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Review

Nuclear export of proteins and drug resistance in cancer

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ABSTRACT

The intracellular location of a protein is crucial to its normal functioning in a cell. Cancer cells utilize the normal processes of nuclear-cytoplasmic transport through the nuclear pore complex of a cell to effectively evade anti-neoplastic mechanisms. CRM1-mediated export is increased in various cancers. Proteins that are exported in cancer include tumor-suppressive proteins such as retinoblastoma, APC, p53, BRAC1, FOXO proteins, INI1/hSNF5, galectin-3, Bok, nucleophosmin, RASSF2, Merlin, $p21^{CIP}$, $p27^{KIP1}$, N-WASP/FAK, estradiol receptor and Tob, drug targets topoisomerase I and IIα and BCR-ABL, and the molecular chaperone protein Hsp90. Here, we review in detail the current processes and known structures involved in the export of a protein through the nuclear pore complex. We also discuss the export receptor molecule CRM1 and its binding to the leucine-rich nuclear export signal of the cargo protein and the formation of a nuclear export trimer with RanGTP. The therapeutic potential of various CRM1 inhibitors will be addressed, including leptomycin B, ratjadone, KOS-2464, and specific small molecule inhibitors of CRM1, N-azolylacrylate analogs, FOXO export inhibitors, valtrate, acetoxychavicol acetate, CBS9106, and SINE inhibitors. We will also discuss examples of how drug resistance may be reversed by targeting the exported proteins topoisomerase IIα, BCR-ABL, and galectin-3. As effective and less toxic CRM1 export inhibitors become available, they may be used as both single agents and in combination with current chemotherapeutic drugs. We believe that the future development of low-toxicity, small-molecule CRM1 inhibitors may provide a new approach to treating cancer.

Contents

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1. Introduction

Drug resistance continues to be the greatest challenge in the treatment of cancer. Despite the significant progress in the development of novel therapies, cancer cells continue to develop mechanisms of escaping the many checks and balances between cell survival and programmed cell death. During cancer development, cells can acquire an intrinsic resistance to the treatment as a result of the breakdown of many normal cellular processes or in response to selection by drug treatment. In addition to various intracellular molecular and biochemical mechanisms, recent studies have shown that resistance can also be attributed to external factors involving the tumor's physical microenvironment. Acquired resistance is a significant problem; cancer cells may develop cross-resistance to multiple drugs that have quite different mechanisms of action [1,2]. Acquired drug resistance can manifest in many ways; for example, chemical inactivation of cisplatinum, carboplatin, or oxalplatin is produced when thiol glutathione forms conjugates with these drugs [3]. DNA repair mechanisms can be altered in response to DNA damage produced by platinum drugs or topoisomerase inhibitors [4–6]. Nucleotide excision repair can remove platinum drug damage, or cell signaling pathways can arrest the cell cycle and delay apoptosis. The amount of free drug available can be reduced or catabolized by overexpression of enzymes that degrade 5-fluorouracil [1]; ATP binding cassette transporter drug efflux pumps can bind to and export drugs such as mitoxantrone, VP-16, doxorubicin, vinblastine, and flavorpiridol [2,7–11]. Cancer cells can downregulate proapoptotic factors such as microtubule inhibitors (vinca alkaloids) [12–15]. The drug target topoisomerase I can be modified by mutation or protein expression downregulated in response to irinotecan and campothecan [16,17]. The tumor microenvironment itself has been shown to contribute to intrinsic drug resistance. In multiple myeloma, the bone marrow stromal cells interact with tumor cells and produce cell adhesion-mediated drug resistance (CAM-DR) to melphalan [18–21]. The tumor microenvironment has been reported to be very hypoxic due to inadequate blood supply and subsequent oxygen diffusion. Hypoxic tumor cells are resistant to both radiotherapy and chemotherapy [22]. In addition, intracellular nuclear export of either tumor suppressive proteins or drug targets can result in drug resistance due to overexpression of CRM1 [23–26]. This latter mechanism, the export of drug targets, tumor suppressors, and cell cycle inhibitors from the nucleus, is the primary focus of this review.

In this review, we discuss in detail the mechanisms of nuclear export, including structure and function of the nuclear pore complex, the specific transport receptor molecules that move large molecules out of the nucleus into the cytoplasm, and the proteins that are targeted for export in cancer. In addition, we provide a summary of potential drug-like inhibitors of nuclear export and how the use of nuclear export inhibitors may contribute to cancer treatment and drug resistance.

2. Nuclear export mechanisms

The nuclear envelope provides a sequestered intracellular environment for DNA replication, RNA transcription, and production of ribosomes. The nuclear envelope membrane bilayer is a selective physical barrier that is involved in regulation of the cell cycle, in addition to apoptosis and proliferation. Nuclear-cytoplasmic trafficking of RNA, ribosomes, important regulators of transcription, cell cycle inhibitors, and specific drug targets (topoisomerases) are tightly regulated by the nuclear pore complex and by the presence of transport receptor molecules known as karyopherins (Fig. 1). Misregulation of nuclear export can result in various pathological conditions, including cancer.

Intracellular movement of proteins is controlled by very specific sequences of amino acids or signaling sequences contained within each protein. Signals exist on proteins for import into the nucleolus [27–29], import into the nucleus (nuclear localization signals) [30], and export from the nucleus to the cytoplasm (nuclear export signals) [31–34]. In addition, amino acid signaling sequences govern protein trafficking to the endoplasmic reticulum [35,36], Golgi apparatus [37], lysosomes [38], mitochondria [39], and peroxisomes [40].

All substances trafficked into or out of the nucleus must traverse structures imbedded in the nuclear envelope called nuclear pore complexes (NPC). Transport of small molecules, proteins, and salts occurs by diffusion through the NPC; however, proteins larger than 40-65 kDa cannot move through the NPC passively. Larger proteins must be transported through the NPC with the assistance of soluble nuclear-cytoplasmic transport receptors or karyopherin proteins. The majority of these nuclear-cytoplasmic transport receptors are members of the karyopherin-β family of proteins [41]. Each karyopherin-β recognizes a unique group of cargo proteins or RNA. This recognition is made by the presence of either a nuclear localization signal (NLS) or a nuclear export signal (NES) in the amino acid sequence of the cargo protein. To date, only three classes of nuclear-cytoplasmic transport signals have been identified; these include the classical basic amino acid NLS sequences recognized by importin α/β [42]. This classical NLS can be monoparite or bipartite [30]. Karyopherin-β2 cargo proteins contain a more complex PY-NLS possessing an N-terminal hydrophobic or basic motif and a C-terminal $RX_{2-5}PY$ motif [43]. The third class of nucleocytoplasmic transport signals is a hydrophobic leucine-rich export signal recognized by the ubiquitous transport receptor chromosome maintenance protein 1 (CRM1/exportin 1). CRM1 binds to and exports both proteins and RNA. At least 19 karyopherin proteins have been identified in humans (importins, exportins, and transportins); however, the NLS/NES recognition signals on the molecules that they transport have not been identified for the majority of these transport molecules [44].

NES for the transport receptor protein CRM1 are sequences of hydrophobic amino acids, including isoleucine, leucine, methionine, phenylalanine, and valine [45]. Although there is limited structural information and a relatively high degree of sequence diversity, a consensus motif for an NES is $HX_{2-3}HX_{2-3}HXH$, where H is a hydrophobic amino acid (isoleucine, leucine, methionine, phenylalanine, or valine) and X is any amino acid [45,46]. The subscripted numbers indicate the potential number of repeats. This consensus sequence can be present in more complex protein structures such as α-helical proteins [47]. In a study using fission yeast, cells were subjected to leptomycin B, a known inhibitor of the nuclear export protein Crm1. Proteomic analysis of these cells identified 285 proteins that were regulated by Crm1 localization. Approximately 45% of these proteins contained the canonical nuclear export consensus sequence; therefore, it may be that CRM1 exported proteins may contain an altered or mismatched nuclear export sequence [48].

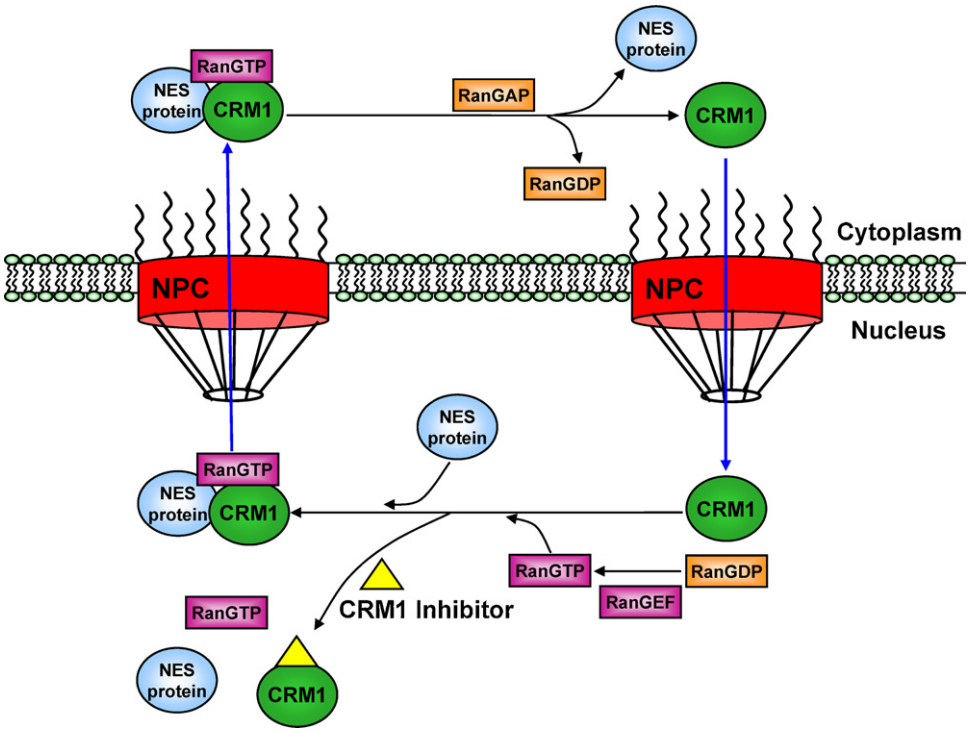

Fig. 1. Nuclear export of a cargo protein occurs by association of its nuclear export signal (NES) with chromosome maintenance protein 1 (CRM1) and Ran-GTP and subsequent transport through the nuclear pore complex (NPC). Ran-GDP is phosphorylated by Ran-GEF, a guanine nucleotide exchange factor. Ran-GTP is dephosphorylated by Ran-GAP (Ran GTPase activating protein).
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The vertebrate nuclear pore complex (NPC) is one of the largest macromolecular complexes in the cell (approximately 125 MDa) [49]. The NPC is made up of multiples of approximately 30 different nucleoporin proteins [50]. The nuclear pore has a central transporter region containing a matrix of nucleoporin proteins that facilitate movement of transport receptor proteins. In addition, the nuclear pore has eight large cytoplasmic fibrils and a nuclear basket structure. The NPC is imbedded in the nuclear envelope bilayers [51]. The nuclear pore is impenetrable to large molecules unless they are associated with a karyopherin transport receptor protein (importin, exportin, or transportin). Each NPC has the ability to move substances into the nucleus (importins), out of the nucleus (exportins), or bidirectionally (transportins), depending on the specific karyopherin and its cargo. The transport mechanism of proteins through the nuclear pore central channel is a topic of intense research. RanGTP is present at high concentrations in the nucleus, and it is thought that a RanGTP concentration gradient provides the energy required for facilitated nuclear export [52]. The high concentration of RanGTP is maintained in the nucleus by the presence of a guanine nucleotide exchange factor (GEF) called regulator of chromosome condensation 1 or RCC1. RCC1 is bound to chromatin and is therefore maintained in the nucleus.

Approximately 12–20% of the nucleoporins in the NPC are made up of phenylalanine/glycine repeat domains (FG-repeats) [53]. CRM1 and other karyopherins of the importin β family possess HEAT domains (*Huntington*, elongation factor 3 (*E*F3), protein phosphatase 2A (PP2*A*), and the yeast PI3-kinase *T*OR1) that are attracted to and interact with the FG-repeat domains of the nucleoporins. It is thought that this interaction, along with facilitated diffusion by the RanGTP gradient, promotes the movement of the complexes through the nucleoporin matrix in the central channel of the NPC (reviewed by Cook et al.) [41].

For nuclear export of a cargo protein to occur, it must bind a RanGTPase-dependent export mediator consisting of a transport receptor protein or exportin (CRM1), which in turn is regulated by a small GTPase molecule, referred to as RanGTP [52,54]. There are several types of RanGTPase-dependent export mediators (exportins) (reviewed in Guttler et al.) [55]. Export complexes are formed in the nucleus. These complexes consist of three components: RanGTP, the exportin transport receptor (CRM1), and the export substrate or cargo protein [56]. This three-member complex binds together with positive cooperativity [57]. Binding of CRM1 to either RanGTP or to the nuclear export signal of a cargo protein is weak; however, when both RanGTP and the cargo substrate protein bind to CRM1 cooperatively, affinity to both substrate and RanGTP is increased by 500–1000 fold [47,58]. This increase in affinity was shown by crystallography to be caused by a change in the global conformation of CRM1. This change in conformation only occurs when both the cargo substrate and RanGTP bind to CRM1 [57,58].

Once formed in the nucleus, the CRM1-RanGTP-cargo substrate complex is exported through the NPC into the cytoplasm. After it arrives in the cytoplasm, RanGTP is hydrolyzed to RanGDP by RanGTPase, and the export complex dissociates into its separate components, releasing the substrate or cargo protein into the cytoplasm. Ran-GTP and the CRM1 receptor proteins are recycled back into the cell nucleus through the NPC to undergo another cycle of nuclear export.

CRM1 is a ubiquitous nuclear export receptor protein that is involved in the export of many substrates, including both proteins and RNA [59,60]. For CRM1 binding to occur, the cargo substrate must have a leucine-rich nuclear export signal present and accessible for CRM1 binding [52,61]. Three-dimensional conformational changes in the cargo protein, caused by protein phosphorylation, dephosphorylation, or mutation can reveal or expose the NES to CRM1 binding [62–64]. Additional protein modifications such as sumoylation [65], ubiquitination [38,62], acetylation [62], or the binding of

protein-specific co-factors can induce export by revealing export signals [45,60,66].

3. Nuclear export inhibitory drugs

Table 1 lists various growth and cell cycle suppressor or chemotherapeutic target proteins that are exported from the nucleus into the cytoplasm of cancerous cells and exhibit an anti-neoplastic effect on tumor cells when retained in the nucleus. In addition, each protein in Table 1 is exported from the nucleus by the exportin receptor protein, CRM1, and may be potential targets for anti-CRM1 drug therapy.

3.1. Leptomycin B

An increasing number of drug-like compounds have been isolated that inhibit nuclear export by CRM1. Leptomycin B (Fig. 2), the first specific inhibitor of CRM1 discovered, was isolated from the bacteria *Streptomyces* by investigators looking for new types of antibiotics [67]. Leptomycin B modifies CRM1 by a Michael-type covalent addition at the reactive site cysteine residue (cysteine 528). Alkylation of cysteine 528 inhibits CRM1 binding to the leucine-rich nuclear export sequence of the cargo protein substrate, preventing the formation of the CRM1-cargo-RanGTP export complex and effectively blocking nuclear export [68]. To date, most CRM1 inhibitors function by modifying, either permanently or reversibly, the reactive site cysteine 528 and prevent CRM1 binding to the nuclear export sequence of cargo proteins. Leptomycin B is a potent inhibitor of CRM1 and is effective at nanomolar concentrations [68]. In vitro studies using leptomycin B have shown acute toxicities at concentrations $<$5 nmol/L for 1 h [69]. However, when tested in a phase I clinical trial as an anti-cancer antibiotic compound, leptomycin B (elactocin) was not found to be clinically useful due to severe toxicities, including anorexia and malaise [70]. Currently, leptomycin B-mediated inhibition of nuclear export of a protein and the presence of leucine-rich nuclear export signals are the standards to define whether a protein is exported by CRM1.

3.2. Ratjadone analogs

Additional anti-cancer/antifungal CRM1 inhibitors have been isolated from myxobacterium *Sorangium cellulosum*; these include ratjadones A, B, C, and D (Fig. 2) [71–74]. Ratjadones, which have a similar chemical structure to leptomycin B, employ an identical molecular mechanism to modify CRM1 at the reactive site cysteine 528 and prevent CRM1 binding to the nuclear export signal of the cargo protein [73]. The IC_{50} values for the ratjadone compounds are in the picomolar range. In addition, cells treated with ratjadones manifest a significant increase in the size of their nuclei, further indicating an effective block of nuclear export [72]. Ratjadone compounds have been shown to inhibit cell growth and proliferation in bacteria, in yeast, and in the human cancer cell lines Jurkat, HepG2, HeLa, and U87-MG [74]. Cell-cycle analysis of these cells showed that ratjadone compounds arrest the cells in G1 phase [74]. We have shown that synthetic ratjadone analog C inhibits nuclear export of topo IIα and sensitizes drug-resistant RPMI-8226 and NCI-H929 human multiple myeloma cells to the topo II inhibitors doxorubicin and etoposide when used at single nanomolar concentrations [26]. To demonstrate that sensitization was topo IIα specific, we knocked down topo IIα protein expression by siRNA. Topo IIα knockdown abrogated this effect.

Table 1
Drug targets, tumor suppressors, and cell cycle inhibitors that undergo CRM1-mediated export in various cancers.

Protein	Functional significance	Export receptor	Modification required for nuclear export	Cancer type where protein is exported to the cytoplasm	Reference
Retinoblastoma	Tumor suppressor	CRM1	Phosphorylation by cyclin-dependent kinases	Retinoblastoma	[114]
APC	Tumor suppressor	CRM1	Single mutation causing frame-shift or premature termination	Colorectal cancer	[64,115–117]
p53	Tumor suppressor	CRM1	Ubiquitinylation by MDM2 E3 ubiquitin ligase	Colorectal cancer; breast cancer	[118–122]
BRCA1	Tumor suppressor	CRM1	BARD1 protein masks NES of BRCA1	Breast cancer	[23,123]
$p21^{CIP1}$	Cell cycle inhibitor	CRM1	HER2/neu mutation and phosphorylation by Akt; BCR-ABL translocation and phosphorylation by Akt; phosphorylation by PKC	Ovarian and breast cancer; chronic myeloid leukemia	[124–128]
Topoisomerase I	DNA topology, drug target	CRM1	Unknown	Anaplastic astrocytoma; neuroblastoma	[129,130]
Topoisomerase IIα	DNA topology, drug target	CRM1	Phosphorylation by casein kinase 2	Multiple myeloma	[93,131]
$p27^{KIP1}$	Cell cycle inhibitor	CRM1	Phosphorylation by human kinase-interacting stathmin (hKIS)	Breast cancer; acute myelogenous leukemia	[132–137]
FOXO	Tumor suppressor	CRM1	Phosphorylation by Akt kinase	Breast, prostate, and thyroid cancer; glioblastoma; melanoma	[62,80]
InI1/hSNF5	Tumor suppressor	CRM1	Mutation of conserved hydrophobic residues within the NES	Malignant rhabdoid tumors	[63]
BCR-ABL	Oncogene, tyrosine kinase	CRM1	Unknown	CML	[112]
Galectin-3	Regulator of cell proliferation and apoptosis	CRM1	Phosphorylation, casein kinase I	Thyroid, prostate, breast cancer	[108,138]
Bok	Pro-apoptotic factor	CRM1	Unknown	Breast cancer, HeLa cells	[139]
N-WASP/FAK	Regulator of actin cytoskeleton	CRM1	Phosphorylation by FAK (focal adhesion kinase), promoted by 17β-estradiol stimulation	Neural Wiskott-Aldrich syndrome, breast cancer, ovarian cancer	[140–142]
Nucleophosmin	Tumor suppressor	CRM1	Mutation of 288 and 290 tryptophan residues (AML), NF-kB/RelA masking (breast cancer)	Acute myeloid leukemia, breast cancer	[143–146]
Hsp90	Molecular chaperone	CRM1	Unknown	Breast cancer	[147]
Estradiol receptor	Blocks S-phase entry, cell-cycle	CRM1	PI3-kinase	Breast cancer	[148,149]
Tob	Cell-cycle inhibitor	CRM1	Unknown	Breast cancer	[150,151]
RASSF2	Tumor suppressor	CRM1	MAPK/ERK-2 phosphorylation	Thyroid cancer, nasopharyngeal carcinoma	[152]
Merlin	Tumor suppressor	CRM1	Unknown	Neurofibromatosis	[153]

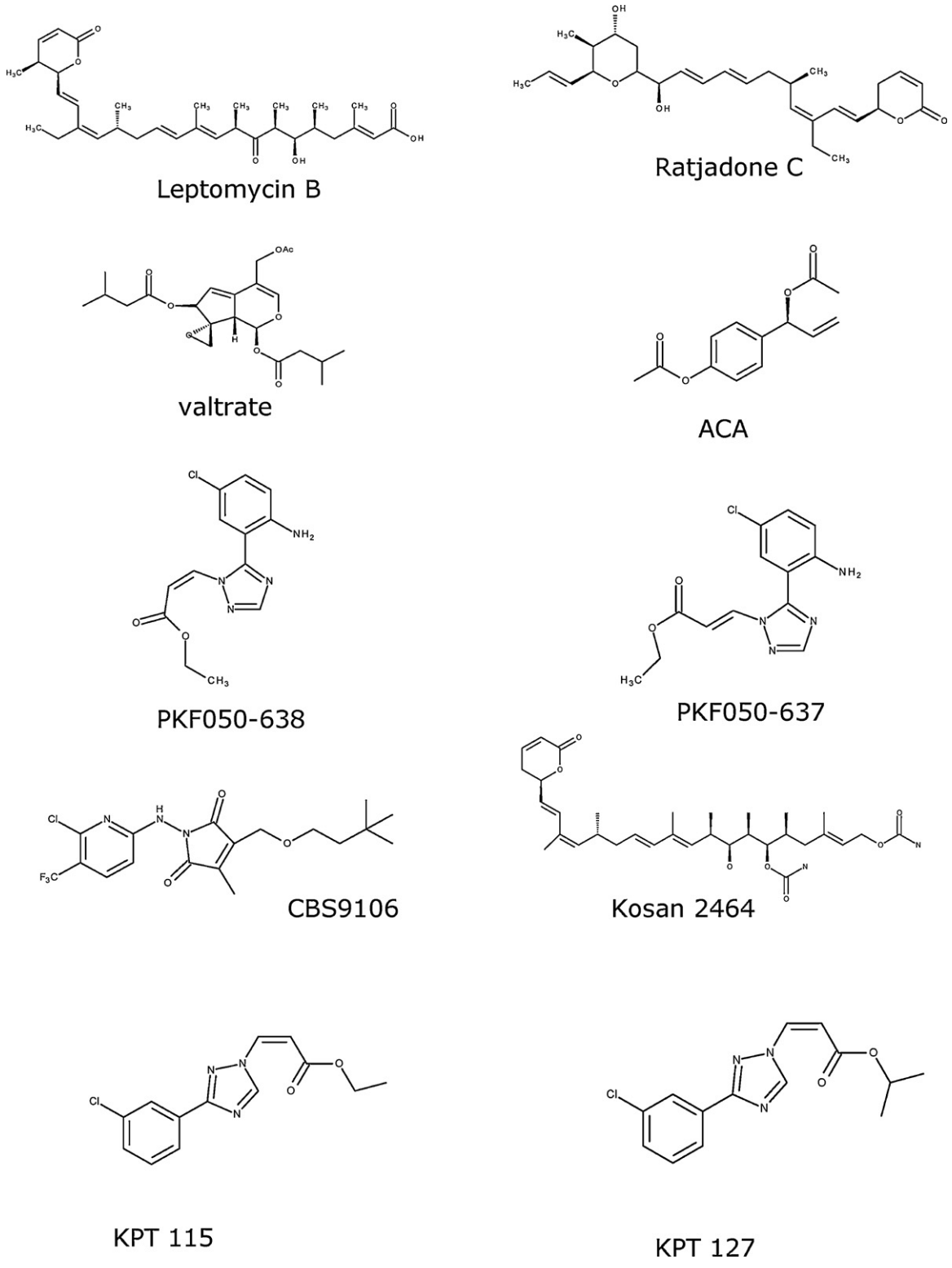

Fig. 2. Chemical structures of CRM1-specific nuclear export inhibitors.

However, we found that ratjadone may also sensitize cells to additional anti-cancer drugs, including the topoisomerase I inhibitor topotecan and the DNA cross-linking agent *cis*-platinum (unpublished data). This indicates that blocking CRM1 may sensitize cancer cells by preventing export of additional tumor suppressors or cell cycle inhibitors (Table 1). To date ratjadones compounds have not been tested in vivo.

3.3. Synthetic leptomycin B derivatives, KOS-2464

A series of semi-synthetic leptomycin B derivatives have been reported that inhibit CRM1 binding at the active site cysteine 528 and induce cell-cycle arrest without the high toxicity associated with leptomycin B [69]. The most effective analog, KOS-2464 (Figure 2) (Kosan Biosciences/Bristol-Myers Squibb), induced rapid

and prolonged block of CRM1-mediated nuclear export and apoptosis at nanomolar concentrations in vitro [69]. It is significant that KOS-2464 induced cell cycle arrest but not apoptosis in normal lung fibroblasts and was reported to have less off-target toxic effects than leptomycin B in vivo while retaining equivalent activity or potency (tumor cell cytotoxicity). In addition, KOS-2464 was tolerated in mice at levels 16-fold higher, and the mice experienced significantly less weight loss than leptomycin B-treated mice. KOS-2464 induced tumor regression and/or tumor growth inhibition in all mouse xenograft models tested, including HCT-116 (colon cancer), SiHa (cervical cancer), NCI-H460 (non-small cell lung cancer), A375 (melanoma), and K563 (CML). This compound also induced p53 expression at 24 and 48 h after in vitro treatment. In our laboratory, we have found that KOS-2464 has very low IC_{50} values (nanomolar) in myeloma (RPMI-8226 and NIH-H929) and leukemia (HL-60 and Kg1a) cell lines. KOS-2464 was also found to sensitize drug-resistant high-density myeloma cells to the topoisomerase inhibitor doxorubicin (unpublished data). In addition, we found that KOS-2464, in combination with anthracyclines, may be effective against myeloma and AML cells isolated from patient bone marrow biopsies.

3.4. N-azolylacrylate analogs, small molecule CRM1 inhibitors

A synthetic small-molecule and CRM1 inhibitor that prevented nuclear export of the HIV-1 Rev protein was developed in a study by Daelemans et al. [75]. This small molecule inhibitor, designated PKF050-638 (Fig. 2), an analog of a class of compounds called *N*-azolylacrylates, is a reversible and highly specific inhibitor of CRM1. Like leptomycin B, PKF050-638 interferes with the reactive site cysteine and prevents binding of the nuclear export signal peptide of the HIV-1 Rev protein to CRM1, preventing the formation of a nuclear export complex. PKF050-638 also exhibited strict molecular structural requirements because a *trans*-enantiomer (PKF050-637, Fig. 2) of the molecule was completely inactive. Jurkat cells transfected with a Rev-GFP expression vector were treated with PKF050-638, and it was determined that CRM1 activity was inhibited in vitro in the micromolar range. Unlike leptomycin B, CRM1 inhibition was found to be reversible in Rev-GFP expressing cells.

3.5. Valtrate and acetoxychavicol acetate, anti-viral CRM1 inhibitors

Additional nuclear export (CRM1) inhibitors are being developed for their anti-viral properties and not specifically for an anti-neoplasm effect. Examples of these include valtrate (Fig. 2) [76] and acetoxychavicol acetate (Fig. 2) [77], which are small-molecule inhibitors of CRM1 that were isolated from the root of *Valeriana fauriei* and *Alpinia galangal,* respectively. Like leptomycin B, these compounds were shown to bind covalently to CRM1 at the reactive site cysteine 528 [78]. In competition binding assays, both compounds have been shown to compete with a biotinylated leptomycin B probe for binding of CRM1; therefore, nuclear inhibition by both valtrate and acetoxychavicol acetate appear to function in a manner similar to leptomycin B. In current studies, valtrate and acetoxychavicol acetate are being developed as viral inhibitors and have not been tested in cancer cells. These small-molecule nuclear export inhibitors prevent export of HIV1 virus and influenza viral RNP without cytotoxicity against the viral host cells [78].

3.6. FOXO family export inhibitors

In a study performed by Kau et al. [79], the investigators sought to develop or screen for nuclear export inhibitors of the FOXO family of transcription factors. FOXO or the Forkhead family of transcription factors includes FOXO1a, FOXO3a, and FOXO4, which when maintained in the nucleus are involved in negative regulation of cell cycle progression and cell survival [80]. The investigators set up a cell-based, chemical genetic screening regimen to identify inhibitors of FOXO nuclear export. The readout of the screening assay was subcellular localization of FOXO1a after drug treatment [79]. The compounds screened (>18,000) were obtained from the NCI Structural Diversity Set, the ChemBridge DiverSetE, and additional NCI marine extracts. Forty-two compounds were identified by this screen to inhibit nuclear export of FOXO1a [79]. FOXO1a nuclear export is mediated by a well-characterized phosphorylation event; therefore, the inhibitory compounds that were identified could be either specific kinase inhibitors or general CRM1 inhibitory molecules. To distinguish between these regarding a possible inhibitory mechanism, the compounds were tested for their ability to inhibit nuclear export of an HIV Rev/GFP fusion protein. From the original library of small molecules, 42 compounds were found to inhibit FOXO1a nuclear export. Of 42 positive compounds, 19 were identified as general CRM1 nuclear export inhibitors. Of the 19 small molecule inhibitors identified, 11 compounds were found to covalently modify CRM1 at cysteine 528 by a Michael-type reaction, similar to leptomycin B, ratjadone, and KOS-2464. Others molecules modified cysteine 528 by nucleophilic attack, by substitution by a good leaving group, or by an unknown chemical rearrangement.

3.7. CBS9106

CBS9106 (Fig. 2) is a small-molecule reversible inhibitor of the nuclear export receptor CRM1 [81]. This CRM1 inhibitor induced cell-cycle arrest and apoptosis as a single agent in 60 different human cancer cell lines, including bladder, breast, colon, CNS, endocrine, lung, kidney, pancreatic, prostate, and skin cancer. Cell-cycle arrest and apoptosis were induced at sub-micromolar concentrations. This effect was reversible by the removal of the drug. In pull-down assays, it was demonstrated that biotinylated-CBS9106 binds directly to the CRM1 protein and is competitively inhibited by leptomycin B for the reactive site cysteine 528. In addition, CBS9106 may interact specifically with thiol groups because it is suppressed by the presence of N-acetylcysteine. CBS9106 did not bind to CRM1 that was mutated at cysteine 528 (replaced by serine); therefore, cysteine 528 binding is essential for inhibitor function. In mouse xenograft models, CBS9106 suppressed tumor growth and increased survival. In vivo administration of CBS9106 reduced CRM1 protein levels in xenograft tissues, whereas no significant mortality or weight loss was observed in mouse studies [81].

3.8. Small-molecule, CRM1-selective inhibitors of nuclear export (SINE)

Commercial/research collaborations are ongoing to develop novel small-molecule, CRM1-selective inhibitors of nuclear export (SINE) [82,83]. These CRM1-specific inhibitors are similar to *N*-azolylacrylates structures developed by Daelemans et al. [75]. Karyopharm Therapeutics has developed two proof-of-concept compounds, KPT-115 and KPT-127, which have been show to inhibit CRM1-mediated nuclear export of HIV-Rev-GFP, FOXO, p53, and topoisomerase IIα [82,83]. These compounds are water-soluble, irreversible inhibitors of CRM1 and bind to the reactive site cysteine 528 residue. The *trans*-enantiomers of these compounds are ineffective as CRM1 inhibitors; therefore, SINE inhibitors are similar to *N*-azolylacrylates in that they are structurally highly specific and exhibit strict molecular structural requirements. SINE compounds have been shown to be effective against 20 genotypically different colorectal cancer cell lines with median IC_{50} values

of approximately 300 nM (MTT assay) [82]. In vivo toxicity was assayed for KPT-127 by 5-day subcutaneous administration of 100 mg/kg in mice. This dose was well-tolerated with some modest lymphopenia and weight loss. In our laboratory, KPT-115 and KPT-127 had IC_{50} values against log-growth phase multiple myeloma cells in the submicromolar range. Hematological cell lines assayed include human myeloma (RPMI-8226, U266, and NIH-H929) and leukemia (HL-60 and Kg-1a). Both KPT-115 and KPT-127 synergize with the anthracycline doxorubicin when tested in the drug-resistant plateau-growth phase human multiple myeloma cell line NIH-H929. Synergy was in the micromolar range and determined by an anti-proliferative/cell viability assay (CellTiter-Blue, Promega) (unpublished data). More effective, new-generation molecules with improved pharmacokinetics, oral bioavailability and lower toxicities are currently being tested in cancer cell lines, human myeloma and leukemia patient cell isolates, and in mouse xenograft models in dogs with de novo tumors.

4. CRM1 expression in human cancer

In the following examples of human cancers listed below, CRM1 protein expression is a negative prognostic indicator. CRM1 overexpression is also correlated with increased metastasis, histological grade, increased tumor size, and decreased progression-free and overall survival.

4.1. Ovarian cancer

Over-expression of CRM1 has been correlated with poor prognosis in cancer. Expression of CRM1 was analyzed in a cohort of 88 ovarian tumor biopsies and 12 ovarian tumor cell lines [84]. Aggressive, late-stage human ovarian tumor biopsies were found to have high levels of CRM1 protein expression [84]. Immunohistochemical staining of cytoplasmic CRM1 protein was correlated with advanced tumor stage and cell proliferation (antibody from Santa Cruz Biotechnology; polyclonal rabbit anti CRM1 [sc-5595]). Increased CRM1 expression was found to be a negative prognostic indicator in ovarian cancer [84].

4.2. Pancreatic cancer

Pancreatic tissue from 69 cancer patients and 10 normal subjects were assayed for CRM1 protein. Cancerous tissue had significantly increased levels of CRM1 protein when compared to normal tissues by Western blot (monoclonal anti CRM1 antibody from Santa Cruz) [85]. High CRM1 levels correlated with increased tumor size ($P < 0.01$), lymphadenopathy ($P < 0.004$), and liver metastasis ($P < 0.003$). When CRM1 protein levels were high, there was a decrease in overall survival ($P < 0.001$) and progression-free survival ($P < 0.006$) [85], making CRM1 expression a negative prognostic indicator in pancreatic cancer.

4.3. Osteosarcoma

Large subgroups of osteosarcoma patients lack prognostic indicators or markers that could help identify treatment choices. CRM1 overexpression has been reported as a prognostic indicator in other cancers; therefore, in a study performed by Yao et al. [86], CRM1 expression levels were assayed by Western blot analysis in 57 osteosarcoma samples (monoclonal anti-CRM1 antibody from Santa Cruz). In a univariate analysis, the expression levels of CRM1 protein were found to be high in samples with histological grades of G1 and G2 ($P = 0.003$). In addition, osteosarcomas with high CRM1 expression correlated with increased tumor size ($P = 0.014$), again shown by univariate analysis [86]. Progression-free survival and overall survival were also correlated to elevated CRM1 protein expression ($P < 0.008$; Kaplan-Meier survival analysis) [86].

4.4. Glioma

The expression levels of both CRM1 and the cell cycle inhibitor/tumor suppressor p27 were assayed in 70 cases of human gliomas and normal brain tissues. Assays performed were Western blot analysis and immunohistochemical staining for both CRM1 and p27. High expression levels of CRM1 were found in 80% of the cancer cell nuclei assayed and was directly related to the grade of the malignancy. In addition, high expression levels of CRM1 protein correlated strongly to poor overall survival of glioma patients ($P < 0.001$) [87]. Patient survival analysis was performed via the Kaplan-Meier method. For cell cycle inhibitor p27 to be exported from the nucleus by CRM1, it must first be phosphorylated at serine 10. High levels of (cytoplasmic) phosphorylated serine 10-p27 were found in glioma samples that also expressed increased levels of CRM1 protein, whereas nuclear (unphosphorylated) p27 levels were low compared with that found in normal tissue. Therefore, high levels of CRM1 and increased cytoplasmic p27 were correlated to glioma grade and poor patient survival.

4.5. Cervical cancer

In a study by van der Watt et al. [88], both nuclear export transport proteins CRM1 and karyopherin-β1 were measured in both cervical tissue and cervical tumor cell lines. Immunofluorescent microscopy, RT-PCR, and microarray analysis showed that both nuclear export proteins were significantly over-expressed in cervical tumor cell lines compared with normal cervical tissues (antibodies from Santa Cruz: polyclonal rabbit anti-CRM1 [sc-5595], rabbit anti-karyopherin-β1 [sc-11367], and goat anti-karyopherin-α2 [sc-6917]) [88]. When CRM1 and karyopherin-β1 levels were decreased by specific siRNA in cell lines, the cancer cells had reduced proliferation and apoptosis was induced, whereas normal tissues were unaffected. In addition, overall expression of the tumor suppressor proteins p53, p27, p21, and p18, were appreciably increased in CRM1-specific siRNA-transfected cervical cell lines compared with normal cervical tissues [88].

These data would indicate that knockdown of CRM1 or decreased CRM1 protein may result in an increase in tumor suppressor proteins and subsequent apoptosis in cancerous tissues.

5. CRM1 and drug resistance

Intracellular location of proteins is essential for their proper functioning in eukaryotic cells. Mislocalization of nuclear proteins can result in various diseases, such as schizophrenia (D3 dopamine receptor) [89], diabetes insipidus (G-protein receptors) [90], and cystic fibrosis (CFTR chloride channel) [91], and is involved in various cancers [92,93]. Specific examples of proteins that must be in the nucleus of cells to produce their anti-cancer effects are listed in Table 1. In this section, we will address specific examples of CRM1 exported nuclear proteins in cancer cells and how they may contribute to drug resistance.

5.1. Topoisomerase II alpha

Topoisomerase II alpha (topo IIα) is a nuclear protein that is essential for cell division, especially in rapidly proliferating cells typical of cancer. Topo IIα is necessary for DNA replication, transcription, chromatid separation, and chromatin condensation

in eukaryotic cells [94]. Topo IIα functions as a homodimer that disentangles DNA and relieves torsional stress produced by DNA replication processes. Topo IIα binds to DNA, producing transient double-strand breaks and then undergoes a conformational change that allows the passage of a double-stranded DNA through the break, making topological isomers of the DNA [94]. After strand passage through the double-strand break, the DNA is religated and a molecule of ATP is hydrolyzed. Topo II chemotherapeutic agents such as doxorubicin and etoposide arrest the topo IIα during DNA cleavage and produce cleavable complexes, resulting in double-stranded DNA breaks and cell death [95]. For DNA damage to occur, topo IIα must be in the nucleus and in contact with the DNA. We have shown that multiple myeloma cells, when grown at high-density plateau conditions, are intrinsically resistant to the topo IIα poisons doxorubicin and etoposide [46,96,97]. Similar high-density conditions are present in the bone marrow of multiple myeloma patients. In previous publications, our group has shown that myeloma cells under high density conditions, both in vivo and in vitro, export topo IIα into the cytoplasm [97]. In addition, we have shown that topo IIα possesses two leucine-rich nuclear export signals [46], that nuclear export of topo IIα contributes to drug-resistance [46,96], and that myeloma cells export topo IIα from the nucleus by a CRM1-mediated mechanism. When topo IIα is trafficked into the cytoplasm, it is not in contact with the cellular DNA; therefore, topo II inhibitors cannot form DNA double-stranded breaks. We have found that drug-resistant high-density myeloma cells can be sensitized to topo IIα inhibitors using either the CRM1 inhibitor ratjadone C or by knockdown of CRM1 protein expression using a CRM1-specific siRNA. Depletion of CRM1 by siRNA or ratjadone C induced a synergistic sensitivity of cells to doxorubicin and etoposide. CRM1 inhibition also sensitized CD-138 isolated plasma cells from multiple myeloma patient bone marrow aspirates, when treated with ratjadone C and doxorubicin. Normal peripheral blood mononuclear cells were not sensitized to topo II inhibitors by CRM1 inhibition. In addition, band depletion assays and comet assays indicated that DNA cleavable complex formation increased along with DNA-double strand breaks [26]. To demonstrate that CRM1-mediated nuclear export of topo IIα may induce drug resistance, we decreased topo IIα expression by siRNA. We found that topo IIα knockdown abrogated the CRM1-topo IIα inhibitor synergistic anti-myeloma effect. These data indicate that CRM1 inhibition may enhance current chemotherapeutic regimens in the treatment of multiple myeloma.

5.2. Galectin-3

Galectin-3 (Gal-3), a 31-kDa member of the β-galactoside-carbohydrate binding protein family, is a multifunctional protein expressed by various types of human cells (Table 1). Gal-3 has been implicated in many aspects of cancer progression such as tumor cell adhesion, proliferation, differentiation, and metastasis [98–101]. Nuclear Gal-3 has been shown to interact directly with the spliceosome complex protein Gemin4 and is essential for mRNA splicing [102]. However, Gal-3 expression and subcellular location are frequently found to be altered in cancer and pre-cancerous cells in vivo [103,104].

Several studies have shown that the subcellular location of Gal-3 appears to be tightly regulated by specific selective mechanisms depending on cell type, growth conditions, or neoplastic transformation [105,106]. Although the size of this protein is small enough to passively diffuse through the NPC (cutoff size of 40–60 kDa), the studies above indicate that specific conditions regulate its transport through the NPC.

Gal-3 has been shown to protect cells against chemotherapy-induced apoptosis and has been implicated in regulating a universal apoptosis commitment step [107]. Nuclear Gal-3 is a regulator of cell proliferation and can induce apoptosis in breast cancer cells. In a study by Takenaka et al. [108], it was demonstrated that Gal-3 is translocated from the nucleus to the cytoplasm when cancer cells were exposed to the anticancer drug cisplatinum. Trafficking of Gal-3 to the cytoplasm prevented induction of apoptosis in these cells. Anti-apoptotic activity was found to be promoted when Gal-3 is phosphorylated at serine-6 by casein kinase I. Immunohistochemical staining demonstrated that phosphorylated Gal-6 is cytoplasmic and that unphosphorylated Gal-3 remains in the nucleus. Phosphorylation of proteins has been reported to either mask nuclear localization signals or expose nuclear export signals in CRM1 transported proteins. In the Takenaka et al. study, breast cancer tumor cells were exposed to cisplatinum and the nuclear export inhibitor leptomycin B. Treatment by both cisplatinum and leptomycin caused Gal-3 to remain in the cell nucleus and induced apoptosis in these cells [108]. Thus, inhibition of nuclear export of an apoptotic-inducing factor (Gal-3) and treatment by a chemotherapeutic agent reversed drug resistance in breast cancer cells.

5.3. BCR-ABL

The primary mutation that arises in the development of chronic myeloid leukemia (CML) is the formation of the Philadelphia chromosome in hematopoietic progenitor cells. This chromosomal translocation produces a chimeric peptide formed from the first exons of the gene BCR with the sequences of ABL kinase gene. This translocation forms an oncogene that codes for a constitutively active tyrosine kinase, BCR-ABL [109]. BCR-ABL protein is exported to the cytoplasm of the cell where it performs its proliferation and anti-apoptotic activities. BCR-ABL tyrosine kinase activity inhibits apoptosis by phosphorylation and activation of PI3-kinase and Akt. BCR-ABL kinase activates signal transduction pathways that abrogate IL-3 growth factor dependence and that are involved in CML cell proliferation [110]. Activation of proliferative and anti-apoptotic pathways induces cellular expansion of leukemic myeloid progenitor cells.

The development of the BCR-ABL kinase inhibitor imatinib mesylate (IM) (Gleevec, Novartis) radically changed the treatment of CML. Imatinib is currently the first-line treatment for CML patients who are in both chronic and blast crisis stages of this disease. However, CML patients may eventually develop drug resistance to this treatment. Resistance to IM therapy occurs due to selective pressure by the IM therapy and the development of IM-resistant BCR-ABL clones. These resistant clones can produce a BCR-ABL kinase that has a much lower affinity to IM or have lost their ability to bind to BCR-ABL. However, some drug-resistant clones develop the ability to overexpress the BCR-ABL protein molecule so that the amount of IM that penetrates cells cannot sufficiently inactivate all copies of the BCR-ABL protein.

The ABL protein contains three putative nuclear localization signals and a single nuclear export signal. In normal cells, the ABL kinase functions in the cell nucleus and is activated by DNA damage to induce the functioning of the tumor suppressor protein p73.

Interestingly, it has been shown that, when BCR-ABL protein is maintained in the nucleus using the export inhibitor leptomycin B, it retains the apoptotic functions of the wild-type ABL protein [111,112]. Therefore, nuclear localization of the oncogenic kinase BCR-ABL can induce apoptosis in leukemic cells. In addition, inactivation of BCR-ABL by IM partially restores nuclear localization of the protein. This additive effect of IM and CRM1 inhibition by leptomycin traps BCR-ABL in the cell nucleus and subsequently will induce apoptosis. An ex vivo study of 35 CML patients was performed where IM and leptomycin B were added to myeloid progenitor cells [112]. The effect of IM and leptomycin B was

examined for cytostatic effects via a colony-forming assay. The combination of IM and leptomycin B induced preferential killing of BCR-ABL-expressing cells, whereas normal progenitor's survived treatment. These proof-of-principle experiments indicate that drug resistance can be addressed by nuclear sequestration of oncogenic proteins.

6. Conclusions

In this review, we discussed in detail the mechanisms of nuclear export, including structure and function of the nuclear pore complex, the specific transport receptor molecules that move large molecules out of the nucleus into the cytoplasm, and the proteins that are targeted for export in cancer. In addition, we provide a summary of potential drug-like inhibitors of nuclear export and how the use of nuclear export inhibitors may contribute to cancer treatment and drug resistance.

Intracellular nuclear export of either tumor suppressive proteins or drug targets can result in drug resistance due to overexpression of CRM1 [23–26]. In this review, we have cited examples of CRM1 overexpression in various malignancies, including breast cancer, cervical cancer, glioma, osteosarcoma, ovarian, and pancreatic cancers. Overexpression of CRM1 protein in tumor cells is an important negative prognostic indicator. CRM1 overexpression is also strongly correlated to increased metastasis, histological grade, increased tumor size, and decreased progression-free survival and overall survival.

The current total of proteins that are known to be exported by the ubiquitous transport molecule CRM1 is a subject of ongoing investigation. Proteins that are exported by CRM1 in cancer include tumor-suppressive proteins such as retinoblastoma, APC, p53, BRAC1, FOXO proteins, INI1/hSNF5, galectin-3, Bok, nucleophosmin, RASSF2, Merlin, $p21^{CIP}$, $p27^{KIP1}$, N-WASP/FAK, estradiol receptor and Tob, drug targets topoisomerase I/IIα and BCR-ABL, and the molecular chaperone protein Hsp90. We contend that the export of drug targets, tumor suppressors, and cell cycle inhibitors from the nucleus may be significant factors in cancer disease progression and drug resistance. Blocking CRM1 nuclear export activity by siRNA or CRM1 inhibitors may restore apoptotic pathways and tumor cell sensitivity to chemotherapeutic drugs such as doxorubicin [26], etoposide [26], cisplatinum [108], and imatinib mesylate [112].

The prototypical inhibitor of CRM1, leptomycin B, effectively and specifically blocks CRM1-mediated export of nuclear proteins by covalent modification of its reactive site cysteine 528. However, this compound has many "off target" toxic effects that makes it unsuitable for use in vivo or in clinical trials [69,70]. New CRM1 inhibitors with fewer off-target side effects are currently being developed. These include the leptomycin derivatives, ratjadone [26] and KOS-2464 [69], and specific small molecule inhibitors of CRM1, N-azolylacrylate analogs [75], FOXO export inhibitors [79], valtrate [78], acetoxychavicol acetate [78], CBS9106 [81], and SINE analogs [82,83]. As effective and less toxic CRM1 export inhibitors become available, they may be used as both single agents or in combination with current chemotherapeutic.

In recent preliminary studies in our lab, we found that less toxic small molecule inhibitors of CRM1 (KOS-2462 and SINE inhibitors) decreased cell viability and proliferation and increased apoptosis in both myeloma and leukemia cancer cell lines (unpublished data). These CRM1 nuclear export inhibitors prevented export of both p53 and topoisomerase IIα in fractionation experiments and when assayed by immunofluorescent microscopy. When assayed in high-density drug-resistant myeloma cell lines, KOS-2464 and SINE analogs were found to synergize with standard chemotherapeutics such as doxorubicin, etoposide, lenalidomide, dexamethasone, bortezomib, and melphalan, This synergy was exhibited in myeloma and leukemia cancer cell lines but not in normal fibroblasts or peripheral blood mononuclear cells. Apoptosis or the ability of the CRM1 inhibitors to induce programmed cell death was also assayed in combination with doxorubicin, where it was found that the CRM1 inhibitors tested induced apoptosis and sensitized myeloma cells to doxorubcin. We very recently have started to test these new compounds in human myeloma and leukemia cells isolated from patient bone marrow samples.

We believe that future development of low-toxicity small-molecule CRM1 inhibitors may provide a new approach to treating cancer and reversing drug resistance.

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