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Research Article

Plant stilbenes induce endoplasmic reticulum stress and their anti-cancer activity can be enhanced by inhibitors of autophagy

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ABSTRACT

Background: Environmental conditions or chemical agents can interfere with the function of the endoplasmic reticulum, and the resulting endoplasmic reticulum (ER) stress can be toxic to the cell if it is not relieved. The classical compensatory response to ER stress is the unfolded protein response (UPR) that reduces protein load in the ER. However, autophagy may also compensate by removing large insoluble protein aggregates. Agents that stress the ER can have anti-cancer activity, and novel applications of ER stress inducing agents are being investigated. Plant stilbenes are a class of stress responsive molecules that includes resveratrol, which are being investigated as potential therapeutics in humans for conditions such as aging or cancer.

Results: We performed a screen of 1726 small, drug like molecules to identify those that could activate an ER-stress responsive luciferase gene. After secondary screening, we determined that the plant stilbenes pterostilbene and piceatannol were the most potent inducers of ER stress from this group. ER stress can be particularly toxic to cells with high ER load, so we examined their effect on cells expressing the Wnt family of secreted glycoprotein growth factors. Molecular analysis determined that these ER stress-inducing stilbenes could block Wnt processing and also induce autophagy in acute lymphoblastic leukemia cells expressing Wnt16. Combining pterostilbene (to induce ER stress) with chloroquine (to inhibit autophagy) lead to significant cellular toxicity in cells from aggressive acute lymphoblastic leukemia.

Conclusions: Plant stilbenes are potent inducers of ER stress. However, their toxicity is more pronounced in cancer cells expressing Wnt growth factors. The toxicity of stilbenes in these ALL cells can be potentiated by the addition of autophagy inhibitors, suggesting a possible therapeutic application.

1. Background

The endoplasmic reticulum (ER) is the organelle in the cell responsible for the synthesis of membrane-embedded and secreted proteins. The ER is also responsible for the proper folding and modification of these proteins. If drugs or environmental conditions interfere with the processing of proteins in the ER, an ER stress is elicited and the cell responds with the Unfolded Protein Response (UPR) [1,2]. The UPR contains three arms: IRE1/XBP1, ATF6, and PERK/ATF4. Together, these three components reduce further protein entry into the ER, assist in folding proteins already in the ER, and remove malfolded proteins to decompress the enlarged ER [3]. If the ER stress persists, or the UPR is insufficient to decompress the ER, prolonged ER stress can result in cell death [4].

We have reported identification of a small molecule that can block the endoribonucease activity of IRE1 and therefore can block activation of the XBP1 arm of the UPR [5]. This molecule was identified through a small molecule screen using an XBP1-responsive luciferase reporter gene. This reporter requires XBP1 splicing to generate a frameshift in the XBP1-luciferase transcript so the luciferase gene is brought into frame and is translated [6]. In this manuscript, we describe the use of this reporter gene to identify novel drug-like agents that induce ER stress, from the library of

Abbreviations: ER, endoplasmic reticulum; UPR, Unfolded Protein Response; AO, acridine orange; LC3, microtubule-associated protein 1 light chain 3; ERAD, ER associated degradation; XBP1, X-box binding protein 1; IRE1, inositol-requiring enzyme-1; PERK, pancreatic eIF2 kinase; CHOP, C/EBP homologous protein 10; ATF4, activating transcription factor 4; LOPAC, library of pharmacologically active compounds; NIHCC, NIH clinical collection

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pharmacologically active compounds (LOPAC) and the NIH clinical collection (NIHCC). We extend these studies to examine the cellular response to the plant stilbenes resveratrol, pterostilbene and piceatannol which all scored as potent ER-stressors in this screen.

Previous work from our group also identified the Wnt family of secreted glycoprotein growth factors as heavily processed in the ER with significant sensitivity to ER-stressing agents like hypoxia or tunicamycin [7]. The processing of Wnt proteins can take up to 18 h in cells, and serve as a molecular marker of ER function. This extended transit time allows for correct intramolecular bonds from the 23–27 conserved cysteine residues, glycosylation, and lipidation [8]. Interference with Wnt processing can result in proteolysis of the growth factor, and reduction in b-catenin signaling [7].

2. Results and discussion

We decided to screen known drug like molecules for their "off target" ability to induce ER stress. We had constructed a reporter cell in the HT1080 fibrosarcoma background that contained a luciferase gene that was fused to the XBP1 open reading frame. In the event of ER stress, the ER-membrane embedded IRE-1 endoribonuclease performs a precise splicing event in the XBP1 mRNA that removes 26 nucleotides and shifts the open reading frame of the final one third of the protein to produce an active transcription factor [9]. In our XBP1-luciferase reporter gene, the luciferase coding sequence is in frame with the carboxy end of XBP1 open reading frame so that after XBP1 splicing, the luciferase protein is translated and its activity detected [5,6]. We also used a separate HT1080 clone that contained a constitutively active CMV-driven luciferase as a control for cell death or non-specific inhibition/activation of transcription or translation. These two cell lines were plated in duplicate in 96 well format and individual wells were exposed overnight to 10 μM of the compounds in the library. The next day, luciferase activity was measured and the relative induction of the XBP1 luciferase was calculated for each compound and normalized to any changes in the CMV-luciferase signal. We calculated the fold induction of the XBP1-luciferase activity for each compound and chose those with greater than 1.5 fold induction for secondary analysis. Secondary screen was performed in cells expressing the XBP1-luciferase, the CMV-luciferase, and also in cells expressing an ATF4UTR-luciferase reporter [10]. This reporter is responsive to activation of the PERK kinase and increased translation of the ATF4 gene.

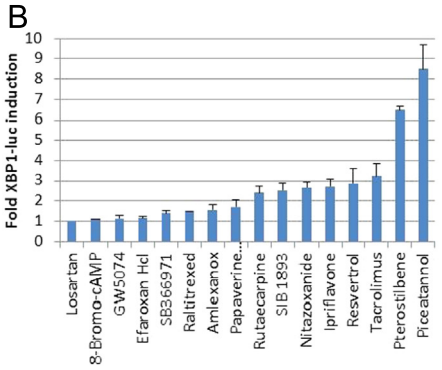
Fig. 1A identifies the 13 top inducers, and shows the normalized fold induction of the reporter genes from the primary screen. These compounds resulted in between 1.5 and 3.9 fold induction of the XBP1-luciferse reporter gene. These compounds were then retested manually in a secondary screen with the XBP1-luciferase and the CMV luciferase reporter HT1080 cells and the normalized results show a qualitatively similar, but significantly higher fold induction. Fig. 1C shows the effects of the same compounds on the ATF4UTR reporter gene in the second reporter HT1080 cell line. Interestingly, resveratrol and the closely related derivatives piceatannol and pterostilbene were all in the original list of ER-stress inducers, and all were near the top of the most potent inducers in the secondary screen. These results support the idea that there is some structural element(s) in these related compounds that interferes with ER function.

We next decided to molecularly confirm the ability of these compounds to stress the ER using western blot analysis to identify changes in markers of the UPR. Fig. 2A shows immunoblot analysis of extracts from parental HT1080 cells treated for 24 h with 20 μM resveratrol, pterostilbene or piceatannol as indicated. In the cells treated with pterostilbene or piceatannol, we detect both splicing of XBP1 and induction of the ATF4 target gene CHOP. We also find modest increase in LC3 processing, indicating a mild autophagic response. Because pterostilbene appeared to be the most potent of the related molecules in these two responses, we focused on its activities. Fig. 2B shows that with increasing time up to 20 h, that there was increasing XBP1 splicing and CHOP induction, indicating continuing ER stress. We also investigated how inhibition of compensatory pathways would affect pterostilbene induced stress. We tested the combination of pterostilbene with either IRE1 inhibitor STF-083010 to block XPB1 splicing [5], or chloroquine to block autophagic turnover. Fig. 2C shows that STF-083010 is indeed a good inhibitor of XBP1 splicing, but this leads to an increase in the PERK arm of the UPR and increased CHOP expression. Addition of chloroquine by itself had a modest increase in LC3 processing; however, LC3 processing was dramatically increased by chloroquine with pterostilbene and/or STF-083010. Finally we compared the ER stress induced by pterostilbene with that of classical ER stressing agents. Fig. 2D shows the induction of GRP78 in response to 24 h of either 20 μM pterostilbene, 2.5 μg/ml tunicamycin, 10 nM of the proteasome inhibitor MG-132, or 1.0 mM DTT. This representative western blot shows that pterostilbene is less able to induce ER stress than these classical ER stressing agents. However, ER stress is dose and time dependent, and these doses of classical ER stressors are somewhat more potent than 20 μM pterostilbene.

Pterostilbene has been reported to be toxic to cancer cells [11,12], so we measured its antiproliferative effect alone and in combination with chloroquine. We first measured the growth inhibition by chloroquine alone to establish a relatively non-toxic dose. Fig. 3A shows that 20 μM chloroquine has only a 30%

A

Drug name	Description	Fold Δ
efaroxam	Imidizoline antagonist	2.0
tacrolimus	mTOR inhibitor	1.6
GW5074	Raf kinase inhibitor	1.6
resveratrol	Sirt activator	2.6
piceatannol	Resveratrol derivative	2.3
pterostilbene	Resveratrol derivative	1.9
nitazoxanide	Anti-protozoal	2.3
Losartan	Hydrochlorothiazide diuretic	1.5
papavarine	Phosphodiesterase inhibitor	1.6
8Br-cAMP	cAMP analogue	1.5
ipriflavone	Calpain inhibitor	2.3
SIB1893	Glutamate receptor agonist	3.1
SB366971	Vanillanoid receptor agonist	2.9
rutaecarpine	K+ channel blocker	3.9



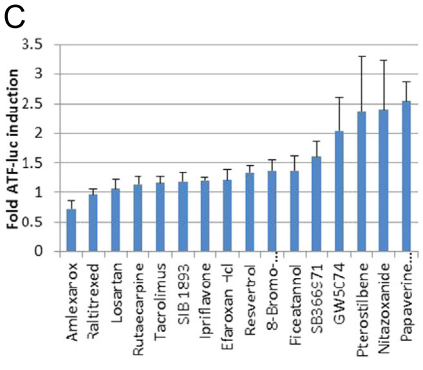


Fig. 1. Identification of small molecules that induce ER stress. Panel A: Results from the primary screen for normalized induction of the XBP1-luciferase reporter gene in cells treated for 24 h at 10 μM of the indicated drug. Panel B: Fold induction of the XBP1-luciferase reporter gene after 24 h of treatment with 20 μM of the indicated compound in manually performed secondary screen of top hits from primary screen. Panel C: Fold induction of the ATF4-luciferase reporter gene in manually performed secondary screen as described in B.