AIDS, Publish Ahead of Print

DOI: 10.1097/QAD.0000000000001808

TMC120 displayed potent cytotoxic effect on human cervical carcinoma through enhancing the polymerization of microtubules

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Abstract

Objective: In the post-highly active antiretroviral therapy (HAART) era, the incidence of some AIDS-defining cancers declined markedly likely reflecting HAART-related improvements in immunity, while incidence of some cancers such as cervical cancer has not been affected.Therefore, it is valuable to find whether antiretroviral drugs or prophylactic microbicides could treat or prevent these cancers, especially thecervical cancer.

Design: We screened the anti-HIV drugs approved or in phase III clinical trials to identify a potential anticancer drug candidate.

Methods:We chosen cervical HeLa and SiHa cancer cells and focused on studying the anti-tumor effect *in vitro*and *in vivo*.Cell proliferation was measured by MTT assay, the cytotoxic effect was obtained through apoptosis as evidenced by Annexin V flow cytometry assay due to the arresting of cancer cells in G2/M phase of cell cycle.Nude mice xenograft model was performed to detect the antitumor effect *in vivo*.

Results: TMC120 was identified as a potential anticancer drug candidate. TMC120 displayed potent cytotoxic effect on various human cancer cells, including cervical carcinoma cell line HeLa and SiHa. Further mechanism study showed that TMC120 enhanced the polymerization of microtubules, which was followed by mitotic arrest, as well as abnormal mitotic spindles. TMC120 also substantially retarded the growth rate of the tumor *in vivo*.

Conclusions: TMC120 is a potential chemoprophylactic and therapeutic agent for cervical cancers in a manner similar to paclitaxel, and could be suitable for helping healthy women to prevent HIV infection and cervical cancer.

Key words: TMC120, HIV, cytotoxic effect, human cervical carcinoma, polymerization of microtubules

Introduction

There was an established relationship between the syndrome and the occurrence of malignancies in patients afflicted by AIDS[1-3]. Of the malignancies, 90% were Kaposi sarcoma (KS) and the rest had mostly non-Hodgkin lymphomas, including primary central nervous system (CNS) lymphoma (PCNSL) and systemic diffuse large B-cell lymphoma (DLBCL)[4]. Also an increased burden of cervical cancer was identified among HIV-positive women [5-7]. Although the incidence of KS, PCNSL, and systemic DLBCL had been a significant reduction after the introduction of highly active antiretroviral therapy (HAART) in 1996, there was no changes in the incidence of cervical cancer after HAART era, even increased in Brazil[8].For HIV sufferers who got cancer complications, anti-cancer drugs, such as cytotoxic agents or targeted antineoplastic agentsshould be used[9, 10].But little is known about combining antiretroviral and antineoplastic agents in patients with HIV who were on HAART therapy[11, 12]. Therefore the use ofantiretroviral drugs with anti-cancer activity is a promising strategy for the prevention and treatment of AIDS-defining cancers.

TMC120 (also known as dapivirine), a nonnucleoside HIV reverse transcriptase inhibitor (NNRTI), was made available to the microbicide field by a landmark agreement between Tibotec and the International Partnership for Microbicides (IPM). It was one of a new generation of NNRTIs that could accommodate some mutations within the NNRTI binding site without significant loss of activity [13-15]. TMC120 is currently investigated for use as a vaginal microbicide in three dosage forms, a semi-solid gel, a vaginal film and a silicone elastomer matrix vaginal ring[13-15]. The vaginal film containing dapivirine was shown to prevent HIV-1 infection *in vitro* and *ex vivo* and had acceptable characteristics which made this film a promising candidate for testing as a vaginal microbicide [16]. Recently, several clinical trials

showed TMC120 vaginal ring was safe and well tolerated. In this regard, the concentration-time plots of TMC120 in vaginal fluid were indicative of a sustained release over the 28 days of use, while plasma TMC120 concentrations were low[13, 14]. These results indicated that the TMC120 vaginal ring had a safety and pharmacokinetic profile that supported its use as a sustained-release topical microbicide for HIV-1 prevention in women.

Here we aimed to find drugs with dual antiretroviral and anti-cancer activities.. Through the screening of more than 20 anti-HIV drugs approved or in phase III clinical trials, TMC120 displayed potent cytotoxic effect on various human cancer cells, including cervical carcinoma cell line HeLa and SiHa. By enhancing microtubule polymerization and arresting cancer cells in mitosis phase,TMC120 induced apoptosis in cancer cells*in vitro* and *in vivo*. Based onits anti-cancer and antiretroviral activities, TMC120 showed a promising chemoprophylactic and therapeutic ability in AIDS-definingcancers.

Materials and Methods

Cell proliferation assay

Cell proliferation was measured by MTT assay. Cells were seeded in 96-well plates overnight for attachment and then incubate for48h with different concentrations of TMC120. After treatment, MTT(0.5 mmol/L) was added for 4h and was then solubilizedby DMSO. The colored formazanproduct was determined at570 nm in a microplate reader (Bio-Rad Laboratories, CA).

Apoptosis assay

The extent of apoptosis was measured by the flow cytometric detection of phosphatidylserine externalization using FITC Annexin V and propidium iodide (PI) staining (BD Biosciences, NJ) according to the manufacturer's instructions[17].Two cervical carcinoma cell lines (HeLa and SiHa) were seeded in 6-well plates overnight for attachment and then incubated for 48 h with differentconcentrations of TMC120. After treatment, the cells were harvested and5 μL of FITC Annexin V and PI were added to the cells respectively and gently vortexed. Stained cells were analyzed by a flow cytometer (BD FACS Canto II, NJ).

Cell cycle analysis

Flow cytometry was performed to study the effect of the TMC120 on cell-cycle distribution. HeLa and SiHa cellswere treated with TMC120 andpaclitaxel for 24h. Then cells were fixed cells and stained with PI(50 μg/mL). DNA cellular content was analyzed with flow cytometer. Data for 10,000 events per sample was collected and cell-cycle distribution was analyzed using FlowJo 7.6 software.

Western blotting

G2/M checkpoint correlative proteinsp21$^{Waf1/Cip1}$, cyclinB1, phospho-cdc2 -Tyr15 and mitosis phase marker proteinsphospho-Histone H3-Ser10, phospho-Aurora A-Thr288/ Aurora B-Thr232/ Aurora C-Thr198 (CST, MA) weredetected by Western blotting. HeLa and SiHa cells were treated with TMC120 or paclitaxel. After treatment, cells were harvested. Then Chemiluminescent signals were developed with Lumiglo reagent and exposed to X-ray film.

Confocal immunofluorescence analysis

Briefly, HeLa and SiHa cells were seeded in Petri dishes with glass bottoms overnight for attachment and then treated with TMC120 or paclitaxel for 12h. At the end of treatment, cells werefixed with 4% (v/v) paraformaldehyde and then incubated with primary antibody against a/β-tubulin at 4°Covernight. After this procedure, cells were incubated withsecondary fluorogenic antibodies the nucleus were stainedwith DAPI [18]. Fluorescent signals were detected using a confocal fluorescence microscope (Nikon EZ-C1, Tokyo, Japan).

Tubulin polymerization assay

The effect of TMC120 on tubulin polymerization was evaluated using the kit (Cytoskeleton Inc., Denver CO) according to the manufacturer's protocol[19]. Briefly,TMC120 (31.3 μmol/L or 62.5 μmol/L) and DMSO (0.5%) were pipetted into the pre-warmed plate and incubated for 2 min at 37°C. After that, the 100 μL reconstituted tubulin above was added directly to the testing wells. Absorbance values were taken every minute during 1h at 340 nm in a microplate reader (Bio-Rad Laboratories,CA).

Nude mice xenograft model

SiHa cells (3×10^6) were suspended in 0.2 mL PBS and injected subcutaneously into the left alar region of 4-6 weeks old female BALB/c nu/nu mice. The tumor volume (mm^3) was calculated by the formula of 1/2 ×length ×$width^2$. After palpable tumors were appeared, the mice were divided into five groups (n=8) randomly:control group which received5% (v/v) DMSO/Physiological saline,TMC120-treated groups which received TMC120 (12.5, 25, 50 mg/kg body weight) dissolved in 5% (v/v) DMSO/Physiological saline three times a week, and paclitaxel (15 mg/kg) was administrated as a positive control. All treatments were administered

by intraperitoneal injections in mice at 0.1 mL/10 g for 18 days. Body weight was monitored.At the end of the experiment, the mice were sacrificed and tumors were excised for TUNEL assays. The research protocol was in accordance with the Institutional Guidelines of Animal Care and Use Committee at Southern Medical University.

TUNEL assay for apoptosis

The terminal deoxynucleotidyl transferasemediated dUTP nick-end labeling (TUNEL) (Roche Diagnostics Corp., Indianapolis, IN) was conducted on 5-μm-thick formalin-fixed paraffin embedded tumor specimens, according to the manufacturer's instructions. The number of TUNEL-positive cells in each treatment samples, and apoptotic cells were visualized with Olympus fluorescence microscope. TUNEL-positiveand Hematoxylin-stained nuclei were counted.

Statistical analysis

All data was shown as means ± s.e.m. Student's *t* test was performed for comparison of 2 groups and one-way ANOVA for comparison of >2 groups by GraphPadPrism5 software.(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control).

Results

TMC120 inhibited cancer cells growth

In order to screen the drugs that affect cancer cells growth, more than 20 anti-HIV drugs approved or in phase III clinical trials(tenofovir alafenamide, lopinavir, pentamidine isethionate, raltegravir potassium, etravirine, darunavir ethanolate, elvitegravir, dolutegravir, rilpivirine, atazanavir, mizolastine, abacavir, rifabutin, atazanavir, efavirenz, nelfinavir, TMC120,

nevirapine, efavirenz, Saquinavir, Ritonavir, T20)were tested in 10 cancer cell lines by MTT assay (data not shown). Among them, TMC120 displayed the most potent cytotoxic effect on various cancer cell lines with IC_{50} values at micromole levels (Table 1).

TMC120 induced apoptosis in cancer cells

TMC120 was investigated in phase 3 clinical trial as a microbicide to prevent women from sexual transmission of HIV.Since cervical cancer was a major problem among HIV-infected women [16, 20],we thus focused on cervical HeLa and SiHa cancer cells. TMC120 displayed cytotoxic effect onboth of the cervicalcancer cells (Table 1). Therefore, we investigated whether TMC120 induced apoptosis in cancer cells by flow cytometric detection of phosphatidylserine externalization using FITC Annexin V/PI staining.TMC120 treatment (6.25, 12.5 and 25 μM) significantly enhanced Annexin V-positive cellscompared with control (Figure1A and 1B). Cisplatin (10 μM) was used as the positive to induce apoptosis. These results suggested that the cytotoxic mechanism of TMC120in cervical cancer cells was through the apoptotic effect.

TMC120 arrested cancer cells in G2/M phase of cell cycle

Deregulation of the cell cycle components has been shown to induce mitotic catastrophe and also may be involved in triggering apoptosis[21]. To investigate whether TMC120 had effect on the cell-cycle distribution, HeLa and SiHa cells were treated with the different concentrations of TMC120 for 24h, and flow cytometry was performed to analyze the cell-cycle distribution using PI staining. As shown in Figure 2, treatment of cells with TMC120 resulted in concentration dependent accumulation of HeLa and SiHa cells in the G2/M phase (Figure 2A and 2B). Namely, compared with the negative control, significantly more G2/M phase cells were detected in the presence of TMC120 from the concentration of 12.5 μM for both HeLa and SiHa

cells. Paclitaxel was used as the positive control for G2/M phase arrest. These results suggested that TMC120 could arrest cancer cells in G2/M phase of cell cycle and subsequently induce cell apoptosis.

TMC120 inactivated the G2/M checkpoint and arrestedcancer cells inmitosis phase

It was an anti-cancer strategy to inactivate the G2/M checkpoint, thus forcing the cancer cells into mitosis with increased DNA damage and finally into mitotic catastrophe and cell death [22]. Takingpaclitaxel for example (Figure 2C)[23], after treatment with paclitaxelat 100 nM in HeLa cells for different time points, G2/M checkpoint negative regulator $p21^{Waf1/Cip1}$, and inactivated factor phospho-cdc2 (p-cdc2) (Tyr15) were down-regulated,while mitosis-promoting factor cyclin B1 was up-regulated significantly from 6 h compared with control (0 h), and cyclin B1 was targeted for degradation by the APC at 48 h. Mitosis phase marker proteins phospho-Histone H3 (Ser10) and phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) were up-regulated significantly since 12 h compared with control. When HeLa cellswere treated with TMC120 at 12.5 μM for different timepoints (Figure2D), cyclin B1 was up-regulated significantly since 1 h, $p21^{Waf1/Cip1}$ and phospho-cdc2 (Tyr15) were down-regulated significantly since 12h compared with control. Phospho-Histone H3 (Ser10) and phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) were up-regulated significantly since 12h compared with control. The analogous tendency was also seen in SiHa cells treated with TMC120 at 12.5 μM (Figure 2E), except that the phospho-cdc2 (Tyr15) was down-regulated slightly. These results suggested that TMC120 inactivated the G2/M checkpoint and arrested cancer cells in mitosis phase, which was similar to paclitaxel.

TMC120 enhanced tubulin polymerization in a dose-dependent manner

Considering the similar effect on M phase arrest and G2/M checkpoint regulation of TMC120 to paclitaxel, the famous anti-cancer drug targeting microtubules, we hypothesized that TMC120 might also interfere with microtubules morphology. To test this hypothesis, a/β-tubulin in response to TMC120 or paclitaxel treatment were examined by immunofluorescent microscopy. As shown in Figure 3, after TMC120 or paclitaxel treatment, both the HeLa cells (Figure 3A)and the SiHa cells (Figure 3B) were poorly spread and the microtubules became dense and concentrated around cell nucleus compared to the control (Figure 3A and 3B). And mitotic cellsexhibited severe alterations, including abnormal and monopolar microtubules arrays(Figure 3B) and rarely normal mitotic spindles were found.

Since TMC120 induced severe alterations on the mitotic microtubule network,we further tested its effect on microtubule polymerization bytubulin polymerization assay (Figure 3C). We compared the behavior of tubulin when exposed to the TMC120 or to the stabilizing agent paclitaxel. As expected, TMC120 (6.25 μM) showed even strongereffect on promoting the microtubule polymerization than paclitaxel (10 μM). These results indicated that TMC120 enhanced tubulin polymerization in a dose-dependent manner and exhibited comparable microtubule- stabilizing effect to paclitaxel.

TMC120 inhibitedcancer growth *in vivo*

The direct anti-cancer activity of TMC120 *in vivo* was evaluated using an 18-day cervical cancer SiHa xenograft model in nude mice. In this study, the growth rate of the tumor was substantially retarded by the treatment of TMC120 compared with control group (Figure4A). Eighteen days of continuous TMC120 treatment (12.5, 25, and50 mg/kg body weight)

significantly reduced the tumor volume by 53.7%, 50.6% and 56.9%, respectively. Meanwhile, treatment of paclitaxel reduced the tumor volume by 32.5% (Figure 4B). No obvious systemic toxicity was observed during the entire period of drug treatment based on the body weight data (Figure 4C).

In addition, TMC120 was shown to inhibit cancer cell growth through inducing apoptosis at cellular level, it was necessary to know whether TMC120 had the same effect on tumor tissues. Therefore, we investigated the effects of TMC120 on apoptosis induction in the tumor sections using TUNEL assays. Representative images from each group and average percentage of TUNEL-positive cells in each group revealed that both TMC120 and paclitaxel significantly increased apoptosis in the tumors in comparison with control group (Figure4D and 4E). It suggested that TMC120 inhibited tumor growth through inducingapoptosis on tumor tissue.

Discussions

AIDS was described in the Western world in the early 1980s, and since its initial description there was an established relationship between the syndrome and the occurrence of malignancies in patients afflicted by it. After the introduction of HAART in 1996 there has been a significant reduction in KS, PCNSL, and systemic DLBCL with increases in Hodgkin lymphoma (HL) (Epstein-Barr virus-related), lung cancer, nonmelanoma skin cancer, and no changes in the incidence, from before to after the introduction of HAART, of cervical cancer and HIV-related Burkitt lymphoma[1].

Previous studies indicated HIV protease inhibitors were considered as a new class of anti-cancer drugs for inhibition of cancer-cell invasion and angiogenesis, and protease inhibitors has

been reported to inhibit malignancies, such as recurrent liposarcoma, solid tumors, stageⅢNSCLC, locally advanced pancreatic cancer, rectal cancer, glioblastoma and acute myelogenous leukemia [24]. Moreover, the endogenous reverse transcriptase was discovered to play roles in proliferation and differentiation of transformed cells [25]. However, whether the HIV reverse transcriptase inhibitors owned anti-cancer effect was still elusive. In this study, we discovered that TMC120 enhanced microtubule polymerization and arrested cancer cells in mitosis phase, thus induced apoptosis in cervical carcinoma cell lines. Furthermore, TMC120 administration reduced tumor growth *in vivo*. These results indicated HIV reverse transcriptase inhibitors could be developed as a potential antitumor agentsfor the first time. Compared with other HIV reverse transcriptase inhibitors, such as nevirapine and efavirenz , TMC120 was a new generation of NNRTIs that has been tested in phase 3 trials as IVRs for vaginal HIV-1 infection [26, 27], and cervical cancer was a major problem among HIV-infected women [20]. Our study disclosed the anti-cancer effect of TMC120, which could be used as a preventive agent for HIV-related cancers, especially the cervical cancer.

In this study, we found TMC120 was a novel anti-cancer agent that inhibited mitotic progression and induced apoptosis in most solid tumor cell lines. We examined whether the impact of TMC120 on cancer cells is cell-cycle dependent specific and then found that TMC120 significantly arrested cancer cells in G2/M phase of cell cyclefrom the concentration of 12.5 μM in both HeLa and SiHa cells. For HeLa cells, cell apoptosis was induced by TMC120 at the concentration of 6.25 μM, but cell cycle arrest was not observed at this concentration, this phenomenon suggested that TMC120 might induce cell apoptosis through other pathways besides the G2/M arrest, which needs further study.In order to maintain genetic integrity, cells were equipped with cell cycle checkpoints that detected DNA damage, orchestrate repair, and if

necessary, eliminate severely damaged cells by inducing apoptotic cell death. The mitotic machinery is now emerging as an important determinant of the cellular responses to DNA damage where it functions as both the downstream target and the upstream regulator of the G2/M checkpoint[15]. The G2/M checkpoint prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA that was damaged in late S or G2 phases prior to mitosis. The critical target of the G2 checkpoint was the mitosis-promoting activity of the cyclin B/cdc2 kinase[28], and their negative regulators including $p21^{Cip1}$ and $p27^{Kip1}$. Weakened G2/M checkpoint under therapeutic setting may trigger cell death via mitotic catastrophe for cells with unrepairable DNA lesions and mitosis machinery [22]. The entry of eukaryotic cells into mitosis was regulated by activation of cdc2/cdk1 at the G2/M transition. This activation is a multi-step process that began with the binding of the regulatory subunit, cyclin B1, to cdc2/cdk1 to form the mitosis-promoting factor (MPF). The critical regulatory step in activating cdc2 during progression into mitosis appeared to be dephosphorylation of cdc2 at Thr14 and Tyr15 [29]. Phosphorylation at Thr14 and Tyr15 resulted in the inhibition of cdc2. Translocation to the nucleus at the G2/M checkpoint, and nuclear accumulation of cyclin B1 were thought to be initiation of mitosis [30]. At the anaphase of mitosis, cyclin B1 was targeted for degradation by the anaphase-promoting complex (APC), allowing for cell cycle progression [31]. The $p21^{Cip1}$ was known as a cyclin-dependent kinase (CDK) inhibitor, and could arrest cell cycle by inhibiting the formation of cyclin-CDK complex [32]. TMC120 showed impressive inactive effect at the progression of G2/M checkpoint.In this scenario, critical mitosis-promoting activity regulatory proteins, p-cdc2/cdk1, $p21^{Cip1}$and cyclin B1, played critical roles in the TMC120 effect. These results strengthened the anti-cancer effect of TMC120.

Phosphorylation of histone H3 was low in interphase cells and occurs almost exclusively during mitosis. Phosphorylation at Ser10, Ser28, and Thr11 of histone H3 was tightly correlated with chromosome condensation during mitosis [33].At prophase and metaphase, Aurora A was highly localized in the centrosomic region and in the spindle poles while Aurora B presented in the centromeric region concurrent with H3 phosphorylation, which then translocated by cytokinesis to the midbody region. Phosphorylation of Aurora A at Thr288 and Aurora B at Thr232 in their catalytic domain increased the kinases activities. Aurora A was involved in centrosome separation, maturation, and spindle assembly and stability. Expression of Aurora B protein also peaked at the transition from metaphase to the end of mitosis. Aurora B regulated chromosome segregation through the control of microtubule-kinetochore attachment and cytokinesis [34]. TMC120 largely enhancedapoptotic indicator proteins levels,this phenomenon further indicated the effect of TMC120 on apoptosis inducing.

Moreover, we further confirmed that TMC120 exhibited anti-cancer activity through inducing apoptosis on tumor tissue*in vivo*without obvious systemic toxicity during the whole period of drug treatment. TMC120 showed a similar anti-cancer effect at several concentrations (12.5, 25 and 50 mg/kg body weight) for 18 days,which indicated that the antitumor effect of TMC120 was concentration independent over the range 12.5-50 mg/kg body weight *in vivo*. TMC120 was a potential chemoprophylactic and therapeutic agent for cancers in a manner similar to paclitaxel, and it could be more effective against some other solid cancers, such as colon carcinomas (colon26, HT29 cell lines), esophageal cancer (EC109 cell line), as there were approximate half inhibitory concentration of TMC120 on cancer cells (Table 1). Furthermore our investigation showed that TMC120 stabilized microtubules in a manner similar to paclitaxel. By binding to β-tubulin and promoting the assembly of microtubules, paclitaxel prevents

microtubule depolymerization and blocks normal cell division [35]. Paclitaxel is not easy to extract and produce, and thetreatment costsmore for the HIV-infected patients. In contrast, the use of TMC120may have a “one stone, two birds” effect on HIV infected patients, in whichTMC120 could not only reduce HIV infection, but also decrease the HIV-related cervical cancer and its treatment cost.

In conclusion, due to the dual antiretroviral and anti-cancer activity, TMC120 could be used as a potent anticancer agent forthe preventionand therapy of cancerin AIDS patients.

Acknowledgements

This work was supported by the Natural Science Foundation of China (31370781), Guangdong Pearl River Scholar Funded Scheme to Shuwen Liu.

Authors’ contributions

Lili Shi performed the drug screening, animal experimentand Western blot assay.Desheng Zhong and Chunping Gu performed cell-cycle and microtubules morphology assay, Lili Shi and Xingang Yao performed statistical analyses. Le Yu, Lin Li and Shuwen Liu contributed to the manuscript preparation and project design.

Conflict of interest

There are no conflicts of interest.

Table 1. Effect of TMC120 on cell proliferation of cancer cells.

Cancer	Cell Lines	IC_{50} (μM)
Lung cancer	A549	9.16±2.14
	95D	7.00±0.51
Liver cancer	HepG2	7.30±0.22
Colon cancer	colon26	3.99±0.95
	HT29	8.81±0.56
Esophageal cancer	EC109	6.61±0.52
Breast cancer	MCF-7	9.83±0.92
Endometrial cancer	HEC-1	7.99±0.24
Cervical cancer	HeLa	6.98±0.93
	SiHa	12.64±0.8

Footnotes: Cells were treated with TMC120 (0–50 μM) for 48 h. And then cell viability was determined by MTT assay. IC_{50} were calculated with Prism software. Data was presented as mean ± s.e.m.

Figure Legends

Figure 1. HeLa (A), SiHa (B) cells were incubatedwith TMC120 (0, 6.25, 12.5, 25 μmol/L), or cisplatin (10, 20 μmol/L) for 48 h. The extent of apoptosis was measured by flow cytometricdetection. Percentage of Annexin V-positive cells was plotted as histograms from 3 independent experiments.

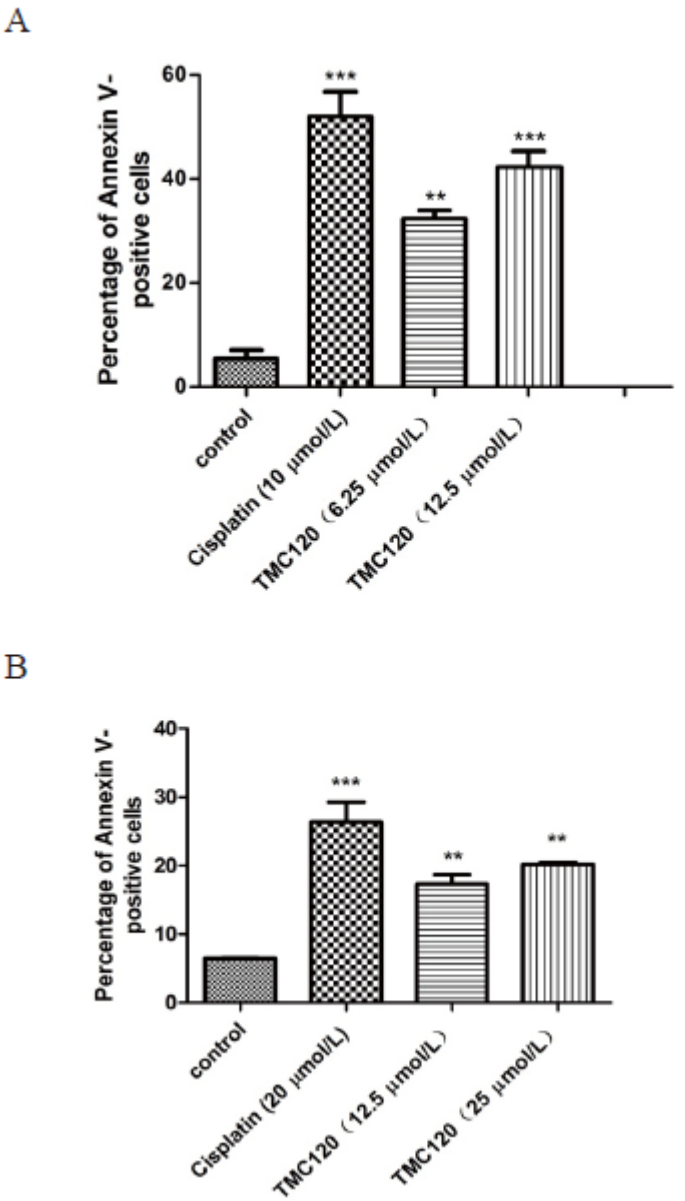

Figure 2. The cell-cycle distribution was measured by the flow cytometricdetection. HeLa (A), SiHa (B) cells were incubated with TMC120 (0, 6.25, 12.5, 25 μmol/L), or paclitaxel (0.03, 0.1, 1 μmol/L) for 24 h. The percentage of G2/M phase cells was from 3 independent experiments.The effects of TMC120 on G2/M checkpoint and M phase marker expression were examined by western blot,(C) HeLa cells were treated with paclitaxel (100 nM) for 0, 1, 3, 6, 12, 24, 48 h. (D) HeLa cells were treated with TMC120 (12.5 μM) for 0, 1, 3, 6, 12, 24, 48 h. (E) Siha cells were treated with TMC120 (12.5 μM) for 0, 1, 3, 6, 12, 24, 48 h.β-actin was used as loading control.

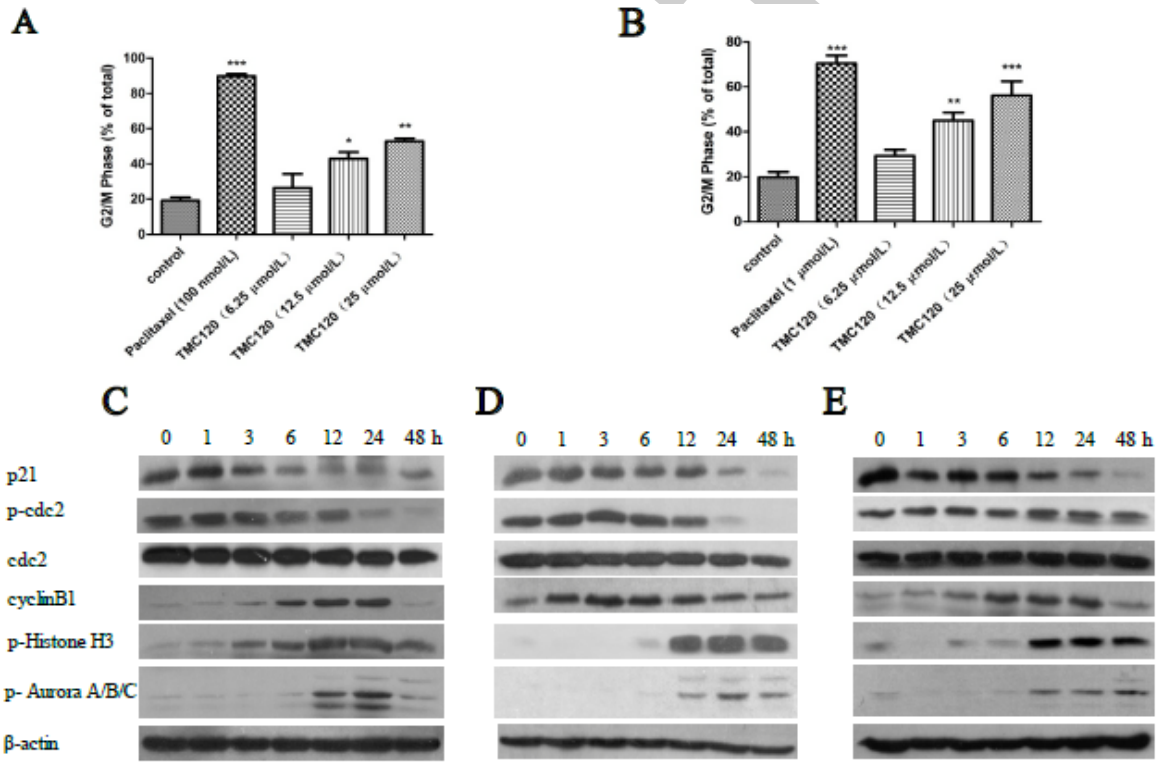

Figure3. Effects of TMC120 on microtubules morphology of HeLa (A) and SiHa (B) cells. Immunofluorescence of cells treated with 12.5 μmol/L of TMC120 and 100 nMor 1 μM of paclitaxel for 12h respectively. For immunodetection of microtubules, cells were stained with an anti-a/β-tubulin antibody to visualize microtubules (red) and DAPI to counterstained DNA (blue). (C) Effect of TMC120 on tubulin polymerization *in vitro*, results were from three independent experiments.

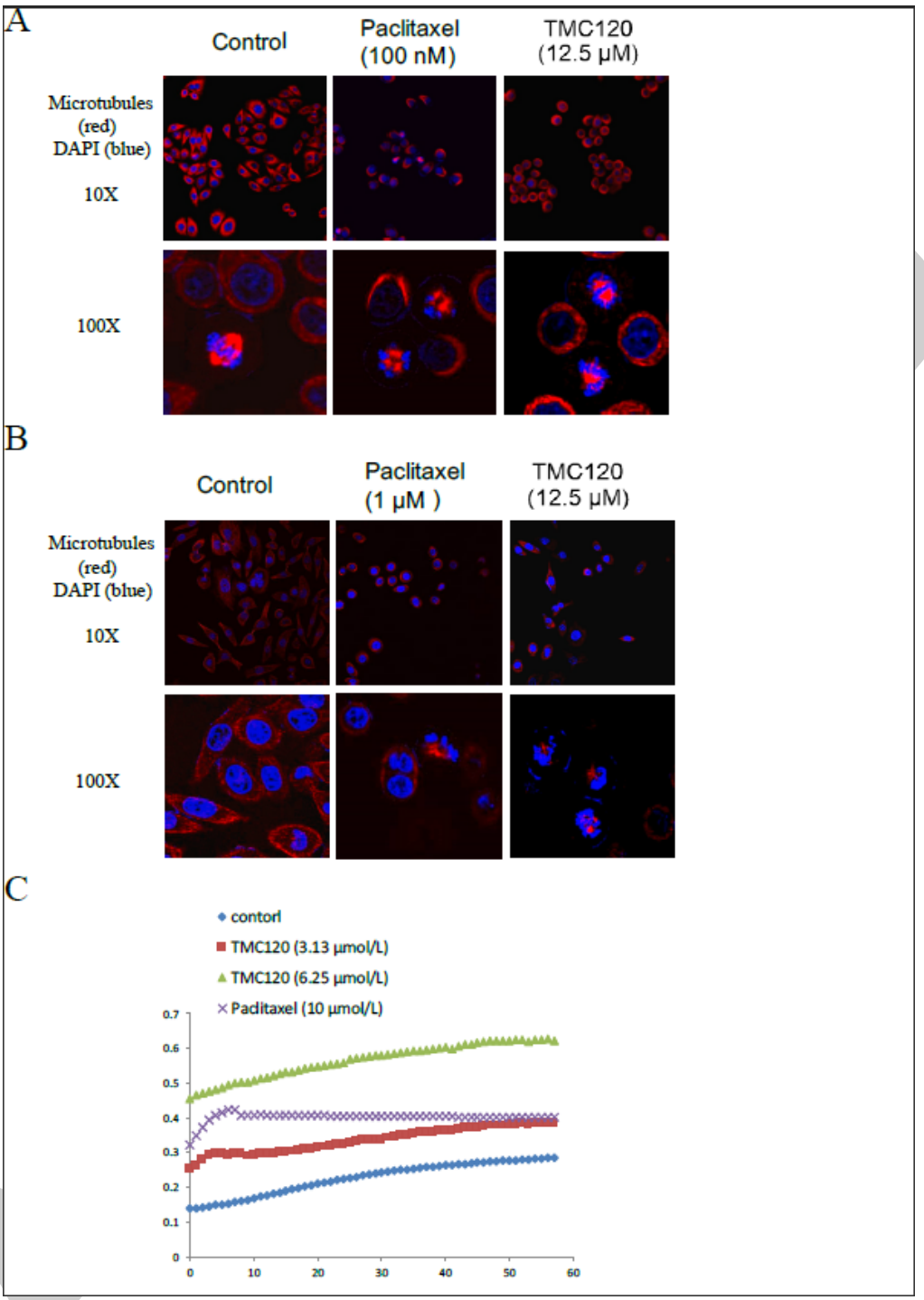

Figure 4. Effects of TMC120 on the growth of cervical cancer cell carcinoma xenografts in nude mice. (A) The tumor of the mice treated with TMC120 (12.5, 25 and 50 mg/kg). (B) Tumor volume was measured every 2 day. (C) The mice body weight was recorded every 2 day. (D) TUNEL assay of cervical cancer cell carcinoma xenografts. (E) The percentage of apoptotic cells (TUNEL-positive cells) was counted from 6 random microscopic fields per sample at the 200 × magnification. Data were plotted as histograms showing five groups.

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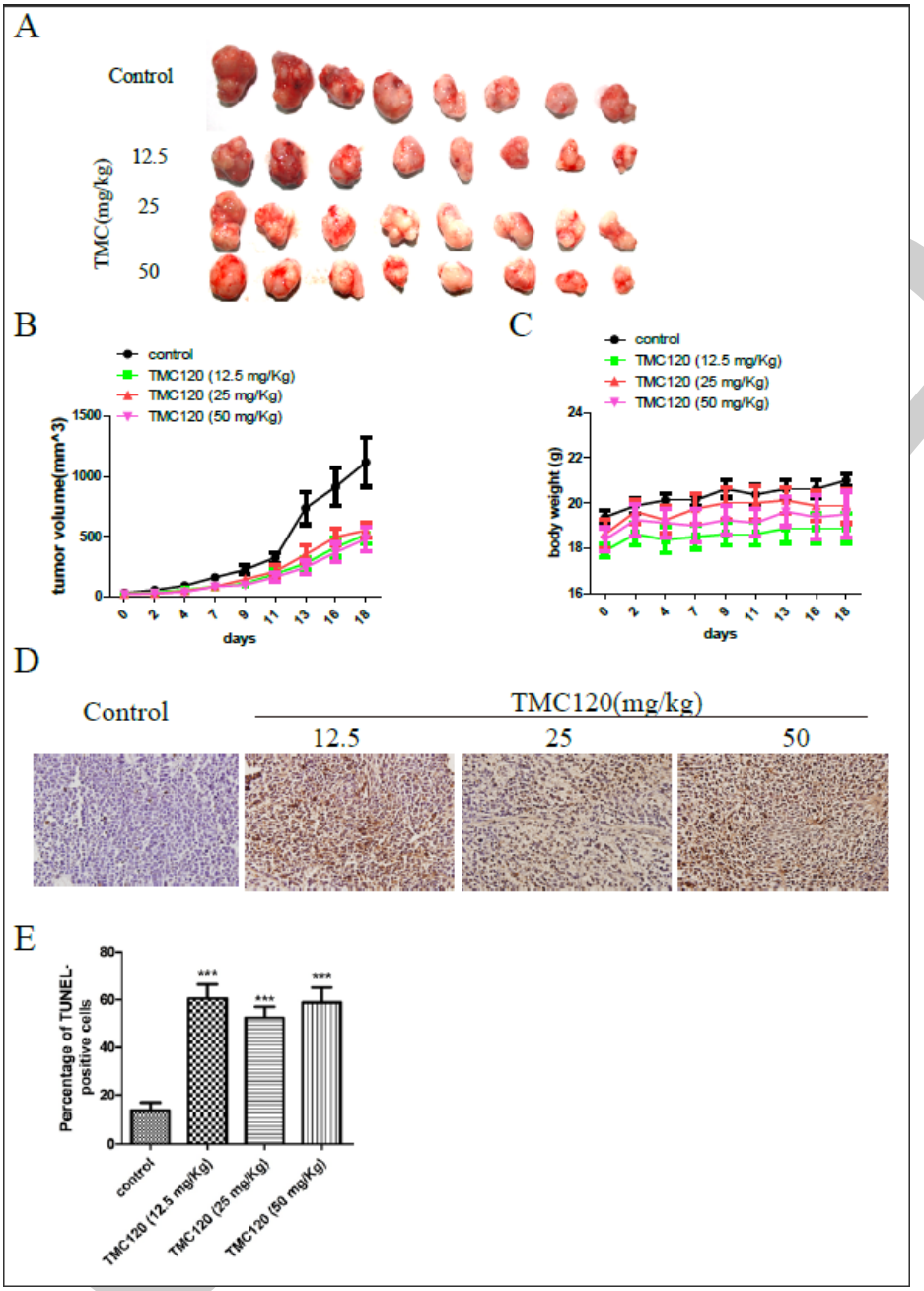

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