Computational Repositioning and Preclinical Validation of Pentamidine for Renal Cell Cancer

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Abstract

Although 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 this 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 anticancer therapy. Here, we demonstrate that one of the drugs predicted to revert the RCC gene signature toward 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 patients with ccRCC 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 Von Hippel-Lindau 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 patients with metastatic ccRCC and support our notion that IBA combined with C-MAP analysis enables repurposing of FDA-approved drugs for potential anti-RCC therapy. Mol Cancer Ther; 13(7); 1–13. ©2014 AACR.

Introduction

Renal cell carcinoma (RCC) consists of multiple subtypes based on histologic 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 pathophysiologic and molecular perturbations among different patients. The main therapeutic approach for metastatic RCC is using high doses of interleukin-2 (IL2). Although 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 antiangiogenic 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 because of 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 IL2 therapy response in patients with RCC (14). Identification of such gene signatures for RCC has not only provided new insights into the molecular mechanisms and biologic 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 7,000 genome-wide expression profiles for 1,309 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 toward 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 patients with cancer. The obvious bottlenecks for any such analysis are the number of variables (10,000 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 and colleagues, 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 patients with cancer. 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 toward a healthy kidney gene expression profile (see flowchart of overall study design in Supplemental Fig. S1). 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 RCC cell lines ACHN, UOK, and 786-O, and human embryonic kidney 293 cells were obtained from American Type Culture Collection. The M5-1 endothelial cell line and the F-12 foreskin fibroblast cell line were kindly provided by Dr. P. Oettgen and Dr. S. Goldring, respectively, Beth Israel Deaconess Medical Center, Boston. RCC4 Von Hippel-Lindau positive [VHL(−)], ECACC Catalog No. 03112702 and RCC4 VHL(+) (ECACC Catalog No. 03112703) RCC cell lines were obtained from Sigma-Aldrich (17). 786-O cells expressing wild-type VHL were kindly provided by Dr. V. Sukhatme, Division of Interdisciplinary Medicine and Biotechnology, Beth Israel Deaconess Medical Center. 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, LKT laboratories, and Calbiochem. The drugs were dissolved in DMSO or ethanol.

Drug treatment

RCC cells [ACHN, UOK, 786-O VHL(−), 786-O VHL(+), RCC4 VHL(−), and RCC4 VHL(+) ] 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

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

Statistical analysis
Student 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).

Apoptosis assays
Apoptosis was measured using the Apoptotic Cell Death Detection ELISA (Roche) 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; ref. 18).

Microarray analysis
Total RNA was isolated from 786-O cells treated with 25 μmol/L pentamidine or vehicle control (DMSO) for 6 hours using Qiagen Rneasy. Biologic 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; ref. 13).

Microarray data deposition
All datasets have been deposited in the Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo (accession no. 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-O cells into NCr nude mice (Charles River). When tumors reached approximately 1.5 cm in diameter, tumors were sterilely 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 mm³. Mice were treated with daily intraperitoneal 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 two-way ANOVA.

Results
IBA selects genes differentially expressed in individual patients with ccRCC as compared with healthy controls
Our 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 patient with cancer 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 patient with ccRCC (22 patients) were generated by identifying the genes that are differentially expressed between every individual patient with ccRCC compared with all normal kidney samples. Genes with more than 2-fold change in gene expression as compared with 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 biologic differences from one patient with RCC to the other. Indeed, IBA analysis of these 22 patients combined with hierarchical clustering of these patients utilizing the IBA-derived gene signatures generates 4 main clusters (branches), indicating the potential existence of at least 4 subtypes among ccRCC (Supplementary Fig. S2). A total of 21 of these 22 ccRCC gene signatures were used as the starting points for C-MAP analysis.

C-MAP analysis of gene signatures for individual patients with ccRCC identifies a set of new candidate therapeutic compounds against RCC
The top 100 upregulated and top 100 downregulated 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 toward normal kidney, that is 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% and 40%, demonstrating some
overlap, but also significant divergence among the different patients with ccRCC. The rationale for using 21 individual ccRCC gene signatures rather than 1 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 C-MAP for every single patient with RCC.

We then compared the negative enrichment scores for all drugs between all patients and used hierarchical clustering and colorgram to determine whether certain drug signatures had a consistent high negative enrichment (score between −0.7 and −1.0) in many or all patients with RCC, 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 and −1.0) across almost all patients with ccRCC (Fig. 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 anticancer efficacy. Another set of drugs had enhanced negative enrichment in certain subsets of patients, potentially reflecting the different subtypes and different biologic pathways affected in different

Figure 1. C-MAP analysis identifies new therapeutic candidate drugs against RCC. Heatmap representation of the top 40 drugs (P < 0.05) with negative enrichment score in the C-MAP analysis across patients with ccRCC. Each column represents a different patient with RCC 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.
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 patients with ccRCC) with a negative enrichment correlation between $-0.7$ and $-1.0$ in more than 50% of all patients with ccRCC (Fig. 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 patients with ccRCC 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 nonrheumatic basis (25). Dopamine, a well-known neurotransmitter, plays a role in various physiologic 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 C-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. All the selected drugs were tested at 2, 5, and 10 times lower concentrations. Significant apoptosis was induced by 10 nmol/L exemestane, 200 nmol/L dopamine, 300 μmol/L oxaprozin, 100 nmol/L oligomycin, and 50 μmol/L pentamidine (Fig. 2B). Although amitriptyline even at 100 nmol/L still rapidly killed all the cells, at 50 nmol/L amitriptyline induced only moderate apoptosis in both ACHN and UOK cells (data not shown and Supplemental Fig. S4). Thus, RCC cells are very sensitive to amitriptyline, because 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 VHL gene are directly linked to VHL 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 tumor 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 8 drugs described in Fig. 1. Four drugs (pentamidine, oligomycin, oxaprozin, and dopamine) strongly induced apoptosis in VHL(−) 786-O cells relative to control DMSO (Fig. 2C), and amitriptyline at 100 nmol/L 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 used 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 μmol/L), oxaprozin (300
molar), and dopamine (1 μmol/L), and to a lesser extent oligomycin (1 μmol/L), oxaprozin (300 μmol/L), and dopamine (1 μmol/L), yohimbine (25 μmol/L), or phenanthridinone (50 μmol/L).

These data further validate 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 (Supplementary Fig. S6). 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 with VHL(+) 786-O cells. To validate these findings, we tested the effects of combinatorial treatment in RCC4 VHL(+) and VHL(−) cells. As demonstrated in Supplementary Fig. S7A, treatment with the selected drugs in combination induces higher level of apoptosis in cells deficient in VHL than its VHL(+) counterpart. In addition, we performed analysis of apoptosis induction by FACS. Using the

Figure 2. Candidate drugs from the C-MAP analysis induce apoptosis in RCC cell lines. A, apoptosis assay for ACHN and UOK RCC cell lines treated with pentamidine (100 μmol/L), oligomycin (1 μmol/L), oxaprozin (300 μmol/L), dopamine (1 μmol/L), yohimbine (25 μmol/L), or phenanthridinone (50 μmol/L). B, ACHN treated with pentamidine (100, 50, 20, and 10 μmol/L), oligomycin (1, 0.5, 0.2, and 0.1 μmol/L), oxaprozin (300, 150, 60, and 30 μmol/L), dopamine (1, 0.5, 0.2, and 0.1 μmol/L), and exemestane (10, 5, 2, and 1 nmol/L). C, apoptosis assay for VHL(−) and VHL(+) 786-O RCC cells treated with pentamidine (100 μmol/L), oligomycin (1 μmol/L), oxaprozin (300 μmol/L), and dopamine (1 μmol/L). All treatments were performed for 24 hours. DMSO 0.05% treatment was used as control through the experiments. Data means ± SD of triplicate independent treatments. Statistical analysis was performed using Student t Test; *, P < 0.01; and **, P < 0.001.
same conditions we observed that combinatorial treatment induces a higher number of cells to apoptose and that this is a VHL-dependent event (Supplementary Fig. 7B).

Using the lowest dose of each drug that minimally induced apoptosis of RCC cells (Fig. 2B and Supplementary Fig. S4), 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 μmol/L olygomycin, 0.2 μmol/L dopamine, 20 μmol/L pentamidine, 25 nmol/L and 50 nmol/L amitriptyline or 150 μmol/L oxaprozin, and combinations thereof (Fig. 3). With these concentrations each of the drugs only induced moderate apoptosis. However, several of the combinations of low doses synergistically enhanced apoptosis (Fig. 3).

Figure 3. Candidate drugs from the C-MAP analysis induced apoptosis effect is higher when used as combined therapies in RCC cell lines. Apoptosis assay for UOK (A), ACHN (B), and 786-O VHL(−) and 786-O VHL(+) (C) RCC cell lines treated with pentamidine (20 μmol/L), olygomycin (0.2 μmol/L), oxaprozin (150 μmol/L), dopamine (0.2 μmol/L), and the combinations of each two drugs in the same concentrations for 24 hours. DMSO 0.05% treatment was used as control through the experiments. Data means ± SD of triplicate independent treatments. Statistical analysis was performed using Student’s t test; **, P < 0.01; and ***, P < 0.001.
Molecular Cancer Therapeutics

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-O 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), 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; Fig. 4A). Mice were treated until tumors reached institutional limits (2,000 mm³), confirming that the antitumor efficacy of pentamidine treatment prolonged overall survival compared with vehicle-treated controls (P = 0.01; Fig. 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 inhibits in vivo RCC progression and increases survival rates in mice**

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. A total of 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 Supplementary Fig. S8.

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 patients with ccRCC. 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 (Fig. 5).

Among the genes that are reduced by pentamidine in 786-O cells, but overexpressed in ccRCC compared with normal kidney are various genes of interest such as inositol-triphosphate 3-kinase A (ITPKA), ribosomal protein S6 kinase, 90 kDa, polypeptide 1 (RPS6KA1), WNT1, and ZEB1. ZEB1 is a key transcriptional regulator of epithelial-to-mesenchymal transition (EMT; ref. 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 IPA 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 (Supplementary Fig. S9A). Pentamidine downregulated genes are significantly linked to cellular development, cellular growth and proliferation, lipid metabolism, and inflammatory response (Supplementary Fig. S9B).

The pentamidine upregulated genes depict significant overrepresentation (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" (Supplementary Fig. S9C).

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 (Supplementary Fig. S9D).

Discussion

Although advances in immunotherapy and antiangiogenic 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 biologic 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 composites 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 pathophysiologic 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 patient with ccRCC 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 with healthy subjects. Despite a significant overlap of differentially expressed genes among all patients with ccRCC, 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 patients with ccRCC 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 patients with ccRCC with regard to compounds predicted to reverse the ccRCC gene signature/phenotype. The advantage of IBA compared with 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 patients with ccRCC, with a typical gene overlap of 30% to 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 patients with ccRCC and to select candidate drugs that are anticipated to elicit antitumor activity in the majority of patients with ccRCC. The demonstration of high-scoring drugs consistent across most patients with ccRCC 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, oxaprozine, 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 2 drugs would elicit higher apoptosis induction rates. Because 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 biologic functions.

None of the drugs with apparent proapoptotic efficacy are currently used in RCC treatment and most of them are not known anticancer drugs. In vivo evaluation of 3 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 antitumor 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 anticorrelated with the ccRCC-specific signature, that is 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 with pentamidine treatment.

Pentamidine has been shown in a small number of publications to affect cancer growth. Pathak and colleagues (33) demonstrated pentamidine anticancer 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 seems 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). Although 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 patients with ccRCC.

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 toward HIF-2α (17). The VHL dependence of pentamidine activity in RCC cells could be because of 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 proapoptotic 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 antitumor 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. Although the described xenograft study revealed no significant antitumor 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.

Disclosure of Potential Conflicts of Interest
T.A. Libermann is a consultant/advisory board member for anXome. No potential conflicts of interest were disclosed by the other authors.

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