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A Novel mTOR Inhibitor; Anthracimycin for the Treatment of Human Hepatocellular Carcinoma

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Abstract. *Background/Aim: Anthracimycin, a secondary metabolite of Streptomyces, has been shown to inhibit the invasion of certain cancer cell lines. Materials and Methods: In this study we evaluated the effect of anthracimycin on cell growth and signaling pathways in hepatocellular carcinoma (HCC). Results: Anthracimycin suppressed cell proliferation and motility and induced apoptosis in human HCC cell lines. Furthermore, anthracimycin had no effect on the enrichment of EpCAM-high liver cancer stem cells (CSCs), while fluorouracil dramatically enriched the CSCs with activation of the stemness-related genes EPCAM and SOX9 in HuH7 cells. Mechanistically, anthracimycin suppressed mammalian target of rapamycin (mTOR) signaling, and was most effective at inhibiting HCC cell proliferation with mTOR activation. Conclusion: Anthracimycin is a novel mTOR inhibitor capable of suppressing the proliferation of CSCs and non-CSCs equally well in HCC, and it is suggested that anthracimycin could be effective in the eradication of HCC associated with mTOR-signaling activation.*

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer-related death in men worldwide (1). Sorafenib tosylate is a multikinase inhibitor reported to prolong overall survival (by a median of ~3 months) in advanced HCC patients; however, its response rate is generally <5% according to evaluation criteria defined by the Response Evaluation Criteria in Solid Tumors (2, 3), indicating that more effective reagents are required for the treatment of advanced HCCs. Recent evidence suggested that some HCCs are hierarchically organized with a subset of cells called cancer stem cells (CSCs) that possess stem cell features (4). CSCs show chemoresistance to cytotoxic reagents and, therefore, are considered critical targets for HCC eradication (5-7).

Anthracimycin, previously designated as TPU-0114, is a novel polyketide compound with a molecular weight of 396 Da ($C_{25}H_{32}O_4$) (Figure 1A) (5-7). This structurally unique compound is produced by an actinomycete strain belonging to the genus *Streptomyces* isolated from a compost sample. This small molecule potentially inhibits the invasion of some cancer cell lines (5), but it remains unclear how the compound affects cancer cell growth and motility. In this study, we evaluated the effect of anthracimycin on cell growth, motility, activated signaling pathways, and stemness in HCC.

Materials and Methods

Cell lines and reagents. Huh1, Huh7, and SK-Hep-1 cells were routinely cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Three hepatocellular carcinoma cell lines established from primary HCC samples were also routinely cultured with DMEM supplemented with 10% FBS, as previously described (8). Anthracimycin was purified from *Streptomyces* strain NITE P-769 as described previously (5). Fluorouracil (5-FU) was purchased from Taiho Pharmaceutical (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan).

Cell proliferation and apoptosis. Briefly, 2×10^3 cells were seeded in 96-well plates, and cell proliferation was evaluated in quadruplicate using the Cell-counting Kit 8 (Dojindo Laboratories, Kumamoto, Japan). Anthracimycin was dissolved in dimethyl sulfoxide (DMSO), and cells were treated with 0.1% DMSO (control) or the indicated

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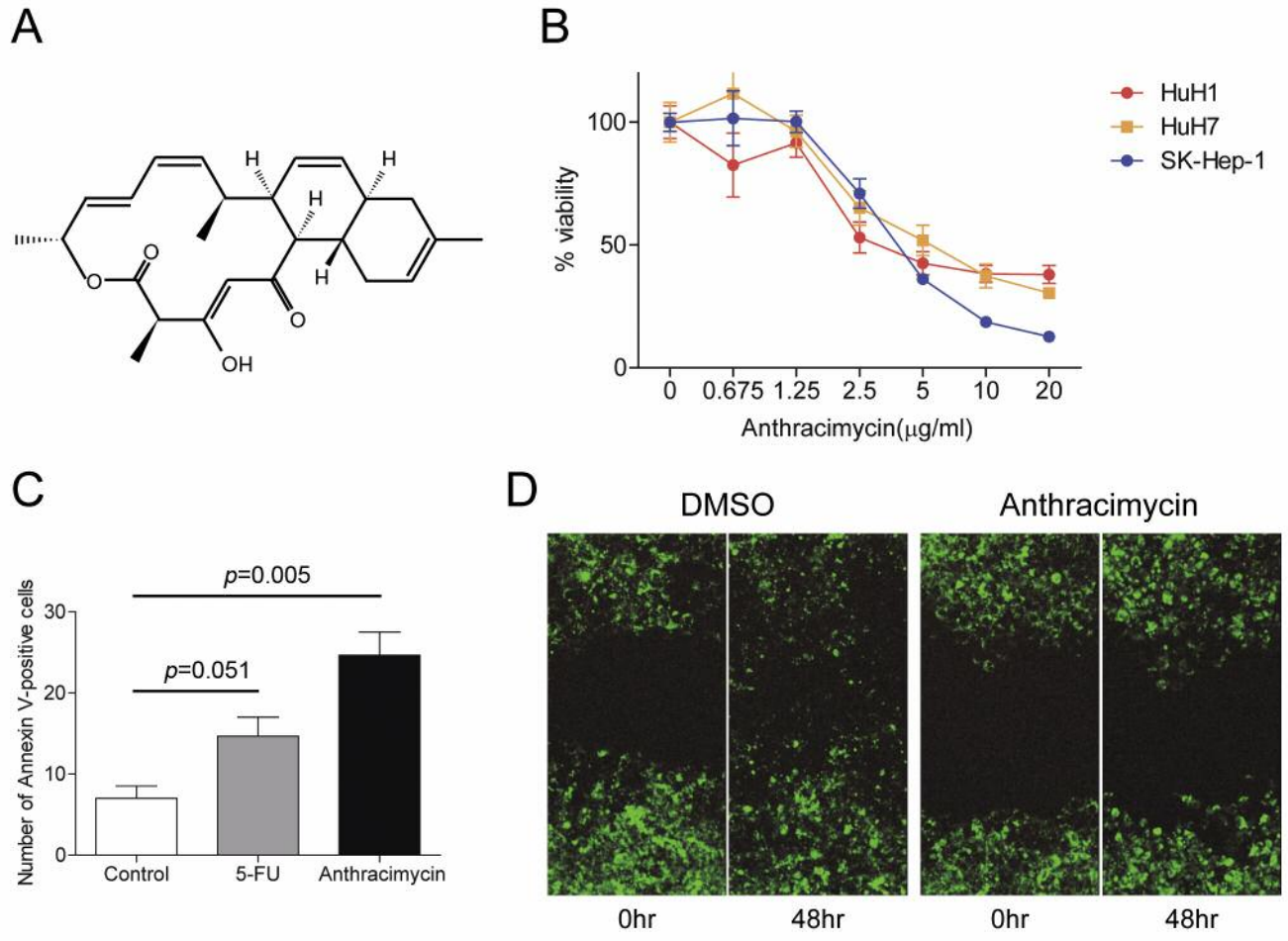

Figure 1. Effect of anthracimycin on cancer cell proliferation, apoptosis, and motility. (A) Structural formula of anthracimycin. (B) Anthracimycin suppressed proliferation of HuH1, HuH7, and SK-Hep-1 HCC cell lines in a dose-dependent manner. (C) Anthracimycin treatment (2.5 μg/ml for 48 h) induced annexin V-positive apoptotic cells compared to the control (0.1% DMSO). (D) Wound-healing assay results showed that anthracimycin (2.5 μg/ml) inhibited cell motility in HuH7 cells.

concentration of anthracimycin for 72 h. The IC_{50} in each cell line was calculated by the GraphPad PRISM software version 5.0.4 (GraphPad Software, San Diego, CA, USA). Annexin V-positive apoptotic cells were evaluated by immunofluorescence using an ANNEXIN V-FITC Fluorescence Microscopy Kit (BD Biosciences, San Jose, CA, USA) according to manufacturer instructions. Briefly, 1×10^5 cells were seeded in a 2-well chamber slide and treated with 0.1% DMSO (control), 2.5 μg/mL 5-FU, or 2.5 μg/ml anthracimycin for 48 h. Membranous staining of annexin V-positive cells was evaluated by a BZ-9000 fluorescence microscope (Keyence Corp., Tokyo, Japan). The number of annexin V-positive cells per 100 cells (evaluated by 4',6-diamidino-2-phenylindole positivity) was measured in quadruplicate.

Wound-healing assay. A wound-healing assay was performed using μ-Slide 8-well chambers and culture inserts (Ibidi, Martinsried, Germany) as described previously (9). Briefly, 1×10^6 HuH7 cells were labeled with DiO for 20 min and washed with phosphate-buffered saline (PBS). Cells (2×10^3 cells/50 μL/well) were then incubated in a culture insert in μ-Slide 8-well chambers overnight. Silicone inserts were removed, and the culture media was immediately replaced with DMEM containing 10% FBS and 0.1% DMSO (control) or 2.5 μg/ml anthracimycin. Time-lapse image analysis was performed for 48 h using a CSU-X1 spinning-disk confocal microscope (Yokogawa, Tokyo, Japan) and an Andor iXon3 EMCCD camera system (Andor Technology, Belfast, UK) (8).

Fluorescence-activated cell sorting (FACS) analysis. FACS analysis was performed to evaluate the expression status of the CSC marker EpCAM. Briefly, 2×10^5 HuH7 cells were seeded in 6-well plates and cultured overnight. Cells were then cultured with DMEM supplemented with 10% FBS and 0.1% DMSO (control), 2.5 μg/ml 5-FU, or 2.5 μg/mL anthracimycin for 72 h. Cells were trypsinized, washed, and resuspended in Hank's Balanced Salt Solution (Lonza, Basel, Switzerland) supplemented with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2% FBS. Cells were then incubated with the fluorescein isothiocyanate (FITC)-conjugated anti-EpCAM monoclonal antibody Clone Ber-EP4 (Dako Japan, Tokyo, Japan) on ice for 30 min and analyzed using a FACSCalibur

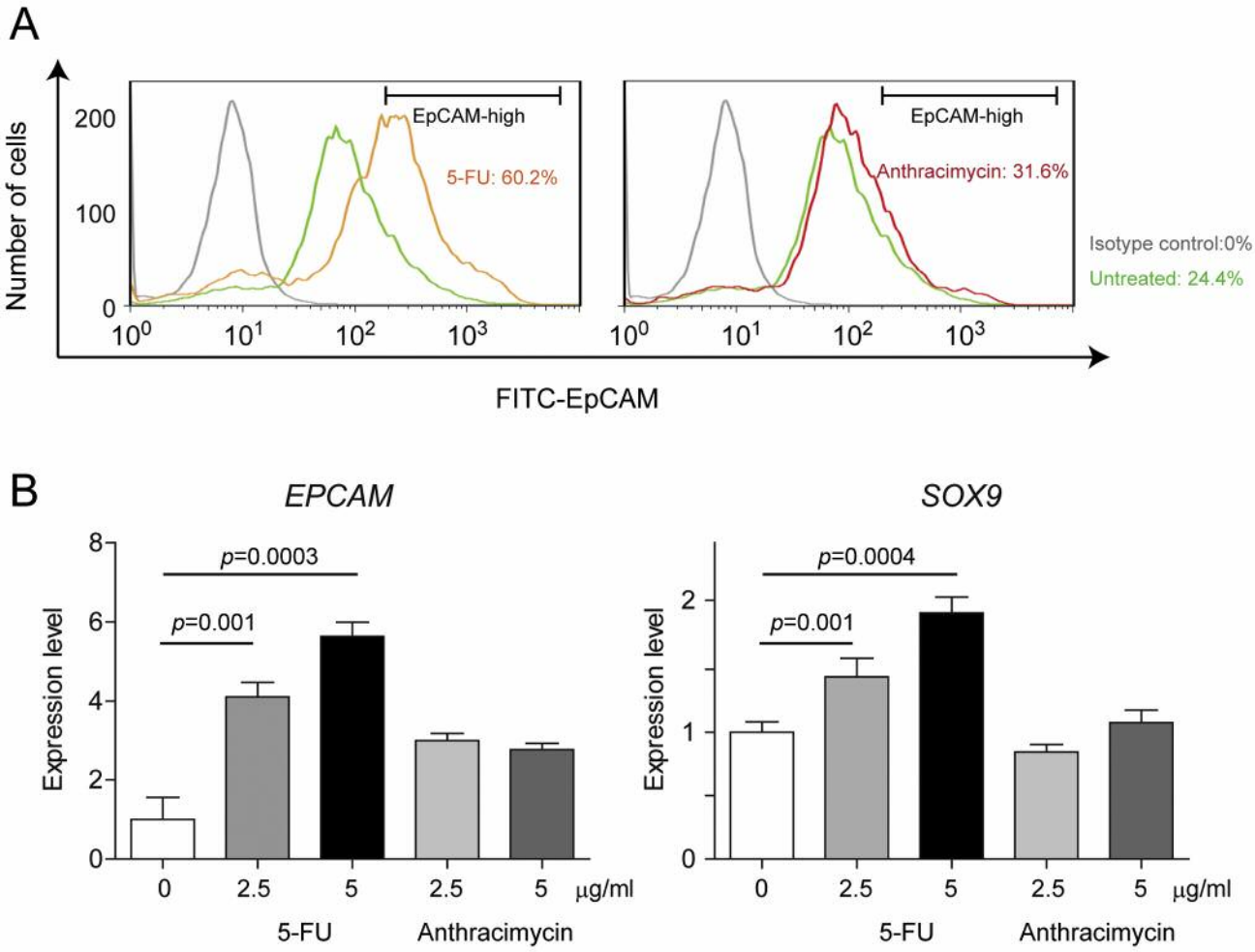

Figure 2. *The effect of anthracimycin on liver cancer stemness. (A) FACS analysis of cancer stem cell marker EpCAM expression in HuH7 cells treated with 5-FU (2.5 µg/ml for 72 h; left panel) or anthracimycin (2.5 µg/ml for 72 h; right panel). (B) qRT-PCR analysis of EPCAM and SOX9 in HuH7 cells treated with 5-FU or anthracimycin at the indicated concentrations for 72 h.*

(BD Biosciences) (10). The FlowJo software version 7.6.5 (FlowJo, LLC, Ashland, OR, USA) was used to analyze the EpCAM-high cell population for each experimental condition.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Expression of *EPCAM* and *SOX9* was determined in triplicate using the 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each sample was normalized relative to 18S ribosomal RNA expression. Probes used were: *EPCAM*, Hs00158980_m1, *SOX9*, Hs00165814_m1, and *18S*, Hs99999901_s1 (Applied Biosystems).

Western blot. Briefly, 2×10^5 cells were seeded in 6-well plates and cultured overnight. Cells were then cultured with DMEM supplemented with 10% FBS and 0.1% DMSO (control), 5-FU, or anthracimycin at the indicated concentrations and for the indicated time. Adherent cells were washed with PBS, and whole-cell lysates were prepared using radioimmunoprecipitation assay lysis buffer. Mouse monoclonal antibody to human $p21^{Waf1/Cip1}$ clone DCS60, rabbit monoclonal antibody to human phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) clone D13.14.4E, rabbit monoclonal antibody to human Bcl-xL clone 54H6, rabbit monoclonal antibody to human mTOR clone 7C10, rabbit monoclonal antibody to human Raptor clone 24C12, and rabbit polyclonal antibodies to human p16 INK4A, NF-κB p65, phospho-NF-κB p65 (Ser468), Akt, phospho-Akt (Thr308), p44/42 MAPK (Erk1/2), Rheb, phospho-mTOR (Ser2448), p70 S6 Kinase, phospho-p70 S6 Kinase (Thr389), and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA) and diluted according to the protocol recommended for each antibody. Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ, USA), and mTOR protein concentrations detected by western blot bands were quantified using ImageJ software version 1.46r (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Student *t*-tests were performed using GraphPad Prism software version 5.0.4 (GraphPad Software) to compare the test groups evaluated by immunofluorescence and qRT-PCR. Correlation between the IC_{50} of anthracimycin and mTOR concentration was also examined by the Pearson correlation coefficient using GraphPad Prism software (GraphPad Software).

Results

Anthracimycin suppressed cell proliferation and motility and induced apoptosis in human HCC. We evaluated the effect of anthracimycin on cell proliferation in three representative human HCC cell lines (HuH1, HuH7, and SK-Hep-1). Anthracimycin suppressed cell proliferation in all cell lines in a dose-dependent manner (Figure 1B), with the IC_{50} in HuH1, HuH7, and SK-Hep-1 cells measured at 2.5 μg/ml, 2.8 μg/ml, and 3.0 μg/ml, respectively. We also measured the number of annexin V-positive cells treated with 0.1% DMSO (Control), 2.5 μg/ml 5-FU, and 2.5 μg/ml anthracimycin and identified a dramatic and statistically significant increase in annexin V-positive cells treated with anthracimycin (Figure 1C). Additionally, evaluation of the effect of anthracimycin on cell motility by wound-healing assay revealed that 2.5 μg/ml anthracimycin strongly suppressed HuH7 motility (Figure 1D).

Anthracimycin suppressed cell proliferation without enrichment of liver CSCs in HCC. CSCs are considered resistant to cytotoxic reagents and, therefore, the CSC population is generally enriched following treatment. Consistently, when HuH7 cells were treated with 2.5 μg/ml 5-FU for 72 h, EpCAM-high liver CSCs were dramatically enriched (60.2%) as compared with the control (24.4%) (Figure 2A, left panel), suggesting that EpCAM-high liver CSCs were resistant to 5-FU treatment. Interestingly, anthracimycin treatment did not enrich EpCAM-high liver CSCs (31.6%) (Figure 2A, right panel). Additionally, although 5-FU treatment resulted in significant up-regulation of genes encoding the CSC markers *EPCAM* and *SOX9*, anthracimycin treatment did not induce similar upregulation of stemness-related genes in a dose-dependent manner according to qRT-PCR analysis (Figure 2B).

We investigated the effect of anthracimycin treatment on cell-cycle arrest using SK-Hep-1 cells lacking p53 mutations, making them susceptible to cytotoxic reagents *via* the p53-p21 pathway. Consistently, 5-FU treatment strongly induced p21 expression in a dose-dependent manner in SK-Hep-1 cells. However, anthracimycin treatment had no effect on p21 induction, suggesting that anthracimycin suppressed cell proliferation and induced apoptosis in a p53-independent manner (Figure 3A). We further evaluated the effect of anthracimycin treatment on NF-κB, Akt, and ERK signaling in HuH7 cells by western blot (Figure 3B). Adherent HuH7 cells treated with anthracimycin for 72 h showed induction of phospho-NF-κB (p65) and phospho-Akt as compared with control or 5-FU-treated HuH7 cells. Anthracimycin treatment had no effect on phospho-ERK1/2 proteins, but resulted in a slight decrease in the concentration of anti-apoptosis protein Bcl-xl relative to levels observed in the control or 5-FU-treated HuH7 cells. These data suggested that anthracimycin treatment may block signaling pathways downstream of Akt- or NF-κB-signaling cascades, resulting in a positive-feedback mechanism to compensate for the inhibited signaling pathways, which might result in Akt or NF-κB phosphorylation in adherent anthracimycin-treated cells.

Anthracimycin suppressed mTOR signaling and HCC cell proliferation in an mTOR concentration-dependent manner. To determine the potential molecular targets inhibited by anthracimycin treatment, we first screened 118 kinases activated in cancer using Life Technologies SelectScreen Profiling Service. We found that anthracimycin inhibited PIK3CA/PIK3R1 expression [p110α/p85α phosphoinositide (PI)-3 kinase] by 46% *in vitro* at anthracimycin concentrations of 20 μM (~8 μg/ml) as examined by the Adapta universal kinase assay (Life Technologies). However, the IC_{50} in HuH1, HuH7, and SK-Hep-1 cells was ~3 μg/ml (Figure 1A), and Akt exhibited enhanced levels of phosphorylation in anthracimycin-treated HuH7 cells (Figure 3B). Therefore, we considered that PI-3 kinase was an unlikely direct molecular target of anthracimycin. We next evaluated the mTOR-signaling pathway, because mTOR is a downstream target activated by PI-3 kinase-Akt signaling, and downstream molecules associated with Akt signaling may be directly inhibited by anthracimycin according to western blot data (Figure 3B). Interestingly, when we treated HuH7 cells with 5-FU or anthracimycin for 8 h, we identified a dramatic, dose-dependent reduction in phospho-p70 S6 kinase protein following anthracimycin treatment (Figure 4A). This effect was not observed following 5-FU treatment. Since expression of Rheb, mTOR, phospho-mTOR, Raptor, and p70 S6 kinase was not affected by anthracimycin treatment, this implied that anthracimycin did not specifically target PI-3 kinase *in vitro* as suggested by the SelectScreen Profiling Service. Rather, these data suggested that anthracimycin might inhibit formation of mTOR complex 1 (mTORC1), thereby inhibiting phosphorylation of p70 S6 kinase. A similar observation was reported involving the mTOR-signaling inhibitor rapamycin, which was discovered in the bacterium *Streptomyces hygroscopius*. We evaluated the concentrations of mTOR and phospho-mTOR in three representative HCC cell lines (HuH1, HuH7, and SK-Hep-1) and three HCC cell lines established from primary HCCs (HCC 1, HCC 2, and HCC 3). The concentrations of mTOR and phospho-mTOR were heterogeneous in each primary HCC cell line, while HuH1, HuH7, and SK-Hep-1 cells exhibited increased activation of mTOR signaling as compared with HCC 1, HCC 2, and

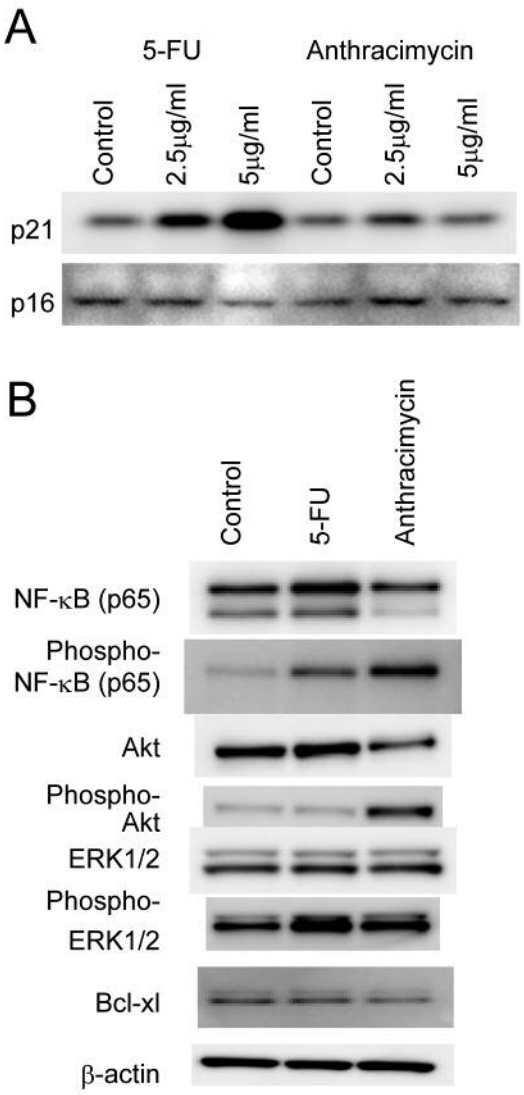

Figure 3. *The effect of anthracimycin on growth-signaling pathways. (A) Western blot analysis of p21 and p16 in SK-Hep-1 cells treated with 5-FU or anthracimycin at the indicated concentrations for 72 h. (B) Western blot analysis of NF-κB-, Akt-, and ERK-signaling proteins in HuH7 cells treated with 5-FU (2.5 μg/ml) or anthracimycin (2.5 μg/ml) for 72 h.*

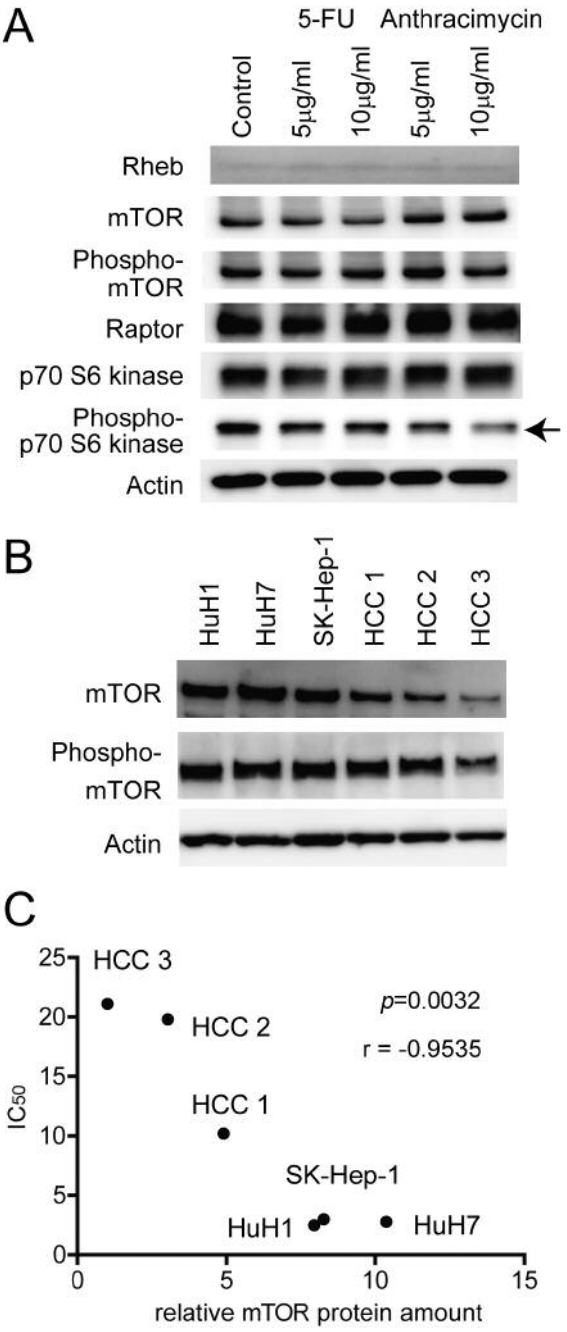

Figure 4. *The effect of anthracimycin on the mTOR-signaling pathway. (A) Western blot analysis of mTOR-signaling proteins in HuH7 cells treated with 5-FU or anthracimycin at the indicated concentrations for 8 h. The black arrow indicates the observed dose-dependent decrease in phosphorylated p70 S6 kinase protein following anthracimycin treatment. (B) Western blot analysis of mTOR and phospho-mTOR proteins in human HCC cell lines and HCC cells established from primary HCC samples. (C) Scatter-plot analysis of mTOR concentration and the IC_{50} of anthracimycin in HCC cells. A negative correlations between mTOR concentration and anthracimycin IC_{50} was observed ($r=-0.9535$, $p=0.0032$). Relative mTOR concentration was calculated using ImageJ software and evaluated against the mTOR concentration measured in primary HCC 3 cells.*

HCC 3 cells (Figure 4B). Interestingly, the sensitivity of the primary HCC cell lines to anthracimycin (IC_{50}) negatively correlated with mTOR concentration (p=0.0032, r=–0.9535) (Figure 4C), suggesting that the effect of anthracimycin on cell proliferation is dependent upon activation of the mTOR-signaling pathway in HCC. Collectively, these data suggested that anthracimycin suppressed HCC cell proliferation and motility possibly through inhibition of the mTOR-signaling pathway. Given that anthracimycin did not affect induction of cancer stemness and was most effective in HCC cell lines

exhibiting abundant mTOR expression, anthracimycin may be a mTOR inhibitor valuable for treating HCC associated with mTOR-signaling activation.

Discussion

Natural microbial products are an important resource for cancer drug discovery and provide significant anticancer reagents, including doxorubicin, mitomycin C, and rapamycin. Here, we discovered that anthracimycin produced by *Streptomyces* strain NITE P-769 inhibited HCC cell proliferation and motility and induced apoptosis potentially through suppression of the mTOR-signaling pathway. Given that previous findings suggested that anthracimycin was less toxic to normal cells relative to cancer cells (unpublished data), anthracimycin may be a good candidate for the treatment of advanced HCCs associated with mTOR-signaling activation.

TOR is a serine/threonine kinase highly conserved from yeast to mammals that regulates cell growth, cell-cycle progression, and metabolism (11). mTOR is a mammalian target of rapamycin, which is a macrolide produced by *S. hygroscopicus*, that belongs to the PI-3 kinase family and interacts with several proteins to form two distinct protein complexes (mTORC1 and mTORC2) that function as kinases to phosphorylate p70 S6 kinase and 4E-BP1 (by mTORC1) or Akt (by mTORC2). Rapamycin mainly inhibits mTORC1-complex formation by binding to FK506-binding protein 12 to suppress phosphorylation of p70 S6 kinase. Our data indicated that anthracimycin inhibited the phosphorylation of p70 S6 kinase without directly affecting mTOR phosphorylation. However, the mechanisms associated with anthracimycin suppression of mTOR signaling remain unclear and should be elucidated in future studies.

Since mTOR plays a fundamental role in cell metabolism, most growth factors activate mTOR signaling mainly through activation of the Akt-signaling pathway. Stimulation of mTOR signaling activates protein synthesis, lipid synthesis, and energy metabolism, which are key metabolic changes related to cancer growth (12, 13). Consequently, mTOR signaling is activated in many types of cancer (14), with its activation correlated with advanced tumor stages and poor survival outcomes in various types of cancers, including HCC (15). Furthermore, accumulating evidence suggested that the PI-3 kinase/Akt/mTOR-signaling pathway may play a crucial role in CSC maintenance (16). Inhibition of mTOR signaling induced a paused pluripotent state in colon, lung, pancreatic and leukemia CSCs (17-21). Our data indicated that anthracimycin equally suppressed liver CSCs and non-CSCs in HuH7 cells, suggesting that mTOR constitutes a viable molecular target for the eradication of HCC CSCs. Consistently, a recent paper indicated the close link between the expression of cancer stem cell markers and the activation of PTEN/AKT/mTOR pathway components in HCC (22).

The mTOR inhibitors temsirolimus and everolimus are approved for the treatment of advanced renal cell carcinoma, breast cancer, and pancreatic neuroendocrine tumors (23). Sirolimus, temsirolimus, and everolimus are currently evaluated for the treatment of advanced HCC as first- or second-line therapeutics (15). Although a recent phase III study evaluating everolimus as a second-line treatment for advanced HCC after sorafenib failure resulted in negative results (24), our data indicated that the evaluation of mTOR-activation status may be a key component in the successful treatment of HCCs using the mTOR inhibitor anthracimycin. Further preclinical studies are required to evaluate the efficacy of anthracimycin treatment of HCC.

Since mTOR inhibition resulted in alteration of metabolic programs and protein synthesis in various types of cells, an mTOR inhibitor, everolimus, is known to suppress the neointimal hyperplasia of coronary arteries (25), angiogenesis in the tumor (26, 27) as well as immune responses (28, 29). Although we confirmed the suppression of mTOR signaling in HCC cell lines, it is still unclear if anthracimycin also suppresses the angiogenesis or immune responses, as observed by everolimus. Because the immune suppression may have a risk to escape cancer cells from immune surveillance, if anthracimycin has a limited effect on immune cells, it would be a better natural compound to eradicate HCC by targeting both CSCs and non-CSCs than everolimus (30). The effect of anthracimycin on angiogenesis and immunosuppression should be clarified in future *in vivo* studies.

Acknowledgements

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