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Tylophorine arrests carcinoma cells at G1 phase by downregulating cyclin A2 expression

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ABSTRACT

Tylophorine, a representative phenanthroindolizidine alkaloid from *Tylophora indica* plants, exhibits anti-inflammatory and anti-cancerous growth activities. However, the underlying mechanisms of its anti-cancer activity have not been elucidated and its effects on cell cycle remain ambiguous. Here, we reveal by asynchronizing and synchronizing approaches that tylophorine not only retards the S-phase progression but also dominantly arrests the cells at G1 phase in HepG2, HONE-1, and NUGC-3 carcinoma cells. Moreover, tylophorine treatment results in down regulated cyclin A2 expression and overexpressed cyclin A2 rescues the G1 arrest by tylophorine. Thus, we are the first to report that the downregulated cyclin A2 plays a vital role in G1 arrest by tylophorine in carcinoma cells.

Introduction

Tylophorine belongs to the small group of plant natural compounds, phenanthroindolizidine alkaloids, which have recently been exploited as potential cancer therapy drugs because of their high potency against various cancer cell lines [1–10]. In the 1960s, trials of tylocrebrine as an anti-cancer agent were discontinued because of central-nervous-system side effects [11]. Therefore, effort has been invested in molecular pharmacology studies [8,12,13] and improving the physical properties, such as polarity and hydrophilicity, of this group of compounds to avoid drug transfer through the blood–brain barrier [2,14].

Cyclin-dependent kinases (CDKs) and cyclins play critical roles in cell cycle regulation [15,16]. Many anti-cancer agents derived from natural products exhibit growth inhibitory activity in carcinoma cells through cell cycle regulation. For instance, paclitaxel inhibits microtubule disassembly, and butyrolactone inhibits CDK activity; both cause cell cycle arrest at the G2/M phase [17,18]. Antofine, a tylophorine analogue, arrests the cell cycle at the G2/M phase in Col2, human colon cancer cells [19]. DCB3500 (tylophorine) and DCB3503 (a tylophorine analogue) cause S-phase accumulation of KB cells but have no effect on specific cell cycle arrest in HepG2 cells [8]. DCB3503's effect on cell cycle progression was further examined in PANC-1 cells [13] and was suggested to be through downregulated cell cycle regulatory proteins. However, more information on specific interactions and relations is needed to elucidate substantial links between phenanthroindolizidine alkaloids and anti-cancer-cell growth and cell cycle interference.

Here, we unravel the effect of tylophorine on cancer cell cycle specific interference and the involvement of downregulated cyclin A2 in anti-cancer cell growth mechanisms of tylophorine.

Materials and methods

Cell culture and reagents. HepG2 (HB-8065; ATCC), HONE-1 [20] (a gift from Dr. Ching-Hwa Tsai at National Taiwan University, Taiwan, ROC) and NUGC-3 cells (the Japanese Cancer Research Resources Bank) were maintained in DMEM (Hyclone Inc.) supplemented with 10% heat inactivated fetal calf serum (Biological Industries Inc.) and penicillin–streptomycin (BioSource, Invitrogen) in a 5% CO_2 atmosphere at 37 °C in a humidified incubator. Tylophorine was prepared as described, as was dehydro-tylophorine, with modification [21]; dehydro-tylophorine was prepared by reacting tylophorine with *N*-bromosuccinimide and then exposure under light. The final product was verified by liquid chromatography/mass spectrometry (Agilent Technologies), with no

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tylophorine detected (data not shown). HPLC-degree dimethyl sulfoxide (DMSO), nocodazole, thymidine, propidium iodide and BrdU were purchased from Sigma–Aldrich.

Cell synchronization procedures were as follows. For double-thymidine blocking, cells growing exponentially were incubated with 2 mM thymidine-containing DMEM complete medium for two sequential 18 h periods, with a 10 h recovery period without thymidine between the 18 h periods. For thymidine-nocodazole blocking, cells growing exponentially were incubated with 2 mM thymidine-containing complete medium for 18 h, then the medium was replaced with one containing 50 ng/ml nocodazole for 8 h incubation before compound treatment. For serum starvation, cells were cultured in medium containing 0.5% FCS for 48 h before compound treatment in DMEM complete medium.

Cell growth inhibition assay. Concentrations for 50% growth inhibitory effect, GI_{50} values, measured for HepG2, HONE-1 and NUGC-3 cells were determined as described [22]. For drug removal experiments, HepG2, HONE-1 and NUGC-3 cells were seeded at 5000, 2500, and 3000 cells/well respectively in 24-well plates. After 24 h, cells were treated with drugs for another 24 h. The drug-containing medium was then removed (day 0), and cells were incubated in drug-free medium for another 1–8 days. At the end of each incubation period, the number of viable cells was estimated by MTS assay [22].

Western blot analysis. Whole cell lysates were prepared with use of PRO-PREP™ protein extraction solution (iNtRON Biotechnology) and quantitated by BCA protein assay (Pierce Biotechnology). Equal amounts of protein per lane were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against cyclin A2, cyclin B1, Cdk1 (Santa Cruz Biotechnology), p27, cyclin E, Cdk2 (Abcam), Cdk4 (Cell Signaling), tubulin (Amersham Life Science), and horseradish peroxidase-conjugated secondary antibodies (PerkinElmer). Detection of antigen–antibody complexes involved ECL detection reagents (Western Blot Chemiluminescence Reagent Plus, PerkinElmer), according to the manufacturers' instructions.

Plasmid construction, transfection and luciferase assay. The human cyclin A2 was amplified from the MGC 132447 clone (Open Biosystems) by use of 5′-CGAATTCCGATGTTGGGCAACTCTGCG-3′ and 5′-GCCTCGAGTTACAGATTTAGTGTCTCTGGTGG-3′, then subcloned into EcoRI/XhoI sites of pCMV-myc (Clontech) to generate the CCNA2-pCMV-myc expression vector. The human cyclin A2 promoter region (fragment from −215 to +245) was amplified from a BAC clone RP11-63B12 (Invitrogen) by PCR and subcloned into a SacI/SmaI site of the pGL3basic luciferase reporter vector (Promega) to create a cyclin A2 promoter-driven luciferase reporter gene construct, pCCNA2-pGL3basic. The sequences of all constructs were verified by DNA sequencing. FuGene 6™ (Roche) and the Steady-Glo luciferase assay system (Promega) were used for transient transfection and luciferase activity assay according to the manufacturers' instructions [12]. The luciferase activity was normalized to protein concentration and presented as relative activity compared to vehicle (0.1% DMSO) control.

Cell cycle analysis. Cells were trypsinized, washed twice with PBS and fixed in 70% ethanol at 4 °C for at least 1 h. Fixed cells were centrifuged and resuspended in 50 μg ml^{-1} RNase and 10 μg ml^{-1} propidium iodide for DNA staining at 37 °C for 1 h. DNA content was measured by use of a FACS flow cytometer (BD Biosciences). DNA histograms were obtained by use of ModFit LT 3.1 (Verity Software House, Topsham, ME). Cell aggregates were gated by analysis, and each profile was compiled from at least 10,000 gated events.

For cyclin A2-myc over-expression experiments, HONE-1 cells were transfected with pCMV-myc or CCNA2-pCMV-myc expression vectors the day before treated with DMSO or 2 μM tylophorine in the presence of nocodazole (25 ng/ml) for further 24 h, respectively. After compound treatment, cells were harvested and fixed by 70% ethanol, then incubated with the monoclonal anti-myc tag antibody (Santa Cruz Biotechnology) in staining solution (1% BSA, 0.1% Triton X-100 in PBS) for 1 h at RT. After being stained, cells were washed by staining solution twice and resuspended in staining solution containing FITC-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) and incubated at RT for 30 min. After being washed, the resulting cells were stained with propidium iodide for DNA as mentioned previously. The FITC signal and propidium iodide were detected in FL-1 and FL-2 channels, respectively, by BD FACS flow cytometry. More than 10,000 FITC-positive cells were gated for cell cycle analysis.

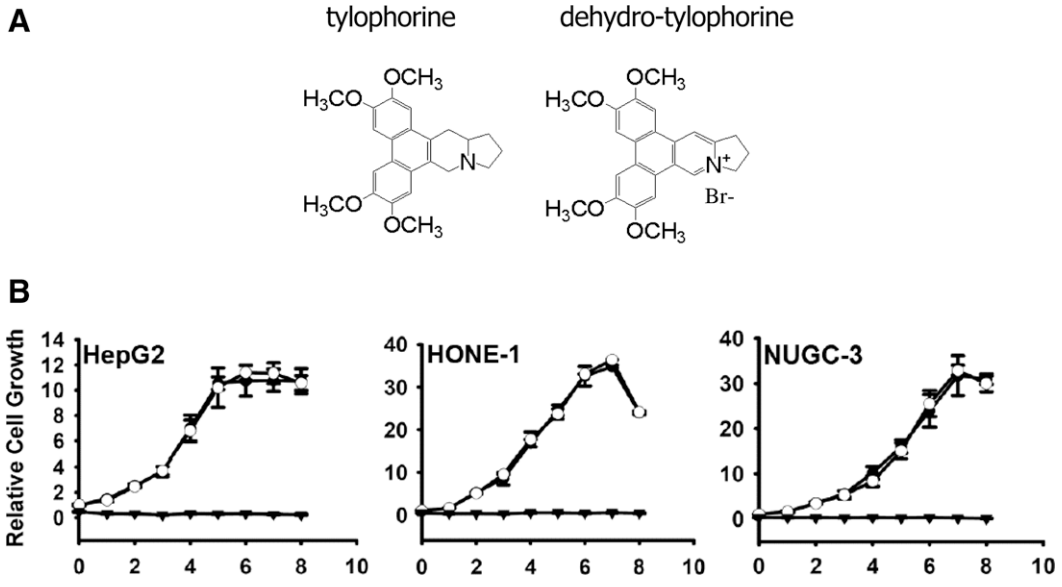

Fig. 1. Effect of tylophorine and dehydro-tylophorine on the growth of carcinoma cells. (A) Chemical structures of synthesized tylophorine and dehydro-tylophorine. (B) Tylophorine irreversibly inhibited the growth of carcinoma cells. Cells were seeded one day before treatment with vehicle (0.2% DMSO, (●), 2 μM dehydro-tylophorine (○), or 2 μM tylophorine (▼) for 24 h. The drug-containing medium was then removed (day 0), and the cells were incubated in drug-free medium for another 1–8 days. Data are representative of three independent experiments and are means ± SD of triplicate samples.