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Glaucarubinone inhibits colorectal cancer growth by suppression of hypoxia-inducible factor 1α and β-catenin *via* a p-21 activated kinase 1-dependent pathway

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ABSTRACT

p-21-Activated kinase 1 (PAK1) enhances colorectal cancer (CRC) progression by stimulating Wnt/β-catenin, ERK and AKT pathways. PAK1 also promotes CRC survival *via* up-regulation of hypoxia-inducible factor 1α (HIF-1α), a key player in cancer survival. Glaucarubinone, a quassinoid natural product, inhibits pancreatic cancer growth by down-regulation of PAK1. The aim of this study was to investigate the effect of glaucarubinone on CRC growth and metastasis, and the mechanism involved. Cell proliferation was measured *in vitro* by [^{3}H]-thymidine incorporation and *in vivo* by volume of tumor xenografts. Protein concentrations were measured by Western blotting of cell extracts. We report here that glaucarubinone inhibited CRC growth both *in vitro* and *in vivo*. The potency of glaucarubinone as an inhibitor of cell proliferation was negatively correlated to PAK1 expression in CRC cells. Glaucarubinone suppressed the expression of HIF-1α and β-catenin. Knockdown of PAK1 by shRNA enhanced inhibition by glaucarubinone while constitutively active PAK1 blocked the inhibitory effect. Our findings indicate that glaucarubinone inhibited CRC growth by down-regulation of HIF-1α and β-catenin *via* a PAK1-dependent pathway.

1. Introduction

Colorectal cancer (CRC) develops through multiple steps involving alterations in cell proliferation, differentiation, survival and cell death. Mutations in the components of the Wnt/β-catenin signal pathway such as the adenomatous polyposis coli (Apc) tumor suppressor gene and the β-catenin (CTNNB1) oncogene occur in over 90% of CRCs, trigger adenomatous polyp formation, and initiate colorectal neoplasia [1]. Loss-of-function mutations of Apc occur in more than 60% of colonic adenomas and carcinomas [2], and cause cytoplasmic accumulation and nuclear translocation of β-catenin leading to constitutive activation of Wnt signaling.

The hypoxia associated with tumor expansion initiates a number of oncogenic responses. Hypoxia suppresses proliferation, initiates angiogenesis and cell migration, and drives the progression to malignant tumor phenotypes [3]. Hypoxia-inducible factor-1α (HIF-1α) plays a central role in the hypoxia-driven changes in tumor phenotype and its expression is often associated with poor prognosis [4–6]. While the aberrant activation of β-catenin promotes cell proliferation and initiates colorectal tumorigenesis through β-catenin/TCF4 signaling, the expansion of tumors and the inadequacy of their local vasculature result in areas of hypoxia, which in turn induce the expression of HIF-1α and stabilize the protein. Under hypoxia, HIF-1α competes with TCF4 for binding to β-catenin, and β-catenin switches from binding with TCF4 and promoting cell proliferation to binding with HIF-1α, thereby enhancing cell survival and adaptation to hypoxia [7]. However, the expression of HIF-1α is not only induced under hypoxic condition. In cancer cells, growth factors, oncogenes, or loss-of-function mutations of tumor suppressor genes can induce HIF-1α expression under normoxic conditions [8,9]. HIF-1α stimulates normoxic growth of CRC cells and prevents premature senescence and aging of cells, particularly following γ-irradiation [10,11].

The p-21-activated kinase family of 6 serine/threonine kinases is characterized by an N-terminal regulatory domain and a highly conserved C-terminal kinase domain [12]. PAKs are upregulated and/or hyperactivated in many human cancers including CRC [13]. The expression of PAK1 increases with progression through the adenoma to carcinoma sequence, with the most dramatic increases in invasive and metastatic CRC [14]. PAK1 promotes CRC growth by activation of ERK and AKT pathways [15]. PAK1 also associates with and phosphorylates β-catenin in CRC cells, and PAK1 knockdown inhibits β-catenin expression and suppresses the activation of the β-catenin signal pathway [16,

Abbreviations: PAK1, p-21 activated kinase 1; CRC, colorectal cancer; Glau, glaucarubinone; HIF-1α, hypoxia-inducible factor 1α; Apc, adenomatous polyposis coli

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17]. In addition PAK1 enhances CRC survival by up-regulation of HIF-1α both *in vitro* and *in vivo* [18]. These lines of evidence support the idea that PAK1 acts as a point of convergence where multiple signals intersect, and plays a key role in CRC progression.

The natural products, quassinoids, have been isolated from various species of the Simaroubaceae plant family, and have a range of biological activities including antitumor properties [19]. Quassinoids suppress tumor growth by inhibition of protein synthesis and the AP-1 transcription factor, induction of apoptosis, and down-regulation of the myc oncogene [20–24]. The quassinoid 6α-tigloyloxychapparrinone inhibits HIF-1α expression by reducing phosphorylation of eukaryotic translation initiation factor 4E [25]. Recently we have reported that glaucarubinone, a member of the quassinoid family suppresses pancreatic cancer growth, synergistically with gemcitabine, by down-regulation of PAK1 and PAK4 [26]. These observations suggest that glaucarubinone may exert its antitumor effect through modulation of PAK-dependent pathways. In the current study, we have further investigated the effect of glaucarubinone on CRC growth and metastasis/invasion and the possible mechanisms involved. We demonstrate here that glaucarubinone inhibits CRC growth *in vitro* and *in vivo* by suppression of HIF-1α and β-catenin *via* a PAK1-dependent pathway.

2. Material and methods

2.1. Cells and transfection

The human colorectal cancer (CRC) cell lines DLD1, HCT116, HT29, SW480 and SW1222 were obtained from the ATCC and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). None of these CRC cell lines have mutations in either PAK1 or PAK2; DLD1 only has a missense mutation in PAK4 [27]. The PAK1 knockdown (KD) clones of HCT116 cells were generated by transfection with plasmid DNAs encoding either shRNA sequences (SABioscience, Frederick, MD) to silence the PAK1 gene specifically or with a scrambled sequence as a negative control using Lipofectin Reagent (Invitrogen, Melbourne, Australia) as described previously [15]. The constitutively active PAK1 constructs (generously provided by Dr. Gary Bokoch, The Scripps Research Institute, La Jolla, CA) were sub-cloned into the pCDNA3.1 vector (Invitrogen, Melbourne, Australia). CA PAK1 plasmid DNA was transfected into SW480 cells using Lipofectin Reagent (Invitrogen) as described previously [18].

2.2. Cell proliferation assay

Cell proliferation was measured using ^{3}H-thymidine incorporation. Cells were seeded in a 96-well plate at 5×10^3 cells/well in DMEM containing 5% FBS, with 1 μCi/well [methyl-^{3}H]-thymidine (Perkin Elmer, Boston, MA) in the presence or absence of glaucarubinone or ailanthinone (prepared as prescribed previously [21], and obtained from the National Cancer Institute) at the indicated concentrations. After 24 h, cells were harvested using a NUNC cell harvester (Nunc, Roskilde, Denmark). The amount of ^{3}H-thymidine incorporated through DNA synthesis was detected with a β-counter (Packard, Meriden, CT).

2.3. Cell migration assay

Cell migration was determined using the Boyden Chamber assay described previously [15]. Membranes (8 μm pore size, Becton Dickinson, NJ) were coated with 3 μg human fibronectin on the lower surfaces and placed into a 24-well plate containing 600 μl/well of serum-free DMEM with 0.1% BSA. Cells ($2–5 \times 10^4$/100 μl/chamber) were added to the upper chambers and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere plus or minus glaucarubinone or ailanthinone. The cells on the upper surface of the membranes were removed by wiping with a cotton swab before the membranes were fixed and stained with Quick-Dip (Fronine, Sydney, Australia). The cells that had migrated to the lower surface of the membranes were counted from 24 fields at 40 times magnification using a NIKON Coolscope (Coherent Scientific, Adelaide, Australia).

2.4. PAK kinase assays

The PAK kinase assay was performed on immunoprecipitates as described previously [28]. CRC cells were treated with glaucarubinone (100 or 500 nM) for 24 h and lysed. The cell lysates were incubated with anti-PAK antibody and protein A beads for 4 h at 4 °C. PAK kinase activity in the immunoprecipitates was assayed using myelin basic protein (MBP, 5 μg/ml) as substrate, and the phosphorylated MBP was visualized in a BAS-3000 phospho-imager (Berthold, Bundoora, Australia). The ratio of phosphorylated MBP to total MBP was calculated and taken to represent the kinase activity. The amount of PAK protein was determined by Western blots.

To generate a GST-PAK1 fusion protein, the wild type (WT) PAK1 construct (generously provided by Dr. Gary Bokoch, The Scripps Research Institute, La Jolla, CA) was sub-cloned into the pGEX-2TH vector (Invitrogen, Melbourne, Australia). The GST-PAK1 fusion protein was purified from bacterial cultures as described previously [29]. 0.1 μg of GST-PAK1 fusion protein was incubated with 5 μg MBP in the presence of different concentrations of glaucarubinone or the PAK1 inhibitor Frax-597 (10 nM SYNthesis Pty. Ltd., Melbourne, Australia) in kinase buffer (40 mM HEPES pH 7.4, 10 mM $MgCl_2$, 20 μM ATP) containing [^{32}P]-ATP. The reaction mixture was subjected to 12% SDS-PAGE, and the phosphorylated MBP was visualized in a BAS-3000 phospho-imager. The ratio of phosphorylated MBP to total MBP was calculated and taken to represent the kinase activity.

2.5. Mouse xenograft study

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Austin Health Animal Ethics Committee. The mouse experiments were conducted at the Austin Bioresource Centre, Austin Health (Melbourne, Australia) with ethics approval (permit number: A2010/04016) from the Austin Health Animal Ethics Committee. SCID mice were purchased from the Animal Resource Centre (Perth, Australia). HCT116 and DLD1 cells (2.5×10^6 cells/100 μl/site) were injected subcutaneously into the flanks of 6 week old SCID mice. When the tumor size reached 50 mm^3 (day 4 after intra-peritoneal injection for HCT116 cells, day 9 for DLD1 cells), glaucarubinone was given by intra-peritoneal injection, every day from Monday to Friday for 3 weeks. There were six mice in both control (5% DMSO in saline) and glaucarubinone-treated (1 mg/kg, dissolved in 5% DMSO in saline) groups. Tumor dimensions were measured with a caliper, and tumor volume calculated as 1/2 l (length) × w^2 (width). At the end of the experiment, mice were sacrificed. Tumors were excised, weighed and frozen for protein extraction and Western blot.

2.6. Western blots

Cells were lysed in SDS sample buffer. The proteins in cell lysates were resolved by SDS-polyacrylamide gel (SDS-PAGE), and detected with antibodies against HIF-1α (BD Biosciences, San Jose, California), PAK1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PAK1 (Ser144), PAK2, PAK4, β-catenin, ERK, pERK, AKT, pAKT and GAPDH (Cell Signaling). Bound antibodies were visualized using ECL reagents (GE Healthcare, Buckinghamshire, UK), and the density of each band was analyzed using Multi-gauge computer software (Berthold, Bundoora, Australia).

50 mg samples of frozen xenograft tumors were homogenized using an ultra Turrax T25 homogenizer (Janke and Kunkel, Staufen, Germany) in 500 μl tissue lysis buffer (150 mM NaCl, pH 7.5, 50 mM HEPES, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, protease inhibitor cocktail (Roche)) as described before [18]. The lysates were then centrifuged at 13,000 rpm for 10 min at 4 °C, and supernatants were collected. Proteins

were separated by SDS-PAGE. Protein expression was determined with the antibodies indicated in the text.

2.7. Quantitative real time PCR

To measure PAK1 mRNA, cells were seeded at 4×10^5/well in 6-well plates, and total RNA was extracted with TRIzol (Invitrogen) and converted to cDNA using the Superscript™ III first strand synthesis system (Invitrogen). The resulting cDNA transcripts were used for real time PCR amplification with an ABI 7700 Sequence Detector (Applied Biosystems, Melbourne, Australia) and Taqman chemistry according to the manufacturer's instructions. Results were normalized to 18S RNA expression.

2.8. Statistical analysis

All values are expressed as means ± standard error. Results were analyzed by one-way analysis of variance or *t*-test as appropriate with the program Sigma Stat (Systat Software Inc., San Jose, CA). The program Sigmaplot 12 was used for regression analysis (Systat Software Inc.). Differences between two means with $p < 0.05$ were considered significant.

3. Results

3.1. Glaucarubinone suppressed CRC cell proliferation and migration

The effects of glaucarubinone and ailanthinone, a structural analogue of glaucarubinone [30], on CRC cell proliferation and migration were determined by ^{3}H-thymidine incorporation and Boyden chamber assay, respectively. Both glaucarubinone and ailanthinone decreased CRC cell proliferation in a dose dependent manner (Fig. 1A & B). The maximal inhibition by glaucarubinone on proliferation was achieved at 500 nM for DLD1, HCT116 and SW480 cells, 1000 nM for SW1222 and >1000 nM for HT29 cells (Fig. 1A). The maximal inhibition by ailanthinone on proliferation was achieved at 200 nM for DLD1, SW480 and HCT116, 300 nM for HT29 cells, and >500 nM for SW1222 cells (Fig. 1B). Similarly both glaucarubinone and ailanthinone suppressed the migration of DLD1 and HCT116 cells (Fig. 1C & D). The maximal inhibition of migration by glaucarubinone and ailanthinone was at 100 nM. The IC_{50} values for inhibition of proliferation and migration by glaucarubinone and ailanthinone are listed in Supplementary Table 1. These results indicated that CRC cell growth and migration/invasion were inhibited by glaucarubinone and ailanthinone in the nM concentration range.

3.2. Inhibition of CRC cell growth by glaucarubinone correlated with PAK1 expression

To determine if there was any connection between the inhibition of CRC cell proliferation by glaucarubinone and PAK expression, the levels of PAK1 and PAK2 in a panel of CRC cells were measured by Western blot. Among five CRC cell lines tested, similar levels of PAK1 were detected in DLD1, HCT116 and SW480 cells while significantly higher levels of PAK1 were expressed in SW1222 and HT29 cells (Fig. 2B). The IC_{50} values for the inhibition of proliferation by glaucarubinone were similar among DLD1, HCT116 and SW480 (all around 100 nM), while the IC_{50} values for glaucarubinone inhibition on SW1222 and HT29 cells were significantly higher, at 376 nM and 595 nM respectively (Fig. 2A). Regression analysis showed that the IC_{50} for the inhibition by glaucarubinone of CRC cell proliferation was significantly correlated

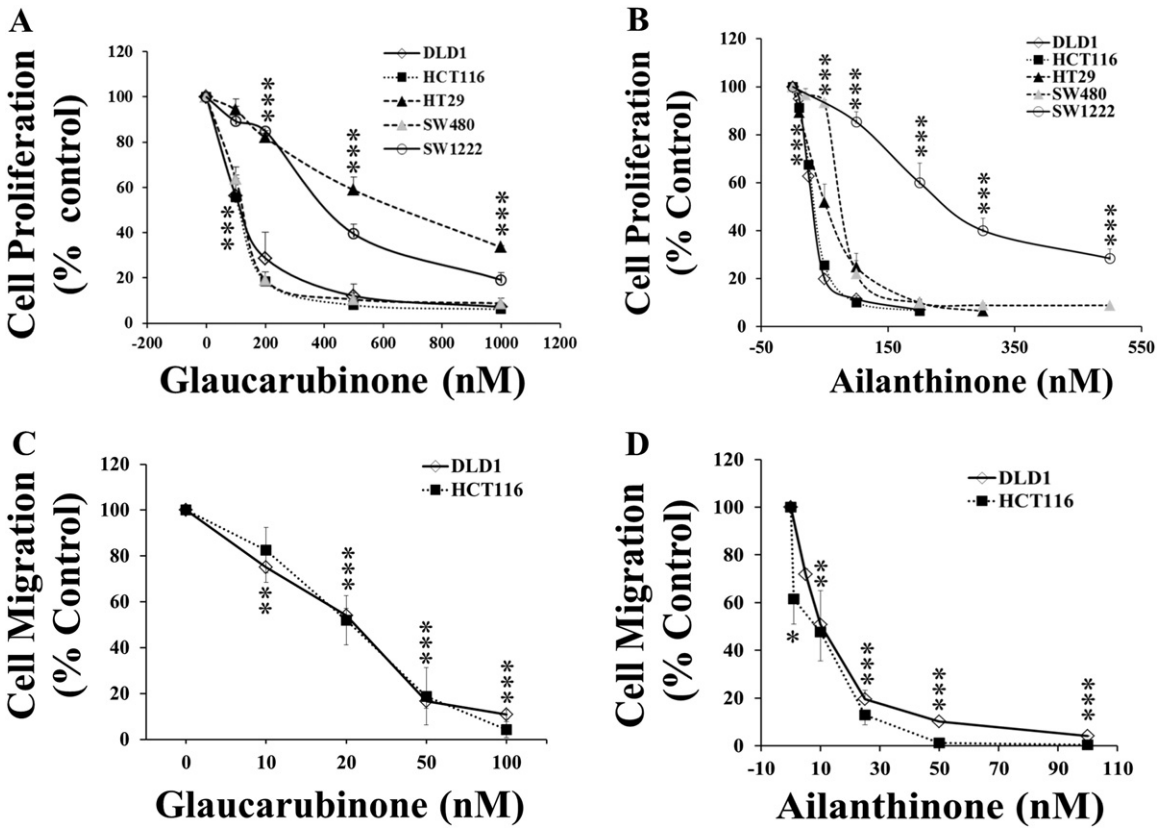

Fig. 1. Glaucarubinone and ailanthinone inhibited CRC cell proliferation and migration. CRC cells were incubated with glaucarubinone (A, C) or ailanthinone (B, D) for 24 h. Cell proliferation (A, B) and migration (C, D) were measured by ^{3}H-thymidine incorporation (A, B) and Boyden chamber assay (C, D) as described in Material and methods. The values from control cells without any treatment were taken as 100%. The data were summarized from three independent experiments and presented as mean ± standard error. Controls were not treated with either glaucarubinone or ailanthinone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the values of non-treated controls.

with the expression levels of PAK1 ($p = 0.0342$, Fig. 2C), but not PAK2 ($p = 0.1507$, Fig. 2D). Furthermore the inhibitory effect of glaucarubinone on proliferation was significantly enhanced in PAK1 knockdown (KD) HCT116 cells with 50% lower IC_{50} values observed in two PAK1 KD clones (Fig. 2E). On the other hand, the inhibitory effect of glaucarubinone on proliferation was significantly decreased in SW480 cells that had been transfected with a plasmid encoding a constitutively active (CA) PAK1 (Fig. 2F). The amounts of PAK1 in KD and CA cell lines are shown at the top of Fig. 2E and F, respectively. The expression and activity of PAK1 in KD or CA cell lines were also presented in our previous studies [16,18]. These results showed that inhibition by glaucarubinone of cell growth correlated with PAK1 expression levels in CRC cells.

3.3. Glaucarubinone inhibited PAK1 activity by decreasing its protein expression

To determine the effect of glaucarubinone on PAK1 activity, 4 CRC cell lines were treated with glaucarubinone at 100 and 500 nM for 24 h. The cells were then lysed and the cell lysates were subjected to an *in vitro* kinase assay using myelin basic protein (MBP) as the substrate. Glaucarubinone reduced the kinase activity of PAK1 in DLD1, HCT116 and SW480 cells, but not HT29 cells which had significantly higher levels of PAK1 (Fig. 3A). Similarly, glaucarubinone decreased the levels of the phosphorylated, active form of PAK1 in DLD1, HCT116 and SW480 cells, but not HT29 cells (Fig. 3B), and the levels of total PAK1 in all CRC lines tested (Fig. 3C). The reduction by glaucarubinone of PAK1 protein expression was not caused by changes in proteasomal degradation of PAK1 protein as glaucarubinone decreased PAK1 protein to similar levels in the presence of a proteasomal inhibitor, MG132 (10 μM, Sigma) (Fig. 3C). Furthermore the observation that glaucarubinone did not reduce the concentration of PAK1 mRNA (Fig. 3D), suggested that glaucarubinone reduced PAK1 protein expression independently of any changes in PAK1 transcription. These observations suggested that the reduction by glaucarubinone of PAK1 kinase activity was due to inhibition of PAK1 protein expression, and that the inhibition of PAK1 protein by glaucarubinone was neither *via* suppression of PAK1 mRNA, nor through promotion of protein degradation.

To determine whether or not glaucarubinone directly inhibited PAK1, the kinase activity of a bacterially expressed GST-PAK1 fusion protein was measured in the presence of increasing concentrations of glaucarubinone (Fig. 3E). PAK1 kinase activity was not affected by glaucarubinone at 500 nM and only decreased to 80% at 1 μM. Glaucarubinone did not affect the kinase activities of either PAK2, or PAK4, or their protein concentrations (Supplementary Fig. 1). These data confirmed that glaucarubinone inhibited PAK1 kinase activity by down-regulation of its protein expression.

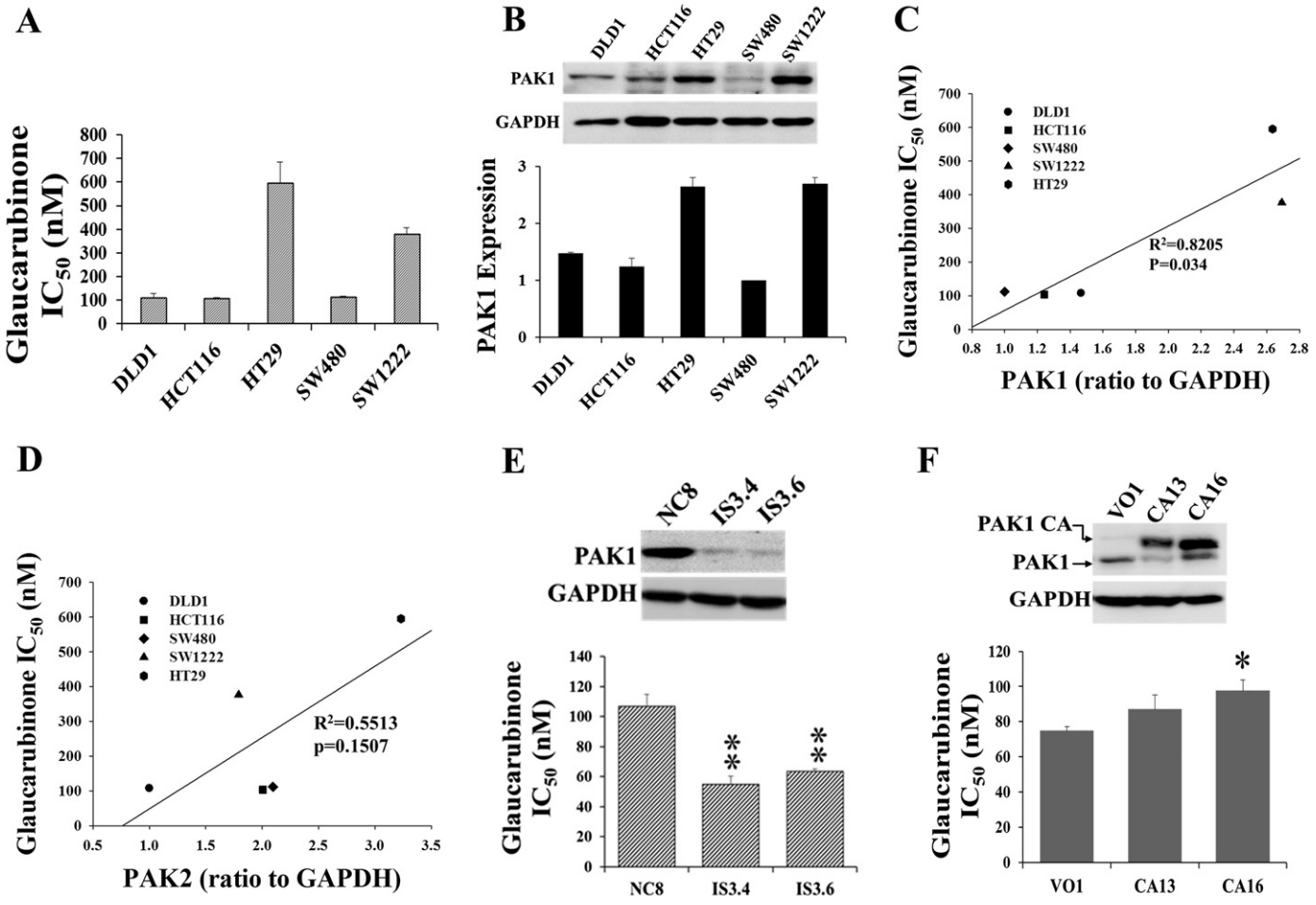

Fig. 2. PAK1 expression correlated with the inhibition of CRC cell proliferation by glaucarubinone. The IC_{50} values for glaucarubinone inhibition of CRC cell proliferation (A) were determined by curve fitting the results shown in Fig. 1A. The concentration of PAK1 in CRC cells was determined by Western blots, and expressed as a ratio of the GAPDH (B). To show the difference in PAK1 protein expression from different CRC cell lines, the PAK1/GAPDH ratio in SW480 cells, which had the lowest PAK1, was taken as 1. The correlation between the glaucarubinone IC_{50} values and the PAK1 (C) or PAK2 (D) concentration was calculated by regression analysis with the program Sigmaplot 12. PAK1 knockdown clones (IS3.4 and IS3.6) and negative control clone (NC8) of HCT116 cells (E) were cultured with different concentrations of glaucarubinone. Constitutively active PAK1 clones (CA13 and CA16) and a vector only-transfected clone (VO1) of SW480 cells (F) were also cultured with different concentrations of glaucarubinone. The IC_{50} values for inhibition by glaucarubinone were calculated using data from three independent experiments.*, $p < 0.05$; **, $p < 0.01$ compared to the values of NC8 (E) or VO1 (F).

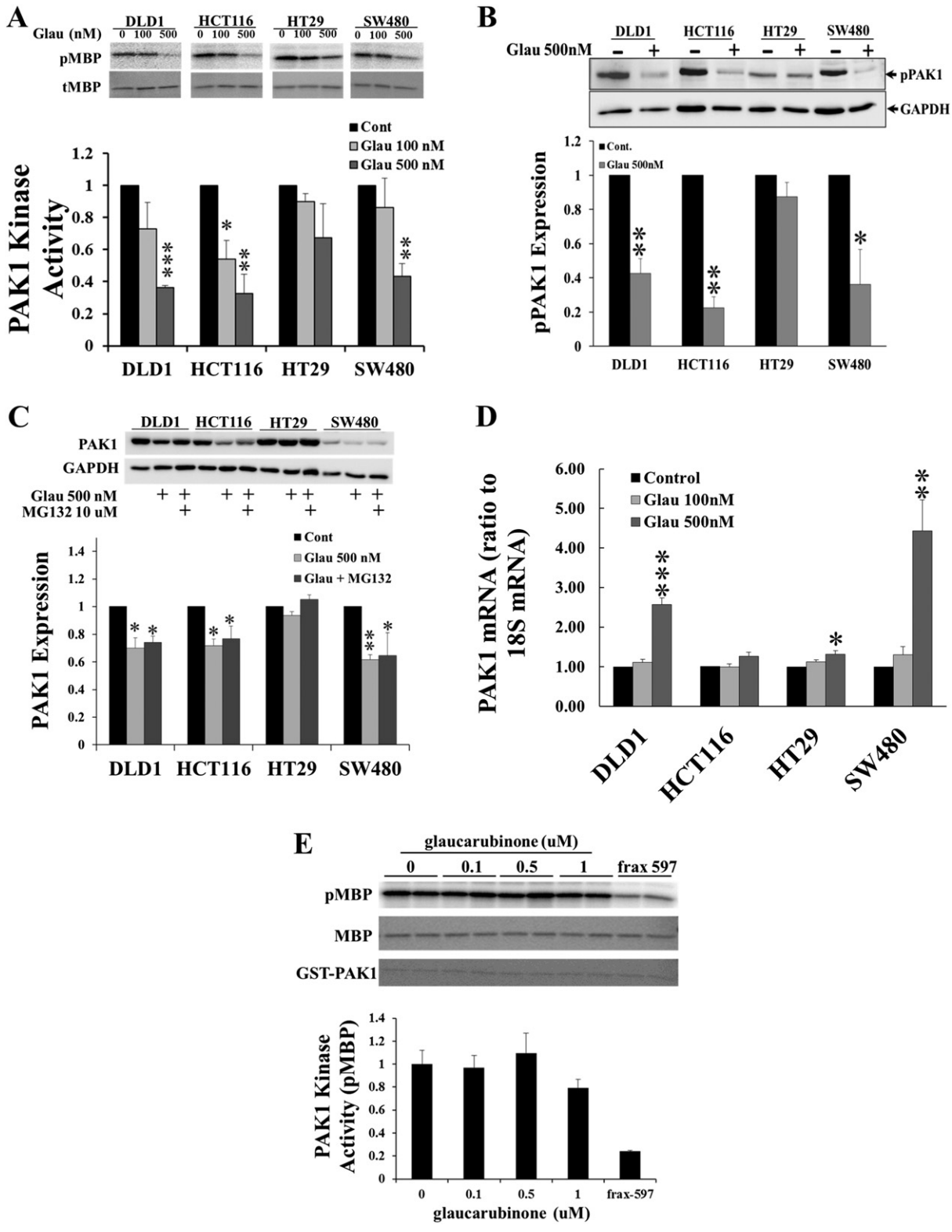

Fig. 3. Glaucarubinone inhibited PAK1 activity by down-regulation of PAK1 expression. CRC cells were cultured with or without glaucarubinone (Glau, 100 nM or 500 nM) for 24 h (A, B, D), in the presence or absence of the proteasomal inhibitor MG132 (10 μg/ml, C), and lysed. The cell lysates were immunoprecipitated with anti-PAK1 antibody, and the *in vitro* kinase activity of the immunoprecipitates was assayed by phosphorylation of myelin basic protein (MBP) as described in the Material and methods section (A). The expression of phosphorylated PAK1 (pPAK1, B) and total PAK1 (C) was determined by Western blot, and the ratios of pPAK1 (B) or total PAK1 (C) to GAPDH in control cells were taken as 1. The amount of PAK1 mRNA and 18S RNA was determined by real time PCR, and the ratio of PAK1 mRNA to 18S mRNA in each untreated cell line was taken as 1 (D). The kinase activity of a bacterially expressed GST-PAK1 fusion protein was measured by phosphorylation of myelin basic protein (MBP) in the presence of glaucarubinone (0.1–1 μM) or the specific PAK1 inhibitor frax 597 (10 nM) (E). The data were summarized from three independent experiments and presented as mean ± standard error. Cont: control, pMBP: phosphorylated myelin basic protein (MBP), tMBP: total MBP, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to control.

3.4. Glaucarubinone inhibited HIF-1α and β-catenin via down-regulation of PAK1

Both HIF-1α and β-catenin play key roles in CRC progression. To determine the effect of glaucarubinone on HIF-1α and β-catenin, the levels of these proteins in CRC cells incubated with or without glaucarubinone were measured by Western blot. Glaucarubinone inhibited the expression of both HIF-1α (Fig. 4A) and β-catenin (Fig. 4B) in DLD1, HCT116 and SW480 cells, but not in HT29 cells which had a significantly higher level of PAK1. To determine whether the effect of glaucarubinone was specific to HIF-1α and β-catenin, the levels of total and phosphorylated ERK and total and phosphorylated AKT in the same CRC cell extracts were measured by Western blot. Glaucarubinone did not reduce the levels of total and phosphorylated ERK – or AKT (Fig. 4C). These data suggested that glaucarubinone inhibited CRC cell growth by suppression of HIF-1α and β-catenin.

To determine the role of PAK1 in the suppression by glaucarubinone of HIF-1α and β-catenin expression in CRC cells, both PAK1 KD HCT116 cells and SW480 cells over-expressing constitutively active (CA) PAK1 were treated with glaucarubinone, and the levels of HIF-1α and β-catenin were measured by Western blot. Over-expression of CA PAK1 reversed the inhibition by glaucarubinone of HIF-1α and β-catenin expression (Fig. 5A & B). On the other hand, the inhibition by glaucarubinone of HIF-1α and β-catenin expression was significantly enhanced in PAK1 knockdown cells (Fig. 5C & D). Glaucarubinone decreased the amount of endogenous PAK1 in vector only transfected cells, but not in CA PAK1 transfected cells (Fig. 5E, top panel). However glaucarubinone reduced the amount of PAK1 not only in negative control (NC) cells but also in PAK1 KD cells (Fig. 5E, bottom panel). These results indicated that PAK1 mediated the inhibitory effect of glaucarubinone on HIF-1α and β-catenin in CRC cells.

3.5. Glaucarubinone inhibited CRC growth in vivo by suppression of PAK1, HIF-1α and β-catenin

The effect of glaucarubinone on CRC cell growth *in vivo* was measured in a mouse xenograft model as described in the Material and methods section. The xenograft growth of HCT116 cells was decreased by glaucarubinone treatment as the tumor volume was reduced significantly from day 6 of glaucarubinone treatment onwards (Fig. 6A). No significant toxicity was observed under the experimental conditions as the body weights of the mice treated with glaucarubinone were not significantly different compared to the control group (Fig. 6B). Similar results were obtained for the xenograft growth of DLD1 cells (Supplementary Fig. 2).

To investigate the mechanisms involved, proteins were extracted from the tumors of control and glaucarubinone-treated mice at the end of the experiment, and the levels of proteins were determined by Western blot. As with the *in vitro* results, glaucarubinone inhibited the expression of HIF-1α, β-catenin, and both total and active PAK1 (Fig. 6C), but had no effect on the expression of either ERK or AKT. These results indicated that glaucarubinone suppressed the *in vivo* growth of CRC cells by down-regulation of HIF-1α and β-catenin, possibly *via* a PAK1-dependent pathway.

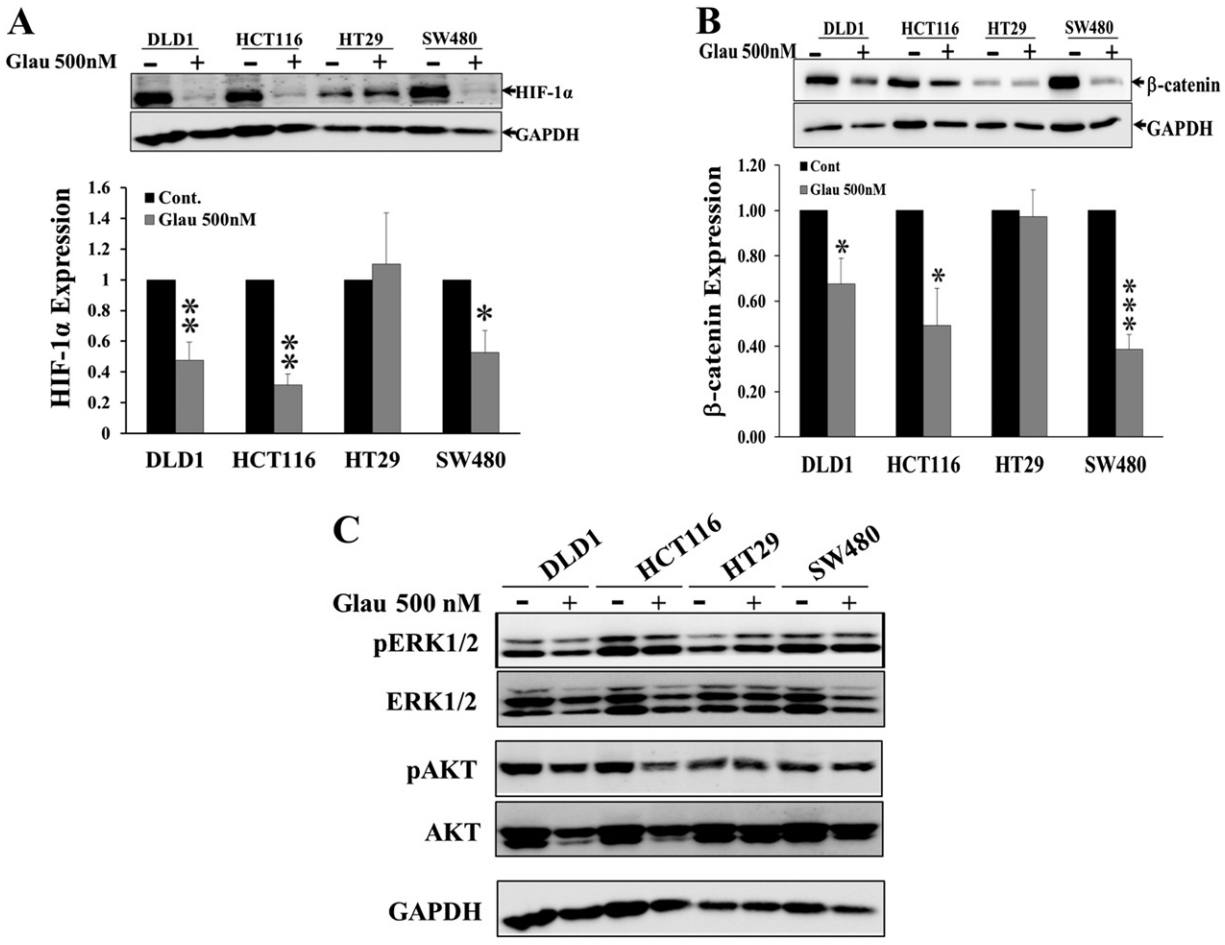

Fig. 4. Glaucarubinone suppressed the expression of HIF-1α and β-catenin, but not of active and total ERK and AKT. CRC cells were incubated with 0 [16], 100 nM or 500 nM glaucarubinone (Glau) for 24 h. The expression of HIF-1α (A), β-catenin (B), phosphorylated ERK (pERK), total ERK (C), phosphorylated AKT (pAKT), and total AKT (C) was determined by Western blot. The expression ratios relative to GAPDH in the control samples were taken as 1. The data were summarized from three independent experiments and presented as mean ± standard error. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to control.

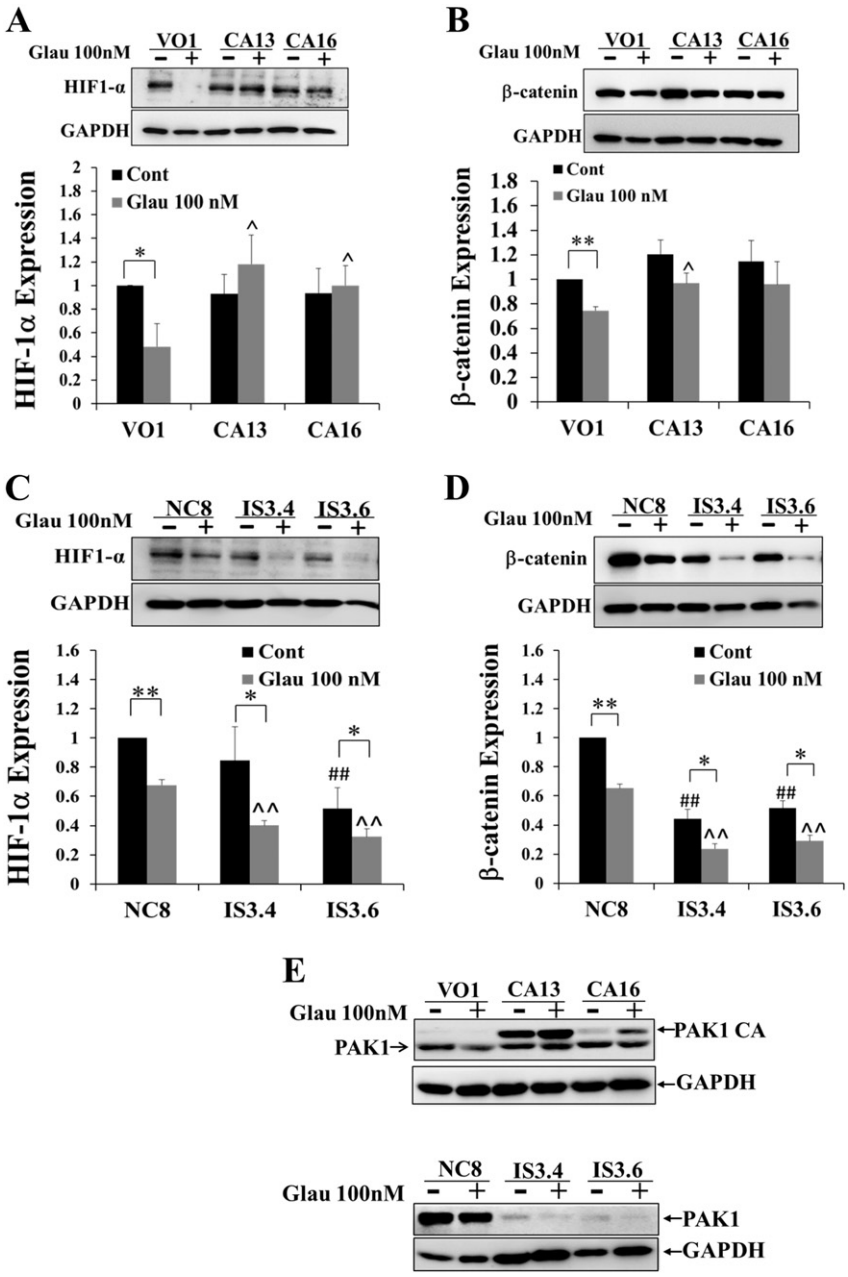

Fig. 5. Inhibition of HIF-1α and β-catenin by glaucarubinone was reversed in CA-PAK1 cells, but enhanced in PAK1 KD cells. Constitutively active PAK1 clones CA13 and CA16 and a vector only clone (VO1) of SW480 cells (A, B), and PAK1 knockdown clones IS3.4, IS3.6 and a negative control clone (NC8) of HCT116 cells (C, D), were cultured with 0 [16] or 100 nM glaucarubinone (Glau). The expression of HIF-1α (A, C) and β-catenin (B, D) was measured by Western blot. The expression ratios relative to GAPDH in the control samples of VO1 or NC8 cells were taken as 1. The amounts of PAK1 and PAK1 CA with or without glaucarubinone treatment are presented in E. The data were summarized from four independent experiments and are presented as mean ± standard error. *, $p < 0.05$; **, $p < 0.01$; control vs glaucarubinone-treatment. ##, $p < 0.01$ compared to control VO1 or control NC8; ^, $p < 0.05$, ^^, $p < 0.01$ compared to glaucarubinone-treated VO1 or NC8.

4. Discussion

We report here that glaucarubinone inhibits CRC growth *in vitro* and *in vivo*. The inhibition by glaucarubinone of CRC cell proliferation correlated with PAK1 expression as lower PAK1 expression rendered CRC cells more sensitive to glaucarubinone (Fig. 2). Furthermore, the inhibitory effect of glaucarubinone on CRC cell proliferation was enhanced by inhibition of PAK1 by knocking down PAK1, and decreased by increasing PAK1 activity *via* expression of constitutively active (CA) PAK1 in CRC cells. In fact, glaucarubinone suppressed PAK1 activity by reducing its protein expression in CRC cells (Fig. 3). These observation are consistent with our recent publication that glaucarubinone and gemcitabine synergistically inhibited pancreatic cancer growth by down-regulation of PAK1 [26]. Although the suppression of PAK1 activity by glaucarubinone in cells is unlikely to be due to a direct inhibition by glaucarubinone of the kinase activity of PAK1 (Fig. 3), it is possible that glaucarubinone may decrease PAK1 activity by reducing PAK1 protein expression *via* suppression of a post-transcriptional pathway. The mechanism involved in down-regulation of PAK1 protein expression and suppression of PAK1 activity by glaucarubinone in cells needs further investigation. Although the inhibitory effect of glaucarubinone on cell proliferation correlated with levels of PAK1 protein in CRC cells, the effect of glaucarubinone may not be mediated entirely by PAK1.

The inhibition by glaucarubinone of CRC growth was also associated with decreased expression of HIF-1α and β-catenin in both CRC cells (Fig. 4) and xenografted tumors (Fig. 6) under glaucarubinone treatment.

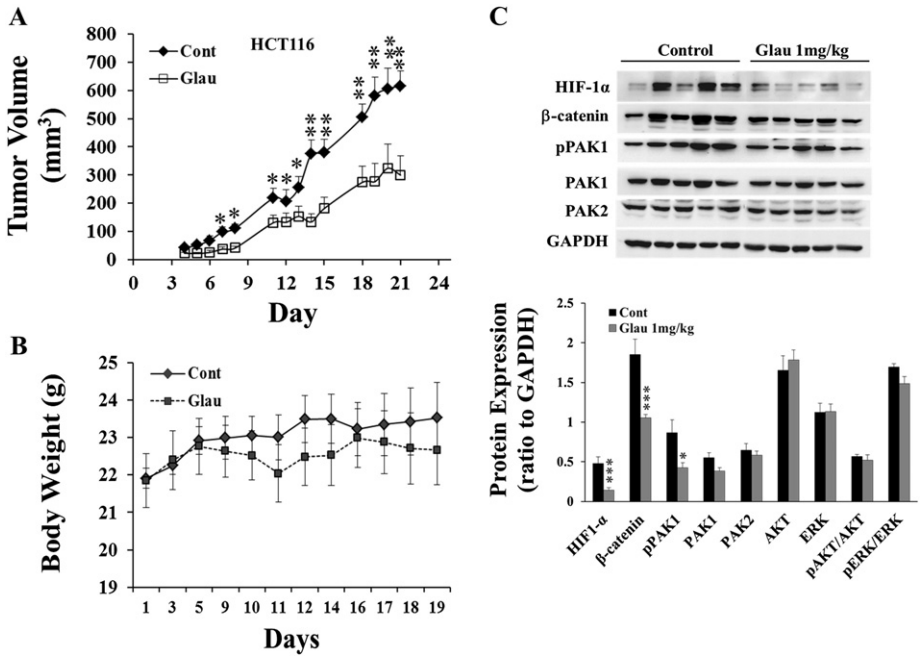

Fig. 6. Glaucarubinone inhibited the xenograft growth of CRC cells by down-regulation of PAK1, HIF-1α and β-catenin. HCT116 cells were subcutaneously injected into the flanks of 10 SCID mice, which were then divided into control (Cont, n = 5) and glaucarubinone-treated (Glau, n = 5) groups. The control mice were injected intra-peritoneally with 5% DMSO in saline and the glaucarubinone-treated mice with 1 mg/kg glaucarubinone dissolved in 5% DMSO in saline for 3 weeks as described in the Material and methods section. The tumor volumes (A), body weights (B) and protein concentrations (C) were measured with a caliper, by weighing and by Western blot, respectively, as described in the Material and methods section. The protein concentrations were expressed as ratios relative to the GAPDH in each sample. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to control.

The inhibition by glaucarubinone of HIF-1α and β-catenin expression is not simply due to its suppression of protein synthesis as neither pERK and ERK, nor pAKT and AKT, were affected by glaucarubinone treatment. Furthermore the inhibition of HIF-1α and β-catenin expression by glaucarubinone was mediated by a PAK1-dependent pathway as the inhibitory effect was enhanced in PAK1 knockdown CRC cells but reduced in CRC cells with over-expression of constitutively active (CA) PAK1 (Fig. 5). Likewise glaucarubinone inhibited HIF-1α and β-catenin expression in xenografted CRC tumors, and the inhibition was associated with reduced activation of PAK1 (Fig. 6). Although the decrease in total amount of PAK1 caused by glaucarubinone *in vivo* did not reach statistical significance, the amount of phosphorylated and active PAK1 was significantly reduced by glaucarubinone. Together these data suggested that glaucarubinone inhibited CRC growth by down-regulation of HIF-1α and β-catenin *via* a PAK1-dependent pathway.

These observations are consistent with previous reports of connections between PAK1 and β-catenin signaling. PAK1 phosphorylates β-catenin and stimulates β-catenin nuclear translocation and activation in CRC cells [16,17]. PAK1 also stimulates CRC growth and metastasis by activation of β-catenin [16]. We have previously reported that PAK1 correlates closely with HIF-1α and β-catenin in intestinal and colorectal cancers in the $APC^{\Delta 14}$ mice model [18]. Inhibition of PAK1 expression by small interfering RNA reduced the expression of both β-catenin and HIF-1α, and the reduction was associated either with a decreased tumor number in $APC^{\Delta 14}$ mice, or with reduced tumor survival in a CRC xenograft model [18]. Conversely PAK1 promoted hypoxia-induced HIF-1α expression leading to increased survival of CRC cells [18]. These findings demonstrate that PAK1 is a key regulator of HIF-1α and β-catenin in CRC growth and survival. In the present study, we show that PAK1 mediated the inhibitory effects of glaucarubinone on HIF-1α and β-catenin expression, which in turn led to inhibition of CRC growth.

Members of the quassinoid family, including glaucarubinone, have been found to affect multiple cellular processes, such as inhibition of proliferation, suppression of DNA and protein synthesis, and induction of apoptosis [19]. Quassinoids act through targeting multiple signaling pathways. For example, NBT-272 induced cell cycle arrest and inhibited proliferation by down-regulation of myc, an oncogene which is frequently over-expressed in various tumors [31]. 6α-tigloxychaparrinone, another quassinoid, decreased hypoxia-induced HIF-1α protein expression and mRNA translation in gastric cancer cells through an ERK-dependent pathway [25]. For the first time, we report here that glaucarubinone inhibited the protein expression of both HIF-1α and β-catenin in CRC cells by modulation of PAK1 activity, leading to decreased CRC growth. HIF-1α and β-catenin play important roles in CRC growth and metastasis. PAK1 has recently been identified as a point of convergence mediating multiple signals important for cancer progression [32]. PAK1 promotes CRC growth and metastasis by activation of β-catenin [16,17], and enhances CRC survival through up-regulation of HIF-1α [18]. The connection from glaucarubinone to PAK1, HIF-1α and β-catenin provides significant insight into the mechanism involved in the anticancer function of quassinoids. Given this mode of action, quassinoids would seem to be worthy of further study as a CRC treatment. However the fact that CRC cell lines with lower PAK1 expression are more sensitive to glaucarubinone implies that glaucarubinone may be more effective in chemoprevention or adjunct chemotherapy after tumor resection.

In summary we have demonstrated in this paper that glaucarubinone inhibited CRC cell growth *in vitro* and *in vivo* by down-regulation of HIF-1α and β-catenin expression *via* a PAK1-dependent pathway. These findings further confirm the central role of PAK1 in CRC progression and provide insight into the mechanism involved in the anticancer activity of glaucarubinone.

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