Original Research Paper

Optimize the combination regimen of Trastuzumab and Nab-paclitaxel in HER2-positive tumors via modulating Caveolin-1 expression by lovastatin

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The combination regimen of trastuzumab (Tras) plus Nab-paclitaxel (Nab) is recommended to treat HER2-positive (HER2+) cancers. However, they exert effects in different mechanisms: Tras need to stay on cell membranes, while Nab need to be endocytosed, therefore the concurrent combination regimen may not be the best one in HER2+ tumors treatment. Caveolin-1 (Cav-1) is a key player in mediating their endocytosis and is associated with their efficacy, but few researches noticed the opposite effect of Cav-1 expression on the combination efficacy. Herein, we systematically studied the Cav-1 expression level on the combination efficacy and proposed an optimized and clinically feasible combination regimen for HER2+ Cav-1 high tumor treatment. In the regimen, lovastatin (Lova) was introduced to modulate the Cav-1 expression and the results indicated that Lova could downregulate Cav-1 expression, increase Tras retention on cell membrane and enhance the in vitro cytotoxicity of Tras in HER2+ Cav-1 high cells but not in HER2+ Cav-1 low cells. Therefore, by exchanging the dosing sequence of Nab and Tras, and by adding Lova at appropriate time points, the precise three-drug-sequential regimen (PTDS, Nab(D1)-Lova(D2)-Lova & Tras(D2+12 h)) was established. Compared with the concurrent regimen, the PTDS regimen exhibited a higher in vitro cytotoxicity and a stronger tumor growth inhibition in HER2+ Cav-1 high tumors, which might be a promising combination regimen for these patients in clinics.

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1. Introduction

Human epidermal growth factor receptor-2 (HER2) has been reported to be overexpressed in many types of solid tumors [1–3]. Overexpression of HER2 has often been associated with high tumor invasiveness and metastatic potential [4,5]. Trastuzumab (Tras) was the first monoclonal antibody drug to target HER2 [6,7]. By binding to the extracellular domain IV of the HER2 receptor on the cell membrane, Tras inhibited the proliferation of HER2+ tumors by inhibiting AKT phosphorylation, GO/G1 phase cell cycle arrests, and ADCC effect [8–10]. Currently, Tras was considered as one of the standard-of-care treatments for many HER2+ tumors including breast cancer [11] and gastric cancer [12]. Taxanes, including paclitaxel, were one of the preferred chemotherapy drugs for breast cancer [13–15]. As microtubule agents, they interfered with the depolymerization of microtubules and hyper-stabilized microtubules inside tumor cells and caused cell apoptosis [16]. The combination regimen of Tras and Nab-paclitaxel (Nab) was recommended as one of the first-line treatment options in salvage therapy for advanced metastatic HER2+ breast cancer [17–20] and was also one of the second-line options for advanced HER2+ gastric cancer [21,22]. The concurrent combination regimen of “Tras first, followed by Nab” was widely used in clinical practice [23,24].

However, considering the heterogeneity of tumor tissues and the difference in gene expression in tumors, it remained unclear whether this concurrent combination regimen is the optimal treatment strategy for all HER2+ tumors. In fact, the current combination regimen may not consider the difference in mechanisms of action between the two drugs. For Tras, the premise for the efficacy was to bind to the extracellular domain IV of HER2 receptors [25] on the surface of the cell membrane and prolong their retention time to initiate subsequent alterations of intracellular signals; for the nano-drug Nab, they should be endocytosed into cells to as much as possible to increase efficacy. Caveolin-1 (Cav-1) was a major component of the Caveolae structure on the cell membrane [26,27] and was one of the key players in mediating endocytosis [28]. It was shown that the Cav-1 expression in tumor cells correlated with the efficacy of Tras [29] and Nab [30,31]. On the one hand, HER2 receptors were reported to co-localize with Cav-1 in Caveolae on tumor cell membranes [32], and the cellular distribution of HER2 receptors was dynamic and would be endocytosed and recycled constantly [33,34]. So, for Tras, when HER2 receptors were Cav-1-mediated endocytosed, they could not bind with it on the cell membrane and exert efficacy. Studies showed that Cav-1 expression level partly affected the cellular distribution of HER2. In gastric cancer tumor tissues, it was found that higher Cav-1 expression indicated higher HER2 cytoplasm distribution and lower HER2 membrane distribution, conversely, gastric tissues with lower Cav-1 expression were prone to keep HER2 receptors residing on the cell membrane [29]. Therefore, low Cav-1 expression or reducing Cav-1 expression may increase the HER2 retention time on the cell membrane, and increase the opportunity to bind to Tras to enhance efficacy [29]. On the other hand, Nab as a commercial nano-drug, several studies demonstrated that Cav-1-mediated endocytosis was critical for their uptake amount and rate [30]. It was reported that higher Cav-1 expression was likely to facilitate Nab endocytosis mediated by this route, and knocking down Cav-1 expression could weaken its efficacy [28,30,35,36]. Therefore, high Cav-1 expression or increasing Cav-1 expression may be beneficial to Nab cytotoxicity [36].

These results from previous studies indicated that there might be an opposite effect of Cav-1 on the efficacy of the two drugs in the concurrent combination regimen. In HER2+ tumor cells, Cav-1 expression varied [29,37–39]. For the current combination regimen of “Tras first, followed by Nab”, Cav-1 expression was not taken into consideration, and we speculated that for HER2+ Cav-1High tumor cells, the efficacy of first-administered Tras may be reduced; for HER2+ Cav-1Low tumor cells, the efficacy of Nab may be restricted. Therefore, the combined efficacy of Nab and Tras would be limited in HER2+ Cav-1Low and HER2+ Cav-1High tumor cells respectively, and the concurrent regimen could not fully achieve the best therapeutic efficacy.

To maximize their own cytotoxicity of Tras and Nab, modulating Cav-1 expression levels to regulate the cellular endocytosis behaviors of the two drugs appeared to be a promising strategy. Statins, as cholesterol-lowering drugs, were widely exploited in treating hyperlipidemia and for the secondary prevention of cardiovascular diseases in clinics. Recent studies reported its potential ability as a Cav-1 pharmacological modulator in downregulating Cav-1 expression levels [29,40,41]. Inspired by these backgrounds, we think introducing statins could affect the combined efficiency, and through the rational design of the lovastatin (Lova) administration scheme, the combination regimens of Tras plus Nab could achieve a better therapeutic effect.

Herein, in this study, we mainly focused on HER2+ Cav-1High tumor cells, and precisely optimized the combination regimen by modulating Cav-1 expression levels with Lova. Our results showed that exchanging the dosing sequence of Tras and Nab, and introducing Lova between the two drugs could be a strategy to increase Nab uptake and Tras membrane retention through Cav-1-related endocytosis control, ultimately increased the therapeutic efficacy of the combination in HER2+ Cav-1High tumors in vitro and in vivo.

2. Materials and methods

2.1. Treatments, regents, antibodies

Nab-paclitaxel (Nab, Paclitaxel for Injection (Albunin Bound), provided by CSPC Pharmaceutical, China) was dissolved in 0.9% saline, Tras (Herceptin, provided by Roche Genentech, US) was dissolved in distilled water. Lova (Targetmol, China) was dissolved in DMSO, and Lova sodium (Clippio, US) was dissolved in ethanol for preparation. RPMI-1640 medium, penicillin (100 U/ml)–streptomycin (100 mg/ml), tryptic–EDTA (0.25%) were purchased from Macgene technologies (China). McCoy 5a medium was purchased from Sigma. Cell counting kit-8, BCA protein assay kit, RIPA lysis buffer, Protease and phosphatase inhibitor cocktail, GAPDH Rabbit Monoclonal Antibody, HER2/ErbB rabbit polyclonal antibody,
HRP-labeled Goat Anti-Rabbit IgG(H + L), Annexin V-FITC apoptosis detection kit, cell cycle, and apoptosis analysis kit were purchased from Beyotime technologies (China). Octyl-b-D-glucopyranoside was purchased from Millipore. Anti-Cav-1 antibody was purchased from Abcam. Alexa Fluor 488 and Alexa Fluor 647 secondary antibodies were purchased from Bios. For transient transfection for Cav-1 knocking down and overexpression, lipofectamine 2000 was purchased from Thermo Fisher, genOFF h-CAV1_1999A was purchased from Ribobio technologies (China), and CAV1_pCDNA3.1(+) were purchased from Genscript technologies (China).

2.2. Cell culture

The HER2+ human breast cancer cell lines SKBR3, and BT474 were obtained from the National Infrastructure of Cell Line Resource, China; the breast cancer cell line HCC1954 were purchased from Meisen cell, China, and the gastric cancer cell line NCI-N87 were obtained from Procell Life Science & Technology (China), and they were all authenticated. SKBR3 cells were cultured in McCoy 5a medium supplemented with 10% FBS and penicillin (100 U/ml)-streptomycin (100 mg/ml); BT474 were cultured in RPMI-1640 medium supplemented with 10% FBS, recombinant insulin (1 μg/ml) and penicillin (100 U/ml)-streptomycin (100 mg/ml); HCC1954 cells and NCI-N87 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin (100 U/ml)-streptomycin (100 mg/ml). Cells were cultured under aseptic conditions at 37 °C in an incubator at 5% CO2.

2.3. Western blot

For Cav-1 analysis, the total protein was extracted with RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail and 60 mM octyl-b-D-glucopyranoside, and the concentration of total protein was quantified using the BCA protein assay kit. The primary anti-Cav-1 antibody (1:2000, Abcam) was used to incubate overnight at 4 °C, and the primary anti-GAPDH antibody (1:2000, Beyotime) was also used as control.

For Nab uptake analysis, albumin was chosen as the intracellular uptake marker. To remove the original albumin in the cells, two acid/salt washes were performed with 0.1 M glycine and 0.1 M NaCl (pH=3.02) on ice for 2 min each and then washed twice with phosphate buffered solution (PBS) before the extraction of the total protein. The primary anti-albumin antibody (1:2500, Beyotime) was used to incubate overnight at 4 °C.

Protein bands were detected with HRP-labeled secondary antibody and visualized by the chemiluminescence method using Immobilon Western Chemiluminescence HRP Substrate (Millipore). The bands were scanned with Tanon-5200 Multi. ImageJ was used to semi-quantify these bands by calculating the optical density ratio of Cav-1 or albumin to GAPDH.

2.4. Immunofluorescence

2.4.1. Cav-1 expression and distribution in different cell lines

Cells were plated on glass-bottom dishes overnight. After siRNA or pDNA transfection or no treatment, the cells were washed gently with PBS 3 times and then fixed with 4% paraformaldehyde solution for 10 min. The cells were then permeabilized with 0.1% Triton X-100 for 10 min and blocked for an additional 30 min. Cells were incubated with a primary antibody to Cav-1 (1:400) overnight at 4 °C and then incubated with an Alexa Fluor 488-labeled secondary antibody (1:300) for 2 h at room temperature, followed by Hoechst 33342 staining of the nuclei. These immunofluorescence stainings were performed and examined by confocal laser scanning microscopy (CLSM) (Leica STED, Germany).

2.4.2. Nab endocytosis mechanism observation

Cells were pretreated for 1 h with different endocytosis inhibitors, namely, 2.5 μg/ml filipin, 2.5 mM methyl-β-cyclodextrin (M-β-CD), 50 μM Chlorpromazine (CPZ), 100 μM Genistein, 100 μM EIPA. After the pretreatment, 50 μM Nab was added to cells and incubated for 2 h. Two acid/salt washes were performed with 0.1 M glycine and 0.1 M NaCl (pH=3.02) on ice for 2 min each and then washed twice with PBS before being fixed and immunofluorescence-stained with albumin to represent Nab, and then cells were observed with CLSM.

2.4.3. Tras endocytosis observation

Cells were seeded in 8-chamber coverslips and cultured overnight. For SKBR3 cells, 25 μM Lova sodium solution was added to the chambers and they were incubated for 4, 12 and 24 h at 37 °C; for NCI-N87 cells, 25 or 50 μM Lova sodium solution was added to the chambers and they were placed in an incubator for 12 and 24 h, respectively. After Lova pretreatment in different time-long or transfection treatments, the drug-containing medium in each well was removed and they were carefully washed with PBS. Then, 1 μM Tras solution diluted with the blank medium was added to these wells and incubated at 37 °C for 15 or 90 min, respectively. At the end of incubation, cells were fixed with 4% paraformaldehyde solution for 10 min, then permeabilized with 0.1% Triton X-100 for 10 min and blocked for another 30 min. Cells were then incubated directly with an Alexa Fluor 647-labeled secondary antibody (1:300) for 2 h at room temperature for Tras immunofluorescence staining.

For 8-h endocytosis behavior observation of Tras in SKBR3 cells, cells were pretreated with 25 μM Lova sodium solution for 24 h, after removing Lova, 1 μM Tras solution diluted with the blank medium was added to these wells and incubated at 37 °C for different time (15 min, 30 min, 60 min, 90 min, 120 min, 180 min, 4 h, and 8 h). Then Tras was immunofluorescence-stained and observed in CLSM.

2.5. Cytotoxicity assay

The in vitro cytotoxicity experiments were performed by CCK-8 kit according to the manufacturer’s instructions. Cells were seeded in 96-well plates at a cell density of 7000 per well and cultured overnight. Then, for Nab cytotoxicity, the medium was removed and replaced with a Nab working solution at a concentration of 2 nM to 200 nM; for Tras cytotoxicity, the medium was removed and replaced with a Tras solution at a concentration of 1 to 100 μg/ml. The drugs were incubated for an additional 72 h. After different treatments, the medium
was removed and 10 μl CCK-8 solution and 100 μl blank culture medium were added to each well and then incubated for approximately 1 h. Finally, the OD values were examined at 450 nm using a microplate reader, and the survival rates were determined.

2.6. Apoptosis analysis

Cells were collected after 72-h treatments for apoptosis analysis. In detail, cells in each group were washed with PBS and collected in tubes, and Annexin V-FITC binding buffer was added to resuspend the cells before incubating in the dark for 15 min. After that, PI was added for another 10 min of incubation. Then the cells were placed on ice, and flow cytometry (BD FACS Calibur, BD) was used to detect them.

2.7. Cell-cycle analysis

Cells were collected after 72 h for cell cycle analysis. Cells in each group were washed and collected in tubes, then 1 ml 70% pre-cooled ethanol was added to each tube to resuspend cells and fix them for 24 h. After fixation, cells were washed and resuspended in pre-cooled PBS before being collected after 5 min centrifugation at 1000 g. Following that, PI staining buffer containing RNase was added to each tube, and cells were gently resuspended before being incubated in the dark for 30 min. Finally, cells were placed on ice and protected by the light, and flow cytometry was used to detect them.

2.8. Transfection assay

2.8.1. siRNA transfection assay

Cav-1 expression was knocked down by siRNA transfection in NCI-N87 cells and SKBR3 cells. NCI-N87 cells and SKBR3 cells were seeded on 6-well plates. After the cells reached 80% confluence, the culture medium was removed and the cells were washed 3 times with PBS. Then, 1750 μl Opti-MEM medium was added to each well and a mixture of siRNA and Lipofectamine 2000 was incubated in Opti-MEM medium for 15 min and also added to each well, and the cells were incubated for 6 h at 37 °C. After removing the siRNA-containing medium, RPMI-1640 medium with Fetal bovine serum (FBS) was added and the cells were incubated for another 42 or 66 h. Then, the cells were washed with PBS and collected for western blot analysis.

2.8.2. pDNA transfection assay

The Cav-1 expression was overexpressed by pDNA transfection in BT474 cells. BT474 cells were seeded on a 6-well plate. After the cells reached 80% confluence, the culture medium was removed and cells were washed with PBS 3 times. Then a mixture of pDNA, P3000, and Lipofectamine2000 in Opti-MEM medium was incubated for 15 min and added into each well with 1750 μl Opti-MEM medium, and cells were then incubated for 6h at 37 °C. After removing the pDNA-containing medium, the RPMI-1640 medium with FBS was added, and the cells were incubated for another 42 or 66 h. After that, cells were washed with PBS and collected for western blot analysis.

2.9. In vivo studies

4-week-old Balb/c nude mice, CB-17 SCID male mice were purchased from the Department of Laboratory Animal Science, Peking University. Before inoculated, they were housed in a pathogen-free SPF environment for another 1–2 weeks, with a certificate of compliance. The animal experiments were approved by the Animal Ethics Committee of Peking University.

2.9.1. NCI-N87 and SKBR3 xenograft model

NCI-N87 cells (5 × 10^6 cells per mouse) or SKBR3 cells (1 × 10^7 cells per mouse) were inoculated subcutaneously at the right armpit of male CB-17 SCID mice or female Balb/c nude mice, respectively. The mice were sacrificed and the tumor tissues were dissected once the tumor volume reached 300 mm^3. NCI-N87 tumor tissues or SKBR3 tumor tissues were then cut into fractions to inoculate again subcutaneously at the right armpit of other male CB-17 SCID mice or female Balb/c nude mice, respectively. After 30 d, the NCI-N87 tumor tissue all reached 100–200 mm^3, and the SKBR3 tumor tissue all reached 50–100 mm^3. The NCI-N87 xenograft mice and SKBR3 xenografted mice were formed for antitumor efficacy experiments.

2.9.2. Antitumor efficacy superiority evaluation

In different treatment groups, Tras (5 mg/kg) in 0.9% saline was administrated via intraperitoneal injection, and Nab (20 mg/kg) in 0.9% saline was administrated intravenously via tail vein injection. Lova (8.3 mg/kg) was suspended in a mixture of DMSO, PEG300, Tween 80, and water (1:8:1:10) according to the manufacturer’s instruction and was administered by oral gavage. During the 3 rounds of the administration period, on every 1st day and the 5th day of each administration cycle, the long and short diameters of tumor tissues were measured with a digital caliper, and the volume of tumor tissue was calculated. The tumor volume change curve of each group was depicted, and the tumor inhibition rate (%) was calculated.

\[ V_{\text{Tumor volume}} = \frac{\text{long diameter} \times \text{short diameter}}{2} \]

2.9.3. TUNEL assay

SKBR3 tumor-bearing mice were sacrificed after all administration, and the intact tumor tissue was excised, fixed with 4% paraformaldehyde, and embedded in paraffin for sections. The prepared tumor tissue sections were stained with TdT-mediated dUTP Nick-End Labeling (TUNEL) to assess the ability of different administration regimens to induce tumor tissue apoptosis.

2.9.4. Combination regimens safety evaluation

Body weights of the mice were monitored and recorded twice a week; after three rounds of dosing, blood routine examination and biochemical tests were performed in all groups, and the major organs in each group were processed for H&E staining.
2.10. Statistical analysis

Data are presented as mean±SD for all cytotoxicity assays and antitumor efficacy evaluations. Statistical comparisons were made between the control and experimental conditions using the unpaired two-tailed Student t-test with significance assessed at P < 0.05. GraphPad Prism 8.0 was used to perform the statistical analysis.

3. Results and discussion

3.1. Cav-1 expression and cellular distribution in different cell lines

We firstly compared the Cav-1 expression level of some HER2+ breast cancer and gastric cancer cell lines. The position and density of Cav-1 protein bands exhibited differently in various cell lines. Cav-1 appeared at 17 kDa and 22 kDa in HCC1954 cells and NCI-N87 cells, whereas appeared mainly at 17 kDa in BT474 cells and SKBR3 cells. HCC1954 and NCI-N87 cell lines had the highest Cav-1 expression level, SKBR3 cells had a moderately high Cav-1 expression, and BT474 cells had the lowest Cav-1 expression (Fig. 1A). The cellular distribution of Cav-1 was further examined by immunofluorescence staining (Supplementary Fig. S1). It showed that the green fluorescence of Cav-1 in BT474 was weakest, presenting an intracellular large punctate distribution; the fluorescence staining of SKBR3 was stronger and Cav-1 was distributed both on cell membranes and inside cells; The staining of Cav-1 in NCI-N87 cells was strongest, mainly distributed on cell membranes, and showed continuous bright staining. The trend of fluorescence intensity of Cav-1 in immunofluorescence staining was consistent with the trend of protein expression in western blot. Therefore, NCI-N87, HCC1954, and SKBR3 were regarded as HER2+Cav-1High cell lines and BT474 as a HER2+Cav-1Low cell line.

3.2. Endocytosis mechanism verification of Nab

Several studies have reported that Cav-1-mediated endocytosis was the major route for Nab internalization. To further verify the mechanism of the cellular internalization of Nab, SKBR3 and NCI-N87 cells were pretreated with different endocytosis inhibitors before treatment with Nab. As shown in Fig. S2, compared with the control group, the cell internalization of Nab were inhibited after pretreatment with Filipin (Inhibitor of lipid raft), M-β-CD (Inhibitor of lipid raft), and Genistein (Inhibitor of Cav-1-mediated endocytosis), and were not inhibited after pretreatment with Chlorpromazine (Inhibitor of Clathrin) and EIPA (Inhibitor of Macropinocytosis). Semi-quantitative analysis showed that the contribution percentage of Cav-1-mediated endocytosis was at least 40% in all cell endocytic routes of Nab (Fig. S3). Similar endocytosis results were also exhibited in NCI-N87 gastric tumor cells (Fig. S4). All these validated that Nab was mainly endocytosed via the Cav-1-mediated pathway.

3.3. In vitro cytotoxicity of Nab and Tras

Next, we tested the cytotoxicity of Tras and Nab in these cells. For Nab, SKBR3 and HCC1954 showed good response sensitivity, and the IC50 value was 4.07 and 10.47 nM, respectively, while the cytotoxicity of Nab in BT474 and NCI-N87 cells were neither more than 50% (Fig. 1B); For Tras, though all cytotoxicity was no more than 50%, BT474 showed best tumor cell inhibition ability, and the survival rate was about 60% when the concentration was only 1 to 5 μg/mL, while the cytotoxicity of Tras in NCI-N87 and SKBR3 cells were weak. Moreover, it did not show apparent cell inhibition ability in HCC1954 cells (Fig. 1C). It could be speculated that in HER2+ tumor cell lines, Nab was more cytotoxic in HER2+Cav-1High cell lines, while Tras was more cytotoxic in HER2+Cav-1Low cell lines. The different cytotoxicity sensitivity of the two drugs in HER2+ cell lines indicated this might result from different Cav-1 expression levels in these cells, and the concurrent combination of Nab and Tras may not exert the best overall tumor-killing efficacy.

3.4. Transfection and cytotoxicity change study

Then, we investigated whether the difference in cytotoxicity of the two drugs in HER2+ tumor cell lines was due to the different Cav-1 expressions. Firstly, Cav-1 expression was knocked down by siRNA transfection assay, and siRNA-003 was chosen because of successfully reducing Cav-1 expression on HER2+Cav-1High NCI-N87 and SKBR3 cells (Fig. 1D). Then the effect of Cav-1 downregulation by siRNA-003 transfection was further verified by immunofluorescence in NCI-N87 cells (Fig. 1E). By transiently downregulation of Cav-1, it showed that the cytotoxicity of Nab was decreased and the cytotoxicity of Tras was enhanced compared with the untreated group in both cells (Fig. 1F and 1G). These demonstrated that the cytotoxicity difference of Nab or Tras in different HER2+ tumor cell lines was indeed caused by various Cav-1 expression levels, and high Cav-1 expression would enhance the cytotoxicity of Nab, while low Cav-1 expression would improve tumor cell inhibition by Tras. Therefore, in HER2+Cav-1High cells, the overall cytotoxicity of this combination regimen may be improved if Nab were firstly administrated and Cav-1 downregulated thereafter, followed by Tras administration.

3.5. Lovastatin-induced Cav-1 downregulation ability study

The above results indicated that modulation of Cav-1 expression might enhance the combination efficacy of Nab and Tras, so we tried to find out a clinically available drug to perform this function. Recent studies showed that statins owned the potential ability to downregulate Cav-1 [40–42]. Compared with hydrophilic rosuvastatin, Lova was a lipophilic statin with good cell membrane permeability [43]. As a prodrug, it was metabolized to an active acid form in the liver to exert efficacy [44]. In our study, the active form of Lova, Lova sodium was chosen to investigate the pharmacological modulation ability of Cav-1 in vitro. In HER2+Cav-1High SKBR3 cells, the expression of Cav-1 decreased when
Fig. 1 – Cav-1 expression affected cytotoxicity of Tras and Nab in an opposite manner. (A) Western blot images of Cav-1 expression in different HER2+ breast cancer cells (SKBR3/4/HCC1954) and HER2+ gastric cancer cells (NCI-N87). (B) In vitro 72-h cytotoxicity of Nab on HER2+ cancer cells detected by CCK-8. (C) In vitro 72-h cytotoxicity of Tras on HER2+ cancer cells detected by CCK-8. (D) Western blot images of Cav-1 expression when knocked down by siRNA transfection and (E) the immunofluorescence staining of Cav-1 cellular distribution before and after siRNA transfection. (F) Cytotoxicity change of Nab caused by Cav-1 knocking-down on HER2+ Cav-1High SKBR3 and NCI-N87 cells. (G) Cytotoxicity change of Tras caused by Cav-1 knocking-down on HER2+ Cav-1High SKBR3 and NCI-N87 cells. Data are mean ± SD. *P < 0.05; **P < 0.001 vs. Control.
Fig. 2 – Lova reduced Cav-1 expression on HER2\(^+\)Cav-1\(^{\text{High}}\) tumor cells and had an opposite effect on the cytotoxicity of Nab and Tras in vitro. Western blot images of Cav-1 expression change when treated with Lova (25 or 50 μM) for 12 and 24 h in (A) SKBR3 and (B) NCI-N87 cells, and semi-quantification of Cav-1 expression were also performed by ImageJ. (C) In vitro cytotoxicity changes of Nab when pretreated with Lova (25 or 50 μM) for 24 h in SKBR3, NCI-N87, and BT474 cells (2–25 nM for SKBR3, 25–100 nM for NCI-N87, and 50–1000 nM for BT474). (D) In vitro cytotoxicity changes of Tras when pretreated with Lova (25 or 50 μM) for 24 h in SKBR3, NCI-N87, and BT474 cells (10–100 μg/ml for SKBR3, 25–100 μg/ml for NCI-N87 and 5–25 μg/ml for BT474). \(*P < 0.05; \#P < 0.01; \#\#P < 0.001, \) Control vs. 25 μM Lova pretreatment; \(\& P < 0.05; \&\& P < 0.01; \&\&\& P < 0.001, \) Control vs. 50 μM Lova pretreatment.

SKBR3, NCI-N87, and HCC1954 cells, and the cytotoxicity of Nab was reduced compared with the untreated group (Fig. 2C and Fig. S6B). In HER2\(^+\) Cav-1\(^{\text{Low}}\) BT474 cells, pretreatment with Lova did not alter the cytotoxicity of Nab (Fig. 2C). For Tras, the results showed that in HER2\(^+\) Cav-1\(^{\text{High}}\) SKBR3 and NCI-N87 cells, pretreatment with Lova could increase cytotoxicity compared with the untreated group (Fig. 2C). In contrast, in HER2\(^+\) Cav-1\(^{\text{Low}}\) BT474 cells, pretreatment with Lova could not increase the cytotoxicity of Tras (Fig. 2D). In addition, it also did not change the cytotoxicity of HER2\(^+\) Cav-1\(^{\text{High}}\) HCC1954 cells because of the Tras-resistance characteristic of the cells [45](Fig. S6B).

3.7. Mechanism analysis of cytotoxicity change

To better understand the detailed mechanisms of cytotoxicity change of Nab and Tras caused by Lova, the cellular endocytosis and transport behaviors of the two drugs were investigated. First, we measured the effect of Lova on albumin uptake by western blot in HER2\(^+\) Cav-1\(^{\text{High}}\) SKBR3 and NCI-N87 cells following the 90-min pulse of high dose Nab, and Nab uptake amount was represented by intracellular albumin amount. In SKBR3 cells, it showed that pretreatment with Lova for 12 or 24 h significantly reduced the endocytosis of Nab within 90 min (Fig. 3A and 3B). In NCI-N87 cells, 25 and 50 μM Lova treatment for 12 and 24 h could also reduce the endocytic uptake of Nab (Fig. 3C and 3D). To better analyze the relationship between Cav-1 expression and Nab uptake amount, western blot bands of Cav-1(Fig. 3E) and Nab were both semi-quantified, and the correlation curve was formed. The correlation between the Cav-1 expression level and the Nab uptake showed that there was a significant positive correlation with R = 0.8688 (Fig. 3F), and it indicated that the higher Cav-1 was expressed, the more Nab was endocytosed. These suggested that the decreased cytotoxicity of Nab was because Lova downregulated Cav-1 expression and thereby inhibited Cav-1-mediated endocytosis of Nab.
Fig. 3 – Mechanisms of the efficacy changes of Nab and Tras affected by Lova in HER2+ Cav-1\(^{\text{HIGH}}\) tumor cells. (A) Western blot image of Nab uptake amount in SKBR3 cells when pretreated with Lova in different time-long. Lova was pre-incubated (25 \(\mu\)M) for 12 or 24 h, and pulse-chased with 50 \(\mu\)M Nab for 90 min followed by measurement of intracellular Albumin expression, and (B) semi-quantitation of Albumin uptake amount in SKBR3 were shown. (C) Western blot image of Nab uptake amount in NCI-N87 cells when pretreated with Lova in different time-long. Lova (25 or 50 \(\mu\)M) was pre-incubated for 12 or 24 h and pulse-chased with 50 \(\mu\)M Nab for 90 min followed by measurement of intracellular Albumin expression, and semi-quantitation of (D) Nab uptake and (E) Cav-1 expression and were performed. (F) Correlation analysis of Cav-1 expression and Nab uptake in NCI-N87 cells. (G, H) Immunofluorescence images of Tras endocytosis behavior change when pretreated with Lova in different time-long in SKBR3 cells. SKBR3 cells were pretreated with Lova (25 \(\mu\)M) for 4, 12 and 24 h, and then pulse-chased with 1 \(\mu\)M Tras for (G) 15 min and (H) 90 min. (I) Endocytic behavior of Tras in SKBR3 cells within 8 h after 24 h of Lova treatment. After pretreatment with 25 \(\mu\)M Lova for 24 h, Tras was incubated for 8 h. The endocytic behavior and cellular distribution of Tras in SKBR3 cells were observed by CLSM at various time points (15 min, 30 min, 60 min, 90 min, 120 min, 180 min, 4 h and 8 h) during the 8 h period. White arrows pointed to endocytosed Tras inside cells.
In addition, we noticed that Cav-1 expression was significantly increased in the control group after this short-term high-dose uptake of Nab (Fig. 3E), this may also contribute to limited efficacy if Tras is concurrently administered. The addition of Lova after Nab may change this limitation for Tras. We then measured the effect of Lova on Tras cellular endocytosis behavior by immunofluorescence in HER2+ Cav-1High SKBR3 and NCI-N87 cells following 15 min- or 90 min- the pulse of Tras. It showed that in SKBR3 cells, after 15 min of uptake, almost all Tras were still on the cell membranes and rarely appeared inside cells (Fig. 3G). However, after 12- and 24-h pretreatment with Lova, compared with a large amount of endocytosis of Tras within 90 min in the control group and the 4-h pretreatment group (white arrows in Fig. 3H), the endocytosis of Tras was significantly slowed down and reduced, and Tras were almost all restricted on the cell membrane (Fig. 3H). Similar cellular behavior was observed in NCI-N87 cells (Fig. S6C).

To further explore the Tras endocytosis behaviors in a longer time, Tras endocytosis within 8 h were also observed after being pretreated with Lova for 24 h. It was showed that in the control group, Tras was gradually endocytosed into cells, and at 4 h and 8 h, apparent red signals representing Tras were in the cytoplasm; while in the Lova treatment group, Tras was almost stay on and restrained the cell membrane instead of endocytosing into cells during the 8 h incubation (Fig. 3I). Therefore, it could be speculated that 25 μM Lova pretreatment for 12 and 24 h reduced Cav-1 expression and increased the amount of HER2 and Tras binding on the cell membrane, thereby enhancing the cytotoxicity of Tras. These results provided us with an idea that the efficacy of combination regimens may be increased when Lova was administered after Nab and before Tras in HER2+ Cav-1High cells.

3.8. In vitro cytotoxicity study of combination regimens

To verify our assumptions, we evaluated the cytotoxicity of different combination regimens by exchanging the dosing sequence or introducing Lova in SKBR3, BT474, and NCI-N87 cells (detailed schedule in Fig. 5A and 5B). In SKBR3 cells, the concurrent regimen of Tras plus Nab (Tras+Nab D1) did not enhance the cytotoxicity (65%) compared with Nab alone (62%), but exchanging the dosing sequence and prolonging the interval to Nab(D1) Tras(D3) (Nab was given on the first day, and Tras was administered on the third day) significantly enhanced the cytotoxicity to 46%. Moreover, adding Lova after 24 h of the Nab administration on the first day and Tras was given on the third day [Nab(D1) Lova(D2) Tras(D3)] would further improve the tumor cell killing ability (41%) (Fig. 4A). In BT474 cells, the Tras+Nab D1 regimen showed the best combination cytotoxicity (40%) while exchanging dosing sequence or adding Lova could not further increase the cytotoxicity and was even weaker than that of the concurrent regimen (Fig. 4B). In addition, the results in NCI-N87 cells showed that compared with concurrent regimens, the cytotoxicity was enhanced after changing the dosing sequence to Nab(D1) Tras(D3) and Nab(D1) Lova(D2) Tras(D3) (Fig. 4C). Interestingly, cytotoxicity was even weaker when the dosing sequence was changed to Tras(D1) Nab(D3) (Fig. 4C). These results showed that Lova could downregulate Cav-1 expression and enhance efficacy by residing Tras on the cell membrane, and by modulating the combination regimen, the three-drug-sequential (TDS) [Nab(D1) Lova(D2) Tras(D3)] combination regimen potentiated cytotoxicity in HER2+ Cav-1High cells but not in HER2+ Cav-1Low cells.

3.9. In vitro mechanism analysis of TDS regimens potentiating cytotoxicity

It was reported that one of the crucial antitumor mechanisms of Nab was mainly the apoptosis inducement, and one of the tumor inhibition mechanisms of Tras was G0/G1 cell-cycle arrest. Therefore, to further clarify the mechanisms of the TDS regimen promoted cytotoxicity in HER2+ Cav-1High tumor cells, we investigated the difference in apoptosis situation and cell-cycle arrest when treated with different regimens in HER2+ Cav-1High SKBR3 cells. First, the effect of only changing the dosing sequence on cell apoptosis was investigated (Fig. 4D). It showed that the apoptosis signal was 20% for the Nab(D1) Tras(D3) regimen, which was 2.54-fold of the concurrent regimen and 2-fold of the Tras(D1) Nab(D3) regimen (Fig. 4E and 4F). These indicated that simply changing the dosing sequence to Nab(D1) Tras(D3) could enhance cytotoxicity by inducing more apoptosis on HER2+ Cav-1High tumor cells. We thought that in these cells, firstly-adminstrated Nab could be uptake more via Cav-1-mediated endocytosis, and more apoptosis-related cell death caused by Nab could occur; while Tras administrated after 48 h (D3) could avoid Tras efficacy reduction caused by Cav-1 expression upregulation when concurrently administered with Nab. Next, we continued to investigate the effect of adding Lova in Nab(D1) Tras(D3) on cell-cycle arrestment (Fig. 4G). It showed that when treated with the TDS regimen, there was a significant increase in the proportion of cells in the G0/G1 phase (from 65.87% to 75.48%), and the proportion of cells in the G2/M phase was drastically reduced from 17.38% to 4.39% compared with the control group. In addition, when compared with Nab(D1) Tras(D3) regimen, the TDS regimen also showed apparent elevation in G0/G1 phase arrest. While the concurrent Tras+Nab D1 regimen did not show apparent cell cycle change compared with the control group (Fig. 4H and 4I). These results indicated that adding Lova in Nab(D1) Tras(D3) in the TDS regimen could further strengthen the G0/G1 cell cycle arrest which was mainly initiated by Tras and increased cytotoxicity, while the concurrent regimen indeed limited Tras efficacy in cell-cycle arrest. Taking together, these mechanisms analyses all suggested that the enhanced cytotoxicity of the TDS regimen was a comprehensive result combined the increasing apoptosis effect and the increasing G0/G1 cell cycle arrest effect maximized by Nab and Tras, and they were mediated by Cav-1 expression change by Lova involvement.

3.10. Refinement of TDS regimens directed by Cav-1 expression

As we could see, effective reduction of Cav-1 expression and thereby promoting Tras efficacy was a crucial step for the overall cytotoxic outcomes. In the previous experiments,
Fig. 4 – In vitro cytotoxicity change and related mechanisms of TDS regimens in HER2\superscript{+}Cav-1\textsuperscript{high} tumor cells. In vitro cytotoxicity of different combination regimens in (A) SKBR3, (B) BT474, and (C) NCI-N87 cells. These cells were treated with concurrent regimen (Tras+Nab D1) or Nab (5 nM for SKBR3, 25 nM for BT474, and 50 nM for NCI-N87) on Day 1 with either Lova (25 μM) on Day 2, Tras (10 μg/ml for SKBR3 and BT474, 50 gμ/ml for NCI-N87) on Day 3 [Nab(D1) Lova(D2) Tras(D3), TDS regimen], or Tras (10 μg/ml) on Day 3 with a 24-h gap of no treatments [Nab(D1) Tras(D3)]. For NCI-N87 cells, additional regimens [Tras(D1) Lova(D2) Nab(D3)] and [Tras(D1) Nab(D3)] were measured. Each treatment was removed after 24 h of treatment. After 72-h administration of different combination regimens, combination cytotoxicity were measured by CCK-8. (D) Administration schedule of different combination regimens in SKBR3 cells for apoptosis analysis. (E) Representative apoptosis flow cytometry images of Annexin V-FITC/ PI in different regimens treated for 72 h. (F) Quantitative comparison of early apoptosis cell percentage in different combination regimens (n = 3). Nab for 5 nM, Tras for 10 μg/ml. **P < 0.01. (G) Administration schedule of different combination regimens in SKBR3 cells for cell cycle analysis. (H) Representative cell-cycle images after treated with different combination regimens for 72 h. (I) Quantitative analysis of cell percentage in G0/G1, S, G2/M cell cycle. *P < 0.05; **P < 0.01; ***P < 0.001.
we noted that short-time high-dose administration of Nab elevated Cav-1 expression. Therefore, to precisely optimize the TDS regimen, we further explored the effect of Nab on Cav-1 expression. According to the drug administration scheme (Fig. 5A), it showed that Nab incubation for 12 h significantly increased the Cav-1 expression, and decreased at 24 h. After the removal of Nab, the Cav-1 expression gradually returned to the original Cav-1 expression level at the interval of 12 and 24 h (Fig. 5B). Next, since the factors “interval between Nab and Lova” and “Lova incubation length” both affected the Cav-1 expression, we refined these two factors, and six different combination regimens (Schedule 4–9, Fig. 5C) were designed and cytotoxicity on SKBR3 cells were investigated. The results in Fig. 5D and 5E showed that Schedule 8 was the most cytotoxic, with a cell survival rate of 43.03%; Schedule 6 and 7 were also exhibited strong cytotoxicity, with cell survival rates of 44.01% and 46.01%, respectively; the cell survival rate of the concurrent dosing regimen on the same day was 59.84% (Schedule 3), and the additional 24 h-interval between Nab and Lova did not significantly enhance cytotoxicity (Schedule 4 and 5).

Next, we continued to investigate the mechanisms associated with Cav-1 expression that affect overall cytotoxicity. We mainly investigated the effect of the interval between Nab and Lova administration on the Cav-1 expression before Tras administration. Among several dosing regimens investigated (Fig. 6A), “12 h of the interval between Nab and Lova administration for 12 h” (Schedule 3) and “no interval with Nab and Lova administration for 24 h” (Schedule 2) were effective in reducing Cav-1 expression before Tras administration (Fig. 6B). These results further proved that the potentiation of cytotoxicity was associated with the reduced Cav-1 expression before Tras administration. However, the additional 24-h interval between Nab and Lova in Schedule 1 did not show an apparent effect on downregulating Cav-1 expression before Tras administration, and this may explain the weaker overall cytotoxicity of this combination regimen (shown as Schedule 4 in Fig. 5D and 5E).

Finally, considering the compliance and convenience of administration, we chose “Nab was incubated for 24 h, and with no interval, Lova incubation for 24 h, followed by Tras” as the precise TDS (PTDS) dosing regimen.

We further validated the cytotoxicity advantage specificity of the PTDS dosing regimen in BT474 cells overexpressing Cav-1. Cav-1 was successfully overexpressed by pDNA transfection for 72 h (Fig. 6C and 6D), and after transiently overexpressing Cav-1 in BT474 cells, it showed enhanced Tras endocytosis within 90 min compared with untreated BT474 cells (Fig. 7C).
Moreover, we continued to evaluate the cytotoxicity change when treated with different regimens in Cav-1 overexpressing BT474 cells (Fig. S7D), it showed that treatment with the PTDS regimen caused stronger cytotoxicity compared with the concurrent Tras+Nab D1 regimen (Fig. 6E), which was a different outcome compared with that in the untreated BT474 cells. These results demonstrated the cytotoxic advantage specificity of the PTDS combination regimen in HER2+ Cav-1\textsuperscript{High} tumor cells.

3.11. \textit{In vivo} adjustment of PTDS regimen directed by Cav-1 expression

Because of the changes from \textit{in vitro} to \textit{in vivo} environments, and the change in the Lova administration route, further adjustment of the Lova administration timepoint was needed. From our above results, cellular Cav-1 expression could be reduced when incubating with 25 $\mu$M Lova for 24 h in SKBR3 cells and NCI-N87 cells, and the PTDS regimens based on Cav-1 modulation showed the strongest cytotoxicity in vitro. Therefore, based on the PTDS regimen in vitro, we modified this regimen according to the effect of Lova oral administration on Cav-1 expression in NCI-N87 tumor tissues \textit{in vivo}. The immunofluorescence staining of Cav-1 in tumor tissues at different time points (Fig. 7A) showed that compared with bright Cav-1 staining at 0 h, Cav-1 expression was significantly reduced at 12 h after the first Lova administration, as well as at 16 h (Fig. 7B). After 24 h, the Cav-1 expression gradually recovered. Similar results were also showed in the larger view of CLSM images (Fig. S8) and scanned in Vectra 3.0 Automated Quantitative Pathology Imaging System (Fig. S9). This experiment indicated the lowest Cav-1 expression at 12 h after the first Lova administration, and these proper timepoints for Lova administration \textit{in vivo} were established thereby. It was reported that oral administration of Lova twice 12 h apart could effectively reduce Cav-1 expression B [29], which was also consistent with our experiment results. Subsequently, the modified PTDS regimen [Nab(D1)-Lova(D2)-Lova & Tras(D2+12 h)]：“Nab on Day 1, Lova on Day 2, and Lova again after 12 h with concomitant Tras” was formulated for the \textit{in vivo} antitumor efficacy experiments.

3.12. \textit{In vivo} antitumor superiority study

As a standard regimen in clinics, the antitumor efficacy of Tras+Nab D1 in advanced HER2$^+$ breast cancer and gastric cancer was verified in previous clinical trials. Therefore, we tested whether the modified PTDS regimen could further enhance the therapeutic efficacy of the drug combination in SKBR3 and NCI-N87 subcutaneous models. After tumors were formed, mice were randomly grouped to four regimens delivered in three cycles in 3 weeks: (1) Saline group; (2) Tras+Nab D1; (3) Nab(D1)-Lova(D2)-Lova & Tras(D2+12 h) (the modified PTDS regimen); (4) Nab(D1) Tras(D2+12 h) (Fig. 8A). For SKBR3 and NCI-N87 xenografts, compared with saline groups, all three regimens exhibited efficacy in tumor inhibition with an inhibition rate over 80%. There was a significant reduction in tumor volume in mice receiving the modified PTDS regimen compared with Tras+Nab D1, the tumor inhibition rate was 95.1% for SKBR3 (Fig. 8B) and 90.89% for NCI-N87 (Fig. 8C), respectively. Moreover, the apoptosis rate of tumor tissues in mice receiving the modified PTDS regimen was as strong as 16.08%, which was 12.27-fold higher than that of Tras+Nab D1 (Fig. 8D). In terms of safety evaluation, there was no significant difference in mice body weight (Fig. S10 and S14), basic experimental values (Fig. S11: S12 and S15-S16), and H&E staining (Fig. S13) in different combination regimens, indicating good safety \textit{in vivo}. All these results indicated that, compared with the concurrent combination regimen, the PTDS regimen [Nab(D1)-Lova(D2)-Lova & Tras(D2+12 h)] had significantly superior antitumor efficiency in the PTDS regimen in HER2$^+$ Cav-1\textsuperscript{High} xenograft mice.

3.13. Discussion

The combination regimen of Nab and Tras was one of the preferred treatment options for advanced HER2$^+$ breast cancer and one of the second-line options for advanced HER2$^+$ gastric cancer. In the clinical, the concurrent regimen of “Tras first, followed by Nab” was usually used, and the sequence setting was thought to reduce the adverse effects of infusion-related reactions(IRR) [46]. In this study, we first noted that the two drugs owned totally different endocytosis requirements to perform their antitumor function: Tras needed to stay and reside on cell membranes and avoid endocytosis, while Nab need to be endocytosed as much as possible. Interestingly, they were all endocytosed mediated by Cav-1. The Cav-1
expression had an opposite effect on the efficacy of the two drugs, but current combination regimens ignored this phenomenon. Therefore, the best efficacy of the combination may not be fully achieved for HER2+ tumors with various Cav-1 expressions. As a result, we exploited the opposite requirement of the two drugs for the maximal efficacy of Cav-1 expression in tumor cells, and explored the optimal combination regimen in HER2+ Cav-1\textsuperscript{High} cell models, and found that the therapeutic efficacy could be improved by exchanging the order of administration of the two drugs and introducing Lova at the appropriate time point. The optimized PTDS regimen rationally modulated the endocytosis process of Nab and Tras and enhanced their antitumor efficacy. Our results suggested that Cav-1 expression was expected to be a biomarker for the adjustment of combination regimens of Nab and Tras, and we provided a simple and clinically feasible optimization for the combination regimen of Nab and Tras in HER2+ Cav-1\textsuperscript{High} tumors.

Several HER2+ breast cancer and gastric cancer cells with different Cav-1 expression exhibited different drug responses to Nab and Tras, and downregulation of Cav-1 by siRNA verified its opposite effect on the cytotoxicity of the two drugs. Therefore, pharmacological modulator Lova was introduced to reduce Cav-1 expression. several regents were reported to reduce Cav-1 expression. Among them, compared with other reagents (e.g., Filipin, M-β-CD, etc.), statins were widely used as commercially available drugs in patients with hyperlipidemia, and secondary prevention of cardiovascular disease at reasonable prices, and statins were reported showing Cav-1 expression depleting ability [47,48], which showed potential clinical application in this field. Lipophilic statins such as Lova and simvastatin have a better effect due to their better cell membrane permeability compared to hydrophilic statins such as rosuvastatin [43]. At the same time, Lova did not affect the overall HER2 expression (Fig. S5A). So, we speculated that the subsequent change in Tras efficacy was not related to the change in the HER2 expression. In HER2+ Cav-1\textsuperscript{High} tumors, Lova pretreatment decreased the cytotoxicity of Nab and promoted the cytotoxicity of Tras. These effects were associated with the Cav-1 expression downregulation by Lova, which reduced its mediated endocytosis to hinder Nab uptake and facilitate Tras retention on the cell membrane. Thereby, a TDS regimen of Nab(D1) Lova (D2) Tras (D3) was designed, and it could promote the combination cytotoxicity in HER2+ Cav-1\textsuperscript{High} tumors compared to the concurrent regimen. Mechanism investigations showed that the TDS regimen induced stronger apoptosis while promoting cell cycle arrest in the G0/G1 cycle. This result was exactly reflecting the result of the enhanced action of Nab and Tras, respectively.

To precisely modulate the Cav-1 expression before Tras administration, we further refine the TDS regimens into 6 regimens based on the interval of Nab and Lova and Lova incubation time. By evaluating the temporal pattern of Cav-1 change in the administration process and the corresponding cytotoxicity, we finally optimized the TDS regimens to a more precise “Nab was incubated for 24 h, and with no interval, Lova incubation for 24 h, followed by Tras” regimen for HER2+ Cav-1\textsuperscript{High} tumors. By overexpressing Cav-1 on BT474 cells, it was
further verified the combination cytotoxicity advantage of the PTDS regimen. The in vivo experiment results were almost consistent with the results of in vitro experiments. In HER2\(^+\) Cav-1\(^{\text{High}}\) tumor-bearing mice, the modified PTDS regimen for in vivo experiment not only inhibited tumor growth and reduce the size of tumors but also induced significantly stronger apoptosis in tumor tissues compared to the standard regimen. These all further demonstrated the superior efficacy of the TDS regimen in HER2\(^+\) Cav-1\(^{\text{High}}\) tumors.

In summary, our data demonstrated that in HER2\(^+\) Cav-1\(^{\text{High}}\) tumors, the combination efficacy of Nab and Tras could be maximized by exchanging dosing sequences and introducing Lova at an appropriate time to improve their individual endocytosis process. Compared with the concurrent regimen, the PTDS regimen exhibited the best therapeutic efficacy in both in vitro and in vivo experiments.

The results of our study showed that Cav-1 expression levels in HER2\(^+\) tumor tissues were promising to be a predictive biomarker for the designing regimens of Nab and Tras. Moreover, the novel strategy that combination regimen efficacy potentiation by statins modulating Cav-1 expression could be applied to more regimens design involved in mAbs [42,49], antibody-drug conjugates (ADCs) [38,50], and nano-drugs [30,51,52], which might relate to Cav-1 mediated endocytosis. For example, due to the colocalization of Cav-1 and HER2 or EGFR, another anti-HER2 mAbs pertuzumab and anti-EGFR mAbs cetuximab were reported to work on the cell membrane and need to prevent from Cav-1-mediated endocytosis [42], while ADC T-DM1 and liposomal drugs needed to endocytosed to exert efficacy. Therefore, by evaluating the Cav-1 expression in tumors and analyzing the characteristics of drugs in patients’ combination regimens, statin-involved combination regimens may be considered: For Cav-1\(^{\text{High}}\) tumor tissues, the efficacy of most mAbs which need to stay on cell membranes may be potentiated by modulating Cav-1 expression with statins pre-administration, so introducing lipophilic statins or changing to lipophilic statin from their daily hydrophilic statins may benefit; while ADCs and nano-drugs may be administered first in the combination treatments in Cav-1 \(^{\text{High}}\) tumors. Considering the clinical accessibility of statins, this could be a simple but promising optimizing strategy to maximize antitumor combination regimens efficacy.

4. Conclusions

Herein, we found the opposite effect of the Cav-1 expression on the efficacy of nano-drug Nab and monoclonal antibody drug Tras, and limited the efficacy of the standard concurrent combination regimen of [Tras+i-Nab D1]. In our study, we aimed at HER2\(^+\) Cav-1\(^{\text{High}}\) tumor cells, and a novel combination regimen was formed by regulating the endocytosis behavior of drugs based on Cav-1 expression level change. By simply exchanging the dosing sequence of Nab and Tras, and by adding pharmacological Cav-1 modulator Lova at appropriate time points, the PTDS regimen was established and the maximized antitumor efficacy of Nab and Tras were achieved via the strongest apoptosis and most G0/G1 phase arrest, respectively. PTDS regimen showed antitumor superiority and specificity in HER2\(^+\) Cav-1\(^{\text{High}}\) tumors and was promising to be an optimized combination regimen choice for these patients in clinics.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.06.002.

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