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Published version

PHILLIPS, Kate, WRIGHT, Nicola, MCDERMOTT, E. and CROSS, Neil (2019).
TRAIL responses are enhanced by nuclear export inhibition in osteosarcoma.
Biochemical and Biophysical Research Communications, 517 (2), 383-389.

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TRAIL responses are enhanced by nuclear export inhibition in osteosarcoma

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Abstract

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising anti-tumour agent that induces apoptosis of malignant cells through activation of death receptors. Death receptor agonistic antibodies are in clinical trials as TRAIL-mimetics, however, along with TRAIL monotherapy, show limited efficacy due to the rapid emergence of TRAIL-insensitive disease. TRAIL-sensitisers, which enhance TRAIL activity or overcome TRAIL resistance, may facilitate death receptor agonists as viable anti-tumour strategies. In this study we demonstrate that the nuclear export inhibitor, Leptomycin B, is a potent *in vitro* TRAIL-sensitiser in osteosarcoma. Leptomycin B works synergistically with both TRAIL and death receptor 5 agonistic antibodies to induce apoptosis in TRAIL sensitive cell lines. Further, Leptomycin B sensitises TRAIL-insensitive cell lines to TRAIL and death receptor agonistic antibody mediated apoptosis. We also confirmed that aldehyde dehydrogenase (ALDH) positive cells are not resistant to the apoptotic effects of TRAIL and Leptomycin B, an important observation since ALDH positive cells can have enhanced tumorigenicity and are implicated in disease recurrence and metastasis. The nuclear export pathway, in combination with death receptor agonists, is a potential therapeutic strategy in osteosarcoma and warrants further research on clinically relevant selective inhibitors of nuclear export.

Key words

Apoptosis; Nuclear export inhibitor, aldehyde dehydrogenase; TRAIL; death receptor; osteosarcoma; Leptomycin B

Funding

This work was supported an award by the Bone Cancer Research Trust, UK (award reference BCRT/34/13).

Introduction¹

Osteosarcoma is the most prevalent primary bone malignancy. It shows a bimodal age distribution affecting a significant number of adolescents and young adults, as well as those aged >55 years. In younger patients, osteosarcoma commonly occurs at the metaphysis of lower extremity long bones and has been associated with hormonal change and bone growth, whereas in older patients disease may also develop in other primary bone locations [1]. Current treatment consists of surgery with adjuvant radiotherapy and chemotherapy yet despite surgical advancements, survival rates remain largely unchanged in recent decades [2]. In adolescents the survival rate for high grade osteosarcoma is ~60% at 5 years [3] although this is drastically reduced in late stage and metastatic patients. Late stage diagnosis is common and therapeutic agents that target local recurring tumours and metastases need to be identified.

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is a member of the tumour necrosis factor family that extrinsically induces apoptosis through binding of death receptor 4 or 5 (DR4 or DR5) to initiate a caspase cascade [4]. TRAIL can also bind to decoy receptors (DcR1 and DcR2) and osteoprotegerin (OPG) thus inhibiting death inducing signalling [5-7]. Therapeutic monoclonal antibodies specific to DR4 or DR5 are shown to induce apoptosis of malignant cells and to bypass DcRs and OPG [8] which offers a potential mechanism to overcome inhibition of death receptor signalling. However, evaluation of TRAIL and agonists of DR4/DR5 in clinical trials showed limited anti-tumour efficacy due to rapid emergence of TRAIL-resistant disease [9-10], mirroring *in vitro* observations [11-12].

Exportin-1 (XPO1) is a nucleo-cytoplasmic transporter responsible for exporting nuclear proteins into the cytoplasm from the nucleus. XPO1 cargo include growth regulatory and

¹ Aldehyde dehydrogenase (ALDH), Cancer stem cell (CSC), Death receptor (DR), Decoy receptor (DcR), Flow Activated Cell Sorting (FACS), Osteoprotegerin (OPG), Exportin-1 (XPO1), selective inhibitor of nuclear export (SINE), Tumour necrosis factor-related apoptosis inducing ligand (TRAIL)

tumour suppressor proteins such as FOXO, STAT3, APC, Rb1, p53 and p21. XPO1 is over-expressed in osteosarcoma [13] and other cancers including glioma, pancreatic cancer and haematological conditions [14]. XPO1 overexpression increases exportation of tumour suppressor proteins inhibiting suppressive activity even in the presence of DNA damage and faulty DNA repair mechanisms [15]. As such, inhibiting nuclear export has the potential to re-establish tumour suppression and may offer therapeutic benefit.

Leptomycin B is a well-characterised inhibitor of XPO1 which is widely used in cellular studies of protein trafficking and has been used to assess apoptotic responses *in vitro* [16, 17]. Leptomycin B monotherapy has previously been used in a Phase I trial as an anti-tumour agent [18]. In this study, we investigated the apoptotic effects of Leptomycin B in combination with TRAIL and agonistic death receptor antibodies. Further, we investigated whether aldehyde dehydrogenase (ALDH) positive osteosarcoma cells were resistant to the apoptotic effects of TRAIL and/or Leptomycin B. ALDH activity is associated with cancer stem cell (CSC) phenotype and drug resistance in other sarcomas [19-21]. Furthermore, ALDH correlates with metastatic potential in osteosarcomas [22] and has been identified as a CSC marker in cultured osteosarcoma cells [23]. To date, no studies have addressed TRAIL sensitisation by nuclear export inhibitors, or the potential role of ALDH⁺ cells in mediating TRAIL-insensitivity in osteosarcomas.

Materials and Methods

MG63, Saos-2 and U2-OS human osteosarcoma cell lines were obtained from ATCC and maintained in basal media (MG63 – RPMI 1640; Saos-2 – MEM-alpha with nucleosides; U2-OS – McCoy's 5A) supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. RPMI-1640 was further supplemented with 1% non-essential amino acids. Cultures were maintained at 37°C, 5% CO₂.

Microscopic assessment of apoptosis

96-well plates were seeded with 2.0×10^4 cells/well (MG63 & U2-OS) or 4.0×10^4 cells/well (Saos-2) and allowed to settle overnight prior to treatment with 0 - 50 ng/mL rhTRAIL (PeproTech EC Ltd, London, UK) or 0 - 50 ng/mL anti-DR5 monoclonal antibody (Mab631, R&D Systems, Abingdon, UK) and/or 0 - 20 nM Leptomycin B (Enzo Life Sciences, Exeter, UK) for 24 hours. Induction of apoptosis was assessed by staining cells with 10 µg/mL Hoechst 33342 and 10 µg/mL Propidium Iodide (PI; Sigma, Poole, UK) at 37°C for 30 minutes. Cultures were visualised using an Olympus IX81 inverted fluorescent microscope and images captured using Cell-F software (Olympus, Tokyo, Japan). Apoptosis was determined manually by assessment of nuclear morphology. Cells were counted and percent apoptosis calculated from duplicate representative fields of view from each well, each containing at least 200 cells. All treatment combinations were performed in triplicate, and in at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett *post-hoc* test for single treatments, or two-way ANOVA with Holm-Sidak *post-hoc* test for combination treatments using Prism v6.07 software (GraphPad Software Inc., CA, USA).

NucView Caspase-3 activity assay

Cells were seeded to 96 well plates and subjected to 24 hour treatment with TRAIL, anti-DR5 and/or Leptomycin B as described above. NucView Caspase-3 activity assay (Biotium, Cambridge Biosciences, Cambridge, UK) was performed as per manufacturer's instructions

for flow cytometry analysis. Briefly, cells were stained with 0.2 mM NucView Caspase-3 substrate and incubated at room temperature for 20 minutes. Data was collected using a Gallios flow cytometer (Beckman Coulter, High Wycombe, UK) measuring fluorescence emission at 525 nm (FL1). Assay control samples were prepared by pre-incubation of cells with the irreversible inhibitor of caspase-3, Z-DEVD-FMK, at 37°C for 20 minutes prior to addition of NucView Caspase-3 substrate. Inhibitor concentrations were determined empirically for each cell line: 100 µM for MG63, 200 µM for Saos-2 and 100 µM for U2-OS. Statistical analysis was performed using one-way ANOVA with Dunnett post hoc test using Prism v6.07 software.

Aldehyde dehydrogenase activity assay and cell sorting

ALDH activity assay was performed using ALDEFLUOR kit (Stemcell Technologies, Cambridge, UK) as per manufacturer's instructions. Briefly, cells were stained in 0.61 µg/mL Aldefluor substrate in Aldefluor buffer at 37°C for 30 minutes. Following staining, cells were washed and re-suspended in Aldefluor buffer supplemented with the efflux inhibitor, verapamil (200 µM), and kept on ice for cell sorting. Assay control samples were prepared by addition of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). Cells were sorted using a FACSARIA flow cytometer (BD, Wokingham, UK) measuring fluorescence emitted at 525 nm (FL1). ALDH positive cells were those emitting fluorescence greater than inhibitor-treated control cells. ALDH positive and ALDH negative populations were collected, washed, assessed by trypan blue exclusion for ≥95% viability, seeded to 96-well plates and subjected to 24-hour treatment with TRAIL, anti-DR5 and/or Leptomycin B as described previously. Induction of apoptosis was assessed microscopically following Hoechst 33342 and PI staining as described above. Statistical analysis was performed using two-way ANOVA with Holm-Sidak *post-hoc* test using Prism v6.07 software.

Results

We assessed sensitivity to TRAIL and anti-DR5 in MG63, Saos-2 and U2-OS cell lines (Figure 1) by Hoechst 33342 and PI nuclear staining. MG63 were exquisitely sensitive to TRAIL with significant induction of apoptosis at ≥ 2 ng/mL stimulation. Induction of apoptosis was observed in approximately 80% of MG63 cells with 50 ng/mL TRAIL treatment. MG63 were also sensitive to anti-DR5 although to a lesser extent than TRAIL, with significant induction of apoptosis at ≥ 10 ng/mL stimulation. Apoptosis was observed in approximately 40% of MG63 cells using 50 ng/mL anti-DR5 treatment. Saos-2 were sensitive to TRAIL only at concentrations ≥ 25 ng/mL and insensitive to anti-DR5 at all concentrations investigated. Maximum dose TRAIL stimulation (50 ng/mL) induced apoptosis in approximately 20% of Saos-2 cells. U2-OS were sensitive to TRAIL and anti-DR5 stimulation with significant induction of apoptosis at ≥ 10 ng/mL. Induction of apoptosis was observed in approximately 30% of cells using 50 ng/mL TRAIL treatment and 10% of cells with 50 ng/mL anti-DR5. Baseline assessment of TRAIL and anti-DR5 sensitivity indicated that TRAIL was the more potent inducer of apoptosis in the 3 cell lines investigated.

To determine whether the nuclear export inhibitor Leptomycin B could effectively sensitise osteosarcoma cells to TRAIL or anti-DR5 we performed combination treatment experiments. A concentration of TRAIL that was shown in our previous experiments to induce $< 30\%$ apoptosis was selected for each cell line; 50 ng/mL for Saos-2 and 25 ng/mL for U2-OS. Additional titration of low dose TRAIL was performed on MG63 using 0.125, 0.25, 0.50, 1.0 and 2.0 ng/mL (data not shown) based on the high TRAIL sensitivity observed for this cell line. Stimulation with 0.25 ng/mL induced significant apoptosis in approximately 10% of MG63 cells and was selected for use in combination experiments. Matched concentrations of

anti-DR5 were used to determine the differential sensitivity between the DR4 and DR5 agonist, TRAIL, and anti-DR5 stimulation alone.

The osteosarcoma cell lines investigated showed only limited sensitivity to Leptomycin B as a single agent. Leptomycin B alone induced significant apoptosis in Saos-2 at ≥ 2 nM, but not in MG63 or U2-OS at any concentration investigated (up to 20 nM) as determined by Hoechst 33342 and PI nuclear staining. Combination treatment with Leptomycin B and TRAIL revealed synergy in the induction of apoptosis in all 3 cell lines (Figure 2). This effect was clearly apparent with all concentrations of Leptomycin B and TRAIL investigated. Leptomycin B was also seen to enhance the apoptotic activity of anti-DR5 although effect sizes were more modest (Figure 2). To confirm nuclear morphology data, we repeated combination treatment experiments for selected Leptomycin B concentrations and measured activity of Caspase-3 by flow cytometry. Low level Caspase-3 activity was observed in MG63, Saos-2 and U2-OS in response to high dose Leptomycin B (Figure 3) although significant increases in cells with apoptotic nuclear morphology were only recorded in our previous experiment in Saos-2. Combining Leptomycin B with TRAIL or anti-DR5 dramatically increased Caspase-3 activity. For MG63, activity was significantly increased when 20 nM Leptomycin B was combined with either TRAIL or anti-DR5. For Saos-2, activity was significantly increased when ≥ 0.5 nM Leptomycin B was combined with either TRAIL or anti-DR5. For U2-OS, activity was significantly increased when ≥ 0.5 nM Leptomycin B was combined with TRAIL, and when 20 nM Leptomycin B was combined with anti-DR5.

Expression of ALDH is associated with cellular pluripotency and may confer stem-like characteristics to cancer cells. To investigate whether sensitivity to Leptomycin B in combination with either TRAIL or anti-DR5 is affected by ALDH expression, we separated ALDH⁺ and ALDH⁻ cells by FACS. In MG63, a significantly lower proportion of ALDH⁺

cells were apoptotic following combination treatment with Leptomycin B and TRAIL when compared to ALDH- cells (Figure 4). This effect was not due to differences in TRAIL sensitivity as no significant difference to TRAIL alone was recorded between ALDH+ and ALDH- cells. Further, this effect was not seen when Leptomycin-B was combined with anti-DR5. In Saos-2 and U2-OS, there was no differential sensitivity to Leptomycin B in combination with either TRAIL or anti-DR5, or to TRAIL or anti-DR5 when used as single agents, between ALDH+ and ALDH- cells (Figure 4).

Discussion

Resistance to TRAIL-based therapies limits the use of rhTRAIL, or agonistic antibodies to DR4/5. It is acknowledged that efficacy is limited with single agent use and that combination treatment with TRAIL sensitisers will be required for sustained anti-tumour activity. Many TRAIL sensitisers have been described, including DNA damaging and microtubule inhibiting chemotherapies, proteasome inhibitors, and histone deacetylase (HDAC) inhibitors [24, 25]. Here we describe the nuclear export inhibitor, Leptomycin B, as a potent TRAIL sensitiser in osteosarcoma cell lines.

Leptomycin B inhibits XPO1, therefore trapping cargo molecules within the nucleus. XPO1 enhances the malignant phenotype in transformed cells as many cargo molecules, when delivered to the cytoplasmic compartment, either limit tumour suppression or enhance oncogenic signalling. Key cargo molecules include the tumour suppressors; p53, p73, APC, Rb, FOXO, BRCA1 and NPM1, and the cell cycle regulators; p21, p27 and galectin-3 [16, 26, 27]. Oncogenic mRNA transcripts such as c-Myc, cyclin D1 and MDM2 are also cargo and delivery to the cytoplasm enhances oncoprotein production [28]. As such, interest in the therapeutic use of nuclear export inhibitors is longstanding. However, the use of Leptomycin B in a single clinical trial in 1996 was associated with dose-limiting toxicity and no responses in 33 patients with advanced refractory cancer [18].

Osteosarcoma cells overexpress XPO1 [13] however, they were not overly sensitive to Leptomycin B alone. Significant induction of apoptosis following single agent treatment was recorded only in Saos-2, although all cell lines showed some increase in caspase-3 activity with high dose stimulation, which may indicate some re-establishment of tumour suppressive or growth regulatory mechanisms. Whilst single agent use did not lead to significant cell death, a potent and synergistic effect was recorded in all cell lines to combination with either

TRAIL or anti-DR5. This effect was observed irrespective of TRAIL sensitivity since our initial experiments confirmed that MG63 were TRAIL sensitive while Saos-2 and U2-OS were not. Caspase-3 activity confirmed dose-dependent sensitisation of osteosarcoma cells to both TRAIL and anti-DR5. Many mechanisms for nuclear export inhibitor anti-tumour activities have been proposed in other cancer types, particularly entrapment of nuclear p53 driving increased Bax expression whereby dominant negative p53 mutant cells are less affected by Leptomycin B than wild-type cells [16, 29]. U2-OS is p53-wild-type, whereas Saos-2 is p53-deleted and MG63 is p53-mutant. All three cell lines were susceptible to Leptomycin B sensitisation, suggesting that in osteosarcoma at least, effects are independent of p53 status. Blockade of p27 nuclear export promotes growth arrest in osteosarcoma [30] but this alone is unlikely to explain apoptotic responses observed here. Interestingly, Kashyap *et al.* (2016) demonstrated siRNA knockdown of I κ B decreases sensitivity to nuclear export inhibition in U2-OS cells [31]. I κ B sequesters NF- κ B in the cytosol, supporting a role for the NF- κ B axis in cell survival in response to nuclear export inhibition [32].

Acquired resistance is a major problem of targeted therapies, particularly monotherapies. Assessment of agents against both the bulk tumour population and the more tumorigenic CSC-like population determines whether the bulk population is preferentially targeted, as in the case of DNA damaging agents. Here we assessed the sensitivity of ALDH positive cells. These may represent the CSC-like population [23] and are associated with aggressive phenotype, resistance to apoptosis [33], and metastasis [22]. We demonstrated that ALDH activity confers no resistance to apoptosis induced by combination treatment of Leptomycin B and TRAIL or anti-DR5 in Saos-2 or U2-OS, although ALDH activity in MG63 cells did lead to lower induction of apoptosis, however potent TRAIL sensitisation by Leptomycin B was seen in ALDH+ cells of all cell lines. TRAIL sensitisation with nuclear export inhibitors

could therefore be a potential therapeutic strategy for elimination of tumorigenic osteosarcoma cells, although it is unlikely that Leptomycin B could be considered for clinical use. Selective inhibitors of nuclear export (SINEs) such as Selinexor, represent the most promising therapeutic candidates with improved safety and tolerability in phase I clinical trials [15, 34]. Future pre-clinical studies using SINE sensitisation and TRAIL therapeutics is therefore warranted.

References

- [1] J. Whelan, A. McTiernan, N. Cooper, Y.K. Wong, M. Francis, S. Vernon, S.J. Strauss, Incidence and survival of malignant bone sarcomas in England 1979-2007, *International Journal of Cancer*, 131 (2012) E508-E517.
- [2] S.S. Bielack, S. Hecker-Nolting, C. Blattmann, L. Kager, Advances in the management of osteosarcoma, *F1000Research*, 5 (2016).
- [3] E. Desandes, Survival from adolescent cancer, *Cancer Treatment Reviews*, 33 (2007) 609-615.
- [4] A. Ashkenazi, R. Pitti, S. Marsters, S. Ruppert, C. Donahue, A. Moore, P. Roberts, Induction of apoptosis by APO-2 ligand, a new member of the TNF cytokine family, *Faseb Journal*, 10 (1996) 1600-1600.
- [5] G.H. Pan, J. Ni, Y.F. Wei, G.L. Yu, R. Gentz, V.M. Dixit, An antagonist decoy receptor and a death domain-containing receptor for TRAIL, *Science*, 277 (1997) 815-818.
- [6] J.P. Sheridan, S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, A.D. Goddard, P. Godowski, A. Ashkenazi, Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors, *Science*, 277 (1997) 818-821.
- [7] J.G. Emery, P. McDonnell, M.B. Burke, K.C. Deen, S. Lyn, C. Silverman, E. Dul, E.R. Appelbaum, C. Eichman, R. DiPrinzio, R.A. Dodds, I.E. James, M. Rosenberg, J.C. Lee, P.R. Young, Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL, *Journal of Biological Chemistry*, 273 (1998) 14363-14367.
- [8] R.M. Locklin, P.I. Croucher, R.G.G. Russell, C.M. Edwards, Agonists of TRAIL death receptors induce myeloma cell apoptosis that is not prevented by cells of the bone marrow microenvironment, *Leukemia*, 21 (2007) 805-812.
- [9] C.S. Fuchs, M. Fakih, L. Schwartzberg, A.L. Cohn, L. Yee, L. Dreisbach, M.F. Kozloff, Y.J. Hei, F. Galimi, Y. Pan, V. Haddad, C.P. Hsu, A. Sabin, L. Saltz, TRAIL Receptor Agonist Conatumumab With Modified FOLFOX6 Plus Bevacizumab for First-Line Treatment of Metastatic Colorectal Cancer, *Cancer*, 119 (2013) 4290-4298.
- [10] M. Reck, M. Krzakowski, E. Chmielowska, M. Sebastian, D. Hadler, T. Fox, Q. Wang, J. Greenberg, R.A. Beckman, J. von Pawel, A randomized, double-blind, placebo-controlled phase 2 study of tigatuzumab (CS-1008) in combination with carboplatin/paclitaxel in patients with chemotherapy-naïve metastatic/unresectable non-small cell lung cancer, *Lung Cancer*, 82 (2013) 441-448.
- [11] S.L. Spencer, S. Gaudet, J.G. Albeck, J.M. Burke, P.K. Sorger, Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis, *Nature*, 459 (2009) 428-U144.
- [12] N.A. Cross, E.A. Waterman, N. Jokonya, A. Fowles, C.H. Buckle, J. Phillips, I. Holen, F.C. Hamdy, C.L. Eaton, Phenotypic variations of TRAIL sensitivity in cloned populations of prostate cancer cells, *Journal of Cellular Biochemistry*, 104 (2008) 1452-1464.
- [13] Y. Yao, Y. Dong, F. Lin, H. Zhao, Z. Shen, P. Chen, Y.J. Sun, L.N. Tang, S.E. Zheng, The expression of CRM1 is associated with prognosis in human osteosarcoma, *Oncology Reports*, 21 (2009) 229-235.
- [14] A. Das, G. Wei, K. Parikh, D. Liu, Selective inhibitors of nuclear export (SINE) in hematological malignancies, *Exp Hematol Oncol*, 4 (2015) 7.
- [15] K. Parikh, S.D. Cang, A. Sekhri, D.L. Liu, Selective inhibitors of nuclear export (SINE)-a novel class of anti-cancer agents, *Journal of Hematology & Oncology*, 7 (2014).
- [16] P.S. Lecane, T.M. Kiviharju, R.G. Sellers, D.M. Peehl, Leptomycin B stabilizes and activates p53 in primary prostatic epithelial cells and induces apoptosis in the LNCaP cell line, *Prostate*, 54 (2003) 258-267.

- [17] L.J. Gray, P. Bjelogrić, V.C.L. Appleyard, A.M. Thompson, C.E. Jolly, S. Lain, C.S. Herrington, Selective induction of apoptosis by leptomycin B in keratinocytes expressing HPV oncogenes, *International Journal of Cancer*, 120 (2007) 2317-2324.
- [18] E.S. Newlands, G.J.S. Rustin, M.H. Brampton, Phase I trial of elactocin, *British Journal of Cancer*, 74 (1996) 648-649.
- [19] B. Fourneaux, A. Bourdon, B. Dadone, C. Lucchesi, S.R. Daigle, E. Richard, A. Laroche-Clary, F. Le Loarer, A. Italiano, Identifying and targeting cancer stem cells in leiomyosarcoma: prognostic impact and role to overcome secondary resistance to PI3K/mTOR inhibition, *Journal of Hematology & Oncology*, 12 (2019).
- [20] L. Martinez-Cruzado, J. Tornin, L. Santos, A. Rodriguez, J. Garcia-Castro, F. Moris, R. Rodriguez, Aldh1 Expression and Activity Increase During Tumor Evolution in Sarcoma Cancer Stem Cell Populations, *Scientific Reports*, 6 (2016).
- [21] O. Awad, J.T. Yustein, P. Shah, N. Gul, V. Katuri, A. O'Neill, Y.L. Kong, M.L. Brown, J.A. Toretsky, D.M. Loeb, High ALDH Activity Identifies Chemotherapy-Resistant Ewing's Sarcoma Stem Cells That Retain Sensitivity to EWS-FLI1 Inhibition, *Plos One*, 5 (2010).
- [22] N. Greco, T. Schott, X. Mu, A. Rothenberg, C. Voigt, R.L. McGough, M. Goodman, J. Huard, K.R. Weiss, ALDH Activity Correlates with Metastatic Potential in Primary Sarcomas of Bone, *Journal of cancer therapy*, 5 (2014) 331-338.
- [23] L. Wang, P. Park, H.N. Zhang, F. La Marca, C.Y. Lin, Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity, *International Journal of Cancer*, 128 (2011) 294-303.
- [24] T.J. Sayers, A.D. Brooks, C.Y. Koh, W.H. Ma, N. Seki, A. Raziuddin, B.R. Blazar, X. Zhang, P.J. Elliott, W.J. Murphy, The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP, *Blood*, 102 (2003) 303-310.
- [25] A. Arhoma, A.D. Chantry, S.L. Haywood-Small, N.A. Cross, SAHA-induced TRAIL-sensitisation of Multiple Myeloma cells is enhanced in 3D cell culture, *Experimental Cell Research*, 360 (2017) 226-235.
- [26] J.G. Turner, D.M. Sullivan, CRM1-Mediated Nuclear Export of Proteins and Drug Resistance in Cancer, *Current Medicinal Chemistry*, 15 (2008) 2648-2655.
- [27] L. Brunetti, M.C. Gundry, D. Sorcini, A.G. Guzman, Y.H. Huang, R. Ramabadran, I. Gionfriddo, F. Mezzasoma, F. Milano, B. Nabet, D.L. Buckley, S.M. Kornblau, C.Y. Lin, P. Sportoletti, M.P. Martelli, B. Falini, M.A. Goodell, Mutant NPM1 Maintains the Leukemic State through HOX Expression, *Cancer Cell*, 34 (2018) 499-+.
- [28] U.H. Gandhi, W. Senapedis, E. Baloglu, T.J. Unger, A. Chari, D. Vogl, R.F. Cornell, Clinical Implications of Targeting XPO1-mediated Nuclear Export in Multiple Myeloma, *Clinical Lymphoma Myeloma & Leukemia*, 18 (2018) 335-345.
- [29] P. Smart, E.B. Lane, D.P. Lane, C. Midgley, B. Vojtesek, S. Lain, Effects on normal fibroblasts and neuroblastoma cells of the activation of the p53 response by the nuclear export inhibitor leptomycin B, *Oncogene*, 18 (1999) 7378-7386.
- [30] A.W. Currier, E.A. Kolb, R.G. Gorlick, M.E. Roth, V. Gopalakrishnan, V.B. Sampson, p27/Kip1 functions as a tumor suppressor and oncoprotein in osteosarcoma, *Scientific Reports*, 9 (2019).
- [31] T. Kashyap, C. Argueta, A. Aboukameel, T.J. Unger, B. Klebanov, R.M. Mohammad, I. Muqbil, A.S. Azmi, C. Drolen, W. Senapedis, M. Lee, M. Kauffman, S. Shacham, Y. Landesman, Selinexor, a Selective Inhibitor of Nuclear Export (SINE) compound, acts through NF-kappa B deactivation and combines with proteasome inhibitors to synergistically induce tumor cell death, *Oncotarget*, 7 (2016) 78883-78895.
- [32] D. Javelaud, F. Besancon, NF-kappa B activation results in rapid inactivation of JNK in TNF alpha-treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-kappa B, *Oncogene*, 20 (2001) 4365-4372.

[33] X. Mu, C. Isaac, T. Schott, J. Huard, K. Weiss, Rapamycin Inhibits ALDH Activity, Resistance to Oxidative Stress, and Metastatic Potential in Murine Osteosarcoma Cells, *Sarcoma*, 2013 (2013).

[34] S.C. Mutka, W.Q. Yang, S.D. Dong, S.L. Ward, D.A. Craig, P. Timmermans, S. Murli, Identification of Nuclear Export Inhibitors with Potent Anticancer Activity In vivo, *Cancer Research*, 69 (2009) 510-517.

Figure Legends

Figure 1. Apoptosis induction following 24-hour stimulation with TRAIL or anti-DR5 in MG63, Saos-2 and U2-OS osteosarcoma cell lines assessed by Hoechst 33342 and PI nuclear staining. Data shown is mean \pm SEM (n=3), statistics by one-way ANOVA with Dunnett *post-hoc* test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

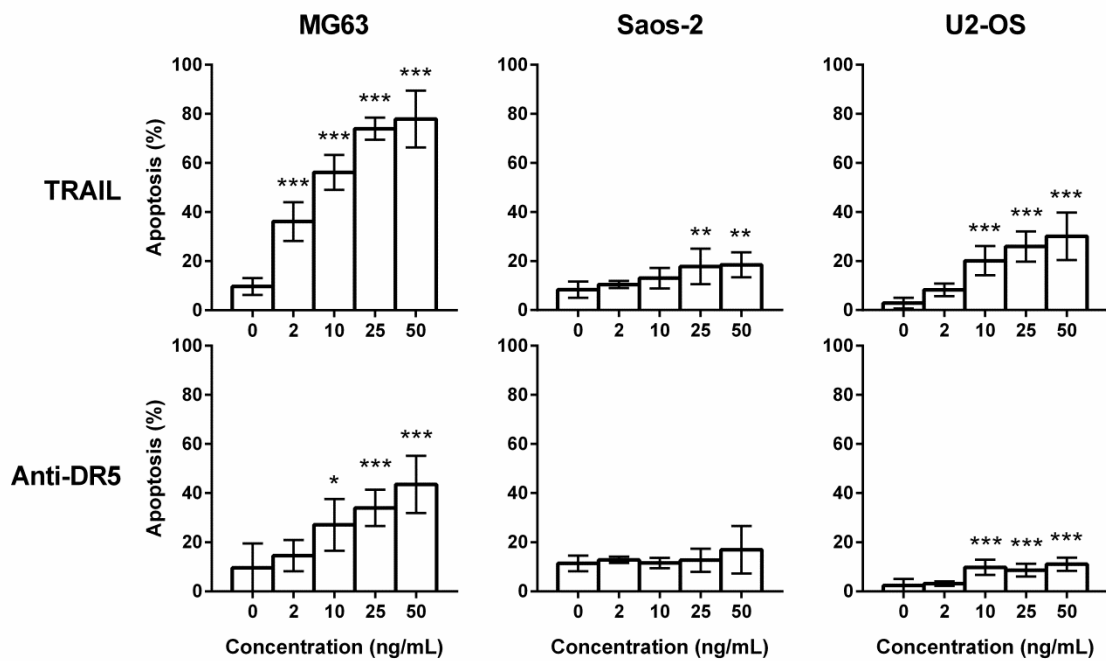


Figure 2. Apoptosis induction following 24-hour stimulation with Leptomycin B in the presence and absence of TRAIL or anti-DR5 in MG63 (0.25 ng/mL), Saos-2 (50 ng/mL) and U2-OS (25 ng/mL) osteosarcoma cell lines assessed by Hoechst 33342 and PI nuclear staining. a) Data shown is mean \pm SEM (n=3), statistics by two-way ANOVA with Holm-Sidak *post-hoc* test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$; ### indicates significant induction of apoptosis by Leptomycin B alone compared to untreated control ($p < 0.001$). b) Representative Hoechst 33342 and PI staining of US-OS cells in response to vehicle control, TRAIL (25ng/mL) Leptomycin B (20 nM), TRAIL + LMB, anti-DR5 (25ng/mL) and anti-DR5 + Leptomycin B. LMB = Leptomycin B

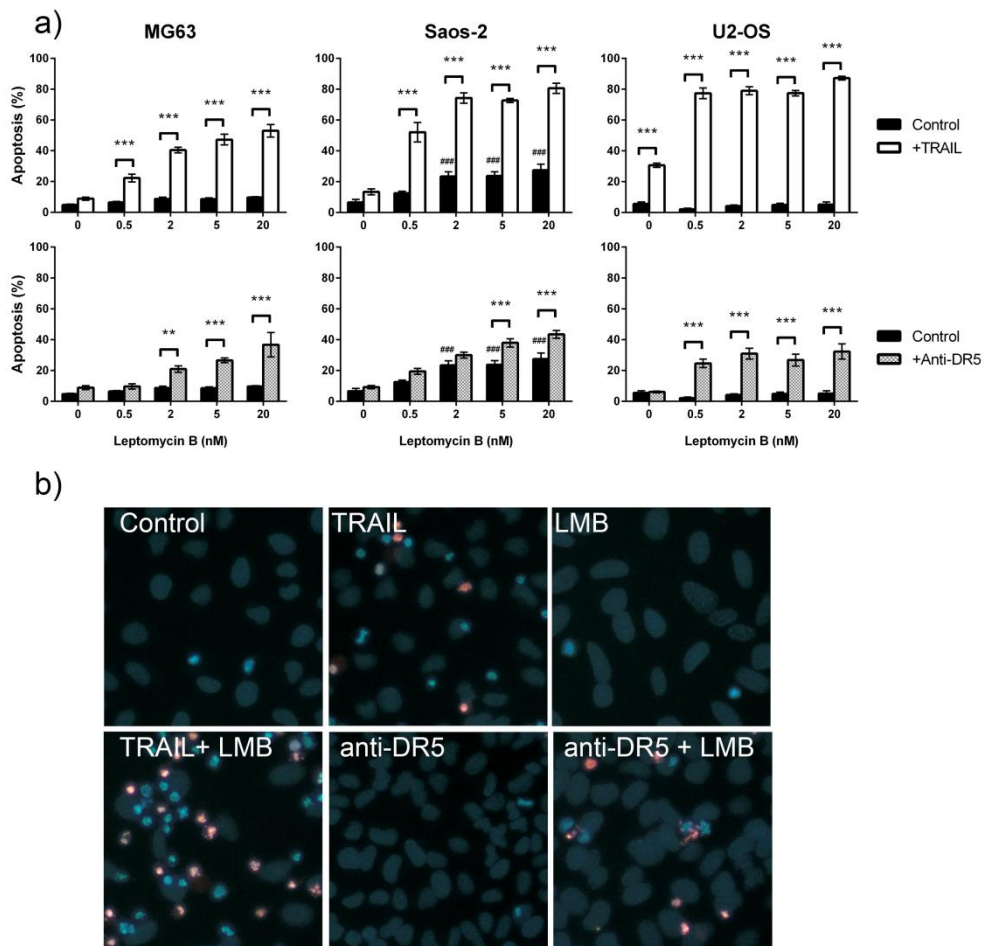


Figure 3. Caspase 3 activity following 24-hour stimulation with Leptomycin B in the presence and absence of TRAIL or anti-DR5 in MG63, Saos-2 and U2-OS osteosarcoma cell lines. Data shown is mean \pm SEM (n=3), statistics by one-way ANOVA with Dunnett *post-hoc* test. * indicates $p < 0.05$. ** indicates $p < 0.01$, *** indicates $p < 0.001$ compared to 0nM control.

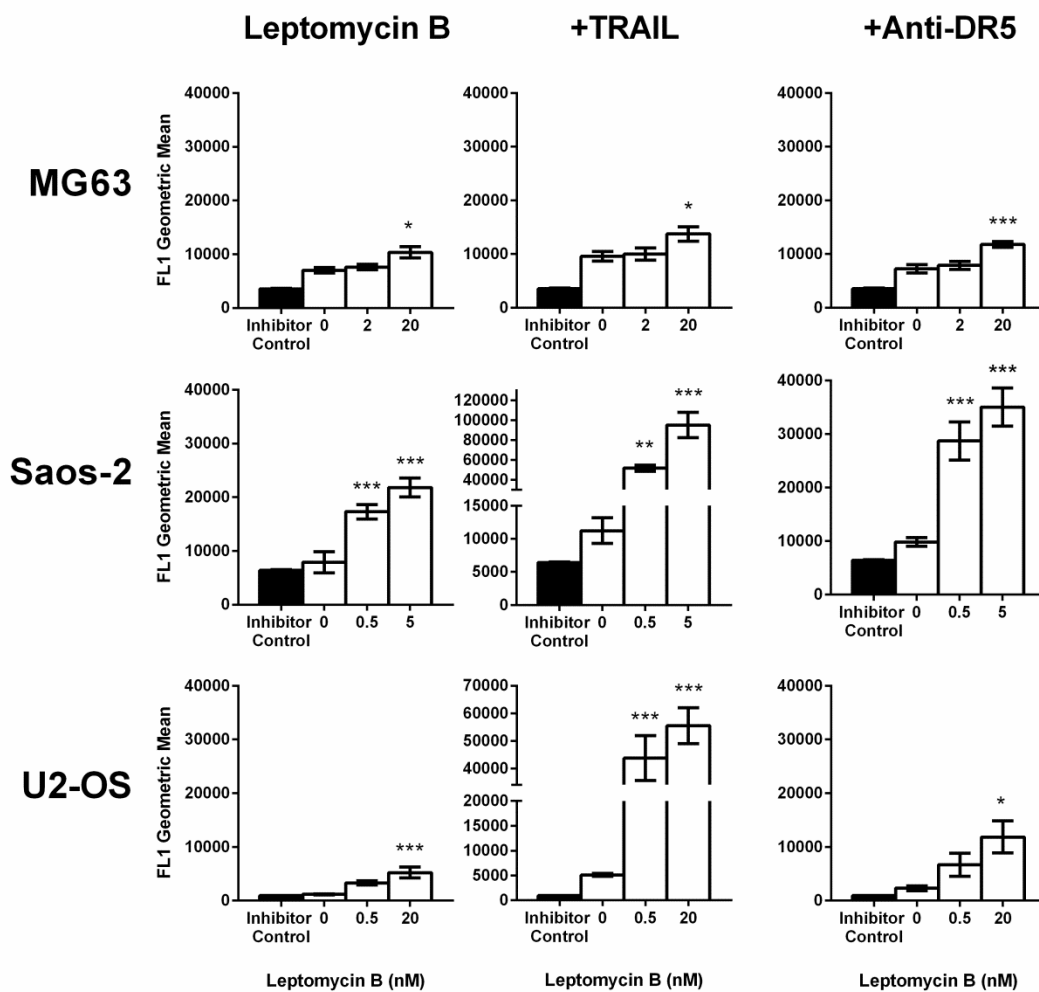


Figure 4. Apoptosis induction following 24-hour stimulation with Leptomycin B in the presence and absence of TRAIL or anti-DR5 in FACS-sorted ALDH- and ALDH+ MG63, Saos-2 and U2-OS osteosarcoma cell lines, assessed by Hoechst 33342 and PI nuclear staining. Data shown is mean \pm SEM (n=3), statistics by two-way ANOVA with Holm-Sidak *post-hoc* test. * indicates $p < 0.05$. ** indicates $p < 0.01$, *** indicates $p < 0.001$ compared to 0nM control. ## indicates significant difference between ALDH- and ALDH+ cells ($p < 0.01$).

