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14. **ABSTRACT Current treatment options for advanced, metastatic, highly heterogeneous, and refractory breast cancer (BC) tumors are extremely limited, contributing to most of the BC-related deaths in USA and around the world primarily due to drug-resistance, and drug-related side effects and toxicities.1-3 The goal of this project is to develop a new class of drugs for the treatment of refractory BC that are safe and effective with the least side effects. Separase, an enzyme that cleaves the chromosomal cohesin complex during mitosis, is overexpressed in more than 60% of BC and 50% of TNBC, and 65% of Luminal-B BC tumors and its overexpression strongly correlates with aneuploidy, high incidence of relapse, metastasis, and a lower 5-year overall survival rate.4-8 Separase is an oncogene,6,7 and overexpression of Separase induces aneuploidy, genomic instability, mammary tumorigenesis, and intratumoral heterogeneity in mice.4,6 We posit that modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting resistant, Separase-overexpressing aneuploid tumors, particularly the hard-to-treat TNBC.9 We hypothesize that pharmacologic modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting Separase-overexpressing aneuploid heterogeneous tumors. We further hypothesize that by decreasing Separase activity to a therapeutically useful degree of inhibition by Sepin compounds, we will effectively reprogram the Separase-overexpressing tumors and that partial Separase inhibition will selectively eliminate Separase-overexpressing tumor cells addicted to elevated Separase expression while sparing normal cells, and thereby preventing aneuploidy and aneuploidy-associated tumor heterogeneity. Following is the final progress report detailing our accomplishments for the year-3 of this project. In the enclosed articles we have described our preclinical works towards developing Separase inhibitor, Sepin-1 for breast cancer treatment. Our results indicate that Sepin-1 is a potential new compound that can be further developed for breast cancer therapy.**

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Introduction

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Separase, an enzyme that cleaves the chromosomal cohesin complex during mitosis, is overexpressed in more than 60% of BC and 50% of TNBC, and 65% of Luminal-B BC tumors and its overexpression strongly correlates with aneuploidy, high incidence of relapse, metastasis, and a lower 5-year overall survival rate.[4-8] Separase is an oncogene,[6,7] and overexpression of Separase induces aneuploidy, genomic instability, mammary tumorigenesis, and intratumoral heterogeneity in mice.[4,6] We posit that modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting resistant, Separase-overexpressing aneuploid tumors, particularly the hard-to-treat TNBC.[9]

We hypothesize that pharmacologic modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting Separase-overexpressing aneuploid heterogeneous tumors. We further hypothesize that by decreasing Separase activity to a therapeutically useful degree of inhibition by Sepin compounds, we will effectively reprogram the Separase-overexpressing tumors and that partial Separase inhibition will selectively eliminate Separase-overexpressing tumor cells addicted to elevated Separase expression while sparing normal cells, and thereby preventing aneuploidy and aneuploidy-associated tumor heterogeneity.

Following is the final progress report detailing our accomplishments for the year-3 of this project. In the enclosed articles we have described our preclinical works towards developing Separase inhibitor, Sepin-1 for breast cancer treatment. Our results indicate that Sepin-1 is a potential new compound that can be further developed for breast cancer therapy.

Key Words

Separase, Separase Inhibitors, Sepin-1, Triple Negative Breast Cancer

Accomplishments

The specific aim for this project is to evaluate Sepin-1 as a potential new agent for breast cancer therapy. The technical objectives for this year was to characterize the mechanisms of Sepin-1 action in inhibiting tumor growth. Following is the highlight of our progress.

- The oncostatic action of Sepin-1 has been studied, and a manuscript was just published (Zhang and Pati, 2018. ***J Cancer Sci Ther*.** 10:517-524). We examined the mechanisms of Sepin-1 action in inhibiting breast cancer cell growth. Our results indicate that Sepin-1 reduces FoxM1, a cell cycle-driving protein in breast cancer cell lines, and may represent a potetial pathway for Sepin-1 mediated growth inhibition.
- The metabolism of Sepin-1 in the human, mouse, and rat liver microsomes was investigated, and a manuscript was published (Li et al. 2018. ***Frontiers in Pharmacology*** 9:313, 1-10). As a preclinical evaluation of sepin-1, here we have profiled the Phase I metabolism and bioactivation of sepin-1 in Human liver microsomes (HLM), mouse liver microsomes (MLM), and rat liver microsomes (RLM), using metabolomic approaches. The metabolic enzymes contributing to the metabolism of sepin-1 were identified using recombinant CYP450s, and the possible reactive metabolites were investigated in HLM using glutathione (GSH) as the trapping agent. The inhibitory effects of sepin-1 on seven common CYP450s were also evaluated. These results can be used further to optimize the structures of Sepin-1, resulting in more suitable pharmacological properties and reduced possible toxicity, as well as to predict the metabolism-mediated possible interactions of sepin-1 with other chemotherapeutic drugs.
- A review article on the role of Separase as an oncogene and as a target for cancer therapy was published (Zhang and Pati, 2017. ***Biol Rev Camb Philos Soc***.; 92(4):2070-2083).
- The potential synergistic effect of Sepin-1 combined with an array of known chemotherapeutic drugs in BT-474 (ER^+ PR^+ HER^+), MCF7 (ER^+ $PR^{+/-}$ HER^-), and MDA-MB-231 and MDA-MB-468 (both triple negative) breast cancer cells were tested. The synergistic effect of Sepin-1 combined with Docetaxel was confirmed in mouse MCF7 xenograft tumor model.
- We have now a new Sepin-1 formulation (10mM Citrate-buffered saline containing, pH4.0 in 10% DMSO), as it was found that Sepin-1, at higher concentration is unstable in basic buffer such PBS pH 7.4.
- We have developed and validated a bioanalytical method for the accurate quantification of Sepin-1 and to estimate pharmacokinetic parameters in animal studies.

Some of the highlights of our accomplishments are described below. For details please refer to the attached manuscripts.

1) In our manuscript Zhang and Pati (2018) we examined the mechanisms of Sepin-1-mediated growth inhibition. Sepin-1 hinders growth of breast cancer cells, cell migration, and wound healing. Inhibition of cell growth induced by Sepin-1 *in vitro* doesn't appear to be through apoptosis but rather due to growth inhibition. Following Sepin-1 treatment caspases 3 and 7 are not activated and Poly (ADP-ribose) polymerase (Parp) is not cleaved. The expression of Forkhead box protein M1 (FoxM1), a transcription factor, and its target genes in the cell cycle, including Plk1, Cdk1, Aurora A, and Lamin B1, are reduced in a Sepin-1-dependent manner. Expressions of Raf kinase family members A-Raf, B-Raf, and C-Raf also are inhibited following treatment with Sepin-1. Raf is an intermediator in the Raf-Mek-Erk signaling pathway that phosphorylates FoxM1. Activated FoxM1 can promote its own transcription via a positive feedback loop. Sepin-1-induced downregulation of Raf and FoxM1 may inhibit expression of cell cycle-driving genes, resulting in inhibition of cell growth (**Fig. 2**).

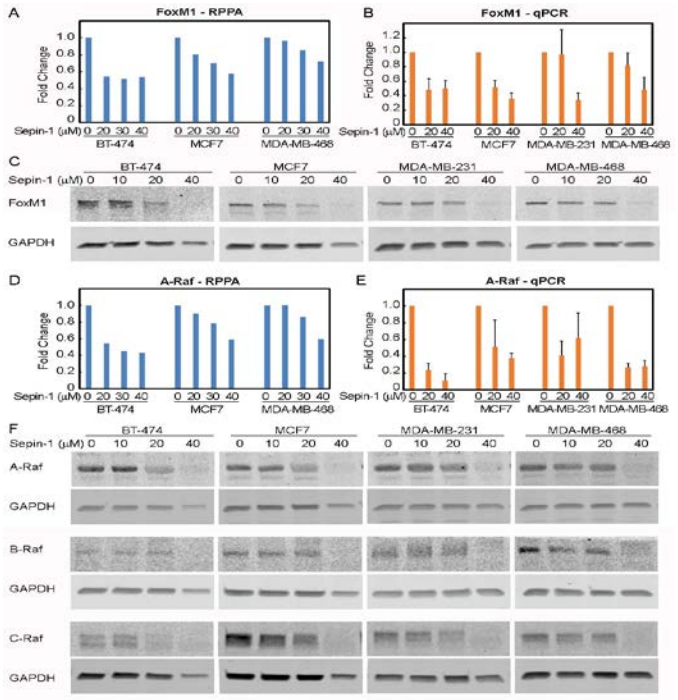

Fig. 2: Sepin-1 reduces expression of FoxM1. Breast cancer cells were treated with Spein-1 for 24h. Protein samples were made and used for RPPA (A, D) and immunoblotting (C, F). Total RNA was prepared and used for qPCR (B, E). Three independent experiments were performed for immunoblotting and qPCR. One representative immunoblotting is shown. qPCR results were average of three experiments (n=3±SE).

2) In our manuscript Li et al. (2018) we investigated the *in vitro* metabolism of Sepin-1 in human, mouse and rat liver micrsomes using metabolomic approaches. In human liver microsomes, we

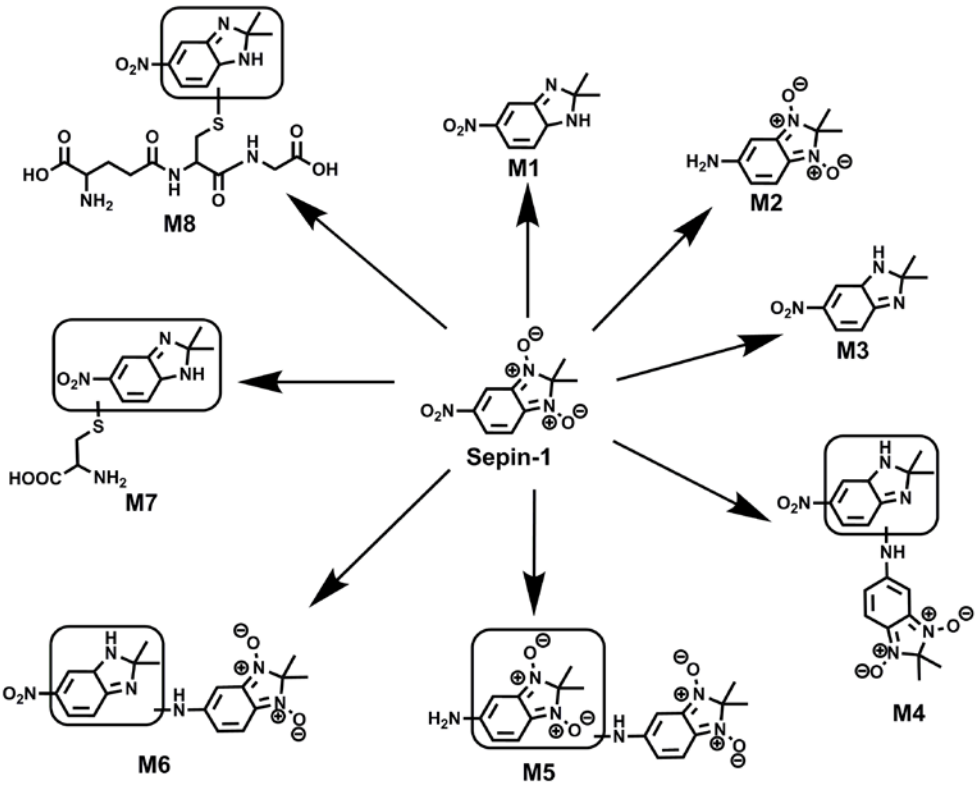

Figure 3. Summary of putative structures of Sepin-1 metabolites and adducts. All structures were determined based on the exact mass (mass error less than 10 ppm) and MS/MS fragments

identified 8 metabolites and adducts of Sepin-1, including one cysteine-sepin-1 adduct and one glutathione-sepin-1 adduct (Fig 3). Most of the Sepin-1 metabolites in HLM were also found in both mouse and rat liver microsomes. Using recombinant CYP450 isoenzymes, we demonstrated that multiple enzymes contributed to the metabolism of Sepin-1, including CYP2D6 and CYP3A4 as the major metabolizing enzymes. Inhibitory effects of sepin-1 on seven major CYP450s were also evaluated using the corresponding substrates recommended by the US Food and Drug Administration (FDA). Our studies indicated that Sepin-1 moderately inhibits CYP1A2, CYP2C19 and CYP3A4 with IC_{50} < 10 μM; but weakly inhibits CYP2B6, CYP2C8/9 and CYP2D6 with IC_{50} > 10 μM. This information can be used to optimize the structures of Sepin-1 for more suitable pharmacological properties and to predict the possible sepin-1 interactions with other chemotherapeutic drugs.

Reportable Outcomes

Manuscript:

1. Zhang N, **Pati D.** (2018). Separase Inhibitor Sepin-1 Inhibits Foxm1 Expression and Breast Cancer Cell Growth. ***J Cancer Sci Ther*.** 10(3):517-524. doi: 10.4172/1948-5956.1000517. PMID: 29780443
2. Li F., Zhang N., Gorantla S., Gilbertson S, **Pati D.** (2018). The Metabolism of Separase Inhibitor, Sepin-1 in the Human, Mouse, and Rat Liver Microsomes. ***Frontiers in Pharmacology***. 9:313, 1-10 doi: 10.3389/fphar.2018.00313.
3. Zhang, N and **Pati D** (2017). Biology and Insights into the Role of Cohesin Protease Separase in Human Malignancies. ***Biol Rev Camb Philos Soc***.;92(4):2070-2083.

Employment:

Following personnel contributed to this project: Debananda Pati, Nenggang Zhang, Siddharth Gorantla, Michael Lewis, Yukimatsu Toh, and Lacey Dobrolecki. One summer studentship was granted to Ms. Valeria Robleto, an undergraduate student from University of the Ozarks in Clarksville, Arkansas by the SMART program of Baylor College of Medicine to work on this project. SMART program is funded by the National Institute of Heath, National Institute of General Medical sciences. *Mrs. Anne Meyn, a projectLEAD graduate with over eight years' experience as an active breast cancer research advocate serves as an honorary members for this project.*

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9. Zhang N, Scorsone K, Ge G, Kaffes CC, Dobrolecki LE, Mukherjee M, Lewis MT, Berg S, Stephan CC and Pati D. (2014). Identification and characterization of Separase Inhibitors (Sepins) for Cancer Therapy. *J. Biomolecular Screening* 19(6): 878-889.

Impact

Therapeutic treatment options for patients with late stage TNBC are very limited and often unsuccessful. There are no targeted agents to supplement cytotoxic chemotherapy. The TNBC phenotype is impervious to therapies commonly used in other breast cancer subtypes, including hormonal therapy and Her-2 receptor antagonism. It is established that in the absence of ER, PR and HER-2, endocrine therapies such as Tamoxifen and aromatase inhibitors and HER-2 directed therapies such as Trastazumab and Lapatinib are not efficacious. We are seeking a new drug applications (NDA) for Sepin-1, an inhibitor of Separase, as a therapeutic target for treatment of patients with hormone refractory breast cancer who have previously received at least two chemotherapeutic regimens for metastatic disease. We believe Sepin-1 would be superior over current therapies for the following reasons.

- TNBC is a heterogeneous disease comprising a spectrum of cancers with distinct activated biological pathways. Overexpression of Separase is a fundamental characteristic shared across all TNBC, and Separase is a novel traget.
- Alternate targeted approaches currently being explored, including PARPi, EGFR inhibitors, antiangiogenic agents, and checkpoint kinase 1 inhibitors have not made a substantial impact on clinical outcomes in metastatic TNBC to date.
- Relatively low cost, straight forward CMC, and manufacturing scalability compared to monoclonal antibody and immunotherapy approaches.
- Separase expression is very high in 25-50% TNBC.
- Small molecule, relatively simple and low cost synthesis.
- Targeted inhibition of a TNBC applicable pathway separate from existing standard of care Mechanism of Action
- Relatively low toxicity.
- Compound amenable to refinement of formulation for future development and extended patent protection (liposomal/pegylation/etc.).

IND-enabling studies for Sepin-1 is funded through a product development grant from CPRIT and the mechanisms of Sepin-1 action has been pursued through this DOD grant.

Changes/Problems

The mouse clinical trial using TNBC tumor xenograft lines were abandoned due to the technical issues described in the year-2 report. Briefly, we used 90% Phosphate Buffered Saline, pH7.4 containing 0.1% Tween 20 (PBST) and 10% DMSO as a formulation for Intra peritoneal injection (IP) and oral gavage. This formulation was based on our preliminary stability analysis using lower concentration of Sepin-1 (100ng/ml). The rationale was that if a compound is stable at very low dose, it should be as well stable at higher concentrations. However, subsequently when we used the therapeutic dose for our animal studies, we found that Sepin-1 is not stable in PBS:DMSO formulation described above, and Sepin-1 stability is pH dependent. We then used a new formulation (CBS, 10mM Citrate-buffered saline containing, pH4.0 in 10% DMSO), which is stable up to 3 hours with <10% degradation. However, one other major technical issue that we also have encounter is that mouse plasma samples contain a peak shown exactly the same retention time as Sepin-1 when the samples are determined using HPLC. Despite our numerous efforts so far, we have not been successful in differentiating these two peaks. Therefore, we have not been able to complete the PK/PD study of Sepin-1 in mice. Without a PK/PD assessment, we could not justify the efficacy studies in mice models. However, as an alternate we have used rats and dogs to study the pharmacokinetics of Sepin-1. Studies in rats and dogs are covered by our accompanying grant from CPRIT.

This project has also been suffered from personnel issues (e.g. difficulty in finding and retaining suitable persons).

Products:
Not Applicable

Participants & Other Collaborating Organizations:
Not Applicable

Special Reporting Requirements
Not Applicable

Appendix-I

Statement of Work
(Work accomplished)

Month	Technical Objectives	Status

1-24 Investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of Sepin-1 in mice Abandoned due to technical issues, but currently focusing on an oral formulation and developing a new method for PK assessment as a part of the no cost extension of this project.

Milestones: PK method was developed. Appropriate route of administration and dose, frequency of dosage, has been determined. Bioavailability of Sepin-1 in SCID-beige mice was investigated in Yr. 2, but could not be done due to technical difficulties. A new formulation for Sepin-1 was developed.

6-30 To assay a therapeutic role for Sepin-1 in Separase-overexpressing breast cancer (BC). Completed, but currently focused on testing two other Sepin compounds.

Milestones: Toxic effect of the Sepin-1 has been investigated. More detailed studies are ongoing, but delayed due to the issues with Sepin-1 formulation, and slow growth of the patient-derived tumor xenografts in mice. Oncostatic effects of Sepin-1 on tumor regression were evaluated.

24-36 Drug combination experiments:
Effect of Separase inhibitors in combination with other anti-BC therapies Completed

Milestones: 1) Safety profile of the combination, including the maximum tolerable dose (MTD) and the dose-limiting toxicities (DLTs) have been determined.
2) Anti-tumor activity, pharmacodynamics (PD), PK will be assayed. Started but progress was hindered due to technical difficulties. Abandoned
3) Modeling of drug interactions will be performed. Completed

1-36 Effects of Separase inhibitors *in vivo* using a four-arm (Vehicle, Sepin-1, and 2 promising Sepin analogs) mouse clinical trial, using TNBC tumor xenograft lines. Abandoned due to technical (PK/PD assessment) issues

9-36 To characterize the mechanisms of Sepin action in inhibiting tumor growth Completed

APPENDIX-II

Manuscripts