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ORIGINAL ARTICLE

Clinically acceptable colchicine concentrations have potential for the palliative treatment of human cholangiocarcinoma

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Abstract Microtubules are an ideal target for anticancer drugs because of their essential role in mitosis. Colchicine is a microtubule destabilizer. Whether the clinically acceptable colchicine concentrations had anticancer effects on human cholangiocarcinoma cells was investigated. Two human cholangiocarcinoma cell lines (C14/KMUH, C51/KMUH) were investigated using clinically acceptable plasma colchicine concentrations (2 ng/mL and 6 ng/mL for the *in vitro* experiment, 0.07 mg colchicine/kg/d × 14 days for the nude mouse experiment). Our results showed that colchicine caused significantly dose-dependent antiproliferative effects on both cell lines (all $p < 0.0001$). Nude mouse (BALB/c-nu) experiments showed that the increased tumor volume ratios in colchicine-treated mice were significantly lower than control mice started from the 11th day of treatment ($p = 0.0167$). The tumor growth rates in colchicine-treated mice after 14 days of treatment were significantly lower than in control mice (0.147 ± 0.004/d vs. 0.274 ± 0.003/d, $p = 0.0015$). In addition to the well-known direct colchicine–tubulin interaction as a common anticancer mechanism of colchicine, microarray and quantitative reverse transcriptase-polymerase chain reaction showed that the antiproliferative effects of both 2 ng/mL and 6 ng/mL colchicine on C14/KMUH cells could be partially explained by downregulations of both *HSD11B2* and *MT-COI*. There was no effect of colchicine

Conflicts of interest: All authors declare no conflicts of interest.

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on *MT-COI* expression in C51/KMUH cells, however, 6 ng/mL colchicine also downregulated *HSD11B2* in this cell line. In conclusion, clinically acceptable colchicine concentrations can inhibit the proliferation of human cholangiocarcinoma cells. This drug has good potential for the palliative treatment of cholangiocarcinoma due to its low cost and our long-standing prescription experience.

Introduction

Cholangiocarcinoma (CC) is one of the most difficult-to-treat intra-abdominal malignancies. Although surgical resection of the tumor and liver transplantation may provide the opportunity for long-term survival, these modalities can only be applied in a small number of patients [1]. For patients unable to receive a surgical approach, several palliative treatments including radiotherapy, chemotherapy, and photodynamic therapy have been applied but the effects were limited [2].

The common characteristic of cancer cells is increased rate of mitosis, which means that cancer cells are more vulnerable to mitotic poison than are normal cells. Microtubules have long been considered as an ideal target for anticancer drugs because of their essential roles in mitosis and forming the dynamic spindle apparatus. Colchicine is a cheap alkaloid agent that has been used in medicine for a very long time [3—5]. It is a microtubule destabilizer that has a very strong binding capacity to tubulin to perturb the assembly dynamics of microtubules [6—9]. It also can increase cellular free tubulin to limit mitochondrial metabolism in cancer cells through inhibition of the voltage-dependent anion channels of the mitochondrial membrane [10]. The cost of colchicine is much more affordable than other microtubule-interfering agents such as paclitaxel and docetaxel. Moreover, oral intake of colchicine is a very convenient and safe treatment when it is appropriately used and contraindications have been excluded [3—5]. The peak plasma concentrations after oral administration of 0.6—1 mg colchicine range from approximately 2 ng/mL to 6 ng/mL [11—13]. The lowest reported lethal doses of oral colchicine are 7—26 mg and acute ingestions of colchicine exceeding 0.5 mg/kg have a high fatality rate [5]. Our recent *in vitro* and *in vivo* experiments showed that the clinically acceptable colchicine concentrations had significantly dose-dependent anticancer effects on hepatocellular carcinoma (HCC) cells [14]. The anticancer effects of colchicine on HCC cells originated not only from the well-known direct colchicine—tubulin interaction [6—10] but also from colchicine-induced differential expressions of several antiproliferative genes [14]. Whether the clinically acceptable colchicine concentrations also had significant anticancer effects on CC cells is still unknown. This study was done to clarify this issue for the investigation of the potential role of colchicine in the palliative treatment of CC. All gene names are according to the official symbols from the HUGO Gene Nomenclature Committee provided by the US National Center for Biotechnology Information.

Materials and methods

Cell lines

Two human CC cell lines (C14/KMUH, C51/KMUH) established by our institution were investigated. The detailed methodology for the establishment of these cell lines was described in our previous study [15]. These cell lines were verified using positive for periodic acid-Schiff stain and stain for cytokeratin 19 but negative for monoclonal mouse antihuman hepatocyte antigen. All procedures to establish these cell lines were approved by the Institutional Review Board of our hospital, and patients provided informed consent. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The serum-containing culture medium consisted of 10% fetal bovine serum, 90% Dulbecco Modified Eagle high glucose medium, supplemented with 20mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, Utah, USA). Colchicine was purchased from Sigma-Aldrich Corporate (St. Louis, MO, USA).

Proliferative experiment

Each cell line seeded in a 96-well culture plate was incubated with serum-containing medium for 24 hours. Next, the medium was replaced with serum-free medium with various concentrations of colchicine (0 ng/mL, 2 ng/mL, and 6 ng/mL). The cells were incubated for an additional 72 hours for proliferative assay. The premixed WST-1 cell proliferation reagent (Clontech Laboratories, Inc., A Takara Bio Company, Mountain View, CA, USA) was applied. The principle for this examination is that the stable tetrazolium salt WST-1 is cleaved to a soluble formazan by viable cells. The amount of formazan dye formed detected by the spectrophotometer expressed as optical density directly correlates to the number of metabolically active cells in the culture. The experimental procedures were carried out following the manufacturer's protocols. The cells were incubated with reagent for 3 hours at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Then, the results were analyzed using an automated microplate reader (MRX, Dynex Technologies, Inc., Chantilly, VA, USA). Absorbance was measured at a wavelength of 450 nm (reference wavelength 630 nm). In all experiments, 16 replicate wells were used for statistical calculation.

Microarray and quantitative reverse transcription-polymerase chain reaction experiments

Each cell line was seeded in three 25-cm^2 plastic culture flasks with serum-containing medium for 24 hours. Then, the medium was replaced with serum-free medium with various concentrations of colchicine (0 ng/mL, 2 ng/mL, 6 ng/mL). The cells were incubated for a further 24 hours. Total RNA in each flask was extracted using Trizol Reagent (Invitrogen, Life Technologies Corporation, Grand Island, NY, USA), and followed by RNAeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Purified RNA was quantified by OD260 nm using a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and qualified using Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). To investigate genes with consistently differential expressions caused by different concentrations of colchicine, two microarrays (Agilent SurePrint G3 Human GE 8 $\times$ 60 k, Agilent Technologies) were applied for C14/KMUH (6 ng/mL colchicine vs. control without colchicine) and C51/KMUH (6 ng/mL colchicine vs. control 2 ng/mL colchicine) cells, respectively. Microarray experimental procedures were carried out following the manufacturer's protocols and the criteria for the selection of differentially expressed genes were the same as in our previous studies [16,17]. For quantitative reverse transcription-polymerase chain reaction (RT-PCR) study, specific oligonucleotide primer pairs were selected from Roche Universal ProbeLibrary (Roche Diagnostics Ltd., Taipei, Taiwan). The procedures for real-time PCR reactions and the calculation of the fold expression or repression of the target gene were the same as in our previous studies [16,17]. The housekeeping gene *TBP* (TATA box binding protein) was used as a reference gene. Two genes were selected for quantitative RT-PCR study. The PCR primers used were 5′-CTTCAAGACAGAGTCAGTGAGAAAC-3′ sense primer and 5′-CCAGGCGTAGCGAGTGC-3′ antisense primer for *HSD11B2*, 5′-AGCAGGAACAGGTTGAACAGTC-3′ sense primer and 5′-AGGAGAAGATGGTTAGGTCTACGG-3′ antisense primer for *MT-COI*, and 5′-CAATTTAGTAGTTATGAGCCAGAG-3′sense primer and 5′-TTCTGCTCT GACTTTAGCAC-3′ antisense primer for *TBP*.

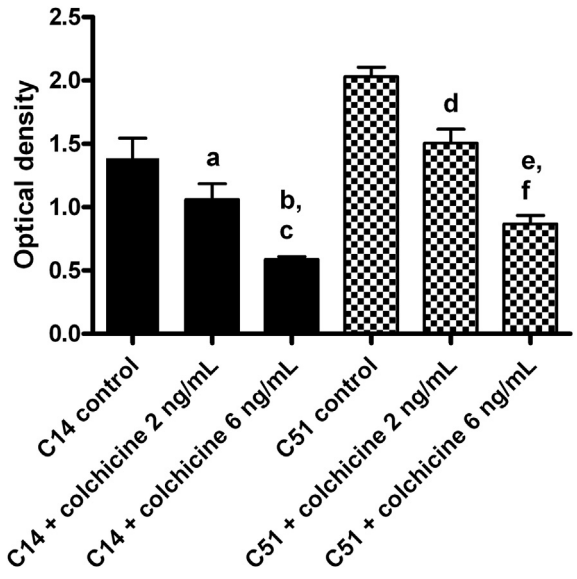

Figure 1. Antiproliferative effects of colchicine on two human cholangiocarcinoma cell lines (C14/KMUH and C51/KMUH). The premixed WST-1 cell proliferation reagent was applied for investigation. The absorbance was measured at a 450-nm wavelength (reference wavelength 630 nm). In all experiments, 16 replicate wells were used to determine the mean and the standard deviation (SD). The unpaired two-tailed *t* test was applied for statistical analysis. Bars indicate SD. a, b, c, d, e, f: $p < 0.0001$. a, d: 2 ng/mL colchicine versus control; b, e: 6 ng/mL colchicine versus control; c, f: 6 ng/mL colchicine versus 2 ng/mL colchicine.

Nude mouse experiment

This study was approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University (Kaohsiung, Taiwan). All mice were kept in the Experimental Animal Center of our hospital. The feeding process was carried out by a qualified staff member using a syringe connected with a feeder. Fourteen male nude mice (BALB/c-nu) purchased from The Taiwan Laboratory Animal Center of National Health Research Institutes, Taipei City, Taiwan were equally divided into the control and two treatment groups. A total of 5 $\times$ 10^6 C14/KMUH cancer cells suspended in 0.2 mL serum-containing culture medium were injected subcutaneously into the flank of each mouse. When tumors reached a diameter of 4–5 mm in the largest dimension, each mouse in the treatment group was continuously fed with 0.07 mg colchicine/kg dissolved in phosphate-buffered saline once/d for 14 days. All mice were sacrificed at the 15th day after the start of colchicine treatment. The tumor volumes were calculated using the formula: length $\times$ width^2 $\times$ 0.5 [18]. The increased tumor volume ratio was calculated as follows: tumor volume at day x (V_x) divided by baseline pretreatment tumor volume (V_0).The tumor growth rates were calculated using the formula: $\ln(V_0-V_{14})/(t_0-t_{14})$, where V_0 and V_{14} are tumor volumes at the start of treatment and at the 14th day of treatment, respectively, and t is the day for the measurement of tumor volume [19]. All tumors were immediately fixed in 24% formalin after sacrifice of the mice. Then, all tumors were embedded in paraffin within 24 hours for further pathological study. Serial sections at a distance of 3 mm for all tumors were performed to detect the percentage of tumor necrosis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA). Results are given as mean values $\pm$ standard deviations (SD) or standard errors of the mean. An unpaired two-tailed *t* test was used to analyze the significance of any difference between two means. Statistical significance was defined as $p < 0.05$.

Results

Proliferative experiment

Colchicine significantly inhibited the cellular proliferation of both cell lines (Fig. 1). The antiproliferative effects of colchicine on both cell lines were dose-dependent. The inhibitory effects of 2 ng/mL and 6 ng/mL colchicine on cellular proliferation were: (1) 23.2% and 57.2% for C14/KMUH cells; and (2) 25.6% and 57.1% for C51/KMUH cells, respectively.

Microarray and quantitative RT-PCR analyses

Colchicine at a concentration of 6 ng/mL caused upregulations of nine genes (*ASAP1-IT1*, *CCDC40*, *KIAA0485*, *LOC401497*, *MAGI2-IT1*, *MGC24103*, *ODF3L1*, *RN7SL1*, and *TAF15*) and downregulations of 21 genes (*ACTRT2*, *AOC2*, *C1QTNF5*, *DLGAP1-AS2*, *DUSP9*, *ECEL1P2*, *EFCC1*, *GRHL3*, *HLA-DPB2*, *HSD11B2*, *LOC100129620*, *LOC100509378*, *LOC399900*, *MAGEE1*, *MT-COI*, *RASL10B*, *SPPL2B*, *TMEM50B*, *TNXB*, *TRIM58*, and *ZNF687*) in C14/KMUH cells compared with the control without colchicine. Six genes (*CREB5*, *GAS7*, *RRAD*, *TPPP*, *TPPP3*, and *UBE2E1*) were upregulated and 12 genes (*ACTRT2*, *C17orf74*, *C1QTNF5*, *C9orf106*, *CNGA1*, *ECEL1P2*, *GJA9*, *HLA-DPB2*, *HSD11B2*, *LOC100129620*, *MAGEE1*, and *MEIOB*) were downregulated caused by 6 ng/mL colchicine in C51/KMUH cells compared with 2 ng/mL colchicine. Seven genes (*ACTRT2*, *C1QTNF5*, *ECEL1P2*, *HLA-DPB2*, *HSD11B2*, *LOC100129620*, and *MAGEE1*) were consistently downregulated caused by different concentrations of colchicine in both cell lines. There was no consistently upregulated gene caused by different concentrations of colchicine in both cell lines.

Among those differentially expressed genes detected by microarray, two genes (*HSD11B2* and *MT-COI*) were selected for further quantitative RT-PCR analysis. The reason for this selection was that differential expressions of these genes could contribute to the anticancer effects of colchicine on CC. There was a good consistency between results from quantitative RT-PCR and microarray. The results of quantitative RT-PCR were shown in Table 1. Both 2 ng/mL and 6 ng/mL colchicine caused downregulations of both *HSD11B2* and *MT-COI* in the C14/KMUH cell line but these effects were not dose-dependent. Colchicine caused downregulation of *HSD11B2* in C51/KMUH cells only at a concentration of 6 ng/mL. Both 2 ng/mL and 6 ng/mL colchicine had no influence on expression of *MT-COI* in C51/KMUH cells.

Table 1 Gene expression fold change caused by colchicine in two human cholangiocarcinoma cell lines.

Gene name	*HSD11B2*			*MT-COI*		
Colchicine (ng/mL)	2/0[a]	6/0	6/2	2/0	6/0	6/2
C14/KMUH	0.57	0.54	0.96	0.15	0.20	1.29
C51/KMUH	1.06	0.67	0.62	0.84	0.91	1.08

Gene expression fold change was determined using quantitative reverse transcription-polymerase chain reaction. The housekeeping gene *TBP* (TATA box binding protein) was used as the reference gene. The value of gene expression fold change ≥ 1.3 was defined as upregulation and ≤ 0.7 was defined as downregulation.

[a] Colchicine concentration used in experimental cells/colchicine concentration used in control cells.

Nude mouse experiment

All the mice survived. The control mice significantly gained more body weight than colchicine-treated mice during the experimental period (2.43 ± 0.05 g vs. 1.33 ± 0.33 g, $p < 0.005$). The increased tumor volume ratios in colchicine-treated mice were significantly lower than control mice started from the 11^{th} day of treatment ($p = 0.0167$; Fig. 2). The tumor growth rates in colchicine-treated mice after 14 days of treatment were also significantly lower than control mice (0.147 ± 0.004/d vs. 0.274 ± 0.003/d, $p = 0.0015$). Pathological analysis showed that control mice had significantly larger percentages of tumor necrotic areas than colchicine-treated mice ($44.3 \pm 5.3\%$ vs. $31.7 \pm 7.5\%$, $p = 0.0075$).

Discussion

The current study showed that the clinically acceptable colchicine concentrations had significantly dose-dependent antiproliferative effects on CC. These effects were similar to those observed in HCC cells as demonstrated in our previous study [14]. This can be explained by the direct colchicine–tubulin interaction to perturb the assembly dynamics of microtubules as a common anticancer mechanism in both CC and HCC cells [6–10]. However, the effects of colchicine on differential expressions of genes that

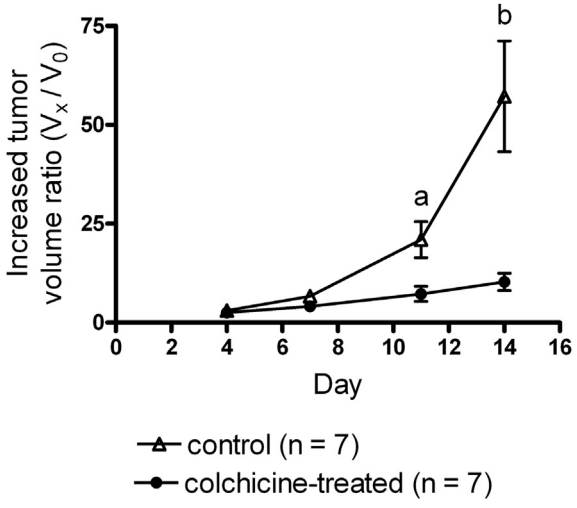

Figure 2. Increased tumor volume ratios in colchicine-treated and control mice. The increased tumor volume ratio $= V_x/V_0$ (V_x: tumor volume at day x, V_0: baseline pretreatment tumor volume). Data are expressed as mean values $\pm$ standard errors of the mean (error bars). The increased tumor volume ratios in colchicine-treated mice were significantly lower than control mice started from the 11th day of treatment. a: $p = 0.0167$, b: $p = 0.0062$, unpaired t test.

contributed to anticancer effects between CC and HCC cells were not the same. The effects of colchicine on the dose-dependent upregulations of two antiproliferative genes (*AKAP12* and *TGFB2*) in HCC cells [14] were not observed in CC cells. *HSD11B2*, which was downregulated by colchicine in cancer-associated fibroblasts but not in HCC cells [14], was also downregulated by 6 ng/mL colchicine in both CC cell lines and by 2 ng/mL colchicine in the C14/KMUH cell line. The protein encoded by *HSD11B2* is a type II isozyme of the corticosteroid 11-beta-dehydrogenase that catalyzes cortisol to the inactive metabolite cortisone. This protein prevents illicit activation of the mineralocorticoid receptor in tissues with expression of mineralocorticoid receptor, and protects cells from the growth-inhibiting and/or proapoptotic effects of cortisol in tissues without expression of the mineralocorticoid receptor [20,21]. Downregulation of *HSD11B2* can contribute to the anticancer effects of colchicine on CC cells. For C14/KMUH cells, colchicine also caused downregulation of *MT-COI* to enhance the anticancer effects of colchicine on these cells. The protein encoded by *MT-COI* known as cytochrome c oxidase subunit I is one of three mitochondrial DNA encoded subunits of respiratory complex IV. Complex IV is the final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation. It plays a crucial role in the regulation of aerobic production of cellular energy to drive all cellular processes [22,23]. Dysfunction of cytochrome c oxidase not only will impair cellular energy supply but also can increase mitochondrial reactive oxygen species production and cellular toxicity [22,23].

Different concentrations of colchicine also caused differential expressions of several genes, which may favor CC progression. These include upregulation of *TAF15* and downregulations of *DUSP9*, *GRHL3*, and *RASL10B* in C14/KMUH cells and upregulation of *RRAD* in C51/KMUH cells. The protein encoded by *TAF15* plays a role in RNA polymerase II gene transcription. Upregulation of *TAF15* can promote cellular proliferation [24]. The protein encoded by *DUSP9* is a member of the mitogen-activated protein kinase phosphatases that act as negative regulators of mitogen-activated protein kinase activity in mammalian cells. *DUSP9* expression can cause microtubule disruption [25] and may be a tumor suppressor [25]. *GRHL3* encodes a member of the Grainyhead family of transcription factors. Decreased *GRHL3* expression has been reported to contribute to tumor progression in squamous cell carcinoma of the skin [26]. The protein encoded by *RASL10B* is a new member of Ras superfamily with tumor suppressor potential [27]. The protein encoded by *RRAD* is a Ras-related GTPase that promotes cell growth by accelerating cell cycle transitions. Knockdown of *RRAD* expression can induce cell cycle arrest and premature senescence without additional cellular stress in multiple cancer cell lines [28,29]. Nevertheless, our animal study showed that the possible effects caused by differential expressions of the aforementioned genes to promote CC progression could be overcome by the direct colchicine—tubulin interaction and concomitantly differential expressions of genes to inhibit proliferation of CC cells. However, two genes (*LOC100129620* and *MAGEE1*) were consistently downregulated caused by different concentrations of colchicine in both cell lines. The functions of these commonly downregulated genes caused by colchicine are still unknown and need to be further investigated.

The pathological results from the nude mouse study showed that control group had larger tumor necrotic areas than the experimental group. Rapid proliferation of cancer cells exceeds the speed of neovasculization for sufficient blood supply in the control group is the explanation. This phenomenon is similar to clinically observed spontaneous tumor lysis syndrome in solid tumors [30]. The significant increase of body weight in the control group was caused by rapid increase of tumor mass in this group.

In conclusion, clinically acceptable colchicine concentrations have significantly dose-dependent antiproliferative effects on CC. This drug has good potential for the palliative treatment of CC due to its low cost and our long-time prescription experience.

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