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# High-throughput screening identifies artesunate as selective inhibitor of cancer stemness: Involvement of mitochondrial metabolism

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ABSTRACT

Cancer stem cells (CSCs) have robust systems to maintain cancer stemness and drug resistance. Thus, targeting such robust systems instead of focusing on individual signaling pathways should be the approach allowing the identification of selective CSC inhibitors. Here, we used the alkaline phosphatase (ALP) assay to identify inhibitors for cancer stemness in induced cancer stem-like (iCSCL) cells. We screened several compounds from natural product chemical library and evaluated hit compounds for their efficacy on cancer stemness in iCSCL tumorspheres. We identified artesunate, an antimalarial drug, as a selective inhibitor of cancer stemness. Artesunate induced mitochondrial dysfunction that selectively inhibited cancer stemness of iCSCL cells, indicating an essential role of mitochondrial metabolism in cancer stemness.



## 1. Introduction

Decades of cancer drug discovery approaches are largely based on cancer cell killing ability and precisely inhibiting specific oncogene. These approaches led to the accumulation of potent cytotoxic drugs that achieve partial or even complete tumor regression, but are frequently followed by resistant relapse [1]. Cancer cells within a solid tumor are heterogeneous [2] and include a small population of differently progressing cancer stem cells (CSCs) which have tumor initiating ability [3]. These CSCs can evade cytotoxicity of chemotherapeutic drugs and drive relapse after chemotherapy. In other words, CSCs are driver cells while the other differentiated tumor cells are passengers. Thus, selective CSC inhibitors are essential for stable cancer cure.

CSCs are maintained by functional abilities to expand in constantly evolving tumor-microenvironment, which also determine therapeutic resistance [4,5]. Such functional abilities are maintained by redundant pathways and inhibition of a single pathway prompts activation of alternative pathways [1,6,7]. Consequently, approaches that target such functional abilities should be used for anti-CSCs drug discovery. Tumorspheres (3D-culture) allow CSCs to activate their functional abilities, including genetic expression, tumor heterogeneity, and drug resistance, conferring ability to mimic CSCs in the physiological tumor microenvironment [8–12]. Thus, CSCs in tumorspheres form a suitable in-vitro functional system to assay functional inhibition of CSCs.

In this study, we focused on inhibiting functional system rather than individual pathways or genes associated with cancer stemness. To generate a functional assay system for CSCs, we utilized a platform of induced cancer stem cell-like (iCSCL) cells. These cells are established by artificial induction of stemness using induced pluripotent stem cells (iPS) technology. They are characterized for cancer stemness and tumorigenicity, and can be stably propagated and used for CSCs selective drug screening [13,14]. Here, we performed large-scale drug screening of approximately 6000 compounds and discovered that the antimalarial drug artesunate is a selective inhibitor of cancer stemness. Further, we found that mitochondrial metabolism is critical for cancer stemness of iCSCL cells and that artesunate selectively inhibited cancer stemness by inducing mitochondrial dysfunction.

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## 2. Materials and methods

### 2.1. Reagents

Artesunate, deferoxamine (DFO), and hydrocortisone were purchased from Tokyo Chemical Industry (Japan). Salinomycin, phenformin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), sodium pyruvate, insulin, and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (USA). Oligomycin, antimycin, rotenone, and small molecules for drug screenings were obtained from RIKEN NPDepo library (Japan) [15,16]. DMEM-F12 and B-27 supplement were purchased from Life Technologies (USA). Seahorse microplates$^{TR}$ and XF Assay media$^{TR}$ were purchased from Seahorse Bioscience (USA). Sox2 antibody, *para*-Nitrophenylphosphate (pNPP) substrate, fibroblast growth factor-basic (bFGF), heparin, flat bottom ultra-low attachment surface plates, Cell Count Reagent SF, and CellTiter-Glo were purchased from Millipore (USA), Kanto Chemical (Japan), Peprotech (USA), Mochida Pharma (Japan), Corning (USA), Nacalai Tesque (Japan), and Promega (USA), respectively.

### 2.2. Serum-free media

Serum-free media for tumorspheres were prepared by using DMEM-Ham's F12 supplemented with insulin (5 μg/ml), hydrocortisone (0.5 μM), B27 (2%), EGF (20 ng/ml), bFGF (10 ng/ml), and heparin 10 U/ml. Media were freshly prepared before use.

### 2.3. Tumorspheres formation

Monolayer cultured iCSCL-10A cells were trypsinized, washed with PBS, suspended in serum-free media and seeded in ultra-low attachment surface plates at a density of $16 \times 10^4$ cells/ml. Tumorspheres formed on the 4th day were drug treated and analyzed on the 7th day.

### 2.4. Cell based assays

**Alkaline phosphatase (ALP) assay** – Extraction solution (0.9% NaCl + 1% NP-40) and ALP Buffer (0.2 M Tris-HCl - pH 9.5, 1 mM $MgCl_2$) were prepared and stored at 4 °C for long-term use. Before ALP assay, pNPP substrate was dissolved in ALP buffer at 12.5 mM to prepare the substrate solution, and substrate solution and extraction solution were brought to room temperature. ALP assay was performed in 96-well plates; culture media were removed and 50 μl extraction solution was added followed by 50 μl of pNPP substrate solution. The cells were incubated at 37 °C for 1 h and the absorbance was measured at 405 ηm.

**WST-8 Assay** and **CellTiter-Glo (CTG) Assay**– Cell viability was assessed using Cell Count Reagent SF (WST-8 assay) and CellTiter-Glo reagent (CTG assay) as described earlier [17].

### 2.5. Extracellular flux analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using the XF Assay media supplemented with 1 mM sodium pyruvate and 25 mM glucose and analyzed by XF96 analyzer (Seahorse). For monolayer (2D) culture analysis, iCSCL-10A cells were seeded on Seahorse microplate for one day and then treated with drugs for indicated time. The culture medium was changed to XF Assay media prior to analysis and incubated in $CO_2$ free incubator for 1 h. For tumorspheres (3D) analysis, tumorspheres were drug treated for indicated condition. Prior to analysis, tumorspheres were dissociated into single cells by pipetting then washed with PBS, suspended in XF Assay media, and seeded at 30000 cells/well. The plates were centrifuged at 1000 rpm for 1 min and incubated in $CO_2$ free incubator for 1 h. Drugs used in these assays were FCCP (0.5 μM), antimycin (1 μM), and rotenone (1 μM).

## 3. Results

### 3.1. Drug screening for cancer stemness inhibitors using iCSCL-10A cells

We analyzed the functional relevance of iCSCL-10A tumorspheres regarding cancer stemness and chemoresistance. Stemness gene Sox2 expression was higher in tumorspheres (3D) than in monolayer cells (2D) (Fig. 1A) suggesting better stemness in 3D cells. Anti-cancer drugs paclitaxel and gemcitabine were less effective in 3D culture than in 2D culture (Fig. 1B). However, the 3D culture was susceptible to CSC inhibitor salinomycin (Fig. 1B) indicating that iCSCL-10A tumorspheres are likely suitable for functional assay to evaluate efficacy and selectivity of cancer stemness inhibitors. Nevertheless, tumorspheres assay requires much time, high costs and has a low-throughput; therefore, not suitable for drug screening of large number of compounds.

Drug screening using ALP assay in iCSCL cells was reported to be a simplified platform to discover cancer stemness inhibitors [13]. Salinomycin, a well-known CSC inhibitor, selectively inhibited ALP in monolayer culture of iCSCL-10A cells (Fig. 1C). This suggests that CSC inhibitors, such as salinomycin, could be easily detected by ALP assay. Further, besides having a high-throughput, ALP assay requires shorter time and lower costs than tumorspheres assay. Such features made ALP assay of iCSCL-10A suitable for primary high-throughput screening of cancer stemness inhibitors.

We performed a primary screening of about 6000 compounds from RIKEN NPDepo library at 2 μg/ml, with salinomycin as a positive control (Fig. 1D). In order to identify the compounds that have selectivity against cancer stemness, we selected the 86 compounds with more than two-fold selectivity for ALP inhibition and not less than 40% viability as primary hits (Fig. 1E).

Next, we analyzed the primary hits at a high dose (5 μg/ml) by comparing their selectivity in tumorspheres assay against monolayer culture (Fig. 2A). We selected compounds which have at least four-fold selectivity towards tumorspheres inhibition (3D/2D viability ratio <0.25) and obtained 25 hits (Fig. 2A). We evaluated these hits at 10 fold lower dose (0.5 μg/ml) to select the potent inhibitor of tumorspheres (Fig. 2B). We found that NPD2604, an artemisinin analog is the most potent and selective inhibitor of tumorspheres (Fig. 2B,C).

We evaluated additional 27 analogs of NPD2604 and found that NPD2604, artemisinin, and artesunate had excellent selectivity and potency against tumorspheres (Fig. 2D). Artesunate was selected as a selective CSC inhibitor for further evaluation because of its common and safe use as antimalarial drugs in human.

### 3.2. Selectivity to cancer stemness inhibition is mediated by mitochondrial dysfunction

Artesunate induced cancer cell death was reported to be mediated by iron and such cell death is prevented by chelation of free iron using DFO [18]. Thus, we analyzed the role of free iron in artesunate induced cell death, ALP inhibition and tumorspheres inhibition. Similar to salinomycin, artesunate also selectively inhibited ALP at low dose (Supplement Fig. 1). Artesunate induced cell death in 2D cells only at a high dose (30 μM) and cell death was prevented by DFO (Fig. 3A). However, ALP and tumorspheres were inhibited at lower doses of artesunate (3 μM and 0.3 μM respectively) but not prevented by DFO (Fig. 3B,C). These results show that