IRE1α is critical for kaempferol induced neuroblastoma differentiation

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Abstract:

Neuroblastoma is an embryonic malignancy arises out of the neural crest cells of the sympathetic nervous system. It is the most common childhood tumor and well known for its spontaneous regression via the process of differentiation. The induction of differentiation using small molecule modulators such as all trans retinoic acid is one of the treatment strategies to treat the residual disease. In this study, we have reported the effect of kaempferol, a phytoestrogen in inducing differentiation of neuroblastoma cells *in vitro*. Treatment of neuroblastoma cells with kaempferol reduced the proliferation and enhanced apoptosis along with the induction of neuritogenesis. Analysis of the expression of neuron specific markers such as β III tubulin, neuron specific enolase and NRDG1 (N-myc down regulated gene 1) revealed the process of differentiation accompanying kaempferol induced apoptosis. Further analysis on understanding the molecular mechanism of action showed that the activity of kaempferol happened through the activation of the endoribonuclease activity of IRE1α (Inositol requiring enzyme 1 alpha), an endoplasmic reticulum (ER) resident transmembrane protein. The *in silico* docking analysis and biochemical assays using recombinant human IRE1α confirms the binding of kaempferol to the ATP binding site of IRE1α and thereby activating ribonuclease activity. Treatment of cells with the small molecule inhibitor STF083010 which specifically targets and inhibits the endoribonuclease activity of IRE1α showed reduced expression of neuron specific markers and curtailed neuritogenesis. The knock down of IRE1α using plasmid based shRNA lentiviral particles also showed diminished changes in the change in morphology of the cells upon kaempferol treatment. Thus our study suggests that kaempferol induces differentiation of neuroblastoma cells via the IRE1α -XBP1 pathway.

1. Introduction:

Neuroblastoma the most common solid extra cranial malignant tumor of infants originates from the precursor cells derived from neural crest tissues (Duckett and Koop, 1977; Hoehner et al., 1996). The clinical representation of the disease varies drastically from a mass with no symptoms to a primary tumor causing critical illness by disseminating into the neck, thorax, abdomen or pelvis and stands as the most commonly diagnosed tumor in early childhood (Brodeur, 2003). The heterogeneity of neuroblastoma tumors makes intense chemoradiotherapy as a therapeutic strategy. Therefore, for minimizing the effect of chemoradiotherapy, alternative therapeutic strategies targeting crucial proteins involved in the tumor progression is needed. The derivatives of retinoic acids are widely used clinically after a cytotoxic therapy as differentiating agents for controlling minimal residual disease in high risk neuroblastoma patients (Matthay et al., 1999).

Retinoic acid, a derivative of vitamin A has been shown to promote differentiation of embryonic, neural and mesenchymal stem cells (Jones-Villeneuve et al., 1982; Takahashi et al., 1999; Wichterle et al., 2002; Zhang et al., 2006). All trans retinoic acid (ATRA) (Watabe et al., 2002), 9-cis RA and 13-cis RA (Chow et al., 1991) are the well-known RA isomers that have been shown to induce differentiation and used in the treatment of neuroblastoma . Resistance to retinoic acid therapy is commonly observed *in vitro* as well as clinically, due to the mutations in RAR gene or RAR receptor (Pemrick et al., 1994). In addition, trichostatin A, inhibitor of HDAC, kenpaullone and SB- 216763 has also been reported to induce neuritogenesis *in vitro* (Balasubramaniyan et al., 2006; Halder et al., 2015; Lange et al., 2011). Recent studies reported alectinib (Anaplastic Lymphoma Kinase inhibitor) and YK-4-279 (mitosis disruptor) as promising candidates for neuroblastoma treatment by emphasizing the lack of effective therapies at present in clinics (Kollareddy et al., 2017; Lu et al., 2017). Inhibitors of HDAC8, Sphingadienes (a growth inhibitory sphingolipid from soy) and p-dodecylaminophenol have been recently reported to have therapeutic efficacy by reducing tumorogenesis *in vitro* and *in vivo* in mice models (Rettig et al., 2015; Takahashi et al., 2017; Zhao et al., 2018b).

IRE1α is an ER resident protein involved in the UPR pathway. It has a luminal N-terminal domain inside ER lumen and a two functional enzymatic cytoplasmic domains having kinase and endoribonuclease activity. The activation IRE1α by the accumulation of misfolded proteins or by other physiological stimuli not limiting to lipid perturbation, viral infection or immune response (Abdullah and Ravanan, 2018b) which leads to the initiation of its kinase or endoribonuclease activity. The kinase activity is known for the recruitment of TRAF2 protein thereby the activation of JNK (c-Jun N-terminal kinase) mediated signalling cascade driving cells towards an apoptotic response is elicited (Urano et al., 2000). The ribonuclease activity of IRE1α can cleave multiple RNA substrates involved in diverse cell response pathways with Xbp1 mRNA being the major substrate (Niwa et al., 2005). Spliced form of Xbp1 mRNA get translated into an active transcription factor XBP1s and involved in the transcription of variety of genes (Acosta-Alvear et al., 2007a). The mouse embryos lacking IRE1α died after 11.5-14.5 days post-

coitum emphasizing the role of IRE1α in the developmental stages of the organism (Iwawaki et al., 2009). Moreover, the IRE1α-XBP1 pathway of gene activation is known to be a critical factor involved in the differentiation of plasma cells, osteoblast and adipocytes (Reimold et al., 2001a; Sha et al., 2009a; Tohmonda et al., 2011).

Kaempferol is a phytoestrogen belongs to the class of flavonoids and known to exhibit antioxidant, anti-inflammatory and anti-cancer activity (Wang et al., 2018). In our attempt for identifying an inhibitor against ER stress induced cell death, we found kaempferol to rescue mammalian cells from apoptosis induced by multiple cell death inducers by acting as an allosteric inhibitor for executioner caspases (Abdullah and Ravanan, 2018a). While performing the experiments we observed the change in morphology of IMR32 cells, a human neuroblastoma cell line with neurite like outgrowths upon prolong incubation in the presence of kaempferol.

In this study, we have investigated the effect of kaempferol on IMR32 and Neuro2a neuroblastoma cell lines. Observations made in our experiments suggest that kaempferol mediated differentiation of neuroblastoma cells happens via the activation of IRE1α endoribonuclease activity. Since Kaempferol is a dietary polyphenol and cohort studies had been performed for understanding its effect on various other cancers (Cui et al., 2008; Gates et al., 2009; McCann et al., 2005), it could also be explored for its effect on neuroblastoma in clinical trials.

2. Materials and Methods

2.1 Cell culture and reagents

IMR32, human neuroblastoma cell line and Neuro2A, mouse neuroblastoma cells were obtained from NCCS, Pune, India. Cells were maintained in DMEM containing 10% FBS (Himedia), 1X glutamine and 1X antibiotic and antimycotic solution at 37^{o}C and 5%CO_2. Kaempferol (HPLC purity≥98.8%) was obtained from Natural remedies (Bangalore, India). Diarylpropionitrile (DPN) and 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*] pyrimidin-3-yl] phenol (PHTPP) were obtained from Alfa Aesar (Hyderabad, India). APY29 was purchased from MedChem express, USA. STF083010 was purchased from Cayman chemicals, USA.

2.2 Cell viability assay

MTT assay was performed for analyzing the cytotoxic effect of kaempferol on IMR32 and Neuro2A cell lines. Briefly, 0.5 x 10^4 cells were seeded per well in a 96 well plate and treated with kaempferol for 48 h, 72 h and 96 h. After the desired incubation time, 20 µl of 5 mg/ml MTT (Himedia) was added to each of the well and incubated for 3 hours. The terazolinium salt formed by the live cells was dissolved by adding 200 µl of DMSO, after the removal of media. The purple color formed was read at 570 nM using ELISA plate reader.

2.3 Cell proliferation assay

For trypan blue dye exclusion assay, 0.5 x 10^5 cells per well was seeded in a 24 well plate. Cells were treated with kaempferol (25, 50 and 100 µM), ATRA (25 µM) and DMSO (vehicle) for a period of 48 h, 72 h and 96 h. Cell counting was done manually using hemocytometer, by staining the cells with trypan blue solution prepared in 0.4% methanol. Live cells that did not take up the dye were counted to quantitate the rate of proliferation under control and treated conditions.

2.4 Differentiation experiments

For differentiation experiments, neuroblastoma cell lines were seeded at a density of 3x10^5 cells in 60mm dish and treated with 50 µM of kaempferol. Induction was given once for a continuous time of 96 hours without any replacement of media. ATRA at a concentration of 25 µM was used as a positive control to compare the morphological changes; images were taken using phase contrast microscope at specified time points.

2.5 Stable lentiviral transductions

The IRE1α (sc-40705-V) shRNA lentiviral particles was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) contain expression constructs encoding target-specific shRNA designed to specifically knock down human IRE1α gene expression. Scrambled shRNA contain lentiviral particles (sc-108080; Santa Cruz Biotechnology) that will not lead to specific degradation of any cellular mRNA was used as negative control. Lentiviral transduction was performed in the presence of polybrene (5 µg/ml, Sigma-Aldrich), and stable transductants were generated via selection with puromycin (1 µg/ml, Sigma-Aldrich) according to the manufacturer's instructions.

2.6 Immunocytochemistry and microscopy

Immunocytochemistry was carried out as described in the Cell signaling Technology (CST) guide. Briefly, 2.5 x 10^4 cells/well were seeded in a 24 well plate. After overnight incubation, cells were treated with the compounds for indicated time. The cells were then washed with 1XPBS and fixed with 4% Formaldehyde for 15 min. The cells were washed again with 1XPBS and incubated with 0.5% skimmed milk containing 0.3% tritonX100 for blocking nonspecific binding, for 1 hour. Immunostaining was done using β-III tubulin (CST #5568) and Synaptophysin (Novus #NB2-25170SS) specific primary antibodies at 4^0C with overnight incubation followed by 1 hour incubation with the secondary antibody, anti-rabbit Alexa Flour 594 (#8889, Cell Signaling Technologies, USA). Phase contrast and fluorescence images of cells under treated and non-treated conditions were captured using EVOS FLoid imaging station (Thermo Fisher, USA) with 20X fluorite objective and LED light cubes containing hard coated filters (blue and red). For a particular protein expression study, imaging parameters were kept constant throughout the imaging and no image modifications were done post imaging.

2.7 Gene expression analysis

Total RNA isolation was performed using RNA isoplus (Takara, India). RNA quantification was performed and 2 µg of total RNA was used for cDNA conversion using Primescript RT reagent kit (Takara, India). Quantitative-PCR (qRT-PCR) was

performed using SYBR green reagent (Takara, India) using Applied Biosystems Quant studio 3 real time PCR machine. Human 18s rRNA was used as an internal control for normalizing the gene expression. The fold induction of mRNA levels was calculated by $2^{-\Delta\Delta CT}$ *method*. The list of primers used in this study is tabulated (Table 1).

2.8 Immunoblotting

Immunoblotting was carried out as described earlier by Ravanan et al (Ravanan et al., 2011). In brief, cells were plated in 60 mm dishes at the density of 1.5 x 10^5 cells/dish. After overnight incubation, cells were treated with mentioned compounds. At 24 ,48, 72 and 96 h time points after the treatment, cells were washed with ice cold PBS and lysed with RIPA buffer containing 1X Protease inhibitor cocktail (Sigma Aldrich). Total protein concentrations were quantified by Folin's assay. Immunoblotting analysis was done by loading 50 µg of protein in each lane for SDS-PAGE. Antibodies used in this study were obtained from Cell Signalling Technology, USA (Beta actin#8457, N-myc#9405, IRE1α#3294 and XBP1s#12782), Novus biological, USA (NDRG1#NBP1-32074, ERα#NB120-3577 and ERβ#NB100-92166) and ABclonal, USA (NSE #A3118 and GRP78 #A0241). Anti-rabbit, HRP linked secondary antibody from Cell Signalling Technology (#7074) was used for chemiluminescence based detection.

2.9 Methylene blue staining and neurite counting

Cells were seeded in 35 mm dish at a density of 1.5 x 10^5 cells and differentiation was induced with kaempferol (50 µM) or APY29 (250 nM). Wherever STF083010 (50 µM) is used as inhibitor, cells were pre-treated with STF083010 for 90 min and then treated with the mentioned test compounds. After 96 h of incubation, cells were fixed using 4% formaldehyde for 15 min and stained using 0.2% methylene blue solution in methanol for 30 min. After repeated washes for unbound stain removal, images were taken using monochrome phase contrast camera of EVOS FLoid imaging station (Thermo Fischer). Total number of cells and cells bearing 2 or more neurites were counted for 10 random focuses and represented as percentage of cells in the graph (Chaudhari et al., 2017b).

2.10 Kinase inhibition studies

Recombinant human IRE1α enzyme was obtained from SignalChem (#E31-11G). ADP Glo kinase assay (Promega) was performed as per the manufacturer's protocol to confirm the inhibitory mechanism of kaempferol. Briefly, 50 ng of enzyme was used with 10 µM or 100 µM of ATP with different concentrations of kaempferol in a white flat bottom 96 well plate. Staurosporine (Sigma Aldrich) a pan kinase inhibitor was used as positive control. The luminescence produced by the conversion of dephosphorylated ADP to ATP was measured using the luminometer (Berthold).

2.11 *In silico* docking analysis

Studies to understand the mode of binding of kaempferol to IRE1α was carried out using AutoDock suite 4.0. Crystal structure of the human IRE1α bound with ADP (PDB ID: 3P23) was used as the target protein. Ligands were obtained from Pubchem as .sdf format and converted to .pdb using smiles translator. Polar hydrogen atoms were

assigned for the macromolecule prior docking. *Gasteiger* charges for ligands and Kollman charges for the receptor molecule was added using AutoDock tools 1.5.6. Throughout the study, the macromolecule was kept rigid with rotatable bonds assigned for the ligands. The grid center made on the macromolecule covering the entire surface of the protein acted as search space. AutoGrid 4.0 was used to produce the map files of the flexible atoms and docking parameter file (DPF) was generated using Lamarckian Genetic algorithm of AutoDock 4.0. Validation of the docking study was carried out by re-docking the ADP with the solved crystal structure of kinase domain of IRE1α. The re-docked complex was compared to the known crystal structure of the ligand-macromolecule complex. Binding energies of the best docked pose of the docked complexes were calculated based on torsional energy, H-bonding, non-bonded interactions and desolvation energies. LigPlot+ v. 1.4 was used to study the interactions at 2D level while UCSF Chimera 1.10.2 and PyMol V. 1.7.4 were used in visualizing 3D interactions.

2.12 Statistical analysis

All data are presented as the Standard error of mean (S.E.M) of at least three independent experiments. Statistical comparisons were performed using one-way/two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPad Prism, version 5.0) and p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Kaempferol induces neuroblastoma differentiation and decreases cell viability

To understand the effect of kaempferol on neuroblastoma cells, we employed IMR32 and Neuro2a cell lines, well-established in vitro models that have been used widely for understanding neuroblastoma differentiation. IMR32 and Neuro2a cells were incubated in the presence of kaempferol (50 μM) for 4 days, with the treatment in the presence of ATRA (25 μM) served as a positive control. After 48 h of incubation, we observed morphological changes including neurite outgrowth associated with decreased proliferation and viability. To confirm whether the neurite outgrowth is due to cellular differentiation, we performed immunocytochemistry to analyze the expression of β-III tubulin, a neuronal differentiation marker. The significant increase in the expression level of β-III tubulin in the kaempferol and ATRA treated cells compared to the controls confirmed the neurite outgrowth to be due to the cellular differentiation (Fig. 1A). A further assessment on the viability of the cells using MTT assay and trypan blue dye exclusion assay revealed that kaempferol decreased the viability of both IMR32 and Neuro2a treated cells (Fig. 1B and Supplement 1A and B). An additional quantitation of the number of cells bearing 2 or more neurites indicated that an incubation period of 4 days is enough for kaempferol to effectively induce a significant change in the morphology (Fig. 1C and D), while in ATRA treated cells there was lesser morphological changes in terms of neurite extensions when compared to kaempferol treated cells. ATRA had been shown to require 10-14 days for a significant change in morphology

(Pence and Shorter, 1990), while kaempferol induced it in 4 days of treatment. Complementarily, we observed an increase in the mRNA and protein levels of certain neuron specific markers genes like synaptophysin (*SYP*), neuron specific enolase (*NSE*) and N-myc down regulated gene 1 (*NDRG1*) (Fig. 2A and B). Although the mRNA transcripts of *NSE* and *NDRG1* in ATRA treated condition didn't show significant up-regulation, the protein levels were up-regulated during day3 and day4 of differentiation. The expression of N-myc, at mRNA level, was down-regulated in kaempferol treated conditions while it was significantly increased in ATRA treated conditions (Fig. 2A). In contrast, the down- regulation of *N-myc* observed at the mRNA levels was not exactly the same at the protein level as we observed no such drastic reduction in N-myc protein levels (Fig. 2B). The reason for this could be the accumulation of N-myc protein due to protein stabilization rather than transcription of the gene (Choi et al., 2010). Moreover, the increase in the expression of N-myc protein is shown to be a required criterion for the onset of differentiation (Guglielmi et al., 2014). However, we observed an increase in NDRG1 expression levels with a decrease in the expression level of N-myc protein between day 2 and day 4 (Fig. 2B); a phenomenon reported to occur during neuroblastoma differentiation (Kang et al., 2006). Altogether, our data suggest that kaempferol promotes differentiation and cell death in neuroblastoma cells.

3.2 Kaempferol induced differentiation is independent of the estrogen receptor signaling

It has been previously reported that kaempferol as a phytoestrogen (estrogen receptor agonist) suppresses the proliferation of breast cancer cells and promotes apoptosis (Kim and Choi, 2013). The use of antagonist against estrogen receptors had shown to reverse the biological activity of kaempferol (Wang et al., 2009). Since, ERβ signaling has been shown to be essential for kaempferol mediated biological responses (Kuiper et al., 1998) we examined the expression of ERα and ERβ in IMR32 cells. In our study, we observed that kaempferol substantially instigates the expression of estrogen receptor β (ERβ) compared to the estrogen receptor α (ERα) both at mRNA and protein level in IMR32 cells (Fig. 3A and B). Later on, intending to investigate whether ERβ signaling is involved in the modulation of kaempferol induced neuroblastoma differentiation, we used PHTPP (4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol), a selective ERβ antagonist to find out if PHTPP pretreatment interferes with the differentiation process. Interestingly, pretreatment of kaempferol treated cells with PHTPP had no effect both on the formation of neurites and β-III tubulin expression suggesting that kaempferol induced differentiation is independent of ERβ signaling (Fig. 3C). Since there was a basal expression of ERα was observed in IMR32 cells upon kaempferol pretreatment in our study and Wang et al., 2009, have used tamoxifen to reverse the activity of kaempferol (Wang et al., 2009). Therefore we performed an experiment with pretreatment of IMR32 cells with tamoxifen at different concentrations. The pre-treatment of IMR32 cells with tamoxifen an ERα antagonist also did not show any observable effects on the process of differentiation induced by kaempferol (data not shown). In addition, Diarylpropionitrile (DPN), another ERβ agonist, failed to induce differentiation of IMR32 cells. Moreover, pretreatment of

PHTPP did not reverse the cell death induced by kaempferol as well (Fig. 3D). Collectively, these findings suggest that kaempferol induced neuroblastoma differentiation and cell death is not mediated by ERβ signaling.

3.3 Kaempferol modulates the expression of IRE1α

Endoplasmic reticulum (ER) stress is known to be associated with various cellular differentiation processes (Ma et al., 2010; Matsuzaki et al., 2015; Wielenga et al., 2015) and kaempferol has been known to induce ER stress in certain cancer cell lines (Guo et al., 2017; Guo et al., 2016; Huang et al., 2010). These research findings prompted us to check the expression level of ER stress marker genes such as *BiP, PERK, IRE1α and ATF6* in kaempferol treated IMR32 cells. The results presented in Fig. 4A show that kaempferol significantly increased the expression of *IRE1α,* while that of the other ER stress marker genes was not significantly changed with time (Fig. 4A). IRE1α is an ER transmembrane sensor of unfolded protein response pathway (UPR) with both kinase and endoribonuclease activity (Bork and Sander, 1993). During ER stress, IRE1α activation initiates the splicing of XBP-1 (X-Box binding Protein-1) mRNA which in turn translated into XBP1s protein and involved in transcription of diverse set of genes including genes involved in the UPR pathway (Acosta-Alvear et al., 2007a). Previous studies have reported the involvement of IRE1α-XBP1 pathway in the differentiation of plasma cells (Todd et al., 2009), osteoclasts (Tohmonda et al., 2013) and adipocytes (Sha et al., 2009b) in addition to the unfolded protein response pathway. This led us to hypothesize whether kaempferol might induce the neuroblastoma differentiation by modulating the IRE1α-XBP1 pathway. We then performed protein analysis of IRE1α for kaempferol treated cells and compared it with brefeldin A (a known inducer of ER stress) treated cells. Our results indicated that kaempferol increased the expression of IRE1α but not BiP at protein level. BiP is a molecular chaperone whose increased level is considered as a hallmark of the ER stress process. In contrast, brefeldin A treatment up regulated the levels of IRE1α as well as BiP protein level indicating the onset of strong ER stress response (Fig. 4B). We have previously showed the bioactivity of kaempferol in ameliorating ER stress induced cell death with a reduction in the level of ER stress markers expression in kaempferol pretreated conditions in IMR32 cells (Abdullah and Ravanan, 2018a). Our present study showed that kaempferol increases the expression of IRE1α without activating ER stress in IMR32 cells.

3.4 Activation of IRE1α endoribonuclease activity by kaempferol

Quercetin, a flavonoid, has been reported to bind to the Q-site in human-yeast chimeric IRE1 and activates endoribonuclease activity to enhance the cleavage of XBP1 mRNA (Wiseman et al., 2010). Kaempferol also belongs to the flavonoid class of phytocompounds. Considering this, we employed computational docking studies using Autodock to check if kaempferol binds to human IRE1α molecule. Blind docking was performed by selecting the complete surface of the protein molecule. The result showed the kaempferol's binding affinity towards the kinase pocket of IRE1α (Fig. 4C). The binding of kaempferol was compared to bound states of indigenous activator of IRE1α, ADP and APY29 (known IRE1 kinase inhibitor and endoribonuclease activator) (Fig.

4D). The bound state of ADP in the IRE1α nucleotide binding site is known to activate the endoribonuclease activity of the molecule (Lee et al., 2008). The docked poses analyzed for amino acid residues occupying the bound state of kaempferol showed that the binding of kaempferol to the kinase pocket of the molecule share the similar amino acid residues for interactions as of ADP and APY29 (Fig. 4D). The interactions of kaempferol with Glu 643, Cys 645 and His 692 residues of human IRE1α had been shown for kinase activity inhibition by staurosporine, a pan kinase inhibitor (Concha et al., 2015). APY29 has been known to interact with amino acid residues of kinase domain to activate the endoribonuclease activity of IRE1α (Korennykh et al., 2009). Moreover, the interaction with Cys 645 at the kinase cleft has been reported for APY29 to activate the IRE1α endoribonuclease activity (Wang et al., 2012). The conformation of DFG (Aspartic acid- Phenylalanine-Glycine) motif of kinase domain in IRE1α with an activator for endoribonuclease activity should be in DFG-in conformation rather than DFG-out (Wang et al., 2012). The ADP bound crystal structure of IRE1α (PDB ID: 3P23), with DFG-in motif showing favorable binding affinity for docking with kaempferol suggests its ability to modulate the IRE1α endoribonuclease activity. Moreover, docked pose of kaempferol falls into the same pocket where ADP and APY29 are known to interact with the kinase domain of IRE1α (Fig. 4E). Collectively, the docking analysis showed that kaempferol binds to the kinase domain of IRE1α and could activate the endoribonuclease activity.

To confirm the docking results further, ADP-Glo kinase assay (Promega) was performed using recombinant kinase active human IRE1α. Incubation of kaempferol with IRE1α showed a dose dependent decrease in the kinase activity of IRE1α (Fig. 4F), confirming its binding to the kinase domain of the protein and thereby inhibiting IRE1α's kinase activity. The kinase inhibition assay performed by increasing the concentration of the substrate ATP from 10 μM to 100 μM, suggested a non-competitive mode of inhibition favored by kaempferol (Fig. 4G), which again correlates with the previously reported weak binding of quercetin (Wiseman et al., 2010) to the nucleotide binding site of human-yeast chimeric IRE1 protein.

3.5 IRE1α knockdown reduces neuroblastoma differentiation

To further confirm the role of IRE1α-XBP1 pathway in neuroblastoma differentiation, we established stable IRE1α knock down clones of IMR32 cells using lentiviral particles containing IRE1α shRNA (4 target specific constructs that encodes 19-25 nt shRNA). Successful knock-down of IRE1α at mRNA and protein level was confirmed by qRT-PCR and immunoblotting respectively (Fig. 5A and B). The functional involvement of IRE1α in neuroblastoma differentiation is confirmed, when neither of kaempferol and APY29 exposure triggered β-III tubulin expression in IRE1α knockdown IMR32 cells (Fig. 5C). Additionally, to compare the expression of neuronal differentiation markers (NDRG1 and NSE) between IRE1α knockdown cells and control shRNA transduced cells, we performed immunoblotting analysis. We observed a decrease in the expression level of these markers in IRE1α knockdown cells when compared to the control shRNA

transduced cells (Fig. 5D). Also we noticed a reduction in the XBP1s protein level in IRE1α shRNA treated conditions on day4, with a reduced level of differentiation observed. To confirm the activity of endoribonuclease activity in inducing the differentiation of neuroblastoma cells, we inactivated the endoribonuclease activity of IRE1α by using STF083010, a small molecule inhibitor. STF083010 is a specific inhibitor which binds to the endoribonuclease domain of IRE1 and inhibits the cleavage of XBP1 mRNA, while the kinase activity is left intact (Papandreou et al., 2011). The extent of differentiation repression in IRE1α knockdown cells, in the presence of kaempferol or APY29 was further exemplified by the addition of STF083010 (the IRE1 endoribonuclease inhibitor), to these cells (Fig. 5E). Similarly, the number of cells with 2 or more neurites was significantly less in IRE1α knockdown condition upon kaempferol or APY29 treatment, which furthermore decreased upon pretreatment with STF083010 (Fig. 5F). These results validate the role of IRE1α's endoribonuclease activity in kaempferol induced neuroblastoma differentiation.

3.6 XBP1s drives differentiation of neuroblastoma cells

Activated IRE1α splices the *XBP1* mRNA which then gets translated into an active transcription factor that regulates diverse set of genes involved in the regulation of various cellular processes including genes related to ER stress, differentiation, cell cycle arrest and immune response (Acosta-Alvear et al., 2007b). The ratio of spliced to unspliced form of XBP1 mRNA corresponds to the extent of IRE1α activation. Small molecule modulators binding to the nucleotide binding site of IRE1α can induce the endoribonuclease activity in the absence of ER stress (Han et al., 2009).

Therefore, we inclined to check the expression level of spliced *XBP1* mRNA by qRT-PCR. IMR32 cells were treated with kaempferol or brefeldin A. Comparing the mRNA expression of *XBP1* unspliced to spliced form, we observed an enhanced presence of XBP1 spliced mRNA levels upon kaempferol treatment. The increase in the expression of *XBP1* (unspliced) mRNA correlates with the enhanced expression of ATF6 (Tsuru et al., 2016). Whereas, this was not the case for brefeldin A treated cells as they displayed a decreased expression level of XBP1 unspliced form (Fig. 6A). To confirm that XBP1s are transcriptionally active, we analyzed its downstream targets such as *SEC63, PDIA2, PDIA3, DNAJC3, ERDJ4 and ERDJ5* mRNA level by qRT-PCR, whose regulation by the transcription factor XBP1s was validated before (Zhao et al., 2018a). These downstream targets of XBP1s transcription factor were not selected randomly; they have certain role in the event of cellular differentiation. High *SEC63* expression had been shown to have involvement in apoptosis and reduced proliferation in hepatic tumors (Casper et al., 2013); ERDJ4 has been shown to have crucial role in B cell differentiation in the event of hematopoiesis (Fritz and Weaver, 2014). Besides, *DNAJC3* is known to get increased during plasma cells differentiation (Shaffer et al., 2004); the expression of *PDIA3* and *DNAJC3* were shown to get up regulated during T-helper cell differentiation and treatment with IRE1α's endoribonuclease activity specific inhibitor 4μ8c down regulated their expression via inhibiting *XBP1* mRNA splicing (Pramanik et al., 2017). Our results suggested that expression of all these genes increased in kaempferol treated conditions,

and get reversed by pretreatment with STF083010 (Fig. 6B). These results confirm that kaempferol activates XBP1s transcriptional activity by activating the endoribonuclease activity of IRE1α and STF083010 blocks the transcriptional activity induced by kaempferol by inhibiting XBP1 mRNA cleavage (Fig. Supplement 2A). Furthermore, we proceeded to check whether APY29, an activator of IRE1α endoribonuclease activity could induce the differentiation of IMR32 cells similar to kaempferol. The western blot analysis of APY29 treated cell lysates showed an increased expression of neuronal differentiation markers similar to that of kaempferol treated conditions (Fig. 6C). The induction of differentiation by APY29 observed in Neuro2a cells was similar to kaempferol treated conditions. There was a marked increase in the β-III tubulin expression and synaptophysin vesicles observed through immunocytochemistry in Neuro2a cells (Fig. Supplement 2B and C). Taken together, these results suggest that kaempferol activates the IRE1α-XBP1 branch pathway to induce the neuroblastoma differentiation. In addition, we analyzed if IRE1α-XBP1 pathway is involved in inducing neuroblastoma differentiation by other agents like CDDO and ATRA (Chaudhari et al., 2017b). However, we found that pre-incubation of cells with STF083010 did not inhibit differentiation induced by CDDO or ATRA (Fig. Supplement 3A and B) suggesting that IRE1α activation is not the only pathway involved in neuroblastoma differentiation. STF083010 (50 μM) did not have cytotoxic effect in IMR32 cells; pretreatment with STF083010 followed by kaempferol treatment also did not modulate cell death (Fig. 6E) suggesting that cell death induction by kaempferol is independent of IRE1α-XBP1 pathway. However, the immunocytochemistry analysis for the expression of β-III tubulin showed reduced differentiation upon STF083010 pre-treatment, in both kaempferol and APY29 treated conditions (Fig. 6D). And also, there was reduced change in morphology and decrease in the number of cells bearing neurites with STF083010 pre-treatment in the presence of kaempferol or APY29 (Fig. 6F and G). Altogether, these results suggest that STF083010 inhibits differentiation but not the cell death induced by kaempferol and APY29. Therefore, the activation of endoribonuclease activity of IRE1α followed by the XBP1 mRNA splicing holds a crucial role in inducing the differentiation of neuroblastoma cells. This could be the reason for the morphological changes and neurite outgrowths observed earlier in other neuroblastoma cell lines upon treatment with flavonoids (Brown et al., 1998a; Sato et al., 1994a).

4. Discussion

Although several studies have been done in improving the prognosis of high risk neuroblastoma with differentiation therapies, the survival rate of patients remains considerably poor (Berthold et al., 2005; Matthay et al., 2009; Simon et al., 2011). The implementation of chemotherapeutic approaches with synthetic molecules also adds up to the detrimental effects on the patients. Therefore an enhanced understanding of differentiation pathways and novel therapeutic approaches are needed. An approach using dietary compounds or plant metabolites could be a better alternative for reducing the damaging effects of synthetic drugs. Retinoic acid and its derivatives are well explored for their effects in inducing differentiation of neuroblastoma cells in clinical therapies. Other than RA, there are reports on the activity of 17β-estradiol in enhancing

the neuritogenesis in neuroblastoma cells, *in vitro* (Mérot et al., 2005). Flavonoids, being known for their anticancer activity, mimic estrogen in physiological systems, while some of them have been reported to induce neuritogenesis in neuroblastoma cells *in vitro* (Brown et al., 1998b; Sato et al., 1994b). Kaempferol had been reported before for its anticancer activity via multiple cellular pathways which includes triggering of ER stress, increasing the expression of DR5 receptor, via estrogen receptor expression and suppressing signal regulated kinases (Guo et al., 2017; Luo et al., 2012; Wang et al., 2013; Zhao et al., 2017).

The role of IRE1α in differentiation of multiple cell types shows its involvement in deciding the fate of cells. *XBP1* mRNA, the major substrate for the IRE1α endoribonuclease activity, had been shown to transcribe genes that are involved in multiple cellular processes, including the genes involved in differentiation of numerous cell types (Cho et al., 2013; Iwakoshi et al., 2003; Reimold et al., 2001b). The role of quercetin, a flavonoid, in activating the endoribonuclease activity of IRE1α gave hint on exploring the anticancer activity of kaempferol in the aspects of IRE1α-XBP1 pathway in the differentiation of neuroblastoma cells (Wiseman et al., 2010). Moreover the inactivation of IRE1α endoribonuclease activity and the hypomorphic expression of XBP1 in intestinal stem cells had been shown to be the reason for intestinal tumorogenesis (Niederreiter et al., 2013).

The difference in the gene expression pattern between kaempferol and ATRA treated conditions shows that they both could activate different pathways for inducing neuroblastoma differentiation. CDDO a triterpenoid was shown to induce differentiation of IMR32 cells via PPAR signalling pathway while ATRA via CREB dependent pathways (Chaudhari et al., 2017a). The difference between the mRNA and protein expression of NSE observed in the ATRA treated condition had been observed in other studies as well. The differentiation of SK-N-DZ cells by overexpressing the Aryl hydrocarbon receptor (AHR) showed no change in the regulation of NSE at transcript levels (Wu et al., 2014). The studies using retinoic acid in IMR32 cell line showed down regulation of NSE mRNA levels at day 5 of differentiation(Maresca et al., 2012). These data suggests that the transcripts of neuronal markers might be regulated via post-transcriptional regulatory mechanisms. Apart from the activation of IRE1α -XPB1 pathway, critical role of activation of other signalling pathways such as cMAP and P38 MAPK pathway (Monaghan et al., 2008), JNK-MAPK pathway and PPAR signalling pathway (Chaudhari et al., 2017a) have been reported to have a critical role in inducing the differentiation of neuroblastoma cells.

In summary, the current study demonstrated the involvement of IRE1α in differentiation of the neuroblastoma cells. The modulation of IRE1α in neuroblastoma cells by kaempferol or by specific modulator entitled for activating the endoribonuclease activity of IRE1α showed enhanced cell death, proliferation repression coupled to differentiation with increase in neuronal marker expressions, an expected outcome for neuroblastoma therapies. The results presented herein demonstrate that kaempferol induces differentiation and cell death of neuroblastoma cells. Our study reporting the differentiation and cell death elicited by kaempferol might suggest new therapeutic

approach via modulation of IRE1α for the treatment of neuroblastoma, the infuriating childhood malignancy.

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Author Contributions

P.R. designed the experiments and A.A. performed all the experiments. Data analysis and manuscript preparation was done by P.R., A.A and PT.

Conflict of interest

No potential conflicts of interests were disclosed by the authors.

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Figure legends:

Figure 1: Kaempferol induces differentiation of neuroblastoma cells

A. Immunocytochemistry images showing the expression of β III tubulin expression in IMR32 and Neuro2a cells treated with kaempferol (KFL) and ATRA for a time period of 96 h. Representative images of experiments performed more than three times. Scale bars = 67 μm.

B. Cell death analysis using MTT cell viability assay with Kaempferol (KFL) 50 μM and ATRA 25 μM. The average ±SEM from 3 independent experiments performed in triplicates. (**p < 0.01; ***p < 0.001; two-way ANOVA)

C. Phase contrast images showing the change in morphology and neurite outgrowth with Kaempferol (KFL) and ATRA treatment in IMR32 and Neuro2a cells for 96 h. Representative images of experiments performed more than three times. Scale bars = 67 μm.

D. Graphs representing the number of cells bearing 2 or more neurites in IMR32 and Neuro2a cells after 96 h of incubation with Kaempferol (KFL) or ATRA. The average ±SEM from 10 independent counting is shown. (*p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA)

Figure 2: Kaempferol enhances the expression of differentiation markers in IMR32 cells

A. Graphs representing the mRNA expression of synaptophysin (SYP), Neuron specific enolase (NSE), NDRG1 and N-myc genes upon Kaempferol (KFL) and ATRA treatment

for 72 and 96 h. The average ±SEM from three independent experiments. (*p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA)

B. Western blots performed for NDRG1, NSE and N-myc protein in IMR32 cells showing the expression upon kaempferol (KFL) and ATRA treatment for 4 days (Day1 – Day4).

Figure 3: Estrogen pathway independent activity of kaempferol

A. mRNA expression levels of estrogen receptor (ER) α and β upon kaempferol (KFL) treatment in IMR32 cells. The average ±SEM from three independent experiments. (*p < 0.05; **p < 0.01; one-way ANOVA)

B. Western blot analysis showing the regulation of ER α and β expression upon kaempferol (KFL) treatment in IMR32 cells.

C. Immunocytochemistry experiment showing expression of β III tubulin with kaempferol and with or without PHTPP pretreatment in IMR32 cells. Representative images of experiments performed more than three times. Scale bars = 67 µm.

D. Cell viability analysis for IMR32 cells using MTT assay showing no effect on the percent viability of cells pretreated with PHTPP (25 µM) in presence of kaempferol (KFL). The average ±SEM from 3 independent experiments performed in triplicates. (*p < 0.05; **p < 0.01; one-way ANOVA)

Figure 4: IRE1α modulation by kaempferol via binding to kinase domain

A. Gene expression analysis for ER stress marker genes with kaempferol pretreatment in IMR32 cells. The average ±SEM from three independent experiments. (*p < 0.05; ***p < 0.001; two-way ANOVA)

B. Western blot analysis showing the expression of IRE1α and BiP expression on treatment with kaempferol 50 µM (KFL) and Brefeldin A 1 µg/ml (BFA) at 3, and 12 hour time points.

C. Docked poses of ADP, kaempferol, and APY29 at the nucleotide binding site of human IRE1α kinase domain obtained using Autodock 4.0 and superimposed representation was made using PYMOL.

D. LigPlot+ analysis (2D representation) of the docked complexes of ADP, kaempferol and APY29. Red circles represent the common amino acid residues making interactions with all three compounds. Dashed green lines represent the hydrogen bonding between the amino acid residue and the compound.

E. Docked poses of ADP, kaempferol and APY29 at DFG-in confirmation (D711, F712 and G713) of human IRE1α (PDB ID: 3P23). Superimposed docked poses and interaction analysis was performed using UCSF chimera.

F. ADP Glo assay for kinase inhibition by kaempferol (KFL). Staurosporine (STS) was used as a positive control for kinase inhibition activity. Graphs representing the ±SEM of experiment performed in triplicate.

G. Kinase inhibition assay performed using 10 µM and 100 µM of ATP with same range of concentrations of kaempferol. No significant change in the IC_{50} value shows non-competitive mode of binding of kaempferol to the kinase domain of human IRE1α.

Figure 5: IRE1α knockdown reduces differentiation of IMR32 neuroblastoma cells

A. Confirmation of knock down of IRE1α in IMR32 cells at mRNA level. The graph represents average ±SEM of experiment performed in triplicates. (*p < 0.05; one-way ANOVA)

B. Western blot analysis showing IRE1α knockdown in IMR32 cells. IRE1α shRNA treated cells (KD) showing reduced expression than control shRNA (CON) treated cells.

C. Immunocytochemistry for expression of β III tubulin showing reduced expression in IRE1α knockdown cells in the presence of kaempferol and APY29. Representative images of experiments performed more than three times. Scale bar = 67 µm.

D. Western blot analysis for the expression of neuronal markers showing reduced expression in IRE1α knockdown cells. Reduction in the expression of XBP1s levels was also observed in IRE1α knockdown conditions.

E. Phase contrast methylene blue stained images of IMR32 cells showing change in morphology and neurite outgrowth upon treatment with kaempferol (KFL) and APY29 for 96 h in control shRNA and IRE1α shRNA treated cells. Pretreatment with STF083010 (50 µM) inhibits the process of neuritogenesis further in IRE1α shRNA treated cells. Representative images of experiments performed more than three times. Scale bar = 100 µm.

F. Graphs representing the number of cells with 2 or more neurite per cell in control shRNA and IRE1α shRNA treated IMR32 cells after 96 h incubation with kaempferol and APY29. STF083010 pretreatment reduces the number of neurite bearing cells in both kaempferol (KFL) and APY29 treated conditions further. The average ±SEM from 10 independent counting is shown.* represents comparison between control and kaempferol/APY29 treated cells; # and @ represents comparison between kaempferol/APY29 treated cells with STF083010 pretreated cells; $ and % represents comparison between kaempferol and APY29 treated conditions in control shRNA and IRE1α shRNA treated cells respectively (*p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA).

Figure 6: XBP1s expression leads to differentiation of neuroblastoma

A. Graphs representing the spliced and unspliced form of xbp1 mRNA present upon kaempferol 50 µM and Brefeldin A (BFA) 1 µg/ml treatment. Ratio represented to point out that kaempferol treated cells maintains high expression levels of xbp1 spliced and

unspliced form. The average ±SEM from three independent experiments. (*p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA)

B. Gene expression studies on downstream transcriptional targets of XBP1s in IMR32 cells. Expression levels of mRNA of six downstream targets upon kaempferol (KFL) 50 µM treatment for 12 h was quantified using qRT-PCR. STF083010 was used at 50 µM concentration to reduce the XBP1s activity. The average ±SEM experiments performed in duplicates (n=3). * represents comparison between control and kaempferol treated cells; # represents comparison between kaempferol treated cells with STF083010 treated cells (*p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA)

C. Western blot analysis showing the increase in expression of neuronal marker proteins NSE and NDRG1 with increased XBP1s levels in kaempferol and APY29 treated conditions.

D. Immunocytochemistry for expression of β III tubulin showing reduced expression upon pretreatment of cells with STF083010 50 µM in the presence of kaempferol and APY29. Representative images of experiments performed more than three times. Scale bar = 67 µm.

E. Cell death analysis using MTT assay showing no effect of STF083010 on rescuing cell viability upon pretreatment in kaempferol and APY29 treated conditions in IMR32 cells. The average ±SEM from 3 independent experiments performed in triplicates. (*p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA)

F. Phase contrast methylene blue stained images of IMR32 cells showing change in morphology and neurite outgrowth upon treatment with kaempferol (KFL) and APY29 for 96 h. Pretreatment with STF083010 (50 µM) inhibits the process of neuritogenesis. Representative images of experiments performed more than three times. Scale bar = 100 µm.

G. Graphs representing the number of cells with 2 or more neurite per cell in IMR32 cells after 96 h incubation with kaempferol and APY29. STF083010 pretreatment reduces the number of neurite bearing cells in both kaempferol (KFL) and APY29 treated conditions. The average ±SEM from 10 independent counting is shown.* represents comparison between control and kaempferol/APY29 treated cells; # represents comparison between kaempferol/APY29 treated cells with STF083010 pretreated cells (*p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA).

Figure 7: Proposed mechanism of action

Binding of kaempferol (K) to the kinase domain of IRE1α activates its endoribonuclease activity. The transcriptional activity of XBP1 spliced form of protein induces the differentiation of neuroblastoma cells. Pre-treatment with STF083010 (S), an inhibitor for IRE1 ribonuclease activity, inhibits the process of cleavage of XBP1 mRNA thereby inhibits differentiation.

Supplementary figure legends:

Fig. S1: Kaempferol reduces proliferation of IMR32 cells

A. Trypan blue dye exclusion assay in kaempferol treated cells showing reduced proliferation associated with cell death in 50 and 100 µM concentrations. Data represented as average ±SEM of counting obtained from three independent experiments performed in triplicates (***$p < 0.001$; two-way ANOVA). Dashed line represents the seeding density of $50x10^3$ cells on day 0.

B. Phase contrast images showing the reduction in confluence and change in morphology with kaempferol (KFL) 50 µM with ATRA 25 µM as positive control. Representative image of experiments performed more than three times. Images were acquired at 200x magnification.

Fig. S2: Modulation of IRE1α endoribonuclease activity by kaempferol

A. Expression of *XBP1s* and *XBP1us* at mRNA level induced by kaempferol (50 µM) in the presence of STF083010 (50 µM) at 12 h. The average ±SEM of three independent experiments. * represents comparison between control and kaempferol treated cells; # represents comparison between kaempferol treated cells with STF083010 treated cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; one-way ANOVA)

B & C. Immunocytochemistry for the expression of β III tubulin and synaptophysin in Neuro2a cells upon treatment with kaempferol (KFL) and APY29 for 96 h. Representative images of experiments performed more than three times. Scale bars = 67 µm.

Fig. S3: STF083010 inhibition of neuroblastoma differentiation is specific for kaempferol

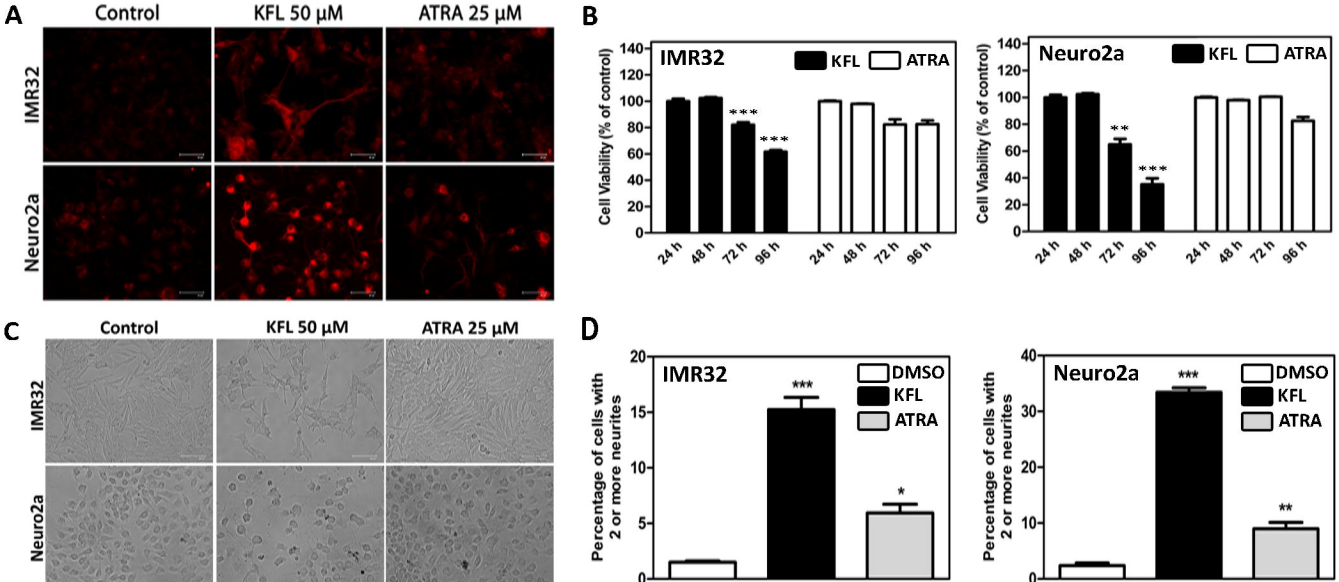
A. Phase contrast images of IMR32 cells treated with ATRA and CDDO in the presence or absence of STF083010 (50 µM) for a period of 5 days. Representative images of experiment performed for three times. Scale bar = 100µm.

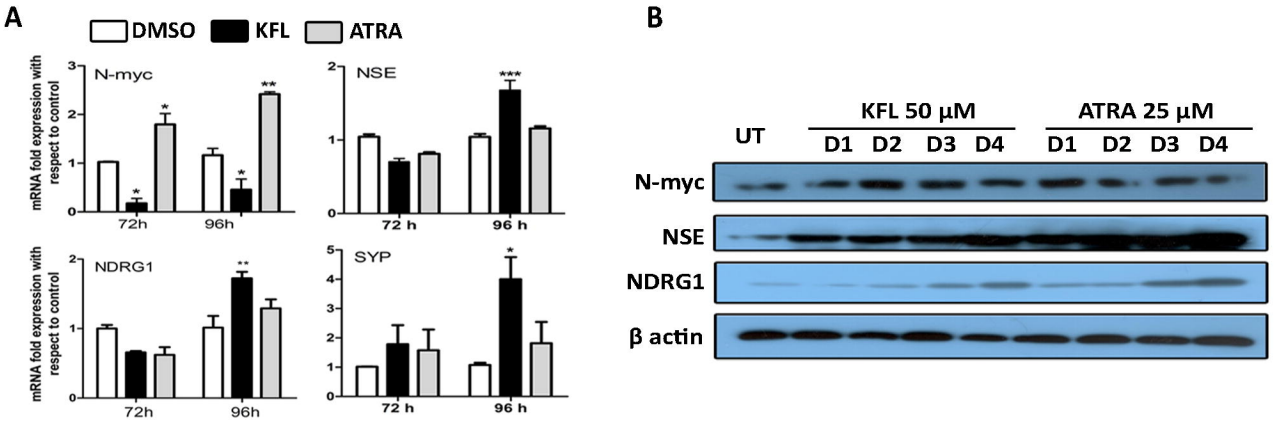
B. Graphs representing the number of cells with 2 or more neurite per cell in IMR32 cells after 5 days of incubation with ATRA (25 µM) and CDDO (500nM). STF083010 (50 µM) pretreatment did not reduce the number of neurite bearing cells in both ATRA and CDDO treated conditions. The average ±SEM from five independent counting is shown (non-significant at $p < 0.05$; one-way ANOVA).

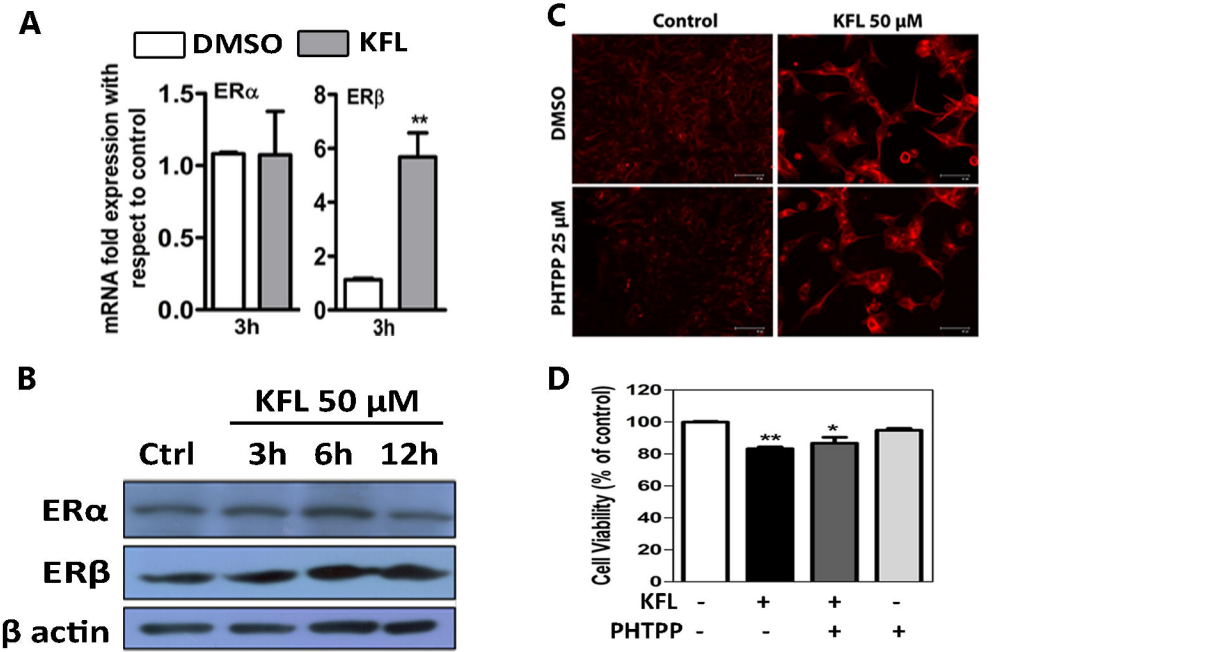
Table 1:

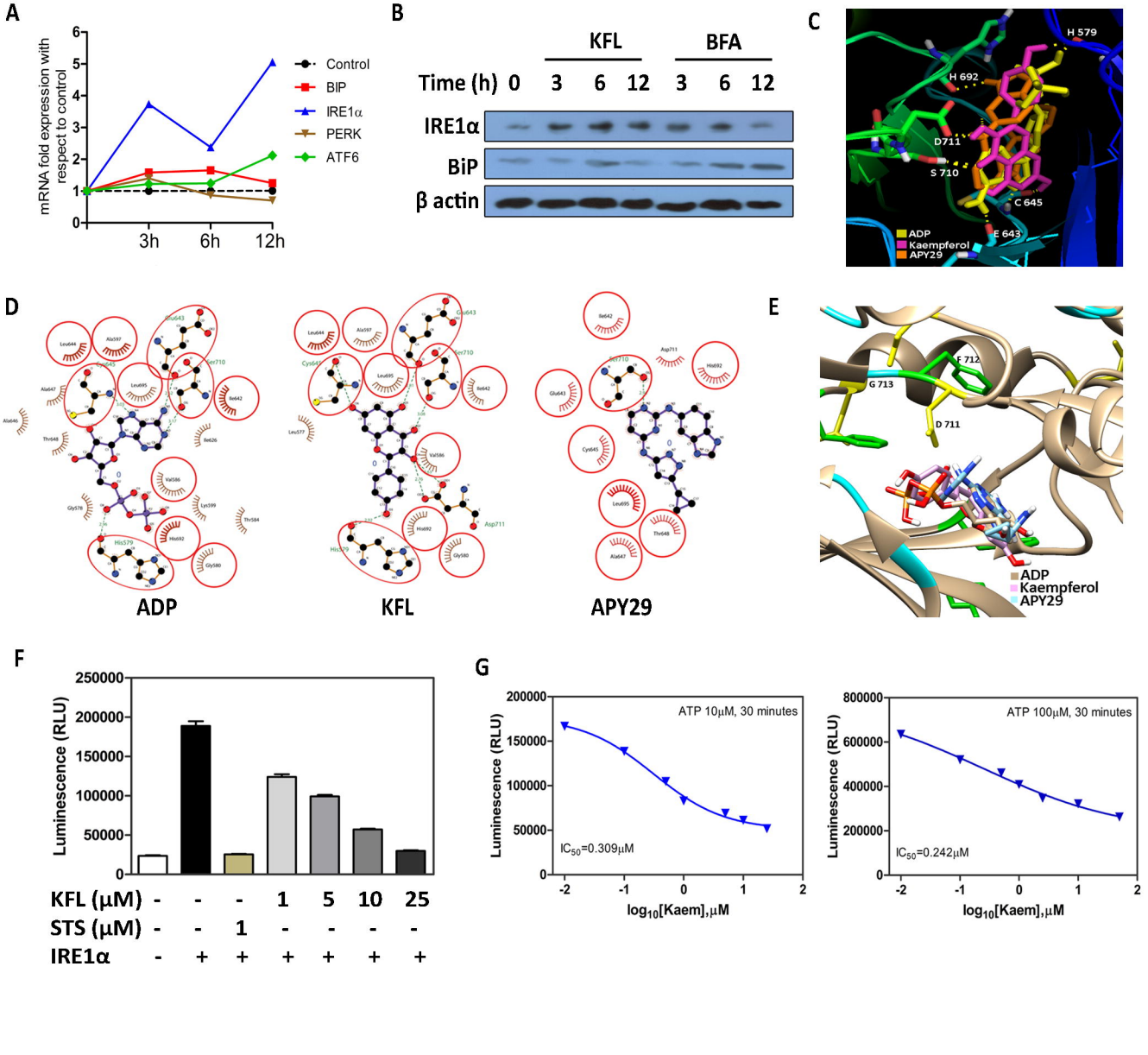
List of primers used in this study.

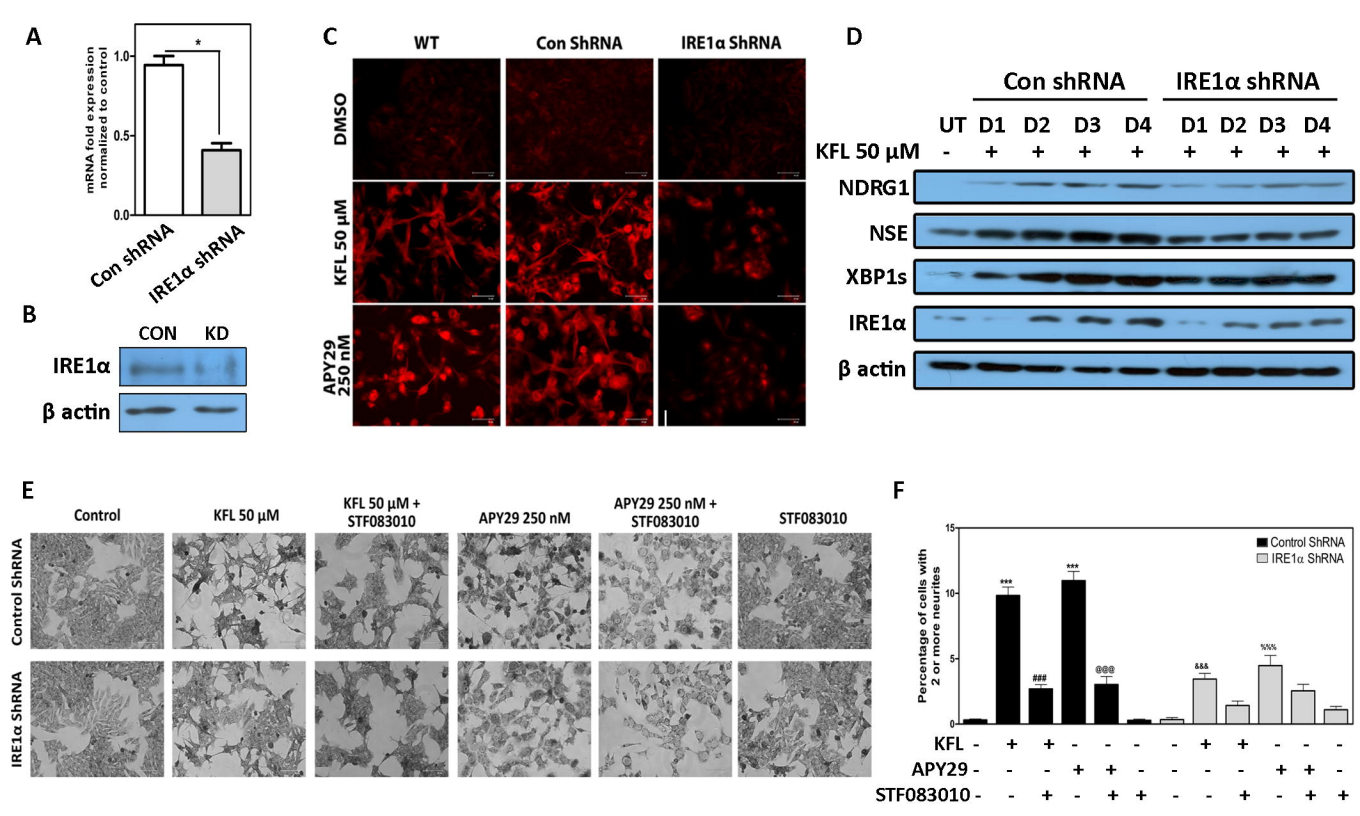
Gene	Sense (5'-3')	Anti-Sense (5'-3')
18s	GCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTC
BiP	CAACCAACTGTTACAATCAAGGTC	CAAAGGTGACTTCAATCTGTGG
IRE1α	CCATCGAGCTGTGTGCAG	TGTTGAGGGAGTGGAGGTG
ATF6	GGCATTTATAATACTGAACTATGGA	TTTGATTTGCAGGGCTCAC
PERK	CAGTGGGATTTGGATGTGG	GGAATGATCATCTTATTCCCAAA
XBP1us	CCGCAGCACTCAGACTACG	ATGTTCTGGAGGGGTGACAA
XBP1s	CCGCAGCAGGTGCAGG	GAGTCAATACCGCCAGAATCCA
Sec63	CCTCCACTTACCTGCCCATA	GGTTCCGGGCCATTACTATT
PDIA2	GATCAGCGGCCAGTTAAGAC	GATGTCCTCGTGGTCTTGGT
PDIA3	AAGCTCAGCAAAGACCCAAA	CACTTAATTCACGGCCACCT
ERdj4	TCGGCATCAGAGCGCCAAATCA	ACCACTAGTAAAAGCACTGTGTCCAAG
ERdj5	GACGGGCAAAGATGTCAGGA	GCCCGTTTGGCCTTTTCTAC
DNAJC3	CTCAGTTTCATGCTGCCGTA	TTGCTGCAGTGAAGTCCATC

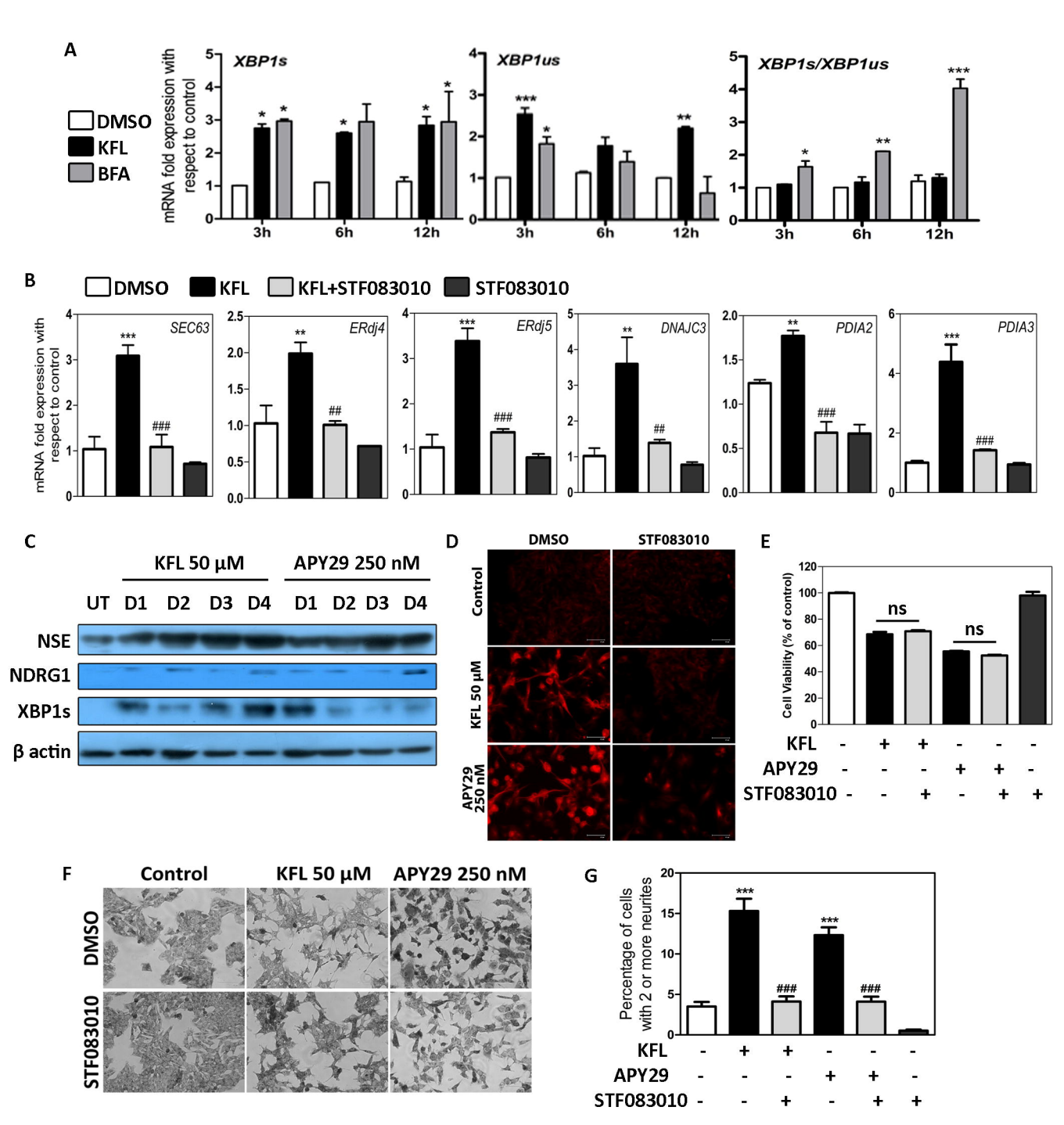

A
Control
KFL 50 μM
ATRA 25 μM
IMR32
Neuro2a
B
IMR32
KFL
ATRA
Cell Viability (% of control)
***
***
24 h
48 h
72 h
96 h
Neuro2a
**
***
C
D
DMSO
Percentage of cells with 2 or more neurites
*
**

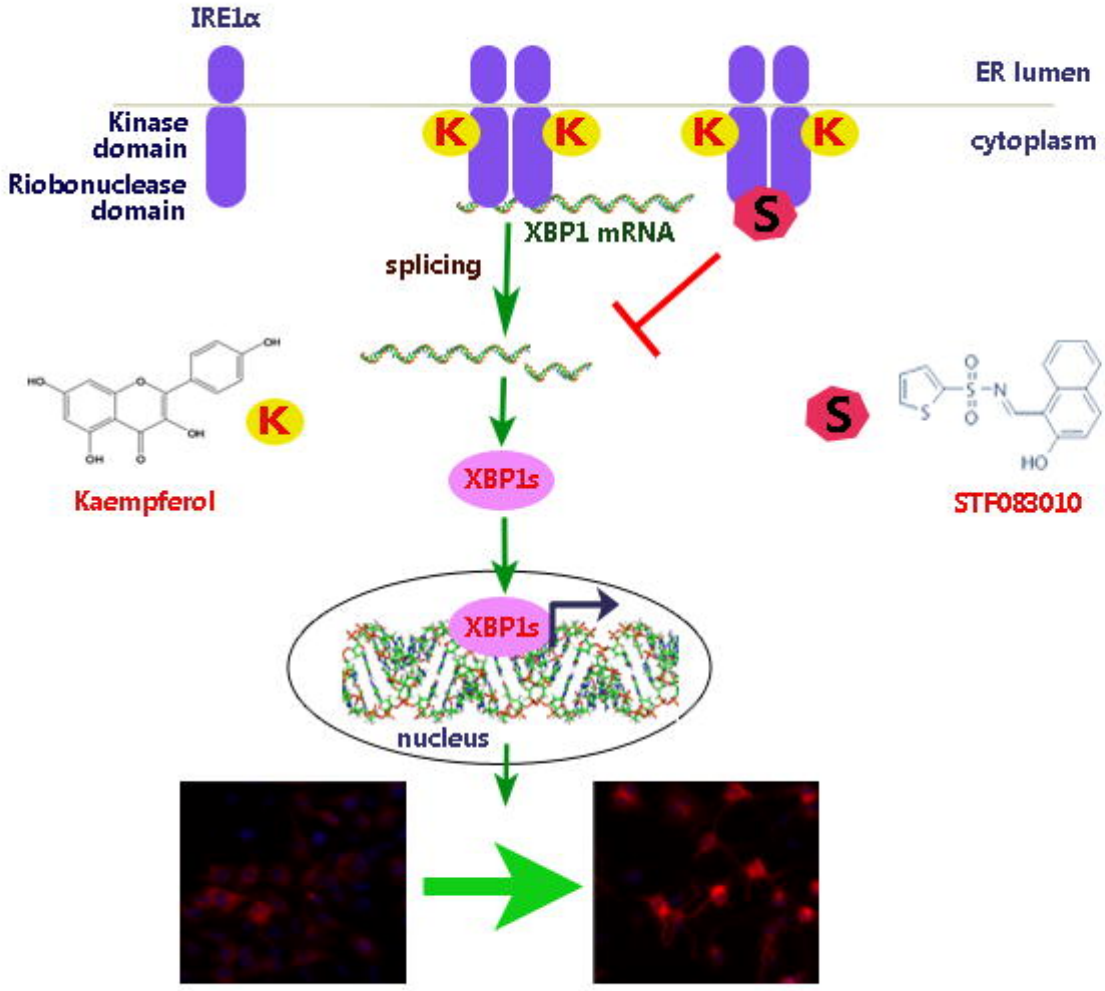

A
DMSO
KFL
ATRA
N-myc
NSE
NDRG1
SYP
mRNA fold expression with respect to control
72h
96h
72 h
96 h
B
UT
KFL 50 µM
ATRA 25 µM
D1
D2
D3
D4
N-myc
NSE
NDRG1
β actin


A
DMSO
KFL
mRNA fold expression with respect to control
ERα
ERβ
1.5
1.0
0.5
0.0
8
6
4
2
0
**
3h
3h
B
KFL 50 μM
Ctrl
3h
6h
12h
ERα
ERβ
β actin
C
Control
KFL 50 μM
DMSO
PHTPP 25 μM
D
Cell Viability (% of control)
120
100
80
60
40
20
0
**
*
KFL - + + -
PHTPP - - + +


A
mRNA fold expression with respect to control
Control
BIP
IRE1α
PERK
ATF6
3h
6h
12h
B
KFL
BFA
Time (h) 0 3 6 12 3 6 12
IRE1α
BiP
β actin
C
H 579
H 692
D711
S 710
C 645
E 643
ADP
Kaempferol
APY29
D
ADP
KFL
APY29
E
F 712
G 713
D 711
ADP
Kaempferol
APY29
F
Luminescence (RLU)
KFL (μM) - - - 1 5 10 25
STS (μM) - - 1 - - - -
IRE1α - + + + + + +
G
ATP 10μM, 30 minutes
IC50=0.309μM
log10[Kaem],μM
ATP 100μM, 30 minutes
IC50=0.242μM
log10[Kaem],μM


A
mRNA fold expression normalized to control
1.0
0.5
0.0
*
Con shRNA
IRE1α shRNA
B
CON
KD
IRE1α
β actin
C
WT
Con ShRNA
IRE1α ShRNA
DMSO
KFL 50 μM
APY29 250 nM
D
Con shRNA
IRE1α shRNA
UT D1 D2 D3 D4 D1 D2 D3 D4
KFL 50 μM - + + + + + + + +
NDRG1
NSE
XBP1s
IRE1α
β actin
E
Control
KFL 50 μM
KFL 50 μM + STF083010
APY29 250 nM
APY29 250 nM + STF083010
STF083010
Control ShRNA
IRE1α ShRNA
F
Control ShRNA
IRE1α ShRNA
Percentage of cells with 2 or more neurites
15
10
5
0
***
###
@@@
&&&
%%%
KFL - + + - - - - + + - - -
APY29 - - - + + - - - - + + -
STF083010 - - + - + + - - + - + +


A
DMSO
KFL
BFA
mRNA fold expression with respect to control
XBP1s
XBP1us
XBP1s/XBP1us
3h
6h
12h
B
DMSO
KFL
KFL+STF083010
STF083010
mRNA fold expression with respect to control
SEC63
ERdj4
ERdj5
DNAJC3
PDIA2
PDIA3
C
KFL 50 µM
APY29 250 nM
UT
D1
D2
D3
D4
NSE
NDRG1
XBP1s
β actin
D
DMSO
STF083010
Control
KFL 50 µM
APY29 250 nM
E
Cell Viability (% of control)
ns
KFL
APY29
STF083010
F
Control
KFL 50 µM
APY29 250 nM
DMSO
STF083010
G
Percentage of cells with 2 or more neurites
KFL
APY29
STF083010


IRE1α
ER lumen
Kinase domain
Riobonuclease domain
cytoplasm
K
K
K
K
S
XBP1 mRNA
splicing
Kaempferol
K
S
STF083010
XBP1s
XBP1s
nucleus