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Cancer Letters 272 (2008) 102–109

CANCER Letters

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Cantharidin reverses multidrug resistance of human hepatoma HepG2/ADM cells via down-regulation of P-glycoprotein expression

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Received 14 May 2008; received in revised form 27 June 2008; accepted 30 June 2008

Abstract

Multidrug resistance (MDR) is a serious obstacle encountered in cancer treatment. In this study, we established an in vitro multiple drug resistant HepG2 cell line (HepG2/ADM), and characterized its MDR. This model was used to screen potential candidate chemosensitisers from over 200 purified naturally occurring compounds extracted from plants and animals. Cantharidin was found to have a significant reversal on MDR in our model. Further, our results showed that Cantharidin could significantly inhibit P-gp (P-glycoprotein) expression, mRNA transcription, as well as MDR1 promoter activity. These results suggest that Cantharidin is a novel and potent MDR reversal agent and may be a potential adjunctive agent for tumor chemotherapy.
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Keywords: Multi drug resistance; P-glycoprotein; Cantharidin

1. Introduction

Drug resistance to chemotherapeutic agents is one of the major obstacles in the treatment of human cancers. After developing resistance to a single drug or a class of drugs, cancer cells show cross-resistance to other functionally and structurally unrelated drugs. This phenomenon known as MDR has a profound effect on successful chemotherapy of cancer [1]. Various mechanisms are involved in drug resistance in cancer, such as cancer cells expressing the ATP-binding cassette (ABC) transporters which include P-gp [1], multidrug resistance protein (MRP) [2,3], and breast cancer resistance protein (BCRP) [4–6]. Lung resistance related protein (LRP) may also be involved in MDR [7]. In addition, other drug resistance mechanisms include changes in metabolizing and detoxifying

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doi:10.1016/j.canlet.2008.06.029

systems, such as DNA repair and the cytochrome P450 oxidases in cancer cells, responsible for drug-induced interruptions in apoptotic pathways [8]. Although many mechanisms of MDR in cancer cells have been studied, the most important and thoroughly studied mechanism of MDR is the efflux mechanism based on the function of P-gp, an ABC transporter, which is a 170 kDa plasma membrane glycoprotein encoded by the human MDR1 gene. This P-gp acts as a drug efflux pump to extrude a wide range of different chemotherapeutic drugs out of MDR cancer cells [9]. Therefore inhibition of P-gp-mediated drug efflux leads to resensitization of MDR cancer cells to treatment with chemotherapeutic agents, and may allow a successful chemotherapy in patients with MDR tumor [10,11]. Up to now, a number of MDR reversal agents have been directed at the problem of tumor MDR, such as verapamil, quinidine, cyclosporine A, PSC388, LY335979, OC1440935, GF120918 [12], tetrandrine [13,14], FG020326 and several of its derivatives [15,16], ONO-1078 [17], 5-*O*-benzoylated taxinine [18], agosterol A [19], and others. Unfortunately, in clinical trials, the efficacy of these MDR reversal agents is difficult to assess, mainly due to adverse pharmacokinetic side-effects.

Therefore, development or discovery of safe and effective MDR reversal agents is urgently required. Natural products from Chinese traditional medicine (CTM) are a fertile area in which to look for novel drugs with activity against MDR. In this study, we established a multiple drug resistant cell line as a screening model, and then screened over 200 natural compounds extracted from plants and animals for reversal potential to MDR. One compound which was isolated from Mylabris phalerate Pallas, Cantharidin (hexahydro-3aa, 7aa-dimethyl-4b, 7b-epoxyisobenzofuran-1,3-dione, Fig. 1) was found to be a potent inhibitor of MDR, which is isolated from Mylabris phalerate Pallas. We further investigate the molecular mechanism of the reversal of MDR by Cantharidin.

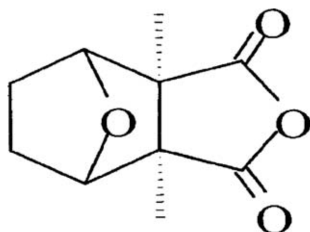

Fig. 1. Structure of Cantharindin.

2. Materials and methods

2.1. Cell lines and cell culture

Human embryonic kidney HEK293T cells, human hepatocyte L02 cells, and human liver carcinoma HepG2 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, TBD, China) and antibiotics (100 U/ml penicillin and 100 μg/ml astreptomycin).

2.2. Development and determination of multidrug resistance

To develop the drug resistant cell line (HepG2/ADM), Adriamycin (Haizheng, China) was added to HepG2 cells in a stepwise increasing concentration, from 0.05 to 2 μg/ml over 8 months. The resistant cells were selected by removing the dead non-resistant cells. Multidrug resistance was maintained by culturing the cells at 1 μg/ml Adriamycin. Then multidrug resistance was tested by treating the HepG2 and HepG2/ADM cells with different concentrations of anticancer drugs (Adriamycin, 5-Fu, Vincristine, Paclitaxel) for 48 h. MTT assay was performed to determine the percentage survival of cells surviving at each concentration. After plotting the dose–response curve, IC_{50} was calculated, from which reversal fold was calculated. All assays were performed in triplicate. RT-PCR was used to assess the MDR1 mRNA levels of HepG2 and HepG2/ADM cells.

2.3. Screening of chemosensitiser from natural compounds

The assay is dependent on the uptake and the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide MTT (Sigma, USA) by mitochondrial dehydrogenase in viable cells to a blue formazan product which can be measured spectrophotometrically. Natural compounds extracted from plants and animals were dissolved in DMSO as a 10 mg/ml stock. HepG2 and HepG2/ADM cells were seeded into culture at 1×10^4 cells/well. A full range of concentrations of Adriamycin combination with or without one microgram per milliliter of natural compounds were added to the cells. After 44 h, 20 μl MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h in a 37 °C incubator containing 5% CO_2,

allowing viable cells to reduce the yellow MTT into dark-blue formazan crystals. Hundred microliters of DMSO was added to each well and agitated for 10 min to dissolve the formazan crystals. Absorbance in each well was read at 570 nm by a Automated Microplate Reader (Bio-Rad).

2.4. Cytotoxicity assay

L02, HepG2, HepG2/ADM, and 293T cells were harvested with trypsin and resuspended in a final concentration of 1×10^5 cells/ml. Aliquots (0.1 ml) of each cell suspension were distributed evenly into 96-well multiplates and incubate for 24 h, designated wells were treated with different concentration of cantharidin. After 44 h, MTT assay was performed as described above.

Since we require concentrations of reversal multidrug agents which are neither inhibitory nor toxic, the inhibition rate of cantharidin on these three cell lines was evaluated as a control, and IC_{10} values were calculated by SPSS method. Cantharidin concentrations of 0.5, 1, and 2 μg/ml were used to study the reversal of MDR. All determinations were carried out in triplicate.

2.5. Analysis of MDR reversal activity

HepG2 and HepG2/ADM cells were seeded into 96-well culture plate at 1×10^4 cells/well. Serial dilutions of Adriamycin with or without 0.5, 1, and 2 μg/ml cantharidin were added to the cells. By using the same MTT assay as above, the degree of resistance was calculated by dividing the IC_{50} for the MDR cells by that of the parental sensitive cells. The reversal fold was calculated by dividing the IC_{50} for cells to Adriamycin in the absence of the cantharidin by that obtained in the presence of the compound. The increase in sensitivity to the Adriamycin was expressed as a gain of sensitivity [20].

2.6. Western blot analysis

HepG2 and HepG2/ADM cells were plated in a 6-well plate in a concentration of 5×10^5 cells in 2 ml of growth medium. After 24 h, 1 μg/ml cantharidin was added to the designated HepG2/ADM cells for another 24 h. Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with lysis buffer (1% Nonidet P-40, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 1 μg/ml aprotinin) for 30 min with occasional rocking followed by centrifugation at 15,000 rpm, for 15 min at 4 °C. Identical amounts (100 μg of protein) of cell lysate were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the resolved proteins were electrophoretically transferred to polyvinylidenefluoride (PVDF) membrane, and blocked with 5% fat-free dry milk in TBST (20 mM Tris–HCl (pH 7.6), 150 mM NaCl and 0.02% Tween 20) for 1 h, at room temperature. The membrane was immunoblotted with rabbit anti-human P-gp polyclonal antibody (1:500) (Santa Cruz, USA) in 1% milk/TBST. To assure equivalent protein loading, the membranes were simultaneously incubated with GAPDH monoclonal antibody (1:1000) (Kangcheng, CO, China) at 4 °C, overnight. Membranes were washed three times, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and washed extensively before detection. The membranes were subsequently developed using ECL reagent (Beyotime, China) and exposed to film according to the manufacturer's protocol.

2.7. Reverse transcription-PCR

Total RNA was extracted from cells by using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were measured with the optical densities at 260 and 280 nm. The reverse transcription (RT) was carried out by a PrimeScript™ RT Reagent kit (TaKaRa, Japan), and the following primers were used to amplify the target genes:

MDR1: 5′-CCCATCATTGCAATAGCAGG-3′, 5′-AGTCCTCGTCTTCAAACTTG-3′; β-actin: 5′-TCGTGCGTGACATTAAGGAG-3′, 5′-ATGCC AGGGTACATGGTGGT-3′. PCR was performed for 35 cycles, each cycle was comprised of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 45 s, before a final extension at 72 °C for 10 min. The amplified PCR products were electrophoresed on 1% agarose gels, and PCR fragments were visualized by ethidium bromide staining and were quantified by Tanon GIS-2000 gel image processing.

2.8. Generation of reporter plasmid

Human genomic DNA was prepared from human whole blood by using the phenol/chloroform

extraction protocol. To obtain the MDR1 promoter fragments, PCR amplification on human genomic DNA; (forward primer 5′-GGGGTACCCCAG TCTCTACG-3′, reverse primer 5′-CAAGCTTGT CCGACCTGAAGAG-3′) was performed in a 50 μl reaction mixture. After an initial denaturation step at 94 °C for 5 min, the PCR reaction were carried out for 30 cycles at 94 °C for 30 s, 60 °C for 50 s, and 72 °C for 2 min, with a final extension of 10 min at 72 °C. The PCR product was cloned into the KpnI/HindIII sites of pGL3-basic vector.

2.9. Transient transfection and reporter gene assay

293T cells were plated in a 24-well plate in a concentration of 5×10^4 cells in 1 ml of growth medium. After 24 h, cells were transfected with 1 μg of pGL3-basic vector or 1 μg of pGL3-MDR1 promoter plasmid by using calcium phosphate cell transfection kit (Beyotime, China) according to the manufacturer's instructions. Cells were also transfected with β-galactosidase vector for normalizing transfection. Twenty-four hours after transfection, cells were incubated in serum-deprived medium in the presence or absence of cantharidin for 24 h, and then were harvested with extraction in buffer (25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1% Triton X-100). Luciferase activity was measured and normalized to the β-galactosidase activity, as described previously [21] using a FLUOstar OPTIMA system. All transfections were repeated at least three times.

3. Statistical analysis

Data were expressed as means ± SD. Statistical analysis of the data was performed using the Student's t test. $p < 0.05$ was considered statistically significant.

4. Result

4.1. Determination of multidrug resistance

Resistant HepG2 cells (HepG2/ADM) were derived by treating the cells with stepwise increasing concentration of Adriamycin over 8 months. When MTT assay was performed, it was found that HepG2/ADM cells were resistant not only to Adriamycin but also to multiple anticancer drugs. Among them, we have tested 5-FU, Vincristine and Paclitaxel. The IC_{50} of these drugs to HepG2/ADM cells increased significantly when compared with non-resistant HepG2 cells (Table 1). HepG2/ADM cells were about 30-fold resistant to Adriamycin in comparison with the parental HepG2 cells. And the MDR1 mRNA level in HepG2/ADM cells increased by RT-PCR assay (Fig. 2).

Table 1
Determination of IC_{50} of different anticancer drugs

Anticancer drugs	HepG2 IC_{50}	HepG2/ADM IC_{50}	Resistant fold
Adriamycin	1.04 ± 0.07	30.6 ± 1.01	29.4
Paclitaxel	0.19 ± 0.11	6.13 ± 1.01	32.3
Vincristine	1.20 ± 0.17	21.3 ± 7.31	17.7
5-FU	2.96 ± 0.12	159.94 ± 14.42	53.9

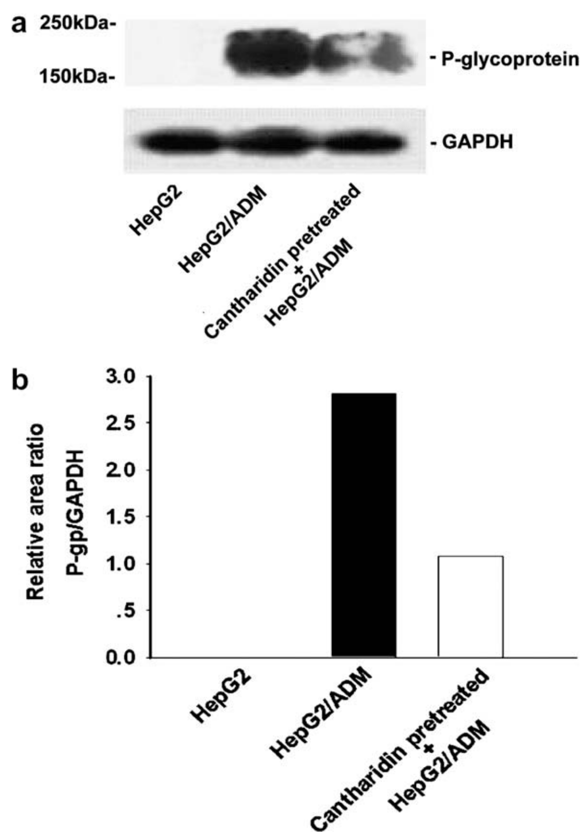

Fig. 2. Effect of cantharidin on P-gp expression in HepG2/ADM cells with western blot analysis. HepG2 and HepG2/ADM cells were plated in a 6-well plate. After 24 h, l ug/ml cantharidin was added to the designated HepG2/ADM cells for another 24 h. Cell extracts were prepared with lysis buffer, identical amounts of cell lysate were resolved by 10% SDS-PAGE, and the resolved proteins were electrophoretically transferred to PVDF membrane. The membrane was immunoblotted with rabbit anti-human P-gp polyclonal antibody in l%milk/TBST. The membranes were subsequently developed using ECL reagent and exposed to film according to the manufacturer's protocol. The P-glycoprotein levels were quantified by Image J software. A representative experiment is show in.(A). The values of relative area (P-gp/GAPDH) shown in (B).

4.2. Screening of chemosensitiser from natural compounds

A series of natural compounds from CTM were screened for their MDR reversal activity in vitro by our cell lines. The results showed that cantharidin was particularly effective in its reversal effect on MDR.

4.3. MTT cytotoxicity assay

To further investigate the effect of cantharidin on MDR, human normal liver cell line L02 was used to determine the non-toxic dose of cantharidin. The cytotoxic effects of cantharidin on four cell lines were measured after a 48 h treatment (Table 2). The IC_{10} of cantharidin in L02, HepG2, HepG2/ADM cells was 6.60, 3.25, and 4.10 μg/ml, respectively. In the 293T cell line it was 14.33 μg/ml. Cantharidin showed lesser cytotoxicity in normal cell lines as has been previously reported [22]. So concentrations of 0.5, 1, and 2 μg/ml cantharidin were used to study the reversal of MDR.

Table 2
The screening results of CTM on the reversal of MDR

Compound	IC_{50} of ADM (μg/ml) ± SD	Fold-reversal of MDR
Control	30.60 ± 1.01	1.00
Cantharidin	2.63 ± 1.18	11.63
Tectoridin	29.80 ± 1.02	1.02
Huperzine A	27.51 ± 0.78	1.11
Sodium houttuyfonate	28.33 ± 0.91	1.08
Cinobufagin	6.70 ± 1.13	4.5
Isorhamnetin	25.32 ± 0.86	1.20
Isoalantolactone	26.78 ± 1.22	1.14
Burnate	19.23 ± 1.09	1.59
Jasminoidin	21.23 ± 1.17	1.44
Catalpinoside	28.78 ± 0.92	1.06
Daphnetin	27.34 ± 1.18	1.12
Aescine	28.12 ± 0.65	1.09
Syringoside	29.10 ± 1.23	1.05
Naringin	30.22 ± 1.65	1.01
Isoalantolactone	30.44 ± 1.78	1.00
Scutellarin	25.38 ± 0.89	1.20
Ammidin	24.34 ± 0.76	1.26
Aurantiamarin	22.30 ± 1.01	1.37
Cordycepin	30.30 ± 1.25	1.01
Soyasaponin	25.31 ± 1.32	1.21
Chrysophanol	28.90 ± 1.06	1.06
Euphol	26.67 ± 0.87	1.15
Rubimaillin	30.20 ± 1.83	1.01
Piperine	27.56 ± 1.92	1.11
Amber acid	23.12 ± 0.81	1.32
Magnolol	19.87 ± 1.09	1.54
Icariin	28.90 ± 1.27	1.06
Asiaticoside	27.65 ± 1.39	1.11
Curcumine	8.45 ± 1.54	3.62
Cyclosan	29.10 ± 0.78	1.05
Forsythin	30.23 ± 0.65	1.01
Capsaicin	30.20 ± 1.83	1.01
Matrine	7.23 ± 1.98	4.2
Coumadin	30.12 ± 1.95	1.01
Sophoridine	30.20 ± 0.67	1.01
Catechin	28.39 ± 1.03	1.08
Menthol	29.30 ± 1.27	1.04
Saikoside	27.54 ± 1.03	1.10

4.4. Reversal of MDR in vitro by cantharidin

To investigate the effect of cantharidin on the sensitivity of cells to chemotherapeutic agent, cells were incubated with 0.5, 1, and 2 μg/ml of cantharidin and a full range of concentrations of Adriamycin. The IC_{50} of Adriamycin for the HepG2 cells and HepG2/ADM cells were 1.04 and 30.6 μg/ml, respectively. So the HepG2/ADM cells in these experiments were approximately 30-fold more resistant to Adriamycin compared with the parental drug-sensitive HepG2 cells (Table 3). Cantharidin concentrations of 0.5, 1, and 2 μg/ml lowered the IC_{50} of Adriamycin to 14.6, 2.63, and 0.56 μg/ml in the HepG2/ADM cells. This gave a 2.09-, 11.63-, and 54.64-fold reversal of MDR, respectively. These results suggest that cantharidin was very effective at reversing MDR in vitro. However, no such activity was found in HepG2 cells (data not shown).

4.5. The effect of cantharidin on P-glycoprotein expression

To assess the effect of cantharidin on P-glycoprotein expression, Western blot analysis was performed (Fig. 3). A high level of P-glycoprotein expression was detected in HepG2/ADM cells. However, when HepG2/ADM cells were treated with cantharidin, the P-glycoprotein level was significantly decreased (Table 4).

4.6. The effect of cantharidin on MDR1 mRNA transcription

RT-PCR was performed to detect the change in mRNA levels of mdr1 gene when the cells were treated with cantharidin. A 157-bp fragment of mdr1 cDNA was detected in each sample. The product of β-actin is 303-bp in length as an internal control (Fig. 4). mRNA of mdr1 increased significantly when multidrug resistance was developed. However, when HepG2/ADM cells were treated with cantharidin, the level of mdr1 mRNA was decreased to almost the same level as non-resistant HepG2 cells. Quantities of RNA in each lane were normalized by β-actin expression.

4.7. Effect of cantharidin on the activity of MDR1 promoter

To further analyze the transcriptional regulation of the MDR1 gene by cantharidin, we transfected HepG2/ADM cells with reporter plasmids containing the 2 kb of MDR1 promoter, followed by treatment with 1 μg/ml cantharidin. As shown in Fig. 4 cantharidin can inhibit the activity of MDR1 promoter dramatically.

Table 3
IC_{10} of cantharidin on L02, HepG2, HepG2/ADM, and 293T cells

	L02	HepG2	HepG2/ADM	HEK293T
IC_{10} (μg/ml)	6.60 ± 1.15	3.25 ± 0.21	4.10 ± 0.20	14.33 ± 1.26

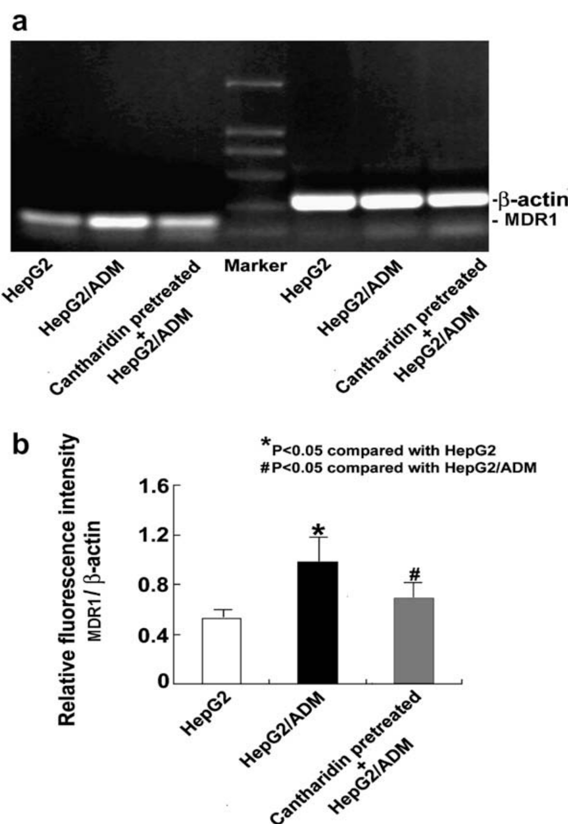

Fig. 3. RT-PCR analysis of MDR1 mRNA expression in HepG2 and HepG2/ADM cells. HepG2 and HepG2/ADM cells were plated in a 6-well plate. 24 h later, lug/mlcantharidin was added to the designated HepG2/ADM cells for another 24 h.The reverse transcription (RT) was carried out by a PrimeScript™ RT reagent kit. The MDR1 PCR product (157-bp) and P-actin PCR product (303-bp) were electrophoresed on 1% agarose gels, and PCR fragments were visualized by ethidium bromide staining and were quantified by Tanon GIS-2000 gel image processing. M: Marker DL2000. Measurements were performed at least three times and a representative experiment is show in (A). The values of relative fluorescence intensity (MDRl/D-actin) shown in (B).

Table 4
Modulation by cantharidin of the sensitivity to ADM of HepG2/ADM cells

Group	Concentration (μg/ml)	IC_{50} of ADM (μg/ml) ± S.D	Fold-reversal of MDR
Control	0	30.6 ± 1.01	1.00
Cantharidin	0.5	14.60 ± 4.17	2.09
	1	2.63 ± 1.18	11.63
	2	0.56 ± 0.07	54.64

5. Discussion

Multidrug resistance of cancer cells is often associated with overexpression of P-gp, a plasma membrane transporter that extrudes chemotherapeutic drugs by using ATP hydrolysis as the energy source. The aim of this paper was to find an effective MDR reversing agent from Chinese traditional medicine, and to gain insight into its reversal effect and the molecular mechanism of that effect.

Various tumor cells were observed to develop multidrug resistance when the cells were treated with the drugs over a period of time [23,24]. This resistance was due to, apart from other causes, the appearance of P-glycoprotein which exerts efficient pumping action to pump the drugs out of tumor cells. This leads to the requirement for a greater amount of antitumor drugs for effective cancer therapy but at the cost of greater side-effects. Recently, there have been reports showing that expression of siRNAs targeting MDR1 gene is able to reverse the P-gp mediated MDR [25]. Therefore, we developed a drug resistant cell line (HepG2/ADM), which expressed high level of P-glycoprotein. Adriamycin is a chemotherapeutic agent used for the treatment of many solid and hematologic malignancies [26]. Clinical resistance is often acquired to Adriamycin, as well as to many other anticancer drugs, resulting in their loss of therapeutic efficacy. So we selected ADM to develop our multidrug resistant HepG2/ADM cell line.

Our HepG2/ADM cell line was developed by treating the cells with Adriamycin only. However, from Table 1, it can be seen that multiple drug resistance was achieved. From Figs. 2 and 3, it can be seen that both mRNA and protein level of P-glycoprotein increased significantly when HepG2 cells developed resistance. By using HepG2/ADM cells, we screened over 200 natural compounds to search for an effective new chemosensitiser. Ultimately,

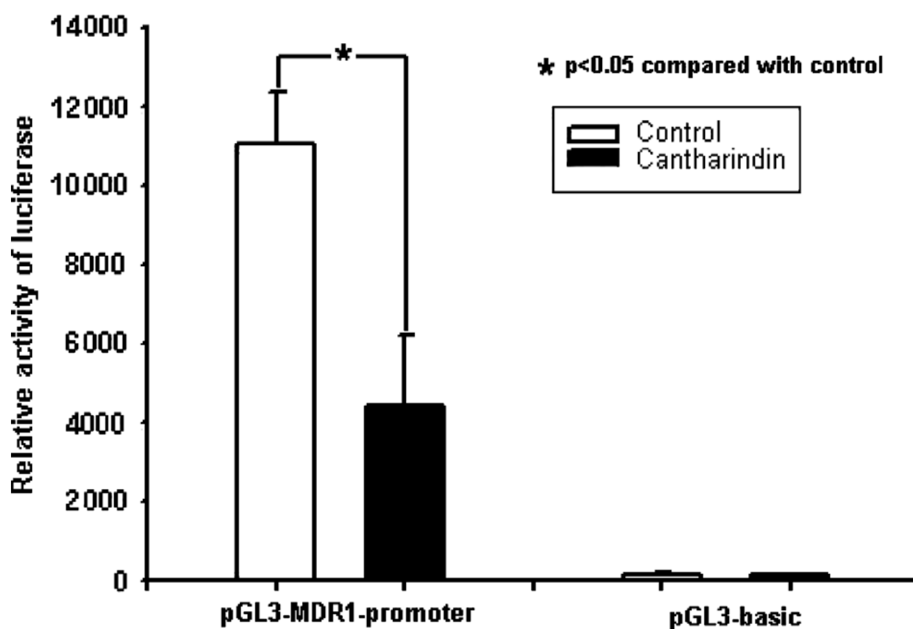

Fig. 4. Effect of cantharidin on the activity of MDR1 promoter. 293T cells were plated in a 24-well plate in a concentration of 5 × 104 cells in 1 ml of growth medium. After 24 h, cells were transfected with pGL3-basic vector or pGL3-MDRl-promoter plasmid by using Calcium phosphate cell transfection kit according to the manufacturer's instructions. Cells were also transfected with (J-galactosidase vector for normalizing transfection. 24 h after transfection, cells were incubated in serum-deprived medium in the presence or absence of cantharidin for 24 h, and then were harvested with extraction in buffer. Luciferase activity was measured and normalized to the β-galactosidase activity by using a FLUOstar OPTIMA system. All transfections were repeated at least three times.

cantharidin was found to have the desired high reversal on MDR1.

Cantharidin is a vesicant produced by beetles in the Order Coleoptera, has a long history in both folk and traditional medicine. Cantharidin inhibits the activity of several PPP family phosphatases, displays antitumor activity, and induces apoptosis in many types of tumor cells [27]. However, the effect of cantharidin on reversal of MDR and its molecular mechanism was unclear. So we further investigate the reversal activity of cantharidin on MDR and the molecular mechanism involved in this process.

Reversal of resistance assays requires cantharidin concentrations which are not inhibitory or toxic by themselves. If more than 90% of the parent cell line appears to be viable after treatment, then the concentration of cantharidin is non-toxic and could be used in reversal experiments. Thus, in the present study, the IC_{10} values of cantharidin on L02, HepG2, HepG2/ADM, and 293T cells were determined. So cantharidin concentrations of 0.5, 1, and 2 μg/ml were used to study the reversal of MDR. The ability of cantharidin to reverse resistance of HepG2/ADM cells to Adriamycin is shown in Table 3. We can see cantharidin gave a significant reversal of resistance to Adriamycin at a concentration of 0.5, 1, and 2 μg/ml. These results suggest that cantharidin was very effective at reversing MDR in vitro. Additionally decreased expression of mRNA and protein were found by RT-PCR and Western blot analysis. Reduction of the expression of P-gp at both the transcriptional and translational levels may certainly be proposed as one of the mechanisms for certain modulators or agents to reverse MDR phenotype [28].

To further elucidate the mechanism of the reduction on mRNA and protein level, we transfected the MDR1 promoter plasmid into 293T and HepG2/ADM cells, and then treated the cells with cantharidin. We found cantharidin can inhibit the activity of MDR1 promoter dramatically.

In conclusion, we have provided evidence here showing that cantharidin can effectively reverse MDR, via down-regulation of MDR1 gene expression. Our results suggest that cantharidin is a novel and potent MDR reversal agent and may be a potential adjunctive agent for tumor chemotherapy.

Conflicts of interest

This is original research that we have done, my co-authors agree with publishing the work, and I

will not submit it to any other journal while it is being considered by Cancer Letters.

Acknowledgments

This study was supported by the National Natural Scientific Foundation of China (Grant 30472169), the Program for New Century Excellent Talents in University (Grant NCET-06-0320), and the Cultivation Fund of the Scientific and Technical Innovation Project of Northeast Normal University (Grant NENU-STB07008).

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