Computational repositioning and preclinical validation of pentamidine for renal cell cancer

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Abbreviations:

C-MAP = Connectivity Map
ccRCC = clear cell renal cell carcinoma
GADD45 = growth arrest and DNA damage inducible 45
GSEA = Gene Set Enrichment Analysis
HDAC = histone deacetylase
HIF-1α = hypoxia-inducible factor 1α
IBA = individualized bioinformatics analysis
IL-2 = interleukin-2
IPA = Ingenuity Pathway Analysis
ITPKA = inositol-triphosphate 3-kinase A
MDA-7/IL-24 = melanoma differentiation associated gene-7/interleukin-24
mTOR = mechanistic target of rapamycin
PARP = poly(ADP-ribose) polymerase
PDGFR = platelet-derived growth factor receptor
RCC = Renal cell cancer
RMA = robust multi-chip analysis
RPS6KA1 = ribosomal protein S6 kinase, 90kDa, polypeptide 1
VEGF = vascular endothelial growth factor
VEGFR = VEGF receptor
VHL = Von Hippel-Lindau
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Abstract

While early stages of clear cell renal cell carcinoma (ccRCC) are curable, survival outcome for metastatic ccRCC remains poor. We previously established a highly accurate signature of differentially expressed genes that distinguish ccRCC from normal kidney. The purpose of the current study was to apply a new individualized bioinformatics analysis (IBA) strategy to these transcriptome data in conjunction with Gene Set Enrichment Analysis of the Connectivity Map (C-MAP) database to identify and reposition FDA-approved drugs for anti-cancer therapy. Here we demonstrate that one of the drugs predicted to revert the RCC gene signature towards normal kidney, pentamidine, is effective against RCC cells in culture and in a RCC xenograft model. ccRCC-specific gene expression signatures of individual patients were used to query the C-MAP software. Eight drugs with negative correlation and p-value <0.05 were analyzed for efficacy against RCC in vitro and in vivo. Our data demonstrate consistency across most ccRCC patients for the set of high scoring drugs. Most of the selected high scoring drugs potently induce apoptosis in RCC cells. Several drugs also demonstrate selectivity for VHL negative RCC cells. Most importantly, at least one of these drugs, pentamidine, slows tumor growth in the 786-O human ccRCC xenograft mouse model. Our findings suggest that pentamidine might be a new therapeutic agent to be combined with current standard-of-care regimens for metastatic ccRCC patients and support our notion that IBA combined with C-MAP analysis enables repurposing of FDA-approved drugs for potential anti-RCC therapy.
Introduction

Renal cell cancer (RCC) consists of multiple subtypes based on histological classification, and RCC incidence continues to increase, with up to 30% of cases with distant metastases at initial diagnosis (1). Clear cell RCC (ccRCC), the most common and malignant RCC subtype, comprises around 70% of all RCC tumors (2). Distinct molecular alterations and different clinical outcomes differentiate the RCC subtypes, indicating multiple mechanisms of RCC pathogenesis (3,4).

Patients with localized RCC tumors usually undergo nephrectomy, an often curative treatment. However, treatment options for patients with metastatic disease are limited and do not take into consideration the individual pathophysiological and molecular perturbations among different patients. The main therapeutic approach for metastatic RCC is using high doses of interleukin-2 (IL-2). While this therapy has a relatively low response rate, a small percentage of patients exhibit complete remission upon treatment (5).

Based on increased elucidation of the molecular mechanisms linked to ccRCC and the high vascularization and angiogenesis associated with ccRCC, several new FDA approved anti-angiogenic therapies have recently entered clinical evaluation for metastatic ccRCC. Current agents demonstrating efficacy include bevacizumab, sunitinib, sorafenib, and pazopanib, which inhibit angiogenic pathways through vascular endothelial growth factor (VEGF) receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and Raf serine/threonine kinase pathways (6-9), and mechanistic target of rapamycin (mTOR) pathway inhibitors, temsirolimus and everolimus (10-11). Nevertheless, almost all patients eventually fail to respond to any of these therapies and succumb to the disease. The variability in RCC clinical outcome among different patients is likely due to the molecular heterogeneity of RCC, which so
far has not been considered for stratification of patients into different treatment protocols.

Transcriptome analysis of RCC has revealed alterations in gene expression specific for RCC subtypes and linked to outcome (4,12). We previously identified specific gene signatures for each RCC subtype, for disease progression and metastasis at time of diagnosis, and reported a distant metastasis gene signature (13). We also reported a protein signature that accurately predicted IL-2 therapy response in RCC patients (14). Identification of such gene signatures for RCC has not only provided new insights into the molecular mechanisms and biological pathways involved in RCC development and progression, but has also opened the door for evaluating more targeted approaches to RCC therapy and for exploring the potential of disease gene signatures to predict which drugs may have an impact on the disease.

Transcriptional profiling of several human cancer cell lines treated with bioactive small molecules (primarily FDA-approved drugs) led to the establishment of the Connectivity Map (C-MAP) database (http://www.broadinstitute.org/cmap) of, originally, build 01 (a compendium of 453 genome-wide gene expression profiles for a spectrum of 164 small molecules) and, recently, build 02 (more than 7000 genome-wide expression profiles for 1309 small molecules) which can be exploited to discover functional connections between the drug gene signatures and disease gene signatures (15). C-MAP analysis involves the ranking of drugs based on the highest inverse correlation with the disease-specific gene signatures, providing a score for each drug. Drug gene signatures that are most opposite to disease-specific gene signatures (ccRCC vs. normal kidney) are predicted to reverse the disease phenotype towards the healthy state. Several recent publications confirmed the use of C-MAP analysis to identify drugs
for diseases based on gene expression signatures and provided proof-of-concept for repurposing drugs with efficacy against a disease based on in silico prediction (16).

Although transcriptome analysis has provided major breakthrough discoveries in cancer, most of the bioinformatics approaches are based on population or group analysis. Few approaches have taken into account gene expression differences in individual cancer patients. The obvious bottlenecks for any such analysis are the number of variables (ten thousands of genes for each patient) and the inability to apply significance estimating statistical approaches to it. While being aware of the shortcomings of analysis of transcriptome data for individual patients, we have developed a novel Individualized Bioinformatics Analysis (IBA) strategy (Bhasin et al., manuscript in preparation) to personalize gene expression analysis and to incorporate the heterogeneity and individual differences in order to identify gene expression changes, signaling pathway alterations as well as potential biomarkers and drug targets or drug signatures in individual cancer patients. In this study, we applied this individualized bioinformatics approach to derive RCC specific gene signatures from patient samples and to identify, by C-MAP analysis of these gene signatures, candidate drugs that are anticipated to revert the ccRCC gene signature towards a healthy kidney gene expression profile (see flowchart of overall study design in Supplemental Figure 1). We clearly demonstrate that several FDA-approved drugs scoring high upon C-MAP analysis strongly induce apoptosis in RCC cell lines, and further enhance apoptosis when used in combinations. We, furthermore, show significant tumor inhibitory effects of pentamidine in a xenograft model of RCC. Overall, our data provide strong evidence for the potential of computationally repurposing FDA-approved drugs for the treatment of RCC.
Materials and Methods

Cell Culture. The renal cell cancer cell lines ACHN, UOK and 786-O, and Human Embryonic Kidney 293 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The MS-1 endothelial cell line and the F-12 foreskin fibroblast cell line were kindly provided by Dr. Peter Oettgen and Dr. Steven Goldring, respectively, Beth Israel Deaconess Medical Center, Boston, USA. RCC4 VHL(-) (ECACC catalogue no. 03112702) and RCC4 VHL (+) (ECACC catalogue no. 03112703) renal cell cancer cell lines were obtained from Sigma-Aldrich (17). 786-O cells expressing wild type VHL were kindly provided by Dr. Vikas Sukhatme, Division of Interdisciplinary Medicine and Biotechnology, Beth Israel Deaconess Medical Center, Boston, USA. Cell culture conditions are provided in Supplementary Methods. All cell lines were obtained from either ATCC or Public Health England and authenticated via short tandem repeat (STR) profiling performed by ATCC or Public Health England. The experiments were carried out within 6 months of their resuscitation.

Reagents. Drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA), LKT laboratories (St. Paul, MN, USA) and Calbiochem (San Diego, CA, USA). The drugs were dissolved in DMSO or ethanol.

Drug Treatment. RCC cells (ACHN, UOK, 786-O VHL negative and positive) were treated in their particular medium for 6 hours (for transcriptome analysis) or 24 hours (for apoptosis analysis). Final concentrations for each compound are provided in Supplementary Methods.
**Individualized Bioinformatics Analysis (IBA).** The RCC gene expression profiles used for generating the individualized analysis have been previously described (13). The high quality arrays were normalized by a robust multi-chip analysis (RMA) package (Bioconductor release 2.0) that consists of background correction, normalization and summarization of the signal values. These normalized signal values were used for the individualized bioinformatics analysis (IBA). IBA details are provided in Supplementary Methods.

**Connectivity Map Analysis.** The top 100 up-regulated and top 100 down-regulated genes from the ranked differential gene expression list of each individual RCC patient (N=21) were used to query the Connectivity Map (C-MAP) software build 01 (http://www.broadinstitute.org/cmap), applying Gene Set Enrichment Analysis (GSEA) as previously described (15). Details of the Connectivity Map dataset and analytics have been previously published (15). C-MAP analysis details are provided in Supplementary Methods.

**Statistical Analysis.** Student’s t-test was used for comparison of apoptosis induction after drug treatment in RCC cell lines and was performed using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA).

**Apoptosis Assays.** Apoptosis was measured using the Apoptotic Cell Death Detection ELISA (Roche, Indianapolis, IN, USA) according to the manufacturer’s protocol. Cells were treated with drugs (alone or in combination) or with DMSO or ethanol as controls as described above.
Detection of apoptotic cells by flow cytometry. Cells were treated with drugs (alone or in combination) or with DMSO or ethanol as control as described above. Detection of apoptosis was performed as previously described (for details see Supplementary Methods) (18).

Microarray Analysis. Total RNA was isolated from 786-O cells treated with 25 μM pentamidine or vehicle control (DMSO) for 6 hours using Qiagen Rneasy. Biological duplicates were used for this analysis. Microarray analysis using the human genome Affymetrix HT U133 A/B array plates was conducted by the BIDMC Genomics, Proteomics, Bioinformatics and Systems Biology Center at the Beth Israel Deaconess Medical Center according to previously described protocols (for details see Supplementary Methods) (13).

Microarray Data Deposition
All data sets have been deposited in the Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo (accession nos. GSE54709).

Pathway and Functional Enrichment Analysis. Ingenuity Pathway Analysis (IPA 8.0) (Qiagen) was used to identify the pathways and functions significantly affected by genes that are altered in response to pentamidine treatment in 786-O cells (for details see Supplementary Methods).

Animals and orthotopic implantation of 786-O tumor cells. RCC xenograft tumors were established by SQ injection of 786-0 cells into NCr nude mice (Charles River,
Wilmington, MA, USA). When tumors reached approximately 1.5 cm in diameter, tumors were stereilely excised and 2 mm fragments used to inoculate naïve NCr nude mice (n=8/group). For treatment studies, mice were entered onto treatment when tumor volumes reached 200 mm$^3$. Mice were treated with daily i.p. injections of amitriptyline 10 mg/kg, oxaprozin 20 mg/kg, or pentamidine 20 mg/kg or PBS (vehicle). The statistical significance of drug treatments was determined by 2-way ANOVA.
Results

Individualized Bioinformatics Analysis selects genes differentially expressed in individual ccRCC patients as compared to healthy controls

Our Individualized Bioinformatics Analysis (IBA) approach is based on first generating a range of gene expression for each gene in the healthy tissue for a significant number of patients and then identifying in each individual cancer patient all the genes whose expressions vary significantly from the expression range in healthy patients. Such an approach enables to identify gene expression changes that are common to the majority of patients with a certain type of cancer, to identify genes differentially expressed in only subsets of patients (potentially reflecting different subtypes) and to identify genes that are uniquely differentially expressed in an individual patient (for potential precision medicine indications).

In this study, the normal range of gene expression for each gene was generated from normalized expression profiles of 23 normal kidney samples, and then the gene signatures for every individual ccRCC patient (22 patients) were generated by identifying the genes that are differentially expressed between every individual patient with ccRCC compared to all normal kidney samples. Genes with more than 2 fold change in gene expression as compared to mean of normal kidney gene expression were considered differentially regulated. This novel IBA approach resulted in gene signatures specific for each patient that are anticipated to also reflect biological differences from one RCC patient to the other. Indeed, IBA analysis of these 22 patients combined with hierarchical clustering of these patients utilizing the IBA-derived gene signatures generates four main clusters (branches), indicating the potential existence of at least
four subtypes among ccRCC (Supplemental Figure 2). 21 of these 22 ccRCC gene signatures were used as the starting points for C-MAP analysis.

**Connectivity Map analysis of gene signatures for individual ccRCC patients identifies a set of new candidate therapeutic compounds against RCC**

The top 100 up-regulated and top 100 down-regulated genes from each of the 21 ccRCC gene signatures were used as input for C-MAP analysis build 01 to identify drug gene signatures that score the highest in reversing the tumor gene signatures towards normal kidney, i.e. the C-MAP highest negative correlations. The overlap of the top 100 genes from one patient to the other fed into this analysis was between 30-40%, demonstrating some overlap, but also significant divergence among the different ccRCC patients. The rationale for using 21 individual ccRCC gene signatures rather than one unified ccRCC gene signatures for the C-MAP analysis was that such an individualized analysis would demonstrate the robustness of the approach as well as the commonality and differences between individual patients. Applying all the individual RCC gene signatures to the C-MAP analysis provided us with a connectivity map for every single RCC patient.

We then compared the negative enrichment scores for all drugs between all patients and used hierarchical clustering and cologram to determine whether certain drug signatures had a consistent high negative enrichment (score between -0.7 to -1.0) in many or all RCC patients, only in subsets of patients or whether we would obtain a completely random set of scores across the patients. Our analysis clearly demonstrates that a small subset of drugs scored (inversely) very high (score between -0.7 to -1.0) across almost all ccRCC patients (Figure 1, bright green, green refers to gene signatures that are reversed by the drug, the brighter the green the higher the inverse correlation).
despite the significant differences observed in input gene signatures used for every individual patient, indicating that these drugs may be the highest priority candidates to be evaluated for anti-cancer efficacy. Another set of drugs had enhanced negative enrichment in certain subsets of patients, potentially reflecting the different subtypes and different biological pathways affected in different patients. Furthermore, multiple drugs demonstrated more pronounced negative enrichment of <-0.7 only in some patients with a lot of variability from one patient to the other, and these drugs may be of less interest or may be useful only for a small subset of patients.

We selected the top scoring drugs (based on consistent reverse high scoring correlation across most or all ccRCC patients) with a negative enrichment correlation between -0.7 and -1.0 in more than 50% of all ccRCC patients (Figure 1, bright green across most patients) to be tested against RCC in vitro and in in vivo models. To potentially rapidly translate a drug into clinical trials, we focused only on FDA-approved drugs and did not further consider small molecules not in the clinic. The selected list of high scoring FDA-approved drugs common to most ccRCC patients included pentamidine, amitriptyline, oligomycin, yohimbine, phenanthridinone, oxaprozin, dopamine and exemestane.

Pentamidine is currently used for the treatment of stage one *Trypanosoma brucei* and *Trypanosoma brucei rhodesiense* infection and *Pneumocystis carinii* pneumonia (19). Amitriptyline is primarily used as an antidepressant, and only recently has been correlated with anticancer effects (20). Oligomycin is a known antibiotic that blocks oxidative phosphorylation and inhibits the electron transport chain (21). Yohimbine is an antagonist of the alpha-2-adrenergic receptors and demonstrates anxiogenic effects in healthy human volunteers (22). Phenanthridinone is a potent poly(ADP-ribose) polymerase (PARP) inhibitor. PARP family inhibitors sensitize cancer cells for drug
and radiation treatment and are in clinical trials as cancer therapeutics (23). PARP proteins have been associated with inflammation, neuronal death and ischemia, and novel phenanthridinone derivatives are potent and selective inhibitors of hepatitis C virus replication in vitro (24). Oxaprozin is a propionic acid derivative widely used in the treatment of inflammatory and painful diseases of rheumatic and non-rheumatic basis (25). Dopamine, a well-known neurotransmitter, plays a role in various physiological processes and, dopamine agonists are clinically being used to ameliorate pain (26). Exemestane, a third-generation irreversible aromatase inhibitor with steroidal structure, has been used in the treatment of estrogen-receptor-positive breast cancer in postmenopausal women (27).

**High scoring candidate drugs from the Connectivity Map analysis induce apoptosis in RCC cell lines**

Eight of the high scoring drugs for ccRCC were tested with regard to their effects on survival of the RCC cell lines ACHN and UOK, using as a starting point the same drug concentration as described in the C-MAP database for the breast and prostate cancer cell lines. Five drugs (pentamidine, oligomycin, oxaprozin, dopamine and exemestane) out of the eight evaluated drugs strongly induced apoptosis in both RCC cell lines (Figure 2A), and two other drugs (yohimbine and phenanthridinone) also slightly induced apoptosis relative to the solvent DMSO (Figure 2A) indicating that this set of structurally unrelated drugs elicits an apoptotic response in RCC cell lines. In contrast, the five drugs did not significantly impact cell survival of three non-cancerous cell lines, MS1, F12 and HEK 293 (Supplemental Figure 3). Moreover, amitriptyline at 1 μM led to complete cell death of both RCC cell lines within 24 hours post treatment (data not shown).
We performed a dose response analysis for the five most consistent inducers of apoptosis as well as amitriptyline in ACHN cells to determine the lowest dose that still induces programmed cell death. The concentrations of the selected drugs were tested at 2, 5 and 10 times lower concentrations than the initial doses. Apoptosis was measured in ACHN cells after 24 hours of treatment with the different doses. Significant apoptosis was induced by 10 nM exemestane, 200 nM dopamine, 300 μM oxaprozin, 100 nM oligomycin and 50 μM pentamidine (Figure 2B). While amitriptyline even at 100 nM still rapidly killed all the cells, at 50 nM amitriptyline induced only moderate apoptosis in both ACHN and UOK cells (data not shown and Supplemental Figure 4). Thus, RCC cells are very sensitive to amitriptyline, since doses used for the C-MAP database rapidly kill all the RCC cells and much higher doses are needed to kill other types of cells.

**Several high scoring candidate drugs induce apoptosis more selectively in VHL (-) than VHL (+) 786-O cells**

Mutations of the Von Hippel-Lindau (VHL) gene are directly linked to Von Hippel-Lindau disease, an inherited cancer syndrome, and VHL mutations or epigenetic inactivation are found in the majority of sporadic ccRCC. Re-expression of VHL efficiently suppresses tumour growth in nude mice and various studies have implicated VHL as a key player in ccRCC development (28). To determine whether any of the above tested drugs induce VHL-dependent apoptosis in RCC cells, VHL (-) and VHL (+) 786-O cells were treated with the eight drugs described in Figure 1. Four drugs (pentamidine, oligomycin, oxaprozin and dopamine) strongly induced apoptosis in VHL (-) 786-O cells relative to control DMSO (Figure 2C), and amitriptyline at 100 nM killed all the cells (data not shown). Pentamidine, oligomycin and dopamine had a
statistically highly significant reduction in apoptosis induction in VHL (+) 786-O cells and amitriptyline as well resulted in reduced cell killing, indicating a correlation with VHL status.

To confirm our findings we evaluated apoptosis induction in a set of additional ccRCC cell lines, RCC4 VHL (+) and RCC4 VHL(-). The original ccRCC cell line RCC4 is VHL-deficient, and the cells employed in this study are RCC4 stably transfected with either pcDNA3 or pcDNA3-VHL which encodes the VHL gene product pVHL (17). Treatment with Pentamidine (100 μM), Oxaprozin (300 μM) and Dopamine (1 μM), and to a lesser extent Olygomycin (1 μM), increases induction of apoptosis in RCC4 VHL (-) relative to RCC4 VHL (+) with statistical significance which indicates a selectivity for VHL (-) cells (Supplemental Figure 5). This data further validates our observations in the 786-O VHL (+) and 786-O VHL (-) cells.

A dose response curve for pentamidine, oligomycin, oxaprozin and dopamine confirmed the VHL-dependent apoptosis induction for pentamidine, oligomycin, and dopamine (Supplemental Figure 6). The concentrations of the selected drugs were tested at 2, 5 and 10 times lower concentrations than the initial doses. Pentamidine and oligomycin resulted in more than 50% reduction of apoptosis in VHL (+) 786-O cells when compared to VHL (-) 786-O cells. To validate these findings, we tested the effects of combinatorial treatment in RCC4 VHL(+) and VHL(-) cells. As demonstrated in Supplemental Figure 7A, treatment with the selected drugs in combination induces higher level of apoptosis in cells deficient in VHL than its VHL (+) counterpart. Additionally, we performed analysis of apoptosis induction by FACS. Using the same conditions we observed that combinatorial treatment induces a higher number of cells to apoptose and that this is a VHL dependent event (Supplemental Figure 7B).
Using the lowest dose of each drug that minimally induced apoptosis of RCC cells (Figure 2B and Supplemental Figure 4), we systematically analyzed apoptosis induction upon combining low doses of drugs. Pentamidine, olygomycin, oxaprozin and dopamine were tested for their abilities to induce apoptosis alone and in combination. ACHN and UOK cells as well as VHL (-) and VHL (+) 786-O cells were treated with 0.2 μM olygomycin, 0.2 μM dopamine, 20 μM pentamidine, 25 nM and 50 nM amitriptyline or 150 μM oxaprozin, and combinations thereof (Figure 3). With these concentrations each of the drugs only induced moderate apoptosis. However, several of the combinations of low doses synergistically enhanced apoptosis (Figure 3 and Supplemental Figure 4). Certain combinations such as pentamidine with olygomycin, amitriptyline or dopamine, amitriptyline with oligomycin, and olygomycin with dopamine were most pronounced in synergizing. Pentamidine with olygomycin or amitriptyline were more effective in apoptosis induction in UOK cells (Figure 3A and data not shown) than ACHN cells (Figure 3B and data not shown). Oxaprozin did not appear to synergize with any of the other drugs in ACHN and UOK cells, but in 786-O VHL (-) cells.

**Pentamidine inhibits in vivo RCC progression and increases survival rates in mice**

Based on the above cell-based results we selected amitriptyline, oxaprozin and pentamidine for in vivo effects on RCC tumor growth. We utilized a xenograft model derived from injection of 786-0 cells into immunodeficient mice. Balanced cohorts (n=8 mice per treatment group) with established RCC xenograft tumors were treated with vehicle, amitriptyline (10 mg/kg), oxaprozin (20 mg/kg), or pentamidine (20 mg/kg) by daily intraperitoneal injection as described in Material and Methods. Treatment with amitriptyline or oxaprozin did not result in significant reduction of tumor growth at the
doses utilized. Mice treated with pentamidine showed a statistically significant reduction in tumor growth (p=0.03; Figure 4A). Mice were treated until tumors reached institutional limits (2000 mm$^3$), confirming that the anti-tumor efficacy of pentamidine treatment prolonged overall survival compared to vehicle-treated controls (p = 0.01; Figure 4B). These results validate the notion that a computational approach to select for drugs that may impact RCC can indeed prioritize and reposition drugs that elicit anti-RCC efficacy.

**Pentamidine reverses expression of a set of genes perturbed in ccRCC in 786-O cells**

To support the hypothesis that pentamidine, predicted by C-MAP analysis to revert expression of genes dysregulated in ccRCC, indeed counteracts altered gene expression in ccRCC we performed transcriptional profiling of 786-O cells treated with pentamidine or vehicle for 6 hours. 365 genes were differentially expressed in pentamidine-treated 786-O cells (Pentamidine Gene Signature) (Excel Spreadsheet 1 in Supplementary Information). A colorgram of the top 40 pentamidine-regulated genes is shown in Supplemental Figure 8.

We tested the Pentamidine Gene Signature on our previously described (13) dataset of primary and metastatic ccRCC tumor samples and normal kidney controls to determine whether pentamidine counter-regulates genes deregulated in ccRCC patients. Hierarchical clustering of these ccRCC and normal kidney samples using 83 genes (22.7% of the 365 pentamidine-regulated genes) included in the Pentamidine Gene Signature demonstrated that (i) all ccRCC samples accurately separated from all normal kidney samples and (ii) the majority of genes exhibited clear differential expression between ccRCC and normal kidney (Figure 5). Among the genes that are reduced by
pentamidine in 786-O cells, but overexpressed in ccRCC compared to normal kidney
are various genes of interest such as inositol-triphosphate 3-kinase A (ITPKA),
ribosomal protein S6 kinase, 90kDa, polypeptide 1 (RPS6KA1), WNT1, and ZEB1.
ZEB1 is a key transcriptional regulator of epithelial-to-mesenchymal transition (EMT)
(29). High WNT1 expression correlates with increased tumor diameter, stage and
vascular invasion (30). RPS6KA1 is overexpressed in breast and prostate cancer and its
inhibition in triple-negative breast cancers eliminates tumor-initiating cells (31).
Overexpression of ITPKA in cancer increases migration and the metastatic potential of
tumor cells (32). These results indicate that pentamidine in fact induces a gene signature
that is anticipated to reverse expression of a set of deregulated genes in ccRCC.

Functional and Pathway Enrichment analysis of pentamidine-regulated
genes

To gain insight into the functional and biological pathways that are significantly
affected by pentamidine altered genes, we performed functional and canonical pathway
enrichment analysis using the Ingenuity pathway analysis software package (IPA 8.0).
The pentamidine upregulated genes are significantly linked to cellular/organ/tissue
development, metabolism (e.g. amino acid, carbohydrate and lipid metabolism), cell
cycle, cellular growth and proliferation (Supplemental Figure 9A). Pentamidine
downregulated genes are significantly linked to cellular development, cellular growth
and proliferation, lipid metabolism and inflammatory response (Supplemental Figure
9B).

The pentamidine upregulated genes depict significant over-representation (P
value <0.05) in multiple biosynthesis and DNA repair related pathways including
“Taurine Biosynthesis”, “Glycine Biosynthesis I”, “Creatine-phosphate Biosynthesis”
and “DNA Double-Strand Break Repair” as well as “RhoA Signaling” (Supplemental Figure 9C).

Pathway analysis of the genes downregulated in response to pentamidine treatment depicted a significant association with pathways involved in metastasis, cell signaling and stemness including “Regulation of the Epithelial-Mesenchymal Transition Pathway”, “Colorectal Cancer Metastasis Signaling”, “Ephrin B Signaling”, “FXR/RXR Activation” and “Wnt/β-catenin Signaling”, all pathways highly relevant to cancer (Supplemental Figure 9D).
Discussion

While advances in immunotherapy and anti-angiogenic therapy increase survival for patients with metastatic ccRCC, the vast majority of patients continue to succumb to the disease. Consequently, new therapeutic agents with enhanced efficacy against metastatic ccRCC are needed. Despite the continuous development of many new drugs targeting cancer, there is a large repertoire of FDA-approved drugs available that were not originally developed for cancer therapy, but may target biological pathways that are drivers of certain types of cancer. The systematic evaluation and screening of drugs for potential therapeutic efficacy against specific types of cancer has only recently become feasible upon the establishment of transcriptome signature databases for compendiums of small molecule drugs, such as the C-MAP database (15).

In this study, we sought to identify new anti-ccRCC properties of FDA-approved drugs not currently used against ccRCC and repurpose such therapeutic compounds for the treatment of ccRCC by integrating an innovative individualized bioinformatics platform, IBA, with drug gene signatures. Our notion was that perturbations of gene expression and pathophysiological pathways in different patients with the same type of cancer, ccRCC, are diverse and heterogeneous, most likely reflecting the presence of divergent initiating and acquired mutations as well as additional inherent individual genetic and epigenetic differences. The most frequent approach to generate gene signatures for a specific type of cancer, ccRCC, is likely identifying the most significant gene expression changes common to the majority of patients, but does not provide the spectrum of heterogeneity and individual differences. To overcome these limitations, we developed an approach, IBA, where we first generated the variability and range in gene expression for each gene in the genome for the healthy counterpart, normal kidney, among a set of 23 patients and then compared expression for all genes of each
individual ccRCC patient to the healthy, baseline range of gene expression. This strategy enabled us to identify for each individual patient the set of genes in the tumor that are differentially expressed compared to healthy subjects. Despite a significant overlap of differentially expressed genes among all ccRCC patients, striking individual differences were also observed and hierarchical clustering indicated the potential existence of several different subtypes of ccRCC. Indeed, using completely different bioinformatics strategies, recent publications have identified several ccRCC subtypes (3,4).

GSEA of the gene signatures for each of the 21 ccRCC patients was applied to the C-MAP database, generating 21 enrichment files that were then compared by hierarchical clustering to determine consistency and divergence among different ccRCC patients with regard to compounds predicted to reverse the ccRCC gene signature/phenotype. The advantage of IBA compared to a group-based C-MAP analysis that utilizes a single across group gene signature is that by performing GSEA on a large number of gene signatures that only partially overlap and reflect the diversity among patients with the same disease, the robustness of the predictions can be validated and the applicability for all or the majority of patients can be explored. In fact, our results demonstrate that despite the divergence of gene signatures used as input for GSEA of the 21 ccRCC patients, with a typical gene overlap of 30-40% between different signatures when using the top 100 up- and downregulated genes as input, a similar enrichment pattern for a subset of drugs was observed across the majority of patients, but clear divergence among different patients were also seen. A small subset of drugs was predicted to reverse the ccRCC gene signature in the majority of patients with high significance, and this set of potential therapeutic compounds, with a focus on FDA-approved drugs, was prioritized as the top candidates for further functional
validation in ccRCC. Compounds that had a high score value only in a small subset of patients indicate that these drugs might be effective in just a few cases. These drugs were considered of less immediate interest and were not currently subjected to further investigation. Thus, by considering the diversity and heterogeneity among individual patients, we were able to visualize the spectrum of similarities and differences between different ccRCC patients and to select candidate drugs that are anticipated to elicit anti-tumor activity in the majority of patients with ccRCC. The demonstration of high scoring drugs consistent across most ccRCC patients provides strong support for this new IBA strategy.

Eight of the top scoring drugs (negative enrichment across the majority of patients) were tested against RCC in vitro and in vivo models, and among them, pentamidine, olygomycin, oxaprozin, amitriptyline and dopamine - alone or in combinations - strongly induced apoptosis in several RCC cell lines. Yohimbine and phenanthridinone at the concentrations used for the C-MAP database were not as effective in apoptosis induction of RCC cells and were not currently further pursued. It is possible that higher concentrations of these two drugs would elicit higher apoptosis induction rates. Since the inverse gene set enrichment of RCC gene signatures and drug signatures only indicates a correlation in gene signatures, the specific RCC gene sets that are reversed by certain drugs may not be involved in cell survival, but other biological functions.

None of the drugs with apparent pro-apoptotic efficacy are currently used in RCC treatment and most of them are not known anti-cancer drugs. In vivo evaluation of three of these drugs, pentamidine, oxaprozin and amitriptyline, in a RCC xenograft model of 786-O cells demonstrated that at least one of the drugs, pentamidine, at the selected dose significantly reduced tumor progression and prolonged survival. These
results most vividly show that our in silico screening strategy is able to prioritize and repurpose drugs not commonly used in cancer therapy for preclinical evaluation. The demonstration of anti-tumor efficacy in small animal cancer models is a key bridge from in vitro studies to human clinical testing.

Transcriptome analysis of pentamidine treated 786-O cells further supports the initial rationale for integrating ccRCC gene signatures with drug signatures to discover drugs that are anti-correlated with the ccRCC-specific signature, i.e. induced a gene expression pattern consistent with normal kidney. Pentamidine-regulated genes accurately differentiated between ccRCC and normal kidney, with many of the genes exhibiting altered expression in ccRCC and inverse expression as compared to pentamidine treatment.

Pentamidine has been shown in a small number of publications to affect cancer growth. Pathak et al. (33) demonstrated pentamidine anti-cancer activity in human cancer cell lines that expressed endogenous PRL tyrosine phosphatases, and tumor growth inhibition of human melanoma xenografts. Pentamidine inhibits activity of endo-exonuclease which is overexpressed in multiple cancer types and kills Lewis lung carcinoma cells (34). Pentamidine also appears to potentiate TRAIL-resistant K562 cells to TRAIL-induced apoptosis (35) and inhibits hypoxia-inducible factor 1α (HIF-1α) expression in prostate and breast cancer cells (36). While the mechanisms and pathways involved in the anti-RCC action of pentamidine are unknown, our transcriptome analysis of pentamidine-treated 786-O and RCC4 cells suggests that the antiproliferative mechanism of action of pentamidine is linked to a distinct set of genes deregulated in ccRCC patients.

Our results demonstrate that VHL(+) 786-O and VHL(+) RCC4 cells, that are unable to induce tumor formation, are less susceptible to pentamidine-mediated
apoptosis induction. These compelling data reinforce the notion of the potential benefits of pentamidine treatment for ccRCC. VHL mutation in RCC results in constitutive activation of hypoxia pathways and a shift towards HIF-2α (17). The VHL-dependence of pentamidine activity in RCC cells could be due to the known effects of VHL on HIF-1α and HIF-2α protein expression and activity.

Several of the other selected drugs may also be of interest for RCC therapy. Administration of the mitochondrial H(+)-ATP synthase inhibitor, oligomycin, to in vitro cancer cell systems inhibited the oxygen consumption responsible for mitochondrial ATP generation (37), an effect restricted to cancer cells (38). The NSAID, oxaprozin, induces apoptosis of activated monocytes in a dose-dependent manner, associated with the inhibition of AKT and NF-κB phosphorylation, and the activation of caspase-3 (39). Several NSAIDs are effective in chemoprevention or treatment of different cancers. Their effects have been attributed in part to cyclooxygenase-2 inhibition (40). We demonstrated that NSAIDs-mediated apoptosis and growth arrest in cancer cells is mediated by the pro-apoptotic cytokine melanoma differentiation associated gene-7/interleukin-24 (MDA-7/IL-24), leading to induction of growth arrest and DNA damage inducible 45 (GADD45) α and γ, c-Jun NH2-terminal kinase activation and inhibition of Cdc2-cyclin B checkpoint kinase (41). Oxaprozin induced strong apoptosis in RCC cells, although in vivo the tested dose did not elicit any significant anti-tumor activity against 786-O xenografts.

The antidepressant amitriptyline reduces viability of HT29 colon carcinoma cells (42). Amitriptyline induces multiple myeloma apoptosis through the inhibition of cyclin D2 expression, decreases histone deacetylase (HDAC) expression and directly inhibits HDAC activity (43). However, the doses used to induce apoptosis in multiple myeloma cells are 500-fold higher than the doses needed for killing RCC cells. Amitriptyline also
induces cellular damage in lung cancer, cervical cancer, and hepatoma via induction of reactive oxygen species (20). Our data demonstrate that amitriptyline even at low doses is a potent inducer of apoptosis in RCC cells and synergism with pentamidine was observed. While the described xenograft study revealed no significant anti-tumor activity, further studies with varying doses or in combination with other therapeutic agents are warranted.

In summary, we developed a novel individualized bioinformatics platform and applied it to public drug gene signatures, enabling us to rationally select drugs typically not used for cancer therapy as preclinical candidates for treatment of ccRCC. **In vitro** and **in vivo** validations highlight the potential repurposing of pentamidine as a high priority drug for combination with standard of care therapy in metastatic ccRCC.
Conflict of Interest

The authors have no conflict of interest to declare.

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References


Figure Legends

**Figure 1 – Connectivity Map analysis identifies new therapeutics candidate drugs against RCC.** Heatmap representation of the top 40 drugs (p<0.05) with negative enrichment score in the Connectivity Map analysis across ccRCC patients. Each column represents a different RCC patient and every row a different drug. Green color intensity refers to the reverse gene signature between the patient and the drug and red color the direct correlation.

**Figure 2 – Candidate drugs from the Connectivity Map analysis induce apoptosis in RCC cell lines.** Apoptosis assay for ACHN and UOK RCC cell lines treated with (A) pentamidine [100 μM], olygomycin [1 μM], oxaprozin [300 μM], dopamine [1 μM], yohimbine [25 μM] or phenanthridinone [50 μM]. (B) ACHN treated with pentamidine [100, 50, 20 and 10 μM], olygomycin [1, 0.5, 0.2 and 0.1 μM], oxaprozin [300, 150, 60 and 30 μM], dopamine [1, 0.5, 0.2 and 0.1 μM] and exemestane [10, 5, 2 and 1 nM]. (C) Apoptosis assay for VHL (-) and VHL (+) 786-O RCC cells treated with pentamidine [100 μM], olygomycin [1 μM], oxaprozin [300 μM] and dopamine [1 μM]. All treatments were performed for 24 hours. DMSO 0.05% treatment was used as control through the experiments. Data means ± S.D. of triplicate independent treatments. Statistical analysis was performed using student t test with ** p< 0.01 and *** p<0.001. RCC = Renal cell cancer.

**Figure 3 – Candidate drugs from the Connectivity Map analysis induced apoptosis effect is higher when used as combined therapies in RCC cell lines.** Apoptosis assay for (A) UOK, (B) ACHN, (C) 786-O VHL (-) and 786-O VHL (+) RCC cell lines.
treated with pentamidine [20 μM], oligomycin [0.2 μM], oxaprozin [150 μM], dopamine [0.2 μM], and the combinations of each 2 drugs in the same concentrations for 24 hours. DMSO 0.05% treatment was used as control through the experiments. Data means ± S.D. of triplicate independent treatments. Statistical analysis was performed using student t test with ** p< 0.01 and *** p<0.001. RCC = Renal cell cancer.

**Figure 4 – Pentamidine inhibits RCC progression and increases survival rate in vivo. (A)** Tumor volume after 20 days of treatment in mice with established disease, treated daily with vehicle, amitriptyline (10 mg/kg), oxaprozin (20 mg/kg) or pentamidine (20 mg/kg) by intraperitoneal injection. Fisher’s exact test was used to compare tumor volume – an indicator of disease progression – between the cohorts. (B) Survival curve of mice treated with above conditions for pentamidine and vehicle.

**Figure 5 – Pentamidine reverses expression of genes that are deregulated in tumors of ccRCC patients.** Hierarchical clustering of pentamidine-regulated genes in ccRCC tumor samples and normal kidney. Red color correlates with relative increased expression and blue color with decreased expression.
Figure 4

(A) 786-0 RCC Xenografts

- Vehicle
- Amitriptyline
- Oxaprozin
- Pentamidine

Tumor Volume (mm^3) vs. Days of Treatment

* p = 0.03

(B) Percent survival vs. Days of Treatment

- Pentamidine
- Vehicle

p = 0.0135
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