

## Original Article

# BRD4 degrader ARV-825 produces long-lasting loss of BRD4 protein and exhibits potent efficacy against cholangiocarcinoma cells

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**Abstract:** BRD4, a member of the bromodomain and extraterminal domain (BET) family and an important epigenetic reader, has emerged as an attractive oncology target. Cholangiocarcinoma is a lethal neoplasm without approved targeted therapies. BET bromodomain inhibitors have shown promising effects in certain cancers including cholangiocarcinoma. Recently developed BRD4 Proteolysis Targeting Chimera (PROTAC) compounds lead to fast and efficient degradation of BRD4 and provides longer-lasting effect than small molecule BRD4 inhibitors. In this study, we investigated the antitumor effect of a newly developed BRD4 degrader ARV-825 in cholangiocarcinoma. Immunohistochemistry and Western blotting were used to determine the expression level of BRD4. CCK-8 assay and BrdU ELISA assay were used to assess cell proliferation. Caspase 3/7 activity and Annexin V/PI staining were used to assess apoptosis. We demonstrated that BRD4 expression was elevated in cholangiocarcinoma tissues compared to normal bile duct or surrounding normal liver tissues. ARV-825 produced fast and long-lasting loss of BRD4 protein, resulting in more inhibition of CCA cell proliferation and induction of apoptosis than BRD4 inhibitors OTX-015 and JQ1. C-Myc is a well-known downstream target of BRD4. We found that ARV-825 suppressed c-Myc levels more effectively than BRD4 inhibitors. However, ARV-825 did not inhibit c-Myc expression in CCA cells with low basal c-Myc levels. Further analysis showed that ARV-825 significantly upregulated p21 expression and arrested cell cycle progression at G1 phase. In conclusion, BRD4 degrader ARV-825 leads to rapid and sustained degradation of BRD4 and is effective against cholangiocarcinoma.

**Keywords:** Cholangiocarcinoma, BRD4, proteolysis targeting chimera, ARV-825, OTX-015, JQ1

## Introduction

Cholangiocarcinoma (CCA) is a highly lethal cancer. Despite gemcitabine in combination with cisplatin is the current standard chemotherapy for patients at advanced stages, most patients were resistant to this therapy either primarily or secondary. Additionally, no targeted therapy is currently available against this neoplasm [1]. Therefore, it is crucial to discover new treatment strategies.

Bromodomain and extraterminal domain (BET) proteins, which consists of BRD2, BRD3, BRD4 and BRDT, are epigenome readers known to associate with acetylated chromatin and transcriptional regulation. Recent studies have revealed important roles for BET proteins in development, inflammation and certain types of cancer. Overexpression of particularly BRD4 has been linked to tumorigenesis and progression in several hematologic malignancies as well as in solid tumors [2-7].

Several structure and activity-based BET protein small-molecule inhibitors (BETi) have been developed, including JQ1 and OTX015. These agents disrupt the binding of BRD4 with acetylated chromatin, inhibit growth and induce apoptosis in a variety of cancers including multiple myeloma [8], pancreatic cancer [9], triple-negative breast cancer [10], glioblastoma [11] and thyroid cancer [12]. BET inhibitors cause selective repression of the potent MYC oncogene in a range of tumors as a consequence of BRD4 depletion at the enhancers that drive MYC expression [13].

Recently, Garcia et al has demonstrated that JQ1 inhibited CCA growth in a c-Myc high expression patient-derived xenograft (PDX) model, but not in c-Myc low expression PDX model [14]. This observation suggests that BRD4 inhibitors may be of therapeutic interest in a subset of CCA cancer patients through downregulation of c-Myc and its downstream targets that are important for cell growth and survival of CCA cells.

However, a number of studies have shown that BETi treatment exhibits reversible binding and incomplete inhibition of BRD4, which may potentially compromise the activity of BETi in cancer cells [15-19]. Unlike BETi, BET-proteolysis targeting chimera (PROTAC) ARV-825 recruits and utilizes an E3-ubiquitin ligase to effectively degrade BRD4 [16]. ARV-825 has been shown to induce more apoptosis than BETi in lymphoma and acute myelocytic leukemia [18, 19].

Here, we compared the anti-CCA activity of the novel BET-PROTAC (ARV-825) with the BETi JQ1 and OTX015. We found that ARV-825 was more potent than the JQ1 and OTX015 in inhibiting proliferation and inducing apoptosis.

### Materials and methods

#### Reagents

ARV-825, JQ1, OTX015, MG132, KJ Pyr-9 and 10058-F4 were purchased from Medchemexpress. Anti- $\beta$ -actin primary antibody was purchased from Sigma Aldrich. Antibody against human BRD4 for Western blotting was purchased from Cell Signaling Technology. Antibody against p21 was from Santa Cruz. Antibody against c-MYC was from Abcam. Antibody against BRD4 for immunohistochemistry was

from Bethyl Laboratories. Immobilon Western Chemiluminescent HRP detection kit was from Millipore. The Bromodeoxyuridine (BrdU) ELISA kit was from Roche. Cell counting kit-8 (CCK-8) was from Dojindo Laboratories. Caspase-Glo 3/7 assay kit was from Promega. cDNA Reverse Transcription Kit and SYBR® Green qPCR detection Kit were from TaKaRa. RNeasy Plus Mini kit was from QIAGEN. Annexin V-FITC Apoptosis Detection kit was from BD Pharmingen. Cell culture medium was obtained from Gibco. Fetal bovine serum (FBS) was from Biological Industries. ARV-825, JQ1, OTX-015 and MG132 were dissolved in Dimethyl sulfoxide (DMSO) to make a stock solution of 10 mM. KJ Pyr-9 and 10058-F4 were dissolved in DMSO to make a stock solution of 50 mM.

#### Patients and specimens

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the ethics committee of the hospital. Human CCA tissues were obtained from specimens of CCA patients undergoing curative surgery in the Department of Hepatobiliary Surgery, Nanjing Drum Tower Hospital. A total of 6 snap-frozen CCA samples and non-tumor liver tissues were collected for detecting protein expression of BRD4. Archived paraffin-embedded tumor tissues from 71 consecutive CCA patients and 57 surrounding non-tumor bile duct tissues were used for immunohistochemistry.

#### Immunohistochemistry (IHC) assay

IHC was performed to determine the protein expression of BRD4 in cholangiocarcinoma and nontumoral surrounding bile ducts according to standard protocol. Staining intensity was scored as follows: 0 (achromatic), 1 (light yellow), 2 (brownish yellow) and 3 (brown). The percentage of staining was scored as follows: 0 (no positive cells), 1 (<25% positive cells), 2 (25%-49% positive cells), 3 (50%-74% positive cells) and 4 ( $\geq$ 75% positive cells). The total score was calculated by combining the two parameters. Immunohistochemical staining was analyzed by two pathologists in a blinded manner.

#### Cell culture

Human CCA cell lines HuCCT1, HuH28 and OZ cells were kindly provided by Lewis R.Roberts

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(Mayo Clinic, MN, USA), which were originally obtained from the Japanese Collection of Research Bioresources. Human CCA cell line RBE was purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Human intrahepatic biliary epithelial cells HIBEpiC was purchased from ScienCell. All cell lines used were cultured in RPMI 1640 with 10% FBS and maintained at 37°C in the presence of 5% CO<sub>2</sub>.

### *BrdU cell proliferation ELISA assay*

Cells were plated in 96-well plates at 3000 cells/well in triplicate. After 24 h, drugs were added and cells were incubated for 72 h. The BrdU ELISA assay was performed according to the manufacturer's instructions.

### *Cell viability assay*

Cells were seeded into 96-well plates at 1000-3000 cells/well in triplicate, cultured overnight then treated with drugs for indicated time. Cell viability was detected by CCK-8 assay according to the manufacturer's instructions.

### *Annexin V-FITC apoptosis assay*

Cells were seeded in 6-well plates at 1×10<sup>5</sup> cells/well and treated with indicated concentrations of ARV-825 for 72 h. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection kit and performed according to the manufacturer's instructions. Data were analyzed using FlowJo software.

### *Colony formation assay*

Cells were plated at 500 cells/well in a 6-well plate. After overnight, drugs were added and cells were incubated for 7 days. Cells were then fixed with methanol solution and stained with 0.5% crystal violet. The number of colonies, defined as ≥50 cells/colony, was counted under light microscopy.

### *Caspase 3/7 activity assay*

3000 cells were seeded into 96-well white opaque plates. The next day, cells were treated with varying concentrations of indicated drugs for 48 h. At the end of the incubation time, Caspase-Glo reagent was added to each well. Caspase 3/7 activity was analyzed using the Caspase-Glo 3/7 assay kit according to the manufacturer's instructions.

### *Western blotting*

Equivalent amounts of protein were separated on a 4-20% Tris-HCl gel and transferred to Polyvinylidene fluoride membranes. Membranes were probed with the appropriate primary antibodies against β-actin (1:5000), BRD4 (1:1000), c-Myc (1:5000) and p21 (1:500) at 4°C overnight. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and signals were visualized using the HRP detection kit. β-actin was used as a loading control. Image J software was used to quantify the signal.

### *Real-time qPCR*

MRNA was extracted using the RNeasy Plus Mini kit and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits according to the manufacturer's instructions. Quantitative real-time PCR was done with the Real-time PCR System (Roche) using the SYBR qPCR detection Kit. 18S was used as the internal control. The primers for c-Myc were 5'-GCTGCTTAGACGCTGGATTT-3' and 5'-TAACGTTGAGGGGCATCG-3'. The primers for 18S were 5'-TTGGAGGGCAAGTCTGGTG-3' and 5'-CCGCTCCAAGATCCAACA-3'.

### *Statistics*

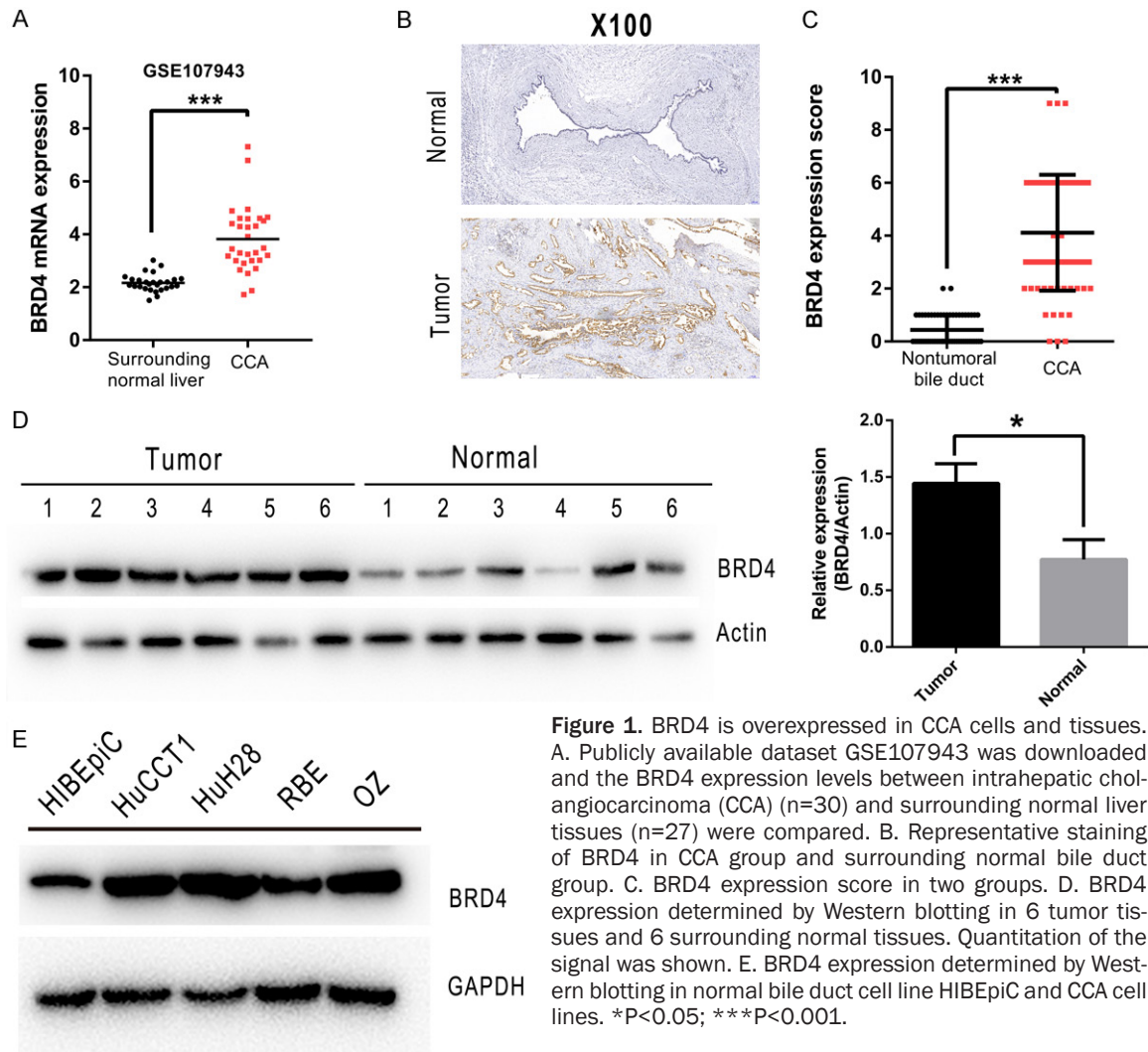
All statistical tests were conducted with GraphPad Prism 6.0. The half maximal inhibitory concentration (IC<sub>50</sub>) was calculated using nonlinear regression analysis in Prism 6.0. The Student's t test was used to compare two groups. In experiments involving more than two groups, one-way ANOVA with a Turkey *post hoc* test was used. Results were considered statistically significant at P<0.05.

## **Results**

### *BRD4 is overexpressed in CCA*

To determine the potential utility of targeting BRD4 for the treatment of CCA, we measured the gene expression levels of BRD4 in CCA. We first analyzed BRD4 mRNA expression in a CCA microarray dataset GSE107943. This dataset contained microarray mRNA gene profiles on intrahepatic CCA (iCCA) (n=30) or human non-cancerous surrounding liver samples (n=27). RPKM-normalized gene expression data were

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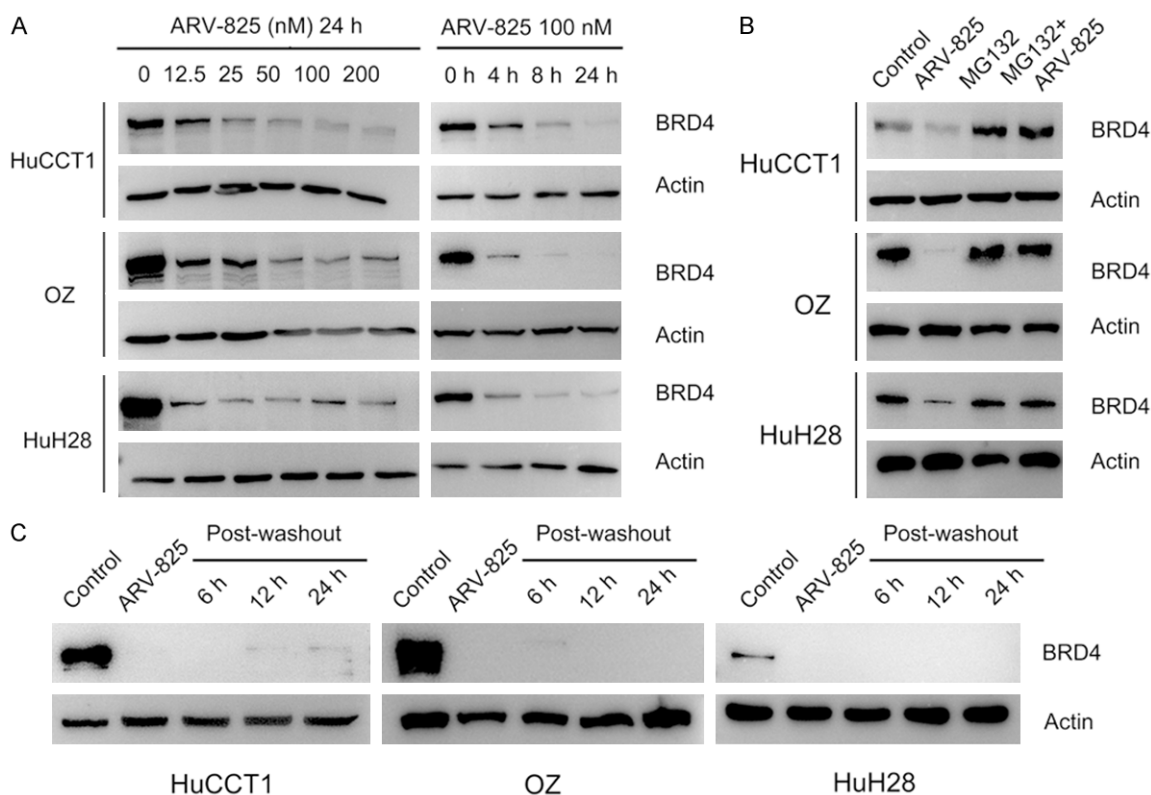


used to compare the BRD4 expression level between surrounding normal livers and iCCA patients. As shown in **Figure 1A**, BRD4 expression was increased in iCCA patients compared to normal subjects ( $P<0.0001$ ). In addition, we measured the protein expression levels of BRD4 in human CCA tissues and surrounding normal bile ducts. As shown in **Figure 1B** and **1C**, IHC score was significantly higher in CCA group (n=71) than that in the normal group (n=57). Western blotting assays in CCA tissues and normal tissues showed similar results (**Figure 1D**). Furthermore, BRD4 protein level was higher in CCA cell lines HuCCT1, HuH28, RBE and OZ than normal biliary cell line HIBEpiC (**Figure 1E**). Collectively, these data demonstrate that BRD4 is overexpressed in CCA cells.

### ARV-825 leads to fast and efficient BRD4 degradation

ARV-825 is a novel BRD4 degrader that exerts superior lethal activity than BETi in hematologic malignancies [16, 18, 19]. As shown in **Figure 2A**, treatment with ARV-825 dose- and time-dependently downregulated BRD4. Co-treatment with a proteasome inhibitor MG132 completely blocked the BRD4 degradation induced by ARV-825 confirming that ARV-825 led to BRD4 degradation through proteasome pathway (**Figure 2B**). CCA cells were treated with ARV-825 for 24 h and then washed with fresh medium three times to remove the compounds. After the removal of ARV-825, BRD4 expression did not recover up to 24 h (**Figure 2C**), suggesting the suppression of BRD4 by ARV-825 is long-lasting. Taken together, these data dem-

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**Figure 2.** ARV-825 induces rapid and long lasting degradation of BRD4 in CCA cells. A. CCA cells were treated with different concentration of ARV-825 for 24 h or 100 nM ARV-825 for different time. BRD4 expression was determined by Western blotting. B. Western immunoblots for BRD4 performed on CCA cell lysates prepared after 12 h treatment with 100 nM ARV-825 in the presence or absence of proteasome inhibitor MG132 (200 nM). C. CCA cells were treated for 24 h with 100 nM ARV-825, followed by three washes to remove compounds and re-seeded in fresh medium for various time points. Lysates were collected and analyzed by immunoblotting for BRD4 and Actin.

onstrate that ARV-825 leads to fast and efficient BRD4 degradation in a proteasome-dependent mechanism in CCA.

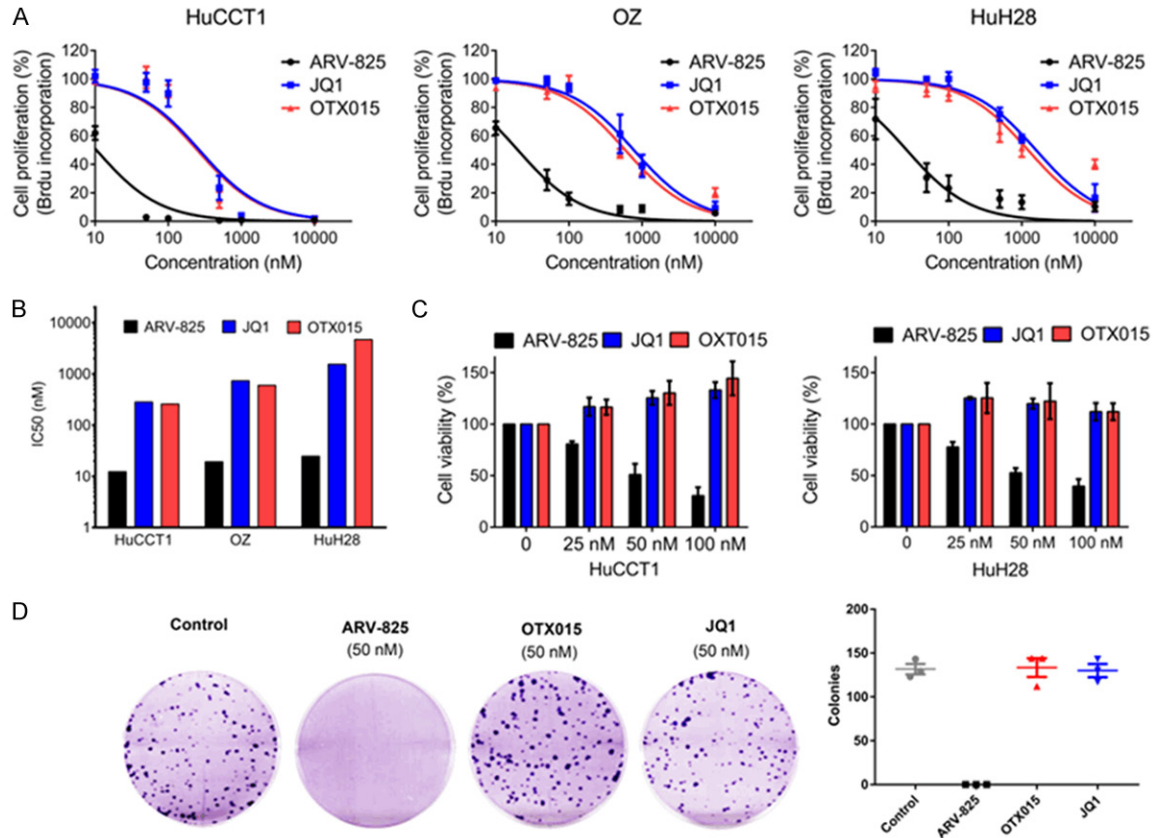
### *ARV-825 leads to a superior effect on cholangiocarcinoma cell proliferation suppression and apoptosis induction than BRD4 inhibitors*

Next, we performed a head-to-head comparison between ARV-825 and the small molecule inhibitors OTX015 and JQ1, to determine their effects on cell proliferation and apoptosis. CCA cells were exposed to increasing concentrations of ARV-825, OTX015 or JQ1 for 72 h and cell proliferation was evaluated by BrdU ELISA assay. All cell lines tested showed a potent dose-dependent growth inhibition after drug treatment. However, ARV-825 was superior on CCA cell proliferation suppression than OTX015 and JQ1 with significantly lower  $IC_{50}$ s (**Figure 3A** and **3B**). Cell viability assay showed that ARV-825 dose-dependently inhibited cell viability of HuCCT1 and HuH28 cells after 96 h treatment,

while the same dose of OTX015 or JQ1 has no inhibitory effect on cell viability (**Figure 3C**). Additionally, ARV-825 was more potent in the clonogenicity assay than OTX015 and JQ1 as 50 nM ARV-825 completely inhibited HuCCT1 cell colony formation while the same dose of OTX015 and JQ1 has no inhibitory effect (**Figure 3D**).

We next compared the effects of ARV-825 versus the BETi OTX015 and JQ1 in inducing apoptosis of CCA cells. We observed increased Caspase 3/7 activity after a 48 h treatment of HuCCT1 and HuH28 cell lines with ARV-825, but not with high dose of OTX015 and JQ1 (**Figure 4A**). By 48 h, HuCCT1 cells also demonstrated significant apoptosis with 50 nM and 100 nM ARV-825, as evidenced by increase of Annexin V positive cells (**Figure 4B**). In contrast, significantly higher dose of inhibitors, OTX015 and JQ1, did not induce apoptosis in HuCCT1 cells. Taken together, these findings corroborate that PROTAC-mediated BRD4 degradation

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**Figure 3.** The inhibition effects of ARV-825, OTX015 and JQ1 on growth of CCA cells. A. HuCCT1, OZ and HuH28 cells were treated with ARV-825, OTX015 and JQ1 at indicated concentrations for 72 h and cell proliferation was determined by BrdU ELISA assay. B. IC<sub>50</sub>s of different CCA cells treated with above-mentioned agents measured by BrdU ELISA assay. C. Relative cell viability of HuCCT1 and HuH28 cells treated with ARV-825, OTX015 and JQ1 for 96 h. D. Plated HuCCT1 cells were treated with different agents at 50 nM for 7 days and colonies were stained with 0.5% crystal violet. The results are presented as mean ± SEM from 3 independent experiments.

is an effective strategy in inhibiting CCA cell growth.

*ARV-825 leads to more significant and longer lasting c-Myc suppression than small molecule inhibitors in c-Myc high expression CCA cells*

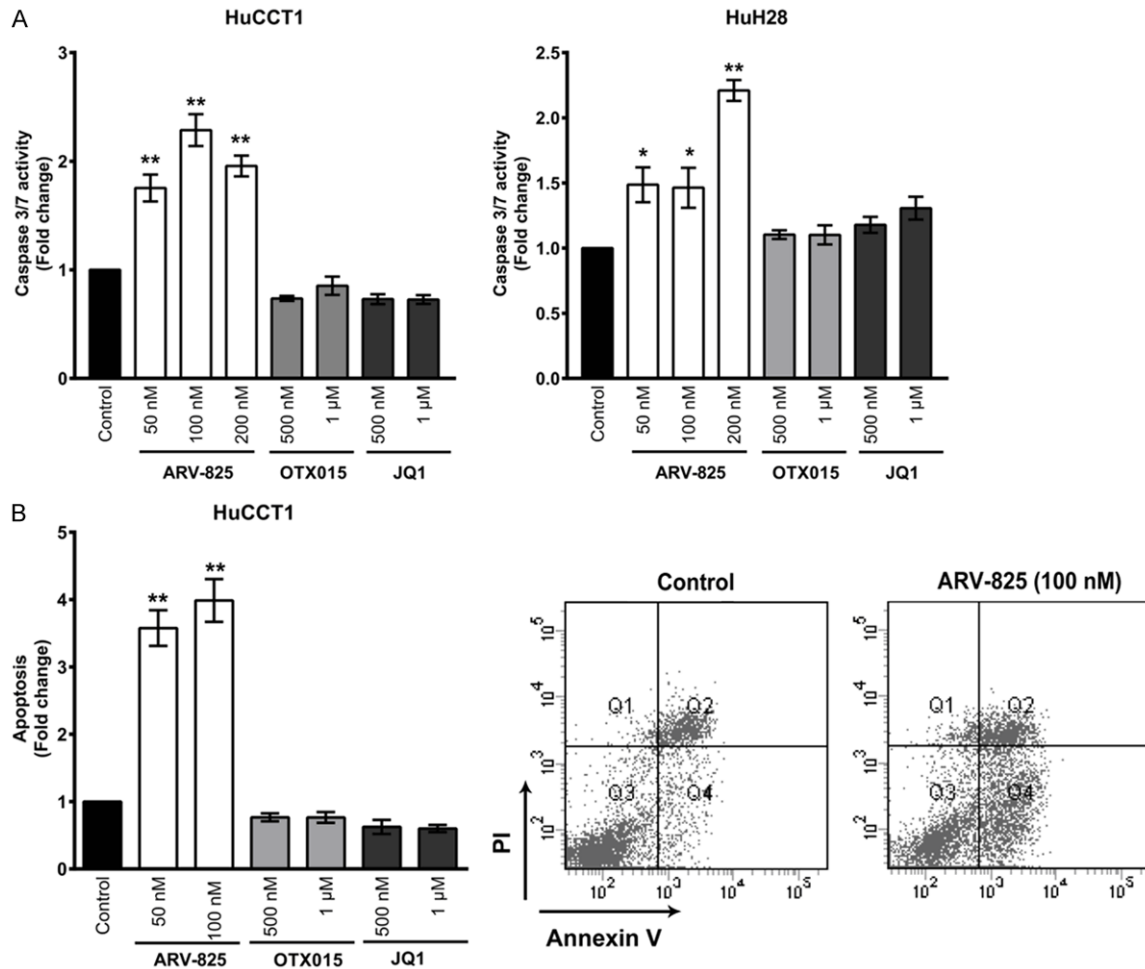
C-Myc is an oncoprotein involved in many hallmarks of cancer, including cell cycle, senescence, proliferation and apoptosis. C-Myc is expressed in 42%-95% of CCA tumors and contributes to malignant biological phenotypes of this tumor [20-24]. ARV-825 treatment led to markedly downregulation of c-Myc mRNA and protein levels in HuCCT1 and OZ cells (Figure 5A and 5B). Treatment with OTX015 and JQ1 also led to a reduction in c-Myc expression in HuCCT1 cells (Figure 5C). However, the suppression of c-Myc protein levels by OTX015 and JQ1 was not long-lasting. HuCCT1 cells were treated with OTX015, JQ1 or ARV-825 for 24 h

and then washed with fresh medium three times to remove compounds. C-Myc protein expression recovered 24 h after the removal of OTX015 and JQ1 (Figure 5D). In contrast, ARV-825 maintained c-Myc suppression at least up to 24 h (Figure 5E). Interestingly, ARV-825 did not inhibit c-Myc expression in HuH28 cells, which has a low basal c-Myc protein expression and was less sensitive to c-Myc inhibitors KJ Pyr-9 and 10058-F4 compared to HuCCT1 and OZ cells (Figure 5F-H). Together, these evidence shows that ARV-825 leads to significant and long lasting c-Myc suppression in CCA cells with high c-Myc expression.

*ARV-825 upregulates p21 and induces G1 phase arrest in cholangiocarcinoma*

Cyclin-dependent kinase inhibitor p21 (also known CDKN1A) functions as a regulator of cell cycle progression at G1 through binding to and

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**Figure 4.** The effects of ARV-825, OTX015 and JQ1 on apoptosis of CCA cells. A. HuCCT1 and HuH28 cells were treated with ARV-825, OTX015 and JQ1 at different concentrations for 48 h. Caspase 3/7 activity was measured by Caspase 3/7-Glow assay. \* $P < 0.05$ ; \*\* $P < 0.01$  compared to Control. B. HuCCT1 cells were treated with ARV-825, OTX015 and JQ1 at different concentrations for 72 h. Apoptosis was then measured by Annexin V-FITC/PI labeling followed by flow cytometry. \* $P < 0.05$ ; \*\* $P < 0.01$  compared to Control.

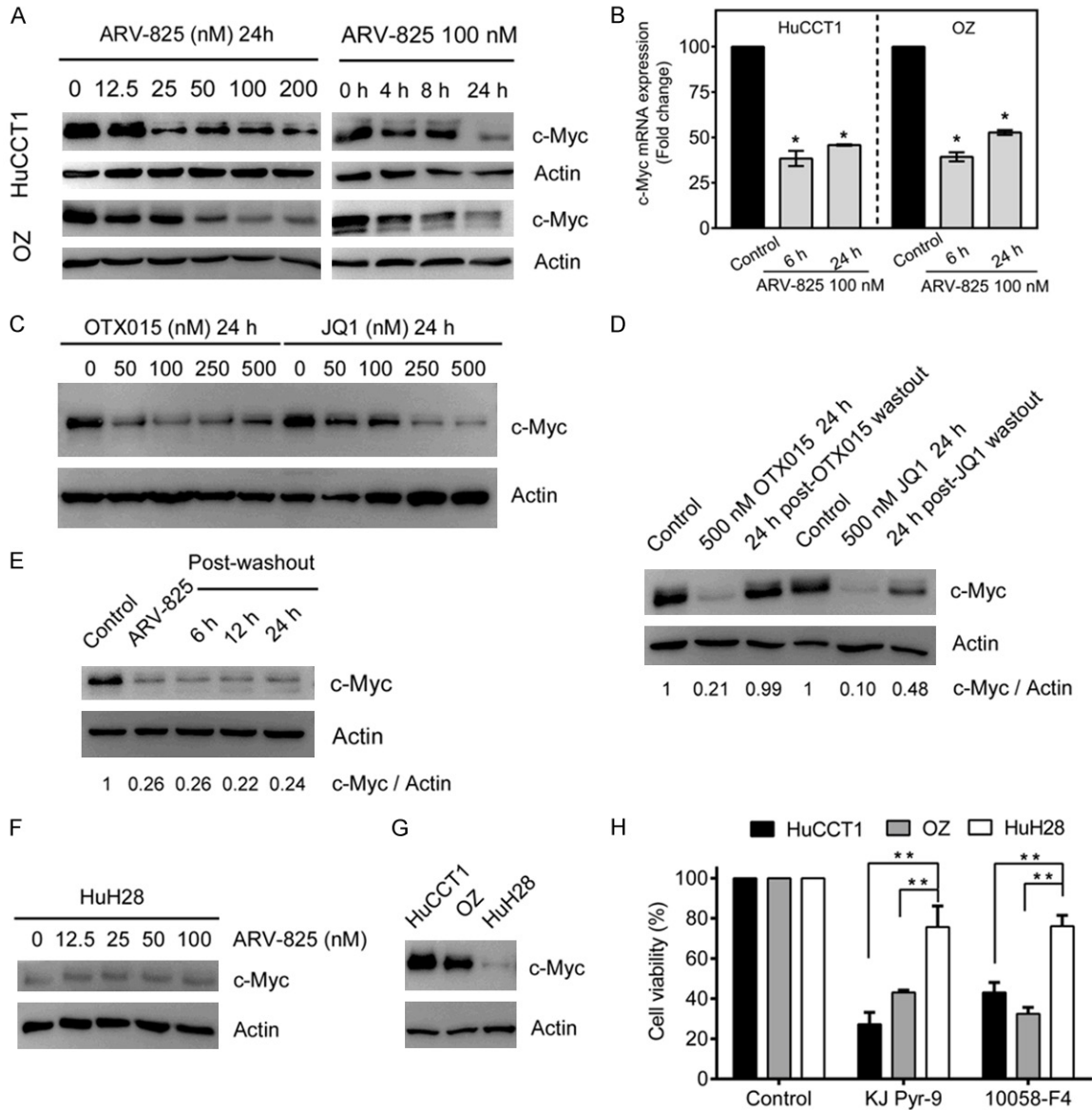
inhibiting the activity of cyclin-cyclin-dependent kinase 2 or -cyclin-dependent kinase 4 complexes. It is reported that upregulation of p21 tumor suppressor gene mediates the antitumor effect of BRD4 inhibitors in various tumors [25-27]. Indeed, a dose-dependent increase in p21 protein expression upon ARV-825 treatment has been detected in all CCA cell lines (Figure 6A). Consistent with the upregulation of p21, cell cycle analysis showed that ARV-825 suppressed G1/S transition (Figure 6B).

### Discussion

BRD4 has captured considerable attention from academia and the pharmaceutical industry due to its great potential as a novel target in

multiple disease settings, particularly in cancer. One key advantage of BRD4 as an oncology target is that it is identified as preferentially clustering at super-enhancer regions in control of pivotal oncogenes such as c-Myc and thus offers an alternative strategy in targeting those oncoproteins which are difficult to inhibit by traditional strategies. Moreover, BRD4's distinct high occupancy of genomic loci proximal to specific oncogenes provides the potential for a therapeutic window that could allow specific targeting of tumor cells while sparing normal tissues. Indeed, BRD4 inhibitors have shown anti-tumor activities with good tolerability in different mouse tumor models including a PDX model of CCA. Currently, BET Bromodomain inhibitors are in Phase I/II clinical trials.

## Antitumor effect of ARV-825 in CCA



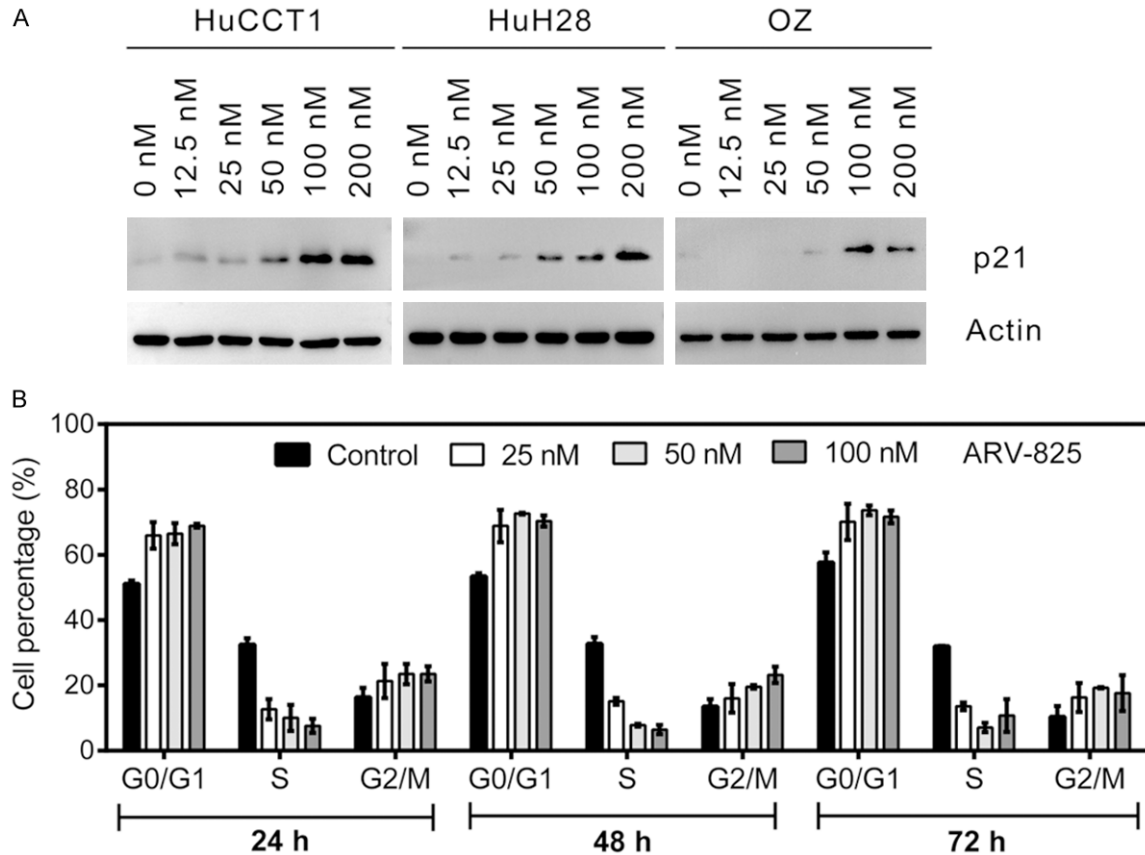
**Figure 5.** ARV-825 inhibits c-Myc expression in CCA cells. A. CCA cells were treated with ARV-825 at indicated concentration or for different time. C-Myc protein expression was determined by immunoblotting. B. CCA cells were treated with ARV-825 at indicated concentration for different time. c-Myc mRNA expression was detected by qRT-PCR. \* $P < 0.05$  compared to Control. C. Western immunoblots for c-Myc performed on HuCCT1 cell lysates prepared after 24 h treatment with OTX015 or JQ1. D. HuCCT1 cells were treated with OTX015 or JQ1 for 24 h, followed by compounds removal and re-seeding in fresh medium for 24 h; lysates were subjected for immunoblot with c-Myc and actin antibodies. E. HuCCT1 cells were treated with ARV-825 (100 nM) for 24 h, followed by compounds removal and re-seeding in fresh medium for 6-24 h; lysates were subjected for immunoblot with c-Myc and actin antibodies. F. Western immunoblots for c-Myc performed on HuH28 cell lysates prepared after 24 h treatment with ARV-825. G. Western immunoblots for c-Myc performed on HuCCT1, OZ and HuH28 cell lysates. H. Cells were treated with c-Myc inhibitors KJ Pyr-9 (10  $\mu$ M) and 10058-F4 (100  $\mu$ M) for 72 h and cell viability was determined by CCK-8 assay. \*\* $P < 0.01$ .

However, a number of studies have found that BRD4 inhibitors lead to accumulation of BRD4 protein in cancer cells. This increase of BRD4 levels, together with the reversible nature of

inhibitor binding, could prevent efficient BRD4 inhibition. One strategy to achieve more effective BRD4 inhibition is to design BRD4 degraders, which have received significant interest in



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**Figure 6.** ARV-825 induces p21 expression and arrests cell cycle at G1 phase. A. Western immunoblots for p21 performed on CCA cell lysates prepared after 24 h treatment with ARV-825 (0-200 nM). B. HuCCT1 cells were treated with ARV-825 for 24-72 h, cell cycle was determined by flow cytometry.

recent years, as they may achieve the desired pharmacological effect at lower drug concentrations [15, 17, 28, 29].

Recently, Lu et al designed a novel chimera molecule, ARV-825, using the PROTAC platform to efficiently degrade BRD4 [16]. Several observations have found that ARV-825 leads to more potent proliferation suppression and robust apoptosis induction, possibly due to its rapid and long-lasting degradation of BRD4 and suppression of downstream targets such as c-Myc [18, 19, 30]. Consistent with these studies, we demonstrate that ARV-825 treatment causes more profound and sustained depletion of the levels of BRD4 in CCA cells. Concomitantly, ARV-825 exerts significantly greater lethality against CCA cells than BRD4 inhibitors OTX015 and JQ1.

High sensitivity to BRD4 inhibitors has been associated with high level of c-Myc in different tumor types. Garcia et al has shown that JQ1

suppressed the growth of the CCA PDX model concomitant with inhibition of c-Myc and c-Myc transcriptional targets expression [14]. While in another JQ1-insensitive CCA PDX model, expression of c-Myc and its downstream targets were unaffected by this agent. Knock down of c-Myc has been shown to inhibit cell proliferation and induce apoptosis in CCA cells [31]. These findings suggest that c-Myc inhibition may contribute to the mechanism of action of BRD4 inhibition in CCA. However, a recent study by Fujiwara et al found that the antigrowth effect of JQ1 in intrahepatic CCA was not attributed to downregulation of the c-Myc gene in RBE cells [31]. In the current study, we found that ARV-825 did not suppress c-Myc in HuH28 cells, even though ARV-825 significantly blocked growth of HuH28 cells, suggesting that the c-Myc suppression seems not to be the mechanism of ARV-825 in HuH28 cells. This was also found to be the case with a number of lung cancer cells, osteosarcoma cells and pancreatic cancer cells that were sensitive to the

effects of BRD4 inhibitors, but showed no downregulation of c-Myc following inhibitor treatment [9, 32, 33]. Interestingly, compared to HuCCT1 and OZ cell lines, HuH28 cell line has lower baseline level of c-Myc and is likely to have low dependence on c-Myc, indicating that ARV-825 may repress CCA cells with low c-Myc expression through c-Myc independent pathways.

Upregulation of p21 was another key antitumor mechanism of BRD4 inhibitors. P21 has been shown to be downregulated in CCA [34]. Overexpression of p21 is capable of suppressing proliferation and invasion of CCA cells [34], proving that p21 is a tumor suppression gene of CCA. We have shown that ARV-825 significantly upregulated p21 expression and arrested cells in G1 phase, suggesting that upregulation of p21 may in part mediate the antitumor effect of ARV-825.

### Conclusions

BRD4 degrader ARV-825 leads to rapid and sustained degradation of BRD4 and is effective against CCA. Inhibition of c-Myc and upregulation of p21 partly mediates the antitumor effect of ARV-825 in CCA.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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