# OSBP-related protein 4L abrogation by OSW-1 results in T-ALL cell death

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## Abstract

**Oxysterol binding protein (OSBP) and OSBP-related protein 4L (ORP4L) were recently identified as targets of the highly potent anti-cancer natural product OSW-1 and suggested to play roles in cancer cell survival. However, the mechanisms of OSW-1/OSBP/ORP4L-induced cancer cell apoptosis remained obscure. Here we show that ORP4L in Jurkat T-leukemia cells interacts with inositol 1,4,5-trisphosphate receptor 1 (ITPR1) and modulates Ca2+ release from the endoplasmic reticulum (ER) to control cell fate. These findings suggest that control of Ca2+release from the ER by ORP4L is important for leukemia cell survival and cytotoxicity of OSW-1, and identify ORP4L as a potential new target of leukemia therapy.**

## Keywords

**ORP4L,OSW-1,T-ALL,Cell death.**

## 1. Introduction

OSBP is a cytoplasmic protein with a high affinity for oxysterols[1]. In humans, 12 genes encode a family of proteins characterized by a C-terminal OSBP-related ligand-binding domain (ORD)[2] that binds oxysterols or cholesterol[3; 4]. This OSBP-related protein (ORP) family has been implicated in vesicle transport, cellular lipid metabolism, and cell signaling, which they regulate in a sterol-specific manner[5; 6]. ORP4 has been shown to bind 7-ketocholesterol[7] and 25-hydroxycholesterol (25-OHC)[8; 9]; There are two major variants of this protein, designated ORP4S and ORP4L, of which ORP4S comprises a sterol binding domain only, while ORP4L has an additional N-terminal extension containing a pleckstrin homology (PH) domain and a so-called two phenylalanines in an acidic tract (FFAT) motif that specifies targeting at the endoplasmic reticulum[8]. The *ORP4/OSBP2* transcript has been reported as a potential marker for solid tumor dissemination and poor prognosis[10], and to be detectable in peripheral blood leukocytes from chronic myeloid leukemia patients but not healthy donors[10; 11].

The naturally occurring compound OSW-1 is highly cytotoxic against tumor cell lines[12]. Recent reports have identified ORP4L as a target of OSW-1 involved in tumor cell proliferation and survival[13; 14; 15]. This compound has a unique mechanism of action, in which structural and functional damage to mitochondria triggers activation of a $Ca^{2+}$dependent apoptosis pathway. However, the underlying molecular mechanisms remain unclear.

## 2. Materials and Methods

### 2.1 Cells and reagents

Jurkat T-lymphocytes were maintained in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% $CO_2$.

### 2.2 Co-immunoprecipitation

For co-immunoprecipitation assays, Jurkat T-cells were stimulated with 10 μg $ml^{-1}$ of anti-CD3, and cell lysates were immunoprecipitated with anti-ITPR1 antibodies.

### 2.3 Western blot analysis

Cells were washed twice with ice-cold PBS, scraped from the dishes, and suspended in lysis buffer (50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 0.5 mM MgCl2, 10% glycerol, 1% Triton-X100, 0.1% SDS)

with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) on ice for 10 min before clearing of the lysates by centrifugation for 5 min at 12000 g. The supernatants were collected and analyzed by SDS-PAGE. Images were captured using Tanon-5200 and the densitometry of each band was quantified using Tanon Gis software.

### 2.4 Calcium fluorometry and single cell calcium imaging

Cells ($2 \times 10^5$ cells/dish) were incubated with 1 μM Fluo4-AM for 30 min at 37°C in extracellular calcium buffer (ECB, 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Hepes, pH 7.5, 1 mg/ml BSA, and 5 mM glucose) in dark, replaced with fresh ECB or ECB lacking $CaCl_2$ and containing 0.1 mM EGTA for an additional incubation at 25°C for 30 min to permit dye de-esterification. Then, cells were excited with low-intensity 488-nm laser excitation. Images were acquired at 2s intervals under time-lapse mode by confocal microscope. Fluorescence was collected for 2 min before histamine (10 μM) or thapsigargin (1 μM) were added into the suspension. Image data were subsequently analyzed using ImageJ (National Institutes of Health) and are presented as a ratio of F/F0 in final results, where F0 represents baseline fluorescence intensity of each cell.

### 2.5 ATP generation measurement

ATP levels were determined ATP Bioluminescence Assay Kit CLS II (Roche, Basel, Switzerland) according to the manufacturer's instructions.

### 2.6 Cell proliferation and cell apoptosis assay

For cell proliferation assay, 5,000 Jurkat T-cells infected with control or shORP4L lentivirus for 24 h were plated in 96-well flat bottom plates with RPMI 1640 medium supplemented with 10% of FBS. After 1–4 days' culture, cell numbers were evaluated by Cell Counting kit-8 (WST-8 assay, Dojindo, Molecular Technologies, Rockville, MD, USA) following the manufacturer's protocol. Cells were stained with Hoechst 33342 for examination of typical apoptotic nuclear morphology by epifluorescence microscopy.

### 2.7 Statistics

The data are expressed as mean±s.d. All comparisons between groups were made by unpaired two-tailed Student's t test. P values of $<0.05$ were considered statistically significant.

## 3. Results

### 3.1 ORP4L interacts with ITPR1

To verify the ORP4L-ITPR1 interaction, we employed the human T-cell leukemia cell line Jurkat expressing high levels of both ITPR1[16] and ORP4L, to conduct co-immunoprecipitation (co-IP). ORP4L was specifically co-precipitated with antibodies against ITPR1(Fig. 1).

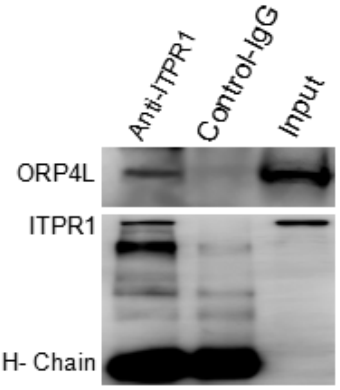


Fig. 1 ORP4L interacted with ITPR1 in Jurkat T cells analyzed by co-immunoprecipitation.

### 3.2 ORP4L regulates Ca2+-dependent bioenergetics and cell fate

In contrast to most cancer cells, which rely on aerobic glycolysis as the major source of ATP, leukocytes from patients with ALL are characterized by high respiratory rates and low aerobic glycolysis[17]. $Ca^{2+}$ released constitutively from the ER via ITPRs is taken up by mitochondria, which

stimulates mitochondrial dehydrogenases required for the maintenance of normal cell bioenergetics[18; 19]. OSW-1 treatment significantly decreased the percentage of Jurkat T-cells displaying $Ca^{2+}$ oscillations (from 56% to 20%), an effect partly reversed by ORP4L overexpression (from 20% to 42%). Moreover, the $Ca^{2+}$ oscillation frequency was lower in OSW-1 treated cells than in controls and was partially restored by ORP4L overexpression (Fig. 2). Consistent with altered $Ca^{2+}$ signaling, generation of ATP was inhibited by OSW-1 and rescued by ORP4L overexpression (Fig. 3). The above results indicated that over-expression of ORP4L leads to increase of $Ca^{2+}$ release and suboptimal bioenergetics.

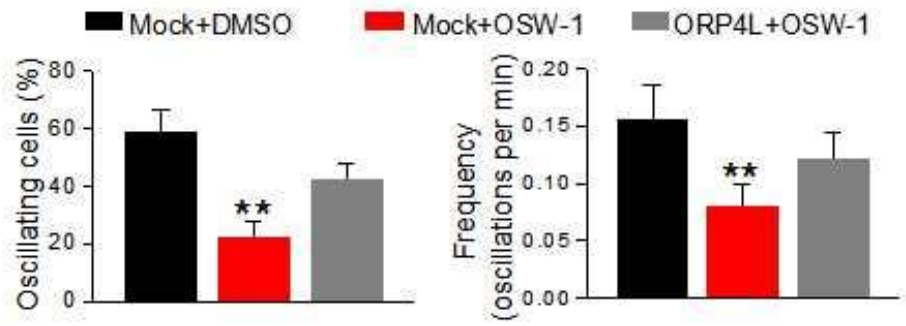


Fig. 2 OSW-1 targeting ORP4L results in Ca2+ oscillations decrease in Jurkat T cells (n=3). Data are presented as mean ± SD. **P<0.01. Student's t test.

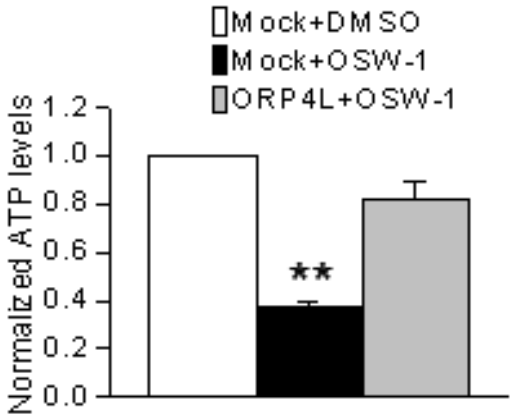


Fig. 3 Generation of ATP was inhibited by OSW-1 and rescued by ORP4L(n=3). Data are presented as mean ± SD. **P<0.01. Student's t test.

### 3.3 ORP4L inhibition by OSW-1 results in cell death

A group of structurally diverse natural products including cephalostatin 1, OSW-1, ritterazine B and two schweinfurthins that act as potent inhibitors of cancer cell growth have been demonstrated to target ORP4L[20]. However, the mechanism by which OSW-1 exerts anti-tumor activity is not well delineated. We observed that ORP4L overexpression desensitized Jurkat T-cells to OSW-1 cytotoxity (Fig. 5). Subsequently, we treated Jurkat T cells with different concentrations of OSW-1, and then detected apoptosis by Hoechst 33342 staining. We found that apoptosis of Jurkat T cells increased with increasing OSW-1 treatment concentration(Fig. 4). And with the increase of OSW-1 treatment time, the apoptosis rate of Jurkat T cells also increased, and the apoptosis rate of Jurkat T cells in the untreated group was almost unchanged. These results show that ORP4L mediates the cytoxic effect of OSW-1 and identify ORP4L as a putative new candidate target for the development of T-ALL therapy.

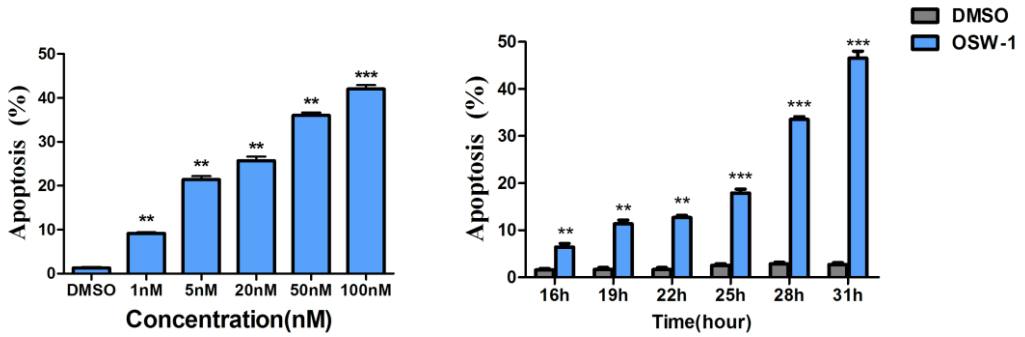


Fig. 4 OSW-1 affects the apoptosis of Jurkat T cells. Data are presented as mean ± SD. ***P<0.001、**P<0.01. Student's t test.

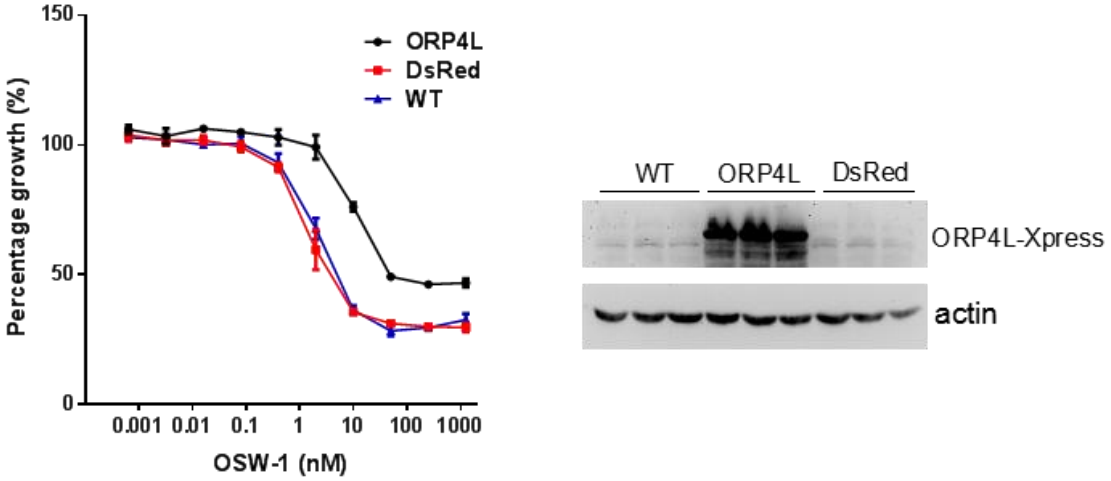


Fig. 5 Effects of ORP4L over-expression on the susceptibility of Jurkat T cells to OSW-1 cytotoxicity(n=3).

## 4. Conclusion

In summary, we have revealed that ORP4L acts as a new controller of ER $Ca^{2+}$ release and a regulator of cell fate decisions in T-leukemia cells. We have further established that ORP4L mediates the pro-apoptotic action of OSW-1. Our results suggest that ORP4L regulates ITPR1 function to facilitate cell viability. Considering the role of ORP4L in cell fate decisions, expression of ORP4L in leukemia cells, and the potency of ORP4L inhibition in reducing tumor cell growth, this protein is a putative new candidate target for the development of leukemia treatments.

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