Bioorganic & Medicinal Chemistry Letters 23 (2013) 2642–2646

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

In silico identification of poly(ADP-ribose)polymerase-1 inhibitors and their chemosensitizing effects against cisplatin-resistant human gastric cancer cells

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ARTICLE INFO

Article history:
Received 5 November 2012
Revised 16 February 2013
Accepted 21 February 2013
Available online 1 March 2013

Keywords:
Cisplatin-resistance
PARP-1 inhibitor
Chemosensitization

ABSTRACT

Poly(ADP-ribose)polymerase-1 (PARP-1) enzyme is involved in the repair of DNA damages made by certain anticancer agents. It is suggested that PARP-1 inhibitors potentiate the cytotoxic effects and circumvent the resistance of DNA-modifying anticancer agents such as cisplatin. In this study, we conducted virtual screening of Korea Chemical Bank database targeting PARP-1 and identified several potent PARP-1 inhibitors with submicromolar IC_{50} values (77–79 nM). We then examined the chemosensitization of cisplatin by pre-treatment of PARP-1 inhibitors in cisplatin-resistant human gastric cancer cells. Our results show that PARP-1 inhibitors suppress the formation of poly(ADP-ribose) and enhance the cytotoxicity of cisplatin.

Poly(ADP-ribose) polymerase-1 (PARP-1) is one of the most abundant nuclear enzymes in the eukaryote and functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks. Upon DNA damage, PARP-1 is activated and binds to DNA breaks, which catalyses the poly(-ADP-ribosyl)ation reactions whereby ADP-riboses are transferred from nicotinamide dinucleotide (NAD^+) to glutamic and less commonly to aspartic and lysine residues in PARP-1 itself and their substrates including histones. Accumulation of negative charges on PARP-1 and histones results in a repulsion force and subsequent dissociation of these components from DNA. The resultant chromatin relaxation facilitates for DNA damage repair.[1–4] Binding of PARP-1 to single strand breaks (SSBs) recruits components of DNA damage repair pathways such as X-ray repair cross complementing protein 1 (XRCC1) and protects them from converting into double strand breaks (DSBs).[1,5] In DSBs repair, the role of PARP-1 has been revealed through the identification of PARP-1-dependent alternative non-homologous end joining (NHEJ) pathway.[6–9] However, in response to genotoxic agents, cells express different behaviors dependent on stimulus intensity which triggers PARP-1 activity. PARP-1 activation by mild to moderate genotoxic stimuli facilitates DNA repair. Thus cells survive without the risk of passing mutated genes. More severe DNA damage induces apoptosis in which caspase inactivates PARP-1, subsequently eliminating cells with severe DNA damage. However, over-activation of PARP-1 by excessive DNA damage leads to depletion of NAD^+ and ATP which prevents apoptotic cell death. Under this condition, the inhibition of PARP-1 preserves NAD^+ and ATP, therefore allow cells either to function normally or die via apoptotic pathway.[10] Based on these observations, PARP-1 inhibitors have been suggested in single or combination therapy of various diseases.[11–18]

Formation of cisplatin–DNA adducts which trigger different downstream signaling pathways is the main cause for cytotoxic effect of cisplatin.[19] Interestingly, PARP-1 showed high affinity to the most common 1,2-d(GpG) and this affinity decreases upon automodification which implicates the role of PARP-1 in repair of cisplatin-induced DNA damage.[20,21] Therefore, combination between PARP-1 inhibitor and cisplatin enhances cytotoxicity effect of cisplatin which has been demonstrated by recent studies.[22–29] In this study, we first reported chemosensitizing effect of PARP-1 inhibitors on long-term cisplatin-resistant gastric cancer cells.

PARP-1 consists of three main domains: the N-terminal DNA binding domain, the auto-modification domain and the C-terminal catalytic domains. Most of PARP-1 inhibitors imitate interaction between PARP-1 catalytic domain with its substrate, NAD^+. Early PARP-1 inhibitors were analogues of 3-amino benzamide because it was observed that the benzamide moiety was crucial for the specific binding to the enzymatic site, forming three key hydrogen bonds to the enzyme. From observations that binding affinity would be significantly increased when the carboxamide group, which is normally free to rotate, was restricted into lactam, many classes of PARP-1 inhibitors have been discovered such as amino-ethyl pyrrolo dihydroisoquinolonone, tricyclic quinoxalinone,

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http://dx.doi.org/10.1016/j.bmcl.2013.02.094

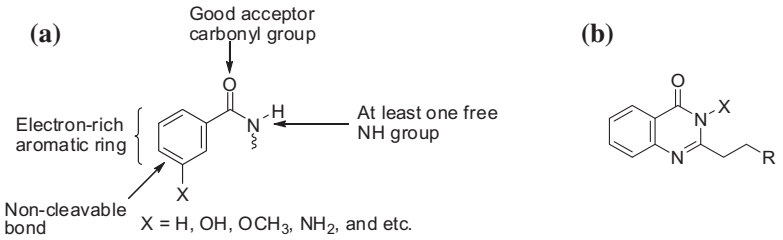


Figure 1. (a) Requirements for PARP-1 inhibitors. (b) Pharmacophore query.

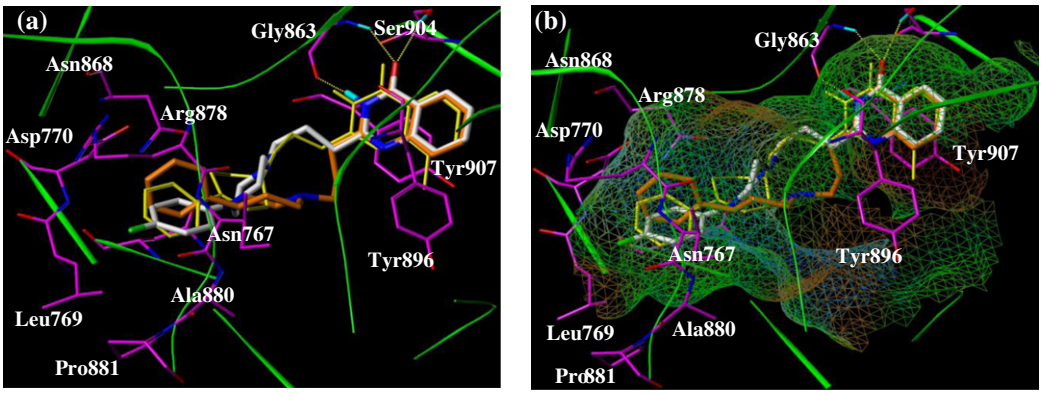


Figure 2. (a) FlexX-docked pose[45] of D31 and D36 into active site of PARP-1. FR257517 (yellow) in the X-ray crystal structure of PARP-1 (PDB ID: 1UK0) are showed for comparison. Hydrogen bonding interaction between D31 (atom type) and D36 (orange) are represented in yellow dotted lines. (b) Lipophilic potential surface map of the catalytic site pocket of PARP-1 is demonstrated in the docking model of D31 and D36. Lipophilicity increases from blue (hydrophilic) to brown (lipophilic).

quinazolinone, phthalazinone, benzimidazole, indole, and isoquinoline derivatives.[35–43] Analysis of different classes of PARP-1 inhibitors had suggested three structural features which must be taken into design of PARP-1 inhibitors: (i) electron-rich aromatic ring, (ii) a non-cleavable bond in the 3 position relative to carboxamide group, (iii) carboxamide moiety which is free to rotate or restricted to ring system.[2] Based on the model, we created a pharmacophore query (Fig. 1b) in which the quinazolinone core makes a sandwiched hydrophobic interaction, including π–π interaction with the phenyl ring of tyrosine residues Tyr907 and CH–π interaction with Cβ of Tyr869, the oxygen of carbonyl group will form hydrogen bonds with Ser904Oγ and Gly863NH whereas NH moiety forms hydrogen bond with Gly863C=O. The side chain consists of at least two carbon units allows the R group to reach the deep pocket located in the auto-modification domain of PARP-1. The pharmacophore-based virtual screening was performed against Korea Chemical Bank (http://www.chembank.org/) chemical database containing about 5 million chemicals using the Unity program in Sybyl 8.1. Only 27 compounds satisfied the pharmacophore query (Fig. 1b), and they were subjected to PARP-1 inhibitory assay.[34] Seven hit compounds were identified and the two most potent compounds showed nanomolar IC_{50} values (Table 1). However, these two compounds, D31 and D36, were registered for patent by KuDo pharmaceutical company for PARP-1 inhibitory activity. The other compounds except D30, have not been patented but they have already been reported in the literatures.[40–43]

Drug resistance is one of the greatest obstacles in cancer therapy. Due to their ability to transform necrotic cell death into apoptotic cell death, PARP-1 inhibitors enhance the effect of different DNA-alkylating agents such as topoisomerase I inhibitors, doxorubicin, cisplatin, oxaliplatin, gemcitabine, etc.[24–31] To examine whether PARP-1 inhibitors enhance the effect of cisplatin in resistant cell lines, we conducted chemosensitizing experiments of PARP-1 inhibitor against cisplatin-resistant cell line. For this experiment, cisplatin-resistant human gastric cancer cell line (YCC-3/D)

Table 1
PARP-1 inhibitors identified from Korean Chemical Bank

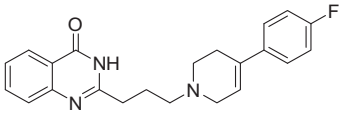
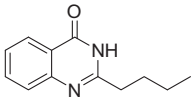
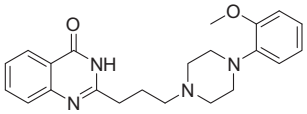
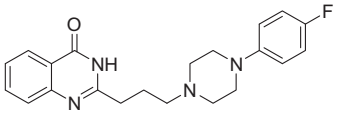
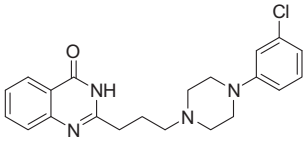
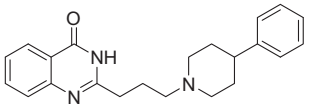
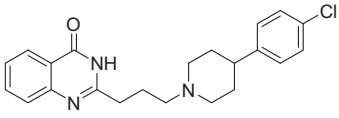
ID	Chembank ID	Hit Structure	IC_{50}[44] (μM)
D04	6338MJ0004		0.304
D07	2139SI0007		8.886
D30	6254SO0030		0.189
D31	6255SO0031		0.077
D32	6256SO0032		1.008
D36	6355SO0036		0.079
D38	6357SO0038		0.388

Table 2
IC_{50} values of cisplatin against YCC-3 versus YCC-3/D after a 36-h treatment[46]

Cell line	IC_{50} (μg/mL)	RF
YCC-3	12.3	2.137
YCC-3/D	26.29	

was established by treating parent cell line (YCC-3) with cisplatin 0.5 μg/mL for 18 months and then increasing cisplatin concentration at each 0.5 μg/mL increment and fixed at 2.5 μg/mL for another 6 months. Dose-dependent analysis against cisplatin of YCC-3/D showed that the YCC-3/D was less sensitive to cisplatin comparable to the parent cell line YCC-3 with RF (resistance factor) value around 2 (RF value[26] was calculated by ratio between IC_{50} value of YCC-3/D and IC_{50} value of YCC-3 against cisplatin) (Table 2). PARP-1 inhibitor D31 and D36 were re-synthesized for chemo-sensitization experiments (see Supplementary data for the details of synthetic methods and structural characterization of compounds). The treatment of D31 or D36 alone was not cytotoxic in YCC-3/D. The cytotoxicity IC_{50} values of D31 and D36 against YCC-3/D are above 200 μM and around 100 μM (Fig. 3). Therefore, the concentration of 50 μM was chosen for the following chemo-sensitization experiments. Pre-treatment of 50 μM PARP-1 inhibitors, D31 and D36, significantly decreased IC_{50} values of cisplatin, from 26.29 to 17.14 μg/mL and 24.59–15.19 μg/mL, respectively, in YCC-3/D cells (Fig. 4). In contrast, no significant sensitization effects were obtained by pre-treatment of PARP-1 inhibitors into the parent YCC-3 cells (Supplementary data). We also tried co-treatment of the same concentration of D31 and D36 with cisplatin,

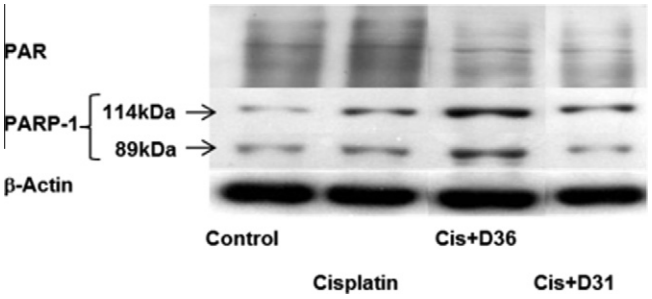


Figure 5. Western blot[50] with PAR antibodies showing the reduction of PAR formation by PARP-1 inhibitors in YCC-3/D cells. The PARP-1 antibody detects two bands, 113 kDa PARP-1 and 89 kDa apoptosis-induced cleavage fragment of PARP-1.

but it did not improve the cytotoxicity of cisplatin in YCC-3/D cells. To verify whether D31 and D36 inhibit the PARP-1 enzymatic activity in YCC-3/D cells, Western blot analysis was conducted. In the Western blot (Fig. 5), the cleavage of PARP-1 into 89-kD and 24-kD fragments by caspase-3 was detected in YCC-3/D cells, which is a characteristic of apoptosis.[48,49] In response to cisplatin treatment, PARP-1 is activated and results in the synthesis of poly(ADP-ribose) polymer (PAR) on acceptor proteins.[1–4] Our results revealed the decrease in PAR formation in the presence of PARP-1 inhibitors, D31 and D36, in YCC-3/D cells.

In summary, we reported here an identification of potent PARP-1 inhibitors through pharmacophore-based virtual screening of Korean Chemical Database. The two most potent PARP-1 inhibitors, D31 and D36, effectively improved the sensitivity of

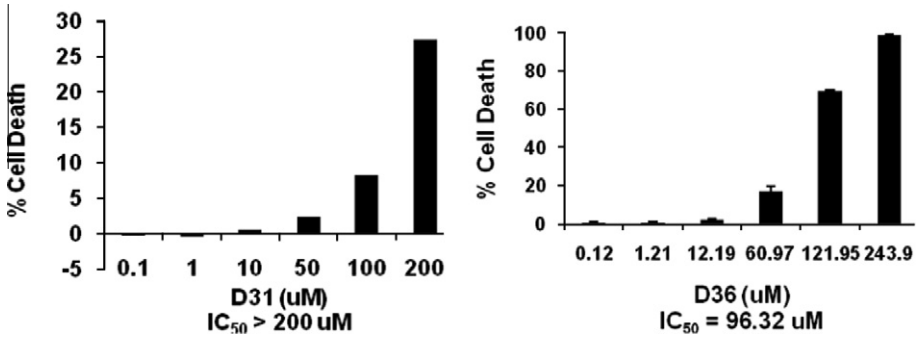


Figure 3. Cytotoxic effect of PARP-1 inhibitors against cisplatin-resistant human gastric cancer cell line YCC-3/D.[46] IC_{50} values were determined after exposure of YCC-3/D to PARP-1 inhibitors for 36 h.

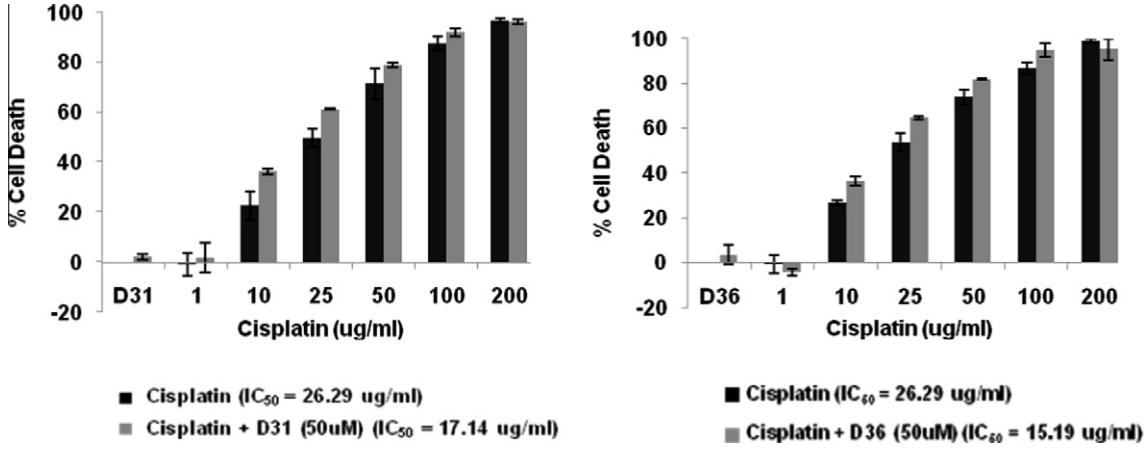


Figure 4. Chemosensitizing effect[47] of PARP-1 inhibitors, D31 and D36 against YCC-3/D. YCC-3/D cells were pre-treated with PARP-1 inhibitors for 8 h, and then treated with cisplatin for another 36 h to determine IC_{50} values.

cisplatin-resistant human gastric cancer cells to cisplatin by inhibiting PARP-1 catalytic activity.

Acknowledgments

This study was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education, Science and Technology (Grant 2010-0029358), and in part by a grant of the Korea Healthcare technology R&D Project, Ministry for Health & Welfare, Republic of Korea (Grant A092006).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.094.

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44. *PARP-1 inhibitory assay*[34]*:* Into the wells of a 96-well plate, 20 μL of a 250 nM solution of NAD^+ in PARP assay buffer (pH 8.00) consisted of 50 mM Tris and 2 mM $MgCl_2$, 10 μL of activated DNA at a concentration of 50 μg/mL dissolved in PARP assay buffer, and 10 μL of the inhibitors at different concentrations in PARP assay buffer were added. The reaction was initiated by adding PARP-1 at a concentration of 2 μg/mL, 10 μg/mL DNA, and 100 nM NAD^+ with various concentrations of inhibitors in a total volume of 50 μL. The plate was incubated for 15 min at room temperature and the amount of NAD^+ remaining was determined by adding 20 μL of KOH 2 N and 20 μL of acetophenone 20%. After being incubated at 4 °C for 10 min, 90 μL of formic acid (88%) was added to the reaction mixture and heated at 100 °C for 10 min. The plate was allowed to cool to room temperature and fluorescent intensity was read on a SPECTRAmax GEMINI XS microplate spectrofluorometer at excitation wavelength of 400 nm and emission wavelength of 445 nm.
45. *Virtual screening and docking experiments:* Protein modeling and docking experiments have been performed with the Sybyl 8.1 software package (Tripos, Inc., St. Louis, MO, USA) based on Linux CentOS 4.0. To select the quinazoline-based candidate PARP-1 inhibitos, the pharmacophore search was conducted against Korea Chemical Bank database using the Unity program in Sybyl 8.1. The structures of D31 and D36 were prepared in MOL2 format using the sketcher module and Gasteiger–Huckel charges were assigned to the ligand atoms. The structure of D31 and D36 were optimized by energy minimization until a convergence value of 0.001 kcal/(Å mol).The X-ray coordinate of PARP-1 complexed with FR257517 inhibitor (PDB ID: 1UK0)[33] was retrieved from the PDB, one monomer and all crystallographic water molecules were removed. After the hydrogen atoms and atomic charges were added to the receptor, side chain amides of PARP-1 were fixed. The active site was defined as all the amino acid residues enclosed within 6.5 Å radius sphere centered by the bound inhibitor FR257517. The docking was performed using the default parameters of the FlexX programs implanted in the sybyl 8.1, and subsequent scoring for FlexX solution was conducted by a consensus scoring function (CScore). One of the conformers of D31 and D36 having the highest consensus score (CScore = 5) were selected and complexed with PARP-1, resulting in a final model (Fig. 2).
46. *Cytotoxicity effect of cisplatin and PARP-1 inhibitors:* YCC-3 and YCC-3/D cells[32] were seeded into 96-well plate at a density of 5000 cells/well and incubated at 37 °C/5% CO_2 overnight. PARP-1 inhibitors or cisplatin were treated to cells and incubated for 36 h at 37 °C/5% CO_2. To determine the proportion of cell death, the cisplatin-containing media was removed and 100 μL of medium containing 10 μL of a 1.90 mg/mL MTS solution (Promega CellTiter $96AQ_{ueous}$ One Solution Cell Proliferation assay) was added to each well, which was followed by incubation for 2 h at 37 °C. The absorbance was determined using a microculture plate reader (ELISA reader) at 490 nm. Each assay was performed in triplicate. The drug concentration required to inhibit cell proliferation by 50% (IC_{50}) was determined by plotting the percentage of cell growth inhibition vs. the drug concentration.
47. *Chemosensitizing effect of PARP-1 inhibitors:* YCC-3/D cells[32] were seeded into 96-well plate at a density of 2500 cells/well and incubated at 37 °C/5% CO_2 overnight. PARP-1 inhibitors were treated to cells for 8 h. After removing media containing PARP-1 inhibitors, cisplatin was added to cells at indicated concentration and incubated for 36 h at 37 °C/5% CO_2. To determine the proportion of cell death, the cisplatin-containing media was removed and 100 μL of medium containing 10 μL of a 1.90 mg/mL MTS solution (Promega CellTiter $96AQ_{ueous}$ One Solution Cell Proliferation assay) was added to each well, which was followed by incubation for 2 h at 37 °C. The absorbance was determined using a microculture plate reader (ELISA reader) at 490 nm. Each assay was performed in triplicate. The drug concentration required to inhibit cell proliferation by 50% (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the chemotherapeutic drug concentration.
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50. *Western blot analysis:* The cell lysates were boiled in a Laemmli sample buffer for 5 min at 95 °C, and 30 μg of the protein lysates from each sample were electrophoresed on an 8% SDS–polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad) with a constant 80 V for 90 min. The membrane was pre-blocked in TBST containing 5% skim milk powder for 1 h at room temperature. The blot was incubated for 3 h at room temperature with a 1:1000-diluted

monoclonal antibody to human PARP-1 (BD Biosciences, Becton Dickinson Korea), 1:1000-diluted PAR antibody (Trevigen, Kormed Corp.), and beta actin (Abcam, Inc.) in TBST. The blot was washed three times for 10 min each time with TBST. The membrane was incubated for 1 h with peroxidase-conjugated anti-mouse IgG whole antibody (Amersharm Bio). Then, the blot was washed three times for 10 min each time with TBST. Protein was detected using an enhanced chemiluminescence Western blot analysis system (Amersharm Bio).