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Inhibition of CRM1-dependent nuclear export sensitizes malignant cells to cytotoxic and targeted agents

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Abstract

Nuclear-cytoplasmic trafficking of proteins is a significant factor in the development of cancer and drug resistance. Subcellular localization of exported proteins linked to cancer development include those involved in cell growth and proliferation, apoptosis, cell cycle regulation, transformation, angiogenesis, cell adhesion, invasion, and metastasis. Here, we examined the basic mechanisms involved in the export of proteins from the nucleus to the cytoplasm. All proteins over 40 kDa use the nuclear pore complex to gain entry or exit from the nucleus, with the primary nuclear export molecule involved in these processes being chromosome region maintenance 1 (CRM1, exportin 1 or XPO1). Proteins exported from the nucleus must possess a hydrophobic nuclear export signal (NES) peptide that binds to a hydrophobic groove containing an active-site Cys528 in the CRM1 protein. CRM1 inhibitors function largely by covalent modification of the active site Cys528 and prevent binding to the cargo protein NES. In the absence of a CRM1 inhibitor, CRM1 binds cooperatively to the NES of the cargo protein and RanGTP, forming a trimer that is actively transported out of the nucleus by facilitated diffusion. Nuclear export can be blocked by CRM1 inhibitors, NES peptide inhibitors or by preventing post-translational modification of cargo proteins. Clinical trials using the classic CRM1 inhibitor leptomycin B proved too toxic for patients; however, a new generation of less toxic small molecule inhibitors are being used in clinical trials in patients with both hematological malignancies and solid tumors. Additional trials are being initiated using small-molecule CRM1 inhibitors in combination with chemotherapeutics such as pegylated liposomal doxorubicin. In this review, we present evidence that combining the

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new CRM1 inhibitors with other classes of therapeutics may prove effective in the treatment of cancer. Potential combinatorial therapies discussed include the use of CRM1 inhibitors and the addition of alkylating agents (melphalan), anthracyclines (doxorubicin and daunomycin), BRAF inhibitors, platinum drugs (cisplatin and oxaliplatin), proteasome inhibitors (bortezomib and carfilzomib), or tyrosine-kinase inhibitors (imatinib). Also, the sequence of treatment may be important for combination therapy. We found that the most effective treatment regimen involved first priming the cancer cells with the CRM1 inhibitor followed by doxorubicin, bortezomib, carfilzomib, or melphalan. This order sensitized both *de novo* and acquired drug-resistant cancer cell lines.

Keywords

CRM1; topoisomerase II α ; nuclear pore complex; drug resistance; multiple myeloma; nuclear export

1. Nuclear export

1.1 Nuclear-pore complex structure and function

The cell nucleus is a sequestered environment due to the highly selective transport of proteins greater than 40 kDa both into and out of the nucleus through the nuclear pore complex (NPC). The NPC is one of the largest protein complex structures (125 MDa) in the cell, containing at least 30 different proteins called nucleoporins [1; 2]. The NPC has a central transporter region containing a nucleoporin matrix that assists movement of macromolecules, eight fibril proteins that extend into the cytoplasm, and a nuclear basket structure. Movement of molecules through the NPC is bidirectional; movement in or out of the nucleus is dependent on the type of receptor protein and the molecule associated with it.

For nuclear export or import of a large protein (>40 kDa) to occur, it must be bound to a nuclear export receptor molecule. The majority of these NPC transport molecules are members of the karyopherin- β family of proteins. There are approximately 19 members of karyopherin- β receptor family proteins, with each recognizing a specific group of cargo proteins or RNA [3]. CRM1 (chromosome region maintenance 1) is a ubiquitous transport receptor protein that binds its cargo via a hydrophobic nuclear export signal (NES) peptide sequence (see Section 1.2). Export complexes are formed in the nucleus and are made up of three components or trimer consisting of, CRM1, RanGTP, and the cargo or exported substrate. Binding of CRM1 to the cargo is weak; however, when the cargo protein and RanGTP bind to CRM1 cooperatively, the affinity of CRM1 to both RanGTP and the cargo substrate is increased 500- to 1,000-fold [4; 5]. Figure 1A shows the trimer formation of CRM1, RanGTP, and the cargo protein (in this case topoisomerase II α (topo II α)) in the nucleus at the NPC and transport through the NPC. The energy required for transport is provided by RanGTP. RanGTP is maintained in high concentrations in the nucleus by the presence of RCC1, a guanine nucleotide exchange factor (GEF). The high RanGTP concentration gradient from the nucleus to the cytoplasm provides the energy required for facilitated nuclear export. After the CRM1/RanGTP/cargo trimer arrives in the cytoplasm, RanGTP is hydrolyzed to RanGDP by a RanGAP (Figure 1B), causing dissociation of the

trimer and release of the cargo protein into the cytoplasm. CRM1 and RanGDP are then recycled back into the nucleus through the NPC for another export cycle (Figure 1C).

1.2 CRM1 and nuclear export signals

CRM1 is a ubiquitous nuclear export receptor molecule that binds to a cargo substrate (protein or RNA) containing a hydrophobic NES. At last count (January 2014), the “NESdb” database of NES-containing CRM1 cargoes published that there are 241 known macromolecules that bind to and are exported by CRM1 [6; 7]. The hydrophobic NES of the cargo protein binds to a hydrophobic groove of CRM1 containing an active site Cys528 [8]. NES were originally characterized as leucine-rich; however, characterization of the NES may be better described as not specifically leucine-rich but that it possesses a general hydrophobic pattern. NES peptides are 8–15 residues long and conform loosely to a consensus sequence of Φ 1-X_{2,3}- Φ 2-X_{2,3}- Φ 3-X Φ 4 (Φ n represents Leu, Val, Ile, Phe, or Met and X can be any amino acid), as reviewed in Xu et al [7].

2. Blocking nuclear export of proteins

Figure 1 demonstrates three potential means of attenuating nuclear export of the cancer drug target topo II α : 1) CRM1 inhibitors, 2) NES small molecule inhibitors, and 3) casein kinase 2 inhibitors, with the last preventing post-translational phosphorylation of topo II α .

2.1 CRM1 inhibitors

Direct inhibition of nuclear export is most commonly achieved with CRM1 inhibitors. There is a large cadre of nuclear export inhibitors from both natural and synthetic sources, as recently reviewed by Turner and Sullivan et al [9] and Hill and Link et al [10]. These reviews include more detailed information than will be covered here, including chemical structures. The majority of these inhibitors bind to the active site Cys528 contained within a hydrophobic groove of CRM1 and irreversibly modify it by a Michael-type covalent addition. Site-directed mutagenesis of Cys528 to Ser528 will prevent binding of CRM1 by leptomycin B [11], ratjadone [12] and other small molecule CRM1 inhibitors [13]. Exceptions to this include the CRM1 inhibitor CBS9106 developed by Sakakibara and Kawabe et al [14]. CBS9106 associates with the active site Cys528, but the binding is reversible. In addition, this compound does not induce CRM1 mRNA expression, whereas other CRM1 inhibitors, including the classic inhibitor of CRM1, leptomycin B, and newer generation N-azolyllacrylate analogs now referred to as SINE (selective inhibitors of nuclear export) increase CRM1 mRNA in a dose-dependent manner [15]. It is interesting that leptomycin B and SINE inhibitors increase CRM1 mRNA, but only the SINE compounds and CBS9106 lead to proteasome-mediated degradation of CRM1 [15]. SINEs are selective for the export molecule CRM1 and do not appear to inhibit other karyopherin- β (import or export) proteins (unpublished observations, Landesman and Shacham et al (Karyopharm Therapeutics)). Currently, only KPT-330 is in clinical trials (see Section 3).

2.2 Nuclear Export Signal Small Molecule Inhibitors

Since CRM1 is a ubiquitous exporter of nuclear proteins (>241 proteins), truly selective inhibitors that bind to the NES and prevent export may produce a more targeted therapy

(Figure 1). We have explored this idea by developing inhibitors that bind to a single specific NES peptide and prevent export of a topo II α . We have shown that topo II α has a NES at amino acids 1017-1028 (site A: DILRDFELRLK) and at amino acids 1054-1066 (site B: FILEKIDGKIIIE) [16]. Disrupting the binding between topo II α -NES and CRM1 could prevent CRM1-mediated nuclear export of topo II α and result in the sensitization of MM cells to topo II inhibitors. We identified, by a virtual screening method, a series of inhibitors of the interaction of CRM1 and topo II α which block the nuclear export of topo II α [17]. A homology model of human topo II α based on *S. cerevisiae* topo II was used for molecular docking. These NES inhibitors are designed to bind specifically to the NES of topo II α and prevent its export. We found that they did not prevent the export of other proteins such as p53. When NES inhibitors are combined with the topo II poison doxorubicin, they sensitize cancer cells to doxorubicin treatment and synergistically induce apoptosis and inhibit proliferation [17].

2.3 Post-translational modification of protein cargoes

Post-translational modification of cargo proteins include phosphorylation or dephosphorylation [18; 19; 20], sumoylation [18; 21], ubiquitination [18; 21], acetylation [18], or protein binding [22; 23; 24]; in effect any biochemical event that will prevent CRM1/cargo protein binding by either masking a NES or by exposing a nuclear localization signal peptide. Examples of proteins that are modified by phosphorylation before import or export include p53 [25], Nrf2 [26], topo II α [27], and PPAR-gamma [28]. We investigated the phosphorylation status of topo II α , isolated from nuclear and cytoplasmic extracts of human myeloma cells by immunoprecipitation [29]. The purified topo II α was digested with trypsin and analyzed with an electrospray ion trap mass spectrometer and liquid chromatography. Comparing the phosphorylation sites of nuclear and cytoplasmic topo II we found that cytoplasmic topo II α but not nuclear topo was highly phosphorylated at serine 1524, a published casein kinase II (CK2) motif [29]. Using site-directed mutagenesis we converted serine 1524 to an alanine and found that mutated recombinant FLAG-tagged TOP2A export was reduced, as compared to wild-type FLAG-tagged topo II α . This was observed by immunofluorescence microscopy in human myeloma cell lines. Serine 1524 is a CK2 phosphorylation site, therefore we used the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) and found by immunofluorescence microscopy, that topo II α nuclear export was blocked in high-density cells. The TBB data was duplicated using a CK2 specific siRNA to knockdown CK2 expression (90% knockdown). In addition, we found that blocking nuclear export of topo II α with TBB or CK2-specific siRNA sensitized drug-resistant myeloma cells to topo II poisons doxorubicin and VP-16 [29].

It may therefore be possible to block nuclear export of a specific cargo by inhibition of a kinase or other specific post-translation event.

3. Clinical Experience with CRM 1 inhibitors

3.1 Leptomycin B Clinical Trial

The first CRM1 inhibitor to enter the clinical arena was leptomycin B. Newlands et al reported on a phase I trial of elactocin (leptomycin B) in patients with various solid tumors

in 1996 [30]. This was based on preclinical evidence of efficacy of this agent in various cell lines and the suggestion that it inhibits DNA synthesis and DNA polymerase. Thirty-three patients were enrolled in the phase I trial, and several schedules of administration were studied in order to mitigate the toxicity: 1 hour infusion every 3 weeks, 1 hour weekly infusion, 1 hour daily infusion for 5 days, a 24-hour continuous infusion, and a continuous 5-day infusion [30]. Regardless of the infusion schedule, the dose-limiting toxicities were nausea, vomiting, and profound anorexia and malaise, often lasting more than 1 week with the need for intravenous hydration. No responses were noted in this trial, and the authors recommended no further investigations given the side effects and lack of benefits noted [30].

3.2 Selinexor or KPT-330 Clinical Trials

More recently, Selinexor (KPT-330), an oral CRM1 inhibitor, has been administered to patients with advanced hematologic malignancies and solid tumors in two phase I clinical trials (KPT-330-001 and KPT-330-002, respectively) [31; 32; 33; 34; 35]. Over 100 patients have been treated as part of those two studies. In the initial design, Selinexor was administered orally three times per week alternating with twice per week (10 doses in a 4-week cycle). Subsequently, Selinexor was administered twice weekly with improvement in tolerability [33]. The maximum tolerated dose of the 10 dose schedule was 30 mg/m², but dose escalation continued on the 8 dose schedule at doses above 30 mg/m² [34]. The elimination half-life was independent of the dose and was about 7.0 hours [31; 32; 33; 34; 35]. In addition, evaluation of tumor tissues confirmed nuclear localization of multiple tumor suppressor protein after therapy with Selinexor [31; 32; 33; 34; 35]. Gastrointestinal adverse events represent the predominant toxicity, which included nausea, vomiting, anorexia, and fatigue. Other notable toxicities included thrombocytopenia, which was seen in patients with advanced hematologic malignancies [33]. Evidence of anti-tumor activity was noted in patients with various tumors and particularly in patients with advanced hematologic malignancies, including patients with acute myeloid leukemia (4 patients of 14 evaluable patients achieved a complete response with or without complete count recovery) [31], non-Hodgkin lymphoma/chronic lymphocytic leukemia (3 patients of 15 evaluable achieved more than 50% reduction in lymph nodes) [33], and multiple myeloma (1 patients achieved a partial response and 6 a minimal response out of 15 evaluable patients) [32]. Solid tumor responses were noted in patients with colorectal cancer (4 of 12 patients had a CEA decrease and 1 patient had a partial response, with 6 patients having stable disease) [34], whereas patients with cervical cancer and endometrial stromal sarcoma had prolonged disease stabilization [35].

3.3 Combination of Doxorubicin and Selinexor in Patients with Multiple Myeloma

Doxorubicin is commonly used in the treatment of patients with multiple myeloma, and pegylated liposomal doxorubicin is approved in combination with bortezomib for the treatment of patients with relapsed and refractory myeloma [36]. KPT-330-001 has demonstrated clinical activity for Selinexor in patients with advanced myeloma [32]. Our group has shown that high-density human myeloma cell lines and patient myeloma cells export DNA topoisomerase II α using CRM1 from the nucleus to the cytoplasm, thus rendering the myeloma cells resistant to the cytotoxic effects of topoisomerase II inhibitors (for example, doxorubicin) [16; 37]. In addition, CRM1 inhibitors (leptomycin B and

ratjadones) and CRM1 siRNA block the nuclear export of topoisomerase II α and re-sensitize these cells to doxorubicin [38]. Based on these findings, we plan a phase I/II trial of Selinexor in combination with pegylated liposomal doxorubicin and dexamethasone in patients with relapsed and refractory myeloma. Specifically, eligible patients will receive a loading dose of Selinexor orally twice weekly for 2 weeks in combination with weekly dexamethasone. After the loading dose, patients will receive escalating doses of weekly Selinexor in combination with pegylated liposomal doxorubicin on day 1 and weekly dexamethasone. After 8 cycles, patients will receive maintenance therapy with weekly Selinexor and dexamethasone until disease progression. The primary endpoint of the phase I portion is determination of the maximum tolerated dose of Selinexor in this combination, and the primary endpoint for the phase II portion will be the overall response rate of the combination. *Ex vivo* drug sensitivity and nuclear localization of topoisomerase II α will be evaluated as part of planned correlative studies.

4. Combination Studies: *In vitro*, *In vivo* Xenografts, and *Ex vivo*

In studies done with CRM1 inhibitors (see table 1), single-agent CRM1 inhibitors induced apoptosis in cancer cell lines, slowed tumor growth in xenograft mice, and improved survival. However, in most cancers, single-agent CRM1 inhibitors were less likely to eliminate established tumors or reduce tumor burden. In addition, the CRM1 inhibitor's ability to induce apoptosis or anti-proliferative effects in cell lines improved synergistically when combined with chemotherapeutics such as ABT-737, AraC, bevacizumab, BRAF inhibitors, bortezomib, carfilzomib, cisplatin, daunomycin, decitabine, docetaxel, doxorubicin, imatinib, melphalan, oxaliplatin, paclitaxel, SN-38, nutlin-3a, or topotecan. CRM1 inhibitors have been shown to sensitize cancer cells both *in vitro*, in xenografts, or *ex vivo* to these chemotherapeutics and in some cases reverse drug resistance.

Listed in this section are several studies where CRM1 inhibitors were used in combination with other cancer therapeutics and in drug-resistant/relapsed cancers. A table of current abstracts (Table 1) summarizes the latest findings in CRM1 combination therapies. In addition, we include a table on drug sequencing in the treatment of multiple myeloma (Table 2).

4.1 BRAF inhibitors

Metastatic melanoma is a highly aggressive tumor with generally poor prognosis. Over half the patients with metastatic melanoma have a constitutively activated BRAF kinase driving proliferation of the cancer [39]. Drugs that target the mutated BRAF kinase have been shown to significantly improve overall survival of metastatic melanoma patients, emphasizing the role of this oncogene in melanoma biology. BRAF inhibitors block melanoma cell growth signals and subsequent proliferation and have shown good clinical results with low toxicity. However, resistance to BRAF inhibitor therapy eventually develops, and subsequent recurrences or relapses regularly occur within a short period after BRAF inhibitor treatment.

CRM1 is over-expressed in malignant melanoma and may prove to be a negative prognostic indicator [40]. Melanoma cells treated with the CRM1 inhibitor leptomycin B had high

levels of apoptosis without negatively affecting normal melanocytes or primary lung fibroblasts. Cell death involved both intrinsic and extrinsic apoptotic pathways and included nuclear retention (entrapment) of anti-proliferative factors p53 and p21 and the down-regulation of the anti-apoptotic factor survivin. CRM1 inhibitor-treated melanoma cells went into G1 cell-cycle arrest, and wild-type p53 expression was increased [40]. These data indicate the potential of new therapies using BRAF and CRM1 inhibitors to overcome both *de novo* resistance and prevent the development of acquired resistance in melanoma.

The synergistic potential of CRM1 and BRAF inhibition was explored in a recent study by Fragomeni et al [41]. Multiple small molecule CRM1 inhibitors obtained from Karyopharm Therapeutics (KPT-185, KPT-251, KPT-276, and KPT-330 (the clinical compound)) were used in combination with BRAF inhibitor analogs PLX-4720 and PLX-4032 in this study. It was found that proliferation of UACC-903, A375, and Mewo melanoma cell lines was inhibited synergistically when CRM1 inhibitors and BRAF inhibitors were used, as measured by the MTT assay. These results were independent of whether the cells had a mutant (UACC-903/A375 cells) or wild-type (Mewo cells) BRAF gene. The CRM1/BRAF inhibitor combination also synergistically induced apoptosis when measured by caspase 3/7 activity. Synergy was very strong with a combinatorial index (CI) of <0.3. The level of synergy is reflected by the CI values. CI values <1 are synergistic, values equal to 1 are additive, and those >1 are antagonistic. In an A375 melanoma BRAF V600E tumor xenograft model, both CRM1 inhibitor KPT-276 and BRAF inhibitor PLX-4720 slowed tumor growth as single agents when compared to untreated controls. However, complete and total tumor regression was observed when CRM1 and BRAF inhibitors were used in combination. The mechanism of synergy was attributed to increased levels of p53 and retinoblastoma proteins and a decrease in survivin by CRM1 inhibition, as well as to abrogation of extracellular signal-regulated kinase phosphorylation normally associated with CRM1 inhibition by the BRAF inhibitors. In conclusion, the authors found that CRM1 and BRAF inhibition synergizes and induces melanoma regression in metastatic melanoma cell lines and mouse xenografts.

4.2 Tyrosine kinase and CRM1 inhibitors in leukemia

Chronic myelogenous leukemia (CML) is a clonal disease of hematopoietic progenitor cells. In the chronic phase, CML is characterized by the abnormal expansion and accumulation of granulocytes in the circulation. However, the disease eventually progresses into a blast crisis phase, where acute leukemias of lymphoid or myeloid cells expand, resulting in patient mortality. Imatinib (STI571 or Gleevec), a tyrosine kinase inhibitor, targets Philadelphia (Ph⁺) chromosome-positive leukemias (both CML and ALL); however, over time, patients generally develop drug resistance. The Ph⁺ genetic mutation produces a chimeric BCR-ABL oncoprotein that is primarily localized in the cytoplasm where it activates proliferative and anti-apoptotic signaling pathways [42; 43]. BCR-ABL can inhibit apoptosis by activating PI3-kinase and Akt. However, when the Abl kinase protein is present in the nucleus, whether alone or in the chimeric form (BCR-ABL), it is activated by the presence of DNA damage and will induce a functional homolog of the tumor suppressor p53, p73. Nuclear activation of Abl kinase and p73 will result in DNA damage-induced apoptosis [44; 45]. In a proof of concept paper by Vigneri and Wang [46], they found that combining the CRM1

inhibitor leptomycin B and the BCL-ABL tyrosine kinase inhibitor imatinib induces nuclear localization (entrapment) of BCR-ABL and promotes apoptosis in CML cells *in vitro* and in BCR-ABL-transformed mouse bone marrow cells *in vivo*, sparing normal bone marrow stem cells. Combinatorial treatment was synergistic and resulted in the irreversible and complete killing of all of the BCR-ABL expressing cells, whereas single-agent treatment with the CRM1 inhibitor or imatinib only produced transient and reversible anti-CML effects.

In a follow-up study [47], the efficacy of the leptomycin B and imatinib mesylate combination was evaluated in human cells. CD34-positive normal cells from 6 healthy donors and myeloid progenitor cells from 35 CML patients were collected for the study. Sequential treatment of CD34+ cells with imatinib followed by leptomycin B induced apoptosis specifically in BCR-ABL-positive CML cells but had little effect on CD34/BCR-ABL double-negative cells from normal donors and normal myeloid progenitors derived from CML patients. Detection of BCR-ABL positive and negative cell colonies was based on molecular assay (RT-PCR). Since leptomycin B or imatinib, when used separately did not kill BCR-ABL CML cells, this would indicate that a combination approach may be of significant therapeutic value for newly diagnosed and imatinib-resistant CML patients.

A recent study by Walker et al [48] in Ph+ CML and ALL demonstrated that the CRM1 inhibitor KPT-330 induced apoptosis in leukemic but not normal CD34+ progenitor cells and increased survival in mice challenged with BCR-ABL+ cells. Survival was 50% after 15 weeks of KPT-330 treatment and BCR-ABL+ cells were significantly reduced *in vivo*. In addition, it was found that the CRM1 inhibitor potentiated the effect of the tyrosine kinase inhibitor imatinib in BCR-ABL+ cells after 24 hours. It was not determined whether this effect was statistically synergistic; however, combinatorial therapy was clearly more effective than either CRM1 inhibitor or tyrosine kinase inhibitor alone.

4.3 Melphalan

Melphalan is an alkylating agent that binds to DNA, inducing DNA damage and resulting in cancer cell death. Melphalan is still commonly used in standard dose induction therapy for the treatment of multiple myeloma and is the backbone for high-dose chemotherapy with autologous stem cell transplantation [49]. Melphalan, when used in combination with the CRM1 inhibitors KOS-2464, KPT-185, KPT-249 or the clinical compound KPT-330, synergistically induced cytotoxicity in human myeloma cell lines [15] (Table 2c). Synergy was stronger with sequential treatment of the CRM1 inhibitor followed 24 hours later by melphalan. The myeloma drugs lenalidomide and dexamethasone did not synergize with CRM1 inhibitors [15] (Table 2c).

4.4 Cisplatin/Oxaliplatin

Cisplatin is a DNA cross-linking agent that is used as a single agent or in combination with other therapeutics to treat numerous cancers, especially advanced or relapsed cancers and cancers that cannot be treated with radiation or surgery. Malignancies treated with cisplatin include bladder cancer, cervical cancer, malignant mesothelioma, non-small cell lung cancer, ovarian cancer, squamous cell carcinoma of the head and neck, neuroblastoma, and testicular cancer. Combination therapy using cisplatin and the classic inhibitor of CRM1

mediated export (leptomycin B) have been shown to induce apoptosis in a galectin-3 mutant and galectin-3 wild-type breast cancer cell line (BT-549) in a study by Takenaka et al [50]. Galectin-3 over-expression and sub-cellular localization have been linked to the development of cancer, including cancer cell growth and proliferation, transformation, apoptosis, angiogenesis, cell adhesion, invasion, and metastasis. The wide range of galectin-3 activities in cancer cells is due to its multiple inter- and sub-cellular localizations and interaction with a wide range of various binding partners [51]. When cancer cells are treated with a pro-apoptotic agent, such as cisplatin, galectin-3 is phosphorylated by a post-translational event and then exported from the nucleus to the cytoplasm. Phosphorylated cytoplasmic galectin-3 activates ERK and JNK, resulting in inhibition of both cytochrome c release and caspase activation with subsequent suppression of apoptosis in cancerous cells. CRM1 inhibition by leptomycin B prevents nuclear export of galectin-3, circumventing the anti-apoptotic function of galectin-3 and restoring cisplatin-induced apoptosis in breast cancer cells [50]. In 2013, three abstracts were published in *Cancer Research* on the use of small molecule CRM1 inhibitors (SINE) in combination with cisplatin (Table 1). In a recent abstract by Azmi et al [52] (Table 1), the platinum drug oxaliplatin synergistically induced apoptosis when used in combination with KPT-185 in a panel of human pancreatic and colon cancer cell lines. All cell lines tested exhibited synergy with the KPT-185/oxaliplatin combination, and cell death was mediated by nuclear PAR-4 expression.

4.5 Daunomycin

In our laboratory, we have tested the use of daunomycin in combination with the CRM1 inhibitors KOS-2464, KPT-185, KPT-249, KPT-276, or the clinical compound KPT-330 in acute myelogenous leukemia (AML). Human AML cell lines and *ex vivo* samples from bone marrow aspirates obtained from newly diagnosed and relapsed patients were treated with both CRM1 inhibitors and daunomycin and assayed for apoptosis by flow cytometry. Combined therapy induced apoptosis synergistically in both human AML cell lines [53] and patient bone marrow biopsies (unpublished data).

4.6 Doxorubicin

Significant progress has been made over the past several years in the treatment of multiple myeloma [54]. Patient median survival rates have increased from approximately 2 years to over 7 years due to improved therapies, including bortezomib/carfilzomib and lenalidomide/pomalidomide-based therapies and high-dose chemotherapy with autologous stem cell transplant [54]. However, patients eventually develop drug resistance, become refractory to all anti-multiple myeloma therapies, and die from progressive disease [55]. The incurable nature of multiple myeloma clearly demonstrates the need for novel agents and treatments. Therapies for patients with newly diagnosed or relapsed multiple myeloma often include treatment with pegylated liposomal doxorubicin or proteasome inhibitors bortezomib and carfilzomib [56; 57]. A very complete picture has emerged for the use of the topoisomerase II (topo II) inhibitor doxorubicin in combination with CRM1 inhibitors. Original observations published by our laboratory and others have strongly indicated that topo II α is exported from the nucleus of drug-resistant high-density multiple myeloma cells (cell lines and patient cells) [37; 58; 59]. High-density multiple myeloma cells (conditions that approximate the bone marrow environment) demonstrate *de novo* resistance to topo II

inhibitors [16; 37; 58; 59] (Figure 2A) because they traffic topo II α to the cytoplasm (Figure 2B and 2C), thus sequestering topo II α from the nuclear DNA and rendering it ineffective in generating lethal DNA strand breaks in the presence of a topo II inhibitor. The nuclear export of topo II α depends on the binding of specific NES on topo II α by CRM1 [16]. Nuclear proteins with a molecular mass >40 kDa are unable to passively diffuse across the nuclear membrane [9]. Those with a hydrophobic amino acid NES bind CRM1 and, in the presence of RanGTP, are transported through the NPC to the cytoplasm and released [9; 60]. We have shown that topo II α has a NES at amino acids 1017-1028 (DILRDFEFELRLK) and at amino acids 1054-1066 (FILEKIDGKIIE) [16]. Thus, disrupting the binding between topo II α -NES and CRM1 prevents the CRM1-mediated nuclear export of topo II α and results in the sensitization of multiple myeloma cells to topo II inhibitors. CRM1 binding to topo II can be inhibited by CRM1 siRNA, CRM1 inhibitors, and topo II α -specific NES inhibitors [16]. We found that CRM1 inhibition/knockdown prevented nuclear export of p53 and topo II α in myeloma cells. In vitro studies in human myeloma cell lines determined that CRM1 inhibitors, including ratjadone C or small molecule inhibitors from Karyopharm Therapeutics (KPT-185, KPT-249, and KPT-330) or CRM1 siRNA sensitized drug resistant multiple myeloma cells to doxorubicin [38]. KPT-185T, the inactive trans-isomer of KPT-185, did not synergize with doxorubicin. *Ex vivo* studies using myeloma cells obtained from both newly diagnosed and relapsed patient bone marrow aspirates treated with SINE drugs and doxorubicin synergistically induced apoptosis [15] (Figure 3). Mononuclear cell fractions isolated from aspirates were treated with the drug combination and myeloma cells were identified by flow cytometry by staining with light-chain and CD138 antibodies. Non-myeloma cells, defined as light-chain/CD138 negative/negative, were unaffected by CRM1 inhibitor/doxorubicin treatment (Figure 3). Nuclear p53 and topo II α proteins were increased by CRM1 inhibition; therefore, the addition of DNA damage by doxorubicin is likely to have produced a synergistic effect. A phase I/II clinical trial of the CRM1 inhibitor KPT-330 (Selinexor) in combination with pegylated liposomal doxorubicin and dexamethasone in patients with relapsed and refractory myeloma is being initiated at the Moffitt Cancer Center.

4.7 Proteasome inhibitors

Proteasome inhibitors, bortezomib and carfilzomib, are used extensively for the treatment of multiple myeloma. In a study by Turner et al [15], bortezomib and carfilzomib were tested in combination with the CRM1 inhibitors KPT-185T, KPT-185, and KPT-249 and the clinical compound KPT-330 in human myeloma cell lines and *ex vivo* bone marrow aspirates. *Ex vivo* cells were obtained from both relapsed and newly diagnosed patients. Ficoll-isolated mononuclear cell fractions were treated with individual agents and in combination, with myeloma cells identified via flow cytometry by staining with light-chain and CD138 antibodies. Myeloma cells treated with the CRM1/proteasome inhibitor combination showed a synergistic decrease in proliferation and increased apoptosis as compared to single agents (Figure 3). Non-myeloma cells, defined as light-chain/CD138 negative/negative, were unaffected by CRM1 inhibitor/doxorubicin treatment (Figure 3). The CRM1 inhibitor KPT-185 has been found to increase I κ B and p53 protein in the nucleus of chronic lymphocytic leukemia cells [61]. I κ B down-regulates NF- κ B, a constitutively active protein in myeloma that stimulates proliferation and cell survival. P53 is a critical

regulator of apoptosis that is suppressed in myeloma and other cancers. We found that KPT-185, KPT-249, KPT-330, leptomycin B, and KOS-2464 increased I κ B and p53 levels in multiple myeloma cells (unpublished results). Proteasome inhibitors are also known to increase expression of p53 and I κ B levels [62]. The overall increase in nuclear p53 inducing apoptosis and down-regulation of NF- κ B may result in enhanced cell death of the myeloma cells while not affecting normal lymphocyte populations.

4.8 Sequential versus concurrent treatment (Table 2)

We have studied the sequencing of drugs in the treatment of multiple myeloma as summarized in Table 2. Drugs connected by plus signs indicate concurrent treatment or simultaneous administration of both drugs to cell cultures and incubation for 48 hours. Drugs connected by arrows indicate that the drug listed first was added for 24 hours followed by the addition of the second drug for an additional 24 hours. The level of synergy is reflected by the CI values. CI values <1 are synergistic, values equal to 1 are additive, and those >1 are antagonistic. Table 2a is a summary of CRM1 inhibitors and proteasome inhibitors bortezomib or carfilzomib. In general, we found that sequential treatment (where the CRM1 inhibitor was given first followed by the proteasome inhibitor) was more effective than concurrent treatment or treatment in the reverse order (proteasome inhibitors followed by CRM1 inhibitor). Table 2b shows that sequential treatment with CRM1 inhibitor followed by doxorubicin is highly synergistic and that sequential treatment of CRM1 inhibitor followed by melphalan is synergistic (Table 2c). Dexamethasone and lenalidomide were not synergistic with the CRM1 inhibitor KPT-249.

We believe that drug sequencing analyses indicate that it is very likely that the CRM1 inhibitor restores, in addition to nuclear topo II α , the function of p53, I κ B, and other antiproliferative factors. This primes the cells for treatment by proteasome inhibitors or DNA damage induced by doxorubicin and melphalan.

5. Discussion

Cancer development is a multi-step process whereby many tumor suppressive proteins or cell cycle inhibitors are either silenced or compartmentalized within the cell where they are no longer effective at detecting DNA damage and/or promoting cell death [63]. CRM1 over-expression has been shown to be a causal factor, whereby oncogenes, tumor suppressive factors, and cell cycle inhibitors are mislocalized from the nucleus into the cytoplasm, resulting in promotion of the cancerous phenotype. Examples of mislocalized factors in cancer include APC [20; 64; 65; 66], BCR-ABL [46; 47; 48], Bok [67], BRCA1 [68; 69; 70], estradiol receptor [71; 72], FOXO [18; 73], galectin-3 [50; 74], HSP90 [75], Inf1/hSNF [21], Merlin [76], nucleophosmin [77; 78; 79; 80], N-WASP/FAK [81; 82; 83], p21 [84; 85; 86; 87; 88], p27 [89; 90; 91; 92; 93; 94], p53 [25; 95; 96; 97; 98], RASSF2 [99], survivin [100; 101], Tob [102; 103], DNA topoisomerase I [104; 105], and topo II α [9; 15; 16; 106; 107]. Recent studies have shown that many cancer types, including AML [108], CLL [61], CML [48], glioma [109], mantle cell lymphoma [110], melanoma [40], multiple myeloma [111], non-Hodgkin lymphoma [112], osteosarcoma [113], ovarian cancer [114], and pancreatic cancer [115; 116], over-express CRM1. CRM1 protein expression may become a

significant biomarker for disease progression in specific malignancies. In the past, we and others have proposed the use of targeting CRM1 in the treatment of cancer [9; 38; 68; 106; 117; 118; 119]; however, only until recent developments of less toxic therapeutics has the potential use of CRM1 inhibitors come closer to being realized. These less toxic small-molecule CRM1 inhibitors, though reasonably effective against cancer cell lines and slowing the growth of xenograft tumors in mice, may need additional help in the form of combinatorial therapies. Potential combinatorial therapies include the use CRM1 inhibitors and the addition of alkylating agents (melphalan), anthracyclines (doxorubicin and daunomycin), BRAF inhibitors, platinum drugs (cisplatin and oxaliplatin), proteasome inhibitors (bortezomib and carfilzomib), SN-38, or tyrosine-kinase inhibitors (imatinib). The idea behind these therapies is that blocking nuclear export by CRM1 small-molecule inhibitors may restore the apoptotic pathways of the tumor suppressive factors listed above and synergistically induce cancer cell death.

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Figure 1. Intracellular export of proteins

A CRM1/RanGTP/Cargo protein (topo IIα) complex is transported through the NPC to the cytoplasm where phosphorylated topo IIα is released. The nuclear export of topo IIα can be blocked by 1) CK2 inhibitors that prevent phosphorylation and binding of topo IIα to CRM1; 2) covalent binding of CRM1 inhibitors to CRM1, preventing binding of topo IIα NES, and 3) specific binding of the NES by small molecule inhibitors. Blocking the nuclear export of topo IIα increases the amount in the nucleus, which is able to cleave DNA in the presence of topo II inhibitors, thus overcoming this *de novo* resistance. **Figure 1A** shows the trimer formation of CRM1, RanGTP, and the cargo protein (topo IIα) in the nucleus at the NPC and transport through the NPC. **Figure 1B**, RanGTP is hydrolyzed to RanGDP by a RanGAP in the cytoplasm (Figure 1B), causing dissociation of the trimer and release of the cargo protein. **Figure 1C**, CRM1 and RanGDP are then recycled back into the nucleus through the NPC for another export cycle. CK2, casein kinase 2; CRM1, chromosome maintenance protein 1; NES, nuclear export signal; NPC, nuclear pore complex.

Figure 2. (A) Intracellular trafficking of topo II α in low- and high-density myeloma cells Human H929, 8226, and U266 MM cells were grown for 16 hours at low or high density and treated with 2 μ M doxorubicin for 4 hours (n = 2). Apoptosis was determined by caspase 3 staining. Log cells that maintained nuclear topo II α were significantly more sensitive to topo II α -targeted chemotherapy. **(B) NCI-H929 topo II α immunofluorescence.** H929 human MM cells were grown at low and high densities and stained for cytoplasm (phalloidin, green), topo II α (red), and DNA (DAPI, blue). Results indicate that topo II α is located in the nucleus of low-density cells and is exported from the nucleus to the cytoplasm in high-density cells. **(C) Myeloma cells export topo II α in patient bone marrow aspirates.** Plasma cells in a bone marrow aspirate from a relapsed myeloma patient were identified by kappa-light chain antibody (green) (b & e). Cells were also stained for topo II α (c) (red), CRM1(f) (red), DNA (blue; dapi) (a & d). Figure reprinted in part by from Cancer Research 2009; 69:6899–905.

Figure 3. KPT-185, KPT-249, and KPT-330 sensitize patient myeloma cells to doxorubicin, bortezomib, and carfilzomib

Bone marrow mononuclear cells were isolated and treated with SINE (300 nM) +/- doxorubicin (2 μ M), bortezomib (10 nM), carfilzomib (20 nM), or DMSO vehicle control (VC) for 20 hours. Treated cells were fluorescently labeled with antibodies against activated caspase 3, CD138, and light chain (kappa or lambda). Results for patient multiple myeloma cells (n=12 patient samples) (A–C) and patient non-myeloma cells (n=12) (D–F) are shown. Apoptosis was induced when SINE was co-incubated with doxorubicin ($P = 0.0004$), bortezomib ($P = 0.003$), or carfilzomib ($P = 0.002$) in CD138/light-chain double-positive myeloma cell populations (A–C) but not in non-myeloma CD138/light-chain double-negative cells ($P > 0.25$) (D–F). This indicates that SINE may specifically inhibit neoplastic cells in MM patients. Figure reprinted by permission from Journal of Cancer 2013; 4:614–625.

Table 1

Combination Therapy with CRM1 inhibitors, AACR, ASH and ASCO 2011–2013 Abstracts

Cancer	Drugs Used	Anti-tumor Drug Effects, In vitro and In vivo Studies	Reference
AML	KPT-185 +/- Nutlin-3a	KPT-185/Nutlin-3a synergistically induced apoptosis and cytotoxicity. p53 knockdown abrogated these effects. No synergy in normal cord blood cells.	Kojima et al. Blood: 2012; 120 Abst # 870
AML	KPT-330 +/- AraC	KPT-330/AraC showed additive antiproliferative effects in acute promyelocytic leukemia cell lines. In vivo KPT-330/AraC prolonged survival compared to monotherapy (P < 0.0001) in mice.	Rettig et al. Blood: 2013; 122 Abstr #237
AML	KPT-330 +/- Decitabine	KPT-330/Dct increased cytotoxicity compared to monotherapy in AML cells. In a mouse xenograft model, treatment with Dct followed by KPT-330 improved survival compared to KPT-330 alone (47 vs 36.5 days) p=0.008.	Ranganathan et al. Blood: 2013; 122 Abstr #1453
CLL	KPT-185 +/- CPG, CD40L, BAFF, TNF- α , IL-6, IL-4	KPT-185 abrogated protection induced by soluble factors in CLL cells.	Lapalombella et al. Blood: 2011; 118 Abst #232
CML	KPT-330 +/- Imatinib	KPT-330 +/- Imatinib reduced colony formation significantly compared to Imatinib alone in primary CML cells.	Sorouri et al. Blood: 2013; 122 Abst #2707
MM & AML	KOS-2464 +/-Dox, Daun	KOS-2464 sensitized MM and AML cells to Dox (CI 0.268) and Daun as shown by apoptosis and cell viability assays.	Turner et al. Can Res: 2012; 72 Abst #1771
Multiple Myeloma	KPT-185 +/- Dex, Len, Mel, Btz	KPT-185/Dex, Len, or Btz synergistically (CI <1.0) induced cytotoxicity in MM cell lines. KPT-185/Mel induced additive effects.	Kong et al. Blood: 2011; 118 Abst #2913
Multiple Myeloma	KPT-185, KPT-276 +/- Btz, Dex	KPT/Btz and/or Dex increased the cellular cytotoxicity in MM lines.	Kandarpa et al. Blood: 2011; 118 Abst #1852
Multiple Myeloma	KOS-2464, KPT-185, KPT-249, KPT-330 +/- Btz, Cfz	CRM1 inhibitors sensitized both MM cell lines and MM patient cells (ex vivo) synergistically (CI <0.5) to Btz and Cfz as shown by apoptosis and cell viability assays.	Turner et al. Can Res: 2013; 73 Abstr #2066
Multiple Myeloma	KPT-330 +/- Cfz	KPT-330/CFZ synergistically increased cell death (CI 0.2–0.6) by both apoptosis and autophagy in MM cell lines. In a xenograft mouse KPT-330/CFZ was more effective than monotherapy, and high dose completely impaired tumor growth with good tolerability.	Rosebecket al. Blood; 2013 122 Abst #279
Multiple Myeloma	KPT-185, KPT-276 +/- Btz, Dex	KPT-185/Btz markedly increased apoptosis in MM1R cells vs monotherapy. Dex/KPT-185 or KPT-276 induced synergistic cytotoxicity in cell lines.	Tai et al. Can Res: 2013; 73 Abstr #2142
Multiple Myeloma	NCI-9138 +/-Dox	NCI-9138 sensitized human myeloma cells H929 drug-resistant to Dox but did not affect normal fibroblast cell lines or human PBMCs.	Turner et al. Blood; 2013 122 Abst #1925
NHL	KPT-330 +/- Cfz	KPT-330/Cfz induced strong synergy (CI<1.0) in aggressive NHL cell lines using MTS assay and Annexin-V apoptosis assays.	Lopez et al. Blood: 2013; 122 Abst #5147
Colon	KPT-185 +/- SN38	KPT185/SN38 resulted in synergistic cell death in colon cancer cells (CI<1). CDDE assay showed KPT-post increased apoptosis (4.3-fold) compared with KPT-pre (4.2-fold), concurrent (3.8-fold) and SN38 alone (1-fold).	Chung et al. J Clin Oncol 2012; 30 Abstr #609
Colon	KPT-251 +/- SN38	KPT-251/SN3S induced synergism in both CPT-11/SN3S-sensitive and CPT-11/SN38-resistant colon cancers cells in vitro and in vivo.	Chung et al. J Clin Oncol 2012; 30 Abstr #396
Colon Pancreatic	KPT-185 +/- oxaliplatin	KPT-185/oxaliplatin synergistically enhanced apoptosis in all the cell lines.	Azmi et al. J Clin Oncol 2012; 30 Abstr #245
NSCLC	LMB +/- Dox	Pre-treatment with Dox improved the cytotoxic effects of LMB 2-fold on A549 cells (P<0.05) as compared to LMB alone. LMB then Dox or	Lu et al. Can Res: 2011; 71 Abst #3515

Cancer	Drugs Used	Anti-tumor Drug Effects, In vitro and In vivo Studies	Reference
		concurrent did not change the cytotoxic effects of either LMB or Dox.	
NSCLC	KPT-185 +/- ABT-737	KPT-185/ABT-737 induced synergistic cytotoxicity in the SINE-resistant A549 cell line & modest killing in the sensitive cell line H-226.	McCauley et al. Can Res: 2012; 72 Abstr #1831
NSCLC	KPT-185, KPT-330 +/- RT	KPT-185/RT clonogenic assays showed an additive effect for SINE-resistant A549 and a dose-related effect for SINE-sensitive H1299 cell lines.	Rashal et al. Can Res: 2013; 73 Abstr #2075
Melanoma	novel CRM1 inhibitor +/- PLX4032	CRM1 inhibitor and BRAF inhibition were strongly synergistic in BRAF mutant cell lines with a statistically significant decrease in cell proliferation and increased apoptosis.	Fragomeni et al. Can Res: 2012; 72 Abstr #1914
Neuroblastoma	KPT-330 +/- Irinotecan, Tpt, Cisplatin, Dox	Combination treatment with KPT-330/Irinotecan, Topotecan, Cisplatin, and Doxorubicin showed additive effects in growth inhibition.	Attieh et al. Can Res: 2013; 73 Abstr #2756
Ovarian	KPT-185, KPT-330 +/- Cisplatin	In vitro KPT/Cisplatin showed additive effect on cell death, overcoming cisplatin resistance in the isogenic cell lines A2780 and CP70.	Chen et al. Can Res: 2013; 73 Abstr #2163
Ovarian	KPT-185 +/- Tpt, Cisplatin, LipoDox, Paclitaxel, Bevacizumab	KPT-185/Tpt, Cisplatin and LipoDox show synergistic (CI<1) reduction in cell viability in human ovarian cancer cell lines. In the A2780 mouse model, KPT-185/Tpt reduced tumor growth by 98% (p<0.01). Significant tumor growth inhibition also was seen with KPT-330/Paclitaxel or Bevacizumab.	Miyake et al. Can Res: 2013; 73 Abstr #5541
Prostate	KPT-185, KPT-251 +/- Cisplatin, Docetaxel	Synergy was seen with KPT in combination with Cisplatin and Docetaxel in vitro and in vivo.	Gravina et al. Can Res: 2012; 72 Abstr #1841
Renal	KPT-330 +/- ABT-737	KPT-330/ABT-737 showed synergism in RCC cell lines and a normal human kidney cell line in vitro.	Inoue et al. J Clin Oncol 2013; 31 Abstr #411

Abbreviations:

AML, Acute Myelocytic Leukemia; BAFF, B-cell activating factor; Btz, Bortezomib; CDDE, Cell Death Detection ELISA assay; CD40L, Cluster of Differentiation 40 ligand; Cfz, Carfilzomib; CLL, Chronic Lymphocytic Leukemia; Daun, Daunorubicin; Dct, Decitabine; Dex, Dexamethasone; Dox, Doxorubicin; IL-6 & IL-4, Interleukin 6 and 4; Len, Lenalidomide; LipoDox, Liposomal Doxorubicin; LMB, Leptomycin B; Mel, Melphalan; MM, Multiple Myeloma; MTS, Microculture Tetrazolium Assay; NHL, Non-Hodgkins Lymphoma; NSCLC, Non-small cell lung cancer; PARP, Poly (ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cells; RT, Radiation Therapy; TNF- α , Tumor necrosis Factor alpha

Tables 2
Combination Index of concurrent or sequential treatment of MM cells

Combination index (CI) values of high density human multiple myeloma cell line NCI-H929 treated sequentially or concurrently with CRM1 inhibitors and either proteasome inhibitors (table 2a), doxorubicin (table 2b), or other chemotherapy agents (table 2c). Cells were placed at high density ($3.0 \times 10^6/\text{ml}$) and incubated with the first drug for 24 hours followed by the second drug for an additional 24 hours for sequential treatment. For concurrent treatment cells were treated with both drugs for 48 hrs. Cytotoxicity was determined by a high-throughput CellTiter-Blue (Promega Corp.) cell viability assay. The combination index value was calculated using the Chou and Talalay method with CI <1 representing synergy, =1 additive, and >1, antagonistic results.

a. Combination Index (CI) Values for CRM1 Inhibitors + Proteasome Inhibitors in High-density H929 MM Cells by CT-Blue Viability Assay					
Drug Administration: concurrent (+) or sequential (→)	Concentration range: Drug 1	Concentration range: Drug 2	Combination Index (mean)	Combination Index (SEM)	n
KOS-2464 + Bortezomib	9.1 nM – 1 μM	1.8 nM – 200 nM	0.711	0.184	2
KPT 185 + Bortezomib	390.6 nM – 100 μM	39 pM – 50 nM	0.922	0.057	2
KPT 249 + Bortezomib	390.6 nM – 100 μM	39 pM – 10 nM	0.887	0.006	2
KPT 330 + Bortezomib	780.4 nM – 20 μM	1.8 nM – 50 nM	0.953	0.065	3
KOS-2464 → Bortezomib	3.9 nM – 5.0 μM	39 pM – 100 nM	0.415	0.134	3
KPT-185 → Bortezomib	390.6 nM – 100 μM	39 pM – 50 nM	0.41	0.019	3
KPT-249 → Bortezomib	390.6 nM – 100 μM	39 pM – 10 nM	1.098	0.259	2
KPT-330 → Bortezomib	780.4 nM – 20 μM	1.8 nM – 50 nM	0.651	0.144	4
Bortezomib → KPT 330	4.5 nM – 50 nM	1.8 μM – 20 μM	0.8	0.072	2
KOS-2464 + Carfilzomib	9.1 nM – 100 nM	1.8 nM – 20 nM	0.713	0.141	2
KPT 185 + Carfilzomib	1.8 μM – 20 μM	1.8 nM – 20 nM	0.408	0.083	3
KPT 249 + Carfilzomib	1.8 μM – 20 μM	1.8 nM – 50 nM	0.469	0.089	2
KPT 330 + Carfilzomib	1.8 μM – 20 μM	1.8 nM – 50 nM	0.552	0.045	2
KOS-2464 → Carfilzomib	9.1 nM – 1 μM	1.8 nM – 200 nM	0.224	0.021	3
KPT-185 → Carfilzomib	780.4 nM – 20 μM	1.8 nM – 50 nM	0.322	0.136	3
KPT-249 → Carfilzomib	780.4 nM – 20 μM	1.8 nM – 100 nM	0.426	0.084	4
KPT-330 → Carfilzomib	780.4 nM – 20 μM	1.8 nM – 50 nM	0.388	0.08	3
Carfilzomib → KPT-249	4.5 nM – 50 nM	1.8 μM – 20 μM	1.269		1
Carfilzomib → KPT-330	4.5 nM – 50 nM	1.8 μM – 20 μM	1.153	0.341	2

b. Combination Index (CI) Values for CRM1 Inhibitors + Topoisomerase II Inhibitor in High-density H929 MM Cells by CT-Blue Assay					
Drug Administration: concurrent (+) or sequential (→)	Concentration range: Drug 1	Concentration range: Drug 2	Combination Index (mean)	Combination Index (SEM)	n
KOS-2464 + Doxorubicin	3.9 nM – 5 μM	19.5 nM – 100 μM	0.674	0.147	2

b. Combination Index (CI) Values for CRM1 Inhibitors + Topoisomerase II Inhibitor in High-density H929 MM Cells by CT-Blue Assay					
Drug Administration: concurrent (+) or sequential (→)	Concentration range: Drug 1	Concentration range: Drug 2	Combination Index (mean)	Combination Index (SEM)	n
KPT-185 + Doxorubicin	195.3 nM – 100 μ M	7.8 nM – 10 μ M	0.807	0.165	4
KPT-249 + Doxorubicin	195.3 nM – 100 μ M	7.8 nM – 10 μ M	0.735	0.094	4
KPT-330 + Doxorubicin	195.3 nM – 100 μ M	7.8 nM – 10 μ M	0.696	0.155	5
KOS-2464 → Doxorubicin	3.9 nM – 5 μ M	19.5 nM – 100 μ M	0.278	0.065	7
KPT-185 → Doxorubicin	391 nM – 100 μ M	390.6 nM – 100 μ M	0.377	0.087	4
KPT-249 → Doxorubicin	3.9 nM – 100 μ M	39.1 nM – 100 μ M	0.628	0.053	7
KPT-276 → Doxorubicin	39.1 nM – 100 μ M	390.6 nM – 100 μ M	0.340	0.134	7
KPT-330 → Doxorubicin	78.1 nM – 20 μ M	390.6 nM – 200 μ M	0.092	0.025	3
Doxorubicin → KOS-2464	97.66 nM – 25 μ M	195.31 pM – 50 nM	1.003	0.010	2
Doxorubicin → KPT 185	97.66 nM – 25 μ M	195.31 nM – 50 μ M	1.197	0.259	3
Doxorubicin → KPT 249	97.66 nM – 25 μ M	195.31 nM – 50 μ M	1.064	0.249	2
Doxorubicin → KPT 330	97.66 nM – 25 μ M	195.31 nM – 50 μ M	1.024	0.233	4

c. Combination Index (CI) Values for CRM1 Inhibitors + Other Drugs in High-density H929 MM Cells by CT-Blue Assay					
Drug Administration: sequential (→)	Concentration range: Drug 1	Concentration range: Drug 2	Combination Index (mean)	Combination Index (SEM)	n
KOS-2464 → Melphalan	3.9 nM – 5 μ M	390.6 nM – 100 μ M	0.362	0.216	3
KPT-185 → Melphalan	78.1 nM – 100 μ M	390.6 nM – 200 μ M	0.849	0.104	4
KPT-249 → Melphalan	390.6 nM – 100 μ M	39.1 nM – 10 μ M	0.979	0.066	3
KPT-330 → Melphalan	78.1 nM – 20 μ M	390.6 nM – 200 μ M	0.549	0.117	3
KPT-249 → Dexamethasone	390.6 nM – 100 μ M	39.1 nM – 200 μ M	1.263	0.238	4
KPT-249 → Lenalidomide	390.6 nM – 100 μ M	39.1 nM – 10 μ M	1.098	0.11	3