Cytotoxic effect of a non-peptidic small molecular inhibitor of the p53-HDM2 interaction on tumor cells

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INTRODUCTION
HDM2 protein regulates the activity of p53 protein in at least three different ways[10]. First, HDM2 inhibits the transcription activity of p53 protein by binding to its transactivation domain[8]. Second, HDM2 protein modulates nucleo-cytoplasmic shuffling of p53 protein as a shuttle protein[11]. Third, it promotes the degradation of p53 protein as an E3 ubiquitin ligase[4]. HDM2 overexpression inactivates p53 protein in 5-10% of human cancers. Since the p53-HDM2 interaction was elucidated by Kussie et al[12], inhibition of this interaction has been of interest for the study of cancer therapy[7-10]. The feasibility of this strategy has been verified by many inhibitors such as modified thioredoxin inhibitors[13], anti-MDM2 monoclonal antibody 3G5[5], peptide inhibitor fused to the glutathione S-transferase protein[6], anti-sense oligonucleotide resistant to HDM2[7], HDM2 alternatively spliced products[8], chalcone and its derivatives[14], chlorofusin[15] and octamer synthetic peptide[16,17]. These inhibitors above inhibit p53-HDM2 interaction, increase p53 accumulation and cause cell cycle arrest or apoptosis in various tumor cells.

Based on the clarification of the crystal structure of p53-HDM2 complex[8], we have obtained a series of non-peptidic small HDM2 inhibitors designed by computer-aided model and synthesized by chemical method. These inhibitors have been proved to release p53 through competing with the binding site of p53 and HDM2 by ELISA and some results were shown previously[21]. Syl-155 is one of these inhibitors (data not shown). In this report, we investigate whether syl-155 could rescue p53 function from the p53-HDM2 interaction and evaluate its activities in tumor cells with various states of p53.

MATERIALS AND METHODS
Cell lines
Human fibrosarcoma cell line HT1080 expressing wild-type p53 protein, human esophageal squamous cancer cell line KYSE510 expressing mutant p53 protein and human osteosarcoma osteoblast-like cell line MG63 which was p53-negative[22] express HDM2 were used in this study. Human embryonic lung fibroblast (HELF) cell line was used as control. HT1080 and MG63 were purchased from cell center, Wuhan University, China; KYSE510 was a gift from Dr. Shimada Y, First Department of Surgery, Faculty of Medicine, Kyoto University, Japan; HELF was a gift from Institute of Material Medica, CAMS, China.

Non-peptidic small molecular HDM2 inhibitor
Non-peptidic small molecular HDM2 inhibitors were designed by computer-aided model and synthesized by chemical method. These inhibitors have been proved to release p53 through competing with the binding site of p53 and HDM2 by ELISA and some results were shown previously[21]. Syl-155 is one of these inhibitors (data not shown). In this report, we investigate whether syl-155 could rescue p53 function from the p53-HDM2 interaction and evaluate its activities in tumor cells with various states of p53.
MTT assay for determination of cell viability and growth

The MTT assay was carried out as described previously with some modifications. HT1080, KYSE510, MG63 and HELF cells were seeded in seven 96-well plates, and each well contained 1.2×10⁶ cells. Triplicate wells were used for each experimental condition. Absorbance was measured in a bio-kinetics reader (Bio-Tek Instruments Inc., Winooski, VT) at a wavelength of 490 nm. The means were obtained on each of 7 d and used to draw a curve of cell proliferation. The viability rates (VR) on the third day were calculated as follows:

\[ VR (%) = \frac{\text{Total absorbance in tested group (3 d)}}{\text{Total absorbance in control (3 d)}} \times 100\% \]

Flow cytometry assay

HT1080, KYSE510 and MG63 cells were harvested at various time points after treatment with 10 μg/mL syl-155 for up to 72 h, stained with 50 μg/mL PI (Calbiochem, San Diego, CA, USA), and analyzed by a FACSCalibur flow cytometer (Becton Dickson, Mountain View, CA, USA) using CELLQUEST software. The position of the cells with sub-G-1 DNA content was indicative of apoptosis.[23]

Western blot assay

HT1080, KYSE510 and MG63 cells were harvested at various time points after treatment with 10 μg/mL syl-155 for up to 120 h. Whole cell lysates were prepared using cell lysis buffer (100 μg/mL PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mL/L NP-40 in cold PBS), and then protein was extracted for Western blot. Western blot analysis was performed as described previously.[24] Akt2, p53 and p21 proteins were detected by mouse monoclonal antibodies D-12, DO-1 and F-5 (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, respectively. β-actin was used as internal control.

RESULTS

Viability of tumor cells detected by MTT

A series of non-peptidic small molecular HDM2 inhibitors were synthesized. Based on the IC₅₀ (<10 μg/mL) calculated by MTT assay (data not shown), syl-155, one of these non-peptidic small molecular HDM2 inhibitors, was chosen for further studies. Cell viability assay by MTT showed that syl-155 could cause cell death in HT1080 cells while its cytotoxic effect on KYSE510, MG63 and HELF cells were not obvious (Table 1).

Changes of cell growth curves by syl-155

HT1080, KYSE510 and MG63 cell lines were treated with syl-155 at various concentrations for 1-6 d, and the cell viability was determined as described above by MTT assay. Syl-155 inhibited the growth of the three cell lines in a dose- and time-dependent manner (Figure 1). With the prolongation of treatment time, the growth of HT1080 cells treated with syl-155 at concentrations 2.5, 5.0 and 10 μg/mL was slower than that of KYSE510 and MG63 cells treated with the same concentration of syl-155 compared with the negative control within 6 d. HT1080 cells incubated with 10 μg/mL of syl-155 grew slower than that incubated with 2.5 or 5.0 μg/mL of syl-155. Syl-155 was synthesized chemically by Yin et al., in Institute of Material Medica, CAMS.[25] These inhibitors could prevent the interaction of HDM2 and p53 proteins through competing with p53 for binding to HDM2. Syl-155 was one of these inhibitors. DMSO was used as assistant solvent for these inhibitors. In this study, the final concentration of DMSO was 0.2 mL/L in cultures. Control cells also received 0.2 mL/L DMSO (<4 mL/L) which had no effect on cell proliferation or viability[23].
at 10 μg/mL had an inhibitory effect of more than 50% on tumor cell growth 3 d after treatment. The inhibitory rate in the next 3 d was 57.86%, 67.54% and 74.98%, respectively, after treatment with 10 μg/mL of syl-155.

Expression of HDM2, p53 and p21 protein induced by syl-155
Previous studies indicate that inhibition of the interaction between p53 and p21 protein can prevent HDM2 degrading p53, which leads to accumulation of p53 and induce apoptosis. In HT1080 cells, 12 h after syl-155 administration, the protein level of p53 increased. At 24 h, p53 protein reached a higher level. Then the protein level of p53 decreased but maintained a higher level than the basal level. At 120 h, p53 protein rose and reached a much higher level than ever before. The p21 protein level also slowly rose (Figure 2A), whereas no accumulation of p53 or p21 protein was observed in tumor cell lines KYSE510 (Figure 2B) and MG63 (data not shown). Syl-155 had no effect on the levels of p53 and p21 proteins in both p53-negative or p53-mutant cells.

Syl-155 induced apoptosis of HT1080 cells
The inhibitory effect of syl-155 on cell proliferation was stronger in HT1080 cells than in KYSE510 or MG63 cells. Western blot results showed that syl-155 induced accumulation of p53 and p21 proteins in HT1080 cells but not in KYSE510 and MG63 cells. The question was raised as to whether syl-155 could induce cell cycle arrest or apoptosis of HT1080 cells through the accumulation of p53 protein by preventing HDM2 from degrading it. Therefore flow cytometry was employed for the investigation. HT1080, KYSE510 and MG63 cells were treated with 10 μg/mL syl-155 for 0-72 h, stained with PI, and analyzed by flow cytometry to quantify cells in the different phases of cell cycle. The results showed that syl-155 induced an increased cell cycle arrest at G0/G1 phase in HT1080 cells (Table 2).

DISCUSSION
The function of p53 is inactivated during the development of most human tumors[27-29]. The main mechanism of p53 inactivation is due to p53 gene mutation which occurs in more than 50% human tumors. Another important mechanism is that p53 protein interacts with other proteins which either promote p53 protein degradation or inhibit its ability to transactivate its downstream genes involved in cell cycle arrest or apoptosis[29]. HDM2 is an oncoprotein that mainly binds to p53 protein functioning as E3 ubiquitin ligase and promotes its proteolysis through a ubiquitination degradation pathway by the 26S proteosome[4-5,30]. Furthermore, HDM2 is a target of the transcriptional factor p53. Therefore, there is an autoregulatory negative feedback loop between p53 and HDM2 protein[31]. In normal cells, HDM2 maintains p53 protein at low levels. HDM2 can also inactivate p53 by masking the transactivation domain of p53 so that the downstream genes of p53 such as p21 cannot be transcribed[32]. HDM2 overexpression can result in excessive inactivation of p53, diminishing its tumor suppression function. HDM2 can affect cell cycle, apoptosis and tumorigenesis by interacting with other proteins, including retinoblastoma 1 and ribosomal protein L5.

HDM2 is amplified in 7% of human tumors, 40-60% of osteosarcomas and approximately 30% of soft tissue...
The inactivation of p53 through the binding to HDM2 is of particular interest in a therapeutic point of view, as the function of p53 protein could be potentially restored by disrupting the interaction of p53 with HDM2. So it has become very significant to seek inhibitors of p53-HDM2 interaction.

The feasibility of this strategy has been verified by previous studies. These inhibitors include HDM2 alternatively spliced products[15], GST fusion protein[11], monoclonal antibody[12], synthetic peptides[11,19,20], chalcone and its derivatives[14], chlorofusin[17,18], and anti-sense oligonucleotide[14]. All these inhibitors can cause p53 accumulation and activate the function of p53 as tumor suppressor in tumor cells. Usually non-peptidic small molecules have higher permeability into cells, and can be synthesized with a lower cost. It is interesting to note whether non-peptidic small molecules can substitute for these inhibitors. Computer-aided design based on the crystal structures makes it possible to screen and choose effective compound on a large scale. Elucidation of the crystal structure of p53-HDM2 complex[8] provides a basis for seeking non-peptidic small molecule HDM2 inhibitors. In the study, we synthesized a series of non-peptidic small molecule HDM2 inhibitors by computer-aided design based on the crystal structure of p53-HDM2 complex. MTT assay initially screened these compounds, and one (syl-155) of them was chosen for further study.

We studied the effect of syl-155-activated p53 pathway on three tumor cell lines with various status of p53. In HT1080 cell line expressing wild type p53, syl-155 induced the accumulation of p53 protein, which consequently increased the levels of p21. However, like AP[16], syl-155 had no effect on p21 levels in both p53-negative or p53-mutant cells. p53-mutant protein was also not affected after treatment with syl-155 in KYSE510 cells expressing mutated p53. Therefore, we can conclude that syl-155 only inhibits p53-HDM2 interaction in cells carrying wild-type p53 protein and elevates p21 protein levels by stimulating the accumulation of wild-type p53 protein. Wild-type p53 protein is a labile protein and its cellular level is mainly regulated by the rate of its proteosomal degradation. p53 degradation is mediated by two alternative pathways that either depend on HDM2 and ubiquitin or they are independent of both. The latter pathway is regulated by NAD(P)H and quinone oxidoreductase 1 (NQO1)[8]. This can explain why p53 protein level decreases after reaching a higher level, which correlates with the changes of p21 protein level.

In cell assay, syl-155 affected proliferation of the three cell lines in a dose- and time-dependent manner. The growth of HT1080 cells treated with syl-155 was slower than that of KYSE510 and MG63 cells. The syl-155-mediated inhibition of tumor cell proliferation may be the consequence of an induction of apoptosis or cell cycle arrest. In our experiment, syl-155 induced more apoptosis of HT1080 cells than of KYSE510 and MG63 cells. Syl-155 also induces G1 arrest of HT1080 cells. Syl-155 induced cell cycle arrest and apoptosis in HT1080 cells which is consistent with the accumulation of p53 and p21 protein. Syl-155 only stimulates the p53 pathway in cells expressing wild-type p53. In combination with MTT assay, syl-155 is more toxic to tumor cells expressing wild-type p53. The more the cell expresses HDM2, the more it is susceptible to AP-mediated apoptosis[29]. In summary, drugs for inhibiting the p53-HDM2 interaction show promise in treatment of tumors expressing wild-type p53.

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