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Review

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

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ABSTRACT

Nuclear-cytoplasmic trafficking of proteins is a significant factor in the development of cancer and drug resistance. Subcellular localization of exported proteins linked to cancer development include those involved in cell growth and proliferation, apoptosis, cell cycle regulation, transformation, angiogenesis, cell adhesion, invasion, and metastasis. Here, we examined the basic mechanisms involved in the export of proteins from the nucleus to the cytoplasm. All proteins over 40 kDa use the nuclear pore complex to gain entry or exit from the nucleus, with the primary nuclear export molecule involved in these processes being chromosome region maintenance 1 (CRM1, exportin 1 or XPO1). Proteins exported from the nucleus must possess a hydrophobic nuclear export signal (NES) peptide that binds to a hydrophobic groove containing an active-site Cys528 in the CRM1 protein. CRM1 inhibitors function largely by covalent modification of the active site Cys528 and prevent binding to the cargo protein NES. In the absence of a CRM1 inhibitor, CRM1 binds cooperatively to the NES of the cargo protein and RanGTP, forming a trimer that is actively transported out of the nucleus by facilitated diffusion. Nuclear export can be blocked by CRM1 inhibitors, NES peptide inhibitors or by preventing post-translational modification of cargo proteins. Clinical trials using the classic CRM1 inhibitor leptomycin B proved too toxic for patients; however, a new generation of less toxic small molecule inhibitors is being used in clinical trials in patients with both hematological malignancies and solid tumors. Additional trials are being initiated using small-molecule CRM1 inhibitors in combination with chemotherapeutics such as pegylated liposomal doxorubicin. In this review, we present evidence that combining the new CRM1 inhibitors with other classes of therapeutics may prove effective in the treatment of cancer. Potential combinatorial therapies discussed include the use of CRM1 inhibitors and the addition of alkylating agents (melphalan), anthracyclines (doxorubicin and daunomycin), BRAF inhibitors, platinum drugs (cisplatin and oxaliplatin), proteosome inhibitors (bortezomib and carfilzomib), or tyrosine-kinase inhibitors (imatinib). Also, the sequence of treatment may be important for combination therapy. We found that the most effective treatment regimen involved first priming the cancer cells with the CRM1 inhibitor followed by doxorubicin, bortezomib, carfilzomib, or melphalan. This order sensitized both *de novo* and acquired drug-resistant cancer cell lines.

1. Nuclear export

1.1. Nuclear-pore complex structure and function

The cell nucleus is a sequestered environment due to the highly selective transport of proteins greater than 40 kDa both into and out of the nucleus through the nuclear pore complex (NPC). The NPC is one of the largest protein complex structures (125 MDa) in the cell, containing at least 30 different proteins called nucleoporins [1,2]. The NPC has a central transporter region containing a nucleoporin matrix that assists movement of macromolecules,

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eight fibril proteins that extend into the cytoplasm, and a nuclear basket structure. Movement of molecules through the NPC is bidirectional; movement in or out of the nucleus is dependent on the type of receptor protein and the molecule associated with it.

For nuclear export or import of a large protein (>40 kDa) to occur, it must be bound to a nuclear export receptor molecule. The majority of these NPC transport molecules are members of the karyopherin-β family of proteins. There are approximately 19 members of karyopherin-β receptor family proteins, with each recognizing a specific group of cargo proteins or RNA [3]. CRM1 (chromosome region maintenance 1) is a ubiquitous transport receptor protein that binds its cargo *via* a hydrophobic nuclear export signal (NES) peptide sequence (see Section 1.2). Export complexes are formed in the nucleus and are made up of three components or trimer consisting of CRM1, RanGTP, and the cargo or exported substrate. Binding of CRM1 to the cargo is weak; however, when the cargo protein and RanGTP bind to CRM1 cooperatively, the affinity of CRM1 to both RanGTP and the cargo substrate is increased 500- to 1000-fold [4,5]. Fig. 1A shows the trimer formation of CRM1, RanGTP, and the cargo protein (in this case topoisomerase IIα (topo IIα)) in the nucleus at the NPC and transport through the NPC. The energy required for transport is provided by RanGTP. RanGTP is maintained in high concentrations in the nucleus by the presence of RCC1, a guanine nucleotide exchange factor (GEF). The high RanGTP concentration gradient from the nucleus to the cytoplasm provides the energy required for facilitated nuclear export. After the CRM1/RanGTP/cargo trimer arrives in the cytoplasm, RanGTP is hydrolyzed to RanGDP by a RanGAP (Fig. 1B), causing dissociation of the trimer and release of the cargo protein into the cytoplasm. CRM1 and RanGDP are then recycled back into the nucleus through the NPC for another export cycle (Fig. 1C).

1.2. CRM1 and nuclear export signals

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

2. Blocking nuclear export of proteins

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

2.1. CRM1 inhibitors

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

2.2. Nuclear export signal small molecule inhibitors

Since CRM1 is a ubiquitous exporter of nuclear proteins (>241 proteins), truly selective inhibitors that bind to the NES and prevent export may produce a more targeted therapy (Fig. 1). We have explored this idea by developing inhibitors that bind to a single specific NES peptide and prevent export of a topo IIα. We have shown that topo IIα has a NES at amino acids 1017–1028 (site A: DILRDFFELRLK) and at amino acids 1054–1066 (site B: FILEKIDGKIIIE) [16]. Disrupting the binding between topo IIα-NES and CRM1 could prevent CRM1-mediated nuclear export of topo IIα and result in the sensitization of MM cells to topo II inhibitors. We identified, by a virtual screening method, a series of inhibitors of the interaction of CRM1 and topo IIα that block the nuclear export of topo IIα [17]. A homology model of human topo IIα based on *Saccharomyces cerevisiae* topo II was used for molecular docking. These NES inhibitors are designed to bind specifically to the NES of topo IIα and prevent its export. We found that they did not prevent the export of other proteins such as p53. When NES inhibitors are combined with the topo II poison doxorubicin, they sensitize cancer cells to doxorubicin treatment and synergistically induce apoptosis and inhibit proliferation [17].

2.3. Post-translational modification of protein cargoes

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