A novel mechanism of XIAP degradation induced by timosaponin AIII in hepatocellular carcinoma

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Abstract

Inducing tumor cell death is one of the major therapeutic strategies in treating cancer. The aim of this study is to investigate the mechanism underlying the involvement of autophagy in cell death induced by timosaponin AIII (TAIII). Cell viability was determined by MTT and cologenic assay; apoptosis was determined by flow cytometry and TUNEL assay; autophagy was examined by immunoblotting and immunofluorescence; ubiquitination was detected by co-immunoprecipitation; mRNA expression was detected by real-time PCR; and determination of necrotic cell death was approached with LDH assay. The in vivo tumor growth inhibition was determined by xenograft model. TAIII exhibits potent cytotoxicity on human hepatocellular carcinoma (HCC) cells without severe hepatic toxicity. TAIII induced caspase-dependent apoptosis in HCC, and the induction of apoptosis was attributed to the inhibition of TAIII on XIAP expression. Repressing XIAP expression allowed cell tolerance toward the treatment with TAIII. The suppression of XIAP by TAIII is under post-transcriptional control and independent of proteasomal-driven proteolysis. Instead, TAIII-induced AMPKα/mTOR-dependent autophagy was responsible for XIAP suppression and triggered the XIAP heading lysosomal degradation pathway. Ubiquitination of IAPs is required for the autophagic degradation induced by TAIII. Blockade of autophagy turns on the switch of necrotic cell death in TAIII-treated cells. Timosaponin AIII induces HCC cell apoptosis through a p53-independent mechanism involving XIAP degradation through autophagy–lysosomal pathway. The possibility of developing TAIII as a new anti-tumor agent is worth considering.

1. Introduction

Successful exploration of paclitaxel, camptothecin and vincristine for chemotherapy has revealed the potential of discovering novel chemotherapeutics from medical plants to combat human cancers. The Chinese medicinal herb Anemarrhena asphodeloides Bunge (Fig. 1A) has a long tradition of use in China to restrict tumor in cancer patients, while a saponin component timosaponin AIII (TAIII) was identified to be an active component. Previous studies of others and us revealed the potential of TAIII as a potent anti-tumor agent in various human cancers [1–3], while mechanisms mediating the anti-tumor effect of TAIII remain unclear.

The inhibitors of apoptosis (IAPs) family members are well-known endogenous inhibitors of caspase activities in HCC [4]. Overexpression of XIAP proteins was observed in HCC tissue allowing HCC cells to escape from apoptotic cell death [5]. In HCC patients, overexpression of XIAP, the most identified IAP protein, was correlated with the increased risk of relapse [6]. These studies define XIAP as an attractive target for HCC therapy. Efforts on developing XIAP antagonists were made in the direction of designing small molecules to mimic the N-terminal end of active second mitochondria-derived activator of caspase (Smac) protein, a mitochondrial cytochrome C-dependent protein that antagonizes XIAP activity and induces caspase activation [7]. The Smac and Smac-like proteins bind to the IAPs and facilitate the auto-ubiquitination of IAPs, which subsequently undergo rapid proteasomal degradation [8]. Recent studies reveal that many ubiquitinated molecules are degraded through selective autophagy–lysosomal pathways [9], however, whether the strictly regulated XIAP could be engulfed by lysosome remains unclear.

In this study, we reported that autophagy-inductive TAIII exhibits potent inhibitory effect on human hepatocellular carcinoma via inducing caspase-dependent apoptosis to HCC cells. Less cytotoxicity of TAIII was found in non-malignant cells. Initiation of apoptotic cell death by TAIII depends on XIAP inhibition in HCC cells. Less cytotoxicity of TAIII was found in non-malignant cells. Initiation of apoptotic cell death by TAIII depends on XIAP inhibition in HCC cells. TAIII could activate AMPKα signaling to suppress the mTOR pathway, which confers its induction of autophagy. Induction of autophagy may be required for XIAP suppression in TAIII-treated cells, which is demonstrated by the...
observation that autophagosome–lysosome fusion inhibitor could attenuate the inhibition of XIAP expression in HCC cells exposed to TAIII. However, this was not observed when the TAII-treated cells were exposed to the proteasome inhibitor. Ubiquitination of XIAP protein is necessary for the degradation driven by autophagy–lysosome dependent pathway. Blockade of autophagy by RNA interference against Atg5 activates necrosis in TAII-treated cells. Our study reveals that using TAII as a novel chemotherapeutic agent for hepatocellular carcinoma should be worth considering.

2. Results

2.1. TAII potently suppresses tumor growth of hepatocellular carcinoma

Potent suppression of tumor growth and reduced cell viability could be observed in HCC cell lines HepG2, MHCC97L, PLC/PRF/5 and Hep3B (Fig. 1B) treated with TAII from A. asphodeloides Bunge (Fig. 1A), in a dose-dependent manner. The IC50 of TAII varies within different cell lines, however, no significant relevance between the cytotoxicity of TAII and p53 status in HCC cell lines could be observed, which is indicated by the phenomenon that TAII may exhibit higher cytotoxicity to p53-deficient Hep3B cells than to p53-proficient HepG2, MHCC97L and PLC/PRF/5 cells. This may suggest that a p53-independent mechanism may be involved in the induction of cell death by TAII in HCC cells. Clonogenic assay presents no significant relapse nor resistance of TAII-treated HCC cells (Fig. S1). In addition, TAII exhibits higher toxicity to carcinoma cell lines than to normal hepatocytes (Fig. 1C), which reveals that TAII could exert its anti-tumor action in hepatocellular carcinoma without inducing potent toxic effect in normal hepatocytes. The toxicity of TAII was monitored in vivo as well. In tumor bearing nude mice generated by subcutaneous injection of MHCC97L, no remarkable body weight loss could be observed (Fig. S2) in mice treated with TAII (7.5 mg/kg/2 days) while tumor growth was dramatically restricted (Fig. 1D and S3). Ki67 expression, as a tumor proliferation marker, was significantly reduced in TAII-treated xenograft (Fig. 1E). These findings suggest that TAII can be a worthy therapeutic agent for HCC without inducing significant toxic effect on normal hepatocytes.

2.2. TAII induces caspase-dependent apoptosis of hepatocellular carcinoma cells

Previous study has reported that TAII could initiate apoptosis in Hela cancer cells [1], which confers the Hela cell death. The apoptosis-inductive effect of TAII could be further confirmed in this study from the observation that increased FITC-conjugated Annexin V binds to the membrane phospholipid phosphatidylserine (PS) at the outer leaflet of plasma membrane along with the PI permeability, which indicates that HCC cells were undergoing apoptosis upon TAII intervention (Fig. S4). Activation of caspase cascade as well as PARP cleavage further confirms the apoptotic-inductive effect of TAII in HCC cells (Fig. 2A). To examine if the caspase-3 activation is responsible for the apoptosis induction, HCC cells were exposed to TAII in the presence of caspase inhibitor. Co-treatment with 50 μM Z-VAD-FMK could significantly ameliorate the cell death induced by TAII treatment in HCC cells, indicating that the apoptosis induction in TAII-treated cells may be attributed to the initiation of caspase activation (Fig. 2B). Furthermore, TAII could initiate apoptosis in xenografted models. Intraperitoneal injection of TAII (7.5 mg/kg/2 days) reduced the size of the tumor in mice through the activation of tumor cell apoptosis. This trait was depicted by DNA fragmentation, the hallmark of apoptosis (Fig. 2C). Cleavage of caspase-3 and PARP could be observed in xenografted tumor from mice with TAII treatment (Fig. 2D). These findings suggest that TAII initiates caspase-dependent apoptosis in HCC cells.

2.3. TAII is chemically similar to autophagy-inducers

By comparing the chemical fingerprint of TAII with those of target sets in the WOBAT database, we carried out chemical similarity analysis for TAII. The top ranked target sets with compounds chemically similar to TAII are found to be “Na+/K+–ATPases” (E-value of 1.82E – 13), based on the pair-wise comparison of the chemical fingerprint of TAII with those of 225 ligands in the “Na+/K+-ATPases” target set. “Na+/K+–ATPases”, such as cardiac glycosides, are known to induce autophagy in human non-small cell lung cancer cells [PMID: 22750415] and induce autolysosome formation and lysosomal degradation [PMID: 21635740]. Biochemical studies were performed to validate these findings.

2.4. Induction of apoptosis by TAII in hepatocellular carcinoma cells requires inhibition of XIAP expression

It has been reported that knockdown of IAP genes by siRNA induces apoptosis and suppresses proliferation in human cancer cells [10]. The inductive effect of IAP suppression on apoptosis is p53-independent, indicating that the p53 status does not affect the grade of apoptosis induced [11]. Expression of XIAP was suppressed upon TAII treatment, however, it seems that in PLC/PRF/5 and Hep3B cells, cIAP1 and cIAP2 could be suppressed while in HepG2 and MHCC97L cells, cIAP1 and cIAP2 could not respond to TAII treatment. However, TAII treatment could effectively inhibit XIAP expression (Fig. 3S). Considering that the cytotoxicity of TAII in four hepatocellular carcinoma cell lines is similar, this may indicate that cIAP1 and cIAP2 may not be responsible as a main mechanism in TAII-induced apoptosis. To further prove this, we detected the mRNA transcripts of cIAP1, cIAP2 and XIAP in non-malignant cell line L-02 and HCC cell lines HepG2, MHCC97L, PLC/PRF/5 and Hep3B. Interestingly, we found that only XIAP is aberrantly expressed in malignant cell lines compared with non-malignant cell line. The expression of cIAP1 and cIAP2 seems not specific with regard to the malignancy of cell lines (Fig. S6). We found that the inhibition of XIAP on XIAP expression incurred in a dose- and time-dependent manner (Fig. 3A), and the suppression of XIAP by TAII treatment could be found in in vivo study (Fig. 2D). Considering the cytotoxicity of TAII in four hepatocellular carcinoma cell lines is similar, this may indicate that cIAP1 and cIAP2 may not be responsible as a main mechanism in TAII-induced apoptosis. Furthermore, when we knock-down the expression of cIAP1 and cIAP2 in HepG2 cells, the cytotoxicity effect of TAII could not be dramatically affected (Fig. S7). To further examine if the inhibition of XIAP was responsible for the cell death induced by TAII in HCC cells, we introduced RNA interference to suppress XIAP in HCC cells. Reduction of XIAP expression could attenuate the cytotoxic effect of TAII on HCC cells (Fig. 3B), which indicated that TAII mainly targeted on XIAP and other IAP family member in inducing cytotoxic effect. Furthermore, it could be observed that the grade of caspase activation was remarkably reduced in TAII-treated HCC cells with XIAP silencing (Fig. 3C). The reduced sensitivity of HCC cells with XIAP silencing toward the TAII treatment reveals that XIAP plays a major role in the TAII-induced apoptosis in hepatocellular carcinoma.

2.5. TAII-induced AMPKε-activated autophagy in hepatocellular carcinoma cells

Autophagy induction was observed in TAII-treated HCC cells. The increase of autophagosomal form of LC3 (LC3-II) along with the decrease of cytoplasmic form of LC3 (LC3-I) reveals the initiation of autophagy in TAII-treated HCC cells (Fig. 4A). Consistent observation could be found in in vivo study (Fig. 2D). No significant
induction of autophagy could be observed in non-malignant L-02 cells after 3 h TAIII-treatment (Fig. 4A). The induction of autophagy by TAIII exposure was further confirmed in HepG2 cells with a stable expression of GFP-LC3. Elevated expression of autophagic punctate dots in TAIII-treated cells could be observed, which presented as the indicative of autophagy when cells were exposed to TAIII (Fig. 4B). Suppression of Atg5 expression by RNA interference significantly attenuated the formation of autophagic punctuation in TAIII-
treated cells (Fig. 4B). Previous study has shown that TAIII is capable of inhibiting mTOR signaling, the negative regulatory of autophagy, in human cancer cells [2]. In our study, we found that AMPKα, one of the upstream regulators of mTOR, was activated by TAIII treatment in HCC cells. Activation of AMPKα may be responsible for the autophagy induction by mTOR inhibition [12]. To further confirm the role of AMPKα in autophagy initiation in cells with TAIII intervention, HCC cells were treated with TAIII in the presence of AMPKα inhibitor Compound C. Inactivation of AMPKα in TAIII-treated cells restored the mTOR activity and blocked the induction of autophagy (Fig. 5A and B). TAIII exerts no significant effect on the activities of Akt and Erk signaling (Fig. 5B). In addition, induction of autophagy in TAIII-treated HCC cells was attenuated by the presence of AMPKα inhibitor Compound C (Fig. 5C). Recovery of mTOR activity under AMPKα inhibition reveals the central role of AMPKα in the regulation of autophagy in TAIII-treated HCC cells.

2.6. Suppression of XIAP expression by TAIII requires autoubiquitination and autophagy induction

We tried to discriminate the role of autophagy in cell death induced by TAIII in HCC cells. With RNA interference against Atg5, we blocked the induction of autophagy in HCC cells exposed to TAIII. Interestingly, we found that the expression of XIAP was restored by autophagy inhibition, which indicated the potential role of autophagy in the XIAP suppression induced by TAIII treatment (Fig. 6A). To explore the underlying mechanism involved in XIAP suppression, the mRNA transcripts of XIAP in HCC cells exposed to TAIII were detected. No potent reduction of mRNA transcript level of XIAP was observed, which indicated that a post-transcriptional regulation mechanism may be involved (Fig. 5B). We blocked the proteosomal degradation by pre-treating HCC cells with protease inhibitor MG-132. However, the presence of MG-132 did not attenuate the XIAP proteolysis induced by TAIII treatment. Interestingly, we observed restoration of XIAP expression in TAIII-treated cells in the presence of lysosome inhibitor Leupeptin (Fig. 6B). Leupeptin is a protease inhibitor that blocks the degradation of lysosome-engulfed proteins. In addition, increasing ubiquitination of XIAP could be observed in TAIII-treated HCC cells (Fig. 6C). This critical role of ubiquitination in IAP protein lysosomal degradation was further confirmed with the observation that presence of ubiquitin E1 enzyme inhibitor PYR-41 blocked the degradation of XIAP in TAIII-treated cells (Fig. 6D). These findings suggest a novel mechanism of XIAP degradation involving ubiquitination-dependent autophagic proteolysis.

2.7. Inhibition of autophagy in TAIII-treated cells initiates necrotic cell death

To determine the role of autophagy in TAIII-induced HCC cell death, we blocked the autophagy induction in TAIII-treated cells with RNA interference against Atg5. Increased cytotoxicity was observed in TAIII-treated cells when autophagy was suppressed (Fig. 7A). Further determination of death mode in autophagy-suppressed HCC cells with TAIII exposure was conducted, and the membrane permeability of PI after 3 h treatment of TAIII was significantly increased in hepatoma cells which autophagy was suppressed, indicating that a quick loss of membrane integrity and induction of necrosis occur in TAIII-treated HCC cells once autophagy is inhibited (Fig. 7B). Initiation of necrosis by TAIII in autophagy-suppressed cells was further confirmed with examining the release of LDH, which was the biomarker of necrotic cell death. Potent increase of LDH activity in culture medium was found in autophagy-suppressed HCC cells, which indicated that cells with autophagy inhibition may undergo necrotic cell death upon TAIII treatment (Fig. 7C). Our data suggests that autophagy is necessary for the induction of apoptosis in TAIII-treated hepatocellular carcinoma cells.

3. Discussions

Autophagy is defined as a biological process that the cell consumes its’ own cellular components through the lysosome machinery [13]. It is a homeostatic process that preserves to maintain the balance between synthesis, degradation and subsequent recycling of cellular components [14]. As a well recognized process, autophagy is now regarded as complicated genetic cascades that at least three distinct forms of autophagy were identified with different cellular and molecular machineries, macroautophagy, microautophagy (mitophagy) and chaperone-mediated autophagy (CMA) [15]. In chaperone-mediated autophagy, the autophagic vacuoles only recognize the particular proteins tagged with chaperones and deliver the selected molecules to the lysosome for proteolysis process [16]. In our study, we observed that the cellular endogenous inhibitors of apoptosis XIAP could undergo selective autophagy induced proteolysis. The protein degradation induced by TAIII in HCC cells is independent of proteasome but relies on lysosomal pathway. The XIAP was previously reported to be degraded through proteasomal degradation [17] and our study concludes that the selective autophagy-signaling may be the complementary machinery for regulating its expression. In addition, we found that the protein degradation of XIAP induced by TAIII requires ubiquitination of the XIAP. Proteosomal degradation of XIAP also requires ubiquitin labeling onto the protein. However, the XIAP degradation by autophagy is an action different from our knowledge that XIAP could be majorly degraded through proteosome pathway. The switch directing the protein to either proteosomal degradation or autophagy–lysosomal degradation pathway is still incompletely unveiled. However, it is known that ubiquitination process of the targeted proteins connects the two complementary but strictly separated pathways [18]. Previous study has found that autophagy could be a compensation mechanism to degrade proteins when the ubiquitin–proteosom system (UPS) is impaired [19]. In our study we could not observe the recruitment of RING, the E3 ligase of XIAP, even though XIAP is ubiquitinated in TAIII-treated cells (data not shown). This may indicate that proteosomal degradation of XIAP is impaired in TAIII-treated HCC cells. The exact mechanism is still undergoing investigation.

The relationship between apoptosis and autophagy in mammalian cells is complicated in the sense that, under some circumstances, autophagy may be either a negative or positive regulator in inducing apoptosis when it occurs spontaneously with apoptosis [20]. The complex interplay between autophagy and apoptosis requires a thorough investigation in each particular case. Currently, it is the common concept that autophagy plays a role in deciding cell fate in a given cell, but it primarily depends on the types and degrees of death stimuli or stress factors which the cell is exposed to [21]. In our study, we identified a new relationship between apoptosis and autophagy in TAIII-treated HCC cells. Autophagy started prior to apoptosis initiation to degrade the endogenous apoptosis inhibitor XIAP through lysosome-dependent pathway. The degradation of apoptosis suppressor facilitates the activation of alternative apoptosis pathway. It could be therefore concluded that the apoptosis in TAIII-treated HCC cells depends on autophagic...
TAIII induces apoptosis in hepatocellular carcinoma. A shows caspase-cascade activation by TAIII in hepatocellular carcinoma cells. Cells with TAIII intervention were lysed and related target proteins were detected by immunoblotting with specific antibodies. Results indicated that 24 h treatment of TAIII induced dose-dependent caspase-3 (pro-form 32 kDa, cleaved form 17 kDa) and PARP (pro-form 112 kDa, cleaved form 89 kDa) cleavage in hepatocellular carcinoma cells. B shows the presence of 50 μM caspase inhibitor Z-VAD-FMK attenuated apoptosis induced by TAIII. Cells were treated with TAIII in the presence and absence of 50 μM Z-VAD-FMK for 24 h. Cell viability was determined by MTT assay. Δp < 0.05 and ΔΔp < 0.01 when compared with control. C shows TAIII induced apoptotic cell death in vivo. The xenograft model was established as indicated in Fig. 1D and the tumor was fixed and the paraffin section was prepared. Apoptotic tumor cells were stained with TUNEL assay kit. D shows that TAIII could induce differential protein expressions in xenografted tumor. The collected tumor was homogenized and protein was collected. Expression of LC3, XIAP, caspase-3 and PARP was detected by immunoblotting. Decreased expression of XIAP and increased cleavage of caspase-3 and PARP by TAIII were observed. Increase of membrane form of LC3 (LC3-II) with decreased cytosolic form LC3-I was found.
degradation of endogenous apoptosis inhibitor. Our previous study presented that autophagy-dependent protein degradation induced by TAIII requests conjugation of poly-ubiquitin to the protein, which leads to the degradation of those particular molecules by lysosome pathway [22]. Here we suggest that ubiquitinated XIAP is degraded through this autophagy-dependent proteolysis pathway leading to induction of apoptosis in HCC cells. It was previously shown that induction of autophagy is required for apoptosis induction in CR4/CXR4-expressing T cells interacting with HIV-1-encode envelope glycoproteins [23], and autophagy induced by anti-tumor agent promotes the apoptotic death in cancer cells [24]. Our study suggests that XIAP can be the mediators in autophagy-related apoptosis initiation.

We noticed that inhibition of autophagy did not attenuate cell death, which was controversial with our expectation since the apoptotic cell death induced by TAIII required the participation of autophagy. By contrast, exacerbated cell death was observed. Further study showed that inhibition of autophagy switched on necrosis in TAIII-treated HCC cells. The switch between apoptosis and necrosis is not completely revealed, but it was observed that primary cultured cortical neurons undergo necrosis in low-density culture while they initiate apoptosis at high density [25], which is mediated by the necrosis-inhibitory molecules prothymosin α [26]. Previous study also showed that induction of autophagy in chemotherapy-injury liver impairs necrosis, which provokes inflammation-related damage of hepatocytes, which reveals the role of autophagy in maintaining cell to undergo apoptotic death [28]. Depletion of LAMP2 which impaired selective autophagy is reported to initiate a switch from apoptosis to necrosis [27], but the involvement of related molecules has not been unveiled. Our study shows that XIAP may be capable in mediating a switch from apoptosis to necrosis. A systemic scheme on the regulatory network of TAIII-induced cell death was shown in Fig. S9.

In conclusion, a novel mechanism involved in XIAP-induced apoptosis was reported in HCC cells exposed to TAIII. TAIII was found cytotoxic to HCC cells independent of p53 status, and in vivo tumor growth of hepatocellular carcinoma cells could be suppressed by TAIII treatment. TAIII exhibits less toxicity to non-malignant cells. TAIII-induced caspase-
dependent apoptosis in HCC cells, which were correlated with its inhibition on XIAP expression. TAIII did not reduce the mRNA transcripts of XIAP, and inhibition of proteasome did not restore the XIAP level in TAIII-treated cells. Instead, TAIII induced AMPKα/mTOR-dependent selective autophagy which brought the XIAP protein to lysosomal degradation pathway. The autophagy-related lysosomal degradation of XIAP required its ubiquitination. Blockade of autophagy accelerated the cytotoxic effect of TAIII in HCC cells, which was attributed to the activation of necrotic cell death in autophagy-suppressed HCC cells exposed to TAIII. Our study reveals the potential of TAIII as a potential therapeutic drug candidate against HCC with novel mechanism involved.

4. Materials and methods

4.1. Cell lines and cell culture

The human hepatocellular carcinoma cell lines HepG2, PLC/PRF/5 and Hep3B were purchased from ATCC (USA); MHCC97L cell line was kindly gifted by Dr. Man Kwan from the Department of Surgery, The University of Hong Kong. The human normal hepatic cell line L-02 was purchased from the Laboratory Animal Center in Sun Yat-Sen University (Guangzhou, P.R. China). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, with high glucose, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, USA). Cells were incubated in a humidified atmosphere containing 5% CO2 at 37 °C.

4.2. Calculation of autophagic flux

Induction of autophagy was measured by calculating the autophagic flux in cells with treatment. In brief, HepG2 cells were transfected with plasmids encoding GFP-LC3 (kindly gifted by Prof. Tamotsu Yoshimori, Osaka University, Japan) and cells with GFP-LC3 stable expression were selected. To measure the autophagic flux, cells with treatment were visualized under fluorescence microscope (Carl Ziess, USA, 40×, CCD camera). Three scopes of each treatment were captured and the FITC-positive autophagic punctate dots were counted. Increased number of autophagic puncta indicated the induction of autophagic flux upon treatment.

4.3. Co-immunoprecipitation assay

The ubiquitination of XIAP was determined by co-immunoprecipitation assay with Pureproteome Protein G Magnetic Beads (Millipore, USA) according to the instruction provided by the manufacturer. In brief, cells were treated with timosaponin AIII in the presence of leupeptin for 6 h. Then cells were collected and lysed with NP-40 lysis buffer (Invitrogen, USA) supplemented with cocktail protease inhibitor (Roche, USA), and 50 μl of cell lysates was collected for input control. The ubiquitinated XIAP was precipitated by binding to magnetic beads pre-incubated with XIAP antibody. The ubiquitinated XIAP was then eluted with 0.2 M glycine (pH 2.5) after washing thoroughly. Eluted protein was mixed with loading buffer and heat at 90 °C. Ubiquitination of XIAP was detected by immunoblotting with antibody against XIAP.
ubiquitin. Expression of XIAP in purified protein and input lysate was detected with antibody against XIAP as loading control.

4.4. Xenograft model

The in vivo anti-tumor activity of TAIll was examined by xenograft model. In brief, female nude mice subcutaneously received \(1 \times 10^7\) MHCC97L cells on its right flank. One week after injection, mice were randomized into two groups. One of which received 7.5 mg/kg TAIll three times per week through intraperitoneal injection for 3 weeks while the other received PBS. Tumor growth and body weight were monitored three times per week. At the end of the study, the mice were sacrificed with overdose of phenobarbital (200 mg/kg). Tumor was dissected out and subjected to histological analysis. All animal protocols have been approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong and complied with international ethical requirement in animal study.

4.5. Chemical similarity analysis

A chemical similarity approach (SEA) was recently developed to predict targets for new drugs [PMID: 19881490]. In brief, SEA is a database of a large number of compounds, whose biological targets are known. These reference compounds are classified according to their biological targets and named as “target sets”. Using a statistical model, the chemical similarity between TAIll and all the compounds in each target sets were computed and ranked.

4.6. Statistical analysis

Statistical analysis was conducted using Student’s t-test. Other supporting information on materials and methods was presented in Supplemental document.

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Fig. 6. Suppression of XIAP by TAI1 in hepatocellular carcinoma cells is autophagy-dependent and requires ubiquitination of XIAP. A shows that inhibition of autophagy by introducing RNA interference against Atg5 attenuated TAI1-suppressed XIAP expression. Cells were transiently transfected with scramble negative control siRNA (scr siRNA) or siRNA against Atg5 24 h prior to TAI1 treatment. Restoration of XIAP expression in cells with autophagy inhibition was observed. B shows that suppression of lysosome but not proteasome attenuated interference against Atg5 attenuated TAIII-suppressed XIAP inhibition. Cells were treated with TAIII with and without MG132 (proteasome inhibitor, 20 μM)/leupeptin (lysosome inhibitor, 50 μM). Significant restoration of XIAP expression in cells with TAI1 treatment in the presence of leupeptin but not MG132 indicated that the degradation of XIAP was dependent on autophagic-lysosome but not proteasome pathway. C shows that TAIII treatment promoted the ubiquitination of XIAP. Cells were treated with TAIII in the presence of 50 μM leupeptin, and CoIP assay was conducted using XIAP antibody. The ubiquitination of XIAP was detected by western blot with specific antibodies against ubiquitin. Precipitated and input level of XIAP was detected as control. D shows that suppression of ubiquitination abolished TAI1-induced XIAP inhibition in HCC cells. Cells were exposed to TAI1 in the presence and absence of PYR-41, an ubiquitin E1 inhibitor (50 μM) for 6 h. Protein was collected and analyzed by immunoblotting.

References

Fig. 7. Inhibition of autophagy leads to necrotic death in HCC cells treated with TAIII. A shows that inhibition of autophagy by suppressing Atg5 expression sensitized HCC cells to TAIII treatment. Cells were transfected with either scr siRNA or siRNA against Atg5 gene. Medium was refreshed after transfection and cells were exposed to TAIII for 24 h. Cell viability was determined by MTT assay. B shows increased PI permeability in TAIII-treated hepatoma cells with suppression of autophagy. Cells with or without Atg5 suppression were exposed to TAIII for 3 h, and then stained with PI (40 μg/mL) for 30 min. The cells were then subjected to flow cytometry analysis. Increased population with bright PI signal could be observed in autophagy-suppressed cells when they are exposed to TAIII. C shows that inhibition of autophagy by either suppressing AMPK activation or inhibiting Atg5 expression led to an increase of cell necrosis upon exposure to TAIII. Medium was collected from B and the LDH release into medium was determined. Increase LDH activity was observed in TAIII-exposed cells with autophagy inhibition.