Kamebakaurin inhibits the expression of hypoxia-inducible factor-1α and its target genes to confer antitumor activity

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Abstract. Hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that mediates the adaptation of tumor cells and tissues to the hypoxic microenvironment, has attracted considerable interest as a potential therapeutic target. Kamebakaurin is a diterpenoid compound isolated from Isodon excia (Maxim.) Hara, which has been used for anti-inflammatory activities. However, its antitumor activity along with molecular mechanism has not been reported. Kamebakaurin showed potent inhibitory activity against HIF-1 activation induced by hypoxia or CoCl2 in various human cancer cell lines. This compound significantly decreased the hypoxia-induced accumulation of HIF-1α protein, whereas it did not affect the expression of topoisomerase-I (Topo-I). Further analysis revealed that kamebakaurin inhibited HIF-1α protein synthesis, without affecting the expression level of HIF-1α mRNA or degradation of HIF-1α protein. Furthermore, kamebakaurin prevented hypoxia-induced expression of HIF-1 target genes for vascular endothelial growth factor (VEGF) and erythropoietin (EPO). However, kamebakaurin caused cell growth inhibition via cell cycle arrest at G1 phase in tumor cells. In vivo studies, we further confirmed the inhibitory effect of kamebakaurin on the expression of HIF-1α proteins, leading to growth inhibition of HCT116 cells in a xenograft tumor model. These results show that kamebakaurin is an effective inhibitor of HIF-1 and provide new perspectives into its anticancer activity.

Introduction

Hypoxia, a reduction in tissue oxygen levels below physiological levels, is a nearly universal hallmark of solid tumors, and commonly develops due to heterogeneous blood flow from structurally and functionally abnormal blood vessels within the tumor (1,2). Intratumoral hypoxia is significantly associated with aggressive tumor progression, resistance to chemotherapy and radiation, and poor prognosis (3,4).

Tumor cells and tissues adapt to hypoxic microenvironment via the activation of numerous hypoxia-related molecules, among which hypoxia-inducible factor 1 (HIF-1) is the predominant one (5). HIF-1 is a heterodimeric transcription factor composed of an O2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, which are basic helix-loop-helix-PAS domain proteins, only HIF-1α is regulated by the oxygen tension (6). In normoxic conditions, the hydroxylase of proline residue 402 and/or 564 by prolyl hydroxylase domain protein 2 (PHD2) promotes the interaction of HIF-1α with the von-Hippel-Lindau (VHL) tumor suppressor protein, which recruits an E3 ubiquitin-protein ligase and thus targets HIF-1α for degradation by the ubiquitin proteasome system (7). In response to physiological hypoxia, HIF-1α becomes rapidly stabilized and is localized to then nucleus, where it binds to HIF-1β to form the HIF-1 complex. HIF-1 specifically binds to a short DNA sequence, 5'-A/GCGTG-3', known as the hypoxia-responsive element (HRE) within target genes (8). In the last decade, significant evidence has accumulated that indicates that HIF-1α overexpression increases the probability of patient mortality (9). HIF-1 plays a key role in tumor progression and angiogenesis because activating the transcription of human VEGF genes allows the encoding of the vascular endothelial growth factor, a critical regulator for vascularization (10). Because of its importance in cancer, HIF-1α is viewed as a novel anticancer target for the development of new anticancer therapeutics (11).

Kamebakaurin, a compound of kaurane diterpenes was isolated from traditional Chinese medicinal plant Isodon excia (Maxim.) Hara. Previous studies have shown its anti-inflammatory actions targeting microglia-mediated neurodegenerative diseases and inhibited the production of...
nitric oxide (NO) and prostaglandin E2 (PGE2) through the inhibition of nuclear factor-xB (NF-xB) signaling in lipopolysaccharide (LPS)-treated RAW264.7 macrophages (12,13). In the present study, we found that kamebakaurin also inhibited hypoxia-induced HIF-1 activation. This compound rapidly downregulates not only HIF-1α but also its synthesis without affecting mRNA levels or protein degradation, but also the expression of HIF target genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which are essential for tumor growth. Based on these research findings, we further confirmed our in vitro observations by showing profound antitumor activity of kamebakaurin in a murine xenograft model with no apparent toxicity to the animals.

Materials and methods

Cell culture and methods. HeLa and KM12C cells were grown in DMEM with penicillin (100 U/ml)-streptomycin (100 U/ml) (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA). HCT116 and SNU638 cells were maintained in RPMI-1640 medium supplemented as above. The cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cobalt chloride (CoCl2), desferrioxamine (DFO), and the structure is shown in Fig. 1A. The purity of kamebakaurin was >98% in HPLC analysis.

Transfection and luciferase reporter assay. The ability of the compound to inhibit hypoxia inducible factor was determined by HRE-dependent reporter assay as previously described (15). In brief, at 50-80% confluence, HCT116 cells were co-transfected with the vectors for pGL3-HRE-Luciferase plasmid containing six copies of HREs derived from the human VEGF gene and with pRL-CMV (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen). Following 24 h of incubation, the cells were treated with various concentrations of kamebakaurin and incubated for 16 h in hypoxia. Luciferase assay was performed using Dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega). Luciferase activity was determined in Microlumat plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 µl of assay buffer containing luciferin and measuring light emission for 10 sec. The results were normalized to the activity of renilla expressed by cotransfected Rluc plasmid to all plates to compensate the same volume of DMSO. After 24 h, the cells were pretreated with different concentrations of kamebakaurin for 24 h. Subsequently, cells were cultured with MTT solution (5 mg/ml) [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) for 4 h. The viable cells converted MTT to formazan, which generated a blue-purple color after dissolving in 100 µl of DMSO. The absorbance at 570 nm was measured by microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. Whole-cell extracts were obtained by lysing cells in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride) supplemented with the protease inhibitor cocktail (Sigma-Aldrich). HIF-1α protein was analyzed in nuclear extracts prepared from cells using NE-PER reagent (Pierce, Rockford, IL, USA), according to the instructions of manufacturer. An aliquot of protein extracts were used to determine protein concentration by the Bradford method. Fifty microgram of whole-cell extracts or 30 µg of nuclear extract protein per lane was separated by SDS-polyacrylamide gels and followed by transferring to a polyvinylidenefluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk, and then incubated with the corresponding antibody. Antibody for HIF-1α was obtained from BD Biosciences. The primary antibodies for VEGF, Topo-I, GLUT1, c-Myc and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody for cyclin D1 was purchased from Cell Signaling Technology (Beverly, MA, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

VEGF ELISA. HCT116 cells were plated in a 96-well plate at a density of 1x10^5 cells per well and treated with various concentrations of kamebakaurin for 12 h under hypoxia conditions. The VEGF levels in the culture supernatant and the serum were determined by ELISA using the Duo-Set ELISA development kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

RT-PCR analysis. Total RNA from HCT116 cells was obtained using RNA Mini kit (Qiagen, Valencia, CA, USA). Total RNA (2 µg) was used to perform reverse transcription-PCR (RT-PCR) using RT-PCR kit (Invitrogen) according to the manufacturer's protocol. The PCR primers for VEGF were 5'-GCTCTACCTCCACCATGCCAA-3' (sense) and 5'-TGGA AGATGTTCCACCGGTCTC-3' (antisense); for EPO were 5'-CAGTTTTCGCAATCCTTGG-3' (sense) and 5'-GTCA CGAGCATTGCCACCTAAG-3' (antisense); for HIF-1α were 5'-CTCAAAGTCCGACAGCCTCA-3' (sense) and 5'-CCCT GCAGTAGGTTTCTGCT-3' (antisense); for GAPDH were 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCA CCACCATGTTGCTGTA-3' (antisense). The oligonucleotide sequences of the reaction products were confirmed by sequencing.
Measurement of cell cycle. Distribution of cells in different stages of cell cycle was analyzed by BD Accuri™ C6 flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). The kit utilizes propidium iodide (PI) staining to allow quantitative measurements of percentage of cells in the G0/G1, S and G2/M phases on the Cell Quest software (Becton-Dickinson). For all assays, 10,000 events were counted. The ModFit LT V4.0 software package (Verity Software, Topsham, ME, USA) was used to analyze the data. The cell cycle analysis was performed according to the manufacturer’s protocol, and as previously described (16). Briefly, HCT116 cells (5x10^5 cells/ml) were treated with different concentrations of kamebakaurin for 12 h and harvested from culture dishes. After washing with PBS, HCT116 cells were fixed with ice-cold 70% ethanol at -4°C for 12 h. The cells were then washed with PBS containing 0.1% Triton X-100, stained with PI/RNase reagent for 30 min and analyzed by BD Accuri™ C6 flow cytometry. The ModFit LT V4.0 software package (Verity Software) was used to analyze the data.

Tumor xenograft assay. All surgical procedures and care applied to the animals were in accordance with IACUC guidelines. Six weeks old specific-pathogen-free Crj:BALB/c nu/nu female athymic nude mice (Vital River, China) were randomly assigned to three groups, each of which consists of five mice (n=5 per group), and then were subcutaneously inoculated with 0.2 ml of HCT116 cells (5x10^7 cells/ml) in the left flank region. Kamebakaurin, dissolved in DMSO, was administered orally every other day for 40 days at a dose of 15 and 50 mg/kg body weight starting from day 10 post cell implantation to mice. Tumor weight was calculated every five days using the equation: [length x (width)^2]/2. Tumors were harvested 4 h after the last treatment, followed by homogenising in RIPA for western blot analysis.

Statistical analysis. All values are expressed as mean ± SD. A comparison of the results was performed with one-way ANOVA and Tukey’s multiple comparison tests (Graphpad Software, Inc, San Diego, CA, USA). Statistically significant differences between groups were defined as p-values <0.05.

Results

Kamebakaurin inhibits HIF-1α protein expression in tumor cells. To investigate whether kamebakaurin (Fig. 1A) inhibited HIF-1α transcriptional activation, we transfected HCT116 cells with a luciferase reporter gene driven by six specific HRE. A substantial increase of luciferase activity was observed in cells cultured in hypoxic conditions, whereas kamebakaurin dose-dependently inhibited hypoxia-induced luciferase activity (Fig. 1B). Given that the inhibition of HIF-1α transcriptional activation might be correlated with kamebakaurin-induced cytotoxicity, parallel studies of cell viability were performed (Fig. 1C). After the HCT116 cells were treated with different concentrations of kamebakaurin for 24 h, no significant alteration of cell viability was observed relative to the untreated control group.

Kamebakaurin decreases HIF-1α protein levels in a dose-dependent manner. To explore the underlying mechanism of kamebakaurin activity, we investigated its effect on HIF-1α protein levels. In HCT116 cells, HIF-1α protein is undetectable under normoxia, whereas it is stabilized under hypoxia or in the presence of CoCl₂, and becomes readily detectable by western blotting. Following 24 h of incubation, the cells were incubated under hypoxia in the absence or presence indicated concentrations of KMBK. Luciferase activities were determined as described in Materials and methods. Data are represented as mean ± standard deviation of three independent experiments. **p<0.01, ***p<0.001, significant with respect to control. HCT116 cells were treated with the indicated concentrations of KMBK. (C) After 24 h of incubation, cell viability was determined by MTT assays.
of HIF-1α by well-characterized hypoxia mimetic reagents, including CoCl₂ and DFO, could also be abrogated by kamebakaurin (Fig. 2B).

We next performed an immunofluorescence assay to evaluate the effect of kamebakaurin on HIF-1α expression in HCT116 cells. Following 12 h of treatment, kamebakaurin (30 µM) exerted almost complete inhibition of HIF-1α protein levels in cell nuclei induced by hypoxia in HCT116 cells (Fig. 2C). To further confirm the effects of kamebakaurin, we conducted both time-course experiments and dose-response experiments to determine the expression of HIF-1α protein in the presence of kamebakaurin. The addition of kamebakaurin to the culture medium remarkably inhibited HIF-1α accumulation, and this inhibition persisted as long as the drug was present, at least up to 12 h (Fig. 2D). Dose-response experiments indicated that kamebakaurin dose-dependently inhibited hypoxia-induced accumulation of HIF-1α in HCT116 cells, with complete abrogation at 30 µM (Fig. 2E).

Kamebakaurin inhibits the protein synthesis of HIF-1α but not its degradation. Generally, the accumulation of HIF-1α is dependent on the balance between its protein synthesis and degradation. To further address the mechanism by which kamebakaurin inhibits HIF-1α protein level, we examined whether kamebakaurin modulates HIF-1α protein synthesis in the presence of a proteasome inhibitor MG-132 to prevent HIF-1α degradation. As expected, addition of proteasome inhibitor caused the increased accumulation of HIF-1α protein levels in the presence of CoCl₂ (Fig. 3A). Kamebakaurin inhibited the accumulation of HIF-1α protein induced by CoCl₂.
despite of the presence of MG-132. No significant effects were observed on Topo-I levels.

To address the effect of kamebakaurin on HIF-1α protein stability, the protein translation inhibitor cycloheximide (CHX) was used to prevent de novo HIF-1α protein synthesis and then the cells were exposed to normoxia for increasing periods up to 60 min. The nuclear extracts were prepared to detect HIF-1α protein by western blotting. Under these conditions, HIF-1α protein levels mainly reflected the rate of HIF-1α degradation. As shown in Fig. 3B, the degradation of HIF-1α was similar in the kamebakaurin-treated and the control cells. Therefore, it is confirmed in our experiments that kamebakaurin does not promote the degradation of HIF-1α.

To determine whether HIF-1α synthesis inhibition by kamebakaurin was a downstream effect from decreased HIF-1α gene transcription or HIF-1α mRNA stability, we analyzed HIF-1α mRNA levels by RT-PCR. Kamebakaurin did not reduce HIF-1α mRNA levels significantly (Fig. 4A). This suggests that kamebakaurin-mediated decrease of HIF-1α synthesis is likely due to downregulation of HIF-1α mRNA translation.

**Kamebakaurin suppresses expression of HIF-1α target genes.** The expression of VEGF and EPO, which are involved in tumor cells proliferation, angiogenesis, invasion and metastasis, is known to be regulated by HIF-1α (5,11). We therefore examined whether kamebakaurin decreases the expression of these genes. VEGF and EPO mRNA levels were measured by RT-PCR analysis in HCT116 cells. Treatment of the cells with kamebakaurin resulted in a dose-dependent inhibition of VEGF and EPO mRNA expression (Fig. 4A). Then, we also
examined the hypoxia induction of VEGF or GLUT1 (glucose transporter 1) protein expression. Consistently, they were dose-dependently inhibited by kamebakaurin (Fig. 4B). The concentrations to inhibit the expression of HIF-1α target genes were comparable with those of HIF-1α protein accumulation. This result led us to measure the VEGF protein concentration in the culture supernatant by ELISA. Consistently, the hypoxic induction of secreted VEGF protein was dose-dependently inhibited by kamebakaurin (Fig. 4C).

Kamebakaurin inhibits the proliferation of HCT116 cells via blocking cell cycle progression in the G1 phase and downregulates cyclin D1 and c-Myc. To evaluate the effect of kamebakaurin on cell proliferation, we tested whether the antiproliferative effect of kamebakaurin is associated with cell cycle arrest by measuring the DNA content of nuclei of HCT116 cells in flow cytometric analysis. As shown in Fig. 5B, treatment with 30 µM kamebakaurin markedly induced G1/S phase cell cycle arrest. FACS analysis revealed that 24 h exposure to kamebakaurin increased the population of G1/S phase cells in a dose-dependent manner. Cells at the G1/S phase increased from 69.74% in medium alone to 76.14, 80.00 and 82.49% in the presence of 3, 10 and 30 µM kamebakaurin, respectively (Fig. 5A). While kamebakaurin treatment retarded the progression of G1 to S/G2 phase.

Next, we determined the specific cell cycle regulators responsible for the cell cycle arrest induced by kamebakaurin by western blot analysis using antibodies specific to cyclin D1 and c-Myc. The result showed that cyclin D1 and c-Myc were decreased by kamebakaurin dose-dependently (Fig. 5B). Taken together, these results clearly suggest that antiproliferative effect of kamebakaurin is associated with its induction of cell cycle arrest at G1 phase, and further confirmed that kamebakaurin downregulates cyclin D1 and c-Myc protein levels on the cell cycle.

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Kamebakaurin inhibits the growth of HCT116 cells in a xenograft tumor model. To further reveal the effect of kamebakaurin on the expression of HIF-1α and VEGF in vivo, we next determined whether these results could be translated into an in vivo xenograft model. HCT116 cells were subcutaneously implanted in athymic nude mice, and the experimental mice were treated with kamebakaurin (15 and 50 mg/kg) every other day until the end of the study. As expected, kamebakaurin (50 mg/kg) produced significant growth inhibition of HCT116 cells in a tumor xenograft model, compared to that of the vehicle-treated control group (Fig. 6A). Due to the key roles of HIF-1α in tumor angiogenesis, we studied its expression in the tumors by western blotting. Consistent with the finding in cultured cells, kamebakaurin significantly decreased the
protein levels of HIF-1α in the tumors, whereas no significant difference was observed in Topo-I levels (Fig. 6B).

VEGF has key roles in tumor angiogenesis, therefore, we measured the concentration of secreted VEGF in the serum of xenograft mouse. Consistent with the findings in cultured cells, kamebakaurin significantly decreased the serum VEGF levels dose-dependently (Fig. 6C). Taken together, our study showed that the downregulation of HIF-1α by kamebakaurin could contribute to the inhibition of tumor growth and VEGF secretion in a tumor xenograft model with HCT116 cells.

Discussion

Regions of hypoxia in tumors are associated with a poor prognosis including treatment failure, metastasis, and inferior survival (17). Therefore, proteins that allow tumors to adapt to hypoxic conditions such as HIF-1 represent critical targets for cancer treatments. HIF-1 plays a central role in tumor progression and angiogenesis in vivo. Exposure to a variety of growth factors has also been shown to increase HIF-1 activity in normoxic and hypoxic conditions. HIF-1α is overexpressed in many human cancers and has been associated with tumor angiogenesis and VEGF in one of the most potent angiogenic factors currently known (18). After VEGF binds to its receptors, it functions not only as a proliferating factor, but also as an anti-apoptotic for vascular endothelial cells (19). In addition, tumor growth and angiogenesis in xenograft tumors also depends on HIF-1 activity and the expression level of HIF-1α (20). In this study, we identified kamebakaurin as an inhibitor of HIF-1α activation and VEGF production.

The level of HIF-1α in cells is dependent on the balance between its protein degradation and protein synthesis. HIF-1α is oxygen-sensitive and is degraded mainly by ubiquitin-proteasome systems (4). We found that kamebakaurin strongly inhibited HIF-1α protein accumulation, without affecting the expression level of HIF-1α mRNA or degradation of HIF-1α protein. These observations may support the hypothesis that the kamebakaurin-dependent reduction of HIF-1α accumulation is due to the decrease of de novo HIF-1α protein synthesis.

Kamebakaurin was also found to inhibit cell proliferation through cell cycle arrest at G1 phase. This inhibition was correlated with a reduction in the expression of cell proliferation proteins such as cyclin D1 and c-Myc. Cyclin D1 is overexpressed in several cancers and is a biomarker of cancer phenotype and disease progression, indicating that targeting of cyclin D1 oncogene appears to be an attractive therapeutic strategy (21). Myc is documented to play a role in tumor initiation and regulation of cell growth and proliferation. Inhibiting Myc function has been shown to be a possible therapeutic strategy (22). c-Myc is one of the myc family of transcription factors which activates the expression of a myriad of genes by binding to consensus sequences and recruiting histone
acetyltransferases. A potent proto-oncogene, c-Myc, is often found to be upregulated in many types of cancers. c-Myc overexpression stimulates gene amplification (23), presumably through DNA overreplication, which can have a profound effect on the control of cell growth. In this regard, our study demonstrates that kamebakaurin downregulates cyclin D1 and c-Myc, leading to cell growth inhibition through G1-phase arrest.

In conclusion, we have shown that kamebakaurin decreased HIF-1α protein levels and inhibited hypoxia-induced VEGF and EPO expression. In addition, our study provides evidence that kamebakaurin can inhibit the proliferation of cancer cells through the cell cycle arrest at G1 phase. These results may provide a rationale for the development of kamebakaurin as an anticancer drug.

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