

REVIEW

Promising SINEs for Embargoing Nuclear–Cytoplasmic Export as an Anticancer Strategy

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ABSTRACT

In cancer cells, the nuclear–cytoplasmic transport machinery is frequently disrupted, resulting in mislocalization and loss of function for many key regulatory proteins. In this review, the mechanisms by which tumor cells co-opt the nuclear transport machinery to facilitate carcinogenesis, cell survival, drug resistance, and tumor progression will be elucidated, with a particular focus on the role of the nuclear–cytoplasmic export protein. The recent development of a new generation of selective inhibitors of nuclear export (XPO1 antagonists) and how these novel anticancer drugs may bring us closer to the implementation of this therapeutic strategy in the clinic will be discussed.

Significance: The nuclear transport mechanism is dysregulated in many malignancies and is associated with dysfunction of many regulatory proteins. Targeting this mechanism as an anticancer strategy has been compelling, and novel agents that selectively inhibit the nuclear export pathway have demonstrated preliminary evidence of clinical efficacy with an acceptable safety profile. *Cancer Discov*; 4(5): 527–37. ©2014 AACR.

INTRODUCTION

Normal cellular function is dependent upon the appropriate localization of proteins within specific intracellular compartments (1). There is growing evidence to suggest that the nuclear–cytoplasmic transport machinery is frequently disrupted by cancer cells, and that the subsequent mislocalization of key regulatory proteins facilitates carcinogenesis, cell survival, drug resistance, and tumor progression (2, 3). In this review, we will discuss the mechanisms of nuclear transport dysfunction in tumor cells, with a particular focus on the nuclear export apparatus as a therapeutic target, and how the recent emergence of a new generation of small-molecule selective inhibitors of nuclear export (SINE), specifically exportin 1 (XPO1) inhibitors, may provide a means to implement this therapeutic strategy in cancer treatment.

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NUCLEAR–CYTOPLASMIC TRANSPORT MECHANISMS

The Nuclear Pore Complex Is a Gateway between the Nucleus and Cytoplasm

In the eukaryotic cell, critical cellular processes, such as DNA synthesis, RNA transcription and translation, and protein processing, are carried out within distinct intracellular compartments (1). Small molecules (<40 kD) can passively diffuse across different compartments, but larger molecules, including most proteins and RNAs, require transport by signal-dependent and energy-dependent mechanisms (2). The site of active transport between the nucleus and the cytoplasm occurs at the nuclear pore complex (NPC), which is composed of basic building blocks called nucleoporin. NPC is a large macromolecular assembly that is embedded in the nuclear envelope and mediates the exchange of >40-kD molecules that pass between the nuclear and cytoplasmic compartments (3). Nuclear transport processes are highly regulated, and there is growing evidence to suggest that the ability to selectively modulate the geographic location of macromolecules within the cell is a key means to modulate cellular function (1, 3).

Nuclear–Cytoplasmic Transport Receptors and Signals

The majority of nuclear–cytoplasmic transport receptors belong to the karyopherin β family of proteins and act as carrier molecules that facilitate transit into (importins) and out

of (exportins) the nucleus via the NPC (4). Each karyopherin transport receptor recognizes a unique group of cargo proteins or RNA 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, which directs them in and out of the nucleus, respectively (4). Three classes of nuclear-cytoplasmic transport signals have hitherto been identified: (i) the classical basic amino acid NLS sequences recognized by a heterodimer composed of importins α and β (5); (ii) a more complex NLS possessing N-terminal hydrophobic/basic motif and C-terminal RX2-5PY motifs found in karyopherin β cargo proteins, which directly binds to their specific carriers (6); and (iii) a hydrophobic leucine-rich NES recognized by the ubiquitous transport receptor chromosome maintenance protein 1 (CRM1, also known as XPO1; refs. 7, 8). Karyopherin β proteins also bind weakly to phenylalanine-glycine (FG) repeats in nucleoporins, thus targeting karyopherin β -cargo complexes to the NPC for translocation. At present, there are at least 19 karyopherin proteins identified in humans (9).

Current evidence suggests that nuclear-cytoplasmic export is mainly regulated by XPO1 in humans (3). XPO1 mediates the nuclear export of a small subset of RNAs, as well as over 200 eukaryotic proteins that possess a canonical hydrophobic leucine-rich NES (9). In addition, XPO1 also seems to regulate the nuclear export of some proteins [e.g., forkhead O transcription factor 3a (FOXO3a) and histone deacetylases] that form complexes with adapter molecules containing an NES, such as 14-3-3 proteins, a family of basic proteins with diverse physiologic functions that are also implicated in the nuclear cytoplasmic shuttling of proteins (3, 10, 11).

Nuclear Export Dynamics

The direction of transport across the NPC is determined by the concentration gradient of the small GTPase Ran. Levels of RanGTP in the nucleus are approximately 100-fold greater than those in the cytoplasm due to the location of the RanGTP exchange factor (RanGEF or RCC1) within the nucleus. Conversely, in the cytoplasm, the majority of Ran is in the GDP-bound form due to the cytoplasmic localization of (i) its GTPase-activating protein (RanGAP), (ii) the RanGTP-binding protein RanBP1, and (iii) homologous RanBP1 domains in cytoplasmic nucleoporin NUP358 (also known as RanBP2; refs. 3, 12). Once XPO1 is bound to its cargo, it forms a complex with RanGTP and exits the nucleus via the NPC (3). Upon leaving the nucleus, RanGTP undergoes hydrolysis by RanGAP and is converted to RanGDP, promoting dissociation of the XPO1 complex, and the cargo is subsequently released into the cytoplasm. Hence, interactions between transport receptors and the Ran GTPase control bidirectional transport, as translocation is dependent on the different nucleotide-bound states of Ran in the nucleus versus the cytoplasm (13, 14). A summary of the nuclear export pathway is depicted in Fig. 1.

XPO1-MEDIATED EXPORT AND CANCER

Dysregulation of growth-regulatory proteins (GRP) and functional inactivation of tumor-suppressor proteins are hallmarks of oncogenesis (15). Given that many tumor-suppressor proteins and GRPs perform their antineoplastic functions within the nucleus, any mechanism that could potentially

enhance their nuclear export and cytoplasmic sequestration would effectively result in their functional inactivation, leading to a protumorigenic cellular state. Known examples of tumor-suppressor proteins and GRP sequestration within the cytoplasm of cancer cells include BRCA1 and FOXO proteins in breast cancer; RUNX3 in gastric cancer; p53 in breast, ovarian, and colorectal cancers, retinoblastoma, and neuroblastoma; adenomatous polyposis coli (APC) or β -catenin in colon cancer; and BCR-ABL in chronic myelogenous leukemia (CML; refs. 2, 3, 16–21). The frequent observation of molecular localization signal alterations and the cytoplasmic mislocalization of these regulatory factors are consistent with functional linkage of nuclear export to cancer.

Overexpression and Alteration of XPO1-Mediated Export in Cancer

Increased expression of XPO1 has been observed and correlated with poor prognosis or resistance to chemotherapy in a number of solid (ovarian, pancreatic, brain, and cervical cancers, osteosarcoma, and melanoma) and hematologic malignancies [acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), multiple myeloma, and lymphoma; refs. 22–27]. Importantly, this increased expression of XPO1 seems to have functional significance in cancer cells. A study by van der Watt and colleagues (26) demonstrated that XPO1 is overexpressed in cervical cancer tissue and cell lines. In these tissue/cell lines, the inhibition of XPO1 significantly reduced cell proliferation and induced apoptosis, while sparing the noncancer cells. The exact mechanisms driving overexpression of XPO1 are unknown, but recent evidence suggests that the nuclear factor Y (NFY) and specificity protein 1 (Sp1) transcription factors may be responsible for XPO1 overexpression in cancer, and that XPO1 transcription is inhibited when p53 interferes with NFY function following DNA damage (28). Given that many tumor-suppressor proteins and GRPs are exported by XPO1 in various cancers (Table 1), upregulation of XPO1-mediated export via XPO1 overexpression may be a common mechanism of enhancing nuclear export in cancer (2, 29).

Cancerous inhibitor of PP2A (CIP2A) is an oncoprotein that inhibits protein serine/threonine phosphatase 2A (PP2A), a protein distributed within the nucleus and cytoplasm that controls the phosphorylation of numerous proteins involved in cell signaling and regulates cell growth and apoptosis, and has recently been identified as a substrate of the XPO1 export pathway (30). CIP2A also binds directly to c-Myc and inhibits PP2A activity toward the c-Myc serine (Ser) 62 residue, thus preventing proteolytic degradation of c-Myc (31). CIP2A has also been shown to transform human cells when overexpressed and to promote anchorage-independent cell growth and *in vivo* tumor formation (31). CIP2A overexpression has been observed in bladder, tongue, hepatocellular, and colon cancers, non-small cell lung carcinoma, head and neck squamous cell carcinoma, and CML (31, 32). Cytoplasmic overexpression of CIP2A has also been associated with higher-grade, advanced-stage, and poorer outcomes in serous ovarian cancer (33).

In addition to nuclear transport, XPO1 is also involved in the regulation of mitotic checkpoints, spindle assembly, and postmitotic nuclear envelope reassembly (34). XPO1 has been shown to localize to kinetochores, where it forms a complex that is essential for RanGTP-dependent recruitment

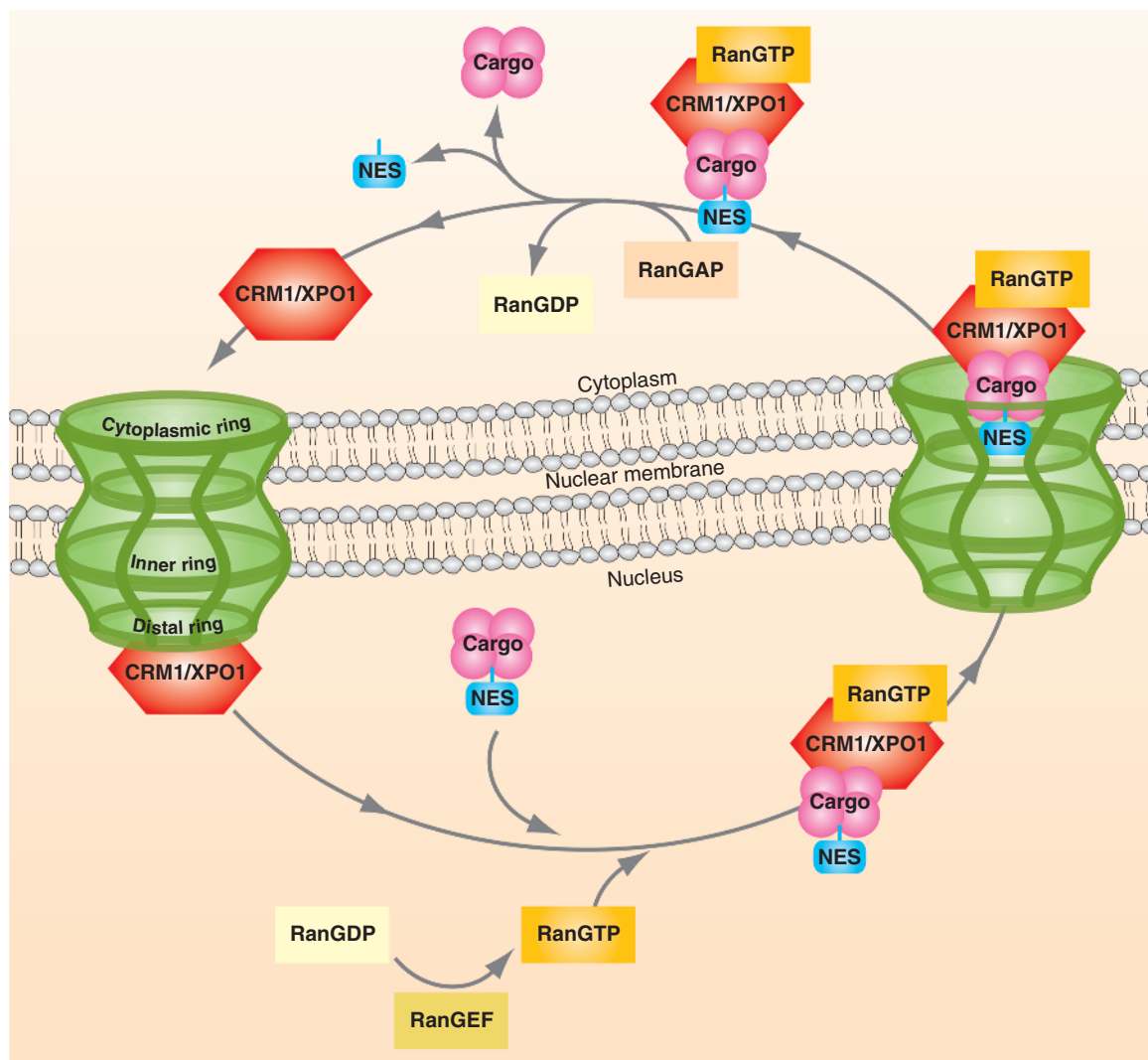


Figure 1. Situated in the nuclear membrane, the NPC (green) is composed of multiple rings held together in a basket configuration that measures 200 nm in length. The complex is open-ended at the nucleus and the cytoplasm and traffics proteins between them. Cargo proteins containing an NES bind to CRM1 and RanGTP in the nucleus, whereupon they are shuttled to the cytoplasm through the NPC (right). Hydrolysis of RanGTP to RanGDP by RanGAP in the cytoplasm dissociates the complex and XPO1 (CRM1) is free to be transported back through an NPC into the nucleus where the process resumes.

of RanGAP and RanBP2 to kinetochores (34). Furthermore, inhibition of XPO1 by the XPO1 inhibitor Leptomycin-B has been shown to disrupt mitotic progression and chromosome segregation at anaphase (34). Hence, XPO1 plays a critical role in mitotic spindle assembly and function and may, therefore, be upregulated to facilitate a dual role of enhancing cell proliferation while exporting tumor-suppressor proteins that have growth-inhibitory and apoptotic functions in tumors. The p53 tumor-suppressor protein regulates multiple signaling pathways to induce cell-cycle arrest for DNA damage repair to maintain genomic integrity and, if necessary, to induce apoptosis (35). It is estimated that about 50% of malignant tumors harbor mutations or deletions in the *TP53* gene (36). In addition, p53 function is also crucially dependent on its subcellular nuclear localization, which is primarily regulated by the XPO1-mediated nuclear export pathway (37, 38). Both p53 and XPO1 seem to be involved in a reciprocal

regulatory loop where, as described earlier, nuclear p53 can repress XPO1 transcription by interfering with NFY function (28), while increased activity of XPO1 can cause p53 mislocalization and dysfunction in cancers (39). Cytoplasmic sequestration of wild-type p53 has been reported in a variety of tumors, including breast, colorectal, and hepatocellular carcinomas (40).

Finally, mutations in a highly conserved region of *XPO1* have been observed in CLL, although the functional significance of this remains unknown (41).

Activation of Oncogenic Pathways and XPO1-Mediated Nuclear Export

Cytoplasmic mislocalization of tumor-suppressor proteins and GRPs can also be induced by activation of oncogenic pathways within cells. The PI3K-AKT oncogenic signaling pathway is activated in many human epithelial cancers,

Table 1. Tumor-suppressor proteins, cell-cycle inhibitors, transcription factors, and drug targets that undergo XPO1-mediated nuclear export

Protein	Role	Brief description of function	Tumors in which XPO-mediated nuclear export may be functionally relevant
p53	Tumor suppressor	Guardian of the genome: induces cell-cycle arrest, activates DNA repair proteins to maintain genomic integrity and, if necessary, initiates apoptosis following DNA damage.	Colorectal, breast, and ovarian cancer, retinoblastomas, and neuroblastoma
APC	Tumor suppressor	Inhibits WNT signaling by targeting β -catenin for proteasome-mediated degradation in the cytoplasm.	Colorectal cancer
Activating transcription factor 2 (ATF2)	Transcription factors	Member of the leucine zipper family of transcription factors that regulates transcription of genes involved in antiapoptosis, cell growth, and DNA damage response.	Melanoma, prostate cancer
BCR-ABL	Tyrosine kinase	Constitutively active BCR-ABL tyrosine kinase. Caused by reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11), resulting in the formation of the <i>BCR-ABL</i> fusion gene.	CML
Bcl2-related ovarian killer protein (BOK)	Proapoptotic protein	Exerts proapoptotic function through the mitochondrial pathway.	Breast and cervical cancer cell lines
BRCA1	Tumor suppressor	Regulates the DNA damage response and repair functions in the nucleus and at centrosomes to inhibit centrosome duplication after DNA damage.	Breast cancer
CIP2A	Oncogenic protein	Inhibits protein PP2A and stabilizes the oncogenic transcription factor c-Myc.	Head and neck and colon cancer
CDK inhibitor 1 (p21, CIP1)	Cell-cycle inhibitor	Regulates cell-cycle progression from G ₁ -phase by inhibiting the activity of cyclin-CDK2 or cyclin-CDK1 complexes.	Ovarian and breast cancer and CML
CDK inhibitor 1B (p27, KIP1)	Cell-cycle inhibitor	Inhibits cyclin E/CDK2, thus blocking G ₁ -S phase transition.	Ovarian, esophageal, thyroid, colon, and breast carcinomas
ERK	Oncogenic protein	Cytoplasmic localization of activated ERK1/2 is required for its antiapoptotic activity.	Melanoma
ER- α	Prevents S-phase entry	Nuclear export of ER- α into the cytoplasm is required for estradiol-dependent activation of DNA synthesis and entry into S-phase of the cell cycle.	Breast cancer
FOXO transcription factors (FOXO1, FOXO3a, FOXO4 and FOXO6)	Tumor suppressor	Negative regulators of cell proliferation, cell survival, and cell-cycle progression.	Breast, prostate, and thyroid cancer, glioblastoma, and melanoma
Galectin-3	Regulator of proliferation/apoptosis	Multifunctional protein that regulates cell proliferation and can induce apoptosis in cancer cells.	Thyroid, prostate, and breast cancer
HSP90	Molecular chaperone	HSP90 chaperone machinery is required to prevent mutated and overexpressed oncoproteins from misfolding and degradation in cancer cells.	Breast cancer
Inl1/hSNF5	Tumor suppressor	Component of the chromatin remodeling SWI/SNF complex whose tumor-suppressor functions include inducing G ₁ arrest and controlling the mitotic spindle checkpoint. Deleted in malignant rhabdoid tumors.	Malignant rhabdoid tumors

(continued)

Table 1. Tumor-suppressor proteins, cell-cycle inhibitors, transcription factors, and drug targets that undergo XPO1-mediated nuclear export (Continued)

Protein	Role	Brief description of function	Tumors in which XPO-mediated nuclear export may be functionally relevant
Moesin-ezrin-radixin-like protein (Merlin/NF2)	Tumor suppressor	Neurofibromatosis type 2 (NF2) gene encodes the tumor-suppressor protein Merlin, which is an ERM (ezrin, radixin, and moesin) protein family member that regulates proliferation, apoptosis, survival, motility, adhesion, and invasion.	Inherited schwannomas, meningiomas, and ependymomas
Nucleophosmin (NPM)	Tumor suppressor	Centrosome-associated protein with aberrant cytoplasmic expression observed in several hematologic malignancies.	AML and breast cancer
Neuronal Wiskott-Aldrich syndrome protein (N-WASP)/focal adhesion kinase (FAK)	Regulators of actin cytoskeleton	Phosphorylation of FAK leads to phosphorylation of N-WASP, which promotes cell motility and invasion.	Neural Wiskott-Aldrich syndrome, breast, esophageal, and ovarian cancer
Ras association domain-containing protein 2 (RASSF2)	Tumor suppressor	Binds KRAS with the characteristics of an effector and promotes apoptosis and cell-cycle arrest.	Thyroid cancer, nasopharyngeal carcinoma
Retinoblastoma (RB1)	Tumor suppressor	Inhibits G ₁ -S phase transition of the cell cycle in response to DNA damage.	Retinoblastoma
RUNX3	Tumor suppressor	Associated with transforming growth factor- β signaling and functions as a tumor suppressor in gastric carcinogenesis. Suppresses the development of colon cancer by forming a complex with β -catenin-TCF4 and attenuating WNT-mediated signaling activity.	Breast, colon, and gastric cancer
Survivin	Inhibitor of apoptosis	Member of inhibitor-of-apoptosis protein family (IAP) that exerts its cytoprotective effects when located in the cytoplasm by inhibiting apoptotic pathways.	Breast cancer, oral, and oropharyngeal squamous cell carcinoma
Transducer of ErbB-2 (TOB)	Tumor suppressor	Cell-cycle inhibitor that induces arrest at G ₁ -S phase, upregulates cyclin-dependent kinase inhibitor p27, and downregulates the antiapoptotic proteins Bcl-2 and Bcl-XL	Breast cancer
Topoisomerase I	Relaxation of supercoiled DNA	Enzyme that catalyzes the relaxation of supercoiled DNA during replication, transcription, recombination, and chromosome condensation. Cellular target of camptothecin.	Anaplastic astrocytoma and neuroblastomas
Topoisomerase II α	Relaxation of supercoiled DNA	Enzyme that catalyzes the relaxation of supercoiled DNA during replication, transcription, recombination, and chromosome condensation. Cellular target of doxorubicin and etoposide.	Multiple myeloma
Vitamin D ₃ upregulated protein 1 (VDUP1)	Tumor suppressor	Mediates nuclear export of the hypoxia-inducible factor-1 α (HIF-1 α), a transcriptional activator of angiogenesis, anaerobic metabolism, and survival pathways, leading to proteasomal degradation of HIF-1 α in the cytoplasm.	Cervical and lung cancer cell lines
WEE1	Tumor suppressor	Inhibitor of CDK1 and key molecule in maintaining G ₂ cell-cycle checkpoint arrest for premitotic DNA repair.	Currently unknown—but reduced expression of WEE1 has been observed in non-small cell lung, colon, and prostate cancer

resulting in the upregulation of several downstream target genes that inhibit apoptosis and promote cell proliferation (42). Several downstream PI3K–AKT signaling substrates that negatively regulate cell proliferation, cell survival, and cell-cycle progression have been shown to be functionally inactivated because of cytoplasmic sequestration following PI3K–AKT pathway activation, including the FOXO transcription factors and the cell-cycle inhibitor p27 (11, 43, 44). The lipid phosphatase PTEN negatively regulates the PI3K–AKT pathway, and mutations in the *PTEN* gene have frequently been found in a variety of cancers, including glioblastoma multiforme, melanoma, renal cell carcinoma, and prostate, ovarian, and endometrial cancers (45, 46). In *PTEN*-null cancer cells, constitutive activation of AKT leads to phosphorylation of FOXO transcription factors at multiple sites, thus preventing FOXO DNA binding and transcriptional activity (47). Hyperphosphorylation of the FOXO proteins also facilitates binding with 14-3-3, which then promotes XPO1-mediated nuclear export of FOXO (11). Colon cancer and renal cancer cell lines that do not express PTEN show constitutive mislocalization and loss of function for FOXO proteins, which in turn result in loss of regulation for metabolism for many cell types (47).

The cell-cycle inhibitor p27 can also be sequestered in the cytoplasm or exported from the nucleus by XPO1 via AKT phosphorylation (48). Under normal conditions, p27 is localized in the nucleus, where it acts as a cell-cycle inhibitor by binding to and inhibiting the activity of cyclin-dependent kinase 2 (CDK2), a serine/threonine protein kinase that promotes G₁-S phase cell-cycle progression (3). Phosphorylation of the p27 NLS by AKT has been shown to impair nuclear import with subsequent cytoplasmic sequestration of p27, leaving unbound CDK2 within the nucleus free to facilitate cell-cycle progression and tumorigenesis (48, 49). In addition, RAS activation may lead to XPO1-mediated nuclear export of p27 via phosphorylation of p27 at Ser10 by the PI3K–AKT and RAF–MEK–ERK pathways (50, 51). Cytoplasmic localization of p27 has been observed in esophageal, thyroid, colon, and breast cancers (3). Wang and colleagues (52) recently demonstrated that increased XPO1 and phosphorylated Ser10 p27 (pSer10p27) expression levels were associated with advanced-stage and high-grade epithelial ovarian cancer and poorer overall survival. Knockdown of XPO1 and pSer10p27 expression levels also leads to cell-cycle arrest and inhibition of cell proliferation in SKOV3 cells *in vitro* and *in vivo* (52).

The homologous recombination DNA repair proteins BRCA1 and RAD51 have also been shown to be functionally inactivated because of cytoplasmic sequestration following PI3K–AKT pathway activation (20). Cytoplasmic localization of BRCA1 has been observed in 60% of primary invasive ductal breast cancers, with a high correlation between cytoplasmic BRCA1 localization and AKT1 activity (based on increased levels of phosphorylated AKT1) in the tumors (20). The exact mechanism of AKT1-induced cytoplasmic sequestration of BRCA1 is currently unknown, but nuclear export of BRCA1 via the XPO1 pathway has previously been described following DNA damage caused by ionizing radiation (53), and XPO1 has also been implicated in the regulation of BRCA1 centrosomal localization, thus suggesting that AKT-induced nuclear export of BRCA1 may be XPO1 mediated (54). The

relationship between AKT1 activity and homologous recombination-related tumor-suppressor protein mislocalization and function has recently been further highlighted by data showing that BRCA1 deficiency in cells may itself lead to activation of AKT1 and impaired CHK1 nuclear localization (55). Impaired CHK1 nuclear import disrupts the interaction between CHK1 and RAD51 in the nucleus, resulting in homologous recombination repair defects (55).

Recent data suggest that the nuclear export of the estrogen receptor α (ER α) into the cytoplasm is required for estrogen-dependent activation of DNA synthesis and S-phase entry, resulting in increased proliferative activity of breast cancer cells (56, 57). Following estrogen stimulation, PI3K–AKT phosphorylates FOXO1, also known as forkhead in rhabdomyosarcoma (FKHR; ref. 57). In addition, estrogen also activates the nonreceptor protooncogene SRC, resulting in tyrosine phosphorylation of ER α , which in turn leads to assembly of an ER α –FKHR complex within the nucleus and its subsequent nuclear export via XPO1. The cytoplasmic localization of the ER α –FKHR leads to downregulation of FKHR transcriptional activity and upregulation of cyclin D1 transcription, resulting in DNA synthesis and S-phase transition (56).

Other examples of intracellular mislocalization of tumor-suppressor proteins and GRPs mediated by activation of oncogenic pathways include the functional inactivation of the RUNX3 tumor-suppressor protein by phosphorylation by SRC kinase activity and subsequent cytoplasmic mislocalization via XPO1-mediated nuclear export in gastric and breast cancers, and aberrant activation of the WNT pathway in more than 90% of colorectal cancers (17, 19). The p53 tumor-suppressor protein is also mislocalized. Hence, the development and progression of cancer mediated by upregulation of oncogenic signaling pathways (e.g., PI3K–AKT, SRC, and WNT) and inactivation of tumor-suppressor proteins may be partly underpinned by nuclear–cytoplasmic mislocalization of key regulatory proteins.

XPO1-MEDIATED NUCLEAR EXPORT AND DRUG RESISTANCE IN CANCER

There is also accumulating evidence to suggest that XPO1 may mediate drug resistance in a variety of tumor types by facilitating the nuclear export of various drug targets. These include topoisomerase I α (topo1 α) and topo2 α , galectin-3, and BCR–ABL (2).

The nuclear protein topo2 α functions as a homodimer that disentangles DNA and relieves the torsional stress in supercoiled DNA caused by the process of DNA replication (58). Chemotherapeutic agents, such as doxorubicin and etoposide, inhibit topo2 α during DNA replication and produce cleavable complexes, resulting in double-stranded DNA breaks and cell death (59). It has been demonstrated that for DNA damage to occur in this manner, topo2 α must be localized within the nucleus and in physical contact with DNA (21). In myeloma cells, nuclear export of topo2 α to the cytoplasm via XPO1 has been associated with resistance to topo2 α inhibitors, and reversal of the topo2 α inhibitor-resistant phenotype can be induced by the XPO1 inhibitors ratjadone C or Leptomycin-B or by siRNA-mediated

knockdown of XPO1 protein expression (21). Depletion of XPO1 by siRNA or ratjadone C has been shown to result in synergistic sensitivity of myeloma cells, obtained from bone marrow aspirates of patients with multiple myeloma and from human multiple myeloma cell lines, to doxorubicin and etoposide (21).

Nuclear galectin-3 is a multifunctional protein that regulates cell proliferation and can induce apoptosis in cancer cells. The subcellular location of galectin-3 seems to be tightly regulated by specific selective mechanisms via the NPC depending on cell type, growth conditions, or neoplastic transformation (60). Nuclear-to-cytoplasmic translocation of galectin-3 has been observed in prostate and breast cancer cells following treatment with cytotoxic agents, with subsequent inhibition of apoptosis in these cells (61, 62). Treatment of the breast cancer cells by both cisplatin and the nuclear export inhibitor Leptomycin-B has been shown to induce nuclear retention of galectin-3 and apoptosis, thus reversing cisplatin resistance (61).

The development of the BCR-ABL kinase inhibitor imatinib mesylate (Gleevec; Novartis) has revolutionized the treatment of CML and has become its standard first-line treatment (2, 16). Development of drug resistance is, however, a common event when the disease progresses to its accelerated or blast phase despite imatinib treatment. The main mechanisms underlying the development of imatinib resistance are point mutations in the kinase domain, BCR-ABL amplification and clonal evolution [i.e., the development of additional nonrandom chromosomal abnormalities besides the balanced translocation of t(9:22)(q34;q11.2) that results in the Philadelphia chromosome; ref. 16]. The ABL protein contains three putative NLSs and a single NES. In normal cells, the ABL kinase functions in the cell nucleus and is activated by DNA damage to induce the activation of the p73 tumor-suppressor protein (63). In fact, the BCR-ABL protein has been found to retain the apoptotic functions of the wild-type ABL protein, and induces apoptosis in leukemic cells when maintained in the nucleus using the export inhibitor Leptomycin-B (64). Furthermore, inactivation of BCR-ABL by imatinib partially restores nuclear localization of the protein (2). A strategy of combined treatment with imatinib and XPO1 inhibition by Leptomycin-B to effectively trap BCR-ABL in the cell nucleus has been shown to induce cell death in imatinib-resistant cells that displayed BCR-ABL amplification or signs of clonal evolution (16).

NUCLEAR EXPORT INHIBITORS AS ANTICANCER THERAPEUTICS

In view of the aforementioned evidence supporting the role of nuclear transport dysregulation in maintaining the neoplastic phenotype and drug resistance, targeting the nuclear transport mechanism as a therapeutic strategy has gained interest within the oncology community in recent years. The main focus of this strategy has been the targeted inhibition of XPO1, with the hypothesis that inhibition of nuclear export will result in forced nuclear retention and thereby activation of several key tumor-suppressor proteins and GRPs, for example, p53 pathway proteins, with consequent induction of cell-cycle arrest and apoptosis in tumor cells while sparing normal cells.

Naturally Derived XPO1 Inhibitors

Inhibition of XPO1 as a therapeutic strategy has been studied for almost two decades. The bacterial toxin Leptomycin-B was the first selective, irreversible XPO1 inhibitor that was discovered and subsequently tested in a clinical trial (65). Leptomycin-B has potent antitumor activity *in vitro*, but is also toxic to normal cells (66). Despite marked toxicities in animals, Leptomycin-B was administered intravenously to patients with refractory solid tumors in a phase I study by Newlands and colleagues (65). Toxicities included profound gastrointestinal side effects, including nausea, vomiting, watery diarrhea along with marked anorexia, fatigue/asthenia, and malaise, whereas typical side effects of cytotoxic agents such as neutropenia, mucositis, and alopecia were not observed. Transient tumor marker responses in patients with ovarian carcinoma and trophoblastic tumor and stable disease in a patient with refractory sarcoma were reported, but the associated severe toxicities led to its development being abandoned (65).

Leptomycin-B exerts its inhibitory effect on XPO1 by irreversibly alkylating the reactive site cysteine residue (Cys528; ref. 29). Recently, it was demonstrated that upon binding to XPO1, Leptomycin-B undergoes hydrolysis of the lactone ring, which in turn enables persistent binding of the drug to XPO1 (67). Alkylation of Cys528 prevents XPO1 from binding to the leucine-rich nuclear export sequence of the cargo protein substrate, hence inhibiting the formation of the XPO1-cargo-RanGTP export complex and effectively blocking nuclear export (3, 29). There are now data suggesting that the severe toxicities observed following treatment with Leptomycin-B involve additional off-target effects caused by binding to cysteine proteases as well as the irreversible blockade of all XPO1 functions *per se* (68). Therefore, a selective and reversible inhibitor might possess potent anticancer activity with an improved tolerability profile. Most of the currently available XPO1 inhibitors function by permanently modifying the reactive site Cys528 and preventing XPO1 binding to the NES of cargo proteins (2). Since the discovery of Leptomycin-B, other naturally derived inhibitors have been identified, including the ratjadone analogs (A, B, C, and D) isolated from *Sorangium cellulosum*, which have a similar chemical structure to Leptomycin-B and use an identical molecular mechanism to modify XPO1, but these compounds have not been tested *in vivo* (2).

Synthetic XPO1 Inhibitors

Kosan Biosciences Inc., before its acquisition by Bristol-Myers Squibb, created derivatives of Leptomycin-B, including KOS-2464, with better pharmacologic properties that showed a much improved therapeutic index across multiple tumor xenografts (69). Significantly better tolerability and less weight loss than with Leptomycin-B were observed in animals, although transient anorexia remained (69). More recently, a small-molecule reversible inhibitor of XPO1, CBS9106, which induced cell-cycle arrest and apoptosis as a single agent in 60 different human cancer cell lines, including bladder, breast, colon, and lung cancer, at submicromolar concentrations has been described (70). Unlike the Leptomycin-B analogs, CBS9106 binds to the Cys528 residue by forming a reversible

covalent bond with it and was found to inhibit tumor growth in xenograft models without significant weight loss or mortality (70). Both of these compounds have not progressed into the clinic but are at least indicative that XPO1 can be inhibited with an adequate therapeutic window.

Perhaps the compounds that herald the most promise for the clinical utility of XPO1 inhibition are the new small-molecule inhibitors of XPO1, also known as selective inhibitors of nuclear export (SINE). These compounds share a similar structure with the N-azolyacrylate analog PKF050-638, a highly specific and reversible small-molecule XPO1 inhibitor that was originally developed to prevent nuclear export of the HIV-1 Rev protein, an essential regulator of the HIV-1 mRNA expression (71). Karyopharm Therapeutics has developed a series of SINE compounds [KPT-115, KPT-127, KPT-185, KPT-251, KPT-276, KPT-330 (selinexor), and KPT-335 (verdinexor)] that have been shown to inhibit XPO1-mediated export of p53, p73, RB1, FOXO proteins, APC, I κ B, NPM, topo2 α , and survivin. Preclinical studies of these compounds result in dose-dependent cytotoxicity for a number of hematologic and solid tumors [i.e., CLL, AML, CML, multiple myeloma, non-Hodgkin lymphoma (NHL), melanoma, and renal, breast, pancreatic, prostate, and colorectal cancer cells; refs. 2, 21, 68, 72–78]. These highly specific inhibitors of XPO1 are water soluble, and they bind to the reactive site Cys528 residue by forming a slowly reversible covalent bond (79). SINEs have been shown to be selectively cytotoxic for neoplastic cells with a half maximal effective concentration (EC₅₀) of 10 to 1,000 nmol/L versus >5 to 20 μ mol/L in nonneoplastic cell lines, and their cytotoxic effects seem to be independent of known genetic alterations such as BRAF^{V600E} mutations in melanoma or TP53, PI3K, AKT, and BRCA1/2 mutation status in breast cancer cell lines (24, 75). Synergistic activity using KPT-185 in combination with SN38 and oxaliplatin in colorectal cancer cell lines and KPT-251 with chemotherapy in prostate cancer cell lines has also been observed (72, 73). In murine and monkey pharmacokinetic studies, KPT-185 showed limited bioavailability and systemic exposure, whereas KPT-276 and selinexor showed >50% bioavailability reaching C_{max} >5 μ mol/L following 10 mg/kg oral administration of drug (24).

Preclinical *in vitro* and *in vivo* data have shown that both hematologic and solid tumor cells are susceptible to single-agent cytotoxicity caused by selinexor, consistent with its restoration of multiple tumor-suppressor and growth-regulatory pathways, leading to the death of cancer cells (24, 75, 79). Interestingly, SINE treatment also results in reduction in expression of multiple oncoproteins, including cMyc in multiple myeloma, cKIT and FLT3 in AML, and cyclin D1 in mantle cell lymphoma, as well as proteasome-mediated degradation of the XPO1 protein itself. The major adverse event across all tested species to date (mice, rats, dogs, and monkeys) is reversible weight reduction accompanied by reduced food intake without significant vomiting or diarrhea (79). The improved toxicity profile of these newer XPO1 inhibitors relative to previous XPO1 inhibitors, such as Leptomycin-B, is thought to be related to their exquisite specificity for XPO1 with no detectable binding to other proteins, including the cysteine proteases, believed to be the cause of poor tolerance to Leptomycin-B (68).

The SINE XPO1 inhibitors have also been investigated in a comparative oncology setting, using dogs with newly

diagnosed and chemotherapy-refractory cancers (80). In one study, the SINE compound verdinexor was administered in 17 dogs with NHL, mast cell tumor, or osteosarcoma. Common toxicities included anorexia, vomiting, and diarrhea. Dose-limiting toxicities were anorexia and vomiting, which occurred at 2 mg/kg twice weekly. Among 16 evaluable dogs, 2 achieved partial responses (both with NHL) and 12 others achieved stable disease, indicating activity of XPO1 inhibitors in large-animal models (80). Verdinexor is currently in phase II studies in dogs with newly diagnosed or recurring lymphoma.

Oral selinexor is currently being developed and evaluated for tolerability and single-agent antitumor activity in phase I clinical trials of patients with solid tumors (trial number NCT01607892; <http://clinicaltrials.gov>, last accessed on February 14, 2014), and the data from these studies have hitherto been presented only in meeting abstracts. In these studies, selinexor is administered orally at either 8 doses or 10 doses in each 28-day cycle. The preliminary results of the phase I solid-tumor study with the 10 doses per cycle were presented recently. At the time of presentation, 2 of 3 patients had experienced dose-limiting toxicities at 40 mg/m², with grade 3 anorexia with dehydration and fatigue in 1 patient, and grade 3 fatigue in another. Commonly observed nonhematologic toxicities included fatigue, nausea, and anorexia that were mostly low grade (grade 1–2), whereas the most common hematologic toxicity was thrombocytopenia. Toxicities were all reversible upon stopping the drug. Among the 39 patients treated, at least 7 patients had disease stabilization of more than 6 months, including patients who were treated at low dose levels. Selinexor has a dose-proportional pharmacokinetic profile and a half-life of 5 to 7 hours, and paired biopsies, done in 12 patients, showed increased nuclear retention of a number of tumor-suppressor proteins, including FOXO1, I κ B, and p53, following treatment (81). The alternative dosing regimen in which the drug is administered twice weekly (i.e., 8 doses every 28 days) is currently being investigated in patients with advanced solid tumors, and the maximum-tolerated dose (MTD) has not been reached to date.

In the parallel phase I study in advanced, progressive hematologic malignancies (NCT01607892), patients with relapsed/refractory NHL, CLL, multiple myeloma, or AML were dosed with oral selinexor (8–10 doses every 28 days; refs. 82–84). The adverse-event profile was similar to that observed in the solid-tumor study, and MTD on the eight doses per cycle schedule has not been reached to date. Durable responses and disease stabilization with single-agent oral selinexor across all disease subtypes have been observed, with some patients remaining on study for over 1 year. In CML, recently published data have demonstrated that treatment with selinexor results in apoptosis and impaired the clonogenic potential of leukemic, but not normal, CD34(+) progenitors (85). As a proof of mechanism, following exposure to selinexor, nuclear accumulation of CIP2A, SET (an oncoprotein that inhibits PP2A activity), I κ B α (an inhibitor of the NF- κ B transcription factor), FOXO3a, p53, and p21, with subsequent reactivation of PP2A within the cytoplasm and reduction in BCR-ABL levels, was noted (85). Antileukemic activity in a CML blast crisis mouse model was observed following treatment with selinexor, and treatment of a single

patient in accelerated-phase CML with Selinexor also led to a reduction in bone pain, splenomegaly, and immature myeloid blasts in the peripheral blood (85).

CONCLUSIONS

There is preclinical evidence to suggest that inhibition of XPO1-mediated nuclear export leading to nuclear retention and functional reactivation of tumor-suppressor proteins and GRPs may be a viable therapeutic anticancer strategy. Until recently, the development of this pharmacologic class of compounds has been hampered by the narrow therapeutic window of XPO1 inhibition (2, 65). New-generation XPO1 inhibitors with improved preclinical efficacy, pharmacokinetics, oral bioavailability, and reduced toxicities in animal models have generated renewed interest in this approach and are now being tested in early-phase clinical trials (79).

Notably, despite the encouraging early signals, and apart from the obvious lack of published clinical toxicity and efficacy data, there are crucial questions that remain unresolved in the context of nuclear export inhibition as a therapeutic strategy. These include the challenges of identifying potential predictive biomarkers of efficacy to optimize patient selection for these drugs, and the possible cumulative toxicities of long-term nuclear export inhibition. Despite these reservations, the emerging data from early-phase clinical studies seem encouraging. In addition, given the noted synergistic effects on cytotoxicity in neoplastic cells, the potential for combining XPO1 inhibition with conventional chemotherapy, such as topoisomerase inhibitors, is open for exploration in the future (21, 73, 86). Moreover, combination strategies with other targeted therapeutics may be another option for consideration, and recent preclinical data showing that combined XPO1 and BRAF inhibition leads to synergistic tumor regression in *BRAF*-mutant melanoma suggest that this may be a viable therapeutic approach as well (87).

Ultimately, it may be overly optimistic to expect that the inhibition of nuclear-cytoplasmic transport will be an effective target in all cancer types, or that the therapeutic efficacy of this approach will not eventually be hampered by intrinsic and acquired resistance mechanisms and/or toxicities engendered by sustained physiologic inhibition of such transport in normal cells. In the context of drug resistance, it is worth noting that the Cys528 residue, which seems to be the main target of XPO1 inhibitors, is not part of an active site of XPO1 and also seems to be nonessential for XPO1/CRM1 function. Mutant forms of XPO1/CRM1 that carry a Cys528-Ser mutation can substitute for wild-type CRM1 in mammalian cells, and *Saccharomyces cerevisiae* express a form of CRM1 that contains a threonine at this position, instead of a cysteine. Human cells that express CRM1 with the Cys528-Ser mutation have been shown to be resistant to the effects of Leptomycin-B (88).

Nonetheless, the strategy of imposing a molecular embargo on nuclear transport as a backbone for new avenues of cancer therapy may be on the verge of becoming a clinical reality.

Disclosure of Potential Conflicts of Interest

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Promising SINEs for Embargoing Nuclear–Cytoplasmic Export as an Anticancer Strategy

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