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Efflux inhibition by IWR-1-endo confers sensitivity to doxorubicin effects in osteosarcoma cells

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ABSTRACT

Osteosarcoma is the most common bone tumor that affects children and young adults. Despite advances in the use of combination chemotherapy regimens, response to neoadjuvant chemotherapy in osteosarcoma remains a key determinant of patient outcome. Recently, highly potent small molecule inhibitors of canonical Wnt signaling through the poly(ADP-ribose) polymerase (PARP)-family enzymes, tankyrases 1 & 2 (Tnks1/2), have been considered as possible chemotherapy sensitizing agents. The goal of this study was to determine the ability of the highly specific Tnks1/2 inhibitor IWR-1-endo to sensitize chemotherapy-resistant osteosarcoma to doxorubicin. We found that IWR-1-endo significantly inhibited cellular efflux, as measured by cellular retention of Calcein AM and doxorubicin. In a model of doxorubicin resistant osteosarcoma, pre-treatment with IWR-1-endo strongly sensitized to doxorubicin. This sensitization reduced the doxorubicin IC_{50} in doxorubicin-resistant cells, but not in chemotherapy naïve cells and caused doxorubicin-treated cells to accumulate at the G2/M checkpoint. Further, we found that sensitization with IWR-1-endo produced increased γH2AX foci formation, indicating increased DNA damage by doxorubicin. Taken together, our findings show that IWR-1-endo increases cellular responses to doxorubicin, by blocking efflux transport in a drug-resistant model of osteosarcoma.

1. Introduction

Neoadjuvant chemotherapy, or rounds of chemotherapy given prior to tumor excision, has been in widespread use in osteosarcoma patients since its efficacy was demonstrated by Rosen et al. and Winkler et al. in 1979 and 1984, respectively [1,2]. Methotrexate, adriamycin (doxorubicin), and *cis*-platin (the MAP regimen) is the current standard of care for osteosarcoma. The MAP regimen as neoadjuvant chemotherapy has yielded substantial improvements in patient morbidity and mortality since its widespread acceptance nearly thirty years ago. Additionally, the prognostic value of response to neoadjuvant chemotherapy, assessed by tumor necrosis grading at the time of tumor resection, has been shown [3,4]. Thus, tumor response to initial rounds of chemotherapy is indicative of patient outcome. Despite these findings, trials which have attempted to exploit the prognostic value of response to neoadjuvant chemotherapy in osteosarcoma have not yet reported significant improvement in outcomes [5]. Non-responsive tumors frequently acquire increased expression of the ATP-binding cassette (ABC) family of efflux transporters, which reduce the intracellular concentration of chemotherapy toxins such as paclitaxel, 5-fluorouracil, etoposide, olaparib, and doxorubicin (dox) by limiting their intracellular concentrations [6–8]. In multiple cancers, several ABC transporters have been shown to facilitate the efflux of dox, including ABC3, MDR1, MDR3, ABC19, MRP1, MRP2, MRP6, and BCRP1 [8–12]. Overlapping selectivity between chemotherapies and ABC transporters is reported, thus dox-resistance through cellular efflux is driven by a multi-modal and adaptable system which generally produces the multidrug resistance (MDR) phenotype. In this manner, acquired resistance to one chemotherapy (such as dox) through ABC overexpression, is reported to confer resistance to other anticancer chemotherapies which are also ABC substrates [7]. This mechanism of resistance is associated with poor outcomes in various human cancers [6–8].

Recently, inhibitors of the canonical Wnt signaling pathway have been explored as chemotherapy sensitizing agents in lung and colorectal cancer models. In these studies, inhibitors of the Axin2-regulating Tankyrase 1 and Tankyrase 2 enzymes (Tnks1/2) have been shown to sensitize to epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K), and AKT inhibition in various cancer models [13–16]. The current literature has not yet clarified whether Tnks1/2 inhibition would sensitize to commonly utilized chemotherapies which facilitate

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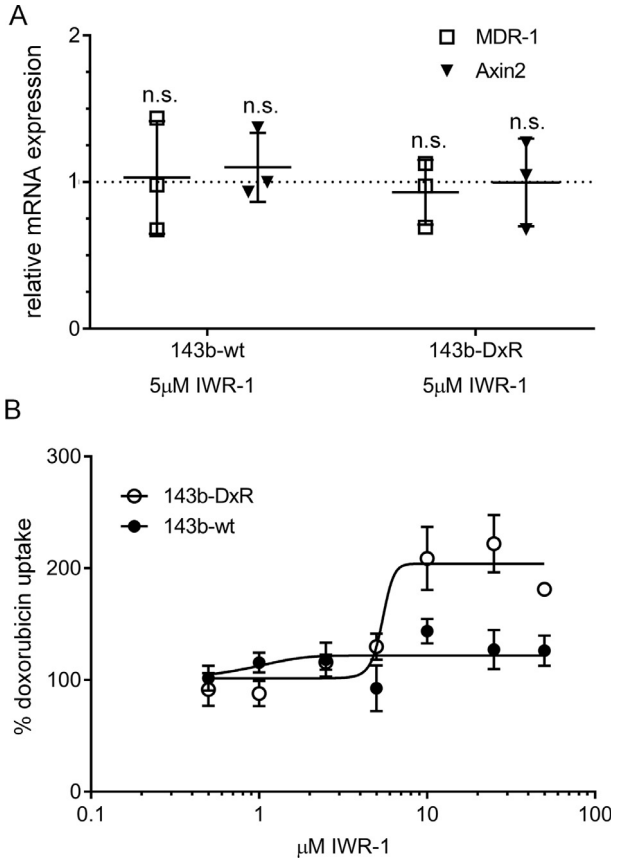

Fig. 1. IWR-1 does not alter Wnt target gene expression but inhibits efflux of doxorubicin in osteosarcoma cells. (A) Relative mRNA expression of MDR-1 and Axin2 in the 143b-wt and 143b-DxR cell lines following 16 h treatment with 5 μM IWR-1. Mean change in expression relative to vehicle control for each cell line is shown, as well as the standard deviation (n = 3). (B) 143b-DxR and 143b-wt cells were treated with increased doses of IWR-1 for 16 h before quantification of doxorubicin uptake by fluorescent emission (535 nm). The average of nine unique measurements from each well was calculated to determine fluorescent values. Results from a representative experiment are shown, error bars represent standard deviation. P-value determined by student's *t*-test, where $p < 0.05$ was considered statistically significant (n = 3).

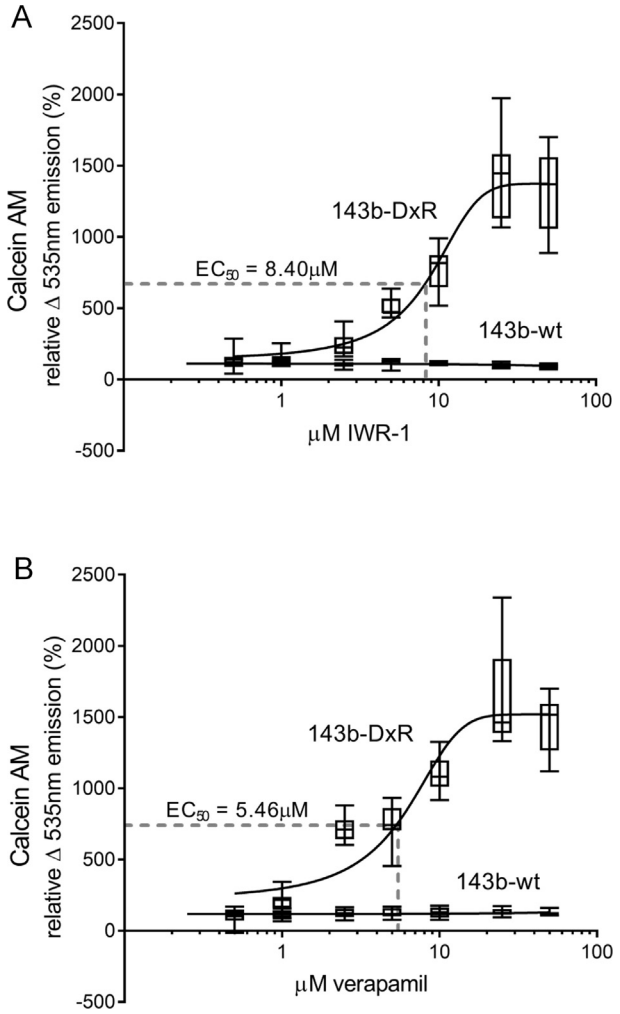

Fig. 2. IWR-1 inhibits efflux of ABC transporter substrate Calcein AM. 143b-DxR and 143b-wt cells were treated with increasing doses of IWR-1 (A) or verapamil (B) before incubation with Calcein AM for 30 min. Fluorescent emission from Calcein AM was quantified following incubation period. The average of nine unique measurements from each well was calculated to determine fluorescent values. The mean of three independent experiments containing technical triplicates is shown for each experimental condition; boxes represent the interquartile range while error bars represent maximum and minimum values.

DNA damage. Moreover, regulation of ABC transporter expression by β-catenin, the primary target of canonical Wnt signaling, has been reported, indicating that transcriptional regulation of the ABC transporters through Wnt inhibition may be possible [17–19]. Thus, we sought to investigate the ability of Tnks1/2 inhibition, via the small molecule Tnks1/2 inhibitor IWR-1-endo (IWR-1), to mitigate resistance to dox in osteosarcoma. We developed a model of chemotherapy resistant osteosarcoma by challenging a naïve cell line with dox, selecting surviving colonies, and expanding the resistant cells for further challenge. Treatment of chemotherapy-resistant osteosarcoma cells with IWR-1 significantly increased intracellular concentrations of Calcein AM and doxorubicin in resistant cells, indicating inhibition of cellular efflux transport. This effect was observed to be independent of regulation of Wnt target genes. Dox-resistant cells were sensitized by pre-treatment with IWR-1, causing increased toxicity from dox and accumulation of cells at the G2/M checkpoint. Additionally, our data show increased numbers of γH2AX foci with IWR-1 sensitization, indicating increased damage to DNA via accumulation of dox in the cell. In sum, we report that IWR-1 inhibits cellular efflux capacity, and sensitizes to dox in a model of chemotherapy resistant osteosarcoma.

2. Materials and methods

2.1. Mammalian cell culture

Human osteosarcoma cell lines 143b (143b-wt, American Type Culture Collection, Manassas, VA, USA), and the derived 143b doxorubicin-resistant cell line (143b-DxR) were cultured in 75 cm^{-2} flasks in Dulbecco's Advanced Modified Eagles Media-F12 (DMEM-F12, Sigma-Aldrich, St. Louis, MO, USA) formulation without the addition of penicillin or streptomycin. The 143b-DxR cell line was developed by challenge with doxorubicin (Sigma-Aldrich, St. Louis, MO, USA), and selection and expansion of colonies which were resistant to the drug. The 143b-DxR cell line was continuously kept in 200 nM doxorubicin. Prior to use, cell lines were kept in liquid phase nitrogen.

2.2. mRNA expression quantitation

Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the Zymo Quick-RNA™ MicroPrep kit (Zymo Research, Irvine, CA, USA) were used to collect and purify RNA from cells. cDNA was produced from 2.0 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied