+)-UA [11].

Numerous studies document that UA exerts antiproliferative potential inducing cell cycle arrest at either G₀/G₁, S or G₂/M phase and/or cell death through apoptosis or necrosis.

For instance, Backorova et al. (2011) showed that UA induced cell cycle arrest in the S phase in A2780 (ovarian), HL-60 (leukemia) and HCT116 (colon cancer cells) and in some of these cells - G₂/M phase arrest which was dependent on a dose (50 and 100 μM) and treatment time (48 or 72 h). Interestingly, the antiproliferative activity of UA did not depend on the presence of a tumor suppressor, p53 protein. This work also revealed that UA induced apoptosis and its extent was cell-line specific [12]. Later, investigating mechanisms of this activity, researchers found that UA decreased mitochondrial membrane potential (MMP) and increased reactive oxygen species (ROS) or reactive nitrogen species (RNS) levels, especially after 48 and 72 h of treatment (in up to 90–100% cells) and dose-dependent changes in p53, Bcl-2 and Bax levels which correlated with apoptosis in A2780 ovarian and HT-29 colon cancer cells [13].

G₂/M arrest may suggest that the microtubule dynamic is disturbed. However, treatment of MCF-7 breast or H1299 lung cancer cells with UA (29 μM for 24 h) did not result in any morphological changes in microtubules or an increase in the mitotic index compared to the effects of vincristine or taxol, drugs targeting microtubules. These results suggested that the antineoplastic activity of UA is not related to alterations in the formation and/or stabilization of microtubules [14].

S phase arrest has been observed in human hepatoblastoma HepG2 treated with growing concentrations of UA (3.13, 6.25, 12.5, 25 or 50 μM). At the highest concentrations, the fractions of subG₀/G₁ cells were elevated which were confirmed to be apoptotic cells. Lower viability was correlated with decreased levels of pro-survival proteins, such as Bcl-2 and Mcl-1 as well as Akt and p-Akt (Thr-308 and Ser-473), mTOR and p-mTOR (Ser-2448), p-S6K (Ser-371), p-4E-BP1 (Thr-37/46).

Additionally, UA elevated autophagy which played a protective role as its inhibition by 3-methyladenine or chloroquine or downregulation of Atg7 potentiated apoptosis. Moreover, autophagy-regulated activation of JNK played a protective role and its inhibition increased apoptotic cell fraction [15].

Another study compared the response of HepG2 (HBV-negative) and SNU-449 (HBV-positive) hepatocellular carcinoma cell lines to UA. Lichen compound reduced viability in a dose- and time-dependent manner and SNU-449 cells were more sensitive to UA than HepG2 cells. UA also induced cell cycle arrest at G₀/G₁ (HepG2) or S and G₂/M (SNU-449) phase, and apoptotic cell death after 48-h treatment. Autophagy induction was detected after 36 hours of treatment. The viability of HUVEC cells (normal endothelial cells) was not affected by UA tested in the concentration range of 6.25-100 μM which shows selectivity of UA toward malignant cells [16].

UA-induced decrease in pro-survival signaling pathways was also reported in other studies. Nguen et al (2014) investigated the impact of *Flavocetraria cucullata* metabolites on a panel of noncancerous and cancer cell lines. UA at concentrations as low as 5 and 10 μM decreased p-Akt (Ser-473), p-ERK1/2 (Thr202/Tyr204 and Thr185/Tyr187) and p-c-Jun (Ser-63) in A549 lung cancer cells. UA at 10 μM modulated the epithelial-mesenchymal transition (EMT) markers: reduced mRNA for Snail, Twist and N-cadherin and elevated E-cadherin (at the transcript and protein level) in these cells, as well as reduced time-dependently migration, invasion and anchorage-independent growth of A549 and AGS gastric cancer cells. UA decreased the viability of prostate CWR22Rv-1, lung A549, colon HT29, and gastric AGS cancer cells and had no impact on the viability of 4 different cell lines representing normal cells. Depending on concentration (25, 50 or 100 μM) and cell line, UA inhibited cell cycle progression after 24-h treatment in G₀/G₁ or S phase (CWR22Rv-1 and A549) or in G₂/M phase (AGS and HT29) and induced apoptosis of cancer cells with an elevation of Bax:Bcl-xL ratio, procaspase-3 and PARP cleavage, especially in AGS and CWR22Rv-1 (after 48 h of exposition) [17].

Ebrahim et al. (2017) showed that UA (15 and 25 μM) induced autophagy in breast cancer cells which was accompanied by a decrease in mTOR activity reflected by a drop in p-Akt, p-4E-BP1, p-S6K in breast MCF-7 and MDA MB 231 cancer cells. UA also inhibited motility and invasion of MDA MB 231 cells (at 10-30 μM and 10 μM, respectively) [18].

In gastric cancer cells, 100-400 μM UA induced G₀/G₁ arrest (in BGC823 cells) or G₂/M (in SGC7901 cells) after 24-h treatment and apoptosis with the rise of Bax:Bcl2 ratio (also *in vivo*) and caspase 3 activation as well as autophagy. *In vivo*, UA (100 mg/kg i.p. for 11 days) was more effective than 5-fluorouracil (5-FU, 25 mg/kg) in the retardation of BGC823 tumor growth in mice [19].

In A-431 squamous carcinoma cells UA also induced cell cycle arrest in G₀/G₁, apoptosis and necrosis (concentration tested within a range of 25-250 μM) which was connected with a reduction of MMP and reduced glutathione level, rise in ROS production, lipid oxidation and structural changes in DNA and surface lipids and proteins [20].

Mechanisms underlying G₀/G₁ arrest induced by UA have been investigated in A549 human lung carcinoma cells. The authors observed arrest at the G₀/G₁ phase in cells treated with 25, 50 or 100 μM UA for 24 or 48 h. It was accompanied by decreased levels of CDK4 and CDK6 cyclin-dependent kinases and cyclin D1, and increased levels of p21/Cip1, CDK inhibitor. UA treatment also enhanced cell death by up to twofold (24-h treatment) and eightfold (48-h treatment). While examining the cell death-associated molecular changes, authors observed that UA induced mitochondrial membrane depolarization and cleavage of PARP [21].

UA suppressed JAK1/2-Src-STAT3 and RAS-RAF-MEK-ERK pathways in HeLa cells. It led to a drop in the production of Programmed death ligand 1 (PD-L1) which not only enhanced the cytotoxic activity of T lymphocytes toward cancer cells but also resulted in a decline in viability and clonogenic potential of HeLa cells and it correlated with reduced levels of c-myc and cyclin D1. UA inhibited mTOR, leading to MiT/TFE nuclear translocation and enhanced lysosomal biogenesis [22].

Recently, using UA-linker-Affi-Gel, 14-3-3 proteins have been identified as UA targets. Interaction between these molecules led to the degradation of 14-3-3 by proteasomal and autophagy pathways in Caco2 colon cancer cells. As 14-3-3 proteins bind to numerous phospho-proteins, their elimination affects cell proliferation, invasion, metabolism and signaling pathways regulating cell

survival. Authors showed that UA (10 μ M) reduced levels of different proteins overrepresented in HCT-116 colon cancer cells expressing different isoforms of 14-3-3. Among the downregulated proteins were cyclin D1, cyclin B1 and p-Cdc2 (Tyr-15) which explains UA-induced G₀/G₁ arrest. Moreover, UA reduced levels of proteins phosphorylated at positions crucial for their activity such as p-mTOR (Ser-2488), p-Akt (Ser-473), p-STAT3 (Tyr-705), p-JNK (Thr183/Tyr185), and levels of EMT markers (Snail, Twist, N-cadherin, β -catenin). UA also reduced the activity of AP-1, STAT, and NF- κ B transcription factors which were elevated by overexpression of 14-3-3 isoforms in HEK293T cells [23].

Interesting mechanisms of UA activity have been noticed in human gastric and colon cancer cells treated with potassium usnate (KU), a water-soluble form of UA. The viability of a panel of cell lines (AGS, MNK45, SNU638, Caco2, HCT116 and HT29) was dose- and time-dependently reduced by KU. Moreover 24-h treatment with KU at IC₅₀ concentrations induced cell cycle arrest in G₀/G₁ or S phase, depending on the cell line, which was accompanied by a drop in CDK4, cyclin D2 and transient elevation of p21 protein levels. More detailed investigations based on gastric SNU638 and colon HCT116 cancer cells revealed that KU induced endoplasmic reticulum (ER) stress with the elevation of intracellular Ca²⁺, ROS and ER stress markers, such as BIP, PERK, IRE1 α , p-EIF2a, CHOP and ATF3. ATF3 is a transcription factor controlling the expression of ER stress (such as ATF3 itself), cell cycle modulating (GADD45) and apoptotic genes (Bak, PUMA, DR5). Its activity appeared crucial for the KU cytotoxic effect. Downregulation of ATF3 by specific siRNA protected against KU-induced elevation of Bak, p-BAD, PUMA, activation of caspase 3 and cell death. Moreover, KU (20 mg/kg i.p. injections for 16 days) applied to mice with CT26 metastatic colon cancer cells reduced the number of metastatic nodules in livers, elevating ATF3 and cancer cell apoptosis levels [24].

UA was shown to be a novel Pim-1 inhibitor (IC₅₀ = 202 nM). This protein serine/threonine kinase is often overexpressed in hematopoietic malignancies and acts as an oncogene supporting myc-driven transcription, 4E-BP-1-dependent translation, and inactivation of pro-apoptotic Bad. The study found that UA inhibited the proliferation of human HL-60 acute myeloid leukemia cells and K562 chronic myeloid leukemia cells (IC₅₀ = 10 and 10.4 μ M, respectively, after 3 days of treatment) and induced apoptosis. HL-60 cells were more responsive and after 18 h of treatment with 20 μ M UA apoptotic fraction increased from 3% to over 30%, while such amount of apoptotic K562 resulted from 48-h treatment with 60 μ M UA. It was accompanied by activation of caspase 3, 9 and 8, a decrease in anti-apoptotic Mcl-1, reduced p-eIF4E, p-4E-BP1 and p-Akt levels in both cell lines, as well as a decrease in c-myc, cyclin D1, p-Bad, Pim-1, MNK1 and increased p27 protein levels in K562 cells, which resulted from an inhibition of MNK1/eIF4F and Pim-1/4E-BP1 signaling pathways [25].

UA also exerted antiproliferative and pro-apoptotic effects in prostate cancer cells, hormone-independent DU-145 and PC-3 [26,27] and hormone-dependent LNCap cell lines [28]. Besides features characteristic for apoptosis (Bax:Bcl-2 mRNA elevation, drop in MMP, caspase activation), a decrease in NF κ B p50 at the protein level and NFKB1 mRNA was reported in DU145 prostate cancer treated with 40 μ M UA [27].

Another study investigated the mechanisms of the anticancer effects of (+)-UA from *Cladonia arbuscula* and (-)-UA from *Alectoria ochroleuca* on two human cell lines, T47D breast cancer cells and Capan-2 pancreatic cancer cells. The study found that both enantiomers were equally effective in inhibiting cell proliferation. (+)-UA at 10 μ g/ml arrested cell cycle at G₀/G₁ after 24-h treatment and decreased MMP; however, apoptosis was not detected. Instead, necrosis was seen in Capan-2 cells treated for a longer (48 h) time [29].

The activity of UA was also investigated against OVCAR-3 and A2780 ovarian cancer cells. The study utilized real-time cell analysis and demonstrated the antiproliferative effect of UA for these cell lines with no impact on non-cancerous L929 cells. UA at a concentration of 20 μ M inhibited the cell cycle at the G₀/G₁ phase and induced apoptosis of OVCAR-3 cells treated for 48 h. Evaluation of expression of apoptosis-related genes showed that UA significantly upregulated Casp-1, Casp-8, TRAF6, CHECK1, CHECK2, RIPK2, Bak1, Bag1, Bag4, BCL2A1, TNFRSF21, TP53, CIDEA, GADD45, BIRC3 and 5 and downregulated some genes of TNF and Bcl-2 family. It also blocked cell migration and invasion [30].

UA also modulated the expression of apoptosis-related genes of the apoptosis pathway in SKBR-3 breast cancer cells with significant elevation of mRNA for caspases 3, 4, 10, TRAF 5 and 6, numerous TNF family members, APAF1, Bik, Bak1, Bax, Bok, MCL1, p53, Chek1, Chek2, DAPK2, RIPK2, GADD45A, and reduction in mRNA for Bcl2, Bcl2L11, Bag1, Bag4. Moreover, Bax, caspase 3 and 9 have also been significantly elevated at the protein levels in cells treated with 7.2 μ M UA for 48 h. Notably, MCF-12A noncancerous breast epithelial cells, were resistant to UA used up to 10 μ M concentration [31].

The impact of UA on breast cancer cells with attention to miRNA expression profile was investigated. MDA MB 231, MCF-7, and BT-474 cells were treated with UA (IC_{50} c.a. 13 μ M) for 48 h and RNA was analyzed using microarrays. The authors identified differentially expressed miRNAs and their number was cell line specific (67 in MDA MB 231, 8 in MCF-7, and 15 in BT-474). MiRNAs were almost unique to each cell line; however, their targets were discovered to play a role mainly in 4 pathways in all 3 cell lines: basal cell carcinoma, neurotrophin signaling pathway, gap junction and Hedgehog signaling pathway. Pathway enrichment analysis revealed that in MDA MB 231 cells most targets of miRNA were transcripts involved in MAPK, Erb, PI3K-Akt and p53 pathways, while in BT-474 – in Erb, mTOR signaling, focal adhesion and gap junctions. UA increased the level of has-miR-185-5p, miRNA downregulated in many cancers, which is connected with their chemoresistance [32]. This miRNA, when overexpressed in BT-474, induced G₀/G₁ cell cycle arrest and apoptosis (with no effect in MCF-12A noncancerous cells), which was connected with the upregulation of pro-apoptotic Bcl-2 members, caspases, kinases related to cell death, death receptors and downregulation of antiapoptotic Bcl-2 [33].

Oncogenic long noncoding RNA urothelial cancer associated 1 (*UCA1*) was identified as another target of UA. *UCA1* is regarded as an oncogene due to its stimulating effects on cancer cell proliferation, migration, and invasion. It was shown to be upregulated in endometrial cancer tissue compared to normal tissue and contribute to cancer development. UA inhibited dose- and time-dependently survival of Ishikawa endometrial cancer cells (IC_{50} = 51,76 μ M after 48-h treatment) which correlated with a 3-fold decrease in *UCA1* level [34].

In the search for the mechanisms of antiproliferative activity of UA against breast cancer cells Zuo et al. (2015) found that it induced ROS generation in MCF-7 cells, which triggered the mitochondrial pathway of apoptosis with activation of c-Jun-N-terminal kinase (JNK), an increase in Bax:Bcl-2 ratio, drop in MMP, the release of cytochrome c, and caspase cascade activation. N-acetylcysteine (NAC) protected against these effects indicating that UA-induced ROS are responsible for them. UA given intraperitoneally inhibited tumor growth in a murine xenograft model with a dose of 100 mg/kg being more effective and less toxic to animals than cyclophosphamide (25 mg/kg bw) [35].

ROS induction was also observed in H520 and Calu-1 lung squamous cell carcinoma treated with (+)-UA (10, 20 or 40 μ M). Authors found that it was caused by inhibition of mitochondrial respiratory chain complexes I and III and lower stability of nuclear factor erythroid 2-related factor 2 (Nrf2). It resulted in a drop in mRNA for heme oxygenase 1 (HO1) and NAD(P)H quinone dehydrogenase 1 (NQO1), enzymes engaged in protection against ROS. Mitochondria-targeted antioxidant, Mito-TEMPO partially protected against UA-induced oxidative stress while Nrf2 agonist, tBHQ, was more effective, also protecting against UA-induced apoptosis. In the xenograft model UA at a dose 50 mg/kg (i.p.) retarded tumor growth which was blocked by NAC (in drinking water), and potentiated anticancer activity of paclitaxel (10 mg/kg) [36].

Different preparations from lichens were tested against glioma cells. Acetone extracts from *Parmelia sulcata*, *Evernia prunastri*, *Cladonia uncialis* more potently reduced the viability of A172 and T89G cells than pure compounds derived from these extracts, i.e. salazinic acid, evernic acid and UA, for instance, IC_{50} values for *C. uncialis* extract were approximately 11 and 3.9 μ g/ml, respectively while IC_{50} values for (-)-UA purified from this extract were 31.5 and 13 μ g/ml, respectively. Although extracts revealed weak free radical scavenging and Cu²⁺ ions reducing activities in vitro, pure compounds had no antioxidant activities. All tested extracts and compounds, including (-)-UA,

inhibited superoxide dismutase (SOD) activity, and (-)-UA revealed inhibitory activity against glutathione reductase (GR) and glutathione peroxidase (GPx), enzymes engaged in ROS defense [37].

UA isolated from *Usnea cornuta* extract was concentration-dependently cytotoxic to MCF-7, A-549 and HeLa cells (IC₅₀ values were 89, 84 and 48,7 μ M, respectively, after 24 h of treatment). More detailed studies on HeLa cells revealed that UA caused a drop in MMP and GSH levels and increased ROS production and lipid peroxidation. It also induced autophagy, and chloroquine, an inhibitor of late stages of autophagy, potentiated ROS production, depletion of GSH, lipid peroxidation and apoptosis induced by UA used at 25 or 50 μ M concentrations [38].

Increased ROS production may lead to DNA damage. However, data on UA-induced genotoxicity in cancer cells are inconsistent. In a study conducted by Mayer et al. (2005), UA showed antiproliferative activity against MCF-7 breast cancer cells (estrogen receptor-positive, wild type for p53) and MDA MB 231 (estrogen receptor-negative, non-functional p53) with an IC₅₀ of 18.9 and 22.3 μ M, respectively [39]. The authors found that the antitumor activity of UA did not involve DNA damage or p53 activation. In MCF-7 cells treated with UA, although there was an accumulation of p53 and p21 proteins, the transcriptional activity of p53 remained unaffected. They also found that there was no phosphorylation of p53 at Ser-15 after treatment of MCF-7 cells with UA, suggesting that the oxidative stress and disruption of the normal metabolic processes of cells triggered by UA did not involve DNA damage. The property of UA as a non-genotoxic anti-cancer agent that works in a p53-independent manner was highlighted as a promising candidate for novel cancer therapy [39].

Emsen et al. (2018) found that UA, although much more toxic to U87MG glioblastoma cells than to primary rat cerebral cortex cells, PRCC (IC₅₀ values by MTT after 24 h were 41.6 and 132.7 μ g/ml, respectively), did not significantly elevate 8-hydroxy-2'-deoxyguanosine (marker of DNA oxidative damage) levels in cells treated with 2.5-40 μ g/ml UA [40].

On the other hand, studies published in 2020 reported that UA induced DNA damage. UA (10-25 μ M) applied to SNU-1 and AGS gastric cancer cells induced apoptosis which was connected with increased Bax:Bcl-2, depolarization of mitochondrial membrane and ROS elevation. ROS in UA-treated AGS cells were responsible for DNA double-strand breaks revealed by alkaline comet assay, increase in γ -H2A.X, DNA-PKcs, p-ATM (Ser-1981), Chk2 and p53 – markers of DNA damage response. Moreover, NAC protected against DNA damage and cell death [41].

Another study showed that UA induced DNA damage response involving ATM kinase and G₂/M cell cycle arrest in RKO colorectal cancer cells pretreated with 400 μ M H₂O₂. Phosphorylation of histone H2AX, which is the marker of DNA double-strand breaks, or ATM activation, was elevated in cells treated with H₂O₂ and UA (0.5, 1, 5, 10 μ M) compared with H₂O₂ alone; however, authors did not show how UA alone impacts DNA integrity and DNA damage response. Interestingly, low concentrations of UA (0.5 and 1 μ M) reduced ROS levels in H₂O₂-treated cells, while 5 and 10 μ M UA - increased ROS levels [42].

Data obtained in vivo indicate that both (+) and (-)- UA enantiomers at doses 100 or 50 mg/kg induced DNA damage observed as increased tails in comet assays in the liver and kidneys of mice. Interestingly it was observed only 1 h after oral administration and not detected after longer times, probably due to rapid DNA repair. The authors also observed increased lipid peroxidation in cells, thus concluding that oxidative stress induced by UA might be involved in the genotoxicity of this compound [43].

DNA damage induced by UA and detected by comet assay was also reported in KB oral carcinoma cells. It was accompanied by increased ROS, reduced MMP, antioxidant enzymes and GSH levels, and induction of apoptosis [44].

Besides DNA damage due to oxidative stress, UA might be a potential inhibitor of key enzymes involved in DNA synthesis and repair. UA has been reported to be a rather weak inhibitor of PARP1 and polymerase β activity (residual activity of 73-77% after incubation with 0.5 mM UA), and some of its derivatives appeared to be much more active [45].

3. UA Inhibits Angiogenesis, Cancer Cell Motility and Invasion

Growth of tumors depends on angiogenesis, thus inhibition of this process can be an effective anticancer strategy. It has been demonstrated that UA inhibited angiogenesis *in vivo* based on chick embryo chorioallantoic membrane assay, in VEGF-induced mouse corneal angiogenesis model and in mice xenografted with Bcap-37 breast cancer cells [46]. Based on *in vitro* research it has been shown that UA dose-dependently (1, 10, 20, 50 μ M) inhibited activating phosphorylation of VEGFR2 and VEGFR2-mediated MEK/ERK1/2 and Akt/p70S6K signaling pathways in HUVEC endothelial cells which resulted in a drop in cell proliferation, migration and tube formation, and induction of apoptosis [46].

(-)UA also inhibited HUVEC cell viability (IC_{50} after 48 h was 50 μ M) and tube formation at concentrations 50 μ M and higher (100 and 200 μ M) [47]. It has also been shown that UA reduced VEGF and MMP-9 levels which was partially dependent on the reduction of PD-L1 in HUVEC cells. These effects resulted in decreased tube formation, migration and invasive potential of endothelial cells [22].

An article published in 2016 reported that (+)-UA inhibited A549, H1650, and H1975 non-small cell lung cancer cell migration and invasion at a concentration of 5 μ M. Authors showed that (+)-UA decreased the level of active, GTP-bound Rac1 (by 22% compared with control) and GTP-RhoA (by 40% compared with control) which are crucial for cell motility regulation. Moreover, UA potentiated the activity of cetuximab, monoclonal antibodies against EGFR used for metastatic colon and lung cancer patients, in reducing the invasive potential of A549 cells [11]. The same team reported the antiproliferative activity of UA using a panel of colorectal cancer cell lines. At the concentration of 5 μ M, UA inhibited invasion of Caco2, HCT116 and CT289 cells *in vitro*; however having no effect in murine orthotopic liver metastasis model (applied at 5 or 10 mg/kg, 6 or 10 times within two weeks, i.p.), while its water-soluble potassium salt (UK) was more effective *in vitro* and *in vivo*. Both UA and UK decreased at the mRNA levels of EMT markers, such as Twist, Snail, Slug, Zeb2, and N-cadherin in Caco2 cells [48].

SCF induces migration of c-KIT-containing colorectal cancer cells. It has been shown that (+)-UA at a concentration lower than 10 μ M inhibited SCF-induced migration of HCT116 and LS174 cells. The mechanism underlying this activity relied on the downregulation of c-Kit gene transcription mediated by sumoylation of Transcription Factor AP-2 alpha (TFAP2A) by upregulated UBC9, and degradation of c-KIT protein due to the induction of autophagy. This, in turn, resulted from decreased ATP level and inhibition of mTOR by 8 μ M (+)-UA in HCT-116 cells. Caspases 3 and 7 were not activated by (+)-UA; however cell's membrane was permeabilized and LDH release after 48 h treatment increased, which suggests necrotic cell death [49].

UA inhibited the motility of prostate (DU145) and melanoma (HTB-140) cells and it was connected with dose-dependent (at 10 or 25 μ g/ml) rearrangements of the actin cytoskeleton [26].

It has been shown that overexpression of PD-L1 in HUVEC endothelial cells led to the elevation of pro-angiogenic proteins, VEGF and MMP-9, enhanced tube formation, migration and invasion; however, UA (100 μ M) protected against these processes decreasing PD-L1 level [22].

4. UA Facilitates the Immune Destruction of Cancer Cells

The programmed cell death 1 (PD-1) and its ligand PD-L1 are responsible for apoptosis and the exhaustion of T cells. PD-L1 is often elevated in cancer cells which leads to avoidance of their destruction by immune cells. UA at 10, 30 and 100 μ M concentrations has been shown to decrease PD-L1 levels in HeLa cervical cancer, A549 lung cancer, HCT116 colorectal cancer and liver cancer Hep3B cells, even if TNF stimulated production of PD-L1- α . It correlated with the enhanced cytotoxicity of co-cultured T lymphocytes toward HeLa, SiHa and CaSKi cervical cancer cells and higher production of TNF- α and ITF- γ by T cells. Authors identified mechanisms underlying diminished production of PD-L1 as reduced STAT3 and Ras signaling pathways, suppression of mTOR and subsequently increased MiT/TFE transcription factor translocation to the nucleus, enhanced biogenesis of lysosomes and proteolysis of PD-L1 [22].

It has been shown that lichen-derived extracts and compounds, including UA, possess inhibitory properties related to kynurenine pathway enzymes. (-)-UA at 100 μ g/ml reduced by almost 22%

indoleamine 2,3-dioxygenases 1 (IDO1), an enzyme involved in the conversion of L-tryptophan to L-kynurenone [37]. Metabolites of this pathway are crucial for the suppression of anti-tumor immune responses and IDO1 is highly expressed in multiple types of cancer [50,51].

5. UA Acts against Tumor-Promoting Inflammation

The anti-inflammatory activity of UA has been recently nicely summarized in publications by Wang et al. [8] and Pazdziora et al. [52]. However, it is worth mentioning a few studies in the context of cancer.

It has been shown that lichen-derived extracts and compounds, including UA, possess inhibitory properties related to kynurene pathway enzymes. (-)-UA at 100 μ g/ml reduced indoleamine 2,3-dioxygenases 1 (IDO1). It also strongly inhibited COX-2 (up to almost 60%), indicating its anti-inflammatory activity. Moreover, it inhibited hyaluronidase with $IC_{50} = 500$ μ g/ml being more potent than β -escin used as a standard in this assay [37]. Hyaluronidase is responsible for generating low molecular weight hyaluronan which displays pro-inflammatory properties, such as stimulation of macrophage activation and production of cytokines [53]. Another study by this team compared activity (+)-UA and (-)-UA enantiomers showing, that right-handed enantiomer is slightly more potent ($IC_{50} = 644.5$ and 676.3 mg/ml, respectively) inhibitor of hyaluronidase [6]. This work also presented that both enantiomers (although to a different extent) decreased levels of pro-inflammatory molecules, such as toll-like receptor 4 (TLR4), cytosolic phospholipase A2 (cPLA2), cyclooxygenases COX-1 and COX-2 in LPS stimulated RAW 264.7 macrophages as well as the release of nitric oxide (NO) and weakly TNF- α and IL-6 [6].

Another study investigated the anti-inflammatory effects of UA in MCF-7 breast cancer and found that it plays a crucial role in regulating the inflammatory response. UA dose-dependently decreased levels of NO, prostaglandin PGE2, cytokines IL-2, IL-6, CXCL10, CXCL8, CCL2, MCP-1 and TNF- α , as well as growth factor VEGF. Moreover, it downregulated the expression of genes coding for cyclooxygenase2 (COX-2) and inducible nitric oxide synthase (iNOS). At the same time, UA revealed pro-oxidant activity in cancer cells as it reduced glutathione and increased malondialdehyde (MDA) levels [54].

Reduction of pro-inflammatory proteins such as TNF- α , NF- κ B or IL-6 was also reported in KB oral carcinoma cells treated with UA at concentrations 10, 20 or 30 μ M [44]. Moreover, the same team showed chemopreventive properties of UA as it protected against DMBA-induced oral squamous cell carcinoma in hamsters. Mechanisms underlying this activity were suppression of inflammatory (COX-2 and iNOS) and proliferation markers (cyclin D1 and PCNA) induced by carcinogen as well as upregulation of antioxidants levels or activity, and modulation of liver detoxification enzymes levels [55].

6. UA Deregulates Energetics in Cancer Cells

As some reports indicated that UA at high concentrations might be hepatotoxic, extensive research was performed to identify mechanisms underlying this activity. Data obtained on rodent primary hepatocytes or isolated mitochondria revealed that UA toxicity might be related to disturbed metabolism, particularly mitochondria functioning and drop in ATP level. These effects concerning normal non-cancerous cells have been beautifully presented in recent review articles [56,57]; however, it is worth mentioning a few studies.

UA is a lipophilic weak acid, thus it can easily pass the mitochondrial membranes and in the matrix, it releases a proton resulting in the generation of a usniate anion. It diffuses into the intermembrane space, binds a proton, and UA is restored. This cycling might cause a proton leak that could dissipate the proton gradient across the membrane, changing the mitochondrial membrane potential. The protonophoric activities of UA were documented using artificial planar bilayer lipid membranes and isolated rat mitochondria [58]. The analysis of biochemical profiles of rat primary hepatocytes showed that high doses of (+)-UA (10 or 30 μ M) decreased ATP levels. It was connected with the depletion of glycogen stores, a drop in glycolysis and the tricarboxylic acid (TCA) cycle. Moreover, the mechanism of UA action resembled the action of mitochondrial uncoupler, FCCP,

which supported the idea that UA is a proton carrier [59]. It has also been demonstrated that UA can carry calcium ions across liposomal, mitochondrial and erythrocyte membranes, thus behaving like a calcium ionophore [60].

UA has also been shown to affect the mitochondrial function in cancer cells. Numerous studies (described in the previous paragraph) show that UA causes a drop in MMP in cancer cells which is connected with apoptosis. However, in some models, although UA decreased the proton gradient across the mitochondrial inner membrane, no release of cytochrome c was observed. UA as a lipophilic weak acid is supposed to act as a proton shuttle and directly dissipate mitochondrial inner membrane potential, affecting oxidative phosphorylation. Indeed, it was reported, that UA (5 and 10 μ g/ml) decreased ATP level in T47D breast cancer cells after 24-h exposition. It activated AMPK, decreased mTOR/S6K signaling, upregulated p-eIF2 α and induced autophagy. However, autophagy flux was impaired due to the disruption of lysosomal acidification and thus degradative processes did not occur [61]. Moreover, it was shown that UA inhibited the mitochondrial respiratory chain complexes (I and III) in lung squamous carcinoma cells, leading to a decrease in ATP production and an increase in the production of ROS [36]. Interestingly, the same team showed that UA affected lysosomal function in breast cancer cells.

Inhibition of glycolysis and mitochondrial respiration was also observed in CaCo2 and HCT116 colon cancer cells. UA at 2.5, 5 and 10 μ M concentrations inhibited glycolysis (basal and compensatory) and mitochondrial functioning (basal respiration, ATP production, proton leak) which correlated with decreased expression of metabolic genes (*SLC2A1*, *SLC2A4*, *HK2*, *PFK1*, *GPI*, *ALDOA*, *PGK1*, *ENO1*, *PKM2*, *LDHA*, *CDH4*, *SRC1*, *PGC-1a*, *TFAM*, *PKM1*, *PDK1*, *ASCT2*, *SLC7A5*, *SLC7A7*, *GLS1*) and drop in *SLC2A1*, *HK2*, *PKM2*, and *LDHA* proteins, even when these processes were elevated by the surplus of an isoform of 14-3-3 [23].

Results of research investigating mechanisms of UA activity toward cancer cells in vitro and in vivo are summarized in Figure 1 and Table 1.

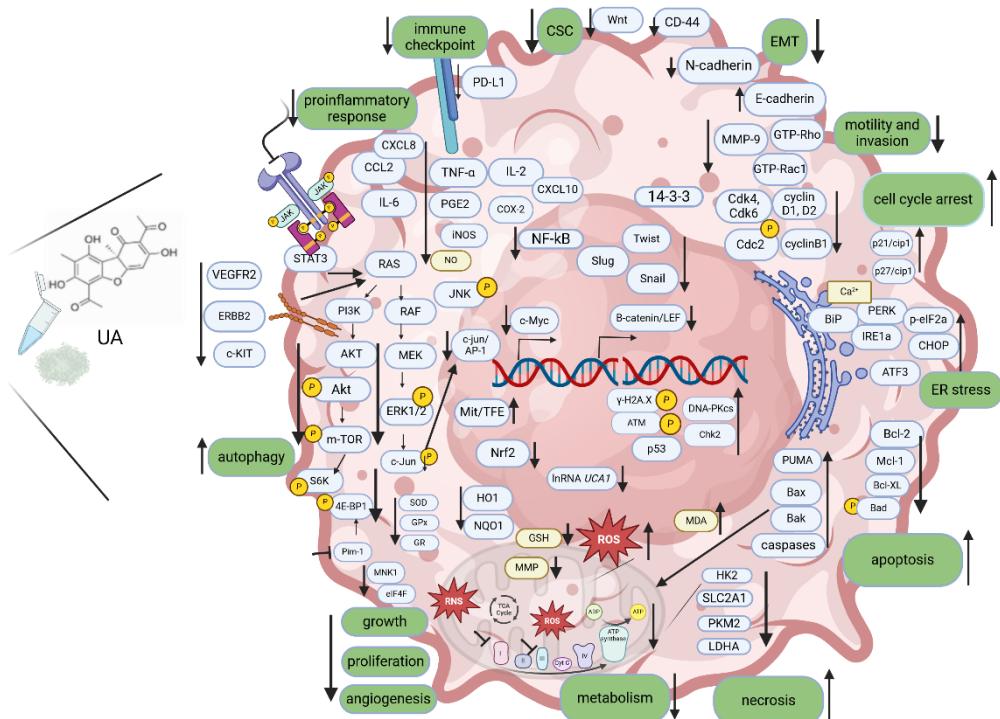


Figure 1. The mechanisms of action of UA in disrupting cancer hallmarks. UA inhibits cell proliferation, induces apoptosis of cancer cells, inhibits angiogenesis, cell motility and invasion, facilitates the immune destruction of cancer cells, acts against tumor-promoting inflammation and deregulates cellular energetics. Additionally, UA induces ER stress and autophagy, inhibits EMT and stem cell features. Created with BioRender.com.

Table 1. Mechanisms of action of UA toward cancer cells of different origin in vitro and in vivo.

Organ/tissue	Cell lines	Compound concentrations tested/ IC_{50}	Effects in vitro	Effects in vivo	References
			UA dose-dependently decreased the viability of cancer cells. (-)-UA extracted from <i>Cladonia uncialis</i> inhibited the activities of IDO1, COX2, hyaluronidase, SOD, GR and GPx in vitro assays. Results of the cell lines: $IC_{50} = 91.4 \pm 2.0 \mu M$ A172 $IC_{50} = 37.8 \pm 3.8 \mu M$ T98G (48 h, MTT) Parallel Artificial Membrane Permeability Assay indicated that UA can cross the blood-brain barrier.	-	[37]
Head	human glioblastoma cells ma U87MG	UA $IC_{50} = 41.55 \mu g/ml$	UA in a dose-dependent manner lowered viability and increased LDH release, especially in cancer cells. It revealed high antioxidant capacity in healthy cells (max at 10 $\mu g/ml$). UA non-significantly increased 8-OH-2'-deoxyguanosine levels in cancer cells.	-	[40]
	human oral carcinoma cells KB Normal fibroblasts HGF-1	UA $IC_{50} = 30 \mu M$ (24 h, MTT)	UA in dose-dependent manner reduced viability of KB cancer cells and normal fibroblasts (HGF-1) were significantly more resistant. UA at concentration 10, 20 or 30 μM elevated ROS level, lipid peroxidation, decreased SOD, CAT, GPx activities and reduced GSH level, MMP. It induced DNA damage, apoptosis with downregulation of Bcl-2 and upregulation of p53, Bax, caspases 9 and	-	[44]

				3. It decreased NF-κB, TNF-α and IL-6 levels		
				UA decreased viability in a dose- and time-dependent manner, induced G ₀ /G ₁ cell cycle arrest with a drop in CDK4, CDK6, cyclin D1 and an increase in p21 levels, mitochondrial membrane depolarization (at 100 μM), induced apoptosis.		[21]
				UA induced S or G ₀ /G ₁ arrest dependent on concentration, apoptosis, decreased Bcl-xL:Bax ratio, reduced clonogenic potential (10 μM) and anchorage-independent growth, motility (5 and 10 μM) and invasion (10 μM). UA elevated E-cadherin (at mRNA and protein level), reduced mRNA for N-cadherin, Twist and Snail (10 μM); reduced p-c-jun, p-Akt and p-ERK1/2.	Tumor-free survival of BALB/c nude mice with subcutaneously injected A549 cells was longer if cells were pretreated with the sublethal concentration of UA (10 μM).	[17]
Lung	non-small cell lung cancer cells (A549 (and a panel of other cancer and noncancerous cells))	UA	12.5, 25, 50, 100 μM	5 μM UA reduced transcriptional activity of β-catenin/LEF and AP-1; reduced mRNA for CD44, cyclin D, c-myc; decreased GTP-Rac1 and GTP-RhoA levels, inhibited motility and invasion of lung cancer cells; potentiated activity of cetuximab in inhibiting invasive potential		[11]
	non-small cell lung cancer cells: A549 H460 H1650 H1975	(+)-UA	IC ₅₀ = 65.3 ± 0.65 μM n/a n/a n/a	UA induced dose-dependently ROS (10, 20 40 μM) and ROS-dependent apoptosis, inhibited mitochondria	Tumor growth in athymic nude mice inoculated with H520 cells was significantly retarded by UA (50 mg/kg, decreased Nrf2 protein	[36]
	lung squamous carcinoma cells H520 Calu-1	(+)-UA	IC ₅₀ = 32.51 ± 0.44 μM IC ₅₀ = 34.25 ± 0.05 μM	respiratory chain complexes I and III, decreased Nrf2 protein		

			level and its transcriptional activity (drop in expression of its target genes, HO1 and NQO1) which was mediated by PI3K/Akt pathway inhibition. UA at 15 μ M enhanced the cytotoxic activity of paclitaxel (at 0.1 μ M) in vitro	thrice weekly i.p.) compared with controls. UA at such a dose enhanced the effect of paclitaxel (10 mg/kg, thrice-weekly i.p.)	
	human breast cancer cell lines	UA	The antiproliferative activity of UA did not involve DNA damage or p53 activation. Although there was an accumulation of p53 and p21 proteins in UA-treated MCF-7 cells, the transcriptional activity of p53 remained unaffected and there was no phosphorylation of p53 at Ser15.	-	[39]
	MCF-7 MDA MB 231	IC ₅₀ = 18.9 μ M IC ₅₀ = 22.3 μ M			
Breast	human breast cancer cells	(+)-UA isolated from <i>Cladonia arbuscula</i> IC ₅₀ = 4.2 μ g/ml (-)-UA from <i>Alectoria ochroleuca</i> IC ₅₀ = 4.0 μ g/ml	Both enantiomers were equally effective in inhibiting cell proliferation. (+)-UA induced G ₀ /G ₁ cell cycle arrest, decreased MMP.	-	[29]
	T47D	(24 h, [3H] thymidine incorporation)	No evidence of apoptosis of cells treated with 20 μ g/ml after 24 h or necrosis of cells treated with 5 and 10 μ g/ml UA for 24 or 48 h.	-	
	human breast cancer cells	(+)-UA isolated from <i>Cladonia arbuscula</i> 5 and 10 μ g/ml tested	UA decreased ATP level after the 24-hour exposition. It resulted in activating phosphorylation of AMPK, decreased mTOR/S6K signaling, upregulation of p-eIF2 α and induction of autophagy. Autophagy flux was impaired due to the disruption of lysosomal acidification.	-	[61]
	human medullary	UA	UA dose-dependently inhibited the	In nude mice xenografted	[46]

breast cancer cells	1-50 μ M	proliferation of Bcap-37 cancer cells and HUVEC endothelial cells. At 1, 10, 20 μ M concentrations UA inhibited migration and capillary structure formation by HUVEC cells, induced their apoptosis and inhibited activation of VEGFR2 and Akt/p70 S6K/S6 and MEK/ERK1/2 signaling pathways	with Bcap-37 cancer cells and treated intrallesionally with 60 mg/kg/day (22 days) of UA tumor growth and angiogenesis were inhibited.
human umbilical vascular endothelia			UA dose-dependently suppressed tumor growth in nude mice xenografted with MCF-7 cells (i.p. at 25, 50 or 100 mg/kg every 2 days for 21 days). [35]
human breast cancer cells	UA	UA decreased the viability of cancer cells in a dose- and time-dependent manner, while up to 25 μ M did not affect normal MCF-10A cells. UA induced apoptosis in MCF-7 cells through the mitochondrial pathway, increased Bax:Bcl-2 ratio, reduced MMP, increased ROS (25 μ M for 24 h) and activated JNK.	The highest dose (100 mg/kg) was more effective for cancer cells and much less toxic than cyclophosphamide CTX (25 mg/kg).
MCF-7 MDA MB 231 SKBR-3 normal human mammary epithelial cells MCF-10A	$IC_{50}=34.12 \pm 1.25 \mu$ M $IC_{50}=38.41 \pm 1.64 \mu$ M $IC_{50}=48.07 \pm 1.52 \mu$ M	(24 h, MTT)	UA at 15 and 25 μ M induced autophagy in MCF-7 and MDA MB 231 cells, which correlated with a drop in p-Akt, p-4E-PB1, and p-S6K. It inhibited migration and invasion (5-30 μ M) of MDA MB 231 cells. Its benzylidene derivative 52 was much more potent in vitro and in vivo.
human breast cancer cell lines	UA		UA benzylidene derivative 52 was tested in vivo. It inhibited MDA MB 231 and MCF-7 cells xenografted to nude mice (10 mg/kg bw, i.p. 3 times per week). [18]
MCF-7 T47D MDA MB 231 MDA MB 468 SKBR-3 BT-474	$IC_{50}=11.2 \mu$ M $IC_{50}=15.9 \mu$ M $IC_{50}=13.1 \mu$ M $IC_{50}=13.7 \mu$ M $IC_{50}=14.4 \mu$ M $IC_{50}=15.1 \mu$ M	(72 h, MTT)	Differentially expressed UA-responsive - [32]

			miRNAs were identified and they appeared almost unique to each cell line. The targets are enriched in basal cell carcinoma, MAPK and Hedgehog signaling pathways (MCF-7 cells), ErbB and mTOR signaling, focal adhesion and gap junctions (in BT474 cells), MAPK, ErbB2, PI3K-Akt and p53 signaling pathways (MDA MB 231). Among the upregulated miRNA was tumor suppressor, has-miR-185-5p.		
human breast cancer cells	UA	IC ₅₀ =7.21 μM (48 h, MTT)	UA dose-dependently decreased cancer cell viability with no effect on normal cells. It modulated the expression of apoptosis-related genes, such as these coding for caspases, BCL-, TRAF- and TNF-family members, increased Bax, caspase 3 and 9 protein levels, when applied at 7.21 μM for 48 h.	-	[31]
human breast cancer cells	UA	LD ₅₀ = 13.11 μM	UA decreased NO, VEGF, PGE2 levels, gene expression levels of COX-2 and iNOS, and cytokines (IL 2, CXCL10, CXCL8, CCL2, TNF- α , IL-6). It decreased glutathione levels and increased MDA levels in a dose-dependent manner.	-	[54]
mouse mammary cancer cells	UA, HA-UA-GNPs and UA-GNPs	IC ₅₀ = 120.04 ± 4.8 μM	The cytotoxicity and cellular uptake of UA loaded into gliadin nanoparticles (GNPs) functionalized with hyaluronic acid (HA, was efficiently reduced by	Tumor (4T1 cells) growth in BALB/C female mice	[62]

			$IC_{50} = 0.56 \pm 2.8 \mu M$ $IC_{50} = 92.64 \pm 3.6 \mu M$, respectively	targeting DC44 receptor) was higher than UA or UA-GNPs (24 h, MTT)	HA-UA-GNPs, compared with an equal dose (100 mg/kg of UA as an i.p. injection every two days for 21 days) of non-targeted UA-GNPs and free UA	
				UA in time- and dose-dependent manner decreased cell viability, at 24 and 48 h it induced LDH release, and after 24 h induced S phase arrest and apoptosis. It decreased antiapoptotic proteins (Bcl-2, Mcl-1), and reduced activating phosphorylation of Akt, PDK1, mTOR and its substrates (S6K and 4E-BP1). UA elevated autophagy (induction and flux) which was a protective mechanism.	-	[15]
Liver	human hepatocellular carcinoma cells HepG-2	UA 1.56-50 μM		UA elevated phosphorylation of ERK1/2, p38 and JNK. The latter kinase was involved in autophagy and apoptosis regulation.		
	human hepatocellular carcinoma cells HepG-2 (HBV(-)) SNU-449 (HBV(+))	UA 6.25,12.5, 25, 50, 75 and 100 μM		UA at lower concentrations, 6.25 and 12.5 μM for HepG2 and 6.25 μM for SNU-449, increased viability measured after 24 h. Longer treatment (48 h) was connected with dose-dependent viability drop. UA induced G ₀ /G ₁ cell cycle arrest in HepG2, S and G ₂ /M arrest in SNU-449, apoptosis and autophagy in both	-	[16]

	endothelia l cells HUVEC		cancer cell lines with limited effect on normal control cells (HUVEC).	
human gastric carcinoma cells	(+)-UA $IC_{50} = 236.55 \pm 11.12 \mu\text{M}$	UA induced G_0/G_1 cell cycle arrest in BCG823 (100, 200, 400 μM , 24 h) and G_2/M arrest in SGC7901 (300, 600, 1200 μM), apoptosis with the rise in Bax, cleaved PARP and caspase 3 and a decrease in Bcl-2 levels. UA induced autophagy (elevated LC3-II and decreased p62 levels).	BGC823- bearing nude mice were treated with 100 mg/kg UA i.p. for 11 days (every 2 days), tumor volume and mass were 2-fold lower than control, PBS-treated mice. UA was more effective than 5-FU (25 mg/kg).	[19]
BGC823 SGC7901	$IC_{50} = 618.82 \pm 1.77 \mu\text{M}$			
Stomach		UA in a dose- and time- dependent manner decreased cell viability, clonogenicity and elevated apoptosis. It reduced MMP and increased Bax:Bcl-2 ratio. In AGS cells UA increased ROS generation in a time- dependent manner and DNA damage was detected by alkaline comet assay after 48-h treatment. UA (15 or 25 μM) in ROS-dependent manner up-regulated p- ATM, γ -H2A.X, DNA- PKcs, p53, Chk-2 levels. NAC protected against these effects.	-	[41]
Pancreas	(+)-UA isolated from <i>Cladonia arbuscula</i> $IC_{50} = 5.3 \mu\text{g/ml}$ (-)-UA from <i>Alectoria ochroleuca</i> $IC_{50} = 5.0 \mu\text{g/ml}$	Both enantiomers were equally effective in inhibiting cell proliferation. (+)-UA induced G_0/G_1 cell cycle arrest, decreased MMP. No evidence of apoptosis of cells treated with 20 $\mu\text{g/ml}$ after 24 h, and necrosis was detected in cells	-	[29]
	Capan-2	(24 h, [^3H] thymidine incorporation)		

			treated with 5 or 10 µg/ml UA for 48 h.	
		(+) UA, human colon adenocarci- noma cells HT-29	UA at 50 or 100 µM in a time-dependent (24, 48 and 72 h) manner decreased MMP and induced apoptosis. It was preceded by ROS elevation (observed after 1, 3 or 6 h post- treatment).	- [13]
		(+)-UA human colorectal cancer cell lines HCT116 LS174	8 µM UA for 24 or 48 h inhibited SCF-induced cell proliferation and migration; decreased cellular ATP content, and increased LDH release. It inhibited mTOR signaling (drop in p-S6K, p-4E-BP) and PKC-A. It elevated autophagy (LC3-II) which was responsible for UA-induced reduction of c-KIT receptor.	- [49]
Colon			Firefly luciferase- expressing CT26 cells were inoculated via splenic injection to form multiple tumor foci in the livers of male BALB/c mice.	
		human colorectal cancer cell lines HCT116 DLD1 SW480 HT29 SW620 Caco2 COLO320	UA reduced viability of cells in a dose- dependent manner, at 5 µM concentration reduced invasion in vitro. KU was more effective in the majority of tested cells. Both, UA and KU decreased mRNA for N-cadherin, Snail, Twist, Slug and ZEB2, and protein levels of Twist, Snail and Slug EMT markers in Caco2 cells. KU decreased expression of genes related to motility (<i>CAPN1</i> , <i>CDC42</i> , <i>CFL1</i> , <i>IGF1</i> , <i>WASF1</i> , <i>WASL</i>) in Caco2 cells (UA only affected <i>CFL1</i> and <i>IGF1</i>)	UA at 5 or 10 mg/ml i.p. (6 or 10 times during 2 weeks) or KU at a dose of 5, 10 or 20 mg/kg/mouse i.p. (6 times during 2 weeks) were applied. KU exhibited more potent anticancer
		Mouse colorectal cancer cells CT26		[48]

				effects (PARP cleavage, caspase 3 activation, reduction in EMT markers) and at 20 mg/kg inhibited liver metastasis in an orthotopic murine colorectal cancer model.
				The viability of a panel of cell lines was dose- and time-dependently reduced by KU. 24-h treatment with KU at IC ₅₀ concentrations induced cell cycle arrest in G ₀ /G ₁ or S phase, depending on the cell line, reduced CDK4, cyclin D2 and p21 protein levels. It induced apoptosis by mitochondrial pathway. In SNU638 and HCT116 cells KU induced ER stress with the elevation of intracellular Ca ²⁺ , ROS and ER stress markers, such as BIP, PERK, IRE1 α , p-eIF2 α , CHOP and ATF3 proteins as well as ATF3-regulated genes. Downregulation of ATF3 by specific siRNA protected against KU-induced elevation of Bak, p-BAD, PUMA, activation of caspase 3 and cell death.
				[24]
human colon carcinoma cells	KU- potassium usnate	IC ₅₀ = 38.9 ± 1.76 μM	KU applied (20 line, reduced CDK4, cyclin D2 and p21 protein levels. It induced apoptosis by mitochondrial pathway. In SNU638 and HCT116 cells KU induced ER stress with the elevation of intracellular Ca ²⁺ , ROS and ER stress markers, such as BIP, PERK, IRE1 α , p-eIF2 α , CHOP and ATF3 proteins as well as ATF3-regulated genes. Downregulation of ATF3 by specific siRNA protected against KU-induced elevation of Bak, p-BAD, PUMA, activation of caspase 3 and cell death.	
RKO	UA tested	0.5, 1, 5, 10 μM	UA at 5 or 10 μM potentiated the inhibitory effect of H ₂ O ₂ (400 mM) on the proliferation and migration of RKO cells.	[42]

				Combined treatment enhanced DNA damage, ATM, p-ATM and γ -H2AX elevation, G ₂ /M cell cycle arrest, apoptosis and autophagy, and elevated ROS. ATM level was controlled by UA-upregulated mir18a-5p.		
human colorectal cancer cells Caco2 HCT116	UA	2.5, 5, 10, 20 μ M tested		UA time- and dose-dependently reduced 14-3-3 proteins which depended on proteasome and autophagy. It correlated with decreased p-cdc2 level and G ₀ /G ₁ arrest. UA at 5 μ M decreased invasion in cells expressing different isoforms of 14-3-3 as well as EMT markers, Snail, Twist, N-cadherin, β -catenin (at 10 μ M). Among other downregulated proteins were cyclin D1, cyclin B1, p-mTOR, p-Akt, p-STAT3, and p-JNK. UA also reduced the activity of AP-1, STAT, and NF- κ B transcription factors which were elevated by overexpression on 14-3-3 isoforms in HEK293T cells. UA inhibited glycolysis and mitochondrial respiration in CaCo2 and HCT116 which correlated with decreased expression of metabolic genes and drop in SLC2A1, HK2, PKM2, and LDHA proteins, even when these processes were elevated by the surplus of an isoform of 14-3-3.	-	[23]

Prostate	human prostate cancer cell lines	UA isolated from <i>Cladonia arbuscula</i> (Wallr.)	UA inhibited the proliferation of both prostate cancer cells, and induced apoptosis of PC-3 cells (cleavage PARP, caspase 7 and 9 elevations). UA induced actin cytoskeleton rearrangements in a dose-dependent manner in both cancer cell lines and reduced DU-145 cell motility.	-	[26]
	normal prostate epithelial cells	EC ₅₀ = 18.2 µg/ml			
	PNT2				
	Skin fibroblasts	EC ₅₀ = 20.5 µg/ml			
	HSF	(48 h, cell number)			
Ovaries	human prostate cancer cells	UA	UA decreased cell viability in a dose- and time-dependent manner, reduced MMP, elevated Bax:Bcl-2 mRNA, activated apoptosis, and downregulated NF-κB p50.	-	[27]
	DU-145	IC ₅₀ = 42.15±3.76 µM (48 h, MTT)			
	human ovarian adenocarcinoma cells	(+)-UA	At 50 or 100 µM in a time-dependently (48 and 72 h) increased S phase cell cycle arrest, decreased MMP, and induced apoptosis. It was preceded by RNS elevation (observed after 3 or 6 h post-treatment).	-	[12,13]
	A2780	IC ₅₀ = 75.9 ± 2 µM (72 h, MTT)			
	human ovarian adenocarcinoma lines	(+)-UA	UA in a dose- and time-dependent manner reduced the viability of cancer cells while normal fibroblasts were more resistant (24 h). 20 µM UA induced G ₀ /G ₁ cell cycle arrest and apoptosis, inhibited migration and invasion of OVCAR-3 cells. UA modulated transcriptome, particularly elevated expression of some genes connected with	-	[30]
	OVCAR-3	IC ₅₀ = 20 µM			
	A2780				
	mouse fibroblasts	xCELLingence system and MTT L929			

			apoptosis (caspase 1 and 8, for instance).		
			UA dose-dependently reduced PD-L1 levels in a panel of cancer cells, including HeLa. It inhibited PD-L1 protein synthesis and enhanced its degradation in HeLa cells which correlated with its lower level at the cell surface and enhanced T-lymphocyte killing activity toward cervical cancer cells. It inhibited mTOR which induced autophagy and autophagic degradation of PD-L1. UA inhibited Jak1/2-Src-STAT3 and Ras-MEK-ERK pathways leading to reduced PD-L1 expression, drop in c-myc and cyclin D1 levels, reduced clonogenic potential. UA diminished PD-L1-mediated angiogenic potential of HUVEC cells.	-	[22]
Uterus	human cervical cancer cell lines HeLa SiHa CaSKi (and other types of cancer cells)	UA 3, 10, 30, and 100 μ M tested (24h, MTT)			
	normal human cervical epithelial cells HcerEpic human umbilical vascular endothelia HUVEC				
	human cervical cancer cells	UA isolated from <i>Usnea cornuta</i> $IC_{50} = 48.65 \mu$ M (24 h, MTT)	UA at 25 and 50 μ M increased ROS levels, lipid peroxidation, decreased MMP, and GSH level, increased caspase3/7 activity and cell death. UA induced protective autophagy – its inhibition by chloroquine increased UA cytotoxicity.	-	[38]
	endometrial cancer cells	UA $IC_{50} = 51.76 \mu$ M Ishikawa (48 h, XTT)	UA inhibited cell proliferation and downregulated the expression of oncogenic lncRNA UCA1.	-	[34]
Blood	human acute myeloid	UA	UA induced apoptosis in human leukemia cells with HL-60 cells being more responsive.	-	[25]

	leukemia cells HL-60	IC ₅₀ 10.00 ± 1.03 μM	It correlated with caspase 3, 9 and 8 activation, PARP cleavage, and drop in Mcl-1. UA inhibited Mnk1/eIF4E and Pim1/4E-BP1 signaling.		
	human chronic myelogenous leukemia cells K562	IC ₅₀ = 10.39 ± 0.60 μM (3 days, cell number)	increased p27 and decreased cyclin D1, p-Bad, c-myc, Pim-1 levels. UA inhibited Pim-1 activity in vitro.		
	human melanoma cells HBT-140	UA isolated from <i>Cladonia arbuscula</i> (Wallr.) EC ₅₀ = 13.7 μg/ml	UA exerted weak cytostatic effects and apoptosis induction. At 10 and 25 μg/ml, it induced rearrangements of the actin cytoskeleton in a dose-dependent manner, 10 μg/ml UA inhibited cell motility.	-	[26]
	Skin fibroblasts HSF	EC ₅₀ = 19.3 μg/ml (72 h, cell number)	Both enantiomers decreased the viability and proliferation of cells in a dose- and time-dependent manner but their potency was enantiomers and cell-line specific. They inhibited cell migration (at 10 μg/ml), and acted synergistically with doxorubicin in A375 cells. They weakly decreased the release of pro-inflammatory TNF-α, IL-6 and NO and significantly reduced the synthesis of TLR4, cPLA2, COX-1 and COX-2 in LPS-stimulated macrophages (10 or 25 μg/ml concentrations tested).	-	[6]
Skin	HTB140 A375 WM793 Murine macrophages RAW264.7	IC ₅₀ = 14.7 and 20.6 μg/ml IC ₅₀ = 11.8 and 22.2 μg/ml IC ₅₀ = 30.1 and 52.1 μg/ml respectively (48 h, LDH)	UA induced dose-dependent cytotoxicity (within the range 25-250 μM) in cancer but not in normal cells. It induced LDH release and PI	-	[20]

normal human embryonic kidney cells HEK293T	accumulation by cancer cells. It correlated with ROS elevation, lipid peroxidation, changes in surface lipids and proteins, drop in GSH level and MMP. UA induced G ₀ /G ₁ cell cycle arrest and apoptosis.
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7. Combination Therapies Using UA

Raising knowledge on mechanisms underlying anticancer activity of UA inclines towards combination therapies using UA and clinically approved drugs. Such combinations aim to enhance the anticancer effect and reduce the toxicity of a drug.

The synergistic effect was observed when UA (at 12.5 or 50 μ M concentration) was combined with low concentrations of tamoxifen (the selective estrogen receptor modulator) or enzalutamide (a second-generation androgen receptor inhibitor) for treating hormone-dependent breast MCF-7 or prostate LNCaP cancer cells, respectively. UA potentiated cell cycle perturbations and apoptosis induced by drugs. These effects were also observed in non-cancerous cells; however, at different extents than in cancer cells. Mechanisms of synergistic activity were not explored [63]. The same team reported that the combination of UA with sorafenib, a drug used in systemic chemotherapy of hepatocellular carcinoma, acted synergistically towards HepG2 and SNU-449 cells. Sorafenib at lower concentrations was not toxic to normal cells but when combined with UA more effectively arrested the cell cycle and induced apoptosis than any compound used alone, and at the same time was less toxic to the HUVEC cell line [64].

It has also been shown that 5 μ M UA and an anti-EGFR antibody, cetuximab, more effectively inhibited the invasive potential of A549 lung cancer cells than any of the compounds alone [11]. UA at a concentration of 15 μ M enhanced the activity of paclitaxel at 0.1 μ M toward H520 and Calu-1 lung cancer cells *in vitro*. Moreover, UA at a dose 50 mg/kg (i.p.) retarded H520 xenograft tumor growth *in vivo*, and potentiated anticancer activity of paclitaxel (10 mg/kg) [36].

Another *in vivo* study investigated the efficacy of (+)-UA and bleomycin combination on hepatoma H22-bearing mice. Bleomycin is widely used to treat malignant ascites, however, it causes pulmonary fibrosis which is connected with excessive inflammatory response and oxidative stress in lung tissue. UA (25, 50, 100 mg/kg, p.o.) combined with bleomycin (15 mg/kg, i.p.) revealed significantly better effectiveness than bleomycin alone in reducing ascites fluid, inhibiting ascites cell viability, arresting the cell cycle at G₀/G₁ phase, and promoting apoptosis. It was associated with the transcriptional upregulation of p53/p21 and downregulation of cyclins E1 and D1. Moreover, UA reduced the side effects of bleomycin, including lung tissue damage. It was connected with a reduction of MDA, hydroxyproline (HYP), TNF- α , IL-1 β , IL-6, TGF- β 1 and p-Smad2/3, and an increase of SOD and Smad7 levels in lung tissues of H22-bearing mice treated with bleomycin [65].

More recently, the combination of (+)-UA or (-)-UA with doxorubicin was tested on HTB140, A375 and WM793 melanoma cells. The synergistic, additive or antagonistic effects were observed depending on the cell line, doses used and treatment time. Interestingly, UA (especially (-)-UA) sensitized to the drug WM793 cells, which are quite resistant to doxorubicin [6].

8. Bioavailability and Pharmacokinetics

The absorption, distribution, metabolism, and excretion (ADME) are important factors in understanding the pharmacokinetics and potential toxicity of the new drug, and in the case of UA such data are limited. The available literature provides some insights into the dosage and administration of UA, particularly in the context of its pharmacokinetics and toxicity. For instance, studies conducted on rabbits indicate that plasma D(+)-UA levels following intravenous (5 mg/kg) administration showed a triexponential elimination with a mean terminal half-life of 10.7 \pm 4.6 h.

Following oral administration at a dose of 20 mg/kg the UA peak plasma concentration of 32.5 ± 6.8 g/ml was reached after 12.2 ± 3.8 hours, and a mean terminal half-life was 18.9 ± 2.9 hours. The mean absolute bioavailability of UA administrated orally was 77.8% [66].

Studies in rats administered intraperitoneally with 25 mg/kg of D(+)-UA have demonstrated that the molecule is distributed in different tissues, with a higher concentration found in lungs and liver, followed by blood (an average tissue-to-plasma concentration ratio was 1.777, 1.503, and 1.192, respectively). Moreover, approximately 99.2% of UA was bound to plasma proteins and showed albumin concentration-dependent binding (up to 6.5 g/l of albumin) [67] which was similar to results obtained by others showing that more than 99% of UA is bound in human or rat plasma [68].

UA metabolism has been studied *in vitro* using human plasma, hepatocytes, and liver subcellular fractions. Three monohydroxylated metabolites and two glucuronide conjugates of UA were identified after incubation with human liver S9 fraction using liquid chromatography/mass spectrometry (LC/MS). Hepatic clearance of UA was estimated as 13.9 ml/min/kg and it was shown that UA is primarily metabolized by CYP1A2 while conjugation of UA with glycuronic acid depends on UGT1A1 and UGT1A3. Moreover, the study revealed that UA is a potent inhibitor of CYP2C19 and CYP2C9 and a less potent inhibitor of CYP2C8 and CYP2C19 [68].

The trapping assay with glutathione and UPLC-MS/MS analysis was used to elucidate reactive metabolites of UA in human, rat and mice liver microsomes. Authors found dehydrogenated and hydroxylated UA metabolic adducts with glutathione. These reactive adducts and/or depletion of GSH by UA might be related to UA hepatotoxicity. Interestingly, differences in metabolites were identified between human and rodent models and between (+) and (-)-UA enantiomers in human microsomes [69].

A more recent study investigated the metabolism of UA and its relationship to toxicity based on *in vitro* experiments using human liver microsomes, rat liver microsomes and S9 fraction combined with UPLC-Q-TOF-MS for metabolite identification. The authors identified 14 phase I metabolites and 4 phase II metabolites of UA and found that the key UA metabolizing enzymes are CYP2C9, CYP3A4, CYP2C8, and UGT1A1. UA was not toxic to human primary hepatocytes when applied at 0.01-25 μ M concentrations for 48 h; however, it was toxic to mouse 3T3 fibroblasts ($IC_{50} = 7.4 \mu$ M). Using a model of coincubation of human liver microsomes with 3T3 cells, authors found that UA (1-50 μ M) or its metabolites were not toxic to 3T3 cells, at least after a short 4-h exposition, thus they concluded that the UA cytotoxicity might be related to chronic exposure [70].

Data on *in vivo* toxicology are scarce. Acute toxicity has been only determined for mice and rabbits and 50 % lethal dose (LD_{50}) values in the case of oral applications were 838 mg/kg and >500 mg/kg, respectively [71]. Intraperitoneal injections of UA suspension at a dose of 15 mg/kg/day for 15 days in male Swiss mice caused a hepatic dysfunction as revealed by a high level of serum transaminase and histological observation of necrotic areas in the livers.

Data in humans are limited to reports on cases of severe hepatotoxicity (hepatonecrosis, fulminant hepatic failure and other complications) after taking dietary supplements containing UA, such as Lipokinetix, weight loss formula [72].

9. Perspectives for the Use of UA as an Anticancer Drug

As shown in previous paragraphs, UA is effective in targeting cancer hallmarks; however, data from *in vitro* and *in vivo* models indicate that rather high concentrations of UA have to be applied which might be toxic to healthy cells, especially hepatocytes. Another problem with the use of UA is its low water solubility. Thus, extensive research efforts go in at least two directions: to get knowledge on structure-activity correlations and receive UA derivatives with enhanced activity and better selectivity toward cancer cells, and to improve UA bioavailability by enhancing its solubility in water or delivery into the cells [73].

Numerous modifications to the UA structure have been reported and screened for antiproliferative activity (rev. in [73]). Some of UA derivatives reveal much better activity than the parent compound, and molecular mechanisms of their action, as well as toxicological and pharmacokinetic studies, are important for their further clinical development. For instance, our

results with a pyrazole derivative of UA, referred to as compound 5, have demonstrated superior anticancer activity compared to the parent compound. This derivative has shown significant inhibitory effects on the growth and proliferation of pancreatic cancer cells both in vitro ($IC_{50} = 0.90$ - $1.35 \mu\text{g/ml}$ after 48 h treatment) and in animal models. The compound induced the release of calcium ions from the ER, leading to ER stress in cancer cells. It also causes G₀/G₁ cell cycle arrest and cell death. When tested in nude mice with xenografted pancreatic cancer cells, the UA derivative 5 successfully inhibited tumor growth without causing apparent toxicity to the kidneys or liver [74].

The hydrophilic potassium salt of UA (KU) shows more favorable characteristics than the parent compound which was comprehensively presented in the review article by de Araujo et al. [75]. Results of Yang et al. clearly indicate that the bioavailability of KU, measured as the amounts in tumor, liver and plasma of CT26 syngeneic tumor xenograft-bearing mice after oral administration (30 mg/kg), was higher than that of UA. Moreover, KU was more potent than UA in the inhibition of invasiveness of the majority of colorectal cell lines in vitro, and at a dose of 20 mg/kg (i.p.), it significantly decreased liver metastasis in an orthotopic murine colorectal cancer model [48]. In the KU acute oral toxicity tests in Swiss Webster mice, LD₅₀ was evaluated as $>200 \text{ mg/kg}$ indicating much lower toxicity than UA [76].

Another way to improve the therapeutic index of UA is to develop drug delivery systems. Data on UA encapsulated into lipid-based nanocarriers (liposomes, nanoemulsions), polymeric nanocarriers or microparticles (of different structure and composition), and nonorganic nanoparticles (magnetic or diamond) are comprehensively presented and discussed in a recent review by Zugic et al. [77]. For instance, Farzan et. al. developed a novel UA delivery system, where UA was encapsulated within nanoparticles of biodegradable gliadin (GNP) functionalized with hyaluronic acid (HA) which targets CD44 receptors overexpressed on breast cancer cells. This approach allowed for targeted delivery of UA specifically to breast cancer cells, increasing efficacy and reducing side effects (UA $IC_{50} = 120.04 \mu\text{M}$, while UA-loaded nanoparticles HA-UA-GNPs $IC_{50} = 0.56 \mu\text{M}$ and UA-GNPs $IC_{50} = 92.64 \mu\text{M}$). Tumor growth in mice treated with HA-UA-GNPs was significantly reduced compared with tumors in UA-GNPs or free UA-treated animals. The study demonstrated successful in vitro and in vivo translational research, bridging laboratory findings with potential clinical applications [62].

10. Overall Conclusions and Future Directions

Presented data indicate the versatility of UA in targeting different cancer hallmarks, including inhibition of abnormal growth signals that drive uncontrolled cell division, induction of apoptosis or other cell death programs, disruption of cancer cell metabolism, inhibition of the new blood vessels formation which are crucial for the sustained growth of tumors, inhibition of migratory and invasive potential of cancer cells, immunomodulatory potential in mobilizing body's immune response against malignant cells, and anti-inflammatory properties, which can be beneficial in addressing a tumor-promoting inflammatory microenvironment (Figure 1). Overall, the multifaceted actions of usnic acid underscore its potential as a promising therapeutic agent in the fight against various types of cancer, offering a range of mechanisms to disrupt cancer hallmarks and inhibit tumor progression. Further research is needed anyway, not only on mechanistic aspects of activity and toxicity of UA or UA-based formulas but most of all biopharmaceutical properties, efficacy and safety in vivo to reconcile the promising great potential of UA with the current lack of its therapeutic use in cancer patients.

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