Cyclin E1 Inhibition can Overcome Sorafenib Resistance in Hepatocellular Carcinoma Cells Through Mcl-1 Suppression

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Abstract

Purpose: To clarify the effects of cyclin E1 suppression on antitumor efficacy of sorafenib in hepatocellular carcinoma cells and to explore the potential of combining sorafenib with cyclin-dependent kinase (CDK) inhibition in therapy.

Experimental Design: The effects of cyclin E1 suppression on sorafenib-induced apoptosis were tested in both sorafenib-sensitive (Huh-7 and HepG2, IC₅₀ 5–6 μmol/L) and sorafenib-resistant (Huh-7R and HepG2R, IC₅₀ 14–15 μmol/L) hepatocellular carcinoma cells. The activity of pertinent signaling pathways and the expression of cell cycle and apoptosis-related proteins were measured using Western blotting. Efficacy of sorafenib combined with the pan-CDK inhibitor flavopiridol was tested both in vitro and in xenograft experiments. The pertinent downstream mediators of antitumor efficacy were tested in transient transfection and RNA interference experiments.

Results: Cyclin E1 mRNA and protein expressions were suppressed after sorafenib treatment in sorafenib-sensitive but not in sorafenib-resistant hepatocellular carcinoma cells. Changes in cyclin E2 or D1 were not correlated with sorafenib sensitivity. The knockdown of cyclin E1 expression reversed the resistance of hepatocellular carcinoma cells to sorafenib in terms of cell growth and apoptosis induction, whereas the overexpression of cyclin E1 increased the resistance to sorafenib. The growth-inhibitory and apoptosis-inducing effects of sorafenib were enhanced by flavopiridol, and Mcl-1 suppression was determined to play a critical role in mediating this enhancing effect.

Conclusions: The cyclin E1 suppression in hepatocellular carcinoma cells may serve as a pharmacodynamic biomarker for predicting sorafenib efficacy. The combination of sorafenib and CDK inhibitors may improve the efficacy of sorafenib in hepatocellular carcinoma.

Introduction

The multikinase inhibitor sorafenib is currently the standard systemic treatment for patients with advanced hepatocellular carcinoma (1, 2). Sorafenib has been found to exert many “off-target effects” that may contribute to its antitumor efficacy, in addition to the inhibition of Raf kinase signaling and tumor angiogenesis (3, 4). Multiple mediators in the apoptosis regulatory pathways, including Mcl-1 and survivin, may regulate the antitumor efficacy of sorafenib in cancer cells (5–7). Identifying these pathways and molecules will greatly contribute to design strategies for overcoming sorafenib resistance (8).

The dysregulation of cell-cycle control is a hallmark of cancer. The overexpression of cyclins is generally associated with advanced-stage disease and poor prognosis in many types of cancers, including hepatocellular carcinoma (9–13). Although molecular agents targeting cyclin-dependent kinases (CDK) and other cell-cycle regulators have been extensively studied, their roles in cancer therapy were not established until recently, when the CDK inhibitor palbociclib was demonstrated to prolong the progression-free survival of breast cancer patients who received hormonal therapy (14–16). Preclinical studies have indicated that cell-cycle inhibitors can reverse the resistance of cancer cells to hormonal, cytotoxic, and molecular targeted therapies (17–19). These preclinical and clinical data suggest that cell-cycle inhibitors may play a crucial role in combination anticancer therapy.

Sorafenib can suppress several key cell-cycle regulators, including E2F1, cyclin D, cyclin E1, and CDKs, which may contribute to its antitumor efficacy (5, 6, 20–22). We determined cyclin E1 expression in hepatocellular carcinoma cells was associated with sensitivity to sorafenib (20). In current study, we sought to clarify the mechanisms of sorafenib-induced cyclin E1 suppression in hepatocellular carcinoma cells, especially the regulatory roles of the E2F1–Rb–cyclin E1 complex (23, 24). The potential for improving the efficacy of sorafenib in hepatocellular carcinoma by combination with CDK inhibition was also explored.

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Translational Relevance
In this study, we demonstrated that the suppression of cyclin E1 by sorafenib in hepatocellular carcinoma cells is correlated with its therapeutic efficacy. Adding of the pan-CDK inhibitor flavopiridol can significantly improve the efficacy of sorafenib both in vitro and in vivo. The suppression of Mcl-1 was found to be a critical mediator for improved therapeutic efficacy. The results suggest the potential of using cell-cycle inhibitors in combination therapy for hepatocellular carcinoma.

Materials and Methods

Cell culture
The hepatocellular carcinoma cell lines tested in this study were HepG2, Hep3B, PLC-5, SK-Hep1, SNU398 (from the ATCC), and Huh-7 (from the Health Science Research Resources Bank). Sorafenib-resistant cell lines, Huh-7R and HepG2R, were generated by continuous treatment of Huh-7 and HepG2 cells combined with sorafenib up to 10 μmol/L to mimic the clinical phenomenon of acquired resistance. No further authentication was conducted in our laboratory.

Chemicals and other reagents
The compounds used in this study were sorafenib (Bayer-Scherering Pharma), U0126 (MEK kinase inhibitor, Calbiochem), ZM336372 (Raf kinase inhibitor, Merck KGaA), and flavopiridol (Selleck Chemicals). The antibodies used for Western blotting, immunohistochemical staining, and chromatin immunoprecipitation (ChIP) assays were cyclin E1 (BD Biosciences), Bcl-XL (Cell Signaling Technology), and CD31 (Abcam). The overexpression or knockdown of cyclin E1, Mcl-1, or E2F1 was confirmed by performing qRT-PCR and Western blotting. The effects of cyclin E1 modulation on the sensitivity of hepatocellular carcinoma cells to drug treatment were measured using the MTT assay and flow cytometry.

Modulation of cyclin E, Mcl-1, and E2F1 in vitro
The overexpression or knockdown of cyclin E1, Mcl-1, or E2F1 was performed through transient transfection or siRNA, respectively. The primer/plasmid sequences are summarized in Supplementary Table S1. The efficacy of overexpression/knockdown was confirmed by performing qRT-PCR and Western blotting. The results suggest that the changes in cyclin E1 levels after sorafenib treatment may serve as a pharmacodynamic marker in hepatocellular carcinoma. Sorafenib suppressed cyclin E1 mRNA expression in sorafenib-sensitive hepatocellular carcinoma cells (Huh-7 and HepG2, IC50 5–6 μmol/L) but not in sorafenib-resistant cells (Huh-7R and HepG2R, IC50 14–15 μmol/L). The effects on cyclins E2 and D1 mRNA expression did not correlate favorably with the sensitivity to sorafenib (Fig. 1B). Cyclin E1 expression was not suppressed by U0126 or the Raf kinase inhibitor ZM336372 in the sensitive hepatocellular carcinoma cells, supporting a Raf kinase-independent mechanism (Fig. 1B). To confirm the effects of cyclin E1 modulation on sorafenib sensitivity, the IC50 of sorafenib was measured after the knockdown or overexpression of cyclin E1. The knockdown of cyclin E1, but not cyclin E2, significantly increased the sensitivity to sorafenib in both sensitive and resistant hepatocellular carcinoma cells (Fig. 1B and Supplementary Fig. S3). Cyclin E1 knockdown of the luciferase gene in the pGL4.17-base luciferase expression plasmid (Promega; Supplementary Fig. S1; ref. 24). Three different deletion fragments of the cyclin E1 promoter (−389 to +747, −192 to +747, and +515 to +747) were generated. Huh-7 cells were transfected with individual cyclin E1 reporter constructs and cotransfected with pGL4.73 [hRluc/SV40], which constitutively expresses Renilla luciferase, to normalize transfection efficiency. The reporter activities with or without sorafenib treatment were determined using a Dual-Luciferase Assay Kit (Promega). The binding activity of E2F1 to cyclin E1 promoter with or without sorafenib treatment was analyzed by ChIP assay.

Tumor xenograft experiments
The protocol for the xenograft experiments was approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University (Taipei, Taiwan). All the animal studies were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the NIH (Bethesda, MD). Male BALB/c athymic (nu/nu) mice were inoculated subcutaneously with Huh-7 or Huh-7R cells. Sorafenib was administered daily by gavage and flavopiridol 3 times per week through intraperitoneal injection. Tumor volume and body weight were recorded every 7 days. At the end of drug treatment, tumor samples were collected for Western blotting, immunohistochemical analysis, and a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (25).

Statistical analysis
All data were representative of at least three independent experiments. Quantitative data were expressed as mean ± SD. Comparisons were analyzed using Student t test and ANOVA. Significance was defined as P < 0.05. Animal survival time was calculated using the Kaplan–Meier method and compared using a log-rank test.

Results
Suppression of cyclin E1 expression in hepatocellular carcinoma cells by sorafenib correlated with sorafenib efficacy
The extent of cyclin E1 suppression after sorafenib treatment correlated favorably with the sensitivity to sorafenib (Fig. 1A and Supplementary Fig. S2A). However, the baseline levels of cyclin E1 were not correlated with sorafenib sensitivity (Supplementary Fig. S2B). The data suggest that the changes in cyclin E1 levels after sorafenib treatment may serve as a pharmacodynamic marker in hepatocellular carcinoma. Sorafenib suppressed cyclin E1 mRNA expression in sorafenib-sensitive hepatocellular carcinoma cells (Huh-7 and HepG2, IC50 5–6 μmol/L) but not in sorafenib-resistant cells (Huh-7R and HepG2R, IC50 14–15 μmol/L). The effects on cyclins E2 and D1 mRNA expression did not correlate favorably with the sensitivity to sorafenib (Fig. 1B). Cyclin E1 expression was not suppressed by U0126 or the Raf kinase inhibitor ZM336372 in the sensitive hepatocellular carcinoma cells, supporting a Raf kinase-independent mechanism (Fig. 1B and Supplementary Figs. S2A and S3). To confirm the effects of cyclin E1 modulation on sorafenib sensitivity, the IC50 of sorafenib was measured after the knockdown or overexpression of cyclin E1. The knockdown of cyclin E1, but not cyclin E2, significantly increased the sensitivity to sorafenib in both sensitive and resistant hepatocellular carcinoma cells (Fig. 2A and Supplementary Fig. S4). Cyclin E1 knockdown
enhanced the apoptosis-inducing effects of sorafenib in hepato-cellular carcinoma cells (Fig. 2B and Supplementary Fig. S5), and cyclin E1 overexpression was associated with reversal of sorafenib-induced apoptosis in sorafenib-sensitive hepatocellular carcinoma cells (Fig. 2C). Cyclin E1 overexpression significantly increased viability in the sorafenib-sensitive hepatocellular carcinoma cells after sorafenib treatment (Fig. 2D). The apoptosis-enhancing effects of cyclin E1 knockdown were associated with suppression of Mcl-1 expression (Fig. 2E). These data indicate that cyclin E1 expression plays a crucial role in mediating antitumor efficacy of sorafenib in hepatocellular carcinoma cells.

Sorafenib inhibited cyclin E1 expression through downregulation of E2F1
To clarify the regulatory mechanisms of cyclin E1 expression by sorafenib, the expression of E2F1 and Rb proteins was measured.
in sorafenib-sensitive and sorafenib-resistant hepatocellular carcinoma cells. Sorafenib suppressed E2F1 and Rb expression only in sorafenib-sensitive hepatocellular carcinoma cells (Fig. 3A). The changes in the levels of CDK2, the primary CDK activated by cyclin E1, was not consistent with the sensitivity of hepatocellular carcinoma cells to sorafenib (Fig. 3A). Sorafenib inhibited the luciferase activity of all the cyclin E1 promoter fragments, which contained the 6 E2F-binding sites previously reported (24), suggesting a transcriptional regulation on cyclin E1 expression (Fig. 3B). The association of E2F1 with the cyclin E1 promoter-enhancer in hepatocellular carcinoma cells was confirmed using a ChIP assay (Fig. 3C), and E2F1 knockdown could suppress the expression of total and phospho-cyclin E1 in hepatocellular carcinoma cells (Supplementary Fig. S6).

Enhancement of the antitumor activity of sorafenib by flavopiridol

The combination of sorafenib and the CDK inhibitor flavopiridol was tested in vitro and in vivo to explore whether the antitumor efficacy of sorafenib can be improved by cell-cycle modulation. Flavopiridol could suppress cyclin E1 and Rb protein levels in hepatocellular carcinoma cells in a dose-dependent manner (Supplementary Fig. S7). Sorafenib-induced apoptosis could be significantly enhanced by the addition of flavopiridol in both sorafenib-sensitive and sorafenib-resistant hepatocellular carcinoma cells (Fig. 4A and Supplementary Fig. S8). However, the change in cell-cycle distribution after addition of flavopiridol did not correlate with the antitumor synergy between the two agents (Supplementary Fig. S9). The apoptosis-enhancing effects of flavopiridol were associated with the suppression of Mcl-1 expression (Fig. 4B), consistent with findings derived using cyclin E1 knockdown (Fig. 2E). Screening using an apoptosis array did not detect changes in other apoptosis-related proteins consistent with the antitumor enhancement (Supplementary Fig. S10). Mcl-1 overexpression reversed the apoptosis induced by sorafenib by flavopiridol (Fig. 4C), whereas Mcl-1 knockdown further enhanced the apoptosis induction (Fig. 4D and Supplementary Fig. S5). These data indicate that flavopiridol can enhance the
antitumor efficacy of sorafenib and that cyclin E1 and Mcl-1 play critical roles in mediating this antitumor enhancement.

In vivo experiments also supported the enhancement of antitumor efficacy by flavopiridol, particularly in the sorafenib-resistant (Huh-7R and HepG2R) cells. Whole-cell lysate was collected after 24-hour treatment of the cells with sorafenib 10 μmol/L and was subjected to Western blot analysis. B, sorafenib-induced E2F1 suppression reduced cyclin E1 transcription. The 5’-deletion constructs of the cyclin E1 promoter (−389 to +474 bp, containing six E2F-binding sites) were transfected into Huh-7 cells, and the relative luciferase activity of each promoter fragment after sorafenib treatment is shown on the right.

**P < 0.05, compared with the control group. C, ChIP assay of E2F1 association with the cyclin E1 promoter-enhancer in Huh-7 and HepG2 cells. Negative control (Neg Con) was chromatin immunoprecipitated with a normal mouse IgG. Positive control (Pos Con) was chromatin immunoprecipitated with an anti-RNA polymerase II antibody. Input was 0.1% of the sonicated chromatin before immunoprecipitation.

Discussion

In this study, we demonstrated that cyclin E1 suppression by sorafenib in hepatocellular carcinoma cells correlated with
Figure 4.
Synergistic antitumor activity between sorafenib and the CDK inhibitor flavopiridol. A, induction of apoptosis by sorafenib and/or flavopiridol, measured using flow cytometry (sub-G1 fraction analysis). B, effects of sorafenib and flavopiridol on apoptosis-related proteins in hepatocellular carcinoma cells. Hepatocellular carcinoma cells were treated with the drugs for 48 hours and whole-cell lysates were subjected to Western blotting. (Continued on the following page.)
therapeutic efficacy of sorafenib. Adding the pan-CDK inhibitor flavopiridol can significantly improve the efficacy of sorafenib both in vitro and in vivo. The suppression of Mcl-1 was found to be a critical mediator of the improved therapeutic efficacy. These findings provide the rationale of design for combination therapy for hepatocellular carcinoma.

Figure 5.
Flavopiridol enhanced the antitumor efficacy of sorafenib in vivo. Huh-7 or Huh-7R cells were injected subcutaneously into male BALB/c athymic nude mice. Mice were treated as indicated (V, vehicle; S10, sorafenib 10 mg/kg/d; Fla-3, flavopiridol 3 mg/kg/d three times a week). A, difference in tumor growth (n = 5 in each group). B, difference in animal survival (n = 10 in each group). C, changes in tumor microvessel density (CD31 staining; MVD), tumor cell apoptosis (TUNEL assay), and tumor cell proliferation (Ki67 staining) after drug treatment. *, P < 0.05; **, P < 0.01 derived using Student t test. Columns, means (n = 5); bars, SD. D, changes in cyclin E1, E2F1, and apoptosis-related proteins in tumor lysates. Western blotting results were scanned and quantified using the ImageJ software (NIH, Bethesda, MD) and expressed as target/actin ratios. Tx, treatment. HPF, high power field; NC, negative control.
Although the dysregulated cell-cycle control in cancer cells has been extensively studied, most clinical trials on single-agent treatment with CDK inhibitors or other cell-cycle regulators have reported significant antitumor efficacy. One reason may be the functional redundancy of cell-cycle control mechanisms, which occur at the genetic, biologic, and biochemical levels (26). However, our finding that only cyclin E1 but not E2 contributes to sorafenib resistance suggests that individual cell-cycle regulators may still play specific functional roles. The suppression of cyclin E1 may serve as a pharmacodynamic marker of sorafenib efficacy in hepatocellular carcinoma, and further study on cyclin E1 expression in hepatocellular carcinoma tumor tissues prospectively collected from patients before and after sorafenib treatment would help validate our findings.

Results from our study and other investigators support Mcl-1 as a key mediator of cell survival and drug resistance in hepatocellular carcinoma (5, 6, 27, 28). Mcl-1 may be suppressed by sorafenib through downregulation of E2F1, which also suppresses cyclin E1 expression in hepatocellular carcinoma cells. Our data indicate that regulation of the E2F1–Rb–cyclin E1 complex may play crucial roles in mediating sorafenib resistance in hepatocellular carcinoma cells. Mcl-1 expression is also regulated by multiple signaling pathways, including MAPK, JAK/STAT, and PI3K/AKT, in cancer cells (29, 30). Therefore, Mcl-1 expression can serve as a pharmacodynamic marker of different types of combination therapy.

Recent developments of CDK inhibitors for cancer treatment have focused on selective CDK4/6 inhibitors including palbociclib, abemaciclib, and ribociclib. Pan-CDK inhibitors such as flavopiridol or dinaciclib may have better antitumor activity through both cell-cycle regulation and the suppression of the expression of antiapoptotic proteins, including Mcl-1, Bcl-XL, and XIAP (32, 33). A potential concern about using pan-CDK inhibitors is the off-target adverse events. However, our data indicate that flavopiridol can enhance the antitumor efficacy of sorafenib in doses without significant single-agent effects on Mcl-1 expression and tumor control. This finding raises the issue of whether the single-agent antitumor activity of CDK inhibitors should be demonstrated to justify their use in combination treatment. Future clinical trials should clarify whether specific or multitargeted CDK inhibitors can yield a more favorable therapeutic index in clinics.

There are several challenges to incorporating cell-cycle regulators in systemic therapy for hepatocellular carcinoma. The first is to identify biomarkers to predict treatment response. It was hypothesized that CDK inhibitors and other cell-cycle regulators may be of greatest benefit in cancer patients with known aberrations in cell-cycle control, such as cyclin D amplification or p16 loss (34). However, results from the palbociclib trial for breast cancer did not support this hypothesis (15). Therefore, although aberrations in cell-cycle control in hepatocellular carcinoma have been repeatedly reported, whether these aberrations can serve as markers for patient enrichment remains to be tested (35–38). The second challenge involves watching for treatment-induced myelosuppression. Although the myelosuppression reported in recent clinical trials on CDK inhibitors has generally been well tolerated, it may pose an increased risk of complications in hepatocellular carcinoma patients because of the patients’ underlying cirrhosis and hypersplenism. Therefore, identifying the biologic effective dose in future clinical trials on hepatocellular carcinoma is critical, especially in trials on combination therapy.

In conclusion, cyclin E1 suppression contributes to sorafenib-induced apoptosis in hepatocellular carcinoma cells. Future studies are necessary to validate its value in predicting sorafenib efficacy.

Disclosure of Potential Conflicts of Interest
C. Hsu reports receiving speakers bureau honoraria from Bayer-Schering Pharma. A.-L. Cheng is consultant/advisory board member for and reports
receiving speakers bureau honoraria from Bayer-Schering Pharma. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Hsu, Y.-C. Cheng, Z.-R. Feng

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Hsu, L.-I. Lin, Y.-C. Cheng, Y.-Y. Shao, D.-L. Ou

Writing, review, and/or revision of the manuscript: C. Hsu, D.-L. Ou

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Hsu, L.-I. Lin, A.-L. Cheng

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