Original Article

In vitro and in vivo study of GSK2830371 and RG7388 combination in liver adenocarcinoma

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Abstract: Intrahepatic cholangiocarcinoma (iCCA) is an adenocarcinoma arising from the intrahepatic bile duct and accounts for the second highest incidence of primary liver cancers after hepatocellular carcinoma. The lack of effective treatment leads to a poor prognosis for advanced iCCA, so new targeted therapy is needed. The impairment of wild-type (WT) p53 tumor suppressor function by its negative regulators frequently occurs in iCCA. Therefore, restoration of WT p53 function by inhibiting its negative regulators is a therapeutic strategy being explored for cancer treatment. Combining an MDM2 inhibitor (MDM2i, RG7388) to stabilize p53 and a WIP1 inhibitor (WIP1i, GSK2830371) to increase p53 phosphorylation enhances p53 function. The combination of MDM2 and WIP1 inhibitors has been reported in several cancer types but in vivo studies are lacking. In the current study, liver adenocarcinoma cell lines, RBE and SK-Hep-1, were treated with RG7388 alone and in combination with GSK2830371. Cell proliferation, clonogenicity, protein and mRNA expressions, and cell cycle distribution were performed to investigate the effect and mechanism of growth suppression. To evaluate the antitumor efficacy of RG7388 and GSK2830371 in vivo, SK-Hep-1 xenografts in NOD-SCID mice were treated with combination therapy for two weeks. The combination of MDM2i and WIP1i significantly increased the growth inhibition, cytotoxicity, p53 protein expression, and phosphorylation (Ser15), leading to transactivation of downstream targets (p21WAF1 and MDM2). The in vivo results demonstrated that the combination treatment can significantly inhibit tumor growth. In this study, the liver adenocarcinoma cell lines responded to combination treatment via reactivation of p53 function evidenced by increased p53 expression, phosphorylation and expression of its downstream targets. This efficacy was also demonstrated in vivo. The current research provides a novel strategy for targeting the p53 pathway in liver adenocarcinoma that warrants further investigation.

Keywords: RG7388, GSK2830371, MDM2, WIP1, p53, cholangiocarcinoma

Introduction

Primary liver cancers include predominantly hepatocellular carcinoma and remaining intrahepatic cholangiocarcinoma (iCCA) [1]. In contrast to extrahepatic cholangiocarcinoma (eCCA), iCCA is an adenocarcinoma arising from bile duct of liver which is a relatively rare but aggressive biliary tract cancer [2]. Prevalence of iCCA is much higher in Asian than in Western countries [3] possibly because hepatitis virus B, C and flukes are the main etiologies of iCCA in Asian populations [4-6]. iCCA has aggressive biological behavior with high recurrence rate after primary surgery [7-10] and is typically diagnosed at an advanced stage with poor prognosis [11, 12].

Gemcitabine-based chemotherapy is still the standard treatment for iCCA [13, 14]. Previous
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Clinical trials have evaluated molecular-targeted therapies in combination with chemotherapy but most of them showed disappointing results in large-scale phase III studies [15-17]. Recently, the TOPAZ-1 trial demonstrated that durvalumab in combination with chemotherapy improved overall survival in patients with biliary tract cancer [18]; however, the benefit was marginal (12.8 vs. 11.5 months, hazard ratio [HR] 0.80; 95% confidence interval [CI], 0.66-0.97; \( P=0.021 \)). Therefore, there is an unmet medical need to explore additional therapeutic agents or strategies for iCCA.

The tumor suppressor protein p53, encoded by the \( TP53 \) gene, is responsible for DNA repair, cell cycle arrest, apoptosis and cell senescence leading to cancer suppression and death [19, 20]. However, cancers frequently have impaired wild-type (WT) p53 function due to either \( TP53 \) mutation or dysregulation of its negative regulators which enhance cancer cell development, survival, and proliferation [21]. The principal negative regulators of WT-p53 are mouse double minute 2 homolog (MDM2) and wild-type p53-induced phosphatase 1 (WIP1). Targeting MDM2 and WIP1 with small molecule inhibitors (MDM2i and WIP1i) to activate the p53 pathway is a novel therapeutic strategy in cancer treatment, particularly in p53 WT cancers [22-24]. The activity of RG7388 (idasanutlin) as MDM2i was explored across various indications was explored across various indications [25, 26]. GSK2830371, a WIP1i, was designed for inhibition of WIP1 protein leading to increased p53 phosphorylation [27]. We have previously reported anti-cancer activity of MDM2i in combination with GSK2830371 in cutaneous melanoma [22] and liver adenocarcinoma [23] by increasing the function and stability of p53 in a p53-dependent manner. This strategy has been studied in a range of cancers [28-30], however, no in vivo studies have been reported for this combination. Therefore, we have investigated the in vitro and in vivo preclinical activity of RG7388 and GSK2830371 in iCCA to explore the potential for future clinical trials.

Materials and methods

Cell lines and compounds

RBE [31] and SK-Hep-1 [32] were purchased from the RIKEN Cell Bank (Ibaraki, Japan) and authenticated by the STR (short tandem repeat) method. These cell lines were cultured in RPMI medium 1640 (Gibco) and DMEM (Gibco) containing 10% FBS respectively. RG-7388 MDM2i was obtained from Roche under a material transfer agreement (MTA). GSK-2830371 WIP1i was purchased from Sigma-Aldrich. All compounds were dissolved in DMSO and used at a final concentration of 0.5% DMSO with minimal cytotoxic effects on cells.

Growth inhibition assay

Cells were seeded as 3000 cells/well in 96-well plates overnight before 72-hour and 96-hour treatment with RG7388 and GSK2830371, both individually and in combination. CCK8 tetrazolium based redox reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used according to the manufacturer's instructions and absorbance optical density (OD) was measured at 450 nm wavelength using a microplate reader. The GI50 value was determined and the detailed calculation of GI50 was presented in our previous report [23].

Clonogenic assay

Cell lines were seeded in 6-well plates with different cell densities depending on the cell lines (RBE 800/well and SK-Hep1 200/well) for 96 hours before treatment with RG7388, combined with or without GSK2830371 for 72 hours. Fresh medium was replaced and the cells were fixed when colonies were visible depending on the growth rates of the cells (usually 3-4 days). The IC50 values for reduction in clonogenic survival were determined [23].

RNA extraction and qRT-PCR

Cell lines were seeded in 6-well plates and treated with RG7388 and GSK2830371, either alone or in combination for 6 and 24 hours. Cells were harvested and total RNA was extracted using TRI Reagent (Sigma). RNA purity and concentration were estimated with an ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, UK). Complementary DNA (cDNA) was generated using the HiScript I™ First Strand cDNA Synthesis Kit (Bionovas) as described by the manufacturer, qRT-PCR was carried out using SYBR green RT-PCR master mix (Life Technologies) as per the manufacturer’s guidelines and the following primers:
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MDM2: F-AGTAGCAGTGAATCTACAGGGA, R-CT-GATCCCAACCATACCTGGAAT; CDKN1A: F-TGT-CCGTCAAGACCTGATG, R-AAAATCGAAGTCTCA-TGCTC; GAPDH: F-CTGCCTCACTGCTCAACAGG, R-ACCAACCGTGTCGAGCCAA. qRT-PCR reactions using a total of 25 ng of the cDNA samples per 20 µL final reaction volume, transcriptional levels were determined by QS5 system using SYBR green system (Applied Biosystems, Thermo Scientific). GAPDH was used as endogenous control and samples of cells exposed to DMSO solvent control were used as the calibrator for each independent repeat experiment. Analysis was carried out using QuantStudio™ Design & Analysis Software.

Immunoblotting

After cells were seeded in 6-well plates and treated with RG7388 alone and in combination with GSK2830371 for 6 and 24 hours, cell lysates were harvested by Pierce™ RIPA Lysis and Extraction buffer (Thermo Scientific) with Protease and Phosphatase Inhibitor (Roche) on ice and stored at temperature of -20°C. Protein concentration was measured by a Pierce™ bicinchoninic acid kit (Thermo Scientific). Equal quantities of protein were then separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were then orthogonally transferred by electrophoresis and immobilized onto Amersham™ nitrocellulose membranes (GE Healthcare Life Science). Primary antibodies against p53 (DO-7) (#GTX34938, GeneTex), phospho-p53 (Ser-15) (#9284, Cell Signaling Technology), MDM2 (2A10) (#MABE281, Merck Millipore), p21WAF1 (12D1) (#2947, Cell Signaling Technology), WIP1 (F-10) (#sc-376257, Santa Cruz Biotechnology), GAPDH (GT239) (#GTX627408, GeneTex) and secondary goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (#115-035-003/#111-035-003, Jackson ImmunoResearch Laboratories) were used, as appropriate for the corresponding primary. All antibodies were diluted in 5% (w/v) non-fat milk in TBS-Tween. Proteins were visualized using enhanced chemiluminescence (Merck Millipore) and image capture system (UVP ChemStudio PLUS).

Fluorescence-activated cell sorting (FACS)

Cells were seeded in 6-well plates and treated with RG7388 alone and in combination with GSK2830371 for 24 and 48 hours. Both floating and adherent cells were collected and fixed using 70% cold ethanol. Samples were incubated in propidium iodide (PI)/RNase Staining Solution (Cell Signaling) for 20 minutes in the dark at room temperature, then were analyzed on a FCSCalibur™ flow cytometer using CellQuest Pro software (Becton Dickinson, Oxford, UK). Cell cycle distribution based on DNA content was determined using FlowJo vX software.

Xenograft tumorigenicity

To evaluate the efficacy of combination treatment in vivo, SK-Hep-1 cells were used to produce xenografts in mice. In brief, 5×10^6 tumor cells in 50 µl PBS were injected into 4-week-old NOD/SCID mice subcutaneously. When the tumors grew to average 100 mm^3, RG7388 (80 mg/kg, two times per day on days 1-5 and 8-12 during the treatment period), GSK2830371 (75 mg/kg, two times per day [27]) and combinations were applied for two weeks. Tumor size, tumor volume, and body weight of mouse were measured during the 2-week treatment and then for 4 weeks after treatment. Tumors were harvested after completion of study (6 weeks).

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) unless otherwise stated. Statistical tests were carried out using GraphPad Prism 6 software and p-values represent unpaired t-tests or two-way ANOVA analysis. A p-value less than 0.05 was considered as statistically significant.

Results

GSK2830371 potentiated the growth inhibitory activity of RG7388

Minimal growth inhibitory activity of 10 µM GSK2830371 was found in RBE and SK-Hep-1 cells in our previous study [23]. A combination index to examine the synergism between two compounds can only be performed when both agents have a dose-dependent effect [33], therefore, potentiation rather than synergy was examined in the following experiments. A fixed concentration of 2.5 µM GSK2830371 was selected to evaluate the potentiation of RG-7388 by GSK2830371 when used in combination. SK-Hep-1 and RBE were treated with RG7388 alone or in combination with 2.5 µM
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GSK2830371 for 72 and 96 hours and cell proliferation was measured by CCK-8 assay (Figure 1A-D). The % growth inhibition of combination treatment was normalized to any effect of GSK2830371 alone. GSK2830371 significantly increased growth inhibitory activity of RG7388, which was evidenced by increased growth inhibition and cytotoxic activity. Numerically lower GI<sub>50</sub> values for combination treatment compared with RG7388 monotherapy was also noted (Figure 1E). Furthermore, we also tested the growth inhibitory effect of RG7388 monotherapy in a panel of BTC cell lines, and most of them had no growth inhibitory activity except SK-Hep-1 and RBE (Figure S1). The small effect of RG7388 at the highest dose (10 µM) was potentially an off-target effect.

GSK2830371 potentiated the cytotoxic activity of RG7388

If the OD value in the proliferation assay after treatment was less than the OD at day 0, subtraction of the day 0 OD value produced negative value, which indicated the compound or combination was not only cytostatic but also cytotoxic. Figure 1A-D demonstrated that combination therapy not only resulted in greater

Figure 1. The growth inhibitory activity was potentiated by GSK2830371 compared with RG7388 treatment alone. SK-Hep-1 (A, B) and RBE (C, D) cells were treated with single agent RG7388 (0 to 10 µM) or in combination with WIP1i (2.5 µM) for 72 (A, C) and 96 (B, D) hours. The GI<sub>50</sub> values for 72 or 96 hours treatment are shown in (E). All data show the mean ± SEM from three independent experiments. *, P < 0.05.
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Figure 2. GSK2830371 combination with RG7388 enhanced the inhibition of colony formation compared with RG7388 monotherapy. (A) Schema for experiment timeline. Colony formation was strongly repressed after MD-M2i and WIP1i treatment in SK-Hep-1 (B, C) and RBE (D, E) cells. The IC\textsubscript{50} values are summarized in (F) and a detailed table of results normalized to DMSO or WIP1i is shown in (G). *, P < 0.05. All data exhibited the mean ± SEM from three independent experiments.

inhibition of proliferation but also greater cytotoxic activity than RG7388 monotherapy. In addition, a clonogenic assay was performed to evaluate whether GSK2830371 potentiation of the cytotoxic activity of RG7388 was also reflected in reduced colony formation (Figure 2). As shown in Figure 2, fewer colonies were formed after combination treatment compared with RG7388 treatment alone, indicating that the addition of GSK2830371 significantly reduced the clonogenic survival of RBE and SK-Hep-1 cells compared with the effect of RG7388 alone (Figure 2B-E). The IC\textsubscript{50} values of RG7388 for colony number reduction were calculated after normalization to either GSK-2830371 or DMSO and shown in Figure 2F, 2G.

<table>
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<tr>
<th>IC\textsubscript{50}</th>
<th>No WIP1i</th>
<th>WIP1i 2.5\textmu M (to WIP1i)</th>
<th>WIP1i 2.5\textmu M (to DMSO)</th>
</tr>
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<tr>
<td>SK-Hep-1 p value</td>
<td>0.157±0.078</td>
<td>0.041±0.008</td>
<td>0.024±0.006</td>
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<tr>
<td>RBE p value</td>
<td>0.066±0.015</td>
<td>0.039±0.006</td>
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These results indicated GSK2830371 not only potentiated the growth inhibition but also the reduction in clonogenic cell survival induced by RG7388.

**GSK2830371 induced p53 phosphorylation and stabilization via WIP1 inhibition**

To confirm the on-target effects of the combination of RG7388 and GSK2830371 and explore the possible mechanism, RBE and SK-Hep-1 were treated with RG7388, GSK2830371, or a combination of both compounds for 6 and 24 hours. Immunoblotting showed RG7388 stabilized p53, leading to increased p53 detection after 6-hour treatment and the increased p53 persisted for 24 hours (**Figure 3A, 3B**). MDM2, p21 and WIP1 are all transcriptional targets of p53 and expression of these proteins increased at 6 hour and the increases persisted for 24 hours. When GSK2830371 was added to RG7388 treatment, decreased WIP1 protein and correspondingly increased phosphorylated p53 (Ser15) was observed, indicating the on-target effect of GSK2830371. Compared to RG7388 monotherapy, no significant changes in total p53 protein after combination treatment were evident, but increases in p53 targets, p21 and MDM2, were found (**Figure 3A, 3B**). Similar findings were noted for both RBE and SK-Hep-1 treated with two doses of RG7388 (0.1 µM and 1 µM) with or without GSK2830371 (2.5 µM) for 6 and 24 hours. Moreover, the p53 transcriptional target gene protein products were evident at the earlier time point of four hours after 0.1 µM RG7388 in combination with GSK2830371 treatment (**Figure S2**).

Dose-dependent increases in p53 target gene transcripts were also found using qRT-PCR (**Figure 3C, 3D**). Furthermore, combination treatment induced significantly more mRNA transcripts of CDKN1A (encoding p21) and
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Figure 4. G2 phase arrest was induced by RG7388 treatment in combination with GSK2830371. The cell cycle distribution was assayed by FACS. The histogram profiles show the cell cycle distribution detected by staining with PI (A, B, D, and E). The cell cycle distributions from at least 3 independent replicates were summarized in (C) (SK-Hep-1) and (F) (RBE). D, DMSO; W, WIP1i; R, RG7388. *, P < 0.05 by two-way ANOVA compared to control group. All data show the mean ± SEM from three independent experiments.

MDM2 than RG7388 monotreatment. The fold increases of p53 target gene transcripts were cell-line and time dependent. For SK-Hep-1, MDM2 increased more than CDKN1A after 6-hour treatment but CDKN1A induction was higher than MDM2 after 24-hour treatment. In contrast, RBE expressed higher levels of CDKN1A mRNA after 6-hour treatment and higher levels of MDM2 mRNA after 24-hour treatment.

GSK2830371 induced more G2 arrest in combination with RG7388

RBE and SK-Hep-1 cells were treated with two doses of RG7388 (0.1 µM and 1 µM) and GSK2830371 (2.5 µM) for 24 and 48 hours and FACS were performed to evaluate the cell cycle distribution after RG7388 with or without GSK2830371 treatment. Comparing to DMSO treatment, GSK2830371 (2.5 µM) demonstrated minimal effects on cell cycle distribution in RBE and SK-Hep-1. For SK-Hep-1 cells, 0.1 µM RG7388 induced both G1 and G2 arrest and decreased S phase, and 1 µM RG7388 induced more G2 arrest. GSK2830371 markedly increased the G2 arrest when it was added to RG7388 (Figure 4A-C). A similar pattern was found with RBE cells. For RBE 0.1 µM RG7388 induced only G1 arrest and 1 µM RG7388 induced G2 arrest. GSK2830371 again induced further G2 arrest when it was added to 0.1 µM RG7388 but not 1 µM RG7388 (Figure 4D-F).

In vivo xenograft evaluation of RG7388 and GSK2830371 combination

The schedule of treatment and sacrifice of mice was summarized in Figure 5A. RG7388 (80 mg/kg, day 1-5 and 8-12) and/or GSK2830371 (75 mg/kg, day 1 to 14) [27] were administered by oral gavage for two weeks. The tumor weight on sacrifice from the mice after 2-weeks treatment and 4-weeks subsequent follow-up are shown in Figure 5B and summarized in Figure 5C. Both RG7388 and GSK2830371
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Figure 5. WIP1i and MDM2i combination restricted tumor growth in vivo. (A) Schema for experiment timeline. Mice were treated with RG7388 on days 1-5 and days 8-12, and GSK2830371 on days 1-14 during the treatment period for two weeks. (B, C) Tumor size and weight showed maximum reduction with the combination treatment. (D) A detailed table of results with the means, standard errors, and p values was displayed from (C). (E) Tumor growth was significantly suppressed to a greater extent with GSK2830371 combined with RG7388 than with either inhibitor alone. The p value was calculated by two-way ANOVA. (F) Waterfall plot of tumor response for each tumor (n=5/group) at week 6 compared with week 0. There was minimal alteration in body weight in all groups; except the RG7388 and/or WIP1i group at week two, which subsequently recovered (G). Scale bar =100 μm. *, P < 0.05 by unpaired t-test in (C). All data exhibited the mean ± SEM (n=5).
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significantly reduced the tumor sizes comparing to control. The combination of RG7388 and GSK2830371 significantly decreased the tumor sizes compared with GSK2830371 monotreatment. The combination treatment resulted in some smaller retrieved tumors than RG7388 monotreatment, even though the average difference did not achieve statistical significance at the 95% confidence level (Figure 5C). Significant reductions in tumor weight were observed for the mice treated with RG7388 or GSK2830371. RG7388 (n=5, P=0.0008) significantly reduced the tumor growth rates compared to GSK2830371 (n=5, P=0.0097), which was compatible with in vitro findings. Furthermore, the combination of RG7388 and GSK2830371 significantly inhibited the tumor growth more than RG7388 monotherapy (n=5, P=0.0398) (Figure 5D). Tumor growth curves were also evaluated from tumor size measurements by calipers every week after the initiation of treatment (Figure 5E). Maximum tumor growth in control and GSK2830371 groups at the endpoint compared with week zero as shown in Figure 5F. The body weight was slightly reduced during the treatment period except for the control group, but they soon returned to normal during the post-treatment follow-up time (Figure 5G).

Discussion

Targeting negative regulators of p53 may provide a novel therapeutic strategy in treatment of advanced BTC [24]. Previously, we reported in vitro studies demonstrating the potential of such a strategy using HDM201 and GSK2830371 treatment of BTC cell lines [23]. RG7388, an MDM2 inhibitor which stabilizes p53 leading to cell cycle arrest and growth inhibition, has been studied in a number of clinical trials. In the current study, we demonstrated that GSK2830371 targeting the WIP1 phosphatase oncoprotein enhanced RG7388 activity via increased p53 phosphorylation and resulted in profound growth inhibition. Importantly, this is the first in vivo study to investigate the combination of MDM2i and WIP1i in cancer.

Both RBE and SK-Hep-1 cell lines we have used are p53 wild-type. It is well established that MDM2 inhibitors are ineffective against mutant p53 cells, and we and others have previously shown that the potentiation of MDM2 inhibitors by inhibition of WIP1 is also p53-dependent [22, 23]. Also, clinical trials of MDM2 inhibitors use wild-type p53 tumor status as a patient selection criterion [24, 34], hence we have chosen these cell lines which are wild-type for p53. Although the basal levels of MDM2 in both cell lines were low (Figure 3A, 3B), targeting p53 by MDM2i is still effective treatment. Except for MDM2-amplified cell lines, the basal levels of MDM2 and p53 are usually low in p53 wild-type cell lines as both MDM2 and p53 are tightly regulated and turning over at a high rate [35, 36]. Elevated p53 activates MDM2 leading to autoregulatory feedback p53 degradation. However, when MDM2i blocks this interaction p53 continues to be produced but is released from degradation without the negative control by MDM2, consequently p53 levels rise and then continue to transcribe MDM2 resulting in elevated MDM2 protein levels. However, the increased MDM2 continues to be prevented by the inhibitor to bind to p53 and the increasing feedback loop becomes futile. The net result is an increase in both the p53 and MDM2 protein levels. Although p53 can regulate many genes based on previous reports [22, 23], we only investigated the major representative genes p21 and MDM2. Based on previous reports, the induction of some p53-regulated genes is cell line dependent, however, CDKN1A and MDM2 are consistently detected major transcriptional targets of p53 intimately involved in its growth inhibitory and autoregulatory function. CDKN1A is the most investigated p53 target gene and has been causally shown to be responsible for p53-induced cell cycle arrest.

GSK2830371 has been reported to enhance p53-mediated tumor suppression by MDM2 inhibitors, nutlin-3 [29, 37], nutlin-3a [38], HDM201 [23] and RG7388 [29] or by chemotherapy [27, 37]. GSK2830371 has been shown to potentiate MDM2 inhibitors via increased p53 phosphorylation, acetylation, and decreased ubiquitination, resulting in increased growth inhibition and cytotoxic activity for melanoma cells [22]. Although p53 phosphorylation (Ser15), the substrate of WIP1, is not essential for p53 activity, it can lead to increased stabilization of p53 as a result of decreased ubiquitination [22]. GSK2830371 has also been shown to potentiate the effect of RG7388 on liver adenocarcinoma cells [23]. In extension of previ-
ous studies, we explored and validated the combination of RG7388 and GSK2830371 in liver cholangiocarcinoma cells using in vitro assays and in vivo models. Although activation of p53 leads to cell cycle arrest and apoptosis, the fate of cells from p53 reactivation may be cancer- or cell-dependent [22, 39]. The underlying mechanisms that determine cell fate following p53 activation and the choice between cell cycle arrest, senescence or apoptosis are incompletely understood. In the current study, no apoptotic responses were found by either FACS analysis or caspase 3/7 activity with either RBE or SK-Hep-1 cells after RG7388 treatment, either with or without combination treatment with GSK2830371 (data not shown) which was consistent with previous findings for liver cancer cell lines [39].

In conclusion, the current study demonstrated that combination treatment with MDM2 and WIP1 inhibitors, RG7388 and GSK2830371, may have some benefit for CCA. This study reports the first in vivo results of this novel combination in a xenograft system. As more than half of BTC are WT p53 [24], these promising results warrant further investigation aimed towards supporting the case for future clinical trials.

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Disclosure of conflict of interest

None.

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References

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Figure S1. The effect of RG7388 (0 to 10 µM) treatment on cell proliferation for 72 hours in a panel of BTC cell lines.

Figure S2. Protein expression of p53, p-p53 (Ser15), p21, MDM2, and WIP1 during RG7388 (0.1 µM) and/or GSK2830371 (2.5 µM) treatment at 0, 2, 4, 6, and 24 hours detected by Western blot in SK-Hep-1 and RBE cells.