Effect of Dicycloplatin, a Novel Platinum Chemotherapeutical Drug, on Inhibiting Cell Growth and Inducing Cell Apoptosis

Guang-quan Li1,2, Xing-gui Chen1, Xing-ping Wu2, Jing-dun Xie2, Yong-ju Liang2, Xiao-qin Zhao2, Wei-qiang Chen1,*, Li-wu Fu2*

1 Department of General Surgery, Chen Xing Hai Hosital, Guangdong Medical College, Zhongshan, People’s Republic of China, 2 State Key Laboratory of Oncology in Southern China, Cancer Center, Sun Yat-Sen University, Guangzhou, People’s Republic of China

Abstract

Dicycloplatin, a new supramolecular platinum-based antitumor drug, has been approved by the State Food and Administration (SFDA) of China. In this study, we investigated the anticancer activity of dicycloplatin in cancer cells and signaling pathways involved in dicycloplatin-induced apoptosis. Dicycloplatin inhibited the proliferation of cancer cells and increased the percentage of apoptosis in a concentration-dependent manner. Besides, some apoptosis related events were observed after treatment with dicycloplatin, including increase of reactive oxygen species (ROS), collapse of mitochondrial membrane potential (Δψm), release of cytochrome c from the mitochondria to the cytosol, upregulation of p53, which were accompanied by activation of caspase-9, caspase-3, caspase-8, and poly (ADP-ribose) polymerase cleavage in a concentration-dependent manner. The role of apoptosis in dicycloplatin-mediated cell death was further confirmed by the concomitant treatment with caspase-8 or caspase-9 inhibitors, which inhibited apoptosis and PARP cleavage. Intracellular glutathione (GSH) was also found to inhibit the cytotoxic effect of dicycloplatin. In conclusion, these findings suggest that dicycloplatin induces apoptosis through ROS stress-mediated death receptor pathway and mitochondrial pathway which is similar to carboplatin.

Introduction

Platinum-based anticancer drugs, such as cisplatin and carboplatin, are the first-line clinical anticancer drugs for the treatment of solid tumors [1], including lung, breast, ovarian and head and neck cancers. Both cisplatin [2,3,4,5] and carboplatin [6,7,8,9] have been shown to inhibit cancer cells growth and induce apoptosis in cancer cells through death receptor pathway and/or mitochondrial pathway. However, the application of these drugs in the clinic is limited by their severe toxicities, such as nephrotoxicity, neurotoxicity and ototoxicity with cisplatin [10,11] and myelosuppression with carboplatin [11]. Therefore, development of novel platinum anticancer drugs with high efficiency and low toxicity is warranted for cancer chemotherapy.

Dicycloplatin is a novel platinum-based anticancer drug which was formed by one molecule of carboplatin and one molecule of 1,1-ring succinic acid interacting intra-molecularly via hydrogen bond. It was shown to be highly soluble and stable, comparable with carboplatin [12]. While the anticancer efficacy of dicycloplatin was found to be more superior than cisplatin, the former has only minimal toxicity [13]. Animal studies also showed that dicycloplatin is much less nephrotoxic than cisplatin and it has similar myelosuppressiove effect as carboplatin [14]. It has been reported recently that dicycloplatin could inhibit the proliferation of a variety of tumor cells in vitro and in vitro via the induction of apoptosis [15,16,17]. More importantly, data from clinical trials indicated that the combination of dicycloplatin with pazlitaxel in advanced non-small cell lung cancer was safe and effective, which further advocating the clinical development of dicycloplatin as a novel anticancer drug [18]. However, the mechanism by which dicycloplatin induced apoptosis in cancer cells remained unclear.

Apoptosis controls the development and homeostasis of multicellular organisms through a highly supervised and organized death process. The death receptor pathway and the mitochondrial pathway are the two major routes of apoptosis [19]. In the mitochondrial pathway, dysfunction of mitochondria induces a cascade of events, including release of cytochrome c from mitochondria to cytosol, binding of cytochrome c to apoptotic protease activating factor 1 (Apaf-1), sequential activation of procaspase-9 and caspase-3 and eventually leading to apoptosis [20]. Increasing evidences indicated that the Bcl-2 family proteins played an important role in regulating the mitochondrial-dependent cell apoptosis [21]. Bcl-2 family proteins comprise anti-apoptotic subfamily and pro-apoptotic subfamily. Bcl-2 and Bcl-xl belong to the anti-apoptotic, whereas Bax, Bak as well as Bid and Bad belong to the pro-apoptotic subfamily [22]. During the apoptosis process, pro-apoptotic subfamily member proteins, such as Bax, Bad and BID, transmit to the mitochondria, and...
Figure 1. Cytotoxic effect of dicycloplatin in cancer cell lines. A, structure of dicycloplatin. B, structure of carboplatin. C–F, cytotoxicity of dicycloplatin and carboplatin in HepG2, A549, BEL-7402 and H460 cells. Cytotoxicity was measured by MTT assay. The cells were exposed to a full range of concentrations of dicycloplatin and carboplatin for 72 h. Cell viability with a model 550 microplate reader after staining with MTT for 4 h. The data presented represent mean ± SD of three independent experiments.

doi:10.1371/journal.pone.0048994.g001
Dicycloplatin Inhibited Cancer Cell Growth

A

**Annexin V**

B

| Dicycloplatin (μmol/L) | Carboplatin (μmol/L) |
|------------------------|----------------------|
| control                | control              |
| 30                     | 30                   |
| 60                     | 60                   |
| 120                    | 120                  |
| (48h) 240μM            | (48h) 200μM          |

Percent apoptosis (%)
promote the release of cytochrome c [21]. The increased cytochrome c activates caspases and induces apoptosis. Additionally, intracellular reactive oxygen species (ROS) could also influence the mitochondrial pathway [23,24,25]. Overproduction of ROS could change the inner membrane permeability and inner membrane potential (ΔΨm) and induce release of cytochrome c from mitochondria to cytosol, finally resulting in apoptosis [26,27]. Fas is the most typical death receptor in the death receptor pathway. After binding with its ligand FasL, Fas is activated and trimerized to recruit the Fas-associated death domain (FADD) and procaspase-8, and finally forming the death-inducing signaling complex (DISC) [28]. Procaspase-8 is activated by autocatalysis, and the activated caspase-8 stimulates apoptosis directly via directly activates caspase-3 [29] or cleaves Bid into truncated Bid (tBid) which transmits to mitochondria and promotes the release of cytochrome c from mitochondria to the cytosol [30].

In our study, dicycloplatin demonstrated superior potency in inhibiting growth and inducing apoptosis than the classical platinum-based anticancer drugs in cancer cells. The detailed mechanism leading to dicycloplatin-induced apoptosis was investigated and compared with that of carboplatin.

**Materials and Methods**

**Chemicals and Reagents**

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescin diacetate were purchased from Sigma. 5,5’,6,6’-tetrachloro-1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and glutathione (GSH) were purchased from Beyotime Institute. Adriamycin was purchased from ZhuHai MingZHi Pharmaceuticals. ApopNexin FITC Apoptosis Detection Kit was purchased from Biovision. Caspase-9 inhibitor (Z-LETD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) were purchased from Biovision. Antibodies against caspase-3, cytochrome c and p53 were obtained from Santa Cruz Biotechnology. Antibodies against caspase-6, caspase-9, poly (ADP-ribose) polymerase (PARP), Bad, Bax, Bid and Cox4 were obtained from Cell Signaling Technology. Antibodies against glyceraldehydes-3-phosphate dehydrogenase, anti-mouse IgG-horseradish peroxidase, and anti-rabbit IgG-horseradish peroxidase were purchased from KangCheng Biotechnology. All tissue culture supplies were purchased from Life Technologies. Other routine laboratory reagents were obtained from Whiga Biotechnology of analytical or high-performance liquid chromatography grade. Supramolecular complexes dicycloplatin was obtained from Beijing Xing DA Science Systems, Inc (Figure 1A).

**Cell Lines and Cell Culture**

The following cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO2, respectively, which contain 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. The human hepatoma cell lines HepG2 and BEL-7402 were obtained from School of Biomedical Sciences, The Chinese University of HongKong [31] and Institute of Medicinal Biotechnology, Chinese Academy of Medical Science and Peking Union Medical College [32], respectively. The human lung cancer cell lines A549 and H160 were obtained from Cancer Hospital Chinese Academy of Medical Science [33] and GuangZhou Institute of Respiratory Disease [34], respectively.

**Cell Viability Assay**

MTT assay measures the activity of mitochondrial dehydrogenase enzymes basing on its ability of cleaving tetrazolium ring to produce formazan; therefore, the assay can be used as an index of cell viability. Cells were harvested during logarithmic growth phase and seeded in 96-well plates at a density of 3.5×104/well. After 24 h incubation, 10 μL dicycloplatin full-range concentration was added to 96-well plates. After 60 h treatment, 20 μL MTT (20 mg/mL stock solution of saline) was added to each well for 4 h. Subsequently, the supernatant was removed, and MTT crystals were solubilized...
Dicyclopatin Inhibited Cancer Cell Growth

**Figure A**

- FL1 Log
- FL3 Log
- Control
- 60 μmol/L
- 120 μmol/L

**Figure B**

- FL1 Log
- FL3 Log
- Control
- 50 μmol/L
- 100 μmol/L

Bar graphs show:

- **Δψ m (% of control)**
  - Dicyclopatin (μmol/L)
  - 60 μmol/L
  - 120 μmol/L
  - **Significant difference**

- **Δψ m (% of control)**
  - Carboplatin (μmol/L)
  - 50 μmol/L
  - 100 μmol/L
  - **Significant difference**

**Legend**
- **control**
- **60**
- **120**
- **50**
- **100**

Significance indicated by ****: p < 0.01
with 200 μL anhydrous DMSO in each well. Thereafter, cell viability was measured with a model 550 microplate reader (Bio-Rad) at 540 nm, with 655 nm as reference filter [35]. The 50% inhibitory concentration (IC50) was determined as the anticancer drug concentration causing 50% reduction in cell viability and calculated from the cytotoxicity curves (Bliss’ software). Cell percent survival was calculated using the following formula: survival (%) = [mean experimental absorbance]/[mean control absorbance] × 100%.

Annexin V/Propidium Iodide Double-Staining Assay
Annexin V and propidium iodide (PI) staining was done using ApoNexin FITC Apoptosis Detection Kit. Cells (6 × 10^5) were seeded in 25 cm^2 flasks and allowed to attach for 24 h. After a 48-h treatment with the desired concentration dicycloplatin, both floating and attached cells were collected, washed with ice-cold PBS twice, and resuspended in 200 μL 1× binding buffer containing Annexin V (1:50 according to the manufacturer’s instruction) and 40 ng/sample PI for 15 min at 37°C in the dark [36]. Then, the number of viable, apoptotic, and necrotic cells was quantified by flow cytometry (Becton Dickinson) and analyzed by the CellQuest software. Cells were excited at 488 nm and the emissions of Annexin V at 525 nm and PI were collected through 610 nm band-pass filters. At least 10,000 cells were analyzed for each sample. Percent apoptosis (%) = [number of apoptotic cells]/[number of total cells observed] × 100%.

Determination of Mitochondrial Membrane Potential
Mitochondrial membrane potential (ΔΨm) was measured by flow cytometry with the mitochondrial tracking fluorescent dye 5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolocarbocyanine iodide [JC-1]. JC-1 was a cell-penetrating lipophilic cationic fluorochrome. Cells containing forming J-aggregates have high ΔΨm, and show green fluorescence (FL3). Cells with low ΔΨm are those in which JC-1 maintains (or reacquires) monomeric form, and show red fluorescence (FL1). Depolarization of ΔΨm was measured by JC-1, which accumulates in mitochondrial matrix, driven by ΔΨm, and expressed as an increase of green to red fluorescence ratio reflecting the transformation of JC-1 aggregates into monomers when mitochondrial membrane becomes depolarized. Bottom, ΔΨm levels of dicycloplatin- and carboplatin-treated HepG2 cells, expressed as units of mean fluorescence intensity, were calculated as percentage of control. Data are mean±SD of three independent experiments (**, P<0.01).

Whole-Cell Lysates and Western Blot Analysis
After HepG2 cells were exposed to 30 to 120 μmol/L dicycloplatin for 48 h, respectively [preincubated with Z-IETD-FMK (a caspase-8 inhibitor) or Z-LETD-FMK (a caspase-9 inhibitor)], whole cells were harvested and washed twice with ice-cold PBS, and the pellet was vortexed and 1× lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10% glycerol, 2% SDS, 0.25% bromophenol blue, and 0.1 mol/L DTT] was added for 100 μL/×10^6 cells. After heated at 95°C for 20 min, the lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected [30]. The protein concentration was determined by the BCA assay (Pierce).

Subcellular Fractionation for Western Blot Analysis of Cytosolic Cytochrome c
After HepG2 cells were exposed to 30 to 120 μmol/L dicycloplatin for 48 h, whole cells were harvested by centrifugation at 1,000 rpm for 5 min. The pellets were washed twice with ice-cold PBS, suspended with 5-fold volume ice-cold cell extract buffer [20 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES-KOH; pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.02 mmol/L aprotinin] and incubated for 40 min at 4°C.
Then, the cells were centrifuged at 1,200 rpm for 10 min at 4°C and the final supernatant was used as cytosolic fraction of cytochrome c. Then, 5×loading buffer [250 mmol/L Tris-HCl (pH6.8), 50% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromphenol blue, and 5% (w/v) DTT] was added to the above-obtained supernatant and the mixture was boiled at 100°C for 15 min. Thus, the protein solution was used for identification of cytosolic cytochrome c by Western blot with 15% SDS-PAGE and blotting onto polyvinylidene difluoride membrane. The cytochrome c protein was detected by using anti-cytochrome c antibody in the ratio of 1:1,000 [39].

Statistical Analysis

Results were done by t-test or one-way ANOVA with SPSS 13.0 software. Data was presented as mean±SD of at least triplicate determinations. *, P<0.05 was indicative of significant difference and **, P<0.01 was indicative of very significant difference.

Results

Dicycloplatin Inhibited Proliferation in a Variety of Tumor Cells

The inhibition of cancer cell proliferation by dicycloplatin was examined by MTT assay. As shown in Figure 1, dicycloplatin inhibited cell proliferation in a concentration-dependent manner, and the IC50 of dicycloplatin was 61.30±6.33 μmol/L, 89.80±6.14 μmol/L, 41.69±4.32 μmol/L and 20.25±3.43 μmol/L in HepG2, A549, BEL-7402 and H460 cells, respectively. Comparable IC50 values were also obtained for carboplatin in these cancer cell lines (Figure 1): Carboplatin’s IC50 was 48.01±2.45 μmol/L, 83.20±2.38 μmol/L, 30.27±3.18 μmol/L and 20.44±1.98 μmol/L, respectively.

Induction of Apoptosis by Dicycloplatin

To assess apoptosis induced by dicycloplatin in cancer cells, we detected the exposure of phosphatidylserine on the cell surface,

Figure 5. Involvement of mitochondrial pathway and death receptor pathway in dicycloplatin-induced apoptosis in HepG2 cells.

After HepG2 cells were treated with 30 to 120 μmol/L dicycloplatin or 25 to 100 μmol/L carboplatin for 48 h, respectively, the mitochondria and cytosolic fractions or the whole-cell lysates were assayed by Western blot analysis with the corresponding antibodies. A, mitochondrial cytochrome c release into cytosol and the cytochrome c in mitochondria. Cox-4 detection was used to confirm that the complete separation of cytochrome c in the mitochondrial and cytosolic fractions. B, activations of caspase-9 and caspase-3 and the cleavage of PARP in dose-dependent manner were observed. In addition, both dicycloplatin and carboplatin could down-regulate the expression of Bcl-2 and up-regulate that of Bad but did not influence the level of Bax. Moreover, activations of caspase-8 and decrease of native Bid were also observed.

doi:10.1371/journal.pone.0048994.g005

Figure 6. Impairment of dicycloplatin-induced PARP cleavage by caspase 8 and caspase 3 inhibition.

HepG2 cells were treated with dicycloplatin alone or pretreated with Z-IETD-FMK (60 μmol/L) or Z-LETD-FMK (60 μmol/L) for 24 h and then incubated with dicycloplatin (60 μmol/L) for 48 h. Glyceraldehyde-3-phosphate dehydrogenase detection was used to confirm equal protein loading. Both Z-IETD-FMK and Z-LETD-FMK could reduce PARP cleavage, whereas Bid cleavage was inhibited by Z-IETD-FMK only.

doi:10.1371/journal.pone.0048994.g006
Dicycloplatin Inhibited Cancer Cell Growth

A

B

C

Annexin V

Percent apoptosis (%)
Dicycloplatin Inhibited Cancer Cell Growth

which is known to be an early event in the initiation of apoptosis, by using ApopNexin FITC Apoptosis Detection Kit. As shown in Figure 2A, the apoptotic cell population increased gradually with concentration in dicycloplatin-treated (30, 60, 120 and 240 µmol/L) HepG2 cells. The percentage of early apoptosis in the untreated cell was 1.73±0.41%, which was increased to 6.29±1.58%, 29.63±1.11%, 33.07±1.1% and 39.60±3.9% after a 48-h treatment with dicycloplatin at 30, 60, 120 and 240 µmol/L, for 48 h, respectively (Figure 2B). Similar results were observed in carboplatin-treated HepG2 cells over a range of different concentrations (25, 50, 100 and 200 µmol/L) (Figure 2).

Dicycloplatin Upregulated the Expression of p53
Dicycloplatin is a novel supramolecular platinum chemotherapeutic drug which was formed by one molecule of carboplatin and one molecule of 1,1-ring succinic acid interacting intramolecularly via hydrogen bonding. Since platinum-based anticancer drugs, such as cisplatin and carboplatin, are to DNA-damaging agents, they were known to induce apoptosis partially by affecting p53 expression in cancer cells. As shown in Figure 3, dicycloplatin, like carboplatin, also upregulated the expression of p53 in a concentration-dependent manner.

Change of Mitochondria Membrane Potential (ΔΨm) Induced by Dicycloplatin
Imbalance of mitochondrial membrane potential (ΔΨm) is a commonly reported phenomenon of the early stage of the apoptosis. To explore whether dicycloplatin changed ΔΨm, JC-1 was used for its detection. As shown in Figure 4A, a decline of ΔΨm was observed in dicycloplatin-treated HepG2 cells in a concentration-dependent manner. ΔΨm was decreased from 70.0±5.51% in untreated cells to 36.4±6.6% and 20.2±4.9% after treatment with 60 µmol/L and 120 µmol/L of dicycloplatin, respectively. As shown in Figure 4B, similar results were obtained for carboplatin in HepG2 cells.

Apoptosis Induced by Dicycloplatin was Dependent on Death Receptor Pathway and Mitochondrial Pathway
Mitochondrial pathway is one of the major apoptosis pathways, in which cytochrome c is the limiting factor. Mitochondrial dysfunction has been suggested to cause the release of cytochrome c. Moreover, increasing evidence suggested that Bcl-2 protein family forms a link between death receptor pathway and mitochondrial apoptotic pathway [40]. The levels of cytochrome c in mitochondria and cytoplasm, caspases, and other apoptotic related proteins were examined by Western blot analysis in HepG2 cells after treatment with different concentration of dicycloplatin (30, 60 and 120 µmol/L). As shown in Figure 5A, cytochrome c was decreased in mitochondrial and increased in cytosolic fraction in HepG2 cells after dicycloplatin treatment. Furthermore, as shown in Figure 5B, the activation of caspase-9 and caspase-3 and the cleavage of PARP were also observed in a dose-dependent manner. It is noteworthy that upregulation of Bad and downregulation of Bcl-2 were observed, but Bax expression was not altered. Additionally, activation of caspase-8 and cleavage of Bid (decrease of native Bid) were also observed. These data implied that both mitochondrial pathway and death receptor pathway were involved in the apoptotic process induced by dicycloplatin in HepG2 cells, which is similar to carboplatin (Figure 5).

Cleavage of Bid Induced by Dicycloplatin were Inhibited by Caspase-8 Inhibitor
It is increasingly believed that the cleavage form of Bid (tBid) caused by caspase-8 can result in the release of cytochrome c from mitochondria into cytosol, and subsequently activating caspase-9. Caspase-8 inhibition study was thus carried out to clarify whether the activation of caspase-9 induced by dicycloplatin is the downstream event of caspase-8 activation. A caspase-8 inhibitor (Z-IETD-FMK, 60 µmol/L) was used to pretreat HepG2 cells for 24 h, and then the cells were treated with 60 µmol/L dicycloplatin for 48 h. Whole-cell lysates were examined by Western blot analysis for the status of the caspase-9 and caspase-8 cascade with the corresponding antibodies. As shown in Figure 6, suppression of caspase-8 activity with Z-IETD-FMK was found to inhibit cleavage of Bid and PARP induced by dicycloplatin. On the other hand, a much less significant inhibition of Bid and PARP cleavage after dicycloplatin was observed when caspase-9 was suppressed by Z-LETD-FMK.

Apoptosis Induced by Dicycloplatin was Dependent on ROS
It is known that ROS plays a certain role in apoptosis induced by some anticancer drugs. In our studies, excessive generation of ROS was observed in dicycloplatin- and carboplatin-treated HepG2 cells (Figure 7A & B). To explore whether apoptosis induced by dicycloplatin was dependent on ROS, GSH (an antioxidant, 5 mmol/L) was used to pretreat HepG2 cells for 1 h, before the subsequent treatment with dicycloplatin at different concentrations (60 to 120 µmol/L) for 48 h. As shown in Figure 7C, the percentage of apoptosis induced by dicycloplatin was remarkably inhibited by GSH. These results indicated that apoptosis induced by dicycloplatin in HepG2 cells was likely dependent on ROS.

Discussion
Cisplatin and carboplatin, the classical platinum-based antitumor drugs, are commonly used as the first-line treatment for a...
number of solid malignancies. However, the clinical application of these drugs is limited by their severe toxicity. Dicycloplatin, a novel platinum chemotherapeutical drug has been reported to exhibit lower toxicity and better anticancer activity compared with cisplatin and carboplatin [13,14,18]. Dicycloplatin is a promising anticancer agent and it has been approved by the SFDA of China. At present, the anticancer mechanism of dicycloplatin is still unclear. This study was aimed to understand the mechanism of action of dicycloplatin and to compare and contrast its mechanism of causing apoptosis versus that of carboplatin.

Our result indicated that dicycloplatin and carboplatin exhibited similar anti-proliferation effect in HepG2 cells with IC50 of 61.30±6.33 μmol/L and 48.01±2.45 μmol/L, respectively (Figure 1C). Moreover, Annexin V/PI double staining assay confirmed that dicycloplatin and carboplatin induced apoptosis in HepG2 cells in a concentration-dependent manner (Figure 2). Taken together, these results indicated that dicycloplatin, like carboplatin, inhibited the proliferation of HepG2 cells via inducing cell apoptosis.

Mitochondrial dysfunctions, such as loss of ΔΨm, permeability transition, and release of cytochrome c from mitochondria into
cytosol are the major causes of apoptosis [41]. In our study, decline of ΔΨm (Figure 4A & B) was detected in dicycloplatin- and carboplatin-treated HepG2 cells. Moreover, excessive emancipation of cytochrome c from mitochondria to cytosol (Figure 5A) and a decrease of cytochrome c in the mitochondria were also observed (Figure 5A). Furthermore, caspase-8, caspase-3, and the cleavage of PARP were also detected by Western blot analysis (Figure 5B). Bcl-2 family proteins play an important role in regulating the mitochondrial-dependent cells apoptosis [21]. It has been demonstrated that cisplatin and carboplatin could change the expressions of Bcl-2 family proteins when inducing apoptosis [9,42,43]. To this end, the increase of Bad and decrease of Bcl-2 were detected in dicycloplatin-mediated apoptosis (Figure 5B). These results indicated that mitochondrial pathway played a role in apoptosis process induced by dicycloplatin and carboplatin in HepG2 cells.

Fas is the most typical death receptor in death receptor pathway, whose binding with FasL can lead to caspase-8 activation. Activated caspase-8 can stimulate apoptosis by directly activating downstream caspase-3 [29] and/or by cleaving Bid [30]. Once Bid is cleaved by caspase-8, the cleavage of Bid (tBid) translocates to the mitochondria from the cytosol and cause mitochondrial dysfunction then promotes the release of cytochrome c from mitochondria to cytosol. Therefore, it is increasingly believed that Bid represents an important biochemical signal bridging the mitochondrial pathways and the death receptor pathways [44]. In our study, an increase of caspase-8 activation and cleavage of Bid (decrease of native Bid protein) were observed after dicycloplatin treatment (Figure 5B). Furthermore, caspase-8 inhibitor Z-IETD-FMK obviously decreased tBid formation (Figure 6). These results suggested that apoptosis induced by dicycloplatin was ROS dependent.

In conclusion, our data suggested that dicycloplatin could induce apoptosis in HepG2 cells via both mitochondrial pathway and death receptor pathway participated in apoptosis process. Moreover, these mechanisms were similar to carboplatin. Furthermore, the mitochondrial pathway was dependent on generation of ROS.

Author Contributions

Conceived and designed the experiments: LwF WqC. Performed the experiments: GqL XqZ YjL Jdx XqC. Analyzed the data: XpW. Contributed reagents/materials/analysis tools: GqL W. Wrote the paper: GqL LwF.

References

1. Giaccone G (2000) Clinical perspectives on platinum resistance. Drugs 59 Suppl 4: 9-17; discussion 37-18.
2. Mese H, Sasaki A, Nakayama S, Alcalde RE, Matsunuma T (2000) The role of caspase family protease, caspase-3 on cisplatin-induced apoptosis in cisplatin-resistant A431 cells. Cancer Chemother Pharmacol 46: 241–245.
3. Blanc C, Deveraux QL, Krajevski S, Janicec RU, Portal AG, et al. (2000) Caspase-3 is essential for pro-caspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 60: 4896–4898.
4. Kuwahara D, Tsunumi K, Kobayashi T, Hasumumi T, Nishihaka K (2000) Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. Cancer Lett 148: 65–71.
5. Seki K, Yoshikawa H, Shiki K, Hammad Y, Akamatsu N, et al. (2000) Cisplatin (CDPP, specifically induces apoptosis via sequential activation of caspase-8, -3 and -6 in osteosarcoma. Cancer Chemother Pharmacol 45: 199–206.
6. Lee CS, Kim YJ, Jang ER, Myung SC, Kim W (2010) Akt inhibitor enhances apoptosis effect of carboplatin on human epithelial ovarian carcinoma cell lines. Eur J Pharmacol 632: 7–13.
7. Floros K, Thomadaki H, Katsaros N, Taleri M, Scordilis A (2004) mRNA expression analysis of a variety of apoptosis-related genes, including the novel gene of the BCL2-family, BCL2L12, in HL-60 leukemia cells after treatment with carboplatin and doxorubicin. Biol Chem 385: 1099–1103.
8. Mishima K, Nariai Y, Yoshimura Y (2003) Carboplatin induces Fas (APO-1/CD95)-dependent apoptosis of human tongue carcinoma cells: sensitization for apoptosis by upregulation of FADD expression. Int J Cancer 105: 593–600.
9. Isho M, Chiba H, Noutomi T, Takada E, Mizuguchi J (2000) Cleavage of Bax-alpha and Bcl-x(L) during carboplatin-mediated apoptosis in squamous carcinoma cell line. Oral Oncol 36: 277–285.
10. Barabas K, Mikher R, Luzie D, Adin C (2000) Cisplatin: a review of toxicities and therapeutic applications. Vet Comp Oncol 6: 1–18.
11. Piccart MJ, Lamb H, Vermeerken JB (2001) Current and future potential roles of the platinum drugs in the treatment of ovarian cancer. Ann Oncol 12: 1195–1203.
12. Yang XQJX, Song QH, Tang KL, Yang ZY, Zhang XF, et al. (2010) Structural relationship and mechanisms involved. Int J Oncol 23: 1661–1667.
13. WANG Chun YR (2005) Assay of the lung cancer cell apoptosis induced by dicycloplatin. Practical Oncology Journal 19: 21–22.
14. YAN DTL, PENG X, Li W, SHEN Z (2007) In Vitro and In Vivo Antitumour Activity of Dicycloplatin, A New Active Platinum Complex. Chin JMAP 24: 352–356.
15. YAN DTL, ZHENG X, et al. (2009) Apoptosis Induced by Dicycloplatin in Human Ovarian Cell Line A2780. Journal of Oncology 15: 667–670.
16. PENG JWH, GUAN Z (2009) Phase II Clinical Study of naive Treatment in Patients with Advanced Non-Small-cell Lung Cancer by using Dicycloplatin plus Paclitaxel and Carboplatin plus Paclitaxel. Chinese journal of Clinical Oncology 12: 711–714.
17. Kaufmann SH, Eashwar WC (2000) Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256: 42–49.
18. Herr I, Debaaih KM (2001) Cellular stress response and apoptosis in cancer therapy. Blood 98: 2603–2614.
19. Desaiger S, Olsen-Sand A, Nichols A, Ersk R, Montesuhti S, et al. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 144: 891–901.
20. Tsujimoto Y (2005) Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 195: 158–167.
21. Hu S, Zhao H, Al-Humadi NY, Yin XJ, Ma JK (2006) Silica-induced apoptosis in alveolar macrophages: evidence of in vivo thiol depletion and the activation of mitochondrial pathway. J Toxicol Environ Health A 69: 1261–1284.
22. Pae HO, Oh GS, Choi BM, Seo EA, Oh H, et al. (2003) Induction of apoptosis by f-acetyl-12,13-epoxy-9-trichloretone-3,13-diol from Iraia japonica Yasutila through intracellular reactive oxygen species formation and caspase-3 activation in human leukemia HL-60 cells. Toxicol In Vitro 17: 49–57.
23. Zuzic J, Gedeonova AI (2006) Serum deprivation-induced HepG2 cell death is potentiated by CYP2E1. Free Radic Biol Med 40: 63–74.
24. Hamseine SE, Begum R, Ramaiah KV, Saldev S, Shajil EM, et al. (2003) Host-pathogen interactions during apoptosis. J Biori 23: 69–70.
25. Hou DX, Ose T, Lin S, Harazoto K, Inumura I, et al. (2003) Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: structure-activity relationship and mechanisms involved. Int J Oncol 23: 705–712.
26. Pelli N, Floreani A, Torre F, Delfino A, Baragiotta A, et al. (2007) Soluble FasL induces apoptosis in human leukemic cells through a novel, C. Clin Exp Immunol 148: 83–89.
29. Yang BF, Xiao C, Li H, Yang SJ (2007) Resistance to Fas-mediated apoptosis in malignant tumours is rescued by KN-93 and cisplatin via downregulation of c-FLIP expression and phosphorylation. Clin Exp Pharmacol Physiol 34: 1245–1251.

30. Blomgran R, Zheng L, Stendahl O (2007) Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. J Leukoc Biol 81: 1213–1223.

31. Tang PM, Zhang DM, Xuan NH, Tsui SK, Waye MM, et al. (2009) Photodynamic therapy inhibits P-glycoprotein mediated multidrug resistance via JNK activation in human hepatocellular carcinoma using the photosensitizer phorbisib a. Mol Cancer 8: 56.

32. Li XQ, Shang BY, Wang DC, Zhang SH, Wu SY, et al. Endostar, a modified recombinant human endostatin, exerts synergistic effects with dexamethasone on angiogenesis and hepatoma growth. Cancer Lett 301: 212–220.

33. Zhang S, Shao K, Zhang CY, Zhou F, Wang W, et al. (2005) Analysis of suppressive role of RASSF1A gene at 3p21.3 in lung cancer cell line A549. Zhonghua Yi Xue Za Zhi 85: 908–911.

34. Wang T, Xu J, Zhong NS (2005) Relationship between the acquired multi-drug resistance of human large cell lung cancer cell line NCI-H460 by cisplatin selection and p53 mutation]. Zhonghua Jie He Hu Xi Za Zhi 28: 102–107.

35. Yan Y, Su X, Liang Y, Zhang J, Shi C, et al. (2008) Emodin azide methyl anthraquinone derivative triggers mitochondrial cell apoptosis involving in caspase-8-mediated Bid cleavage. Mol Cancer Ther 7: 1688–1697.

36. Sewell JM, Mayer I, Langdon SP, Smyth JF, Jodrell DL, et al. (2005) The mechanism of action of Kahalalide F: variable cell permeability in human hepatoma cell lines. Eur J Cancer 41: 1657–1664.

37. Zhang JY, Wu HY, Xia SK, Liang YJ, Yan YY, et al. (2007) Anthracenedione derivative 1403P-3 induces apoptosis in KB and KBv200 cells via reactive oxygen species-independent mitochondrial pathway and death receptor pathway. Cancer Biol Ther 6: 1415–1421.

38. Rubio S, Quintana J, Lopez M, Eiroa Jls, Triana J, et al. (2006) Phenylbenzopyrones structure-activity studies identify betulatetal derivatives as potential antitumoral agents. Eur J Pharmacol 548: 9–20.

39. Lin S, Fuji M, Hou DX (2003) Rhein induces apoptosis in HL-60 cells via reactive oxygen species-independent mitochondrial death pathway. Arch Biochem Biophys 418: 99–107.

40. Lavrik IN, Golea A, Baumann S, Krammer PH (2006) Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. Blood 108: 559–565.

41. Wang Y, Perchellet EM, Tamura M, Hua DH, Perchellet JP (2002) Induction of poly(ADP-ribose) polymerase-1 cleavage by antitumor triptycene bisquinones in wild-type and daunorubicin-resistant HL-60 cell lines. Cancer Lett 168: 73–83.

42. Del Belo R, Valentini MA, Zumino F, Comporti M, Madraro E (2003) Cleavage of Bcl-2 in oxidant- and cisplatin-induced apoptosis of human melanoma cells. Oncogene 20: 4591–4595.

43. Singh S, Chhipa RR, Vijayakumar MV, Bhat MK (2006) DNA damaging drug-induced down-regulation of Bcl-2 is essential for induction of apoptosis in high-risk HPV-positive HEp-2 and KB cells. Cancer Lett 236: 213–221.

44. Yin XM (2006) Bcl-2, a BH3-only multi-functional molecule, is at the cross road of life and death. Gene 369: 7–19.

45. Xia Z, Landgren B, Bergstrand A, DePierre JW, Nussberger L (1999) Changes in the generation of reactive oxygen species and in mitochondrial membrane potential during apoptosis induced by the antidepressants imipramine, clomipramine, and citalopram and the effects on these changes by Bcl-2 and Bcl-X(L). Biochem Pharmacol 57: 1199–1208.

46. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12: 913–922.

47. Zhang R, Humphreys I, Sahu RP, Shi Y, Srivastava SK (2008) In vitro and in vivo induction of apoptosis by capsaicin in pancreatic cancer cells is mediated through ROS generation and mitochondrial death pathway. Apoptosis 13: 1463–1478.