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Research Article

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Antitumor effects of a new compound TW9183 on human A549 lung cancer and SMMC-7721 hepatoma cell lines

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ABSTRACT

TW9183 is a new compound synthesized in our laboratory that has been entered preclinical trials in China for developing it into a new anticarcinogenic medicine. In this study, we aimed to investigate the antitumor effects of the compound on A549 and SMMC-7721 cells. We found that the TW9183 showed better proliferation-inhibitory effects on SMMC-7721 tumor cells (IC50 = 1.586 μmol/l) than A549 cells with time and dose dependent. TW9183 also inhibited colony formation (7.06% at 2 μmol/l and 0.72% at 8 μmol/l respectively) and cell migration in A549 and SMMC-7721 cell lines. TW9183 induced G2/M arrest in A549 cells and SMMC-7721 cells. The study indicated that TW9183 may have potent antitumor effects on human lung and hepatitis cancers.

Keywords: tumor cell, apoptosis, TW9183, A549, SMMC-7721

INTRODUTION

Malignant tumor is a serious threat to human life safety and quality of life，Direct and indirect involvement of protein kinases in tumor growth, metastasis and apoptosis make them the most promising targets for treating cancer[1]. Small molecular targeted kinase inhibitors with high antitumor activity and low side effects are developing rapidly in these years, which are promising for patients that can not be cured by traditional chemotherapy. Intensive researches in the field of tyrosine kinase inhibitors have led to the development of enormous active compounds, including Geftinib (ZD1839, Iressa®) [2] and Lapatinib (Tykerb®)[3] approved by the U.S. Food and Drug Administration (FDA). These medicines are derivatives of quinazoline that exert their antitumor effects by competing with ATP for binding to catalytic domain of EGFR tyrosine kinase, to inhibit PKC, Ras/Raf/MEK and PI3K/Akt signaling pathways for inhibition of proliferation, differentiation and angiogenesis of tumor cells[4-7]. Because of the complexity of the pathogenesis of tumor, kinase inhibitors only show clinical effects on limited type of tumors [8]. The small molecule drug TW9183 is one of derivatives of quinazoline, enzymology experiments in vitro have indicated that its inhibitory rates for kinases including VEGFR2, PDGFα, Aurora-B are more than 80% in human lung cancer and liver cancer xenograft models [9]. In the present study, we evaluated the antitumor effects of TW9183 *in vitro* using A549 and SMMC-7721 cell lines. The study provides experimental evidence for clinical treatment of lung and liver cancer with the compound.

EXPERIMENTAL SECTION

Chemicals and Cell culture

TW9183 (batch number: 20100610, purity: 99.2%), Gefitinib, MTT. HUVEC, A549 and SMMC-7721 cells were grown in RPMI-1640 supplemented with 10% calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator at 37 °C and 5% CO2.

Cytotoxicity assay and Colony formation assays

Cell viability was measured by MTT assay [10]. Cells were seeded in 96-well plates and exposed to various

concentrations with TW9183(0.5~8 μmol/L) for 24, 48 and 72 h. After incubation with TW9183, MTT solution was added. Plates were further incubated at 37 °C for 4 h. The metabolite of MTT dissolved in DMSO were quantified by spectrometer at 570nm. Cell were plated into 6-well tissue culture plates at a density of 300 cells/well in complete medium [11]. 24 h after seeding, various concentrations of TW9183 was added .The medium was changed 48 h after plating, and further incubated for 1～2 weeks in complete medium. Cells were stained with 600 μl crystal violet staining solution and colonies >0.05 mm were counted by Image Pro Plus 6.2. Colony formation efficiency.

In vitro wounding (scratch) assay

Cells were plated into 24-well plates (1×105 cells) in complete medium and grew for 24 h to obtain a confluent monolayer, the monolayer was scraped in a straight line to create a "wound (scratch)". The debris was removed and the edge of the wound (scratch) was made smooth by washing the cells twice with 1 ml PBS [12].Various concentrations of TW9183 was added. The distance between one sides of the scratch to the other side was measured under a TE-2000U inverted fluorescence microscope. The distance between the edges was measured 48 h after the treatments.

Viability measurement of A549 and SMMC-7721 cancer cells by Hoechst 33342

Cells were treated with various concentrations of TW9183 , the medium was removed 48 h after incubation, cells were fixed with 100 μl of 4% paraformaldehyde each well, washed twice with PBS, and stained with 80 μl Hoechst 33342 for 10 min at room temperature, removed the staining solution, washed with PBS twice, observed under a fluorescence microscopy [13-14].

Cell cycle distribution measurement by flow cytometry

Cell were plated into 6-well plates(2×105 cells) and exposed to various concentrations of TW9183 was added for 48 h after incubation, cells were collected, washed and incubated in 70% ice-cold ethanol, kept at 4 °C overnight for fixation. Cells were centrifuged (4 °C, 1000 g, 5 min), and then incubated with 0.5 ml PI solution containing RNase A provided in the Cell cycle and apoptosis analysis kit at 37 °C for 30 min in the dark[15]. Cell cycle distribution was measured by flow cytometry (BD).

Statistical Analysis

Statistical analysis was performed with SPSS17.0. Results are presented as the average ± standard deviation (SD) from at least two in-dependent experiments. A p-value less than 0.05 is considered to be statistically significant.

RESULTS AND DISCUSSION

Inhibitory effects of TW9183 on proliferation of A549 and SMMC-7721 tumor cell lines

To define the inhibition of proliferation, MTT assay was performed. As a result, there was no significant effect for HUVEC after TW9183 treatment（**Fig.1-a**）, but TW9183 could inhibit proliferation for both cancer cell lines （**Fig.1-b,c**）, and there was significant differences compared with the negative control（P<0.001）. TW9183 reduced cell proliferation in a dose and time-dependent manner. Between the two cell lines, TW9183 showed the better inhibition effects on SMMC-7721 cells with the IC50 =1.586 μmol/l for 72h (**Table-1**).

Table 1 Cytotoxicity of TW9183 against A549 and SMMC-7721 cell lines

cell line	TW9183 IC50（μmol/L）			Gefitinib IC50 (μmol/L)
	24 h	48 h	72 h	72 h
A549	19.116	11.313	6.182	20.458
SMMC-7721	9.891	5.949	1.586	15.741

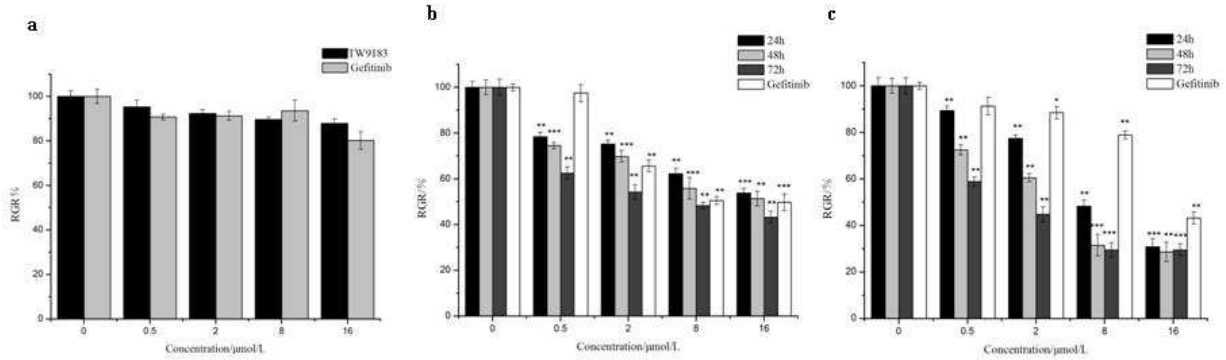

Fig 1. The growth-inhibitory effects of TW9183 on HUVEC、A549 and SMMC-7721 cell lines. The proliferation status of a: HUVEC were measured by MTT assay after treatment with TW9183 for 72h, b:A549 cells, c: SMMC-7721 cells after treatment for 24、48、72 hours, and normalized by NC(negative control). RGR: Relative growth ratio. n = 3.*P < 0.05, ***P < 0.001 vs NC group

Inhibitory effects of TW9183 on colony formation of A549 and SMMC-7721 cell lines

Colony formation ability of cancer cells reflects colony dependence and proliferation, it's an important factor for the formation of solid tumors *in vivo*. Colony formation ability of both of the cell lines decreased in a dose-dependent manner after treated with TW9183, showing more remarkable effect than MTT assay **(Fig.2-b**). In the case of A549, colony formation rates were only 7.06% for the 2 μmol/L TW9183 treatment, and colonies formation was rarely observed for 8 μmol/L and 16 μmol/L TW9183 treatments (**Fig.2-a**).

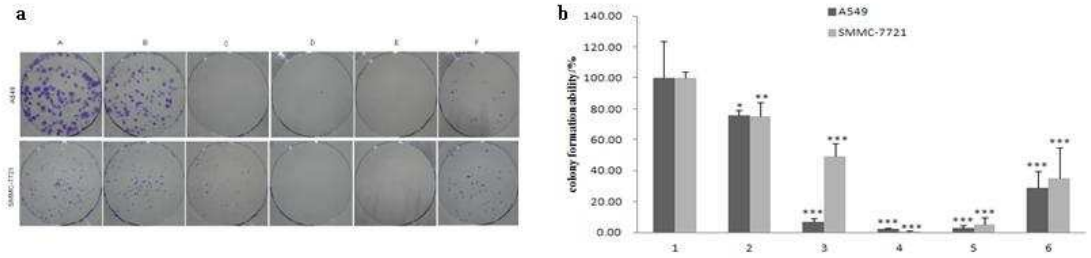

Fig 2. Effects of TW9183 on colony formation of A549 and SMMC-7721 cell lines. (A) 0.0, (B) 0.5, (C) 2.0, (D) 8.0, (E) 16.0 μmol/L TW9183, and (F) 16.0 μmol/L gefitinib, respectively treated for 48 hours.(a). Rep[resentative figure showing the effects of Tw9183. (b). Semiquantification of the effect of TW9183. (1) Negative control . (2) 0.5, (3) 2.0, (4) 8.0, (5) 16.0μmol·L−1TW9183, and (6) 16.0 μmol/L gefitinib. n = 3, *P<0.05、P<0.01、 ***P < 0.001 vs NC group**

Effects of TW9183 on migration

Tumor cell migration is a critical factor in the formation of solid tumors and is necessary for their spread to distant organs. As shown in **Fig.3-b**, in the case of A549, the size of the wound (scratch) before treatment was 566.19±9.55 μm，which was decreased to 195.05±15.37、247.44±22.49、323.96±21.46、347.24±13.07、452.91±20.93 and 99.43±15.19μm for 0.5、2.0、8.0、16.0μmol·L−1 TW9183, 16.0 μmol·L−1 gefitinib and negative control treatment. As shown in **Fig.3-c**, for SMMC-7721 cells, the size of the wound (scratch) prior to treatment was 496.02±12.85 μm，which was decreased to 350.75±24.66、396.78±15.94、383.79±33.06、421.54±38.25、455.56±14.45 and 250.36±19.52 μm for 0.5、2.0、8.0、16.0μmol/L TW9183, 16.0 μmol/L gefitinib and the negative control respectively 48 h after the treatments, indicating inhibition of migration of A549 and SMMC-7721 cell lines, especially A549 cells.

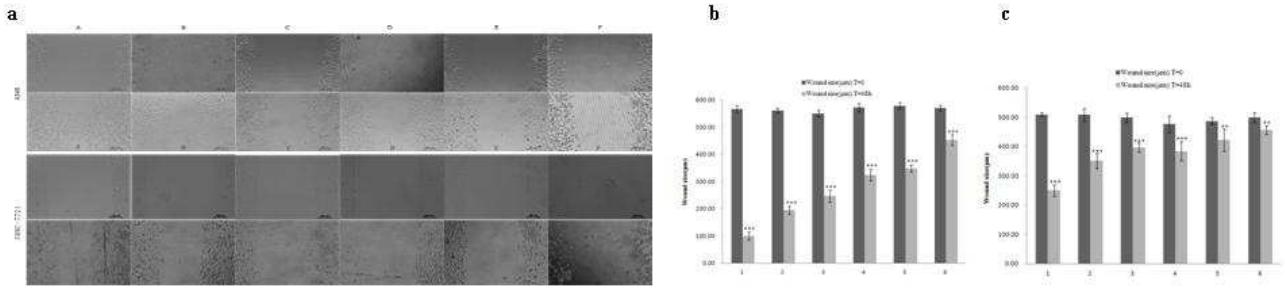

Fig 3. Effects of TW9183 migration of A549 and SMMC-7221 cell lines. The width of the wound was recorded again after 48 h post-wounding by light microscope (×100). (a). Photomicrographs of cells treated with (A) 0.0, (B) 0.5, (C) 2.0, (D) 8.0, (E) 16.0 μmol/L TW9183, and (F) 16.0 μmol/L gefitinib. Six random widths along the wound before and 48 h post-treatment for (b) A549 cells and (c)SMMC-7721 cells. P-values were calculated by unpaired t-test for the same treatment prior to and after 48 h, **P<0.01, ***P<0.001

Effects of TW9183 on viability of A549 and SMMC-7721 cancer cells

As shown in **Fig.4**, in negative control treatment, cells appeared with regular contours, round and large in size. Rarely cells with smaller nuclei and condensed chromatin were seen. In contrast, most nuclei of TW9183 treated cells appeared brightly stained. Note that the numbers of apoptotic nuclei containing condensed chromatin increased significantly as the result of concentration increase.

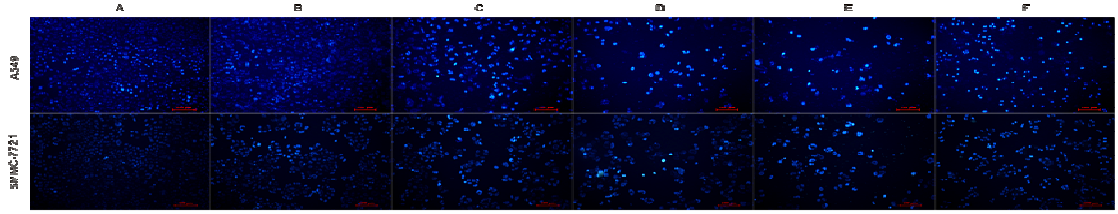

Fig 4. Effects of TW9183 on viability of A549 and SMMC-7721 cells. Fluorescence photomicrographs of cells with Hoechst 33342 staining after treated with (A) 0.0, (B) 0.5, (C) 2.0, (D) 8.0, (E) 16.0 μmol/L TW9183, and (F) 16.0 μmol/L gefitinib for 48 h respectively (×100)

Effects of TW9183 on cell cycle distribution of A549 and SMMC-7721 cancer cells

To determine the effects of TW9183 on cell cycle distribution of A549 and SMMC-7721 cancer cells, flow cytometry was applied. TW9183 induced G2/M-phase accumulation in both cell lines compared to the negative control. In A549 cells, a strong G2/M arrest (50.78%) was observed at 2.0 μmol/L TW9183 treatment (**Fig.5-a** and **Fig.5-b**), and the effect diminished at 16 μmol/L, which leads to a moderate G2-M arrest (20.09%). In SMMC-7721 cells, 8.0 μmol/L TW9183 treatment had the strongest G2/M arrest (83.65%)(**Fig.5-c** and **Fig.5-d**).

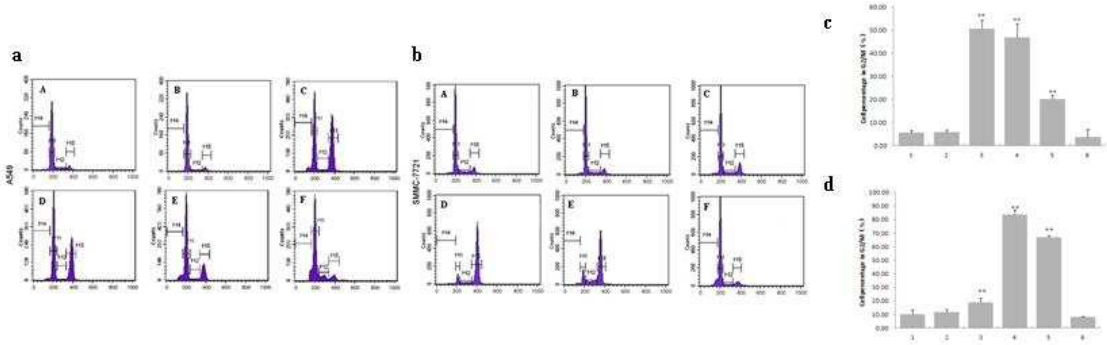

Fig 5. TW9183induces accumulation of A549 cells and SMMC-7721tumor cells at the G2/M phase of the cell cycle. The cells were stained with PI solution and subsequently measurements by flow cytometry. (a) A549 cells were treated with (A) 0.0, (B) 0.5, (C) 2.0, (D) 8.0, (E) 16.0 μmol/L TW9183, and (F) 16.0 μmol/L gefitinib for 48 h respectively. (c) The statistical datas of (a), and normalized by (1) negative control, (2) 0.5、(3) 2.0、(4) 8.0、(5) 16.0μmol/L TW9183, and (6)16.0 μmol/L gefitinib. (b) SMMC-7721 cells were treated with (A) 0.0, (B) 0.5, (C) 2.0, (D) 8.0, (E) 16.0 μmol/L TW9183, and (F) 16.0 μmol/L gefitinib for 48 h respectively. (d) The statistical datas of (c), and normalized by (1) negative control, (2) 0.5、(3) 2.0、(4) 8.0、(5) 16.0μmol/L TW9183, and (6)16.0 μmol/L gefitinib.n = 3, **P < 0.01 vs NC group

In this research we evaluated the antitumor effects of TW9183 in vitro for A549 and SMMC-7721cell lines respectively. TW9183 inhibited proliferation in a time-dose dependent manner, and inhibited migration, promoted apoptosis and induced cell-cycle arrest at G2/M for A549 and SMMC-7721 cell lines. The cells swelled as the

concentration elevated from 0.5 μmol/l to 8 μmol/l, and at the highest concentration 16 μmol/l, cells shrank, debris increased and floating dead cells were observed, indicating a greater cytotoxic effect. In addition, cells swelling and spread were characteristics of cellular senescence[16], which was one of the tumor development mechanisms got a conclusive proof in 2005[17-19]. So further researches are needed to know whether the anti-proliferation effects of TW9183 on A549 and SMMC-7721 cell lines were caused by cellular senescence, e.g. the activity of β-galactosidase and the expression of landmark molecule P21 need to be detected [20]. The anti-proliferation effect of TW9183 on SMMC-7721 cell lines was better than on A549 cell lines, this result was consistent with the animal experimental results showed in a patent [9]. Colony formation is another method to detect cell proliferation, colony formation ability of both cell lines decreased in a dose-dependent manner after treated with TW9183, more remarkable effect than MTT assay. Further, we found that TW9183 inhibited migration of A549 and SMMC-7721 cell lines, which was observed specifically in A549 cells, this maybe one of the anti-tumor mechanisms. Fluorescence photomicrographs of cells with Hoechst 33342 staining showed that TW9183 decreased viability of A549 and SMMC-7721 cells in a dose-dependent manner, especially for A549 cells, indicating a connection with its anti-proliferation effect. TW9183 showed better inhibitory effects on SMMC-7721 cells with the IC50 1.586 μmol/L in 72 h, it hence indicated that other anti-proliferation mechanisms maybe exist for SMMC-7721 cells. In addition, the condensed chromatin fragment may be due to the degradation of DNA in cells, which need to be detected by agarose gel electrophoresis experiment. Flow cytometry analyzed the cell cycle distribution of both cell lines, TW9183 induced stronger G2/M arrest in SMMC-7721 cells, which was 83.65% at 8 μmol/L as compared to 50.78% in A549 cells at 2 μmol/L. This result indicated a cytotoxic effect of TW9183 on SMMC-7721 cells, and a similar anti-tumor mechanism as sorafenib[21].Theoretically, the toxic effects of kinase inhibitors with high selectivity used as antitumor drugs should be lower. In practice, the toxic effects of inhibitors of protein kinases need to be proven in preclinical and clinical trials. The acute and chronic toxic effects of the medicine are being tested now. In conclusion, the present study indicate that TW9183 has strong antitumor effects, and it may be used for treating cancers in clinical trials.

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