ウェブサイト公開用

（様式 10）
（海外特別研究員事業）

令和　2　年　4　月　1　日

海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

採 用 年 度　平成 30 年度
受 付 番 号　201860009
氏　　名　森田 剣

（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　ボストン　（国名：　アメリカ　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
急性白血病における super-enhancer の網羅的解析及び標的治療薬の開発

3. 派遣期間：平成　30　年　4　月　1　日　～　令和　2　年　3　月　31　日

4. 受入機関名及び部局名
ハーバード大学　ダナ・ファーバー癌研究所　小児腫瘍学

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

1. Major goals of this project.

The primary goal of this project is to analyze and develop drugs that targets the super enhancer networks in leukemia cells. We have recently identified core molecules that constitute the super enhancers in T-cell Acute Lymphoblastic Leukemia (T-ALL) cells. There molecules form self-sustaining feed forward loops in cancer cells to maintain the homeostasis. This is called “core regulatory circuitry (CRC)” and can been seen in virtually every types of cancer cells. In T-ALL cells, RUNX1, GATA3, TAL1 and MYB are the essential CRC members (T. Sanda et al. *Cancer Cell* 2012). Interestingly, these transcription factors are often phosphorylated in cancer cell, and dephosphorylation leads to the loss of function either by conformational change or destabilization and degradation mediated by specific ubiquitination. Considering the available super enhancer targeting drugs such as JQ1 exerts its efficacy by inhibiting BRD4, other than phosphorylation, developing compounds that specifically dephosphorylate these vital transcription factors in cancer cells is a novel strategy to target and inhibit the super enhancers in cancer cells. Dephosphorylation of these CRC members are usually mediated by protein phosphatase 2 A (PP2A). Thus, the initial achievable goal of this project is to develop orally available analogues of perphenazine (PPZ), which is a potent activator of PP2A as we have reported (A. Gutierrez et al. *J Clin Invest*. 2014), with increased potency against T-cell Acute Lymphoblastic Leukemia (T-ALL) cells due to protein phosphatase 2 A (PP2A) activation and reduced neurologic side effects due to inhibition of a second target protein, dopamine receptor D2 (DRD2). Drugs with these properties will provide a novel therapeutic approach for patients with refractory ALL.

2. Things that were accomplished under these goals.

1) Major activities

PP2A is a heterotrimeric phosphatase that is assembled in the cell from three classes of subunits: 1) a catalytic C subunit (2 genes), 2) a scaffold A subunit (2 genes), and 3) a regulatory

B subunit (15 genes), each of which are coded by separate families of genes. To identify the important subunits of PP2A for the drug action of PPZ in T-ALL cells, I began by testing two gRNAs targeting each subunit and treated these knockout human T-ALL cell lines with PPZ. Through this approach, I found three key subunits of the PP2A that are necessary for the PPZ-mediated T-ALL cell killing. Biological assays such as co-immunoprecipitation assay and phosphatase activity assay complemented my finding and indicated that PPZ facilitates the assembly of these three subunits into the active PP2A holoenzyme. Next, I developed two independent biochemical reporter assays to measure the phosphatase activity of PP2A and activity of dopamine receptor signaling, and evaluated many analogues of PPZ to identify the most active analogue in PP2A activation but lacks inhibitory activities on DRD2. Based on these findings, I also set up protein crystallization experiments and crystalized the PP2A composed of identified three subunits with PPZ in place. I am currently working on deconvoluting the dataset obtained from X-ray crystallography. This will profoundly help demonstrate how PPZ acts on PP2A, which will guide us to design better analogues of PPZ in PP2A activation.

2) Specific objectives

a) Identify PP2A subunits responsible for PPZ's activity as a PP2A activator in T-ALL.
b) Clarify the effects of PPZ on PP2A activity and develop biochemical assays.
c) Analyze compounds that were designed and produced in vivo in T-ALL models.
d) Co-crystalize PP2A protein and PPZ to determine the structure of ligand-protein interface in detail for structure-based drug designing.

3) Significant results and key outcomes

a) The scaffolding subunit A is encoded by either of two genes PPP2R1A or PPP2R1B. Similarly, the catalytic Subunit C is also encoded by one of two different genes, PPP2CA and PPP2CB. The regulatory B subunit is much more diverse, with 15 possible proteins encoded by separate genes, and these proteins are recruited into complexes with the A and C subunits to confer substrate specificity. In the previous work leading up to this project (A. Gutierrez et al. *J Clin Invest*. 2014), we identified the scaffolding subunit PPP2R1A as a preferential target for PPZ binding by mass spectrometry. Crispr-Cas9-based knockout of the PPP2R1A subunit showed that the effects of PPZ were mediated by this subunit of PP2A and PPZ was shown to directly bind to PPP2R1A by in vitro pulldown using PPZ-fused agarose beads. To address **Specific objective a)**, I designed two gRNAs targeting each B and C subunit. Then I treated these knockout human T-ALL cell lines (KOPT-K1 and RPMI8402 cells) with PPZ one by one to prioritize identification of the important B and C subunits of PP2A. Intriguingly, cells became relatively but specifically resistant to killing by PPZ when PPP2CA, one of the C subunits, and PPP2R5E, one of the B subunits, were knocked out (**Figure 1**). I verified that the phosphorylation status of the major targets of PP2A, such as p-AKT and p-ERK levels, remain at pretreatment levels in these resistant cells, supporting the fact that PPZ was unable to activate the PP2A phosphatase without the implicated subunits (PPP2R1A, PPP2CA and PPP2R5E).

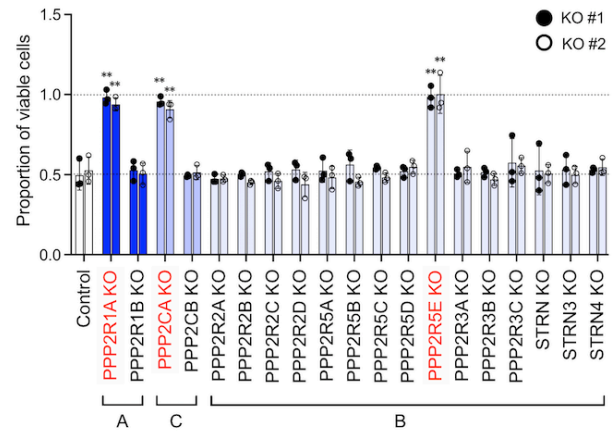

Figure 1. Identification of the subunits of PP2A required for the phosphatase activation and the antitumor activities of PPZ.
Sensitivity to PPZ as tested in KOPT-K1 cells with selective inactivation of PP2A subunits. Each subunit was knocked out by CRISPR-Cas9 with use of two unique gRNAs designed for each subunit (#1 and #2). Control gRNA targeted the luciferase gene. Cells were treated with PPZ at 5 μM for 72 hours and then examined for viability. ** $P < 0.01$ by Student's t-test, comparing the means ± SD of three biological replicates versus controls.

In **Specific objective b)**, I prepared pure PP2A subunit proteins from insect cells and conducted co-immunoprecipitation assay with or without PPZ treatment. In this experiment, I found that these 3 subunits form a complex only when PPZ was added. This PPZ-induced assembly of PP2A was specific to these three subunits, since this assembly was not observed when PPP2R2A was used instead of PPP2R5E.

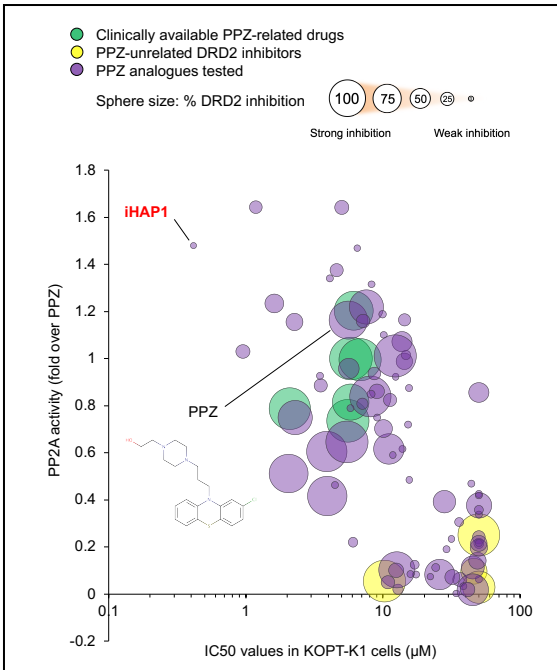

Figure 2. **Comparison of PPZ analogues based on their antileukemia potency, PP2A activation capacity and DRD2 inhibition.**
A diagram showing the relationships among three parameters for PPZ and 82 analogs including iPAP. The axes represent i) IC50 values and ii) activation potency of PP2A in KOPT-K1 cells, and iii) inhibitory concentration of DRD2 examined in HEK293T cells. For PP2A phosphatase activity assay, KOPT-K1 cells were treated with each compound at 10 μM for three hours before the activity of PP2A was quantified. DRD2 activity was monitored in HEK293T cells expressing DRD2, modified G protein and SRE luciferase reporter. Cells were treated with each compound at 10 μM for three hours, then lysed for luciferase reporter assay.

In **Specific objective c)**, I analyzed totally 91 different compounds, including PPZ itself and five structurally related FDA-approved phenothiazines (fluphenazine, chlorpromazine, prochlorperazine, thioridazine and trifluoperazine), 81 commercially available PPZ analogues, and four DRD2 inhibitors that are structurally unrelated to PPZ (sulpiride, domperidone, olanzapine and clozapine. **Figure 2** shows the potencies of each compound, including: i) growth inhibition of KOPT-K1 cells (IC_{50} values, x-axis), ii) activation of PP2A phosphatase activity (y-axis), and i) inhibition of dopamine signaling through DRD2 (represented by the size of each sphere). The clinically available phenothiazines (large green circles), each showed a moderate capacity for PP2A activation accompanied by potent inhibition of DRD2 signaling. Finally, the 81 PPZ analogues (purple circles) varied widely in their ability to either inhibit DRD2 or activate PP2A; in general, their ability to inhibit T-ALL cell growth correlated with their ability to activate PP2A. In this assay, I discovered a class of very potent small molecule PP2A activators, most prominently iHAP1, that do not interfere dopamine signaling. iHAP1 was also significantly more potent than PPZ in two other T-ALL cell lines (RPMI8402 and SUPT-13), with IC_{50} values in the submicromolar range, compared to an IC_{50} of 6 μM for each cell line with PPZ.

To test the enhanced anti-leukemic activity of iHAP1 in vivo and verify that it did not induce movement disorders through inhibition of dopamine signaling, I carried out a comparative analysis of PPZ and iHAP1 activities in vivo in human T-ALL cell xenografts in immunodeficient NSG (NOD/Scid/ IL2Rγnull) mice. To assess toxicity and determine the maximum tolerated dose (MTD), I administered PPZ and iHAP1 by daily oral gavage to 8-week-old female C57BL/6 mice (n = 3 per cohort) and monitored the animals at 15 minutes, 1 hour, 4 hours and 24 hours after each of the first seven administrations for three recognized types of DRD2-mediated toxicity: i) general activity, ii) reactivity to touch and iii) fear/startle response to sound. The abdominal muscle tone was also examined at the end of the study. During this monitoring period, all mice treated with PPZ (5 mg/kg of body weight per dose or higher amounts) showed DRD2-mediated toxicity, resulted in an MTD of 2.5 mg/kg per day. By contrast, mice treated with iHAP1 at concentrations as high as 80 mg/kg per dose showed no evidence of neurological toxicity, or any other toxicity, consistent with our biochemical reporter assays in **Figure 2** showing that iHAP1 does not inhibit DRD2 signaling. I next measured antitumor activity using immunodeficient NSG mice xenotransplanted with KOPT-K1 cells. As shown in **Figure 3**, mice treated with PPZ at its MTD of 2.5 mg/kg per day did not show any survival advantage over controls. By contrast, treatment with iHAP1 at 2.5 mg/kg per day significantly extended the mean overall survival over that of either the control or PPZ cohort. This improvement was more pronounced with higher dose of iHAP1 (80 mg/kg per day), which extended the mean survival by 3-fold, from 8 to 24 days after the start of treatment (P = 0.001). Histologic analysis of tissues sectioned after 7 days of treatment, including the femur, liver and spleen, revealed elimination of detectable hCD45+ leukemia cell growth in iHAP1-treated mice. The bone marrow cells of iHAP1-treated mice (80 mg/kg per day for seven days) showed normal hematopoietic precursor cell growth and differentiation, with all

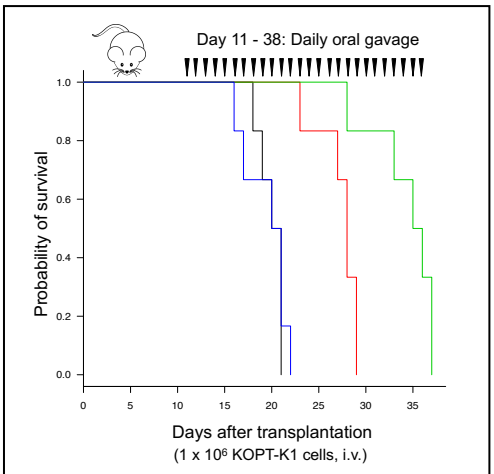

Figure 3. **Antitumor activity of iHAP1 in vivo in T-ALL preclinical models.**
Kaplan–Meier curves for NSG mice xenotransplanted with 1 million KOPT-K1 human T-ALL cells (day 1). Treatment with i) PPZ 2.5 mg/kg/day, ii) iHAP1 2.5 mg/kg/day or iii) iHAP1 80 mg/kg/day or iv) vehicle control (90% corn oil and 10% DMSO) was started on day 11. Overall survival of the xenotransplanted mice was recorded until the mice showed signs of leukemia development (n = 6 for each cohort, P values determined by the log-rank test.).

hemopoietic cell lineages represented in normal proportions. Thus, in the context of in vivo preclinical testing of anti-tumor activity and toxicity with prolonged oral treatment of T-ALL xenografts, iHAP1 emerged as a much more promising PP2A activator than PPZ, showing improved antitumor activity and a lack of toxicity due to phenothiazine-related movement disorders or any other adverse effects. These results have recently been published to stimulate scientific community for further discussion and future drug development (K Morita et al. *Cell*, in press).

Since PP2A crystallization conditions have been established, I am now attempting to co-crystallize PP2A with PPZ with help from Dr. Eric S. Fischer, who is helping me to design better analogues of PPZ [**Specific objective d)]**. Based on these results, Dr. Nathanael S. Gray will help us synthesize many additional analogues to optimize structure-function relationships. The most active PPZ analogues in killing T-ALL cells that have the least CNS toxicity that I discover during this project will be prioritized for further preclinical studies using human T-ALL PDX models in immunodeficient mice to establish pharmacokinetics, antitumor activity and toxicity in mammals. These approaches are currently established in Dr. Look's laboratory. The best molecules in these advanced preclinical studies will be developed as candidates for testing in clinical trials of T-ALL patients. My studies in T-ALL capitalize on optimal in vivo and in vitro systems for assessment of activity/toxicity relationships during drug discovery, and they are helping me to discriminate the best lead compound for clinical drug development.