Disulfiram Treatment of NUP98-PHF23 AML Is Not Effective In Vivo: Potential Role for Hematopoietic Stem Cells Niche

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Background: NUP98 has numerous partner genes of which plant homeodomain (PHD) finger protein 23 (PHF23) fusion with NUP98 (NP23) can be detected by RT-PCR in patients with cytogenetically normal acute myelogenous leukemia (AML). In this fusion transcript of NP23 PHD of PHF23 is known to specifically bind H3K4me3 residues and act as a chromatic modifier. Disulfiram (DSF) which inhibits the binding of PHD to H3K4me3 residues selectively killed NP23 myeloblasts in vitro and therefore, we planned to evaluate the efficacy of DSF in vivo.

Methods: Cultured 961C cells (CD45.2), NP23 myeloblast cells were transplanted into B57BL/6 mice (CD45.1). Using limit dilution assay the number of leukemic stem cells (LSCs) could be calculated. A certain amount of 961C cells were transplanted into B57BL/6 mice and DSF was treated after 1 week. The engraftment level was monitored with CD45.2. Kaplan Meier survival curve was used to compare the survival between therapeutic and control group.

Results: 961C cells could be transplanted without radiation in recipient mice. Calculated LSC was estimated to be 1 out of 184 cells (95% CI range, 56-609). When treated with DSF of different doses and administration routes in 961C recipient mice no survival advantage of DSF was observed in 961C transplanted immunocompetent mouse, however it was evident that engraftment level was consistent in both groups.

Conclusion: No survival advantage of DSF in 961C transplanted immunocompetent mouse was observed, however it was evident that 961C cells shared niche with normal hematopoietic stem cells (HSCs). We expect that 961C cells and transplanted recipient mice have the potential to be used as in vitro system for new drugs development as well as for research dealing with niche for normal HSCs and LSCs.

Key Words: Acute myelogenous leukemia, NUP98, PHF23, DSF, Leukemic stem cell, Hematopoietic stem cell
Introduction

Acute myelogenous leukemia (AML) is a hematologic malignancy of myeloid stem and precursor cells which is often accompanied by genetic abnormalities. Nucleoporin 98 kDa (NUP98) and plant homeodomain (PHD) finger protein 23 (PHF23) genes are involved in a translocation t(11;17)(p15;p13) that generates an in-frame fusion between NUP98 exon 13 and PHF23 exon 4 [1]. Since NUP98 was shown to be a fusion partner with homeobox (hox)A9 in AML patients with a t(7;11)(p15;p15), over 28 distinct NUP98 partner genes have been identified in AML and T cell ALL, NUP98-PHF23 (NP23) fusion transcript was first reported in an adult AML patient with normal cytogenetic in 2007 [2] and the frequency was reported as 2.6% in pediatric cytogenetically normal-AML (CN-AML) by an Italian group [3]. The PHD of PHF23 binds to di- and tri-methylated histone3 lysine 4 (H3K4me2/3), a marker for active transcription, and the NP23 fusion is hypothesized to function as an aberrant transcription factor. Expression of NP23 has been demonstrated to impair the differentiation of myeloid progenitor cells and promote leukemia development in vitro and in vivo [4,5].

Gough et al. generated a mouse model for the NP23 fusion by expressing the NP23 fusion from a pan-hematopoietic promoter: transgenic mice expressing the NP23 fusion developed a wide array of leukemias including AML, T cell ALL, NUP98-PHF23 (NP23) fusion transcript was first reported in an adult AML patient with normal cytogenetic in 2007 [2] and the frequency was reported as 2.6% in pediatric cytogenetically normal-AML (CN-AML) by an Italian group [3]. The PHD of PHF23 binds to di- and tri-methylated histone3 lysine 4 (H3K4me2/3), a marker for active transcription, and the NP23 fusion is hypothesized to function as an aberrant transcription factor. Expression of NP23 has been demonstrated to impair the differentiation of myeloid progenitor cells and promote leukemia development in vitro and in vivo [4,5].

Disulfiram (DSF) is a U.S. Food and Drug Administration (FDA) approved drug which has been used clinically for the last 60 years as an alcohol-abuse deterrent. In addition, it has been studied as a potential anticancer drug, with apoptotic effects and proteosomal inhibition shown in several types of cancers including breast cancer [6], prostatic cancer [7], non-small cell lung cancer [8], and melanomas [9]. In term of hematologic malignancy, a recent study demonstrated that DSF is effective in a B ALL patient-derived xenograft model [10].

DSF was recently shown to inhibit binding of H3K4me3 residues to PHD domain proteins in vitro [11]. DSF showed dramatic and selective killing of the NP23 myeloblast cell line, with no evidence of increased cell death in four different control myeloblast cell lines [5]. Cell deaths was preceded by decreased binding of the NP23 protein to H3K4Me3 residues at the Hoxa and Meis1 loci, accompanied by decreased expression of Hoxa, Hoxb, and Meis1. Of note, the concentration used in the NP23 myeloblast cell line (961C) was 300nM, below clinically achievable levels. Based on the above in vitro study, we wished to evaluate the efficacy of DSF in vivo using the NP23 mouse model. We first transplanted 961C cells into C57BL/6 mice and established transplantation kinetics and leukemia initiating cell number. Subsequently 961C cells transplanted C57BL/6 mice were treated with DSF.

Materials and Methods

1) 961C cell culture

961C cells were established from single-cell suspensions prepared from NP23 AML sample [5] and maintained in Iscove’s Modified Dulbecco’s medium (IDDM) supplemented with 20% fetal bovine serum (FBS), 100 mmol/L L-glutamine, and 100 µg/mL penicillin/streptomycin (Invitrogen).

2) Transplantation of 961C cell into B57BL/6 mouse

To evaluate the possibility of transplantation of 961C cell in B57BL/6, C57BL/6 mice were pretreated with ciprofloxacin one week before transplantation and a number of 10^5 961C cells were injected via the tail vein with 600 cGy radiation (N=4) and without radiation (N=4) on the day of transplantation (Fig. 1). CD45.2 antigen expression was used as a marker for...
Fig. 1. 10^6 961C cells (CD45.2 allele) were injected via tail vein into C57BL/6 recipients (CD45.1 allele) that were (N=4) or were not sub-lethally irradiated (N=4) (left). CD45.2 positive cells were detected in peripheral blood indicating that the leukemic cells were engrafted. The higher proportion of engraftment in the irradiated group (right) suggests that greater availability of hematopoietic “niches” may accelerate engraftment of the 961C cells.

961C cells; as the NP23 transgenic mice express the CD45.2 isoform of CD45, whereas the recipient C57BL/6 mice express the CD45.1 isoform of CD45. To evaluate engraftment kinetics, 10^6 961C cells were injected in C57BL/6 mice (N=15) without irradiation and three mice were euthanized every week to evaluate engraftment within the hematopoietic compartment (Fig. 2). At each week complete blood count (CBC) was performed and the level of CD45.2 in peripheral blood (PB), BM, spleen and thymus was measured using flow cytometry. Engraftment levels are dis-

Fig. 2. (A) The CD45.2 ratio of bone marrow to peripheral blood after transplantation of 961C cells. Horizontal axis indicates weeks after transplantation and vertical axis showed CD45.2 (%) in each organ (B). The pattern of double positive cell (Mac1^-/B220^+) was similar between bone marrow and peripheral blood.
played as mean±Standard Error of Mean (SEM).

3) Limiting dilution assay

The leukemia initiating cell (LIC) frequency for 961C cells was determined by a limiting dilution assay using immunocompetent C57BL/6 mice. A series of dilutions of the 961C cells (100, 1000, 1000) were introduced via tail vein and the number of mice negative for reconstitution at each cell dose was measured, and the frequency of LICs was estimated using Poisson statistics [12].

4) Flow cytometry

To evaluate the characteristics of transplanted cells Fluorescence-activated cell sorting (FACS) analysis was performed with the B220-fluorescein isothiocyanate (FITC), Mac1-phycoerythrin (PE) and CD45.2-allophycocyanin (APC) (BD Bioscience).

5) In vivo DSF study

All animal studies were approved by the National Cancer Institute (NCI) Intramural Animal Care and Use Committee; C57BL/6 mice were intravenously injected via tail vein with 10^6 961C cells without radiation. Seven days after injection, DSF (Sigma-Aldrich) was dissolved in Dimethyl sulfoxide (DMSO) and administered at different doses (12.5-100 mg/kg) via the intraperitoneal route in treatment group, whereas DMSO only was treated in control group using same route. Additional studies evaluated oral and subcutaneous route at different doses of DSF (7 mg/kg, 28 mg/kg) together with control group. Tumor burden was monitored every 7 days using flow cytometry after staining PB obtained from tail vein with an anti-mouse CD45.2.

6) Survival analysis

Kaplan Meier survival curve was plotted and the survival between DSF treated and control group was compared using long rank test, P value <0.05 was considered as significant.

Results

1) Transplantation of 961C cells in C57BL/6

To determine the feasibility of using the 961C cell line as a model for NP23-mediated AML, 10^6 961C cells were injected via tail vein with (N=4) or without (N=4) 600 cGy ionizing radiation. Within 6 weeks post transplantation (time point for initial engraftment assay) 2 of 4 mice in each group (radiation vs without radiation) were found dead. The 961C cells were identified using the CD45.2 antibody; all recipient mice had CD45.2 positive cells in PB. These findings demonstrated that 961C cells can be transplanted without ionizing radiation (Fig. 1).

To evaluate the kinetics of the 961C engraftment, 10^6 961C cells were injected into 15 mice, and 3 mice were euthanized on a weekly basis. As early as one week after injection, CD45.2 cells were detectable in the BM (mean, 4.7±1.0%), and rapidly increased to 63.0±1.5% by the 2nd week after injection. CD45.2 cells were barely detectable in PB at the 1st week time point (mean, 0.5±0.2%), but gradually increased to 74.2±7.7% by the 4th week (Fig. 2A), at which time the mice were moribund and showed anemia and leukocytosis (Table 1). Although the ratio of

### Table 1. Change of hematologic values and CD45.2 in each organ after transplantation of 10^6 961C cells in non-irradiated B57BL/6 mouse

| Time to TPLT | Hb (g/dL) | WBC (K/μL) | Monocyte (%) | Eosinophil (%) | Platelet (K/μL) | Mean CD45.2, mean±SEM (%) |
|--------------|-----------|------------|--------------|----------------|-----------------|--------------------------|
| C1wk         | 14.9±0.8  | 5.0±0.8    | 6.4±0.7      | 0.8±0.3        | 817±492.9       | 4.6±1.0                 |
| C2wk         | 14.3±0.9  | 3.9±0.4    | 7.4±0.4      | 5.1±0.3        | 477±105.1       | 63.0±1.5                |
| C3wk         | 14.1±1.1  | 7.2±2.6    | 5.3±1.1      | 5.4±1.9        | 406.0±77.7      | 59.0±9.4                |
| C4wk         | 12.4±0.2  | 16.5±2.8   | 2.6±0.3      | 17.8±1.6       | 478.3±29       | 79.0±3.7                |
| C5wk         | 10.9±1.6  | 25.8±12.6  | 2.2±0.6      | 15.8±7.5       | 827±29         | 82.7±9.6                |

Mean±SEM (%) | BM | Spleen | Thymus | PB | BM/PB ratio |
-------------|----|--------|--------|----|-------------|
4.6±1.0     | 0.8±0.2 | 0.2±0.1 | 0.5±0.2 | 9.6 |
63.0±1.5    | 57.7±2.2 | 1.0±0.1 | 20.3±6.3 | 3.1 |
59.0±9.4    | 44.0±19.2 | 1.0±0.5 | 26.9±12 | 2.3 |
79.0±3.7    | 79.0±3.8 | 1.0±0.7 | 74.0±7.7 | 1.1 |
82.7±9.6    | 71.3±5.5 | 19.4±15.6 | 63.1±30.6 | 1.3 |
961C cells in the BM compared to PB was initially 9.6:1, this ratio gradually decreased as the leukemia progressed, such that it was close to 1:1 when the mice were euthanized (Fig. 2A). As expected, the vast majority of CD45.2 cells were positive for Mac1 and B220, similar to the 961C cells grown in vitro (Fig. 2B).

2) Limiting dilution assay of 961C cells

To identify the frequency of leukemic initiating cell (LIC) in vivo, a limiting dilution assay was performed by transplantation $10^2$, $10^3$, $10^4$ cells per group (N=5). Three mice in the $10^2$ cell group were alive with no evidence of disease at 16 weeks post injection (Fig. 3A), thus, the frequency of LIC was estimated to be 1 out of 184 cells (95% CI range, 56-609) (Fig. 3B). Thus, we have established and characterized an in vivo platform that can be used to evaluate the efficacy of DSF in vivo.

3) Disulfiram in vivo xenograft mouse model

Following injection of $10^3$ 961C cells in non-irradiated C57BL/6 mice, CD45.2 was measured weekly to evaluate engraftment. For the initial experiment, two DSF doses (7 or 28 mg/kg body weight, 3 days per week) via subcutaneous injection were evaluated (each group, N=5). No significant difference in terms of survival and CD45.2 in each group was detected (Fig. 4A, supplementary data). For the second experiment, the drug was administered orally, again, no survival benefit was achieved (Fig. 4B).
experiment evaluated a higher dose of DSF (N=8), 100 mg/kg intraperitoneal route, 5 days per week. However, one day after treatment half of those of DSF treated mice were found dead and the other four appeared ill, DSF treatment was stopped due to the toxicity, and resumed at a reduced dose (data not shown). For the 4th experiment reduced dose of DSF (50 mg/kg/dose, 25 mg/kg) with intra-peritoneal route, 5 days per week was tried sequentially but there was no survival benefit compared to control group (Fig. 4C).

**Discussion**

NUP98 genes have been reported in a wide range of hematopoietic malignancies and is considered to be one of the most promiscuous fusion partner genes. The NP23 fusion retained the PHF23 PHD domain, binds to H3K4me3 and is thought to function as an aberrant chromatin modifier. Although only a limited number of AML patient have an NP23 fusion, this rare subtype showed an aggressive clinical course with unfavorable clinical outcome (Table 2). Recent published gene expression profiles of NP23 positive AML patients are enriched in gene sets of leukemic stem cells and other subsets of AML patients. The gene expression profiles from NP23 leukemic mouse showed a gene expression similar to that of NP23 positive AML patients [4], supporting the use of the NP23 mice as a preclinical platform to study therapies.

Several putative mechanisms for an anti-cancer effect of DSF or DSF-copper mixture (DSF-Cu) have been reported, including inhibition of proteosomal activity, regulation of transcription factors and activation of the stress-related JNK signaling pathways [13]. Recently, Deng et al. reported efficacy of DSF-Cu in vitro using an adult B-ALL cell line as well as in vivo patient-derived xenograft (PDX) models of adult B-ALL. $1 \times 10^5$ mononuclear cell were intravenously injected in NOD-Scid-IL2Rg−/− (NSI) mice and 7 days after inoculation DSF-Cu were administered by oral gavage in the morning (1.5 mg/kg Cu) and afternoon (150 mg/kg DSF) from Monday to Friday for consecutive 4 weeks. They showed remarkable reduction in leukemic infiltration in hematopoietic tissue (blood, BM and spleen) treated DSF-Cu, compared with control. In addition down-regulated expression of Bcl-2 and Bcl-xL in BM of the DSF-Cu treated mice was demonstrated, which was consistent with the in vitro observation [10].

Gough et al. reported that DSF resulted in rapid and dramatic apoptosis of the 961C cell line (AML cells from NP23 mouse BM) at 300 nM, which was observed only in AML cell lines that contains a PHD motif compared with non-NP23 myeloid leukemia cell lines (188G3, 189E6, 32D, and BA/F3) [5]. Six hours after treatment with 300 nM DSF, more than 70% of 961C cells showed apoptotic death. However, we were unable to demonstrate efficacy of DSF in vivo using a mouse model based on 961C cells. There are several plausible explanations for this inconsistency. First, the 961C cells were engrafted without irradiation in C57BL/6 recipients. In contrast to irradiated recipients, or

### Table 2 Characteristics and treatment outcome in published patients with NUP98-PHF23 fusion transcript

| No. | Age, years | Gender | FAB | CR duration (mo) | HSCT | Relapse | Survival duration (mo) | Karyotype |
|-----|------------|--------|-----|------------------|------|---------|------------------------|-----------|
| 1   | 42         | Male   | M0  | 11               | No   | Yes     | 14                     | $^{10}$46,XY,i(1117)(P15;13) |
| 2   | 14         | Female | M0  | 7                | No   | Yes     | 16                     | $^{10}$46,XX,+3,i(1117)(P15;13) |
| 3   | 2.9        | Male   | M1  | 5                | Yes (AUTO) | Yes | 30         | CN-AML |
| 4   | 9.0        | Male   | M0  | 65               | Yes (MUD) | No | 66$^{ab}$ | CN-AML |
| 5   | 9.7        | Male   | M4  | 40               | Yes (MUD) | No | 41$^{ab}$ | CN-AML |
| 6   | 7.0        | Male   | M5  | 103              | Yes (MUD) | No | 104$^{ab}$ | CN-AML |

$^{ab}$Lived patient.

$^{a}$Above karyotyping was confirmed by conventional karyotyping together with FISH targeting terminal band of 17P in patient No1. and 2 [4].

FAB, French American British; CR, complete remission; mo, month; HSCT, hematopoietic stem cell transplantation; CN-AML, cytogenetically normal acute myelogenous leukemia; auto, autologous; MUD, matched unrelated donor.
immunodeficient recipients (such as NOD-Scid-IL2Rg−/− (NSI) mice), the recipients in these experiments had normal hematopoietic and immune systems. Alternatively, an in vitro-in vivo discrepancy might be caused by drug binding protein(s) present in vivo that effectively decrease the bio-availability of free DSF, and/or rapid washout of DSF. Actually investigators attempted to account for in vitro-in vivo discrepancy through preceding studies. There are at least 3 possible mechanisms: existence of long-lasting inhibition in vivo, involvement of protein-bound forms of drugs, and substrate dependent Ki values (unbound fraction of an inhibitor in blood) [14]. However, these plausible mechanisms have not yet been comprehensively analyzed in this study.

Ablation of myeloid and/or lymphoid-lineage before transplantation has been considered as prerequisite condition to cause the niche available for engraftment. In vivo imaging study has shown that leukemic stem cells can hijack normal BM vascular niche spaces by exploiting signals such as CXCL12 and E-selectin that are important for the homing of healthy HSCs [15]. LSCs are less dependent than healthy HSCs on certain niche signals for their survival, proliferation, and anchoring in the niche. Otherwise LSC are more dependent on CD44 extracellular anchoring and on various selectins and their ligands for homing and engraftment in the marrow cavity compared to normal HSCs as shown in human AML xenograft transplantation [16,17]. However, 961C cells engrafted without radiation, suggesting that this cell line may have potential to be used for the study of LSC and their niche.

In this study, we demonstrated that 961C AML cells could be rapidly engrafted in wild-type, immunocompetent recipients, without the use of ionizing radiation or other myelo/lympho-ablative strategies, providing a transplantable, immunocompetent model of AML. However, despite the in vitro sensitivity of 961C cells to DSF, no therapeutic efficacy of DSF in 961C transplanted immunocompetent mice was demonstrated. Despite this failure, we anticipate that 961C murine model will be useful for drug development as well as for study of competition of normal HSC and LSC for available niches in vivo.

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