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Preclinical Development

Therapeutic Targeting the Loss of the Birt-Hogg-Dubé Suppressor Gene

Xiaohong Lu1,2, Wenbin Wei3, Janine Fenton1,2, Michael S. Nahorski1,2, Erzsebet Rabai2,4, Anne Reiman1,2, Laurence Seabra1,2, Zsuzsanna Nagy2,4, Farida Latif1,2,3, and Eamonn R. Maher1,2,3

Abstract

Birt-Hogg-Dubé (BHD) syndrome, an autosomal dominant familial cancer, is associated with increased risk of kidney cancer. BHD syndrome is caused by loss-of-function mutations in the folliculin (FLCN) protein. To develop therapeutic approaches for renal cell carcinoma (RCC) in BHD syndrome, we adopted a strategy to identify tumor-selective growth inhibition in a RCC cell line with FLCN inactivation. The COMPARE algorithm was used to identify candidate anticancer drugs tested against the NCI-60 cell lines that showed preferential toxicity to low FLCN expressing cell lines. Fifteen compounds were selected and detailed growth inhibition (SRB) assays were done in paired BHD RCC cell lines (UOK257 derived from a patient with BHD). Selective sensitivity of FLCN-null over FLCN-wt UOK257 cells was observed in seven compounds. The most selective growth-inhibitory sensitivity was induced by mithramycin, which showed an approximately 10-fold difference in GI50 values between FLCN-null (64.2 ± 7.9 nmol/L, n = 3) and FLCN-wt UOK257 cells (634.3 ± 147.9 nmol/L, n = 4). Differential ability to induce caspase 3/7 activity by mithramycin was also detected in a dose-dependent manner. Clonogenic survival studies showed mithramycin to be approximately 10-fold more cytotoxic to FLCN-null than FLCN-wt UOK257 cells (200 nmol/L). Following mithramycin exposure, UOK257-FLCN-null cells were mainly arrested and blocked in S and G2-M phases of the cell cycle and low dose of rapamycin (1 nmol/L) potentiated mithramycin sensitivity (1.5-fold in G2-M population and 2-fold in G2-M period time, 2xGI50, 48 hours). These results provide a basis for further evaluation of mithramycin as a potential therapeutic drug for RCC associated with BHD. Mol Cancer Ther; 10(1); 80–9.
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Introduction

Renal cell carcinoma (RCC) accounts for 2% to 3% of all cancers and although familial forms of RCC account for only about 5% of all cases, investigations of rare inherited forms of RCC have provided seminal insights into the molecular pathogenesis of both familial and sporadic RCC. Thus the identification of the gene for von Hippel-Lindau (VHL) disease (a dominantly inherited multisystem familial cancer syndrome characterized by the development of hemangioblastomas, clear cell renal cell carcinoma, pancreatic lesions and pheochromocytoma) led to (a) the recognition that the most frequent genetic event in the evolution of sporadic clear cell renal cell carcinoma is somatic inactivation of the VHL tumor suppressor gene (TSG) and (b) that VHL TSG inactivation leads to dysregulation of the HIF-1 and HIF-2 transcription factors and activation of hypoxia-responsive gene pathways (1–5). These findings provided a rationale for the use of drugs such as sorafenib and sunitinib (inhibitors of HIF target gene pathways) in the treatment of metastatic RCC (6, 7). Birt-Hogg-Dubé (BHD) Syndrome is a dominantly familial cancer syndrome associated with susceptibility to RCC and also benign skin fibrofolliculomas and multiple lung cysts and spontaneous pneumothorax (8, 9). BHD syndrome results from inactivating mutations in the folliculin (FLCN) gene (10–12). Although the precise function of the FLCN gene product is still being elucidated, FLCN (and the folliculin interacting proteins FNIP1 and FNIP2) have been linked to the mTOR and AMPK signaling pathways (13–15). In mice with kidney-targeted homozygous inactivation of Flnk renal tumors and cysts developed with activation of mTOR and the mTOR inhibitor rapamycin diminished kidney pathology and increased survival (16, 17).

Authors’ Affiliations:
1Medical and Molecular Genetics, School of Clinical and Experimental Medicine, 2Centre for Rare Diseases and Personalised Medicine, 3The Cancer Research UK Cancer Centre, and 4Division of Neuropharmacology and Neurobiology Section, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

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Corresponding Author: Eamonn R. Maher, Department of Medical and Molecular Genetics, School of Clinical and Experimental Medicine, University of Birmingham; Edgbaston, Birmingham B15 2TT, The United Kingdom. Phone: 0044-121-6272741; Fax: 0044-121-6272618. E-mail: E.R.MAHER@bham.ac.uk

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Interestingly, mTOR inhibitors drugs (e.g., Temsirolimus, Everolimus,) have shown promise as treatments for metastatic RCC (18). To develop novel therapeutic approaches for BHD-associated RCC, and possibly also sporadic RCC, we adopted a strategy to identify tumor-selective growth inhibition in a RCC cell line with FLCN inactivation. Thus a bioinformatic approach using publicly-available data on FLCN expression and response to anticancer agents of NCI-60 panel of cancer cell lines was used to identify candidate anticancer agents that showed preferential toxicity to FLCN mutant cell lines.

Materials and Methods

**NCI compounds**

Morpholino-ADR (NSC 354646), cyanomorpholino-ADR (NSC 357704), echinomycin (NSC 13502), chromomycin A3 (NSC 58514), brucetin (NSC 67574), vincristine sulfate (NSC 165563), didemnin B (NSC 325319), paclitaxel (Taxol, NSC 125973), mithramycin (NSC 24559), phyllanthoside (NSC 266492), bisantrene hydrochloride (NSC 337766), doxorubicin (Adriamycin, NSC 24559), phyllanthoside (NSC 337766), doxorubicin (Adriamycin, NSC 123127), VM-26 (teniposide, NSC 122819), menogaril (NSC 269148), N,N-dibenzyldaunomycin (NSC 268242), and rapamycin (NSC 226080) were kindly provided by the Developmental Therapeutics Program of the National Cancer Institute (NCI)/NIH (http://dtp.nci.nih.gov).

**Cell lines and cell culture**

Human renal carcinoma of BHD origin cells UOK-257 (UOK-FLCN<sup>+</sup>) and FLCN-transfected UOK-257 cells UOK-FLCN<sup>+</sup>/C0 (19) were characterized and kindly provided by Dr Marston Linehan and Dr. Laura S Schmidt (Urologic Oncology Branch, Centre for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD) at the beginning of this project (2008). No further authentication of the UOK-257 cell lines was done apart from confirming the previously reported FLCN mutation in the cell lines at the strat of the project and the expected expression of folliculin in the 2 cell lines (see Results section). FTC-133 cells, originally derived from a lymph node metastasis of a follicular thyroid carcinoma from a 42-year-old male, were purchased from ECACC and a FLCN containing construct and an empty vector were introduced into parental FTC-133 cells respectively and stably transduced cells with/without FLCN expression were selected with neomycin. All cell lines were cultured in Dulbecco’s modified Eagle’s medium containing DMEM and Ham’s F12 (1:1).

**Growth inhibition assay**

The sensitivity of the cell lines to drug-induced cell growth inhibition was determined using the sulforhodamine B (SRB) assay as described previously (20). Briefly, adherent exponentially growing cells were seeded into 96-well plates at 3 x 10<sup>3</sup> to 5 x 10<sup>3</sup> cells/100 µL/well. After 20 to 24 hours at 37°C, drugs were added at the appropriate drug concentrations to the wells [final dimethyl sulfoxide (DMSO) concentration 1%] as indicated in the Results section. After drug treatment for 72 hours, the cells were fixed in situ by adding equal volume of Carnoy’s fixative (methanol:acetic acid = 3:1), washed, air dried, and stained with SRB (0.4%, Sigma-Aldrich). The absorbance per well was measured at 570 nm on a Victor X3 Multilabel Plate Reader (PerkinElmer).

**Caspase-Glo 3/7 assay and cell viability assay**

The ability to induce caspase 3/7 activation after exposure to compounds was measured by Caspase-Glo 3/7 Assay (Promega) according to the manufacturer’s instruction. The cells were seeded and dosed as described in growth inhibition assay. At the end of incubation, 70 µL of medium was removed and 30 µL of assay reagent was added to the remaining medium. Additional 1 hour incubation with shaking was carried out at room temperature. The resultant luminescent light was measured in a Victor X3 Multilabel Plate Reader (PerkinElmer). Cell viability after compound exposure was determined by CellTiter-Blue Cell Viability Assay (Promega) according to the manufacturer’s instruction. At the end of drug treatment, 20 µL of reagent was added to the medium in 96 wells and the cells were incubated for additional 4 hours. The cell viability was measured by fluorescence with a 570-nm excitation and 590-nm emission set in a Victor X3 Multilabel Plate Reader (PerkinElmer).

**Clonogenic cell survival assay**

The cytotoxicity of mithramycin was determined in UOK-257 cells with/without FLCN expression. Exponentially growing cells were seeded into 100-mm Petri dishes at densities ranging from 250 to 1 x 10<sup>5</sup> cells/dish, the cell seeding density being adjusted to give an estimated 10 to 300 colonies/dish following drug exposure. The cells were left to attach for 24 hours and freshly made mithramycin was added at the appropriate concentrations to the dishes. The final concentration of DMSO in the medium was 1%. Four dishes with 2 seeding densities for each drug concentration were used and at least 3 experiments were carried out under each set of conditions. The cells were exposed to mithramycin for 72 hours, after which the medium was aspirated, the dishes were washed once with warm PBS, and fresh drug-free medium was added. The cells were incubated for an additional 10 to 16 days until visible colonies appeared, which were fixed with Carnoy’s fixative (methanol:acetic acid = 3:1, v:v), and visualized by staining cells in SRB (0.4%). Colonies with over 30 cells were counted. Cloning efficiencies of untreated cells were UOK257-FLCN<sup>+</sup> 26% and UOK257-FLCN<sup>-</sup> 34%. Cell survival following drug exposure was expressed as percent control cloning efficiency or survival.

**Western blot analysis**

Cellular protein expression in UOK Cells with/without FLCN expression in response to drug exposure was
determined using total cell extracts at 48 hours after treatment, according to standard procedures. Protein concentration was determined using DC protein assay kit according to the manufacturer's instructions (Bio-Rad Laboratories Ltd.). Protein (20 μg) from each sample was electrophoresed on 12.5% (w/v) SDS-PAGE gels and electroblotted on to nitrocellulose membrane (Amersham Pharmacia Biotech UK Ltd.). Antibodies against FLCN (a gift from Prof. Armin Pause, Rosalind and Morris Goodman Cancer Centre Montreal, Canada), caspase 3 and pTEN (Cell Signalling Technology), p53 (DO; Novacstra), and actin (Sigma-Aldrich Company Ltd.) were used. The signal was detected using the enhanced chemiluminescence (ECL Plus; Amersham) system after addition of anti-mouse IgG–HRP conjugate (DAKO).

Cell cycle analysis

UOK-257 cells with/without FLCN expression were seeded at 2,000 cells per well in 96-well plates either with 0.1% DMSO or with 1 nmol/L Rapamycin and incubated at 37°C/5% CO2 overnight to adhere. The drugs were added at the appropriate concentrations to the wells in replicates of 8 as indicated in the Results section. At the end of the time course, media was carefully removed and cells were fixed in 85% ice-cold ethanol. After removal of ethanol, cells were incubated in the dark at 37°C for 20 minutes in PBS buffers containing 0.1% Triton X-100, 100 μg/mL RNase A and 10 μg/mL Propidium iodide (PI). The 96-well plates were subsequently scanned using the Acumen eX3 cytometer (TTP LabTech).

Results

Identification of candidate drugs by COMPARE algorithm based on FLCN expression patterns on the NCI-60 panel

This NCI-60 panel consists of 60 cancer cell lines derived from 9 different human tissue types. These cell lines have been characterized extensively for a range of attributes including microarray gene expression profiles and have been screened for cytotoxic sensitivity for over 14,000 compounds.

To identify candidate anticancer agents that might differentially affect cancer cell lines with differing levels of FLCN expression, drugs with GI₅₀ positively correlated with the FLCN expression values as measured using Affymetrix U133 array and U95 array by Gene Logic, Inc. were identified using the DTP COMPARE website (http://dtp.nci.nih.gov/compare/), which implements the COMPARE algorithm (21–23). The seed pattern for drug sensitivity was designed similar to that described previously to identify anticancer agents that preferentially affecting VHL-deficient cell lines (23). Thus the aim was to identify drugs to which cell lines with low FLCN expression were most sensitive and cell lines with high FLCN expression were most resistant. The COMPARE algorithm was used to compare the FLCN seed pattern with individual compound in the DTP database and 15 compounds were selected with the similar sensitivity pattern to the FLCN seed.

Screen Compound Sensitivities in UOK-257 Cells with/without FLCN expression

UOK-257 is the only RCC cell line available that has been derived from a patient with BHD and harbored a germline FLCN frameshift mutation [c.1285dupC; predicted, in the absence of nonsense mediated mRNA decay, to lead to premature protein truncation (p.His429ProX27); ref. 19]. Both UOK257-FLCN⁺ and UOK257-FLCN⁻ cells were examined for their sensitivities to growth inhibition induced by 15 compounds selected from the COMPARE analysis. Various concentrations of compounds were introduced into the cells 24 hours after seeding, incubation was continued for 72 hours and growth inhibition was assessed using the SRB assay. GI₅₀ values, defined as the concentration of compounds required to inhibit growth by 50%, were then calculated. The ratio of GI₅₀ value from UOK257-FLCN⁺ and UOK257-FLCN⁻ were calculated and used as an indicator for the differential sensitivity of the FLCN mutant cells to the drug. As shown in Figure 1 and Table 1, 7 compounds were relatively more inhibitory to UOK257-FLCN⁻ cells (ratio > 1) and the most selective growth-inhibitory sensitivity was induced by mithramycin with almost 10-fold difference in the GI₅₀ values between UOK257-FLCN⁻ cells (64.2 ± 7.9 nmol/L; n = 3) and UOK257-FLCN⁺ cells (634.3 ± 147.9 nmol/L; n = 4). Whereas the UOK257-FLCN⁻ cells were less sensitive to the other 8 compounds (ratio < 1) and cyanomorpholino-ADR induced the least growth inhibition with 6-fold difference in the GI₅₀ values between UOK257-FLCN⁻ cells (0.62 ± 0.16 nmol/L; n = 3) and UOK257-FLCN⁻ cells (3.79 ± 0.39 nmol/L; n = 3). The data indicated that although these compounds were selected from COMPARE algorithm with the same criteria, there was a broad variation between the differential sensitivity of the cells with/without FLCN expression to different drugs.
especially in bruceantin (ratio < 1) at all 3 concentrations. However, there was no concentration dependent increase of caspase 3/7 activity observed. Whereas all 3 UOK257-FLCN⁻ sensitive compounds showed at least 2-fold induction of caspase 3/7 activity, only mithramycin induced a concentration dependent increase and produced with at least 8-fold higher caspase 3/7 activity in UOK257-FLCN⁻ cells than in UOK257-FLCN⁺ cells (200 nmol/L, 48 hours). Cell viability assay was also used to determine whether compound-induced reduction in cell viability (as measured by CellTitre Blue assay) correlated with cell growth inhibition (as measured by SRB assay) and of active caspase 3/7 induction. As shown in Figure 2B, the effects of the individual compounds were similar to the results of the growth inhibition assay (Fig. 1), although the range of differential sensitivities was less (up to 2-fold). However, only mithramycin reduced cell viability in a concentration-dependent manner (2-fold, 200 nmol/L, 48 hours; with a very similar pattern to that for induction of caspase 3/7 activity by mithramycin).

Western blot analysis was done to examine the change of cellular caspase 3 protein expression after treatment of mithramycin for 48 hours in both UOK257-FLCN⁻ and UOK257-FLCN⁺ cells. As shown in Figure 2C, the appearance of active forms (17 and 19 kDa) of cleaved caspase 3 were found at 100 and 200 nmol/L, but not in low concentrations of mithramycin (25 and 50 nmol/L) in treated UOK257-FLCN⁻ cells (which was corresponded to the induction of caspase 3/7 activity shown in Fig. 2A). It was interesting to note that a high molecular weight caspase 3 band (21 kDa) was found both in UOK257-FLCN⁻ and UOK257-FLCN⁺ cells whereas the 19kDa active form of caspase 3 was present in the cellular lysates of UOK257-FLCN⁻ irrespective of mithramycin treatment. The time course of mithramycin treatment in UOK257-FLCN⁻ cells showed that the cleaved active caspase 3 bands appeared as
higher concentration of 500 nmol/L less than 0.1% which was a 10-fold difference between 2 cell lines. At a \(\text{UOK}^{+}\) cell survival was counted in UOK257-FLCN cells without FLCN expression. In contrast, more than 25% nmol/L less than 8% viable cells were observed for UOK-257 cells with/without FLCN expression.

Mitochondrial cell survival. After being exposed to mithramycin and rapamycin (13) and in transgenic mice (14, 16). Hence, we proceeded to investigate whether inhibition of the mTOR pathway by rapamycin influenced the differential mithramycin sensitivity found in UOK cells with/without FLCN expression. Preliminary data (not shown) showed that a low concentration of rapamycin (1 nmol/L) inhibited mTOR activity (as measured by S6R phosphorylation) but had only a small effect (10%-15% reduction) on cell proliferation. Both UOK257-FLCN- and UOK-FLCN- cells were incubated in the presence or absence of 1 nmol/L rapamycin overnight before adding mithramycin at various concentrations for 72 hours and \(G_{50}\) values were determined from growth inhibition curves. As indicated in Figure 4A, rapamycin at 1 nmol/L can potentiate mithramycin sensitivity by 1.5-fold in UOK257-FLCN- cells (69 \pm 10 nmol/L, \(n = 6\) for mithramycin and 47 \pm 14 nmol/L, \(n = 4\) for mithramycin and rapamycin) suggesting that mithramycin in

### Table 1. Compound-induced growth inhibition in UOK cells with/without FLCN

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(G_{50}) (nmol/L) in UOK-FLCN</th>
<th>(G_{50}) (nmol/L) in UOK-FLCN-</th>
<th>(G_{50}) Ratio of UOK-FLCN-/UOK-FLCN-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morpholino-ADR</td>
<td>0.45 \pm 0.03 (3)</td>
<td>0.44 \pm 0.10 (3)</td>
<td>0.97</td>
</tr>
<tr>
<td>Cyanomorpholino-ADR</td>
<td>3.79 \pm 0.39 (3)</td>
<td>0.82 \pm 0.16 (3)</td>
<td>0.16</td>
</tr>
<tr>
<td>Echinomycin</td>
<td>5.72 \pm 0.22 (3)</td>
<td>4.03 \pm 0.89 (3)</td>
<td>0.71</td>
</tr>
<tr>
<td>Didemnin B</td>
<td>7.94 \pm 1.66 (4)</td>
<td>6.53 \pm 1.78 (4)</td>
<td>0.82</td>
</tr>
<tr>
<td>Vincristine sulfate</td>
<td>30.1 \pm 10.3 (5)</td>
<td>56.4 \pm 17.5 (5)</td>
<td>1.88</td>
</tr>
<tr>
<td>Chromomycin A3</td>
<td>37.4 \pm 6.9 (3)</td>
<td>33.0 \pm 5.7 (3)</td>
<td>0.88</td>
</tr>
<tr>
<td>Mithramycin</td>
<td>64.2 \pm 7.9 (3)</td>
<td>634 \pm 148 (4)</td>
<td>9.88</td>
</tr>
<tr>
<td>Brucine</td>
<td>68.4 \pm 14.9 (3)</td>
<td>43.6 \pm 5.1 (3)</td>
<td>0.64</td>
</tr>
<tr>
<td>Paclitaxel (taxol)</td>
<td>75.5 \pm 9.2 (5)</td>
<td>514 \pm 86 (3)</td>
<td>6.81</td>
</tr>
<tr>
<td>Phyllanthoside</td>
<td>199 \pm 79 (5)</td>
<td>427 \pm 97 (4)</td>
<td>2.15</td>
</tr>
<tr>
<td>Doxorubicin (adriamycin)</td>
<td>423 \pm 54 (3)</td>
<td>581 \pm 109 (4)</td>
<td>1.37</td>
</tr>
<tr>
<td>Menogaril</td>
<td>588 \pm 57 (4)</td>
<td>757 \pm 117 (4)</td>
<td>1.29</td>
</tr>
<tr>
<td>VM-26 (teniposide)</td>
<td>773 \pm 162 (3)</td>
<td>849 \pm 358 (3)</td>
<td>1.10</td>
</tr>
<tr>
<td>Bisantrene hydrochloride</td>
<td>1753 \pm 288 (6)</td>
<td>1345 \pm 602 (5)</td>
<td>0.77</td>
</tr>
<tr>
<td>N,N-dibenzyldaunomycin</td>
<td>5739 \pm 918 (4)</td>
<td>1273 \pm 549 (4)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

**NOTE:** Data are mean \(\pm\) SE with the number of the experiments given in parenthesis. \(G_{50}\) values were calculated by fitting a sigmoidal concentration/inhibition curve to the results using nonlinear least square regression (GraphPad PRISM) to data generated by the SRB assay.
A combination of rapamycin was more potent than mithramycin alone in the cells \((P = 0.025, \text{unpaired } t\text{ test, Supplementary Fig. 1})\). Although there was a slight difference \((1.1\text{-fold})\) in \(\text{UOK257-FLCN}^+\) cells, the combination drugs-induced growth inhibition was not as profound \((P = 0.44, \text{unpaired } t\text{ test, Supplementary Fig. 1})\) as seen in \(\text{UOK257-FLCN}^+/\text{C0}\) cells when higher mithramycin concentrations were applied (Fig. 4A).

To study the underlining mechanism of differential sensitivities of mithramycin or in combination with rapamycin in 2 cell lines, cell cycle analysis was carried out using cytometry (Acumen Explorer). To compare cell cycle change at the same degree of mithramycin inhibition, mithramycin was applied in a range of GI\(_{50}\) concentrations (ranging from 0.5-fold to 4-fold) to both \(\text{UOK257-FLCN}^+\) and \(\text{UOK-FLCN}^+\) cell lines in the presence/absence of 1 nmol/L rapamycin. As shown in Figure 4B, in the absence of 1 nmol/L rapamycin, there was 60% increase in cell population of both S and G\(_2\)-M phases in comparison with DMSO control in \(\text{UOK257-FLCN}^+\) cells (4xGI\(_{50}\) mithramycin, 48 hours). However, only 15% increase in S and G\(_2\)-M phases was found in \(\text{UOK-FLCN}^+\) cells at 4xGI\(_{50}\) mithramycin [a 10-fold higher concentration (2.5 \(\mu\)mol/L) than that used in its counterpart \(\text{UOK257-FLCN}^+\) cells (0.25 \(\mu\)mol/L)]. In the presence of 1 nmol/L rapamycin, a more pronounced increase in G\(_2\)-M phase was observed at a lower mithramycin concentration (40% at 2xGI\(_{50}\) and 90% at 4xGI\(_{50}\) in \(\text{UOK257-FLCN}^+\) cells, indicating that rapamycin potentiates the cell cycle inhibitory effect (G\(_2\)-M arrest) of mithramycin. Moreover, the cell cycle inhibition was associated with lengthening of the S and G\(_2\)-M time in \(\text{UOK257-FLCN}^+\) cells (100% at 4xGI\(_{50}\) mithramycin alone; Figure 4C). The effect of mithramycin on the G\(_2\)-M time was also potentiated by rapamycin (70% at 2xGI\(_{50}\) mithramycin alone; Figure 4C). In contrast, the modest effect of mithramycin on the G\(_2\)-M phase in \(\text{UOK-FLCN}^+\) cells was not substantially influenced by the presence of rapamycin (25% at 4xGI\(_{50}\)).

In summary, in response to mithramycin exposure, \(\text{UOK257-FLCN}^+\) cells were arrested in the S and G\(_2\)-M phases of the cell cycle and low dose of rapamycin (1 nmol/L) strongly potentiated mithramycin sensitivity by promoting the G\(_2\)-M arrest.

**Comparison of mithramycin sensitivity in non-RCC Cells and gene status of \(p53\), \(PTEN\) in the cells examined**

Mithramycin sensitivity was also examined in a non-RCC cell lines (FTC-133 a human thyroid carcinoma).
with/without FLCN expression. A FLCN containing construct was introduced into parental FTC-133 cells and stably transfected cells were selected. SRB growth inhibition assay was carried out in cells transfected with empty vector and low FLCN and high FLCN expression. It was interesting to note that there was no apparent differential mithramycin sensitivity to FTC-133 cells with different levels of FLCN expression (59.3 ± 8.9 nmol/L, n = 5 for empty vector; 38.7 ± 10.4 nmol/L, n = 3 for FLCN expressed at low levels; and 35.3 ± 7.3 nmol/L, n = 5 for FLCN expressed at high levels). Similarly, there was no selective vincristine sensitivity in FTC-133 cells with different FLCN levels (data not shown).

We next investigated the p53 and PTEN status of the UOK-257 and FTC-133 cell lines. Direct genomic sequencing and Western blot were used to investigate gene mutation status and protein expression levels. As shown in Table 2, in addition to inactivation of FLCN gene, both cell lines also harbored p53 gene mutations. A c.153C→T mutation resulted in an early truncation of p53 protein in UOK-257 and a point mutation of p53 gene led to inactivation of p53 function by accumulation of the protein in FTC-133 cells (Fig. 5). It is interesting to note that there was PTEN gene mutation resulting in a truncated protein in FTC-133 cells whereas wild type PTEN was present in the UOK-257 cell line.

Discussion

We sought to identify compounds that might differentially impair the viability of a FLCN-deficient RCC cell line. We detected selective sensitivity of UOK257-FLCN− over UOK257-FLCN+ cells for 7 compounds. Most notably mithramycin showed an approximately 10-fold difference in the GI50 values between UOK257-FLCN− cells and UOK257-FLCN+ cells. The drug also preferentially induced caspase 3/7 activity UOK257-FLCN− cells in a dose dependent manner and was almost 10-fold more cytotoxic to UOK257-FLCN− cells than to UOK257-FLCN+ cells (200 nmol/L, clonogenic survival assay). To date, UOK-257 is the only RCC cell line available that is derived from a patient with BHD and we are not aware of any sporadic RCC cell lines with homozygous FLCN inactivation. In our hands, knockdown of FLCN expression by siRNA in sporadic RCC with wild-type FLCN was incomplete and so was an unsuitable model for replicating the findings in UOK257-FLCN null and expressing cell lines. In addition, such an approach would not replicate folliculin inactivation as the initiating event in tumorigenesis. We note that previously, using a similar strategy to one that we employed to identify candidate agents that might show differential activity between high and low FLCN expressing cancer cell lines, Sutphin et al. (23) used the COMPARE algorithm to identify 10 compounds that showed differential activity against VHL low and high expressing NCI60 cell lines. Four of these compounds were tested against paired VHL null and VHL expressing RCC cell lines and ChA3 was found to exhibit differential toxicity, in clonogenic survival studies, to VHL-deficient cell lines relative to VHL-positive RCC cell lines. ChA3 is an aureolic acid compound that binds DNA in the minor groove and inhibits transcription. Although mithramycin (an aureolic acid such as ChA3) exhibits differential cytotoxicity depending on FLCN expression status, we did not find any differences between the growth-inhibitory activity of ChA3 in FLCN deficient cell lines when compared with FLCN expressing UOK-257 cells.

Mithramycin binds to GC-rich regions and inhibits the transcription of genes with GC-rich promoters and has been used to treat several types of cancer, including testicular carcinoma and leukemia (24). Inhibition of Sp1 activity has been implicated in mithramycin cytotoxicity, but we did not find any consistent differences between the mithramycin-induced changes in expression of Sp1 target genes between FLCN deficient and positive UOK257 cell lines. siRNA knockdown of Sp1 in FLCN-deficient and positive UOK257 cell lines did not differentially inhibit cell growth or induce caspase 3/7 activity (data not shown). In response to mithramycin exposure, UOK257-FLCN− cells were mainly arrested and blocked in S and G2-M cell cycle (Fig. 4) and low dose of rapamycin (1 nmol/L) potentiated mithramycin sensitivity (1.5-fold in G2-M population and 2-fold in G2-M period time; 2×G0, 48 hours). It was reported that p53 status played an important role in modulation of
mithramycin-induced cell polyploidy and cell death in colon carcinoma cells (25). Mithramycin SK, a novel analog of mithramycin, results in polyploidization and mitotic catastrophe in HCT116 cells with wt p53 and most cell populations died by necrosis, whereas HCT-116 (p53/C0/C0) cells died mainly from G2-M block through early p53-independent apoptosis. These observations led us to speculate that in response to mithramycin exposure, UOK-257 cells with inactivated p53 gene might also die through a p53-independent apoptosis pathway when arrested in G2-M block. In FLCN-replete UOK257 cells; however, little G2-M cell cycle block was induced by mithramycin.

RCC from BHD patients may show increased HIF-2 expression (26) and Sutphin et al. (23) found that overexpression of HIF-2 in VHL-positive clear cell RCC cell lines phenocopied the effect of VHL inactivation on susceptibility to ChA3 toxicity. However, the observation that ChA3 did not show selective toxicity to FLCN-deficient and positive UOK257 cell lines suggests that other factors are, at least in part, implicated in FLCN-related differential response to mithramycin. We note that FLCN expression did not relate to mithramycin sensitivity in a non–renal cancer cell line (FTC-133). There are several possible explanations for this observation. First, whereas

**Table 2. Gene status in human tumor cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>UOK-257</th>
<th>FTC-133</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer type</td>
<td>BHD</td>
<td>Thyroid</td>
</tr>
<tr>
<td>FLCN</td>
<td>Codon change</td>
<td>Amino acid change</td>
</tr>
<tr>
<td>FLN</td>
<td>c.1285delC</td>
<td>p.His429ProfX27</td>
</tr>
<tr>
<td>PTEN</td>
<td>wt/wt</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Cell growth inhibition of UOK257-FLCN+/– and UOK257-FLCN−/− was determined by the SRB assay in response to exposure of mithramycin alone (solid line) and mithramycin in combination of 1 nmol/L rapamycin (dotted line) for 72 hours (A). After UOK257-FLCN+/– and UOK257-FLCN−/− cells were incubated with mithramycin alone or in combination of 1 nmol/L rapamycin for 48 hours, the percentage of each cell cycle population (B) and of each cell cycle time (C) were calculated. Cells were incubated with either DMSO or 1 nmol/L rapamycin overnight and then exposed continuously to various concentrations of mithramycin. At the end of incubation, cells were fixed and stained with PI as indicated in Materials and Methods before being scanned with the Acumen eX3 cytometer.
FLCN inactivation is an initiating event in BHD RCC (e.g., in UOK257 cells), FLCN inactivation in the FTC-133 thyroid carcinoma cell line may have occurred at a late stage of tumorigenesis and so have different functional consequences. Also, the functional consequences of TSG inactivation may differ according to cell tissue type (e.g., VHL inactivation or hypoxic induction of HIF-2 expression induces oncogenic CCND1 expression in RCC cell lines but CCND1 expression is not hypoxia-inducible in non-RCC cancer cell lines), possibly explaining the very restricted cancer susceptibility phenotypes seen in inherited cancer syndromes such as VHL disease and BHD syndrome (we note that renal cancer, but not thyroid cancer, is a major complication of BHD suggesting that folliculin has a gatekeeper role in the renal but not thyroid cells). Finally, the response of a FLCN-deficient cell line to mithramycin treatment may be modified by coexisting mutations (or epimutations) in additional TSG/oncogenes.

Patients with BHD syndrome are offered annual renal imaging to facilitate early detection of RCC (9). However, some patients may only be diagnosed after presentation with advanced RCC. Treatment of metastatic RCC is challenging for both familial and sporadic cases. Although occasional patients may respond to immunotherapy with the cytokines interferon-α and interleukin-2, recently treatment with targeted therapies to HIF downstream targets (e.g., sunitinib, sorafenib, bevacizumab) and the mTOR pathway (e.g., Temsirolimus, Everolimus) has emerged as the most frequent management strategy. However, these agents, whilst prolonging life, are not cytotoxic and so the identification of targeted cytotoxic agents would be a significant advance. Our findings suggest that mithramycin merits further evaluation as a potential therapy for folliculin deficient RCC.

Disclosure of Potential Conflicts of Interest

A patent application regarding the use of mithramycin in Birt-Hogg-Dube-related tumors was filed by the Myrovlytis Trust with X. Lu and E.R. Maher named as inventors. The other authors disclose no potential conflicts of interest.

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References


Figure 5. Basal level of cellular protein expression in UOK and FTC cells with/without FLCN Expression. Western blotting was carried out by using antibodies as described in Materials and Methods.


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