Clitocine Reversal of P-Glycoprotein Associated Multi-Drug Resistance through Down-Regulation of Transcription Factor NF-κB in R-HepG2 Cell Line

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Abstract

Multidrug resistance (MDR) is one of the major reasons for failure in cancer chemotherapy and its suppression may increase the efficacy of therapy. The human multidrug resistance 1 (MDR1) gene encodes the plasma membrane P-glycoprotein (P-gp) that pumps various anti-cancer agents out of the cancer cell. R-HepG2 and MES-SA/Dx5 cells are doxorubicin induced P-gp over-expressed MDR sublines of human hepatocellular carcinoma HepG2 cells and human uterine carcinoma MES-SA cells respectively. Herein, we observed that clitocine, a natural compound extracted from *Leucopaxillus giganteus*, presented similar cytotoxicity in multidrug resistant cell lines compared with their parental cell lines and significantly suppressed the expression of P-gp in R-HepG2 and MES-SA/Dx5 cells. Further study showed that the clitocine increased the sensitivity and intracellular accumulation of doxorubicin in R-HepG2 cells accompanying down-regulated MDR1 mRNA level and promoter activity, indicating the reversal effect of MDR by clitocine. A 5′-serial truncation analysis of the MDR1 promoter defined a region from position −450 to −193 to be critical for clitocine suppression of MDR1. Mutation of a consensus NF-κB binding site in the defined region and overexpression of NF-κB p65 could offset the suppression effect of clitocine on MDR1 promoter. By immunohistochemistry, clitocine was confirmed to suppress the protein levels of both P-gp and NF-κB p65 in R-HepG2 cells and tumors. Clitocine also inhibited the expression of NF-κB p65 in MES-SA/Dx5. More importantly, clitocine could suppress the NF-κB activation even in presence of doxorubicin. Taken together, our results suggested that clitocine could reverse P-gp associated MDR via down-regulation of NF-κB.

Introduction

Cancer cells can develop resistance against structurally and mechanistically unrelated chemotherapeutic agents, a phenomenon named as multidrug resistance (MDR) [1]. Although numerous resistant mechanisms are known, a large number of evidence strongly supports the important role of energy-dependent efflux systems (e.g., P-glycoprotein (P-gp)) that pump anti-cancer agents out of the cells [2]. P-gp is a 170 kDa protein that belongs to the ATP-binding cassette (ABC) superfamily of membrane transporter proteins and is encoded by the MDR1 gene [3,4].

Since P-gp was first identified three decades ago, its structure and function have been extensively characterized. However, time has clearly demonstrated that P-gp induced multidrug resistance is much more complex than initially supposed. Until recently, scientists have just got the high resolution x-ray structure of mouse P-gp but not human’s [5]. Efforts to circumvent the P-gp associated MDR in clinic mainly focus on the use of modulators which block the P-gp mediated efflux of anticancer agents. In general, P-gp can be inhibited by: i) blocking the drug-binding site(s) either competitively, non-competitively or allosterically; ii) interfering ATP hydrolysis and iii) altering the integrity of cell membrane lipids [6]. A large number of chemical agents including calcium blocker, calmodulin inhibitors, coronary vasodilators, indole alkaloids, quinolines, hormones, cyclosporins, surfactants and antibodies are modulators which could reverse the pump function of P-gp [7]. For example, the calcium channel blocker verapamil has been well-known to reverse multidrug resistance by directly binding to P-gp protein on special sites [8,9]. Cyclosporine such as cyclosporin A could modulate the efflux function of P-gp by interfering with both the substrate-binding sites and the ATP hydrolysis cycle [10]. However, most of these agents necessitated high doses and produced unacceptable toxicity because of their low affinity with P-gp. Although new generation of P-gp inhibitors with high affinity at very low doses, such as elacridar (GF120918), tarirudar (XR9576) and OC144-093 (ONT-093) have been developed, novel approaches in overcoming P-gp associated MDR are still needed.

P-gp is highly regulated, especially at the transcriptional level. This indicates a promising approach in blocking of MDR, by
inactivation of P-gp expression than by blocking its function. Although the mechanism for transcription regulation of MDR1 is still not fully understood, a large number of transcription factors such as Ras [11], Sp1 [12], p53 [13], NF-kB [14] and PKC [15] were reported to be involved. Some extracellular stimuli such as heat shock and chemotherapeutic agents could induce mdr1 mRNA expression [16,17]. Fujita, T. et al indicated that paclitaxel induced nuclear translocation of YB-1 followed by increased expression of MDR1 in MCF-7 cells [18]. Recently, it is reported that DNA methylation and histone acetylation may be involved in the MDR1 regulation by changing chromatin configuration [19,20]. For example, histone deacetylase (HDAC) inhibitors treatment leads to an increase in MDR1 expression through dynamic changes in chromatin structure and transcription factor association within the promoter region [21].

Nature compounds are gaining increasing interest in cancer therapy. Some agents extracted from fruits, vegetables, oilseeds, and plant herbs were able to modulate the activity of P-gp [22,23,24]. Clitocine, a nucleoside firstly isolated from the mushroom Clitocybe inversa in 1980s, was found to be a substrate and inhibitor of adenosine kinase [25]. Recent years, clitocine was reported to exert an anti-tumor effect in varies cancer cell lines such as Hela and MCF-7 cells, and many derivatives of this compound were also synthesized and examined for their biological properties [25,26,27,28]. In the present study, we found that clitocine reverse the P-gp associated multidrug resistance in cancer cells. Furthermore, clitocine inactivates MDR1 expression through down-regulation of NF-κB as demonstrated in vitro and vivo.

Materials and Methods

Drugs and antibodies

Clitocine was extracted from Lecapaxillus giganteus and its chemical structure was presented in Fig. 1. Its' formula is CaH12N2O6 and molecular weight is 287. The purity of compound used in this study was >99%. Additionally, we also provided HPLC data (Figure S1) and NMR data (Table S1) of clitocine.

Doxorubicin (Dox) was purchased from Sigma Chemical Co. (St. Louis, MO), Bay 11-7082 was from Calbiochem (San Diego, CA). The antibodies mouse monoclonal anti-P-gp, rabbit polyclonal anti-NF-κB p65 (REL) were cloned by PCR using total cDNA synthesized from R-HepG2 cells as template, with the forward primers: 5’- CCCAGCTTTAGGAAGACTTTCCCC -3’and the reverse primer 5’- CCAGCTGATTTAGAGCTGATCTGACTC- 3’. The PCR products were digested with HindIII and XhoI (sites underlined in the primers) and subcloned into pGL3 [lac+] Basic Vector (Promega). The open reading frame sequence of human NF-κB p65 (REL) was cloned by PCR using total cDNA synthesized from R-HepG2 cells as template, with the forward primers: 5’- CCCAGCTTTAGGAAGACTTTCCCC -3’and the reverse primer 5’- CCAGCTGATTTAGAGCTGATCTGACTC- 3’. The PCR products were digested with HindIII and XhoI (sites underlined in the primers) and subcloned into pcDNA3.1. The putative NF-κB binding site on pGL3 (~988/+525) was mutated by site-directed mutagenesis using Quikchange II site-directed kit (Stratagene, La Jolla, CA) with forward primer 5’-TAAATGCGAATCCCGAGAAAAAATTTCCTATC-3’ and reverse primer 5’-GGACGAGTCTTGTTTCTCGAATTTTGG-3’, mutated nucleotides were indicated as italic letters. The integrity of the respective plasmid constructs was confirmed by DNA sequencing.

Cell culture

Human hepatoma HepG2 and SMMC-7721 cells, human cervical cancer HeLa cells, human gastric cancer SGC-7901 cells, human uterine cancer MES-SA and MES-SA/Dx5 cells, human breast carcinoma MCF-7 and Bcap37 cells were obtained from American Type Culture Collection, were maintained in DMEM medium (Invitrogen, Inc., Carlsbad, CA) supplemented with 5% heat-inactivated 10% CO2 incubator. Doxorubicin-induced multidrug resistant human hepatoma cells R-HepG2 was a kind gift provided by Prof. Kwok-pui Fung from the Chinese University of Hong Kong. To maintain the Dox-resistance, R-HepG2 and MES-SA/Dx5 cells were cultured with 1.2 μM Dox during passages. From time to time, the sensitivity of cells to Dox was analyzed for their resistance to cell death. Cells were confirmed as mycoplasma free using DAPI staining assay.

Drug sensitivity assay

Cell survival after exposure to the anti-tumor agents was examined by MTT cytotoxicity assay. Cells were seeded in each well of a 96-well plate for 24 h. After incubation with various concentrations of doxorubicin, clitocine, or both for 48 h, the medium was discarded. Then, 50 μl of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical) was added to each well for 4 h incubation at 37°C. The purple formazan formed was then solubilized by DMSO and absorbance at 570 nm was read by a
bicin alone or together with 0.2 m were washed and lysed with the passive lysis buffer from the Dual-

normalize transfection efficiency. 24 h after transfection, the cells was used as a negative control. 24 ng of pRL-CMV, which luciferase-reporter vector containing MDR1 promoter sequence was measured by a FACSort flow cytometer (Becton Dickison) and then analyzed with CellQuest software.

Cellular doxorubicin accumulaton assay

Cells were seeded in 35 mm culture dish for 24 h to allow attachment. Then, the cells were incubated with 2 μM doxorubicin alone or together with 0.2 μM clotocine for 24 h. After being washed twice with PBS, the cells were measured by a FACSort

flow cytometer (Becton Dickison) and then analyzed with phenol/chloroform extraction. Immunocomplexes were collected using Protein A Agarose/ Salmon Sperm DNA for 1 h at 4°C. Following the wash, the immunocomplexes were recovered by resuspending in elution buffer at room temperature for 15 min. DNA-protein complexes as well as the input DNA were reverse cross-linked at 65°C for 4 h and treated with proteinase K at 45°C for 1 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Thereafter, the DNA was subjected to PCR with primers: forward, 5'-TGCTGAGAGAAGACCCACTGC-3' and reverse, 5'-GCTTCGCTGACACCTCTCT-3'. Amplification was carried out for 35 cycles with denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 70°C for 40 s. PCR products were analyzed on a 1.5% agarose gel.

Western blot analysis

After incubation with clotocine for 48 h, the cells were lysed in lysis buffer [2.1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 4.9 mM MgCl2, 1 mM orthovanadate, 1% Triton X 100, and 1 mM phenylmethylsulfonyl fluoride]. Proteins in cell lysate were resolved in denaturing SDS-PAGE gel and transferred to Immobilon PVDF membrane (Millipore, Billerica, MA). After blocking with 5% nonfat dry milk, the membranes were washed by PBS containing 0.1% Tween 20 and incubated with primary antibodies followed by respective horseradish peroxidase conjugated secondary antibodies. Signals were visualized with enhanced ECL chemiluminescence detection reagents (Amersham Life Sciences, Inc., Buckinghamshire, United Kingdom) and visualized on X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometric analysis of the protein bands was done using Image J (NIH). β-actin was used as reference to determine the protein relative level.

Quantitative RT-PCR analysis

Total RNA was extracted by lysing cells with TRI reagent (Molecular Research Center, Cincinnati, OH). Total RNA was used to synthesize the first strand cDNA by M-MLV reverse transcriptase (Promega, Madison, WI). Real-time PCR amplification was performed in ABI 7500 Fast Real-Time PCR system (Applied Bioscience, Foster City, CA) according to manufacturer’s procedure for relative quantification. The amplification reactions were carried out with 1× Power SYBR Green PCR Master Mix (Applied Biosystems). PCR primers for MDR1 (forward, 5'-GGACGTCGGGAGAAGACCAACA-3'; reverse, 5'-CCCCAACATCGTGCACTGC-3'), β-actin (forward, 5'-ACACCCCAACGGCATATGAGTC-3'; reverse, 5'-TCACCGGGAGTCCATGACGAT-3') were designed by using the Primer Express 3.0 (Applied Biosystems). The standard temperature profile included initial denaturing at 95°C for 15 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing and extension at 60°C for 1 min. A DNA dissociation curve was generated to confirm the specificity of amplification. Relative Standard Curve Method (2-ΔΔCt) was used to determine the relative mRNA expression using β-actin as reference. Validation experiment was carried out to ensure that amplification efficiency of the target genes and the reference gene was approximately equal.

Luciferase reporter activity assay

R-HepG2 cells cells were seeded into 24-well plates for 24 h before transfection. The cells were transfected with 1.2 μg of luciferase-reporter vector containing MDR1 promoter sequence using Lipofectamine 2000 (Invitrogen) and pGL3 empty vector was used as a negative control. 24 ng of pRL-CMV, which encoding Renilla luciferase, was included in all transfections to normalize transfection efficiency. 24 h after transfection, the cells were washed and lysed with the passive lysis buffer from the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured in each cell lysate using a FLUOstar Galaxy plate reader.

Chromatin immunoprecipitation (CHIP) assay

ChiP assays were performed using the Chromatin Immuno-precipitation Assay kit based on the manufacturer’s instruction (Upstate Group, Charlottesville, VA). Briefly, cells were fixed in 1% formaldehyde at 37°C for 10 min to cross-link histones to DNA. Cells were washed and detached from the dish by scraping following by addition of SDS lysis buffer. After 10 min incubation on ice, cells were sonicated to shear DNA. After centrifugation at 14,000 rpm for 10 min at 4°C, the sonicated cell supernatants were diluted with ChiP Dilution Buffer and aliquots of samples were saved as the input DNA for quantization of the amount of total DNA. For immunoprecipitation, 1 μg of NF-kB p65 antibodies or normal rabbit IgG was added to the preclared supernatants and incubated overnight at 4°C with rotation. Immunocomplexes were collected using Protein A Agarose/ Salmon Sperm DNA for 1 h at 4°C. Following the wash, the immunocomplexes were recovered by resuspending in elution buffer at room temperature for 15 min. DNA-protein complexes as well as the input DNA were reverse cross-linked at 65°C for 4 h and treated with proteinase K at 45°C for 1 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Thereafter, the DNA was subjected to PCR with primers: forward, 5'-TGCTGAGAGAAGACCCACTGC-3' and reverse, 5'-GCTTCGCTGACACCTCTCT-3'. Amplification was carried out for 35 cycles with denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 70°C for 40 s. PCR products were analyzed on a 1.5% agarose gel.

Electrophoresis Mobility Shift Assay (EMSA) of NF-kB activation

NF-kB activation was analyzed using EMSA with NF-kB probe (AGT TGAGGGGACTTTCCCAGGC) as previously described [29]. Briefly, the Biotin-labeled probes were incubated with nuclear extracts for 60 min at room temperature. For specificity controls, unlabeled probe was added to the reaction at a 1:100 molar excess. Anti-p65 subunit antibody (from Cell signaling) was also included to identify NF-kB-specific DNA binding. DNA-protein complexes were separated by electrophoresis in 5% polyacrylamide gels and signals were visualized with Luminol/ Enhancer Solution detection reagents (thermo pierce) and visualized on X-ray film (Fuji Photo Film, Tokyo, Japan).

Immunocytochemical staining method

R-HepG2 cells were seeded into 24-well plates for 24 h incubation. After incubation with 0.2 μM clotocine for 48 h, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature. 100% ice-cold methanol was then used to permeabilize the cells at −20°C for 10 min. After blocking with 3% BSA in PBS at room temperature for 45 min, the cells were incubated with anti-P-gp and anti-NF-kB p65 antibodies in blocking buffer for 2 h at room temperature and rinsed 3 times in PBS. The primary antibody was detected with goat anti-mouse IgG conjugated with FITC (for P-gp, Santa Cruz, CA) and goat anti rabbit antibody conjugated with RBITC (for NF-kB p65, Santa Cruz, CA). Nuclei were stained with DAPI (Roche). The levels of these proteins were observed with a LSM 510 fluorescence microscope fitted with appropriate filters and images were captured with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ).
Immunohistochemical staining method

R-HepG2 cells (1×10^7 cells/mouse) were inoculated into nude mice and the tumors grew to up to appropriate size (i.e. tumor volume reached 100 mm^3). Tumor bearing animals were divided into the two groups: intravenous injection of vehicle alone (control) or clitocine (10 mg/kg), each day for one week. Each group consisted of 5 animals. After 7 days, tumors were excised and fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (0.1 M PBS, pH 7.4) overnight. The tissues were rinsed three times in PBS, incubated overnight in 0.5 M sucrose in PBS, then embedded in Tissue-Tek® O.C.T. Compound, and cut into 6 μm frozen sections. The sections were washed with TBST for 15 min at room temperature and then blocked in 3% BSA in TBST (20 mM Tris, pH 7.5, 154 mM NaCl, 2 mM EGTA, 2 mM MgCl2, 0.1% Triton X-100) for 1 h. Tissue sections were incubated with anti-P-gp and anti- NF-κB p65 antibodies in blocking buffer (3% BSA, 0.1% azide in TBST) for 1 h, then rinsed 3 times in TBST. The primary antibody was detected with goat anti-mouse IgG conjugated with FITC (for P-gp, Santa Cruz, CA) and goat anti Rabbit conjugated with RBITC (for NF-κB p65, Santa Cruz, CA). Nuclei were stained with DAPI (Roche).

Statistical analysis

In all experiments, data were expressed as mean ± standard deviation (SD). A significant difference of the sample’s value from that of the respective controls in each experiment condition was assessed using Student’s unpaired t-test with p value <0.05 being regarded as statistically significant.

Results

Anti-proliferation effect of clitocine on human cancer cell lines

The anti-proliferation effect of clitocine on human cancer cell lines was assessed by the MTT assay, including HepG2, R-HepG2, MES-SA, MES-SA/Dx5, SMMC7721, Bcap37, MCF-7, HeLa, and SGC-7901 for 48 h. The IC_{50} values were estimated as shown in Table 1. The treatment of the nine human cancer cell lines with clitocine exhibited a marked inhibition on the survival of these cells dose-dependently. Among them, drug resistant cells R-HepG2 and MES-SA/Dx5 showed similar sensitivity compared with their parental cells HepG2 and MES-SA.

Reversal effect of clitocine in drug resistant cancer cells

P-gp was reported to be over-expressed in the R-HepG2 [30] and MES-SA/Dx5 cells which was confirmed as in Fig. 2A. Surprisingly, clitocine can down-regulate the P-gp expression in both cell lines. More interestingly, clotocine can more effectively suppress the P-gp expression in R-HepG2 cells (about more than 50% at 0.2 μM). Although it seemed that higher level of P-gp was expressed in R-HepG2 cells than MES-SA/Dx5 cells.

Additionally, Clitocine could increase doxorubicin toxicity in R-HepG2 and MES-SA/Dx5 cells. As shown in Fig. 2B and D, clotocine exerted toxicity on R-HepG2 and MES-SA/Dx5 cells with IC_{50} of about 0.35 μM and 0.7 μM respectively. To check whether clotocine may affect the sensitivity of drug resistant cancer cells to doxorubicin, the cells were treated with various concentrations of doxorubicin together with 0.05, 0.1, 0.2 or 0.4 μM clotocine respectively. As shown in Fig. 2C and E, co-incubation with ≥0.2 μM clotocine remarkably increased the response of R-HepG2 and MES-SA/Dx5 cells to doxorubicin. The synergistic effect of clotocine and doxorubicin was analyzed using the Chou-Talalay method [31]. As shown in Figure S2, combined treatment of clotocine and doxorubicin presented well as synergism (combination index; CI<1). Furthermore, clotocine also induced a detectable increase in cellular doxorubicin accumulation in R-HepG2 cells (Fig. 2F).

Next we measured the MDR1 mRNA level in R-HepG2 cells with Real-time PCR analysis. As shown in Fig. 2G, the clotocine treatment significantly suppressed the mRNA level of MDR1. Using the dual-luciferase reporter assay, clotocine was also found to effectively down-regulate the activity of reporter with MDR1 promoter (Fig. 2H).

Truncation analysis of MDR1 promoter sequence in relation to clotocine suppression effect

In order to understand how MDR1 is suppressed by clotocine, a series of 5’-end truncated human MDR1 promoter fragments were cloned into the firefly luciferase reporter vector. These constructs were transiently transfected into R-HepG2 cells followed by incubation with 0.2 μM clotocine for 24 h. The luciferase activities were measured by dual reporter luciferase assay. As shown in Fig. 3A, clotocine remarkably decreased (by about 50%) the luciferase activities for the reporters with fragments −998/+525, −732/+525 and −450/+525 but showed no effect for those with fragments −193/+525, +44/+525 and +270/+525. The results indicated that the region between −193 and −450 may be responsible for the clotocine suppression effect on MDR1 promoter activity. Computational analysis for the putative transcription factor binding sites in the full length (−998/+525) MDR1 promoter sequence was performed using MATCH™ 1.0, MatInspector and Transcription Element Search Software (TESS). Interestingly, within the region −450 to −193, only one NF-κB putative binding site (−324 to −315) was identified by all three softwares (Fig. 3B). By mutating the NF-κB putative binding motif (−324 to −315), the inhibitory effect of clotocine on the activity of reporter with full length MDR1 promoter was significantly reversed (Fig. 3C). The results suggest that NF-κB may mediate the clotocine suppression on MDR1. Additionally, the binding of NF-κB to the putative binding site (−324 to −315) within the MDR1 promoter was confirmed by chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 3D, a 277 bp PCR product was amplified from the DNA

| Table 1. IC_{50} of clotocine for nine human cancer cell lines. |
|-----------------|-----------------|
| **Cell lines**  | **IC_{50}(μM)** |
| Cervical cancer | HeLa            | 14.9           |
| Breast cancer   | Bcap37          | 10.9           |
|                 | MCF-7           | 43.0           |
| Liver cancer    | HepG2           | 0.45           |
|                 | R- HepG2        | 0.35           |
| Gastric cancer  | SGC-7901        | 2.2            |
| Uterine cancer  | MES-SA          | 0.62           |
|                 | MES-SA/Dx5      | 0.7            |

*IC_{50} (1×10^4) were incubated with various concentrations of clotocine for 48 h. Cell viability was measured by MTT assay. IC_{50} represents clotocine concentration causing a 50% growth inhibition.

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samples of R-HepG2 cells immunoprecipitated by anti-NF-kB p65 but not in the samples with normal rabbit IgG.

NF-κB mediated the clitocine suppression on MDR1

To elucidate the ways that NF-κB mediates the clitocine suppression on MDR1, the functional role of NF-κB in MDR1 expression was first examined. R-HepG2 cells were treated with NF-κB inhibitor BAY-11-7085 for 48 h followed by Western blot analysis. As shown in Fig. 4A, BAY-11-7085 treatment suppressed the expression of P-gp and also the activity of reporter with full length MDR1 promoter (Fig. 4B), indicating that NF-κB will act as a transcription activator of MDR1.

To confirm whether NF-κB is involved in the suppression of MDR1 by clitocine, the effect of clitocine on NF-κB expression was examined. As shown in Fig. 4C, clotocine treatment could only inhibit the expression of NF-κB p65 but not other NF-κB members such as NF-κB p50. Furthermore, transfection with vector carrying NF-κB p65 increased the level of P-gp and also counteracted the clotocine effect on P-gp in R-HepG2 cells (Fig. 4D). More interestingly, clotocine can also effectively suppress the expression of NF-κB p65 in HepG2, MES-SA and MES-SA/Dx5 cells (Fig. 4E).

Inhibitory effect of clotocine on XIAP expression and NF-κB activation

It was reported that NF-κB was involved in regulation of a large number of genes that control cell proliferation, cell survival and immune responses [32]. At the present, we also examined the important downstream targets of NF-κB such as Inhibitors of apoptosis (IAPs) by Western blot assay. As shown in Fig. 5A, doxorubicin treatment could up-regulate NF-κB level while this effect can be reversed by clotocine; clotocine alone or in combination with doxorubicin significantly down-regulate the expression of NF-κB. Similar result was also observed that doxorubicin activated the XIAP expression while clotocine alone or in combination with doxorubicin suppressed the XIAP expression (Fig. 5A).

It was well known NF-κB may enter into nucleus under multiple stimuli to activate its' target genes [33]. Electrophoresis Mobility Shift Assay (EMSA) was chosen to verify the inhibitory effect of clotocine on NF-κB activation. The data of EMSA indicated that R-HepG2 cells exhibited spontaneously activated NF-κB activity while clotocine could rapidly inactivated NF-κB and its’ nuclear translocation significantly (Fig. 5B). However, doxorubicin treatment induced the activation of NF-κB (Fig. 5B). More interestingly, it looked like that clotocine could effectively overcome the activation of NF-κB resulted by doxorubicin treatment (Fig. 5B) and same trend can be seen in the XIAP expression (Fig. 5A).

Discussion

Development of MDR reflects not only the multiple genetical and epigenetical changes occuring inside the cells under cytotoxic conditions, but also a normal physiological response of cells to struggle for survival. A great number of studies have been carried out over the last 3 decades to understand the pharmacological and toxicological effect of ABC efflux transporters. Among them, the P-gp is an important membrane transporter that has been recognized as the most vital barrier to effective drug delivery and plays a key role in the development of MDR. An attractive strategy to improve the drug delivery and overcome drug resistance is inhibition of the efflux pump P-gp transporter. The aim of this study was to find a more effective MDR-reversing compound and get insight into its underlying molecular mechanism. In the present study, we demonstrated that clotocine, a nucleoside extracted from Leucopaxillus giganteus can circumvent MDR in drug resistant R-HepG2 and MES-SA/Dx5 cells by suppressing the P-gp expression.

R-HepG2 and MES-SA/Dx5 cells showed over-expression of P-gp and clotocine could down-regulate P-gp expression in both cell lines (Fig. 2A). However, it seemed that the P-gp level in R-HepG2 cells was much higher than that in MES-SA/Dx5 cells and clotocine exerted more effective regulatory activity in the former (Fig. 2A). R-HepG2 and MES-SA/Dx5 cells presented much lower sensitivity to doxorubicin compared with parental cells (data not shown). Interestingly, the compound clotocine was found to effectively enhance the anticancer activity of doxorubicin in drug resistant cells at the dose of ≥0.2 μM (Fig. 2C and E). Here we chose R-HepG2 cells for the further research on clotocine’s inhibitory effect in P-gp expression. It was observed clotocine also increased the doxorubicin accumulation in R-HepG2 cells (Fig. 2F), suggesting that the P-gp related pump may be inhibited by clotocine. The hypothesis was supported as P-gp was

Figure 2. Reversal effect of clotocine in drug resistant cancer cells. (A) P-gp was detected in HepG2, R-HepG2, MES-SA and MES-SA/Dx5 cells by Western blot analysis as described in Materials and Methods. (B) After treatment with different concentrations of clotocine in R-HepG2 cells for 48 h, cell viability was determined by MTT assay. Data are mean ± SD, N = 3. (C) R-HepG2 cells were treated with different concentrations of doxorubicin together with 0, 0.05, 0.1, 0.2 or 0.4 μM clotocine respectively for 48 h. Cell viability was determined by MTT assay. Data are mean ± SD, N = 3. (D) R-HepG2 cells were treated with different concentrations of clotocine in MES-SA/Dx5 cells for 48 h, cell viability was determined by MTT assay. Data are mean ± SD, N = 3. (E) MES-SA/Dx5 cells were treated with different concentrations of doxorubicin together with 0, 0.05, 0.1, 0.2 or 0.4 μM clotocine respectively for 48 h. Cell viability was determined by MTT assay. Data are mean ± SD, N = 3. (F) Cellular doxorubicin accumulation level in R-HepG2 cells was measured by flow cytometry. The cells were incubated with 2 μM doxorubicin (Dox) alone or together with 0.2 μM clotocine for 24 h. The amount of doxorubicin accumulated in treated cells was quantified by flow cytometric method. (G) MDR1 mRNA level in R-HepG2 cells after clotocine treatment. After treatment with 0.2 μM clotocine for 48 h, total RNA in R-HepG2 cells was extracted. The MDR1 mRNA level in R-HepG2 cells was then measured by qRT-PCR analysis. Data are mean ± SD, N = 6. (H) The relative luciferase activity of full length MDR1 promoter reporter as determined by Dual-Luciferase Reporter Assay System. Cells were transiently transfected with full length MDR1 promoter reporter followed by treatment with 0.2 μM clotocine for 24 h. After that, the cells were lysed and the luciferase activity was measure. Data are mean ± SD, N = 6. *P<0.05 vs. control, **P<0.01 vs. control.

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NF-κB and P-gp are tightly regulated by clotocine in R-HepG2 cells and in tumor tissues

Animal experiment revealed that clotocine treatment could significantly inhibit the growth of human hepatocellular carcinoma HepG2 and R-HepG2 tumor (data not shown). The effect of clotocine on the expressions of NF-κB and P-gp in R-HepG2 cells in vitro and tumour tissue in vivo was examined by immunohistochemistry. As shown in Fig. 6A, the expressions of NF-κB p65 and P-gp were both suppressed by clotocine, showing a positive relationship between these two proteins in R-HepG2 cells upon clotocine treatment. Similar results were observed in R-HepG2 tumor tissue from nude mice with clotocine treatment (Fig. 6B). The pixel intensity was analyzed with ImageJ (NIH) and the data was normalized with signal of DAPI. The relative value was labeled at the upper right corner of the figures.
Significantly decreased at both mRNA and protein levels in R-
HepG2 cells under clitocine treatment (Fig. 2A and G). Subsequently, clitocine was found to remarkably inhibit the
activity of MDR1 gene promoter, indicating that the effect of
clitocine on MDR1 is at the transcriptional level (Fig. 2H).

The proximal promoter region of the human MDR1 gene was
firstly identified in 1987. The promoter contains a consensus
CAAT box and two GC box-like sequences while lacks many
sequence elements associated with more active cellular and viral
promoter, such as canonical TATA or CCAAT sequences
upstream of initial site [34]. To get more in-depth into the
mechanism for transcriptional regulation of clitocine on MDR1, a
series of 5'-end truncated MDR1 gene promoter constructs were cloned
and their transcriptional activities under the clitocine treatment
were examined. The results suggested that the fragment
2450 to 2193 was indispensable in the suppression effect of clitocine on
MDR1 (Fig. 3A).

By computer-based sequence analysis, a single
consensus binding site (GAAAATTTCC at 2324 to 2315) for
transcription factor NF-kB was identified in the region from
2450 to 2193 (Fig. 3B). Mutation in the NF-kB putative binding motif

Figure 3. Truncation analysis of the MDR1 promoter with clitocine treatment. (A) The relative luciferase activity of 5'-end truncated MDR1
promoters was detected by Dual-Luciferase Reporter Assay System. R-HepG2 cells were transiently transfected with the truncated MDR1 promoter
reporters followed by treatment with 0.2 μM clitocine for 24 h. After that, the cells were lysed and the luciferase activity was measured. Data are
mean ± SD, N=8. (B) Computational analysis of the putative transcription factor binding sites in the full length MDR1 promoter was performed. A
single consensus binding site for transcription factor NF-κB was identified in the region −450 to −193. (C) R-HepG2 cells were transiently transfected
with wild type (WT) and mutated type (MT) full length (−988 to +525) MDR1 promoter reporter followed by treatment with 0.2 μM clitocine in R-
HepG2 cells for 24 h. After that, the cells were lysed and the luciferase activity was measured. Data are mean ± SD, N=4, *P<0.05 vs. control. (D)
Immunoprecipitation (CHIP) assay was performed as described in Materials and Methods. Chromatin from R-HepG2 cells was cross-linked, sheared
and immunoprecipitated with anti-NF-κB p65 antibody. Normal rabbit IgG was included as the negative control (IgG) and the input DNA from fragmented
chromatin before immunoprecipitation was used as internal control. The recovered chromatin was subjected to PCR analysis using primers covering
the putative NF-κB binding motif of the MDR1 promoter and the PCR products were resolved in 1.5% agarose gel. A representative experiment is
shown, and similar results were obtained from three independent experiments.

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(-324 to −315 bp) could significantly reverse the inhibitory effect of clotocine on the activity of reporter with full length MDR1 promoter (Fig. 3C). Furthermore, the interaction of NF-κB with the region was confirmed by chromatin immunoprecipitation (CHIP) assay (Fig. 3D). The results therefore supported that NF-κB may mediate the clotocine suppression on MDR1.

NF-κB is a family of ubiquitous transcription factors which can be activated by a large number of stimuli such as cytokines or DNA damaging agents including chemotherapeutic drugs [33,35]. Normally, NF-κB members are kept inactive in the cytoplasm via binding with inhibitory molecules of IκB family. In response to multiple stimuli, the IκB molecules become phosphorylated on two critical serine residues by IKK; free NF-κB enters into the nucleus and activates transcription of a variety of genes participating in the immune and inflammatory response, cell adhesion, growth control, and protection against apoptosis [36,37,38,39]. NF-κB is the central regulator of immune responses through inducing various genes; some of them act as potential inhibitors of apoptosis especially cellular inhibitors of apoptosis (c-IAPs) [32]. Several studies indicated that NF-κB inhibition could increase cellular

Figure 4. NF-κB mediated the clotocine suppression on MDR1. (A) P-gp in R-HepG2 cells with BAY-11-7085 (10 μM) treatment was assessed by Western blot assay. After treatment with 10 μM BAY-11-7085 for 48 h, P-gp protein level in R-HepG2 cells was measured by Western blot analysis. (B) The relative luciferase activity of reporter carrying the full length MDR1 promoter was determined by Dual-Luciferase Reporter Assay System. R-HepG2 cells were transiently transfected with full length MDR1 promoter reporter followed by treatment with 10 μM BAY-11-7085 for 24 h. After that the cells were lysed and the luciferase activity measured. Data are mean ± SD, N=3. **P<0.01 vs. control. (C) After treatment with 0.2 μM clotocine for 48 h, Western blot assay was performed in R-HepG2 cells. (D) R-HepG2 cells were transiently transfected with pcDNA3-RELA and empty pcDNA3 (negative control) followed by treatment with 0.2 μM clotocine for 48 h. P-gp protein level was measured by western blot analysis. Data are mean ± SD, N=3. (E) After treatment with 0.2 μM clotocine for 48 h, Western blot assay was performed in R-HepG2, HepG2, MES-SA and MES-SA/Dx5 cells to measure the NF-κB p65 level.

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response to cytotoxic agents [40,41,42,43], but this activity was probably dependent on the cell lines, the agents used and the extent of NF-κB suppression [44,45]. The activation of NF-κB signaling could up-regulate MDR1 gene expression in human hepatoma cells [46]. Moreover, NF-κB might directly bind the MDR1 gene promoter and induce drug resistance through MDR1 over-expression in HCT15 colon cancer cells [47]. To confirm the relationship between NF-κB and P-gp expression in R-HepG2 cells, an NF-κB inhibitor, Bay 11-7082 was used. Bay 11-7082 remarkably down-regulated both the P-gp protein level and transcriptional activity of full length MDR1 promoter in R-HepG2 cells (Fig. 4A and B). These results suggested that NF-κB may be involved in the regulation of MDR1 in R-HepG2 cells. Furthermore, NF-κB p65 was down-regulated under clitocine treatment in R-HepG2 cells (Fig. 4C) while no significant decrease was seen for NF-κB p50 (data not show). Interestingly, NF-κB p65

Figure 5. Clitocine inhibits the expression of XIAP and activation of NF-κB in R-HepG2 cells. (A) After treatment with 0.2 μM clitocine, 1.2 μM doxorubicin or combined agents for 48 h, Western blot was performed to assess the expression of NF-κB p65 and its' target mediator XIAP. (B) After treatment with 0.2 μM clitocine, 1.2 μM doxorubicin or combined for 60 min, nuclear protein was extract and analyzed using ESMA with a double-stranded oligo nucleotide probe containing NF-κB consensus sequence. Excess molar ratio of cold probe (lane 6), anti-p65 subunit of NF-κB antibody (lane 7) and IgG (lane 8) were used for the specificity assay.
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Figure 6. Clitocine inhibits the expressions of NF-κB p65 and P-gp in R-HepG2 cells in vitro and in vivo tumor tissue from nude mice. (A) After treatment with 0.2 μM clitocine for 48 h, immunocytochemistry assay was performed in R-HepG2 cells using antibodies: NF-κB p65 and P-gp. (B) After intravenous injection of clitocine (10 mg/kg, once a day for one week), tumors were excised from tumor bearing animals. Tumors were fixed with 4% paraformaldehyde in PBS and cut into 6 μm frozen sections and immunocytochemistry assay was performed. The expression levels of these two proteins were observed with a LSM 510 fluorescence microscope fitted with appropriate filters and the images captured with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ). The pixel intensity was analyzed with Image J. A representative experiment was shown.
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over-expression can reverse the inhibitory effect of clitocine on P-gp (Fig. 4D). The regulatory effect of clitocine on NF-κB p65 expression in other cell lines was also measured by western blot analysis. As shown in Fig. 4E clitocine can also down-regulate the NF-κB p65 expression in other cell lines including MES-SA/Dx5 and their parental HepG2 and MES-SA cells. Furthermore, clitocine could also suppress the expression of NF-κB and its downstream genes such as XIAP even in presence of doxorubicin in RD cells (Fig. 5A). More importantly, clitocine significantly overcame the activation of NF-κB by doxorubicin which was characterized as nuclear translocation of NF-κB (Fig. 5B). By immunocytochemistry assay, the expressions of NF-κB p65 and P-gp were found to be tightly coupled in R-HepG2 cells and both could be suppressed by clitocine in vitro and in vivo (Fig. 5). These data indicated that NF-κB p65 was the target via which clitocine could transcriptionally regulate MDR1 in R-HepG2 and maybe in MES-SA/Dx5 cells.

In conclusion, our data strongly support that clitocine can suppress P-glycoprotein over-expression in doxorubicin induced multidrug resistant R-HepG2 and MES-SA/Dx5 cells via inhibition of NF-κB p65 protein level and it’s activation. Therefore, clitocine could be a potent NF-κB p65 inhibitor for cancer treatment as sensitizers to anticancer drugs [48] and have potential therapeutic application in cancer and inflammatory diseases.

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Supporting Information
Figure S1 The HPLC analysis of clitocine. (TIF)
Figure S2 Synergy of combination of clitocine and doxorubicin in the proliferation of R-HepG2 cells. The MTT data was analyzed by Chou-Talalay method, (combination index >1 indicates antagonism, =1 indicates additivity, and <1 indicates synergy). (TIF)
Table S1 The NMR data of clitocine. (TIF)

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Author Contributions
Conceived and designed the experiments: FL TTK PW KPF. Performed the experiments: JS CAY KL JGYW. Analyzed the data: NNC TYT. Contributed reagents/materials/analysis tools: EY. Wrote the paper: FL TTK.
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