Identification of Nuclear Export Inhibitors with Potent Anticancer Activity In vivo

Sarah C. Mutka, Wen Qing Yang, Steven D. Dong, Shannon L. Ward, Darren A. Craig, Pieter B.M.W.M. Timmermans, and Sumati Murli

Kosan Biosciences, Inc., Hayward, California

Abstract

The export protein CRM1 is required for the nuclear export of a wide variety of cancer-related “cargo” proteins including p53, c-Abl, and FOXO-3A. Leptomycin B (LMB) is a highly specific inhibitor of CRM1 with significant in vitro potency but limited in vivo efficacy due to toxicity. We now report a series of semisynthetic LMB derivatives showing substantially improved therapeutic windows. Exposure of cancer cells to these compounds leads to a rapid and prolonged block of nuclear export and apoptosis. In contrast to what is observed in cancer cells, these agents induce cell cycle arrest, but not apoptosis, in normal lung fibroblasts. These new nuclear export inhibitors (NEI) maintain the high potency of LMB, are selective for cancer cells, these agents induce cell cycle arrest, but not apoptosis, in normal lung fibroblasts. These new nuclear export inhibitors (NEI) maintain the high potency of LMB, are up to 16-fold better tolerated than LMB in vivo, and show significant efficacy in multiple mouse xenograft models. These NEIs show the potential of CRM1 inhibitors as novel and potent anticancer agents. [Cancer Res 2009;69(2):510–7]

Introduction

Transport of macromolecules across the nuclear membrane is fundamental to the proper functioning of a living cell. For example, the ability to localize to the nucleus is essential for transcription factor activation, and spatial separation of proteins is commonly used as a mechanism for preventing spontaneous signal activation. Many important tumor suppressors and transcription factors protect cells by regulating cell growth and apoptosis, and their cytoplasmic localization can serve as an inactivation mechanism resulting in uncontrolled growth and the onset of disease (reviewed in ref. 1).

One strategy to prevent cytoplasmic localization of important transcription factors is to inhibit the proteins responsible for their nuclear export. The exportin CRM1 is absolutely required for the nuclear export of a wide variety of cancer-related “cargo” proteins including p53, c-Abl, and FOXO-3A (2). CRM1 recognizes its cargo proteins through their leucine-rich nuclear export sequence. The CRM1-cargo complex is then actively transported across the nuclear pore complex to the cytoplasm where the cargo is released after RanGAP-catalyzed GTP hydrolysis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Sarah C. Mutka, N30 Pharmaceuticals, 3122 Sterling Circle, Suite 2800, Boulder, CO 80301. S.C. Mutka and W.Q. Yang contributed equally to this work.

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The role of CRM1 as an exportin was first elucidated through the use of a highly cytotoxic polyketide natural product known as leptomycin B (LMB; refs. 2, 3). LMB was found to be very potent in vitro against various cancer cell lines (IC50 values in the 0.1–10 nmol/L range). The potent activity of LMB stems from its highly specific binding and inhibition of CRM1, thereby blocking CRM1-mediated protein export from the nucleus. LMB covalently inhibits CRM1 through a Michael addition of Cys528 onto LMB, and mutation of this cysteine residue prevents the covalent interaction and provides resistance to LMB (4, 5).

Based on its nanomolar potency against cancer cell lines, the in vivo activity of LMB was examined in a number of murine xenograft cancer models. It was found to show only modest efficacy (6). Despite its relatively narrow therapeutic window in mouse tumor models, a single phase I trial of LMB was done. Its clinical development was subsequently discontinued due to the significant toxicity observed without apparent efficacy (7).

Notwithstanding its initial failure in the clinic, LMB could serve as the paradigm for a novel class of cancer therapeutics. These compounds would derive their activity by preventing cytoplasmic localization and inactivation of important tumor suppressors that are dependent on CRM1 for nuclear export, such as p53. It has been estimated that roughly 50% of cancers maintain wild-type p53 (8). In many of these cases, the tumor suppressor function is compromised by overexpression or inactivation of cellular factors that regulate the levels of p53 in the nucleus or lead to its enhanced export out of the nucleus (1). When p53 is activated in the nucleus, it can promote either cell cycle arrest or apoptotic cell death, depending on the environment and level of cell stress. p53 function is negatively regulated in part by a murine double minute-2–dependent pathway that results in both nuclear export and ubiquitin-dependent degradation of p53. In many cancer cells, such as human papilloma virus (HPV)–positive cancers, aberrant cytoplasmic localization and/or degradation of p53 prevents the activation of pathways that would lead to cell death (9–11). Consequently, a means of relocating the antioncogenic wild-type p53 to the nucleus in these aberrant cell types is a promising approach to regaining control of cell proliferation (12). In fact, previous work has shown that treatment with LMB and actinomycin D leads to the accumulation of transcriptionally active p53 in the nucleus of HPV-positive cervical cancer cell lines resulting in apoptotic death (9). Furthermore, when human keratinocytes were treated with LMB, induction of apoptosis was selectively induced in primary cells expressing the HPV oncoproteins (13). Such potent antitumor effects are not limited to HPV-positive cancers. LMB treatment of prostate cancer cells (14) as well as neuroblastoma cell lines (15) induces p53 activation, leading to growth arrest and induction of apoptosis.

To establish the potential utility of nuclear export inhibitors (NEI) as anticancer drugs, we synthesized derivatives of LMB and
now report a series of NEIs with substantially improved therapeutic
windows. These new NEIs maintain the high potency of LMB and are up to 16-fold better tolerated than LMB in mouse models. We
show that exposure of cancer cells to these compounds leads to a
rapid and prolonged block of nuclear export, which is further
associated with an increase in multiple markers of apoptosis. In
contrast to what is observed in cancer cells, these agents induce
cell cycle arrest, but not apoptosis, in normal lung fibroblasts.
These novel CRM1 inhibitors show significant efficacy in multiple
mouse xenograft models, including models of colon and cervical
cancer. Identification of molecules such as these that target CRM1
but with a wider therapeutic window than LMB is of great interest
as a potential novel anticancer therapy.

Materials and Methods

Cell lines and materials. The U2OS RevGFP cell line was obtained from
the laboratory of Prof. Pamela Silver (Dana-Farber Cancer Institute, Boston,
MA). All other cell lines were obtained from American Type Culture
Collection. A general method for formation of 24-LMB-amides is described
in Supplementary Materials.

Cytotoxicity assays. Cytotoxicity assays were done using the CellTitre-
Glo Luminescent Cell Viability Assay (Promega) according to the
manufacturer's instructions. Approximately 5,000 cells per well were treated
with drug either continuously or for a 1-h pulse, and cell viability was
measured using the assay after 72 h. For the short-exposure experiments,
the cells were washed with prewarmed medium after 1 h and then
maintained in drug-free medium until viability was assessed at 72 h.
Luminescence was measured on a Wallac Victor2 multilabel plate reader
(Perkin-Elmer).

Cell extracts and Western blot analysis. For extracts from cell culture,
cells were washed with PBS and resuspended in radioimmunoprecipitation
assay buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1 mmol/L
EDTA, 1% (w/v) NP40, 0.1% (w/v) SDS, 12 mmol/L sodium deoxycholate,
0.9 mmol/L Na2VO4, Roche Complete Protease Inhibitor Cocktail]. Cells were
disrupted by several passages through a syringe; centrifuged for 15 min at
40,000 × g, 4°C; and stored at −20°C until analysis by SDS-PAGE and
Western blotting. Antibodies used include p53 (Bp53-12) and glyceralde-
hyde-3-phosphate dehydrogenase (GAPDH; both from Santa Cruz Biotech-
nology) and cleaved caspase-3 (Cell Signaling).

Immunofluorescence. Approximately 5 × 105 cells were seeded onto
black, tissue culture–treated, optical-bottom 96-well plates (Nalge Nunc).
Approximately 4,000 cells were treated with serial dilutions
of compound for 72 h, and luminescence was measured on a Wallac Victor2
multilabel plate reader (Perkin-Elmer).

Apoptosis assays. Annexin V staining was done using the Annexin V-
FITC Apoptosis Detection Kit (BD Biosciences) according to the
manufacturer's instructions. Analysis by flow cytometry was done with a
Becton Dickinson FACSCalibur.

Determination of maximum tolerated dose of NEIs. C57Bl/6 mice (7–
10 per dose group) were given vehicle or escalating dose levels of NEIs i.v.
via the lateral tail vein either once or on a weekly schedule. Mice were
monitored at least twice a week for body weight change and clinical
symptoms for 2 to 6 wk. Maximum tolerated dose (MTD) was defined as the
highest dose level (of each drug for each schedule) that induced <20% body
weight loss and/or ≤10% mortality.

Xenograft tumor models. Human cancer cell lines were grown in their
recommended medium and harvested. The cells were resuspended in PBS.
For tumor inoculation, cancer cells (3 × 106–10 × 106 per mouse; 0.1 mL/
mouse) were s.c. implanted into the hind flanks of female nu/nu athymic mice.
Animals were monitored at least twice a week for the duration of the
study. Drug treatment was usually initiated when tumors had reached 
~100 to 150 mm3. For efficacy studies with large tumors, tumors were allowed to
reach >450 mm3 before the drug treatment was initiated. Tumor-bearing
mice were randomized and dosed i.v. via the lateral tail vein with either
vehicle or NEIs on the indicated schedule. The NEIs were formulated in 4% Creomorph,
6% propylene glycol, 10% ethanol, 80% saline. Tumor
dimensions were measured with calipers and tumor volume was estimated
using the following formula: volume = (length × width × width)/2.
Statistical analyses were carried out with the use of the GraphPad Prism 4
software. All reported P values were from two-way ANOVA tests and P <
0.05 was considered statistically significant.

Results

NEIs are potent cytotoxic agents. LMB is a polycytidine natural
product that rapidly induces cytotoxic effects in cancer cell lines
via the covalent inhibition of CRM1. For most cancer cell lines, in vitro
IC50 values for a 72-hour exposure are in the subnanomolar
range (Table 1 and data not shown). We reasoned that the covalent
interaction of LMB with CRM1 would result in effects of the drug
that persist beyond the initial drug exposure. To confirm this
hypothesis and to more closely mimic in vivo drug exposures, we
performed cytotoxicity assays with a short drug exposure period.
Drug treatment was followed by continued incubation in drug-free
medium and subsequent measurement of cytotoxicity after 72
hours. With this assay, LMB exposure as short as 1 hour still results
in substantial cytotoxicity. IC50 values under such assay conditions
are <5 nmol/L (Table 1), supporting the conclusion that the effects
of the drug are rapid and long lasting.

To determine whether the potential exists to generate analogues
that could retain potency but with better in vivo tolerance, we
designed a series of semisynthetic LMB analogues, which we
generically term NEIs. This series of LMB derivatives maintain
in vitro IC50 values for a 72-hour exposure in the subnanomolar
range (Table 1 and data not shown). We next determined if our semisynthetic LMB
analogue from our first series, compound 3, has a >15-fold higher
tolerance, we

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dosing schedule (data not shown). We focused on LMB and compound 3 to further characterize the mechanism of action and therapeutic potential of this class of compounds.

**Short exposures to NEIs cause an extended block of nuclear export.** To define the kinetics of nuclear export inhibition for this class of compounds, we examined the subcellular localization of a protein, RanBP1, which is dependent on CRM1 for nuclear export. In untreated SiHa (cervical cancer) cells, RanBP1 is localized to the cytoplasm (Fig. 1A), but treatment with LMB or compound 3 causes rapid accumulation of RanBP1 in the nucleus (Fig. 1B).

Using RanBP1, we showed that nuclear export is completely blocked by 30 minutes after exposure to 10 nmol/L compound 3 (Fig. 1C). Furthermore, consistent with the covalent nature of the drug interaction with CRM1, the nuclear export block persists well beyond the initial drug exposure. On removal of the drug, nuclear export remains inhibited and does not completely recover until ~24 hours after the initial exposure (Fig. 2). Similar results for both extent of nuclear export block and time frame of recovery were seen with exposure to 5 or 10 nmol/L LMB (Fig. 2 and data not shown). This long recovery time suggests that recovery from

### Table 1. NEI analogues retain the potency of LMB with better *in vivo* tolerance

<table>
<thead>
<tr>
<th>Analogue</th>
<th>SiHa (IC50, nmol/L) 1 h</th>
<th>SiHa (IC50, nmol/L) 72 h</th>
<th>HCT-116 (IC50, nmol/L) 1 h</th>
<th>HCT-116 (IC50, nmol/L) 72 h</th>
<th>SKNSH (IC50, nmol/L) 1 h</th>
<th>SKNSH (IC50, nmol/L) 72 h</th>
<th>Mouse MTD (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R= − OH</td>
<td>LMB</td>
<td>2.1</td>
<td>0.4</td>
<td>4.3</td>
<td>0.3</td>
<td>4.1</td>
<td>0.4</td>
</tr>
<tr>
<td>R=</td>
<td>1</td>
<td>1.9</td>
<td>0.4</td>
<td>4.4</td>
<td>1.5</td>
<td>3.2</td>
<td>0.9</td>
</tr>
<tr>
<td>R=</td>
<td>2</td>
<td>0.4</td>
<td>0.5</td>
<td>2.6</td>
<td>0.6</td>
<td>1.7</td>
<td>0.4</td>
</tr>
<tr>
<td>R=</td>
<td>3</td>
<td>1.6</td>
<td>0.5</td>
<td>3.3</td>
<td>0.8</td>
<td>2.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**NOTE:** IC50 was measured for various human cancer cell lines 72 h after either continuous drugging or a 1-h drug exposure using the Promega CellTiter-Glo Assay system. For MTD experiments, female C57Bl/6 mice were used. Seven to 10 animals per dose group were given vehicle or compound i.v. as a single bolus injection via the lateral tail vein. The MTD was defined as the maximum dose that induced <20% body weight loss for LMB, compound 2, and compound 3. We lost one mouse on day 3 for compound 1, so the MTD for this molecule was defined by mortality. There was no other mortality observed at the MTD dose in this study.

**Figure 1.** Nuclear export is rapidly blocked by treatment with LMB or compound 3. SiHa (human cervical cancer) cells were treated with 10 nmol/L compound 3 or LMB for the indicated time points up to 2 h. Cells were formaldehyde fixed and stained for RanBP1 (a biomarker for CRM1 inhibition; red) and Hoechst (to define the nucleus; blue). A, RanBP1 is localized to the cytoplasm in untreated cells. B, after drug treatment, nuclear localization of RanBP1 is detected in essentially 100% of the cells. C, for each time point, images were acquired and analyzed using a Cellomics ArrayScan Vti with Molecular Translocation software. By 30 min, RanBP1 is localized to the nucleus in the entire population. A minimum of 500 cells per well were analyzed.
nuclear export block requires de novo CRM1 synthesis, and this is supported by the lack of nuclear export recovery in cells incubated in the presence of the protein synthesis inhibitor, cycloheximide, after removal of the NEIs (Supplementary Fig. S1). The kinetics of nuclear export inhibition and recovery are consistent among multiple cell types tested including HCT-116 (colon cancer), U2OS (osteosarcoma), A549, NCI-H460 (lung cancers), LNCaP (prostate cancer), and others (data not shown).

Nuclear export inhibition leads to apoptosis in tumor cell lines but reversible cell cycle arrest in normal lung fibroblasts.

We next explored the downstream effects of treatment with NEIs. After a 1-hour exposure to compound 3, persistent cell cycle arrest is observed in tumor cell lines (SiHa cells) and in normal human lung fibroblasts (MRC5; Supplementary Table S1). The treated cells arrest in the G2 and/or G1 phase of the cell cycle, and the percentage of proliferating cells (S phase cells) is dramatically reduced in both tumor cells and normal lung fibroblasts, consistent with what has been previously observed for LMB (15, 16). Moreover, although normal lung fibroblasts respond to NEIs with a persistent cell cycle arrest, they remain viable after nuclear export inhibition. These results are consistent with previous work using multiple human normal fibroblast cell lines, which showed that cells regain their ability to proliferate after LMB-induced growth arrest on drug removal and passage by trypsinization (15). Similar results were observed in MRC5 cells exposed to compound 3 (Fig. 3A) or LMB; Fig. 3B and data not shown). In contrast, nuclear export inhibition in normal lung fibroblasts does not lead to the induction of apoptosis, consistent with the transient growth arrest observed on exposure to NEIs (13, 15).

In contrast to what was observed in normal lung fibroblasts, inhibition of nuclear export in cancer cells leads to an induction of multiple markers of apoptosis, including plasma membrane alterations and caspase activation. SiHa cervical cancer cells were treated for 1 hour with 10 nmol/L compound 3 followed by drug removal and continued growth. Seventy-two hours after the initial drug exposure, ~30% of the population is Annexin V positive, indicative of plasma membrane alterations, which occur in the early stages of apoptosis (Fig. 3A). Similarly, caspase activation is also observed in SiHa cells 72 hours after a 1-hour exposure to 25 or 500 nmol/L of compound 3 or LMB (Fig. 3B and data not shown for 25 nmol/L). Induction of apoptosis is observed in many other human tumor-derived cell lines [e.g., HCT-116 (colon), LoVo (colon), LNCaP (prostate), A498 (kidney), and others; data not shown]. In contrast, nuclear export inhibition in normal lung fibroblasts does not lead to the induction of apoptosis, consistent with the transient growth arrest observed on exposure to NEIs (13, 15).

Although a control compound, staurosporine, caused dramatic induction of caspases in MRC5, Wi-38, and IMR-90 normal lung fibroblast cell lines, no caspase activation was observed in any of these cell lines in response to high doses of NEIs (500 nmol/L compound 3 or LMB; Fig. 3B). Similarly, no Annexin V staining was observed in MRC5 cells exposed to compound 3 (Fig. 3A) or LMB (data not shown).

Nuclear export inhibition correlates with sustained p53 overexpression. We showed in multiple p53 wild-type cell lines that inhibition of nuclear export strongly correlates with up-regulation of p53. Using an immunofluorescence assay in a p53 wild-type osteosarcoma cell line (U2OS), we showed that p53 is up-regulated and localized to the nucleus in a dose-dependent manner and that p53 up-regulation correlated with nuclear export block (Fig. 4A). These results are consistent with previous work that showed that LMB treatment leads to p53 nuclear localization and

**Figure 2.** Nuclear export block persists after removal of NEI. SiHa (human cervical cancer) cells were treated with 5 nmol/L compound 3 for 1 h. The drug was removed by washing, and cells were allowed to recover in drug-free medium for the times indicated. Cells were formaldehyde fixed and stained for RanBP1 (a biomarker for CRM1 inhibition; red) and Hoechst (to define the nucleus; blue). A, in untreated cells, RanBP1 is localized to the cytoplasm. After 1 h of drug treatment with compound 3, nuclear localization of RanBP1 is detected in essentially 100% of the cells. B, for each time point, images were acquired and analyzed using a Cellomics ArrayScan Vti with Molecular Translocation software. A minimum of 500 cells per well were analyzed. Columns, average of two independent experiments. Nuclear export remains blocked in >80% of the cell population 8 h after the drug is removed, and a minority (~25%) of the cells remain at least partially blocked 24 h after the drug is removed.
activation in various cell types (9, 13–15, 17). Furthermore, in the HCT-116 colon cancer cell line, a 1-hour exposure to compound 3 leads to increased cellular p53 expression for up to 48 hours (Fig. 4B). This result establishes that a downstream response to CRM1 inhibition, in this case p53 up-regulation, can persist even after the nuclear export block is reversed at ~24 hours (see Fig. 2).

Previous studies have shown that the cytotoxic effects of LMB are reduced in human neuroblastoma and cervical cancer cell lines when the activity of endogenous p53 is abrogated by overexpression of a dominant negative p53 mutant (9, 15). Consistent with this result, addition of a small-molecule inhibitor of p53, pifithrin-α (18), blocked compound 3–induced apoptosis in SiHa cells (Fig. 4C). These results show that p53 can play an important role in mediating NEI-induced apoptosis.

**Novel NEIs show robust efficacy in multiple tumor models.** Because of the strong induction of apoptosis observed in compound 3 treated cells in culture (see Fig. 3), we harvested tumor samples to determine if this effect was also observed in vivo. HCT-116 tumor–bearing mice were treated with a single i.v. dose of either vehicle or compound 3, and tumors were harvested 24 hours later. Consistent with the induction of apoptosis observed in vitro, we observed evidence of increased apoptosis in vivo, as shown by an increase in the levels of cleaved caspase-3 (Supplementary Fig. S2).

We next compared the antitumor activities of compound 3 and LMB in mouse xenograft models. Given the strong biological rationale and supporting in vitro data showing that NEI induced activation of p53 in HPV-infected cells (9, 13), we first studied efficacy in the HPV-positive SiHa cervical cancer model. Tumor regression was induced by weekly compound 3 treatment for 3 weeks (Fig. 5A), and tumors began to shrink shortly after the mice had been exposed to compound 3 (day 4). At day 25,
tumor volumes were still significantly below those observed when dosing began, and in fact, two mice had no detectable tumors. In contrast, only a growth delay was found in the LMB-treated group (Fig. 5A), consistent with the limited in vivo efficacy previously observed for LMB (6). To further assess the duration of the antitumor effect of compound 3, we treated SiHa tumor–bearing mice with either vehicle or compound 3 once a week for 2 weeks and then monitored tumor response for a longer period. Compound 3–treated tumors again responded rapidly after the first dose, and 100% of tumors regressed for at least 4 weeks. Furthermore, tumors could not be detected in three of seven mice in the compound 3–treated group between days 26 and 40. Two of these mice remained tumor-free for >450 days and are considered to be therapeutically cured (Supplementary Fig. S3).

Many anticancer agents are less effective against larger tumors. To assess the antitumor activity of compound 3 in mice with a greater tumor burden, we allowed the SiHa tumors to grow to \( \sim 500 \text{ mm}^3 \) before treating with a single i.v. injection of compound 3. We found that a single administration of compound 3 was able to show antitumor activity in a dose-dependent manner, with 10 mg/kg causing tumor growth inhibition and 40 mg/kg causing significant tumor regression (Fig. 5B).

To determine if compound 3 is active in other tumor models, we tested for antitumor activity using an HCT-116 colon cancer xenograft model. Using this model, we showed that compound 3 significantly inhibited tumor growth when dosed at 30 mg/kg weekly (Fig. 5C). Furthermore, consistent with results in the SiHa model, 30 mg/kg of compound 3 was found to be significantly more efficacious and less toxic than 2.5 mg/kg of LMB (data not shown). Compound 3 at 30 mg/kg was found to be similarly efficacious as the 35 or 40 mg/kg dose in the SiHa and HCT-116 models but better tolerated (inducing only \( \sim 10\% \) body weight loss), and hence, 30 mg/kg was chosen as an appropriate dose at which to examine the activity of compound 3 in additional xenograft models. Antitumor activity was also observed in NCI-H460 (non-small-cell lung cancer), A375 (melanoma), and K562 [chronic myelogenous leukemia (CML)] cancer models (Supplementary Table S2). Thus, NEIs like compound 3, which have a significantly improved tolerability relative to LMB, show robust efficacy in multiple xenograft models.

**Discussion**

In this study, we have synthesized analogues of LMB, the prototypical NEI, which show potential as novel anticancer therapeutics. LMB itself has significant in vitro potency but is poorly tolerated in vivo (6, 7). The covalent nature of the interaction with CRM1 needs to be considered when comparing compounds. In vitro \( IC_{50} \) values for covalent inhibitors determined after prolonged drug exposure cannot be interpreted as indicators of target binding affinity, as they would be for reversible inhibitors, because covalent inhibitors have essentially infinite binding affinity for their target. The \( IC_{50} \) values for NEIs determined after short drug exposure serve as an indirect measure of the rate of CRM1 inactivation and thereby reflect the relative efficiencies of a series of compounds at inactivating CRM1. The observation that our series of NEIs retain essentially equivalent cytotoxicities to LMB after a 1-hour exposure (Table 1) shows that they retain the potency of LMB.

To define the kinetics of nuclear export inhibition for this class of compounds, we wanted to find an endogenous protein that is dependent on CRM1 for nuclear export and is ubiquitously expressed. Such a protein could serve as a useful biomarker for nuclear export in all cell types without the need for additional inducers of expression or nuclear import. We chose RanBP1, an
in vitro experiments show that exposure of cancer cells to NEIs downstream cytotoxic effects. Using RanBP1 as a biomarker, our in vitro experiments show that exposure of cancer cells to NEIs leads to a rapid and prolonged block of nuclear export in all cell types tested.

The observation that the induction of p53 is detectable at 24 or 48 hours (Fig. 4B) despite the fact that nuclear export has recovered in the majority of the cell population by 24 hours (Fig. 2B and data not shown) shows that the downstream effects of nuclear export inhibition can persist longer than the actual nuclear export block. Nuclear entrapment of p53 turns on the p53 transcriptional program, including activation of transcription of p53 itself. As the data show, this leads to a significant increase in p53 protein levels in the cell (Fig. 4; refs. 9, 15, 19). Once this program is turned on, because it is part of an autoactivation loop, its regulation need no longer depend on nuclear export block and will instead depend on factors such as the stability of p53 and the dynamic point at which p53 levels drop to the range in which the positive feedback loop is no longer engaged. Thus, a downstream consequence of nuclear export inhibition, in this case p53 induction, can persist independently of the original signal, which was the nuclear export block.

The inhibition of nuclear export is associated with an increase in multiple markers of apoptosis in cancer cells. In contrast to this, NEIs induce cell cycle arrest, but not apoptosis, in normal lung fibroblasts. Thus, although NEIs cause the inhibition of CRM1 in both tumor and normal cell types, a difference is observed in the downstream consequences of this inhibition. The basis of this difference in response remains under investigation.

We have synthesized novel NEIs that are up to 16-fold better tolerated than LMB in mouse models while retaining significant potency. These results suggest that the limited in vivo efficacy of LMB was likely due to off-target effects because our NEIs retain the potent inhibition of CRM1, but are clearly better tolerated in vivo. The reasons why the novel NEIs are better tolerated are currently under investigation. Areas of exploration include the tissue distribution profile of these molecules as well as an investigation of their in vivo on-target and off-target protein binding properties. The better tolerance enables these novel NEIs to be dosed at higher levels in vivo. As Fig. 5B shows, 10 mg/kg of compound 3, which is 4-fold higher than the MTD of LMB, shows only modest efficacy, whereas a dose of 40 mg/kg results in regression. This supports the conclusion that the low MTD of leptomycin limits its efficacy and higher doses are required for robust efficacy for compounds such as these, which show comparable activity in vitro. Doses above 30 mg/kg of compound 3 are associated with significant efficacy in multiple mouse xenograft models, thereby validating nuclear export as a potentially useful therapeutic target in cancer.

In this study, we have focused on p53 wild-type cancer models including an HCT-116 colon model and a SiHa cervical cancer model. In these models, the p53 tumor suppressor becomes trapped in the nucleus on inhibition of CRM1. In HPV-positive cancer types, such as SiHa, this prevents the aberrant cytoplasmic localization and degradation of p53 and leads to activation of pathways that cause cell cycle arrest and apoptotic cell death. We show that such effects are not limited to HPV-positive cancers because NEIs induce p53 activation (Fig. 4B) and show antitumor efficacy in the HCT-116 colon cancer model (Fig. 5C). Furthermore, we have also tested for antitumor activity of compound 3 in a variety of other tumor models including NCI-H460 (non–small-cell lung cancer), A375 (melanoma), and K562 (CML; Supplementary Table S2). Compound 3 showed antitumor activity in all of these models, ranging from induction of tumor regression to tumor growth inhibition. Thus, in contrast to the poor in vivo activity of LMB, the NEI analogue compound 3 shows robust efficacy in all xenograft models examined to date and is of great interest as a potential cancer therapeutic.

Although we have focused here on p53 wild-type cancer types, CRM1 mediates the nuclear export of numerous other proteins that are also important therapeutic targets. Various lines of evidence provide a strong biological rationale for the use of NEIs in multiple cancer types. For example, in CML, the oncogenic BCR-Abl tyrosine kinase is found in the cytoplasm where it activates a number of mitogenic signaling pathways (20–22). Treatment of CML cells with the tyrosine kinase inhibitor imatinib not only inhibits BCR-Abl but also promotes its shuttling into and out of the nucleus (23). Coadministration of LMB with imatinib was shown to cause nuclear accumulation of BCR-Abl, ultimately resulting in the activation of programmed cell death both in vitro (24) and in vivo experiments (24). Given the poor in vivo tolerance of LMB, a significant therapeutic benefit could be gained by combining a potent but better tolerated CRM1 inhibitor with an inhibitor of BCR-Abl. This combination has the potential to overcome the problem of drug resistance by eradicating, rather than inhibiting, the growth of CML cells.

Similarly, LMB inhibition of CRM1 has been shown to promote the nuclear buildup of the Forkhead family of transcription factors (FOXOs; refs. 25, 26). These transcription factors are regulated by multiple signaling pathways that play critical roles in tumorigenesis, including the PI3K/PTEN/Akt pathway (reviewed in refs. 27, 28). In PTEN-deficient cells, the Akt pathway is activated and FOXO transcription factors are rendered inactive by localization to the cytoplasm. Restoring PTEN function in these cells blocks Akt activity and restores nuclear localization of FOXO and, therefore, its ability to activate downstream factors. Prolonged FOXO residence in the nucleus leads to the induction of proapoptotic genes and ultimately to growth arrest and death in PTEN-null tumor cells (29, 30). In addition, it has recently been shown that localization of FOXO to the nucleus in PTEN-null cells inhibits Hif1 transcriptional activity (31), thereby potentially interfering with the ability of PTEN-null cells to survive under hypoxic conditions. It will thus be of great interest to examine the efficacy of NEIs in PTEN-deficient cancers.

In conclusion, the NEIs we have synthesized have enabled us to validate CRM1 as a target for anticancer therapeutics. These data show that the limited in vivo efficacy observed for LMB was a result of its poor tolerance. The identification of NEIs that are significantly better tolerated has shown that molecules with this mechanism of action can show robust in vivo efficacy. These results provide strong evidence supporting the development of NEIs as a novel anticancer therapy.
Disclosure of Potential Conflicts of Interest

All authors have ownership interest in Kosan Biosciences.

Acknowledgments

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References

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