Review

Structural Basis of Targeting the Exportin CRM1 in Cancer

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Abstract: Recent studies have demonstrated the interference of nucleocytoplasmic trafficking with the establishment and maintenance of various cancers. Nucleocytoplasmic transport is highly regulated and coordinated, involving different nuclear transport factors or receptors, importins and exportins, that mediate cargo transport from the cytoplasm into the nucleus or the other way round, respectively. The exportin CRM1 (Chromosome region maintenance 1) exports a plethora of different protein cargoes and ribonucleoprotein complexes. Structural and biochemical analyses have enabled the deduction of individual steps of the CRM1 transport cycle. In addition, CRM1 turned out to be a valid target for anticancer drugs as it exports numerous proto-oncoproteins and tumor suppressors. Clearly, detailed understanding of the flexibility, regulatory features and cooperative binding properties of CRM1 for Ran and cargo is a prerequisite for the design of highly effective drugs. The first compound found to inhibit CRM1-dependent nuclear export was the natural drug Leptomycin B (LMB), which blocks export by competitively interacting with a highly conserved cleft on CRM1 required for nuclear export signal recognition. Clinical studies revealed serious side effects of LMB, leading to a search for alternative natural and synthetic drugs and hence a multitude of novel therapeutics. The present review examines recent progress in understanding the binding mode of natural and synthetic compounds and their inhibitory effects.

Keywords: XPO1; exportin1; nuclear transport; NES; cancer; CRM1; drugs; inhibitor
1. Introduction

In the early days of cancer treatment, shotgun approaches using drugs interfering with DNA replication in a more general way were used, with the consequence of massive unwanted side effects. About 30 years ago, identification of the individual proteins involved in specific cancers and an understanding of their biochemistry incited hype about having found the cure for cancer. In subsequent years, inhibitors identified to block these proteins allowed specific treatment of cancers, but the problem of resistances arose concurrently. At present, a multitude of proteins interfering with cell regulation have been described, but the specific amount of proteins required/involved to trigger cell cancerogenesis and the specific functions including the complex interplay of these proteins is still poorly understood. Recent understanding of interactions in the intermingled cellular pathways revived the discussion about “the” cure for cancer or the need for a more personalized and cancer-specific treatment targeting the individual deregulating mechanisms in each patient.

Recent developments in cancer therapy reveal that the effects of specific drugs may be increased by interfering with additional macromolecular machineries in the cell [1]. Due to the differences in cell metabolism between normal and cancer cells, such machineries are more stringently required by the latter. One example is the transport machinery, which is required for exchange of proteins and RNAs between the nuclear and the cytoplasmic compartment in all eukaryotic cells. This review focuses on CRM1-dependent export deregulation and effects of drugs on CRM1 function. For further reading as introduction to the complexity of cancer development, regulation and treatment, we refer to excellent reviews and perspective articles [1–5].

2. Observation: Alteration of Distribution of Proteins Related to Cancer

In cancerogenic cells, tumor suppressor proteins and oncoproteins are often aberrantly mislocalized. Mislocalization may be either due to any kind of deregulation of the protein biosynthesis pathway, malfunctioning of the protein itself or aberrations in the transport processes that are required to shuffle proteins from the cytoplasmic compartment into the nuclear compartment and vice versa. The malfunctioning of the latter process may result in a deregulation either by inactivation or by over-activation of the critical proteins for cell cycle regulation or growth and division. Such pathway-specific deregulation may cause an overall deregulation of the cell, ultimately leading to the establishment of cancer.

A large variety of proteins involved in human cancers, like APC (adenomatous polyposis coli protein), NFAT (nuclear factor of activated T-cells), β-catenin or Survivin, Rb (retinoblastoma protein), p53 and Bcr-Abl mislocalize in different cancer cells (Figure 1) and are reviewed in: [6–8]. The proteins mentioned are also representative examples of the different possibilities of distributional changes that could occur within a cell.

The tumor suppressor protein/transcription factor p53, named according to its apparent molecular weight, is localized in the nucleus in normal cells. It is often inactivated in cancer cells due to mutations leading to a “loss of function” (e.g., in its DNA-binding capabilities) or p53 is delocalized into the cytoplasm due to NES unmasking and active export [9,10] reviewed in [11,12]. Similarly, the tumor
suppressor retinoblastoma protein (Rb) is localized in the nucleus in normal cells, but has been shown to be delocalized to the cytoplasm in specific cancers [13–16].

**Figure 1.** Spatial relocalization of (proto-) oncoproteins in cancer cells compared to normal cells. In cancer cells, proteins mislocalized into the nucleus are depicted in blue; in the cytoplasm, they are colored green; and those shifting from nuclear and cytoplasmic distribution to either cytoplasmic or nuclear are depicted in grey and white, respectively. The arrows indicate the direction of the shift in cancer cells. The function of the proteins depicted here is described in the main text.

Another group of proteins populates both compartments in normal cells, but is shifted towards or excluded from one compartment in cancer cells. For example, the tumor suppressor APC regulates many cellular functions and in complex with two other proteins (Glycogen Synthase Kinase (GSK)-3β and Axin) promotes the degradation of β-catenin in the cytosol. In non-transformed cells, APC is found in the nucleus and the cytoplasm, albeit with a more pronounced accumulation in the cytoplasm, as it bears both import and export signals [17,18]. The mutated forms of APC, present in more than 60% of all colon cancer patients [19] are most commonly C-terminally truncated versions. Such truncations are incapable of binding to Axin and to accumulate in the nucleus, as the mediating residues are located in the C-terminal region. Thus, regulation of the proto-oncogene β-catenin (cadherin-associated protein), a key mediator of the canonical Wnt signaling pathway, is lost. Instead of phosphorylation by GSK-3β and proteasome-dependent cytoplasmic degradation, it exhibits an increased import in many cancer cells and accumulates in the nucleus. β-catenin’s structural properties resembling those of nuclear transport receptors strengthen recent evidence suggesting a transport receptor-independent nuclear accumulation. Interestingly, β-catenin functions as a moonlighting (dual function) protein involved in regulation of cell-cell adhesion as well as gene transcription. Its binding to transcription factors causes gene transactivation and leads to tumor formation; reviewed in: [20,21].
The transcription factor family NFAT (nuclear factor of activated T-cells) is also found in both cellular compartments in normal cells. The import of NFAT is dependent on calcineurin, a serine/threonine phosphatase. Upon dephosphorylation in NFATs serine-rich region (SRR), a nuclear localization signal (NLS) is exposed and import can occur. Export of NFAT is stimulated by PKA and, interestingly, by the nuclear fraction of GSK-3β. In human solid tumors and hematological malignancies, isoforms of NFAT are constitutively activated and/or overexpressed, leading to increased nuclear accumulation and activation of the downstream targets reviewed in: [22–26].

An example of the accumulation of a protein distributed between both compartments in normal cells, but enriched in the cytoplasm in cancer cells, is the proto-oncogene Bcr-Abl, a ~200 kDa protein resulting from a fusion of parts of the ABL1 (Abelson Murine Leukemia Viral Oncogene Homolog 1) and BCR (breakpoint cluster region) genes. The transcript of the Bcr-Abl gene fusion is functional as an abnormal kinase and stimulates proliferation of myeloid cells into chronic myelogenous leukemia cells [27]; reviewed in: [28,29]

The underlying mechanism for nucleocytoplasmic exchange of all of these proteins mentioned above requires soluble transport receptors that specifically recognize their cargoes by signals and transport them to the opposing compartment. The most versatile export factor CRM1 is required for the export of a plethora of proteins e.g., Rb [13] or the proto-oncogene p53 [9]. Their localization shift towards the cytoplasmic compartment is often an important prerequisite to stabilize the deregulation of the tumor cell and enable uncontrolled/permanent cell proliferation.

3. The Nucleocytoplasmic Transport Machinery

The interchange of metabolites between the nuclear and cytoplasmic compartment occurs by passive diffusion. In contrast, an active, receptor-mediated transport is required for proteins to enter the nucleus in order to regulate and transcribe DNA or for transport of RNA (protein complexes) into the cytoplasm. Furthermore, certain proteins that have to perform their function only at specific time points during the cell cycle are therefore imported or exported in a highly regulated manner.

The site of transfer is the nuclear pore complex (NPC), a large supramolecular complex composed of more than 30 different proteins, the nucleoporins. They assemble into the structural framework of the NPC and form the meshwork gating the central aqueous channel of the NPC [30–32]. While this meshwork is no hindrance for small proteins and metabolites, large molecules require specific receptors for transition [30–32]. The receptors are classified in importins and exportins depending on their direction of transport with the nucleus as reference point. Many of them share structural properties to the first receptor identified, Importin-β (Impβ). Proteins of the Impβ superfamily of transport receptors are all composed of a common structural motif, the HEAT repeats named after the first proteins identified bearing this motif, namely Huntingtin, Elongation factor 3 (EF3), Protein Phosphatase 2A and the yeast P3 kinase Tor (Target of rapamycin). A single HEAT repeat covers 40–50 residues and is composed of two antiparallel α-helices, which are connected by a short linker loop [33,34]. The slight angular shift between the two helices and the overall stacking of the HEATs results in a superhelical protein conformation with a high intrinsic flexibility.

Directionality of transport depends on the small GTPase Ran that comes in two types, either in a GTP or GDP bound state, which are asymmetrically distributed in the nucleus and the cytoplasm [35]. In the
nucleus, the Ran Guanine nucleotide Exchange Factor (RanGEF) RCC1 (Regulator of Chromosome Condensation 1) is bound to chromatin [36–38] and ensures a high nuclear concentration of RanGTP [39,40]. In the cytoplasm, the Ran-GTPase Activating Protein (RanGAP) and stimulatory factors Ran Binding Proteins 1 and 2 (RanBPs 1/2) are located, thereby resulting in high concentrations of RanGDP compared to RanGTP [41–43]. Interestingly, the eukaryotic translation initiation factor eIF4E, a potent oncogene, is not only involved in translation of bulk mRNA but additionally plays a role in the CRM1-mediated export of a subset of oncogene mRNAs. It was shown that eIF4E overexpression and dysregulation increases RanBP1 levels and reduces the amount of RanBP2. This leads to a faster and more efficient export of the eIF4E/CRM1-dependent mRNAs and their subsequent translation and thus an increased oncogenic potential [44,45].

Importins bind their cargo in the cytoplasm and release it upon binding of RanGTP in the nucleus, while exportins bind cargo in the nucleus only in the presence of RanGTP and release it in the cytoplasm upon Ran-driven GTP hydrolysis stimulated by RanBPs and RanGAP. The export receptor returns empty into the nucleus for another round of export (Figure 2).

**Figure 2.** Schematic drawing of the steps within the CRM1 transport cycle. The steps depicted highlight the different states of CRM1 with respect to the overall shape as well as points of cargo and RanGTP binding and release. See text for details.

3.1. Bi-Functional CRM1: Discovery as an Export Receptor and Cell Cycle Control Factor

CRM1 has first been identified in a cold-sensitive strain of the budding yeast *Schizosaccharomyces pombe* where its mutation causes abnormal chromosome morphology at restrictive temperatures [46]. Later,
CRM1 was shown to interact with Can/Nup214 [47,48], a protein located at the cytoplasmic side of the nuclear pore complex (NPC).

Since then, *in vitro* and *in vivo* experiments clearly demonstrated the role of CRM1 as a major nuclear export receptor [49–54] and identified its cargoes as proteins, which carry a leucine-rich—classical—nuclear export signal (NES). The first NESes were identified in the human immunodeficiency virus type 1 (HIV-1) protein Rev (regulator of expression of virion proteins) and in the cellular protein kinase A inhibitor PKI [55–58]. More complex export events, like the export of m^7^G-capped snRNAs may require additional proteins: e.g., the Cap Binding Complex (CBC; consisting of the two cap binding proteins 20 and 80) in addition to PHAX (phosphorylated adaptor of RNA export), which provides the NES [59,60]. In fact, the aforementioned HIV-1 regulatory protein Rev is another example for a cofactor required for mRNA export. In its absence, unspliced or incompletely spliced viral mRNAs coding for the proteins Gag, Pol and Env are not transported into the cytoplasm and thus viral replication fails, making Rev-mediated RNA export in HIV infection an interesting process to interfere with by drug treatment [61,62].

Besides the established role in nucleocytoplasmic trafficking, further investigations clarified the role of CRM1 in different cellular processes. Additional functions include opposing the effects of Impβ in mitosis [63] and a role in mitotic progression as it localizes to kinetochores and binds to RanGAP1 and RanBP2 in a RanGTP-dependent manner. Moreover, CRM1 has additional effects on the definition of kinetochore fibers and in chromosome segregation during mitosis. In particular, CRM1 activity in metaphase and later anaphase changes repartitioning of RanGTP and consequently also of effectors on kinetochores and centrosomes [63–69].

### 3.2. Conformational States of CRM1 during Nucleocytoplasmic Transport

Structural investigations of CRM1 in different assembly states enabled insight into the local structural rearrangements of CRM1 that stabilize overall conformational changes of CRM1 between the individual steps of a nucleocytoplasmic transport cycle.

CRM1 consists of 21 HEAT repeats, in such an arrangement that the A helices form the convex outer surface of the protein, and the B helices form the concave inner surface [70–72]. Their slightly tilted, consecutive arrangement results in an overall superhelical twist with a flexible pitch [72–74]. Structural investigations of CRM1 in the free state (e.g., cargo- and Ran-unbound form) have shown that it adopts various conformations at equilibrium [75,76]. Multiple conformations of the extended (free) form have recently been observed in crystal structures at reasonable resolution [75,77], revealing a superhelical conformation with no interaction of the N- and C-terminal regions (Figure 3, left panel). Interestingly, the last HEAT repeat, 21, is unusual, as it arranges in two different states. In the extended, cargo-free form of CRM1, helix 21B spans the molecule reaching the opposing side of the superhelix (Figure 3, left panel).
Figure 3. Structural changes of CRM1 between the extended and compact conformation. Structural overview illustrating the conformational changes of CRM1 during an export cycle. The conformations depicted here thus highlight the changes in two conformational states of CRM1 (grey) with respect to the overall shape (extended versus compact) as well as the positional changes of the CRIME-domain (green), the acidic loop (blue), the C-terminal helix (HEAT helix 21B, red) and the conformation of the NES-binding cleft (orange) during the transport cycle. See main text for details.

Here, it touches the base of HEAT 9 helices A and B [70,75], and thus CRM1 is incapable of RanGTP binding, which attaches within the superhelix (see below). Further investigations showed that a negatively charged stretch within the C-terminal residues following HEAT 21B forms electrostatic interactions with a basic patch on HEAT 12B in the vicinity of the acidic loop binding site and contributes to modulation of NES-binding cleft properties [78]. The acidic loop itself is a stretch of 26 residues and located between HEAT helices 9A and B. In the extended forms of CRM1, it is oriented in a “flipped back” conformation closely binding to the B-helices of CRM1 in the NES-binding region formed by HEATs 11 and 12 (Figure 3) [70,71,76,79,80].

The highest sequence identity between CRM1 and other members of the Impβ superfamily has been detected in the first three HEAT repeats, the CRIME domain (Figure 3), indicating its functional importance [48,49,72,79,81]. This region is required for the binding of RanGTP. In the nuclear compartment, binding of RanGTP to the CRIME-domain could trigger progressive encircling of RanGTP by CRM1. The binding is accompanied by a reduction of the helical pitch of CRM1 that leads
to a displacement of helix 21B from its “crossing” orientation to a “parallel” orientation at the outside of CRM1 [71,82]. This in turn, results in a closed CRM1 structure and tight interaction of N- and C-terminal regions (Figure 3, right panel). The acidic loop is released from its “flipped back” conformation and arranges like a “seatbelt” with the tip of the loop contacting residues of HEATs on the opposing side of CRM1, thereby locking RanGTP on the N-terminal part of CRM1 (Figure 3) [71]. Consequently, mechanical strain on the NES-binding cleft decreases, leading to opening of the cleft and increased accessibility for NES-cargos. As a result, a stable export complex assembles, which may then traverse the NPC (Figure 2).

In the cytoplasm, this ternary complex encounters RanBPs, which increase RanGAP binding and the GTP-hydrolysis rate of Ran. One of them, namely RanBP1, is soluble, whereas RanBP2 (Nup358), is localized directly at the pore bound to the filaments emanating from the NPC. Modification of RanGAP by the Small Ubiquitin-like Modifier (SUMO) tethers it to RanBP2 [42,83]. Structural analysis revealed that binding of RanBPs to the CRM1-RanGTP-cargo complex induces significant changes in CRM1 [82]. The binding of RanBP1 fixes the C-terminal acidic DEDDDL sequence of Ran in a position leading to displacement of the acidic loop from CRM1 and in turn interacts with the Ran switch I loop and the adjacent CRM1 surface [82]. The relocalization of the acidic loop in the proximity of HEAT helices 11B and 12B on the inside of the CRM1 ring is thought to induce structural changes at CRM1 HEATs 11 and 12, leading to a constriction of the NES-binding cleft and thus release of the NES-cargo [82].

The RanGTP-RanBP1 subcomplex has to dissociate from the export complex in order to interact with RanGAP. Subsequently, GTP is hydrolyzed to GDP by Ran aided by RanGAP and RanBPs with the resulting RanGDP exhibiting a lower affinity towards CRM1. CRM1 lacking any binding partners translocates back into the nucleus for another round of export.

3.3. NES Recognition by CRM1 and Export of (Proto-) Oncoproteins or Tumor Suppressors

The crystal structures of full-length CRM1 in complex with RanGTP and/or the cargo Snurportin1 (SPN1) revealed for the first time how CRM1 and the NES of cargo interact on a structural level (Figure 4A,B) [70,71].

The CRM1 cargo SPN1 facilitates the import of UsnRNP core particles by bridging the interaction between the UsnRNPs and the actual import receptor Impβ. In this pathway, SPN1 specifically binds the modified 5'-cap of the UsnRNP core particle via its cap-binding domain (CBD) [84]. For relocation into the cytoplasm, SPN1 bears an N-terminally localized CRM1-dependent NES, which forms an amphipathic α-helix [71,80,85]. Within that α-helix, five hydrophobic key residues dock into corresponding hydrophobic pockets (named Φ0–Φ4) of the NES-binding cleft of CRM1 (Figure 4, left panels) [70,71]. In fact, the hydrophobic character, the size and the position of these Φ residues are important and essential for high-affinity binding of NES to CRM1. This is underlined by the observation that a single mutation of any of the Φ residues to a polar amino acid leads to a significantly weaker binding of a given NES [80]. Most strikingly, removal of the first methionine of the SPN1-NES occupying the Φ0 position entirely abolishes binding to CRM1, thereby reflecting its importance [71]. Cys528 (in human CRM1), which is known to be modified by Leptomycin B (LMB) and many other CRM1-blocking compounds, is located in the vicinity of the Φ3 position and thus in the central region of the CRM1 NES-binding cleft.
Figure 4. Cargo and inhibitor binding via the NES-binding cleft. Structural features of the NES-binding cleft of CRM1 is shown at the top. The five hydrophobic binding pockets for the interacting residues of the NESes are indicated by circles, with blue and yellow indicating the occupied sites and red circles highlighting the sites unused by the inhibitors depicted in the panels below. Additionally, the yellow circle indicates the position of Cys528 in human CRM1 and the S539C mutation in yeast CRM1 used for covalent binding of the inhibitors.

Left panels: three NESes differing with respect to the spacing in between the residues determining binding within a rigid NES-binding pocket are shown. Detailed view of the NES-binding clefts of CRM1 bound to the SPN1-NES (A,B) (PDBid: 3GJX), PKI-NES; (C) (PDBid: 3NBY); and Rev-NES (D) (PDBid: 3NBZ). The SPN1-NES has been removed in (A) for clarity reasons to show the dimensions of the cleft and the respective Φ-pockets. Note that the key residues of all three NESes occupy identical Φ-pockets and differences in the Φ-spacing are compensated by a different arrangement of the NES-peptide main chain. Natural inhibitors of cargo binding bound to yeast CRM1 are shown in the middle panels. Blocking of the NES-binding cleft by CRM1-specific natural inhibitors: LMB (E,F) (PDBid: 4HAT); as well as Ratjadone (G) (PDBid: 4HAU); and Anguinomycin (H) (PDBid: 4HAV). LMB has been removed in (E) to show the dimensions of the occupied NES-binding cleft. Small synthetic inhibitors and their orientation in the NES-binding cleft are depicted on the right. KPT185 (I,J) (PDBid: 4GMX), KPT251; (K) (PDBid: 4GPT) and KPT276 (PDBid: 4WVF). KPT185 has been removed in (I) to show the dimensions and highlight the individual pockets of the occupied NES-binding cleft.
Subsequent structural analyses of additional NESes, fused to the CBD of SPN1 (SPN1-chimeras) revealed that the identical pockets Φ0–Φ4 within a rigid NES-binding cleft of CRM1 are used for binding of the PKI and Rev NES [80]. As the five hydrophobic key residues of the three different NESes exhibit different spacings on the amino acid sequence level, this requires a different arrangement of the NES-peptide chain to compensate for such differences (Figure 4, left panels).

Databases like “NESdb” and “ValidNESs” are available, which archive NES-containing CRM1 cargoes. At last count (July 2015), there were 265/262 entries in these databases referring to macromolecules that bind to CRM1 and hence are exported [57,58]. Since all of them are assumed to bind in the same hydrophobic Φ pockets, they most likely apply a similar binding mode.

In recent years, CRM1 has been identified as an export receptor for various (proto-) oncoproteins and tumor suppressor genes like p53 [9], BRCA1 [86], p21CIP [87], cyclin D1 [88], APC [17,89,90], Bok [91], forkhead box (FoxO) proteins [92–94], nucleophosmin [66,95,96], N-WASP [97], as well as the established drug target topoisomerase I/II [98–100]. The observed differences between normal and malignant cells with respect to the localization of proteins that function as oncoproteins makes CRM1 itself an interesting target in molecular oncology and therapeutics (Figure 1). Such changes in the localization of (proto-) oncoproteins and their deregulation may be caused either by an increase of cellular CRM1 levels that influence their distribution pattern by competition or due to any interference with the protein-CRM1 interaction. There are several possibilities for the latter case, like mutations (e.g., nucleophosmin, CRM1 [101]), phosphorylation (e.g., p27KIP, Rb, p53 [13,102–105]), ubiquitination and sumoylation (e.g., p53 [106–108]) or NES unmasking (e.g., INI1, NF-AT, p53, BRCA2/RAD51 [9,109–111]). However, as a consequence, both possibilities may finally lead directly or indirectly to a deregulation of nuclear export of tumor suppressor proteins or (proto-) oncoproteins (see Figure 1 and below).

A well-known drug target (e.g., targeted by doxorubicin and etoposide) against multiple myeloma is Topoisomerase IIα (Topo IIα), a nuclear protein, which is essential for DNA replication, transcription, chromatin separation as well as chromatin condensation [112–114]. For nucleocytoplasmic shuttling, Topo IIα contains both an NLS in its C-terminal domain, as well as two NES sequences in the central catalytic domain [99]. It has been shown that at increased cell densities and in myeloma cells, Topo IIα is exported to the cytoplasm in a CRM1-dependent fashion rendering the cells resistant to Topo IIα-specific inhibitors, which rather act on the DNA-bound nuclear protein [99,115,116]. Using a combination of Topo IIα inhibitors and efficient, non-acute toxic CRM1 inhibitors allows keeping the protein in the nucleus and hence sensitize it for Topo IIα inhibitor treatment [117,118].

The breast cancer-associated protein, BARD1 (BRCA1-associated RING domain protein), co-localizes with BRCA1 in nuclear foci [119]. After DNA damage, the two proteins form a stable heterodimer implicated in multiple nuclear functions like DNA repair, protein ubiquitination and control of mRNA processing [120–122]. Additionally, it has been observed that BRCA1 mislocalizes to the cytoplasm in cancer cells but not in normal cells. Later it was shown that BARD1 has BRCA1-independent pro-apoptotic activity in the cytoplasm. Both, BRCA1 as well as BARD1 harbor NESes [123,124], which are part of the BRCA1/BARD1 dimerization surface and thus are masked when both proteins bind to each other. Disruption of this interaction leads to cytoplasmic accumulation and increased apoptosis. However, it seems that in such cells, nuclear import of BRCA1/BARD1 is impaired rather than nuclear
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Export of BARD1, thereby explaining its cytoplasmic accumulation and thus cancer development [121,123,125].

The inhibitor of apoptosis Survivin is highly abundant in human tumors and in fetal cells but absent in normal cells. It has been shown that Survivin contains a leucine-rich NES (amino acids 89–98) for CRM1 export [126,127] but lacks a classical import signal (NLS). Survivin has different functions in cell viability and cell division. The cytoplasmic (e.g., exported) form controls cell viability as it inhibits caspase activation and thus prevents apoptosis. This effect is thought to contribute significantly to the fast growth and apoptotic resistance of tumor cells. In contrast, nuclear Survivin regulates cell division as it is part of the chromosomal passenger complex, which coordinates essential chromosomal and cytoskeletal events during mitosis [126–130].

The cyclin-dependent kinase inhibitor p27Kip is an important regulator of the cell cycle. During cell cycle progression, it binds and inhibits cyclin/cyclin-dependent kinase (CDK) complexes in the nucleus and thus stops or slows down cell division at the G1 stage [131–133]. For example, interaction of p27Kip with cyclin D and CDK4 inhibits the kinase activity and thus prevents phosphorylation and inactivation of the transcriptional repressor, retinoblastoma tumor suppressor protein (Rb) [134,135]. Notably, the activity of p27Kip itself is subjected to regulation on multiple levels like transcription, translation, proteolysis and nuclear export [104]. For CRM1-dependent export, phosphorylation of p27Kip Ser10 by other kinases plays an essential role [136]. As cytoplasmic p27Kip is no longer able to inhibit cyclin/CDK complexes, Rb is consequently phosphorylated and inactivated, resulting in expression of multiple factors and promotion of fast cell cycle progression [137]. This, in turn, is highly correlated with a high tumor grade, poor prognosis and increased metastasis in different subsets of carcinomas like breast-, cervix, esophagus and uterus carcinomas as well as in lymphomas and leukemia [138–140].

Apart from a direct interference of CRM1 (proto-) oncogene interaction by the mentioned effects, elevated CRM1 expression levels in tumor cells can cause nuclear export to be deregulated. Indeed, CRM1 protein expression level was shown to be a prognostic indicator for various cancers and is also correlated with increased metastasis, histological grade, increased tumor size, and decreased progression-free and overall survival. In particular, elevated CRM1 expression correlates with poor clinical outcome in ovarian- [141], pancreatic- [142,143], kidney- [144] and cervical cancers [145], as well as gastric carcinomas [146], osteosarcoma [147], glioma [148] as well as leukemia [149,150]. In addition, mantle cell lymphoma [151], multiple myeloma [152,153] and melanoma [154] have been shown to be accompanied by elevated CRM1 levels [118,143,150,155,156].

Although it is a more global strategy to interfere with CRM1-dependent transport processes as it affects all proteins bearing a canonical NES in all cells, it seems to be a promising idea to use natural or synthetic compounds to block CRM1 and thus interfere with transport and localization of cancer-related proteins. Interestingly, the effect of CRM1 inactivation seems to have a more pronounced effect on cancer cells leading to increased apoptosis than on normal cells that tolerate such compounds to a certain degree.

Not only CRM1 but also other transport factors exhibit altered expression and functions in cancer cells. A role as prognostic biomarker has been determined for Exportin 7 in ovarian cancers [157]. Recently, it has been shown that Impβ expression is increased in several malignant tumors such as cervical tumors and malignant peripheral nerve sheath tumors (MPNSTs) as well as in breast, gastritic, neck, lung and ovarian cancers [145,158–162]. Impβ up-regulation has been shown to promote cell
proliferation in gastritic and cervical cancer cells [145,158]. Also, one of the adaptor molecules Karyopherin α2 bridging the interaction between Impβ and NLS-bearing cargo is a potential biomarker in multiple cancers reviewed in [163]. A role as prognostic biomarker has been determined for the small GTPase Ran in ovarian and colorectal cancer [157,164]. Ran promotes cancer cell metastasis by interaction with Txl-2 [165]. Moreover, it has been shown to promote proliferation of pancreatic cancer cells [166] and is a potential therapeutic target in diffuse large B-Cell lymphoma and other cancer cells with specific defects [167,168].

In line with these observations, progress with respect to altering transport processes by drugs has been made. For example, treatment of MPNST cells with an inhibitor of S-adenosyl-methionine-dependent methyl-transferases, 3-deazaneplanocin A (DZNep) impaired cell viability and proliferation and reduced Impβ protein levels [159]. The Impβ1-specific inhibitor Importazole inhibits Impβ1’s role in nuclear import [169] and decreases the viability of malignant breast tumor cells much more than that of the non-transformed counterpart [162].

3.4. Drug Binding to CRM1

The first compound identified as the most potent CRM1 inhibitor is the drug Leptomycin B (LMB) [49,50,61,170], which is able to block Rev function and HIV-1 replication [61,171].

All the natural compounds identified thus far have an α,β-unsaturated δ-lactone ring in common. The Streptomyces spp. natural products LMB and Kazusamycin were originally characterized as antifungal and antitumor agents [61,172–175]. An additional member of the family, Anguinomycin, was isolated as a natural product from Streptomyces spec. and later on also produced by total chemical synthesis [176–179]. Interestingly, a simple α,β-unsaturated lactone analog with a truncated polyketide chain was shown to retain most of the biological activity [178].

Ratjadone belongs to another group of inhibitors and was isolated from the myxobacterium Sorangium cellulosum strain So ce360 [180,181]. Chemical total synthesis was established, providing the tools for the production of variants [182,183]. Ratjadone blocks export by employing the same mechanism as LMB [184,185], hence crystal structure analyses revealed that it uses the identical binding pockets of the CRM1 NES-binding domain. In addition, it has recently been shown to block the Rev/CRM1 export pathway [186].

Structure analysis revealed the mode of binding of LMB and the related inhibitors Anguinomycin A and Ratjadone A to the NES-binding pocket (Figure 4E–H). Moreover, deeper analysis revealed that all three show an unexpected mechanism of inhibition, involving covalent conjugation of the α,β-unsaturated δ-lactone ring to Cys539 of CRM1 [172,185]. The subsequent CRM1-catalyzed hydrolysis of the natural products’ lactone ring, which is mediated by basic residues (Lys or Arg) positioned near the reactive cysteine, renders the binding irreversible (Figure 5) [187]. All three natural compounds occupy the same space in the NES-binding cleft of CRM1, namely four of the five hydrophobic pockets used by the SPN1-NES (Φ0 to Φ3) for binding, leaving Φ4 vacant (Figure 4, middle panels, red circle). The reactive cysteine (aa 528 in human and aa S539C mutation in yeast) lies in the vicinity of the hydrophobic pocket Φ3, and binding of LMB restricts binding of NESes by spatial competition (Figure 3) [172,173,187]. Interestingly, covalent conjugation is not strictly required for
LMB binding to the CRM1 groove, because the groove is also open in a complex of LMB with CRM1 that lacks the reactive cysteine, but seems highly flexible, as indicated by weak electron density [187].

**Figure 5.** Covalent binding of inhibitors to the NES-binding cleft of CRM1. All inhibitors characterized thus far are covalently attached to the reactive cysteine C539 by a Michael-type addition. (A) Mechanism of the Michael addition of the cysteine to the lactone ring of CRM1 inhibitors. Subsequently, in the α,β-unsaturated δ-lactone ring containing compounds, a nucleophilic attack of a water molecule leads to lactone hydrolysis in a subsequent step due to the neighboring basic residues that form an oxyanion hole stabilizing the transition state of the reaction (not depicted). For compounds containing an α,β-unsaturated δ-lactone ring, binding is irreversible due to the CRM1-catalyzed opening of the lactone ring. Structural arrangement of the NES-binding cleft of CRM1 shown in cartoon mode (grey); (B) Binding of LMB (cyan) to CRM1 results in hydrolysis of the lactone ring due to neighboring basic residues indicated (teal). The mFo-DFc omit map contoured at a level of 3.0 sigma, clearly shows the arrangement of the hydrolyzed LMB; (C) Mutation of the three basic residues prevents ring opening of LMB (cyan); (D) By contrast, binding of the novel compounds lacking the lactone ring, is slowly reversible. KPT185 interacts with Cys528, but no other changes are observed.

For LMB, murine xenograft cancer models revealed a modest efficacy (CI-940) [188] and clinical phase I trial confirmed the results but also pinpointed severe toxicities, including anorexia and malaise [189]. Moreover, the identification of the specific inhibition of CRM1 export function by the drug LMB [49,50,61,170] and recent structural understanding of the mode of cargo recognition within...
the NES-binding cleft and the inhibition of exactly that binding cleft by LMB [70,71,187], led to the
development of novel therapeutics [149,156,190]).

Semisynthetic products of LMB coined Nuclear Export Inhibitors (NEIs) revealed improved
therapeutic capabilities by maintaining the high potency observed for LMB, as they are better tolerated
in vivo, and show significant efficacy in multiple mouse xenograft models. These NEIs are thought to
have potential as CRM1 inhibitors and potent anticancer agents [190].

Another synthetic small-molecule and CRM1 inhibitor, an analog of a class of compounds called N-
azolylacrylates, was developed in a study by Daelemans et al. [191]. It exhibits the same cellular effects,
namely prevents nuclear export of the HIV-1 Rev protein and is a highly specific inhibitor of CRM1.
Like LMB, this compound (PKF050-638) interferes with the NES-binding cleft cysteine and prevents
binding of the nuclear export signal [191]. The further development led to Small Inhibitors of Nuclear
Export (SINEs) that are similar to the N-azolylacrylate structures [192]. Three of those SINEs (KPT185,
KPT251 and KPT276), which all share a trifluoromethyl phenyl triazole scaffold, have been crystallized
in a complex with CRM1 [149,156,193]. Crystal structure analysis revealed that they occupy only three
of the five hydrophobic pockets (Φ2–Φ4) centered on the reactive cysteine leaving Φ0 and Φ1 vacant
(Figure 4J–L). The structural comparison of the interaction network of the natural compounds like LMB,
Anguinomycin A and Ratjadone A with CRM1 on the one side [187] and synthetic compounds e.g.,
KPT185 and KPT251 on the other, revealed an additional important property of the synthetic
compounds. In the synthetic compounds, the Michael acceptors (an isopropyl acrylate in KPT185 and
an alkyl-oxadiazole in KPT251) are not hydrolyzed when bound to wild-type CRM1, thus they may bind
in a slowly reversible fashion into the NES-binding cleft [187]. This reversibility of binding could
contribute to the reduced side effects observed in in vivo studies, which is in contrast to the irreversible
binding of the natural compounds. The putative weak interactions of NESes to the vacant sites Φ0 and
Φ1 of the NES-binding cleft could increase the rate of synthetic inhibitor release, enabling cargo binding
to an extent that is sufficient for normal, but not for malignant, cell survival.

These small molecule inhibitors or derivatives thereof are being used in clinical trials in patients with
both hematological malignancies [139,149] and solid tumors [153]. Antitumor effects of KPT185 and
its clinical equivalent KPT276 have been shown in cancer cells and xenografts [194]. An improved
version is KPT330, an oral drug currently undergoing phase I studies in patients with advanced, relapsed,
and refractory solid tumors, hematological malignancies, and sarcoma [139,153,195]. Preclinical
evaluation of bioavailable SINE KPT335 has been performed in canine cancer cells and dogs [196].

There is a large number of additional nuclear export inhibitors from both natural and synthetic sources
available. Natural compounds include Prostaglandins [197], the spice curcumin from the plant
Curcuma longa, which is already in clinical trials [198–200], or compounds from the plants
Valerianae sp. [171,201,202]. In addition, Plumbagin, a bicyclic naphthoquinone [203], and Piperlongumine,
a natural alkaloid of the long pepper [204], have been shown to inhibit CRM1-mediated nuclear export.
Moreover, the cytotoxic styryl-lactone Goniothalamin from the family of Annonaceae [205–209],
19S-19-Acetoxychavicol acetate isolated from Alpinia galangal [210,211], Callystatin A from the marine
sponge Callyspongia truncata [212–214] and Leptofuranins from Streptomyces tanashiensis [215,216]
have been described. Compounds resulting from screening experiments are CBS9106, a novel reversible
oral CRM1 inhibitor with CRM1-degrading activity [217], as well as multiple compounds that have been
identified in screens examining nuclear export of FOXO proteins or inhibit the activation-dependent nuclear export of the p38 kinase substrate MAPK-activated protein kinase 2 (MK2) [218,219].

As these compounds block CRM1-dependent export and influence the cellular distribution pattern of the proteins/oncoproteins/tumor suppressor proteins, they modulate the fate of cancer cells by decreasing their survival rate [139,143,144,149,151,154,155,220]. To this end, treatment with KPT330 has been shown to result in the nuclear enrichment of various proteins e.g., p53, and p21, which then are able to perform their cellular function decreasing the survival rate of the cancer cells as shown in diffuse malignant peritoneal mesothelioma, renal cell carcinoma and leukemias [195,221–223]. Recently, a novel drug (S109, a derivative of CBS9106) was shown to inhibit proliferation and arrest colorectal cancer cells by nuclear retention of tumor suppressor proteins like p21, p27 and FOXO, by reversibly binding to CRM1 and to decrease the CRM1 level using the proteasomal pathway [224]. For detailed information on the compounds and their effects on various cancers, we refer to excellent recent reviews [225–228].

As an additional effect, the CRM1 inhibitors may sensitize resistant cancer cells for other drugs, e.g., as shown by Topoisomerase in multiple myeloma, which has to be localized in the nucleus in order to be sensitive for doxorubicin and etoposide treatment [117]. Along this line, CRM1 inhibition by KPT330 enhances the antitumor activity of Gemcitabine in pancreatic cancer [229] or of Ibrutinib in chronic lymphocytic leukemia [230]. Overall, this supports the observation that blocking CRM1 sensitizes cancer cells to other drugs by preventing export of additional tumor suppressors or cell cycle inhibitors.

4. Conclusions, Outlook, Pending Issues

The idea of using CRM1 as a drug target to battle cancer is based on the observation that treatment of cancer cells with natural drugs leads to a prolonged block of nuclear export and subsequent apoptosis of cancer cells. In contrast, in normal cells, these drugs, although they induced cell cycle arrest, do not lead to apoptosis. Recent investigations identified novel synthetic and semi-synthetic compounds with reduced side effects.

Understanding the molecular differences of CRM1-dependence (and influence of inhibitors) in healthy and cancerogenic cells will help to design compounds that are more specific.

In the future, improved understanding of the NPC-CRM1 interaction and the transition process through the NPC itself might also be used for selective inhibition of nucleocytoplasmic transport. This could be achieved by specifically targeting the CRM1-Nup interactions, e.g., Nup214, Nup98 or hCG1 or by modulation of export of only a subset of NES-cargoes [231–239].

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Author Contributions

A.D., T.M. and R.F. reviewed publications and wrote the manuscript.
Conflicts of Interest

The authors declare no conflict of interest.

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