Identification of Novel Therapeutic Targets for Fibrolamellar Carcinoma Using Patient-Derived Xenografts and Direct-from-Patient Screening

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To repurpose therapeutics for fibrolamellar carcinoma (FLC), we developed and validated patient-derived xenografts (PDX) from surgical resections. Most agents used clinically and inhibitors of oncogenes overexpressed in FLC showed little efficacy on PDX. A high-throughput functional drug screen found primary and metastatic FLC were vulnerable to clinically available inhibitors of TOPO1 and HDAC and to napabucasin. Napabucasin’s efficacy was mediated through reactive oxygen species and inhibition of translation initiation, and specific inhibition of eIF4A was effective. The sensitivity of each PDX line inversely correlated with expression of the antiapoptotic protein BCL-xL, and inhibition of BCL-xL synergized with other drugs. Screening directly on cells dissociated from patient resections validated these results. This demonstrates that a direct functional screen on patient tumors provides therapeutically informative data within a clinically useful time frame. Identifying these novel therapeutic targets and combination therapies is an urgent need, as effective therapeutics for FLC are currently unavailable.

SIGNIFICANCE: Therapeutics informed by genomics have not yielded effective therapies for FLC. A functional screen identified TOPO1, HDAC inhibitors, and napabucasin as efficacious and synergistic with inhibition of BCL-xL. Validation on cells dissociated directly from patient tumors demonstrates the ability for functional precision medicine in a solid tumor.
INTRODUCTION

Fibrolamellar carcinoma (FLC) is a fatal liver cancer that affects adolescents and young adults without underlying liver disease. Although rare (1 in 5 million), it is well defined and consistent across patients, offering a favorable tool for characterizing pathogenesis and for testing the efficacy of therapeutics. Characterization of the FLC transcriptome by RNA sequencing (RNA-seq) revealed a fusion transcript of exon 1 of the heat shock protein DNAJB1, and exons 2–10 of PRKACA, the catalytic subunit of protein kinase A (PKA; ref. 1) in all patients tested. The resulting fusion kinase retains the catalytic activity of PKA (1). Use of CRISPR/Cas9 to create the deletion, chimeric fusion gene, and kinase produces tumors that recapitulate the histopathology and the transcriptome of FLC (4, 5). Expression of the fusion gene from a transposon also produces tumors, demonstrating that pathogenesis depends on the DNAJB1–PRKACA chimeric protein, not on the loss of genes in the deletion (4). Thus, the chimeric protein is both correlated with and causative of FLC.

FLC is classified as a subset of hepatocellular carcinoma (HCC; ref. 6) and treatments for HCC are often used for FLC. However, FLC differs from HCC in its genome, coding and noncoding transcriptome, proteome (7–9), and molecular pathogenesis, suggesting these cancers may require distinct therapies. Currently, patients with FLC are included in the Pediatric Hepatic Malignancy International Therapeutic Trial (PHITT), receiving surgery plus cisplatin and doxorubicin combination therapy. If the tumors are unresectable or metastatic, patients are also given sorafenib, and they are randomized to evaluate the utility of intercalating cycles of gemcitabine and oxaliplatin (NCT03533582). Although the use of these drugs in FLC has been reported, their efficacy is not yet known (10). Surgery is the only established curative therapy for FLC, and is usually only palliative for advanced disease (11). For metastatic or anatomically unresectable disease, current chemotherapeutics offer minimal survival advantage (12, 13), and the overall five-year survival ranges between 30% and 45% (14–18). There are no clinically applicable inhibitors of the DNAJB1–PRKACA chimera.

Here, we investigated whether approved or late-phase therapeutics could be repurposed for FLC. We generated a...
collection of FLC patient-derived xenografts (PDX). Resected tumor tissue was implanted without interim cell culture. The PDX were validated for expression of the fusion transcript and protein, and a histopathology and transcriptome that recapitulated the tumor of origin. We tested a repurposing library of >5,000 drugs on cells dissociated from the PDX. The top hits included napabucasin, inhibitors of epigenetic modulators, topoisomerase 1 (TOPO1), and antiapoptotic proteins. The efficacy of these hits was further validated on PDX implanted in mice. Cells taken directly from freshly resected patient tumors provided additional validation. We show the activity of napabucasin is mediated through inhibition of translation initiation and by activation of reactive oxygen species (ROS). We also demonstrate that drug sensitivity inversely correlates with expression of the antiapoptotic protein BCL-xL, and that inhibitors of BCL-xL synergize with other drugs. This is the first large-scale drug screen for FLC using PDX made from fresh tissue without interim cell culture. This screen identifies four novel classes of therapeutics for FLC as well as synergistic drug combinations. Finally, our direct-from-patient drug screening provides a rapid assay for the personalized profile of therapeutic efficacy against a solid tumor.

RESULTS

PDX of FLC Recapitulate the Original Tumor

Fresh tumor tissue was donated by patients (age range, 17–36 years old, 4 female, 2 male) undergoing surgery for FLC. Tumors were cut into pieces and implanted subcutaneously, intrahepatically, or under the kidney capsule. A portion was dissociated into single cells and injected intrasplenically, intraperitoneally, or subcutaneously in NOD/SCID-gamma (NSG) mice (see Methods). Tumors were detected by observation and palpation (range, 3–12 months). Xenografts were derived from a primary liver tumor (FLC1), a liver recurrence (FLC4), or metastases (FLC2, 3, 5, and 6). The tumors came both from patients who were treatment-naïve and patients who had received prior chemotherapy (Supplementary Table S1).

To evaluate whether the PDX model recapitulates the patient tumor, several criteria were assessed. First, the original tumors and the derived PDX were tested by reverse transcriptase PCR (RT-PCR) for expression of the DNAJB1–PRKACA fusion transcript (Fig. 1A). In all cases, the fusion transcript was found in the patient tumor and in the derived PDX, but never in the adjacent nontumor tissue. Through multiple rounds of passaging, xenografts never lost expression of the fusion transcript. The second validation was by Western blotting for the DNAJB1–PRKACA fusion protein (Fig. 1B). An antibody that recognizes the shared carboxyl end of PRKACA and DNAJB1–PRKACA demonstrated that although the native protein is found in all samples, the fusion protein was found in every primary tumor and metastasis and PDX, but not in adjacent nontumor tissue. The third validation was histologic analysis. Individual PDXs closely resembled the corresponding tumor of origin and showed typical features of FLC, including large cells with eosinophilic cytoplasm, prominent nucleoli, “pale bodies” (cytoplasmic inclusion bodies), and areas of fibrosis (Fig. 1C). The fourth validation test was based on the transcriptome. We characterized the transcriptome of primary FLC tumors and paired adjacent nontumor liver (dbGaP Study Accession: phs002435.v1.p1) and identified 509 differentially expressed genes (|log2| fold change ≥1, FDR ≤5%; Supplementary Table S2). Unsupervised clustering of tumors, PDX, and nontumor liver showed that tumors clustered with their derived PDX and separated from adjacent nontumor liver (Fig. 1D). The |log2| fold change of the same differentially expressed genes showed excellent correlation (R2 = 0.944) between tumors and derived PDX (Fig. 1E). Principal component analysis of these differentially expressed genes demonstrated FLC tumors clustered with their derived PDX and away from adjacent nontumor tissue (Fig. 1F). These four criteria support the conclusion that the PDX faithfully replicate important characteristics of their tumors of origin.

In Vitro Screening for Drug Repurposing

Cells dissociated from the PDX were screened with >5,000 compounds (Fig. 2A; Supplementary Table S3; data from the high-throughput screens are at https://osf.io/wzd6u/). We tested compounds eligible for repurposing, including drugs that are approved for clinical use or are in clinical trials. Preclinical and tool compounds were included to help differentiate putative on-target from off-target activities of the clinical drugs.

The screen identified 175 compounds for further evaluation that induced ≥40% killing after treatment with 1 μmol/L for 72 hours. To this list, we then added compounds that are in clinical use against FLC or inhibit targets relevant to the biology of FLC to assess their efficacy (full list is at https://osf.io/wzd6u/). For further validation, these compounds were freshly prepared from powder and then screened using an 11-point dose–response curve (10 nmol/L–10 μmol/L) on six FLC PDX. Our control cells were primary human hepatocytes (PHH) propagated in immune-deficient FNRG mice (19). Drug efficacy was ranked by the average −log10EC50 for FLC1–5, minus the −log10EC50 for the PHH. There was no loss of cell viability during the course of the screen (Supplementary Fig. S1A).
Therapeutics for Fibrolamellar Carcinoma

A

FLC1
N T p0 p1 p2 p3 p4 - +
FLC3
M p0 p1 - +
FLC4
N T p0 p1 - +
FLC2 FLC5 FLC6
T p4 T p4 T p4 + -

B

FLC1
DNAJB1–PRKACA
FLC4
DNAJB1–PRKACA
FLC3
FLC5

C

Patient tumor
Derived PDX

FLC1
FLC2
FLC3
FLC4
FLC5

D

Sample_id
Tissue_type
Sample_id
20
1N
1T
1p0
1p4
1p5
1p4
1p5
2N
2p0
2p2
2T

3T
3p0
3p2
3p0
3p2
4N
4p0
4p2
4p0
4p2
5T
5p0
5p2
5p0
5p2

E

Log2 (fold change) in tumor vs. normal

Log2 (fold change) in PDX vs. normal

R² = 0.9443

F

PC1: 53% variance

PC2: 16% variance

Tissue type
Normal
Tumor

DNAJB1–PRKACA

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Figure 2. Napabucasin is potent and selective against FLC. A, Workflow for generation of PDX and high-throughput drug screening. B, Normalized percent survival of FLC1-6 and PHH treated with napabucasin, additional inhibitors of the EIF4F complex (SBI-0640756 and CR–1–31–B) or ryuvidine [a SETD8 protein lysine methyltransferase (PKMT) inhibitor]. C, Expression levels of STAT3 by differential expression analysis from RNA-seq in nontumor liver (N), tumor’s (T), and PDX (P). D, IHC staining for pSTAT3 on a section of FLC1 showing positive staining only in the stroma (scale bar, 50 μm). E, Normalized percent survival of cells from FLC1 treated with the STAT3 inhibitors C188-9 (red) and HO3867 (blue). F, Differential expression analysis of oxidoreductases (NQO1, CBR1, POR, NFE2L2, TXN) in nontumor liver (N), tumors (T), and PDX (P) from RNA-seq. G, Normalized percent survival of cells from FLC1 treated with different drugs in the presence (red) or absence (blue) of NAC 10 mmol/L. H and I, Effect of napabucasin (1 μmol/L or 2 μmol/L) on eIF4F-sensitive protein levels of (H) c-MYC with GAPDH control and (I) MDM2, BCL-xL, MCL1, Cyclin D1, C/EBP with GAPDH control, in FLC6 cells.
The drug sensitivity of the liver tumors (FLC1, 4) segregated from the metastases, which were more resistant (FLC2, 3, 5; Supplementary Fig. S1B). The top hit in our screen was napabucasin; the second and third were the epigenetic modulators JIB-04 and ryuvidine; and the fifth was navitoclax, which blocks antiapoptotic proteins.

## Validation of Agents Identified in the Primary Screen

### Napabucasin

In our efficacy ranking, the top hit was napabucasin, which was potent against all FLC PDX in vitro with an EC₅₀ that ranged from <10 nmol/L to ~300 nmol/L. There was no detectable effect on PHH even at 10 μmol/L (Fig. 2B; Supplementary Fig. S1B). The antitumor effect of napabucasin is reported to be mediated by inhibiting pSTAT3 (20, 21). However, when we probed for expression levels, STAT3 transcripts were decreased 4- to 5-fold in FLC tumors relative to the adjacent nontumor liver (Fig. 2C). When protein levels were probed using IHC, FLC cells were negative for pSTAT3 staining, and positive cells were observed only in the stroma (Fig. 2D). Additionally, specific inhibitors of STAT3, C188-9, and HO3867 had no effect on FLC cells in vitro (Fig. 2E). These results are not consistent with napabucasin affecting FLC through inhibition of STAT3.

The cytotoxicity of napabucasin has alternatively been proposed to be mediated by generation of ROS. The NAD(P)H dehydrogenase [quinone]1 (NQO1) bioactivates napabucasin to form a hydroquinone, which, in turn, becomes a semiquinone releasing cytotoxic ROS (22). NQO1 is increased 32-fold in the transcriptome of FLC relative to adjacent normal (ref. 7; Fig. 2E), while the levels of other oxidoreductases such as carbonyl reductase 1 (CBR1) or cytochrome P450 oxidoreductase (POR), nuclear factor erythroid 2-related factor 2 (NFE2L2), and thioredoxin (TXN), which do not bind as effectively to napabucasin, were not similarly elevated (Fig. 2F). This mechanism is consistent with the efficacy of elesclomol, which induces ROS (23) and is sixth in our screen.

To test the role of ROS in napabucasin-induced cytotoxicity against FLC, we pretreated the cells with N-acetyl cysteine (NAC), a precursor of cysteine and glutathione, which scavenges ROS. NAC partially attenuated napabucasin toxicity (Fig. 2G). In contrast, NAC had no effect on SN38, the active metabolite of irinotecan, and NAC completely abrogated the toxicity of other agents, including ryuvidine, VLX1570, and SBI-0640756, NSC697923, and mildly attenuated that of panobinostat, quisinostat, and LY2877857 (Fig. 2G; Supplementary Fig. S2). These results indicate that ROS generation contributes to some, but not all, of the effects of napabucasin.

Napabucasin has also been reported to inhibit protein synthesis through the eukaryotic initiation factor 4E (eIF4E) and eIF4E-binding protein 1 (4E-BP1), components of the eIF4F complex involved in the initiation of protein synthesis for certain mRNA (21). One of the best-studied oncogenes transcriptionally regulated by the eIF4F complex is c-MYC (24). FLC cells treated with 1 μmol/L napabucasin showed a dramatic decrease in c-MYC levels with no effects on GAPDH (Fig. 2H). Treatment with napabucasin lowered the levels of additional proteins whose synthesis is known to be sensitive to the eIF4F complex, including MDM2, BCL-xL, MCL1, and Cyclin D1 (Fig. 2I). SBI-0640756, which also disrupts the complex, was the 19th-best compound in the screen (Supplementary Fig. S1B) and showed a clear therapeutic window against FLC relative to PHH (Fig. 2B). Derivatives of silvesterol, such as CR-1-31-B, specifically inhibit the eIF4A RNA helicase and block the translation of mRNA-containing a G-quadruplex (25). Inhibiting eIF4A blocks the synthesis of several key oncogenes, including c-MYC and antiapoptotic proteins such as BCL-xL and MCL1 (25). CR-1-31-B was cytotoxic to FLC in the low nanomolar range, with no effect on PHH (Fig. 2B). These results indicate that inhibition of eIF4F may substantially contribute to the effects of napabucasin, and eIF4A, and perhaps other members of the initiation complex, may be important therapeutic targets in FLC.

### Epigenetic Modulators

Two of the three highest-scoring agents in our screen were epigenetic modulators: JIB-04, a pan-jumonji histone demethylase inhibitor (second in efficacy), and ryuvidine, a SETD8 protein lysine methyltransferase (PKMT) inhibitor (third). Both were effective against all PDX lines (EC₅₀ 20–200 nmol/L, respectively) and did not affect PHH (EC₅₀ > 10 μmol/L; Supplementary Fig. S1B; Fig. 3A; Fig. 2B). Clinical-stage epigenetic modulators had a variable effect on different PDX. Quisinostat (46th on the list) and panobinostat (103rd) are pan-histone deacetylase inhibitors (HDACi) that were more effective against the primary liver (FLC1, 4) than the metastatic FLC lines (FLC2, 3, 5; Fig. 3A), while fimepinostat (22nd), another pan-HDACi, affected FLC1, 3, and 4.

### Topoisomerase Inhibitors

SN-38, a metabolite of the TOPO1 inhibitor irinotecan, was one of our most efficacious compounds (13th). However, similarly to clinical-stage epigenetic modulators, it also showed a variable response and was effective only against FLC1, 3, and 4. SN-38 was more potent than topotecan or camptothecin, while irinotecan, which requires hepatic metabolism, showed no effect in vitro (Fig. 3B; Supplementary Fig. S3A). The TOPO2 inhibitor idarubicin showed an effect on some of the FLC PDX, whereas others (aclarubicin, daunorubicin, and doxorubicin) did not (Fig. 3B; Supplementary Fig. S3A).

### Other Modulators of Protein Levels

The top hits in our screen included compounds that, like napabucasin, modulate protein levels independent of transcription. These include NSC697923, the seventh most efficacious compound; an E2 ubiquitin conjugating enzyme (UBE2N) inhibitor, VLX1570, at #31; a deubiquitinase (USP14) inhibitor, PS901, at #38; a deubiquitinase (USP7), and NMS873, an AAA ATPase (VCP/p97) inhibitor at #72. These compounds were effective against different subsets of the FLC PDX (Fig. 3C). We did not see any effect of proteasome inhibitors such as bortezomib, ixazomib, delanzomib, ONX-0914, LDN-57444, Quisinostat (46th on the list) and panobinostat (103rd) are pan-histone deacetylase inhibitors (HDACi) that were more effective against the primary liver (FLC1, 4) than the metastatic FLC lines (FLC2, 3, 5; Fig. 3A), while fimepinostat (22nd), another pan-HDACi, affected FLC1, 3, and 4.

### Inhibitors of PKA

The oncogenic activity of DNAJB1–PRKACA requires its kinase activity (4). Uprosertib, a PKA inhibitor currently...
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Figure 3. Dose–response curve validation of drugs from high-throughput screen. A–I, Dose–response curve of drugs tested in triplicate against cells from PDX (FLC1–6) and PHH. Error bars, standard deviation. Drugs were tested at 10 μmol/L–10 nmol/L with 2-fold serial dilution (apart from specific drugs tested at higher concentrations based on the published EC50). The y-axis shows normalized percent survival calculated as 100 – [(positive control – drug response)/(positive control − negative control) × 100]. A, Epigenetic modulators: HDAC inhibitors panobinostat, fimepinostat, quisinostat; and HDM inhibitor JIB-04. B, TOPO1 inhibitors SN38, irinotecan, topotecan, and TOPO2 inhibitor idarubicin (more in Supplementary Fig. S3A). C, Inhibitors of the protein degradation pathway: NSC697923, NMS873, VLX-1570, and P5091. D, Drugs proposed for FLC that were tested in the preliminary screen, but did not meet criteria for validation, including inhibitors of MEK/ERK, HSP70, CA12, aromatase, proteasome, and receptor tyrosine kinases. y-axis shows normalized percent survival of cells after 1 μmol/L treatment for 72 hours. Blue, FLC1; orange, FLC5. E, Protein kinase A inhibitors (more in Supplementary Fig. S3B). F, The PAK1-3 inhibitor FRAX597. G, The Aurora kinase A inhibitor ENMD-2076 (more in Supplementary Fig. C). H, The ERBB2 inhibitor neratinib (more in Supplementary Fig. S3D). I, The tyrosine kinase inhibitors sorafenib (regorafenib in Supplementary Fig. S3E). J, The CDK inhibitors dinaciclib and flavopiridol (more in Supplementary Fig. S3F). K, PI3K/mTOR inhibitors (more in Supplementary Fig. S3G).

Table 1. PKA inhibitors tested

<table>
<thead>
<tr>
<th>Drug</th>
<th>K, PKA</th>
<th>K, against other kinases</th>
<th>EC50 FLC</th>
<th>EC50 PHH</th>
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<tbody>
<tr>
<td>H89</td>
<td>48 nmol/L</td>
<td>PKG 480 nmol/L</td>
<td>FLC1 5.82 μmol/L</td>
<td>41.5 μmol/L</td>
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<td></td>
<td></td>
<td></td>
<td>FLC2 23.88 μmol/L</td>
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<td></td>
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<td></td>
<td>FLC3 18.17 μmol/L</td>
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<td></td>
<td></td>
<td></td>
<td>FLC4 20.94 μmol/L</td>
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<td></td>
<td></td>
<td>FLC5 9.49 μmol/L</td>
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<tr>
<td>KT5720</td>
<td>60 nmol/L</td>
<td>PKG/PKC &gt;2 μmol/L</td>
<td>FLC1 1.61 μmol/L</td>
<td>&gt;10 μmol/L</td>
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<td></td>
<td></td>
<td></td>
<td>FLC2 2.14 μmol/L</td>
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<td></td>
<td>FLC3 2.71 μmol/L</td>
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<td></td>
<td>FLC4 1.48 μmol/L</td>
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<td></td>
<td></td>
<td></td>
<td>FLC5 3.87 μmol/L</td>
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<tr>
<td>A-674563</td>
<td>16 nmol/L</td>
<td>Akt1 11 nmol/L</td>
<td>FLC1 0.34 μmol/L</td>
<td>3.45 μmol/L</td>
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<td></td>
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<td>CDK2 4.6 nmol/L</td>
<td>FLC2 0.7 μmol/L</td>
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<td></td>
<td></td>
<td>GSK-3β 110 nmol/L</td>
<td>FLC3 0.88 μmol/L</td>
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<td></td>
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<td>ERK2 260 nmol/L</td>
<td>FLC4 0.16 μmol/L</td>
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<td></td>
<td></td>
<td>PKCζ 360 nmol/L</td>
<td>FLC5 3.45 μmol/L</td>
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<tr>
<td>Capivasertib (AZD5363)</td>
<td>7 nmol/L</td>
<td>Akt1 3 nmol/L</td>
<td>FLC1 2.98 μmol/L</td>
<td>&gt;10 μmol/L</td>
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<td></td>
<td></td>
<td>Akt2 8 nmol/L</td>
<td>FLC2 3.85 μmol/L</td>
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<td></td>
<td></td>
<td>Akt3 8 nmol/L</td>
<td>FLC3 5.15 μmol/L</td>
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<td></td>
<td></td>
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<td>FLC4 2.09 μmol/L</td>
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<td></td>
<td></td>
<td>ROCK2 580 nmol/L</td>
<td>FLC5 12.6 μmol/L</td>
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<td>AT13148</td>
<td>3 nmol/L</td>
<td>Akt1 38 nmol/L</td>
<td>FLC1 0.039 μmol/L</td>
<td>4.05 μmol/L</td>
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<td></td>
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<td>Akt2 402 nmol/L</td>
<td>FLC2 6.14 μmol/L</td>
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<td></td>
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<td>FLC3 0.59 μmol/L</td>
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<td></td>
<td></td>
<td>p70s6k 8 nmol/L</td>
<td>FLC4 4.29 μmol/L</td>
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<td></td>
<td></td>
<td>ROCK1 6 nmol/L</td>
<td>FLC5 0.096 μmol/L</td>
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<td></td>
<td></td>
<td>ROCK2 4 nmol/L</td>
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<tr>
<td>Uprosertib (GSK2141795)</td>
<td>2 nmol/L</td>
<td>Akt1 180 nmol/L</td>
<td>FLC1 0.049 μmol/L</td>
<td>&gt;10 μmol/L</td>
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<tr>
<td></td>
<td></td>
<td>Akt2 328 nmol/L</td>
<td>FLC2 5.039 μmol/L</td>
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<td>Akt3 38 nmol/L</td>
<td>FLC3 0.87 μmol/L</td>
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<td></td>
<td></td>
<td>p70s6k 8 nmol/L</td>
<td>FLC4 0.91 μmol/L</td>
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<tr>
<td></td>
<td></td>
<td>ROCK2 4 nmol/L</td>
<td>FLC5 1.72 μmol/L</td>
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in clinical trials (NCT02093546) was #28 in efficacy and AT13148 was #51. AT13148 and uprosertib showed efficacy in the nanomolar range against FLC1, and AT13148 showed nanomolar efficacy for FLC1 and 5. Capivasertib and KT5720 showed uniform efficacy against all FLC PDX lines, but in the micromolar range (Fig. 3E). H89 and A-674563 showed no therapeutic window for FLC over PHH (Supplementary Fig. S3B). Thus, inhibitors of PKA showed mixed results (Table 1).

Oncogenes in FLC

Several oncogenes are increased in FLC tumors including aurora kinase A (AURKA), aromatase, p21 activated kinase 3 (PAK3), and members of the epidermal growth factor receptor
(EGFR) pathway including ERBB2 (HER2; ref. 7). Consequently, some are targets of clinical trials for FLC. FRAX567, a preclinical group-1–specific PAK inhibitor (IC₅₀ = 8.13/19 nmol/L for PAK 1/2/3), was #12 in our efficacy ranking, showing consistent inhibition against all PDX (Fig. 3F). We have previously shown that PAK3 is one of the most highly expressed oncogenes in FLC (7). The effects of inhibiting the other oncogenes that are increased in expression in FLC were not as strong. ENMD-2076, an AURKA inhibitor (IC₅₀ = 14 nmol/L) in a clinical trial for FLC (NCT02234986), was #123 on the list, and had an EC₅₀ > 5 μmol/L against the PDX cells (Fig. 3G). Aurora kinase inhibitor I, a preclinical inhibitor (IC₅₀ = 3.4 nmol/L), was #51 with an EC₅₀ of 1–4 μmol/L. However, alisertib, a more potent inhibitor of AURKA (IC₅₀ = 1.2 nmol/L) in phase III trials for small cell lung cancer and other malignancies, showed no effect on FLC (Supplementary Fig. S3C), suggesting that the effect of aurora kinase I is likely off-target. Neratinib, an inhibitor both of EGFR (IC₅₀ = 2 nmol/L), and CP-724714, in trials for tumors expressing HER2 (IC₅₀ = 92 nmol/L) and E₂ RB (IC₅₀ = 14 nmol/L), was potent against HER2-positive breast cancer cells in vitro, but not in our panel of PDX (Supplementary Fig. S3A). To test whether BCL-xL was responsible for upregulation of antiapoptotic proteins, including NOXA, PUMA, and BID, we measured apoptosis in FLC1 and 5 in the micromolar range (Fig. 3H). However, we performed an anchor screen on the relatively resistant FLC5. Transcripts for BCL-xL were increased 160% compared with the adjacent nontumor liver. In PDX that were more resistant to therapeutics based on average IC₅₀ of compounds tested, BCL-xL was increased 160% compared with the adjacent nontumor liver (Fig. 4B). Thus, BCL-xL, but not BCL2, may provide a mechanism of apoptosis evasion in FLC. Transcripts for other antiapoptotic proteins, including NOXA, PUMA, and BID, were not significantly altered, BAX and BAK were increased 50% (7), and MCL1, BIM, and BCL2 were downregulated (Fig. 4C; ref. 7), further strengthening this hypothesis. This led to the suspicion that some of the variability in the response of PDX might be the consequence of differential expression of antiapoptotic proteins.

Panobinostat, a pan-HDACi (see epigenetic modulators above), was one of the drugs that demonstrated a highly variable response among the sensitive and resistant PDX (Supplementary Fig. S1; 3A). To test whether BCL-xL was responsible for the variable responses to epigenetic modulators, we used A1331852, a selective BCL-xL inhibitor. A1331852 synergized with panobinostat, and the extent of synergy correlated to BCL-xL expression. A more modest synergy was seen with MCL1 inhibition (AZD5991), and no synergy was observed with BCL2 inhibition (venetoclax, ABT199; Fig. 4D). An alternative assay using dose–response curves (28) similarly demonstrated synergy between panobinostat and A1331852 in FLC (Fig. 4E). This included sensitizing the more resistant lines for panobinostat as an active single agent. Importantly, treatment with A1331852 did not increase the toxicity of panobinostat in PHH (Fig. 4E) and was thus specific for FLC.

### Effect of Inhibiting BCL-xL on Response to Other Agents

To further explore the effect of inhibition of BCL-xL in FLC, we performed an anchor screen on the relatively resistant FLC5. We tested different compounds in combination with either the BCL-xL inhibitors A1331852 and navitoclax or the BCL2 inhibitor venetoclax. These included inhibitors of mTOR (visutserib and everolimus), MEK (trametinib), AURKA (ENMD-2076), PKA (AT13148 and uprosertib), TOPO1 (topotecan), and TOP2 (idarubicin). All drugs, apart from ENMD-2076 and uprosertib, showed some synergy with A1331852. Results were similar when drugs were combined with navitoclax, except for uprosertib which now synergized. No drug showed synergy with venetoclax (Fig. 4F). Thus, the sensitivity of FLC to a selective group of compounds is enhanced by blocking BCL-xL.

### In Vivo Validation of Therapeutics For FLC

In vitro drug efficacy data may be unrepresentative of outcomes in animal studies. We therefore tested some of our top hits from the in vitro screens in vivo, in mice bearing
Figure 4. Inhibiting BCL-xL sensitizes FLC but not PHH. A, Normalized percent survival of FLC1-6 and PHH with antiapoptotic inhibitors navitoclax (BCL2/BCL-xL), obatoclax (BCL2/BCL-xL), and venetoclax (BCL2). B, Differential expression analysis of BCL-xL in nontumor liver (N), tumors (T), and PDX (P) from RNA-seq (left). Differential expression of BCL-xL in nontumor liver, sensitive (FLC1 and 4) versus resistant (FLC2, 3, and 5) tumors and derived PDX (right). C, Differential expression analysis of additional antiapoptotic (BCL2 and MCL1) and proapoptotic (BIM, BID, NOXA, and PUMA) genes in nontumor liver, tumors, and PDX from RNA-seq. D, The ZIP synergy score of combination therapy with panobinostat and A1331852 (BCL-xL inhibitor), AZD5991 (MCL1 inhibitor), and venetoclax (BCL2) for FLC1, 3, 5, and PHH. E, Dose–response curves of panobinostat in the absence (full circles) and presence (empty circles, dotted lines) of A1331852, showing the normalized percent survival for FLC1, 3, 5 (blue, green, and orange, respectively) and PHH (black). F, Synergy scores (ZIP, Bliss, and HSA) for A1331852, navitoclax, and venetoclax anchor screens.
PDX (see Methods). Napabucasin, the top hit in our screen (Supplementary Fig. S1), reduced growth at 7 and 14 days (44% and 52%) versus vehicle control (70% and 118%, \( P < 0.01 \); Fig. 5A). There was a decrease in mitotic figures, Ki-67 and pH3-positive cells in the tumors of the treated mice, and a small increase in TUNEL-positive cells (Fig. 5A and B). Mice remained active and alert during the course of the napabucasin treatment and experienced an average weight loss of 6.7% compared with 6.2% in the vehicle control–treated group. The ratio of tumor volume to mouse weight was stable or decreased for most of the napabucasin-treated mice but consistently increased in those treated with vehicle control. To assess the effect of inhibiting eIF4A, CR-1-31B was tested on FLC6, our most aggressive PDX. A statistically significant reduction of growth was observed starting within two weeks versus vehicle control–treated mice (Supplementary Fig. S4A). It is possible that some of the agents that were negative in the in vitro tests, such as a pan-kinase inhibitor, sorafenib, might function by blocking processes present only in intact tissue. However, when sorafenib was tested on FLC6 in vivo, there was no detectable effect on tumor growth (Supplementary Fig. S4B).

HDAC inhibitors that scored well in vitro and are available in the clinic were tested in mice. Panobinostat led to complete inhibition of tumor growth (~7% growth at 7 days; 4% at 14 days) compared with vehicle control (45% at 7 days and 102% at 14 days, \( P < 0.0001 \); Fig. 5C). Mice remained active and alert during the treatment. Those treated with panobinostat experienced an average weight loss of 11% compared with 6% in the vehicle control–treated group. The tumor volume to mouse weight ratio was stable or decreased in panobinostat-treated mice, but increased in mice treated with vehicle control. Panobinostat led to a dramatic decrease in mitotic figures and in Ki-67 and pH3-positive cells. However, there was no increase in apoptosis, as assayed by TUNEL (Fig. 5C and D).

We used the HDAC inhibitor quisinostat to further test the effect of such inhibitors. Treatment of mice with quisinostat led to complete inhibition of tumor growth compared with vehicle control treatment (7% vs. 110% growth at 14 days, respectively, \( P < 0.0001 \); Supplementary Fig. S4C). Treatment with quisinostat led to an almost complete disappearance of mitotic figures, Ki-67 and pH3-positive cells, but no significant increase in apoptotic cells as assayed by TUNEL (Supplementary Fig. S4C and S4D). Macroscopic assessment showed necrosis in the drug-treated tumors (Supplementary Fig. S5).

In treatments of dissociated cells from PDX in vitro, panobinostat synergized with A1331852, an inhibitor of BCL-xL (Fig. 3D–F). Administration of these drugs showed synergism in vitro, and after two days of treatment, many apoptotic cells were observed with the combined treatment of panobinostat and A1331852 (Fig. 5E and F). However, consistent with the previous longer treatments (Fig. 5D), no evidence of increased apoptosis was detected in tumors from mice treated with panobinostat alone compared with vehicle (Fig. 5F). Furthermore, in FLC5, the most drug-resistant PDX, panobinostat synergized with navitoclax, resulting not only in inhibition of tumor growth, but in partial response, with a significant reduction in tumor size (Fig. 5G).

### Testing in Cells Directly Isolated from Patient Tumors

To evaluate whether our screening results in PDX-derived FLC cells were recapitulated in tumor tissue, some of the most efficacious compounds were tested on cells directly isolated from patient tumors. Within a few hours of resection, cells were dissociated and screened against a panel of compounds (Supplementary Fig. S6A). All tumor cells were derived from lymph node metastases, with the exception of FLC.T9, which was from ascites fluid (Supplementary Table S5). All cells tested directly from patients were sensitive to the top hits of our in vitro screen including the three inhibitors of the eIF-4F complex (napabucasin, CR-1-31-B, and SBI-0640756), the two HDAC inhibitors (panobinostat and quisinostat), the TOPO1 inhibitor (SN-38), and the dual PI3K (p110α)/DNA-PK inhibitor (PIK-75). There was some sensitivity to proserotinib, dinaciclib, flavopiridol, and LY2857885. There were some compounds that did not fare well on PDX, that showed response on a few of the direct-from-patient testing. Most notable is neratinib, currently in a clinical trial for FLC, which had a \( K_s \) of 300–700 nmol/L for three of the direct-from-patient tumor cells, but no effect on others (Fig. 6A).

There was a striking similarity among the response profiles of cells isolated from lymph nodes of different patients with FLC. The cells from the ascites fluid were more sensitive to some of the drugs in this panel, but their response otherwise resembled that of the lymph nodes. Compounds that inhibit protein degradation pathways (NSC697923 and VLX1570) showed a blunted response in the direct-from-patient testing (Fig. 6A). However, it should be noted that these compounds were not active against cells from all PDX lines either. FLC.T6 was the only tumor for which cells were compared directly from the patient tumor and the derived PDX. Drug sensitivity for FLC.T6 and FLC6 correlated well for most drugs tested and specifically for napabucasin, panobinostat, and SN-38, as well as neratinib. Notable outliers were NSC697923, VLX1570, and AT13148 (Fig. 6B; Supplementary Fig. S6B).

### Figure 5.

In vivo validation of drugs against FLC. A and B, Effect of napabucasin (10 mg/kg) or vehicle control after 14 days on FLC1. A, Effect of napabucasin on tumor volume in mice (n = 6; average ± SE; \( P < 0.05 \)) and quantification of the IHC for TUNEL, Ki-67, and pH3. B, H&E of tumors for mitotic bodies and IHC for TUNEL, Ki-67, and pH3. C and D, Effect of panobinostat (10 mg/kg) or vehicle control after 14 days on (C) tumor volume (n = 5; average ± SE; \( P < 0.001 \)) and quantification of the IHC for TUNEL, Ki-67, and pH3 and (D) H&E of tumors for mitotic bodies and IHC for TUNEL, Ki-67, and pH3. E, H&E of tumors and IHC for TUNEL for mice implanted with FLC1 were treated with vehicle, panobinostat (10 mg/kg), A1331852 (25 mg/kg), or both for 2 days. Scale bar, 50 μm. F, Quantification of the TUNEL IHC. G, Mice implanted with FLC5 were treated with panobinostat (2.5 mg/kg), navitoclax (50 mg/kg), or both (n = 4; average ± SE; \( P < 0.01 \)).

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Therapeutics for Fibrolamellar Carcinoma

A

B

C

D

E

F

G

H&E TUNEL Ki-67

Panobinostat

H&E TUNEL Ki-67

Panobinostat

H&E TUNEL Ki-67

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H&E TUNEL Ki-67

Panobinostat
**Figure 6.** Direct-from-patient in vitro screening recapitulates findings from PDX screen. **A,** Dose–response curve of drugs tested in triplicate against cells dissociated directly from patient tumors (T6–T11) versus PHH. Error bars, standard deviation. Drugs were tested at 10 μmol/L–10 nmol/L with 2-fold serial dilution. The y-axis shows normalized percent survival calculated as 100 − [(positive control – drug response)/(positive control − negative control)] × 100. Cells were treated for 72 hours, and normalized percent survival was quantified using CellTiter-Glo. **B,** Comparison of the EC50 in cells dissociated from patient tumor FLC.T6 vs. cells dissociated from its derived PDX FLC6.
DISCUSSION

The development of efficacious therapies for FLC has been stymied by the lack of faithful models. As a result, clinicians have had to rely either on therapies used for other liver cancers, such as HCC, or on therapies that target pathways that are upregulated in FLC. Both approaches are potentially problematic. First, FLC and HCC are distinct diseases. There is little resemblance in the coding and noncoding transcriptome of HCC and FLC (7, 8), and the pathogenesis of FLC is rooted in dysregulation of the ecology of PKA signaling (1, 29), which has not been implicated in HCC. In addition, there is a lack of published data showing that agents used in treating HCC are efficacious in treating FLC. Finally, pathways that are activated in FLC, or at least increased in expression, may be bystanders irrelevant to pathogenesis or therapy. For example, the increase of PKA activity may be increasing expression of aromatase or AURKA, but these are not necessarily oncogenic in FLC.

To facilitate therapeutic development, we generated several PDX that were validated at the histologic, genomic, transcriptomic, and proteomic levels. These were used to test therapeutics that have been used clinically for FLC, target pathways activated in FLC, and, in an agnostic screen of clinical-stage drugs, identify those that could be repurposed for FLC. As a controlscreen we used PHH, similarly propagated and pas-saged in immune-deficient mice. The top hits were validated in vivo and then confirmed on tumor cells isolated directly from patient tissue after resection.

Several novel compounds and targets were identified in our screen. Napabucasin was potent against all FLC PDX and did not cause toxicity in the PHH up to the highest dose tested (Fig. 2B). Napabucasin inhibits tumor progression and metastasis in osteosarcoma (21) and is being studied in phase III clinical trials against several solid tumors. A proposed target of napabucasin is the eIF4F complex. Two other compounds that affect this pathway, SBI-0640756 and CR-1-31-B, also demonstrated specific toxicity in FLC PDX and direct-from-patient screens (Figs. 2B and 6A). SBI-0640756 inhibits eIF4G1 and disrupts the eIF4F complex, blocking the growth of BRAF-resistant melanoma (30). CR-1-31B was effective in vitro of BRAF-resistant melanoma (30). Navitoclax with panobinostat led to a partial response of the combination was consistent with data from monotherapy studies (NCT01009073; ref. 37). Many drugs, which had no detectable activity on their own, synergized with blockers of BCL-xL. In vivo A1331852 synergized with panobinostat to induce apoptosis (Fig. 5E and F), and the combination of navitoclax with panobinostat led to a partial response of the most resistant PDX (Fig. 5G). This suggests that strategies for choosing combination therapies should account for the specifics of the mechanisms of drug sensitivity in FLC.

Various drugs were consistently efficacious against cells dissociated from PDX, preclinical mouse models, and cells dissociated from patients. In contrast, most of the drugs currently used in the clinic had minimal activity against FLC. Sorafenib, a multikinase inhibitor that is first-line therapy for HCC, as well as other tyrosine kinase inhibitors, did not demonstrate efficacy against FLC in vitro or in vivo (Fig. 3F; Supplementary Fig. S4B). Chemotherapeutics such as cisplatin, carboplatin, oxaliplatin, etoposide, fluorouracil, gemcitabine, and ifosfamide that are used to treat FLC in the clinic, or are currently in clinical trials, showed no utility. Several of the oncogenes found to be upregulated in the FLC transcriptome have been tested in clinical trials, including ENMD-2076 for AURKA, everolimus for mTOR, and neratinib for EGFR/ ERBB2. Our screen showed no selective toxicity against FLC over human hepatocytes for any of the drugs in clinical trials for these targets (Fig. 3D, G, and H). Experimental compounds with even higher specificity for the targets showed no selective toxicity over PHH. Thus, it will be informative to see the published outcomes of these clinical trials. Recently, inhibition of MEK was proposed for treating FLC (27). We
tested a panel of 18 MAPK/ERK inhibitors, none of which were efficacious against the FLC cells tested (Fig. 3D). These results suggest a general caution when using therapeutics to target oncogenes that are increased in expression in a tumor.

PDX in mice are a powerful system for preclinical screening of drugs and have been proposed for precision medicine. However, there are some limitations to the use of the PDX for this purpose. First, the success rate for implantation is 30% to 35% in our hands. Thus, the PDX would help only a subset of the patients. Second, the PDX took several months to a year to grow. This limits their utility for providing information within a clinically relevant time frame. Third, the PDX may not recapitulate the response of the patient. The physiology of mice is not identical to that of humans, and the human tumor cells are surrounded by mouse stromal cells whose interacting ligands and receptors may not be completely homologous. Mouse strains used for PDX lack a functional adaptive immune system and may not recapitulate the effect of therapeutics on antitumor immunity. Fourth, an implanted tumor may not respond to drugs in the same way as a tumor that has developed spontaneously. Fifth, passing the tumor in a mouse could lead to changes in drug response compared with the original tumor. Finally, it is possible that only a select small subset of the tumor cells implanted in mice can survive, and these do not recapitulate the full response of the tumor cells in the patient.

The PDX data have identified promising novel targets and the mechanisms through which they may work. Clinical trials are needed for validation. The screens on tumor cells, isolated from patient tumors shortly after resection, were on the entire set of tumor cells and not subgroups potentially selected by mouse passage. The responses in the direct-from-patient screens showed similar results across all patients tested, except for drugs inhibiting protein degradation, which were potent against cells only from ascites (n = 1) but not from lymph node metastases (n = 5). Similarly, both the PDX and the direct-from-patient cells were sensitive to some of the compounds that seem most promising therapeutically: blockers of initiation of translation, HDAC, or TOPO1. There were some exceptions. Neratinib showed no effect on PDX, but efficacy in the submicrolar range on three direct-from-patient screens and no effect on cells from the other three patients. Is this the consequence of differences in assaying PDX or direct-from-patient cells, or is it patient variability?

We compared the results for cells dissociated from PDX FLC6 in Figs. 2–4 with samples derived from the same patient for PDX or direct-from-patient cells, or is it patient variability? The screening of cells directly from patient tumors has a potential advantage. First, the results are available within days of resection or biopsy rather than the months to a year required for PDX. This is critical for informing therapeutic decisions. Second, there is the concern that only a subset of cells have grown to form organoids or a PDX. Thus, tests on these model systems may represent the response of a subset of cells. The direct-from-patient test is done directly on a sample, with no intermediate passaging. Third, the direct-from-patient testing gives a personalized profile of a specific patient tumor. This is a step to fulfilling the promise of precision medicine. We have observed that the FLC tumors fall into distinct classes of response. As we accumulate further data from direct-from-patient screens, individual patient characteristics may become apparent that predict differential responses to individual drugs, much as was revealed by BCL-xl stratification of the PDX results. Validation of this approach will initially come from clinical case reports of otherwise intractable disease that will test the utility of this method.

Finally, our demonstration that the transcriptome is stable and that the expression of the chimeric oncogene remains stable through consecutive passages of PDX in mice strongly suggests that FLC has become oncogenically addicted to DNAJB1–PRKACA (38, 39). Thus, elimination of the oncogene, oncotranscript, or oncoprotein may be an effective therapeutic approach for FLC.

**METHODS**

**Human Tissue Samples**

With Institutional Review Board approval (Rockefeller IRB45503-0797, SSI-0855), written informed consent was obtained from patients scheduled for tumor resection. All studies were conducted in accordance with recognized ethical guidelines. The diagnosis of FLC was determined by a pathologist at each institution, and the demonstration of the DNAJB1–PRKACA fusion transcript by RT-PCR and DNAJB1–PRKACA fusion protein by Western blot, as described below. Tumor tissue and adjacent nontumor liver (if available) were collected for the study. After resection, the tissue was placed into cold PBS on ice, cut into 2–3 × 0.5 cm portions, and placed into 50-mL tubes in Roswell Park Memorial Institute (RPMI-1640 with glucose) media supplemented with 2% penicillin/streptomycin. Pieces of tumor were prepared for implantation into mice (see below). The rest of the tumor tissue was cut into 2-mm pieces (without mincing), and connective tissue blood vessels, clots, and necrotic tissue were discarded. Pieces of tumor were also fixed in formaldehyde for histologic analysis, flash-frozen or placed into Optimal Cutting Temperature compound (OCT), and frozen for later RNA/protein analysis.

**Tumor Dissociation**

The 2-mm pieces of tissue were placed into 50-mL Falcon tubes with RPMI, collagenase 4 (Worthington 1 mg/mL), and DNase (Roche, 1 µg/mL), and digested while rotating at 37°C until digestion was complete (Benchmark scientific Rototherm). All following steps were done on ice or at 4°C. The digested tissue was passed through a 200-µm (Pluriselect) strainer using a syringe plunger for remaining pieces, and then through a 100-µm strainer (Fisher). The cells were spun down at 300 × g for 5 minutes at 4°C and the pellet depleted of red blood cells by a 10-second exposure to 1 mL of water followed by the addition of 49 mL of PBS. The cells were counted and either implanted into mice or used for in vitro experiments. For in vitro experiments, the cells from PDX tumors were subjected to mouse cell depleton according to the manufacturer’s instructions (Miltenyi Biotec). For PDX studies, NSG mice were purchased from Jackson Laboratories (NOD.Cg-Prkdcsid B2gptg1WJr/Nj, strain 005537) and bred at The Rockefeller University animal facility specific pathogen-free (SPF) immune-core. Mice were kept in 12 hours light/dark cycle, fed an amoxicillin diet, and had ad libitum access to food and water. Both...
male and female mice were used for initial implantation of tumors and for passaging PDXs. Female mice 5–8 weeks old were used for in vivo experiments. Mice were inspected at least twice a week for health and tumor growth. Tumors were passaged if they reached 2 cm or the mouse displayed signs of illness or weight loss. For passage of PHH, \( F_{ab}^{-}\) NOD \( Rag^{-}\) II2regnull (FNRR) mice (40) were transplanted with PHH and subjected to intermittent liver injury (41). After humanization of the liver had plateaued, as determined by human albumin levels in mouse serum, PHH were isolated as described previously (19). All experiments were conducted under animal-use protocols approved by Rockefeller University.

**Implantation into Mice**

With Institutional Animal Care and Use Committee approval (#20027-H), mice were anesthetized using isoflurane and given buprenorphine for analgesia. Pieces of tumor were cut and placed into RPMI on ice. Tumor pieces were implanted subcutaneously, under the kidney capsule or directly into the liver. For subcutaneous implantation, a small skin incision was made in the flank area, and pieces of tumor were placed between the skin and fascia, before the skin was stapled. For implantation under the kidney capsule, the kidney and retroperitoneum were exposed via abdominal approach, and a very small incision was made in the kidney capsule. Small pieces of tumor (<0.5 mm) were placed under the kidney capsule and gently advanced away from the incision. The kidney was then placed back into the abdominal cavity, before the abdominal wall was sutured and the skin stapled. For implantation directly into the liver, the liver was exposed via abdominal incision, and a small area was cauterized. Thin forceps were then used to make a small tunnel in the liver parenchyma and a narrow piece of tissue was placed into the tunnel. The surface edge of the tunnel was then cauterized, and the liver was placed back into the abdominal cavity. The abdominal wall was sutured, and skin was stapled. Cells dissociated from patient or PDX tumors were implanted subcutaneously into the liver or the spleen. For subcutaneous implantation 5 \( \times \) \( 10^6 \) cells were mixed at a 1:1 ratio with Matrigel (Corning) and injected into the subcutaneous space over the flank. For intraperitoneal implantation, the 5 \( \times \) \( 10^5 \) cells were mixed with Matrigel at a 1:1 ratio and injected directly into the liver. For splenic injection, 5 \( \times \) \( 10^4 \)–5 \( \times \) \( 10^5 \) cells in RPMI were injected into the spleen.

**Primary Screen.** Cells dissociated from PDX tumors and depleted of mouse stromal cells were used for high-throughput/ screening. For the primary screen, 5,093 compounds were dispersed with Janus 384 pintool (PerkinElmer) into 384-well plates. Cells were plated into the 384-well plates containing screening compounds at 2,000 cells/well in Kubota’s medium (PhoenixSangs Biologicals) supplemented with 2% penicillin/streptomycin and incubated at 37°C for 72 hours. Compounds were screened at a final concentration of 1 \( \mu \text{mol/L} \). Each plate contained negative control wells treated with DMSO and positive control wells treated with 20 \( \mu \text{mol/L} \) chaetocin (Selleckchem #8068). Plates with a Z’-factor <0.5 were disregarded or repeated. The screen was performed in triplicate for FLC1 and FLC5. After 72 hours of incubation CellTiter-Glo reagent (Promega) was added using Thermo Multidrop Combi (Thermo Scientific) according to the manufacturer’s instructions, and the plates were read for luminescence (BioTek Synergy Neo). The normalized percent cell survival was calculated by:

\[
\% \text{ survival} = 100 - \left( \frac{(I_{\text{positive}} - I_{\text{compound}})}{(I_{\text{positive}} - I_{\text{negative}})} \right) \times 100.
\]

**Validation Screen.** Compounds from the primary screen that produced a survival of <60% at 1 \( \mu \text{mol/L} \) after 72 hours were selected for further validation. To these we added inhibitors of protein kinase A inhibitors or pathways upregulated in FLC, and drugs either suggested or in use for FLC. Compounds were plated in 384-well plates from 10 nmol/L–1 \( \mu \text{mol/L} \) using an 11-point, 2-step serial dilution. Each plate was prepared in triplicate. Cells were plated into the 384-well plates containing the compounds at 2,000 cells/well in Kubota’s medium (PhoenixSangs Biologicals) supplemented with 2% penicillin/streptomycin and incubated for 72 hours. Each plate contained negative control wells treated with DMSO and positive control wells treated with 20 \( \mu \text{mol/L} \) chaetocin (Selleckchem #8068). Plates with a Z’-factor <0.5 were disregarded or repeated. Compounds were similarly screened against PHH grown in humanized mice and isolated as previously described (19). PHH were plated in 384-well plates containing compounds at 5,000 cells/well in W10 media [William’s E medium supplemented with ITS (BD), penicillin/streptomycin/gentamycin]. PHH were similarly incubated and assayed for luminescence as described for FLC cells above. To measure the role of ROS in the toxicity of compounds, FLC cells were incubated with compounds for 72 hours in the presence or absence of 10 nmol/L NAC. Cells were assayed for luminescence as above. Dose-response curves were generated using GraphPad PRISM 8 (GraphPad Software; www.graphpad.com).

**Synergy Assay**

For synergy assays, compounds were plated in a two-combination matrix. Compound A (visusertib, everolimus, trametinib, topotecan, ENMD-2076, Idarubicin, AT13148, or upreotinib) was plated in a 10-point, 2-fold dilution from 10 \( \mu \text{mol/L} \) to 20 \( \mu \text{mol/L} \). Compound B (A-1331852, AZD5991, navitoclax, or venetoclax) was plated in an 8-point, 3-fold dilution from 10 \( \mu \text{mol/L} \) to 5 \( \mu \text{mol/L} \). Each plate contained negative control wells treated with DMSO and positive control wells treated with 20 \( \mu \text{mol/L} \) chaetocin. Each plate was prepared in triplicate. Cells were plated into the 384-well plates containing the compounds at 2,000 cells/well in Kubota’s StemCell Growth Medium (PhoenixSangs Biologicals) supplemented with 2% penicillin/streptomycin, incubated for 72 hours, and assayed for luminescence as described above. Dose-response matrix data were analyzed using the web-based application SynergyFinder (42). Synergy results are expressed as ZIP, HSA, and Bliss scores (43, 44).

**Compounds**

Compounds for the primary screen were from the High-throughput and Spectroscopy Center at The Rockefeller University. These are compound screening libraries that include preclinical drugs, clinically approved drugs, and annotated bioactive compounds with pharmaceutically relevant structures. They were part of the following commercially available libraries: Selleck; Tocris; Pharmakon, Microsource; LOPAC, Sigma; Prestwick Chemical; HTSARC Chemical Collection; Rockefeller University; and NIH Clinical Collection (provided through the NIH Molecular Libraries Roadmap Initiative and distributed by Compound Focus, Inc). Compound stocks were stored in propylene glycol/water wells at a final concentration of 5 nmol/L in DMSO at −20°C. AZD5991 and A1331852 and navitoclax were purchased from Chemietek. Sorafenib was purchased from MedChemExpress. CR-1-318 was a gift from the Wendel lab. All other compounds were purchased from Selleckchem in powder form and diluted in DMSO to make a stock solution. These were then further diluted in media to reach appropriate final concentrations.

Data from all screening studies are archived and analyzed using the CDD Vault from Collaborative Drug Discovery (http://www. collaborativedrug.com). MarvinSketch (ChemAxon, version 18.28.0) was also used for creating structure spreadsheets and tables. GraphPad PRISM (version 8) was used for curve fitting and data analysis.

**Drug Formulation for In Vivo Experiments**

Napabucasin, panobinostat, and quisinostat were purchased from Selleckchem and A1331852 and navitoclax from Chemieket, diluted...
in DMSO to make stock solutions, aliquoted, and stored at ~80°C. Napabucasin was formulated by heating to 50°C for 10 minutes and then sequentially adding 45% PEG300 (Sigma), 5% Tween80 (Sigma), and 45% sterile water, with vortexing after adding each component (Selleckchem). Panobinostat was formulated by sequentially adding 48% PEG300 (Sigma), 2% Tween80 (Sigma), and 48% sterile water, with vortexing after adding each component (Selleckchem). Quisinstat was formulated in 10% hydroxypropyl-b-cyclodextrin (Sigma), 25 mg/mL mannitol (Sigma), in sterile water (45 g). A1331852 was formulated by sequentially adding 10% Ethanol (Fisher), 60% Phosal 50 PG (Lipoid), and 30% PEG400 (Sigma), and vortexing (MedChem-Express). Navitoclax was formulated in 10% ethanol (Fisher), 30% PEG400 (Sigma), and 60% Phosal 50 PG (Lipoid), with vortexing after adding each component (MedChemExpress). Sorafenib was formulated in 90% corn oil (Selleckchem) with vortexing (MedChem-Express). Working solutions were made fresh prior to administration.

**In Vivo Drug Testing**

Female NSG mice age 5–8 weeks were used for *in vivo* studies. Pieces of PDX were implanted subcutaneously in the flank. Mice were followed biweekly. Treatment was initiated when tumors reached an average volume of 150 mm³ as measured by ultrasound or caliper. Quisinstat 10 mg/kg was administered by daily intraperitoneal (i.p.) injection for 14 days. Napabucasin or panobinostat were given at 10 mg/kg by daily i.p. injection 5 days on and 2 days off, for a total of 14 days. Navitoclax 50 mg/kg or sorafenib 30 mg/kg were given by daily oral gavage 5 days on and 2 days off, for a total of 14 days. CR-1 31B was given at 0.1 mg/kg by i.p. injection twice per week for a total of 24 days. A1331852 was given at 25 mg/kg by daily i.p. injection for 2 days. Mice received a daily subcutaneous injection of saline and received Nutra-Gel diet bacon flavor (Bio Serv) or DietGel 76A chocolate flavor (ClearH2O). Mice were monitored daily for health and weight. Tumor size was measured at days 0, 7, and 14 by ultrasound (Vivo 3100, Visual Sonics) or by electronic calipers at day 0 and then twice per week until the end of treatment. Tumor volume was calculated by measuring (length*width*height)/2 for ultrasound or (length*width^2)/2 for caliper. Mice were sacrificed by ketamine xylazine injection and cervical dislocation. Tumors were photographed and divided, and pieces were fixed in formalin, frozen as OCT blocks, or flash-frozen for further analysis. GraphPad Prism (version 8) was used for data analysis.

**IHC Staining**

The IHC analysis by TUNEL, Ki-67, PHH3, and pSTAT3 antibodies were performed at Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using the Discovery XT processor (Ventana Medical Systems, Roche-AZ).

**TUNEL.** Slides were manually deparaffinized in xylene, rehydrated in a series of alcohol dilutions (100%, 95%, and 70%) and tap water, placed in a Discovery XT autostainer, treated with protease 3 (Ventana Medical Systems; catalog no. 760-2020) for 8 minutes, incubated with avidin–biotin blocking reagent (Ventana Medical Systems) for 12 minutes and then incubated with TdT (Roche; catalog no. 0333536001, 1000 U/mL) and biotin-DUTP (Roche; catalog no. 110930/09/10, 4.5 nmol/mL) labeling mix for 2 hours. Detection was performed using a DAB detection kit (Ventana Medical Systems) according to the manufacturer’s instructions. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific).

**Ki-67.** Tissue sections were blocked with Background Buster (Innovex; catalog no. NB306-50) for 30 minutes. Sections were incubated with primary mouse monoclonal Ki-67 antibody (Dako; catalog no. M7240) at 0.5 μg/mL concentration for 6 hours, followed by a 60-minute incubation with a biotinylated mouse secondary antibody (Vector Labs, MOM Kit BKM-2202) at 5.75 μg/mL. Detection was performed with secondary antibody blocker, Blocker D, streptavidin–HRP D (Ventana Medical Systems), according to the manufacturer’s instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific).

**PHH3.** Tissue sections were blocked with Background Buster (Innovex; catalog no. NB306-50) for 30 minutes, followed by a 4-hour incubation with rabbit polyclonal anti-PHH3 antibody (Millipore; catalog no. 06-570; 1 μg/mL). Secondary antibody incubation was performed for 32 minutes with biotinylated goat anti-rabbit IgG (Vector Labs; catalog no.: PK6101; 5.75 μg/mL). Blocker D, streptavidin–HRP, and a DAB detection kit (Ventana Medical Systems) were used according to the manufacturer’s instructions.

**Imaging**

Hematoxylin and eosin and IHC slides of original patient tumor tissue and derived FLC PDX tumor tissue were imaged on an Olympus IX83 microscope using a 10× and 60× objective. Images were acquired using an Olympus DP26 camera and cellSence software (Olympus).

**RNA Isolation, Generation of cDNA, and PCR**

Total RNA from original patient tumors, adjacent nontumor liver (when available), and derived FLC PDXs was extracted using the miRNeasy Mini Kit (Qiagen). RNA concentration levels were measured using a Nanodrop 2000c (Thermo Fisher), and purity was assessed by the 260/280 ratio. The miScript II RT Kit (Qiagen) was used to convert total RNA into cDNA according to the manufacturer’s instructions. PCR for the DNAJB1–PRKACA fusion transcript was run for 40 cycles (forward primer: TTAACAGGAGA TCGCTGAGGC; reverse primer: CTGTGTTCTGAGCGGGACTT, expected amplicon 148 kb). Electrophoresis of the PCR product was performed at 100 V for 45 minutes using a 2% agarose gel and visualized with SYBR Safe (Life Technologies). Images were acquired with a Gel Doc EZ imager (Bio-Rad).

**RNA-seq and Bioinformatics**

RNA concentrations and 260/280 ratios were measured using a Nanodrop 2000c (Thermo Fisher), and RNA quality was assessed by RIN values (Agilent BioAnalyzer and TapeStation). RNA-seq libraries were prepared using a TruSeq Stranded Total RNA Sample Prep Kit with Ribo-Zero Gold ribosomal RNA depletion (Illumina). Ribosomal RNA-depleted libraries were sequenced on an Illumina HiSeq X-Ten (BGI Sequencing Services). Quality assessment and trimming were performed using FastQC v0.11.7 and BBDDuk (included in BBDMap v38.22). Reads were mapped to the human reference genome hg38 supplemented with the ENSEMBL GRCh38.92 gene annotations using STAR v2.6.1 (46). Analysis of differential gene expression was conducted in R version 3.5 using DESeq2, excluding rRNA and mt-rRNA genes as well as the immune and stromal signature genes (47). Principal component analysis and heat maps were generated using the set of genes consistently dysregulated in FLC primary tumors versus adjacent nontumor liver tissue (FDR 5%) across three independent batches of samples (283 upregulated and 226 downregulated genes; see Supplementary Table S2).
Protein Isolation and Immunoblotting

Total protein from the original patient tumors, adjacent nontumor liver (when available), and derived FLC PDX was extracted using RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (Complete EDTA-free and PhosSTOP, Roche). Supernatants were collected, and protein concentrations were measured by a modified Lowry assay (DC protein assay, Bio-Rad). Protein (10 μg) per sample was diluted with 4x Nupage LDS sample buffer (Life Technologies) containing 10% β-mercaptoethanol. Samples were heated at 100°C for 5 minutes and then loaded on 4%–12% Bis–Tris gels (Nupage, Invitrogen) and run in MOPS buffer for 50 minutes at 200 V. Transfer was performed using the iBlot (Life Technologies). Membranes were blocked for 1 hour in 5% milk (Carnation powdered milk) in Tris-buffered saline with Tween (TBST), washed in TBST, and then probed with primary antibodies against PRKACA (Santa Cruz Biotech, PKA α, sc-903, 1:200) in 5% milk and incubated overnight shaking at 4°C. After washing in TBST, membranes were incubated with horseradish peroxidase–conjugated appropriate secondary antibodies (Sigma, A0545 goat anti-rabbit, A9917, goat anti-mouse, 1:100,000) in 5% milk in TBST for 1 hour. Membranes were washed in TBST and then incubated with Amersham ECL prime Western blotting detection reagent (GE Healthcare), exposed to film in a dark room and then developed.

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REFERENCES


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