JQ1 Induces DNA Damage and Apoptosis, and Inhibits Tumor Growth in a Patient-Derived Xenograft Model of Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is a fatal disease with a 5-year survival of <30%. For a majority of patients, chemotherapy is the only therapeutic option, and virtually all patients relapse. Gemcitabine is the first-line agent for treatment of CCA. Patients treated with gemcitabine monotherapy survive ~8 months. Combining this agent with cisplatin increases survival by ~3 months, but neither regimen produces durable remissions. The molecular etiology of this disease is poorly understood. To facilitate molecular characterization and development of effective therapies for CCA, we established a panel of patient-derived xenograft (PDX) models of CCA. We used two of these models to investigate the antitumor efficacy and mechanism of action of the bromodomain inhibitor JQ1, an agent that has not been evaluated for the treatment of CCA. The data show that JQ1 suppressed the growth of the CCA PDX model CCA2 and demonstrate that growth suppression was concomitant with inhibition of c-Myc protein expression. A second model (CCA1) was JQ1-insensitive, with tumor progression and c-Myc expression unaffected by exposure to this agent. Also selective to CCA2 tumors, JQ1 induced DNA damage and apoptosis and downregulated multiple c-Myc transcriptional targets that regulate cell-cycle progression and DNA repair. These findings suggest that c-Myc inhibition and several of its transcriptional targets may contribute to the mechanism of action of JQ1 in this tumor type. We conclude that BET inhibitors such as JQ1 warrant further investigation for the treatment of CCA.

Introduction

Cholangiocarcinoma (CCA), a neoplasm of epithelial cells lining bile ducts, originates in any portion of the biliary tree (1). Tumors located within the bile ducts of the liver are designated as intrahepatic CCA (IHCC); tumors arising in the biliary tree of the peripancreatic region are termed extrahepatic CCA (EHCC). The incidence of IHCC is increasing worldwide, while the incidence of EHCC has remained stable or has decreased (2). Approximately 30% of CCA patients present with disease amenable to surgical resection, the only curative treatment for CCA. The remaining 70% present with advanced or metastatic disease, and undergo systemic chemotherapy with gemcitabine or gemcitabine and cisplatin. This combination extends median overall survival from ~8 months for gemcitabine alone to ~11 months, but is not curative (3).

To facilitate evaluation of novel therapies for CCA, we developed 5 CCA patient-derived xenograft (PDX) models from primary human tumor specimens and verified that early-passage tumors in murine hosts retain specific molecular, histologic, and genetic characteristics of their human tumors of origin. The models were derived from four independent EHCC specimens and from a metastatic lesion of a patient with IHCC. These are the first PDX models for EHCC and metastatic IHCC.

While the molecular etiology of CCA is incompletely understood, several key characteristics common to this tumor type have been identified. These include mutations in codons 12 and 13 of the KRAS gene, as well as elevated levels of epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and the oncogenic transcription factor c-Myc (4, 5). KRAS encodes a GTPase of the Ras family. Gain-of-function mutations of KRAS occur in 23% to 50% of IHCC and 30% to 40% of EHCC tumors.
These mutations constitutively activate the Ras pathway and accelerate tumor progression (6). A subset of CCA tumors (13%–15%) harbors gain-of-function mutations in the EGFR gene, and this growth factor receptor is overexpressed in 25% to 31% of IHCC and 21% to 58% of EHCC tumors (7–10). Detectable EGFR expression has been identified as a prognostic factor for tumor recurrence (5). Similarly, while HER2 is overexpressed less frequently than EGFR (0.9%–8.5% of CCA tumors), its overexpression is associated with poor prognosis (5). The oncogenic transcription factor c-Myc is a basic helix-loop-helix zipper protein that complexes with Max to bind to consensus E-box enhancer sequences. c-Myc regulates transcription of gene products that control diverse cell functions that include proliferation, apoptosis, and cell-cycle progression (11). c-Myc is expressed in 94% (59/63) of EHCC tumors and 42% (10/24) of IHCC tumors (4, 12). Experimental downregulation of c-Myc expression decreases the invasiveness of CCA cells in vitro, suggesting that c-Myc contributes to progression of this tumor type (13).

Because a majority of CCA tumors overexpress c-Myc and the bromodomain and extraterminal domain (BET) inhibitor JQ1 downregulates expression of c-Myc in some tumor types, we sought to determine if JQ1 had antitumor activity in preclinical CCA models. JQ1 inhibits the activity of BET family members (BRD2, BRD3, BRD4, and BRDT; ref. 14). This family of proteins binds to acetylated lysine residues of histones. Interaction of BET proteins, a component of BET-dependent transcriptional complexes, with acetylated lysine residues at specific loci regulates transcription of gene products that depend on this mechanism for expression (14, 15). JQ1 binds to the recognition pocket for acetylated lysine residues of BET proteins, predominantly BRD4, thereby competitively inhibiting BET-histone binding and recruitment of transcriptional complexes to genomic loci (14). JQ1 suppresses tumor growth in preclinical models of multiple tumor types, but has not been evaluated in preclinical CCA models (14, 16–18).

**Materials and Methods**

**Ethics statement**

Human subject and animal use were approved by the Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham (Birmingham, AL). All patients who provided tumor specimens gave informed consent.

**Establishment of CCA PDX models**

As published previously, primary human tumor tissue not needed for diagnosis was confirmed to contain tumor cells (LNC), and implanted subcutaneously into immunocompromised mice (Female SCID CB 17®-nude mice, 4-6 weeks old [Taconic Farms]) within 1 to 6 hours of resection (19). PDX models were deemed viable if F1 tumors demonstrated progressive growth. Five of five specimens (CCCA1–5) met these criteria. In addition to tissue specimens implanted, specimens were also embedded in paraffin (FFPE), snap frozen, or archived in DMSO as viable tissue.

**Mutational status of codons 12 of KRAS**

Detailed methods have been published previously (19). Briefly, DNA was isolated from F0 (primary tumor) and F1 generation tissue using a DNA/RNA extraction kit (Epicentre). Concentration and quality of nucleic acids were determined with an ND-1000 spectrophotometer and Nanodrop 3.0.1 software (Nanodrop, Coleman Technologies, Inc.). PCR reactions were done with 400 ng DNA, using conditions and primers as published to generate a 214-bp reaction product that included codons 12 of the KRAS gene (19). Following gel electrophoresis and DNA extraction (Fermentas-Fisher Scientific), PCR products were sequenced by the Center for AIDS Research (CFAR) DNA sequencing core at UAB. Electropherograms were analyzed using Finch TV (version 1.4.0; www.geospiza.com).

**Histologic analysis: Hematoxylin and eosin (H&E) staining**

Slides containing thin sections of FFPE specimens were stained with hematoxylin and counterstained with eosin using standard methods (19).

**Immunohistochemistry (IHC)**

IHC was performed using published standard methods (18). Tissue was incubated with primary antibody overnight to detect human c-Myc (AB0062; Invitrogen), EGFR (Santa Cruz Biotech), HER2, LCK, and MSH2 (Cell Signaling Technology), Chk1 and E2F1 (Bethyl Laboratories), BRCA2 (Santa Cruz Biotech), or TEK (Proteintech). Antibody binding was detected using immPRESS secondary reagents (Vector Laboratories) and DAB High Contrast chromogen (Systek Laboratories) according to the manufacturers’ instructions. Slides were counterstained with Harris Hematoxylin (Fisher Scientific), and photomicrographs were taken using an Olympus BH-2 microscope with DP70 camera and DPP-BSW v3.1 software. Expression indices (EI) were calculated as a percentage of tumor cells expressing detectable protein (1–100) multiplied by staining intensity (1, weak; 2, moderate; 3, strong), with the highest possible score of 300 (20).

**Nanostring gene expression analysis**

Total RNA (400 ng) was isolated from frozen specimens by Triazol-chloroform and analyzed in the UAB NanoString Laboratory (http://www.uab.edu/medicine/radonc/en/nanostring) using nCounter Analysis System (NanoString Technologies) as described previously (18).

**Drug efficacy studies**

We prepared JQ1 according to published procedures (14). When subcutaneous tumors reached ~100 to 200 mm³, tumor bearing mice (7–10 tumors/group) received 50 mg/kg JQ1 or vehicle (vehicle control, VC) intraperitoneally daily for 20 days. Tumors were measured with Vernier calipers (Fowler/Slyvac) twice weekly, and tumor volumes were calculated using the equation $v = (π/6)d^3$. Twenty-four hours after completion of treatment, mice were euthanized and tumor tissue fixed in formalin and paraffin embedded (FFPR) or frozen in liquid nitrogen and archived.

**Statistical analysis**

Unless analyzed by NanoString (above), tumor volumes were compared by two-way analysis of variance followed by Bonferroni posttest (GraphPad Prism 5.0). Values presented equal mean ± SEM. $P < 0.05$ was considered statistically significant.
Results

Clinical characteristics of patients from whom specimens were obtained to develop PDX models

We obtained CCA tumor specimens from each of 5 consented patients undergoing standard-of-care resection or biopsy. Of the five specimens, four (CCA1–4) were obtained from primary tumor loci and were EHCC subtype and stage IIa or IIb (Table 1). All four EHCC specimens were obtained by the Whipple procedure, which involves partial pancreatic resection. The specimen from which CCA5 was derived was obtained at biopsy of a metastatic peritoneal lesion and is IHCC subtype and stage IV. Tumor differentiation status ranged from well differentiated (G1) to moderately (G2) or poorly differentiated (G3). Microscopic evaluation confirmed lymph node involvement and perineural invasion in three of the four EHCC patients. Limited clinical information was available for the CCA5 tumor of origin.

All CCA specimens produced viable PDXs that retained histological characteristics of the primary tumors from which they were derived

CCA1-4 surgical specimens were implanted subcutaneously into immunocompromised mice, within an hour of resection. Tumor progression was monitored weekly. For model CCA5, the interval between surgery and implantation was ~6 hours. Growth rates for CCA1, CCA2, and CCA3 allowed for transplantation of F1 generation tumors within 9 to 16 weeks (Supplementary Fig. S1A). CCA4 and CCA5 tumors grew more slowly, with F1 tumors transplanted at 32 to 37 weeks. CCA3 F1 generation tumors were viable but highly vascularized and bloody, making precise quantitation of tumor size difficult. All five models were readily evaluable regarding histology of F1 tumors.

We compared the histologic characteristics of FFPE preparations of F0 and F1 tumors, to determine whether the model tumors retained the histology of the tumors of origin. In CCA tumors, cells that line biliary ducts are usually low cuboidal, with a relatively high nuclear-cytoplasmic (N:C) ratio, as seen for CCA1, CCA2, and CCA3 (Supplementary Fig. S1B). Additional histologic and morphologic features and comparison between F0 and F1 tumors are detailed in Supplementary data for each model (Supplementary Fig. S2).

The histopathological analysis of each of the F1 generation PDXs demonstrates that each model retains defining morphology and histology of the primary tumor from which it was derived. We did observe minor discrepancies in some of the models, such as relatively small differences in the amount of peritumoral stroma or in differentiation status. A plausible explanation for the decrease in peritumoral stroma in the PDX is the increase in tumor cellularity composing the PDX. This type of change in stromal content has been documented previously in other types of PDX models including pancreatic cancer, lung cancer, and breast cancer models (18, 21, 22).

Overall, PDX models retain predominant characteristics of tumors of origin.

PDX models retain the mutational status of KRAS codons 12 of primary tumors of origin

Mutations in the KRAS gene are among the most frequent genetic alterations in human cancers, with mutations in this gene estimated to occur in 17% to 25% of solid tumors (23). KRAS mutations typically (>90%) occur as point mutations in codons 12 and/or 13, and these mutations result in constitutive activation of Ras/Raf/Mek/Erk or PI3K signaling pathways that promote cell growth and survival (24).

Human-specific primers were used to amplify a 214-base pair genomic sequence that included codons 12 of KRAS (Fig. 1A). No PCR product was obtained with murine DNA from mouse normal pancreas (NP-mouse), confirming the specificity of primer set for human KRAS. Three of the five primary tumors and their corresponding PDX models contained mutations in the sequence encoding codon 12 (Fig. 1B). CCA2 and CCA3 harbor a G/A (GGT → GAT) transition resulting in substitution of aspartic acid (D) for the normal glycine (G). CCA4 possessed a G/T (GGT → GTT) transversion resulting in substitution of valine (V) for glycine (G). The apparent attenuation of several peaks in the tracings for CCA3 and CCA4 most likely reflects a low ratio of tumor cell:desmoplastic cell components in the samples received from resection. Surgical specimens from which CCA3 and CCA4 models were derived contained <30% tumor cells. Specimens from which CCA1, CCA2, and CCA5 models were derived contained >50% tumor cells. We detected no mutations in codon 12 in CCA1 or CCA5 F0 or F1 specimens. The data show that PDX models retain the KRAS status of the tumors from which they were derived (Table 2).

Table 1. Clinical characteristics of tumor from which CCA PDX models were derived

<table>
<thead>
<tr>
<th>ID</th>
<th>Subclass</th>
<th>Stage</th>
<th>Grade</th>
<th>LNM</th>
<th>P.I.</th>
<th>eSize (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA1</td>
<td>pT2N1</td>
<td>IIA</td>
<td>G3</td>
<td>Y</td>
<td>Y</td>
<td>1.8</td>
</tr>
<tr>
<td>CCA2</td>
<td>pT3N0</td>
<td>IIa</td>
<td>G2-G3</td>
<td>Y</td>
<td>Y</td>
<td>3.5</td>
</tr>
<tr>
<td>CCA3</td>
<td>pT3N1</td>
<td>IIb</td>
<td>G3</td>
<td>Y</td>
<td>Y</td>
<td>1.3</td>
</tr>
<tr>
<td>CCA4</td>
<td>pT3N1</td>
<td>IIb</td>
<td>G1</td>
<td>Y</td>
<td>N</td>
<td>1.2</td>
</tr>
<tr>
<td>CCA5</td>
<td></td>
<td>IV</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

NOTE: The tumor tissue used to generate PDX models were obtained from consented patients undergoing surgical resection (CCA1–CCA4) or biopsy (CCA5) as part of standard of care for treatment of CCA.

*aTNM staging system. T represents the size and extent of the primary tumor; N represents the number of lymph nodes involved; M indicates presence of metastasis.

*bLymph node metastases present: Yes/No.

*cLymphovascular invasion.

*dPerineural invasion.

*eSize of primary tumor.

*fMetastases.

n/a: not available.
Immunohistochemical analysis for expression of genes reported to contribute to progression of CCA tumors: EGFR, HER2, and c-Myc

As stated above, an elevated level of expression of EGFR, HER2, or c-Myc has been reported to support CCA tumor progression or to be associated with poor prognosis (4, 5). Immunohistochemical (IHC) analyses demonstrated that EGFR, HER2, and c-Myc were expressed at similar levels in PDX tumors and tumors of origin (Supplementary Fig. S2A). IHC expression data are summarized and expression indices presented below each image and detailed in Supplementary Fig. S2.

CCA1. The F0 primary tumor tissue shows moderate to strong staining for EGFR, while F1 PDX tissue has a moderate staining pattern. EGFR localizes to cell membranes in F0 and F1 tissue. F0 and F1 tumors express minimal to undetectable HER2 and moderate levels of c-Myc.

CCA2. F0 and F1 specimens are characterized by minimal to moderate patchy membranous EGFR. HER2 is undetectable in both F0 and F1 specimens. The F0 and F1 PDX show weak nuclear and moderate cytoplasmic expression for c-Myc protein.

CCA3. F0 and F1 specimens display similar moderate membranous staining for EGFR. F0 and F1 tissues have similar moderate basolateral membranous staining patterns for HER2. Both the F0 and F1 tissues have weak to undetectable nuclear c-Myc, with fewer than 10% of tumor cells showing positive reactivity.

CCA4. F0 and F1 tumor cells show strong basolateral expression of EGFR. HER2 expression is not detectable in either specimen. F0 tissue shows discrete moderate nuclear and cytoplasmic expression of c-Myc, while tumor cells in F1 specimens have minimal to moderate nuclear staining for c-Myc with increased staining for cytoplasmic c-Myc.

Table 2. PDX models of CCA retain the KRAS sequence in codon 12 of the primary tumors from which they were derived

<table>
<thead>
<tr>
<th>Specimen ID</th>
<th>KRAS codon 12 sequence change (F0)</th>
<th>Mutation conserved in PDX (F1)</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA1</td>
<td>GGT</td>
<td>Yes</td>
<td>WT</td>
</tr>
<tr>
<td>CCA2</td>
<td>GGT → GAT</td>
<td>Yes</td>
<td>G12D</td>
</tr>
<tr>
<td>CCA4</td>
<td>GGT → GAT</td>
<td>Yes</td>
<td>G12V</td>
</tr>
<tr>
<td>CCA5</td>
<td>GGT</td>
<td>Yes</td>
<td>WT</td>
</tr>
</tbody>
</table>

NOTE: The sequence of codon 12 of the KRAS gene was determined for F0 and F1 tumors.
expression of EGFR and c-Myc varied by expressed at similar levels in F0 and F1 tumors, while levels of (CCA3) showed a weak to moderate level of HER2. HER2 is fold higher levels of EGFR than normal pancreas. One model (Supplementary Fig. S2B). IHC data also indicate that 3 of 5 normal pancreas, and a moderate level of EGFR expression staining.

F0 tumor tissue displays weak immunoreactivity for nuclear also shows weak nuclear staining for c-Myc, with no cytoplasmic rate to moderate cytoplasmic staining for EGFR, and also mild membranous staining.

IHC data demonstrated no detectable c-Myc or HER2 in normal pancreas, and a moderate level of EGFR expression (Supplementary Fig. S2B). IHC data also indicate that 3 of 5 PDX models showed moderate levels of c-Myc (CCA1, 2, and 4) and 3 of 5 models (CCA1, 3, and 4) showed ~1.6- to 1.9-fold higher levels of EGFR than normal pancreas. One model (CCA3) showed a weak to moderate level of HER2. HER2 is expressed at similar levels in F0 and F1 tumors, while levels of expression of EGFR and c-Myc varied by >10% in the PDX compared with the original corresponding tumor. Overall, the data demonstrate that first-generation PDX tumors (F1) retained expression patterns of their primary tumors of origin (F0) for c-Myc, EGFR, and HER2, and that each tumor expressed at least one key protein associated with CCA tumor progression.

The bromodomain inhibitor JQ1 inhibited tumor growth selectively in JQ1-sensitive CCA2 tumors

Published work demonstrates that downregulation of c-Myc in CCA cells inhibits the invasiveness of this type of tumor cell in vitro, that JQ1 downregulates c-Myc expression in some tumors, and that c-Myc expression may be BET dependent (13, 14, 25, 26). As CCA1 and CCA2 models had similar growth rates and similar endogenous levels of c-Myc, we evaluated the effect of JQ1 on tumor growth and on c-Myc expression in these two models (Fig. 2 and Table 3). If inhibition of c-Myc is an independent indicator of JQ1 efficacy in CCA tumors, we would expect inhibition of c-Myc to occur concomitantly with inhibition of tumor growth.

We administered the well-tolerated regimen of 50 mg/kg JQ1 daily for 20 days and monitored the growth of tumors in mice treated with vehicle alone (VC) or JQ1 (Fig. 2A and 2D; refs. 14, 16). Tumor volumes were normalized to day 0, with the average tumor volume for each group designated as 1.0. Figures 2B and 2E demonstrate individual tumor growth in each group. We also conducted IHC analyses for the proliferation marker Ki-67 in tumor tissue.

The regimen of JQ1 used did not inhibit CCA1 tumor growth (Fig. 2A and 2B), and Ki-67 immunostaining demonstrated that JQ1 had no effect on expression of this proliferation marker in this model (Fig. 2C). In contrast, JQ1 inhibited the growth of CCA2 tumors (Fig. 2D and 2E), evident as >50% decrease in tumor volume on the final day of treatment; and the Ki-67 index reflected the JQ1-mediated inhibition of tumor progression as a ~70% reduction in the percentage of tumor cells expressing this proliferation marker (Fig. 2F). The time course of quantitated values of tumor volumes and P values comparing these volumes calculated using GraphPad Prism software are shown in Supplementary Table S1. For comparison, we evaluated the efficacy of gemcitabine 100 mg/kg weekly for ~16 weeks in our model (A.L. Miller and K.J. Yoon, unpublished observation). We observed that gemcitabine induced partial tumor regressions within initial 6 weeks of treatment (~50% decrease in tumor volume), but tumors regrew in spite of continued gemcitabine treatment. This observation with our preclinical model reflects clinical observations in that while gemcitabine is the most effective agent for CCA and sometimes induces temporary remissions, relapse is virtually inevitable. Addition of other conventional agents such as cisplatin to gemcitabine regimens induces only an incremental increase in duration of survival.

Given that JQ1 inhibits BET proteins and that BET-associated transcriptional complexes regulate expression of a myriad of genes, we assessed the in vivo effect of JQ1 on a panel of PanCancer genes (14, 15). By comparing expression profiles of CCA2 tumor tissue from JQ1- and VC-treated mice, we identified RNAs downregulated by JQ1, using the NanoString nCounter platform of 230 PanCancer genes.

JQ1 decreased the expression in vivo of 24 genes by ≥2.4-fold

Heatmapping showed that JQ1 decreased the expression of RNA encoding 24 genes by ≥2.4-fold, including RNA encoding MYC family members c-MYC, MYCN, and MYCL1, which decreased by 5.78-, 5.14-, and 3.03-fold, respectively (Fig. 3A and Table 3). Expression of each of these MYC family members has been reported to be BRD4-dependent in some tumor types (14, 17, 27). A subset of the other 21 genes affected are direct (TP53, MSH2, E2F1, CDK4, CDK6, BRCA2, and FAS) or indirect (CHEK1) transcriptional targets of c-Myc (28–32). Whether expression of the remaining 13 genes depends on c-Myc in this tumor type is unknown. The fold change in 16 of the 24 genes downregulated by ≥2.4-fold by JQ1 in CCA2 tumors was compared with the fold downregulation of these genes in CCA1 tumors (Table 3). This comparison revealed that cell-cycle regulatory proteins Chk1 and E2F1 were selectively downregulated in CCA2 tumors, suggesting that inhibition of Chk1 and E2F1 expression contributes to the cytotoxicity of JQ1. (Gene products downregulated by ≥2.4-fold by JQ1 in CCA1 tumors are shown in Supplementary Table S2.)

STRING v10 analysis (Search Tool for the Retrieval of Interacting Genes/Proteins) database (www.string-db.org) identified pathways likely to be impacted by JQ1-induced downregulation of these 24 gene products. Results of this analysis are presented graphically in Fig. 3B, with individual genes represented as nodes. Lines connecting various nodes are based on confidence scores, with darker lines denoting higher confidence scores. Confidence scores represent probabilities (performance predictions) that connected proteins are functionally linked (KEGG mapping; ref. 33). Seven of the 24 downregulated genes contribute to cell-cycle regulation [MYC, TP53, CHEK1, WEE1, CDK4, CDK6, E2F1 (red nodes); false discovery rate; FDR = 9.71 × 10⁻¹⁷], five are involved in the p53 signaling pathway (CDK4, CDK6, CHEK1, FAS, and TP53; FDR = 4.37 × 10⁻¹), seven are involved in PI3K–AKT signaling (CDK4, CDK6, FGFR2, FGFR3, MYC, TEK, and TP53; FDR = 1.82 × 10⁻¹⁰; Supplementary Table S3), and one contributes to DNA repair (BRCA2).

We then reasoned that if inhibition of c-Myc expression by JQ1 comprised a primary mechanism of action of JQ1 or an independent indicator of JQ1 efficacy, JQ1-sensitive CCA2 tumors exposed to this BET inhibitor would have lower levels of c-Myc than VC-treated tumors, and JQ1 would not affect c-Myc expression in CCA1 tumors that were unaffected by JQ1 (14–16).
Figure 2. JQ1 inhibited CCA2 tumor growth in vivo. Tumor-bearing mice were treated with JQ1 (50 mg/kg) or vehicle (VC) daily for 20 days. Average tumor volume (A, D) or individual tumor volumes (B, E) for CCA1 or CCA2 tumor volumes (mm³) were normalized to tumor volume on day 0. Tumor tissue was harvested 24 hours after completion of treatment, and IHC done to detect the proliferation marker Ki67 (C, F). IHC data were quantitated and results are reported as proliferation indices, calculated as a percentage of nuclei with grade 1-3 level of detectable Ki67 protein. Scale bar, 10 μm.
JQ1 altered tumor histology and downregulated expression of c-Myc protein and multiple c-Myc transcriptional targets selectively in the JQ1-sensitive CCA2 model.

Histopathological analysis (H&E) demonstrated no gross differences in differentiation status or cytological features (N:C ratio, nuclear polarity, or nuclear pleomorphism) between VC- and JQ1-treated CCA1 tumors (Fig. 4). CCA2 tumors exposed to JQ1 displayed a decrease in N:C ratio, an increase in peritumoral stroma, and a decrease in overall tumor cellularity, reflecting the decreased tumor cell viability of this JQ1-sensitive model.

Table 3. Fold change in expression of genes downregulated by ≥2.4-fold by JQ1 in CCA2 tumors, compared with CCA1 tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>JQ1</th>
<th>VC</th>
<th>Fold change</th>
<th>JQ1</th>
<th>VC</th>
<th>Fold change</th>
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<tbody>
<tr>
<td>FAS</td>
<td>NM_000043.3</td>
<td>13.23</td>
<td>31.45</td>
<td>–2.38</td>
<td>32.41</td>
<td>82.71</td>
<td>–2.55</td>
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<td>BRCA2</td>
<td>NM_000059.3</td>
<td>41.73</td>
<td>102.2</td>
<td>–2.45</td>
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<tr>
<td>WEE1</td>
<td>NM_003390.2</td>
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<td>17.69</td>
<td>–2.48</td>
<td>508.96</td>
<td>467.08</td>
<td>1.09</td>
</tr>
<tr>
<td>CDK6</td>
<td>NM_001259.5</td>
<td>132.33</td>
<td>354.13</td>
<td>–2.53</td>
<td>115.55</td>
<td>189.77</td>
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<tr>
<td>MYBL2</td>
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<tr>
<td>CHEK1</td>
<td>NM_001274.2</td>
<td>9.61</td>
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<td>CTGF</td>
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<td>1505.53</td>
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<td>MMP9</td>
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<td>27.48</td>
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NOTE: Exposure to JQ1 in vivo downregulated expression of 24 genes by ≥2.4-fold in the relatively sensitive CCA2 tumors. In contrast, JQ1 downregulated only two genes to this extent (FAS and FGF2) in a subset of 16 of these 24 genes in CCA1 tumors. These data indicate inhibition of expression of FAS and FGF2 are likely not associated with sensitivity to JQ1 and that expression of BRCA2, WEE1, CDK6, CHEK1, CDK4, MMP9, PPARG, E2F1, PPARG, E2F1, MSH2, TP53, FGFR2, MYCN, and MYC may be BET dependent in CCA tumors.

Figure 3. JQ1 downregulated c-Myc and its downstream targets in a JQ1-sensitive CCA2 model. A, JQ1 inhibited c-Myc and cell-cycle-related gene products in CCA2 tumors. Genes downregulated ≥2.4-fold by NanoString analyses are displayed by heatmap. B, Twenty-four genes downregulated ≥2.4-fold were entered into the STRING database and a protein–protein interaction network generated. The red nodes indicate proteins involved in cell-cycle regulation.
H&E-stained sections of tumors exposed in vivo to vehicle (VC) or JQ1 demonstrate JQ1-induced changes in histology. JQ1 downregulated c-Myc, BRCA2, Chk1, LCK, TEK, E2F1, and MSH2 protein expression in CCA2 tumors, but not in CCA1 tumors. JQ1 did not inhibit BRD4 or EGFR expression in either tumor model. Total expression index values are shown below each image. Scale bar, 20 µm (H&E) or 10 µm (all others). Expression indices for BRD4, EGFR, c-Myc, BRCA2, Chk1, LCK, TEK, E2F1, and MSH2 were calculated as described in Materials and Methods and are shown in Supplementary Table S4.

<table>
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<th>CCA1 JQ1</th>
<th>CCA2 VC</th>
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JQ1 did not affect c-Myc protein level in CCA1 tumors: EI of 200 in control-treated tumors versus 180 in JQ1-treated tumors; Fig. 4; Supplementary Table S4). In contrast, JQ1-sensitive CCA2 tumors expressed ~2.5-fold less c-Myc protein than tumors from VC-treated mice (EI of 260 vs. 105) at completion of therapy. If we consider cytoplasmic c-Myc to represent nascent protein, then CCA2 tumors exposed to JQ1 expressed ~5-fold less c-Myc (EI of 170 vs. 30) than VC-treated tumors (Fig. 4; Supplementary Table S4). These data indicate that JQ1 concomitantly suppressed tumor growth and c-Myc expression selectively in CCA2 PDX tumors, in agreement with NanoString analyses showing that tumors harvested from JQ1-treated mice expressed ~5.7-fold less c-Myc RNA than tumors from control mice. Consistent with the established mechanism of action of JQ1, JQ1 did not alter BRD4 expression as assessed by IHC analysis (Fig. 4). Additional IHC images and expression indices for BRD4 expression in CCA3–CCA5 are shown in Supplementary Fig. S3.

As an indirect indication of c-Myc function, we determined whether specific transcriptional targets of c-Myc were also downregulated. IHC data showed that JQ1 selectively inhibited expression in CCA2 tumors of Chk1 by ~2-fold and BRCA2 by ~4-fold (Fig. 4; Supplementary Table S4), each of which has been reported to be regulated by c-Myc (28, 29, 34). IHC analyses also demonstrated downregulation of four additional proteins that were identified by NanoString to be downregulated in CCA2 (Fig. 4; Supplementary Table S4): JQ1 inhibited expression of LCK, TEK, E2F1, and MSH2 proteins by 1.4- to ~10-fold. In contrast, JQ1 did not affect BRD4 and EGFR expression in CCA1 or CCA2 tumors. We performed immuno-blot to corroborate IHC results. These analyses were consistent with IHC data and showed that JQ1 inhibited expression of TEK, LCK, Chk1, and MSH2 in CCA2 tumors (Supplementary Fig. S4). As mentioned above, exposure to JQ1 in vivo downregulated expression of 24 genes by ≥2.4-fold in sensitive CCA2 tumors, but downregulated only 2 genes to this extent (FAS and FGFR2) in the subset of 16 of these genes in CCA1 tumors (Table 3). The data suggest that inhibition of expression of FAS and FGFR2 are unlikely to be associated with sensitivity to JQ1 and that expression of CHEK1, BRCA2, WEE1, CDK6, CDK4, MMP9, FLT3, PPARγ, E2F1, MSH2, TP53, FGFR2, MYCN, and MYC may be BET dependent in CCA tumor cells. We propose that proteins selectively downregulated in the more drug-sensitive CCA2 model may contribute to the cytotoxicity of JQ1.

**JQ1 induced DNA damage and apoptosis in CCA2 tumors**

Based on observations in the literature that BET proteins are essential to the DNA damage response, we hypothesized that JQ1 would induce DNA damage and further apoptosis (35). To address this hypothesis, we performed IHC staining for the DNA double strand break marker phospho-H2AX (Ser-139-γH2AX) and the apoptosis indicators cleaved caspase 3 and cleaved PARP (Fig. 5; ref. 36). IHC results indicated increased levels of γH2AX, cleaved caspase 3 and cleaved PARP in JQ1-sensitive CCA2 tumors and no change in levels of these proteins in CCA1 tumors. JQ1-induced DNA damage in vivo has not been reported previously. Quantitation of IHC results demonstrated that JQ1-treated CCA2 tumor specimens had a greater percentage of γH2AX-positive cells and higher apoptotic indices than VC-treated CCA1 specimens (P < 0.0001; Fig. 5A–C).

In summary, the data show that selective to CCA2 tumors, JQ1 inhibited tumor growth, induced DNA damage and apoptosis, and inhibited expression of c-Myc and c-Myc transcriptional targets associated with cell-cycle regulation and DNA repair. The data suggest that the inhibition of expression c-Myc and selected downstream transcriptional targets may contribute to the mechanism of action of JQ1 in JQ1-sensitive CCA tumors.

**Discussion**

CCA is a uniformly fatal disease (1). To evaluate potential novel therapies for CCA, we developed and characterized five independently derived PDX models from primary EHCC and metastatic IHCC tumor specimens (CCA1–5), the first such models reported. The models retained specific genetic, histologic, and molecular characteristics of tumors of origin. Using two of these models to evaluate the efficacy of the BET bromodomain inhibitor JQ1, the data showed that inhibition of tumor growth was associated with inhibition of c-Myc protein expression, as has been reported for some other types of solid tumors (14, 25, 37, 38). The data also show the novel observations that the BET inhibitor JQ1 concomitantly and selectively suppressed tumor growth, induced DNA damage, and inhibited Chk1, BRCA2, LCK, TEK, E2F1, and MSH2 protein expression in the JQ1-sensitive in vivo model.

An emerging literature documents recent interest in the use of BET bromodomain inhibitors for treating hematologic and solid malignancies (39). BET inhibition binds directly to proteins that harbor BET bromodomains (14). A consequence of this binding is the inhibition of association of BET-dependent transcription complexes to a profile of genetic loci and inhibition of expression of gene products dependent on this transcription mechanism (15). As c-Myc contributes to the phenotype of many tumor types and its transcription is thought to be BET protein dependent, early studies postulated that the primary mechanism of action of JQ1 was indirect inhibition of c-Myc expression (14–16). Interestingly, recent data indicate that gene products dependent on BET-regulated transcription may be cell type specific (18, 40). In multiple myeloma, B-cell–acute lymphoblastic leukemia, and medulloblastoma cells, JQ1 inhibited expression of c-Myc in vitro and in vivo (16, 25, 41, 42). A recent study from our lab demonstrated that JQ1 did not inhibit c-Myc expression in pancreatic ductal adenocarcinoma PDX models in vivo (18). The current study demonstrates that JQ1 inhibits c-Myc selectively in a drug-sensitive CCA tumor model.

C-Myc is expressed in up to 94% of CCA tumors (12). Experimental downregulation of c-Myc decreases the invasiveness of QBC939 bile duct carcinoma cells in vitro and inhibits CCA tumor progression in a mouse model of chronic cholestasis (13, 43). Conversely, upregulation of c-Myc supported cholangiocarcinogenesis in vivo. c-Myc is estimated to regulate ~15% of human gene products, with diverse functions that include cell-cycle checkpoint and DNA damage response proteins (Chk1), cyclin dependent kinases (CDK4/6), and DNA repair proteins (BRCA2; refs. 29–31). Consistent with the proposed mechanism of action of BET inhibitors, JQ1 inhibited expression of c-Myc and multiple c-Myc transcriptional targets, but also inhibited gene products whose expression is c-Myc-independent such as FOSL1 (40). Our findings that JQ1 induces DNA damage and apoptosis in vitro is consistent with a recent in vitro study demonstrating that
Figure 5. JQ1 induced DNA damage and apoptosis in CCA2 PDX tumors in vivo. **A**. Tissue sections were immunostained to detect γH2AX as an indicator of DNA damage or **B** cleaved caspase 3 or **C** cleaved PARP as an indicator of apoptosis, each of which is involved in DNA damage response. VC, vehicle control. Scale bar, 10 μm. Quantitation of γH2AX immunostaining comparing data from CCA1 vs. CCA2 tumors is shown in graphs below images. Apoptotic indices were calculated by counting the number of apoptotic cells per total number of tumor cells (50) and shown as graphs. """, P < 0.0001.
JQ1 induced apoptosis and delayed the DNA damage response in leukemic cells by interfering with the association of the BET protein BRD4 and p53 (44). While the observed difference in sensitivity of independently derived CCA1 and CCA2 tumors to JQ1 is undoubtedly multifactorial, several observed differences between the two models are consistent with published reports linking a given molecular characteristic with JQ1 sensitivity. Because c-Myc regulates expression of many genes, multiple c-Myc targets could contribute to the efficacy of JQ1, and the expression profile comparison between treated and untreated CCA1 and CCA2 tumors (Table 3 and Supplementary Tables S2 and S3) represents a first step toward identifying such proteins. Our hypothesis is that the efficacy of JQ1 involves simultaneous downregulation of expression of multiple transcriptional targets of c-Myc. More specifically, we postulate that simultaneous downregulation of the c-Myc transcriptional targets Chk1 and E2F1 is essential for JQ1-induced growth inhibition of CCA2 tumors. This postulate is based on: (i) our observation that JQ1 inhibited these two transcripts in CCA2 but not CCA1 tumors, (ii) data in the literature demonstrating that each of these proteins regulate cell-cycle transition from $G_2$ to M or $G_1$ to S; and (iii) that inhibition of expression or dysfunction of these proteins decreases tumor cell proliferation (45–48). Further, CCA1 tumors express wild-type KRAS; CCA2 tumors harbor a G12D KRAS mutation. Notably, Shimamura and colleagues suggested that non–small cell lung cancer cells harboring KRAS mutations were more sensitive to JQ1 than cells expressing the wild-type protein (49). Work to directly address the effect of KRAS status on the antitumor efficacy of JQ1 is ongoing.

In summary, we established five novel PDX models of CCA that retain specific genetic and histologic characteristics of primary tumors of origin. JQ1 selectively inhibited the growth of CCA2 compared with CCA1 tumors and concomitantly inhibited expression of c-Myc and several of its known transcriptional targets including Chk1, BRCA2, E2F1, and MSH2 in CCA2 tumors. JQ1 also induced DNA damage and apoptosis in vivo. Induction of DNA damage in vivo by JQ1 has not been demonstrated previously. The observed induction of DNA damage suggests that JQ1 may enhance the efficacy of agents that are selectively toxic to tumor cells deficient in DNA repair. We conclude that BET inhibitors such as JQ1 warrant further investigation for the treatment of CCA.

Disclosure of Potential Conflicts of Interest

M.J. Heslin is a consultant/advisory board member for Best Doctors. E.S. Yang is a consultant/advisory board member for Nanostring Technologies. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Development of methodology: R.C.A.M. van Waardenburg, E.S. Yang, K.J. Yoon

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.L. Garcia, A.L. Miller, L.N. Council, J.D. Christein, J.P. Arnoletti, M.J. Heslin, S. Reddy, R.C.A.M. van Waardenburg, J.E. Bradner, E.S. Yang, K.J. Yoon

Writing, review, and/or revision of the manuscript: P.L. Garcia, A.L. Miller, J.P. Arnoletti, M.J. Heslin, S. Reddy, R.C.A.M. van Waardenburg, J.E. Bradner, E.S. Yang, K.J. Yoon

Study supervision: K.J. Yoon

Other (animal care): T.L. Gamblin

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References


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Study supervision: K.J. Yoon

Other (animal care): T.L. Gamblin

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Patrick L. Garcia, Aubrey L. Miller, Tracy L. Gamblin, et al.


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