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Reversal Effects of 20(R)– and 20(S)–Ginsenoside-Rg3 on Daunorubicin Uptake in Multidrug Resistant Leukemia Cells Studied in the Single-Cell Biochip

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Abstract

Multidrug resistance (MDR), frequently mediated by over-expression of the P-glycoprotein (P-gp/ABCB1) efflux transporter, remains a major challenge in the treatment of leukemia by limiting intracellular accumulation of chemotherapeutic agents such as daunorubicin (DNR). This study evaluates the applicability of a microfluidic-based single-cell biochip to investigate the reversal effects of microgram-level ginsenosides on daunorubicin uptake in multidrug-resistant leukemia cells. Pure ginsenosides are difficult to obtain in bulk and are typically available only in milligram quantities, which restricts their evaluation using conventional MDR assays such as flow cytometry that require large cell populations and substantial amounts of compound. To address this limitation, a microfluidic single-cell biochip (SCB) requiring microgram quantities of ginsenosides (<100 µg) and fewer than ten cells was employed. Intracellular DNR accumulation was measured in the CEM/VLB1000 leukemia cell line following treatment with DNR alone or in combination with ginsenoside Rg3-R, ginsenoside Rg3-S, 20(S)-protopanaxatriol (PPT), and 20(S)-protopanaxadiol (PPD), in order to compare their relative efficacy in enhancing drug accumulation. Although Rg3-R and Rg3-S share highly similar chemical structures and are glycosylated derivatives of the PPD aglycone, Rg3-S exhibited greater potency in increasing intracellular daunorubicin accumulation than Rg3-R, and both were more effective than PPD. These findings underscore the importance of ginsenoside stereochemistry modulating P-gp associated drug resistance and demonstrate the utility of the SCB platform for quantifying daunorubicin accumulation in multidrug resistant leukemia cells at single cell resolution.

Keywords: microfluidic biochip; single cell fluorescence; leukemia drug uptake; multidrug resistance; daunorubicin efflux reversal; ginsenosides

1. Introduction

Multidrug resistance (MDR) remains a major obstacle to effective cancer chemotherapy, posing a substantial challenge to disease modulation and long-term treatment success. One of the most extensively studied mechanisms underlying MDR is the overexpression of the ATP-binding cassette transporter P-glycoprotein (P-gp/ABCB1), which actively effluxes a wide range of chemotherapeutic agents from cancer cells, thereby reducing intracellular drug accumulation and therapeutic efficacy (Hu, 1990). In leukemia, elevated P-gp expression has been strongly associated with resistance to anthracycline drugs such as daunorubicin (DNR), often leading to treatment failure and disease relapse.

We have previously reported a same-single-cell approach for studying the modulation of drug efflux in multidrug-resistant cancer cells using a microfluidic platform (Li et al., 2008). This approach was later extended to evaluate drug modulation by directly measuring intracellular drug

accumulation rather than efflux (Li et al., 2011). Compared with the earlier efflux-based method, the accumulation-based approach is both faster and simpler while maintaining single-cell resolution. This methodology has since been adopted as the microfluidic single-cell biochip (SCB) platform for subsequent MDR studies.

In parallel with advances in microfluidic technologies, increasing attention has been directed toward traditional medicinal compounds due to their reported anticancer properties and potential to enhance chemotherapy outcomes, including improved therapeutic response and enhanced quality of life (Wen-jing et al., 2006). Among these compounds, ginsenosides, bioactive saponins derived from *Panax ginseng*, have attracted significant interest. Several studies have suggested that MDR in cancer cells can be reversed by ginsenosides, potentially through interactions with the P-gp transporter that reduce drug efflux (Chai et al., 2010). As a result, intracellular accumulation of chemotherapeutic agents such as DNR may be enhanced in MDR cells.

Despite their therapeutic potential, purified ginsenosides are difficult to obtain in bulk and are typically available only in trace amounts at the milligram level. This limitation presents a significant challenge for conventional MDR assays, such as flow cytometry, which require large numbers of cells and milligram quantities of compounds. To overcome this constraint, the microfluidic single-cell biochip (SCB) method was developed to enable MDR evaluation using microgram quantities of compounds like ginsenosides of less than 100 μg and fewer than ten cells. In this study, the SCB platform was applied to measure intracellular DNR accumulation in single MDR leukemia cells using microgram quantities of ginsenosides, including Rg3.

Ginsenoside Rg3 has been reported to possess anticancer activity (Mochizuki 1995; Yun 2001) and is present at low levels in white (air-dried) ginseng but at higher concentrations in steamed (red) ginseng (Kim et al., 2000). Elevated levels of Rg3 have also been identified in certain commercial ginseng products (Uhr et al. 2014). There were four ginsenosides studied in this paper: 20(S)-ginsenoside Rg3 (Rg3-S), 20(R)-ginsenoside Rg3 (Rg3-R), 20(S)-protopanaxadiol (PPD) and 20(S)-protopanaxatriol (PPT). Rg3-S and Rg3-R are structurally similar as they are the two stereoisomers at the C-20 position of Rg3. In addition, PPD and PPT share similar core frameworks, with PPD serving as the aglycone of both Rg3 stereoisomers. While these compounds are all structurally similar, they exhibit distinct biological activities (Yue et al., 2006).

Moreover, only Rg3 and PPD have been reported to have the MDR-reversal effect (Park 1996; Kim 2003; Zhao 2009). However, differences in MDR reversal activity between the Rg3 stereoisomers, Rg3-R and Rg3-S, have not been systematically investigated, particularly at the single-cell level. In this work, we employed the SCB platform to evaluate how four structurally related ginsenosides, Rg3-S, Rg3-R, PPD, and PPT, affect intracellular DNR accumulation in individual multidrug-resistant leukemia cells. This study aims to clarify the influence of ginsenoside stereochemistry on MDR modulation while further demonstrating the applicability of microfluidic single-cell analysis for drug resistance studies using scarce natural products.

2. Materials and Methods

Cell cultures: The drug-sensitive human leukemia CCRF-CEM cell line (CEM/WT) and the multidrug-resistant Vinblastine (VLB) subline (CEM/VLB 1000) were obtained from BC Cancer Agency. Both CEM/WT and the resistant subline, CEM/VLB 1000, were cultured in α -MEM supplemented with 10% fetal bovine serum (FBS) and penicillin (5%). The cell lines were maintained at 37 °C in a humidified atmosphere containing 5.0% CO₂ and were passaged once a week. In Addition, the drug-resistant subline, CEM/VLB1000, was sub-cultured with 100 $\mu\text{g}/\text{ml}$ vinblastine solution to maintain resistance of 1000 $\mu\text{g}/\text{ml}$ (CEM/VLB).

Microchip fabrication: Figure 1 depicts the design of the microfluidic single-cell chip. As shown in figure 1(A), the microfluidic chip consists of four reservoirs connected by four microchannels to a central cell-retention chamber. In the chamber, there is a V-shaped structure, which can retain a single cell. Reservoir 1 is the α -MEM solution inlet, reservoir 2 is the reagent inlet, reservoir 3 is the cell inlet,

and reservoir 4 is the waste inlet. A microfluidic chip was prepared and cleaned with NOX soap, purified water and 90% ethanol, respectively.

Drug accumulation: Prior to the experiment, an aliquot (~100 μL) of CEM/VLB1000 cell culture suspension was taken for each of the experiments. Microfluidic chip was mounted on microscope slide, and the equipment was connected to a CCD camera, interfaced with an inverted fluorescence microscope and computer. Before introducing the vial with the cells, the chip was sterilized by introducing 70% ethanol through reservoir 4 and allowing it to flow through the microchannels. After 70% ethanol was pipetted out of reservoir 4, a-MEM was added to remaining chip reservoirs. Next, about 10 μL of the cell sample was drawn by a micropipette and added into a reservoir 3 of the microfluidic chip reservoir. By manually adjusting the pressure inside the microfluidic chip, one cell was selected for and retained within the central chamber for the experiment. A measurement window was set up, and drug accumulation was monitored by repeatedly positioning the cell within a defined measurement window over a total period of 3100 s (the first 100 s were used for background measurement, after which drug solutions were introduced at 1000 s intervals.). Accumulation curves were processed and reformatted. Fold increases were calculated by normalizing DNR accumulation in the presence of inhibitors to baseline DNR accumulation measured in untreated cells.

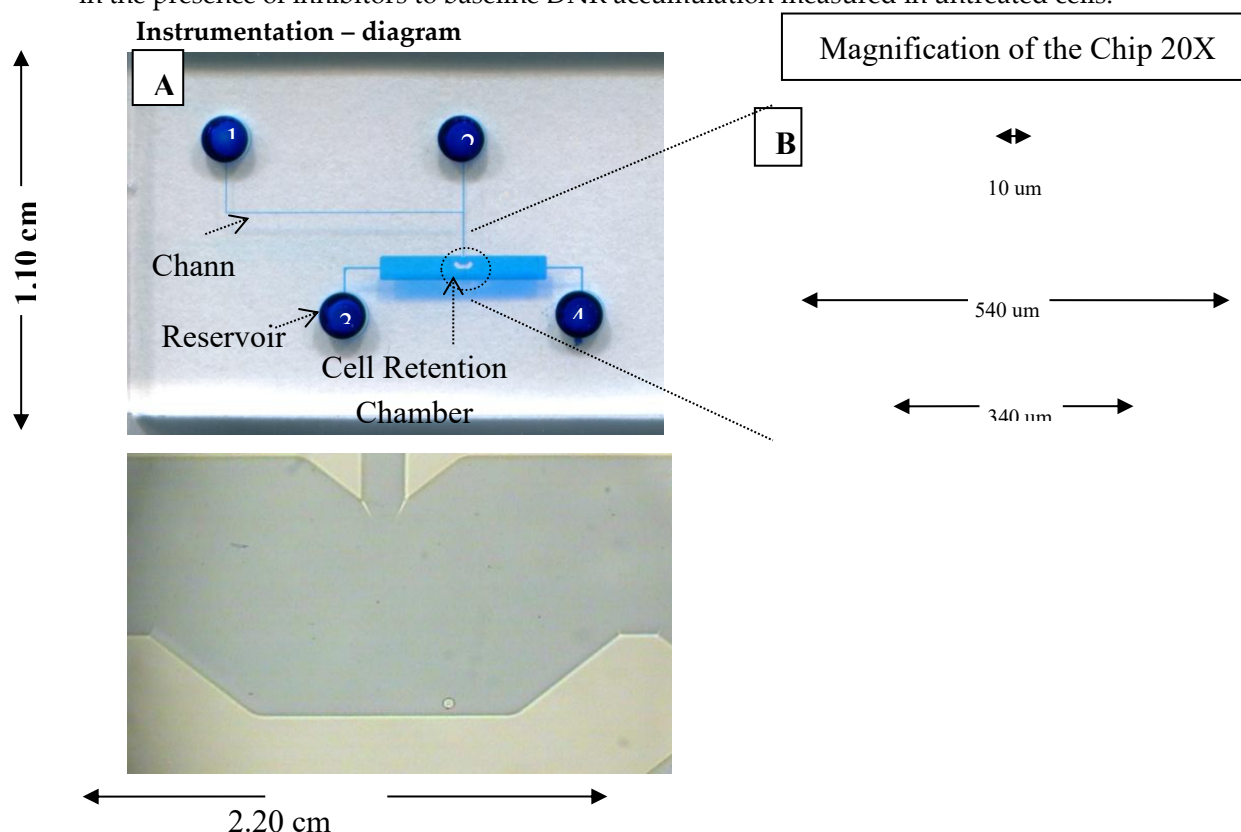


Figure 1. Design of the microfluidic single-cell chip. (A) A photograph image of the microchip colored with blue food dye. The microchip consists of 4 solution reservoirs and 1 cell retention chamber. The dimensions of the microchip are 1.10 cm x 2.20 cm. (B) An image of the retained CEM/VLB1000 cell under 20x magnification, with chamber and channel dimensions shown in μm .

3. Results and Discussion

MDR reversal effect on DNR accumulation in the MDR cancer cells

We first evaluated the effect of PPD on intracellular DNR accumulation in MDR cancer cells. As shown in Figure 2A, when only DNR solution was given to the cell, cell fluorescence increased, indicating continuous DNR accumulation. After ~700 s, fluorescence reached a steady state, reflecting equilibrium between DNR influx and efflux via P-gp transporters. Upon addition of DNR solution

containing Rg3-R, intracellular DNR accumulation increased, indicating MDR reversal. After background correction (Figure 2B), DNR accumulation increased 1.6-fold in the presence of PPD, demonstrating MDR reversal.

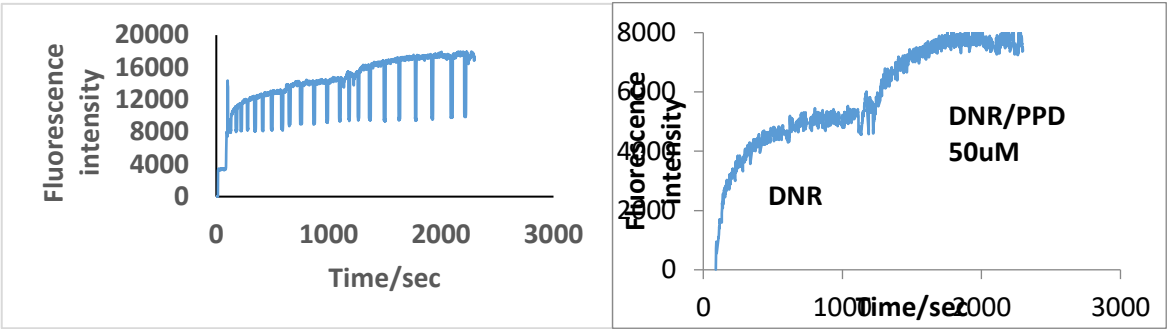


Figure 2. Uptake enhancement of DNR due to PPD 50 μ M on a single-cell.

We next applied the same uptake procedure to Rg3-R, Rg3-S, and PPT. The results of the uptakes are summarized below. As shown in Table 1, the ginsenosides have a dose-dependent effect on CEM/VLB1000 cells. Intracellular DNR accumulation doubled with rising ginsenoside concentrations, indicating a dose-dependent effect. This result is superior to a previous study where the effective Rg3 concentration for MDR reversal as measured by the rhodamine 123 accumulation assay was observed not at 100 μ M, but at 320 μ M level, though that as measured by the cytotoxicity assay was observed at 5-40 μ M level (Kim et al. 2003).

Although Rg3-R and Rg3-S share similar chemical structures, Rg3-S was more potent at modulating MDR. The more potent effect of Rg3-S was also observed in its effect on insulin secretion (Park et al 2008) and anti-apoptotic effect (Min 2006).

Table 1. Mean/SD of the Ginsenosides and CEM/VLB Cell.

		Compound					
		DNR 35 μ M	PPT/ DNR 35 μ M	PPD/ DNR 35 μ M	Rg3-S/ DNR 35 μ M	Rg3-R/ DNR35 μ M	
[]	[35] μ M (1)	1.35 $\pm$ 0.06 (n=4)	[50] 2.14 $\pm$ 0.67 (n=5)	1.68 $\pm$ 0.15 (n=4)	2.05 $\pm$ 0.07 (n=2)	2.07 $\pm$ 0.29 (n=3)	
	[35] μ M (2)	1.38 $\pm$ 0.22 (n=4)	[100] 3.52 $\pm$ 1.06 (n=5)	2.30 $\pm$ 0.36 (n=4)	5.55 $\pm$ 0.35 (n=2)	3.67 $\pm$ 1.16 (n=3)	

DNR accumulation control experiments inside the MDR cancer cells

To confirm that increased DNR accumulation was not caused by fluctuations from solution switching, we used DNR only solution to replace DNR/Rg3 solution. Specifically, DNR solution was added sequentially three times to the same cell, and accumulation was measured after each addition. The rate of accumulation curve of Daunorubicin 35 μ M in CEM/VLB1000 subline is shown in Graph 1. DNR was added into reservoir 2 at 100 seconds of the drug into the cell. After addition, internal DNR accumulation increased until 500s, after which the accumulation reached a plateau of 940.04 and the efflux of the drug out of the cell equated to intracellular accumulation. As well, switching drugs (DNR 35 μ M) at 1100s and at 2100s did not increase intracellular accumulation of the drug significantly.

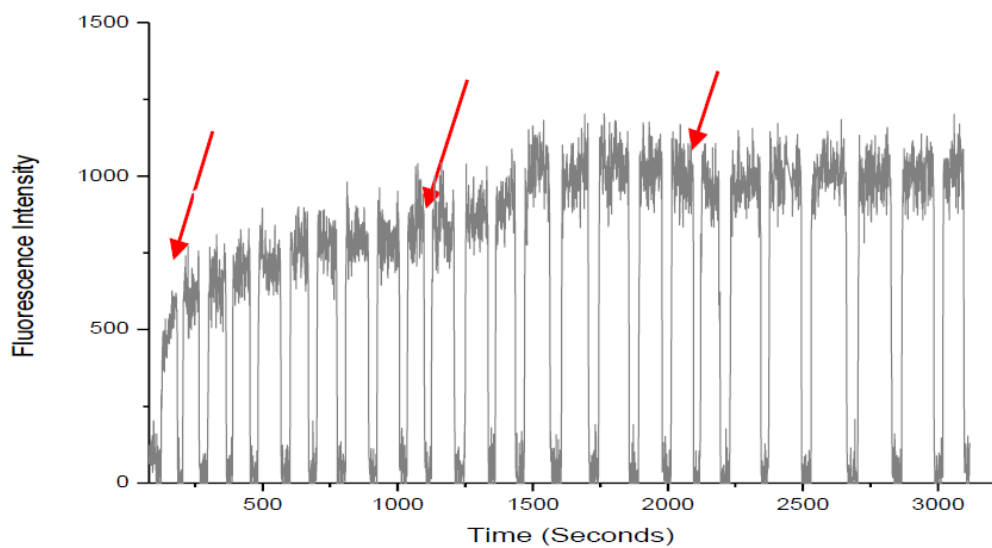


Figure 3. Negative control in Single-Cell Chip.

We repeated the same experiments on 3 different cells, and the results were shown in table 2. The results clearly showed that DNR accumulation will not increase dramatically after it reaches the steady state simply by repeating adding DNR to the cell, thus confirmed that the enhanced DNR accumulations were because of the MDR reversal effects of Rg3-R and Rg3-S in the above section.

Table 2. DNR accumulation control experiments inside the MDR cancer cells.

	DNR 35 μ M initial accumulation		
	Cell 1	Cell 2	Cell 3
DNR 35 μ M 1	1.4	1.3	1.3
DNR 35 μ M 2	1.3	1.2	1.3
Cell image			

When we compared the ginsenosides between two cell lines, CEM/VLB1000 and CEM/wt, it was evident that the dose-dependent relationship is not as strong in the CEM/wt, i.e. 1.70 ± 0.141 ($n=2$) for 50 μ M and 2.35 ± 0.495 ($n=2$) for 100 μ M. This may be due to the evidence that CEM/wt does not possess as many P-gp pumps as the CEM/VLB1000 subline.

4. Conclusions

We evaluated intracellular DNR accumulation in the presence of four ginsenosides: Rg3-S, Rg3-R, PPD, and PPT. Using the CEM/VLB1000 leukemia cell line, we compared intracellular DNR accumulation in the presence of each ginsenoside to evaluate their relative efficacy at reversing MDR-mediated drug efflux. It was found that although Rg3-R and Rg3-S share similar chemical structures and are aglycones of PPD, Rg3-S exhibited greater potency in enhancing intracellular DNR accumulation, and both Rg3 stereoisomers were more effective than PPD.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

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Conflicts of Interest: The authors declare no conflict of interest

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