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To:

My Grandparents,
Mum, Dad, Antonio,
Angelo and Daniele.

Index

Summary

Osteosarcoma (OS) is the most aggressive type of primary solid tumor that develops in bone. Whilst conventional chemotherapy can improve survival rates, the outcome for patients with metastatic or recurrent OS remains poor, so novel treatment agents and strategies are required. Research into new anticancer therapies has paved the way for the utilisation of natural compounds as they are typically less expensive and less toxic compared to conventional chemotherapeutics. Previously published works indicate that Agave exhibits anticancer properties, however potential molecular mechanisms remain poorly understood. In the present study, we investigate the anticancer effects of Agave leaf extract in OS cells. Here, we observe that Agave inhibits cell viability, colony formation, and cell migration, and can induce apoptosis in OS cell lines. Moreover, Agave sensitizes OS cells to cisplatin (CDDP), to overcome chemoresistance. Considering these effects, we investigated whether Agave extract modulates the Hippo signalling pathway, revealing a marked decrease of Yes associated protein (YAP) and Tafazzin or WWTR1 (TAZ) mRNA and protein expression upon treatment. We demonstrate that YAP/TAZ down-regulation inhibits OS cell viability and migration. Furthermore, we reveal that the effects of Agave could be partially recapitulated by saponins, a class of chemical compounds abundantly expressed by the Agavaceae family of plants. We propose an initial mechanism of action in which Agave induces YAP/TAZ protein degradation, followed by a secondary event whereby Agave inhibits YAP/TAZ transcription, effectively deregulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65:p50 heterodimers responsible for transcriptional induction of YAP and TAZ.

1. Introduction

1.1. Osteosarcoma: origin and common therapies

Osteosarcoma (OS) is the most frequent primary tumor affecting bone, and typically originates in the extremities of the long bones in the legs e.g., femur or tibia, or in arm bones such as the humerus. Less frequently, it develops in the hip bones, shoulders or jaw, and is always associated with increasing pain [135; 5]. OS shows a bimodal age occurrence, arising frequently in children and teens aged 10-16 years and in older adults usually over 40. OS originates from mesenchymal stem/stromal cells (MSCs) or from the derived osteogenic lineage represented by the osteoblast cells 112 (Fig. 1).

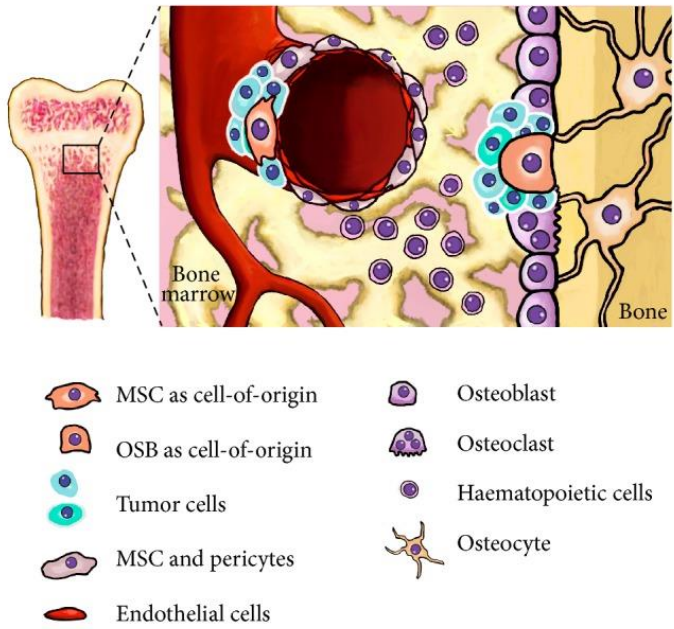

Fig. 1. Bone microenvironment in OS development. MSCs and osteoblasts are the main candidates to acquire pro-tumorigenic mutations and to promote OS development. Modified from Abarrategi et al., 2016 [1].

Many evidences report translocation and genetic mutations with cell-of-origin models based frequently on *TP53* mutations and/or retinoblastoma (Rb) protein down-regulation that occur in MSCs [133; 112]. It was reported that also bone microevironmental signals, such as bone morphogenic protein 2 (BMP2), and calcified substrates could play a key role in promoting oncogenic transformation [136]. Many groups generate OS through the inactivation of *TP53* and/or *Rb* genes in Cre-Lox-based mice models [16; 162] underlining their critical function in regulating bone development. Another study suggest that OS could originate also from differentiated osteocyte that exhibit constitutive Notch signaling activation; moreover, the combined loss of p53 accelerates OS development [152]. Likewise, in mice osteoblasts the upregulated Hedgehog (Shh) signaling, combined with p53 mutations, induce OS development [29]. Anyway, undifferentiated or osteogenic-committed MSC, and human OS samples exhibit common gene signatures supporting both

osteogenic or MSC as cell-of-origin for OS onset under proper epigenetic or microevironmental signals [38].

Besides p53 and Rb inactivation, OS neoplastic cells show a complex karyotype associated with chromosome instability, copy number variations and deregulations of many other signalling pathways such as vascular endothelial growth factor-receptor (VEGF-R), transforming growth factor beta (TGFβ), Wnt/β-catenin, Hippo/Yes associated protein (YAP), phosphatidylinositol 3 kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR), mitogen-activated protein kinases (MAPKs) and erythroblastic leukaemia viral oncogene homologues (ERBBs) [44; 1]. Moreover, many studies report deletions in *p16^{INK4a}* and *p19* genetic locus [92], *cyclin-dependent kinase 4* (*Cdk4*), *mouse double minute 2 homolog* (*Mdm2*), *cyclins D1* and *E* amplifications [95; 90; 89] and c-Fos, c-Jun, c-avian myelocytomatosis viral oncogene homolog (c-Myc) overexpressions [168; 158; 122]. Gene expression data reveal that *minichromosome maintenance complex component 4* (*MCM4*), *large tumor suppressor* 2 (*LATS2*), *baculoviral IAP repeat-containing protein 2 and 3* (*Birc2/3*), *chaperonin containing TCP1 subunit 3* (*CCT3*), *COP9 signalosome subunit 3* (*COPS3*) and *WW domain containing E3 ubiquitin protein ligase 1* (*WWP1*) mutated gene expressions may play a role in OS pathogenesis [79; 173]. In addition, ossification factor genes such as *MET*, *twist family BHLH transcription factor 1* (*Twist*) and *adenomatous polyposis coli* (*APC*) are frequently mutated especially in paediatric high-grade patients suggesting an important role in OS outcome [51]. Other genetic or epigenetic alteration include deletion of *protein kinase CAMP-dependent type I regulatory subunit alpha* (*PRKAR1A*) gene and mutations/overexpressions of *Runt-related transcription factor 2* (*RUNX2*) and *RecQ helicases LA* (*RECQLA*) genes [114; 34], reduced expression of WW domain containing oxidoreductase (WWOX) and hypermethylation of *hypermethylated in cancer 1* (*HIC1*) gene [129] or loss of heterozygosity of budding uninhibited by benzimidazoles 3 (BUB3) and fibroblast growth factor receptor 2 (FGFR2) [100]. All these observations delineate a very complex mutational genetic background in OS patients suggesting that multiple oncogenic pathway deregulations delineate chromosome instability acquired during OS development.

Several studies report the isolation of OS cells subpopulation with cancer stem cells (CSCs)-like features [110], these cells are able to self-renew and sustain tumor formation promoting metastasis and chemoresistance [2; 150]. These characteristics are due to an increase in DNA repair ability, inhibition of apoptotic signaling, increased levels of lysosomal activity and overexpression of ATP-binding cassette (ABC) transporters [57; 65]. Thus, it is clear that OS-CSCs exhibit peculiar properties that make them more resistant to therapies and also the bone microenvironment may play a role in regulating them self-renewal, growth, metastatic potential and drug resistance [5]. It is worth to mention that bone is a suitable niche for OS-CSCs because highly vascularized, favouring migration and metastasis of CSCs, because it represent an hypoxic environment in which hypoxia-induced signaling can promote stemness and drug resistance [78; 187], and because it is rich of calcium and growth factors such as BMP, insulin-like growth factor 1 (IGF1) and FGF that support tumorigenesis [22].

First line treatment for OS is an intravenous combinational chemotherapy (cisplatin (CDDP)/doxorubicin) [1]. For patients with localized OS, surgical resection is an essential component of the therapy, however if not feasible, radiation therapy can be used to improve prognosis [18; 63]. As previously described, OS is frequently associated with chemo- and radio-resistance due to the presence of CSCs subpopulations and also to Hippo/YAP signalling alterations [1; 163]. Several ongoing clinical trials employ drugs that target cell membrane receptors (i.e. Trastuzumab, Cixutumumab, Erlotinib, Sorafenib and Bevacizumab), intracellular signaling (i.e. Everolimus and Curcumin) or niche and their signaling (i.e. Zolendronic acid, Denosumab and Mifamurtide) (reviewed in Abarrategi et al. [1]). Moreover, many other drugs are able to reduce OS-CSCs subpopulations targeting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (i.e. Parthenolide), PI3K, TGF-β, AMP-activated protein kinase (AMPK)/mTOR signaling (i.e. Metformin), Wnt and Notch1 signaling (i.e. Salinomycin and Diallyl trisulfide), histone deacetylase (i.e. Vorinostat) or can stimulate macrophage phagocytosis (i.e. Anti-CD47 antibody) (reviewed in Abarrategi et al. [1]). However, whilst the overall survival rate for OS has increased, the five-year survival rate for pa-

tients with metastatic or recurrent disease has essentially remained unchanged at approximately 20% [6; 67] highlighting the urgent need to develop new and more efficacy anticancer strategies.

1.2. The Hippo pathway

The Hippo pathway is an evolutionarily conserved signalling pathway that plays a key role in development, stem cell maintenance, regeneration, cancer onset, and chemoresistance [56; 163; 185; 166] (Fig. 2). It interacts with many other signaling pathways creating a complex network in which YAP and its paralog TAZ are emerging as a critical node integrating and decoding both oncogenic and tumor suppressor inputs [64] (Fig. 2).

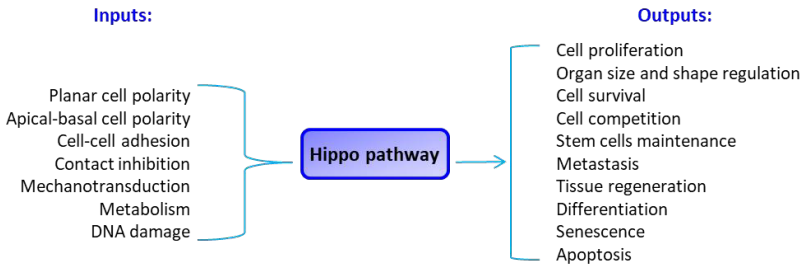

Fig. 2. Hippo pathway regulators and regulation. Hippo signalling integrates different inputs signalling (on the left) and regulates several processes (on the right). Modified from Ferraiuolo et al., 2016 [56].

Hippo signaling was first discovered in *Drosophila melanogaster* and human orthologue counterparts exhibit almost the same functions. The Hippo kinase cassette comprises the tumor suppressor proteins Mammalian Sterile-20 family Serine-Threonine kinases 1 and 2 (Mst1/2) that phosphorylate the adaptor protein Salvador 1 (Sav1) [153], also termed WW45, the Large tumor suppressor 1 and 2 (Lats1/2) AGC family kinases [175] and the Mps one binder kinase activator-like A and B (MOBKL1A and MOBKL1B) also known as Mob1. Mst1/2 activate Lats1/2 by phosphorylation, the interaction between Lats1/2 and Mob1 is necessary for Lats1/2 activation. This interaction is enhanced by Mst1/2 phosphorylation of Mob1. Mst1/2 kinase activity is enhanced through interaction with Sav1 [175]. Thus, Mst1/2 and Lats1/2 trigger a

kinase cascade regulated by Sav1 and Mob1 and phosphorylate the oncogenic Hippo transducers YAP and TAZ resulting in their cytoplasmic retention and/or protein degradation [190] (Fig. 3). In response to cell density, YAP and TAZ phosphorylation at Ser127 and Ser89 respectively creates a 14-3-3 binding site and consequent YAP/TAZ cytoplasmic retention and inhibition. Thus, cell density-dependent activation of the Hippo pathway is involved in the cell contact inhibition [190]. Moreover, Lats1/2 can phosphorylate YAP at Ser381 triggering a consequent phosphorylation by the Casein kinase 1 delta/epsilon (CK1δ/ε) at an additional residue. This event generates a "phosphodegron" signal (conserved also in TAZ which is phosphorylated at Ser311) inducing the recruitment of the E3 ubiquitin ligase SCFβ-TRCP and consequentially YAP ubiquitin-mediated proteasome degradation [190] (Fig. 3). Since YAP and TAZ lack a DNA binding domain, they interact through their WW domains with various DNA-binding transcription factors including TEA domain family members/Transcription enhancer factors (TEADs/TEFs), β-catenin, RUNX1/2 and Smads to drive transcription of their oncogenic target genes [176; 189; 68]. YAP/TAZ can function also as transcriptional co-repressors for the tumor suppressor genes *DDIT4* and *TRAIL* recruiting the Nucleosome remodelling deacetylase (NuRD) histone complex onto the promoters of selected genes [75] (Fig. 3).

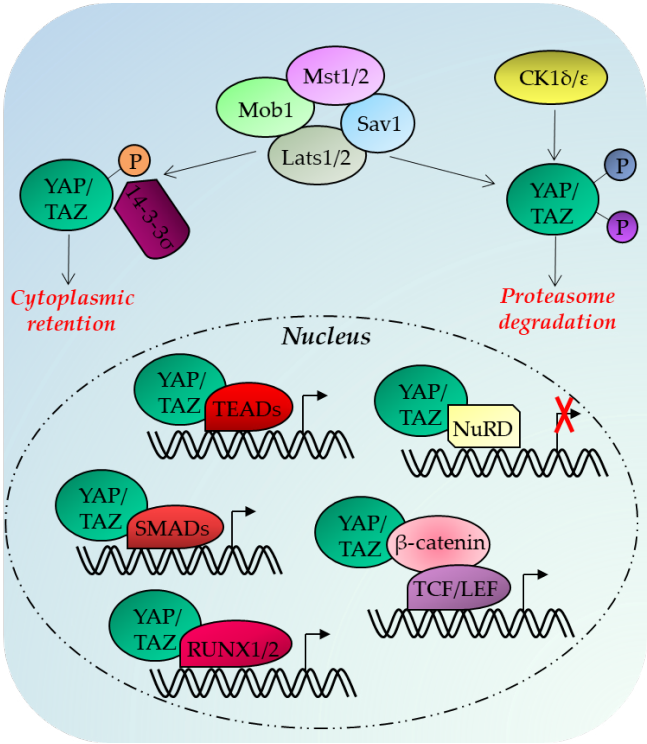

Fig. 3. The Hippo pathway. The core kinases induce YAP/TAZ cytoplasmic retention or protein degradation. When activated, YAP/TAZ translocate into the nucleus and interact with different transcription factors activating or repressing transcription of target genes. Modified from Ferraiuolo et al., 2017 [55].

YAP and TAZ chief functions are to regulate cell proliferation, invasion, stem cell maintenance, and epithelial to mesenchymal transition (EMT) [44; 56]. Interestingly, the Hippo pathway has been shown to cooperate with p53 signalling to induce apoptosis, DNA damage repair, and senescence in response to genotoxic stress as previously reviewed [55]. Dysregulation of Hippo signalling and YAP/TAZ overexpression or hyperactivation are reported in many types of human cancers including metastatic osteosarcomas, brain tumors (meningiomas, schwannomas, and acoustic neuromas), hepatocellular carcinoma (HCC), bile duct tumors, mesothelioma, lung, colon, and breast cancers [64; 185]. This leads to increased cell proliferation, acquisition of cancer stem cell features, EMT and metastasis formation, inhibition of senescence, reduced apoptosis and drug resistance [64]. Invasion and

metastasis are common in advanced solid tumors and are responsible for the majority of deaths from cancer. Loss of cell polarity promotes YAP/TAZ activation in a positive feed-forward loop inducing EMT and metastasis formation [41]. Increasing cell survival and reduced cell death are mediated by YAP/TAZ transcriptional up-regulation of anti-apoptotic B cell lymphoma 2 (Bcl-2) family members, cIAP1/2 anti-apoptotic proteins, c-Myc, connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (CRY61) targets [120]. Moreover, YAP overexpression can suppress anoikis cell death induced by anchorage-dependent cells detaching, promoting dissemination of invading cells and inducing metastasis formation; loss of E-cadherin (linked to EMT) function might also induce YAP/TAZ activation in metastatic cells [180]. YAP/TAZ genes are amplified and localized preferentially in the nucleus of several tumors: lung, pancreas, esophagus, gastric, skin, colon, prostate, liver, ovarian and mammary gland carcinomas, medulloblastomas, gliomas, and oral squamous-cell carcinomas [120; 64; 185]. Recently it was demonstrated that YAP could regulate non-coding RNAs biogenesis associated with cancer. In detail, at low cell density, nuclear YAP binds to and sequesters a regulatory protein of the micro-RNA (miRNA)-processing machinery, p72. This leads to widespread miRNA suppression in cells and tumors with concomitant c-Myc post-transcriptional induction [108]. In lung cancer, YAP elicits its oncogenic activity sustaining the aberrant expression of the *MCM7* gene and its hosted miR-25, 93, and 106 cluster [88]. Moreover, nuclear YAP/TAZ elicit cytoplasm Dicer processing of miRNA precursors (pre-miRNAs) [30]. In liver cancer, YAP/RUNX2 complex binds to the promoter of the tumor suppressor long non-coding (lncRNA) pseudogene metallothionein 1D pseudogene (MT1DP) inhibiting its expression. Down-regulation of MT1DP enhances forkhead box protein A1 (FoxA1) activity with consequent induction of the oncogenic factor alpha-fetoprotein (AFP), a classic liver cancer biomarker [182]. It is also reported that YAP up-regulates lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) expression at both transcriptional and post-transcriptional level encouraging proliferation and enhancing cell migration [164]. Another two lncRNAs have been investigated in colon and renal cancers. YAP cooperates with β-catenin in colon tumorigenesis, also activating the transcription of the lncRNA RNA component of mitochondrial RNA processing endoribonuclease

(RMRP) involved in ribosomal RNA processing [123]. The LncARSR and YAP axis form a feed-forward loop in renal cancer carcinomas (RCCs) in which, forced expression of lncARSR, enhances tumor renal initiating cells and is associated with a poor prognosis in patients [128].

1.2.1. Hippo signaling in osteogenic differentiation and osteosarcoma development

Hippo signaling plays a key role in bone tissue homeostasis. Osteogenic differentiation is regulated by the transcription factor RUNX2 that induces the expression of osteoblast-specific genes such as osteocalcin [70]. It was demonstrated that TAZ binds to RUNX2 working as transcriptional co-activator. Indeed, osteogenesis is promoted by TAZ overexpression and inhibited in TAZ knocking down conditions [70]. TAZ overexpression in mice models induces increased bone formation, higher bone mineral density, higher expression of osteocalcin, alkaline phosphatase (ALP), Sp7 transcription factor, also called osterix, and obviously RUNX2 [177]. Moreover, TAZ could be activated by Wnt/β-catenin pathway resulting in TAZ dephosphorylation and nuclear translocation with consequent induction of osteogenic differentiation [23]. YAP involvement in osteogenic differentiation is more complex as YAP function both as activator and repressor for RUNX2 under specific conditions [184; 50].

Many studies report that aberrations in Hippo signaling are important in OS development. In particular, YAP, Ras-association domain family proteins (RASSFs), neurofibromin 2 (NF2) and Mob1 mutations are involved. RASSFs and NF2 are upstream regulators of the Hippo pathway and act as tumor suppressors activating the Hippo kinases [125; 17]. *RASSF5* and *RASSF10* are frequently hypermethylated and down-regulated at protein levels resulting in OS development associated with distal metastasis [132; 192]. *NF2* mutations are associated with neurofibromatosis type 2 disease but also with meningioma, schwannomas and OS in 63% of cases [99]. Mob1 down-regulation induces OS formation in mice models [115] and aberrant mitosis in *in vitro* experiments [69]. YAP protein is overexpressed in OS tissue compared to normal one and its expression correlate with tumor staging [188]. In murine OS cell lines, YAP and TAZ are overexpressed and primarily localized into the nucleus [131], moreover, YAP suppression induces RUNX2, Cyc

D1 and matrix metallopeptidase 9 (MMP-9) down-regulation as well as reduced cell proliferation, migration and tumor growth in murine xenografts [178] and transgenic mice models [29]. It was demonstrated that Sox2 transcription factor overexpression is responsible of YAP upregulation. Indeed, Sox2 represses NF2 and Kibra activators of the Hippo kinases leading to enriched YAP expression and osteosphere formation *in vitro* [15].

1.3. The NF-κB pathway

NF-κB family of transcription factors are key regulators of immune development, immune responses, inflammation, differentiation, survival and cancer [103]. NF-κB signalling is activated by inflammatory stimuli or ribo- and geno-toxic stresses (NF-κB essential modulator (NEMO, also known IκB kinase γ (IKKγ))-dependent pathway, canonical signalling) or via sensing developmental stimuli (NEMO-independent pathway, non-canonical signalling) [103]. These pathways control NF-κB dynamic constitutively or in response to different stimuli regulating the entire expression programs [144]. Upstream regulators are tumor necrosis factor receptor (TNF-R), toll-like receptor (TLR), interleukin receptor 1 (IL-1R), developmental signals, genotoxic and ribotoxic stresses that regulate NF-κB expression, processing of NF-κB precursors or NF-κB activation [116; 52; 72; 9].

NF-κB family members act as homo- or hetero-dimers of different subunits: p65 (or RelA), RelB, p50 (obtained from p105 precursor), p52 (produced from p100 precursor), and cRel; as monomers are unstable and quickly degraded. Thus, NF-κB dimer formation and repertoire are dynamic and change depending on different cell type during cell differentiation and development [8]. NF-κB monomers RelB and cRel are differentially expressed, whereas p65 is ubiquitously expressed and plays a crucial role in inducing the transcription of all NF-κB subunits [21; 8]. Some NF-κB monomers must be processed before working as transcription factors. This is the case of p50 and p52 that are released after cleavage of p105 and p100 precursors respectively [179]. The strongest binding affinities between NF-κB monomers exist between p65:p50 hetero-, and p50:p50 and p65:p65 homo-dimers [157]. NF-κB p65:p50 and

p65:p65 complexes are transcriptional activators, whereas p50:p50 dimers function as inhibitors [13]. NF-κB dimers are unstable and rapidly degraded, especially for p65, if activating stimuli stop [137; 97]. Different stimuli activate canonical or non-canonical signalling.

The canonical signaling is mediated by the scaffold protein NEMO. In the absence of stimuli, NF-κB is inactivated and sequestered in the cytoplasm through the binding to the inhibitor of kappa B proteins (IκBα/β/ε/δ); in the presence of inflammation (i.e. TNF and IL-1 production), NEMO interacts with the activated IκB kinases (IKKα/β) that phosphorylate IκBs, promoting their proteasomal degradation and NF-κB release. In detail, NEMO contains an ubiquitin binding domain that allows IKKα/β non-degradative ubiquitination (K63-linked) as marker of inflammation and directs IKKα/β-dependent phosphorylation of IκBs on specific serines, leading them to ubiquitination and proteasomal degradation [141]. Thus, NF-κB becomes activated and translocates into the nucleus to promote transcription of its target genes such as inflammatory cytokines (i.e. IL-6 and IL-8), inducible nitric oxide synthase (iNOS) and cyclooxygenases (i.e. COX-2) [31] (Fig. 4). NF-κB can also transcribe directly IκBα/β/ε or p100 that bind to IκBδ promoting NF-kB cytoplasm retention. These mechanisms represent two auto-regulatory negative feedback loops that prevent activation in response to transient stimuli and poise the system for reactivation status [102], or attenuate persistent signals and sequential stimulations [143]. NF-κB is also regulated by genotoxic and ribotoxic stresses that induce NEMO activation and IκBs degradation promoting NF-κB activation, nuclear translocation and transcription of its target genes [94; 117]. Non-canonical signaling is activated by developmental stimuli that promote NF-κB-inducing kinase (NIK) and IKKα stabilization and accumulation [45; 149]. NIK exerts a dual function: it activates IKKα by phosphorylation inducing IκBδ degradation and release of NF-κB, this results in the activation of inflammatory or developmental responses depending on the existing NF-κB dimer repertoire [14]; moreover, NIK promotes a late response inducing p100 processing and so p52:RelB heterodimers formation [87] (Fig. 4).

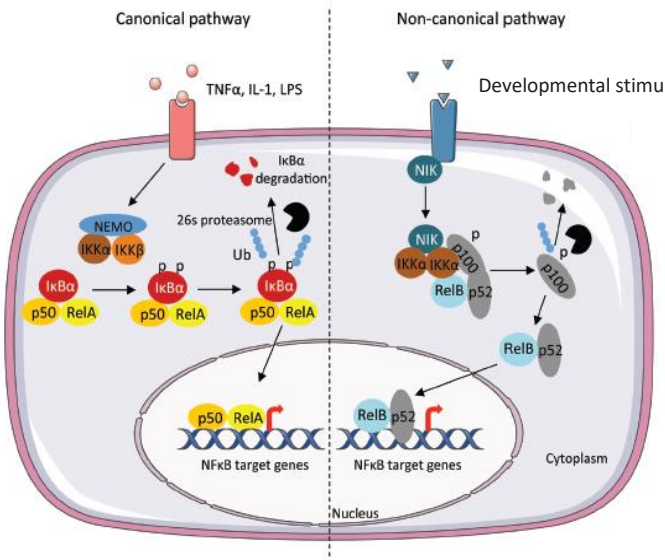

Fig. 4. NF-κB signaling. NF-κB can be activated by inflammatory stimuli, geno- or ribotoxic stresses (canonical pathway, NEMO-dependent) or via developmental signals (non-canonical pathway, NEMO-independent). Modified from Viennois et al., 2013 [161].

NF-κB signalling is aberrantly regulated and frequently activated during onset and progression of several cancer types to promote inflammation and angiogenesis [37], cancer cell survival [48], migration [73], chemo- and radio-resistance and is associated with poor patient prognosis [193; 171]. Indeed, NF-κB promotes cell proliferation by upregulating cyclins D1, D2, D3, E and c-Myc and induces the expression of proteins involved in invasion (i.e. intercellular adhesion molecule 1 (ICAM-1), E-selectin, MMPs) and angiogenesis (i.e. VEGF) in many tumors such as breast cancer and colorectal cancer as reviewed in Dolcet et al. [48]. Many lymphomas and leukemias present constitutively active NF-κB proteins produced by translocation chromosome mutations [101; 24]. Moreover, are also reported IκBα loss-of-function mutations in bladder, breast, colon, ovarian, pancreatic, prostate carcinomas and melanoma cancers [130] and cRel, p52, p65 and p50 nuclear accumulation in breast and nasopharyngeal carcinomas [39; 134; 154]. In detail, p65 nuclear localization and activity is frequently increased in cancer and is associated with worsening tumor progression and metastases in cervical squamous cell carcinomas and gastric cancer [140; 113].

1.3.1. NF-κB pathway deregulation in OS development

NF-κB signaling alterations are involved in OS pathogenesis and progression, in particular many studies report that NF-κB upregulation induces proliferation, metastasis and chemoresistance in OS patients. Zinc finger E-box binding protein 1 (ZEB1) is a transcription factor involved in NF-κB and iNOS activation in OS cells with consequent increase in cell proliferation and invasion 174. NF-κB activation induces chemoresistance and it was demonstrated that polyphenols could inhibit IKKα/β activation and thus NF-κB nuclear translocation with consequent apoptosis induction in Saos-2 cell line 62. Moreover, OS shows a radioresistant subpopulation that induces relapse and metastasis following treatment. It was demonstrated that targeting NF-κB with parthenolide treatment could re-sensitize OS cell population to radiotherapy inducing cell death 194. Many other studies report that down-regulating NF-κB signaling directly (i.e. thymoquinone, tetramethylpyrazine, aspirin, celastrol, tetrandrine and amentoflavone treatments) 124; 165; 84; 183; 93; 121 or indirectly 151; 91; 191; 66; 54; 186; 181, results in reduced proliferation, invasiveness and chemo- and radio-resistances. Moreover, NF-κB induction promotes transcription of target genes involved in migration 167; 86 and down-regulates the tumor suppressor miR-506 71.

1.4. Agave natural extracts properties

Agaves species are succulent monocotyledons that belong to the Agavaceae family which form distinctive rosettes and flower spikes. Most Agaves consist of rosettes of thick, hard, rigid but succulent leaves often with marginal teeth and usually with a lethally sharp terminal spine. Leaves contains carbohydrates (in particular inulin is known to reduces cholesterol and glycaemia), mineral salts and oligo-elements (i.e. Fe^{3+}, Mg^{2+} and Ca^{2+}), the alkaline serine protease EC 3.4, flavones and saponins [32; 145]. Agave is widely employed in Tequila production (from *Agave tequilana*) [27], as drought-tolerant biofuels feedstock [83] and for steroidal hormone synthesis [42] but exhibits also antimicrobial [43], anti-helminthic [19], anti-inflammatory [58], anti-tumoral [109], neuroprotective [148] and prebiotic [26] properties. The use of medical plants in medicine is practised across time immemorial. In Caribbean

traditional medicine, *Agave intermixta* has been used as anti-tuberculotic, anti-arthritic and anti-carcinogenic agent [138] and it was demonstrated to exert a mitotic inhibiting effect over 24h of treatment in plant and mammalian cancer cells [138]. Hexanic non-toxic fraction (riches of saponins content and in particular of hecogenin and tigogenin) from *Agave sisalana*, exerts anti-inflammatory and analgesic properties in acute and chronic mice models reducing oedema extension through myeloperoxidase (MPO) inhibition and pain through probably inhibiting neutrophil infiltration [49]. *A. sisalana* contains also a phenol and flavonoid fraction that inhibits IL-2 and interferon-γ (INF-γ) release thus reducing inflammation and proliferation of mononuclear cells in human peripheral blood [33]. *Agave tequilana* lignin exhibits photoprotection properties, so it was conjugated with zinc oxide nanoparticles to increase the sun protection factor in sunscreens. Indeed, it was demonstrated that lignin nanoparticles increase absorption in the UVB and UVC spectra [61]. Fructans obtained from *A. tequilana* exert a metabolic action decreasing serum glucose and triglyceride levels in mice models and thus preventing weight gain, diabetes and liver steatosis [98]. Another study reports that are saponins responsible of this metabolic effect. In detail, authors demonstrate that saponins can reduce weight, fat mass, serum glucose, insulin, LDL-cholesterol levels and hepatic lipid levels in mice models. Furthermore, saponins increase fatty acid oxidation, mitochondrial activity in skeletal muscle and promote gut microbiota proliferation [80]. Fructans from *A. angustifolia* are rich in garbs component (in particular fructooligosaccharides and inulin) that increase saccharolytic fermentation in proximal and distal colon exerting a prebiotic function that prevent colon cancer [7].

1.5. Saponins compounds and properties

Saponins are amphipathic polycyclic aglycones (hydrophobic) attached to one or more sugar side chains (hydrophilic) characterized by their strong foam-forming properties in aqueous solutions. The aglycone part, which is also called sapogenin, is either a steroid (C27) or a triterpene (C30). Agave plants are an important source of steroidal sapogenins that can be isolated from leaves, flowers, leaf juice, rhizomes and callus [3]. On the basis of the aglycone part, saponins can be classified as spirostanol or furostanol glycosides. These compounds,

depending on the number of sugars attached, may further be classified as mono- until hexa-glycosides [3]. Saponins exhibit a number of well-known important bioactivities like anticancer, adjuvant, immunostimulant, analgesic, anti-inflammatory, gastroprotective, antimicrobial, hypocholesterolemic and antioxidant properties [145]. Despite this, at high concentration, saponins exert hemolytic and cytotoxic activities due to the amphipathic nature that confer the capacity to alter membrane permeability [147; 32]. As mentioned, saponins act as anti-inflammatory agents, indeed they can down-regulate a plethora of inflammation promoting factors such as IL-1β, IL-2, IL-6, IL-10, IL-12, IL-18, iNOS, NF-κB, PGE2, TNF-α, TGFβ1 and COX-2. Diosgenin extracted from Fenugreek is demonstrated to exert anti-inflammatory function in adipose tissue suppressing macrophages infiltration and activation; moreover, it is shown to exert also a metabolic role promoting adipocyte differentiation by *CCAAT/enhancer binding protein* (*C/EBP*) mRNA increased expression that results in reduced serum glucose uptake [159]. Platycodin saponins are known to prevent IκBs degradation and NF-κB activation, thus iNOS, COX-2 and inflammatory cytokines are inhibited after bacterial lipopolysaccharide (LPS) treatment [4]. Likewise, caulosaponin, present in Blue Cohosh, inhibits iNOS, TNF-α, COX-2, IL-1β and IL-6 production after LPS treatment [82], ginenosides, present in *Panax ginseng*, inhibits TNF-α-induced NF-κB transcription of COX-2 and iNOS [146] and hecogenin shows a potent gastroprotective effect in gastric ulcer mice models suppressing NO production and inducing K^+_{ATP} channel activation [28]. Cantalasaponin-1 from *A. tequilana*, *A. angustifolia* and *A. americana* shows anti-inflammatory properties reducing edema in mice models in a dose dependent manner [107].

Saponins exhibit also anticancer functions [96; 126]. Diosgenin from fenugreek is demonstrated to inhibit human telomerase reverse transcriptase (hTERT) expression and so telomerase activity and cell growth in lung cancer cell lines [105]; chlorogenin affects cell cycle inhibiting the G2/M transition in Hela cells [118]. Moreover, diosgenin is able to activate p53, the apoptosis-inducing factor (AIF), and Bax proteins resulting in cell cycle arrest (in S and G2/M phases) and caspase-3-dependent apoptosis in osteosarcoma, laryngocarcinoma and melanoma cell lines [40]. Specifically in osteosarcoma, other studies demonstrate that diosgenin inhibits cell growth, induces *TP53* and $p21^{Waf1/Cip1}$ mRNA

levels and reduces Bcl-2 and COX-2 protein levels [104]; moreover, diosgenin, hecogenin, tigogenin, sarsasapogenin, smilagenin and solasodine saponins can block cell proliferation and induce apoptosis [155]. Paris saponin VII reduces viability, proliferation and migration blocking p38 MAPK activation and thus inhibits MMP-2 and -9 synthesis in a time- and dose-dependent manner [35]. Methyl protodioscin (MPD) suppresses cell growth and induces apoptosis. In detail, MPD inhibits anti-apoptotic protein expression Xiap and Survivin, induces reactive oxygen species (ROS) production that in turn activate p38 MAPK and inhibit Bcl-2 and MMP expression. This regulation results in caspase-3 and -9 activation and apoptosis induction. Moreover, MPD decreases extracellular signal–regulated kinases (ERKs) activation and induces G0 and G2/M arrest inhibiting cell proliferation [156]. Considering all these data, we can summarize saponins function, in particular their anticancer activity. As reviewed in Koczurkiewicz et al., at high concentration (>100μM) saponins induce cell membrane permeabilization exerting cytotoxic and hemolytic effects. At low concentration, saponins inhibit MMPs expression via MAPK/ERK pathway; impair NF-κB and AP-1 transcription down-regulating PI3K/Akt pathway; reduce Mdm2 levels, activate p38 and prevent IκBα phosphorylation and thus NF-κB nuclear translocation. In addiction saponins down-regulate Cdk2/Cyc D1 and Cdk4/Cyc E complexes and Cyc B levels. Finally, they activate TNF and FAS receptors, p53 and Bcl-2-associated X protein (Bax) pro-apoptotic factors. All these modulations result in the inhibition of cell proliferation and invasion and intrinsic and extrinsic apoptosis induction (Figure 5) [77].

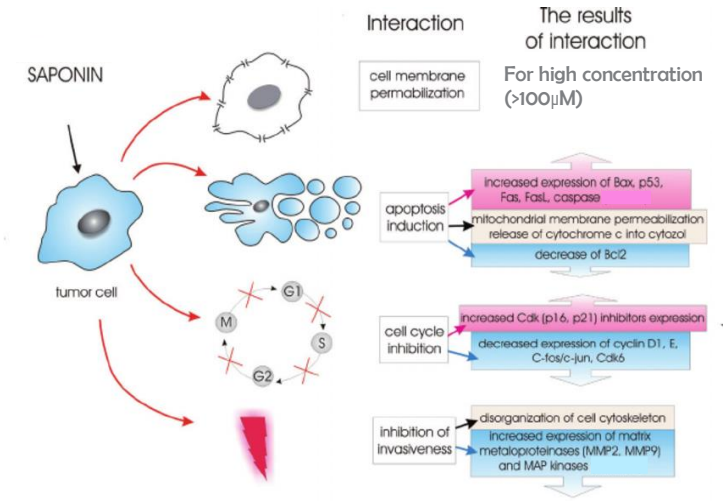

Fig. 5. Saponins effects in cancer. Modified from Koczurkiewicz et al., 2015 [77].

2. Aim of the project

OS is a tumor that develops in bones with an unknown etiology. It is mainly a pediatric cancer frequently associated with Li-Fraumeni, Rothmund Thomson and hereditary retinoblastoma genetic syndromes. Patients with OS exhibit a very low survival rate spanning from 1 to 5 years and 50% of them encounter limb amputations. Because its radio- and chemo-resistances, new effective OS anticancer strategies are urgently required, paving the way for research into less-toxic, cost-effective natural compounds that could be useful novel OS therapeutics. To this end, we tested several natural extracts and found that Agave promoted cell death of OS cell lines. Then, we aimed to dissect the Agave's mechanism of action. We also evidenced how Agave could be useful alone or in combined anticancer treatments potentiating the killing effects of conventional OS therapies and thus overcoming chemoresistance. Agave's effect is also demonstrated in other cancer cell lines opening future prospective in treating many different cancers. We propose a model depicting Agave anticancer effects that occur through the impairment of two well-known oncogenic pathways (YAP/TAZ and NF-κB). Both pathways are frequently hyper-activated in OS and many other cancers. We propose to test Agave anticancer effects *in vivo* to highlight the potential use of Agave as adjuvant treatment for OS.

3. Results

3.1. Agave reduces cell viability, colony formation, cell migration, and sensitizes cells to CDDP

Searching for potential alternative OS therapeutics, we performed cell viability assays testing 15 natural compounds on U-2 OS and Saos-2 OS cell lines (data not shown). We noted that of the 15 compounds tested, Agave natural extract (from A. sisalana) showed the greatest reduction in cell viability. We therefore focused on this extract and extended our analysis to four OS cell lines: U-2 OS, Saos-2, HOS and MG-63. Dose-response assays were performed using Agave treatment for 72 h (Fig. 1A). Table 1 shows the half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) for each cell line. EC50 values ranged from 3.67 μg/mL in U-2 OS to 6.16 μg/mL in HOS cells (Table 1). For subsequent functional analyses, we selected the lower dose of 3.12 μg/mL, which reduced cell viability by approximately 25% (Fig. 1A). We tested cell migratory ability after 24 h of Agave treatment using wound healing- (Fig. 1B-C) and transwell- (Fig. 1D-E) migration assays, and found that Agave impairs cell migration in both U-2 OS and Saos-2 cell lines.

The biggest challenge in OS therapy is overcoming chemoresistance [1]. To determine whether Agave can improve the effectiveness of CDDP as an apoptotic agent, we assayed Agave in combination

with increasing doses of CDDP for 72 h (Fig. 1F-G). Agave extract sensitized OS cells to CDDP, effectively reducing the EC50 (by 2.7- and 2.2-fold) and LC50 (3.7- and 2.7-fold) for U-2 OS and Saos-2 cells, respectively (Table 2). Clonogenic assays were then performed to assess colony formation ability using U-2 OS and Saos-2 cell lines treated with Agave and CDDP either alone or in combination (Fig. 1H-I). Whilst CDDP was more effective than Agave in reducing colony numbers, Agave potentiated CDDP's inhibitory effect in both cell lines. Agave's inhibitory effect on cell viability was also tested in a variety of other cancer cell lines (lung, mesothelioma and breast) (Fig. 2) demonstrating a consistent dose-dependent effect.

Figure 1

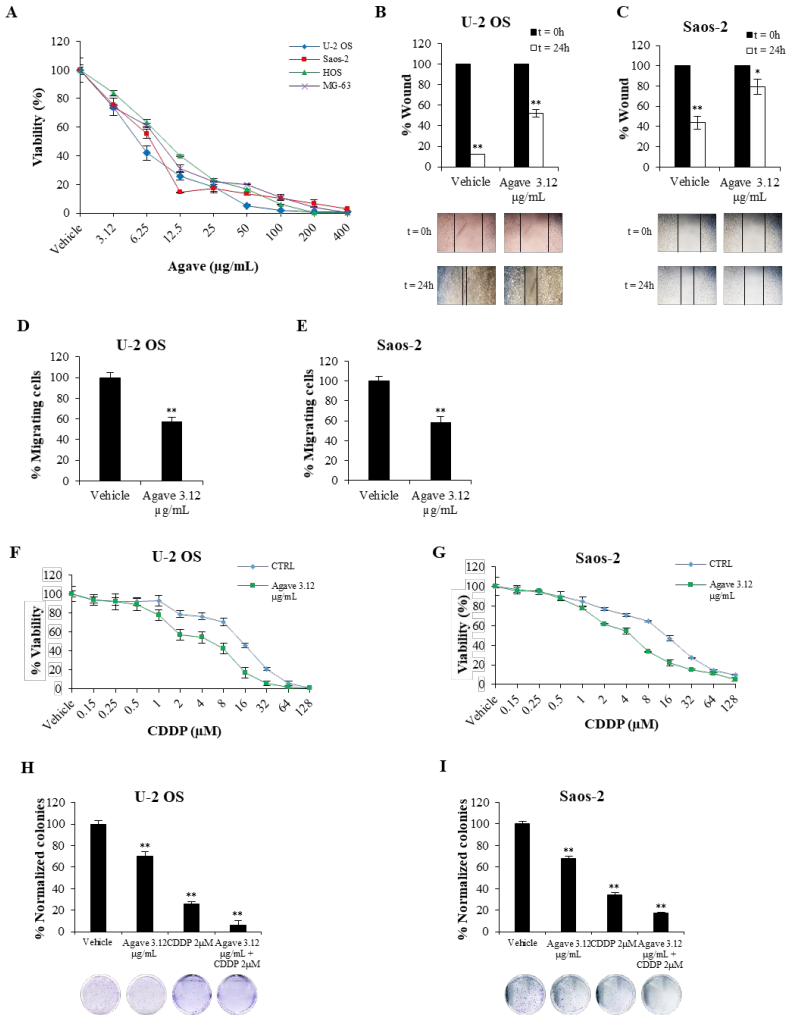

Fig. 1. Agave exerts anticancer effects on osteosarcoma cell lines. (A) Osteosarcoma cell lines were treated with Vehicle control (EtOH) or increasing concentrations of Agave for 72 h before being analyzed by ATPlite assay. The percentage cell viability normalized to control is shown, with values representing mean ± STDEV of n=3 independent experiments. Wound healing- (**B-C**) and transwell- (**D-E**) migration assays were performed on U-2 OS (B, D) and Saos-2 (C, E) cell lines after treatment with Agave (3.12 µg/mL) or Vehicle for 24 h. Histograms represent the percentage of wound width (B-C) or migrated cells (D-E) normalized to the relevant control. Values represent mean ± STDEV from n=3 independent experiments, *p<0.01 **p<0.001. Osteosarcoma cell lines U-2 OS **(F)** and

Saos-2 **(G)** were treated with either Vehicle (CTRL) or Agave (3.12 μg/mL) in combination with increasing doses of cisplatin (CDDP) for 72 h. The percentage viability of Agave treatment alone was subtracted from each CDDP or CTRL value (corrected value), then these data were normalized to the CTRL corrected value. Values shown represent mean ± STDEV from n=3 independent experiments. U-2 OS **(H)** and Saos-2 **(I)** cell lines were treated with Vehicle, Agave (3.12 μg/mL), and/or CDDP (2 μM) fter seeding for clonogenic assays. Histograms represent the mean percentage of colonies ± STDEV normalized to Vehicle control from n=3 independent experiments, **p<0.001. Images underneath histograms are representative stained culture dishes used for counting colonies.

Table 1

	U-2 OS		Saos-2		HOS		MG-63	
Natural product phytocompound	*EC50 (μg/mL)*	*LC50 (μg/mL)*	*EC50 (μg/mL)*	*LC50 (μg/mL)*	*EC50 (μg/mL)*	*LC50 (μg/mL)*	*EC50 (μg/mL)*	*LC50 (μg/mL)*
Agave	*3.67*	*5.21*	*4.8*	*6.3*	*6.16*	*9.38*	*4.39*	*7.81*

Tab. 1. Treatment with Agave reduces cell viability. The half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) values for osteosarcoma cell lines treated with Agave, calculated using Compusyn software from the dose response curves in Fig. 1A.

Table 2

	U2-OS		SAOS-2	
Samples	*CDDP EC50 (μM)*	*CDDP LC50 (μM)*	*CDDP EC50 (μM)*	*CDDP LC50 (μM)*
CTRL	*5.27*	*13.85*	*9.3*	*13.33*
Agave 3.12 μg/mL	*1.94*	*3.79*	*4.3*	*5*

Tab. 2. Agave sensitizes osteosarcoma cells to cisplatin. The half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) values for osteosarcoma cell lines treated with cisplatin (CDDP) in combination with Agave (3.12 μg/mL) or EtOH control (CTRL), calculated using Compusyn software from the dose response curves in Fig. 1F-G.

Figure 2

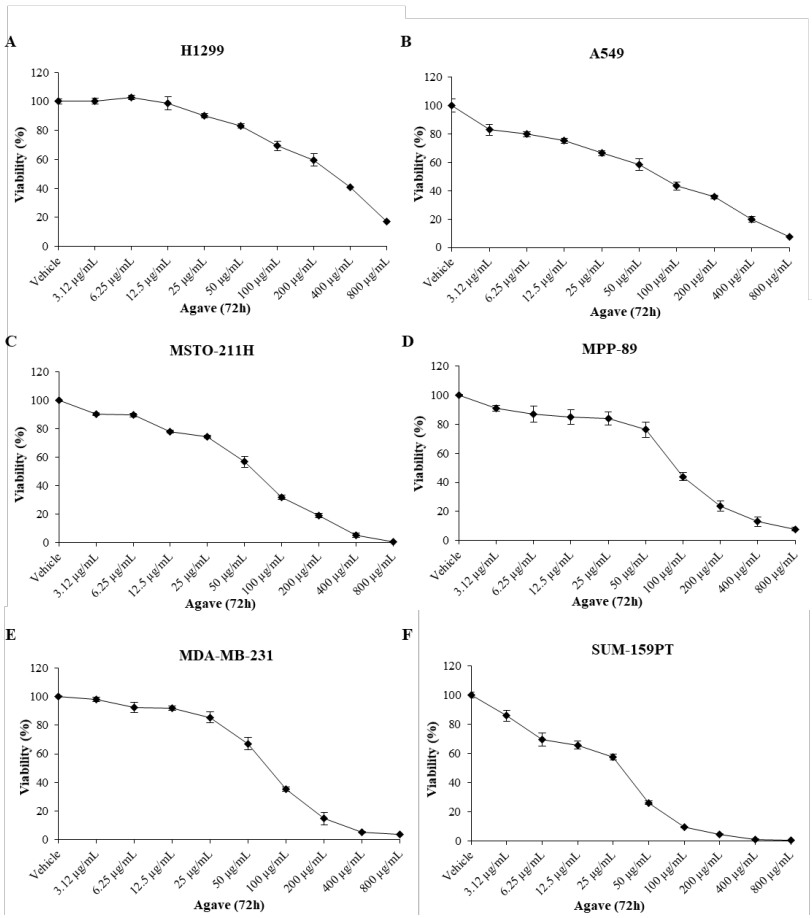

Fig. 2. Agave impairs cell viability in different tumor cell lines. H1299 (A), A549 (B), MSTO-211H (C), MPP-89 (D), MDA-MB-231 (E) and SUM-159PT (F) cells were treated with Vehicle (EtOH) or increasing concentrations of Agave for 72 h before being analyzed by ATPlite assay. The percentage cell viability normalized to control is shown, with values representing mean ± STDEV of n=3 independent experiments.

3.2. Agave decreases oncogenic YAP and TAZ protein levels and induces apoptosis

As previously described, OS typically exhibits dysregulation of multiple signalling pathways, including Hippo/YAP [44]. Alterations in this pathway have also been demonstrated to be a driver of chemo- and radio-resistance in OS patients [163]. To determine the mechanism of Agave's anticancer effects we focused on alterations within the Hippo pathway, specifically the Hippo transducers YAP and TAZ. Agave decreased YAP and TAZ protein expression after 24 h in U-2 OS cells (Fig. 3A). Notably, Lats1 protein level was unchanged, and TEAD1 only mildly down-regulated. Reduced YAP and TAZ expression was also observed in Saos-2, HOS and MG-63 lines after 72 h of Agave treatment (Fig. 3B). Treatment with CDDP alone did not affect YAP and TAZ protein levels (Fig. 3C). Moreover, Agave induced apoptosis by increasing Bax protein expression, as well as caspase 3 and Poly (ADP-ribose) polymerase (PARP) cleavage (lanes 1-2, Fig. 3C), this effect is stronger compared to that mediated by CDDP treatment alone (lanes 1 and 3, Fig. 3C), and appears to enhance CDDP's effect increasing caspase 3 cleavage in combined treatment (lanes 3-4, Fig. 3C). These data suggest that Agave reduces cell viability by down-regulating YAP and TAZ that are well known to function as oncogenes in OS cells [44; 163]. Indeed, YAP/TAZ silencing sensitized OS cell lines to Agave (Fig. 3D), reducing the EC50 and LC50 concentrations by 4.2- and 2.2-fold, respectively (Table 3). Moreover, silencing of YAP and TAZ reduced the clonogenic and migratory capabilities of both U-2 OS and Saos-2 cell lines (Fig. 3E-G and Fig. 4A-B).

Figure 3

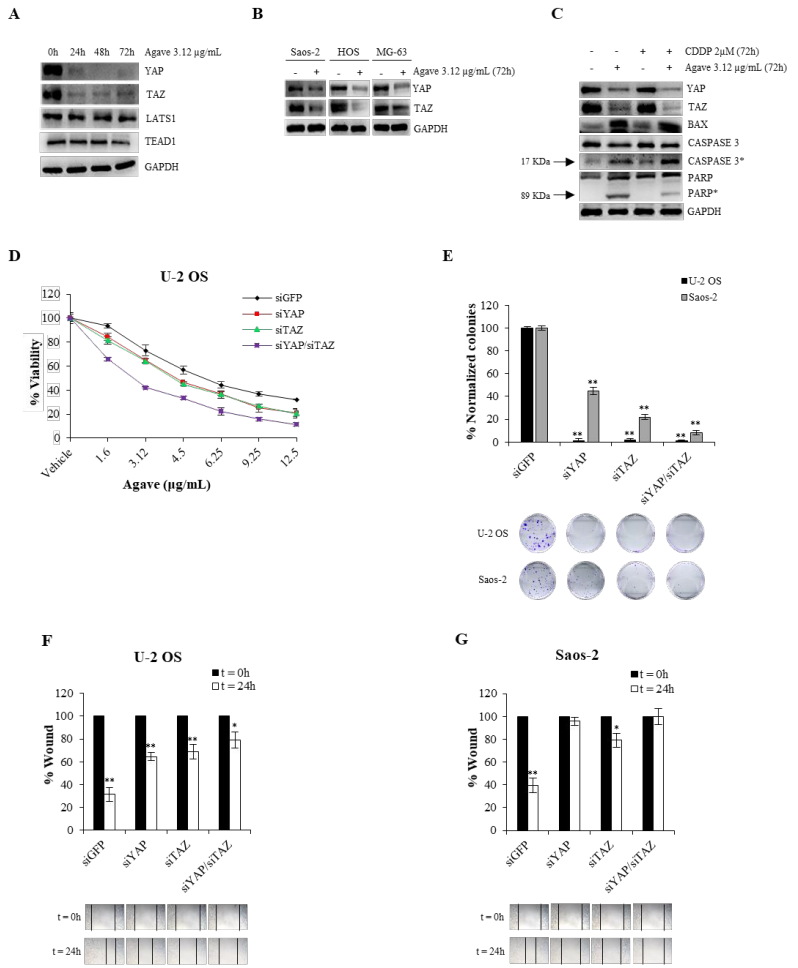

Fig. 3. Agave down-regulates YAP and TAZ oncogenic proteins. (A) U-2 OS cells were treated with Agave (3.12 µg/mL) for 24, 48 or 72 h, or EtOH as Vehicle control (0 h), then subjected to Western Blot analysis as indicated. GAPDH was used as a loading control. (B) Saos-2, HOS and MG-63 cell lines were treated with Agave (3.12 µg/mL) or Vehicle for 72 h, and (C) U-2 OS cells were treated with Vehicle, Agave (3.12 µg/mL) and/or CDDP (2 µM) for 72 h before being subjected to Western Blot analysis as indicated. (D) U-2 OS cells transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ were treated with increasing doses of Agave diluted in Vehicle. Values represent the mean percentage viability ± STDEV for each condition (n=3), determined by ATPlite assay. (E)

U-2 OS and Saos-2 cells were transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ and subjected to clonogenic assays. Histograms show the mean percentage of colonies ± STDEV normalized to siGFP from n=3 independent experiments, **p<0.001. Images underneath histograms are representative stained culture dishes used for counting colonies. U-2 OS (F) and Saos-2 (G) cells were transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ and subjected to wound healing assays. Histograms show the mean percentage of wound width ± STDEV normalized to siGFP from n=3 independent experiments, *p<0.01 **p<0.001. Images underneath histograms are representative of the wounded areas used for measuring wound opening.

Table 3

Samples	*Agave EC50 (µg/mL)*	*Agave LC50 (µg/mL)*
siGFP	*7.6*	*5.2*
siYAP	*4*	*4*
siTAZ	*3.8*	*4*
siYAP/siTAZ	*1.8*	*2.4*

Tab. 3. YAP and TAZ depletion sensitizes osteosarcoma cells to Agave. The half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) values for Agave treatment of siGFP (control), siYAP, siTAZ, or siYAP/siTAZ U-2 OS cell lines, analyzed using Compusyn software from the dose-response curves in Fig. 3D.

Figure 4

Fig. 4. Silencing efficiency in OS cell lines. Western Blot analyses of U-2 OS (A) and Saos-2 (B) cells transfected with siGFP (control), siYAP, siTAZ or siYAP/siTAZ.

3.3. Saponins in Agave extract down-regulate YAP and TAZ protein levels

To dissect the molecular mechanism by which Agave extract functions, we investigated the effect of saponins, which are the most abundant compounds present in Agave natural extract among that with recognised anticancer effects (Table 4) [77; 145]. We assayed Diosgenin, Sarsasapogenin and Solasodine synthetic steroidal saponins using viability assays (Fig. 5A and Table 5), then treated cells with sub-lethal doses of each compound to test their effect on YAP and TAZ. Although less effective when compared to Agave, Diosgenin and Solasodine reduced both YAP and TAZ protein abundance, whilst Sarsasapogenin significantly reduced YAP at the selected concentrations (Fig. 5B). Lats1 and TEAD1 down-regulation were not observed (Fig. 5B).

3.4. Agave enhances YAP and TAZ protein degradation as an early event in OS tumorigenesis

To investigate how Agave can modulate YAP and TAZ protein levels we performed protein stability assays by treating cells with cycloheximide (CHX) in the presence or absence of Agave, then harvesting cells at different time points. Agave treatment resulted in significantly reduced YAP and TAZ protein levels at 24 and 16 h, respectively (Fig. 5C-E). Agave-induced YAP/TAZ reduction was mediated by ubiquitin-dependent proteasomal degradation as indicated by the increased abundance of ubiquitinated YAP/TAZ (Fig. 5F-G). The rapid loss of YAP and TAZ proteins suggests that ubiquitin-mediated YAP/TAZ degradation is an early event following Agave administration.

Table 4

Compounds	*% compound*
Phenols, total	***0.1904***
Of which Flavonoids, total	*0.0806*
Of which Acid Phenol, total	*0.0159*
Of which Phenylpropanoid derivatives, total	*0.0708*
Of which Quinones, total	*0.0025*
Of which Salicylates, total	*0.0113*
Of which Simple Phenols, total	*0.0093*
Terpenes, total	***0.2273***
Of which Monoterpenes , total	*0.1061*
Of which Sesquiterpenes, total	*0.0506*
Of which Triterpenes, total	*0.03397*
Diosgenin	*0.00105*
Sarsasapogenin	*0.00152*
Of which Apocarotenids, total	*0.0302*
Of which Norisoprenoids, total	*0.0064*
Polysaccharides, total	***62.1***
Saccharides, total	***2.17***
Aromatic alcohols, total	***0.0416***
Aromatic acids, aromatic esters and lactones, total	***0.0627***
Nitrogen compounds, total	***6.01099***
Aromatic Ketones, total	***0.0170***
Esters, total	***0.0040***
Lactones, total	***0.0687***
Organic acids, total	***5.7487***
Fats, total	***0.1459***
Sulphur compounds, total	***0.0028***
Vitamins, total	***0.0886***
Water	***6.6***
Minerals, total	***8.4100***
Total	***91.74504***

Tab. 4. Agave composition. All compounds identified by liquid or gas chromatography and mass spectrometry in Agave natural extract (from *A. sisalana*) divided into classes (bold) and subclasses (italics). Diosgenin and Sarsasapogenin, used for subsequent functional assays, are highlighted in blue. Saponins belong to the terpenes class, which are the most abundant of the bioactive compounds.

Figure 5

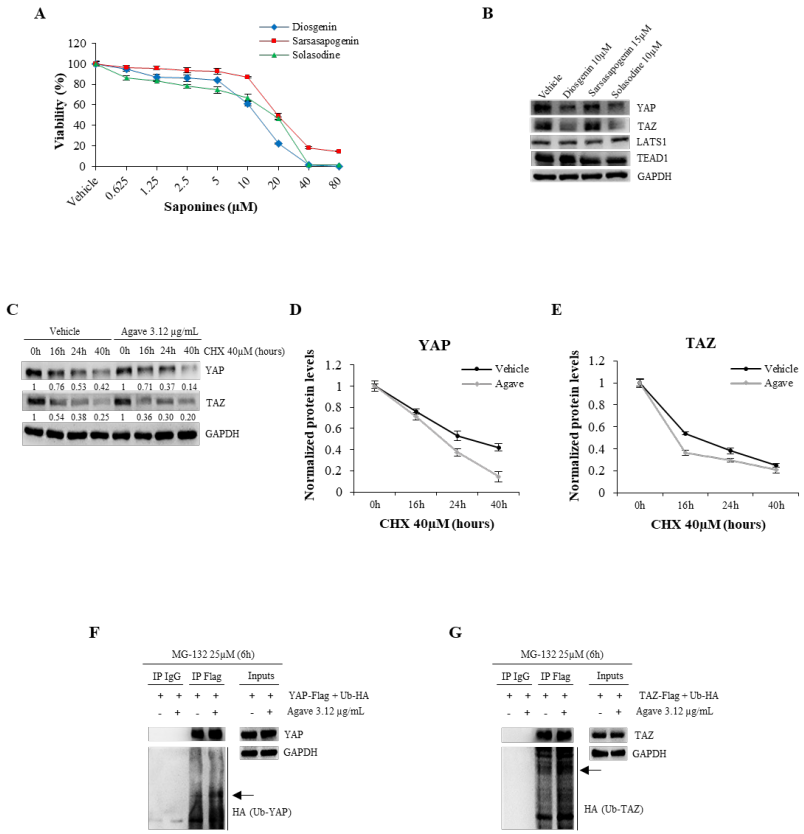

Fig. 5. Agave induces YAP and TAZ protein degradation. (A) U-2 OS cells were treated with Vehicle (EtOH) or increasing concentrations of Diosgenin, Sarsasapogenin and Solasodine synthetic saponins for 72 h before being analyzed by ATPlite assay. The percentage cell viability normalized to control is shown, with values representing mean ± STDEV of n=3 independent experiments. (B) U-2 OS cells were treated with Vehicle, Diosgenin (10 µM), Sarsasapogenin (15 µM) or Solasodine (10 µM) for 72 h before being subjected to Western Blot analysis as indicated. GAPDH was used as a loading control. (C) U-2 OS cells were treated with Vehicle or Agave (3.12 µg/mL) in combination with cycloheximide (CHX, 40 µM) for 16, 24 or 40 h, or DMSO (0 h). Cell extracts were then subjected to Western Blotting with the indicated antibodies and quantified using Alliance (UVITEC) Software. The quantitated protein levels at each time point for YAP (D) and TAZ (E) were normalized to that of GAPDH. U-2 OS cells were co-transfected with Flag-tagged YAP (F) or TAZ (G) as well as HA-tagged Ubiquitin before being treated with Vehicle or Agave (3.12 µg/mL) in combination with MG-132 (25 µM) for 6 h. Protein

lysates were then subjected to immunoprecipitation using anti-IgG or anti-Flag antibodies. Inputs recovered from anti-IgG IPs were loaded onto gels and subjected to immunoblot together with IP samples. Arrows indicate ubiquitinated proteins.

Table 5

Synthetic compounds	*EC50 (μM)*	*LC50 (μM)*
Diosgenin	*15.93*	*12.5*
Sarsasapogenin	*29.29*	*20*
Solasodine	*4.40*	*17.5*

Tab. 5. Saponin treatment reduces osteosarcoma cell viability. The half-maximal effective concentration (EC50) and the half-maximal lethal concentration (LC50) values for U-2 OS cells treated with synthetic saponins Diosgenin, Sarsasapogenin and Solasodine, calculated using Compusyn software from the dose response curves in Fig. 5A.

3.5. Agave reduces YAP and TAZ mRNA levels as a late event in OS tumorigenesis

In addition to decreasing YAP/TAZ protein abundance, we investigated whether Agave reduces *YAP* and *TAZ* mRNA expression. Treatment with Agave for 72 h resulted in a significant decrease in *YAP* (2.3-fold, $p<0.001$) and *TAZ* (1.9-fold, $p<0.001$) mRNA (Fig. 6A-B). This reduced the ability of YAP and TAZ to function as transcriptional co-activators since the expression of their target genes: *CTGF*, *ANKRD1* and *MCM7* were also significantly down-regulated (Fig. 6C-E). Interestingly, treatment with CDDP alone mildly decreased *YAP* and *TAZ* expression, as well as their transcriptional function (determined by target gene expression) by 96 h (Fig. 6A-E). This is consistent with the results showed in Figure 3C in which CDPP alone did not modulate YAP and TAZ protein levels after 72h. Importantly however, the observed effect was less than for Agave either alone or in combination with CDDP (Fig. 6A-E). *TEAD1* mRNA was slightly decreased (1.2 fold, $p<0.01$) after 72 h of Agave or CDDP treatment (Fig. 7A), suggesting non-specific regulation. *LATS1* and *LATS2* were not affected by any treatment (Fig. 7B-C).

Figure 6

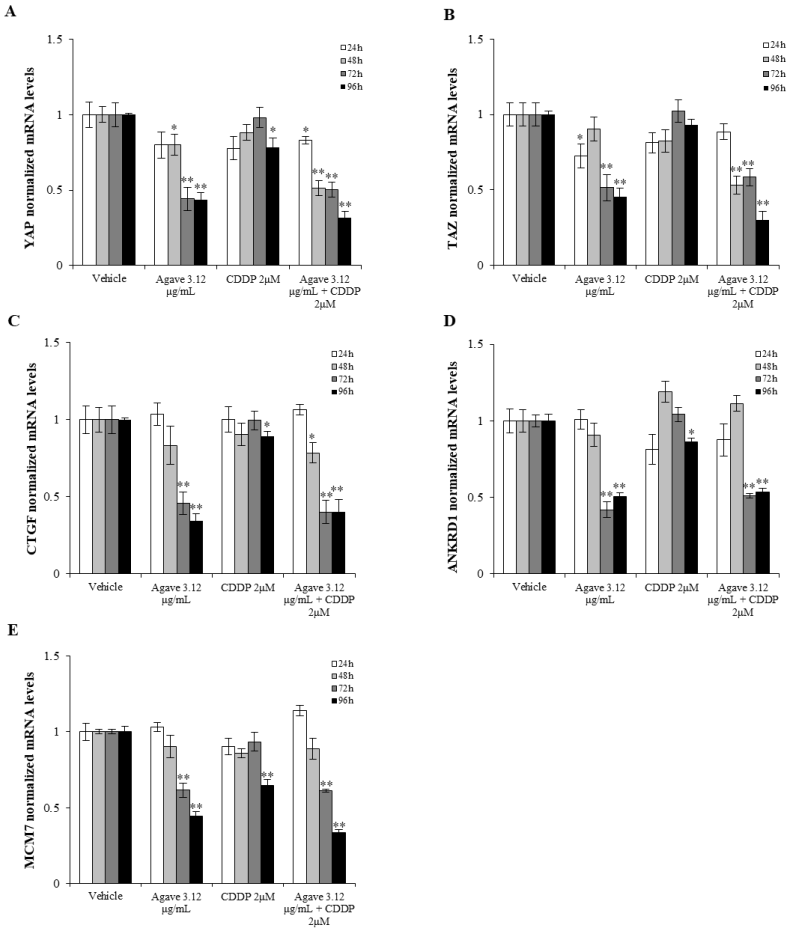

Fig. 6. Agave transcriptionally down-regulates YAP and TAZ. U-2 OS cells were treated with Vehicle (EtOH), Agave (3.12 µg/mL), and/or CDDP (2 µM) for the indicated times before being subjected to Real Time qPCR analysis. Histograms show the mean ± STDEV mRNA level of YAP (A), TAZ (B), CTGF (C), ANKRD1 (D), and MCM7 (E) normalized to GAPDH, (n=3) *p<0.01 **p<0.001.

Figure 7

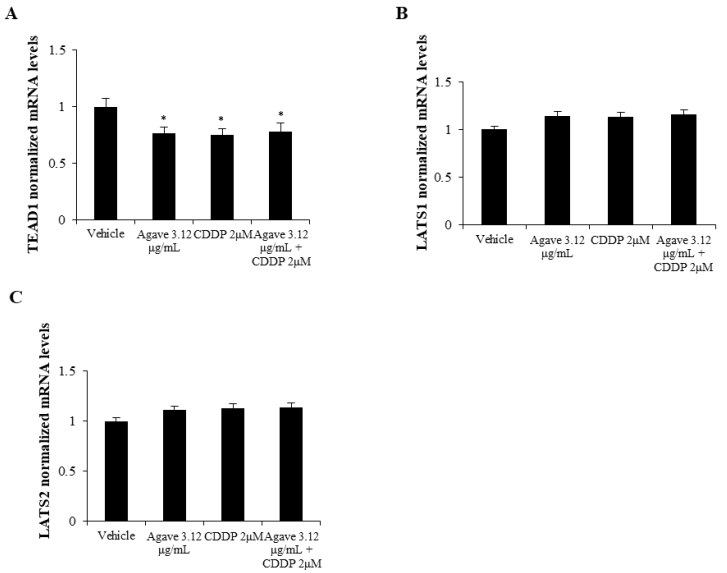

Fig. 7. Agave mildly down-regulates TEAD1 transcript and does not affect LATS1/2 expression. U-2 OS cells were treated with Vehicle (EtOH), Agave (3.12 µg/mL), and/or CDDP (2 µM) for 72 h before being subjected to Real Time qPCR analysis. Histograms show the mean ± STDEV mRNA level (n=3) for TEAD1 (A), LATS1 (B) and LATS2 (C), with cells treated with Vehicle normalized to 1, *p<0.01.

3.6. Agave down-regulates YAP and TAZ mRNA by inhibiting NF-kB transcriptional activator function

To investigate the observed transcriptional inhibition mediated by Agave, we analysed the promoter sequence of YAP and TAZ for transcription factor binding sites using LASAGNA-Search 2.0. Notably, we found multiple consensus binding sites for NF-κB p65/p50 homo- and hetero- dimers within YAP (Fig. 8A) and TAZ (Fig. 8B) promoters. This was very intriguing because, as previously described, NF-κB transcription factors play an oncogenic role in various cancers and have been shown to promote metastasis and chemoresistance in OS [73; 1; 174].

Figure 8

A

Name	Sequence	Position(0-based)	Strand	Score	p-value	E-value
NF-kappaB (p50)(M00051)	ggtgattccc	2923	+	10.9	7.5E-5	0.37
NF-kappaB (p50)(M00051)	gggaagtcc	1698	+	6.15	0.000725	3.6
NF-kappaB (p65)(M00052)	tggacttccc	1698	-	10.86	0.0001	0.50
NF-kappaB (p65)(M00052)	ggtgattccc	2923	+	6.96	0.000925	4.6

B

Name	Sequence	Position(0-based)	Strand	Score	p-value	E-value
NF-kappaB (p50)(M00051)	ggcaatttcc	2640	+	8.12	0.0002	1.00
NF-kappaB (p50)(M00051)	ggggtttcac	1170	-	6.13	0.000725	3.6
NF-kappaB (p50)(M00051)	ggggtttcac	3567	-	6.13	0.000725	3.6
NF-kappaB (p65)(M00052)	ggcaatttcc	2640	+	10.82	0.0001	0.50
NF-kappaB (p65)(M00052)	tggaatgtcc	4221	-	9.56	0.00015	0.75
NF-kappaB (p65)(M00052)	ggactttcc	2798	+	9.01	0.0002	1.00
NF-kappaB (p65)(M00052)	aggactttcc	2797	+	9.01	0.0002	1.00
NF-kappaB (p65)(M00052)	cggaaattgc	2641	-	7.86	0.000475	2.37
NF-kappaB (p65)(M00052)	gggcctttcc	1927	+	7.63	0.00065	3.2

Fig. 8. YAP and TAZ promoter analysis for NF-κB binding sites. Putative NF-κB binding sites within the YAP (A) and TAZ (B) promoters analysed using LASAGNA-Search 2.0 software. Some sequences are present on both DNA strands, and at multiple overlapping sites. We preferentially selected sites that were closer to TATA/TBP boxes that we hypothesized might be more transcriptionally active. The two YAP (A) and three TAZ (B) NF-κB putative binding sites that were subsequently used for ChIP analysis are highlighted in different colors, corresponding to those used in Fig. 9A.

To determine whether the NF-κB consensus-binding sites identified within the YAP and TAZ promoters are linked to their transcriptional regulation by Agave, we performed ChIP analysis using NF-κB p65 and p50 subunits, and p300 histone acetyltransferase as control of transcriptionally active chromatin for two (YAP) and three (TAZ) predicted sites (Fig. 9A). Using the H1H2BA sequence as a non-modulated control (Fig. 9B), we observed that treatment with Agave decreased NF-κB p65 and p50 recruitment to YAP's first putative binding site (Fig. 9C), and reduced p65 recruitment, whilst simultaneously increasing p50 binding, at YAP's second binding site (Fig. 9D). Agave treatment reduced p300 histone acetylase recruitment to both YAP binding sites (Fig. 9C-D), indicating impairment of YAP transcription. Similarly, Agave reduced NF-κB p65, p50 and p300 recruitment to all three TAZ binding sites (Fig. 9E-G). Of particular note, TAZ's first binding site was strongly regulated by NF-κB p65:p65 homo-dimers, and Agave treatment abolished this recruitment (Fig. 9E).

Figure 9

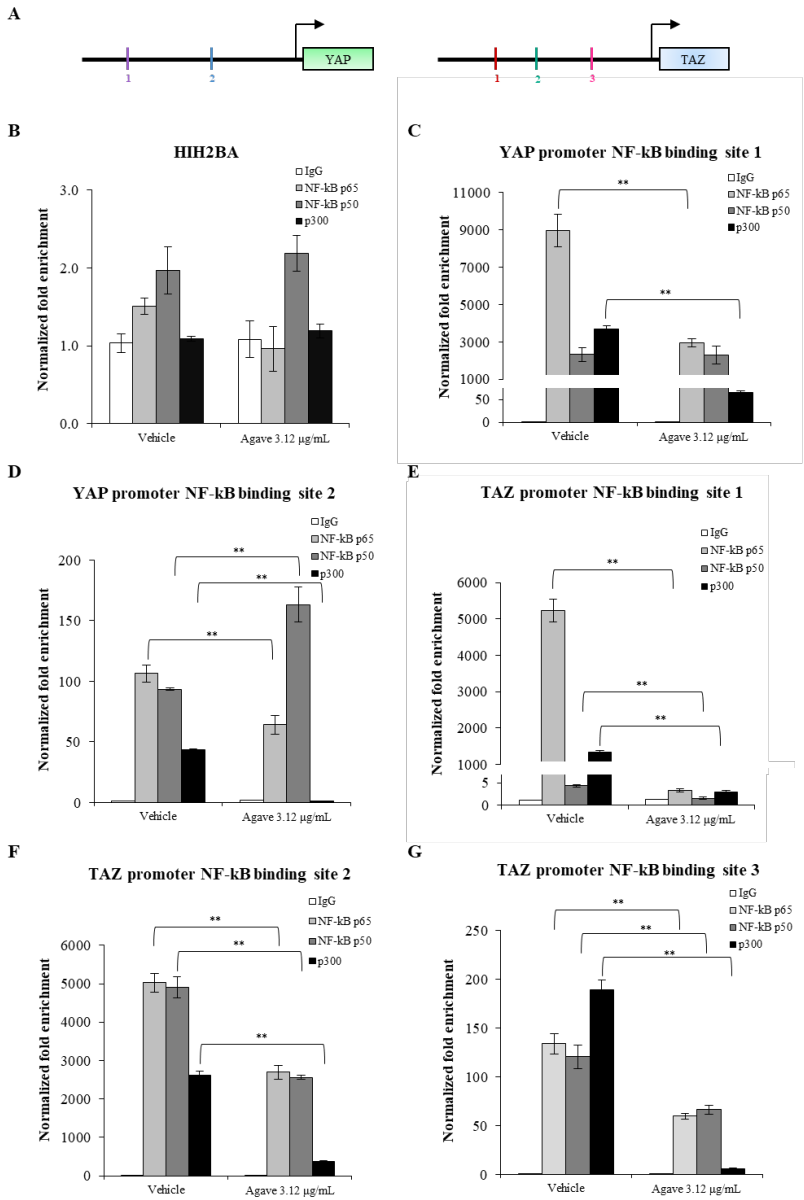

Fig. 9. Agave inhibits NF-κB recruitment onto YAP and TAZ promoters. (A) Schematic representation of the relative position of the putative NF-κB binding sites within the YAP and TAZ promoters. Different sites are highlighted in different colors, which are consistent with that used for sequences showed in Fig. 8. U-2 OS cells were treated with

Vehicle (EtOH) or Agave (3.12 μg/mL) for 72 h before being analyzed by ChIP. Samples were immunoprecipitated with antibodies against IgG, NF-κB p65, NF-κB p50 or p300 and then subjected to qPCR analysis. Histograms shows the mean fold enrichment ± STDEV (n=3) for H1H2BA (B), and the putative NF-κB binding sites within the YAP (C-D) and TAZ (E-G) promoters, **p<0.001.

Since NF-κB transcription factors bind to and regulate YAP and TAZ transcription (Fig. 9), we subsequently investigated whether Agave regulates NF-κB protein expression. Treatment of U-2 OS cells with Agave for 48 h resulted in significant accumulation of NF-κB p50, and a slight decrease of NF-κB p65 (Fig. 10A). Treatment of Saos-2, MG-63 and HOS cell lines showed a consistent pattern of p65 and p50 expression after 72 h (Fig. 10B). As described previously, NF-κB functions as dimers to either activate (p65:p65 and p65:p50) or repress (p50:p50) gene transcription [103]. In addition to total abundance, the relative ratio of different subunits determines the outcome of NF-κB signalling [103]. Quantitation of p65/p50 protein ratios revealed that Agave treatment strongly favours the accumulation of NF-κB inhibitory subunit p50 in all tested cell lines (Fig. 10C).

NF-κB dimers need to be localised to the nucleus to exert their transcriptional effect. We thus investigated the sub-cellular localization of NF-κB subunits following Agave treatment using nuclear/cytoplasmic fractionation. Agave treatment increased nuclear accumulation of p105 (p50 precursor) and p50, and simultaneously reduced p65 localization to the nucleus (Fig. 10D-E). These data are consistent with immunofluorescent staining for NF-κB p105/p50 in the absence and presence of IL-6 (to activate NF-κB signalling) (Fig. 10F-G), and NF-κB p65 (Fig. 10H-I), following treatment with Agave. Finally, consistent with the altered expression and localization of NF-κB subunits induced by Agave, the expression of various NF-κB target genes was significantly reduced (Fig. 10J).

Figure 10

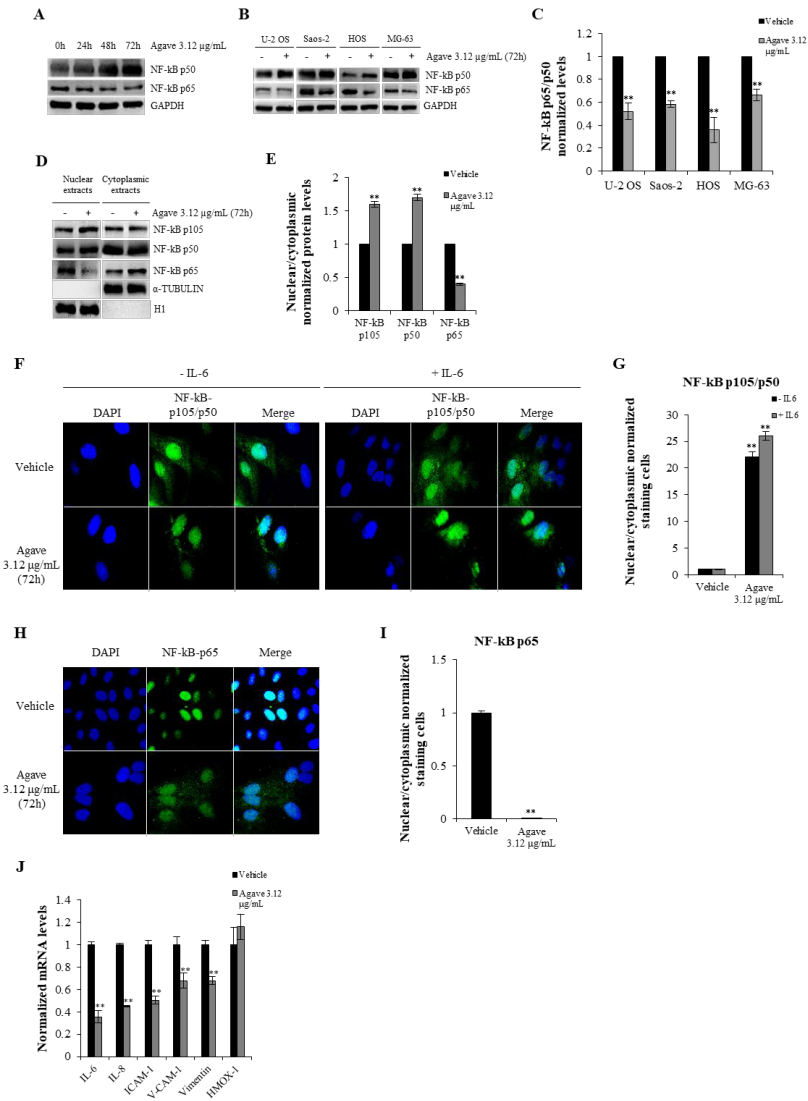

Fig. 10. Agave down-regulates NF-κB transcriptional function. (A) U-2 OS cells were treated with Agave (3.12 µg/mL) for 24, 48 or 72 h, or EtOH as Vehicle control (0 h), then subjected to Western Blot analysis as indicated. GAPDH was used as a loading control. (B) U-2 OS, Saos-2, HOS and MG-63 cells were treated with Agave (3.12 µg/mL) or Vehicle for 72 h before being subjected to Western Blot analysis. The abundance of NF-κB p65 and NF-κB p50 was quantified using Alliance (UVITEC) Software and the NF-κB p65/p50 ratio was determined. The relative ratios are shown in (C). Values represent mean ± STDEV (n=3), with U-2 OS cells treated with Vehicle normalized to 1, **p<0.001. (D) U-2 OS cells were treated with Vehicle or Agave (3.12 µg/mL) for 72 h before being

subjected to nuclear/cytoplasmic fractionation followed by Western Blot analysis. H1 (nuclear) and α-Tubulin (cytoplasmic) were used as loading controls. The nuclear/cytoplasmic ratios for NF-κB p105, p50 and p65 were determined (E). Values represent mean ± STDEV (n=3), with U-2 OS cells treated with Vehicle normalized to 1, **p<0.001. (F, H) U-2 OS cells were treated with Vehicle or Agave (3.12 μg/mL) either in the absence or presence of IL-6 (100 ng/mL) for 72 h. Cells were then stained for NF-κB p105/p50 (F), or p65 (H) with nuclei marked by DAPI staining. Nuclear and cytoplasmic signal was quantified using ImageJ software, and the relative mean ± STDEV for NF-κB p105/p50 (G) and NF-κB p65 (I) is shown, with cells treated with Vehicle normalized to 1, n=3 **p<0.001. (J) U-2 OS cells were treated with Vehicle or Agave (3.12 μg/mL) for 72 h before being subjected to Real Time qPCR analysis. The mean mRNA level ± STDEV (n=3) for each target gene is shown, with cells treated with Vehicle normalized to 1, **p<0.001.

4. Discussion

Agave natural extract is known to exert prebiotic and immunomodulatory effects, as well as putative anticancer activity in colorectal and cervical cancer cells [7; 109; 61]. Though the mechanism/s of Agave's action remain poorly understood, Agave fructans have been shown to contribute to increased fermentation and increased bifidobacterium populations, with decreased ammonia and inflammation in colon cancer cells [7]. Here, we tested the anti-tumorigenic potential of Agave in OS cell lines, demonstrating that it can reduce cell viability, impair migration and colony formation, and can sensitize cells to CDDP. Investigation of the molecular mechanism of action revealed that YAP and TAZ are down-regulated by Agave treatment. This could explain Agave's anticancer effects since direct silencing of YAP/TAZ in OS lines reduced cell survival, colony formation and migration capability. Importantly, Agave treatment induced an apoptotic response, even potentiating CDDP cytotoxicity, identifying Agave as a promising anticancer compound for OS. Agave-induced apoptosis appears to be p53-independent since various cell lines analysed harbour wild-type (U-2 OS, A549, MSTO-211H, MPP-89), depleted (Saos-2, MG-63, H1299) or mutant p53 (HOS, MDA-MB-231, SUM-159PT). This is consistent with a previous publication that identified a p53-independent induction of apoptosis by Agave in cervical cancer cells [109]. In this study, Agave increased Bax and down-regulated Bcl-2 protein levels, with no change in p53 abundance [109].

Our findings support that of multiple reports identifying an oncogenic role for YAP and TAZ in OS [44; 163]. YAP/TAZ are frequently overexpressed in human OS samples, correlating with target gene expression, OS staging and overall patient survival [44; 20]. Further, YAP suppression impairs OS cell proliferation and migration *in vitro*, and reduces tumor growth *in vivo* [44]. Mechanistically, up-regulation of YAP in osteo-tissues is mediated by Sox2 inhibition of NF2 and Kibra, which are activators of the Hippo pathway [142], and by activation of Shh signalling via Hippo pathway crosstalk [44]. In addition, RASSFs, NF2 and Mob1 tumor suppressor proteins are frequently mutated in OS, resulting in increased YAP and TAZ activation [44]. Importantly, there is good evidence for the involvement of YAP/TAZ in inducing chemo-, radio- and molecular targeted therapy-resistant OS [163; 76].

Agave extract is a complex blend of compounds amongst which saponins stand out as bioactive components with multiple functions. Unlike Agave, which is poorly studied, saponins have been widely investigated, with numerous reports citing their antimicrobial [83], pro-apoptotic [32], immunomodulatory [159], neuroprotective [148], anti-proliferative [145] and anti-migratory properties in various cancer cell lines [96; 80; 139]. At high doses (>100 μM), saponins exert a cytotoxic effect by disrupting cell membranes. At low doses however, they induce apoptosis by multiple mechanisms including activation of TNF-R and FAS-L receptors, cleavage of caspase 3 and 9, and induction of p53 by inhibiting Mdm2 [40; 118; 155; 146]. They also block cell proliferation via down-regulation of Cdk2/Cyc D1, Cdk4/Cyc E and Cyc B complexes [104; 170], impair migration and EMT [77], and regulate various signalling pathways including PI3K/Akt, MAPK and NF-κB [105; 36; 172; 35; 156]. Consistent with saponins' tumor suppressive properties, we demonstrate that synthetic saponins reduce cell viability, and down-regulate YAP/TAZ abundance, although to a lesser extent than Agave itself. This is consistent with previous reports demonstrating that the anti-inflammatory properties of Agave extract, mediated by NF-κB modulation, could be partially recapitulated by saponins [4; 81; 169]. In detail, saponins reduce NF-κB activity and its binding to DNA either by preventing IκBs degradation [4; 81], or increasing sirtuin 1 (SIRT1) levels, resulting in reduced NF-κB p65 acetylation and subsequent transcriptional inhibition [169]. It is possible that this is the mechanism of Agave's action on NF-κB in this

study. However, due to the complexity of Agave extract, it is likely that a combination of active components could activate/inhibit multiple signalling pathways simultaneously, to produce a greater overall effect.

From our study, we propose that Agave initially induces YAP and TAZ protein destabilization by promoting ubiquitin-dependent proteasome degradation. As a secondary mechanism, Agave impairs YAP and TAZ transcription by regulating NF-κB expression, and consequently NF-κB subunit recruitment to YAP and TAZ promoter sequences. Indeed, we suggest for the first time that NF-κB subunits bind directly to YAP/TAZ promoters to activate their transcription.

We considered that Agave's modulation of NF-κB could directly regulate apoptosis and chemosensitivity, bypassing YAP/TAZ transcriptional regulation [1; 103]. Saponins cooperate with CDDP to induce reactive oxygen species production, resulting in apoptosis [77]. Chemical inhibitors of NF-κB (parthenolide and BRM270) sensitize OS to chemo- and radio-therapies, and activate apoptosis [25; 194; 106]. Moreover, NF-κB inhibition can sensitize cells to chemotherapeutics via up-regulation of IL-6, an activator of NF-κB signalling [25].

Data presented here, indicate that YAP/TAZ as well as NF-κB are mediators of Agave-induced anti-proliferative, anti-migratory and pro-apoptotic effects in OS cell lines, and possibly in OS of animals and human patients. OS could therefore potentially be targeted by drugs that have recently emerged as inhibitors of the critical YAP/TAZ-TEAD complexes, which drive transcription of proliferative and anti-apoptotic genes. Among YAP/TAZ inhibitors is verteporfin, a photosensitizer that is used clinically to treat macular degeneration that was shown to inhibit YAP-induced overgrowth of liver in animal models [85]. Another potent YAP-TEAD inhibitor is a peptide mimic of VGLL 4 [74]. Translocation of YAP/TAZ to the nucleus was shown to be blocked by two FDA-approved tyrosine kinase inhibitors, dasatinib and pazopanib [119], and the egress of YAP from the nucleus was facilitated by dobutamine, sympathomimetic drug used in the treatment of heart failure [12].

Based on publically presented talks and conference posters over the past two years, in addition to on-going drug discovery programs at

several biotech companies including Inventiva Pharma, Genentech and Novartis, it is anticipated that new therapeutic compounds that inhibit YAP/TAZ-TEAD will emerge soon. Such new drugs for YAP/TAZ could be complemented with selected saponins of Agave to better control and manage OS. Agave/saponin-mediated NF-κB inactivation could be of interest for the treatment of other tumours in which NF-κB is known to play an oncogenic role, including myeloid malignancies [37], breast [193], lung [171], liver [160] and ovarian [10] cancers. Similarly, for tumours in which YAP/TAZ play critical oncogenic roles [64; 185]. Future experiments should test the effectiveness of Agave using *in vivo* cancer models, and focus on delineating the mechanism/s of NF-κB inactivation and YAP/TAZ protein degradation, and identification of additional chemical components of Agave that contribute to its anticancer effectiveness.

5. Conclusions

Our results show that Agave induces YAP/TAZ protein degradation as early event, and subsequently impairs YAP and TAZ transcription by inactivating NF-κB (Fig. 1). We propose that Agave-mediated YAP/TAZ down-regulation and NF-κB inactivation results in a pro-apoptotic, anti-migratory phenotype with enhanced chemosensitivity to CDDP. Natural compounds offer advantages over traditional chemotherapies as they typically exhibit low toxicity, are associated with low production costs, and offer the potential for multi-faceted mechanisms of action. We advocate that Agave natural extract is a promising agent to treat OS, either independently, or as an adjuvant to reduce the intrinsic toxic side effects of chemotherapeutics.

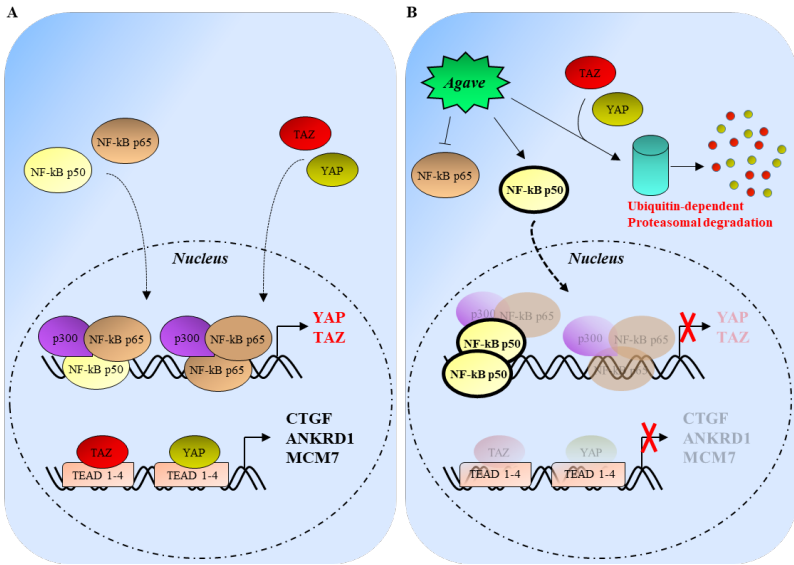

Fig. 1. . Proposed model of Agave's anticancer activity in osteosarcoma. (A) NF-κB p65:p50 and/or NF-κB p65:p65 dimers bind to YAP and TAZ promoters inducing p300 recruitment and transcriptional activation. YAP and TAZ activated proteins, translocate into the nucleus to activate transcription of oncogenic targets genes *CTGF*, *ANKRD1* and *MCM7*. (B) Agave treatment promotes YAP/TAZ ubiquitin-dependent proteasomal degradation, reducing their nuclear translocation. Moreover, Agave down-regulates NF-κB p65 and promotes its cytoplasmic sequestration, whereas NF-κB p50 is up-regulated and enriched in the nuclear compartment. YAP and TAZ transcription is therefore strongly diminished, as it is for their downstream targets.

6. Matherials and Methods

6.1. Cell culture and reagents

Cell lines were obtained from the ATCC (Manassas, US) and cultured either in DMEM (U-2 OS, Saos-2, HOS, MG-63, H1299, A549 and MDA-MB-231 cell lines) or DMEM-F12 (SUM-159-PT, MSTO-211H and MPP-89 cell lines) (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Life Technologies), 100 Units/mL Penicillin and 100 μg/mL Streptomycin (Life Technologies). Cell lines were grown at 37°C in 5% CO_2. DPBS (EuroClone, Milano, Italy) and Trypsin 0.05% (GE Healthcare Hyclone, Little Chalfont, UK) were used to wash and detach cells. Agave total extract was provided in powder form the Italian Aboca Society and resuspended to generate a stock solution at 50 mg/mL in absolute EtOH. Agave extract was obtained from frozen leaf samples of *A. sisalana* plant and were extracted (DER from 5 to 6:1) by hydro-alcoholic procedure (ETOH 50%) and complete characterized as previously described in Pulito et al. [127]. Saponins Diosgenin (Cat. D1634), Sarsasapogenin (Cat. S8534) and Solasodine (Cat. SML1141) were purchased from Sigma (Saint Louis, Missouri, US) and resuspended in absolute EtOH at 10 mM. Cisplatin (CDDP) dissolved in saline solution was provided by the Pharmacy of the "Regina Elena National Cancer Institute" in Rome. DAPI (4', 6-diamidino-2- phenylindole, dihydrochloride, Sigma Cat. 32670) was used to stain cell nuclei. Protease Inhibitor Cocktail (CI) (Cat. P8340), MG-132 (Z-Leu-Leu-Leu-al, Cat. C2211), Cycloheximide (CHX) (Cat. 01810) and

IL-6 (Cat. I1395) were purchased from Sigma. CHX was dissolved in DMSO solution (Cat. 907201418, Carlo Erba, Cornaredo, Milan, Italy).

Osteosarcoma (OS) is the most frequent primary tumor affecting bone, and typically originates in the extremities of the long bones in the legs e.g., femur or tibia, or in arm bones such as the humerus. Less frequently, it develops in the hip bones, shoulders or jaw, and is always associated with increasing pain [135; 5]. OS shows a bimodal age occurrence, arising frequently in children and teens aged 10-16 years and in older adults usually over 40. OS originates from mesenchymal stem/stromal cells (MSCs) or from the derived osteogenic lineage represented by the osteoblast cells 112 (Fig. 1).

6.2. Cell transfection

Transfections were performed using Lipofectamine 2000 (for plasmids) and Lipofectamine RNAiMax (for siRNA) (Life Technologies) according to the manufacturer's recommendations.

siRNAs were purchased from Eurofins MWG (Ebersberg, Germany): siGFP 5′-AAGUUCAGCGUGUCCGGGGAG(dTdT)-3′ [47], siYAP 5′-GACAUCUUCUGGUCAGAGA(dTdT)-3′ [53], siTAZ is a pool of two independent siRNAs mixed in equal amount: siTAZ_1 5′-AAAGUUCCUAAGUCAACGU(dTdT)-3′ and siTAZ_2 5′-UGAUUGAGGAAGUACCUCU(dTdT)-3′ [46]. To exclude unspecific effects, we previously performed YAP and TAZ silencing with two different siRNAs. Representative data are showed in figures. Plasmids utilized were: pCDNA3-YAP-Flag [60], pCS2-TAZ-Flag [11], and pCS2-Ub-HA generated by fusing an epitope of HA with the entire open reading frame of ubiquitin [111].

6.3. Viability assays

Cells (800 per well) were seeded into 96-well plates in 200 μl of media. After 24 h, cells were treated for the indicated times. Where indicated, gene silencing was performed by transfecting cells in suspension immediately prior to plating for experiments. Cell viability assays were performed using the ATPlite assay (Perkin Elmer, Massachusetts, USA) according to the manufacturer's instructions. Plates were evaluated using an EnSpire Technology microplate reader (Perkin Elmer).

Each condition was assayed in triplicate.

6.4. Clonogenic assay

Cells (1000) were plated into 6-well dishes (Corning-Costar, Tewksbury, MA, USA) and treated as indicated. Fresh media (25% of the total volume) with treatments are added every three days. After 15–21 days, cells were stained with crystal violet and colonies counted using OpenCFU Free Open-Source Software [59]. Each condition was assayed in triplicate.

6.5. Wound healing migration assay

Cells were grown to 80% confluence in 6-well tissue culture plates and scratched (wounded) with a sterile 10 μL pipet tip (t=0), before the relevant treatment was applied. Where indicated, gene silencing was performed by transfecting cells in suspension immediately prior to plating for experiments. The progression of migration was photographed at time points t=0 and t=24 h using a light microscope. The wound opening was measured and data normalized to t=0 points. Samples were assayed in triplicate.

6.6. Transwell migration assay

Migration assays were performed using 24-well Boyden chambers with a non-coated 8 μm pore size filter in the insert chamber (BD Falcon, Franklin Lakes, NJ, USA). $5x10^4$ cells, in a volume of 100 μl DMEM containing 0.1% FBS, were seeded into the upper chamber while the bottom chamber was filled with 500 μl of DMEM supplemented with 10% FBS. Either Agave or Vehicle was added to the upper chamber. Chambers were incubated for 24 h before any cells remaining in the upper chamber were removed with a cotton pad, and cells on the bottom layer of the upper chamber were subjected to DAPI staining. In detail, the upper chamber was washed twice with PBS and then cells were fixed with 4% formaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 25 min and, after being washed with PBS, stained with

5 μM DAPI for 5 min. Membranes were cut and mounted on microscopy slides. All cells on each membrane were counted under a Zeiss LSM 510 laser scanning fluorescence confocal microscope (Zeiss, Wetzlar, Germany). Each sample was assayed in triplicate.

6.7. Cell extracts, Immunoprecipitation and Western blotting

Cell lysis was performed on ice for 30 min in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 10 mM $MgCl_2$, 5 mM KCl) supplemented with protease and phosphatase inhibitors (1 mM CI, 5 mM PMSF, 1 mM DTT, 1 mM Na_2VO_4). Cell lysates were sonicated and protein concentration determined by colorimetric assay (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were resolved by 10% denaturing SDS polyacrylamide gel electrophoresis, and transferred for 2 h onto polyvinylidene difluoride membrane (PVDF, Immobilon-P, Merck-Millipore, Darmstadt, Germany). Membranes were blocked with 5% milk-TBS 0.05% Tween-20 for 3 h and incubated overnight with specific primary antibodies. Secondary anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad. Chemiluminescent images were captured using the Alliance 4.7 by UVITEC Cambridge (Eppendorf) detector. For immunoprecipitation (IP) assay, we used 1 μg of antibody or relative IgG control (Santa Cruz Biotechnology) for 1 mg of cell lysate. Lysates were incubated on a rotor at 4°C for 3 h. IPs were washed three times in wash buffer before elution with 50 μl of SDS sample buffer. Then, 25 μl (50% of the total IP, 500 μg of total cell extract) was loaded onto gels in duplicate. Western Blotting was performed using the following primary antibodies: mouse monoclonal anti-GAPDH (sc-47724, Santa Cruz Biotechnology), rabbit polyclonal anti-Lats1 (Cat. 9153, Cell Signaling Technology), mouse monoclonal anti-YAP1 (ab56701, Abcam), rabbit polyclonal anti-TAZ (anti-WWTR1, Cat. HPA007415, Sigma), mouse monoclonal anti-TEAD1 (anti-TEF-1 Cat. 610923, BD-Transduction Laboratories,), rabbit monoclonal anti-NF-κB p65 (Cat. D14E12, Cell Signaling Technology), rabbit polyclonal anti-NF-κB p105/p50 (H-119 sc-7178, Santa Cruz Biotechnology), mouse monoclonal anti-HA (anti-HA-probe F-7 sc-7392, Santa Cruz Biotechnology), rabbit polyclonal anti-Bax (N-20 sc-493, Santa Cruz Biotechnology), mouse monoclonal

anti-caspase 3 (Cat. 31A1067, Enzo Life Science), rabbit polyclonal anti-PARP (Cat. 9542, Cell Signaling Technology). ECL reagent (Amersham, GE Healthcare, Piscataway, NJ, USA) was used for chemo-luminescence detection. All Western Blot analyses were performed at least in triplicate with the most representative blots shown.

6.8. RNA extraction, Reverse transcription and Quantitative Real-Time PCR

RNA was extracted with Trizol (Life Technologies) following the manufacturer's instructions and quantified using a Nanodrop (Thermo Scientific). RNA (500 ng) was reverse-transcribed with M-MLV reverse transcriptase (Life Technologies) following the manufacturer's instructions and 1/10 of the resultant cDNA was subjected to Real-Time PCR using FAST SYBR Green master mix (Applied Biosystems, Carlsbad, CA, USA) and the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Data were analyzed using the relative standard curve method and normalized to GAPDH. The following primers were used:

GAPDH_Fw 5'-GAGTCAACGGATTTGGTCGT-3';
GAPDH_Rv 5'-GACAAGCTTCCCGTTCTCAG-3';
YAP_Fw 5'-TACACCCACAGCTCAGCATC-3';
YAP_Rv 5'-GCCATGTTGTTGTCTGATCG-3';
TAZ_Fw 5'-GGAGAGAGAAAGGATTCGAATGC-3';
TAZ_Rv 5'-TGTCGACAGAGGGCAGCTT-3';
TEAD1_Fw 5'-CCACCAAAGTTTGCTCCTTTGGGA-3';
TEAD1_Rv 5'-ACTTCAAACACACAGGCCATGCAG-3';
CTGF_Fw 5'-GCCACAAGCTGTCCAGTCTAATCG-3';
CTGF_Rv 5'-TGCATTCTCCAGCCATCAAGAGAC-3';
ANKRD1_Fw 5'-AGTAGAGGAACTGGTCACTGG-3';
ANKRD1_Rv 5'-TGGGCTAGAAGTGTCTTCAGAT-3';
MCM7_Fw 5'-CAGAACTCGGATTAGGGCTG-3';
MCM7_Rv 5'-GCTTGGGAAGTGAGTCAAAACT-3';
LATS1_Fw 5'-CTCTGCACTGGCTTCAGATG-3';
LATS1_Rv 5'-TCCGCTCTAATGGCTTCAGT-3';
LATS2_Fw 5'-ACATTCACTGGTGGGGACTC-3';
LATS2_Rv 5'-GTGGGAGTAGGTGCCAAAAA-3';

IL-6_Fw 5′-CTGCAGAAAAAGGCAAAGAATCTAG-3′;
IL-6_Rv 5′-CGTCAGCAGGCTGGCATT-3′;
IL-8_Fw 5′-CTGGCCGTGGCTCTCTTG-3′;
IL-8_Rv 5′-GCAAAACTGCACCTTCACACA-3′;
ICAM1_Fw 5′-AGACAGTGACCATCTACAGCTTTCC-3′;
ICAM1_Rv 5′-CACCTCGGTCCCTTCTGAGA-3′;
V-CAM1_Fw 5′-CATGACCTGTTCCAGCGAGG-3′;
V-CAM1_Rv 5′-CATTCACGAGGCCACCACTC-3′;
Vimentin_Fw 5′-AAATCCAAGTTTGCTGACCTCTCT-3′;
Vimentin_Rv 5′-CTCAGTGGACTCCTGCTTTGC-3′;
HMOX-1_Fw 5′-CAGAGGGTGATAGAAGAGGCC-3′;
HMOX-1_Rv 5′-GGCTCTGGTCCTTGGTGT-3′;
H1H2BA_Fw 5′-ACTCTCCTTACGGGTCCTCTTG-3′;
H1H2BA_Rv 5′-AGTGCTGTGTAACCCTGGAAAA-3′;
ChIP_NF-κB_YAPprom1_Fw 5′-TCTTAGTCTGTTTGTGCTGCT-3′;
ChIP_NF-κB_YAPprom1_Rv 5′-CTGCCTTGATCTTGGACTTCC-3′;
ChIP_NF-κB_YAPprom2_Fw 5′-GGGACTACAGAACATGCCA-3′;
ChIP_NF-κB_YAPprom2_Rv 5′-CCAAGGCAGGGGAATCAC-3′;
ChIP_NF-κB_TAZprom1_Fw 5′-TCAGGATTTGAGACCAGCCT-3′;
ChIP_NF-κB_TAZprom1_Rv 5′-TAGGATTAAGACGCCTGCCA-3′
ChIP_NF-κB_TAZprom2_Fw 5′-TGTGTTAAGGGCAATTTCCGT-3′;
ChIP_NF-κB_TAZprom2_Rv 5′-GTTGGAACTGGATGGGCAAG-3′;
ChIP_NF-κB_TAZprom3_Fw 5′-CTGGGCAGCTGGACTTTTC-3′;
ChIP_NF-κB_TAZprom3_Rv 5′-GGAGACCTGAGCCACTAAGT-3′.

6.9. Protein stability assay

Cells were treated with Vehicle or Agave as indicated and simultaneously treated with CHX 40 μM for the indicated times. Cells were subsequently lysed and subjected to Western Blotting as previously described. Quantification and normalization were performed using Alliance (UVITEC) Software.

6.10. Ubiquitination assay

$1.6x10^6$ cells were transfected with 5 μg of the specified plasmids as previously described and simultaneously treated with Vehicle or Agave as indicated. After 18 h, cells were treated with 25 μM MG-132 for a further 6 h. Protein extracts were immunoprecipitated as described and subjected to Western Blotting. We used mouse monoclonal anti-FLAG (F1804 clone M2, Sigma) primary antibody for the IP, and rabbit polyclonal anti-YAP (H-125 sc-15407, Santa Cruz Biotechnology) and rabbit polyclonal anti-TAZ (anti-WWTR1 HPA007415, Sigma) primary antibodies for Western Blot detection.

6.11. Nuclear/Cytoplasmic protein extraction

Harvested and washed cells were lysed in Buffer A (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA pH 8, 1 mM DDT, 0.5% NP-40, 1 mM CI) and centrifuged at 9800 RCF, for 30 min at 4°C. Cytoplasmic extracts (supernatant) were transferred to a fresh tube and nuclear pellets washed twice in Buffer A before being lysed in Buffer B (20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA pH 8, 1 mM DDT, 1 mM CI). Lysates were then centrifuged at 19000 RCF for 10 min at 4°C and supernatants recovered. Nuclear and cytoplasmic extracts were processed as previously described for Western Blot analysis. For nuclear and cytoplasmic protein normalization, we used mouse monoclonal anti-αTubulin (B-7 sc-5286, Santa Cruz Biotechnology) and goat polyclonal anti-Histone H1 (N-16 sc-34464, Santa Cruz Biotechnology). Quantification and normalization were performed using Alliance (UVITEC) Software.

6.12. Immunofluorescent staining

Cells (800 per well) were seeded into 8-well culture slides (Cat. 354108, Corning BD Falcon) and treated as indicated. Cells were then rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min at room temperature before being permeabilized with 0.5% Triton X-100 for 25 min. Lysates were incubated overnight with the indicated antibodies before being washed three times with cold PBS for 3 min each, stained for 2 h with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific), and counterstained with 5 μM DAPI for 5 min. Cells were examined under a Zeiss LSM 510 laser scanning fluorescence confocal microscope. We used rabbit monoclonal anti-NF-κB p65 (Cat. D14E12, Cell Signaling Technology) and rabbit polyclonal anti-NF-κB p105/p50 (H-119 sc-7178, Santa Cruz Biotechnology) primary antibodies. IL-6 treatments were performed using 100 ng/mL for 30 min before recovering cells. Each sample was assayed in triplicate.

6.13. Chromatin Immunoprecipitation assay (ChIP)

Cells were cross-linked using 1% formaldehyde for 10 min at room temperature before the reaction was stopped by the addition of 0.125 M Glycine, and washed three times with ice-cold PBS. Cells were lysed in Buffer A (5 mM Pipes pH 8, 85 mM KCl, 0.5% NP-40, 1 mM CI), centrifuged at 400 RCF for 10 min at 4°C, resuspended in Buffer B (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) and sonicated to shear DNA to lengths of approximately 200 bp. The chromatin solution was immunoprecipitated with rabbit polyclonal anti-NF-κB p65 (C-20 sc-372, Santa Cruz Biotechnology), rabbit polyclonal anti-NF-κB p105/p50 (H-119 sc-7178, Santa Cruz Biotechnology), rabbit polyclonal anti-p300 (N-15 sc-584, Santa Cruz Biotechnology), or with IgG (Santa Cruz Biotechnology) as negative control. IPs were performed using Pierce ChIP-grade Protein A/G magnetic beads (Thermo Fisher Scientific, Rockford, IL, USA). The immunoprecipitated and purified chromatin was subjected to quantitative PCR analysis (qPCR). Primer sequences for qPCR analysis are listed above in 'RNA extraction, Reverse transcription and Quantitative Real-Time PCR'. Data were normalized

to the amount of Input chromatin and these data were further normalized by subtracting the qPCR signal of a negative sequence control that was not modulated (H1H2BA).

6.14. Promoter analysis

FASTA sequences of human YAP1 and WWTR1 (TAZ) promoters (4000 bp upstream of the TSS) were downloaded from UCSC Genome Browser on-line database and pasted into LASAGNA-Search 2.0 to identify predicted transcription factor binding sites. TRANSFAC matrices were used for the analysis.

6.15. Statistical analyses

All experiments were performed at least three independent times. No samples were excluded from the analysis. All p-values were determined using two-tailed t-tests and statistical significance was set at $*p<0.01$, $**p<0.001$.

7. Appendix

(list of pubblications)

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Osteosarcoma (OS) is the most aggressive type of primary solid tumor that develops in bone. Whilst conventional chemotherapy can improve survival rates, the outcome for patients with metastatic or recurrent OS remains poor, so novel treatment agents and strategies are required. Previously published works indicate that Agave exhibits anticancer properties. In the present study, anticancer effects of Agave leaf extract were investigated in the OS cells, discovering that Agave inhibits cell growth and cell migration, and sensitizes OS cells to cisplatin (CDDP), to overcome chemoresistance. Agave's mechanisms of action include an initial process in which Agave induces YAP/TAZ oncogenic protein degradation, followed by a secondary event whereby Agave inhibits YAP/TAZ transcription through NF-κB p65:p50 heterodimers deregulation.

Maria Ferraiuolo is a biologist experienced in Cancer Research, Viral Vector Production applied to vaccines development or gene therapy applications, and Clinical Research. She obtained her Master's degree in 2014 and PhD degree in 2018. From 2012 to 2018 she worked at Regina Elena National Cancer Institute (IRE-IFO, Rome) in cancer research field, and in 2018 she moved to pharmaceutical environment becoming part of ReiThera's team (Rome) until 2021, with the role of Senior process development scientist. Moved by an increasing interest in Clinical Research, she joined the CRO PRA Health Sciences' team (Milan) acting as Site Activation Specialist since 2021.

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