Berbamine Inhibits the Growth of Liver Cancer Cells and Cancer-Initiating Cells by Targeting Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II

Zhipeng Meng\(^1,2\), Tao Li\(^2\), Xiaoxiao Ma\(^1,2\), Xiaoqiong Wang\(^2,8\), Carl Van Ness\(^2\), Yichao Gan\(^6,7\), Hong Zhou\(^6,7\), Jinfen Tang\(^6,7\), Guiyu Lou\(^2,5\), Yafan Wang\(^3\), Jun Wu\(^4\), Yun Yen\(^1,3\), Rongzhen Xu\(^6,7\), and Wendong Huang\(^1,2\)

Abstract
Liver cancer is the third leading cause of cancer deaths worldwide but no effective treatment toward liver cancer is available so far. Therefore, there is an unmet medical need to identify novel therapies to efficiently treat liver cancer and improve the prognosis of this disease. Here, we report that berbamine and one of its derivatives, bbd24, potently suppressed liver cancer cell proliferation and induced cancer cell death by targeting Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CAMKII). Furthermore, berbamine inhibited the in vivo tumorigenicity of liver cancer cells in NOD/SCID mice and downregulated the self-renewal abilities of liver cancer–initiating cells. Chemical inhibition or short hairpin RNA–mediated knockdown of CAMKII recapitulated the effects of berbamine, whereas overexpression of CAMKII promoted cancer cell proliferation and increased the resistance of liver cancer cells to berbamine treatments. Western blot analyses of human liver cancer specimens showed that CAMKII was hyperphosphorylated in liver tumors compared with the paired peritumor tissues, which supports a role of CAMKII in promoting human liver cancer progression and the potential clinical use of berbamine for liver cancer therapies. Our data suggest that berbamine and its derivatives are promising agents to suppress liver cancer growth by targeting CAMKII. Mol Cancer Ther; 12(10); 2067–77. ©2013 AACR.

Introduction
Liver cancer is the third leading cause of cancer deaths globally due to its high incidence and the lack of effective treatments. In the United States, it is now among the 10 most common cancers, and its prevalence has been increased dramatically in the recent years (1). The major type of liver cancer is hepatocellular carcinoma (HCC), and the risk factors for HCCs include infection of hepatitis B or C virus, overconsumption of alcohol, and insults from xenobiotics (2).

Surgical removal of liver tumor is a primary therapy for patients who have relatively small tumors and a still well-functioning liver (3). For patients at late stages or with cirrhosis, other therapies such as arterial chemomoblation, radiofrequency ablation, percutaneous ethanol injection, proton beam, chemotherapy, and liver transplantation might be applied (4). However, the outcomes of these therapies are not satisfying. Therefore, novel treatments such as target therapies are in urgent need to improve the prognosis of the patients with liver cancer (5). Currently, sorafenib is a widely used target therapy drug that specifically blocks angiogenesis and other growth signaling of liver tumor (6). Nevertheless, this drug only prolongs the patient life by 3 months in average and even shorter in patients under poor conditions potentially due to the resistance of cancer stem cells to this drug (7).

Many compounds in use today for cancer medicine are derived from natural products of plants and marine organisms (8, 9). Berbamine is a natural bisbenzylisoquinoline product isolated from traditional Chinese herbal medicine Berberis amurensis and has been used to treat inflammatory and other diseases for centuries (10). Recent studies suggest that berbamine and its derivatives also possess antitumor activities for chronic myeloid leukemia, breast cancer, and melanoma (11–13).
In this study, we report that berbamine and its derivative bbd24 (Fig. 1) are potent to suppress the growth of liver cancers, as well as cancer-initiating cells. The Ca	extsuperscript{2+}/calmodulin-dependent protein kinase II (CAMKII) is identified as a berbamine target in liver cancer. These results implicate that targeting CAMKII by berbamine and its derivatives may provide a novel approach to treat liver cancer.

Materials and Methods

Cell culture, survival/proliferation assay, and sphere formation assay

HepG2, PLC/PRF/5, SK-Hep-1, and SNU398 cells were ordered from the American Type Culture Collection. MHCC97H cells were from the Cell Resources of Shanghai Institutes for Life Sciences, Chinese Academy of Sciences. Huh7 cells were from Japanese Collection of Research Bioresources Cell Bank. CL48 was a gift from Dr. Yun Yen’s laboratory at the City of Hope Medical Center. All these cell lines were authenticated by the providers and were frozen in liquid nitrogen soon after arrival. The experiments with these cells were carried out within 6 generations after resuscitation.

CL48, Huh7, PRL/PRF/5, and MHCC97H cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS. HepG2 cells were maintained in Minimum Essential Medium (MEM) containing 10% FBS. SK-Hep-1 and SNU398 cells were maintained in RPMI-1640 containing 10% FBS. MTS assay measuring amount of survival cells was conducted with the CellTiter 96 Aqueous Cell Proliferation Kit (Promega). The IC	extsubscript{50} was defined as the drug concentration that induced 50% viability decrease. The sphere formation assay followed a protocol described elsewhere (16). The maintenance of the cells before the sphere formation assay was conducted with digestion and cell passage every 3 days and a subculture ratio for 70% to 80% confluence before passage.

Compound

Cisplatin and 5'-fluorouracil (FU) were from Sigma-Aldrich and were dissolved in dimethyl sulfoxide (DMSO). For 	extit{in vitro} experiments, berbamine and bbd24 were dissolved in DMSO. Berbamine was dissolved in pure sterile water for animal experiments. About 500 ng/mL of the tetracyclin derivative doxycycline (Clontech) was used for induction of CAMKII	extsubscript{g} expression in cell cultures.

Xenograft

A total of 5 x 10	extsuperscript{6} Huh7 cells in 50% Matrigel (BD Bioscience) dissolved in PBS were inoculated in a NOD/SCID mouse. A total of 5 x 10	extsuperscript{6} SK-Hep-1 cells were applied for each xenograft without Matrigel. About 100 mg/kg of berbamine was orally treated to mice with a regimen of twice a day for 5 consecutive days after the tumors reached a size of 2 mm in diameter. After 2 days’ withdrawal, the regimen was repeated once. All the procedures followed the NIH guidelines for the care and use of laboratory animals.

Cell death analysis and flow cytometry

The cell death analysis was conducted with the FITC Annexin V Apoptosis Detection Kit I from BD Pharmigen according to the manufacturer’s instruction. Phycoerythrin (PE)-conjugated anti-human CD133/1 antibody was ordered from Miltenyi Biotec for flow cytometry [fluorescence-activated cell sorting (FACS)] analysis. The purity of the sorted cells was tested with PE-conjugated anti-human CD133/2 antibody. The fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 antibody for MHCC97H cells was from Biolegend.

CAMKII	extsubscript{g} overexpression and knockdown

The human CAMKII	extsubscript{g} coding sequence with a kozak site was cloned into the retroviral vectors pMSCV-puro (Addgene 24828) and pRetroX-Tight-puro (Clontech). A multiplicity of infection (MOI) of 3 to 5 was used for retroviral transduction of the liver cancer cells. The retroviral experiments were carried out following the manual of Retro-X Tet-On Advanced Inducible Expression System. A lentiviral vector pLKO.1-TRC (Addgene 10878) was used for the knockdown of CAMK2	extsubscript{g}. The following targets in the coding sequences were selected for the design of short hairpin RNA (shRNA): GGATATGTCGACTTCTGAAAC, GGAGCCTATGATTTCCCATCA, GCCACAAACCACGTGTTACA, GCATCCATGATGCATCGTCA-GGA. A MOI of 3 was applied for the infection of the target cells. Puromycin (Sigma-Aldrich) was used to select the cells after lentiviral infection. The stable cells were used for the following animal experiments. Both retroviruses and lentviruses were packaged in Hek293T cells and titrated with HT1080 cells.

In this study, we report that berbamine and its derivative bbd24 (Fig. 1) are potent to suppress the growth of liver cancers, as well as cancer-initiating cells. The Ca	extsuperscript{2+}/calmodulin-dependent protein kinase II (CAMKII) is identified as a berbamine target in liver cancer. These results implicate that targeting CAMKII by berbamine and its derivatives may provide a novel approach to treat liver cancer.

Materials and Methods

Cell culture, survival/proliferation assay, and sphere formation assay

HepG2, PLC/PRF/5, SK-Hep-1, and SNU398 cells were ordered from the American Type Culture Collection. MHCC97H cells were from the Cell Resources of Shanghai Institutes for Life Sciences, Chinese Academy of Sciences. Huh7 cells were from Japanese Collection of Research Bioresources Cell Bank. CL48 was a gift from Dr. Yun Yen’s laboratory at the City of Hope Medical Center. All these cell lines were authenticated by the providers and were frozen in liquid nitrogen soon after arrival. The experiments with these cells were carried out within 6 generations after resuscitation.

CL48, Huh7, PRL/PRF/5, and MHCC97H cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS. HepG2 cells were maintained in Minimum Essential Medium (MEM) containing 10% FBS. SK-Hep-1 and SNU398 cells were maintained in RPMI-1640 containing 10% FBS. MTS assay measuring amount of survival cells was conducted with the CellTiter 96 Aqueous Cell Proliferation Kit (Promega). The IC	extsubscript{50} was defined as the drug concentration that induced 50% viability decrease. The sphere formation assay followed a protocol described elsewhere (16). The maintenance of the cells before the sphere formation assay was conducted with digestion and cell passage every 3 days and a subculture ratio for 70% to 80% confluence before passage.

Compound

Cisplatin and 5'-fluorouracil (FU) were from Sigma-Aldrich and were dissolved in dimethyl sulfoxide (DMSO). For 	extit{in vitro} experiments, berbamine and bbd24 were dissolved in DMSO. Berbamine was dissolved in pure sterile water for animal experiments. About 500 ng/mL of the tetracyclin derivative doxycycline (Clontech) was used for induction of CAMKII	extsubscript{g} expression in cell cultures.

Xenograft

A total of 5 x 10	extsuperscript{6} Huh7 cells in 50% Matrigel (BD Bioscience) dissolved in PBS were inoculated in a NOD/SCID mouse. A total of 5 x 10	extsuperscript{6} SK-Hep-1 cells were applied for each xenograft without Matrigel. About 100 mg/kg of berbamine was orally treated to mice with a regimen of twice a day for 5 consecutive days after the tumors reached a size of 2 mm in diameter. After 2 days’ withdrawal, the regimen was repeated once. All the procedures followed the NIH guidelines for the care and use of laboratory animals.

Cell death analysis and flow cytometry

The cell death analysis was conducted with the FITC Annexin V Apoptosis Detection Kit I from BD Pharmigen according to the manufacturer’s instruction. Phycoerythrin (PE)-conjugated anti-human CD133/1 antibody was ordered from Miltenyi Biotec for flow cytometry [fluorescence-activated cell sorting (FACS)] analysis. The purity of the sorted cells was tested with PE-conjugated anti-human CD133/2 antibody. The fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 antibody for MHCC97H cells was from Biolegend.

CAMKII	extsubscript{g} overexpression and knockdown

The human CAMKII	extsubscript{g} coding sequence with a kozak site was cloned into the retroviral vectors pMSCV-puro (Addgene 24828) and pRetroX-Tight-puro (Clontech). A multiplicity of infection (MOI) of 3 to 5 was used for retroviral transduction of the liver cancer cells. The retroviral experiments were carried out following the manual of Retro-X Tet-On Advanced Inducible Expression System. A lentiviral vector pLKO.1-TRC (Addgene 10878) was used for the knockdown of CAMK2	extsubscript{g}. The following targets in the coding sequences were selected for the design of short hairpin RNA (shRNA): GGATATGTCGACTTCTGAAAC, GGAGCCTATGATTTCCCATCA, GCCACAAACCACGTGTTACA, GCATCCATGATGCATCGTCA-GGA. A MOI of 3 was applied for the infection of the target cells. Puromycin (Sigma-Aldrich) was used to select the cells after lentiviral infection. The stable cells were used for the following animal experiments. Both retroviruses and lentviruses were packaged in Hek293T cells and titrated with HT1080 cells.
Western blot analysis
Cell/liver lysis and lyase preparation were previously described (17). Antibodies that identified phospho-CAMKII, CAMKIIy, Bcl-2, and HSP70 were from Santa Cruz Biotechnology. Anti-β-actin and anti-LC3 antibodies were from Sigma-Aldrich. All the other antibodies were from Cell Signaling Technology.

Human HCC specimen analyses
The frozen samples of the patients with HCCs were provided by the City of Hope National Medical Center, and the pathologic description and analyses were conducted by the pathologists in the Department of Pathology. The lysates of tumor and nontumor adjacent tissues were prepared with the T-PER tissue Protein Extraction Reagent (Pierce). The pCAMKII signals were quantified with the software ImageJ.

Statistical analyses
All the data were reported as mean ± SEM. Two-tailed Student t test was used to determine the significance of differences between data groups.

Results
Berbamine inhibits the growth of liver cancer cells in vitro and in vivo
We first determined the effect of berbamine (Fig. 2A and B) on the growth of a variety of liver cancer cells from different origins or different stages of cancer progression (18). The results show that berbamine potently inhibited the growth of epithelial liver cancer cell lines including Huh7, HepG2, MHCC97H, and PLC/PRF/5, with an IC50 as low as 5.2 μg/mL (Fig. 2A). In addition, berbamine also suppressed the growth of mesenchymal-like liver cancer cell lines either from an endothelial origin (SK-Hep-1) or from the cells that has undergone epithelial-to-mesenchymal transition (SNU398; Fig. 2B). In contrast, an embryonic liver cell line, CL48, which is from a normal fetus liver, was much less sensitive to berbamine. The high IC50 (55.3 μg/mL) of CL48 indicated that the effects of berbamine were more specific to the transformed liver cells in a dose-dependent manner (Supplementary Table S1).

We then further determined the antitumor effects of berbamine on a xenograft animal model. Two liver cancer cell lines, Huh7 (epithelial) and SK-Hep-1 (mesenchymal-like), were inoculated into NOD/SCID mice by subcutaneous injection. The oral berbamine treatment greatly suppressed the growth of Huh7-xenografted tumors over the time (Fig. 2C) and led to a tumor reduction by 70% based on the tumor weight (Fig. 2D; Supplementary Fig. S1A). Consistent with the in vitro experiments, the growth of SK-Hep-1 cells in NOD/SCID mice was less sensitive to berbamine than that of Huh7 (Fig. 2E). Nevertheless, there was still a significant suppression of the growth of the SK-Hep-1 xenograft with more than 50% reduction of the tumor weight (Fig. 2F; Supplementary Fig. S1B). These results clearly show that berbamine is a very potent natural compound to suppress the growth of liver cancer.

Berbamine and its derivative, bbd24, induced deaths of liver cancer cells by targeting CAMKII
To determine the mechanisms by which berbamine inhibits the growth of liver cancer cells, we examined whether berbamine inhibited the liver cancer growth by inducing cell deaths. Huh7 cells were analyzed by flow cytometry with the propidium iodide (PI) and Annexin V staining at 72 hours after berbamine treatment. Eighty percent cells underwent cell deaths by either apoptosis or necrosis (Fig. 3A). A biotinylated berbamine was used to study whether berbamine bound to plasma membrane receptors or penetrated plasma membrane to affect cytoplasmic molecules. Accompanying with significant cell death morphology of nucleus fragmentation, berbamine appeared to be able to enter plasma membrane and stay in the cytoplasm, indicating that the major targets of berbamine are probably cytoplasmic and/or membrane receptor-activated signaling molecules (Supplementary Fig. S2).

Our previous study in chronic myeloid leukemia showed that berbamine targeted CAMKII by blocking its ATP-binding pocket (15). Indeed, among many signaling pathways that were examined in different berbamine-treated liver cancer cell lines, including Huh7, SK-Hep-1, and MHCC97H cells, phosphorylation of CAMKII was universally inhibited by berbamine (Fig. 3B). The strongest inhibition of CAMKII phosphorylation was observed in Huh7 cells, which is the cell line that was the most sensitive to berbamine. Berbamine also suppressed CAMKII downstream signaling pathways in these cancer cells. In Huh7 cells, activities of the CAMKII downstream target genes Bcl-2 and STAT3 were reduced by berbamine treatment. While in SK-Hep-1 and MHCC97H cells, activities of AKT, extracellular signal–regulated kinase (ERK), and STAT3 were suppressed to different extents. Of note, the ablation of β-actin expression implied that berbamine might directly affect cytoskeleton and thereby impact cell deaths. In addition, berbamine induced cleavage of LC3, caspase-3, and PARP, which implied that berbamine caused autophagy and apoptosis in all the 3 liver cancer cell lines.

A derivative of berbamine as well as a more potent CAMKII inhibitor (15), bbd24, has a low IC50 of 1.69 μg/mL for Huh7 cells and can induce cell detachment as soon as 6 hours after the treatment (Fig. 3C). This derivative quickly induced Huh7 cell deaths, such as apoptosis, at a very low concentration of 2 μg/mL (Fig. 3D). A significant reduction of phosphorylation of CAMKII and its downstream target ERK was observed with 1 μg/mL bbd24, a dose that also strongly reduced β-actin levels (Fig. 3E).

Berbamine and bbd24 inhibit the growth of the liver cancer–initiating cells
Recent research implies that cancer stem cells or cancer-initiating cells are responsible for cancer initiation,
chemoresistance, and reinitiation of cancer after therapy (19). Therefore, effects of berbamine on this population of liver cancer cells were evaluated. CD133 (AC133, promin-1), a membrane-associated glycoprotein first identified in mouse neuroepithelium, has been widely used as a marker to enrich liver cancer–initiating cells (20, 21). Therefore, this marker was chosen for sorting Huh7 stem cells. Consistent with previous studies, the CD133+ Huh7 cells expressed high levels of β-catenin and Bcl-2 (Supplementary Fig. S3A), the key genes for stem cell survival and self-renewal. However, the CD133+ Huh7 cells did not show increased survival capabilities under berbamine treatment than the CD133− population (Supplementary Fig. S3B). More interestingly, this population appeared to be equally, if not more, sensitive to berbamine treatment than the parental Huh7 cells did, as 4 to 8 μg/mL berbamine was sufficient to induce cell deaths of them (Fig. 4A; Supplementary Fig. S3C).

Figure 2. Berbamine (BBM) suppresses liver cancer cell growth and inhibited CAMKII phosphorylation. A and B, cell proliferation was assayed 72 hours after berbamine treatment for (A) liver cancer cells with epithelial morphology and (B) 2 mesenchymal-like cell lines and a normal embryonic liver cell line, CL48. C, Huh7 and SK-Hep-1 cells were used to generate xenografts. On the indicated days after starting berbamine treatment, the sizes of Huh7 xenografts were measured. D, on day 26, the mice were euthanized and the tumors were weighed. E, on the indicated days, the SK-Hep-1 xenograft tumors were measured. F, on day 30, the tumors were weighed. *, P < 0.05.
The regular chemotherapy drugs can enrich the CD133\(^{+}\) population, which is regarded as a reason of liver cancer recurrence after chemotherapy. In contrast, the berbamine derivative bbd24 decreased the percentage of the CD133\(^{+}\) cells in a dose-dependent manner (Fig. 4B). Because cancer stem cells are able to survive under anchorage-independent conditions (16, 21), the sphere formation assay is used to evaluate the characteristics and behaviors of stem cells, which excludes the potential bias and controversy caused by the application of certain stem cell markers, such as CD133. Berbamine was indeed also an agent that strongly inhibited hepatosphere formation of different liver cancer cell lines (Fig. 4C; Supplementary Table S2), with the doses (Huh7: 1.5 \(\mu\)g/mL; MHCC97H: 1.5 \(\mu\)g/mL; SNU398: 2.5 \(\mu\)g/mL) that were much lower than its IC\(_{50}\) on these cell lines (Huh7: 5.2 \(\mu\)g/mL; MHCC97H: 13.7 \(\mu\)g/mL; SNU398: 14.2 \(\mu\)g/mL). These results suggest that berbamine preferentially targets liver cancer stem cells. Furthermore, Western blot analysis showed that berbamine inhibited CAMKII signaling in the CD133\(^{+}\) Huh7 cells (Fig. 4D), indicating that berbamine also targets CAMKII in liver cancer stem cells.

Direct inhibition of CAMKII recapitulates berbamine effects on liver cancer cells and cancer-initiating cells

To investigate whether the effects of berbamine can be attributed to its effect on downregulation of CAMKII activity, we first examined whether direct inhibition of CAMKII by a chemical inhibitor KN93 could mimic the cytotoxicities of berbamine on liver cancer cell lines. Compared with its structural analog control KN92, KN93 exerted the survival inhibition to a series of liver cell lines in a dose-dependent manner (Fig. 5A), which further confirmed the critical role of CAMKII in liver cancer progression. In addition, the effects of the CAMKII inhibitor were tested on the liver cancer–initiating cells. KN93 significantly minimized the stem cell populations, the CD133\(^{+}\) Huh7 population and the CD90\(^{+}\) MHCC97H population, in their parental cells (Fig. 5B; Supplementary Figs. S4 and S5). Moreover, KN93, but not KN92, also exhibited strong capabilities to inhibit the hepatosphere formations of liver cancer cells at 5 \(\mu\)mol/L, a concentration that did not significantly inhibit the proliferation of these liver cancer cells (Fig. 5C; Supplementary Table S2).
Our previous studies showed that berbamine interacted with the gamma isoform of CAMKII (CAMKII$_g$), and another study showed that the most abundant CAMKII in liver was also the gamma subtype (22). However, there has been no report about the role of CAMKII$_g$ in hepato-
carcinogenesis. Four shRNAs targeting CAMKII$_g$ were
designed and verified for their knockdown efficiency
individually in Huh7 cells by transduction of lentiviral
vectors (Fig. 6A). Then, the shRNAs were pooled and used
to transduce HepG2, Huh7, and MHCC97H cells. The
knockdown of CAMKII$_g$ greatly inhibited the cell prolif-
eration in vitro (Fig. 6B) and led to slight morphologic
changes compared with the cells transduced with the
scramble control shRNA. The transduced Huh7 cells were
further selected by puromycin and used to generate stable
cells, which thereafter were inoculated into NOD/SCID
mice. Although no significant difference was observed in
the initial phases of xenograft growth, the knockdown of
CAMKII$_g$ in Huh7 cells showed a considerable reduction
of tumor volume and weight in the later stages (Fig. 6C;
Supplementary Fig. S6).

**Overexpression of CAMKII$_g$ promotes liver cancer regrowth and resistance to 5'-FU and berbamine**

To verify that CAMKII$_g$ is the major target of berba-
micine, we analyzed the function of CAMKII$_g$ in liver
cancer cells by overexpressing CAMKII$_g$ with retroviral
vectors. An MSCV retroviral vector increased CAMKII$_g$
expression by 3 to 10 times in HepG2 and Huh7 cells
(Fig. 6D). This overexpression slightly enhanced the can-
cer cell proliferation in vitro (Fig. 6B) and promoted
the chemoresistance to 5'-FU, a conventional chemother-
apy drug (Fig. 6F). To exclude the interference from
antibiotic/antiseptic selection during generation of stable
cell lines, especially on the phosphorylation of CAMKII$_g$,
2 tetracycline-inducible CAMKII$_g$ expression cell lines
were further generated with Huh7 and HepG2 cells to
evaluate whether CAMKII$_g$ expression diminished the
effects of berbamine (Fig. 6G). As expected, doxycycline-
induced CAMKII$_g$ expression successfully reduced the
suppression of liver cancer growth by berbamine, which
was more prominent in HepG2 cells probably due to the
more robust induction of CAMKII$_g$ expression in this cell
CAMKIIγ is hyperphosphorylated in human liver tumors

As CAMKII has not been characterized before in liver cancer, it is potentially an unidentified proto-oncogene for human liver cancer. Therefore, we examined whether CAMKII is hyperphosphorylated in liver tumors compared with nontumor adjacent tissues. The phosphorylation of CAMKII (pCAMKII) in liver tumors was upregulated in 8 of 14 pairs of liver cancer specimens (Fig. 7A; Supplementary Table S3). More interestingly, the upregulation of pCAMKII in tumors is more frequent in the patients with liver cancer with higher stages of hepatocarcinomas (Fig. 7B), classified by the TNM Classification of Malignant Tumours Staging System (the American Joint Committee on Cancer 2009). In average, the levels of pCAMKII in high-stage tumors (T = 3) were at least 2 times higher than that in low-stage tumors (T = 1 or 2; Fig. 7C). The phosphorylation of the potential CAMKII substrates and oncogenic proteins, ERK1/2, AKT1/2, and
Figure 6. Knockdown of CAMKII suppresses liver cancer cell growth, and overexpression of CAMKII antagonizes the effect of berbamine. A, shRNAs were expressed by a lentiviral vector, pLKO.1-puro. An MOI of 3 was applied for infection of Huh7 cells. Each of the 4 pairs of shRNAs targeting CAMKII and a scramble control was tested in Huh7 cells by Western blotting. B, cell proliferation assay with MTS was conducted 96 hours after infection of Huh7, SK-hep-1, and MHCC97H by the pool of the 4 lentiviral vectors. C, transduced Huh7 cells were selected by puromycin. The stable cells were used for the xenograft on NOD/SCID mice. The tumor sizes were measured on days 7, 15, and 28 after the s.c. injection. The tumors were weighed on day 28. * P < 0.05. D, overexpression of CAMKII by infecting HepG2 and Huh7 cells with a retroviral vector pMSCV. E, enhanced growth of the CAMKII overexpression HepG2 and Huh7 cells. F, reduced cytotoxicity of 5-FU by the CAMKII overexpression in HepG2 cells. G, tetracycline-derivative doxycycline (DOX) induction of CAMKII by infecting HepG2 and Huh7 cells with a retroviral vector pRetroX-Tight-puro. H, DOX-induced CAMKII expression antagonized the cytotoxicity of 72 hours of berbamine treatment. * P < 0.05. I, stable HepG2 and Huh7 cells with CAMKII knockdown were treated with berbamine at indicated concentrations. The cell survival was measured by MTS assay.
STAT3 (Y705), were mostly correlated with the pCAMKII levels between tumors and non-tumor tissues (Fig. 7A). In contrast, mTOR phosphorylation and β-catenin overexpression in these HCC specimens were not greatly changed in the tumors compared with the paired peritumor tissues.

**Discussion**

Natural compounds and their derivatives are emerging attractive new generation of anti-cancer drug candidates. Berbamine is a traditional natural product that has been used for centuries to treat inflammatory diseases. However, the anti-cancer activities of berbamine are just recently being elucidated. The most promising results are from the studies of leukemia, where the IC₅₀ of the natural form of berbamine after 72-hour treatment was from 4.80 to 7.50 µg/mL (14). More importantly, a clinical study has shown the efficacy of berbamine (12). In contrast, for other cancer cells such as melanoma and breast cancer cells, the IC₅₀ of berbamine is usually higher than 15.0 µg/mL (11, 13). In our studies, the lower IC₅₀ values of the natural form of berbamine were observed for liver cancer cell lines, and the berbamine derivative bbd24 has an IC₅₀ as low as 1.67 µg/mL. Moreover, the xenograft studies showed the berbamine reduced the liver tumor growth by 70% at a very tolerable dose for animals (15). Therefore, berbamine and its derivatives have great potentials to target liver cancer.

CAMKII is a target of berbamine in suppressing liver tumor growth. Furthermore, through targeting CAMKII, the inhibition of berbamine on cancer cells might have broader effects beyond affecting JAK/STAT3 and p210bcr-abl as previously reported (11, 14, 15) because CAMKII directly impacts many other signal pathways including STAT1, NF-κB, JNK, ERK1/2, FOXO1, and Wnt/β-catenin (22, 30–32). These genes, together with STAT3, form a signal network that can play critical roles in hepatocarcinogenesis, which is shown by many experimental animal studies. CAMKIIγ, as an upstream molecule that directly responds to extracellular stimuli and modulates many receptor/adaptor/kinase/transcription factor interactions, could be also involved in liver cancer initiation and progression. Particularly, ERK, AKT, and STAT3, which play central roles in hepatocarcinogenesis (33), seem to be the major downstream of CAMKII in human HCC specimens based on this study. However, this point needs to be verified with systemic statistical analyses of a bigger cohort of patients with HCC. Nevertheless, hyperphosphorylation of CAMKII is significantly correlated with human HCC stages, which indicates that CAMKII may enhance HCC progression by promoting cell cycling and preventing cell deaths. These results, together with our *in vivo* xenograft studies and *in vitro* cell culture experiments, implicate an oncogenic role of CAMKII in liver cancer.

More importantly, we also observed that liver cancer-initiating cells were more sensitive to berbamine than non-cancer-initiating cells based on the CD133 expression...
and the hepatosphere formation assays. Recurrence after surgical liver tumor removal is a long-lasting clinical issue. Chemoresistance of liver cancer cells is also an important reason for the high death rates of the patients. Recent studies have shown that liver cancer stem cells may be responsible for cancer recurrence and chemoresistance. Therefore, berbamine and its derivatives could be of great value to treat the patients with advanced progression. Application of berbamine alone or in combination with other drugs may provide new approaches for liver cancer therapies. In addition, a variety of CAMKII inhibitors have been developed recently (34, 35), which also possess potential clinical values.

In conclusion, we have shown that berbamine and its derivative bbd24 strongly suppress the growth of liver cancer cells by targeting CAMKII. Therefore, targeting CAMKII by natural products may hold great promise to develop new strategies for liver cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Z. Meng, R.-Z. Xu, W. Huang
Development of methodology: Z. Meng, T. Li, H. Zhou

References


Molecular Cancer Therapeutics

Berbamine Inhibits the Growth of Liver Cancer Cells and Cancer-Initiating Cells by Targeting Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II

Zhipeng Meng, Tao Li, Xiaoxiao Ma, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0314

Supplementary Material
Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2013/08/16/1535-7163.MCT-13-0314.DC1

Cited articles
This article cites 34 articles, 7 of which you can access for free at: http://mct.aacrjournals.org/content/12/10/2067.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.