

Design real-time reversal of tumor multidrug resistance cleverly with shortened carbon nanotubes

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Abstract: Multidrug resistance (MDR) in tumors renders many currently available chemotherapeutic drugs ineffective. Research in nanobiotechnology-based therapeutic alternatives has provided innovative and promising strategies to overcome MDR. The aim of this study was to investigate whether the new strategy of a co-loaded reversal agent and chemotherapeutic drug with shortened carbon nanotubes (CNTs) would show useful effects on the real-time reversal of tumor MDR. CNTs were cut and purified via ultrasonication and oxidative acid treatment to optimize their length for drug-delivery vehicles, then verapamil (Ver) and doxorubicin (Dox) were co-loaded on shortened CNTs (denoted as Ver/Dox/shortened CNTs), which acted as a drug delivery system. The multidrug resistant leukemia K562/A02 cells were treated with the denoted Ver/Dox/shortened CNTs. The real-time reversal of tumor MDR were evaluated by flow cytometer, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, acridine orange/ethidium bromide staining, and Western blot analysis. In the same MDR tumor cells the new strategy of a co-loaded reversal agent and chemotherapeutic drug with CNTs could inhibit the function of P-glycoprotein in real-time by Ver as reversal agent, significantly increase the uptake of Dox, enhance the sensitivity of the MDR cancer cells to the chemotherapeutic agent, and induce apoptosis. It was therefore concluded that a co-loaded reversal agent and chemotherapeutic drug with shortened CNTs could have real-time reversal ability of MDR in tumors, which could represent a promising approach in cancer therapy.

Keywords: multidrug resistance, carbon nanotubes, drug delivery system, tumor

Introduction

As one of the most serious obstacles in cancer chemotherapy, multidrug resistance (MDR) phenotype associated with a massive overexpression of P-glycoprotein (P-gp) in neoplastic cells results in insufficient response to a spectrum of plural structurally and functionally unrelated anticancer agents.^{1,2} P-gp acts as drug-efflux pumps to remove drugs from cancer cells, maintaining the intracellular levels of drugs below a cell-killing threshold.³ Therefore, suppression of P-gp function by its inhibitor may increase the effectiveness of cancer chemotherapy in the treatment of tumor MDR.

Verapamil (Ver), the best known P-gp inhibitor, binds P-gp competitively with respect to antineoplastic drugs, which can inhibit the excretion of anticancer drugs, thus overcoming MDR in tumors.⁴ However, Ver is normally used as an antiarrhythmic drug, leading to the possibility of a cardiotoxicity side effect when used as an MDR-reversing agent with antitumor agents,⁵ which constrains its application. To date no effective reversal of tumor MDR is used in cancer therapy. These limitations have spurred efforts to search for new, efficient strategies which not only minimize the toxicity of reversal agents, but also improve the efficacy of the anticancer drugs to overcome MDR in tumors.

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Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure. These cylindrical carbon molecules have unusual properties, which are valuable for nanotechnology, electronics, optics, and other fields of material science and technology. Nanotubes are categorized as single-walled nanotubes and multi-walled nanotubes. CNTs represent a novel class of nanomaterials in the nanomedicine arena.^{3,6–10} Ever since their emergence on the “nano platform”, their unique properties such as high aspect ratio, surface area, and ease of drug loading via π - π stacking interactions have made them potential candidates for a drug delivery system (DDS).^{11–14} They have been shown to deliver various biomolecules including protein, DNA, and ribonucleic acid to cells. Despite excellent progress in using CNTs as drug-delivery vehicles, their size in length, similar to the diameter of cells, obviously makes them unsuitable carriers for cell endocytosis, thus more research is needed to further optimize their length for medical application. Shortened CNTs, therefore, are crucial for intracellular drug delivery.

From the synthesis point of view, development of multi-functional CNTs, tethered with a reversal agent and chemotherapeutic drug, is highly challenging, and what is more challenging is their precise characterization and biological evaluation. It is known that a molecule with a big conjugated structure can stack stably on the surface of CNTs by π - π stacking.^{15–17} In the present work, two kinds of molecules, Ver as reversal agent and chemotherapeutic drug doxorubicin (Dox), were co-loaded on shortened CNTs at the same time as a new vehicle to overcome tumor MDR. Ver in this case would block the transport activity of P-gp, and enable Dox to enter the same multidrug resistant cancer cells. In addition to examining the effectiveness of delivering Dox to the multidrug resistant leukemia K562/A02 cells, extensive studies were undertaken to observe the bio-effects on the cell viability, investigate the induced apoptosis, and assess the real-time reversal ability and the mechanism of MDR *in vitro*. To the best of our knowledge, this is the first report in which shortened CNTs have been successfully exploited for co-delivery of a reversal agent and chemotherapeutic drugs, improving the anticancer efficacy and overcoming MDR in tumors.

Materials and methods

Chemicals and apparatus

Multi-walled CNTs were purchased from Shenzhen Nanotech Port Co., Ltd. (People's Republic of China). Ver was purchased from Tianjing Centralpharm Co., Ltd.

(People's Republic of China). Dox was obtained from Hisun Pharmaceuticals, Zhejiang, People's Republic of China. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], acridine orange (AO) and ethidium bromide (EB) were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Phosphate buffered saline (0.1 M, pH 7.2) was prepared by using double distilled water.¹⁸ The P-gp antibody, β -actin antibody, and horseradish peroxidase-conjugated immunoglobulin G antibody were obtained from Nanjing KeyGen Biotech. Inc (Nanjing, People's Republic of China). All other reagents were of analytical grades. The scanning electron microscope images were obtained by a SU8000 scanning electron microscope (Hitachi Ltd., Tokyo, Japan). The optical density at 492 nm was recorded by the multi-well spectrophotometer reader (Thermo LabSystems Oy, Vantaa, Finland).

Ver and Dox co-loaded on shortened CNTs

Multi-walled CNTs were cut and purified via ultrasonication and oxidative acid treatment to obtain shortened multi-walled CNTs according to the reported study.^{19,20} Briefly, multi-walled CNTs were added to a mixture of 98% sulfuric acid and 65% nitric acid (volume:volume = 3:1) and then sonicated at 0°C for 24 hours. The shortened multi-walled CNTs were dispersed in ultrapure water (1 mg/mL) and pre-sonicated for 30 minutes. Dox (2 mg/mL) and Ver (2 mg/mL), the ratio of Ver/Dox 1:1, were added into the suspension dropwise, namely the ration of Ver/Dox/CNTs 1:1:0.5, and then magnetically stirred for 24 hours at room temperature to construct the nanocomposites of Ver and Dox co-loaded onto shortened multi-walled CNTs (denoted as Ver/Dox/shortened CNTs), which acted as a DDS. The DDS samples were collected by centrifugation at 5,000 *g* for 20 minutes. Unbound Ver and Dox in the supernatant were calculated by measuring the absorbance at 278 nm and 490 nm, respectively, allowing the estimation of the drug encapsulation efficiency and loading efficiency.

Cell culture

Multidrug resistant leukemia cells K562/A02, Dox selected and P-gp overexpressing, were obtained from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, People's Republic of China). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, People's Republic of China), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified

atmosphere of 5% carbon dioxide, maintaining the presence of Dox (1 µg/mL).

Cell internalization studies

Multidrug resistant cancer cells were treated with different treatments, shortened CNTs, Ver, Dox free, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs for 6 hours. The cells without treatment were used as control. Then, the cells were resuspended in phosphate buffered saline after being washed three times. Dox uptake was analyzed by FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA); the gate was arbitrarily set for the detection of green fluorescent Dox (λ_{ex} 488 nm, λ_{em} 515 nm).²¹

Cell viability assay

Multidrug resistant cancer cells K562/A02 were seeded at 1×10^5 cells per mL in 96 well plates and incubated for 24 hours. Then the cells were separated into different treatments, shortened CNTs alone, Ver alone, free Dox, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs (the concentration of both Dox and Ver is 0.5 µg/mL, CNTs 0.25 µg/mL). Cells without treatment were used as control. Cells were further incubated for 48 hours, and their relative viability was assessed using MTT assays. Briefly, MTT solutions were added after treatments and incubated for an additional 4 hours. Dimethyl sulfoxide (Sinopharm Chemical Reagent Co Ltd, Shanghai, People's Republic of China) was added to solubilize the formazan crystal, and optical density of 492 nm was recorded. Cell viability (%) was calculated as follows:

$$\frac{\text{Optical density}_{[492 \text{ nm in test cells}]}}{\text{Optical density}_{[492 \text{ nm in control cells}]}} \times 100.22 \quad (1)$$

Morphological analysis by AO/EB staining

Multidrug resistant cancer cells were treated with free Dox, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs for 48 hours. Then cells were stained with AO (200 µg/mL) and EB (200 µg/mL) for 10 minutes. After that, cells were observed under a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Western blot analysis

After different treatments, the cells were lysed at 4°C for 1 hour in a lysis buffer containing 50 mM Tris-HCl (Hoffman-La Roche Ltd., Basel, Switzerland), pH8.3, containing 1% Triton X-100 (Sigma-Aldrich), 1 mM

phelylmethylsulfonyl fluoride (Sigma-Aldrich), 10 µg/mL leupeptin (Sigma-Aldrich) and 100 U/mL aprotinin (Sigma-Aldrich). Then the isolated protein was quantified using the Bradford method with a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. After being blocked, the membrane was incubated with primary polyclonal antibodies either anti-P-gp or anti-β-actin overnight at 4°C, and subsequently incubated with horseradish peroxidase-conjugated immunoglobulin G antibody as the secondary antibody for 1 hour at room temperature. The protein bands were detected by an enhanced electrochemiluminescence detection system (Amersham ECL, GE Healthcare UK Ltd, Little Chalfont, UK). After normalization by corresponding β-actin expression, P-gp expression levels were determined by densitometry scans.²²

Statistical analysis

All the data are presented as the mean ± standard deviation. The *F*-test was used for significance testing, and *P*<0.05 was considered to be statistically significant. All tests were performed using the Statistical Package for Social Science version 13.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Characterization and drug-delivery vehicles of shortened CNTs

A major technological barrier, which obscures the application of CNTs in biomedicine, is their length. As shown in Figure 1A, we can easily deduce the size of CNTs to be similar to the diameter of cells, which is too long and difficult to be taken up by the cancer cells. The present work thus stemmed from the need to construct new vehicles by shortening multi-walled CNTs for drug cargo. After shortened, multi-walled CNTs had a purity of 95% and their dimensions were suitable for drug delivery as evident from the scanning electron microscope images in Figure 1. Obviously, shortened CNTs as shown in Figure 1B which are less than 300 nm can obviously easily cross the cell membrane facilitating the internalization of various drugs inside the cells. The characterization made shortened multi-walled CNTs a promising candidate for a DDS. It is known that molecules with a big conjugated structure can stack stably on the surface of CNTs via π-π stacking interactions.¹⁵⁻¹⁷ Therefore, both Ver as reversal agent and chemotherapeutic drug Dox tend to strongly interact with the shortened CNTs by π-π stacking interactions due to their conjugate plane structure nature.

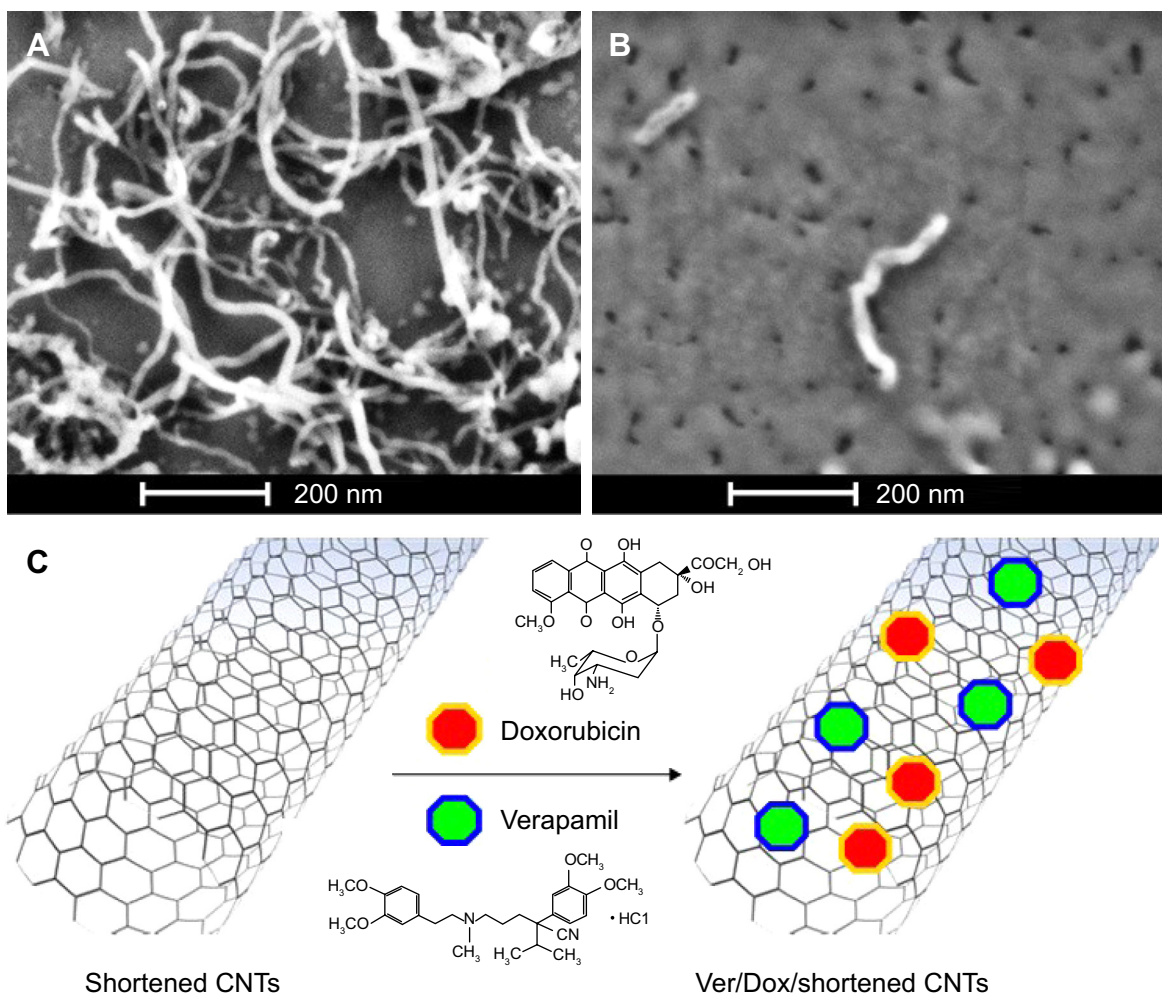


Figure 1 The SEM images of CNTs (A), shortened CNTs (B), and schematic representation of Ver and Dox co-loaded onto shortened CNTs (C).
Abbreviations: SEM, scanning electron microscope; CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin.

The loading efficiency of Ver and Dox (defined as the weight ration of the drug to the shortened CNTs) were assessed and calculated as $148.53\% \pm 10.62\%$ and $164.03\% \pm 12.78\%$, respectively. Figure 1C shows a proposed schematic representation of Ver and Dox co-loaded onto shortened CNTs through the formation of Ver/Dox/shortened CNT nanocomposites as a DDS which promises to expand the therapeutic window for real-time reversal of tumor MDR.

Enhancement of intracellular Dox accumulation with shortened CNTs

The decrease in intracellular drug concentrations, a result of the efflux of anticancer drugs from tumor cells into the surrounding tissue by P-gp which functions as drug-efflux pumps, is believed to be a common cause of MDR.^{23–25} Since Ver is a well-known substrate and inhibitor of P-gp, its administration could induce a significant decrease in transport activity. To investigate whether Ver/Dox/shortened

CNTs could inhibit the function of P-gp, the intracellular accumulation of the substrate of P-gp, Dox, was examined in multidrug resistant K562/A02 cells. As shown in Figure 2, the intracellular relative fluorescence intensity of Dox analyzed by flow cytometry in multidrug resistant K562/A02 leukemia cells incubated with Dox, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs were 19.35 ± 1.38 , 35.64 ± 2.66 , 37.26 ± 3.15 , and 50.22 ± 4.49 , respectively. No fluorescence could be detected in the cells treated by Ver and shortened CNTs, that is to say, the relative intracellular fluorescence resulted from Dox, and permitted a quantitative comparison of cellular uptake. Only low levels of intracellular fluorescence were observed after incubation with free Dox in multidrug resistant cancer cells, indicating only a small amount of drug had entered the cells due to be extruded from the multidrug resistant cancer cells by P-gp. Compared to free Dox, Ver/Dox and Dox/shortened CNTs significantly enhanced the intracellular Dox accumulation, respectively.

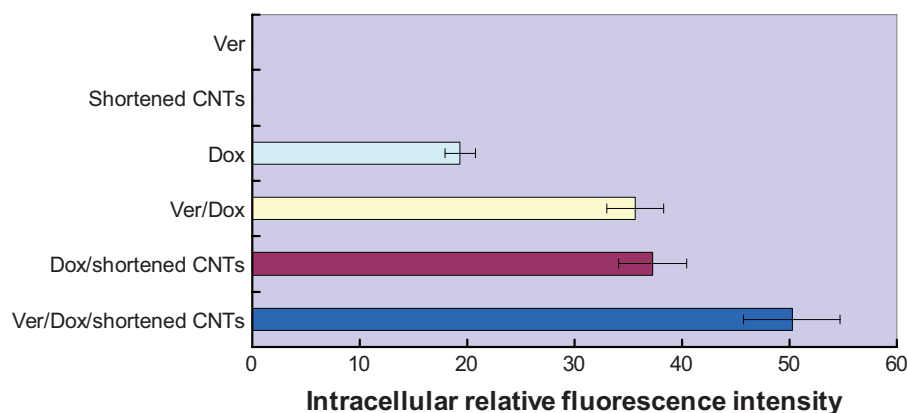


Figure 2 The intracellular relative fluorescence intensity in multidrug resistant leukemia cells K562/A02.

Notes: Concentrations of Dox and CNTs are 5 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respectively. Data expressed as mean \pm standard deviation ($n=3$).

Abbreviations: CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin.

Furthermore, the intracellular relative fluorescence intensity treated with Ver/Dox/shortened CNTs was higher than that treated with both Ver/Dox and Dox/shortened CNTs. These results clearly demonstrate that shortened multi-walled CNTs provide significant properties of co-delivery of reversal agents and chemotherapeutic drugs, inhibiting the function of P-gp by Ver as reversal agent and enhancing intracellular chemotherapeutic drug Dox accumulation, which represents a promising approach to real-time reversal of tumor MDR.

Real-time reversal of tumor MDR with shortened CNTs

The most common MDR involves cell membrane transporters such as P-gp, which function as drug-efflux pumps and keep cytotoxic drugs out of the cells, consequently, the cytotoxic effect of these agents is abolished.²⁶ Based on the above studies, we have further explored the reversal ability of the novel strategy of reversal agent and chemotherapeutic drug co-loaded onto shortened multi-walled CNTs at the same time as a new vehicle to overcome MDR. As shown in Figure 3, when treated with Dox at the concentration of 5 $\mu\text{g}/\text{mL}$ alone, the multidrug resistant cancer cell viability of K562/A02 was only inhibited at 25.51% \pm 3.21%. Both Ver/Dox and Dox/shortened CNTs showed similar cytotoxicity against cells, at the equivalent drug dose of Dox, Ver/Dox showed enhanced cytotoxicity of 38.23% \pm 3.56%, and Dox/shortened CNTs 40.34% \pm 3.98%. Although Ver and shortened CNTs could increase the inhibition of the K562/A02 cells, 58.84% \pm 6.56% of the multidrug resistant cancer cells were inhibited while treated with Ver/Dox/shortened CNTs. Noting that non cytotoxic to cells both Ver and shortened CNTs in the DDS, more multidrug resistant cancer cells were killed due to the cytotoxic action of the enhanced

internalized Dox by the strategy of Ver/Dox/shortened CNTs as a DDS. Thus Ver/Dox/shortened CNTs could alter the sensitivity of multidrug resistant leukemia K562/A02 cells to chemotherapeutic agents, suggesting that the resistance against Dox in the multidrug resistant cancer cells could be significantly reversed by Ver/Dox/shortened CNTs.

Apoptosis induced by Ver/Dox/shortened CNTs

Since obvious cell viability inhibition appeared in the MTT assay of Ver/Dox/shortened CNTs, we further detected the possibility of apoptosis occurrence in multidrug resistant cancer cells K562/A02 using AO/EB double staining. The differential AO/EB staining is capable of distinguishing between viable and nonviable cells based on membrane integrity. When the cell is viable, the AO intercalates into

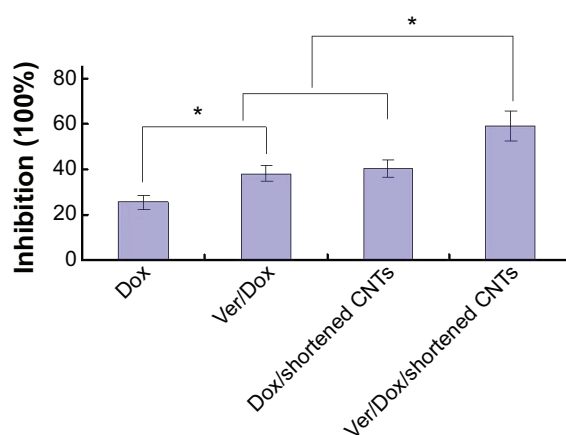


Figure 3 Inhibition effect of different treatments of Dox, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs.

Note: Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$.

Abbreviations: CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin.

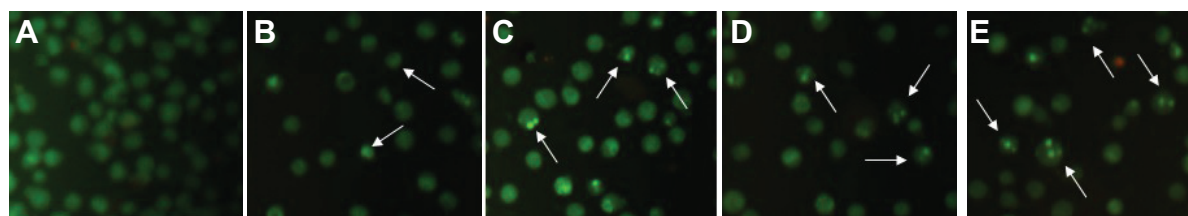


Figure 4 Nuclear morphologic changes of multidrug resistant K562/A02 leukemia cells after different treatments for 48 hours.

Notes: (A) Untreated cells as control, (B) free Dox, (C) Ver/Dox, (D) Dox/shortened CNTs, and (E) Ver/Dox/shortened CNTs. Arrows indicate cells with apoptotic nuclear condensation and fragmentation.

Abbreviations: CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin.

the DNA, giving the cell a green appearance. Early apoptotic cells with intact plasma membranes appear green, with “dots” of condensed chromatin that are highly visible within. Late apoptotic cells are stained bright green–orange because membrane blebbing has started to occur and EB can enter the cells. Nuclei in nonviable cells would fluoresce bright orange. As shown in Figure 4, multidrug resistant leukemia K562/A02 cells treated with the above methods presented typical apoptosis features, such as obvious chromatin condensation and slight nuclear fragmentation. There was almost no apoptotic evidence in the control group without any treatment (Figure 4A). When cells were treated with Ver/Dox/shortened CNTs (Figure 4E), typical apoptotic morphology was more apparent than that of cells treated with Dox alone (Figure 4B), Ver/Dox (Figure 4C), and Dox/shortened CNTs (Figure 4D). The increase of apoptosis induced the above decrease of cell proliferation, that is to say, the decrease of cell proliferation is associated with the increase of apoptosis. These findings strongly indicate that Ver/Dox/shortened CNTs reverse tumor MDR by inducing apoptosis rather than necrosis.

P-gp activity

The overexpression of P-gp is the major cause of MDR development. Suppression of the expression of P-gp at either a transcriptional or a translational level is a critical approach to overcome MDR.²⁷ After the different treatments, the expressions of P-gp were detected by Western blot. As shown in Figure 5, when multidrug resistant leukemia K562/A02 cells were treated with Dox, Ver/Dox, Dox/shortened CNTs, and Ver/Dox/shortened CNTs for 48 hours, levels of P-gp protein were significantly downregulated compared to the control group without treatments. Meanwhile, the downregulated levels of P-gp protein in the Ver/Dox/shortened CNTs group were slightly higher than those in the groups of Dox, Ver/Dox, and Dox/shortened CNTs.

Consequently, based on the above studies, Figure 6 schematically illustrates the possible processes of real-time

reversal of tumor MDR with shortened multi-walled CNTs. Ver and Dox co-loaded on shortened multi-walled CNTs, forming a Ver/Dox/shortened CNT nanocomplex as a DDS, integrates a reversal agent and a chemotherapeutic drug for overcoming MDR of cancer. Ver in this case would block the transport activity of P-gp, and could enable Dox to enter the same multidrug resistant cancer cells, thus increasing the intracellular concentration of anticancer Dox dramatically, and enhancing the inhibition of multidrug resistant cancer cells. The apoptosis initiation is an important event for the cytotoxic effects of anticancer agents after more Dox molecules have moved to the nucleus. Finally, the MDR of the K562/A02 cells was dramatically reversed.

Conclusion

In conclusion, the transport activity of P-gp of multidrug resistant cancer cells has been successfully inhibited by Ver to dramatically increase the intracellular concentration of Dox with shortened multi-walled CNTs. The accumulation of anticancer drugs in multidrug resistant cancer cells enhances the cytotoxicity to cells and induces more apoptosis. A co-loaded reversal agent and chemotherapeutic drug with shortened CNTs could be a real-time reversal strategy for overcoming MDR.

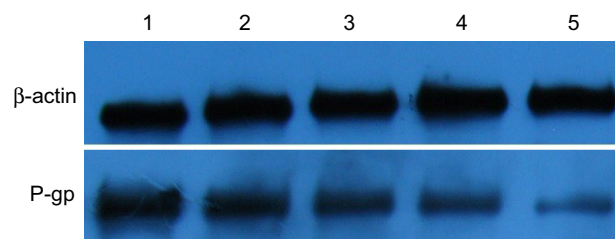


Figure 5 Expression of P-gp in the multidrug resistant leukemia cells by Western blot after different treatments for 48 hours.

Notes: Line 1, control; line 2, free Dox; line 3, Ver/Dox; line 4, Dox/shortened CNTs; line 5, Ver/Dox/shortened CNTs.

Abbreviations: CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin; P-gp, P-glycoprotein.

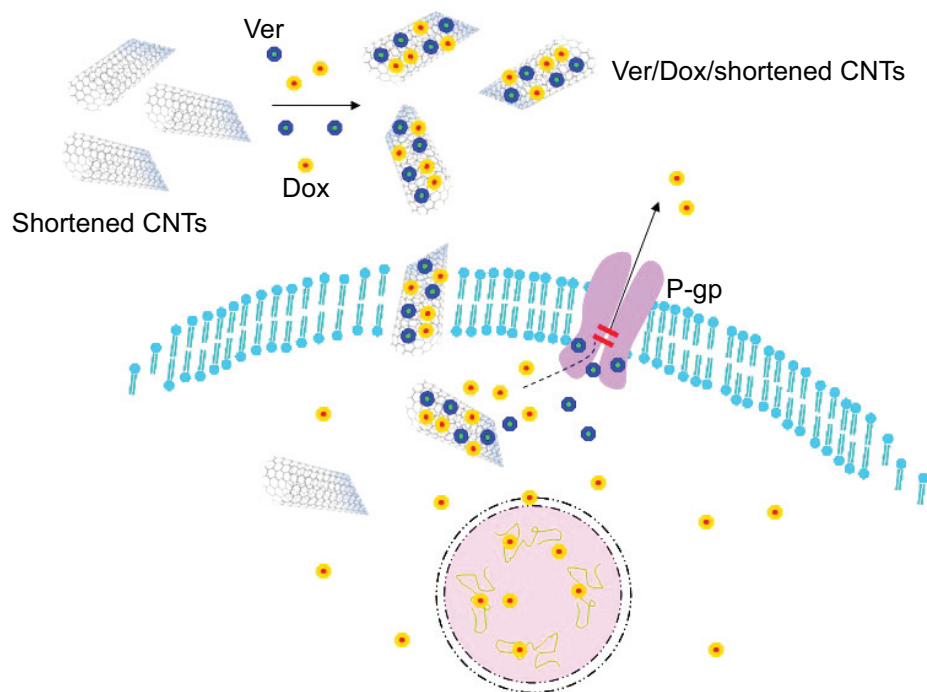


Figure 6 Schematic illustration of the possible processes of real-time reversal of tumor multidrug resistance with shortened carbon nanotubes.
Abbreviations: CNTs, carbon nanotubes; Ver, verapamil; Dox, doxorubicin; P-gp, P-glycoprotein.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31200750, 81371678) and the Natural Science Foundation of Jiangsu Province (BK2012332).

Disclosure

The authors report no conflicts of interest in this work.

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