【総説】

第20回 高安賞最優秀論文賞受賞

論文 「Treatment of RB1-intact hepatocellular carcinoma with CDK4/6 inhibitor combination therapy」

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Introduction

Synthetic cyclin-dependent kinase 4 and 6 (CDK 4/6) inhibitors keep retinoblastoma 1 (RB1) tumor suppressor gene product in an unphosphorylated form for long time, causing cellular senescence, apoptosis and enhanced immunogenicity in RB1-intact tumor cells. These medications are now approved for patients in advanced stages of hormone receptor (HR)-positive; HER2-negative breast cancers. Aromatase inhibitors or estrogen receptor (ER) antagonist fluvestrant doubled progression-free survival in these patients[2]. These agents are tested in clinical trials for a variety of advanced solid tumors including liver, lung, colon and brain malignancies[1]. However, single administration of these agents mostly resulted in suboptimal outcomes in these cancers.

RB family member genes are rarely mutated or deleted in hepatocellular carcinoma (HCC). However, many lines of evidence indicate that the loss of RB function is mechanistically involved in the development of HCC. The HCV-derived NS5B protein accelerates the degradation of RB1, as well as RBL1 and RBL2, by increasing the recruitment of the E6AP ubiquitin ligase[3]. The constitutive production of preS2 and HBx generated from HBV activates cyclin-CDK complexes, causing RB1 inactivation that is reversible[4]. Based on these findings, HCC has been supposed to be one of the most promising targets of CDK4/6 inhibitors.

In the study reviewed here, we first demonstrated that loss of all Rb family members in $Trp53^{-/-}$ mouse liver resulted in development of liver tumor reminiscent to human HCC. Then we discovered that re-expression of RB1 sensitized these tumors to a CDK 4/6 inhibitor, palbociclib. In multiple human HCC cell lines, an unphosphorylatable thus constitutively activated version of RB1 mutant (RB7LP) elicited effects similar to palbociclib, i.e., partial cellular senescence and partial apoptosis. By screening chemical compounds that enhance the efficacy of RB7LP in HCC, we discovered an IKKβ inhibitor, Bay11-7082. This agent significantly enhanced RB7LP function to increase IKKα/β phosphorylation and following NF-κB activation. Subsequently, palbociclib in combination with Bay11-7082 was found to be much more successful than single administration in the treatment of hepatoblastoma and HCC. Furthermore, blockade of IKK-NF-κB or AKT pathways significantly enhanced therapeutic

effects of palbociclib in RB1-intact K-Ras mutant lung and colon cancers. Therefore, we propose that CDK4/6 inhibitors have a good potential to cure varieties of RB1-intact malignancies including HCC and KRAS cancers when combined with a suitable kinase inhibitor[5].

Results

RB pathway inactivation drives HCC

We employed the hydrodynamic injection method to transduce $Rb1^{lox/lox}$; $Rbl1^{-/-}$; $Rbl2^{lox/lox}$; $Trp53^{-/-}$ (QKO) mice with the PiggyBac transposon vector construct (pCMV-Puro-Cre-IRES2-GFP) and pCMV-hyPBase vector expressing a transposase in order to investigate the role of RB1 in the development of HCC. Multiple liver tumors were observed in QKO mice typically within 2.5 months after injection with no overt expression of Rb1, Rbl1, Rbl2, or p53. $Rb1^{lox/lox}$; $Rbl1^{-/-}$; $Rbl2^{lox/lox}$; $Trp53^{+/+}$ (TKO) mice developed tumor typically after 12 months following injection as described previously by other researchers. Liver tumors developed in injected QKO mice exhibited high cellularity and frequent expression of Ki67 and alpha feto protein (AFP). Then, we established primary HCC cell lines from the liver tumor. We introduced hemagglutinin (HA)-tagged wild-type RB1 or V5-tagged RB7LP mutant into the QKO mouse-derived primary HCC cells. Both the wild-type RB1 and the RB7LP mutant showed inhibition of cell proliferation, but RB7LP was superior to wild-type RB1 in terms of growth suppression (Figure 1).

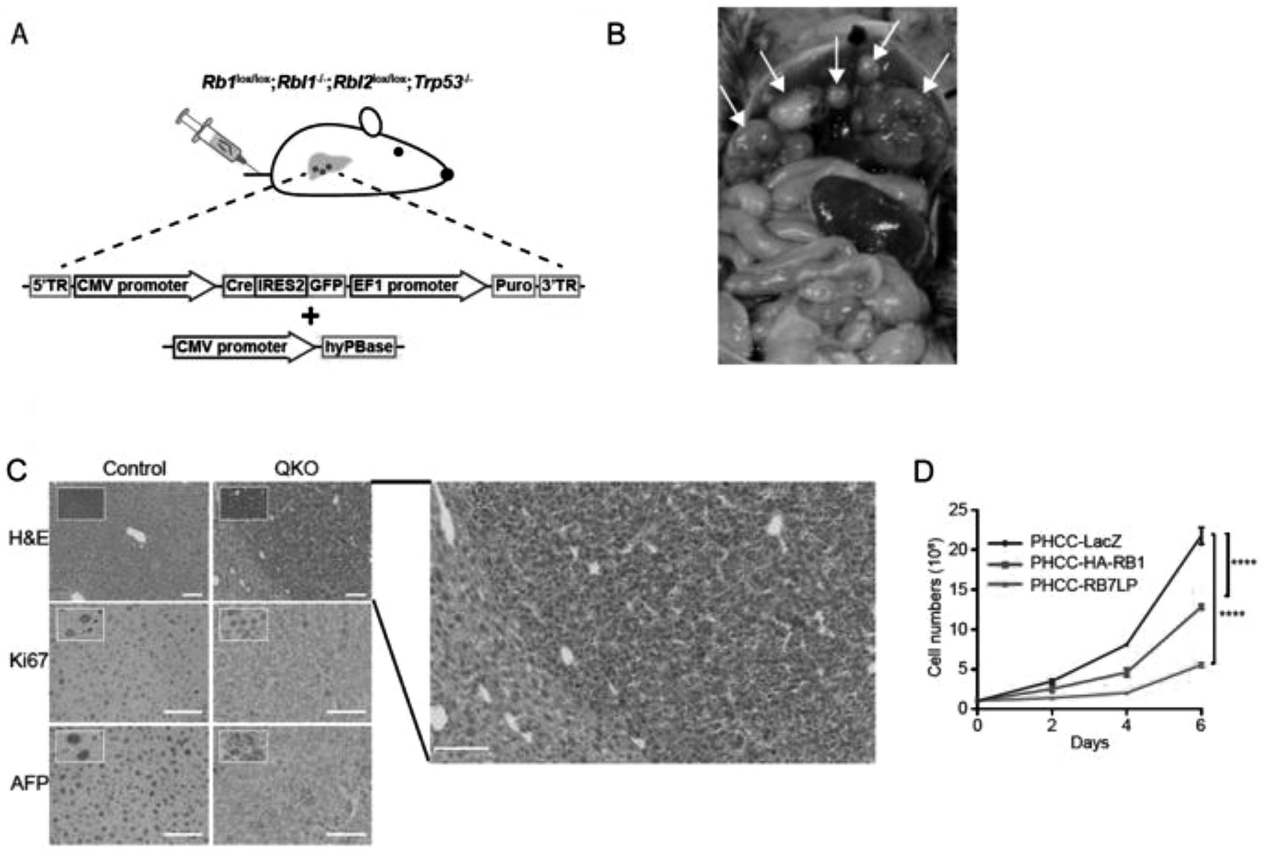

Figure 1. RB pathway inactivation drives HCC.
(A) The strategy used to generate QKO mice. (B) A representative image of liver tumor developed in QKO mouse. (C) Representative images of H & E staining (upper) and immunohistochamical (IHC) analysis of the indicated proteins (middle and lower) of the liver of a control mouse or a tumor developed in a QKO mouse 3 month after Cre-GFP-puro injection. (D) WST-8-based assessment of growth property in primary HCC cells transduced with the indicated protein.

RB7LP mimics the action of CDK4/6 inhibitors

We found that wild-type RB1 and the RB7LP mutant induced partial cellular senescence and partial apoptosis in QKO mouse-derived primary HCC cell. Furthermore, we observed that compared with RB1 negative cells, RB1 positive cell lines are more sensitive to the palbociclib treatment. Next, we treated an RB1-intact human hepatoblastoma cell line HepG2 with palbociclib. It showed an effect similar to the RB1-reconstituted mouse-derived primary HCC cell. Then, we employed a Tet-ON system to overexpress the RB7LP mutant in various human HCC cell lines. We found that the RB7LP mutant induced partial cellular senescence and partial apoptosis in these cells as well (Figure 2).

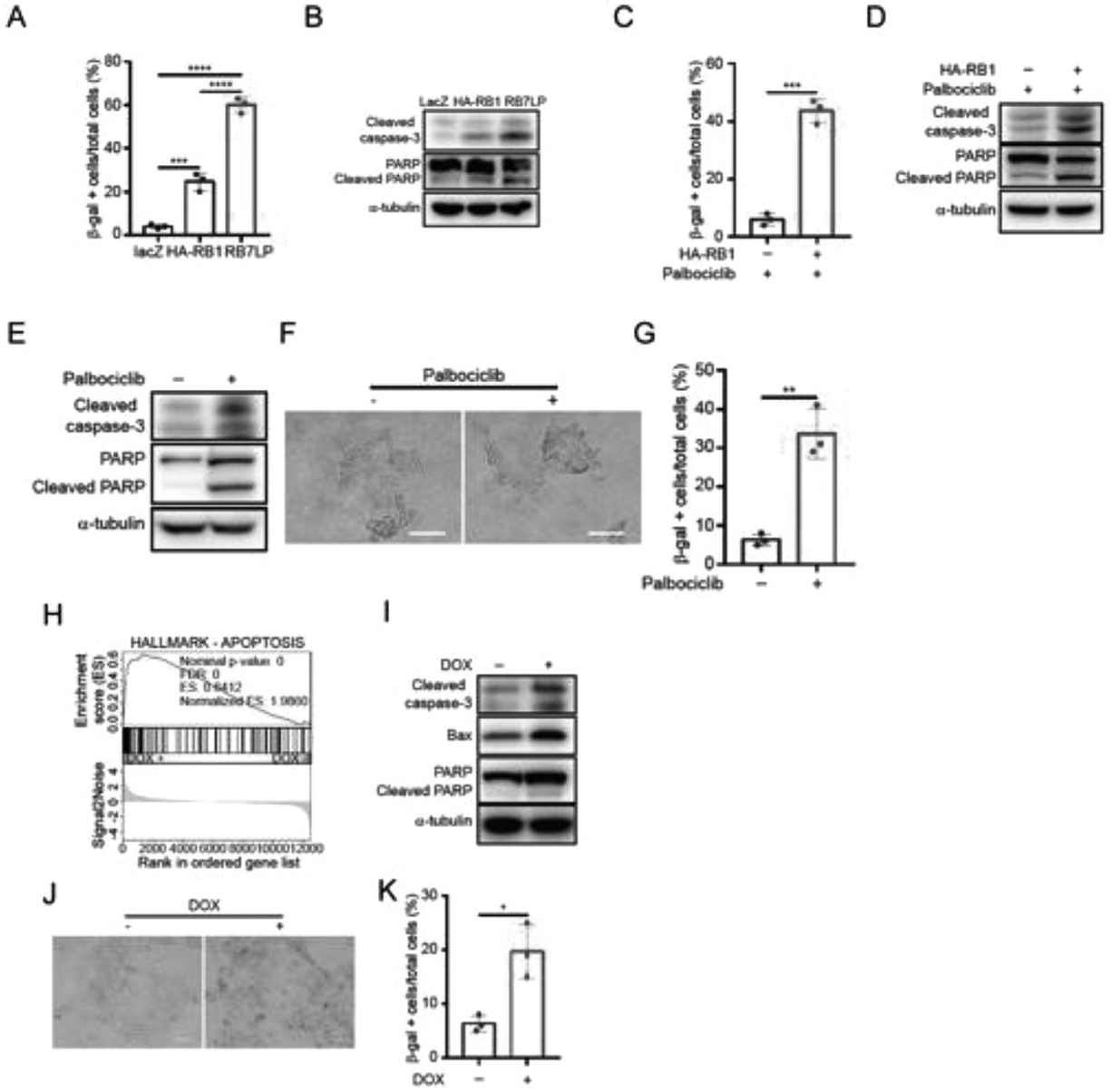

Figure 2. RB7LP mimics the action of CDK4/6 inhibitors

(A, F, G, J, K) Representative images of senescence-associated β-galactosidase (SA-β-gal) staining in the indicated cells and SA-β-gal staining and quantification. (B, D, E, I) Immunoblotting (IB) of the indicated proteins in primary HCC and HepG2 cells infected with the lentivirus expressing the indicated proteins.

IKKβ inhibition sensitizes hepatoblastoma and HCC cells to CDK4/6 inhibitors

Palbociclib when singly administrated exhibited a significant but an unsatisfactory therapeutic effect on HA-RB1(+) QKO HCC and HepG2 cells. We screened chemical libraries seeking for compounds that enhance the efficacy of RB7LP in HepG2 cells. We detected Bay11-7082, known as a chemical inhibitor for IκB kinase β (IKKβ), which appeared to synergize with either palbociclib or RB7LP to induce apoptosis. Furthermore, RB7LP increased IKKα/β phosphorylation and following activation of NF-κB in DOX (+) HepG2 cells. The Luciferase assay revealed that IKKβ inhibitors reduced NF-κB activation. We then treated HepG2 by palbociclib together with an NF-κB inhibitor, which significantly promoted cell apoptosis. In addition, combined use of palbociclib and IKKβ inhibitor exhibited synergistic effects in human HCC cell lines both in vitro and in immuno-deficient mice (Figure 3).

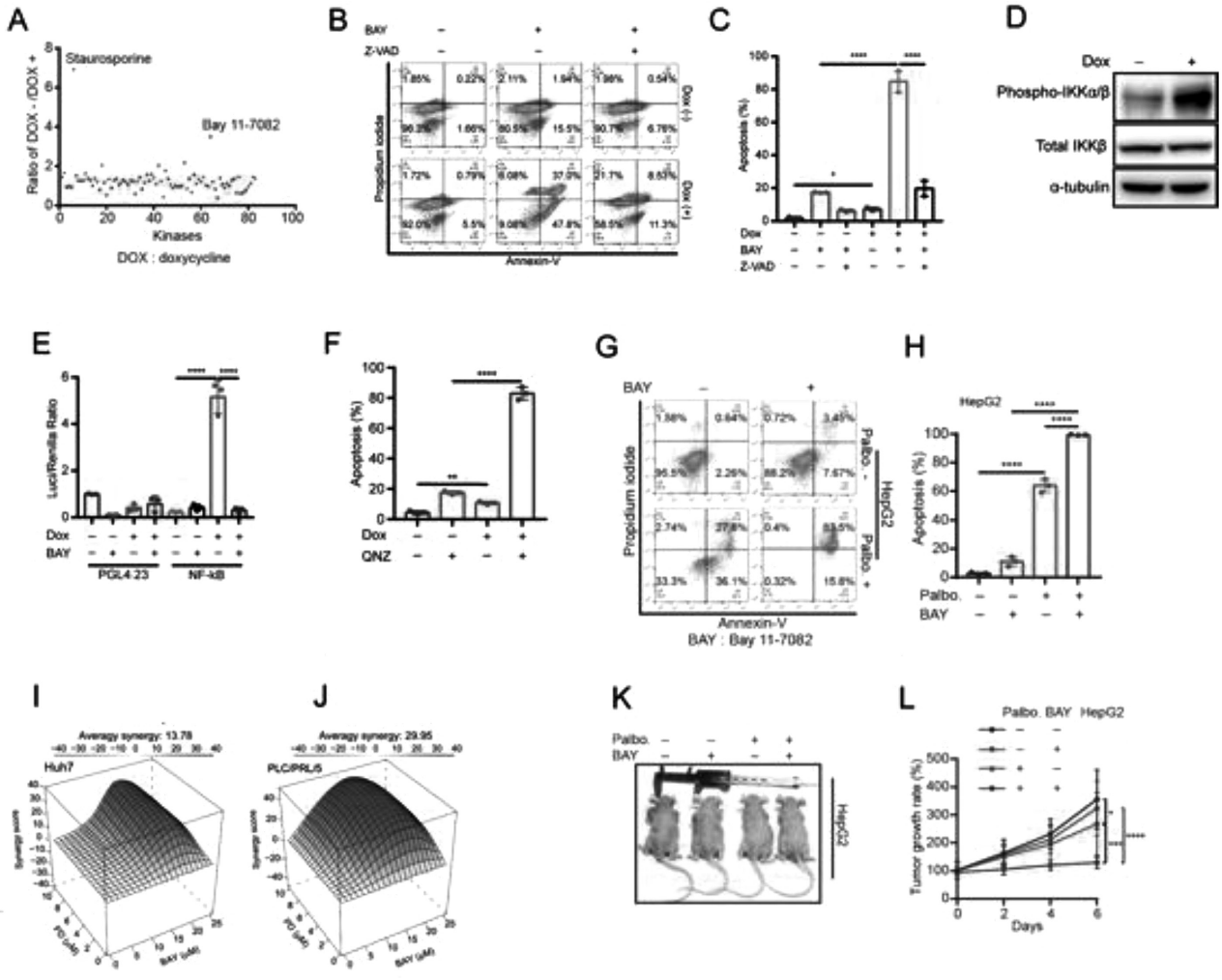

Figure 3. IKKβ inhibition sensitizes hepatoblastoma and HCC cells to RB7LP and a CDK4/6 inhibitor.
(A) Screening of a chemical library that covers 80 kinase inhibitors was performed to seek the compounds that selectively kill HepG2 cells in which RB7LP was overexpressed. (B, C, F, G, H) Representative results of flow cytometry analysis and quantification of apoptotic cells treated in the indicated manners. (D) IB of the indicated proteins in HepG2 cells infected with the lentivirus expressing the indicated proteins. (E) The NF-kB activity assessed by a luciferase reporter assay in HepG2 cells treated with the indicated chemicals. (I, J) Combination index was assessed in the indicated cells treated with the indicated chemicals. (K, L) A representative result of xenograft assay.

The mechanism whereby palbociclib IKKα/β phosphorylation

To determine the mechanism whereby palbociclib increases IKKα/β activities in RB1-intact cells, we measured the concentrations of nucleotides and metabolites (ADP, ATP, AMP, GDP, GMP, G1P, G6P, F6P, NAD and NADH), and found that induction of RB7LP reduced the abundance of all of them, implying nucleotide deficiency. Furthermore, we observed increased expression of p53 and its target genes, higher expression of γ-H2AX and increased phosphorylation of CHK2 following RB7LP induction. These findings suggest that RB7LP induces DNA damage in cells. Next, we detected a similar metabolic shift was achieved by the treatment with palbociclib. In addition, we found that supplementation of dNTPs antagonized the activation of γ-H2AX caused either by RB7LP induction or palbociclib treatment. We therefore reasoned increased IKKα/β phosphorylation and NF-kB activity by nucleotide deficiency owing to RB1 hypophosphorylation followed by DNA damage responses (Figure 4).

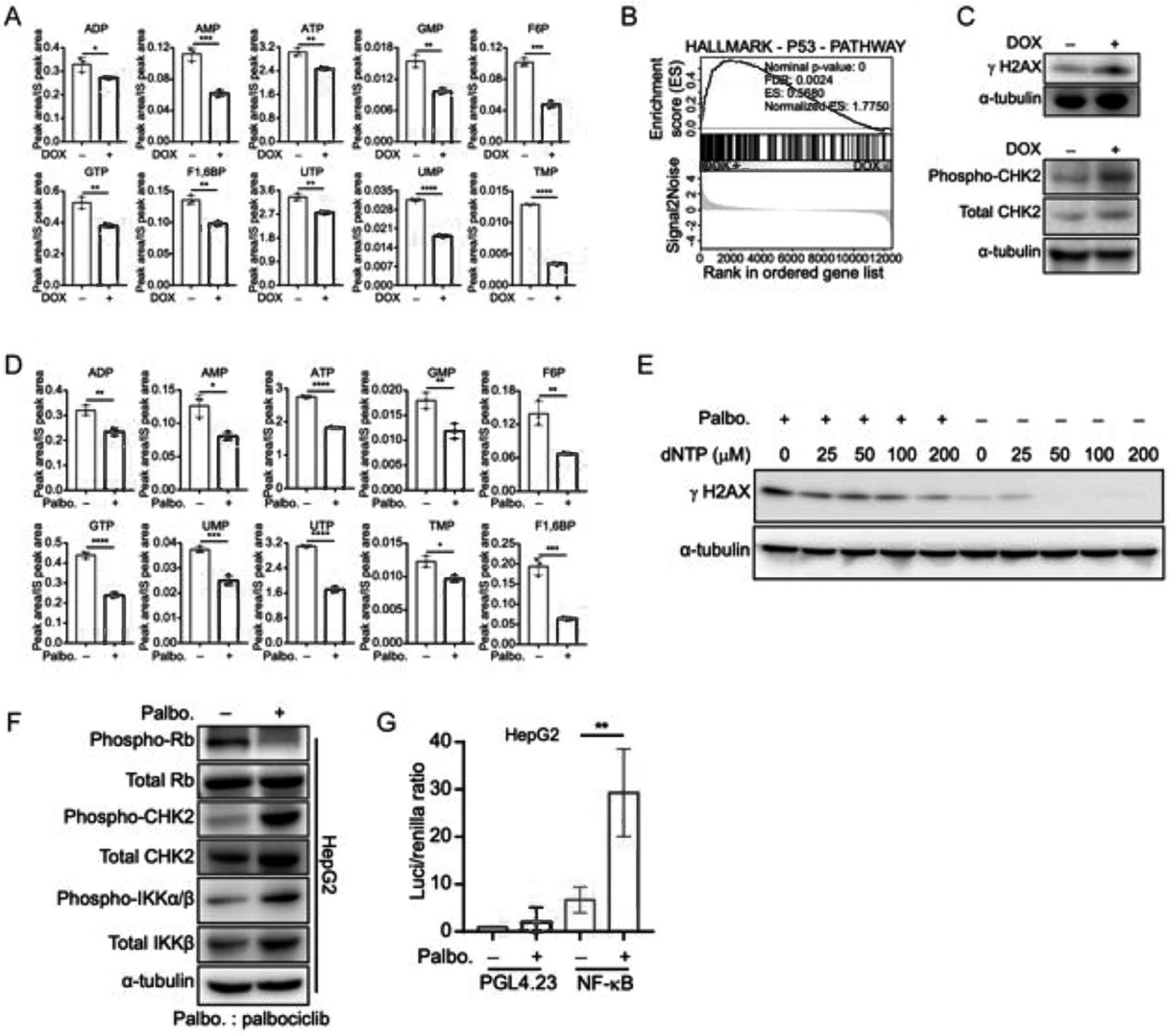

Figure 4. The mechanism whereby palbociclib and RB7LP increases IKKa/b phosphorylation.
(A, D) The cellular concentration of the indicated nucleotides and metabolites was determined in the indicated cells treated with the indicated chemicals. (B) GSEA results for hallmark gene sets in DOX (+) versus DOX (-) cells. (C, E, F) IB of the indicated proteins in HepG2 cells infected with the lentivirus expressing the indicated proteins. (G) Luciferase reporter assay in indicated cells.

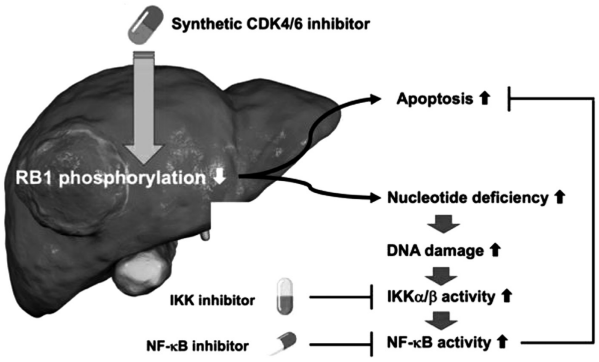

Figure 5. Schematic presentation of our hypothesis.

Conclusion

Synthetic CDK4/6 inhibitors limit RB1 phosphorylation inducing G1 arrest, cellular senescence and apoptosis. On the other hand, these agents cause DNA damage response following nucleotide deficieny and stimulate the survival signals depending on the IKK-NF-κB pathway. Our findings suggest that the use of a synthetic CDK4/6 inhibitor in combination with an inhibitor of the IKK-NF-κB pathway may provide a therapeutic benefit to HCC patients (Figure 5). We also proposed that synthetic CDK4/6 inhibitors are applicable to KRAS mutated cancers when combined with an appropriate kinase inhibitor[5].

References

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