The Synergistic Effect of Melphalan and XPO1 Inhibition in Pre-Clinical Models of Multiple Myeloma

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Abstract

Introduction:
Significant increases in response/survival have been seen over the past several years; however, multiple myeloma (MM) remains incurable. In this study we have demonstrated that the XPO1 inhibitors (XPO1i), selinexor (SEL) and KPT-8602, sensitize both wild-type and drug-resistant MM cells to melphalan (MEL) in pre-clinical models.

Materials and Methods:
We used the XPO1i SEL (300nM), KPT-8602 (300nM), and KOS-2464 (10nM) +/- MEL (5-20 μM) to treat human 8226, H929 and U266 MM cells, and MEL resistant 8226LR5 and U266LR6 cell lines and assayed for apoptosis. DNA damage was assayed by comet assay and g-H2AX in H929 human MM cells. XPO1i/MEL-treated MM cells were assayed for P53, NFkB, IKKα, and Fanconi Anemia (FANC)/BRCA DNA repair proteins by Western blot and electrochemiluminescent immunoassay(ECL-I). We also treated cells from patients with newly diagnosed or relapsed/refractory MM with the XPO1i +/- MEL and assayed for apoptosis. NOD/SCID-g (NSG) mice challenged with U226 or U266LR6 MM tumors were treated with XPO1i/MEL and assayed for tumor growth, survival, and toxicity.

Results:
The addition of the XPO1i's SEL, KPT-8602 or KOS-2464 ($P \leq 0.03$) sensitized drug-resistant 8226LR5 and U266LR6 cells to MEL when compared to single-agent MEL. The XPO1i/MEL drug combination increased DNA damage (Comet assay/g-H2AX) more than single agent MEL or XPO1i alone ($P \leq 0.005$). Western blot and ECL-I showed that XPO1i treatment can increase P53 and decrease NFkB, IKKα, FANC/BRAC pathway proteins including FANCD2 in MM cells. CD138+/light chain+ MM cells from newly diagnosed and relapsed/refractory MM patients were sensitized (20-fold and 5-10 fold respectively) by XPO1i to MEL. XPO1i/MEL combination treatment demonstrated a strong synergistic anti-tumor effect when compared to single-agent MEL (SEL/MEL, $P = 0.0024$ and KPT-8602/MEL, $P = 0.0030$) in NSG mice challenged with U266, with little toxicity as assessed

Conclusions:
XPO1i's sensitized human MM cell lines, both parental and MEL resistant, and patient MM cells to MEL both *in vitro* and *ex vivo*, and in *in vivo* NSG mouse models. Our data show that the synergistic cell kill may be due to increased XPO1i/MEL-induced DNA damage. The mechanism of this synergy may be due to increased nuclear P53, in combination with decreased NFkB and IKKα, and decreased DNA repair activity of the FANC/BRCA pathway. Thus, combination therapies of XPO1i, especially the clinical compounds SEL and KPT-8602 +/- MEL, may have potential to improve the treatment outcomes of MM. The combination of XPO1i and melphalan are being investigated in the context of high-dose chemotherapy and autologous transplant (NCT 02780609).

Small-molecule inhibitors of XPO1 sensitize human MM cell lines to MEL and 4-hydroperoxy-cyclophosphamide (4HC).

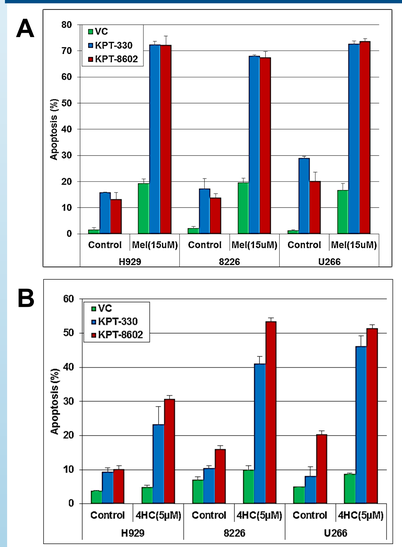

Figure 1: **Small-molecule inhibitors of XPO1 sensitize human MM cell lines to MEL and 4-hydroperoxy-cyclophosphamide (4HC).** High-density H929 ($3x10^6$ cells/ml), 8226 ($2x10^6$ cells/ml) and U266 ($4x10^6$ cells/ml) human MM cells were treated concurrently with KPT-330 (300nM) or KPT-8602 (300 nM) +/- (A) MEL (15 μM) or (B) 4HC (5 μM) for 20 hours. (A) The combination of KPT-330/MEL ($P = 2.2x10^{-6}$ to 0.0032) or KPT-8602/MEL ($P = 1.2X10^{-7}$ to 0.0031) synergistically increased apoptosis (activated caspase 3) in MM cell lines over single agent MEL (n=3). (B) In addition the combination of KPT-330/4HC ($P = 0.004$ to 0.019) or KPT-8602/4HC ($P = 1.2X10^{-7}$ to 0.0031) synergistically increased apoptosis (activated caspase 3) in MM cell lines over single agent 4HC. (n=4).

XPO1 Inhibition Sensitizes MEL-Resistant Myeloma

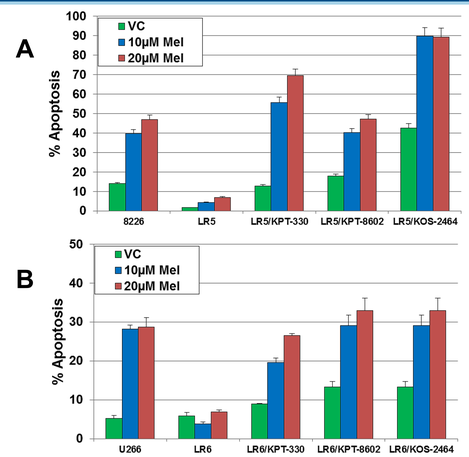

Figure 2:. XPO1 inhibition sensitizes MEL-resistant human MM cell lines to MEL. Human 8226(**A**) and U266(**B**) drug-resistant (LR5 and LR6) and parental MM cell lines were treated concurrently for 20 hr with KPT-330 (300 nM), KPT-8602 (300 nM) or KOS-2464 (10 nM) +/- MEL (10 or 20 μM) and assayed for apoptosis by flow cytometry (activated caspase 3). Resistant MM cell lines were 4.1 to 9.5-fold resistant to single agent MEL when compared to parental cells. The addition of the XPO1i's KPT-330, KPT-8602 or KOS-2464 (p = 0.015, 0.029 and 0.030 respectively) sensitized drug resistant LR5 cells to MEL when compared to single agent MEL. XPO1i's KPT-330, KPT-8602 or KOS-2464 (p = 0.003, 0.009 and 0.0078 respectively) sensitized drug resistant LR6 cells to MEL when compared to single agent MEL. Parental cells treated with KPT-330 or KOS-2462 + MEL were 100% apoptotic/necrotic (data not shown).

XPO1 inhibitor/MEL Induced DNA Damage in MM.

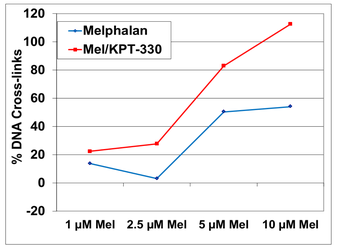

Figure 3: Alkaline Comet Assay showing increased MEL-induced DNA ICL's from the addition of KPT-330. High-density H929 MM cells (3×10^6/ml) were exposed to the XPO1i KPT-330 (0.1 μM) for 20 hr followed by MEL (1, 2.5, 5, & 10 μM) for 2 hr. Drugs were removed and cells incubated another 3 hr to allow cross-links to form. Cells were exposed to 900 rads (X-Rad 160 X-ray biological irradiator) and alkaline comet assays were performed immediately (Trevigen Inc).

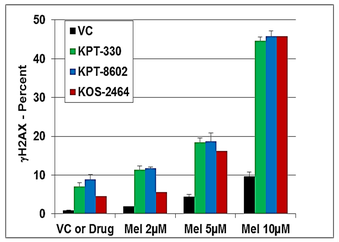

Figure 4: XPO1/melphalan induced γ-H2AX expression. Human H929 MM cells were treated with XPO1i's +/- MEL and assayed for H2AX. Data are expressed as the percentage of treated cells expressing high-levels of H2AX. XPO1 inhibition by KPT-330, KPT-8602 and KOS-2464 increased the effectiveness of 10 μM MEL to induce double strand DNA breaks versus MEL control as shown by H2AX expression ($P = 0.005$, $P = 0.001$ *and* $P = 0.0005$ respectively) and in a dose-dependent manner.

Selinexor/MEL combination treatment decreases NFkB, IKKα, FANCF, FANCL and may prevent DNA repair.

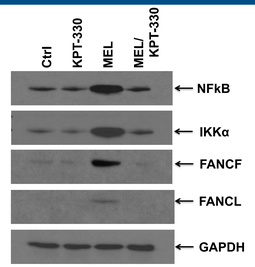

Figure 5: Western blot of FA and NFκB pathway members with XPO1i + MEL. High-density H929 MM cells (3×10^6/ml) were treated for 6 hr with MEL (10 μM), KPT-330 (300 nM) or in combination. Whole cell lysates were separated by SDS PAGE and assayed by Western blot. MEL was found to increase DNA repair enzymes FANCF and FANCL, and cell proliferation proteins NFkB and IKKα. However, when the XPO1i KPT-330 was added, total cellular FANCF, FANCL, IKKα, and NFkB were decreased. MEL/KPT-330 synergy may be due, in part, to an increase in DNA damage by MEL and a decrease in both DNA repair enzymes and cell proliferation proteins by KPT-330.

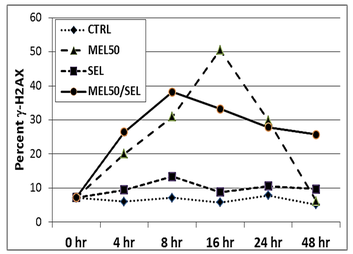

Figure 6: γ-H2AX Expression. XPO1 inhibitors DNA repair. RPMI8226 MM Cells were treated for 2 hours with 50 μM MEL, washed and then incubated with SEL for 4, 8, 16, 24 and 48 hours. MM cells were fixed and stained with anti-γ−H2AX washed and stained with Alexa488- anti-rabbit secondary antibody. Cells were analyzed by FACS for γ−H2AX. MEL caused DNA damage which peaked at 16 hours and was fully repaired by 48 hours. The addition of SEL prevented DNA repair provided a mechanism for SEL/MEL synergy

Ex Vivo Patient Data

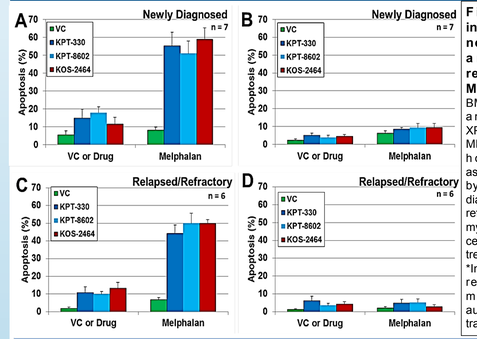

Figure 7 XPO1 inhibitors sensitize newly diagnosed and relapsed/refractory patient MM cells to MEL. BMMC were isolated and treated with XPO1i's (300 nM) +/- MEL (10 μM) for 20 hours. MM cells assayed for apoptosis by FACS **(A)** Newly diagnosed **(C)** relapsed/refractory. **(B, D)** Non-myeloma BMMC patient cells.**(E)** Table of last treatment before biopsy *Indicates patient had received high dose melphalan and autologous stem cell transplant

Patient		Treatment before BM	Time from last treatment to BM	Treatments Received
RO1-3	Relapsed	high-dose melphalan/ASCT	11 months	3*
RO1-5	Refractory	lenalidomide	8 days	3*
RO1-6	Refractory	carfilzomib/pomalidomide	20 days	6*
RO1-10	Refractory	cyclophosphamide/bortezomib/dexamethasone	During	7*
RO1-12	Relapsed	lenalidomide/bortezomib/dexamethasone	5 months	1
RO1-18	Relapsed	lenalidomide/dexamethasone	2.5 years	2

NOD/SCID In vivo Treatment

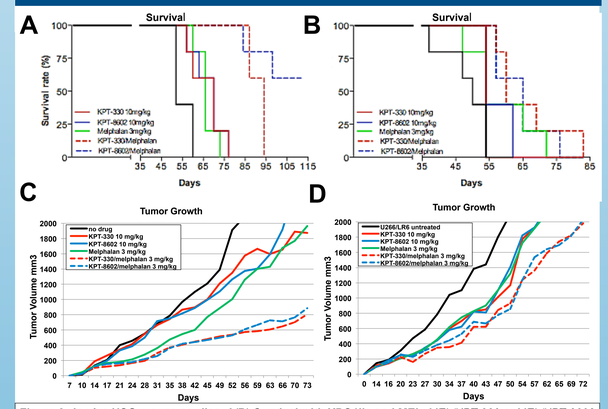

Figure 8: *In vivo* NSG mouse studies. A/B) Survival with XPO1i's and MEL. MEL/KPT-330 or MEL/KPT-8602 treatment improved survival vs MEL alone in both U266(A) human MM xenografts ($P = 0.0296$ and $P = 0.0419$) and MEL resistant U266LR(B) 6 xenografts ($P = 0.0481$ and $P = 0.0481$) **C) Tumor growth with XPO1i's and MEL**. MEL/KPT-330 or MEL/KPT-8602 treatment reduced human MM U266 tumor growth when compared to single-agent MEL ($P = 0.00024$ and 0.00025, respectively).**D)** MEL resistant U226/LR6 tumor growth was also slowed by MEL/KPT-330 ($P = 0.00014$) or MEL/KPT-8602 ($P = 0.00067$) when compared to MEL alone.

Conclusions

- **XPO1 inhibitors improved the response of human drug resistant MM cell lines and patient MM cells to MEL *in vitro* and *ex vivo*.**
- **XPO1 inhibitors increased nuclear p53 (data not shown) in combination with decreased NFkB and IKKα and DNA repair proteins FANCL and FANCF preventing DNA repair of MEL induced crosslinks.**
- **XPO1 inhibitors augment MEL-induced DNA damage and may also block the repair of the DNA damage, resulting in synergistic cell kill.**
- **Combination therapies using XPO1 inhibitors, especially the clinical compound KPT330 (selinexor) or KPT-8602 +/- MEL, may significantly improve the treatment outcomes of MM.**