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REVERSAL OF MULTI-DRUG RESISTANCE IN MDR CANCER CELL BY 3113, A NEW DIPEPTIDOMIMETIC OF P-AMINOBENZOIC ACID

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ABSTRACT

In this study, we reported for the first time that a new compound named 3113, which is the dipeptidomimetic of p-aminobenzoic acid showed multi-drug resistance (MDR) reversal activity. In MDR cancer cells, no matter acquired or inherent, combination with 3113 enhanced doxorubicin (DXR) cytotoxicity by 8 to 10 folds, which was slightly higher than the effect of verapamil (VPL). 3113 was indicated to enhance the cytotoxicity of anticancer drugs through increasing its intracellular accumulation in a time-dependent manner. According to the result of FCM (flow cytometry), the intracellular fluorescence representing the concentration of intracellular DXR was improved by 10 folds as compared with the MDR cells without 3113. Western blotting analysis of P-glycoprotein (P-gp) confirmed that 3113 can alter P-gp expression in a time-dependent way. In conclusion, MDR reversal activity of 3113 depends on, at least in part, the inhibition of P-gp expression.

Keywords: MDR; P-glycoprotein; p-aminobenzoic acid; dipeptidomimetic;

INTRODUCTION

Multiple drug resistance (MDR) is a condition enabling a disease-causing organism to resist distinct drugs or chemicals of a wide variety of structure and function targeted at eradicating the organism. Organisms that display multidrug resistance can be pathologic cells, including bacterial and cancer cells. Chemotherapy often fails in cancer patients because of inherent or acquired MDR, a phenomenon demonstrated as resistance of cancer cells to anticancer agents with diverse structure and mechanism [1-3]. The search for effective MDR reversal agents is now into the third generation. First-generation agents include verapamil (VPL), cyclosporine A, and progesterone [4-9]. Chemical derivative of first-generation compounds results in second- and third-generation molecules [10-28]. Here we report for the first time that the dipeptidomimetic of p-aminobenzoic acid, named 3113, acts as an effective chemosensitizer.

3113 was synthesized straightly through p-aminobenzoic acid coupling with some amino acid esters aided by N,N'diisopropylcarbodiimide (DIC) or N,N' dicyclohexylcarbodiimide (DCC) in order to find the new anti-diabetic compound [29]. On the basis of spectral data including IR, ^{1}H NMR, ^{13}C NMR, MS and HRMS, the chemical structure of 3113 was elucidated as 4-(1-(3-aminobenzoate)-3-hydroxyl-5-

(6-methoxylnaphtalin-2-yl) pentamino) ethyl p-amino benzoate (shown in Fig.1).

Preliminary in vitro bioassay test demonstrated that the compound had limited anti-diabetic activity. However we found accidentally 3113 possesses multi-drug resistance (MDR) reversal activity against a MDR breast cancer cell line, MCF-7/ADR. In this study, we demonstrated that 3113 can induce the intracellular accumulation of doxorubicin via down-regulating P-glycoprotein (P-gp) expression in MDR cancer cells. Verapamil was included in our whole study as the positive control for its acknowledged activities as a MDR reversal agent and its availability. MCF-7 cell was drug-sensitive cells while MCF-7/ADR and C26 cells were drug-resistant cells (acquired and inherent MDR respectively).

MATERIALS AND METHODS

***Materials**:* 3113 was gifted by Chengdu Di Ao pharmaceutical group and its chemical structure is shown in Fig.1. Stock solutions of 3113 (10mg/ml, 20mM) were prepared in Dimethyl sulfoxide (DMSO). Doxorubicin (DXR) were purchased from Zhejiang Haizheng Pharmaceutical Ltd., and dissolved in PBS at the concentration of 2.55mM. Verapamil, DMSO, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Corporation. DMEM and RPMI1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Corporation. Verapamil (VPL) was dissolved in PBS at the concentration of 22mM. Stock solutions of all drugs were aliquoted and stored at -20℃.

***Cell culture**:* MCF-7 (Human breast cancer cell line) and C26 (Mouse colon cancer cell line) were obtained from the American Type Culture Collection (ATCC), and MCF-7/ADR was supplied by Chengdu Di Ao pharmaceutical group. MCF-7 and MCF-7/ADR cells were cultured in DMEM with 10% FBS, 100U/ml antibiotics at 37℃ under 5% CO_2, while C26 cells in RPMI1640 medium. The medium was replaced every 2-3 days, and passaging was performed until 80-90% confluency was reached. To MCF-7/ADR cells, MDR phenotype was maintained by growing the cells in the presence of 5μM DXR every 4 passage. DXR was withdrawn from the culture medium 1 week before using MCF-7/ADR cells for the further study.

***In vitro cytotoxicity assay**:* In order to learn the safe dosage of 3113, MTT assay was conducted. MCF-7, MCF-7/ADR and C26 cells were seeded into 96-well plate at a density of 1×10^4/well. On the next day, two-fold serial dilutions of 3113 (200μM down to 0.78μM) were added and incubated at 37℃ under 5% CO_2 for 48 h. Each concentration repeated 3 times. 10 μL MTT (5mg/ml) was added 48 h later. Medium was removed after 2-3 h and 100μL DMSO was added to dissolve formazan precipitate for 10 min under room temperature. The absorbance at 570nm was measured on a spectra max MS (MDC, Sunnyvale, CA, USA). Inhibition rate was determined by $(OD_{blank}-OD_{treated}) / OD_{blank}$. The safe dose of 3113 on the cancer cells for our further experiments is the highest concentration with inhibition rate below 10%.

***IC_{50} evaluation with MTT assay**:* Cells were seeded into 96-well plate at a density of 1×10^4/well. The chemosensitizer and DXR were added on the second day. The upper half of the plate was added with 10μM 3113 or 15μM VPL and the lower half without 3113 or VPL. The plate was added with serial dilutions of DXR and incubated at 37℃ under 5% CO_2 for 48 h. Each data point was repeated in triplicate.

Inhibition rate were determined as mentioned above. Based on the inhibition curves, IC_{50} values were obtained using non-linear regression program in Microsoft Excel 2003. RF represents fold-change in drug sensitivity and is expressed as: IC_{50} values without reversal agent /IC_{50} values with reversal agent. Different dose ranges of DXR were prepared for different cell lines. For MCF-7/ADR cells, it was 2-fold serial dilution of DXR (255μM down to 1μM with reversal drugs, and 1360μM down to 5.31μM without reversal agents). Due to the background color of DXR over the concentration of 200μM, controls with all reagents except cells were set. For C26 cells, DXR was 1.5-fold serial diluted starting from 17μM to 0.663μM with reversal reagent and 51μM to 1.989μM without reversal reagent. To MCF-7 cells, DXR was 2-fold serial diluted from 170μM to 0.3315μM.

FCM (flow cytometry) analysis of intracellular DXR accumulation: To estimate intracellular DXR, MCF-7 and MCF-7/ADR cells were seeded in a 24-well plate (5×10^5 cells/well). MCF-7/ADR cells were added with 10 or 20μM 3113, 15μM VPL respectively. After varying incubation time (1h, 4h, 24h, 48h, 72h), 2μL DXR stocking solution (2.55mM) was added to the cells, gently mixed and incubated at 37℃ for 45min. Then intracellular fluorescence was determined by a flow cytometer, Cytomics FC500 (Beckman-Coulter, USA) after washing cells three times with PBS. Excitation and

emission were at 488nm and 575nm respectively. Sample sizes were 10^4 cells. For each time point, there were 6 samples including MCF-7 cells with DXR, MCF-7 cells without DXR, 15μM VPL in MCF-7/ADR+DXR, 10 or 20μM 3113 in MCF-7/ADR +DXR, MCF-7/ADR cells+DXR.

Western blotting analysis: MCF-7/ADR cells were seeded in a 6-well plate (1×10^6 cells/well) and cultured for 24h in DMEM supplemented with 10% FBS followed by a further culture time (4h, 24h, 48h) with 20μM 3113. Total cell protein was extracted with RIPA buffer [10mM Tris-Cl (pH 8.0), 1mM EDTA, 0.5mM EGTA, 1%Triton X-100, 0.1%sodium deoxycholate, 0.1% SDS, 140mM NaCl, protease inhibitor cocktail]. The extracts (30μg) were separated by 12% SDS-PAGE, and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with anti-Pgp antibody (1:2000, Cell Signaling Technology, Beverly, MA, USA) or anti-β-actin antibody (1:200, Zhongshang Jingqiao, Beijing, CHINA), and then with anti-rabbit IgG conjugated with horseradish peroxidase (1:5000, Cell Signaling Technology, Beverly, MA, USA) respectively. Immunoreactive bands were detected using Pierce ECL detection system (Rockford, IL, USA).

RESULTS

In vitro Cytotoxicity of 3113 compound: Three cell lines were used in this study: MCF-7 (Human breast carcinoma) is drug-sensitive cells; MCF-7/ADR (derived from MCF-7) is the acquired resistant cells and C26 (Colon carcinoma) is the inherently resistant cells, both depending on the multidrug resistance extrusion pump: P-gp protein [30]. The effects of 3113 alone were tested on the three cell lines over the dose range from 200μM to 0.78μM. As shown in Figure 2, taking 10% inhibition rate as the threshold, 3.125μM 3113 treatment resulted in less than 10% inhibition rate in MCF-7 and C26 cells, while 25μM 3113 compound demonstrated little negative effect on the MCF-7/ADR cells. Consequently 10μM and 20μM 3113 in MCF-7/ADR cells, 3μM 3113 in MCF-7 and C26 cells were applied in our subsequent experiments. Meanwhile, 15μM VPL was chosen as the positive control in our experiments based on the other's publications [30, 31].

Sensitization of resistant cells by 3113: The MDR phenotype of the resistant cell lines were confirmed firstly by the measure of IC_{50} value in MCF-7/ADR, C26 and MCF-7 cancer cell lines. In table 1, IC_{50} value of MCF-7/ADR (328.63μM) was significantly higher than that of sensitive cell line: MCF-7 (12.21μM). The IC_{50} of the inherent resistant cell line: C26 (26.23μM), was also higher than MCF-7 (12.21μM), the DXR sensitive cell line (Table 1).

Approximately 10.11 and 7.98-fold increases in DXR sensitivity were observed in MCF-7/ADR and C26 cells upon adding 3113 (Table 1, RF columns), which were slightly higher than the positive control, VPL (8.28, 6.22- fold change in the two DXR-resistant cell lines respectively). At the same time, the IC_{50} value of the drug-sensitive cell line was unaffected by the combination treatment of 3113+DXR (Table 1).

Effect of 3113 on DXR accumulation in MCF-7/ADR cells: The mechanism(s) by which 3113 modulates MDR were explored through the effect of 3113 on drug accumulation within MCF-7/ADR cells by FCM analysis. Different incubation time of 3113 (1h, 4h, 24h, 48h) were adopted for the possibility that 3113 may slow down the DXR efflux via inhibiting the P-gp transport activity directly. It was demonstrated that there was no significant change of intracellular fluorescence within 1h and 4h exposure to 3113 or VPL. But exposure to 10μM 3113 or 15μM VPL for 24h generated a substantial, 10-fold increase in intracellular fluorescence while extending the exposure time to 48h had little effects on the intracellular fluorescence (see the supplement data). As shown in table 2, the mean intracellular fluorescence increased to 1.4 with the higher concentration of 3113 (20μM).

Compared with the result of 10μM 3113, doubling the concentration of 3113 to 20μM led to a slight increase of intracellular fluorescence (the mean fluorescence values were 1.4 for 20μM 3113, 1.1 for 10μM 3113, and 1.1 for VPL, 1.85 for MCF-7 cells). However it is noteworthy that higher concentration of 3113 led to higher percentage of positive cells, and it reached 79.28% in 20μM 3113 treated cells, similar as the values of sensitive cells: MCF-7 (79.59%). The numbers were 48.08% and 48.55% respectively in lower concentration of 3113 and VPL treated cells. These results showed that 3113 was able to increase the DXR accumulation in a time- and dose-dependent manner. Given significant accumulation of intracellular DXR after 24h incubation with 3113, we speculate that it may increase the intracellular DXR amount through down-regulating P-gp expression. As a consequence, the level of P-gp protein in MCF-7/ADR cells upon exposure to 3113 was checked.

Modulation of P-gp expression by 3113: Western blotting was applied to detect if 20μM 3113 is able to down-regulate P-gp level in MCF-7/ADR cells. In Fig.3, 4h exposure time to 3113 is not long enough to

change the protein level, P-gp level was similar as the untreated cells. However, as the exposure time extended to 24h and 48h, it was decreased remarkably by about 3 folds. These data indicated that 3113 was able to down-regulate P-gp expression in MDR breast cancer cells in a time- dependent way.

DISCUSSION

First-generation reversal candidates including verapamil (VPL), cyclosporine A, and progesterone lead to serious adverse effect when applied in clinic. Consequently the search for the new MDR reversal agents, also called chemosensitizers has been in urgent demand. So far, many drugs have been proved to reverse the MDR pattern in cancer cells, including calcium channel blockers (such as VPL), H_1- receptor blockers, steroids and some natural compounds such as estramustine, curcuminoids [30-33]. Here we add a new kind of compound, dipeptidomimetic of p-aminobenzoic acid with similar activities. As a chemosensitizer, it is expected to overcome the poor response of MDR cancer cells to cytotoxic agents and at the same time to have little or no effect on drug-sensitive cells. 3113, the dipeptidomimetic of p-aminobenzoic acid, tested in our *in vitro* experiments, met both expectations. In MDR cancer cells, no matter acquired and inherent, combination with 3113 enhanced DXR cytotoxicity by 8 to 10 folds, which was slightly higher than the effect of VPL.

Like most of other reversal reagents, 3113 was demonstrated to enhance the cytotoxicity of anticancer drugs through increasing its intracellular accumulation which was confirmed by FCM analysis. The intracellular fluorescence representing the concentration of DXR within the MDR cells was stimulated by at least 10 folds upon 3113 treatment relative to the untreated cells.

Several mechanisms are initiated by the reversal molecules, but the dominant one is through the inhibition of the drug extrusion operated by ATP-dependent pumps, P-gp. Many reversal compounds irrespective of natural or synthesized, have been shown to own the ability of modulating the expression of P-gp such as honokiol [33], diallyl sulfide [34]. However P-gp's drug-pump activity can be inhibited by different mechanisms. For instance, the reversal agents either down-regulate its expression, or occupy the substrate combining site on P-gp, or both. As a result, different incubation times were designed in our intracellular accumulation experiment. To 1 hour and 2 hour incubation time, the fluorescence showed little change in contrast to the positive control, which means 3113 could not inhibit the enzyme activity of P-gp directly. It prevented DXR efflux via inhibiting the protein expression or competitively combining with P-gp as its substrate like VPL. P-gp protein levels indicated by Western blotting in different treated time of 3113 confirmed that 3113 may reverse MDR through altering the P-gp expression. It is very interesting to explore the pathways of 3113 action on the P-gp gene expression. Based on the chemical structures of these compounds, it was reported that they shared the similarity in having a head composed of two aromatic rings with a spacer between them [35]. The size and nature of the spacer and substituents on the head rings may be different between these chemosensitizers. In-depth theoretically structural analysis of these molecules is required to understand the structural difference and the reversal effect. Now we can not exclude the possibility that 3113 is able to combine with P-gp as VPL does.

To our knowledge, it is the first report that new compounds like the dipeptidomimetic of p-aminobenzoic acid (3113 in this study), acts as an effective chemosensitizer. Although the effect of 3113 is slightly higher than the first-generation reversal reagent (VPL), further modification or change based on the structure of dipeptidomimetic of p-aminobenzoic acid may enhance its activity as a chemosensitizer. Furthermore, it is intriguing that conduct the drug-resistant reversal experiment *in vivo*. For example, to observe the therapeutic responses in nude mice bearing MCF-7/ADR xenografts or the syngeneic resistant mouse tumor models.

CONCLUSION

The dipeptidomimetic of p-aminobenzoic acid (3113 in our study) showed multi-drug resistance (MDR) reversal activity in MDR cancer cells through increasing intracellular accumulation of doxorubicin by the inhibition of P-gp expression.

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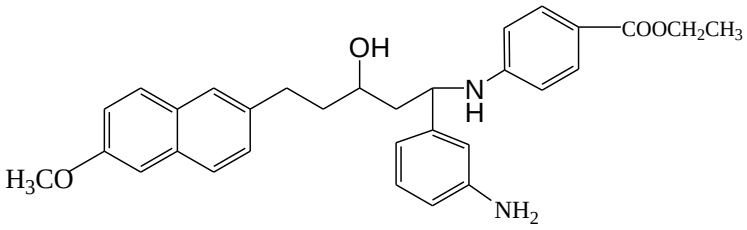

Fig. 1 Chemical structure of 3113 compound (MW:498).

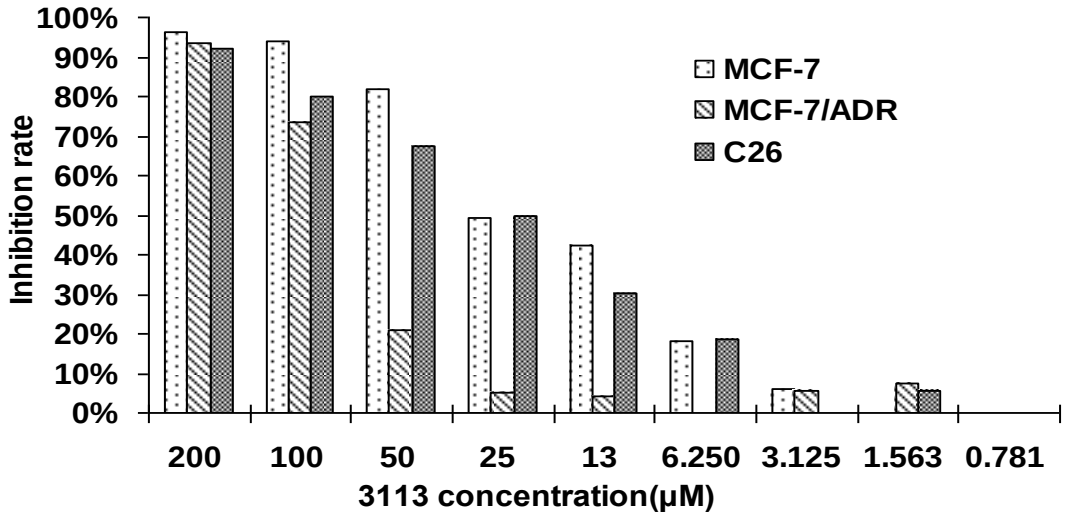

Fig. 2 *In vitro* Cytotoxicity. To learn the safe dosage of 3113, MTT assay was conducted. Three cell lines were used in this study: MCF-7 (Human breast carcinoma) is drug-sensitive cells; MCF-7/ADR (derived from MCF-7) is the acquired resistant cells and C26 (Colon carcinoma) is the inherently resistant cells. Cells were seeded into 96-well plate at a density of 1×10^4/well. On the next day, the plate was added with 2-fold serial dilution of 3113 (200μM down to 0.78μM), and incubated at 37℃ under 5% CO_2 for 48 h. Each concentration repeated 3 times. The dose of 3113 in our further experiments is the highest concentration with inhibition rate below 10%.

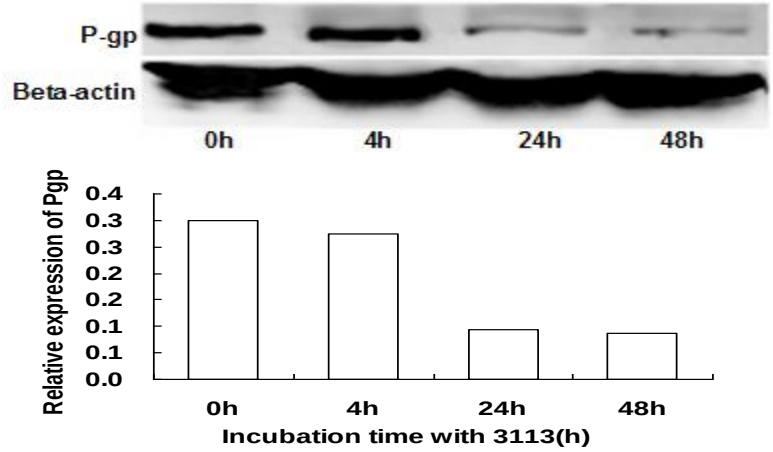

Fig.3 Modulation of P-gp expression by 3113. MCF-7/ADR cells were seeded in a 6-well plate (1×10^6 cells/well) and cultured for 24h in DMEM supplemented with 10% FBS followed by a further culture time (4h, 24 h, 48h) with 20μM 3113. P-gp and β-actin were detected with Western blotting. The upper panel is P-gp protein (170KD) while the lower is β-actin (42KD). From left to right, the samples are MCF-7/ADR without 3113, MCF-7/ADR incubated with 3113 for 4h, 24h, 48h. The bottom is the densitometric analysis of Western blotting to show the reduced level of P-gp protein normalized by internal control.

Table 1: Sensitization of resistant cells to DXR by 3113

Cell line	IC50* (μM)		RF#
	DXR	DXR+drug	
MCF-7/ADR+3113	328.63±9.86	32.52±1.50	10.11
MCF-7/ADR+VPL	328.63±9.86	39.68±0.96	8.28
C26+3113	26.23±1.05	3.32±0.07	7.90
C26+VPL	26.23±1.05	4.22±0.11	6.22
MCF-7+3113	12.21±0.66	13.68±0.73	0.89

**The IC_{50} values were counted for all cell lines upon exposure to series of DXR concentrations with/without reversal agent. For MCF-7/ADR cell line, 10μM 3113 and 15μM VPL were chosen. To C26 and MCF-7 cell lines, the concentration was 3μM 3113, 6μM VPL respectively. SD were less than 5% of the IC_{50} value (n=3). # RF stands for the fold change in DXR sensitivity.*

Table 2 Intracellular fluorescence and percentage of positive cells after 24h incubation with chemosensitizers

Groups	**Mean fluorescence value**	**Percentage of positive cells**
MCF-7	1.85	79.59%
MCF-7/ADR with 20μM 3113	1.4	79.28%
MCF-7/ADR with 10μM 3113	1.1	48.08%
MCF-7/ADR 15μM VPL	1.1	48.55%

Note: *Intracellular fluorescence was determined by the flow cytometer, Cytomics FC500 (Beckman-Coulter, USA) after washing cells three times with PBS. Excitation and emission were at 488nm and 575nm respectively. Sample sizes were 10^4 cells.*

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