RESEARCH ARTICLE

ZD1839 and Cisplatin Alone or in Combination for Treatment of a Nasopharyngeal Carcinoma Cell Line and Xenografts

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Abstract

This study evaluated the effects of ZD1839, an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, on nasopharyngeal carcinoma (NPC) both *in vitro* and *in vivo*. Influence of ZD1839 alone or combined with cisplatin on the NPC cell line CNE2 was detected by MTT assay with flow cytometry assessment of cell cycle distribution and apoptosis rates. Nude mice NPC xenografts were also used to evaluate the effects of ZD1839 alone or combined with cisplatin. The Student's t test evaluated statistical significance. ZD1839 alone or combined with cisplatin inhibited CNE2 cell line proliferation. ZD1839 induced CNE2 cell cycle arrest in the G1 phase, and higher concentrations induced apoptosis. Xenograft tumors were significantly smaller when treated with 200 mg/kg ZD1839, cisplatin, or cisplatin combined with 100 mg/kg ZD1839 than untreated controls. ZD1839 (200 mg/kg) alone showed good tumor inhibition effects, reduction of tumor weights, and smaller tumor volume without loss of body weight. ZD1839 (200 mg/kg) might provide a good and effective therapeutic reagent for NPC.

Keywords: Combined chemotherapy - cell proliferation/drug effects - cisplatin - EGFR - protein kinase inhibitors

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Introduction

Nasopharyngeal carcinoma (NPC), a cancer of epithelial origin, has its highest incidence rates in southern China and southeastern Asia (Jemal et al., 2011). Radiotherapy, the main treatment modality for NPC, has a high local control rate (Lee et al., 2012). Unfortunately, the rapid growth of NPC often results in distant metastases, which leads to a high treatment failure rate.

Currently, much attention in anticancer research is focused on targeting the epidermal growth factor receptor (EGFR) (Chan and Ma, 2012). Among agents targeting EGFR, ZD1839 (gefitinib), IMC-C225 (cetuximab), and OSI-774 (erlotinib) are the furthest along in development (Ma et al., 2012; You et al., 2012). ZD1839 is a novel, low molecular weight, synthetic anilinoquinazoline that is a potent and highly selective EGFR tyrosine kinase inhibitor (Barker et al., 2001; Wakeling et al., 2002), which has with minimal activity against other tyrosine kinases and serine/threonine kinases (Woodburn et al., 2000).

ZD1839 inhibits in vitro growth of a range of human tumor cell lines including head and neck, prostate, breast, and colon cells (Ciardiello et al., 2000; Woodburn et al., 2000; Ranson, 2002). ZD1839 also shows antitumor activity against a range of human tumor xenografts including prostate, breast, ovarian, colon, small-cell lung cancer and non-small-cell lung cancer (Ciardiello et al., 2000; 2001). Furthermore, it has been approved as monotherapy to treat advanced non-small-cell lung cancer (Cohen et al., 2003). Currently, clinical trials of ZD1839 are investigating its application for patients with prostate, breast, head and neck, and colon cancer (Kindler et al., 2005; Gregoire et al., 2011; Adelstein et al., 2012; Engebraaten et al., 2012; Joensuu et al., 2012; Lewis et al., 2012).

As compared to evaluate the abilities of ZD1839 to inhibit the proliferation of NPC cells both in vitro and in vivo, we examined ZD1839 with cisplatin combinatory treatments of nasopharyngeal carcinoma cell line (CNE2) and xenographs.

Materials and Methods

Chemicals and ZD1839

We obtained the cell culture medium PRMI-1640 from Life Technologies, Inc. (Grand Islands, NY); dimethylsulfoxide (DMSO) and MTT from Sigma Chemical Co. (St. Louis, MO); cisplatin from Faulding Company (Australia); and ZD1839 (IRESSA) from AstraZeneca (Macclesfield, United Kingdom).

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Cell Line and Cell Culture

The NPC cell line CNE2 was gifted from Dr Mu-Sheng Zeng (State Key Laboratory of Oncology in South China, Guangzhou).

The CNE2 cell line was routinely cultured in PRMI-1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2. Cells from exponentially growing cultures were used in all experiments.

Growth Inhibition Assay

We evaluated how ZD1839 either alone or combined with cytotoxic drugs affected the proliferation of the CNE2 cell line with the MTT assay. Briefly, exponentially growing NPC cells (1×10^4 cells/ml, 200 μl/well) were seeded into 96-well plates and incubated for 12 hours. Then, the drug was added, and the cells were incubated for another 72 hours. Subsequently, 5% (v/v) of a solution of 5 mg/ml MTT was added to each well and incubated for 4 hours at 37°C. The plates were centrifuged for 5 minutes at 1000 rpm, and the medium was carefully discarded. The formazan crystals that formed were dissolved in 100 μl of DMSO, and the absorbance of each well was measured in a microplate reader at 570 nm. The percentage of cell growth was calculated by comparing the A570 reading from treated cells to the control cells. Experiments were repeated three times to obtain average values. The half maximal inhibitory concentration (IC_{50}) was calculated based on the Bliss equation. Proliferation inhibition rate was calculated as $(1-[A_{treatment} - A_{blank}]/[A_{control} - A_{blank}]) \times 100\%$. Experiments were repeated three times, and the data represent mean values.

Flow Cytometric Analysis of Cell Cycle and Quantitation of Apoptosis

A total of 1×10^6 control cells or cells treated with ZD1839 at 1.95 μmol/L, 3.9 μmol/L, 7.8 μmol/L, 15.6 μmol/L, or 31.25 μmol/L were harvested by trypsinization, washed twice with PBS, fixed in 95% ethanol, and then stored at 4°C for up to 7 days before DNA analysis. The ethanol was removed by centrifugation, then the cells were washed twice with PBS, and then the cells were incubated with 100 µl 1% RNAase at 37°C for 30 minutes. The cells were then stained with a solution containing 50 μg/ml propidium iodide (PI). The stage of the cell cycle and the proportion of cells that underwent apoptosis were analyzed with a Becton Dickinson FACScan flow cytometer. Each treatment was performed in triplicate, and the data represent mean values.

Tumor Xenografts in Nude Mice

Ethics statement: this study was approved by the Institutional Animal Care and Use Committees (IACUC) of Center for Prevention and Treatment of Cancer of Sun Yat-sen University.

A total of 40 BALB/C nu/nu mice, age five to six weeks old, specific-pathogen free (SPF), with a male to female ratio of 1:1 were purchased from the Animal Center of the Center for Prevention and Treatment of Cancer of Sun Yat-sen University (License No: 26-2002A005 for SPF BALB/C nude mice). The animal center was approved by the Guangdong province and licensed as Yue2002A009 for SPF BALB/C nude mice and Yue2002B008 for animal environment (SPF).

Logarithmic-phase CNE2 cells were suspended at 107/ml and 0.2 ml of the cell suspension was subcutaneously inoculated into nude mice at their right armpits. Seven days after the cell inoculations, the tumors were 100-200 mm3. The mice were stratified by gender and then divided on the basis of tumor size into five treatment groups with 8 mice (4 male, 4 female) in each group: untreated control group, 100 mg/kg ZD1839 group, 200 mg/kg ZD1839 group, cisplatin group, and 100 mg/kg ZD1839 + cisplatin group. ZD1839 was administered by oral gavage at the specified doses on days 1-5 of each week for four weeks, while 5 mg/kg cisplatin was administered intraperitoneally once each week for four weeks. The mice were sacrificed by first deeply anesthetized by sevoflurane then euthanized by cervical dislocation two days after the treatments ended. Their tumors were collected and weighed. The tumor inhibition rates were calculated as:

Tumor inhibition rate (%) = (tumor $weight_{control}$ – tumor $weight_{treatment}$)/tumor $weight_{control} \times 100\%$. Tumor volumes were calculated as ½ × (large diameter) × (small diameter)^2.

Statistical Analysis

Data is expressed as mean ± standard deviation (SD). The Student's t test evaluated statistical significance.

Results

The anti-proliferative effects of ZD1839 in CNE2 cell line were done by MTT assay. The IC_{50} value of ZD1839 on CNE2 cell line was calculated as 5.6 μmol/L. The antitumor effect of cisplatin on CNE2 cells was enhanced by ZD1839 (Figure 1). Cell cycle progress was inhibited by ZD1839, with cells treated with 3.9 µM ZD1839 for 48 hours accumulating in G1 rather than S phase compared with control cells (Figure 2).

Among the mice receiving the CNE2 xenografted

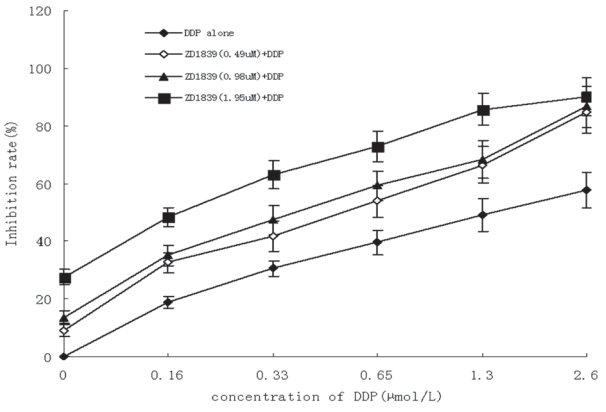

Figure 1. Growth Inhibition of CNE1 Cells Treated with Cisplatin Alone (♦), Cisplatin and 0.49 µM ZD1839 (◊), Cisplatin and 0.98 µM ZD1839 (▲), or Cisplatin and 1.95 µM ZD1839, as Detected by the MTT Assay. Data represent mean ± S.D. of three independent experiments, each performed in triplicate. Error bars indicate standard deviation

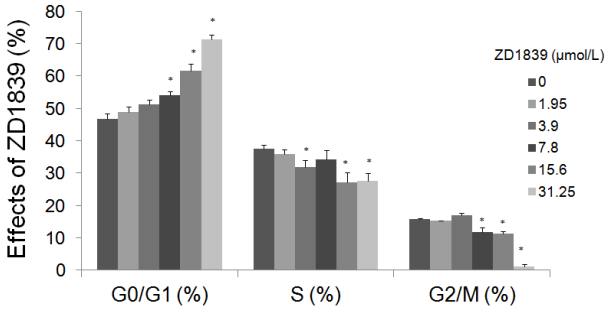

Figure 2. Effects of ZD1839 on Cell Cycle in CNE2 Cells were Detected by Flow Cytometric Analysis. A total of 1×106 CNE2 cells treated with ZD1839 at 1.95 μmol/L, 3.9 μmol/L, 7.8 μmol/L, 15.6 μmol/L, or 31.25 μmol/L. Cell cycle progress was inhibited by ZD1839, with cells treated with 3.9 μM ZD1839 for 48 hours accumulating in G1 rather than S phase compared with control cells. Data represent mean ± S.D. of three independent experiments, each performed in triplicate. Error bars indicate standard deviation

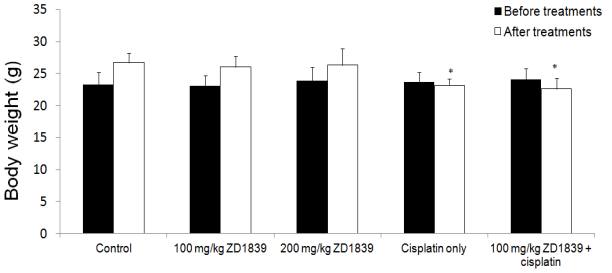

Figure 3. Body Weights s in Groups Before and after Treatments. Five groups of animals were used for comparison, including Control, 100 mg/kg ZD1839, 200 mg/kg ZD1839, Cisplatin only, and 100 mg/kg ZD1839 + cisplatin. Data represent mean ± S.D. of three independent experiments, each performed in triplicate. Error bars indicate standard deviation

tumors, body weights and tumor sizes were balanced and comparable in each group before their treatments, based on analysis of variance. Compared with the untreated control tumors, the tumors were significantly smaller when treated with 200 mg/kg ZD1839 ($P = 0.02$), cisplatin ($P = 0.007$), or cisplatin and 100 mg/kg ZD1839 ($P = 0.001$).

The growth of the xenograft tumors was transiently reduced by treatment with 100 mg/ml ZD1839 combined with cisplatin during the first days of treatment, but the tumor growth subsequently increased (Table 1). The tumors were markedly smaller in the mice treated with cisplatin or with 100 mg/kg ZD1839 and cisplatin compared with the untreated control group ($P = 0.007$ and 0.001, respectively). The tumors of the group treated with cisplatin did not have a significant difference from those treated with 100 mg/kg ZD1839 combined with cisplatin. Compared with the untreated control tumors, the growth of the tumors was inhibited 26.3% by 100 mg/kg ZD1839, 30.6% by 200 mg/kg ZD1839, 45.7% by cisplatin, and 54.8% by 100 mg/kg ZD1839 combined with cisplatin.

After the treatments, the average tumor weights were significantly smaller for treatment with 200 mg/kg ZD1839, cisplatin, or 100 mg/kg ZD1839 combined with cisplatin (Table 2). However, treatment with 100 mg/kg ZD1839 was not significantly different from the untreated control tumors. Also, tumors treated with cisplatin were not significantly different from those treated with 100 mg/kg ZD1839 combined with cisplatin.

Table 1. Tumor Sizes after Treatments

Days of Treatment	Tumor size (mm^3)				
	Control	100 mg/kg ZD1839	200 mg/kg ZD1839	Cisplatin	100 mg/kg ZD1839 + Cisplatin
0	155±45	153±41	147±43	143±43	153±41
4	382±130	286±97	237±67	141±48	96±61
8	651±194	377±109	353±109	267±89	208±117
12	1124±415	744±314	577±202	476±133	343±194
16	1768±651	1125±505	821±286	600±195	549±293
20	2264±840	1573±780	1076±332	838±301	734±269
24	2551±858	1857±904	1484±292	1061±390	993±305
28	2874±968	2133±1045	1916±355*	1557±682**	1383±253**

*indicates $P < 0.05$ and **indicates $P < 0.01$ compared with the control group

Table 2. Mean Tumor Weights after Treatments

Treatment Group	Mean tumor weight (g)	*P*-value
Control	1.86 ± 0.64	
100 mg/kg ZD1839	1.37 ± 0.71	0.176
200 mg/kg ZD1839	1.29 ± 0.32	0.041
Cisplatin	1.01 ± 0.51	0.01
100 mg/kg ZD1839 + Cisplatin	0.84 ± 0.14	0.001

Though body weights were similar between the treatment groups before their treatments, both the group treated with cisplatin and the group treated with cisplatin combined with 100 mg/kg ZD1839 had significantly lower body weights than the untreated control group (Figure 3). Treatment with 100 mg/kg or with 200 mg/kg ZD1839 resulted in body weights that were similar to the untreated controls.

Discussion

ZD1839 had been shown to inhibit the proliferation of the NPC cell lines CNE2. These IC_{50} values are in good agreement with previous work that found ZD1839 has IC_{50} values with most tumor cell lines having an IC_{50} of less than 1 μmol/L (Ranson, 2002).

ZD1839 induced the arrest of CNE2 cells in the G1 phase in our study, with increasing concentrations of ZD1839 resulting an increasing proportion of cells undergoing apoptosis. Our results are in agreement with the work of Ciardello, who has reported that ZD1839 induces cell cycle arrest and apoptosis in lung and colon cancer cell lines (Ciardiello et al., 2000; Sirotnak, 2003). We also found that ZD1839 enhanced the antitumor activity of cisplatin on CNE2 cell line. This finding is in agreement with previous work which found that combining ZD1839 with cytotoxic drugs may produce additive or synergistic effects (Ciardiello et al., 2000; Ciardiello and Tortora, 2001).

Similarly, treatment with ZD1839 reduced the volume of the xenograft tumors that we studied, and the tumors were smaller in the mice treated with 200 mg/kg ZD1839 than in those treated with 100 mg/kg. Tumor volume of the xenograft tumors in our study was smaller in the mice treated with only cisplatin than in the mice treated with 100 mg/kg ZD1839. This implies that ZD1839 may potentiate the antitumor activity of cisplatin on NPC. We found that tumors treated with cisplatin alone or with 100

mg/kg ZD1839 combined with cisplatin had similar rates of tumor inhibition. ZD1839 has been found to enhance the cytotoxicity of cisplatin on lung cancer, prostate cancer, and colon cell xenografts (Sirotnak, 2003).

Notably, cisplatin alone treatment already cause body weight loss. A poor response rate occurred in a phase II trial examining the use of ZD1839 alone to treat recurrent and metastatic NPC that had been pretreated with platinum-based chemotherapy (Chua et al., 2008). However, we found that ZD1839 (200 mg/kg) alone treatment showed good tumor inhibition effects, reduction of tumor weights, and smaller tumor volume without loss of body weight.

Overall, we have demonstrated that ZD1839 inhibits the proliferation of NPC cells, enhances the effect of cytotoxic drugs in vitro, and inhibits the growth of NPC CNE2 xenografts in vivo. Our work supports ZD1839 (200 mg/kg) might provide as good and effective therapeutic reagents for NPC.

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