Ampelopsin suppresses breast carcinogenesis by inhibiting the mTOR signalling pathway

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The mammalian target of rapamycin (mTOR), which is a master regulator of cellular catabolism and anabolism, plays an important role in tumourigenesis and progression. In this study, we report the chemopreventive effect of the dietary compound ampelopsin (AMP) on breast carcinogenesis in vivo and in vitro, which acts by inhibiting the mTORsignalling pathway. Our study indicates that AMP treatment effectively suppresses 1-methyl-1-nitrosoureac (MNU)-induced breast carcinogenesis in rats and inhibits 4-(methylamino)anilino-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP)-induced cellular carcinogenesis. Additionally, AMP inhibits the growth of breast cancer cells in vitro and in vivo. The activity of mTOR kinase was found to be significantly increased in a time-dependent manner during chronic breast carcinogenesis, and this increase can be suppressed by AMP co-treatment. AMP also effectively suppresses mTOR activity in breast cancer MDA-MB-231 cells. We also demonstrated that AMP is an effective mTOR inhibitor that binds to one site on the mTOR target in two ways. Further studies confirmed that AMP inhibits the activation of Akt, suppresses the formation of mTOR complexes (mTORC)1/2 by dissociating regulatory-associated protein of mTOR and rapamycin-insensitive companion of mTOR and, consequently, decreases the activation of the downstream targets of mTOR, including ribosomal p70-S6 kinase, ribosomal protein S6, eukaryotic translation initiation factor 4B and eukaryotic translation initiation factor 4E-binding protein 1. These finding suggest that AMP is a bioactive natural chemopreventive agent against breast carcinogenesis and is an effective mTOR inhibitor that may be developed as a useful chemotherapeutic agent in the treatment of breast cancer.

Introduction

Breast cancer is the leading cause of cancer death among women in Europe and North America (1). The National Cancer Institute has recognized that prevention is a critical component in minimizing the number of individuals who are afflicted with breast cancer (2). The mammalian target of rapamycin (mTOR) is an important component of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway that is frequently overactivated in various cancers, particularly in breast carcinogenesis and metastasis (3,4). The PI3K/Akt/mTOR signalling pathway acts as a key integration point between the extrinsic and intrinsic cellular environments (5,6), regulates a broad spectrum of cellular processes and is one of the major signalling pathways in tumourigenesis and development (7,8). The mTOR protein is a highly conserved 289 kDa protein serine/threonine kinase, and 95% of its amino acid composition is conserved from yeast to human (9). mTOR functions downstream of the PI3K/Akt pathway and positively regulates the phosphorylation of ribosomal p70-S6 kinase (S6K70), ribosomal protein S6 (rpS6), eukaryotic translation initiation factor 4B and eukaryotic translation initiation factor 4E-binding protein (4EBP)1 to control protein translation (10,11). Recent studies suggest that targeting the mTOR signalling pathway may be an effective strategy for chemoprevention and cancer treatment (12,13). The results from several studies support the hypothesis that mTOR acts as a master switch of cellular catabolism and anabolism and thereby determines whether cells, particularly tumour cells, grow, proliferate and migrate (14,15). Currently, mTOR has emerged as one of the most significant intracellular signalling enzymes that regulate cell growth, survival and autophagy in breast cancer progression (16,17). Indeed, mTOR inhibitors have entered preclinical studies and clinical trials for various human cancers (18). The mTOR signalling pathway therefore represents an attractive and promising target for preventive and therapeutic intervention. Therefore, isolating, selecting and developing inhibitors of mTOR signalling, especially from natural products, has become a widely investigated area of research.

Epidemiologic studies and systematic analyses suggest that diets rich in fruits, vegetables and tea are associated with a reduced risk of cancer, particularly cancers of epithelial origin, such as those of the mouth, colon, rectum, lung and breast (19,20). Dietary flavonoids, a group of polyphenolic compounds, have been identified as potential cancer-preventive components of plant food. Bioactive components in botanicals and herbal medicines may provide effective and safe candidates for the chemoprevention of cancer (21,22). Ampelopsin [AMP, 3,5,7,3′,4′-5′-hexahydroxy 2,3 dihydroflavonol], which is also referred to as dihydroxyricetin, is one of the most common dietary flavonoids isolated from the tender stem and leaves of the plant species Ampelopsis grossedentata (Hand-Mazz) WT. Wang. This species is widely distributed and consumed as a type of tea in Southern China and is also used to treat the common cold and tinea corporis. More than 27% of the AMP in these plants resides in the tender stem and leaves, and >40% is found in the cataphyll. AMP is reported to possess some important pharmacological activities, such as anti-inflammatory, cough relief and hepatoprotection (23,24). Moreover, recent studies showed that AMP possesses certain anti-cancer activities that include inhibiting the growth and metastasis of prostate cancer and melanoma cells (25). However, it is unclear whether AMP possesses effective chemopreventive activities against mammary carcinogenesis. In the present study, we evaluated the chemopreventive effect of AMP on breast carcinogenesis in vivo and in vitro and investigated the inhibitory effect and mechanism of AMP on the mTOR signalling pathway.

Materials and methods

Chemicals and reagents

AMP was purchased from Mansite Bio-technology Co (Chengdu, China); DMEM/F12 medium and fetal bovine serum were purchased from HyClone (Beijing, China); Trizol reagent, horse serum, gentamicin, insulin, Lipofectamine 2000, Opti-Mem were purchased from Invitrogen (Carlsbad, CA); Epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ); K-LISA mTOR activity assay kit was purchased from Calbiochem (San Diego, CA); and PathScan Phospho-Akt ELISA assay kit and all antibodies were purchased from Cell Signaling Technology (Danvers, MA). Other chemicals and reagents were of the highest purity obtainable and were used directly without further purification. Flavonoids were isolated from the leaves of Ampelopsis grossedentata and then purified by silica-gel column chromatography (26). The characteristics of AMP are as follows: the molecular weight of AMP is 275.2, with 12.8% of its mass being composed of nitrogen and its main peaks are at 292.1, 295.1 and 313.1 by ESI-MS/MS. The results of NMR and HPLC are consistent with those of the previous literature (27). These data indicate that AMP is a flavonoid with a structure similar to quercetin (28). The present study, we evaluated the chemopreventive effect of AMP on breast carcinogenesis in vivo and in vitro and investigated the inhibitory effect and mechanism of AMP on the mTOR signalling pathway.
were purchased from Cell Signaling Technology (Danvers, MA). 1-methyl-1-nitrosourea (MNU), 3,4-(5,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4-[(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK), benz[a]pyrene (BaP), cholera enterotoxin, hydrocortisol, protein A/G-agarose, dimethylsulfoxide (DMSO), phosphate-buffered saline (PBS) and other chemicals were purchased from Sigma–Aldrich (St Louis, MO). All cell lines were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and the authentication of all cell lines was confirmed by them.

**Animals**

Female Sprague-Dawley rats (aged 42–48 days, 145–165 g) and BALB/c nude mice (aged 42–48 days, 15–20 g) were obtained from the Medical Experimental Animal Centre of the Third Military Medical University [SCXK (army)-2007-015]. These animals were bred and maintained in accordance with our institutional guidelines for the use of laboratory animals. The animal rooms were maintained at 25°C with 50% relative humidity and a 12 h light/12 h dark cycle. All of the animal procedures were approved by the Animal Ethics Committee of the Third Military Medical University.

**HPLC analysis of AMP in rats**

Twelve female Sprague-Dawley rats were acclimatized to an AIN-93G diet for 3 days and randomly divided into two groups: control (n = 3) and AMP treatment group (n = 9). The rats in the AMP treatment group were intragastrically (i.g.) fed 100 mg/kg AMP, and the rats in the control group were i.g. fed the vehicle alone (normal saline). After treatment for 0.5, 1.0 and 2.0 h, three rats in each AMP treatment group were killed, and the rats in the control group were killed at 1.0 h. The plasma of the rats was collected and analysed by high-performance liquid chromatography (HPLC). The plasma sample (1.0 mL) was added with 5 μl β-glucuronidase (≥ 2000 units/ml), 5 μl sulfatase (≥ 28500 units/ml) and 1.5 ml sodium acetate solution (0.2 M), and then incubated in water for 30 min at 37°C. The mixed solution was added with 5.0 ml ethyl acetate (0.2 M), and centrifuged (3000 rpm) for 10 min at 4°C. The supernatant solution was dried under reduced pressure for 5 h at 40°C. The residue was dissolved with methanol and treated with 0.45 μm membrane filtration, and then analysed by HPLC. HPLC analyses were carried out on a Waters 1525 series liquid chromatography system equipped with a dual UV absorbance detector. The analyses were performed with a Hyper OD S2 C18 column (4.6 mm × 50 mm, 1.8 μm) with 5.0% ethanolic acid (A) and methanol (B) as the mobile phase. Elution started at 15% (B), increasing to 67% within 40 min at a flow rate of 1.0 mL/min. AMP was quantified based on the peak area of the respective standard curves measured at 290 nm. Typically, 10 μl was injected for the analyses.

**Cell line authentication**

Cell lines used in this study including human breast epithelial cell MCF10A, human breast cancer cell MCF7-7 and MDA-MB-231, were all purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) 22 May 2013. All cell lines have been tested and authenticated by DNA (short tandem repeat genotyping) profiling. The last test was performed in 12 March 2013.

**mTOR kinase assay and p-Akt ELISA**

The activities of mTOR and p-Akt (S473) were measured with a colorimetric K-LISA mTOR activity assay kit and a PathScan Phospho-Akt (S473) ELISA kit, respectively. Briefly, 10^5 cells were washed twice with Tris-buffered saline, and the cell lysates were prepared by the addition of 1 ml of lysis buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol (wt/vol), 1% Tween-20 detergent (wt/vol), 1 mM EDTA, 20 mM microcystin-LR, 25 mM NaF, and a cocktail of protease inhibitors). For the mTOR activity assay, the mTOR proteins in the cells were immunoprecipitated with mTOR antibody and protein A/G-agarose. The resulting immunocomplex and adenosine triphosphate were added to S6K70-coated wells. After the phosphorylation of S6K70 at T389 by the active mTOR in the sample, the phosphorylated substrate can be detected with anti-S6K70-T389 antibody. The mTOR activity can be measured in terms of its absorbance using a microplate reader. For the mTOR inhibitor testing, 50 μl of mTOR standard (provided by the kit) was incubated with different concentrations of AMP (0, 10, 25 and 50 μM) on ice for 20 min, and the mixtures were then transferred to the S6K70-coated wells. The remaining steps of the protocol are the same as those mentioned above. For the p-Akt assay, the p-Akt(S473) proteins in the cell lysate were captured by the corresponding antibody, which was coated in the microplate. After the addition of the horseradish peroxidase-linked secondary antibody and chemiluminescence substrate, the magnitude of the light emission, which is proportional to the quantity of p-Akt(S473) protein, was measured.

**Western blot analysis**

Cell lysates were prepared using radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with protease and phosphatase inhibitors. Equal amounts of cellular proteins were resolved by electrophoresis in 10% or 12% sodium dodecyl sulfate-polyacrylamide gels for western immunoblotting with specific antibodies. The antigen–antibody complexes on the filters were detected by chemiluminescence.

**Docking study**

The blind docking of AMP to the mTOR target was performed with Autodock 4 by setting grid sizes that included the entire mTOR molecule. The receptor site was prepared with Sybyl (Tripos, St Louis, MO) using the NMR structure 2NPU model 1 from the Protein Data Bank (www.pdb.org). The grid size for the docking site was expanded to include the entire mTOR molecule, and AMP was docked.

**Immunoprecipitation**

Treated cells were lysed in ice-cold lysis buffer (40 mM HEPES, pH 7.4, 2 mM EDTA, 10 mM β-glycerophosphate, 0.3% (wt/vol) CHAPS and protease...
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We first detected the bioavailability of AMP in rats by HPLC analysis. The results are presented as the means ± SD from at least three independent experiments. The tumour incidences were compared using the χ² test. The other data were analysed by one-way analysis of variance followed by Tukey’s test for multiple comparisons. The difference significance was set at P < 0.05.

Results

Chemopreventive effects of AMP against breast carcinogenesis in rats

We first detected the bioavailability of AMP in rats by HPLC analysis. Our study showed that the serum concentration of AMP in rats 0.5, 1.0 and 2.0 h after the oral administration of AMP (100 mg/kg, i.g.) was 1.3 ± 0.8, 11.4 ± 2.7 and 7.6 ± 1.7 μg/ml, respectively.

Using the classical experimental model of MNU-induced breast carcinogenesis in rats, we then evaluated the chemopreventive effects of AMP in vivo. As shown in Table I, the tumour incidence in the AMP administration group (100 mg/kg) was significantly lower than that observed in the control. The cancer incidence was decreased by 37.4%, indicating that the oral administration of AMP can effectively suppress MNU-induced mammal breast carcinogenesis. The histopathological sections of breast tissue and tumours showed evidence of carcinogenesis (Figure 1A). No evidence of adverse effects was observed in the rats that were administered AMP orally, and these animals grew at the same rate as the controls.

Table I. Effect of AMP on tumorigenesis endpoints and body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats in all</th>
<th>Number of rats with tumour</th>
<th>Incidence</th>
<th>Multiplicity (average number/rat)</th>
<th>Latency (d)</th>
<th>Final body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>281.3 ± 5.1</td>
</tr>
<tr>
<td>MNU only</td>
<td>16</td>
<td>9</td>
<td>56.2%</td>
<td>1.7 ± 0.6</td>
<td>60.4 ± 10.5</td>
<td>270.2 ± 6.4</td>
</tr>
<tr>
<td>MNU plus 50 mg/kg AMP</td>
<td>16</td>
<td>5</td>
<td>31.2%</td>
<td>1.6 ± 0.7</td>
<td>73.2 ± 5.0</td>
<td>276.4 ± 5.7</td>
</tr>
<tr>
<td>MNU plus 100 mg/kg AMP</td>
<td>16</td>
<td>3*</td>
<td>18.8%*</td>
<td>1.3 ± 0.4</td>
<td>80.7 ± 7.5*</td>
<td>273.6 ± 5.6</td>
</tr>
</tbody>
</table>

Values are means ± S.D. The tumour incidence in MNU plus 100 mg/kg AMP treatment group were significantly lower than that in MNU only treatment group, indicating that oral administration of AMP (100 mg/kg/day) can effectively suppress MNU-induced mammal breast carcinogenesis. *P < 0.05 compared with MNU only treatment group.
Fig. 1. AMP suppresses breast carcinogenesis and inhibits the growth of breast cancer cells in vitro and in vivo. (A) Histopathological sections of breast tissue (0, 2, 8 w) and tumours (18 w) in MNU-treated rats stained with haematoxylin and eosin. (B) Photographs of xenografted MDA-MB-231 cells in athymic mice at the end of the 28-day treatment period. (C) Viability of MCF10A, MCF-7 and MDA-MB-231 cells treated with different concentrations of AMP for 48 h. The cell viability was detected through the MTT assay. (D) AMP administration (100 mg/kg, i.g.) significantly inhibits MDA-MB-231 cell growth in athymic mice. The data are presented as the means ± SD (n = 6). *P < 0.05 and **P < 0.01 compared with the control.

Fig. 2. AMP suppresses environmental carcinogen-induced chronic cellular breast carcinogenesis. (A) Results of the RDGF assay in MCF10A cells, cells treated with carcinogens (CarT) for 30 days, and cells co-treated with carcinogens and AMP (C-AMP) for 30 days. (B) Results of the AIG assay in MCF10A cells, 30-day CarT and 30-day C-AMP cells. (C) Scratch/wound healing assay in MCF10A cells, 30-day CarT cells, and 30-day C-AMP cells. The arrows indicate the width of the wounded areas. The data are presented as the means ± SD (n = 3). *P < 0.05 and **P < 0.01 compared with MCF10A cells; *P < 0.05 and **P < 0.01 compared with CarT cells.
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Ampelopsin suppresses breast carcinogenesis in a time-dependent manner, confirming the carcinogen-induced activation of mTOR. AMP co-treatment can potently suppress the activation of mTOR in cellular carcinogenesis. Accordingly, the results (Figure 3D) showed that AMP treatment also suppresses mTOR activation in MDA-MB-231 cells. Additionally, the immunohistochemistry detections of p-mTOR(S2448) in breast tissues and tumors showed that AMP administration can significantly inhibit the activation of mTOR in MNU-treated rats (Figure 4A) and in xenografted breast tumors (Figure 4B) in vivo.

We then performed mTOR inhibitor testing of AMP, and the data (Figure 3E) showed that AMP is an effective mTOR inhibitor that can significantly inhibit mTOR activity in a dose-dependent manner. We also performed blind docking of AMP to the mTOR target using Autodock4 by setting grid sizes that included the entire mTOR molecule. The results (Figure 3F) indicated that AMP binds to one site on the mTOR target in two ways with binding energies of −7.1 and −7.0 kcal/mol, respectively.

AMP represses the activation of Akt in carcinogenesis and in cancer cells

Given the intimate cross-talk between mTOR and Akt, we investigated the effects of AMP on Akt activation. Western blots (Figure 5A) showed that the levels of total Akt and p-Akt(T308, S473), particularly the ratio of p-Akt to Akt, in CarT cells was increased in a time-dependent manner, indicating that Akt is activated in carcinogenesis. The p-Akt enzyme-linked immunosorbent assay results (Figure 5C) confirmed the significant promotion of Akt activity in CarT cells. The results showed that AMP co-treatment can effectively suppress Akt activation in cellular carcinogenesis. AMP treatment also significantly decreased Akt activity in MDA-MB-231 cells in a dose-dependent manner (Figure 5B and D). Furthermore, the immunohistochemistry detections of p-Akt(T308) in MNU-treated rats (Figure 4C) and in xenografted tumors (Figure 4D) showed that AMP administration inhibits the activation of Akt in vivo.

AMP suppresses the formation of mTORC1/2 and downstream targets of mTOR

We performed immunoprecipitation experiments combined with a western blot assay to determine the effects of AMP on the formation of mTOR complexes (mTORC)1/2. First, the western blot results (Figure 6A and B) showed no apparent difference in the levels of mTOR, regulatory-associated protein of mammalian target of rapamycin (raptor), rapamycin-insensitive companion of mammalian target of rapamycin (rictor) and mLST8 between 30-d CarT and 30-d C-AMP cells. Similarly, AMP treatment showed no effects on the expression of these proteins in MDA-MB-231 cells. Further study (Figure 6C and D) showed that significantly lower amounts of raptor and rictor but not of mLST8 were isolated with an mTOR antibody in 30-d C-AMP and AMP-treated MDA-MB-231 cells compared with
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CarT and the control, respectively. These data indicate that AMP disrupts mTORC1/2 formation by dissociating raptor/rictor from mTOR instead of decreasing protein expression.

Moreover, we assessed the effects of AMP on the downstream targets of mTOR. Our study showed that the levels of p-S6K70(T389 and T421), p-rpS6(S235/236), p-eIF4B(S422) and p-4EBP1(S65) were all significantly decreased in 30-d C-AMP (Figure 6E and F) and AMP-treated MDA-MB-231 cells (Figure 6F and G) compared with CarT and the control, respectively. These data indicate the substantial suppressive effects of AMP on the downstream targets of mTOR in carcinogenesis and in cancer cells.

Discussion

Previous studies have suggested that more than 70% of sporadic breast cancers are attributable to long-term exposure to environmental factors, particularly various chemical carcinogens, and this chronic pathological process involves accumulated genetic and epigenetic alterations.
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μ
37
38
 μ
33
 μ
34
 μ
35
 C-AMP (50 μM AMP) cells. (D) Effect of AMP treatment (50 μM) for 24 h on mTORC1/2 formation in MDA-MB-231 cells detected by immunoprecipitation. (E) Phosphorylation of S6K70, rpS6, eIF4B and 4EBP1 in 30-day CarT and 30-day C-AMP (50 μM AMP) cells. (F) Effect of AMP treatment for 24 h on the phosphorylation of S6K70, rpS6, eIF4B and 4EBP1 in MDA-MB-231 cells. (G) Immunofluorescence analysis of p-4EBP1(S65) in MDA-MB-231 cells treated with different concentrations of AMP for 24 h.

Fig. 6. AMP suppresses the formation of mTORC1/2 and downstream targets of mTOR. (A) Levels of mTOR, raptor, rictor and mLST8 in 30-d CarT and 30-day C-AMP (50 μM AMP) cells, as detected by western blots. (B) Effects of AMP treatment for 24 h on the expression levels of mTOR, raptor, rictor and mLST8 in MDA-MB-231 cells. (C) Results of the combination of immunoprecipitation (using mTOR antibody) and western blot assays in 30-d CarT and 30-day C-AMP (50 μM AMP) cells. (D) Effect of AMP treatment (50 μM) for 24 h on mTORC1/2 formation in MDA-MB-231 cells detected by immunoprecipitation.

Similar to endocrine therapy, cancer cells can also develop resistance to mTOR inhibitors through a negative feedback loop in which mTOR inhibition leads to Akt activation (37,38). Indeed, mTOR and Akt exhibit an intimate cross-talk for mutual promotion. On the one hand, mTOR functions downstream of the PI3K/Akt pathway and is phosphorylated in response to stimuli that activate Akt. On the other hand, Akt is also a direct target of mTORC2, which contributes to its activation. Therefore, it is important for cancer prevention and therapy to dually inhibit the mTOR pathway and Akt activation. Rapalogs specifically inhibit mTORC1 and hardly affect mTORC2, which leads to Akt activation and drug resistance. Our findings revealed that AMP can repress the aberrant expression and activation of Akt, which may contribute to the suppression of the mTOR signalling pathway in breast cancer and in carcinogenesis. In addition, AMP also inhibits the formation of mTORC2, negating one of the disadvantages of rapalog treatments, which is the negative feedback activation of Akt. Additionally, their potential toxicities, such as pneumonitis, mucositis and metabolic changes, limit the benefits of mTOR inhibitors in cancer therapy. Therefore, our findings suggest that AMP, a natural dietary compound, may serve as a useful supplement for improving the efficacy of mTOR inhibitors in breast cancer treatment.

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