Published in final edited form as:

Biochem Pharmacol. 2018 April ; 150: 141–149. doi:10.1016/j.bcp.2018.01.037.

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

Carl T. Gustafson¹, Tewodros Mamo¹, Avudaiappan Maran^{2,3}, and Michael J. Yaszemski^{1,2,3}

¹Mayo Clinic College of Medicine, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota, USA

²Mayo Clinic College of Medicine, Department of Orthopedic Surgery, Mayo Clinic, Rochester, Minnesota, USA

³Mayo Clinic College of Medicine, Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota, USA

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 IC₅₀ 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 γ H2AX 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

Corresponding author: Avudaiappan Maran, Mayo Clinic College of Medicine, Department of Orthopedic Surgery, Mayo Clinic, 200 1st SW, Rochester, Minnesota, USA 55905. maran.avudai@mayo.edu.

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 multi-drug 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 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 γ H2AX 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 cm² 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 200nM 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 Biosystems, Fisher Scientific, Waltham, MA, USA). A 10uL reaction volume containing a total of 25 ng of sample cDNA was used for each qRT-PCR reaction. qRT-PCR was performed on an ABI HT7000 Thermocycler using SYBR GreenER (Thermo-Fisher, Waltham, MA, USA). The quantitation of mRNAs has been carried out by normalizing the data using GAPDH as described in the manufacturer's protocol (Thermo-Fisher, Waltham, MA). Primer sequences used: GAPDH F 5' –ATGTTTCGTCATGGGTGTGAA–3', R 5' –TGTGGTCATGAGTCCTTCCA–3'; Axin2 F 5' –AGAAATGCATCGCAGTGTGAAG–3', R 5' –GGGTTCCTCGGGAAATGAGGTA–3'; MDR-1 F 5' –GAGCCCATCCTGTTTGACTGC–3', R 5' –TGTATGTTGGCCTCCTTTGCTG–3'.

2.3 Cytotoxicity assay

5×10⁴ cells were plated per well in 24-well polystyrene dishes and allowed to adhere for 16 hours before treatment with vehicle or drug. All treatment solutions were prepared in DMEM-F12 as described above without the addition of fetal bovine serum (Thermo-Fisher, Waltham, MA, USA), and incubated at 37°C for the entirety of the treatment. To measure cell viability, wells were rinsed with Dulbecco's phosphate buffered saline (DPBS, Corning, Corning, NY, USA), and Cell Titer 96® MTS reagent assay (ProMega, Madison, WI, USA) was used according to manufacturer recommendation.

2.4 Calcein AM and doxorubicin retention assay

1×10⁴ cells were plated per well in 96-well round bottom polystyrene dishes and allowed to adhere for 16 hours before treatment with vehicle or drug. IWR-1-endo (Calbiochem, San Diego, CA, USA) or verapamil (Sigma-Aldrich, St. Louis, MO, USA) were added to the wells for 16 hours (IWR-1-endo) or 15 minutes (verapamil). After treatment, Calcein AM (Thermo-Fisher, Waltham, MA, USA) was reconstituted in DPBS and added to each well to reach a final dilution of 1:3000 (333nM), and incubated at 37°C for 30 minutes. For doxorubicin retention assays, following treatment with IWR-1, doxorubicin was added to each well to reach a final concentration of 10µM, and incubated at 37°C for 2 hours.

Solutions were then removed, wells were rinsed with DPBS, and total fluorescent emission in each well was measured with a TECAN Infinite F200 Pro plate reader (Tecan, Zurich, Switzerland).

2.5 Dose-response curve fitting

The GraphPad Prism 7 software was used to perform non-linear fitting of the dose-response curves for excitation (Calcein AM or doxorubicin retention) and inhibition (doxorubicin toxicity) experiments. These models additionally provided the reported values for the E_{MAX} , EC_{50} , and IC_{50} , where appropriate.

2.6 Cell cycle analysis

143b-wt or 143b-DxR cells were seeded onto 10 cm dishes at a density of 5×10^5 cells per dish. 16 hours after plating, cells were sensitized with vehicle or treatment for 16 hours, then treated with vehicle or treatment for 24 hours. Following treatment, whole cells were harvested, washed with phosphate buffered saline (PBS), and fixed in 95% ethanol. Samples were treated with RNase A (1.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and stained with propidium iodide (100 μ g/ml; Roche, Indianapolis, IN) prior to cell cycle analysis (Flow Cytometry Core at the Mayo Clinic).

2.7 γ H2AX immunofluorescence

Treated glass coverslips placed in wells of 6-well polystyrene dishes were seeded with 2×10^5 cells per well. Cells were sensitized with vehicle or treatment groups for 16 hours. Following sensitization, wells were rinsed, and cells were treated with vehicle or doxorubicin for 1 hour. Cells were then fixed in 4% formalin, permeabilized with 0.5% Triton-X (Sigma-Aldrich, St. Louis, MO, USA), and blocked with 3% w/v bovine serum albumin in PBS for 1 hour. Blocked cover slips were rinsed twice with PBS, and stained with antibody against phospho-Histone H2A.X (1:400, 2577S, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Stained cover slips were washed with PBS, and incubated with Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:300, A-11008, Thermo-Fisher, Waltham, MA, USA) for 1 hour. Prepared cover slips were then stained with DAPI (Invitrogen, Carlsbad, CA, USA), fixed to glass slides with ProLong antifade mountant (Thermo-Fisher, Waltham, MA, USA), and imaged with a confocal laser scanning microscope (Nikon, Melville, NY, USA) with a 200X objective.

3. Results

Expression of Wnt target genes Axin2 and MDR-1 was not reduced by IWR-1 treatment for 16 hours in either the 143b-wt or the 143b-DxR cell lines (Figure 1A). Incubation with doxorubicin (dox) following IWR-1 treatment caused increased fluorescence from intracellular dox (Figure 1B). Significantly stronger fluorescent emission from doxorubicin was observed in the 143b-DxR cell line, compared to the 143b-wt cells. Treatment with IWR-1 for 16 hours significantly stimulated fluorescent staining with Calcein AM in the 143b-DxR cell line, with no strong effect on the 143b-wt cells (Figure 2A). For comparison, and to evaluate the dynamic range of the assay, 143b-DxR and 143b-wt cells were treated with verapamil for 15 minutes, and intracellular Calcein AM fluorescence was measured

(Figure 2B). The EC₅₀ values for IWR-1 and verapamil in the Calcein AM retention assays were 8.40 μ M and 5.46 μ M, respectively, as calculated from non-linear fitting of the dose response curves (Table 1).

The capacity for IWR-1 to act as an inhibitor of cellular efflux led us to hypothesize that these cell lines could be sensitized to the DNA damaging effects of dox by IWR-1 treatment. Thus, we treated the 143b-DxR and 143b-wt cell lines with IWR-1 for 16 hours prior to addition of dox for 24 hours. We found that the addition of IWR-1 as a sensitizer, in combination with dox, significantly reduced the viability of the 143b-DxR cells, compared to dox alone (Figure 3A). In the 143b-wt cells, we did not observe any significant reduction in dox IC₅₀ with the addition of IWR-1 sensitization (Figure 3B), findings which were consistent with the lack of effect on Calcein AM retention or dox retention in the 143b-wt cells. IWR-1 treatment alone inhibited cell viability in both the 143b-wt and 143b-DxR cell lines (Figure 3C). Sensitization by IWR-1 reduced the IC₅₀ of dox to 55.2% (11.76 μ M, down from 21.31 μ M) compared to dox alone in the 143b-DxR cells (Table 2), while there was no significant difference in dox IC₅₀ between the IWR-1 sensitized and vehicle treated 143b-wt cells.

To determine whether this sensitization effect was due to increased dox toxicity or to IWR-1 toxicity, we further explored the functional effects of IWR-1 in combination with dox. Treatment with IWR-1 alone did not induce any detectable disruption in the cell cycle distribution of 143b-DxR, or 143b-wt cells (Figure 4A-B and 5A-B, respectively). We observed a significant increase (1.48 fold greater than vehicle control, $p < 0.05$) in G2/M cells with dox treatment at 1 μ M in the 143b-DxR cell line (Figure 4C). Addition of IWR-1 as a sensitizer, 16 hours prior to dox treatment, induced a statistically significant increase in G2/M cells (1.50 fold greater, $p < 0.0001$) relative to dox alone (Figure 4C-D). In the 143b-wt cell line, 20nM dox induced cells accumulating at the G2/M checkpoint as well (Figure 5C, 1.72 fold increase relative to vehicle control, $p < 0.05$), yet 143b-wt cells which were sensitized with IWR-1 did not display any further G2/M phase cells from dox treatment, compared to the dox treated cells (Figure 5C-D).

A hallmark of dox activity in the cell is the induction of DNA double-stranded breaks (DSBs). Thus, we studied the ability of dox, and the combination of IWR-1 and dox, to induce DNA damage in the cells, by analysis of γ H2AX foci formation. Treatment with IWR-1 did not induce γ H2AX foci formation relative to vehicle control in the 143b-DxR or 143b-wt cells (Figure 6 and Figure 7). Dox treatment for 1 hour induced γ H2AX foci in the 143b-DxR (20 μ M dox) and 143b-wt (1 μ M dox) cell lines, with mean foci-positive cells reaching 23.15%, and 21.94% of total cells, respectively (Figure 6A, Figure 7A). An increase in γ H2AX positive cells was observed (1.89 fold increase, relative to dox alone, $p < 0.005$) when 143b-DxR cells were sensitized with IWR-1 prior to treatment with dox (Figure 6A-B). IWR-1 sensitization prior to dox treatment in the 143b-wt cell line did not induce a significant increase in foci-positive cells (Figure 7A-B). This indicated that dox toxicity due to DSBs was increased by IWR-1 in the 143b-DxR cell line, with no effect on the 143b-wt cells.

4. Discussion

Osteosarcoma patients suffer a striking reduction in 5-year event free survival if neoadjuvant chemotherapy is ineffective [5]. Resistance to many cancer chemotherapies is driven by the efflux activity of the family of ATP-dependent transporters [20]. Among cohorts of osteosarcoma patients, tumor expression of MDR-1 (P-gp) is an adverse prognostic factor which is significantly associated with poor outcomes [21,22]. Thus, means to reduce expression or activity of the ABC transporters are of great interest for the improvement of cancer chemotherapy efficacy among osteosarcoma patients. In this study, we demonstrate that IWR-1 reduces efflux of doxorubicin and Calcein AM in doxorubicin-resistant osteosarcoma cells, and sensitizes these cells to doxorubicin by inhibiting their ability to remove doxorubicin; thus increasing the effect of doxorubicin on cell cycle disruption and DNA damage.

Canonical Wnt signaling is regulated by the Tankyrase 1 and Tankyrase 2 enzymes (Tnks1/2) through post-translational modification of Axin2 with poly-ADP-ribose (PAR). Axin2 acts as a scaffold that facilitates the assembly of a protein complex which targets β -catenin, the main effector of canonical Wnt signaling, for destruction. Thus, inhibition of Tnks1/2 activity results in stabilized Axin2, and reduced β -catenin-mediated Wnt signaling. Small molecule inhibitors of Tnks1/2 have been shown to regulate Wnt signaling and tumor growth in models of colorectal, breast, and non-small cell lung cancers [23–25]. Studies which have utilized the Tnks1/2 inhibitor IWR-1 in cancers have observed strong tumor suppressing activity, while our data demonstrate only weak effects on cell viability as a single-agent therapy in osteosarcoma cells (Figure 2) [26,27]. The IWR-1 molecule demonstrates endo / exo isomerism, where the -exo compound has been shown to be inactive, in contrast to the -endo compound (utilized exclusively in this study) which strongly regulates Wnt signaling both *in vitro* and *in vivo* [28]. While Tnks1/2 inhibitors are being considered as candidate molecules for primary chemotherapy, their application as adjuvant therapy may have an even greater impact. Combination therapy with Wnt-blocking compounds has been evaluated in the context of radiation therapy, EGFR inhibitors, and PI3K inhibitors [13,16,25,29]. While these studies have focused on Wnt influence on the DNA damage response, or genome wide screens for efficacious targets, our study is the first to concentrate on the ability of a Tnks1/2 inhibiting small molecule to influence chemotherapy resistance through direct inhibition of cellular efflux. Due to the large number of cancer chemotherapy molecules which are substrates of ABC transporters, and the widespread reports of ABC family transporter overexpression in drug-resistant cancers, our results indicate that IWR-1 mediated inhibition of cellular efflux may well sensitize to a large number of molecules in resistant tumor models.

We chose to work primarily with the 5 μ M concentration of IWR-1 for several reasons which are supported by the current literature. In their seminal paper, Huang *et al.* report the IC₅₀ for IWR-1 in targeting the Tnks1/2 enzymes, as well as the PARP1, and PARP2 enzymes which are structurally similar to Tnks1/2 in their catalytic ADP-ribose transferase domain [30]. While IWR-1 selectively inhibits Tnks1 and Tnks2 at concentrations of 131nM and 56nM, respectively, the PARP1 and PARP2 enzymes are targeted by IWR-1 at concentrations greater than 18.75 μ M. Thus, treatment concentrations of IWR-1 which reach

greater than 18.75 μ M risk significant inhibition of PARP1 and PARP2, confounding conclusions which may be made regarding the specificity of the effects induced by IWR-1. Published reports show treatment concentrations of IWR-1 ranging from 10nM to 50 μ M, with several studies reporting effects on Wnt signaling and sensitization in the range of 5–20 μ M [25,27,30,31]. Indeed, a recent report demonstrated that 10 μ M IWR-1 strongly inhibited both Tnks1 and Tnks2 with no effect on PARP1 and only weak effects on nine additional PARP enzymes [32]. Moreover, our data demonstrate that IWR-1 significantly increased Calcein AM accumulation in the cell at a concentration of 5 μ M (508% increase – Figure 1A). Thus, we chose to continue with the sensitization experiments at the 5 μ M concentration of IWR-1 based upon the limited likelihood of unintended effects on PARP1/2, consistency with adjacent reports, and data which we have reported in this manuscript.

Further investigations with patient-derived osteosarcoma tissues, and testing in additional resistant cell lines and animal models will more thoroughly establish IWR-1 and other Tnks1/2 inhibitors as chemotherapy sensitizing agents. Our data clearly demonstrate that IWR-1 inhibits cellular efflux of doxorubicin, and suggest that this inhibition is independent of Wnt signaling responses, as Axin2 and MDR-1 mRNA expression remained unchanged. While our data indicate inhibition of cellular efflux with effects only on a chemotherapy resistant cell line, this effect may be mediated through a number of possible mechanisms which are currently unclear. Further research might elucidate whether IWR-1 directly interacts with an ABC transporter to competitively inhibit its activity, and if so, which transporters are inhibited by IWR-1. Conversely, IWR-1 may act to reduce efflux of doxorubicin and Calcein AM through other mechanisms, such as by affecting proper trafficking of the ABC transporters through the cell, or by blocking their ability to embed within the cell membrane. Additional research using Tnks1/2 targeting drugs and genetic interference have demonstrated consistent and strong sensitization through adjacent, Wnt-intersecting pathways [14,16,25]. Thus, while our study uncovers inhibition of cellular efflux activity as an effect of IWR-1 treatment, sensitization to chemotherapeutic agents, such as doxorubicin, by IWR-1 may be driven through multiple pathways. The doxorubicin-resistant cell line which we have developed is a single clone of a resistant cell line. Thus, it likely represents a small subset of doxorubicin resistant osteosarcoma, limiting the conclusions which may be drawn regarding osteosarcoma treatment from the findings in this study. Models of resistance to methotrexate and cisplatin, as well as additional models of doxorubicin resistance in osteosarcoma will facilitate further validation of current findings and broader interpretation of conclusions regarding response to chemotherapies such as IWR-1 in the context of osteosarcoma treatment.

In sum, this report uncovers the doxorubicin sensitization effects of IWR-1 in chemotherapy resistant osteosarcoma. Highly potent and specific targeting of Tnks1/2 continues to demonstrate success as adjuvant chemotherapy in multiple models of cancers, the characterization and improved design of these candidate drugs is imperative for their progress towards the clinic. The use of sensitizing agents in osteosarcoma could make a significant positive impact in outcomes among the population of chemotherapy non-responsive osteosarcoma patients.

Acknowledgments

We thank Dr. Martin Fernandez-Zapico, Dr. Nicole Murray, and Dr. Joel Reid for their thoughtful advice and discussion throughout the performance of this work. The Mayo Clinic Microscopy and Cell Analysis Core lab is thanked for expert assistance with cell cycle analysis. We acknowledge funding support from the Mayo Clinic Orthopaedic Research Review Committee (CG), the Predoctoral Training Program in Molecular Pharmacology (NIH T32 GM072474–10, CG; Director: Larry Karnitz), the Predoctoral Training Program in Musculoskeletal Research (NIH 5T32 AR56950–7, TM; Director: Jennifer Westendorf), the NIH/NCI Predoctoral National Research Service Award (1F31CA206388–01A1, TM), Riviera Foundation (AM), and the John and Posy Krehbiel Endowed Professorship in Orthopedic Surgery and Biomedical Engineering (MY).

6. References

- [1]. Rosen G, Marcove RC, Caparros B, Nirenberg A, Kosloff C, Huvos AG, Primary osteogenic sarcoma. The rationale for preoperative chemotherapy and delayed surgery, *Cancer*. 43 (1979) 2163–2177. doi:10.1002/1097-0142(197906)43:6<2163::AID-CNCR2820430602>3.0.CO;2-S. [PubMed: 88251]
- [2]. Winkler K, Beron G, Kotz R, Salzer-Kuntschik M, Beck J, Beck W, Brandeis W, Ebell W, Erttmann R, Göbel U, Neoadjuvant chemotherapy for osteogenic sarcoma: Results of a cooperative German/Austrian study, *J. Clin. Oncol* 2 (1984) 617–624. [PubMed: 6202851]
- [3]. Winkler K, Beron G, Dellling G, Heise U, Kabisch H, Purfurst C, Berger J, Ritter J, Jurgens H, Gerein V, Graf N, Russe W, Gruemayer ER, Ertelt W, Kotz R, Preusser P, Prindull G, Brandeis W, Landbeck G, Neoadjuvant chemotherapy of osteosarcoma: Results of a randomized cooperative trial (COSS-82) with salvage chemotherapy based on histological tumor response, *J. Clin. Oncol* 6 (1988) 329–337. [PubMed: 2448428]
- [4]. Meyers PA, Schwartz CL, Krailo M, Kleiner ES, Betcher D, Bernstein ML, Conrad E, Ferguson W, Gebhardt M, Goorin AM, Harris MB, Healey J, Huvos A, Link M, Montebello J, Nadel H, Nieder M, Sato J, Siegal G, Weiner M, Wells R, Wold L, Womer R, Grier H, Osteosarcoma: A randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate, *J. Clin. Oncol* 23 (2005) 2004–2011. doi:10.1200/JCO.2005.06.031. [PubMed: 15774791]
- [5]. Isakoff MS, Bielack SS, Meltzer P, Gorlick R, Osteosarcoma: Current treatment and a collaborative pathway to success, *J. Clin. Oncol* 33 (2015) 3029–3035. doi:10.1200/JCO.2014.59.4895. [PubMed: 26304877]
- [6]. Vaidyanathan A, Sawers L, Gannon A-L, Chakravarty P, Scott AL, Bray SE, Ferguson MJ, Smith G, Vaidyanathan G, Zalutsky MR, ABCB1 (MDR1) induction defines a common resistance mechanism in paclitaxel- and olaparib-resistant ovarian cancer cells, *Imaging drug resistance with radiolabeled molecules*, *Br. J. Cancer* 115 (2016) 431–441. doi:10.1038/bjc.2016.203. [PubMed: 27415012]
- [7]. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM, Targeting multidrug resistance in cancer, *Nat Rev Drug Discov*. 5 (2006) 219–234. doi:nrd1984[pii]r10.1038/nrd1984. [PubMed: 16518375]
- [8]. Xia CQ, Smith PG, Drug efflux transporters and multidrug resistance in acute leukemia: therapeutic impact and novel approaches to mediation., *Mol. Pharmacol* 82 (2012) 1008–21. doi: 10.1124/mol.112.079129. [PubMed: 22826468]
- [9]. Steinbach D, Gillet J-P, Sauerbrey A, Gruhn B, Dawczynski K, Bertholet V, de Longueville F, Zintl F, Remacle J, Efferth T, ABCA3 as a possible cause of drug resistance in childhood acute myeloid leukemia., *Clin. Cancer Res* 12 (2006) 4357–4363. doi: 10.1158/1078-0432.CCR-05-2587. [PubMed: 16857811]
- [10]. Turton NJ, Judah DJ, Riley J, Davies R, Lipson D, Styles J. a, Smith a G., Gant TW, Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance., *Oncogene*. 20 (2001) 1300–1306. doi:10.1038/sj.onc.1204235. [PubMed: 11313874]
- [11]. Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W, Frank MH, ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma, *Cancer Res*. 65 (2005) 4320–4333. doi:10.1158/0008-5472.CAN-04-3327. [PubMed: 15899824]

- [12]. Ikeda R, Vermeulen LC, Lau E, Jiang Z, Sachidanandam K, Yamada K, Kolesar JM, Isolation and characterization of gemcitabine-resistant human non-small cell lung cancer A549 cells, *Int. J. Oncol* 38 (2011) 513–519. doi:10.3892/ijo.2010.866. [PubMed: 21152861]
- [13]. Scarborough HA, Helfrich BA, Casás-Selves M, Schuller AG, Grosskurth SE, Kim J, Tan A-C, Chan DC, Zhang Z, Zaberezhnyy V, Bunn PA, DeGregori J, AZ1366: An Inhibitor of Tankyrase and the Canonical Wnt Pathway that Limits the Persistence of Non-Small Cell Lung Cancer Cells Following EGFR Inhibition, *Clin. Cancer Res* 23 (2017). doi:doi: 10.1158/1078-0432.CCR-16-1179.
- [14]. Okada-Iwasaki R, Takahashi Y, Watanabe Y, Ishida H, Saito J, Nakai R, Asai A, The Discovery and Characterization of K-756, a Novel Wnt/ β -Catenin Pathway Inhibitor Targeting Tankyrase, *Mol. Cancer Ther* 15 (2016) 1525 LP–1534. doi:10.1158/1535-7163.MCT-15-0938. [PubMed: 27196752]
- [15]. Wang H, Castillo J, Zhang Y, Yang Z, McAllister G, Lindeman A, Reece-Hoyes J, Tallarico J, Russ C, Hoffman G, Xu W, Schirle M, Cong F, Tankyrase inhibitor sensitizes lung cancer cells to endothelial growth factor receptor (EGFR) inhibition via stabilizing angiomotins and inhibiting yap signaling, *J. Biol. Chem* 291 (2016) 15256–15266. doi:10.1074/jbc.M116.722967. [PubMed: 27231341]
- [16]. Arqués O, Chicote I, Puig I, Tenbaum SP, Argilés G, Dienstmann R, Fernández N, Caratù G, Matito J, Silberschmidt D, Rodon J, Landolfi S, Prat A, Espín E, Charco R, Nuciforo P, Vivancos A, Shao W, Tabernero J, Palmer HG, Tankyrase Inhibition Blocks Wnt/ β -Catenin Pathway and Reverts Resistance to PI3K and AKT Inhibitors in the Treatment of Colorectal Cancer, *Clin. Cancer Res* 22 (2016) 644–656. doi:10.1158/1078-0432.CCR-14-3081. [PubMed: 26224873]
- [17]. Flahaut M, Meier R, Coulon A, Nardou KA, Niggli FK, Martinet D, Beckmann JS, Joseph JM, Muhlethaler-Mottet A, Gross N, The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/ β -catenin pathway, *Oncogene*. 28 (2009) 2245–2256. doi:10.1038/onc.2009.80; 10.1038/onc.2009.80. [PubMed: 19421142]
- [18]. Correa S, Binato R, Du Rocher B, Castelo-Branco MT, Pizzatti L, Abdelhay E, Wnt/ β -catenin pathway regulates ABCB1 transcription in chronic myeloid leukemia, *BMC Cancer*. 12 (2012) 303. doi:10.1186/1471-2407-12-303. [PubMed: 22823957]
- [19]. Hung T-H, Hsu S-C, Cheng C-Y, Choo K-B, Tseng C-P, Chen T-C, Lan Y-W, Huang T-T, Lai H-C, Chen C-M, Chong K-Y, Wnt5A regulates ABCB1 expression in multidrug-resistant cancer cells through activation of the non-canonical PKA/ β -catenin pathway., *Oncotarget*. 5 (2014) 12273–90. doi:10.18632/oncotarget.2631. [PubMed: 25401518]
- [20]. Gottesman MM, Fojo T, Bates SE, Multidrug Resistance in Cancer: Role of Atp-Dependent Transporters, *Nat. Rev. Cancer* 2 (2002) 48–58. doi:10.1038/nrc706. [PubMed: 11902585]
- [21]. Serra M, Pasello M, Manara MC, Scotlandi K, Ferrari S, Bertoni F, Mercuri M, Alvegard TA, Picci P, Bacci G, Smeland S, May P-glycoprotein status be used to stratify high-grade osteosarcoma patients? Results from the Italian/Scandinavian Sarcoma Group 1 treatment protocol., *Int. J. Oncol* 29 (2006) 1459–68. <http://www.ncbi.nlm.nih.gov/pubmed/17088985>. [PubMed: 17088985]
- [22]. Serra M, Scotlandi K, Reverter-Branchat G, Ferrari S, Manara MC, Benini S, Incaprera M, Bertoni F, Mercuri M, Briccoli A, Bacci G, Picci P, Value of P-glycoprotein and clinicopathologic factors as the basis for new treatment strategies in high-grade osteosarcoma of the extremities, *J. Clin. Oncol* 21 (2003) 536–542. doi:10.1200/JCO.2003.03.144. [PubMed: 12560446]
- [23]. Quackenbush KS, Bagby S, Tai WM, Messersmith WA, Schreiber A, Greene J, Kim J, Wang G, Purkey A, Pitts TM, Nguyen A, Gao D, Blatchford P, Capasso A, Schuller AG, Eckhardt SG, Arcaroli JJ, The novel tankyrase inhibitor (AZ1366) enhances irinotecan activity in tumors that exhibit elevated tankyrase and irinotecan resistance, *Oncotarget*. 7 (2016) 28273–28285. doi: 10.18632/oncotarget.8626. [PubMed: 27070088]
- [24]. Bao R, Christova T, Song S, Angers S, Yan X, Attisano L, Inhibition of Tankyrases Induces Axin Stabilization and Blocks Wnt Signalling in Breast Cancer Cells, *PLoS One*. 7 (2012). doi: 10.1371/journal.pone.0048670.
- [25]. Casás-Selves M, Kim J, Zhang Z, Helfrich BA, Gao D, Porter CC, Scarborough HA, Bunn PA, Chan DC, Tan AC, DeGregori J, Tankyrase and the canonical Wnt pathway protect lung cancer

- cells from EGFR inhibition, *Cancer Res.* 72 (2012) 4154–4164. doi: 10.1158/0008-5472.CAN-11-2848. [PubMed: 22738915]
- [26]. Busch AM, Johnson KC, V Stan R, Sanglikar A, Ahmed Y, Dmitrovsky E, Freemantle SJ, Evidence for tankyrases as antineoplastic targets in lung cancer., *BMC Cancer.* 13 (2013) 211. doi:10.1186/1471-2407-13-211. [PubMed: 23621985]
- [27]. Lee SC, Kim O-H, Lee SK, Kim S-J, IWR-1 inhibits epithelial-mesenchymal transition of colorectal cancer cells through suppressing Wnt/ β -catenin signaling as well as survivin expression, *Oncotarget.* 6 (2015) 27146–27159. [PubMed: 26450645]
- [28]. Lu J, Ma Z, Hsieh JC, Fan CW, Chen B, Longgood JC, Williams NS, Amatruda JF, Lum L, Chen C, Structure-activity relationship studies of small-molecule inhibitors of Wnt response, *Bioorganic Med. Chem. Lett* 19 (2009) 3825–3827. doi:10.1016/j.bmcl.2009.04.040.
- [29]. Jun S, Jung Y, Suh HN, Wang W, Kim MJ, Oh YS, Lien EM, Shen X, Matsumoto Y, McCrea PD, Li L, Chen J, Park J, LIG4 mediates Wnt signalling-induced radioresistance, *Nat. Commun* 7 (2016) 10994. doi:10.1038/ncomms10994. [PubMed: 27009971]
- [30]. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Zhang Y, Wiessner S, Hild M, Shi X, Wilson CJ, Mickanin C, Myer V, Fazal A, Tomlinson R, Serluca F, Shao W, Cheng H, Shultz M, Rau C, Schirle M, Schlegl J, Ghidelli S, Fawell S, Lu C, Curtis D, Kirschner MW, Lengauer C, Finan PM, Tallarico JA, Bouwmeester T, Porter JA, Bauer A, Cong F, Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling, *Nature.* 461 (2009) 614–620. doi:10.1038/nature08356. [PubMed: 19759537]
- [31]. Wang S-H, Chang J-S, Hsiao J-R, Yen Y-C, Jiang S-S, Liu S-H, Chen S-H, Shen J-Y, Chang S-H, Chen Y-W, Tumour cell-derived WNT5B modulates in vitro lymphangiogenesis via induction of partial endothelial-mesenchymal transition of lymphatic endothelial cells, *Oncogene.* 36 (2017) 1503–1515. doi:10.1038/onc.2016.317. [PubMed: 27593938]
- [32]. Kulak O, Chen H, Holohan B, Wu X, He H, Borek D, Otwinowski Z, Yamaguchi K, Garofalo LA, Ma Z, Wright W, Chen C, Shay JW, Zhang X, Lum L, Disruption of Wnt/ β -Catenin Signaling and Telomeric Shortening Are Inextricable Consequences of Tankyrase Inhibition in Human Cells, *Mol. Cell. Biol* 35 (2015) 2425–2435. doi:10.1128/MCB.00392-15. [PubMed: 25939383]

Figure 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 hour treatment with 5 μ M 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 hours before quantification of doxorubicin uptake by fluorescent emission (535nm). 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).

Figure 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 minutes. 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.

Figure 3. Sensitization with IWR-1 increases doxorubicin toxicity in doxorubicin resistant cells but not in wild-type cells.

143b-DxR cells (A), or 143b-wt cells (B) were treated with vehicle or 5 μM IWR-1 for 16 hours before addition of increasing doses of doxorubicin. Cell viability was measured after 24 hours of doxorubicin treatment. C. Toxicity from 16 hour IWR-1 treatment in 143b-wt and 143b-DxR cells. Cell viability determined by MTS assay is represented as a percentage of control (vehicle treated) cells. The average of three independent experiments is shown for each experimental condition; error bars represent the standard deviation. Statistical significance was determined using the Bonferroni-Dunn method to correct for multiple comparisons with $\alpha = 0.05$. A $p < 0.00833$ was considered to be statistically significant (*).

Figure 4. IWR-1 sensitizes to doxorubicin-induced G2/M arrest in doxorubicin-resistant cells. A-D. Cell cycle distribution of 143b-DxR cells (PI stain) after treatment with vehicle (A, C) or 5μM IWR-1 (B,D) for 16 hours, then treated with media (A,B) or 1μM doxorubicin (C,D) for 24 hours. Histograms shown in A-D are representative images of triplicate experiments. In laid graphs demonstrate cell distribution with averages and standard deviations (parenthesis) as indicated. Statistical significance was determined using two-way ANOVA corrected for multiple comparisons.

Figure 5. Doxorubicin induces G2/M accumulation in wild-type cells with no additional effect from IWR-1.

Cell cycle distribution of 143b-wt cells (PI stain) after treatment with vehicle (A, C) or 5μM IWR-1 (B,D) for 16 hours, then treated with media (A,B) or 20nM doxorubicin (C,D) for 24 hours. Histograms shown in A-D are representative images of triplicate experiments. In-laid graphs demonstrate cell distribution with averages and standard deviations (parenthesis) as indicated. Statistical significance was determined using two-way ANOVA corrected for multiple comparisons.

Figure 6. Increased γ H2AX foci are observed following doxorubicin induced DNA damage in IWR-1 sensitized doxorubicin resistant cells.

A. Quantification of γ H2AX positive cells. Three images were taken of four independent replicates using a laser scanning confocal microscope. Greater than 60 cells were imaged and counted in each field of view. Cells containing greater than 20 γ H2AX foci were considered positive. Total positive cells in each field of view were counted and represented as a ratio of the total number of cells in the field of view (n=4). p-value determined by one-way ANOVA corrected by Tukey's multiple comparison test. B. Representative images of 143b-DxR cells treated with vehicle or 5 μ M IWR-1 for 16 hours, then treated with media or 20 μ M doxorubicin for 1 hour.

Figure 7. γ H2AX foci are not induced by IWR-1 sensitization in doxorubicin treated 143b-wt cells.

A. Quantification of γ H2AX positive cells. Three images were taken of four independent replicates using a laser scanning confocal microscope. Greater than 60 cells were imaged and counted in each field of view. Cells containing greater than 20 γ H2AX foci were considered positive. Total positive cells in each field of view were counted and represented as a ratio of the total number of cells in the field of view. p-value determined by one-way ANOVA corrected by Tukey's multiple comparison test. B. Representative images of 143b-wt cells treated with vehicle or 5 μ M IWR-1 for 16 hours, then treated with media or 1 μ M doxorubicin for 1 hour.

Table 1.
Comparison of Calcein AM retention in doxorubicin-resistant osteosarcoma cells.

Quantification of E_{MAX} , EC_{50} and R^2 of non-linear fitting performed by the GraphPad Prism 7 software in cells treated with IWR-1, or verapamil (a positive control inhibitor of cellular efflux) prior to incubation with Calcein AM.

143b-DxR Calcein AM signal	IWR-1	Verapamil
E_{MAX} (std err)	1374% (41.4)	1520% (52.9)
EC_{50} (std err)	8.40 μ M (0.65)	5.46 μ M (1.32)
R^2 of Non-lin fit	0.9065	0.8659

Table 2.
Quantification of wild-type and doxorubicin resistant osteosarcoma cell lines' response to doxorubicin following IWR-1 treatment.

The IC₅₀ for doxorubicin among vehicle or IWR-1 treated wild-type or resistant osteosarcoma cells is shown. Dose-response curve fitting by GraphPad Prism 7 provided IC₅₀ values and standard error (shown in parantheses).

doxorubicin IC ₅₀ (μM)	143b-wt	143-DxR
+Vehicle (std err)	0.727μM (0.064)	21.32μM (1.50)
+5μM IWR-1 (std err)	0.975μM (0.124)	11.76μM (0.94)