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Structure-Activity-Relationship Study of the Novel p21/waf1 Inhibitor for Anti-Cancer Agents against Renal Cell Carcinoma

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ABSTRACT
The p21/waf1, especially cytoplasmic or phosphorylated p21/waf1, is one of cancer prognostic marker and known to have the anti-apoptotic function in renal cell carcinoma. Thus, it would be a good therapeutic option to impair the anti-apoptotic activity of p21/waf1 in renal cell carcinoma by small molecule inhibitors. In this study, we are trying to find more potent small molecule inhibitors derived from the previous candidates to bind and block the anti-apoptotic function of p21/waf1. After synthesizing the 24 new derivatives that are modified in their two imidazole chain, it has been successfully found that the No. 13 lead compound has the most cytotoxicity by MTT assay as well as p21 attenuating activity in ACHN and A498 cells. Moreover, the cytotoxicity of No. 13 was increased by the relatively low dose of doxorubicin treatment, a kind of conventional chemotherapies which can help to reduce the side-effect induced from the high dose use of doxorubicin treatment in patients with renal cell carcinoma.

Key words: Structure-activity-relationship, p21/waf1, inhibitor, anti-cancer, renal cell carcinoma

INTRODUCTION

Worldwide every year kidney cancers are being newly diagnosed about 190,000 people (Weiss and Lin, 2006). Kidney cancer is slightly more common in men than in women and from 50-70 years of age mainly occurs in the elderly. Early detection of kidney cancer can result in the survival rate of 79-100% (De Mulder *et al*., 2004). Renal Cell Carcinoma (RCC) is the most common type of kidney cancer arising from the renal tubule (Tuma, 2004). To date, initial therapy for RCC is with surgery followed by traditional radiation therapy and chemotherapy. But it is known to be resistant to the conventional radiation therapy and chemotherapy (Stadler *et al*., 2003). Thus, the development of novel targeted therapy that retains activity against chemotherapy-resistant RCC is urgently needed.

Basically, p21/waf1 induced by p53 has been known to have the activity to arrest cell cycle in the G1-S transition state by inhibiting Cyclin/CDK complex. As a result, cell cannot proliferate (El-Deiry *et al*., 1993; Matsushita *et al*., 1998). However, recently, many reports has shown that increased cytoplasmic or phosphorylated p21/waf1 is one of the cancer prognostic markers in specific cancer such as breast, colon and kidney cancer by immunohistochemical staining of tissue from patients with those kinds of cancer (Asada *et al*., 1999; Tian *et al*., 2000). Many reports have shown that Akt in cancer cells to phosphorylate and translocate p21/waf1 from nucleus into cytoplasm (Zhou *et al*., 2001). This cytoplasmic and phosphorylated p21/waf1 has longer half-life than normal p21/waf1 because the phosphorylation site on the p21/waf1 is the binding site for 20S proteasome and the cleavage site by Caspase3. Recently, one study has shown that cytoplasmic and phosphorylated p21/waf1 can inhibit apoptosis by forming a complex with the ASK1 (apoptosis signal-regulating kinase 1) (Zhan *et al*., 2007). They also showed that expression of a deletion mutant of p21/waf1 lacking the nuclear localization signal did not induce cell cycle arrest but led to an apoptosis-resistant phenotype by binding to and inhibition of ASK1. The ASK1 is a member of the MAPKKK group and can mediate apoptosis via MKK6, JNK and its various down-steam transcription factors regulating apoptosis gene. Thus, p21/waf1, especially cytoplasmic and phosphorylated form, could be good therapeutic target to treat RCC.

For these reasons we questioned whether the chemical inhibitor could disrupt p21/waf1 levels in ACHN and A498, two kinds of kidney cancer cell lines and cause the ACHN and A498 cell death which can be increased with the doxorubicin treatment. In this study, we are trying to develop and identify the stronger chemical inhibitors against p21/waf1 for the anti-cancer therapeutic ways to RCC by structure-activity-relationship study of the previous p21/waf1 inhibitor screened by one-bead-one-compound screening system (Park *et al*., 2008a, b).

MATERIALS AND METHODS

Cell lines: The ACHN and A498 human RCC cells (from ATCC) were maintained in MEM 1X media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid and 0.75% sodium bicarbonate at 37°C in a humidified incubator containing 5% CO_2 in air.

Chemical reagents: Mouse anti-β-actin monoclonal antibody and the following chemicals and solvents (dimethyl sulfoxide (DMSO), glycerol, glycine, sodium vanadate, sodium chloride, Thiazolyl Blue Tetrazolium Bromide, Trizma base, Tween 20 and doxorubicin) were from Sigma (St. Louis, MO). Mouse monoclonal anti-p21/waf1 antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-PARP1 antibody was obtained from Cell Signaling (Danvers, MA). Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG were obtained from Bio-Rad (Richmond, CA). The ECL Western Blotting Detection Reagents were obtained from Amersham Biosciences (Buckinghamshire, United Kingdom).

Measurement of cell viability: A 200 mL aliquot of cells (1×10^3 cells in serum free media) was added to a 96 well plate and incubated for 18 h at 37°C in a humidified incubator containing 5% CO_2 in air. After incubation, each condition of small molecule derivatives was added into each well for 48 h and for combined assay with doxorubicin, after incubation for 24 h with compound No. 13, doxorubicin dissolved in DMSO was added to each of the 96 wells and incubated for 24 h more. Control cultures were treated with DMSO. After incubation, a 20 mL MTT solution (5 mg mL^{-1} in phosphate buffer) was added to each well and the incubation continued for 4 h, after which time the solution in each well was carefully removed. The blue crystalline precipitate in each well was dissolved in DMSO (200 mL). The visible absorbance at 560 nm of each well was quantified using a microplate reader.

Immunoblotting: Cells were washed with PBS and lysed in lysis buffer (50 mM HEPES, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA) at 0°C. Cell lysates were centrifuged (10,000x g, 4°C, 10 min) and the supernatants were electrophoresed and immunoblotted. The membranes were blocked in 3% non-fat dry milk for 1 h at room temperature and probed with appropriate antibodies. Membranes were then probed with HRP-tagged anti-mouse or anti-rabbit IgG antibodies diluted 1:5,000-1:15,000 in 5% non-fat dry milk for 1 h at room temperature. Chemiluminescence was detected using enhanced ECL.

RESULTS

New derivative (entry No. 13) was screened by the cell viability assay. Based on the previous finding, as shown in the Table 1, the totally 16 new derivatives were synthesized. These compounds have the different chemical structure in the R1 and R2 group from the parent compounds. To select more potent candidates which have stronger cell cytotoxicity in ACHN cell line than the parent compound, MTT assay was performed at 100 μM dose of the new 16 derivatives. From MTT assay results (Table 1), we could find that entry No. 11 and 13 have the strongest cell cytotoxicity among these 16 new compounds. Next, in order to compare the relative cell cytotoxicity of the No. 11 and 13 compounds in ACHN and A498 cell line, the dose-dependent MTT assay for No. 11 and 13 was done and showed that No. 13 was little bit stronger cytotoxicity than No. 11 in both cell lines.

Number 13 could enhance the anti-cancer activity of doxorubicin in ACHN and A498 cell lines. Then based on the chemical structure of No. 13 compound, new 8 derivatives were further synthesized and tested by MTT assay. As shown in Table 1, No. 17, 20, 22, 23 and 24 have shown the similar or little bit stronger cytotoxicity with No. 13 compound in ACHN cell lines. In the previous studies, we have shown that the attenuation of p21/waf1 expression level by anti-sense oligonucleotide to p21/waf1 and small chemicals could cause sensitization of kidney cancer cell lines to the conventional DNA-damaging chemotherapy. So, we tested our new derivative has the same activity in two kinds of kidney cancer cell lines (ACHN and A498). It seemed that incubation of ACHN and A498 with No. 13 compound in the presence doxorubicin showed an additive cell cytotoxicitic effect rather than synergistic decrease in cell survival and increase in apoptosis (Fig. 1a-b).

DISCUSSION

The p21/waf1 as a potential therapeutic target against kidney cancer has been proposed by us and others (Weiss, 2003). Previously, this hypothetical concept has been supported by the results that were proved with antisense oligonucleotides to p21/waf1 in breast and kidney cancers (Fan *et al*., 2003). However, no antisense has been adapted to a clinical success. Also the clinical use of siRNA is still in very early stage. A main reason for the poor outcome of these kinds of techniques seems hard to delivery oligonucleotides or siRNA materials into target cancer cells (Ozcan *et al*., 2015). Thus, in the regard of the clinical purpose, it will be better consideration to try to develop small molecule inhibitor to p21/waf1 described in this study since they are more likely to be able to penetrate cell membranes by the more hydrophobicity.

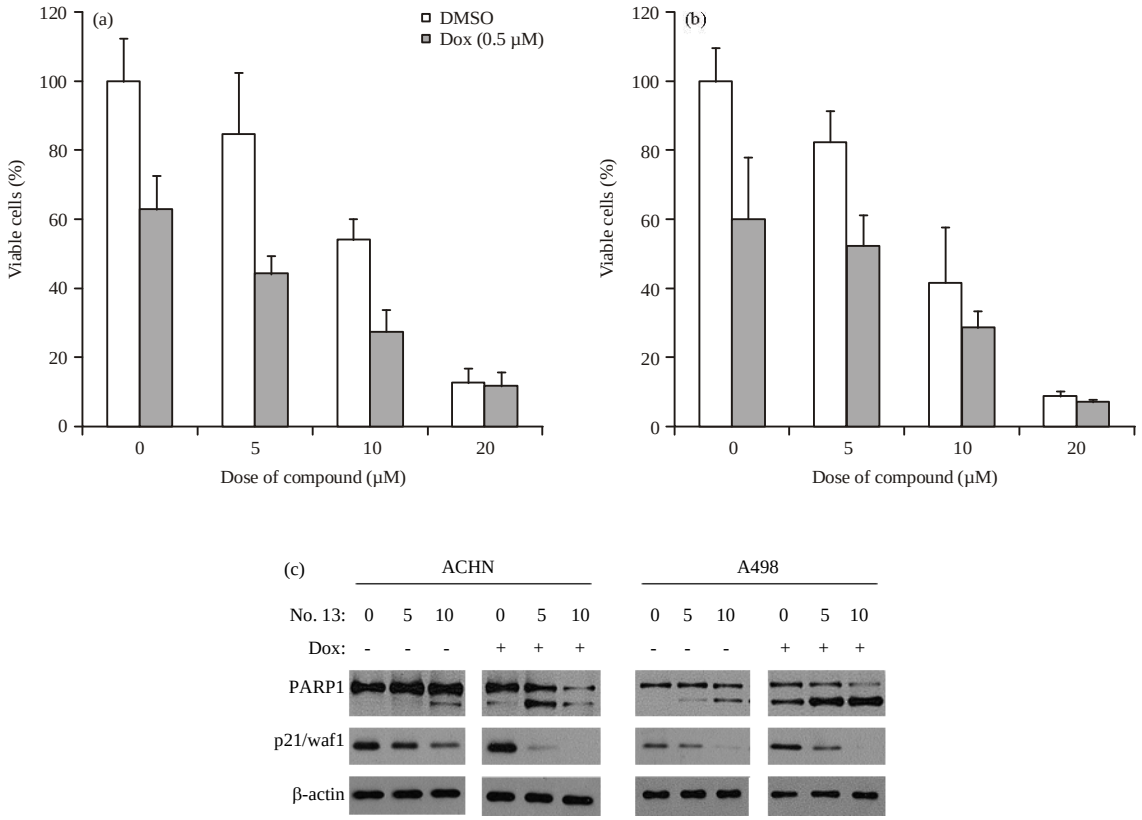
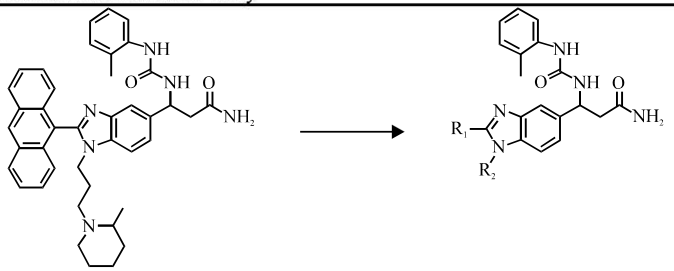


Fig. 1(a-c): Increased cell cytotoxicity of No. 13 combined with doxorubicin, (a) MTT assay of No. 13 followed by doxorubicin in ACHN and (b) A498 cells. ACHN and A498 cells were incubated in serum-free media for 16 h and treated with vehicle (DMSO control) and No. 13 at the indicated concentration in serum-containing media. After 24 h, 0.5 μM of doxorubicin was added to the indicated well and incubated for 24 h. The MTT assay was performed as described in Materials and Methods. Data is presented as Average±Standard Error (SE) of three independent experiments and (c) PARP1 cleavage assay of No. 13 followed by doxorubicin in ACHN and A498 cells. ACHN and A498 cells were incubated in serum-free media for 16 h and treated with vehicle (DMSO) and No. 13 at the indicated concentration in serum-containing media. After 24 h, 0.5 μM of doxorubicin was added to the indicated well and incubated for 24 h. Cells were harvested and immunoblotted with PARP1 and p21/waf1. β-actin blotting is used for a gel-loading control

Initially, p21/waf1 as the Cyclin-Dependent Kinase (CDK) inhibitor has been reported to have a potent activity to regulate cell proliferation. However, recently, p21/waf1 has been recognized to play an opposite role in apoptosis in cancer cells by us as well as other laboratories (Stivala *et al*., 2012). Thus, p21/waf1 is now considered a protein with tumor-suppressive as well as oncogenic activity depending on cancer type. Previously, we identified three novel chemicals that has very similar chemical structure and has an ability to induce apoptosis in kidney cancer cells by degrading and ubiquitinating p21/waf1. In fact, there have been lots of studies showing that p21/waf1 can have oncogenic activities. Recently, Kim *et al*. (2012) reported that Salinomycin as a p-glycoprotein inhibitor can sensitize the anti-cancer activity of the radiation treatment by increasing DNA damage in cancer cells through reducing p21/waf1 expression level. After this study, they have reported another study that has the similar conclusion. In that reports, they showed Salinomycin could sensitizes cancer cells to the anti-cancer effects of doxorubicin and etoposide treatment, two conventional chemotherapy, by increasing DNA damage and reducing p21/waf1 protein (Kim *et al*., 2011). Taken together, the results from our study as well as other studies may contribute to the novel modality by the development of p21/waf1 targeted therapy for cancer patients treated with DNA-damaging drugs or radiation therapy.

The Structure-Activity Relationship (SAR) study is to investigate the relationship between the chemical structure of an active molecule and its biological activity in a specific assay system (Liu *et al*., 2013). By the analysis of SAR, we might determine which chemical groups/atoms play a critical role on modulating its biological effect in the specific system.

Table 1: Structure of the new derivatives and the result of MTT assay



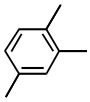
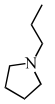
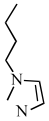
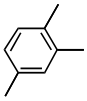
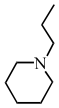
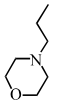
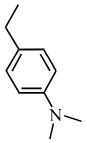
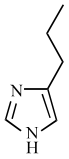
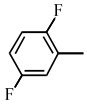
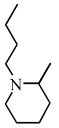
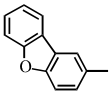
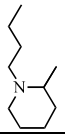
Entry	R1	R2	Calculated M.W.	MALDI-TOF MS M.W. (MH+)	Viable cells (%)[a]
No. 1			538.31	539.28	84.07±14.13
No. 2			538.31	539.28	69.11±17.08
No. 3			549.29	550.26	68.02±13.61
No. 4			532.26	533.22	72.34±13.61
No. 5			552.32	533.28	64.72±11.61
No. 6			554.30	555.28	74.54±13.39
No. 7			574.31	575.28	53.62±7.01
No. 8			535.27	536.25	63.28±8.47
No. 9			588.3	589.29	84.57±16.46
No. 10			642.33	643.32	60.91±6.49

Table 1: Continue

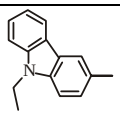
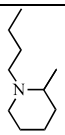
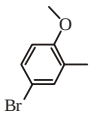
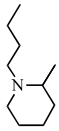
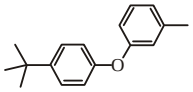
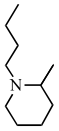
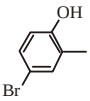
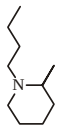
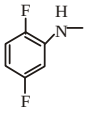
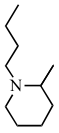
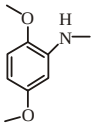
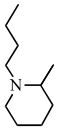
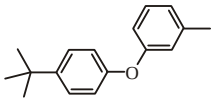
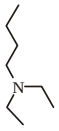
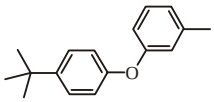
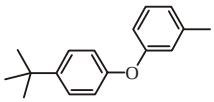
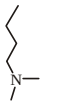
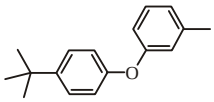
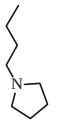
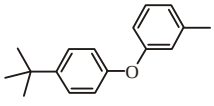
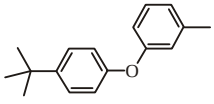
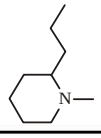
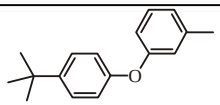
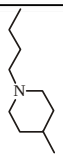
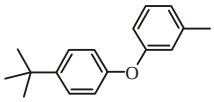
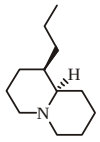
Entry	R1	R2	Calculated M.W.	MALDI-TOF MS M.W. (MH+)	Viable cells (%)[a]
No. 11			669.38	670.35	19.17±6.07
No. 12			660.24 662.24	661.22 663.22	74.37±13.41
No. 13			700.41	701.38	20.66±7.35
No. 14			646.23 648.23	647.2 649.2	78.19±12.37
No. 15			603.31	604.28	87.56±15.74
No. 16			627.35	628.32	91.82±17.65
No. 17			674.39	675.42	16.40±3.7
No. 18			632.35	633.39	74.90±16.7
No. 19			646.36	647.38	59.10±8.9
No. 20			672.38	673.38	18.40±12.9
No. 21			618.33	619.37	63.30±11.9
No. 22			686.39	687.39	28.50±10.3

Table 1: Continue

Entry	R1	R2	Calculated M.W.	MALDI-TOF MS M.W. (MH+)	Viable cells (%)[a]
No. 23			700.41	701.37	10.60±3.3
No. 24			726.43	727.41	12.08±5.5

[a]Viable cell (%) ±Standard error at 100 μM of each compound was calculated by the O.D. of each compound treated well/O.D. of no treatment well from the results of MTT assay

This usually results in the efficient synthesis scheme as well as the more potent compound with the modified chemical structure. In this study, we tried to screen the potent p21/waf1 inhibitor with the stronger cell cytotoxicity in ACHN and A498 cells by performing SAR study of the previously developed compound.

When we did dose-dependant MTT assay of No. 13 compound, it has killed about 80% of cells (ACHN cells at 25 μM and A498 cells at 10 μM). Although No. 11 compound has showed weaker cell cytotoxicity than No. 13 compound, it has still stronger cell cytotoxicity than the parent compound in both of cell lines. Interestingly, it seemed that these compounds seemed to cause more apoptosis in A498 than ACHN. On the other hands, chemical structure of the two new candidates have longer R1 group than the others (Table 1). When R1 group was substituted with the shortest group, the cytotoxicity of almost compound has not been significant increased even though R2 group was replaced with various functional structures which suggested that R1 is more responsible than R2 in causing cell cytotoxicity.

In order to find that compound to have the strongest cytotoxicity in ACHN cell lines, the dose dependent MTT assay was used with these 6 derivatives. When using three different kinds of concentration, these new derivatives have the similar dose-dependent activity with No. 13 compound in ACHN cell lines. There is tendency that R2 group should be kind of longer than 6 carbon chain and the end of chain need to be ring type of structural motif.

Although we do not have any structural information how these chemical inhibitors bind to p21/waf1, we believe our current study could provide the considerable clue for solving the possible working mechanism by that the candidates showed anti-tumor function through p21/waf1 protein in renal cell carcinoma cells. In conclusion, we have synthesized and elucidated about 5-folds stronger chemical inhibitor to p21/waf1 from new derivatives than the previous candidates that has caused apoptosis-related cell death in two kinds of renal cell carcinoma cell lines (ACHN and A549) as well as an additive effect with relatively low dose of doxorubicin treatment. Other detail regulatory mechanism study for the anti-tumor activity of the selected new compound, including the p21/waf1 protein ubiquitination assay, will be required for further investigation.

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