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14. ABSTRACT An endocrine therapy is the most effective means to (initially) manage many hormone-dependent breast cancers. Unfortunately, almost 50% of estrogen receptor positive (ER+) breast cancers fail to respond (de novo resistant) and many others recur following an apparently initial response. We have now shown that cells respond to the stress of these therapies by activating the unfolded protein response (UPR). A central component of the UPR, and that which confers its prosurvival activities, is driven by the unconventional (cytosolic) splicing of XBP1 by the endoribonuclease function of IRE1. We screened small molecule libraries against IRE1 <i>in silico</i> and tested the top predicted "hits" for their abilities to inhibit XBP1s production and breast cancer cell proliferation, and to potentially reverse resistance to AEs. The most effective compound (N-(4-Phenoxy-phenyl)-2-(5-pyridin-3-yl-2H-[1,2,4]triazol-3-ylsulfanyl)-acetamide; NPPTA) was selected for further study and is the initial focus of this IDEA Expansion application.					
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Introduction

In 2008, over 40,000 American women will die of breast cancer [1]. In the same period, there will be over 178,000 newly diagnosed cases of invasive breast cancer, almost 70% of which will be estrogen receptor- α positive (ER+) [2,3]. However, 50% of all ER+ breast tumors will not respond to endocrine therapy [4]. Tamoxifen produces an overall 26% proportional reduction in mortality [5] but many ER+ tumors that show an initial response to tamoxifen eventually recur [4].

In this Idea Expansion (IDEX) Grant, we propose to target IRE1 in endocrine resistant breast cancer cells with N-(4-Phenoxy-phenyl)-2-(5-pyridin-3-yl-2H-[1,2,4]triazol-3-ylsulfanyl)-acetamide (NPPTA; lead compound), or its analogs. These compounds are expected to block pro-survival signaling from the unfolded protein response (UPR) and prevent survival (via pro-survival autophagy and an inhibition of apoptosis). We **hypothesize** that these effects will be mediated in part by the inhibition of XBP1 splicing and its ability to regulate BCL2 family members and NF κ B. Furthermore, a combination of NPPTA and AEs will interact synergistically to selectively kill AE resistant breast cancer cells *in vitro* and *in vivo*. We will achieve our goal by addressing three integrated specific aims, each supported by published or preliminary data. If successful, the data will support a subsequent IND submission in preparation for a clinical trial. This study builds upon the accomplishments of the previously BCRP-funded work and, if successful, has the potential to contribute to the eradication of some breast cancers within the next decade.

Aim 1: We will use *in silico* modeling of NPPTA:IRE1 interactions and quantitative structure-activity relationship analyses (QSAR) to develop rationally designed NPPTA analogs with increased potency and optimized pharmacologic properties.

Aim 2: We will determine the ability of NPPTA and its analogs to sensitize responsive breast cancer cells, and re-sensitize resistant cells, to both estrogen withdrawal (analogous to treatment with an AI) and to two different classes of AE (TAM and ICI). These studies will be done initially *in vitro*, with the strongest candidates being studied *in vivo* to provide preclinical safety, efficacy, and toxicology data to support later first-in-human studies.

Aim 3: We will explore the mechanism(s) of action of NPPTA and its analogs in inducing cell death, focusing initially on its effects on BCL2 family members and NF κ B. We will use high throughput transcriptome analyses to study the effects of NPPTA (or its analogs) on cell survival signaling.

Key Words

Antiestrogen resistance, estrogen receptor positive, breast cancer, unfolded protein response (UPR), BCL2, NF κ B, apoptosis, autophagy, drug resistance.

Overall Project Summary

In Year 1 of this grant, we were able to complete the tasks that are in direct alignment with our approved SOW. A schematic diagram that outlines the synthetic steps for analogs of NPPTA (R1=H) is shown in Figure 1 (panel on the right). Among the analogs made and tested *in vitro*, JS-1-20 (CF3) and JS-1-14 (R1=Cl) were more potent in inhibiting proliferation of LCC1 (MCF7-derivative; antiestrogen sensitive) and LCC9 (Tamoxifen and ICI182,780/ Faslodex resistant) breast cancer cells (Figure 2). Cells were grown in IMEM media supplemented with charcoal-stripped serum (5%) for 24 h and treated with 10 μ M of NPPTA, JS-1-20 or JS-1-14 for 48 h. Cell numbers were determined by crystal violet staining. After treatment, media were removed, and plates were stained with a solution

containing 0.5% crystal violet and 25% methanol, rinsed, dried overnight, and resuspended in citrate buffer (0.1 M sodium citrate in 50% ethanol). Intensity of staining, assessed at 570 nm and quantified using a VMax kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA), is directly proportional to cell number.

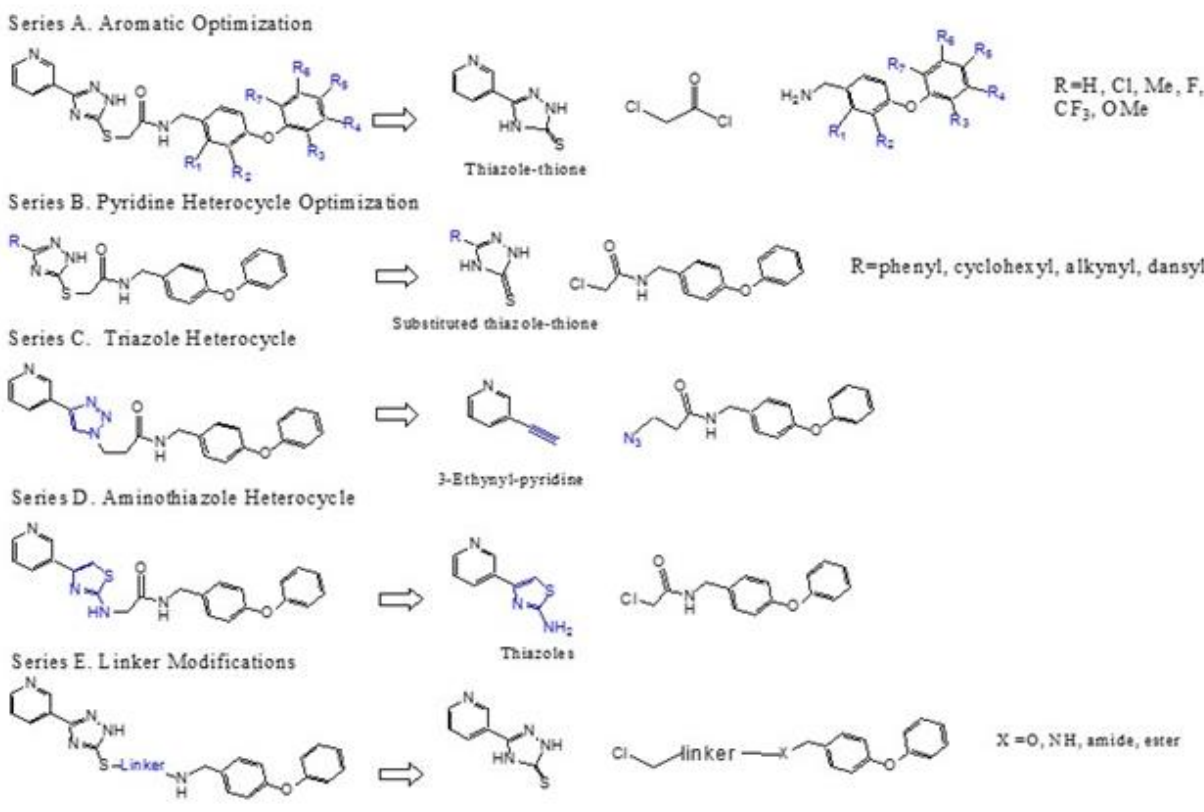


Figure 1: Scheme for synthesis of NPPTA analogs.

JS-1-20 was further tested in LCC2 (Tamoxifen resistant), MCF7 (antiestrogen sensitive), MCF7RR (Tamoxifen-resistant), MCF10A (non-tumorigenic breast epithelial cells) and HFF1 (human foreskin fibroblasts). As shown in Figure 3, JS-1-20 inhibited cell proliferation in breast cancer cells, irrespective of endocrine responsiveness, more potently than in normal cells. Cell number was determined by crystal violet staining.

During **Year 1** of the Idea Expansion award, we also investigated the molecular mechanism of NPPTA-induced cell death in ER+ endocrine resistant breast cancer cell lines. While the initial *in silico* screening for small molecules was conducted for their ability to block XBP1 splicing, we were not able to validate specific inhibition of XBP1 splicing *in vitro*. For analysis of XBP1 splicing, a polymerase chain reaction (PCR) product of 601 bp was amplified spanning the 26 bases cut out in the processed XBP1 mRNA by the endonuclease IRE1. Within this sequence, a site specific for the restriction enzyme Pst I is present. The extent of XBP1 processing can thus be investigated by Pst I restriction analysis of the XBP1 PCR product. After treatment of LCC9 cells with 0, 10 or 20 μ M of NPPTA for 48 h, mRNA samples were extracted and RT-PCR was conducted using the following XBP1 primers: upper strand primer: 5'-AAACAGAGTAGCAGCTCAGACTG-3', and lower strand primer: 5'-GGATCTCTAAACTAGAGGCTTGGTG-3'. PCR products were incubated with Pst I for 5 h at 37°C separated by agarose gel electrophoresis.

To investigate the mechanism of cell death by NPPTA and its analogs, we tested the levels of IRE1a and other proteins of the unfolded protein response (UPR) in LCC9 cells. Cells were grown in IMEM media supplemented with charcoal-stripped serum (5%) for 24 h and treated with increasing concentrations of NPPTA (Figure 4). While total levels of IRE1a protein did not change, phosphorylation of S724 on IRE1a was decreased, consistent with potential activity against the primary target. In addition, total protein levels of GRP78/Bip, the primary upstream regulator of IRE1a in the UPR, was also reduced with $>5 \mu\text{M}$ NPPTA, implying a possible feedback effect on GRP78 from inhibition of IRE1a phosphorylation. Cell cycle analysis for LCC1 and LCC9 cells treated with did not show any changes in LCC9 cells while LCC1 cells were arrested in G1 phase with $10 \mu\text{M}$ NPPTA (Figure 5) and JS-1-20 (Figure 6) for 48 h. To determine cell cycle profiles, cells were cultured at 60-80% confluence in growth medium for 24 h. The following day, cells were treated with vehicle, NPPTA or JS-1-20 or FAS (100 nM) or the combination for an additional 48 h. Cells were then fixed in ethanol, and analyzed by the Flow Increased levels of apoptosis (Figure 7) and necrosis (Figure 8) were detected in LCC9 cells treated with NPPTA and JS-1-20. To measure apoptosis and necrosis, cells were treated for 48 h, and stained with an Annexin V-fluorescein isothiocyanate kit (Trevigen, Gaithersburg, MD).

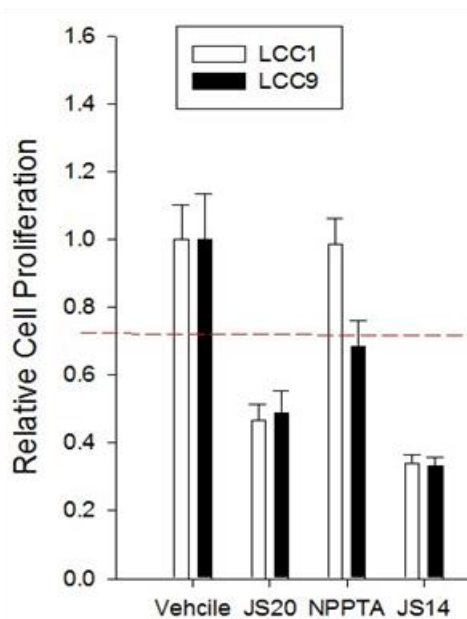


Figure 2: Analogs of NPPTA, JS-1-20 and JS-1-14 inhibits cell proliferation in LCC1 and LCC9 cells.

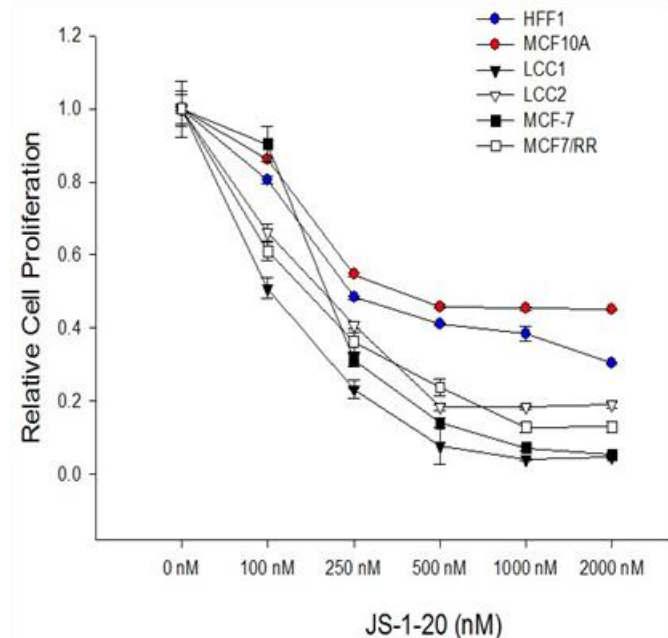


Figure 3: JS-1-20 inhibits cell proliferation more potently in both endocrine sensitive and resistant ER+ breast cancer cells compared

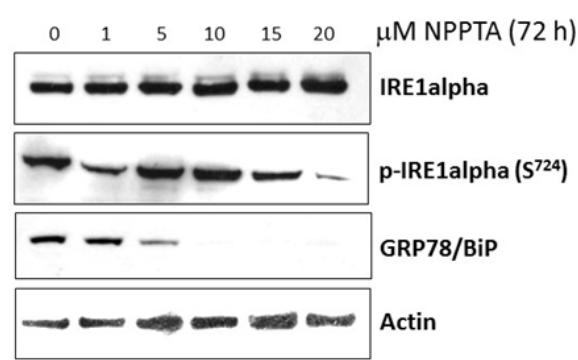


Figure 4: NPPTA inhibits phosphorylation of IRE1a at S724 and downregulates GRP78/Bip.

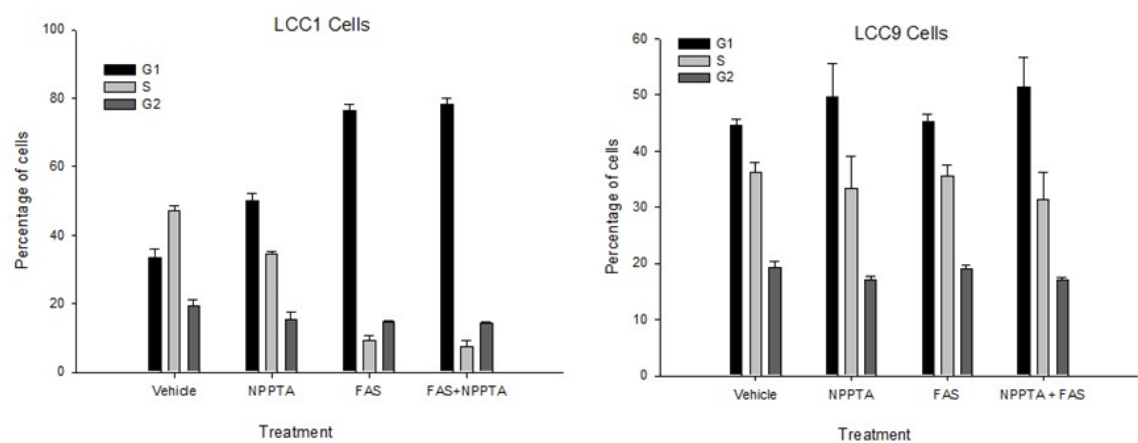


Figure 5: Cell cycle analysis with 10 μM NPPTA, 100 nM Faslodex (FAS) or the combination at 48 h in LCC1 and LCC9 cells.

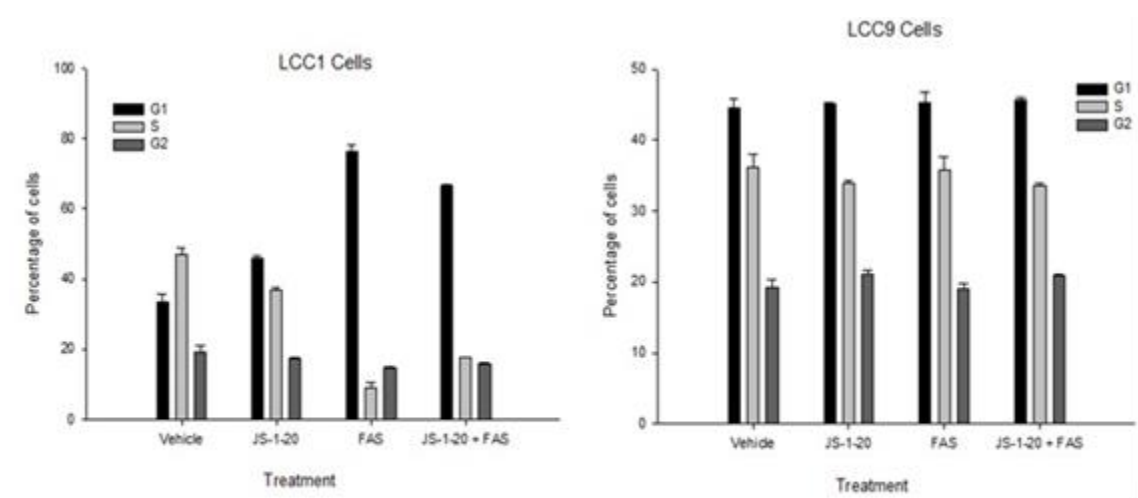


Figure 6 Cell cycle analysis with 10 μM JS-1-20, 100 nM Faslodex (FAS) or the combination at 48 h in LCC1 and LCC9 cells.

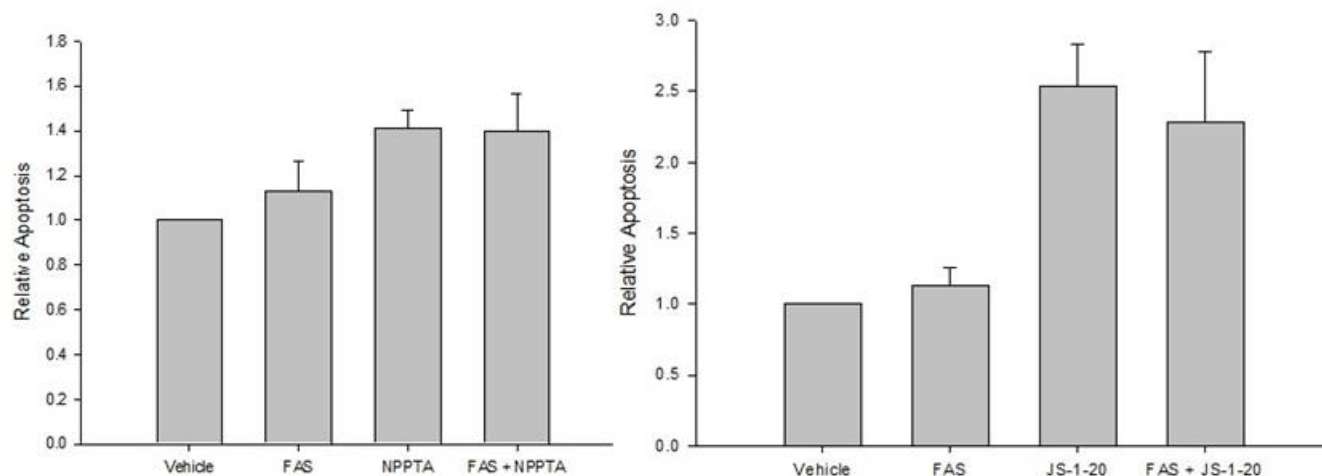


Figure 7: Annexin V (apoptosis) staining following treatment with 10 mM NPPTA, 100 nM Faslodex or the combination in LCC9 cells at 48 h.

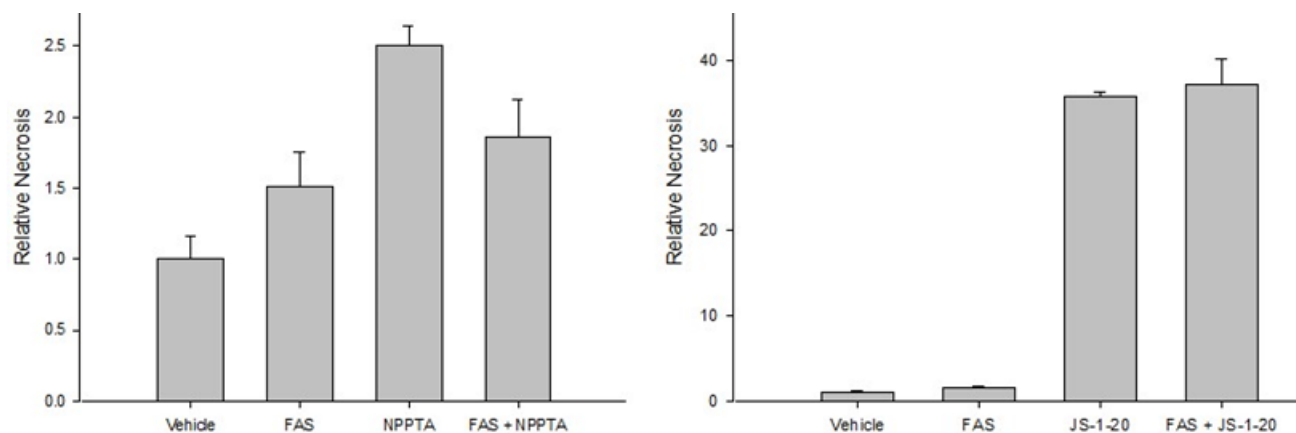
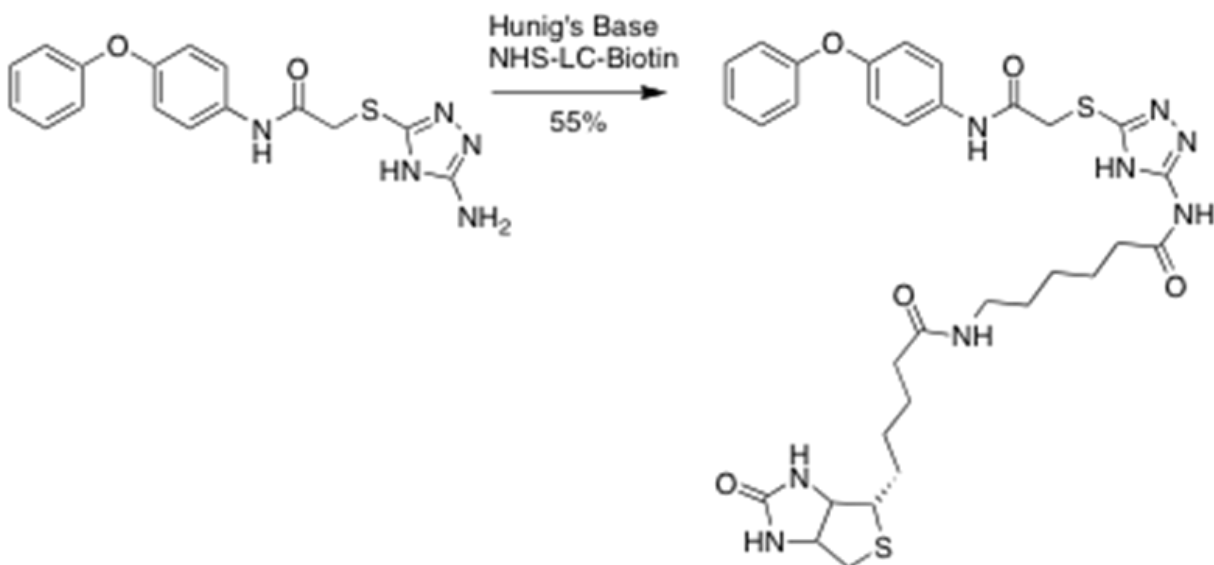
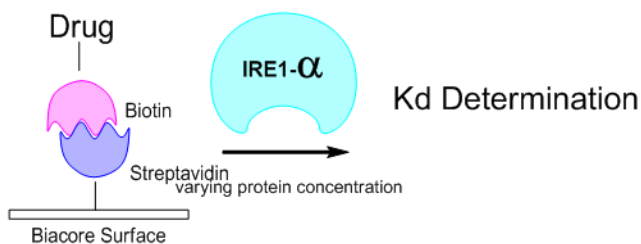


Figure 8: Propidium iodide (necrosis) staining following treatment with 10 mM NPPTA, 100 nM Faslodex or the combination in LCC9 cells at 48 h.

Synthesis of Biotinylated Inhibitor: An improved NPPTA biotinylated inhibitor was constructed THAT contained 5 carbon-linker. This compound was constructed by treating JS-1-61 with NHS-LC-biotin. After purification the resulting compound was obtained with 55%.

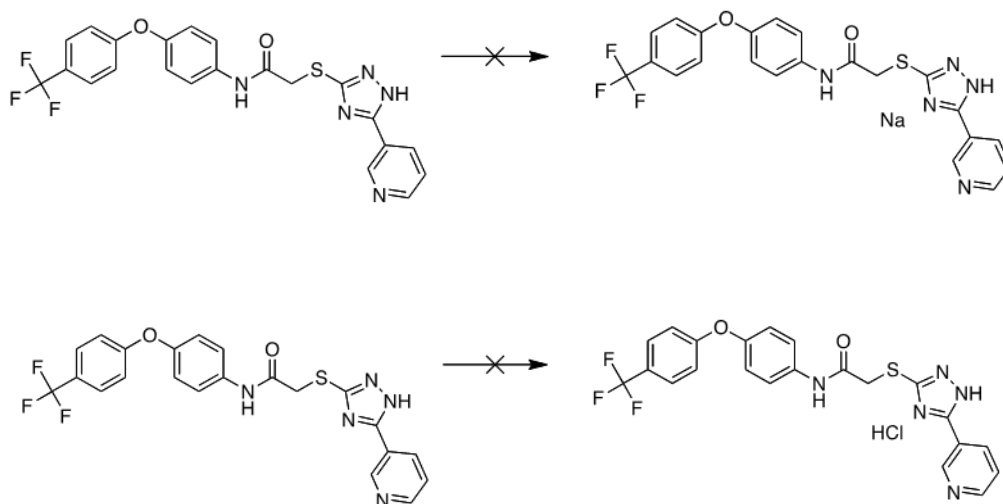


Reverse Biacore Experiment: In an effort to determine the binding affinity of NPPTA for IRE1alpha we attached the synthesized biotinylated compound to the surface of an avidin-coated surface Plasmon resonance chip. Recombinant protein was flowed over the surface to detect binding. Initial binding was observed but results were not reproducible. Studies are ongoing to develop a reproducible assay.

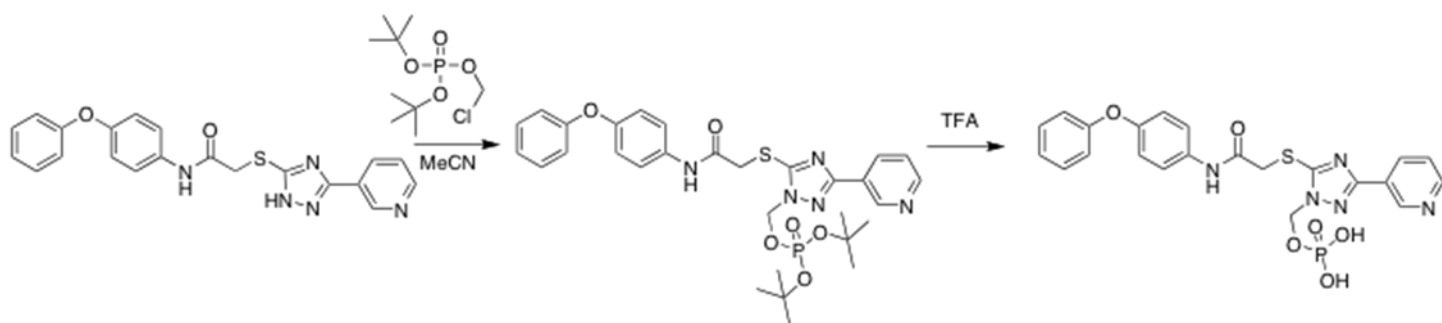


Biotin-Avidin Pull Down: We have attempted experiments to pull down IRE1alpha with our Biotinylated NPPTA. Biotinylated NPPTA was attached to streptavidin-coated beads and treated with cell lysate and recombinant IRE1alpha. Insufficient quantities of protein were captured therefore no conclusion was made. Studies are currently on going.

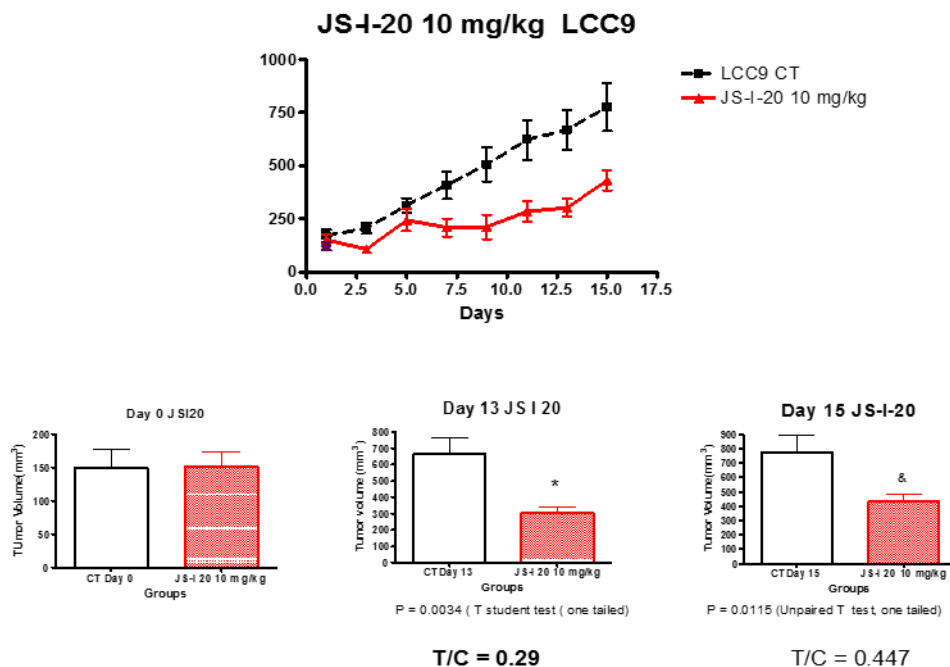
JS20 Salt Synthesis: Synthetic efforts have been made toward creating a more water soluble analog. However due to the amphoteric nature of the 1,2,4-triazole, neither acidic or basic conditions yielded a salt. Synthetic studies are ongoing to resolve this issue.



Pro-drug Synthesis: We are also pursuing another method to create a more soluble compound which involves covalently attaching a phosphate group onto NPPTA. This compound is soluble in water and a large-scale synthesis is underway.



Effects on JS-I-20 on LCC9 tumor growth: We have evaluated JS-I-20 for inhibition of xenograft (LCC9) tumor growth. Tumors were allowed to grow to 200 mm³ and then 10 mg/kg of JS-1-20 was dosed by i.p. injection (N= 5 mice) every other day for 7 doses. At day 15 tumor size in the treated group was reduced by approximately 50%.



Key Research Accomplishments

- Lead compound, NPPTA, induces cell death by inhibiting phosphorylation of IRE1a and inducing necrosis.
- NPPTA analog, JS-1-20, inhibits growth of LCC9 (antiestrogen resistant) xenografts *in vivo*.

Conclusion

Our studies show that NPPTA and its analog, JS-1-20, inhibit breast cancer cells but not normal mammary epithelial cells. This finding is consistent with our overall hypothesis that endocrine resistant breast cancer cells have increased levels of UPR-regulated autophagy to promote cell survival. Furthermore, we also report the first preclinical *in vivo* validation of NPPTA analog, JS-1-20 in inhibiting growth of endocrine resistant human breast cancer xenografts. Collectively, these findings are promising in establishing the NPPTA class of compounds as anti-tumor agents in treating drug resistant breast cancer.

Publication, Abstracts, and Presentation

Annual AACR Meeting (2014), San Diego, CA. Title: A novel small molecule inhibitor of IRE1alpha reverses endocrine resistance in breast cancer cells (#5869). Authors: Ayesha N. Shajahan-Haq, Jacqueline Smith, Siva Dakshanamurthy, Ahreej E. Eltayeb, Milton Brown and Robert Clarke.

Innovation, Patens, Licenses

International Application No. PCT/US12/032110. "Small Molecule Inhibitors of XBP1 Splicing" (pending: application filed)

Reportable Outcomes

- NPPTA analogs, JS-1-20 and JS-1-14 as potent inhibitors of cell growth of endocrine resistant breast cancer cells

Other Achievements

Nothing to report

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Appendices

Nothing to report