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RESEARCH PAPER

WTC-01, a novel synthetic oxime-flavone compound, destabilizes microtubules in human nasopharyngeal carcinoma cells *in vitro* and *in vivo*

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BACKGROUND AND PURPOSE

Dynamic polymerization of microtubules is essential for cancer cell growth and metastasis, and microtubule-disrupting agents have become the most successful anti-cancer agents in clinical use. Besides their antioxidant properties, flavonoids also exhibit strong microtubule-disrupting activity and inhibit tumour growth. We have designed, synthesized and tested a series of oxime/amide-containing flavone derivatives. Here we report the evaluation of one compound, WTC-01 for its anti-proliferative effects in human cancer cells.

EXPERIMENTAL APPROACH

We used a range of cancer cell lines including two human nasopharyngeal carcinoma (NPC) cell lines, measuring proliferation, cell cycle and apoptosis, along with caspase levels and mitochondrial membrane potentials. Assays of tubulin polymerisation *in vitro* and computer modelling of the colchicine binding site in tubulin were also used. In mice, pharmacokinetics and growth of NPC-derived tumours were studied.

KEY RESULTS

WTC-01 was most potent against proliferation of NPC cells (IC_{50} = 0.45 μM), inducing accumulation of cells in G_2/M and increasing apoptosis, time- and concentration-dependently. The colchicine competition-binding experiments and computer modelling results suggested that WTC-01 causes microtubule disruption via binding to the colchicine-binding site of tubulin resulting in mitochondrial membrane damage and cell apoptosis via activation of caspase-9/-3 without noticeable activation of the caspase-8. Notably, our *in vivo* studies demonstrated that at doses of 25 and 50 $mg \cdot kg^{-1}$, WTC-01 exhibited good pharmacokinetic properties and completely inhibited the growth of NPC-TW01 cells in a xenograft nude mouse model.

CONCLUSIONS AND IMPLICATIONS

WTC-01, a new synthetic oxime-containing flavone, exhibited potent anti-tumour activity against NPC cells and merits further investigation.

Abbreviations

MTX, methotrexate; NPC, nasopharyngeal carcinoma; SPA, scintillation proximity assay

Tables of Links

TARGETS
Enzymes
Caspase 3
Caspase 8
Caspase 9

LIGANDS
Colchicine
MTX, methotrexate
Paclitaxel
Vinblastine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck cancer that occurs in the upper rear area of throat and nose. While NPC is uncommon in North American and most other countries, the incidence of NPC in the southern regions of China is 25 times higher than the rest of the world (Chang and Adami, 2006) and it is also highly prevalent in Taiwan (Hsu *et al.*, 2006). It is the 10th most common cause of death among cancer patients in Taiwan. The high NPC incidence found in this area may be due to ethnicity (Hu *et al.*, 2007; Luo *et al.*, 2007), Epstein–Barr virus infections (Fachiroh *et al.*, 2004; Ho *et al.*, 2009; Li *et al.*, 2009), and high salt or consumption of irritating foods (Yu *et al.*, 2009). While many clinical results have shown that pre-radiation chemotherapy with methotrexate (MTX), cisplatin and 5-fluorouracil could significantly improve the five-year survival rate of metastatic NPC patients (Mertens *et al.*, 2005; Komatsu *et al.*, 2012), a considerable number of NPC patients develop drug resistance and succumb to NPC as a result of disease progression. Therefore, new and effective pharmaceutical treatments are urgently needed for treating individuals with NPC. Because the incidence of NPC in Western societies is relatively low and therefore development of new therapeutic agents for NPC has not been a high priority for many pharmaceutical companies, for the scientific community and governmental health agencies in Southeast Asia identifying new pharmacological agents that target NPC has a high priority.

Recently, anti-tubulin activity has been identified in many natural products and their derivatives, leading to microtubule inhibitors such as taxol, colchicine, chalcones, combretasatin, phenstatins and *Vinca* alkaloids (Nepali *et al.*, 2014). Flavonoids as compounds abound in nature, with a phenylbenzopyrone (C6-C3-C6) basic structure, include more than 4000 compounds and are rich in many edible and medicinal plants. Many studies have demonstrated that flavonoids have many biological activities, such as anti-allergic, anti-inflammatory, antioxidant, anti-mutation, regulation of enzyme activity and anti-cancer activity (Craig, 1999; Galati *et al.*, 2000; Middleton *et al.*, 2000; Lu *et al.*, 2001). Recently, several studies had shown that flavonoids bind to the colchicine-binding sites of tubulin and disrupt the formation of microtubules (Kim *et al.*, 2012) and can be used for the treatment of lung cancer (Choudhury *et al.*, 2013) and breast cancer (Pedro *et al.*, 2005).Therefore, flavonoids and their derivatives have become one of the major sources for the development of new anti-cancer pharmaceuticals (Wang, 2000; Birt *et al.*, 2001; Ren *et al.*, 2003).

To improve the therapeutic efficacy of flavonoids, We have synthesized a series of oxime-bearing flavone and isoflavone derivatives and tested them for their anti-proliferation activity against human cancer cell lines. Among them, (Z)-6-[2-hydroxyimino-2-(4-methoxyphenyl)-ethoxy]-2-phenyl-4H- 1-benzopyran-4-one (WTC-01) exhibited significant anti-proliferative activities in *in vitro* assays (Wang *et al.*, 2005). Therefore, in this study, we focused on identifying the anti-proliferative mechanisms and evaluated the *in vivo* anti-tumour efficacy of WTC-01. Collectively, our results suggested that WTC-01 could effectively inhibit NPC tumour growth and might be useful in treating patients with paclitaxel- and MTX-resistant cancers in the clinic. We therefore consider WTC-01 to be a promising new anti-cancer agent that merits further development.

Methods

Synthesis of WTC-01

WTC-01 (Figure 1) was synthesized according to the procedure described (Wang *et al.*, 2005). The white solid was purified by flash column chromatography and recrystallized from CH_2Cl_2. The structure of WTC-01 was confirmed by ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6).

Cell lines

Human cancer cell lines including human leukaemia (Jurkat); non-small cell lung carcinoma (NCI-H226); nasoalpharyngeal carcinoma (HONE-1); human fibrosarcoma cell line HT-1080, human fibroblast (Detroit 551) and nasopharyngeal carcinoma (NPC-TW01) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). All the

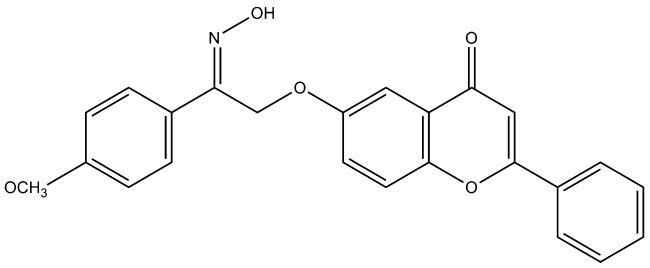

Figure 1
The chemical structure of WTC-01 (Z)-6-[2-hydroxyimino-2-(4-methoxyphenyl)ethoxy]-2-phenyl-4H-1-benzopyran-4-one (WTC-01).

tumour cell lines were maintained in either MEM or RPMI 1640 or DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2/95% air in the presence of penicillin/streptomycin/L-glutamine (Invitrogen, Waltham, MA, USA; Cat. No. 10378-016).

Growth inhibition assay

We used the colorimetric assay for cellular growth and survival as described by Hansen *et al.*, (1989) with modification. Briefly, logarithmic growth phase cells were seeded in a 96-well microtiter plates and incubated overnight prior to the addition of the designated compounds with various concentrations for 72 h. Two hours before the end of treatment, 15 μL of MTT solution (5 mg·mL^{-1}) was added to each well, and then cells were incubated at 37°C for 3 h. Then, 75 μL lysis buffer (20% SDS-50% *N, N*-dimethyl formamide) was added to each well, and the culture plate was incubated at 37°C overnight to dissolve the dark blue crystals. Reduced formazan was measured by absorbance at 570 nm on a Molecular Device plate reader (Sunnyvale, CA, USA). The percentage of conversion by mock-treated control cells was used to evaluate the effect of the chemicals on cell growth and to calculate the IC_{50}.

Preparation of cell lysates and Western blot analysis

Cells were initially seeded at 1×10^5 cells per well in a 6-well plate. After treatment for the indicated time with various concentrations of WTC-01, cells were washed twice with cold PBS, lysed in ice-cold lysis buffer [100 mM Tris (pH 7.4), 1% NP40, 0.01% SDS, 1 mM PMSF, 10 μg·mL^{-1} pepstatin, and 30 μg·mL^{-1} leupeptin], gently scraped from the dishes, and then harvested by centrifugation. Protein concentrations of lysates were determined using the BCA Protein Assay Reagent (PIERCE Biotechnology, Rockford, IL, USA). Equal amounts were separated on SDS-PAGE gels and transferred to PVDF membrane. After soaking in a blocking solution consisting of Tris-buffered saline [TBS: 50 mM Tris (pH 7.5) and 150 mM NaCl] with 0.05% Tween 20, and 5% skimmed milk, the blot was incubated with the primary antibody and antibody binding was detected using the appropriate secondary antibody coupled with horseradish peroxidase according to the instructions of the manufacturer. Enhanced chemiluminescence was used to detect the relevant proteins following protocols suggested by the manufacturer and then images were taken on LAS-4000 (FUJIFILM, Tokyo, Japan).

Caspase inhibition assay

In the caspase inhibition experiments, cell-permeable and specific irreversible inhibitors of caspases-8 and -9 were added to the medium, 1 h prior to WTC-01 administration. Cell growth inhibition was determined by MTT assay as described previously, to evaluate the effects of the compounds on the cells. The stock solution of caspase inhibitors were dissolved in 0.5% v/v DMSO or less.

Flow cytometric analysis

For cell cycle analysis, exponentially growing cells were treated with WTC-01 for the indicated times, harvested and fixed with ice-cold alcohol at –20°C. After 16 h fixation, cells were harvested and resuspended in PBS containing RNase, followed by staining with 50 μg·mL^{-1} propidium iodide. The DNA content of each sample was evaluated on a Becton Dickinson (Franklin Lakes, NJ, USA) Canton II flow cytometer. For each analysis, 10 000 events were recorded and the percentages of events in each cell cycle phase were determined with ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Mitochondrial potential transition was determined by the proportion of cells that retained the mitochondria-specific dye $DiOC_6$. Cells were treated with WTC-01 for the selected treatment duration, 100 nM $DiOC_6$ was added and incubated at 37°C following the manufacturer's instructions. Cells were harvested and resuspended in PBS and the retention of $DiOC_6$ was measured by flow cytometry.

For the measurements of Annexin V–PI binding, the Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instructions. Briefly, WTC-01-treated or control cells were trypsinized, collected by centrifugation and resuspended in 400 μL 1X Binding Buffer at a concentration of 1×10^6 cells per mL and 5 μL of purified recombinant Annexin V and PI reagent were added. After incubation at room temperature for 15 min in the dark, flow cytometry analysis was performed immediately and the results were analysed by De Novo software (MultiCycle AV Plug-in for FCS Express; De Novo Software, Glendale, CA, USA). The Annexin V–PI binding assay was determined at least three times.

Intracellular polymerization assay

Cells were seeded at a density of 1×10^6 cells per well in a 6-well plate and incubated at 37°C overnight then treated with various concentrations (11.25, 22.5 and 45 μM) of WTC-01, colchicine (800 nM) or paclitaxel (40 nM) for 5.5 h. After incubation, cells were washed twice at room temperature in PBS, lysed in microtubule lysis buffer (20 mM Tris-HCl pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% MP-40, 1 mM $NaVO_4$, 1 mM PMSF and 20 μg·mL^{-1} aprotinin/leupeptin), and lysate was collected by centrifugation. Finally, Western blots were performed to detect the levels of soluble and polymerized tubulin in each sample.

In vitro *microtubule assembly assay*

The *in vitro* microtubule assembly assay was performed according to Bollag *et al*. (1995) with alterations. In brief, MAP-rich tubulin in 100 μL buffer containing 100 mM PIPES (pH 6.9), 2 mM $MgCl_2$, 1 mM GTP and 2% (v/v) DMSO was

placed in 96-well microtitre plates in the presence of test agents. The increase in absorbance was measured at 350 nm in a PowerWave X Microplate Reader (BIO-TEK Instruments, Winooski, VT, USA) at 37°C and recorded every 30 s for 30 min. The AUC over 20 min was used to determine the concentration of WTC-01 that inhibited tubulin polymerization by 50% (IC_{50}). The AUC of the untreated control was set to 100% polymerization, and the IC_{50} was calculated by non-linear regression.

Tubulin competition-binding scintillation proximity assay (SPA)

The colchicine competition-binding SPAs were conducted as described previously (Tahir *et al.*, 2000) using biotin-labelled tubulin and streptavidin-labelled poly (vinyl toluene) SPA beads. Briefly, radiolabelled colchicine (final concentration 0.08 μM), unlabelled compound and 0.5 μg special long-chain biotin labelled tubulin were incubated together in 100 μL binding buffer (80 mM PIPES (pH 6.8), 1 mM EGTA, 10% glycerol, 1 mM $MgCl_2$ and 1 mM GTP) for 2 h at 37°C. Streptavidin-labelled SPA (80 μg) was added to each reaction mixture. The inhibition constants (K_i) were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Molecular docking study

The X-ray crystal structure of tubulin in complex with colchicine was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb, PDB code 1SA0; Ravelli *et al.*, 2004) for a docking study of WTC-01 and colchicine. The A and B chains were retained, small molecules and metal ions were removed, hydrogen atoms were added, and the resultant protein structure was used in the docking simulation. The 3D structure of WTC-01 was built and optimized by energy minimization using the MM2 force field and a minimum RMS gradient of 0.05 in the software Chem3D 6.0 (CambridgeSoft Corp. Cambridge, MA, USA). Docking simulation was performed using the GOLD 3.1 program (Jones *et al.*, 1997) on a Silicon Graphics Octane workstation with dual 270 MHz MIPS R12000 processors. The GOLD program utilizes a genetic algorithm (GA) to perform flexible ligand docking simulations. In the present study, for each of the 30 independent GA runs, a maximum number of 100 000 GA operations were performed on a single population of 100 individuals. Operator weights for crossover, mutation and migration were set to 95, 95 and 10 respectively. The annealing parameters for hydrogen bonding and van der Waals were set to 4.0 Å and 2.5 Å respectively. The GoldScore fitness function was applied for scoring the docking poses. The docking region was defined as encompass the colchicine-binding site of tubulin. The best docking solution for WTC-01 was chosen to represent the predicted binding mode to the colchicine site in tubulin.

Wound healing assay

To assess the alterations of cell motility and migration after treatment with WTC-01, wound-healing assays were performed as previously described by Ongusaha *et al.* (2004). Briefly, 10^6 cells were seeded into a 6-well plate 18 h before the experiment. An artificial 'wound' was carefully created at zero hour using a pipette tip to scratch the monolayer of cells. Floating and loosely attached cells were carefully removed by washing with PBS and the tested compound was added at predetermined concentrations. Microphotographs were taken every 3 h and the area of each wound area was calculated by Image J software (Wayne Rasband, NIH, Bethesda, MD, USA). All experiments were conducted in triplicate.

Animals

All animal care and experimental studies strictly adhered to 'The Guidebook for the Care and Use of Laboratory Animals' and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the China Medical University, Taichung, Taiwan (protocol 98-12-NH). The results involving animals in this study are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 48 animals were used in the experiments described here.

Pharmacokinetics and tumour implantation

For the WTC-01 pharmacokinetic studies, male BALB/c mice 6 to 8 weeks of age obtained from the National Laboratory Animal Center (Taipei, Taiwan). Animals were fasted for 15 h before compound administration, water was supplied *ad libitum* but food was supplied 3 h after dosing. The mice received 25 or 50 $mg \cdot kg^{-1}$ of WTC-01 by i.p. injection. All blood samples, each 75 μL taken from the tail vein and restricted to two samples per animal, were centrifuged at 10 000× *g* for 15 min at 4°C, and the serum obtained was stored at −30°C for later analysis. The serum (about 40 μL) was acidified with 25 μL of 0.1 N HCl and extracted with 100 μL of ethyl acetate (containing 5 $\mu g \cdot mL^{-1}$ of amyl paraben as an internal standard). The ethyl acetate layer was evaporated under nitrogen to dryness and reconstituted with 25 μL of mobile phase and then 10 μL was subjected to HPLC-photodiode array analysis. The mobile phase consisted of methanol (A) and 0.1% phosphoric acid (B) and the isotonic elution program was operated as A/B: 80/20 for 15 min. The concentration of WTC-01 in serum was determined using a standard curve that was plotted by linear regression of the peak area ratios (WTC-01 to amyl paraben) against known concentrations of WTC-01. Values represent the mean (± SD) for four animals per group.

For *in vivo* anti-proliferative experiments, pathogen-free male BALB/c nude mice, 6–8 weeks of age, were purchased from the National Laboratory Animal Center (Taipei, Taiwan). To prepare tumour cells for inoculation, cells in exponential growth phase were harvested and only single cell suspensions of >90% viability were used. Solid tumours were produced by subcutaneous inoculation of 3×10^6 cells into the flank region of nude mice (n = 5). Tumour-implanted mice were treated i.p. with vehicle (5% DMSO/10% cremophor/85% saline) or with 25 or 50 $mg \cdot kg^{-1}$ WTC-01 every 3 days. Vincristine (10 $mg \cdot kg^{-1}$, once a week) was used as a positive control. Tumour size and body weight of mice were measured twice a week. Tumour size was calculated based on the formula V = (1/6) × (larger diameter) × (smaller diameter)^2 (Dong *et al.*, 1998).

Data analysis

All assays were carried out in triplicate. Data were expressed as means with standard deviations (SD). Student's *t*-test was

used to compare the mean of each group with that of the control group. A P value < 0.05 was considered statistically significant.

Materials

Primary antibodies to caspase-3 (diluted 1:1000, Cat. No. 9662) and -9 (diluted 1:1000, Cat. No. 9501) were purchased from Cell Signaling Technology (Danvers, MA, USA); Caspase-8 (diluted 1:1000, Cat. No. sc-5263) and actin (diluted 1:2000, Cat. No. sc-1616) as well as horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell culture reagents were obtained from Hyclone (South Logan, UT, USA). Mitochondria membrane potential dye ($DiOC_6$) was purchased from Life Technology (Cat. No. D-273; Thermo Fisher Scientific, Waltham, MA, USA). Western blot chemiluminescent reagent was purchased from Millipore (Boston, MA, USA). All of the other chemicals were from USB (Darmstadt, Germany), Bio-Rad (Richmond, CA, USA) or Sigma Chemical (St. Louis, MO, USA), and were standard analytical grade or higher.

Results

WTC-01 exerts potent anti-proliferative activity against various human carcinoma cell lines.

Initial experiments were conducted to evaluate the anti-proliferative activity of WTC-01 against a range of human cancer cell lines, including nasopharyngeal carcinoma (NPC-TW01 and HONE-1), leukaemia (Jurkat), lung carcinoma (NCI-H226) and fibrosarcoma cell lines (HT-1080). Although WTC-01 could inhibit the growth of all the tested cancer cell lines, results clearly showed that the two NPC cell lines, NPC-TW01 and HONE-1, were the most sensitive to WTC-01 treatment with an IC_{50} of about 0.45 μM compared with HT-1080 (IC_{50} = 2.1 μM), Jurkat (IC_{50} = 7.4 μM) and NCI-H226 (IC_{50} = 15.6 μM). However, WTC-01 was much less potent against the non-cancer, human fibroblast Detroit 551 cell line (IC_{50} >20 μM). Notably, WTC-01 was as potent against paclitaxel and MTX-resistant NPC-TW01 cell lines, as it was against the sensitive NPC-TW01 cells (Table 1). Our results indicated that WTC-01 possessed a clear selectivity against the proliferation of human NPC cells and were also effective against NPCs resistant to paclitaxel and MTX.

WTC-01 induced G_2/M phase arrest and triggered the mitochondrial pathway of apoptosis in NPC cells.

To further investigate the anti-proliferative mechanisms of WTC-01, WTC-01-treated NPC cells were examined by cell cycle analysis using flow cytometry. After 6 h treatment with WTC-01, significant G_2/M cell accumulation was detected in NPC-TW01 and HONE-1 cells and the maximal G_2/M arrest was found at 12 h following WTC-01 treatment. By 24 h of treatment, 14% of NPC-TW01 and ~10% of HONE-1 events were found in the hypo-diploid DNA peak (sub-G_1) respectively (Figure 2A, 2B). Similarly the percentage of G_2/M cells was found in both NPC cell lines to correlate with increasing doses of WTC-01treatment (Figure 2C, 2D). These results indicated that WTC-01 can induce cell arrest in the G_2/M phase.

To further identify whether WTC-01 induced apoptotic cell death, WTC-01-treated NPC-TW01 and HONE-1 cells were subjected to Annexin-FITC Apoptotic analysis. The results indicated that after 18 h of WTC-01 treatment, the apoptotic proportion increased more than 40-fold in NPC-TW01 cells and more than 25-fold in HONE-1 cells (Table 2).

In general, apoptotic cell death can be mediated via two pathways: the extrinsic death receptor and the intrinsic mitochondrial pathway. In order to clarify which pathway was involved, the expression levels and activity of caspases of WTC-01-treated cells were analysed. Western blot analysis showed an increase in pro-caspase-3 and a decrease in pro-caspase-9 proteins in the NPC-TW01 and HONE-1 cells after 12 h of culture with WTC-01. However, no change in the levels of caspase-8 expression was observed in both WTC-01-treated NPC cell lines (Figure 3A). Moreover, a decrease in the anti-apoptotic protein, BCL-2, and an increase in the pro-apoptotic protein, Bax, was observed in WTC-01-treated NPC cells (Figure 3A). Furthermore, caspase activity analysis also showed a time-dependent activation of caspase-9 after 6 h treatment with slight activation of caspase-8 in both NPC cell lines (Figure 3B). Pretreatment with a specific inhibitor of caspase-9 but not with a specific inhibitor of caspase-8, blocked the effects of WTC-01 on survival in NPC-TW01 cells (Figure 3C).

Additionally, we examined the mitochondrial integrity of NPC cells treated with WTC-01. Using retention of the dye $DiOC_6$, our findings suggested that WTC-01 induced a loss of membrane potential over time. Following treatment with WTC-01, the geometric mean value of the dye intensity fell in

Table 1

IC_{50} values for WTC-01 against growth of a range of human cell lines

	Cancer cell lines					Normal cell line	Drug-resistant cell lines	
Cell lines	NPC-TW01	HONE-1	HT-1080	Jurkat	NCI-H226	Detroit 551	TW01-Taxol-Res	TW01-Met-Res
WTC-01 (μM)	0.45	0.43	2.12	7.42	15.61	>20	0.57	0.66
Paclitaxel (nM)	4.8	5.3	ND	<0.6	0.95	ND	30.5	5.5
MTX (nM)	49.8	53.1	14.1	18.5	54.2	>2000	85.6	2500
Vincristine (nM)	8.9	1.9	ND	130.5	>2000	ND	6.3	14.2

Cancer cells were treated with various concentrations of WTC-01, paclitaxel and MTX for 72 h and cell survival was determined using an MTT colorimetric assay. Representative data from three independent experiments performed in quadruplicate are shown.

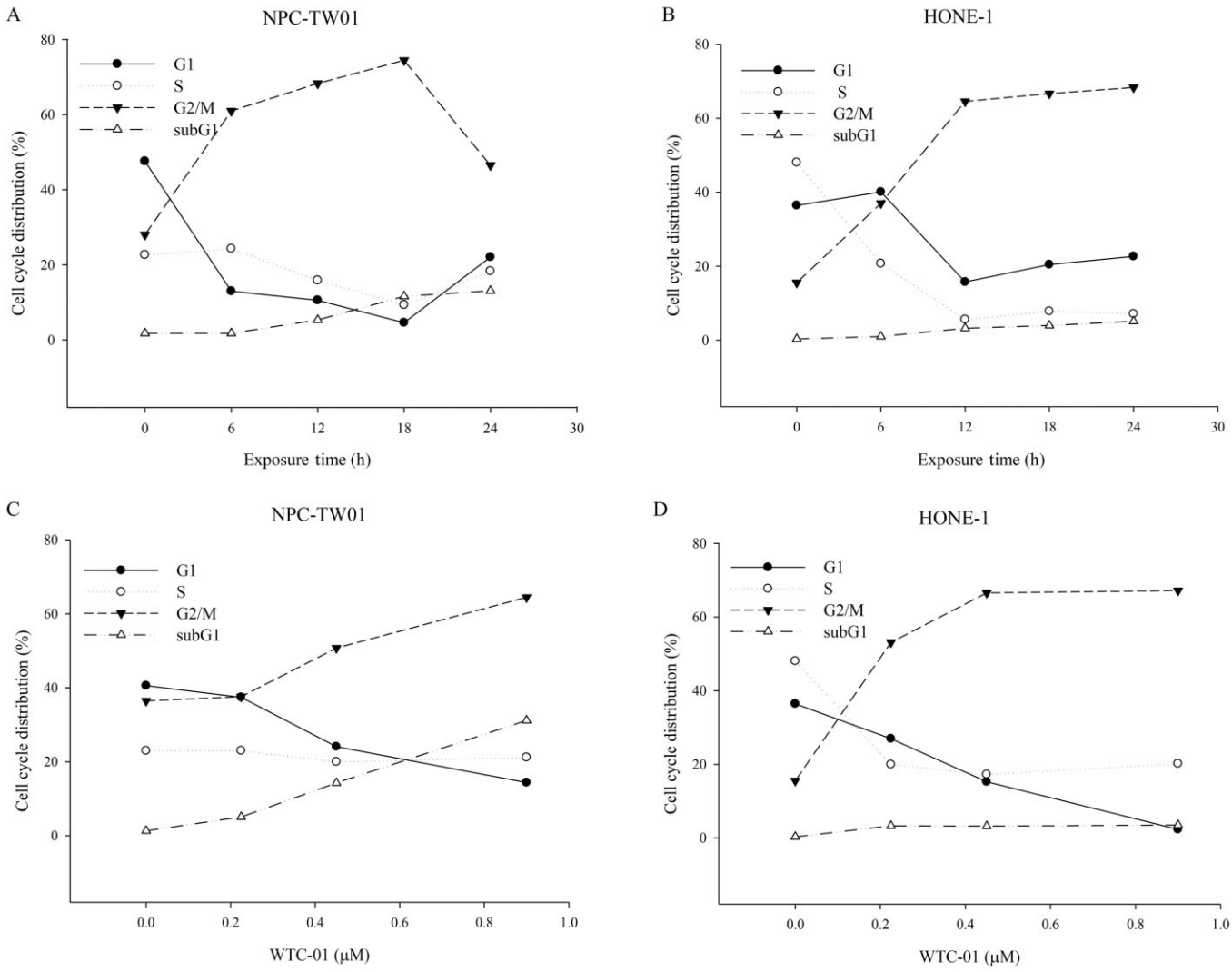

Figure 2

WTC-01 induced a concentration- and time-dependent G2/M cell arrest in human NPC lines.
NPC-TW01 (A, C) and HONE-1 (B, D) cells were treated with IC_{50} (0.45 μM) of WTC-01 (A, B) for the indicated times or different concentrations (0.225, 0.45 and 0.9 μM) of WTC-01(C, D) for 24 h. The cell cycle population ratios were determined by flow cytometry.

Table 2

WTC-01 induced early apoptosis in two human NPC cell lines

	NPC-TW01			HONE-1		
Hours	E	L	E + L	E	L	E + L
0	0.55	0.06	0.51	0.21	0.04	0.25
6	0.91	0.03	0.94	0.33	0.01	0.34
12	12.52	5.78	18.30	4.72	1.03	5.75
18	14.37	11.99	26.36	8.04	1.23	9.27

NPC cells were treated with 0.45 μM of WTC-01 for the period indicated then WTC-01-treated cells were harvested, incubated with Annexin V and PI and subjected to flow cytometric analysis. Data shown are from a single experiment and indicate the population percentage of each stage. E, early phase apoptosis; L, late phase apoptosis.

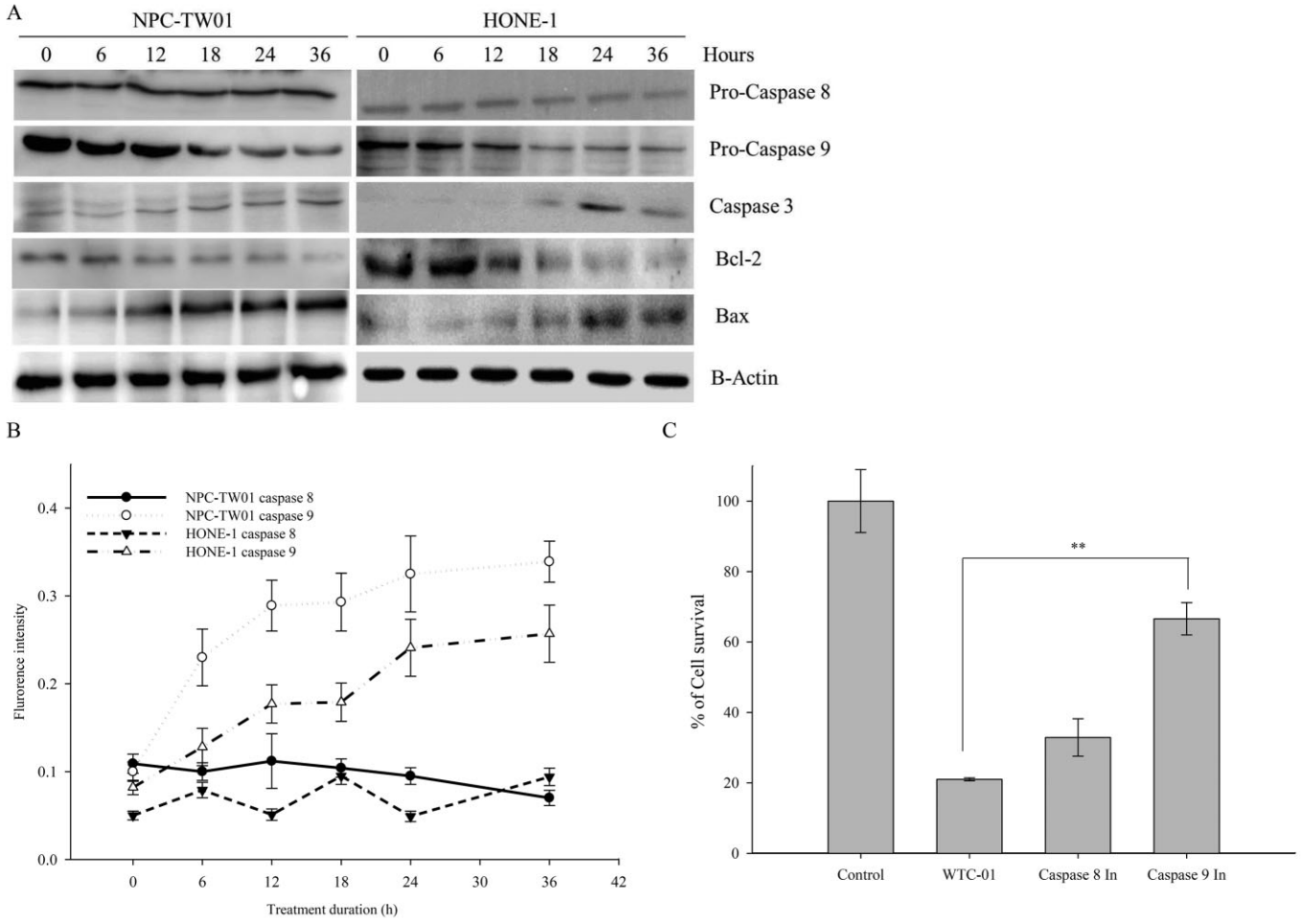

Figure 3

WTC-01 triggers caspase-9-dependent apoptosis in human NPC cells.
(A) Time-dependent activation of pro- and anti-apoptotic proteins following WTC-01 treatment. NPC-TW01 and HONE-1 cells were treated with 0.45 μM of WTC-01 for the indicated times. The protein levels of apoptosis-related proteins were evaluated using Western blot analysis with β-actin as an internal control. (B) Kinetics of caspase-8 and -9 activation in NPC-TW01 and HONE-1 cells. Cells were treated with 0.45 μM of WTC-01 for different periods and caspase activity was analysed. Data represent means ± SD for three determinations. (C) Caspase inhibition affects the cytotoxicity of WTC-01 in NPC-TW01 cells. NPC-TW01 cells were pretreated with 100 μM of caspase-8 inhibitor (Z-IEHD-FMK-TFA) or caspase-9 inhibitor (Z-IETD-FMK), respectively, for 1 h followed by WTC-01(4.5 μM, at 10-fold the IC_{50}) for another 72 h. Cell viability was determined by MTT assay.

both NPC-TW01 and HONE-1 cells (Figure 4A and B). Collectively, these results suggest that the apoptosis in response to WTC-01 was associated with the intrinsic mitochondrial pathway.

WTC-01 disturbs microtubule assembly by binding to the tubulin colchicine-binding site.

Several reports have suggested that many G_2/M arresting chemicals also interfere with microtubule assembly (Dumontet and Sikic, 1999; Jordan and Wilson, 2004; Zhou and Giannakakou, 2005; Schmidt and Bastians, 2007). Our results showed that WTC-01 inhibited polymerization of tubulin in a concentration-dependent manner (Figure 5A). Furthermore, when NPC cells were treated with high concentrations of WTC-01 for 5.5 h, WTC-01 significantly reduced the levels of polymerized microtubules (Figure 5B). The ability of WTC-01 to disrupt the polymerization of pure MAP-rich tubulins *in vitro* was tested. Moreover, the competition-binding SPA results also showed that WTC-01 strongly competes with [^{3}H]colchicine binding to tubulin. The inhibition constant for tubulin binding of WTC-01 was determined to be 3.23 μM (Figure 5C). However, SPA experiments showed that WTC-01 did not compete with [^{3}H]paclitaxel or [^{3}H]vinblastine for binding to tubulin (data not shown).

To predict the possible binding mode of WTC-01 in the colchicine-binding site of tubulin, we performed molecular docking studies using the docking program GOLD 3.1 and the crystal structure of tubulin bound with colchicine. As illustrated in Figure 5D, the docking model reveals that WTC-01 forms three hydrogen bonds to tubulin. Two of the hydrogen bonds, to residues Leu^{248} and Ala^{250}, arise from the oxime hydroxyl group of WTC-01, highlighting the importance of this group for tubulin binding. In addition, the

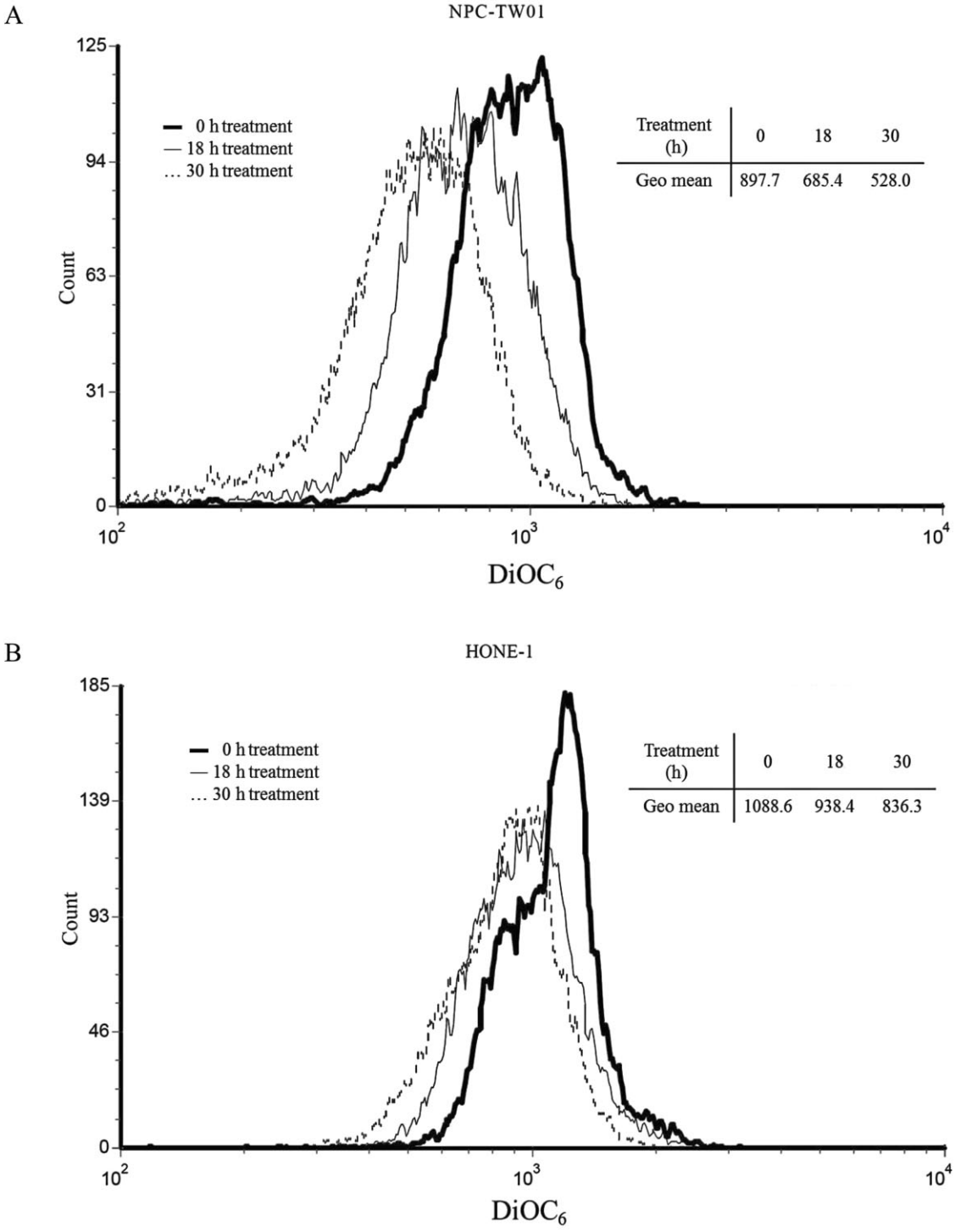

Figure 4

WTC-01 treatment disrupts the mitochondrial membrane potential in human NPC cells.
NPC-TW01 (A) and HONE-1 (B) cells were treated with 0.45 μM of WTC-01 for the indicated times and mitochondrial membrane potential was measured with the dye, $DiOC_6$. Data are presented as log fluorescence intensity. Inset table is the geometric mean value.

flavone and *p*-methoxyphenyl moieties of WTC-01 make hydrophobic interactions with the surrounding residues Val^{181}, Leu^{248}, Leu^{255}, Met^{259}, Ala^{316} and Lys^{352}. Our computational prediction suggested that WTC-01 could adopt a π-stacked conformation to fit into the binding pocket. In the bound conformation, the *p*-methoxyphenyl group of WTC-01 is sandwiched between the benzopyran ring and residue Leu^{248}. These findings identify WTC-01 as a novel

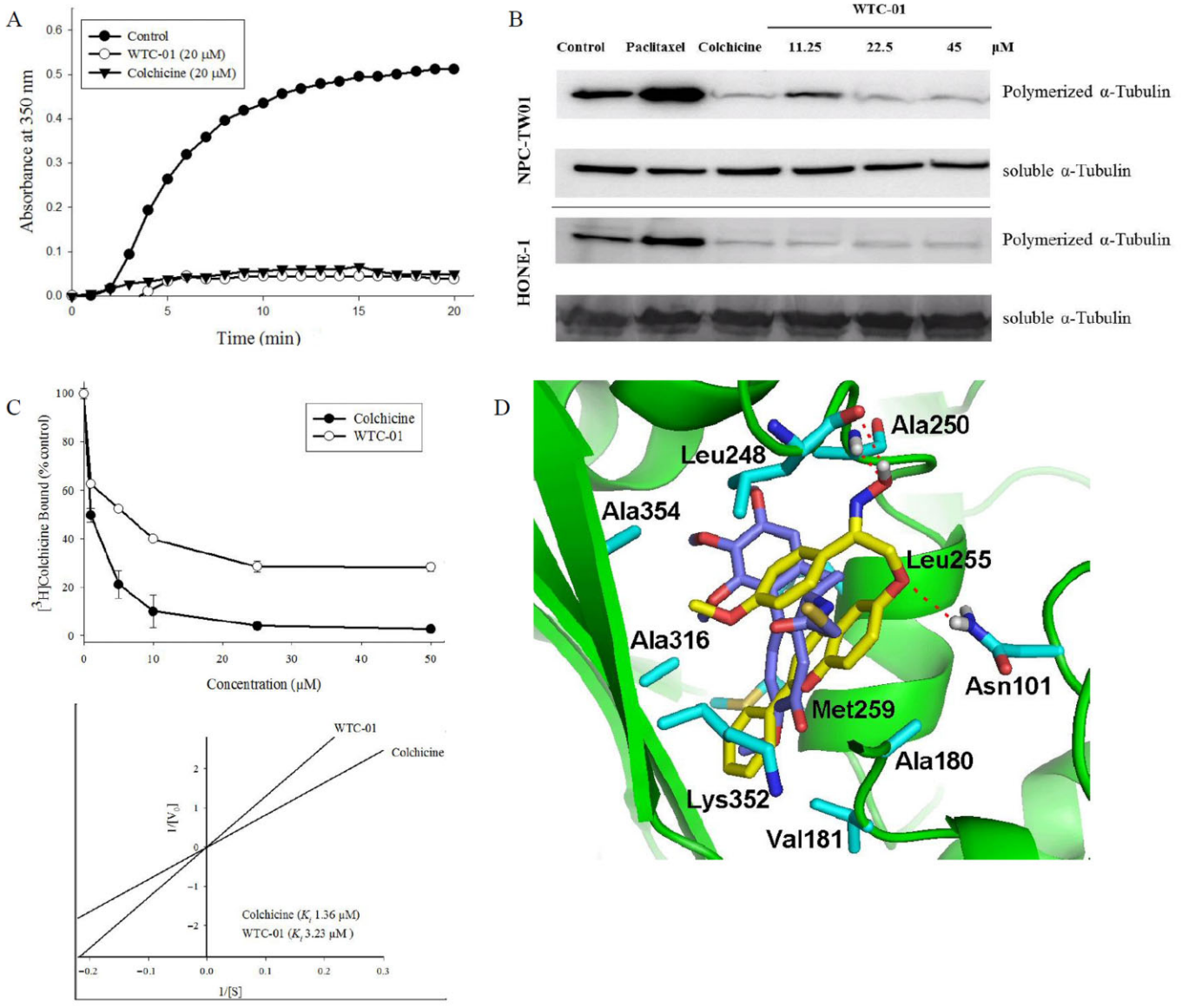

Figure 5

WTC-01 treatment interferes with microtubule assembly in NPC cells.

In vitro (A) and *ex vivo* (B) analysis of WTC-01 inhibition of tubulin polymerization. (A) *In vitro* tubulin polymerization was performed in the absence or present of 20 nM colchicine or WTC-01 and level of polymerized tubulin was measured. (B) Cancer cells were treated either with paclitaxel (40 nM), colchicine (800 nM) or various concentrations of WTC-01 for 5.5 h and the amount of polymerized tubulin was measured by Western blots. (C) The colchicine competition-binding SPAs (upper panel) were conducted and the inhibition constants (K_i; lower panel) were calculated using the Cheng-Prusoff equation. WTC-01 strongly competes with [^{3}H] colchicine for binding to the same pocket in tubulin (D). Overlay of the docking pose of WTC-01 (yellow) with the bound orientation of colchicine (blue) in the crystal structure of tubulin. Computer simulation results suggest WTC-01 is able to dock into the colchicine-binding site of tubulin. Some tubulin amino acid residues interacting with WTC-01 are shown as stick structures. The dashed lines indicate hydrogen-bonding interactions.

anti-tubulin cancer agent that disrupts tubulin polymerization, similar to the effects of colchicine.

WTC-01 treatment significantly reduces cancer cell mobility.

While previous results showed that WTC-01 could interfere with microtubule assembly, whether disruption of microtubule assembly could also inhibit migration of these cells was not determined. Therefore, to assess the effects of WTC-01 on human NPC cell mobility, the migratory potential of WTC-01-treated NPCTW-01 and HONE-1 cells was assessed via a wound-healing assay. In untreated cells, the wound area was closed in 12 h, whereas cells treated with WTC-01 required a longer wound healing time that was concentration-dependent (Figure 6). These results indicated that WTC-01 treatment could retard the migration of tumour cells.

In vivo evaluation of WTC-01 treatment in mice.

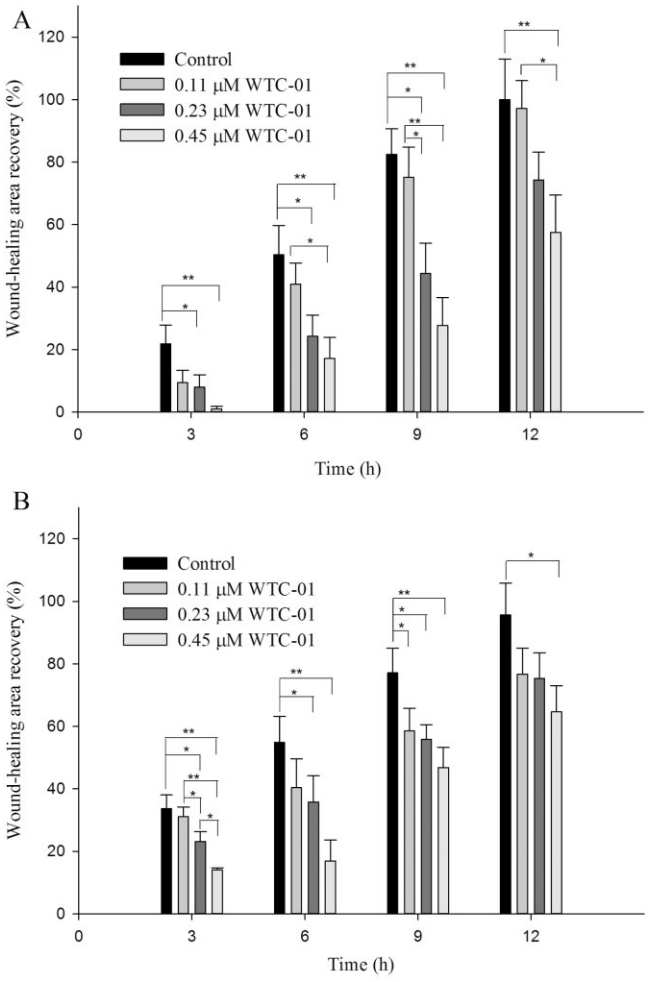

Figure 6

WTC-01 treatment reduces the migratory ability of human NPC cells. Confluent cell cultures of NPC-TW01 (A) and HONE-1 (B) were wounded by scratching with a plastic micropipette tip, suspended cells were then removed by PBS washes and then treated with different concentrations (0.25,0.5 and 1 x IC_{50}) of WTC-01. Cells were photographed at the indicated times after wounding by phase contrast microscopy (×100). The percentage of area wound healing of cells was quantified, and values shown are means ± SD from triplicate experiments. *$P < 0.05$, **$P < 0.01$, significantly different as indicated; one-way ANOVA.

To evaluate the bioactivity and potential clinical utility of WTC-01, additional *in vivo* experiments were performed to investigate its pharmacokinetics and its effect on tumour growth in mice. For the pharmacokinetic studies, 25 or 50 mg·kg^{-1} of WTC-01 was injected i.p. in BALB/c mice and the concentration of WTC-01 in blood samples was measured by HPLC analysis. The data showed that the Cmax of WTC-01 in the serum was about 30-fold higher than the *in vitro* IC_{50} values for both NPC cell lines, for either dose (Figure 7A). Moreover, 24 h after administration of WTC-01, its serum concentration level was still around 4 μM, which is about 10 times higher than the *in vitro* IC_{50} values for WTC-01 against NPC-TW01 cells.

Finally, we examined the potential clinical utility of WTC-01. The tumour-bearing mice that received treatment with 25 or 50 mg·kg^{-1} of WTC-01 showed almost no tumour growth over the treatment period (7–52 days), results identical to those in vincristine-treated, tumour -bearing mice (Figure 7B). Additionally, NPC-TW01 xenograft mice that received 50 mg·kg^{-1} of WTC-01 remained tumour-free for up to 8 weeks after discontinuation of WTC-01 treatment (data not shown). In all experiments, except for a slight body weight increase in the WTC-01-treated animals, no differences were found between the non-drug-treated control group and WTC-01-treated animals in food consumption and clinical signs of toxicity.

Discussion

The NPC is one of the most predominant cancers in Southeast Asia, including Taiwan and along the southeastern coast of China. Because of the low incidence of NPC in countries outside of Southeast Asia, the search for more effective treatment strategies and related basic research of NPC is much less intense, compared with that for other cancers (Fang *et al.*, 2009). Currently, there are two clinical approaches for the treatment of NPC, one is to surgically remove the tumour, and the other is local radiation therapy (Lin *et al.*, 2010; Marcus and Tishler, 2010) followed by chemotherapy. Although the 5 years survival rate can reach around 50%, treatment failure due to metastasis to distant organs and drug resistance to chemotherapy still remains a major problem. Therefore, the development of an effective and highly specific anti-NPC drugs is a high priority among countries with a high incidence of this tumour type.

Several lines of evidence suggest that flavonoids possess many biological activities including anti-inflammatory, antioxidant and anti-cancer effects (Craig, 1999; Galati *et al.*, 2000; Middleton *et al.*, 2000; Lu *et al.*, 2001). Therefore, flavonoids and their derivatives have become a major source for the development of new anti-cancer pharmaceuticals (Wang, 2000; Birt *et al.*, 2001; Ren *et al.*, 2003). Recently, our laboratory synthesized a series of flavonoid derivatives and, among those compounds, WTC-01, which contains an oxime-bearing flavone, exhibited high levels of anti-proliferative activity against a range of cancer cell lines. Here, we have shown that WTC-01 exhibited a highly potent anti-proliferative activity against human NPC cell lines (HONE-1 and NPC-TW01) with an IC_{50} below 0.5 μM (Table 1). Both MTX and paclitaxel have been widely used as first-line chemotherapy for metastatic or recurrent NPC but cancer patients quickly develop resistance and succumb, with treatment failure. Our present results showed that WTC-01 maintained anti-proliferative potency against NPC cells highly resistant to paclitaxel and MTX (Table 1), suggesting that WTC-01 might be of benefit to those NPC patients with paclitaxel and MTX-resistant tumours.

Our mechanistic studies showed that WTC-01 induced NPC cell arrest at the G_2/M phase (Figure 2) in a time- and concentration-dependent manner, and triggered caspase-9-dependent cell apoptosis (Figure 3), along with mitochondrial damage (Figure 4).

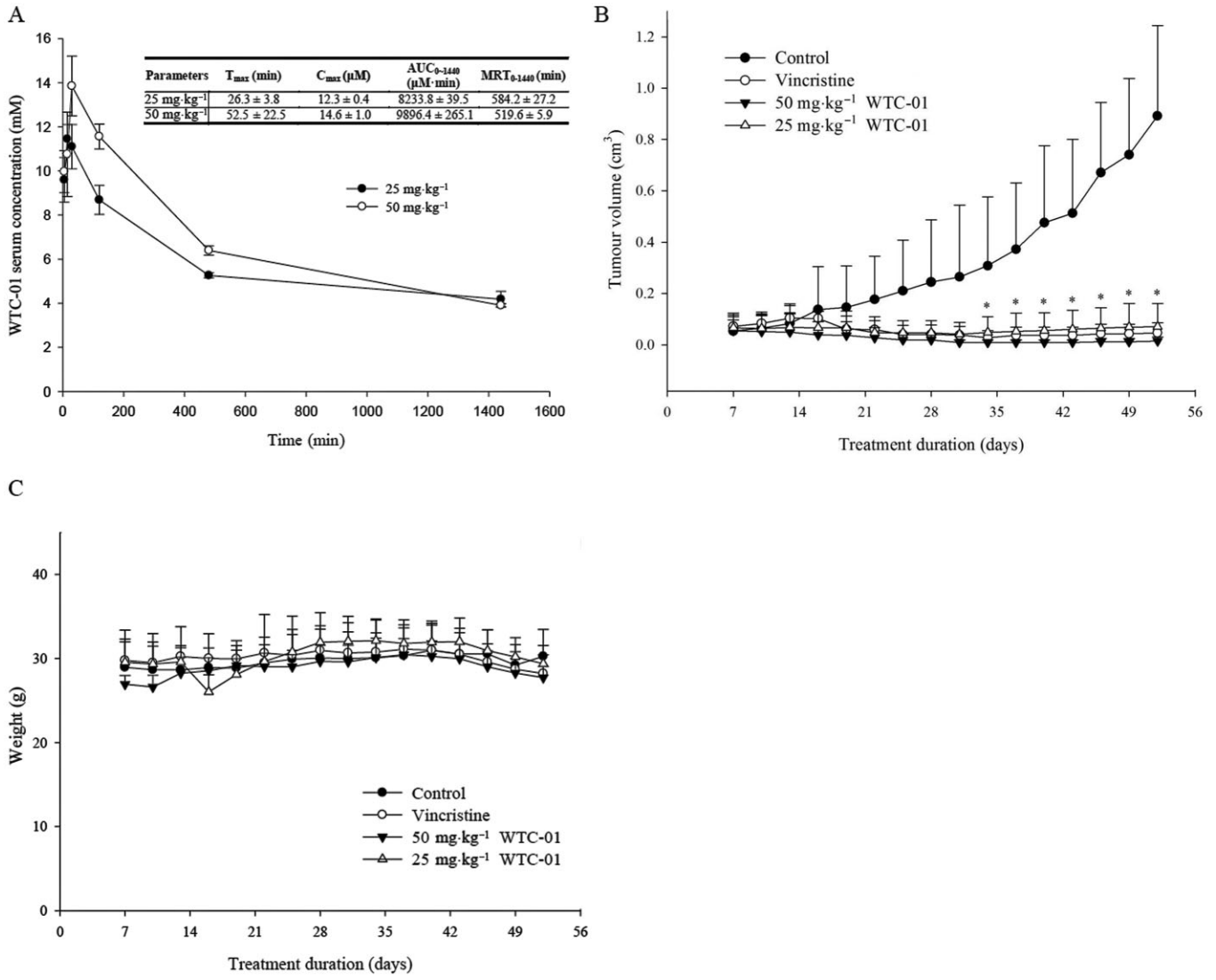

Figure 7

In vivo anti-tumour activity of WTC-01 in a human NPC-TW01 xenograft model. (A) The pharmacokinetics of WTC-01, after i.p. injection. Mice ($n = 4$) were injected with 25 or 50 mg·kg^{-1} of WTC-01 and blood samples were drawn at the indicated times. Serum was isolated and the amount of WTC-01 in the serum sample was measured. (B) Nude mice ($n = 5$) were subcutaneously injected with NPC-TW01 cells. After 7 days, the mice received i.p. injection of 25 or 50 mg·kg^{-1} WTC-01 once every 3 days. Vincristine was used as a positive control at a dose of 10 mg·kg^{-1} once a week. Tumour size was measured every three day Data shown are means ± SD. *$P < 0.05$ compared with control group; Student's *t*-test.

Microtubule polymerization experiments also showed that WTC-01 significantly inhibited tubulin polymerization in NPC cells both *in vitro* and *ex vivo* (Figure 5A, 5B). Competition-binding scintillation assays demonstrated that WTC-01 could compete with colchicine for binding to tubulin (Figure 5C) but not with pacilitaxel or vinblastine. The computer modelling analysis further suggested a tight association of WTC-01 with tubulin in the same pocket as colchicine (Figure 5D). Collectively, our data clearly suggest that WTC-01 is able to disrupt microtubules assembly and function. Additionally, the microtubule disruption function of WTC-01 may also contribute to the retardation of cell mobility observed in our wound-healing assays (Figure 6).

To confirm the bioavailability of WTC-01, *in vivo* experiments, including pharmacokinetic properties and anti-tumour growth efficacy experiments were performed in a murine model. The pharmacokinetic results indicated that the serum levels of WTC-01 could reach the desired concentration and can be maintained for at least 24 h after a single i.p.injection of WTC-01 (Figure 7A). Further experimental results from the tumour xenograft model clearly showed that WTC-01 (25 mg·kg^{-1}), given every 3 days, significantly retarded NPC-TW01 cell growth and completely prevented tumour growth at a higher dose (50 mg·kg^{-1}) (Figure 7B). These mice remained Tumour-free for >8 weeks after the conclusion of WTC-01 treatments.

In conclusion, our data demonstrated that WTC-01, a new synthetic oxime-containing flavonoid derivative, induced NPC cell death through disrupting intracellular microtubule formation, G_2/M phase accumulation, disrup-

tion of mitochondria and caspase 9-dependent apoptosis. The anti-proliferative effects of WTC-01 were mediated at least in part by interference with microtubule assembly through binding to the colchicine-binding site of tubulin, resulting in G_2/M cell arrest, mitochondrial damage and activation of a caspase-9/-3-dependent apoptotic pathway. These findings indicate that WTC-01 may a represent new microtubule targeting compound for management of various human NPCs, and possibly also for patients with paclitaxel- or MTX-resistant tumours.

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Author contributions

S.-H. J. made contributions to conception, design of the study and revision of the manuscript. T.-C. W., C.-H. L., C.-S. C., S.-H. W., Y.-C. L. performed the experimental work and data analysis. C.-Y. C. participated in interpreting data and formulating the article.

Conflicts of interest

None.

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