Original Paper

Cinnamaldehyde Derivative (CB-PIC) Sensitizes Chemo-Resistant Cancer Cells to Drug-Induced Apoptosis via Suppression of MDR1 and its Upstream STAT3 and AKT Signalling

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Key Words

MDR-1 • Drug resistance • Apoptosis • Synergy

Abstract

Background/Aims: Our group reported that cinnamaldehyde derivative, (E)-4-((2-(3-oxoprop-1-enyl)phenoxy)methyl)pyridinium malonic acid (CB-PIC) induced apoptosis in hypoxic SW620 colorectal cancer cells via activation of AMP-activated protein kinase (AMPK) and extracellular signal regulated kinase (ERK). Herein, sensitizing effect of CB-PIC was investigated in resistant cancer cells such as paclitaxel (PT) resistant lung cancer cells (H460/PT), and Adriamycin (Adr) resistant breast cancer (MCF7/Adr) and colon cancer (HCT15/cos) cells. **Methods:** Various drug resistant cell lines were treated with CB-PIC, and the signalling pathway and functional assay were explored by Western blot, Rhodamine assay, FACS, RT-PCR and MTT assay. **Results:** We found that CB-PIC effectively exerted cytotoxicity, increased sub G1 population and the cleaved form of poly (ADP-ribose) polymerase (PARP) and caspase 9 in drug resistant cancer cells. Furthermore, CB-PIC sensitized resistant cancer cells to adriamycin via downregulation of survival proteins such as survivin, Bcl-xL and Bcl-2, along with MDR1 suppression leading to accumulation of drug in the intracellular region. Of note, CB-PIC transcriptionally decreased MDR1 expression via suppression of STAT3 and AKT signalling in three resistant cancer cells with highly expressed P-glycoprotein. Nonetheless, CB-PIC did not affect transport activity of

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P-glycoprotein in a short time efflux assay, while epigallocatechin gallate (EGCG) accumulated Rhodamine 123 into intracellular region of cell by direct inhibition of MDR1 transport activity. **Conclusions:** These data demonstrate that CB-PIC suppresses the P-glycoprotein expression through inhibition of STAT3 and AKT signalling to overcome drug resistance in chemo-resistant cancer cells as a potent chemotherapeutic sensitizer.

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Introduction

Resistance of chemo-therapeutic drugs such as adriamycin and paclitaxel is one of the well-known failures of cancer therapy. Over 90% of patients with metastatic cancer are known to fail cancer therapy because of drug resistance [1]. To evade toxic effect, drug resistant cancer cells usually increase the rates of drug efflux, alterations in drug metabolism and mutation of drug targets in their intracellular regions [2]. MDR1, also known as P-glycoprotein, is a 170kDa protein that belongs to the sub-family of the ATP-binding cassette (ABC) transporters as one of drug resistance molecules [3]. The function of MDR1 has been described as an ATP-dependent efflux pump of chemo-therapeutic drugs. Actually, overexpression of MDR1 is one of the common phenotype of drug resistant cancers in which intracellular accumulation of chemo-therapeutic drugs is significantly decreased [4, 5]. For this reason, recent studies have focused on the suppression of MDR1 expression or function to overcome the MDR1-mediated drug resistance [6]. In general, three options to control ABCB1 gene encoding MDR1 protein are regulation of several oncogenes including Ras, c-Raf, and related protein kinases, epigenetical change of ABCB1 promoters, and transcriptional factors such as C/EBP- β in malignant cancer cells [7].

Recently some natural compounds are on the spotlight due to their safe and efficient antitumor activities in several resistant cancers by single or combination treatment with classical anti-cancer agents. Tannic acid showed synergistic antitumor effect by modulating efflux pump system with chemo-therapeutic drugs [8]. Epigallocatechin gallate (EGCG), the most abundant polyphenol compound in green tea, mediated cell growth arrest and inhibited MDR1 expression in adriamycin-resistant cancer cells [9]. Though our group reported that CB-PIC induces AMP-activated protein kinase (AMPK) mediated apoptosis in hypoxic colon cancer cells [10], the underlying antitumor mechanism of CB-PIC was not yet examined in resistant cancer cells. Thus, in the current study, the potential role of CB-PIC targeting MDR1 was elucidated in paclitaxel resistant lung cancer (H460/PT), adriamycin or adriamycin resistant breast cancer (MCF7/Adr) and colon cancer (HCT15/cos) cells.

Materials and Methods

Chemicals and reagents

CB-PIC (Fig. 1) was kindly given by Dr. BM Kwon. (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). HCT15 human colorectal adenocarcinoma cells, MCF7 human breast adenocarcinoma cells, H460 human non-small cell lung cancer cells were purchased from American Type Culture Collection. (ATCC, Manassas, VA, USA). The cells were maintained in RPMI 1640 supplemented with fetal bovine serum (FBS), penicillin and streptomycin. (Welgene, Korea). Adriamycin and paclitaxel were purchased from Sigma Aldrich. (St. Louis, MO, USA)

Culture for drug resistant cell lines

H460/PT cells were kindly given by Dr. JK Rho (Department of Pulmonary and Critical Care Medicine, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, Korea.) and HCT15/cos and MCF7/Adr cells were kindly given by Dr. MN Park. (National Center of Applied Microfluidic Chemistry, Department of Chemical Engineering, POSTECH (Pohang University of Science and Technology)). The cells were maintained at 37°C in 5% CO₂ in RPMI 1640 medium containing 10% FBS and antibiotics (Welgene, South Korea).

Cytotoxicity assay

To determine the sensitizing effect of CB-PIC with chemo-therapeutic drugs in drug resistant cells, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, the cells (1×10^4 cells / well) were seeded onto 96-well culture plates. After incubation for 24 h, cells were exposed to various concentrations of CB-PIC, adriamycin, paclitaxel alone or combination for 48 h. Then, 100 μ l of 1 mg/ml MTT (Sigma Aldrich, USA) was added to each well for 4 h at 37°C. The purple formazan formed was solubilized by DMSO and absorbance at 590 nm was read by a microplate reader (Molecular Devices Co, Sunnyvale, CA, USA). Cell viability was calculated as a percentage of viable cells versus untreated cells by following equation. Cell viability (%) = [OD (drug) – OD (Blank)] / [OD (untreated) – OD (Blank)] $\times$ 100

Western blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 1% TritonX-100) containing protease inhibitors (Roche, Germany), and phosphatase inhibitors (Sigma Aldrich, USA). Protein samples were quantified by Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA). The protein samples were separated on 8 to 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies of MDR1, p-AKT, AKT, p-STAT3, STAT3, Bcl-2, survivin, Bcl-XL, p53, β -actin (Cell Signalling, Beverly, MA) diluted in 5% BSA in TBS-Tween 20 (1:1000-1:5000) for overnight at 4°C, washed with TBS-Tween 20, and incubated with HRP-conjugated secondary antibodies (1:5000-1:20000) for 30 min at RT. Expression was visualized by using ECL Western blot detection reagent (GE Healthcare, UK).

RT-PCR

Total RNA was isolated with QIAzol (Invitrogen, USA) and cDNA was reverse-transcribed with MML-V reverse transcriptase kit (Enzynomics, Daejeon, Korea). Primer sequences used for PCR are as follows: MDR1 forward 5'-AGGCCAACATACATGCCTTC-3', MDR1 reverse 5'-GCTCCTTGACTCTGCCATTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-TATAAATTGAGCCCGCAGCC-3', GAPDH reverse 5'-TTCCCGTTCTCAGCCTTGAC-3'. The PCR was performed as follows: 5 min at 95°C, 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 45 s, and 5 min incubation at 72°C. The PCR products were loaded onto 1% agarose gel electrophoresis and visualized by loading STAR solution (Dyne-bio, Sungnam, Korea) and UV illumination.

Rhodamine 123 efflux assays

-Short term direct effect on MDR1 enzyme activity; H460/PT cells (1×10^5 cells / well) were exposed to 2 μ M of Rhodamine 123 in growth medium for 30 min to allow uptake of Rhodamine 123. Then the cells were washed twice with PBS, resuspended in fresh medium with or without 10 μ M of CB-PIC or EGCG and incubated for 60 min. Cells were immediately moved on ice to stop reaction. Rhodamine 123 uptake at baseline and efflux was immediately measured by flow cytometry.

-Long term accumulation assay; H460/PT or H460 cells (1×10^5 cells / well) were pre-treated with or without 20 μ M of CB-PIC for 24 h and incubated with 5 μ M of Rhodamine 123 for another 6 h at 37°C. Cells were then pelleted, washed twice, and resuspended in FACS buffer. Rhodamine 123 uptake at baseline and efflux was immediately measured by flow cytometry.

Cell cycle detection

Cell cycle detection was performed by Propidium iodide (PI) staining. Cells were fixed in 70% Ethanol for 1 h at 4°C and incubated in PBS with 0.1% RNase A for 1 h at 37°C and suspended in PBS containing 50 μ g / ml PI for 1 h at room temperature. Stained cells were analyzed by using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA)

Apoptosis detection

Apoptosis detection was performed by Annexin-V and PI double staining. Cells were stained Annexin-V and PI using the Annexin V-Apoptosis Detection kit (Biovision, USA) according to the manufacturer's instructions. Stained Cells were analyzed by FACSCalibur.

MDR1 expression detection

Cells were harvest and suspended by FACS buffer (5% FBS in PBS). Then, FITC-conjugate MDR1 primary antibody (BD Pharmingen™, USA) was added to the cells for 30 min at 4°C. After washed three times with FACS buffer, MDR1 expression was analyzed by FACSCalibur.

Combination index

To determine the synergistic effect of CB-PIC and chemo-therapeutic drugs, MTT assay was performed in three resistant cell lines. Following the determination of IC_{50} for each drug, the synergy between CB-PIC and adriamycin or paclitaxel was evaluated through combination index values by the method of Chou Talalay [11] using CalcuSyn software (Biosoft, USA).

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). The statistically significant differences between sample and untreated control cells were calculated by Student's t-test. All experiments were carried out at least thrice.

Fig. 1. Characterization of Drug Resistant (DR) cancer cells. (A) Effect of Adr on viability in MCF7, MCF7/Adr, H460 and H460/PT cells. The cells were treated with indicated concentrations of Adr for 48 h (B) Effect of PT on viability in H460 and H460/PT cells. H460 and H460/PT cells were treated with indicated concentrations of PT for 72 h. MTT assay was performed to determine the cytotoxicity of Adr or PT in parental or resistant cancer cells. Data represent means $\pm$ SD. (C) MDR1 expression level in several cancer cell lines by Western blotting. (D) MDR1 expression on the surface of HCT15, MCF7 and H460 cells by FACS analysis. IgG; immunoglobulin G, P; parents cells, R; Drug resistant cells. (E) MDR1 activity was determined by Rhodamine123 efflux assay using FACS. Parental and DR cells were pre-incubated with or without 2 μ M Rhodamine123 for 30 min at 37°C. Cells were washed with cold PBS and then analyzed by FACS for verification of the Rhodamine accumulation in DR cancer cells.

Fig. 2. Cytotoxic effect of CB-PIC in DR cancer cells. (A) Chemical structure of CB-PIC. (B) Cytotoxic effect of CB-PIC on MCF7 and MCF7/Adr cells by MTT assay. (C) Cytotoxic effect of CB-PIC in H460 and H460/PT cells. The cells were treated with CB-PIC for 48 h and MTT assay was performed to determine the cytotoxicity of CB-PIC in parental and DR cells. Data represent means $\pm$ SD. (D-E) Effect of CB-PIC on cell cycle of MCF7/Adr cells treated with 20 μ M CB-PIC or 1 μ M Adr for 48 h. Propidium iodide staining was used for FACS analysis to confirm the effect of CB-PIC on cell cycle of cancer cells. Adr was used as a positive control.

Results

MDR1 was overexpressed in drug resistant (DR) cancer cells compared to their parental cells

In the drug sensitivity assay of the drug resistance cell lines such as HCT15/cos, MCF7/Adr and H460/PT, most drug resistant cells were survived even in high concentration of Adr or PT, while IC₅₀ of parental cells was less than 1 μ M (HCT15 and MCF7) of Adr or 0.5 μ M of PT (H460) (Fig. 1A and B). To confirm whether MDR1 is overexpressed in DR cells, HCT15/cos, MCF7/Adr and H460/PT cells, immunoblotting and FACS analysis were carried out. Western blotting showed overexpression of MDR1 protein (Fig. 1C) and flow cytometry analysis revealed cell surface expression of MDR1 in resistant cells compared to parental cells (Fig. 1D). Next, we measured Rhodamine 123 accumulation by flow cytometry to determine the efflux capacity of DR cells. As shown in Fig. 1E, Rhodamine 123 was highly accumulated in parental cancer cells compared to DR cancer cells, indicating that DR cells actively pump MDR1 substrate Rhodamine 123 out of cells compared to parental cells (Fig. 1E). These data demonstrate that all of tested DR cancer cells exhibit the upregulation of MDR1 protein and resistance to chemotherapeutic agents.

CB-PIC exerted cytotoxicity in DR cells

To verify whether CB-PIC affects the viability of DR cells, we conducted MTT assay in CB-PIC treated DR cancer cells. We found that CB-PIC dramatically decreased the viability of DR cells (Fig. 2B and C). Especially, cell death in H460/PT was significantly occurred more than parental cells by CB-PIC treatment (Fig. 2C). Next, we confirmed the effect of CB-PIC on

Fig. 3. Effect of CB-PIC on apoptosis and cleavages of PARP and caspase 9 in DR cancer cells. (A) Effect of CB-PIC on apoptosis in H460/PT and HCT15/Cos DR cells. DR cells were treated by CB-PIC for 48h. The stained DR cells with Annexin V-FITC and PI were analyzed by a dot-plot using a flow cytometer. (B) Quantified bar graph for apoptosis. Data represent means $\pm$ SD. p values compared to untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (C) Effect of CB-PIC on the cleavages of PARP and caspase 9 in HCT15/Cos and H460/PT DR cells. Cell lysates were subjected to Western blotting to verify the effect of CB-PIC on the cleavage of PARP and activation of caspase 9. β -actin was used as an internal control.

apoptosis in DR cancer cells by PI staining. FACS analysis showed that sub-G1 population was dramatically increased in CB-PIC treated H460/PT cells compared to non-treated control or Adr (Fig. 2D and E). Also, to confirm whether CB-PIC mediated cytotoxicity and sub-G1 accumulation are via apoptosis induction, we performed Flow cytometry analysis using Annexin V-PI double staining. Apoptosis was significantly increased in CB-PIC treated DR cells in a dose dependent manner compared to untreated control (Fig. 3A and B). Consistently, CB-PIC increased the cleaved form of PARP and caspase 9 in DR cell lines (Fig. 3C).

CB-PIC transcriptionally regulated MDR1 expression, but had no direct effect on MDR1 enzyme activity

To confirm whether CB-PIC directly affects MDR1 activity in DR cells, we conducted the Rhodamine 123 accumulation assay which is used for validation of drug effect on MDR1 activity via direct inhibition [12]. As shown in Fig. 4A, CB-PIC did not accumulate Rhodamine 123 in H460/PT whereas EGCG significantly enhanced the accumulation of Rhodamine 123 in DR cells by short term exposure at 37°C, indicating that CB-PIC did not directly influence MDR1 activity. To perform functional analysis, we tested MDR1 expression in CB-PIC treated DR cells by immunoblotting. CB-PIC treatment dramatically inhibited MDR1 expression in DR cells in a dose dependent manner (Fig. 4B). Furthermore, we uncovered that the downregulation of MDR1 by CB-PIC was induced at transcriptional stage (Fig. 4C). Also, the surface expressions of MDR1 on DR cell membrane were also consistently suppressed in MCF7/Adr and H460/PT cells by CB-PIC treatment (Fig. 4D and E). Finally, to confirm

Fig. 4. Effect of CB-PIC on Rhodamine accumulation and MDR1 at protein, mRNA and cellular levels in DR cells. (A) Effect of CB-PIC or EGCG on Rhodamine accumulation in H460/PT cells. Cells were incubated with 2 μ M Rhodamine 123 in cold medium for 30 min to allow uptake, and then were washed twice with cold PBS. The cells were suspended in cold PBS with or without 10 μ M of CB-PIC or EGCG and incubated for 60 min at 37°C. Rhodamine 123 uptake was immediately measured by flow cytometry. (B) Effect of CB-PIC on MDR1 at protein level in MCF7/Adr and H460/PT cells. Cells were exposed at 0 μ M (-), 5 μ M (+), 10 μ M (++) of CB-PIC for 24 h. Cell lysates were subjected to Western blotting to confirm the change of MDR1 expression. β -actin was used as an internal control. (C) Effect of CB-PIC on MDR1 at mRNA level in H460/PT cells. Cells were treated with the indicated concentrations of CB-PIC for 24 h. Reverse transcriptase PCR was performed to determine mRNA levels of MDR1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Effect of CB-PIC on MDR1 surface expression of (D) MCF7/Adr cells and (E) H460/PT cells. MCF7/Adr and H460/PT cells were exposed in 10 μ M of CB-PIC for 24 h and MDR1 expression on cell surface was detected by FITC conjugated anti-MDR1 Ab using FACS. Data represent means $\pm$ SD. *p* values compared to untreated control. **p* < 0.05, ***p* < 0.01. (F) Overall effect of CB-PIC on intracellular Rhodamine accumulation in H460/PT or H460 cells under same experimental condition in Fig. 4A. Cells were immediately harvested and Rhodamine level was analyzed in the cells by using FACS.

whether CB-PIC can increase intracellular drug accumulation in DR cells, DR cells were exposure at CB-PIC added media for 24 h in 37°C. CB-PIC increased intracellular Rhodamine accumulation in H460/PT cell more than 20 fold compared to untreated cells. These data mean that CB-PIC transcriptionally inhibits MDR1 expression in various DR cell lines.

CB-PIC showed synergistic effect with Adr or PT through inhibition of STAT3/AKT pathway

As shown in Fig. 5A, CB-PIC downregulated the phosphorylation of AKT and STAT3, and attenuated the expression of anti-apoptotic protein such as Bcl-2 and Bcl-xL, but not survivin compared to untreated control or Adr (Fig. 5A). Additionally, Combination Index (CI) analysis showed that co-treatment of CB-PIC and Adr or PT synergistically decreased cell viability in HCT15/cos and H460/PT cells (Fig. 5B, C, D and E), although all of tested

Fig. 5. Synergistic antitumor effect of CB-PIC and Adr on DR cells via inhibition of AKT and STAT3 signal. (A) Effect of CB-PIC on expression of survival genes such as AKT, STAT3, Bcl-xL and Bcl-2 and Survivin in HCT15/Cos cells. The cells were treated with indicated concentrations of CB-PIC and/or Adr for 48 h. Western blotting was performed for detection of AKT, STAT3 and their downstream proteins. β-actin was used as an internal control. (B-C) Synergistic effect of CB-PIC and Adr on the viability of HCT15/cos cells. (D-E) Synergistic effect of CB-PIC and PT on the viability of H460/PT cells. MCF7/Adr or H460/PT cells were incubated with CB-PIC and/or Adr or PT for 48 h and MTT assay was performed. Data represent means ± SD. Combination index was calculated using Calcsyn Software. (F) Proposed model for transcriptional suppression of MDR1 expression by CB-PIC.

Fa points were not below 1. These data suggest that combination treatment of CB-PIC with chemo-drug synergistically show the effect on various DR cells to overcome resistance.

Discussion

Though many chemo-therapeutic agents contributed to several cancers for years, acquired resistance is a hot issue especially in relapsed cancers [13-15]. MDR1, also known as p-glycoprotein and encoded by ABCB1 gene, is a subunit of ATP-dependent transporter and a molecular target for multidrug resistance [16]. MDR1 transports substrates like microtubule poisons with ATP hydrolysis across membrane [2, 17]. Though MDR1 inhibitors including verapamil have been used with chemo-therapeutic drugs [18], development of new MDR1 inhibitors with little toxicity and better efficacy are required [19].

Accumulated evidences show that natural compounds such as quercetin, (-)-epigallocatechin gallate (EGCG), curcumin, capsaicin, and 6-gingerol are effective in drug resistance in a variety of cancers [20-23]. Among them EGCG and curcumin regulated MDR1 via direct inhibition of active region of p-glycoproteins and its substrates [21, 22]. Amphiphilic chemical structure of EGCG facilitated the interaction with P-glycoprotein [21] and curcumin suppressed P-glycoprotein in KB-C2 (KB-C2/PG) cancer cells ectopically

expressing MDR1 [22]. However, in the current study, though CB-PIC did not directly inhibit MDR1 activity (Fig. 4A), it attenuated the expression of MDR1 at protein and mRNA levels in all tested DR cells in a time and dose dependent manner. Furthermore, CB-PIC suppressed the viability of resistant cancer cells, such as MCF7/Adr, H460/PT and HCT15/cos compared to parental cells, indicating the potent sensitizing effect of CB-PIC in DR cells. Consistently, CB-PIC effectively induced the active form of PARP and caspase 9, increased sub G1, in PT and Adr resistant cancer cells, implying the cytotoxic effect of CB-PIC is exerted via apoptosis induction and MDR1 inhibition.

It is well documented that many transcription factors, such as Ap-1, NF- κ B, Akt and STAT3 are involved in activation of MDR1 gene in various cancers [24-29]. STAT3 binds to the potential promoter region, +64 and +72 of MDR1 in myeloid leukemia. Based on our results, we validated that transcriptional repression of MDR1 gene is mediated by suppression of phosphorylation of Akt and STAT3. In addition, CB-PIC sensitized resistant cancer cells to Adr via downregulation of survival proteins such as survivin, Bcl-xL and Bcl-2 in HCT15/cos cells, indicating inhibition of survival genes mediates apoptosis induction of CB-PIC in resistant colorectal cancer cells. However, further studies to show how CB-PIC regulates Akt and STAT3 are required.

Taken together, our findings suggest that CB-PIC suppresses MDR1 expression via inhibition of STAT3 and AKT signalling to overcome drug resistance in chemo-resistant cancer cells as a potent chemotherapeutic sensitizer.

Disclosure Statement

The authors disclose no potential conflicts of interest.

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