Original Article

Panduratin A induces protective autophagy in melanoma via the AMPK and mTOR pathway

Siew-Li Lai, Mohd Rais Mustafa, Pooi-Fong Wong

Abstract

Background: Targeting autophagy is emerging as a promising strategy in cancer therapeutics in recent years. Autophagy can be modulated to drive cancer cell deaths that are notoriously resistant to apoptotic-inducing drugs. In addition, autophagy has been implicated as a prosurvival mechanism in mediating cancer chemoresistance. Our previous study has demonstrated that Panduratin A (PA), a plant-derived active compound exploits ER-stress-mediated apoptosis as its cytotoxic mechanism on melanoma.

Purpose: Our previous proteomics analysis revealed that treatment with PA resulted in the upregulation of an autophagy marker, LC3B in melanoma cells. Therefore, the present study sought to investigate the role of PA-induced autophagy in melanoma cells.

Methods: Transmission electron microscopy was performed for examination of autophagic ultra-structures in PA-treated A375 cells. Cytoplasmic LC3B and p62/SQSTM1 punctate structures were detected using immunofluorescence staining. Expression levels of LC3B II, p62/SQSTM1, ATG 12, Beclin 1, phospho S6 (ser235/236), phospho AMPK (Thr172) and cleaved PARP were evaluated by western blotting.

Results: Autophagosomes, autolysosomes and punctuates of LC3 proteins could be observed in PA-treated A375 cells. PA-induced autophagy in A375 melanoma cells was found to be mediated through the inhibition of mTOR signaling and activation of AMPK pathway. Furthermore, we showed that PA-induced apoptosis was increased in the presence of an autophagy inhibitor, signifying the cytoprotective effect of PA-induced autophagy in melanoma cells.

Conclusion: Taken together, results from the present study suggest that the inhibition of autophagy by targeting mTOR and AMPK could potentiate the cytotoxicity effects of PA on melanoma cells.

Introduction

Melanoma, the most aggressive and deadliest skin cancer, remains a daunting public health problem owing to its high incidence, morbidity and mortality rates. Global incidence of melanoma continues to increase, in contrast to the stable or declining trends for most cancer types (Siegel et al., 2014). Despite recent breakthroughs in the treatment of melanoma, clinical outcomes from available therapies are still of unsatisfactory with low response rates, overall survival and the development of chemoresistance. These problems have necessitated the search for novel agents and therapeutic targets for improving clinical outcome of melanoma.

Autophagy is an evolutionally conserved catabolic process that maintains cellular homeostasis by removing protein aggregates and damaged organelles (Mizushima, 2007). Autophagy is characterized by the formation of double membrane vesicles (autophagosome) that engulf cytoplasmic components, follow by the fusion with lysosomes which ultimately leading to degradation of the sequestered contents by acidic lysosomal hydrolases. Autophagosome formation is driven by the concerted actions of Beclin 1 and autophagy-related gene (ATG) proteins. The degraded products are recycled as energy source or building blocks for the synthesis of new macromolecules (Boya et al., 2013; Glick et al., 2010). Autophagy could be triggered by stressors including hypoxia, deprivation of nutrient and energy as well as toxic insults induce by chemotherapy agents via several signaling pathways. mTOR is a key mediator of cellular nutritional level, hypoxia and growth factor signaling to autophagy. The suppression of mTOR pathway is one of the most important pathways leading to autophagy induction (Jung et al.,
in addition, AMPK activation by mitochondrial dysfunction or a decrease in ATP/AMP ratio is also one of the upstream activators of autophagy signaling. Activated AMPK can lead to autophagy induction either via suppression of mTOR signaling or through direct activation ofULK1 protein (Mihaylova and Shaw, 2011; Russell et al., 2014).

Several studies have shown that autophagy represents a potential target for cancer therapeutics intervention. Drug-induced autophagy is regarded as double-edged sword, implicating in cytotoxicity or cytoprotective effects, which would either lead to the increase in drug efficacy by inducing autophagic cell death or development of resistance towards chemotherapeutics agents, respectively. Therefore, complete understanding of drug-induced autophagy is vital for the development of promising therapeutics strategy to enhance the effects of chemotherapy and improve clinical outcomes of cancer patients (Sui et al., 2013; Yang et al., 2011). As such, study on the role of autophagy in potential anti-cancer drug candidates is emerging and warranted.

Panduratin A (PA) is the major bioactive phytochemicals found in Boesenbergia rotunda. We and others have previously shown that PA has anti-cancer effects on various cancer types, including melanoma, colon adenocarcinoma, and prostate cancer (Lai et al., 2015; Liu et al., 2018; Yen et al., 2006,2005). Mechanistically, our in-depth temporal quantitative proteomics analysis revealed that the cytotoxicity of PA on melanoma cells involves dysregulation of mitochondrial oxidative phosphorylation, secretory pathway and ER stress-induced apoptosis. In this regard, it has been shown that ER stress can result in the induction of autophagy as a secondary response to alleviate ER stress (Sano and Reed, 2013). Furthermore, many natural products with anti-cancer property have been reported to induce either cytoprotective or cytotoxic autophagy in cancer (Wang and Feng, 2015). Therefore, the present study sought to investigate whether PA could induce autophagy in A375 melanoma cells and the feasibility of targeting autophagy as a strategy in further potentiating the anti-melanoma efficacy of PA.

Materials and methods

Extraction and isolation of PA

PA was isolated from rhizome of Boesenbergia rotunda (L.) Mansf. (a voucher specimen with accession number KU0098 is deposited in the Phytochemistry Herbarium, University of Malaya, Kuala Lumpur) as previously described (Lai et al., 2012,2015). The identity and purity of isolated PA were confirmed by LCMS and NMR (Supplementary figure).

Cell culture

A375 cells were purchased from American Type Culture Collection (ATCC; VA, USA). A375 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, CA, USA) supplemented with 10% heat inactivated FBS (Sigma-Aldrich, MO, USA) and 1% penicillin/ streptomycin (Gibco) at 37 ºC in humidified incubator with 5% CO2.

Transmission electron microscopy for autophagosome visualization

Transmission electron microscopy was performed according to a previously published method with modifications (Yla-Anttila et al., 2009). Briefly, A375 cells were seeded in 100 mm petri dish and allowed to attach and proliferate overnight, before treated with PA for 24 h. Thereafter, PA-treated cells were fixed with 2.5% glutaraldehyde in 0.2 M HEPES buffer, pH 7.4 for 30 min, harvested by using a cell scraper and fixed as a pellet at 4 ºC. Cell pellets collected from three biological replicates were pooled for the subsequent procedures. The pellets were post-fixed in cacodylate buffer and incubated in OsO4 and cacodylate buffer, in 1:1 ratio for 2 h at 4 ºC. Cell pellets were dehydrated stepwise in a series of graded ethanol solutions. The pellets were infiltrated with resin by incubation with propylene oxide and resin mixture at room temperature and finally 100% resin at 60 ºC. Ultrathin sections were obtained by ultramicrotome and stained with 0.1% lead citrate and 10% uranyl acetate. Samples were visualized under a LEO-Libra 120 transmission electron microscope (Carl Zeiss).

Immunofluorescence staining for autophagosome detection

The formation of autophagic vesicles in cells was detected by immunofluorescence staining of autophagy markers, LC3B and p62/SQSTM1. Briefly, A375 cells at ~ 80% confluency were treated with PA for 24 h. Thereafter, cells were fixed, permeabilized and incubated with primary antibody for LC3B or p62/SQSTM1. After 1 h, the cells were washed and incubated with secondary antibody and Hoechst 33342 dye for nuclei staining. Images of fluorescent labeled cells were acquired with the Cellomics Array Scan High Content Screening (HCS) Reader (Thermo Fisher Scientific). It is known that A375 cells are resistant to autophagy induction by Rapamycin, a widely used autophagy inducer (Armstrong et al., 2011). Therefore, Chloroquine (CQ) a late stage autophagy inhibitor, which blocks the fusion of autophagosome with lysosome was included as positive control of the assay for the visualization of accumulated autophagosomes. Experiments were performed in duplicate and three independent experiments were performed.

ELISA

The level of phosphorylated AMPK at Thr172 was analyzed using Phospho-AMPKα (Thr172) Sandwich ELISA Kit (Cell Signaling Technology (Beverly, MA)). Briefly, A375 cells treated with PA at 15 µM for 2, 4, 12, and 24 h were harvested and lysed with cell lysis buffer provided by the kit. Three biological replicates of cell lysates were collected. Thereafter, 100 µg of the resulting protein lysates were added into the AMPKα rabbit antibody coated wells and incubated overnight at 4 ºC. Wells were washed extensively with wash buffer followed by incubation with AMPK (Thr172) mouse detection antibody, HRP-Linked secondary antibody and TMB substrate. The reaction was halted by the addition of STOP solution. Absorbance at 450 nm was read and recorded using Plate Chameleon V microplate reader (Hidex, Turku, Finland).

Western blot analyses

A375 cells at ~ 80% confluency were treated with either PA (15 µM) or CQ (25 µM) for indicated duration before being harvested and lysed with lysis buffer containing 25 mM triethylammonium bicarbonate (TEAB) buffer, 8 M urea, 2% Triton X-100, 0.1% SDS, protease and phosphatase inhibitors (Roche, Basel, Switzerland). For the investigation of the role of autophagy in PA-induced cell death, A375 cells were pre-treated with 3-Methyladenine (3-MA; 10 mM) for 1 h, followed by treatment with either PA (15 µM), CQ (25 µM) or Cisplatin (CDPP; 25 µM) for 24 h. At least three biological replicates of protein lysates were collected for western blot analyses. Protein lysates were resolved on SDS-PAGE followed by electrotransfer onto a PVDF membrane according to the instructions of the manufacturer (Bio-Rad). PVDF membranes were then blocked with 5% BSA in TBST and probed with different primary antibodies for an overnight at 4 ºC. The primary antibodies against LC3B, p62/SQSTM1, phosphor S6 Ribosomal Protein (Ser235/236), AMPK and phosphor AMPK (Thr172) were purchased from Cell Signaling Technology; β actin was obtained from Sigma. Thereafter, membranes were probed with horseradish peroxidase-conjugated mouse or rabbit secondary antibodies followed by visualization using ECL prime detection system (Amersham, Buckinghamshire, UK). Quantitation was done by estimating the optical density of the bands using Quantity One software (Bio-Rad).
Statistical analysis

Statistical significance was analyzed by either paired t test or one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison post test using Graphpad Prism v5.01 software (Graphpad Software, San Diego, CA, USA). Statistical significance is expressed as *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

PA deregulates expression of proteins associated with autophagy

One of the advantages of proteomics analysis is that it allows the identification of global protein changes for future interpretation and investigation. Previously, our proteomics analysis has identified 296 differentially regulated proteins in A375 cells upon treatment with PA (15 µM) for 4, 12 and 24 h. These include proteins responsible for cytotoxic mechanisms as well as other cellular processes triggered in response to the treatment with PA (Lai et al., 2015). In addition, the expressions of 3 proteins involved in the autophagy process, including microtubule-associated protein 1A/1B-light chain 3 (LC3B), microtubule-associated protein 1B (MAP1B) and Sequestosome-1 (p62/SQSTM1) were found to be significantly upregulated in PA-treated A375 melanoma cells (Table 1). These observations prompted us to further investigate the implication of PA treatment in autophagy induction on A375 cells.

PA induces autophagy on A375 melanoma cells

LC3B protein is associated to autophagosomal membranes upon autophagy induction. p62/SQSTM1 functions as an autophagy adaptor, which binds directly to LC3 and GABARAP family proteins via a specific sequence motif. Therefore, the presence of intracellular LC3 and p62/SQSTM1 punctate structures are indicative of increase autophagy. Immunofluorescence assay was performed to visualize the cellular localization of LC3B and p62/SQSTM1. When compared with untreated cells with diffuse staining, A375 cells treated with PA for 24 h displayed multiple punctate structures of LC3B and p62/SQSTM1 in the cytoplasm (Fig. 1A). These observations show that the up-regulations of LC3B and p62/SQSTM1 at protein levels as revealed by proteomics study were associated with increased autophagosome formation. Similarly, these punctate structures were also observed in CQ-treated A375 cells. CQ is an inhibitor of the fusion of autophagosome with lysosome which would result in the accumulation of LC3B and p62/SQSTM1 was included as positive control for the assay.

Following translation, LC3B protein will be processed to form LC3B I. Upon autophagy induction, LC3B I protein will be further processed and conjugated with phosphatidylethanolamine moiety to generate LC3B II and recruited to autophagosomal membranes (Tanida et al., 2008). Fig. 1B depicts the time-dependent upregulation of LC3B II in PA-treated A375 cells, which correlates with the increased in the number of autophagosomes. On the other hand, proteomics analysis revealed that p62/SQSTM1, an autophagy substrate that should be degraded was instead found to be upregulated at 24 h post-treatment with PA (Table 1). Further validation by immunoblotting analysis showed that the p62/SQSTM1 protein expression was decreased in parallel with increased LC3B II level at 4 h post-treatment with PA (Fig. 1B), suggesting its degradation via autophagy. Paradoxically, the expression of p62/SQSTM1 was restored at 12 h post-treatment and further increased at 24 h post-treatment with PA (Fig. 1B). It is noteworthy that the intracellular level of p62/SQSTM1 is dependent on both transcriptional regulation and post-translational autophagic degradation. In addition, autophagic degradation of p62/SQSTM1 was reported with be cell- and condition-specific (Alegre et al., 2017; Liu et al., 2016).

Transmission electron microscopy, a gold standard for the observation of ultra-structures of various autophagic vacuoles was performed to identify autophagic vacuoles in the PA-treated A375 melanoma cells. As shown in Fig. 2, the hallmarks of autophagy which include double membrane autophagosome and single membrane autolysosome with uneven dense masses, could be observed in the electron micrograph of PA-treated A375 cells. The observed autophagosomes are indicative of early stage of autophagy while autolysosomes represent the fusion of autophagosomes with lysosomes at the later stage of autophagy (Mizushima et al., 2010). Collectively these results indicate that PA induces autophagy in A375 cells.

PA induces autophagy via the AMPK-mTOR signaling

We next attempted to elucidate the signaling mechanisms underlying the induction of autophagy by PA. We first examined the level of AMPK phosphorylation at Thr172 site as a readout of AMPK pathway activation. In addition, the effects of PA on the expressions of other common upstream autophagy regulators including ATG 12, Beclin 1 and S6 protein phosphorylation at Ser235/236 were also investigated. As shown in Fig. 3A, treatment of PA resulted in increased phosphorylation level as early as 2 h post-treatment in A375 cells. In addition, the phosphorylation of S6 protein, a downstream substrate of mTOR signaling was suppressed upon 2 h post-treatment with PA (Fig. 3B). On the other hand, the expression of ATG 12 was not affected by the treatment of PA while a downregulation of Beclin 1 was observed at 24 h post-treatment with PA on A375 cells (Fig. 3B). Taken together, these results show that PA-induced autophagy on A375 cells was associated with the activation of AMPK and suppression of mTOR pathway.

mTOR suppression of PA is independent of AMPK activation

To further investigate whether the suppression of mTOR signaling is a result of AMPK activation, A375 cells treated with PA for 15, 30, 60 and 120 min were subjected to western blot detection of phosphor AMPK (Thr172) and phosphor S6 (Ser235/236). Results showed that PA suppressed the phosphorylation of S6 protein as early as 15 min post-treatment (Fig. 4) and the effects sustained up to 24 h (Fig. 3B). On the other hand, activation of AMPK was only observed after 60 min post-treatment with PA (Fig. 4). These observations suggest that PA induced-autophagy is a collaborative result of mTOR signaling suppression and AMPK activation. Indeed, the LC3B II levels were higher at 60 and 120 min post-treatment with PA when suppression of mTOR signaling was coupled with activation of AMPK signaling (Fig. 4).

Table 1

List of differentially regulated autophagy-related proteins in response to PA treatment on A375 cells A375 cells.

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Gene Symbol</th>
<th>Protein Name</th>
<th>Peptides (95%)</th>
<th>4 h Avg ratio</th>
<th>P value</th>
<th>12 h Avg ratio</th>
<th>P value</th>
<th>24 h Avg ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.81 16.8</td>
<td>Q96ZQ8</td>
<td>LC3B</td>
<td>3</td>
<td>1.11</td>
<td>0.1822</td>
<td>1.51</td>
<td>0.0021</td>
<td>1.75</td>
<td>0.0162</td>
</tr>
<tr>
<td>18.37 8.3</td>
<td>P468Z1</td>
<td>MAP1B</td>
<td>17</td>
<td>1.06</td>
<td>0.0737</td>
<td>1.28</td>
<td>0.0000</td>
<td>1.70</td>
<td>0.0001</td>
</tr>
<tr>
<td>35.13 52.3</td>
<td>Q13501</td>
<td>SQSTM1</td>
<td>32</td>
<td>1.01</td>
<td>0.8388</td>
<td>1.20</td>
<td>0.0072</td>
<td>1.43</td>
<td>0.0525</td>
</tr>
</tbody>
</table>

*Avg ratio denotes average protein ratio of PA-treated cells at indicated time-point against untreated control at 0 h.
Inhibition of autophagy induces PA-mediated apoptotic cell death

Next, we sought to determine whether PA-induced autophagy confers cytoprotective or cytodestructive effects on A375 cells. We examined the outcome of PA-mediated cytotoxicity when autophagy was inhibited by autophagy inhibitor, 3-MA. Cisplatin (CDPP), an apoptosis inducer was included as positive control. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases (PI-3K). As shown in Fig. 5, treatment with 3-MA led to accumulation of p62/SQSTM1, confirming inhibition of autophagy by 3-MA under our experimental conditions. In addition, treatment of A375 cells with CQ, a late stage autophagy which inhibits fusion of autophagosome with lysosome also showed reduce accumulation of LC3B II in the presence of autophagy inhibition by 3-MA. Pre-treatment with 3-MA followed by PA resulted in the suppression of autophagy as evidenced by decreased LC3B II expression when compared with PA treatment alone in A375 cells. Notably, the suppression of autophagy was accompanied with increased cleaved PARP expression, signifying the induction of cytoprotective autophagy in A375 cells by PA. On the other hand, as expected, there was no change in cleaved PARP level observed in CDPP-treated A375 cells in the presence of autophagy inhibition by 3-MA.

Discussion

Accumulating studies have documented that inhibition of
Fig. 2. Representative electron micrograph of A375 cells treated with Panduratin A for 24 h. White arrowhead denotes autophagosomes with double membranous structures. Red arrowhead denotes uneven dense masses of partially degraded materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. PA induces autophagy via the AMPK-mTOR signaling. (A) A375 cells were treated with Panduratin A at 15 µM for 2, 4, 12 and 24 h. Cell lysates were subjected to the detection of the phosphorylated level of AMPK (Thr172) using ELISA assay. (B) Western blot detection of the expression levels of LC3B II, ATG 12, Beclin 1 and phospho S6 (Ser235/236). β actin was probed to demonstrate equal protein loading. Fold change was computed by comparing Panduratin A treatment versus untreated control at respective time-point. Data are expressed as means ± SEM of three independent experiments. Statistical significance is expressed as *P < 0.05, **P < 0.01.
Fig. 4. mTOR suppression of PA is independent of AMPK activation. A375 cells were treated with Panduratin A at 15 µM for 15, 30, 60 and 120 min. Cell lysates were analyzed by western blot for the detection of LC3B II, p S6 (Ser235/236) and p AMPK (Thr172). Total S6 and AMPK proteins were probed to demonstrate equal protein loading. Fold change was computed by comparing Panduratin A treatment at different time-points versus untreated control at 0 h. Data are expressed as means ± SEM of three independent experiments. Statistical significance is expressed as *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 5. Inhibition of autophagy increases apoptotic-inducing effect of PA on A375 cells. A375 cells were pre-treated with 3-MA for 1 h, followed by Panduratin A, Chloroquine, CDPP treatment for 24 h. Cell lysates were analyzed by western blot for the detection of cleaved PARP, LC3B II and p62/SQSTM1. β actin was probed to show equal protein loading. Fold change was computed by comparing drug treatment alone versus with 3-MA pre-treatment. Data are expressed as means ± SEM of three independent experiments. Statistical significance is expressed as *P < 0.05, **P < 0.01.
autophagy sensitizes cancer cells to chemotherapeutic agents (Chen et al., 2010; Fulda and Kogel, 2015; Guo et al., 2013; Kumar et al., 2015; Sharma et al., 2014; Sui et al., 2013; Thorburn et al., 2014; Zhang et al., 2012), advocating the manipulation of autophagy as a promising strategy for cancer treatment. Indeed, multiple clinical trials combining autophagy inhibitors and chemotherapeutic agents for diverse cancer types are underway (Chude and Amaravadi, 2017; Jiang and Mizushima, 2014; Thorburn et al., 2014; White, 2015). Phase 1 clinical trial of anti-melanoma drug temozolomide and temsirolimus with hydroxycamoraquine, an autophagy inhibitor showed promising outcome with prolonged stable disease in melanoma patients (Ndyoe and Weeraratana, 2016; Rangwala et al., 2014a; Rangwala et al., 2014b). Anti-cancer agents from natural products are potential drug leads for chemotherapeutics development. It is noteworthy that many anti-cancer agents from natural products have been shown to activate autophagy and result in either death promoting or prosurvival effects depending on the cancer types (Wang and Feng, 2015; Yang et al., 2011; Zhang et al., 2012). The investigation of autophagy activation by an anti-cancer agent and most importantly, the understanding of its implication in its anti-cancer efficacy have thereby attracted great interest.

We have previously shown that the cytotoxicity of PA on A375 melanoma cells was mediated by prolonged ER stress-induced apoptosis via the PERK/eIF2α/ATF4/CHOP pathway. Intriguingly, previous proteomics analysis revealed the upregulation of autophagy modulators, LC3B, MAP1B and p62/SQSTM1 in PA-treated A375 cells. LC3B is a ubiquitin-like protein that becomes lipidated and tightly associated with the autophagosomal membranes (Nakatogawa et al., 2009). The MAP1B has been reported as co-factor in DAPK1-induced autophagy and membrane blebbing (Harrison et al., 2008). MAP1B is composed of heavy- and light-chain subunits, of which proteolytic cleavage will generate light chain that would assemble and form complexes with LC3B protein (Zou et al., 2008). p62/SQSTM1 may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the autolysosome (Bjorkoy et al., 2009; Pankiv et al., 2007). The present study further demonstrated that PA induces autophagy in A375 melanoma cells as evidenced by the elevation of LC3B II protein expression, LC3B punctate formation and the presence of various autophagic vacuoles when examined using transmission electron microscopy.

Autophagosome formation is dependent on the activity of Beclin 1 and ATG proteins. Beclin 1 plays a central role in autophagy initiation by mediating phagophore formation in response to stress signaling (Glick et al., 2010; Kang et al., 2011). On the other hand, ATG 12 is associated with ER-stress induced autophagy. Unfolded protein response and PERK/ eIF2α activation can induce autophagy and LC3 conversion via the ATF4-dependent upregulation of ATG 12 (Kouyoku et al., 2007; Verfaillie et al., 2010). Therefore, we attempted to examine if the observed induction of autophagy in PA-treated A375 cells was mediated by Beclin 1 and ATG 12. Results from the current study showed that the expression of Beclin 1 was downregulated at 24 h instead of upregulated while ATG 12 level remained unchanged in contrast to the increasing accumulation of LC3B II in PA-treated A375 cells. These findings suggest that the induction of autophagy by PA is independent of the Beclin 1 and is not secondary to ER stress induction by PA. Autophagy induction via beclin-independent pathway is also well documented (Scarlatti et al., 2008; Sun et al., 2015). The mTOR and AMPK pathways are two important pathways regulating the induction of autophagy. In relation, we have previously shown that PA impairs mitochondrial production of ATP (Lai et al., 2015). Disturbed AMP/ATP ratio is an activator of AMPK signaling, which could subsequently lead to the suppression of mTOR signaling (Hardie, 2011). In the present study, we showed that the treatment of PA leads to autophagy activation by suppressing mTOR signaling as early as 30 min post-treatment in A375 cells. We further showed that the AMPK signaling was also activated in a later time-point and contributed to PA-induced autophagy. Importantly, we showed that pharmacological inhibition of autophagy by 3-MA potentiates the cytotoxicity and pro-apoptotic effects of PA on A375 cells as evidenced by increased cleaved PARP level.

However, it is still unclear why was the degradation of autophagic substrate, p62/SQSTM1 protein only observed at 4 h post-treatment with PA, but its expression levels were restored and increased significantly at 24 h post-treatment. In this regard, Sahani et al. reported similar observation whereby the expression of the p62/SQSTM1 is restored during prolonged starvation-induced autophagy (Sahani et al., 2014). It is also noteworthy that endogenous p62/SQSTM1 is not produced at a constant level and therefore, its expression is not solely determined by its turnover via autophagic degradation (Alegre et al., 2017; Moscat and Diaz-Meco, 2009; Rikishi, 2012). Furthermore, the expression of p62/SQSTM1 could also be induced by stress, and involved in regulation of inflammatory responses and redox homeostasis in addition to its role in autophagic clearance. Interestingly, it is reported that eIF2α/CHOP pathway activation could lead to the upregulation of p62/SQSTM1 (B’Chir et al., 2013). A recent study by Alegre et al. (2017) found that efavirenz, an anti-retro viral and potential anti-cancer drug induces p62/SQSTM1 transcriptionally via CHOP and that the inhibition of autophagy with 3-MA or silencing of ATG5 did not affect p62/SQSTM1 expression level in efavirenz-treated cells (Alegre et al., 2017). Collectively, these studies depict the role of autophagy-independent mechanisms in regulating p62/SQSTM1 expression level. Results from our previous study which show the activation of PERK/eIF2α/ATF4 pathway and upregulation of CHOP protein at 12 and 24 h by PA could be one of the underlying mechanisms leading to the increase p62/SQSTM1 observed in the present study.

We have previously provided comprehensive mechanistic insights of PA-induced apoptotic-mediated cell death via prolonged ER stress. In this study, we further revealed that PA could also induce cytoprotective autophagy in A375 cells via the suppression of mTOR signaling and activation of AMPK pathway. This cytoprotective autophagy response is not secondary to ER stress but importantly, it can be combined with an autophagy inhibitor to increase the apoptotic effects of PA in melanoma cells, when used in combination. Hence, exploring the use of combination therapy with autophagy inhibitors to modulate prosurvival autophagy to control cell fate can be considered as a potential mechanism to increase the anti-cancer efficacy of natural compounds.

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

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Supplementary materials
Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.03.027.

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