Published in final edited form as:

J Urol. 2013 June ; 189(6): 2317–2326. doi:10.1016/j.juro.2012.10.018.

CRM1 Blockade by Selective Inhibitors of Nuclear Export (SINE) attenuates Kidney Cancer Growth

Hiromi Inoue^{1,2}, Michael Kauffman⁵, Sharon Shacham⁵, Yosef Landesman⁵, Joy Yang³, Christopher P. Evans^{3,4}, and Robert H. Weiss^{1,2,4,6}

¹Division of Nephrology, Dept. of Internal Medicine, University of California, Davis, CA, USA, 95616

²Comparative Pathology Graduate Group, University of California, Davis, CA, USA, 95616

³Department of Urology, University of California, Davis, CA, USA, 95616

⁴Cancer Center, University of California, Davis, CA, USA, 95616

⁵Karyopharm Therapeutics Inc., Natick, MA, 01760

⁶Medical Service, Sacramento VA Medical Center, Sacramento, CA, USA, 95655

Abstract

Since renal cell carcinoma (RCC) often presents asymptotically, patients are commonly diagnosed at the metastatic stage when treatment options are limited and survival is poor. Given that progression-free survival with current therapies for metastatic RCC is only one to two years and existing drugs are associated with a high rate of resistance, new pharmacological targets are desperately needed. We identified and evaluated the nuclear exporter protein, chromosome region maintenance protein 1 (CRM1), as a novel potential therapeutic for RCC.

Purpose—To evaluate novel, selective inhibitors of nuclear export as potential RCC therapeutics.

Materials and Methods—Efficacy of the CRM1 inhibitors, KPT-185 and -251, was tested in several RCC cell lines and in a RCC xenograft model. Apoptosis and cell cycle arrest were quantified, and localization of p53 family proteins was assessed using standard techniques.

Results—KPT-185 attenuated CRM1 and showed increased cytotoxicity in RCC cells *in vitro*, with evidence of increased apoptosis as well as cell cycle arrest. KPT-185 caused both p53 and p21 to remain primarily in the nucleus in all RCC cell lines, suggesting a mechanism of action of these compounds dependent upon tumor-suppressor protein localization. Furthermore, when administered orally in a high-grade RCC xenograft model, the bioavailable CRM1 inhibitor

Please address editorial correspondence and reprint requests to: Dr. Robert H. Weiss, Division of Nephrology, Department of Internal Medicine, Genome and Biomedical Sciences Building, Room 6312, University of California, Davis, CA. 95616, rhweiss@ucdavis.edu, Tel: 530-752-4010; FAX: 530-752-3791.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

KPT-251 significantly inhibited tumor growth *in vivo* with the expected on-target effects and with no obvious toxicity.

Conclusions—The CRM1 inhibitor family of proteins are novel therapeutic targets RCC and deserve further intensive investigation in this and other urologic malignancies.

Keywords

kidney cancer; nuclear transport; p53; p21; tumor suppressor

INTRODUCTION

Renal cell carcinoma (RCC) is the sixth most common cancer in the U.S. and one of the few cancers whose incidence is increasing.¹ Five year survival of patients with metastatic RCC is dismal.^{1, 2} For the one-third of patients who present at the metastatic stage, there are several FDA-approved drugs available, among them the multi-kinase inhibitors,³ some of which (e.g. sorafenib) also inhibit p21,⁴ and the mTOR inhibitors.⁵ Since progression-free survival even with these new drugs is only one to two years,² it is imperative that novel therapeutic targets for patients with metastatic RCC be identified and validated.

Chromosome region maintenance protein 1 (CRM1) is the receptor for the canonical nuclear export sequences (NES) and mediates nuclear export of NES containing proteins including the tumor suppressor proteins (TSP) p53 and p21.^{6, 7} Overexpression of CRM1, leading to increased nuclear export of the TSPs, has been shown to be a poor prognostic factor for many cancers including ovarian, liver, and pancreatic.^{8, 9} In addition, multiple studies show that inhibition of CRM1 induces apoptosis and inhibits tumor growth in several cancer cell lines.^{10, 11} However, despite abundant knowledge of subcellular TSP localization being important in RCC behavior,¹² there are no published studies of CRM1 inhibition in RCC.

We now show that the CRM1 inhibitor KPT-185 causes cell cycle arrest and apoptosis in RCC cell lines in association with p21 and p53 nuclear sequestration and that the orally available analog of KPT-185, KPT-251, inhibits tumor growth in RCC xenograft mice with minimal toxicity. To our knowledge, this is the first study to introduce CRM1 as a novel and promising target for RCC therapy, and our work suggests that further study of these compounds may revolutionize RCC treatment.

MATERIALS AND METHODS

Cell lines

ACHN, Caki-1, and 786-O were obtained from ATCC (Rockville, MD). ACHN cells were maintained in Dulbecco's modified Eagle's medium and 786-O and Caki-1 cells were supplemented maintained in RPMI with 10% fetal bovine serum (FBS), and pen/strep. The primary normal human kidney cell line, NHK, was provided by Darren Wallace (Univ. Kansas) and maintained in Dulbecco's modified Eagle's F12 medium supplemented with 5% FBS and pen/strep. Cells were maintained at 5% CO₂ at 37°C.

Materials

Kidney cancer tissues were obtained appropriate IRB approval. KPT-185 and -251 were synthesized by Karyopharm Therapeutics (Natick, MA). Sorafenib was obtained from LC Laboratories (Woburn, MA). KPT-185 and 251 and sorafenib free base were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies. KPT-251 was combined with vehicles Poloxamer 188 (Pluronic® F68; Spectrum Laboratory Products, Inc.) and PVP K-29/32 (Plasdone® K-29/32; ISP technologies, Inc.) in solution for the *in vivo* study. Sorafenib tosylate was dissolved in Cremophor EL and 95 % ethanol (1:1, v/v). Mouse monoclonal anti- β -actin antibody was obtained from Sigma (St. Louis, MO, USA); rabbit polyclonal anti-CRM1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal anti-p21^{WAF1/Cip} antibody from Millipore (Billerica, MA, USA); rabbit monoclonal anti-PARP antibody, rabbit monoclonal anti-phospho-extracellular-signal-regulated kinase (ERK) antibody, rabbit monoclonal anti-p21^{WAF1/Cip} antibody, mouse monoclonal anti-phospho-MDM2 antibody, mouse monoclonal anti-p53 antibody, anti-rabbit IgG (Alexa Fluor® 488 Conjugate), and anti-mouse IgG (Alexa Fluor® 488 Conjugate) from Cell Signaling Technology (Beverly, MA, USA); Ki67 antibody rabbit monoclonal SP6 from Cell Marque (Rocklin, CA, USA); goat anti-mouse and goat anti-rabbit HRP conjugated IgG from Bio-Rad (Hercules, CA, USA). VECTASHIELD HardSet Mounting Medium with DAPI and DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine were from Vector Laboratories (Burlingame, CA, USA) and BD Cycletes Plus from BD Biosciences (Sparks, MD, USA)

Immunohistochemistry

Human RCC (grades 3–4) and adjacent normal formalin fixed paraffin embedded tissues were deparaffinized and blocked in the blocking buffer (5 % normal goat serum and 0.3 % Triton X-100 in PBS). The slides were incubated with primary and secondary antibodies, and DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine was applied. Hematoxylin was the counterstain. All photos were analyzed using ImageJ¹³ under the same settings (contrast and brightness).

Immunoblotting

Immunoblotting was done as described previously.⁴ Briefly, after appropriate treatments, the cells were lysed, and cell lysates immunoblotted and the signal was detected using enhanced chemiluminescence solutions.

MTT assay

Cell viability assay was conducted as described previously.⁴ Briefly, after appropriate treatments for 72 hours, the cells were incubated in MTT solution/media mixture. Then, the MTT solution was removed and the blue crystalline precipitate in each well was dissolved in DMSO. Visible absorbance of each well at 540 nm was quantified using a microplate reader. The 72 hour incubation time was chosen following the NCI60 cell line screening protocol.

Colony formation assay

1.5×10^4 cells were seeded, treated in triplicate with KPT-185 (300 nM), and then stained with crystal violet (Ricca Chemical company, Texas) at 3, 5, 7, 9 and 12 days following compound treatment. Then, crystal violet from stained cells was dissolved with 10% acetic acid and its absorbance quantified at OD595.

Immunofluorescence

After appropriate treatments, immunofluorescence was conducted as described previously.⁴ Briefly, the cells were fixed in 2% paraformaldehyde and blocked in the blocking buffer. After blocking, the cells were incubated with appropriate antibody, incubated with the appropriate secondary antibody, and coverslipped with vectashield with DAPI. The specimens were examined by confocal microscopy.

Cell cycle analysis by FACS

After appropriate treatment, the cells were collected and washed with PBS. After washing, BD Cycles Plus was applied according to the manufacturer's instructions to stain the cells with propidium iodide. DNA content was determined by flow cytometry using a FACScan flow cytometer and the data were analyzed by software FlowJo.

Caki-1 xenograft mice experiment

All animal procedures were performed in compliance with the University of California Institutional Animal Care and Use Committee. Male athymic Nu/Nu mice were injected with 1×10^6 Caki-1 cells subcutaneously into the flank region. Tumor progression was monitored weekly with a caliper. When tumor sizes reached around 80–100 mm³, the treatments were started. Vehicle solution (Pluronic F-68 and PVP K-29/32 mixture) was given orally (n = 8). Low dose of KPT-251 (25 mg/kg (week 1), 30 mg/kg (week 2), 35 mg/kg (week 3), and 40 mg/kg (week 4)) was given orally (n = 8). High dose of KPT-251 (75 mg/kg (week 1 – 4)) was given orally (n = 8). Sorafenib tosylate (30 mg/kg (week 1 – 4)) was given orally (n = 7). Vehicle and KPT-251 groups were treated three times a week. The sorafenib group was treated everyday. The tumor volume was measured by calipers twice a week using the formula tumor volume (mm³) = length*width²/2. The tumor growth rate was calculated as tumor volume on day X/tumor volume on day 1.

Statistical analysis

Comparisons of mean values were performed using the independent samples t-test. A p-value of < 0.05 was considered significant. XLfit software (Idbs, UK) was used for the analysis of cytotoxicity and the calculation of EC50.

RESULTS

CRM1 is overexpressed in high grade RCC; Selective Inhibitors of Nuclear Export (SINE) compounds KPT-185 and -251 specifically reduce levels of CRM1 in RCC cells

CRM1 overexpression has been shown to correlate with tumor grade, as well as to be an indicator of a poor prognosis, in many non-kidney cancers.^{8,9} To test this issue in kidney

cancer, we evaluated several archived human RCC tissues. While CRM1 was seen both in the nucleus and the cytosol in the normal tubule epithelial cells, CRM1 was localized mainly to the cytosol in the cancer cells (Supplemental Data Fig. 1). High grade (Fuhrman 3–4) RCC tissues showed substantially higher levels of CRM1 expression as compared to normal tissues (Supplemental Data Fig. 1), suggesting that CRM1 levels indeed correlate with transformation in RCC and further may play a role in RCC oncogenesis as has been proposed for other cancer types.^{8, 9}

For the majority of *in vitro* experiments in this study, we utilized the novel CRM1 inhibitor KPT-185 (Supplemental Data Fig. 2a), which blocks HIV-Rev nuclear export with an IC50 of ~20nM.¹⁴ KPT-185 has shown inhibition of CRM1 in non-RCC cancer cell lines as well as in normal cells.¹⁵ When incubated with KPT-185, both von Hippel-Lindau (VHL) - negative (786-O) and VHL-wt (ACHN and Caki-1) cell lines showed marked reduction of CRM1 levels (Fig. 1).

KPT-185 caused cytotoxicity in RCC cells

We next examined the cytotoxicity of CRM1 as compared to sorafenib, one of the conventional targeted RCC therapeutics that we have shown to affect the TSPs.⁴ KPT-185 caused higher cytotoxicity than sorafenib at 0.1 and 1 μ M (serum concentrations commonly achievable when sorafenib is used clinically¹⁶) in all RCC cell lines (Fig. 2). In addition, the VHL negative cell line 786-O showed higher levels of toxicity at a given concentration of KPT-185, a significant finding because the majority of ccRCCs are VHL negative.

Of interest with regard to possible adverse effects, sorafenib showed higher cytotoxicity in the “normal” renal epithelial cell line NHK as compared to KPT-185 at its EC50: 80% NHK growth inhibition was seen at sorafenib’s EC50 for RCC cells, while only 50% NHK growth inhibition was seen at KPT-185’s EC50 for RCC cells, suggesting a possible clinical advantage of KPT-185 over sorafenib (Fig. 2). Colony formation assays, done in parallel with the MTT assays, support this data (Supplemental Data Fig. 3).

The concentration of KPT-185 utilized in our cell lines, 0.1 and 1 μ M, are those achievable in serum with the related orally bioavailable SINE compound, KPT-251 (*vide infra*). The latter compound showed minimal systemic toxicity, and no renal toxicity has been observed with any of the SINE compounds tested thus far in multiple animal species (Shacham et al., unpublished observations).

In order to determine whether the MTT results were due principally to reduced cell proliferation or to cell death (apoptosis or lysis), we performed cell cycle analyses after the cells were incubated with KPT-185 for 24 hours, a time chosen since the cell cycle duration for most cells is 16 to 24 hours. All of the cell lines showed G₂/M arrest, while G₁ arrest also occurred in the VHL negative cell line (786-O), and there was an increase in the sub-G₀ cell population in all RCC cell lines tested (Fig. 3). In addition, as measured by PARP cleavage, apoptosis was induced in a concentration-dependent manner in all of the RCC cell lines examined at concentrations of KPT-185 starting at 1 μ M (Fig. 4) which is a concentration readily achievable *in vivo* (Shacham et al., unpublished observations).

Both p53 and p21 were confined to the nucleus after KPT-185 incubation

Both p53 and its downstream protein p21 require nuclear localization to mediate their effects on cell cycle arrest and subsequent apoptosis.^{12, 17–19} All of these proteins are exported to the cytosol by CRM1 where they can be degraded and/or sequestered.^{6, 20, 21} Thus, we evaluated the expression levels of selected TSPs, p53 and p21, as well as of phospho-MDM2 (p-MDM2), which is induced by p53 and negatively regulates its stability and transcription.²² KPT-185 increased levels of p53 and p21 in all three RCC cell lines evaluated (Fig. 4). Although KPT-185 increased p-MDM2 level in all three cell lines examined, the changes in ACHN and Caki-1 cells were more pronounced than those in 786-O cells.

In light of the nuclear transport function of CRM1, subcellular localization of p53 and p21 was evaluated when the cells were exposed to the CRM1 inhibitors. When examined by immunofluorescence, p53 was confined to the nucleus by KPT-185 in VHL-wt cells (ACHN and Caki-1), as is readily apparent by decreased fluorescence in the cytosolic compartment (Fig. 5). p21 was similarly nuclearly confined in these cell lines (Fig. 6). VHL-negative cells (786-O) showed similar changes in p53 and p21 (Fig 5 and 6), except that p53 localization did not change, consistent with p53 being mutant in this cell line. These data are consistent with data in other cell lines in other studies,^{8, 9, 23, 24} suggesting that nuclear localization of the TSPs is involved in CRM1 inhibition mediated tumor growth inhibition.

The mechanism of cell cycle arrest and apoptosis induction by KPT-185 and sorafenib were distinct

Due to the differences observed in the cytotoxicity between KPT-185 and sorafenib in RCC cell lines, we compared their molecular mechanisms. Sorafenib inhibits p21 levels⁴ as well as other kinases including those in the Raf/MAPK pathway.³ In all three cell lines examined in the present study, sorafenib reduced levels of p21 and p-ERK, as expected (Supplemental Data Fig. 4). In contrast, KPT-185 markedly increased nuclear p21 levels in ACHN, Caki-1, and 786-O, and p-ERK in 786-O (Figs. 4, 5, and 6); the disparity in p-ERK levels among cell lines is likely related to their VHL status, as HIF-1 α (which is destabilized by wild-type VHL) lies upstream of ERK.²⁵ Additional differences were apparent decreased CRM1 and increased nuclear p53 levels by KPT-185 (Fig 1, 4, and 5); sorafenib did not change these protein levels in all three cell lines (Supplemental Data Fig. 4). Thus, the mechanisms of sorafenib- and CRM1-induced RCC cell growth inhibition are distinct.

KPT-251 inhibited tumor growth in vivo by proliferation inhibition and apoptosis induction

Since KPT-185 is not orally available in animals, we utilized the highly related, orally bioavailable CRM1 inhibitor KPT-251 (Supplemental Data Fig. 2b) for an *in vivo* study. Both compounds have markedly similar structures (Supplemental Data Fig. 2a and b) and activities (see Figs. 1, 2, and 4, and Supplemental Data Fig. 5). KPT-251 shows ~30% oral bioavailability in mice (compared with <5% for KPT-185) with essentially identical selectivity, and has been tested in other xenograft systems (Shacham, unpublished observations).

Caki-1 cells were implanted subcutaneously in nude mice and KPT-251 was administered orally thrice weekly. The low and high doses of KPT-251 both dose-dependently inhibited tumor growth, and inhibition of tumor growth in the KPT-251 high dose group was higher ($p = 0.07$) than the sorafenib group (Fig. 7). Animals administered KPT-251 showed no adverse effects, while the sorafenib animals showed skin abnormalities (photographs, Fig. 7) as is seen in patients.²⁶ Tumor tissues obtained at the end of the experiments showed grade four morphology (Supplemental Data Fig. 6), and immunohistochemistry yielded fewer Ki67 positive cells in the KPT-251 high dose group as compared to the vehicle group, confirming inhibition of proliferation by KPT-251 *in vivo* (Fig 8) and consistent with the *in vitro* data. KPT-251 also increased nuclear p53 in the tumor tissues (Fig. 8), consistent with the *in vitro* data (see Supplemental Data Fig. 5), and showed markedly increased apoptosis as evidenced by increased TUNEL staining (Fig. 8). These data demonstrate the efficacy and expected on-target effects of KPT-251 in the grade four xenograft mice.

DISCUSSION

Kidney cancer is diagnosed in 36,000 patients and is the cause of death of 11–13,000 individuals per year in the US²⁷ and survival of patients with metastatic RCC is truly dismal (<10% five-year survival). For the patients with metastatic RCC, progression-free survival is only up to two years even with newer FDA approved targeted therapeutics such as VEGF inhibitors and mTOR inhibitors.² In this study, we introduce CRM1 as a novel potential target in this poorly treatable disease.

Here, we show improved RCC growth inhibition by the CRM1 inhibitor KPT-185 and -251 when compared to sorafenib both *in vitro* and *in vivo*, and an association of KPT-185 with increased nuclear and decreased cytosolic p53 and p21. While causal effects of CRM1 inhibition and CRM1 target proteins have not yet been confirmed, our data strongly suggest that KPT-185's enhanced antitumor potential, in contrast to sorafenib, is occurring at least partially by restricting p53 and p21 localization to the nucleus where they arrests the cell cycle. In addition, by reducing p21's levels in the cytosol where it is known to inhibit apoptosis,¹² these inhibitors induce apoptosis thereby taking a two-pronged salutary approach (cell cycle inhibition and apoptosis induction) to RCC therapy. By contrast, sorafenib reduces both nuclear and cytosolic p21,⁴ and only induces apoptosis but not cell cycle arrest (Fig. 9), findings which might explain the differential toxicity profiles of these compounds. Indeed, KPT-251 showed no toxicity in mice in contrast to sorafenib (see Fig. 7, photographs).

We have shown that p21 can direct cells into either the growth suppressive or anti-apoptotic pathways, and that p21 is a poor prognostic marker when located in the cytosol in both RCC and breast cancer.²⁸ In addition, forced cytosolic localization of p21 results in growth promotion and antagonism of apoptosis.¹² Indeed, and likely for this reason, p21 induction with cytosolic localization has been shown to be an early event in oncogenesis.²⁹ Our results showed that KPT-185 increased *nuclear* p21 and decreased *cytosolic* p21 in all RCC cells tested. Since it is known that p21 transcription is activated by p53 and that p21 is translocated by CRM1, this finding is likely due to both increased p53 activity as well as inhibition of CRM1 activity (with decreased CRM1 expression) by KPT-185. These results

show that CRM1 inhibition can modulate p21 localization with dual anti-neoplastic effects: restoration of TSP/cell cycle inhibition *nuclear* functions of p21, and reduction in anti-apoptotic *cytosolic* functions of p21 (Fig. 9).

We noted that p-ERK levels were increased in the VHL negative cell line 786-O (see Fig. 4). However, the cell viability assay data (see Fig. 2) showed better efficacy of KPT-185 in VHL negative cells than in VHL wild type cells, suggesting that increased p-ERK by KPT-185 does not decrease its toxicity towards RCC cells. It is possible that p-ERK level in VHL negative cells is more sensitive to CRM1 inhibition than in VHL wild type cells since CRM1 is one of the major regulators when VHL is absent.²⁵

CONCLUSIONS

In this study we introduce CRM1 as a novel therapeutic target for the treatment of RCC, a disease currently with severely limited treatment options. We show that the nuclear exporter CRM1 is overexpressed in RCC as compared to normal tissue and that inhibition of CRM1 with KPT-185 and -251 is associated with nuclear retention, and therefore increased levels, of several key p53-pathway proteins, leading to proliferation inhibition and apoptosis induction in RCC both *in vitro* and *in vivo*. Thus, CRM1 inhibitors are exciting new targets ripe for further investigation of novel treatment options for kidney cancer.

Acknowledgments

This work was supported by NIH grants 5U01CA86402 (Early Detection Research Network), 1R01CA135401-01A1, and 1R01DK082690-01A1 (to R.H.W.), and the Medical Service of the US Department of Veterans' Affairs (R.H.W.). This study was also partially funded by Karyopharm Therapeutics.

ABBREVIATIONS

CRM1	chromosome region maintenance protein 1
SINE	selective inhibitors of nuclear export
RCC	renal cell carcinoma
TSP	tumor suppressor protein(s)

References

1. Cairns P. Renal cell carcinoma. Cancer biomarkers: section A of Disease markers. 2010; 9:461.
2. Belldegrun AS, Klatte T, Shuch B, et al. Cancer-specific survival outcomes among patients treated during the cytokine era of kidney cancer (1989–2005): a benchmark for emerging targeted cancer therapies. Cancer. 2008; 113:2457. [PubMed: 18823034]
3. Motzer RJ, Bukowski RM. Targeted therapy for metastatic renal cell carcinoma. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2006; 24:5601. [PubMed: 17158546]
4. Inoue H, Hwang SH, Weckslar AT, et al. Sorafenib attenuates p21 in kidney cancer cells and augments cell death in combination with DNA-damaging chemotherapy. Cancer biology & therapy. 12:2011.
5. Voss MH, Molina AM, Motzer RJ. mTOR inhibitors in advanced renal cell carcinoma. Hematology/oncology clinics of North America. 2011; 25:835. [PubMed: 21763970]

6. Turner JG, Sullivan DM. CRM1-mediated nuclear export of proteins and drug resistance in cancer. *Current medicinal chemistry*. 2008; 15:2648. [PubMed: 18991627]
7. Koyama M, Matsuura Y. An allosteric mechanism to displace nuclear export cargo from CRM1 and RanGTP by RanBP1. *The EMBO journal*. 2010; 29:2002. [PubMed: 20485264]
8. Noske A, Weichert W, Niesporek S, et al. Expression of the nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 is a prognostic factor in human ovarian cancer. *Cancer*. 2008; 112:1733. [PubMed: 18306389]
9. van der Watt PJ, Maske CP, Hendricks DT, et al. The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. *International journal of cancer Journal international du cancer*. 2009; 124:1829. [PubMed: 19117056]
10. Sakakibara K, Saito N, Sato T, et al. CBS9106 is a novel reversible oral CRM1 inhibitor with CRM1 degrading activity. *Blood*. 2011
11. Shao C, Lu C, Chen L, et al. p53-Dependent anticancer effects of leptomycin B on lung adenocarcinoma. *Cancer chemotherapy and pharmacology*. 2011; 67:1369. [PubMed: 20803015]
12. Asada M, Yamada T, Ichijo H, et al. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *The EMBO journal*. 1999; 18:1223. [PubMed: 10064589]
13. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012; 9:671. [PubMed: 22930834]
14. Turner JG, Dawson JL, Shacham S, Kauffman M, Sullivan DM. Novel inhibitors of nuclear export as a treatment for multiple myeloma (Ref Type: Abstract). *AACR-EORTC*. 2011
15. Azmi AS AKA, Aboukameel A, Kauffman M, McCauley D, Mohammad RM. Development of a Novel Small Molecule CRM-1 Inhibitor for Non Hodgkin's Lymphoma. *Blood*. 2011; 118
16. Awada A, Hendlisz A, Gil T, et al. Phase I safety and pharmacokinetics of BAY 43-9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. *British journal of cancer*. 2005; 92:1855. [PubMed: 15870716]
17. Zhang Y, Fujita N, Tsuruo T. Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene*. 1999; 18:1131. [PubMed: 10022118]
18. Zhou BP, Liao Y, Xia W, et al. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature cell biology*. 2001; 3:245. [PubMed: 11231573]
19. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature*. 2002; 419:729. [PubMed: 12384701]
20. Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. *Nature reviews Cancer*. 2004; 4:106. [PubMed: 14732865]
21. Wang D, He F, Zhang L, et al. The role of p27(Kip1) phosphorylation at serine 10 in the migration of malignant glioma cells in vitro. *Neoplasma*. 2011; 58:65. [PubMed: 21067268]
22. Alarcon-Vargas D, Ronai Z. p53-Mdm2—the affair that never ends. *Carcinogenesis*. 2002; 23:541. [PubMed: 11960904]
23. Kanai M, Hanashiro K, Kim SH, et al. Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly(ADP-ribosyl)ation. *Nature cell biology*. 2007; 9:1175. [PubMed: 17891139]
24. Naniwa J, Kigawa J, Akeshima R, et al. Leptomycin B enhances CDDP-sensitivity via nuclear accumulation of p53 protein in HPV-positive cells. *Cancer science*. 2003; 94:1099. [PubMed: 14662026]
25. Irisarri M, Lavista-Llanos S, Romero NM, et al. Central role of the oxygen-dependent degradation domain of Drosophila HIFalpha/Sima in oxygen-dependent nuclear export. *Molecular biology of the cell*. 2009; 20:3878. [PubMed: 19587118]
26. Lee WJ, Lee JL, Chang SE, et al. Cutaneous adverse effects in patients treated with the multitargeted kinase inhibitors sorafenib and sunitinib. *The British journal of dermatology*. 2009; 161:1045. [PubMed: 19558553]
27. Weiss RH, Lin PY. Kidney cancer: identification of novel targets for therapy. *Kidney international*. 2006; 69:224. [PubMed: 16408110]

28. Weiss RH. p21Waf1/Cip1 as a therapeutic target in breast and other cancers. *Cancer cell*. 2003; 4:425. [PubMed: 14706334]
29. Biankin AV, Kench JG, Morey AL, et al. Overexpression of p21(WAF1/CIP1) is an early event in the development of pancreatic intraepithelial neoplasia. *Cancer research*. 2001; 61:8830. [PubMed: 11751405]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. Decreased CRM1 in RCC cells by KPT-185 and -251

VHL wild type (ACHN and Caki-1) and VHL negative (786-O) cells were grown to confluence in 10% serum-containing media and treated with KPT-185, KPT-251, or vehicle (DMSO) at the indicated concentrations for 24 hours. The cells were harvested and immunoblotted with the antibodies indicated as described in Materials and Methods. β-actin is a loading control. The experiment shown is representative of at least 3 separate experiments.

Figure 2. KPT-185 and -251 had higher toxicity than sorafenib in RCC and NHK cells
VHL wild type (ACHN and Caki-1) and VHL negative (786-O) cells were grown to confluence as described in Fig. 1 and treated with KPT-185, KPT-251, sorafenib, or vehicle (DMSO) for 72 hours in their growth media. Subsequently, an MTT assay was performed as described in Materials and Methods. Error bars indicate standard deviation. The experiment shown is representative of at least 3 separate experiments.

Figure 3. KPT-185 arrested cell cycle and increased the cells in apoptotic phase

VHL wild type (ACHN and Caki-1) and VHL negative (786-O) cells were incubated in serum reduced media (5% serum) for 24 hours and treated with KPT-185 or vehicle (DMSO) for 24 hours in regular growth media. After treatment, the cells were prepared for cell cycle analysis as described in Materials and Methods.

Figure 4. KPT-185 and -251 induce PARP cleavage, p53, and p21

VHL wild type (ACHN and Caki-1) and VHL negative (786-O) cells were grown to confluence as described in Fig. 1 and treated with KPT-185, KPT-251, or vehicle (DMSO) at the indicated concentrations for 24 hours. The cells were harvested and immunoblotted with the antibodies indicated. β -actin is a loading control. The experiment shown is representative of at least 3 separate experiments.

Figure 5. KPT-185 kept p53 in nucleus

VHL-wt (ACHN and Caki-1), and VHL negative (786-O) cells were grown to confluence on 8 well chamber slides and treated with KPT-185 or vehicle (DMSO) at the indicated concentrations for 24 hours. The cells were subjected to immunofluorescence visualization by confocal microscopy as described in Materials and Methods. p53 in the cell lines are shown; comparison of the merged panels shows markedly less cytosolic p53 in the KPT-185 treated cells as compared to the vehicle controls. The signaling proteins are stained green and the nuclear dye (DAPI) is blue.

Figure 6. KPT-185 kept 21 in nucleus

VHL-wt (ACHN and Caki-1), and VHL negative (786-O) cells were grown to confluence on 8 well chamber slides and treated with KPT-185 or vehicle (DMSO) at the indicated concentrations for 24 hours. The cells were subjected to immunofluorescence visualization by confocal microscopy as described in Materials and Methods. p21 in the cell lines are shown comparison of the merged panels shows markedly less cytosolic p21 in the KPT-185 treated cells as compared to the vehicle controls. The signaling proteins are stained green and the nuclear dye (DAPI) is blue.

Figure 7. KPT-251 inhibited tumor growth in vivo

Caki-1 cells were injected subcutaneously and tumor volume was measured as described in Materials and Methods. While the mice in the sorafenib treatment group evidence of toxicity (including a skin rash), the mice in KPT-251 treatment groups (both low and high dose) had no such signs. Error bars indicate standard error; * $p < 0.05$ compared to Vehicle group. $^{\dagger}p = 0.051$ (251 low vs 251 high).

Figure 8. KPT-251 showed the expected on-target effects in Caki-1 xenograft tissues
Representative images of Caki-1 xenograft tissue after sacrifice of the experimental mice. The tissue was subjected to immunohistochemical analysis of the proliferation marker Ki67, as well as of p53 and TUNEL.

Figure 9. Increased p21 by CRM1 inhibition induced apoptosis and inhibited proliferation
Cytosolic p21 inhibits apoptosis, while nuclear p21 arrests the cell cycle and may initiate a “genome survey”. By inhibiting both cytosolic and nuclear p21, sorafenib induces apoptosis. In contrast, by attenuating cytosolic p21 *and* increasing nuclear p21, KPT-185 results in a two-pronged attack by inducing apoptosis *and* arresting proliferation.