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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this Idea Expansion (IDEX) Grant, we propose that targeting 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, will block pro-survival signaling from the 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 NFκB. Furthermore, a combination of NPPTA and AEs will interact synergistically to selectively kill AE resistant breast cancer cells in vitro and in vivo.

15. SUBJECT TERMS

Nothing listed

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INTRODUCTION

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, will block pro-survival signaling from the 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 NFκB. Furthermore, a combination of NPPTA and AEs will interact synergistically to selectively kill AE resistant breast cancer cells in vitro and in vivo.

KEYWORDS

Breast Cancer, Drug Development, Endocrine Pathogenesis, Endocrine Resistance

ACCOMPLISHMENTS

What were the major goals of the project?

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.

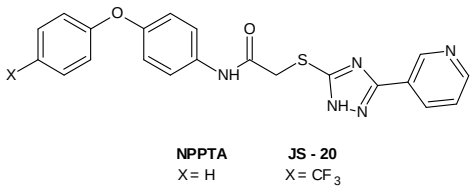
Aim 3: We will explore the mechanism(s) of action of NPPTA and its analogs in inducing cell death, focusing initially on its effects on BCL2 family members and NFκB. We will use high throughput transcriptome analyses to study the effects of NPPTA (or its analogs) on cell survival signaling.

What was accomplished under these goals?

In the final year of the IDEX award, we resolved the issue with insolubility of our lead compound, NPPTA and its analog, JS-1-20 for testing *in vivo*. Through a fee-for-service collaboration with Southwest Research Institute (SwRI, San Antonio, TX), we were able to make tosylic acid (TsOH) soluble salts of both compounds.

Prodrug Synthetic chemistry

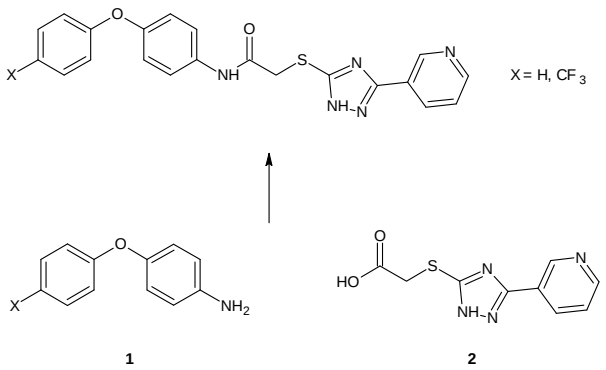
Our initial synthetic efforts in the NPPTA project are to generate gram quantities of **NPPTA** and **JS-20** as seen in Figure 1.



NPPTA X = H **JS - 20** X = CF_3

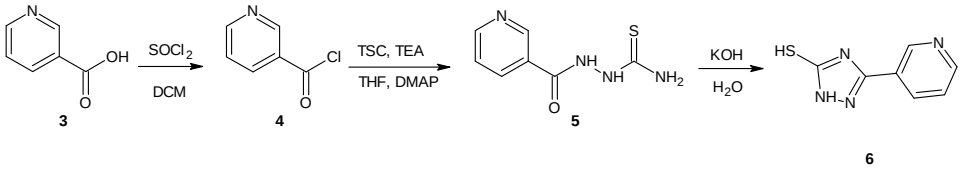
Figure 1. Target analogs for further analysis

Our synthetic plan to generate the initial targets will utilize a converget route that introduces the anilino bi-aryl ether (**1**) in the latter stages as summarized in Scheme 1. This synthetic strategy favors diversity on the bi-aryl ether moiety of the target analogs. This plan requires the preparation of gram quantities of acid **2**.



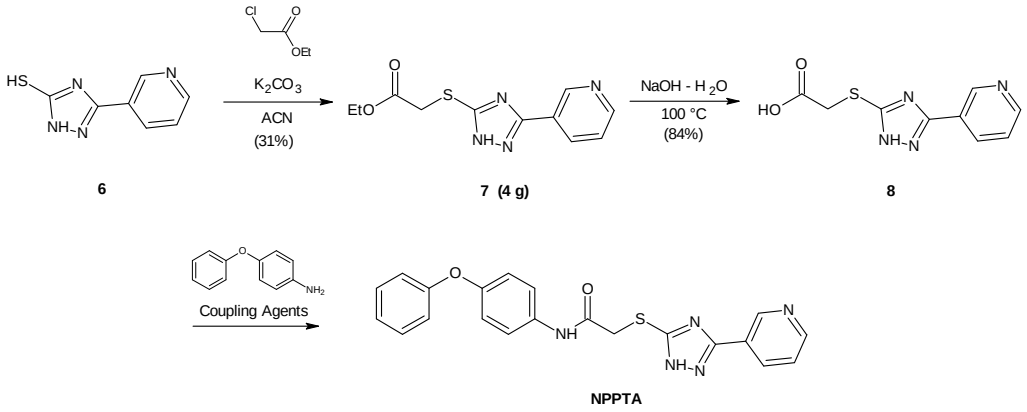
Scheme 1. Proposed synthesis of NPPTA and JS - 20

We have generated the heterocycle **6** in quantities necessary to generate 5 g of the two target analogs. The route is shown in Scheme 2.



Scheme 2. Synthesis of triazole heterocycle 6

During the past week we developed a method to generate the ester intermediate **7** in moderate yield as seen in Scheme 3. In addition, the acid **8** was generated and we are currently attempting to generate NPPTA in small scale. These studies are expected to generate NPPTA which we will characterize and compare versus the authentic sample. These studies are expected to be completed in the coming week.



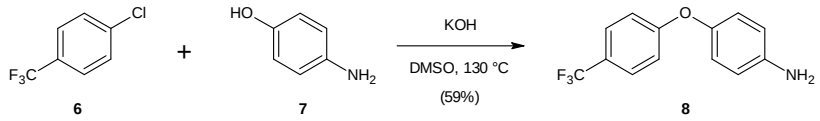
Scheme 3. Proposed synthesis of NPPTA

Our goal in this project is to prepare five grams of **NPPTA** and **JS20** as seen in Figure 1. Both compounds possess anti-cancer properties and will be evaluated *in vivo* as the tosic acid salts.

Scale-up Synthetic chemistry for TsOH salts of NPPTA and JS20: Our synthetic plan to generate the target analogs utilizes a converget route that introduces the anilino bi-aryl ether (**1** and **2**) in the latter stages as summarized in Scheme 1. This synthetic strategy favors diversity at the bi-aryl ether moiety of the target analogs. This plan requires the preparation of gram quantities of acid chloride **2**.

The preparation of the key acid chloride **2** was prepared as described in Scheme 2. Selective alkylation of commercially available **3** (Sigma/Aldrich) using ethylbromoacetate furnished the ethylester intermediate **4**. Ester saponification followed by treatment with thionylchloride furnished the key acid chloride intermediate in excellent yield.

The successful preparation of **2** set up the final amide coupling step with the desired analines as described in Scheme 1. The preparation of **JS20** required the preparation of anailine **1** (X = CF_3) and its preparation was achieved as seen in Scheme 3. SNAr chloride displacement was accomplished in moderate yield to provide the analine **8**.

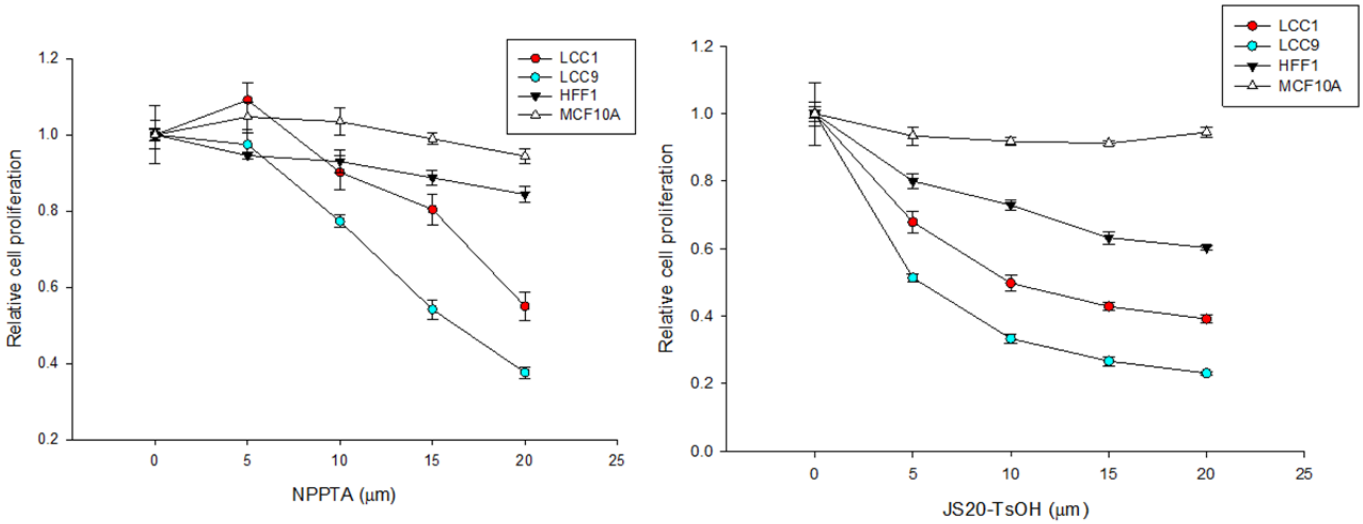


6 + 7 → 8

Scheme 4. Synthesis of analine 8

The final steps in the preparation of **NPPTA** and **JS20** was accomplished as described in Scheme 4. Treatment of acid **2** with analines **9** and **8** in pyridine furnished the target amides in moderate yield. Analysis of the targets verified clean formation the desired products (^{1}H NMR and LCMS). The free bases are converted to the tosyl acid salts (1.0 equiv, DCM-MeOH) to provide the target salts (NPPTA-TsOH; JS-20-TsOH). The target amounts (5 g each) were completed and shipped on September 20, 2016.

NPPTA-TsOH and JS20-TsOH salts significantly decreased cell proliferation in cancer cells but not in non-malignant cells: LCC1 (sensitive) and and LCC9 (antiestrogen resistant) were grown in phenol red–free IMEM with 5% charcoal-stripped calf serum (CCS) at 8000 cells per well in 96-well plates. 24 h later, cells were treated with vehicle alone (0.5% DMSO) or 5, 10, 15 or 20 μM of NPPTA-TsOH or JS20-TsOH for 72 h. 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. Our data shows that NPPTA-TsOH treatment for up to 20 uM decreased cell proliferation in LCC1 and LCC9 cells but did not affect proliferation in non-malignant MCF10A (breast epithelial cells) or HFF1 (human foreskin epithelial cells). Similarly, JS20-TsOH showed increased sensitivity in LCC1 and LCC9 cells compared with MCF10A or HFF1 cells (**Figure 2**).



NPPTA-TsOH and JS20-TsOH salts significantly decreased tumor size: Five week old LCC1 ovariectomized athymic nude mice LCC9 (Harlan, Fredrick, MD) were injected orthotopically with 1.0 × 10^6 LCC1/LCC9 cells in 50% Matrigel into mammary fat pads. We tested the efficacy of NPPTA-TsOH and JS-1-20-TsOH in LCC1 and LCC9 xenograft models in athymic nude mice. Total 30 (5 mice per group with 2 tumors per mouse=10 tumors per group: LCC1 and LCC9 for vehicle, NPPTA or JS-1-20) 5-week of nude mice were inoculated with $1X10^7$ cells. When tumors were about 30 mm^2 in size, mice were injected (i.p.) with 20mg/kg of NPPTA-TsOH, JS-1-TsOH or

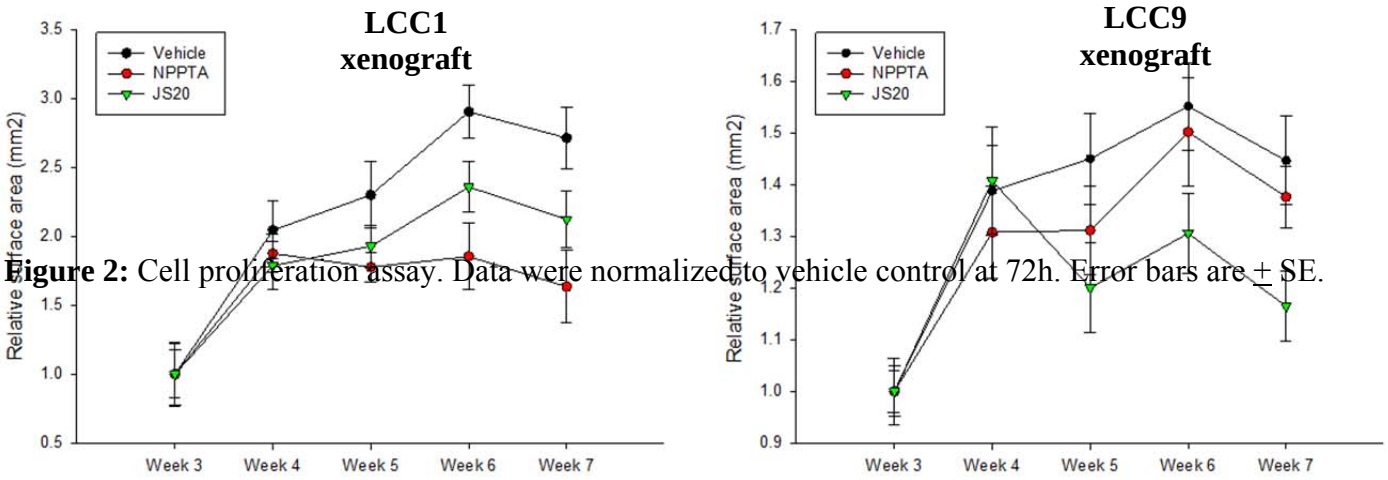


Figure 2: Cell proliferation assay. Data were normalized to vehicle control at 72h. Error bars are ± SE.

Figure 3: Growth of LCC1 and LCC9 xenografts was inhibited by NPPTA-TsOH and JS20-TsOH. N=10 tumors per group. Error bars are ± SE.

vehicle alone (30% Propylene glycol+5% Tween 80) 5 days a week for 3 weeks. Tumors were measured once a week. Interestingly, our data shows (**Figure 3**) that while both NPPTA and JS-1-20 significantly decreased growth of LCC1 tumors at 3 week post-treatment compared with vehicle alone, only JS-1-20, but not NPPTA, significantly decreased growth of LCC9 tumors at 3 weeks compared with vehicle alone.

Possible targets for NPPTA-TsOH and JS20-TsOH: While NPPTA was originally proposed to target the unfolded protein response (UPR) sensor IRE1alpha *in silico*, our *in vitro* data did not confirm IRE1alpha's kinase or endoribonuclease activity to be inhibited by NPPTA (or JS20). It was noted by our team that the structure of NPPTA may suggest a kinase inhibitor function. A kinase screening platform (KinomeScan, DiscoverX) that has a panel of 468 known kinases identified the following hits for NPPTA-TsOH and JS20-TsOH:

NPPTA-TsOH: **c-KIT<PDGFRB<FLT-3**

JS20-TsOH: **MEK6<NEK1**

Future directions and publication(s): Currently, we are conducting the validation studies using siRNA for c-KIT and other possible targets identified above. The possible targets identified above are of interest because c-KIT, PDGFRB, FLT-3 are potential anti-cancer targets in various cancer types (PMID: 27128408; PMID: 27944502; PMID: 27949996).

What opportunities for training and professional development has the project provided?

Dr. Ayesha Shajahan-Haq, PhD, is a junior faculty member (co-investigator) who supervised the work for this project. She has now transitioned to becoming an independent investigator.

How were the results disseminated to communities of interest?

US Appl. No. 14/009,969 as previously reported; nothing additional to report.

What do you plan to do during the next reporting period to accomplish the goals?
Not applicable.

IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

    We were not able to validate whether NPPTA and JS20 were indeed targeting IRE1alpha activation in the UPR. In the last year, we were able to generate soluble salts of these compounds and showed that both salts inhibited growth of LCC1 and LCC9 tumors in vivo. In vitro studies showed that NPPTA and JS20 TsOH salts did not inhibit growth of normal cells. We were able to identify possible new targets of NPPTA (c-KIT, PDGFRB, FLT3) and JS20 (MED6, NEK1). All of these molecules are promising targets in various cancer types and may provide useful tools for the research community. Therefore, we look forward to further validation studies of the above mentioned targets and publishing our findings within the next 6 months.

- **What was the impact on other disciplines?**

  Nothing to Report

- **What was the impact on technology transfer?**

US Appl. No. 14/009,969

- **What was the impact on society beyond science and technology?**

  Nothing to Report

CHANGES/PROBLEMS

Changes in approach and reasons for change

None

Actual or anticipated problems or delays and actions or plans to resolve them

None

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS

Publications, conference papers, and presentations

Nothing to Report

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

      ***Example**:*

Name:	*Robert Clarke, PhD, DSc*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*ORCID:* 0000-0002-9278-0854
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Clarke is the PI of this project and as such is primarily responsible for the overall direction and operation of this application. He directs, coordinates and integrates the proposed studies, prepares reports and manuscripts to disseminate results.*
Funding Support:	*N/A*

Name:	*Ayesha Shajahan-Haq*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Shajahan-Haq supervises the research assistant and helps perform the experiments specified in the proposal. She also participates in the preparation and presentation of the reports/results and the preparation of manuscripts for publication.*
Funding Support:	*N/A*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report

- **What other organizations were involved as partners?**

  Nothing to Report

SPECIAL REPORTING REQUIREMENTS

None

APPENDICES

None.