Berberine Reverses Breast Cancer Multidrug Resistance Based on Fluorescence Pharmacokinetics In Vitro and In Vivo

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ABSTRACT: Exploring the mechanism through which berberine (Ber) reverses the multidrug resistance (MDR) of breast cancer is of great importance. Herein, we used the methyl thiazolyl tetrazolium assay to determine the drug resistance and cytotoxicity of Ber and doxorubicin (DOX) alone or in combination on the breast cancer cell line MCF-7/DOX<sub>Fluc</sub>. The results showed that Ber could synergistically enhance the inhibitory effect of DOX on tumor cell proliferation in vitro, and the optimal combination ratio was Ber/DOX = 2:1. Using a luciferase reporter assay system combined with the bioluminescence imaging technology, the efflux kinetics of D-luciferin potassium salt in MCF-7/DOX<sub>Fluc</sub> cells treated with Ber in vivo was investigated. The results showed that Ber could significantly reduce the efflux of D-luciferin potassium salt in MCF-7/DOX<sub>Fluc</sub> cells. In addition, western blot and immunohistochemistry experiments showed that the expression of P-glycoprotein (P-gp/ABCB1) and multidrug resistance protein 1 (MRP1/ABCC1) in MCF-7/DOX<sub>Fluc</sub> cells was downregulated upon Ber treatment. Finally, high-performance liquid chromatography was used to investigate the effect of Ber on DOX tissue distribution in vivo, and the results showed that the uptake of DOX in tumor tissues increased significantly when combined with Ber (P < 0.05). Thus, the results illustrated that Ber can reverse MDR by inhibiting the efflux function of ATP-binding cassette transporters and downregulating their expression levels.

1. INTRODUCTION

Breast cancer is the number one killer of women and is the second-most prevalent cancer worldwide, accounting for about one-fourth of the confirmed female cancer cases. At present, chemotherapy is one of the important means to treat breast cancer. However, the ability of cancer cells to rapidly develop resistance to chemotherapy is the major reason for chemotherapy failure. Therefore, the study of tumor multidrug resistance (MDR) has become a major focus within cancer research, with the goal of discovering a method to reverse MDR. According to current research results, the possible mechanisms of MDR include high expression of ATP-binding cassette (ABC) transporters, abnormal enzyme expression, changes in genes and proteins that control apoptosis, and changes in related signaling pathways. Multiple mechanisms may co-exist at the same time, or the dominant role may be played by only one mechanism. In recent years, combining traditional Chinese medicine (TCM) with chemotherapeutic drugs to combat MDR has been proposed. TCM can act on multiple components of interest at once, which is likely key to reversing MDR.

At present, the research on TCM agents used to reverse MDR cannot unify the reversal effects observed in vivo and in vitro in tumor cells. It is difficult to establish the correlation of the research results between in vivo and in vitro because of the different pharmacodynamic evaluation indexes of drugs. In vitro experiment drugs directly act on tumor cells; however, the tumor microenvironment in vivo is complex and diverse. Therefore, it is necessary to find a specific real-time dynamic monitoring technology or method which could evaluate the drug efficacy on tumor cells, both in vivo and in vitro. Previously, our team conducted research on the MDR reversal effect of many TCMs in doxorubicin (DOX)-resistant breast cancer in vitro by a real-time, quantitative, and dynamic detection method in vitro, and berberine (Ber) was chosen. Ber is an active alkaloid extracted from medicinal plants such as <i>Coptis chinensis</i>. To investigate whether Ber would keep the MDR reversal effect in vivo as in vitro and to understand how Ber could reverse MDR, we designed and conducted this study.

Bioluminescence imaging (BLI) is a widely used tool to study the biological process of living animals, and it can directly and sensitively monitor the activity of luciferase gene cells in vivo. BLI can integrate the firefly luciferase (Fluc) gene into the chromosomal DNA of cells to express luciferase. When its
2. RESULTS AND DISCUSSION

2.1. Multiple Drug Resistance of MCF-7/DOX<sup>Fluc</sup>. The cell viability of MCF-7 and MCF-7/DOX<sup>Fluc</sup> was calculated (Figure 1A,B). IC<sub>50</sub> of 0.55 μg/mL for MCF-7 and 72.46 μg/mL for MCF-7/DOX<sup>Fluc</sup> giving a multiple drug resistance of MCF-7/DOX<sup>Fluc</sup> was 131.75.

2.2. Combined Treatment of DOX and Ber Inhibited the Proliferation of MCF-7/DOX<sup>Fluc</sup> Cells In Vitro. To observe the proliferation rate of MCF-7/DOX<sup>Fluc</sup> cells treated with DOX and Ber alone or in combination, a synergistic ratio of DOX and Ber was calculated. When the combination ratios of Ber and DOX were 1:1, 2:1, 5:1, and 10:1, the IC<sub>50</sub> values after 48 h were 6.6, 3.2, 4.6, and 6.1 μg/mL, respectively, lower than that of DOX alone (IC<sub>50</sub> = 12.6 μg/mL, P < 0.05) and Ber alone (IC<sub>50</sub> = 20.0 μg/mL, P < 0.05) (Figure 2A). The combination index (CI) values were 1.11, 0.51, 0.76, and 1.70, respectively, and there was statistical difference when the combination ratios were 2:1 and 5:1 (P < 0.05) (Figure 2B). These findings indicated that Ber could enhance the inhibitory effect of DOX on cell proliferation through the synergistic effect. The optimal combinatory ratio of Ber to DOX was 2:1.

2.3. Combined Treatment of Ber and DOX Enhanced the Inhibition of MCF-7/DOX<sup>Fluc</sup> Breast Cancer Xenogenic Model In Vivo. The tumor volume in the group of Ber combined with DOX was smaller than that of other groups (Figure 2C). Compared with the PBS group, the group treated with Ber (10 mg/kg, i.p.) or DOX (5 mg/kg, i.v.) alone exhibited a mild reduction of tumor growth, whereas the combination of DOX and Ber exhibited a significant tumor growth inhibition efficiency (P < 0.05) (Figure 2D). The BLI results showed that the quantity of MCF-7/DOX<sup>Fluc</sup> cells was significantly reduced in the combined group compared with the PBS group (P < 0.05). The BLI results showed a decrease in the quantity of MCF-7/DOX<sup>Fluc</sup> cells treated with either Ber alone (10 mg/kg, i.p.) or DOX alone (5 mg/kg, i.v.) compared with the PBS group, whereas a significant decrease in the quantity of MCF-7/DOX<sup>Fluc</sup> cells was observed after DOX and Ber treatment (P < 0.05) (Figure 2E). The body weight of nude mice in the DOX group was significantly reduced, maybe because DOX could cause toxicities and side effects. After treated by the combination of Ber and DOX, the weight change of nude mice was similar to that treated by DOX alone (Figure 2F). It seemed that Ber could not reverse the toxic side effects of DOX at a high dose (5 mg/kg). Therefore, the concentration of DOX was decreased from 5 to 2 mg/kg to observe the synergistic effect and the toxicities in a later experiment.

2.4. Ber Could Combine with DOX of Low Dose to Reduce Toxicity In Vivo. Compared with the PBS group, the group treated with DOX at a low dose (2 mg/kg, i.v.) alone exhibited a mild reduction of tumor growth, whereas DOX (2 mg/kg, i.v.) combined with Ber (4 mg/kg, i.p.) produced a significant tumor growth inhibition effect, which was similar to the treatment with DOX alone at a high dose (5 mg/kg, i.v.) (P < 0.05) (Figure 3A). The body weight of nude mice in the DOX group showed a significant decrease compared with that in the

![Figure 1](https://doi.org/10.1021/acsomega.0c06288)
PBS group, whereas the body weight in the Ber + DOX group showed no significant decrease, indicating that Ber could reduce the toxicity induced by DOX at a low dose (Figure 3B). The variation in tumor mass showed a similar trend (Figure 3C).

2.5. Ber Inhibited the Function of ABC Transporter in MCF-7/DOXFluc Cells In Vitro.

The fluorescence efflux kinetics of D-luciferin potassium salt was monitored by BLI in vitro after the MCF-7/DOXFluc cells were treated by Ber for 48 h. The results showed that Ber could significantly inhibit the efflux of D-luciferin potassium salt (Figure 4A). The fluorescence intensity of MCF-7/DOXFluc cells treated with the ABCB1 inhibitor (Verapamil (Vera), 10 μg/mL) or Ber in different concentrations (9 and 18 μg/mL) for 48 h was detected by in vivo imaging system (IVIS). PK parameters in each group were obtained from the bioluminescent signal intensity of D-luciferin potassium salt, and the area under curve (AUC) and mean residence time (MRT) were fitted into the noncompartment model. The results showed that both Ber and Vera could significantly increase AUC and decrease MRT (P < 0.001). These data indicated that Ber could decelerate the clearance and efflux rate of D-luciferin potassium salt in MCF-7/DOXFluc cells. It meant that Ber could inhibit the function of the ABC transporter and thus reduce the efflux (Table 1).

2.6. Ber Inhibited the Function of ABC Transporters in MCF-7/DOXFluc Cells In Vivo.

On the 7th and 14th days after receiving Ber (10 mg/kg, i.p.), the MCF-7/DOXFluc tumor-bearing nude mice were injected with D-luciferin potassium salt (10 μg/mL, i.p.), and the luminescence signal was detected by BLI. Within 130 min, the bioluminescence signal value of D-luciferin potassium salt decreased gradually with time, and the...
Toxicity to Main Organs in Mice.

The results indicated that DOX caused damage to the myocardium, and the area of apoptosis and necrosis of tumor cells increased significantly compared with the control group (PBS). Fortunately, compared with the DOX group, the area of apoptosis and necrosis significantly decreased in the DOX + Ber group. The density of nuclei in the DOX group was significantly less than that in the control group, which meant that Ber could not reduce the toxic reaction too much.

Furthermore, biochemical indexes, such as blood urea nitrogen, creatinine, alanine amino transaminase, and aspartate transaminase were also assessed. There were no significant differences among all groups (Table 2).

2.7. Combination of Ber and DOX Had No Obvious Toxicity to Main Organs in Mice. The results of histopathological sections after hematoxylin and eosin (H&E) staining showed that the cells of each organ in the PBS group were closely arranged, the nucleus was complete, the tumor cells were dense, and there was almost no apoptosis or necrosis. Compared with the PBS group, the gap between myocardial cells became larger and the cell morphology changed significantly in the DOX group. The density of nuclei in tumor cells decreased significantly, and some tumor cells died of apoptosis and necrosis. In the Ber + DOX group, the morphology of myocardial cells changed significantly too, which meant that Ber could not reduce the toxic reaction caused by DOX at a high dose. Fortunately, compared with the DOX group, the area of apoptosis and necrosis of tumor cells increased and the nuclei decreased significantly (Figure 5A).

Furthermore, biochemical indexes, such as blood urea nitrogen, creatinine, alanine amino transaminase, and aspartate transaminase were also assessed. There were no significant differences among all groups (Figure 5B).

2.8. Ber Could Increase the Distribution of DOX in Tumor Tissue. After intravenous injection, the distribution of DOX in tumor tissues at 0.5, 2, and 4 h was measured by high-performance liquid chromatography (HPLC). Compared with the DOX group, the Ber + DOX group significantly increased DOX uptake at tumor sites after 0.5, 2, and 4 h (Figure 5C).

2.9. Ber Could Reduce the Expression of P-Gp and MDR Proteins In Vitro and In Vivo. The results of western blot analysis showed that Ber could downregulate the expression of P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1), which indicated that the ability of Ber in reversing drug resistance was partly related to the quantity of key proteins (Figure 6A,B).

The results of IHC experiments showed that the expression of P-gp and MRP1 in the DOX group was significantly upregulated when compared with the PBS group, whereas it was significantly downregulated in the DOX + Ber group (Figure 6C).

The emergence of MDR during chemotherapy is an important reason that restricts clinical treatment and leads to chemotherapy failure. P-gp and MRP1, both of which belong to the ABC transporter family, can combine with chemotherapeutic drugs and pump intracellular drugs to extracellular cells based on the energy released by ATP hydrolysis, thus inducing MDR.27–30 As an adjuvant drug for chemotherapy, TCM has been proven to be effective in reversing MDR by downregulating the expression of ABC transporters in tumor cells. For example, Yanhusuo can effectively reverse the drug resistance of adriamycin and mitoxantrone by inhibiting P-gp, reducing MRP1-mediated efflux, and activating ATPase activity.31 Quercetin can significantly improve the chemotherapy effect, and its main mechanism is to downregulate the expression of P-gp and MRP1, increasing the accumulation of therapeutic drugs and improving the therapeutic effect of DOX.32 A lot of papers reported the MDR reversal ability by Ber, with an emphasis on its mechanism. For example, Ber could enhance the drug sensitivity and induce apoptosis of breast cancer through different doses of regulation of AMPK signaling pathway. A low-dose Ber could improve the sensitivity of drug-resistant breast cancer cells to DOX through the AMPK-HIF-1𝛼-P-gp pathway, whereas a high-dose Ber could directly induce apoptosis through the AMPK-p53 pathway.33 Ber could regulate the expression and function of pgp-170, a gene product of MDR1, to weaken the response of digestive tract cancer cells to paclitaxel.34 Ber was also able to slightly upregulate the mRNA levels of MDR1a and MDR1b, thereby affecting the expression and function of MDR proteins.35 In our research, the MDR reversing mechanism of Ber was studied from both the function and the quantity of the ABC transporter protein. In previous studies related to drug-resistant proteins, people often used flow cytometry and real-time fluorescence quantitative polymerase chain reaction to detect the expression level of drug-resistant proteins.36,37 The advantage of this method is that it can clearly characterize the cell surface and intracellular protein expression, but it is susceptible to the influence of fluorescence intensity. The relative difference of fluorescence intensity depends on the combination difference of laser and filter on the instrument. Here, with the luciferase reporter gene system combined with
Figure 4. (A) BLI rel signal photon–time curve after being treated with Ber or Vera for 48 h in vitro. (B) BLI rel signal photon–time curve of MCF-7/DOX\textsuperscript{Fluc} tumor-bearing nude mice after being treated with Ber for 7 d. (C) BLI rel signal photon–time curve of MCF-7/DOX\textsuperscript{Fluc} tumor-bearing nude mice after being treated with Ber for 14 d. (D) BLI of MCF-7/DOX\textsuperscript{Fluc} tumor-bearing nude mice at different time points within 130 min after being intraperitoneally injected with D-luciferin potassium salt at the dose of 10 mg/kg.

Table 1. Effect of Vera and Ber on the PK Parameters of D-Luciferin Potassium Salt\textsuperscript{a}

| group       | \(t_{1/2}\) (min) | MRT (min) | \(C_{\text{max}}\) \((\times10^6)\) photon/s | AUC\textsubscript{0–120min} \((\times10^8)\) photon/s/min |
|-------------|-----------------|-----------|---------------------------------|---------------------------------|
| Ber (9 \(\mu\)g/mL) | 237.03 ± 53.41 | 254.59 ± 68.54 | 6.73 ± 0.24\*** | 6.36 ± 0.13\*** |
| Ber (18 \(\mu\)g/mL) | 264.79 ± 60.78 | 270.73 ± 71.61 | 7.82 ± 0.22\*** | 8.16 ± 0.35\*** |
| Vera        | 480.24 ± 81.58 | 211.43 ± 90.25 | 8.03 ± 0.29\*** | 8.33 ± 0.37\*** |
| PBS         | 191.94 ± 49.83 | 289.41 ± 70.53 | 3.88 ± 0.30 | 3.97 ± 0.18 |

\textsuperscript{a}Compared with the PBS group, * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\) (\(x \pm \sigma, n = 6\)).

Table 2. PK Parameters of Treatment with Ber In Vivo\textsuperscript{a}

| group    | MRT    | AUC     |
|----------|--------|---------|
| PBS      | 7 d    | 14 d    |
| Ber (10 mg/kg) | 45.78 ± 0.41 | 41.32 ± 0.63 |
| Ber (10 mg/kg) | 37.77 ± 0.52 | 36.59 ± 0.18 |
| Ber (10 mg/kg) | 84.40 ± 0.78 | 98.67 ± 0.52 |
| Ber (10 mg/kg) | 196.15 ± 0.83\** | 142.34 ± 0.47\* |

\textsuperscript{a}Note: Compared with the PBS group, * \(P < 0.05\), ** \(P < 0.01\).
BLI, we established a method for detecting the ABC transporter substrate content in tumor cells by a real-time quantitative and dynamical way \textit{in vivo} and \textit{in vitro}. To study the efflux function caused by the ABC protein, Rhodamine 123 was always used as a marker, which could not be dynamically tracked in real time, especially \textit{in vivo}. D-luciferin potassium is a specific substrate for the ABC transporter. When the intracellular luciferase enzyme is unsaturated, the bioluminescent intensity will follow a linear correlation with the content of D-luciferin potassium salt in cells.\textsuperscript{38,39} It provides the possibility to establish a relationship between the pharmacokinetic parameters of fluorescence \textit{in vivo} and \textit{in vitro}. Our results showed that, in the Ber group, there was a positive correlation between the \textit{in vitro} and \textit{in vivo} AUC of the fluorescence in tumor cells, and the Pearson correlation coefficient values were more than 0.7 (\(P < 0.05\)) (Table 3). This means that the AUC of fluorescence has a strong \textit{in vitro}—\textit{in vivo} correlation. Therefore, it is feasible to screen MDR reversal agents \textit{in vitro} by using the luciferase reporter gene system combined with BLI.

3. CONCLUSIONS

In this work, Ber was demonstrated as an ideal MDR reversing agent. The MDR reversal effect is achieved by inhibiting P-gp and MRP1 functions and their expression in MCF-7/DOX\textsuperscript{Fluc} tumor cells. When combining Ber with DOX to treat DOX-resistant breast cancer, Ber enhanced the intracellular concentration and retention of DOX in tumor cells, which occurred \textit{via} facilitating the cellular drug uptake and reducing the drug efflux rate in MCF-7/DOX\textsuperscript{Fluc} tumor cells. The DOX—Ber combination significantly enhanced the \textit{in vivo} anticancer efficacy of DOX in a drug-resistant MCF-7/DOX\textsuperscript{Fluc} xenograft model.
4. EXPERIMENTAL SECTION

4.1. Materials. DOX was purchased from Hisun Pfizer (Hangzhou, China). Ber was obtained from Macklin Biochemical Co., Ltd. (Shanghai, China, purity ≥ 97%). Fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were purchased from Gibco Corporation (USA); d-luciferin potassium salt was provided by Science Light Biology Science & Technology Co., Ltd. (Shanghai, China). Both methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich (St. Louis, MO, USA). Bicinchoninic acid (BCA) kit and radioimmunoprecipitation assay buffer were purchased from Beyotime Biotechnology (Shanghai, China). Vera was purchased from Apexbio (Houston, USA). Primary antibodies such as P-gp/ABCB1, MRP1/ABCC1, and secondary antibodies were purchased from Abcam Corporation. All chemicals were of analytical reagent grade.

4.2. Cell Lines and Experimental Animals. The MCF-7/DOX human breast cancer cells were kindly provided by West China Pharmacy School of Sichuan University and routinely cultured using RMPI 1640 (from Gibco, USA), supplemented with 10% FBS and 1% penicillin/streptomycin (from Gibco, USA) at 37 °C with 5% CO2 atmosphere in a humidified incubator. The MCF-7/DOXFluc cell line, stably expressing the Fluc reporter gene, was constructed by lentivirus infection as in our former studies.39

Female BALB/c nude mice (4 weeks old, weighing 18–22 g) were purchased from Slake Laboratory Animal Company (Shanghai, China) and bred by Laboratory Animal Center at Zhejiang Chinese Medical University (Hangzhou, China). To establish the xenograft models, 6 × 10^6 of MCF-7/DOXFluc cells and Matrigel (from BD Biosciences, USA) were suspended in PBS at a ratio of 1:1 (v/v) and subcutaneously inoculated on the right side of each mouse. All procedures involving animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

4.3. Determination of Multiple Drug Resistance of MCF-7/DOXFluc. A standard MTT assay was applied to calculate the multiple drug resistance of MCF-7/DOXFluc. DOX with concentrations of 0.05, 0.10, 0.50, 1.00, and 10.00 μg/mL was applied to MCF-7, and DOX with concentrations of 0.10, 1.00, 10.00, 50.00, and 100.00 μg/mL was applied to MCF-7/DOXFluc. As shown in Figure 6C, after incubation at 37 °C for 4 h, the MTT-containing culture medium was discarded, and then 150 μL of DMSO was added. The absorbance of each well was read at 490 nm using a microplate reader (Bio-Tek, USA). Each sample was repeated in triplicate. Cell viability was calculated by the following equation:

\[ \text{cell viability} (\%) = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{control}} - OD_{\text{blank}}} \times 100\% \]

The tumor cell proliferation rate and half-maximal inhibitory concentration (IC50) values were calculated by CompuSyn software. Multiple drug resistance = IC50 of MCF-7/DOXFluc/IC50 of MCF-7.

4.4. In Vitro Cell Viability Studies. A standard MTT assay was applied to evaluate the MCF-7/DOXFluc cell cytotoxicity and MDR reversal effect of Ber. MCF-7/DOXFluc cells were seeded into 96-well plates at a density of 8 × 10^3 cells per well, 24 h prior to drug treatment. In vitro experiments were set up into four groups (PBS group, DOX group, Ber group, and Ber + DOX group), and each group was set up with six wells. DOX-containing culture medium was added to each well of the DOX group, so that the final concentrations of DOX were 62.50, 31.25, 15.63, 7.813, 3.906, 1.953, 0.9766, 0.4883, and 0.04883 μg/mL. The Ber-containing culture medium was added to each well of the Ber group, so that the final concentrations of Ber were 125.0, 62.50, 31.25, 15.63, 7.813, 3.906, 1.953, 0.9766, and 0.09766 μg/mL. The culture medium containing Ber and DOX was added to each well of the Ber + DOX group, so that the final concentrations of Ber were 125.0, 62.50, 31.25, 15.63, 7.813, 3.906, 1.953, 0.9766, and 0.09766 μg/mL, and the culture medium containing Ber and DOX was added to each well of the Ber group, so that the final concentrations of Ber were 125.0, 62.50, 31.25, 15.63, 7.813, 3.906, 1.953, 0.9766, and 0.09766 μg/mL. The culture medium containing Ber and DOX was added to each well of the Ber + DOX group, so that the final concentrations of DOX were 62.50, 31.25, 15.63, 7.813, 3.906, 1.953, 0.9766, and 0.04883 μg/mL, and the culture medium containing Ber and DOX was added to each well of the Ber group, so that the final concentrations of Ber were adjusted according to the concentrations of DOX, so that the combined ratios of Ber and DOX were 10:1, 5:1, 2:1, and 1:1, respectively. The same volume of culture medium was added to each well in the PBS group. The CI analysis of Ber combined with DOX based on the Chou and Talalay method was conducted using CompuSyn software. For the experimental processing and data analysis, refer to Section 4.3.

4.5. In Vivo Antitumor Efficacy and Systemic Toxicity. The xenografts of human breast cancer were used as described in Section 2.2. MCF-7/DOXFluc tumor-bearing nude mice were
used to evaluate the tumor inhibition efficacy of the different concentrations of Ber in combination with DOX. Ber and DOX were dissolved in PBS. The nude mice were randomized into four groups (n = 6 per group) and treated with PBS, DOX (5 mg/kg, i.v.) alone, Ber (10 mg/kg, i.p.) alone, and DOX (5 mg/kg, i.v.) in combination with Ber (10 mg/kg, i.p.). DOX and Ber were administered every other day for 12 days, with a total of six doses. Body weights and tumor volumes were recorded the day after administration. The tumor length (L) and width (W) were used to calculate the volume (V), based on the following equation: 

\[ V = (L \times W^2 \times 1/2) \]

On every second day post administration, 100 μL of d-luciferin potassium salt (50 mg/kg, dissolved in PBS) was intraperitoneally injected into each group (PBS group, DOX group, Ber group, and Ber + DOX group) and immediately imaged by IVIS (Xenogen, USA) to record the peak value of the fluorescence. The BLI intensity of in vivo tumor cells was detected at \( E_l = 328 \text{ nm} \) and \( E_m = 533 \text{ nm} \). At the end of treatment, the tumors and organs (heart, liver, spleen, lungs, and kidneys) were collected for H&E staining to evaluate specific toxicity.

4.6. In Vivo Monitoring of Body Weight and Tumor Volume of MCF-7/DOXfluor Tumor-Bearing Nude Mice Given Low-Dose DOX. Once the tumors in the tumor-bearing nude mice grew to about 100 mm³, the mice were randomized into four groups (n = 6 per group) and treated with PBS, DOX (5 mg/kg, i.v.) alone, DOX (2 mg/kg, i.v.) alone, and DOX (2 mg/kg, i.v.) in combination with Ber (4 mg/kg, i.p.). The drug was administered six times in succession every other day, during which the body weights and tumor volumes were recorded every other day. The tumor volume was calculated using the calculation formula described in Section 2.4.

4.7. In Vitro Fluorescence Kinetics of d-Luciferin Potassium Salt in MCF-7/DOXfluor Cell Analysis. To investigate the effect on the function of ABC transporter-mediated efflux by Ber in vitro, the efflux of d-luciferin potassium salt in MCF-7/DOXfluor cells was noninvasively monitored by BLI in real time and quantitatively. Our main aim was to use Ber to decrease the MDR on MCF-7/DOXfluor but not to kill the cells; Ber of 90% IC50 and 45% IC50 was selected to carry out the in vitro experiment. MCF-7/DOXfluor cells were inoculated into a 96-well plate with a cell density of \( 8 \times 10^4 \) cells per well. After attachment for 24 h, the cells were treated with Ber at two different concentrations (9 and 18 μg/mL) for another 48 h. Vera (an inhibitor of ABCB1, 10 μg/mL) alone was used as a positive control. Before detection, d-luciferin potassium salt (10 μg/mL) was added to each well and immediately kinetically imaged using IVIS. Excited extracellular signals were captured every 5 min, and the kinetics of d-luciferin potassium salt was observed within 130 min. The photon signaling intensity of each group was then normalized by the total protein content as relative BLI (BLIrel) in order to eliminate the confounding influence of the increasing cell populations of photon signaling intensity. The BLIrel versus time curves were plotted. According to the noncompartment model method, the dynamic parameters (AUC and MRT) of d-luciferin potassium salt in cells were calculated.

4.8. In Vivo Fluorescence Kinetics of d-Luciferin Potassium Salt in MCF-7/DOXfluor Cell Analysis. The tumor-bearing nude mice were weighed and intraperitoneally injected with d-luciferin potassium salt (10 mg/kg). The BLI signals of each time point were quantitatively recorded within 130 min to obtain the kinetics of d-luciferin potassium salt in vivo. The photon signaling intensity of each time point was normalized by the volume of tumors in each treatment group and considered as BLIrel. To obtain the kinetics of d-luciferin potassium salt under the intervention of Ber (10 mg/kg), the BLI signals were taken at every time point for 0–130 min by using the IVIS kinetic imaging system after being treated with Ber at different time points (7 d and 14 d). The BLI signal was also normalized as above. According to the noncompartment model method, the dynamic parameters (AUC and MRT) of d-luciferin potassium salt in cells were calculated.

4.9. Expression of P-Gp and MRP1 Proteins by Western Blot Analysis In Vitro. MCF-7/DOXfluor cells were inoculated into a 10 cm tissue culture dish with a cell density of \( 4 \times 10^6 \) cells/mL. The culture medium containing Ber with different concentrations (9 and 18 μg/mL) was given. After 48 h, all cells were washed twice with ice-cold PBS, centrifuged at 14,000 rpm for 15 min, and all supernatants were discarded. A volume of 100 μL of cell lysis buffer containing Protease Inhibitor Cocktail was added to the precipitate and incubated on ice for 30 min, then centrifuged at 14,000 rpm for 15 min, and all supernatant was sucked. The protein concentration of cells in the supernatant was determined by BCA assay. A volume of 50 μL of supernatant was taken from each group, added to 5× protein loading buffer, boiled at 100 °C for 5 min, and run on 8% SDS-PAGE gel, and then the protein was transferred from the gel to the PVDF membrane. TBS-T (1×) was used to prepare 5% nonfat milk, and the cell membrane was sealed for 1 h. At 4 °C, it was incubated with primary antibodies (P-gp and MRP1 antibodies) overnight. The membrane was washed three times with 1× TBS-T and then incubated with secondary antibodies (HRP Affini pure goat antirabbit IgG) for 2 h at room temperature. The quantitative analysis of western blot results was conducted using image J software.

4.10. Expression of P-Gp and MRP1 Proteins by IHC In Vivo. All tumor tissues were stored with 4% paraformaldehyde and embedded in paraffin. The sections were deparaffinized in xylene and then hydrated with gradient ethanol. The antigen was recovered by microwave heating. The sections were incubated with 3% hydrogen peroxide (H2O2) for 30 min at room temperature to eliminate the activity of endogenous peroxidase. The sections were incubated with 10% normal goat serum for 30 min at room temperature. The sections were incubated with primary antibodies (P-gp and MRP1) overnight at 4 °C. After washing with TBS three times, the sections were incubated with labeled polymer-HRP antimonous (DAKO) secondary antibody for 1 h at room temperature. The sections were then exposed to 3, 3′-diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin.

4.11. In Vivo Distribution of DOX. The tumor-bearing nude mice were randomly assigned into two groups (DOX group and Ber + DOX group) (DOX dose of 5 mg/kg, i.v., Ber dose of 10 mg/kg, i.p.). Before the experiment, the tumor-bearing nude mice in each group fasted for 12 h and drank water freely. The tumor-bearing nude mice were separately euthanized at 0.5, 2, and 4 h after treatment, and the tumor tissues of each group were excised, rinsed with 0 °C saline, and dried using a filter paper. The tumor weights were measured and recorded. The tissue samples were homogenized in saline (0.25 g/mL) in an ice bath. Then, 0.5 mL of the homogenate was taken from each tumor tissue and placed in a 1.5 mL centrifuge tube. The samples were extracted with 2 mL of methanol–chloroform (1:4, v/v) mixed solution, vortexed for 5 min, centrifuged at 14,000 rpm for 5 min, and the supernatant was harvested and transferred to a clean tube. The supernatant was dried under
nitrogen at room temperature. A volume of 100 μL methanol–chloroform (1:4, v/v) was used to resuspend the sample. The sample was then vortexed for 2 min and centrifuged at 14,000 rpm for 5 min. The supernatant was collected, and the DOX concentration was measured by HPLC (Waters, USA).

4.12. Statistical Analysis. Statistical analysis was performed using SPSS 22.0 software. Data are represented as mean ± SD. The statistical analysis was determined using one-way ANOVA or the t test. Multiple comparison between the groups was performed using the Tukey method. * P < 0.05, ** P < 0.01, # P < 0.05, and # # P < 0.01 were considered to indicate a statistically significant difference.

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Notes
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■ REFERENCES

(1) Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca-Cancer J. Clin. 2018, 68, 394–424.
(2) Yang, Y.; Liu, X.; Ma, W.; Xu, Q.; Chen, G.; Wang, Y.; Xiao, H.; Li, N.; Liang, X.-J.; Yu, M.; Yu, Z. Light-activatable liposomes for repetitive on-demand drug release and immunopotentiation in hypoxic tumor therapy. Biomaterials 2021, 265, 120456.
(3) Yang, Y.; Yu, Y.; Chen, H.; Meng, X.; Ma, W.; Yu, M.; Li, Z.; Li, C.; Liu, H.; Zhang, X.; Xiao, H.; Yu, Z. Illuminating platinum transport while maximizing therapeutic efficacy by gold nanoclusters via simultaneous near-infrared-Ul imaging and glutathione scavenging. ACS Nano 2020, 14, 13536–13547.
(4) Yang, C.-X.; Xing, L.; Chang, X.; Zhou, T.-J.; Bi, Y.-Y.; Yu, Z.-Q.; Zhang, Z.-Q.; Jiang, H.-L. Synergistic Platinum(II) Prodrug Nanoparticles for Enhanced Breast Cancer Therapy. Mol. Pharm. 2020, 17, 1300–1309.
(5) Sun, Y.; Ma, W.; Yang, Y.; He, M.; Li, A.; Bai, L.; Yu, B.; Yu, Z. Cancer nanotechnology: Enhancing tumor cell response to chemotherapy for hepatocellular carcinoma therapy. Asian J. Pharm. Sci. 2019, 14, 581–594.
(6) Kamaljeet, K.; Goutam, R.; Saket, C.; Ranjit, S.; Amit, K. G. Chemotherapy with si-RNA and anti-Cancer drugs. Curr. Drug Delivery 2018, 15, 300–311.
(7) Lu, J. F.; Pokharel, D.; Bebawy, M. MRPI and its role in anticancer drug resistance. Drug Metab. Res. 2015, 47, 406–419.
(8) Barbuti, A.; Chen, Z.-S. Paclitaxel Through the ages of anticancer drug resistance: mechanisms involved and strategies for circumvention using a drug delivery system. Arch. Pharmacol. Res. 2014, 37, 4–15.
(9) Kibria, G.; Hatakeyama, H.; Harashima, H. Cancer multidrug resistance: mechanisms involved and strategies for circumvention using a drug delivery system. Arch. Pharmacol. Res. 2014, 37, 4–15.
(10) Bekele, R. T.; Venkatraman, G.; Liu, R. Z.; Tang, X.; Mi, S.; Benesch, M. G.; Mackey, J. R.; Godbout, R.; Curtis, J. M.; McMullen, T. P.; Brindley, D. N. Oxidative stress contributes to the tamoxifen-
induced killing of breast cancer cells: implications for tamoxifen therapy and resistance. Sci. Rep. 2016, 6, 21164.

(11) Hermanson, D. L.; Das, S. G.; Li, Y.; Xing, C. Overexpression of Mcl-1 confers multidrug resistance, whereas topoisomerases IIβ down-regulation introduces mitoxantrone-specific drug resistance in acute myeloid leukemia. Mol. Pharmacol. 2013, 84, 236–243.

(12) Wang, Y.; Wang, X.; Zhao, H.; Liang, B.; Du, Q. Clusterin confers resistance to TNFα-induced apoptosis in breast cancer cells through NF-κB activation and Bcl-2 overexpression. J. Invest. 2012, 24, 348–357.

(13) Zhou, D.; Liu, W.; Liang, S.; Sun, B.; Liu, A.; Cui, Z.; Han, X.; Yuan, L. Apoptin-derived peptide reverses cisplatin resistance in gastric cancer through the PI3K-AKT signaling pathway. Cancer Med. 2018, 7, 1369–1383.

(14) He, W.-t.; Zhu, Y.-h.; Zhang, T.; Abulimiti, P.; Zeng, F.-y.; Zhang, L.-p.; Luo, L.-j.; Xie, X.-m.; Zhang, H.-l. Curcumin reverses 5-fluorouracil resistance by promoting human colon cancer HCT-8/5-FU cell apoptosis and down-regulating heat shock protein 27 and p-glycoprotein. Chin. J. Integr. Med. 2019, 25, 416–424.

(15) Lin, F.-Z.; Wang, S.-C.; Hsi, Y.-T.; Lo, Y.-S.; Lin, C.-C.; Chuang, Y.-C.; Lin, S.-H.; Hsieh, M.-J.; Chen, M.-K. Celastrol induces vincristine multidrug resistance oral cancer cell apoptosis by targeting JNK1/2 signaling pathway. Phytomedicine 2019, 54, 1–8.

(16) Tian, L.; Zhang, Y.; Wang, Y.; Dang, R.; Fu, Z.; Gu, B.; Wen, N. Triptolide reduces proliferation and enhances apoptosis in drug-resistant oral human cancer cells. Int. J. Clin. Exp. Pathol. 2019, 12, 1204–1213.

(17) Zhang, W.; Li, M.; Du, W.; Yang, W.; Li, G.; Zhang, C.; Liang, X.; Chen, H. Tissue distribution and anti-lung cancer effect of 10-hydroxycamptothecin combined with platycodon radix and glycyrrhiza radix ET rhizoma. Molecules 2019, 24, 2068–2081.

(18) Su, F.; Wang, J. Berberine inhibits the MEXXY-OptMEpplexum pump to reverse multidrug resistance in a clinical carbapenem-resistant pseudomonas aeruginosa isolate in a planktonic state. Exp. Ther. Med. 2018, 15, 467–472.

(19) Scarfe, L.; Taylor, A.; Sharkey, J.; Harwood, R.; Barrow, M.; Comenge, J.; Beeken, L.; Astley, C.; Santeramo, I.; Hutchinson, C.; Ressel, L.; Smythe, J.; Austin, E.; Levy, R.; Rosseinsky, M. J.; Adams, D. J.; Poptani, H.; Park, B. K.; Murray, P.; Wilm, B. Non-invasive imaging of leukemia cell lines in vitro and in mouse xenografts: effects of monoclonal and polyclonal cell populations on intensity and kinetics of photon emission. J. Hematol. Oncol. 2013, 6, 10–19.

(20) Thompson, S. M.; Callstrom, M. R.; Knudsen, B. E.; Anderson, J. L.; Sutor, S. L.; Butters, K. A.; Kuo, C.; Grande, J. P.; Roberts, L. R.; Woodrum, D. A. Molecular bioluminescence imaging as a noninvasive tool for monitoring tumor growth and therapeutic response to MRI-guided laser ablation in a rat model of hepatocellular carcinoma. Invest. Radiol. 2013, 48, 413–421.

(21) Close, D. M.; Xu, T.; Sayler, G. S.; Ripp, S. In vivo bioluminescent imaging (BLI): noninvasive visualization and interrogation of biological processes in living animals. Sensors 2011, 11, 180–206.

(22) Christoph, S.; Schlegel, J.; Alvarez-Calderon, F.; Kim, Y.-M.; Brandao, L. N.; DeRyckere, D.; Graham, D. K. Bioluminescence imaging of leukemia cell lines in vitro and in mouse xenografts: effects of monoclonal and polyclonal cell populations on intensity and kinetics of photon emission. J. Hematol. Oncol. 2013, 6, 10–19.

(23) Zhang, Y.; Pullambhatla, M.; Laterra, J.; Pomper, M. G. Influence of bioluminescence imaging dynamics by D-luciferin uptake and efflux mechanisms. Mol. Imaging 2012, 11, 499–506.

(24) Huang, R.; Vider, J.; Serganova, I.; Blasberg, R. G. ATP-binding cassette transporters modulate both coelenterazine- and D-luciferin-based bioluminescence imaging. Mol. Imaging 2011, 10, 215–226.

(25) Zhang, Y.; Bressler, J. P.; Neal, J.; Lal, B.; Huang, H.-E. C.; Laterra, J.; Pomper, M. G. ABCG2/BCRP Expression Modulates D-Luciferin-Based Bioluminescence Imaging. Cancer Res. 2007, 67, 9389–9397.

(26) Close, D. M.; Hahn, R. E.; Patterson, S. S.; Baek, S. J.; Ripp, S. A.; Sayler, G. S. Comparison of human optimized bacterial luciferase, and green fluorescent protein for continuous imaging of cell culture and animal models. J. Biomed. Opt. 2011, 16, 047003–047014.

(27) Liu, S.; Chen, S.; Yuan, W.; Wang, H.; Chen, K.; Li, D.; Li, D. PD-1/PD-L1 interaction up-regulates MDR1/P-gp expression in breast cancer cells via PI3K/AKT and MAPK/ERK pathways. Oncotarget 2017, 8, 99901–99912.

(28) Shapira, A.; Livney, Y. D.; Broxterman, H. J.; Assaraf, Y. G. Nanomedicine for targeted cancer therapy: Towards the overcoming of drug resistance. Drug Resist. Updates 2011, 14, 150–163.

(29) Zhang, H.; Fu, L. W. Multidrug resistance-associated proteins and their roles in multidrug resistance. Acta Pharmacol. Sin. 2011, 46, 479–486.

(30) Lei, Y.; Tan, J.; Wink, M.; Ma, Y.; Li, N.; Su, G. An isoorientin alkaloid from the Chinese herbal plant corydalis yanhusuo W.T. Wang inhibits p-glycoprotein and multidrug resistance-associated protein 1. Food Chem. 2013, 136, 1117–1121.

(31) He, Z.; Xiao, X.; Li, S.; Guo, Y.; Huang, Q.; Shi, X.; Wang, X.; Liu, Y. Oridonin induces apoptosis and reverses drug resistance in cisplatin resistant human gastric cancer cells. Oncol. Lett. 2017, 14, 2499–2504.

(32) Pan, Y.; Zhang, F.; Zhao, Y.; Shao, D.; Zheng, X.; Chen, Y.; He, K.; Li, J.; Chen, L. Berberine enhances chemosensitivity and induces apoptosis through dose-orchestrated AMPK signaling in breast cancer. J. Cancer 2017, 8, 1679–1689.

(33) Lin, H.-L.; Liu, T.-Y.; Wu, C.-W.; Chi, C.-W. Berberine modulates expression of mdr1 gene product and the responses of digestive track cancer cells to paclitaxel. Br. J. Cancer 1999, 81, 416–422.

(34) Hiroka, S.; Hiroki, T.; Hajime, M.; Makoto, I. Selective regulation of multidrug resistance protein in vascular smooth muscle cells by the isoorientin alkaloid coptisine. Biol. Pharm. Bull. 2010, 33, 677–682.

(35) Cheng, J.; Dai, J. Y.; Chen, B. A.; Cai, X. H.; Wang, S.; Gao, F. Mechanisms of tetrandrine and 5-bromotetrandrine in reversing multidrug resistance may relate to down-regulation of multidrug resistance associated protein 7 expression. J. Exp. Hematol. 2012, 20, 558–563.

(36) Xu, W.-J.; Jiang, Y. w.; Wang, F. c. Reversal mechanism of multidrug resistance in leukemia cell line K562/ADM. Practical J. Cancer. 2003, 18, 347–349.

(37) Sun, Y.; Gu, M.; Zhu, L.; Liu, J.; Xiong, Y.; Wei, Y.; Li, F. Gencitabin upregulates ABCG2/BCRP and modulates the intracellular pharmacokinetic profiles of bioluminescence in pancreatic cancer cells. Anticancer Drugs 2016, 27, 183–191.

(38) Rettig, G. R.; Mcanuff, M.; Liu, D.; Kim, J.-S.; Rice, K. G. Quantitative bioluminescence imaging of transgene expression in vivo. Anal. Biochem. 2006, 355, 90–94.

(39) Tang, C.-γ.; Zhu, L.-x.; Yu, J.-d.; Chen, Z.; Gu, M.-c.; Mu, C.-f.; Liu, Q.; Xiong, Y. Effect of β-elemene on the kinetics of intracellular transport of d-luciferin potassium salt (ABC substrate) in doxorubicin-resistant breast cancer cells and the associated molecular mechanism. Eur. J. Pharm. Sci. 2018, 120, 20–29.