

Long-term exposure in high viscous microenvironment facilitates acquired drug resistance of colon cancer cells to doxorubicin

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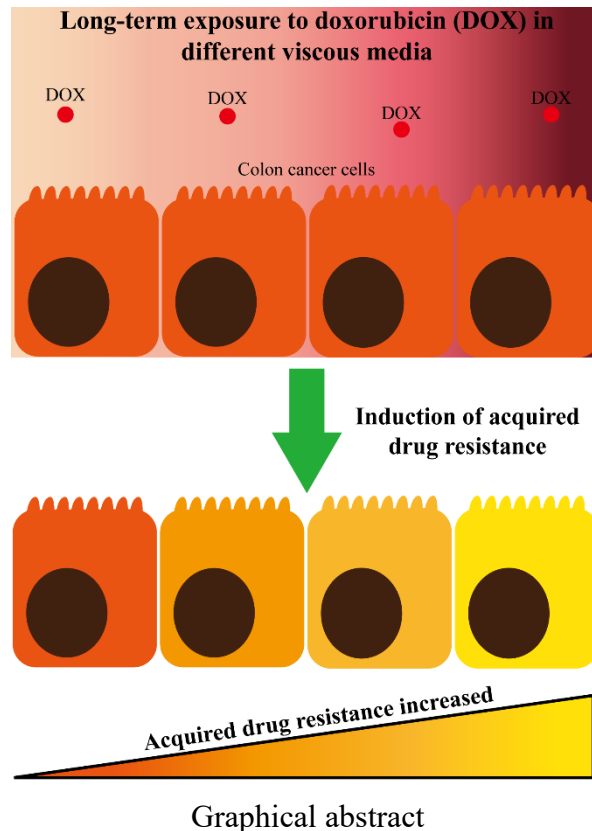
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KEYWORDS: cellular microenvironment; viscosity; acquired drug resistance; colon cancer cells; cancer therapy.

Highlights:

1. This study is the first to demonstrate the accelerating effect of microenvironmental viscosity on acquired drug resistance (ADR) of colon cancer cells to DOX.
2. High viscosity facilitates the ADR of colon cancer cells.
3. The influence of microenvironmental viscosity on ADR is mediated by the expression of drug resistance-related genes.

1 ABSTRACT: Chemotherapy is one of the most common strategies for treating
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3 colorectal cancer (CRC). However, acquired drug resistance (ADR) impairs the
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5 efficiency of chemotherapy. For CRC treatment, the long-term administration could
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7 affect cancer microenvironment and induce environment-mediated drug tolerance that
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9 contributes to ADR. Nevertheless, it is elusive that how the alteration of
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11 microenvironment affects the ADR of colon cancer cells. In this study, the effect of
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13 microenvironmental viscosity on ADR of colon cancer cells to doxorubicin (DOX) was
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15 investigated by long-term culture of colon cancer cells in different viscous media
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17 supplemented with DOX. The 50% inhibitory concentration (IC₅₀) value of drug-
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19 resistant cells established by culturing in high viscosity media was significantly higher
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21 than that of cells cultured in low viscosity media. The drug-resistant colon cancer cells
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23 established in high viscosity media exhibited higher expression levels of drug
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25 resistance-related genes (ABCC2 and ABCG2), higher migration ability and lower
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27 proliferation ability than the cells established in low viscosity medium. Long-term
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29 exposure in DOX-containing high viscosity media made the cells more tolerant to DOX
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31 and facilitated the ADR of colon cancer cells to DOX. The results disclosed the
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33 importance of microenvironmental viscosity on the ADR development and should
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35 provide useful information for cancer therapy.
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Introduction

Oncological resection with central ligation of the supplying vessels and radical lymphadenectomy is the standard operation in colorectal cancer (CRC) surgery. Although it is often viewed as a simple training procedure, the recurrence rate of CRC is largely determined by the quality of the surgery, which can significantly affect the prognosis of patients [1]. For CRC patients who cannot achieve a curative resection (R0) by surgery, adjuvant treatment such as chemotherapy or radiotherapy is necessary [2].

Chemotherapy by using cytotoxic pharmaceuticals to eradicate the cancer cells is one of the most frequently used clinical therapeutic methods for colorectal cancer (CRC) [3]. However, some types of cancer, such as acute myeloid leukemia, ovarian cancer

1 and colon cancer, have the problem of acquired drug resistance (ADR), which describes
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3 the ability of cancer cells to initially respond to chemotherapy but gradually become
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5 resistant to drugs after a period of treatment [4,5]. ADR starts after chemotherapy and
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7 is considered as a comprehensive result of time- and therapy-dependent impact, which
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9 impairs the efficiency of chemotherapy and results in treatment refractory [4,6].
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15 Clinically, duration of colon cancer chemotherapy is approximately 3 to 6 months and
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17 prolonging the treatment time has not been shown to be more effective than a shorter
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19 cycle [7]. Although the treatment time can be different depending on the treatment
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21 conditions (patient characteristics, geographical location, dosage regimen and disease
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23 stage), the occurrence of chemoresistance is hard to avoid [8]. To mimic this process in
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25 laboratory, long-term exposure to anticancer drugs is a common method that is widely
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27 known for establishing drug-resistant cells [9,10]. Differing from clinical treatment, *in*
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29 *vitro* construction cycle is around 2 to 3 months [11]. Moreover, the drug-resistant
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31 ability merely be adjusted by exposure conditions of drug molecules, such as exposure
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33 times and exposure dose [9,12,13] but without consideration of drug excipients.
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43 The reason for emphasizing this point is that the excipients has been reported to affect
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45 some aspects of tissues in a concentration- or time-dependent manner, even directly
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47 affect the drug resistant-related protein [14]. Traditionally, mechanisms of ADR have
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49 been believed as acquired changes in the expression or function of specific genes
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51 products [15]. However, in addition to mechanisms of drug resistance within the cells,
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58 there are dynamic and de novo mechanisms coordinated by tumor microenvironment,
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1 known as environment-mediated drug resistance [16]. For example, lung cancer cells
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3 co-cultured with microenvironment-associated factors exhibit high tolerance to various
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5 chemotherapies [17]. Breast cancer cells cultured in rigid hydrogels display higher
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7 resistance to doxorubicin (DOX) than the cells cultured in soft hydrogels [18].
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12 In our previous research, the high viscosity of the microenvironment promoted de novo
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14 chemoresistance of colon cancer cells to DOX, which was due to the high expression
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16 levels of ABCC2 (ATP-binding cassette sub-family C member 2) and ABCG2 (ATP-
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18 binding cassette super-family G member 2) mRNAs when cells were cultured in the
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20 high viscosity medium. The high viscosity medium also facilitated cell migration and
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22 inhibited cell proliferation, which further promoting the chemoresistance of colon
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24 cancer cells to DOX [19]. These results demonstrated the effect of viscosity on colon
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26 cancer cells provided an initial stimulation that contributes to the emergence of de novo
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28 drug resistance to DOX. However, there is still no direct evidence that the high viscosity
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30 of the microenvironment can affect the acquired drug-resistant ability of colon cancer
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32 cells after long-term treatment.
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44 Given that long-term administration can lead to ADR, and the effects of pharmaceutical
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46 excipients on ADR through altering the microenvironment remain unclear, it is essential
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48 to firstly investigate the cellular responses to the alteration of microenvironment for
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50 further understand the role of excipients during long-term treatment, as it holds
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52 significant clinical implications for guiding future drug administration. Therefore, in
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54 this study, the effect of viscosity on ADR of CRC cells was elucidated by culturing
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CRC cells in media of different viscosity for a long period. Polyethylene glycol (PEG) was added in DMEM culture media to adjust the viscosity of media. CRC cells were cultured in the viscous DMEM media for an extended period (12 weeks) in the presence of DOX. The change of drug-resistant ability of CRC cells was analyzed and the underlying mechanism was discussed.

Materials and methods

Preparation of culture medium of different viscosity

A high-molecular weight PEG (PEG 8M, Mw 8,000,000, Sigma-Aldrich, USA) and a low-molecular weight PEG (PEG 35K, Mw 35,000, Sigma-Aldrich, USA) were used to adjust viscosity of cell culture media. PEG solution (1.5% (wt/v)) was prepared by dissolving PEG powder in Milli-Q water and sterilized by using a Millipore syringe filter with 0.45 μm mesh size (Merck Millipore). 5.75-fold concentrated Dulbecco's modified Eagle's medium with high glucose (Sigma-Aldrich) was prepared by dissolving DMEM powder with high glucose (DMEM) in Milli-Q water, and sterilized by using a Millipore syringe filter with a mesh size of 0.45 μm . The complete DMEM of different viscosity was prepared by mixing the PEG solution (1.5% (wt/v)) and DMEM solution (5.75-fold) at a volume ratio of 8:2, supplemented with fetal bovine serum (10% (v/v)), L-glutamine (584 mg L^{-1}) and antibiotics (100 U mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin) to achieve a final concentration of 1-fold DMEM and 1.0% (wt/v) PEG. The culture medium prepared by adding PEG 8M, PEG 35K and their 3:1

1 mixture was designated as H-DMEM, L-DMEM and M-DMEM, respectively. The
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3 DMEM without PEG addition was designated as N-DMEM. The culture medium
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5 containing DOX was prepared by adding DOX solution (initial concentration 10 mg
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7 mL⁻¹) to the culture medium to achieve required concentration and then mixed well by
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9 stirring overnight.
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13 **Measurement of viscosity**

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19 An MCR 302 rheometer (Anton Paar, Germany) was used to measure the viscosity of
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21 the cell culture medium by a rotational shear mode as previously described [20]. 2 mL
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23 of the sample was placed between two parallel plates (PP-50) with a testing gap of 1.0
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25 mm. Low-viscosity silicone oil was used to cover the edge of the sample to prevent
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27 evaporation during measurement. The measurement was conducted at shear rates
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29 ranging from 0.1 to 100 s⁻¹ at 37 °C. The viscosity at a shear rate of 0.1 s⁻¹ was
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31 considered as the zero-shear viscosity. Quadruplicate samples were used for each
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33 measurement to calculate the means and standard deviations.
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42 **Cell Culture**

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46 Colorectal cancer cell line SW480 (CCL-228, ATCC, USA) was cultured in DMEM
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48 serum medium containing 10% fetal bovine serum (FBS, Gibco), L-glutamine (Sigma)
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50 and antibiotics (100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin) in a humidified
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52 incubator (5% CO₂, 37 °C). The cells were subcultured after reaching a confluence
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54 around 75%.
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Cellular uptake of DOX

To investigate whether medium viscosity affects the diffusion of DOX, the cellular uptake of DOX in SW480 cells cultured in different viscous media for 6 to 24 h was compared using fluorescence observation and intensity quantification. For fluorescence observation, sub-cultured cells were seeded in a 6-well plate at a cell density of 5×10^4 cells per well. After 12 h of seeding, the culture medium was replaced with different viscous culture media containing DOX (0.1 mg/L), and the cells were continuously cultured. At predetermined time points (6 h, 12 h and 24 h after incubation), the culture media were withdrawn, and the cells were washed by $1 \times$ PBS thrice to remove excess DOX. Intracellular DOX was then observed using a confocal microscope (ZEISS LSM 900, German).

For quantification analysis, the sub-cultured SW480 cells were seeded into a 6-well plate at a cell density of 5×10^4 cells per well. After seeding for 12 h, the culture medium was replaced with different viscous media containing DOX (0.1 mg/L), and the cells were continuously cultured. After 6h, 12h, and 24 h culture, the culture media were withdrawn, and the cells were washed by $1 \times$ PBS thrice to remove excess DOX. The cells were then suspended in $1 \times$ PBS, and cell numbers were counted by using a cell counting chamber. Afterward, the cells were dissolved in HCl solution (0.5 mol/L) for 1 h at room temperature and the fluorescence intensity of the solution was measured using a fluorescence spectrophotometer (FP8500, JASCP, Japan) to calculate the intracellular uptake of DOX per cell. Triplicate samples were used for each

measurement to calculate the means and standard deviations.

Establishment of drug-resistant colon cancer cells

The drug-resistant colon cancer cells (SW480/D) were established using a pulse strategy by gradually increasing the concentration of DOX as previously described [12,13]. Briefly, sub-cultured SW480 cells were detached from the culture plate using trypsin-EDTA solution and seeded in T25 cell culture flask with 1×10^6 cells. After cell seeding for 12 h, the culture medium was withdrawn and fresh culture media of different viscosity supplemented with DOX were added. The DOX concentration was initially 0.1 mg L^{-1} and then gradually increased. After 72 h of culture, the culture medium was withdrawn and the cells were continuously cultured in the drug-free medium of different viscosity. After the cells were passaged once, the process was repeated with the same DOX concentration. Then, the DOX concentration was doubled and the process was repeated until a concentration of 1.6 mg L^{-1} DOX was reached. The construction process lasted at least 5 cycles (12 weeks). Afterward, the surviving resistant colon cancer cells were cultured in N-DMEM without the addition of DOX for at least 2 weeks before conducting the following experiments. The drug-resistant colon cancer cells established by culturing the cells in N-DMEM, L-DMEM, M-DMEM and H-DMEM were named as SW480/D/N, SW480/D/L, SW480/D/M and SW480/D/H, respectively. The parental cells (SW480) were used as a control.

Measurement of 50% inhibitory concentration of doxorubicin (DOX) to drug-resistant colon cancer cells

The anticancer effect of DOX to the parental and the established drug-resistant colon cancer cells was investigated by the water-soluble tetrazolium salt-1 assay (WST-1 assay) as described previously [21]. DOX with concentrations ranging from 100.00 $\mu\text{g mL}^{-1}$ to 0.01 $\mu\text{g mL}^{-1}$ were prepared by dissolving DOX·HCl powder in sterilized Milli-Q water and diluting to the required concentration. The sub-cultured cells were seeded in a 96-well plate at a cell density of 5×10^3 cells per well. After culture for 12 h, the culture medium was replaced with fresh culture medium containing different concentration of DOX or without DOX and the cells were continuously cultured for 72 h. And then, the culture medium was withdrawn and 100 μL WST-1 working solution, which was diluted by fresh DMEM at a volume ratio of 1:10, was added to each well. Afterwards, the plate was placed in a 37 °C incubator and incubated for 2 h. The absorbance of the sample at 440 nm was measured as the optical density value (OD) by using a microplate reader (Spark Multimode Microplate Reader, Tecan). The anticancer effect of DOX to colon cancer cells was calculated using the following formula:

$$\text{inhibition rate (\%)} = [(\text{OD}_{\text{medium without DOX}} - \text{OD}_{\text{medium with DOX}}) / \text{OD}_{\text{medium without DOX}}] \times 100.$$

The concentration of DOX that induced a 50% inhibition rate of colon cancer cells was defined as the 50% inhibitory concentration (IC_{50}) value [22]. Triplicate samples were used for each measurement to calculate the means and standard deviations.

Real-time PCR analysis (RT-qPCR analysis) of drug resistance-related genes

The mRNA expression levels of ABCC2 and ABCG2 in the cancer cells were analyzed by real-time PCR as previously described [18]. The sub-cultured cells were detached

from the culture plate using trypsin-EDTA solution and 1 mL Sepasol solution (Nacalai Tesque, Kyoto, Japan) was added for total RNA extraction. A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used for the reverse transcription of complementary DNA (cDNA) from 2 µg of purified total RNA. The cDNA was used as a template for RT-qPCR analysis. The amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ABCC2 and ABCG2 was conducted using a QuantStudios 3 Real-Time PCR system (Thermo Fisher Scientific). GAPDH (Hs99999905_m1) was used as a housekeeping gene (Thermo Fisher, Japan). The pre-designed primer and probe sequences of ABCC2 (Hs00960489_m1) and ABCG2 (Hs01053790_m1) were used. The relative expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the endogenous control. Triplicate samples were used for each measurement to calculate the means and standard deviations.

Immunofluorescence staining of drug resistance-related genes

Immunofluorescence staining for ABCC2 and ABCG2 was performed to evaluate the expression levels of drug resistance-related proteins in colon cancer cells after long-term culture in different viscous media. In brief, following long-term culture as described above, sub-cultured SW480/D/N, SW480/D/L, SW480/D/M, SW480/D/H, and parental SW480 cells were seeded into 6-well plates at a cell density of 5×10^4 cells per well. After 12 h of culture in normal viscous medium, the culture medium was removed, and the cells were fixed with 4% paraformaldehyde for 10 min at room

temperature. The cells were then washed by 1 × PBS thrice and blocked by incubation in blocking buffer (1 × PBS containing 1% BSA and 0.1% Tween 20) overnight at 4 °C. Following blocking, the cells were washed by 1 × PBS thrice and incubated overnight at 4 °C with either anti-ABCC2 antibody (MRP2/ABCC2, Cell signalling technology, CST12559) or anti-ABCG2 antibody (BCRP/ABCG2, Abcam, ab207732) diluted 1:500 in antibody dilution buffer (1 × PBS containing 1% BSA). After incubation, the cells were washed with 1 × PBS thrice and incubated with a secondary antibody (Alexa fluor 488 donkey anti-rabbit IgG, 1:1000 dilution, H + L, A21206, Invitrogen) for 1 h at room temperature in the dark. After incubation, the cells were then washed by 1 × PBS thrice and stained with Hoechst 33342 (1:1000 dilution, 343-07961, FUJIFILM Wako Pure Chemical) for 10 min at room temperature. Finally, the stained cells were observed and recorded using a fluorescence microscope (BZ-X710).

Cell migration assay

The cell migration ability was analyzed by a scratch assay. The sub-cultured cells were seeded in a 6-well plate (5×10^6 cells per well) to obtain approximately 95% confluence in each well after culture for 12 h. The cell monolayer was wounded by scratching with a 10 µL pipette tip. The cells were washed with 1 × PBS to remove the detached cells and the culture medium was replaced with fresh culture medium. Images of the scratch were taken immediately after wounding and every 24 h thereafter by using a microscope (Olympus, Japan) until 72 h. 8 to 10 fixed positions of each well were imaged. The average area of each scratch was analyzed by an imageJ software. The wound recovery

rate was calculated using the following formula: recovery rate (%) = $\frac{((\text{area at 0 hour}) - (\text{area at 24 to 72 hours}))}{(\text{area at 0 hour})} \times 100$. Quadruplicate samples were used for each measurement to calculate the means and standard deviations.

Actin Filaments staining

Actin filaments are the major component of the cytoskeleton which play a key role in regulating cell function. Immunofluorescence staining for actin filaments was performed to evaluate the alteration of cytoskeleton management in colon cancer cells after long-term culture in different viscous media. In brief, following long-term cultured as described above, sub-cultured SW480/D/N, SW480/D/L, SW480/D/M, SW480/D/H, and parental SW480 cells were seeded into 6-well plates at a cell density of 5×10^4 cells per well. After 24 h of culture in normal viscous medium, the culture medium was removed, and the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were then washed by $1 \times$ PBS thrice, blocked and permeabilized by incubation in blocking buffer ($1 \times$ PBS containing 1% BSA, 0.2% Triton X-100 and 0.1% Tween 20) overnight at 4 °C. Following blocking, the cells were washed by $1 \times$ PBS thrice and stained with Alexa Fluor VR 488 phalloidin (1:40 dilution), while stained with Hoechst 33342 (1:1000 dilution, 343-07961, FUJIFILM Wako Pure Chemical) for 10 min at room temperature. Finally, the stained cells were observed and recorded using a fluorescence microscope (BZ-X710).

Analysis of cell proliferation in drug-free medium

The proliferation ability of the established drug-resistant colon cancer cells was

analyzed by using WST-1 assay. Briefly, the sub-cultured cells were seeded in a 48-well plate at a concentration of 5×10^3 cells mL⁻¹. After an initial culture for 12 h, the culture medium was replaced with fresh culture medium. After culture for 24, 48 and 72 h, the culture medium was removed and 250 µL WST-1 working solution was added to each well. Afterwards, the cells were cultured in a 37 °C incubator for 2 h and the absorbance of the sample at 440 nm was measured as described above. The cell number was calculated by a standard curve between cell number and OD value (Supplementary Figure S1). Triplicate samples were used for each measurement to calculate the means and standard deviations.

Statistical analysis

All quantitative experiments were repeated in triplicate to quintuplicate to calculate the means and standard deviations (S.D.). Statistical analysis of the experimental data was performed by a GraphPad Prism software. Two-way ANOVA with Dunnett's multiple comparison test was used to compare the samples. The p value was used to determine the level of significance: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. $p > 0.05$ indicates that there is no significant difference, denoted as ns.

Results

Viscosity of cell culture medium supplemented with PEG of different molecular weight

The viscosity of cell culture medium was analyzed using a rheometer at a shear rate ranging from 0.1 to 100 s⁻¹ at 37 °C. As shown in Figure 1A, the viscosity of all samples decreased with increasing shear rate. The H-DMEM showed the highest viscosity throughout the shear rate range. The zero-shear viscosity of all samples was measured at a shear rate of 0.1 s⁻¹. As shown in Figure 1B, the zero-shear viscosity of the culture medium increased with PEG molecular weight. By using low molecular weight PEG, high molecular weight PEG and their combination, the viscosity of culture medium could be adjusted from 92.5 to 759.2 mPa·s (Figure 1B). The PEG content supplemented in the L-DMEM, M-DMEM and H-DMEM was the same (1.0% (wt/v)).

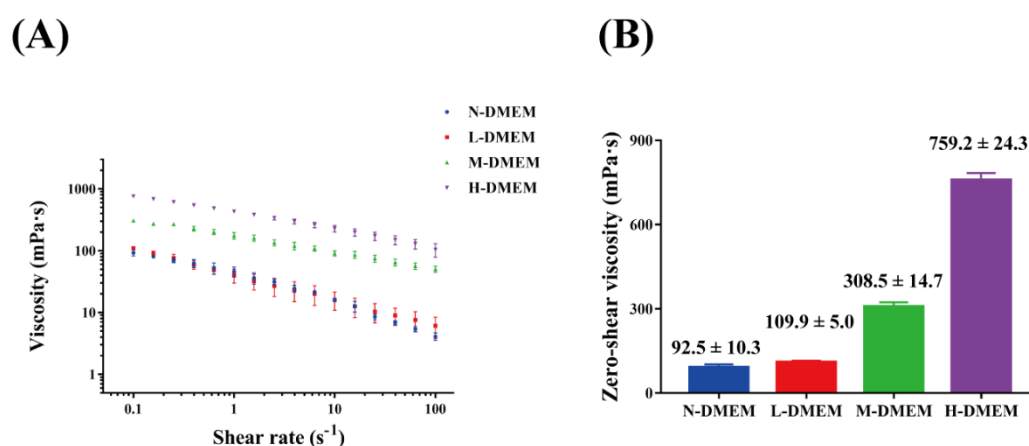


Figure 1. Viscosity of cell culture media supplemented with different molecular weight PEG. **A.** Apparent viscosity curve of cell culture media at shear rate ranging from 0.1 to 100 s⁻¹ at 37°C. **B.** Zero-shear viscosity of cell culture media measured at 0.1 s⁻¹ of shear rate. Data are the means ± S.D. (n = 4).

Effect of viscosity on the acquired drug resistance ability of colon cancer cells

during long-term exposure

After the colon cancer cells were cultured in different viscous media under the presence of DOX for 12 weeks, the drug-resistant colon cancer cells were established. Their proliferation characteristics and IC₅₀ values to DOX were analyzed by WST-1 assay to confirm the effect of viscosity on ADR. As presented in Figure 2A, the inhibition rate (%) of DOX to the parental colon cancer cells was higher than the drug-resistant colon cancer cells. The inhibition rate (%) at each concentration of DOX showed a decreasing trend with the increase of viscosity used to establish the drug-resistant colon cancer cells. The inhibition rate of DOX to the drug-resistant colon cancer cells established in H-DMEM was the lowest, while that of cells established in N-HDEM was the highest.

The IC₅₀ value was also dependent on the viscosity used to establish the drug-resistant colon cancer cells (Figure 2B). After being cultured in DOX-containing medium for 72 h, the IC₅₀ value of DOX to the parental colon cancer cells was 0.13 mg L⁻¹, which was significantly lower than that of all the established drug-resistant colon cancer cells. The IC₅₀ value showed an increasing trend with the viscosity of culture medium used for establishment of the drug-resistant colon cancer cells. The results suggested that long-term exposure to DOX contributed to ADR in colon cancer cells. When the cells underwent long-term exposure to DOX in high viscosity medium, their acquired drug-resistant ability was higher than that of the cells cultured in low viscosity medium (Figure 2B).

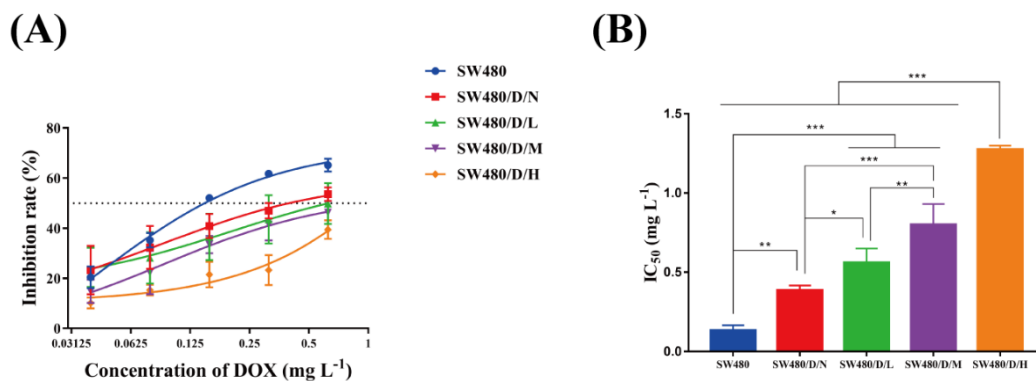


Figure 2. Anticancer effect of DOX to the parental and the established drug-resistant colon cancer cells. **A.** Inhibition rate of different concentration of DOX. **B.** IC₅₀ value of DOX. Data are the means $\pm$ S.D. (n = 3). Significant differences: * p < 0.05, ** p < 0.01, and *** p < 0.001.

Effect of viscosity on expression of ABC transporter genes during long-term exposure

The IC₅₀ value of colon cancer cells cultured in H-DMEM for a long time was the highest compared with the cells cultured in other viscous media, indicating that long-term exposure in a high viscosity medium promoted ADR. To investigate the possible mechanism of this effect, the expression levels of ABCC2 and ABCG2 mRNAs in the cells were analyzed by RT-qPCR and immunofluorescence staining (Figure 3). The expression level of ABCC2 mRNA in the parental colon cancer cells was significantly lower than that in all other drug-resistant colon cancer cells (Figure 3A). And the expression level of ABCG2 mRNA in the SW480 cells was significantly lower than

1 that in SW480/D/M and SW480/D/H cells (Figure 3B). The high expression levels of
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3 these genes could induce resistance to DOX because both ABCC2 and ABCG2 can
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5 trigger DOX efflux out of the cells, thereby decreasing the effective concentration of
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7 intracellular DOX [23]. Besides, the expression levels of ABCC2 and ABCG2 mRNAs
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9 varied among the drug-resistant colon cancer cells. The expression level of ABCC2
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11 mRNA in SW480/D/H and SW480/D/M was significantly higher than that in
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13 SW480/D/N and SW480/D/L (Figure 3A). The expression level of ABCG2 mRNA in
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15 SW480/D/M was also significantly higher than that in SW480/D/N and SW480/D/L
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17 (Figure 3B). Moreover, the immunofluorescence intensities of ABCC2 and ABCG2
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19 proteins in SW480/D/M and SW480/D/H cells were significantly higher than that in
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21 other cells, indicating higher expression levels of ABCC2 and ABCG2 proteins (Figure
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23 3C-D). The high viscosity of culture medium corresponded to high expression levels of
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25 ABCC2 and ABCG2 mRNAs and proteins. These results indicated that increasing the
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27 viscosity of culture medium during long-term culture could promote the expression
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29 levels of ABCC2 and ABCG2, which might be related to the promotive effect of
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31 viscosity on the ADR induction.
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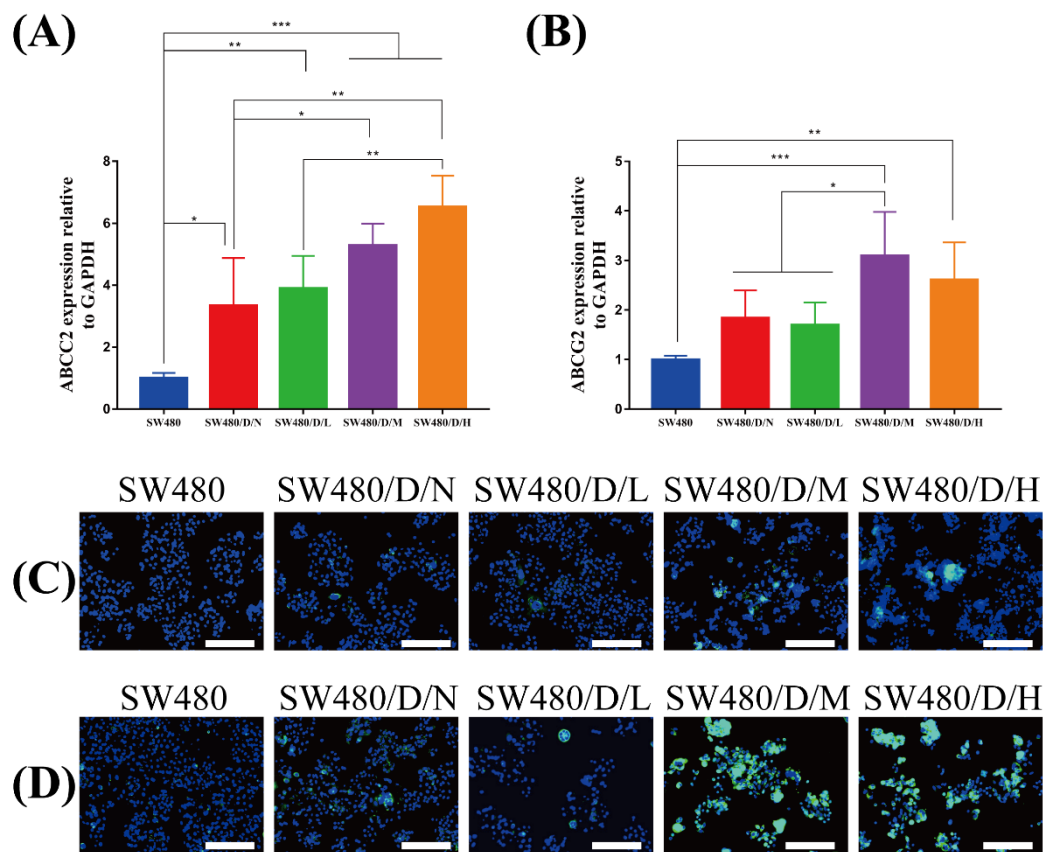


Figure 3. Quantified expression levels of **A.** ABCC2 and **B.** ABCG2 mRNAs in the parental and the established drug-resistant colon cancer cells after the cells were cultured in N-DMEM for 72 h. The data relative to GAPDH were normalized to the expression level in the parental colon cancer cells. Data are the means $\pm$ S.D. (n = 3). Immunofluorescence staining of **C.** ABCC2 and **D.** ABCG2 proteins in the parental and the established drug-resistant colon cancer cells after the cells were cultured in N-DMEM for 72 h. blue fluorescence: nuclei, green fluorescence: ABCC2 or ABCG2 protein. Scale bar: 200 μ m. Significant differences: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Migration ability of the established drug-resistant colon cancer cells

Cell migration is one of the critical characteristics of cancer cells, and alterations in this process are frequently observed in malignant cancer cells. The observed changes in the cytoskeleton of cells after long-term culture in different viscous media (Figure S3) suggested potential alterations in the migration ability of these cells. To investigate the migration ability of the parental and the established drug-resistant colon cancer cells, the scratch test was used to analyze cell migration speed. As shown in Figure 4, all the cells reached approximately 95% confluence before scratching. After scratching, the wound appeared in the monolayer of cells and the wound area decreased with the extension of incubation time (Figure 4A-E). Both the parental colon cancer cells and the established drug-resistant colon cancer cells could migrate to the scratched areas to heal the wounds. However, the established drug-resistant colon cancer cells showed faster movement toward the wound than did the SW480 cells (Figure 4B-E). Analysis of the recovery rate indicated that the SW480 exhibited the lowest recovery rate and the recovery rate increased with the increase in viscosity used for establishing the drug-resistant colon cancer cells (Figure 4F). The drug-resistant colon cancer cells established in high viscosity medium performed a higher migration ability than those established in low viscosity medium. The cells with higher acquired drug-resistant ability showed higher migration ability.

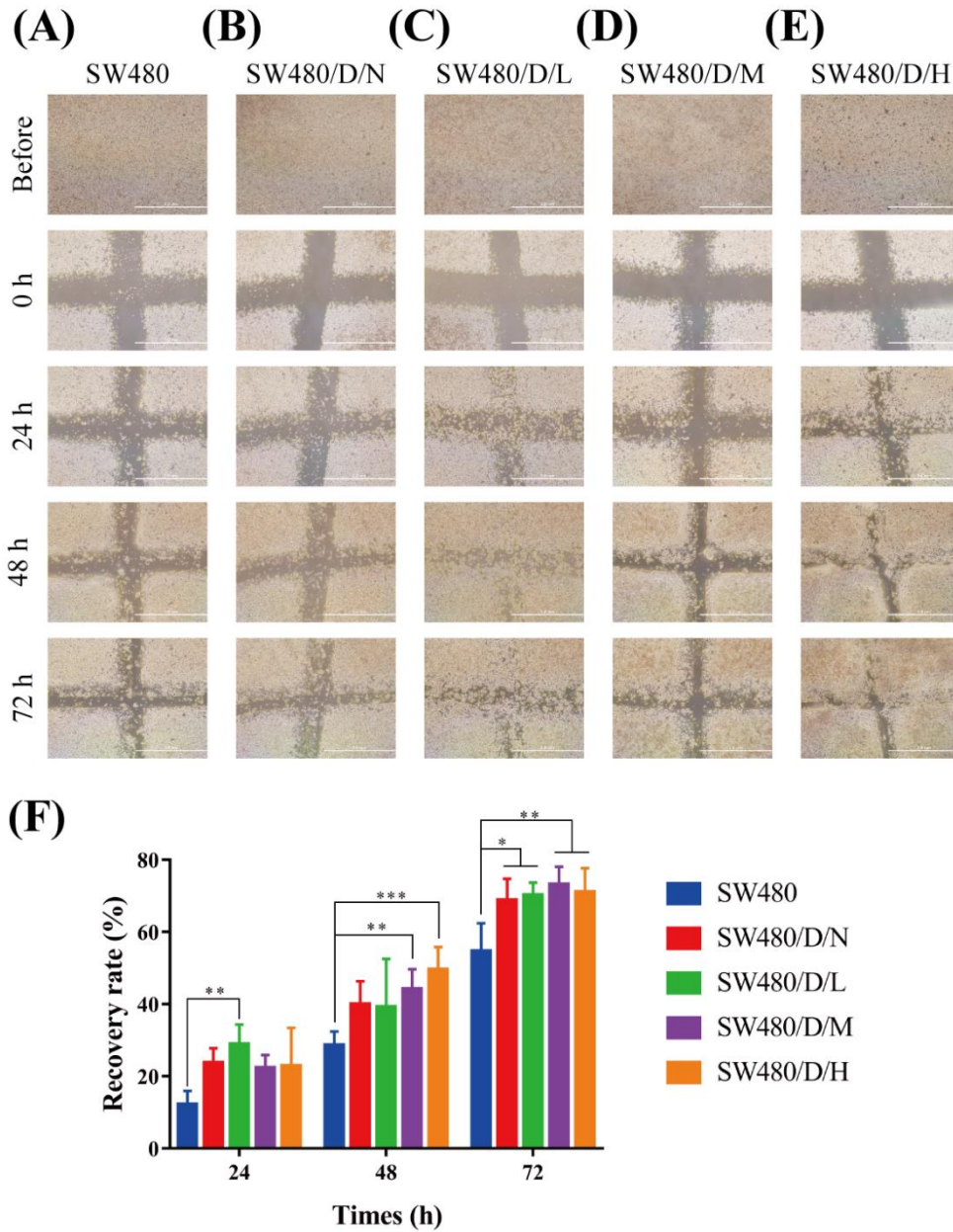


Figure 4. Migration ability of **A.** SW480, **B.** SW480/D/N, **C.** SW480/D/L, **D.** SW480/D/M and **E.** SW480/D/H cells during culture in N-DMEM for 72 h. Scale bar: 2 mm. **F.** Recovery rate of the colon cancer cells during culture in N-DMEM for 72 h. Data are the means $\pm$ S.D. (n = 4). Significant differences: * p < 0.05, ** p < 0.01 and *** p < 0.001.

Proliferation ability of the established drug-resistant colon cancer cells

To investigate whether the established drug-resistant colon cancer cells had different proliferation ability, cell proliferation was evaluated after 72 h culture in N-DMEM (Figure 5). The cell number in all the groups gradually increased with the extension of incubation time. After 24 h culture, the cell number among the groups was not significantly different. However, after culture for 48 h and 72 h, the cell number among the different groups became significantly different. The number of SW480 cells was the highest, while that of SW480/D/H cells was the lowest. The cell number decreased in an order of SW480, SW480/D/N, SW480/D/L, SW480/D/M and SW480/D/H. The result indicated that the proliferation ability of established drug-resistant colon cancer cells was inversely correlated with their acquired drug-resistant ability.

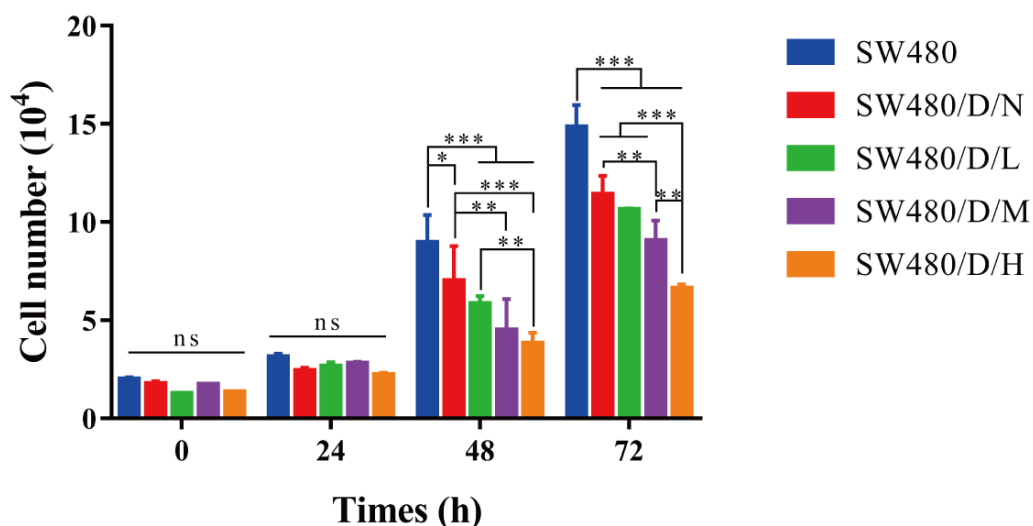


Figure 5. Proliferation ability of the parental and the established drug-resistant colon cancer cells after culture in N-DMEM for 72 h. Data are the means $\pm$ S.D. (n = 3).

Significant differences: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. ns: no significant different.

Discussion

Excipients are materials added to drug molecules during the manufacturing process, playing a crucial role in the efficacy, safety, and stability of medications [24]. Compared to short-term administration, the chemotherapy required long period not only tests the sustained efficacy of drug molecules but also poses significant challenges to the safety and therapeutic impact of excipients. Since the excipients could influence the tumor microenvironment either [14], the effects of microenvironmental changes on cellular drug sensitivity warrant attention. Understanding these effects has significant implications for guiding chemotherapy regimens. In this study, polyethylene glycol (PEG), one of the most commonly used pharmaceutical excipients [25], was utilized to alter the microenvironmental viscosity, and the CRC cells were cultured in medium of different viscosity under the present of DOX for a long-term period to investigate the influence of alteration of microenvironmental viscosity on the ADR development of CRC cells during long-term culture. The viscosity of the culture medium was adjusted by using the same concentration but different molecular weight PEG. The addition of 1% PEG with different molecular weights adjusted the zero-shear viscosity of the culture medium from 92.5 to 759.2 mPa·s. Since the mucus of colon tissue exhibits a viscosity ranging from 150 mPa·s to 250 mPa·s [26], the PEG-containing cell culture

media covered the viscosity range of intestinal mucus. Furthermore, as the diffusion of DOX showed minimal differences across culture media with varying viscosities (Figure S2), this culture system can be used to specifically study the effect of microenvironmental viscosity on the ADR of colon cancer cells.

The IC₅₀ value defines the concentration of anticancer drugs that can inhibit 50% of cancer cells growth, which has been used to evaluate the sensitivity of cancer cells to drugs [10,22]. The higher IC₅₀ value, the lower sensitivity of cells to drugs [10]. After long-term culture in viscous media under the presence of DOX, the colon cancer cells acquired resistance to DOX and exhibited a significantly higher IC₅₀ value than that of the parental cells. This might be attributed to the genetic alterations or epigenetic changes induced by DOX stimulation in viscous medium [27]. Furthermore, the drug-resistant colon cancer cells established in high viscosity medium exhibited a higher IC₅₀ value than the cells established in low viscosity medium. The SW480/D/H and SW480/D/M cells exhibited higher gene expression levels of ABC transporter family (ABCC2 and ABCG2 mRNAs), faster migration ability and lower proliferation ability compared to the SW480/D/N and SW480/D/L cells. The enhancement of ABCC2 and ABCG2 mRNA levels plays a crucial role in inducing drug efflux, which results in ADR [28]. Moreover, the increased cell migration ability and restricted cell proliferation ability indicated higher malignancy of the cancer cells. This is because malignant colon cancer typically displays higher invasion and metastasis ability [29], while low proliferation ability is one of the biological features that characterize aggressive colon cancer cells and is associated with poor prognosis [30]. These results suggested that

1 long-term exposure in high viscosity microenvironment could facilitate ADR
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3 development.
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7 So far, many studies have reported the relationship between ADR and cellular or
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9 molecular changes within individual cells selected under prolonged exposure to
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11 cytotoxic insult [12,22,31]. Since colon cancer cells constantly interact with their
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13 surrounding microenvironment [32], the effect of the cancer microenvironments on cell
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15 functions after long-term exposure should be particularly considered. In this study, we
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17 first demonstrated that colon cancer cells acquired higher drug-resistant ability after
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19 culture in high viscosity medium for an extended period, which verified that a high
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21 viscosity microenvironment could promote ADR of colon cancer cells to DOX and
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23 induce malignant translation of colon cancer cells. Based on the results, the
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25 pharmaceutical preparations which could affect cancer microenvironment should be
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27 regard a lot during long-term treatment of cancer. Besides, from the perspective of cell
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29 model establishment, viscosity of culture medium could be considered as a new
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31 adjustable condition for establishing drug-resistant cells during long-term exposure and
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33 utilization of high viscosity culture media may assist in obtaining chemo-resistant
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35 cancer cells with stronger resistance.
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53 **Conclusions**

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57 In this study, culture media with a viscosity range from 92.5 ± 10.3 mPa·s to $759.2 \pm$
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59 24.3 mPa·s were prepared by using 1% (wt/v) PEG of different molecular weights. The
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colon cancer cell line SW480 was cultured in the different viscosity media in the presence of DOX for an extended period (12 weeks) for ADR establishment. The ADR was dependent on the addition of DOX and the viscosity of culture medium. The addition of DOX induced ADR after long-term culture. The cells cultured in the highest viscosity medium obtained the highest drug-resistant ability. The cells cultured in high viscosity medium performed high malignancy with increased migration ability and decreased proliferation ability. The results suggested that a high viscosity microenvironment increased drug resistance-related genes expression, promoted cell migration ability and decreased cell proliferation ability in colon cancer cells after long-term culture.

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Authorship contribution statement

Guoping Chen and Naoki Kawazoe: Conceptualization; **Guoping Chen, Naoki Kawazoe, Toru Yoshitomi, Yingnan Yang, and Tianjiao Zeng:** Software; **Tianjiao Zeng and Naoki Kawazoe:** Validation; **Tianjiao Zeng, and Chengyu Lu:** Formal analysis; **Tianjiao Zeng, and Chengyu Lu:** Investigation; **Guoping Chen, Naoki Kawazoe, Toru Yoshitomi:** Resources; **Tianjiao Zeng, Chengyu Lu, Man Wang,**

and Huajian Chen: Data curation; **Tianjiao Zeng and Guoping Chen:** Writing-original draft preparation; **Tianjiao Zeng, Chengyu Lu, Man Wang, Huajian Chen, Naoki Kawazoe, Toru Yoshitomi, Yingnan Yang, and Guoping Chen:** Writing-review and editing; **Tianjiao Zeng and Chengyu Lu:** Visualization; **Guoping Chen:** Supervision; **Guoping Chen and Naoki Kawazoe:** Project administration.

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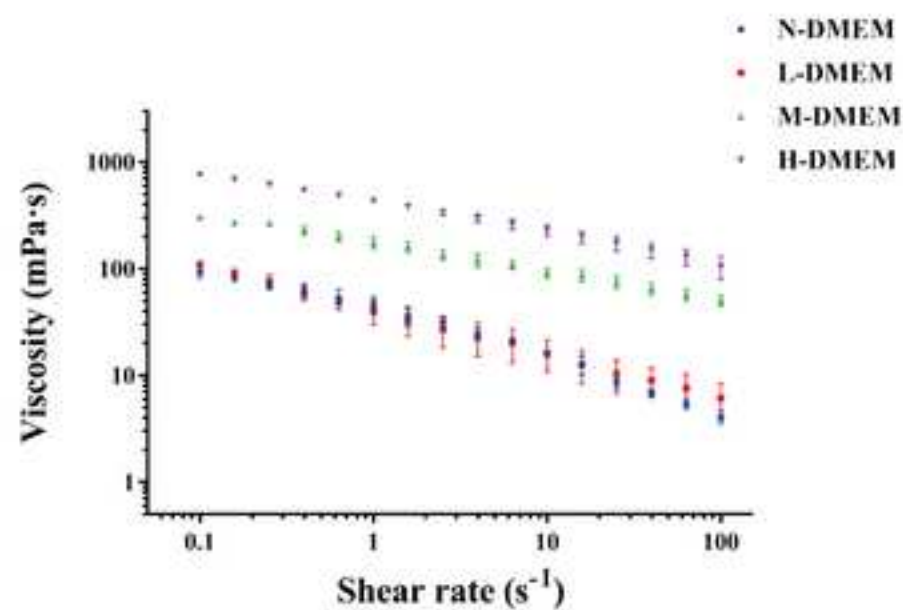
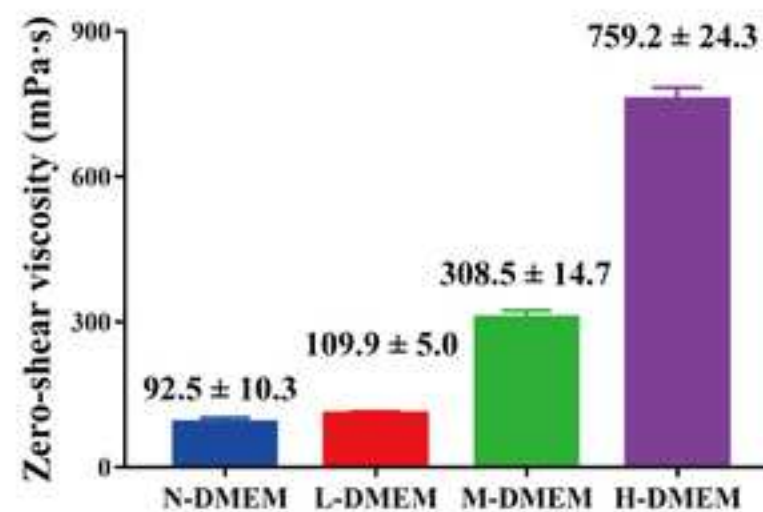
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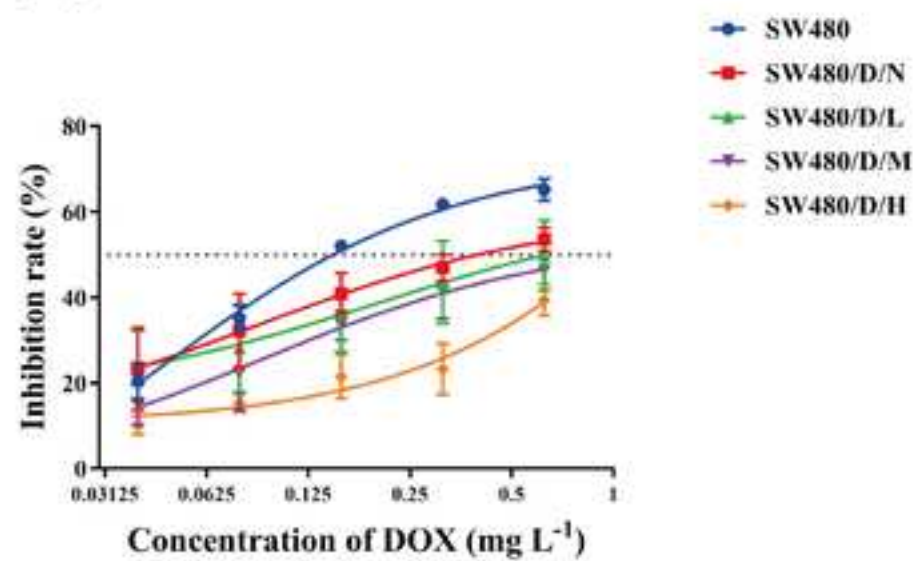
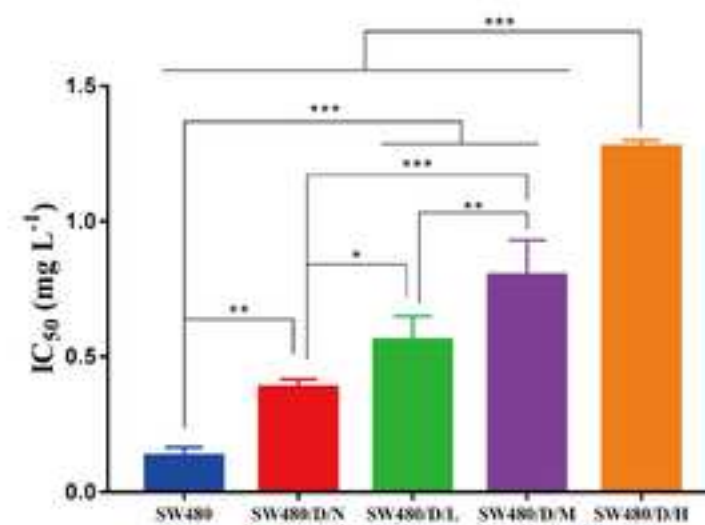
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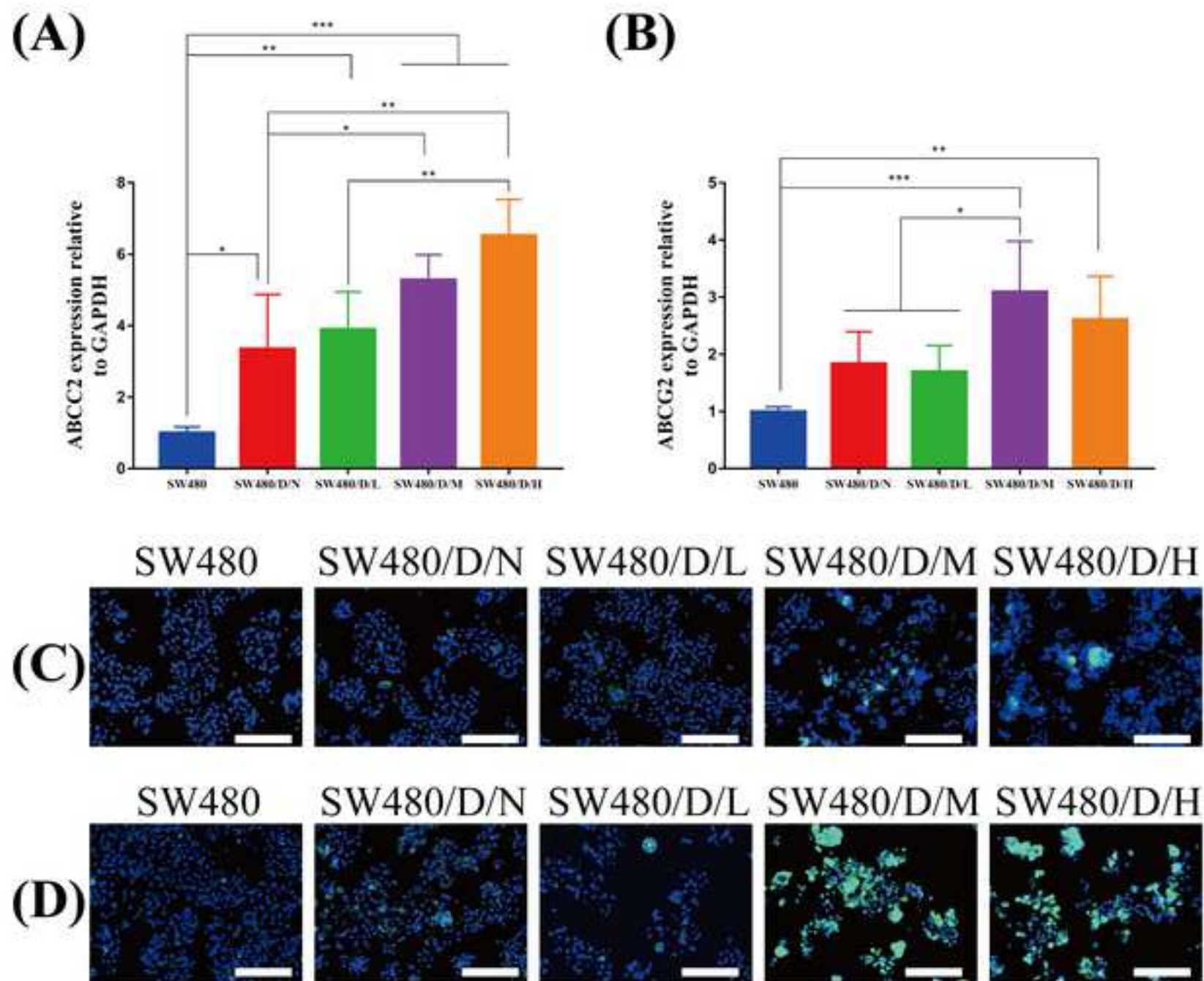
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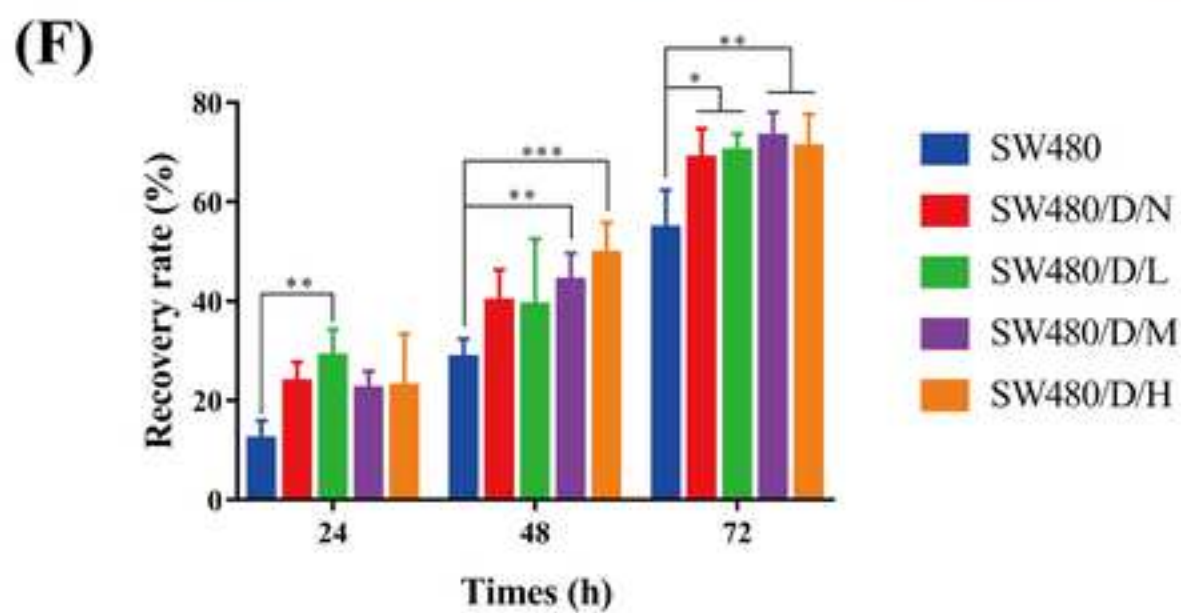
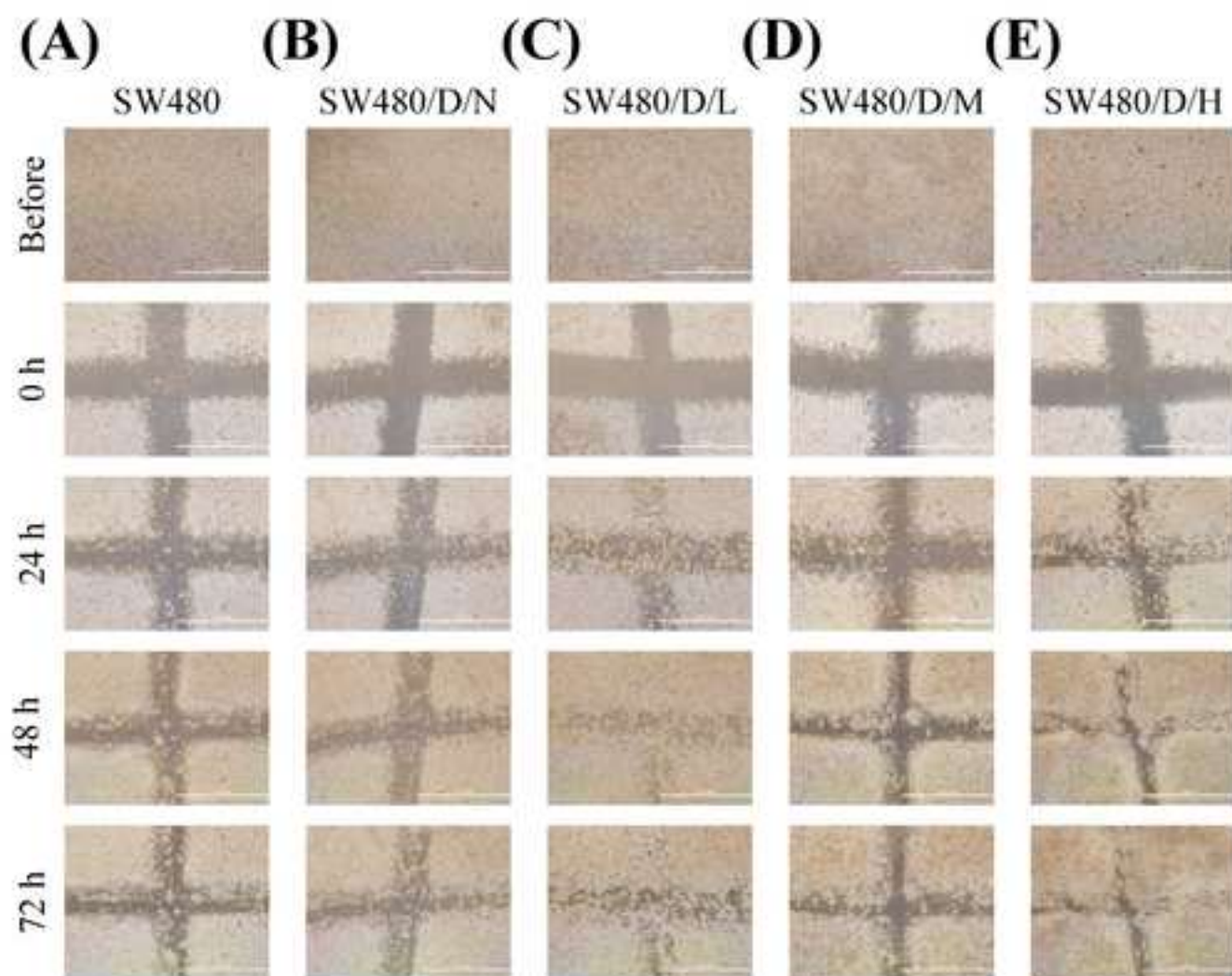
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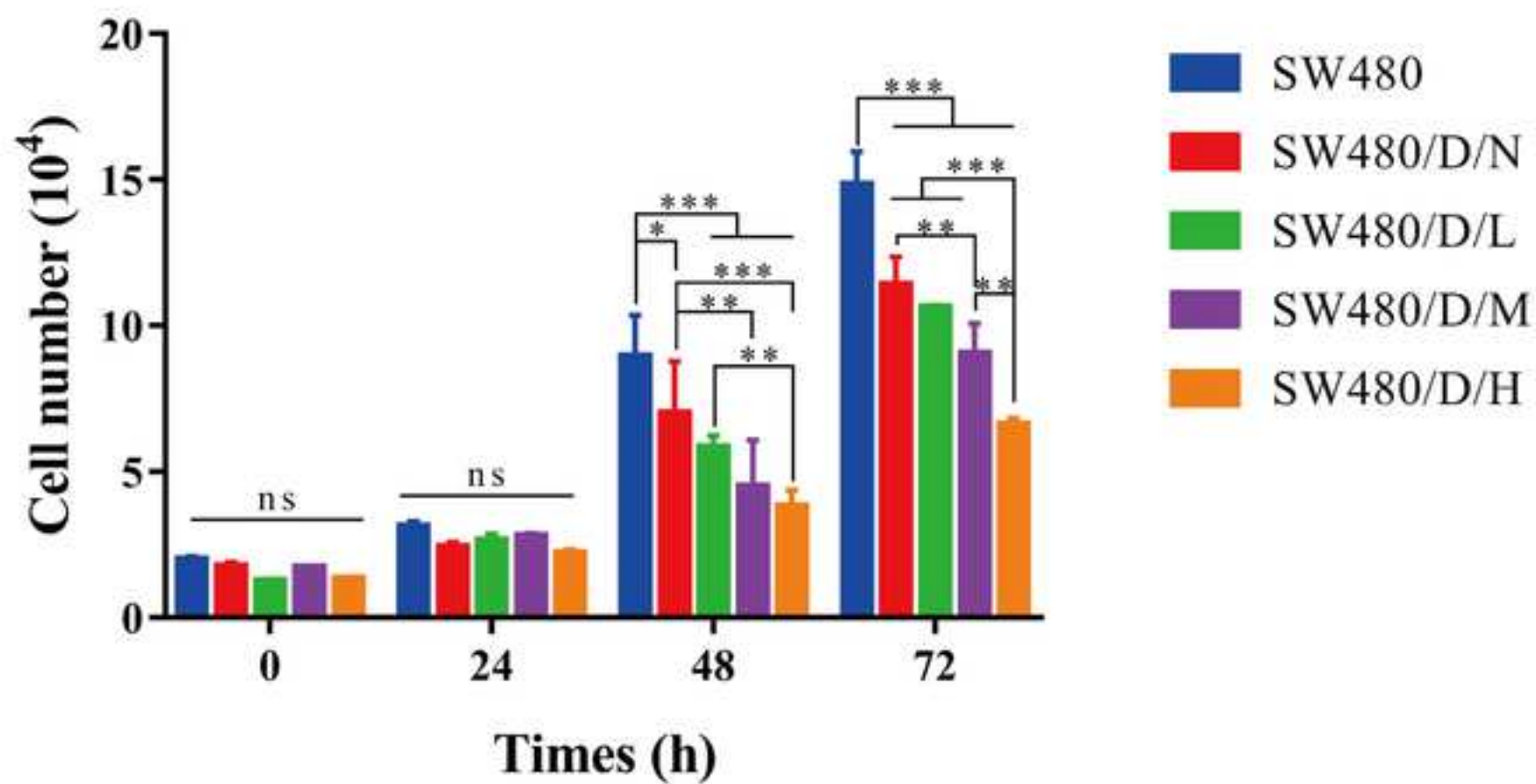
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(A)**(B)**

(A)**(B)**







Long-term exposure in high viscous microenvironment facilitates acquired drug resistance of colon cancer cells to doxorubicin

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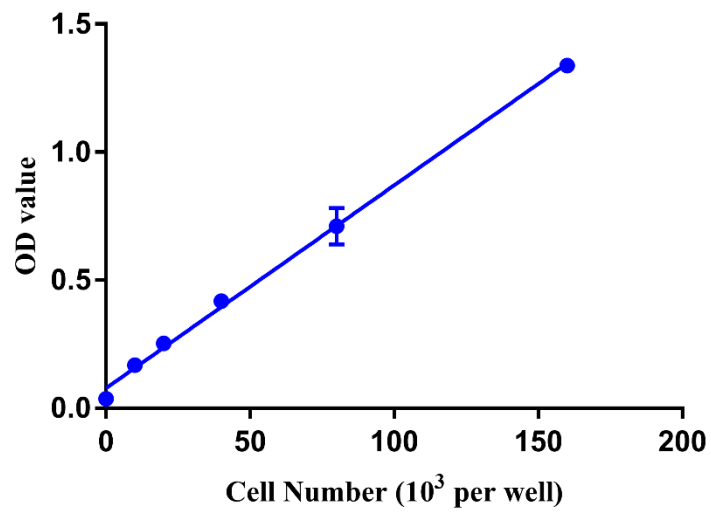
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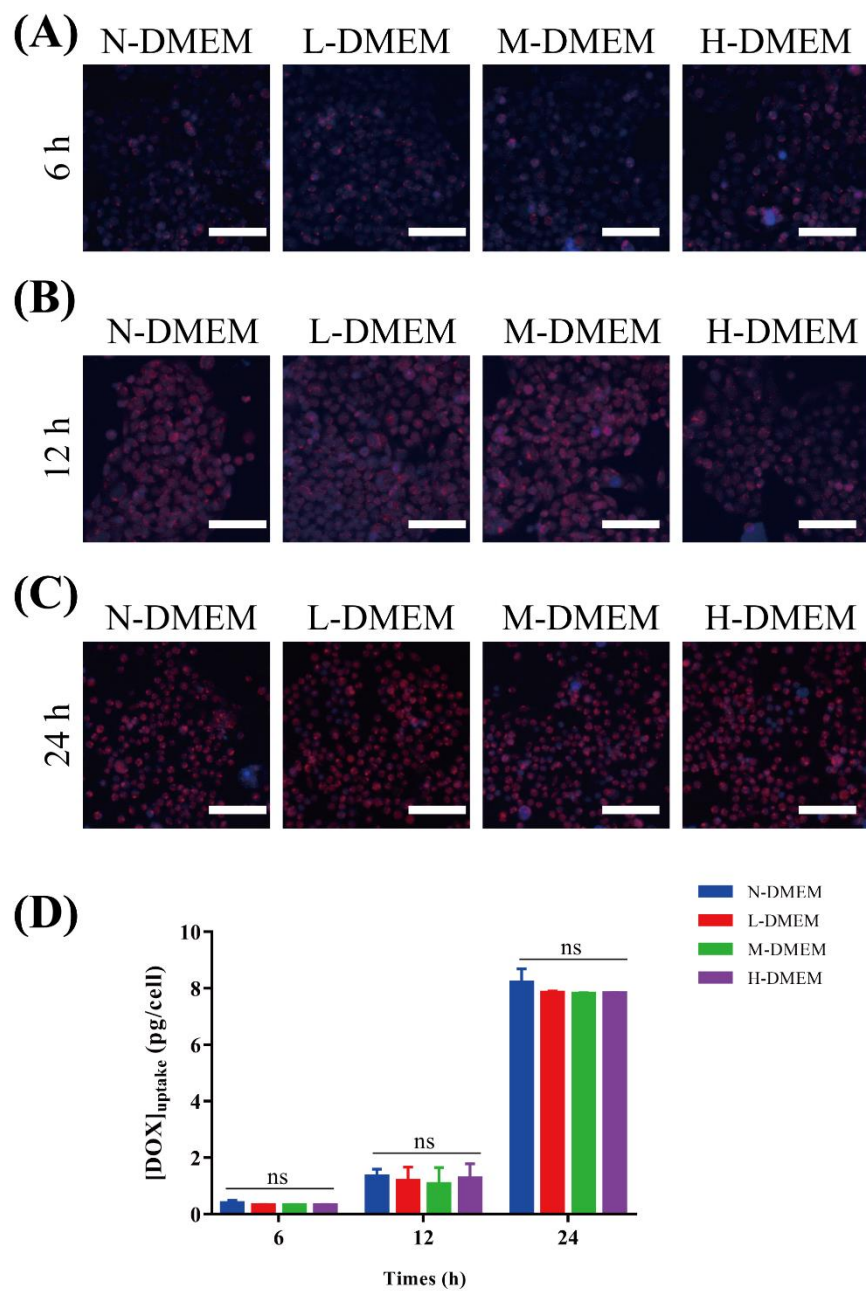
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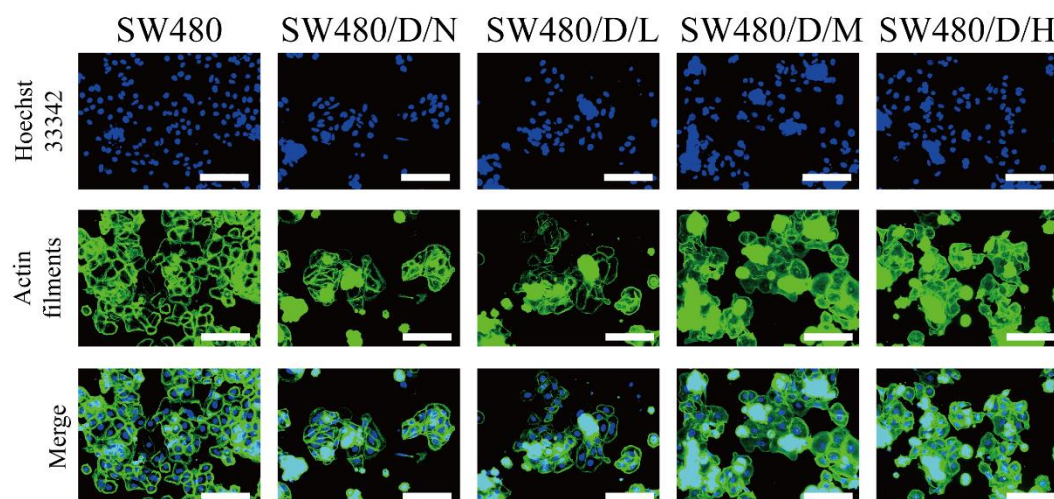
Supplementary data:



Supplementary Figure S1. Standard curve between colon cancer cell number and OD value by WST-1 assay. Triplicate samples were used for each measurement to calculate the means and standard deviations. Data are the means $\pm$ S.D. ($n = 3$).



Supplementary Figure S2. The uptake intensity of DOX in SW480 cells after cells were cultured in different viscous media for 6 h (A), 12 h (B), and 24 h (C). Scale bar: 50 μm . Quantification results of DOX uptake of per cell (D).



Supplementary Figure S3. Cell cytoskeleton assembly. F-actin staining images of SW480, SW480/D/N, SW480/D/L, SW480/D/M, and SW480/D/H. Scale bar: 200 μ m.

Table S1. Primer sequence of the genes used for RT-qPCR analysis.

Gene symbol	Forward primer	Reverse primer
ABCC2	CCATAGCTTCATTCCTGAGTAGC	TCAGAGGACGCTTGTAGCCTT
ABCG2	ACGAACGGATTAACAGGGTCA	CTCCAGACACACCACGGAT
GAPDH	GTCATTGAGAGCAATGCCAG	GTGTTGCTACCCCCAATGTG