Key structure of Brij for overcoming multidrug resistance in cancer

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Abstract

Multidrug resistance (MDR) is a major barrier to the chemotherapy treatment of many cancers. However, some non-ionic surfactants, for example Brij, have been shown to restore the sensitivity of MDR cells to such drugs. The aim of this study was to explore the reversal effect of Brij on MDR tumor cells and elucidate its potential mechanism. Our data indicate that the structure of Brij surfactants plays an important role in overcoming MDR in cancer, i.e. modified hydrophilic-lipophilic balance (MHLB, the ratio of the number (n) of hydrophilic repeating units of ethylene oxide (EO) to the number (m) of carbons in the hydrophobic tail (CH_2).). Cell viability of cells treated with paclitaxel (PTX) nanocrystals (NCs) formulated with Brij showed positive correlations with MHLB ($R^2 = 0.8195$); the higher the ratio of Brij to PTX in NCs, the higher cytotoxicity induced by the PTX NCs. Significant increases in intracellular accumulation of ^{3}H-PTX (P-gp substrate) were observed in an MDR cell line (H460/taxR cells) treated with Brij 78 (MHLB=1.11) and Brij 97 (MHLB=0.6). After treatments with Brij 78 and Brij 97, the levels of intracellular ATP were decreased and verapamil induced ATPase activities of P-gp were inhibited in multidrug resistant cells. The responses of the cells to Brij 78 and Brij 97 in ATP depletion studies correlated with the cell viability induced by PTX/Brij NCs and intracellular accumulation of ^{3}H-PTX. Brij 78 and Brij 97 could not alter the levels of P-gp expression detected by western blotting. These findings may provide some insight into the likelihood of further development of more potent P-gp inhibitors for the treatment of MDR in cancer.

Keywords

Brij; multidrug resistance (MDR); Nanocrystals; paclitaxel; P-glycoprotein

INTRODUCTION

Multidrug resistance (MDR) is the leading cause of treatment failure in cancer therapy. One of the most common mechanisms of MDR is drug efflux mediated by transporters such as P-glycoprotein (P-gp), one of the most typical and the most widely investigated ATP-binding cassette (ABC) transporters. P-gp can use the energy from ATP-hydrolysis to pump

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substrates like doxorubicin and paclitaxel (PTX) out of tumor cells, resulting in a reduction of the accumulation of drugs in tumor cells.[1, 2] To address this problem, P-gp inhibitors such as verapamil, quinine and cyclosporin A have been introduced to reverse MDR since the early 1980s. However, clinical trials have been disappointing due to the high inherent toxicities of P-gp inhibitors and/or pharmacokinetic interactions between anticancer drugs and inhibitors.

Non-ionic surfactants are extensively used in pharmaceutical formulations as solubilizers and emulsifiers to improve dissolution and absorption of poorly soluble drugs. Several non-ionic surfactants exhibited the ability to reverse P-gp-mediated MDR. For example, Pluronic sensitizes MDR tumors by inhibiting the P-gp drug efflux system through ATP depletion.[3-9] D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS, a water-soluble succinate ester of vitamin E) is an amphiphilic compound consisting of a lipophilic (water-insoluble) α-tocopherol portion and hydrophilic polar (water-soluble) PEG chain that can inhibit P-gp-mediated drug efflux and increase the oral bioavailability of anticancer drugs.[10-12] Our laboratory has previously developed paclitaxel (PTX) nanocrystals (NCs) formulations using TPGS as the sole excipient for overcoming MDR.[13]

Brij® molecules, commercially available nonionic surfactants, have also been shown to inhibit efflux pumps. In a study by Lo (2003), Brij 30 significantly increased apical to basolateral absorption and substantially reduced basolateral to apical efflux of epirubicin across Caco-2 monolayers. The addition of Brij 30 also markedly enhanced mucosal to serosal absorption of epirubicin in the rat jejunum and ileum.[14] Similarly, Brij-35 effectively enhanced bis (12)-hupyridone absorption in Caco-2 cells at a concentration lower than its critical micelle concentration.[15] Mumper *et al.* has prepared doxorubicin and paclitaxel-loaded nanoparticles using Brij 78 as an emulsifying agent to overcome MDR by inhibiting P-gp and depleting ATP.[16] With these encouraging results, it was reasonable to assume that other Brij molecules might produce similar or more efficient reversal of P-gp-mediated MDR.

While the structures of all Brij molecules contain a polar head group consisting of PEG chains with different lengths and a hydrophobic tail consisting of an alkyl chain, some exhibit the ability to inhibit P-gp[14-16] and others do not. Therefore, we hypothesized that the structural properties of different Brij molecules may play a critical role in inhibiting P-gp. In this study, we investigated PTX NCs formulations using a series of Brij surfactants to identify structures or features required for overcoming MDR. Each Brij surfactant had different PEG chain lengths and alkyl chain structures. The effects of different Brij formulations on the physicochemical characteristics of NCs were also investigated. The cytotoxicity of NCs against PTX resistant human lung carcinoma cell line (H460/taxR) was examined by MTS assay. The P-gp function, intracellular ATP level, P-gp ATPase activity and P-gp expression levels were determined to evaluate the effects of Brij on the reversal of MDR.

EXPERIMENTAL SECTION

Material

Paclitaxel (PTX) was bought from Lc Laboratories (Woburn, MA). TPGS was bought from Eastman (Anglesey, U.K.). Brij 700, Brij 78, Brij 98, Brij 97, Brij 52, Brij 72, Brij 30 and Brij 35 were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) were from Promega Corporation (Madison, WI). H^3-PTX was obtained from PerkinElmer Life Sciences. ATPlite™ Luminescence ATP Detection Assay System was bought from PerkinElmer (Waltham, MA). Monoclonal antibodies including the MDR1 (sc-55510), glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) (sc-20357) and the secondary antibody, anti-mouse or anti-rabbit IgG with HRP, were products of Santa Cruz Biotechnology, Inc.

Tumor cell lines and cell culture

Resistant human lung cancer cell line H460/taxR was obtained from National Cancer Institute. H460/taxR cells were maintained in RPMI-1640 medium supplemented with 10% heated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA).

Preparation of NCs

The NCs were prepared through stabilization of the nanocrystals.[17] PTX and TPGS or Brij molecules were first dissolved in chloroform (in a glass tube) with different ratios (1:5, 1:3, 1:2, w:w) and coprecipitated by evaporating the chloroform with a steady flow of nitrogen gas. A trace amount of chloroform was removed by keeping the precipitates under a vacuum in a desiccator for 2 to 4 h. Following 20 min hydration in water and vortex, suspensions were sonicated for 10 to 15 min in a bath-type sonicator (output 80 kC, 80 W) to form the NCs.

Characterization of NCs

The particle size and distribution of NCs were measured using a submicron particle sizer (NICOMP particle sizing systems, Autodilute-PAT model 370, Santa Barbra, CA) in the NICOMP mode. Particle size and morphology were determined using a transmission electron microscope (TEM) with an acceleration voltage of 100 kV. To prepare the samples, PTX/Brij NCs (5 μL) were deposited onto a 200 mesh copper grid coated with carbon (Ted Pella, Inc., Redding, CA) and allowed to stand for 5 min, excess liquid was then wicked off. Samples were stained with 1% uranyl acetate (5 μL), followed by overnight air drying at room temperature. Images were acquired using a JEOL 100CX II TEM.

Cytotoxicity on Resistant Cancer Cells

To evaluate cytotoxicity of PTX/TPGS or PTX/Brij NCs, an MTS ([3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed on H460/taxR cells. Cells were seeded into 96-well plates at a concentration of 1×104 cells in a volume of 200 μL per well. After 24 h, various formulations containing PTX were added, including PTX/TPGS NCs, PTX/Brij NCs, free Brij and free PTX (the final paclitaxel concentration in the cell culture medium was 5 μM). Free Brij was prepared using the methods to stabilize nanocrystals described above, but without PTX. The free PTX dissolved in DMSO, free Brij and NCs were diluted using water before being added to the medium. Following a 48 h period of incubation, the medium was removed and 100 μL of fresh medium and 20 μL of the combined MTS/PMS solution was added into each well of a 96-well assay plate. The plates were incubated for an additional 2 h at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance values were read on a microplate reader using a Bio-Rad microplate imaging system (Hercules, CA) at a wavelength of 490 nm. Cell viability was calculated using the following formula: cell viability (%) (A490 nm for the treated cells/A490 nm for the control cells) × 100, where A490 nm is the absorbance.

Cellular uptake of ^{3}H-PTX in H460/taxR cells

H460/taxR cells were seeded in 24-well plates at a density of 1×10^5 cells per well and incubated overnight. Confluent cell monolayers were treated with PTX or PTX/Brij NCs (5 μM PTX containing 32 nCi ^{3}H-PTX per well) and incubated at 37 °C for 5 h. After treatment, cells were washed three times with ice-cold PBS and lysed. The radioactivity in

cell lysates was then quantified on a liquid scintillation analyzer (TRI-CARB, Packard Bioscience Company, Waltham, MA). Celluar uptake efficiency of ^{3}H-PTX (%) was calculated by the amount of intracellular ^{3}H-PTX/added ^{3}H-PTX per well $\times$ 100.

Determination of intracellular ATP

The intracellular ATP content was measured by ATPlite™ Luminescence ATP Detection Assay System with modifications from the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight. Brij or TPGS were added to cells and incubated at 37 °C for 2 h. After treatment, cells were washed twice with ice-cold PBS and then an equal volume of the single-one-step reagent provided by the kit was added to each well and rocked for 15 minutes at room temperature. Cellular ATP content was measured by a luminescent plate reader.

P-gp ATPase assay

ATPase activity is potentially affected by Brij or TPGS; therefore, it was measured using a P-gp-Glo™ assay system (Promega). The System provides the necessary reagents for performing luminescent P-gp ATPase assays. These assays are valuable screening tools for determining if a drug interacts with P-gp. The activity of P-gp ATPase is measured in the presence or absence of Na_3VO_4 and Verapamil (positive reference). The assay method is based on the dependence of ATP on the light-generating reaction of firefly luciferase. This reaction is measured by detecting ATP consumption as a decrease in luminescence. The effect of Brij on the ATPase activity of P-gp was measured using the manufacturer's protocols. In a 96 well plate, recombinant human P-gp (25 µg) was incubated with P-gp-Glo™ assay buffer (20 µl), verapamil (200 µM), sodium orthovanidate (100 µM), and test compounds (Brij and TPGS at 50 µM). Each compound was loaded in five individual wells. The reaction was initiated by the addition of MgATP (10 mM), then halted 40 min later by the addition of 50 µL of the firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later using a Bio-Rad microplate imaging system (Hercules, CA).

Western Blot Analysis

Total protein in the lysate was quantified using a Pierce BCA protein assay kit (Thermo Scientific Inc., Rockford, IL). Approximately 40 µg of proteins from each sample were separated by NuPAGE 12% gel and then transferred onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA). The mouse monoclonal antibody to P-gp was used at a 1:2000 dilution and the rabbit monoclonal anti-GAPDH clone at a 1:200 dilution. The secondary antibodies used were an anti-mouse conjugated with HRP at a dilution of 1:10000 or an anti-rabbit IgG at a dilution of 1:2000. The specific protein bands were visualized using a chemiluminescence kit (Pierce, Rockford, IL), and the chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare).

Statistical analysis

All data were represented as mean value ± standard deviation (SD). Statistical comparisons were made using a one-way analysis variance (ANOVA) Tukey's-test. Results were considered significant when a confidence interval of 95% ($p < 0.05$) was achieved.

RESULTS

Characterization of NCs

The hydrophilic–lipophilic balance (HLB) concept was proposed by Griffin (1954) as follows: "emulsifiers consist of a molecule that combines both hydrophilic and lipophilic

groups and the balance of the size and strength of these two opposite groups is called HLB. For the purpose of convenience, the effective balance of these groups is assigned a numeric value." For the polyoxyethylene alkyl ethers and polyoxyethylene esters, Griffin considered the chain unities of ethylene oxide as the hydrophilic group. According to Griffin, $HLB=20*M_h/M$, where M_h is the molecular mass of the hydrophilic portion of the molecule, and M is the molecular mass of the whole molecule.[18]

The structures, composition, and HLB of the different Brij molecules are shown in Table 1.[19] PTX/Brij NCs can be formed when the Brij has an HLB 9 at a ratio of 1:5, w:w or an HLB 15 at ratios of 1:3 and 1:2, w/w. PTX/Brijs NCs can be formed at ratios from 1:2-1:5 when MHLB (the ratio of the number (n) of hydrophilic repeating units of ethylene oxide (EO) to the number (m) of carbons in the hydrophobic tail (CH_2)) of Brij is greater than 0.3. The stable NCs could not be formulated with Brij 52 and Brij 72 with 2 PEG repeating units (formation of aggregates). Therefore, they were not selected for the further study. Brij 700 with highest HLB and PEG repeating units (n=100) formed NCs of the smallest particle size (Table 2). PTX/Brij NCs with PTX:Brij ratios of 1:2 are significantly larger than NCs with a ratio of 1:5, but the morphology of both was rod-shaped (Figure. 1).

Effect on Resistant Cancer Cells in Vitro

The antiproliferation effect of various formulations of PTX on H460/taxR cells that overexpress P-gp and are resistant to PTX, were measured by MTS. Data (Figure. 2) were compared using an ANONA and Tukey's-test, which showed a significant difference ($p < 0.05$) between PTX/TPGS, PTX/Brij 58, PTX/Brij 78, PTX/Brij 98, PTX/Brij 97, PTX/Brij S10, PTX/Brij 30 NCs and the free PTX (Figure. 2A). PTX/Brij 58, 78 and 98 NCs with the ratio of 1:5 (w:w) exhibited an enhanced anti-proliferation effect similar to that of PTX/TPGS, which inhibited cell growth by 40%. The free Brij 58, 78 and 98 were virtually ineffective (Figure. 2B). There was a significant difference ($p < 0.05$) between the group of free Brij 30 (1:5), Brij S10 (1:5-1:3), Brij 97 (1:5) and the negative control (DMSO). Notably, PTX/Brij 30 NCs with a ratio of 1:5 (w:w) exhibited an enhanced antiproliferation effect similar to that of PTX/Brij 78 and 97 NCs, which could be the result of the cytotoxicy of free Brij 30 (Figure. 2B). Cell viability for PTX/Brij 30 NCs was 48.0%, but it was 69.3% for free Brij 30.

When the data are re-plotted with MHLB on the x-axis and percentage of cell viability (from Figure 2) on the y-axis, a positive correlation is shown between MHLB of Brij surfactants and viability with a coefficient (R^2) of 0.8195. Cell viability of PTX/ Brij NCs was more related to MHLB than HLB (Figure 3). This observation suggests that Brij molecules with a lower ratio of MHLB show a stronger ability to overcome MDR. Although Brij 35 has a similar HLB to Brij 58, 78 and 98, PTX/Brij 35 NCs did not significantly inhibit cell growth. The composition of Brij 35 contains fewer hydrophobic repeating units (m=12), potentially causing its ineffectiveness. PTX NCs of Brij 700 and Brij 35 with the ratio of 1:5 (w/w) containing a ratio of hydrophilic repeating units to hydrophobic repeating units (MHLB) > 1.5 were also ineffective in reversing the resistance of H460/taxR to PTX (Figure 3).

Cellular uptake of ^{3}H-PTX in H460/taxR cells

The cellular uptake of ^{3}H-PTX in the presence of Brij 78, 97, 30, 35 and TPGS was also determined in H460/taxR cells. Brij 78, 97 and TPGS increased the intracellular uptake of ^{3}H-PTX by 4.5-, 7.6- and 3.4-fold in H460/taxR cells, respectively. Brij 30 and 35 had less effect on intracellular uptake of ^{3}H-PTX (Fig. 4A). However, PTX/Brij 30 NCs with a ratio of 1:5 (w:w) exhibited an enhanced antiproliferation effect similar to that of PTX/Brij 78 and 97, which could be the result of the cytotoxicity of free Brij 30 (Fig. 2B). Brij 78, 97

and TPGS increased the uptake of ^{3}H-PTX in H460/taxR cells significantly, suggesting that these could inhibit P-gp.

Determination of intracellular ATP and P-gp expression

Because drug efflux is an energy-dependent process, the intracellular ATP levels of cells after they are treated with Brij 78, 97, 30 and 35 without loading drugs were investigated. We found that exposing cells to Brij 78 and 97 could induce a significant decrease in ATP in drug resistant cells ($p < 0.05$, Fig. 5A). Brij 78 and 97 are more active in the process of ATP depletion and displayed higher efficacy in sensitizing the cytotoxic activity of the drugs in resistant cells. On the contrary, Brij 30 and 35 are less active in ATP depletion are also the least efficacious in sensitizing the drug cytotoxic activity.

Nanoparticles can also protect loaded drugs from efflux by P-gp. The reversal of P-gp mediated MDR can usually be achieved either by down-regulating P-gp expression or inhibiting its function. To assess the effect of P-gp protein expression levels, Western blot analyses were performed. After pretreatment by Brij 78, 97 and TPGS (in the same concentration as cytotoxicity), the expression level of P-gp protein (Fig. 5B) was not significantly altered compared to the control in H460/taxR cells. The results indicate that inhibiting the expression of P-gp is not involved in the reversal of P-gp MDR by Brij78, 97 or TPGS.

P-gp ATPase activity

Drug stimulated P-gp ATPase activity was estimated by P-gp-Glo assay system (Promega, Madison, WI) and the results were shown in Figure 6. This method relies on the ATP dependence of the light-generating reaction of firefly luciferase, where ATP consumption is illustrated as a decrease in luminescence; the greater the decrease in signal the higher the P-gp activity. Sodium orthovanadate was used as a P-gp ATPase inhibitor, whereas Verapamil was used as a positive control. Test compounds that are significantly lower than the control (NT) are P-gp substrates, whereas test compounds that are significantly higher than the NT are P-gp inhibitors. Data were compared using an ANOVA and Tukey's-test. Brij 78, 97 and TPGS (all tested at 50 µM) did not significantly influence ATPase activity on their own ($p > 0.05$). None induced additional ATPase activity nor inhibited basal ATPase function (Fig. 6A). However, in the presence of Verapamil (200 µM), which stimulated ATPase activity; Brij 78, 97 and TPGS were able to significantly inhibit Verapamil-induced ATPase activity ($p < 0.05$, Fig. 6B).

DISCUSSION

MDR is a major clinical problem that significantly reduces the efficacy of many chemotherapeutic agents. The most extensively studied mechanism of MDR is the overexpression of P-gp as cell surface efflux pumps that can successfully purge a wide spectrum of chemotherapeutic agents from cells, thereby decreasing their intracellular accumulation and creating resistance. The inhibition of P-gp in order to reverse MDR in cancer cells has been studied extensively. In addition to small molecule inhibitors, surfactants such as several Pluronics and TPGS are known to modulate efflux pump activity. The therapeutic nanoparticles have emerged as an innovative and promising alternative over conventional small molecule chemotherapeutics aimed at specifically targeting the therapeutic payload to tumors and overcoming MDR.[20-26] Previous work conducted in our laboratories demonstrated that PTX NCs prepared using TPGS as the sole excipient can overcome the drug-resistance in human ovarian NCI/ADR-RES cancer cells. We are able to compare TPGS and Brij molecules because of their similarities; both TPGS and Brij have linear amphiphilic structures with varying lengths of PEG creating the hydrophilic head and

varying lengths of linear hydrocarbon tails. We hypothesize that the structure of nonionic surfactants may be crucial to overcoming MDR.

Brij surfactants have different molecular structures, some of which have been reported to be P-gp inhibitors. Therefore, the Brij surfactants (see Table 1 for their structures, compositions and HLB[19]) had been systemically explored in the present study. The cytotoxicity of various formulations of PTX/Brij NCs on H460/taxR cells, which overexpress P-gp and are resistant to PTX are summarized in Figure 2. A Brij molecule with a lower ratio of MHLB shows a greater ability to inhibit P-gp, resulting in the higher cytotoxicity of PTX/Brij. Tween (Tween 20, 40 and 80) and DSPE-PEG have also been included in our studies. However, while paclitaxel (PTX) can be formulated into NCs with Tween and DSPE-PEG surfactants, these surfactants did not show the activity required to overcome MDR (data not shown).

Previously we studied PTX/F127 (pluronic F127) and PTX/TPGS nanocrystals.[13] PTX/ TPGS nanocrystals can overcome MDR, but PTX/F127 nanocrystals cannot, despite the fact that both crystals are rod shaped. These observations lead us to the conclusion that the shape of the NPs has very little to do with MDR. The reason we used nanocrystals is their high PTX loading efficiency and their structural simplicity (nanocrystals only require one type of non-ionic surfactant as an excipient, which has advantages in completing mechanism studies).

When compared with some Brij molecules, Tween surfactants are shown to have the same hydrophobic tails, for example, Tween 60 compared to Brij 78 and S10 or Tween 80 compared to Brij 97 and 98. However, the hydrophilic portions of the two molecule types are different. Brij molecules have a linear PEG chain as their hydrophilic region, whereas Tween has three branched, shorter PEG chains. Consequently, Brij molecules show a significant inhibition of P-gp and Tween surfactants do not. Collnot et al. (2007) revealed that the rate of permeation of rhodamine 123 (a P-gp substrate) through monolayers of Caco-2 cells was strongly influenced by the length of PEG chains, with an optimum molecular weight of these linear PEG chains occurring between 1000 and 1500 Da.[11] Therefore, we conclude that the hydrophilic head group of Tween does not play an important role in inhibiting P-gp. This finding provides some insight into the role of the structures of Brij-based surfactants in overcoming MDR, which is useful for synthesizing or screening new P-gp inhibitors.

Reduction of the intracellular concentration of chemotherapeutic agents is a major cause of MDR. It is a widely held hypothesis that intracellular levels of anticancer drugs are reduced below lethal thresholds by active extrusion through the operation of ATP-dependent pumps such as P-gp.[27] Thus, the activity of P-gp can be studied by measuring the transportation of P-gp substrates. In our study, we found that Brij 78, Brij 97 increased the accumulation of ^{3}H-PTX in P-gp overexpressed MDR cells. These dates were consistent with our cytotoxic results, collectively suggesting that Brij 78, Brij 97 can inhibit the transport function of P-gp, increasing the intracellular concentration of its substrate anticancer drugs.

The increased accumulation of intracellular drugs may be a result of the decreased expression of P-gp or decreased function of P-gp. Since incubation of MDR cells with Brij 78 or Brij 97 up to 48 h did not significantly change the expression levels of P-gp protein, it is unlikely that Brij 78 or Brij 97 reverses P-gp-mediated MDR.

The exact mechanism by which excipients inhibit efflux transporters remains unknown. Pluronic P85 has been reported to significantly reduce intracellular ATP levels, and this correlates with the inhibition of efflux transporters.[28] The mechanism of inhibition of the cellular efflux pumps by TPGS involves inhibition of ATPase rather than a competitive inhibition or a nonspecific effect on the fluidity of the cell membrane.[10-12] For example,

TPGS 1000 is similar to polyoxyethylene (40) stearate (Myrj 52), a material known to affect P-gp ATPase while not influencing intracellular ATP or mitochondrial function. As a result, there does not appear to be a common inhibitory mechanism for all nonionic surfactants; the interaction with P-gp needs to be investigated for each individual surfactant until a broader relationship between structure and inhibition is defined.

It is quite possible that the chemosensitization phenomenon is a direct result of the decrease in the ATP pools available within the drug resistant cells. In our results, there is a clear correlation between the levels of uptake of P-gp substrate, H^3-PTX, and the ATP levels in the P-gp overexpressing H460/taxR cells after treatment by Brij78 and Brij 97. Brij 78 could also enhance doxorubicin uptake and decrease the efflux and depletion of ATP after NCI/ADR-RES cells were pretreated with various concentrations of Brij 78.[16] Our results indicated that TPGS did not deplete ATP significantly, which is in accordance with the research performed by Dong (2009).[16] Brij 30 increased the uptake of mitoxantrone, but it did not change the intracellular ATP levels in the P-gp/MDCK-II cells.[29] In the present study, Brij 30 did not reduce the intracellular ATP levels and produced only a minor increase in the intracellular accumulation of ^{3}H-PTX in H460/taxR cells, which is contradictory to previous findings.[14, 29] However, this might be because of a difference in the cell lines used. Furthermore, the expression level of P-gp was not affected by the treatments with the addition of Brij78 and Brij 97 (in the same concentration as cytotoxicity).

P-gp has an ATP-binding region that is essential for substrate transport and the hydrolysis of ATP by P-gp ATPase is critical for restoring the transporter to its active conformational state. Thus, monitoring ATPase activity in cell membrane preparations or in purified membrane proteins allows for identification of those compounds that interact with the drug efflux transporters. P-gp exhibits a highly drug-dependent ATP hydrolysis activity, and a variety of P-gp inhibitors, as well as P-gp substrates, can stimulate ATPase activity.[30] When incubated on their own with artificial P-gp containing membranes (Fig. 6A), Brij78, Brij 97 and TPGS neither stimulated ATPase activity nor inhibited basal ATPase function at the concentration 50 μM. These results indicate that there is no direct interaction between the transport sites and the surfactants. Brij78, Brij 97 and TPGS therefore appeared not to be a substrate of P-gp, making a competitive inhibition of substrate binding unlikely. However, a significant inhibition of P-gp after Verapamil-induced ATPase activation was indeed observed.

CONCLUSION

This study identified structures or features required for overcoming MDR and provided the first in vitro evidence that Brij 78 and Brij 97 decreased intracellular ATP levels (energy depletion) and inhibited Verapamil-induced ATPase activity of P-gp in multidrug resistant cells. The combination of these two effects is essential for inhibition of P-gp drug efflux system and chemosensitization of these cells. In addition, the Brij or TPGS did not affect the protein expression of P-gp. Further understanding of the mechanisms of the Brij effects will be crucial for the successful development and use of Brij formulations to improve chemotherapy of resistant tumors in the future.

Acknowledgments

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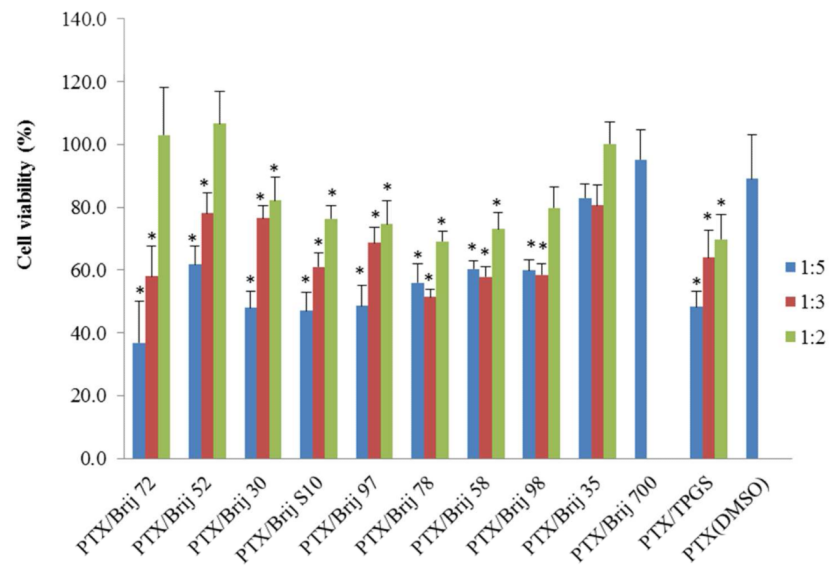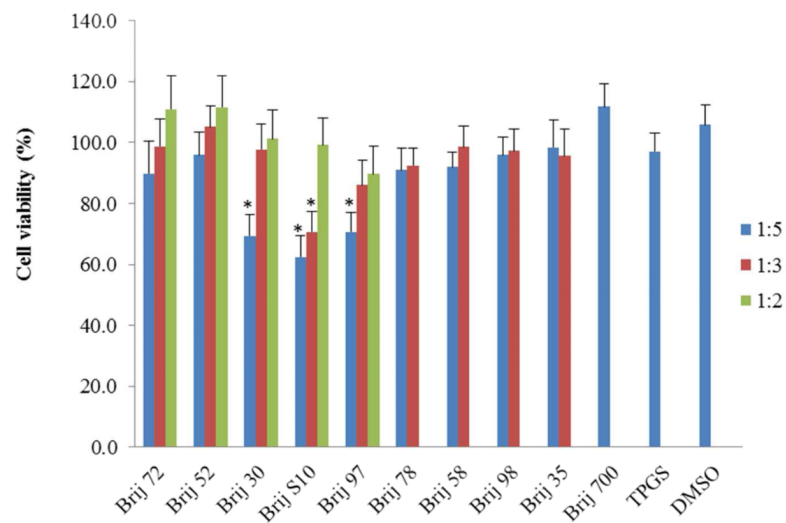30. Li PY, Lai PS, Hung WC, Syu WJ. Biomacromolecules. 2010; 11:2576–2782. [PubMed: 20722436]
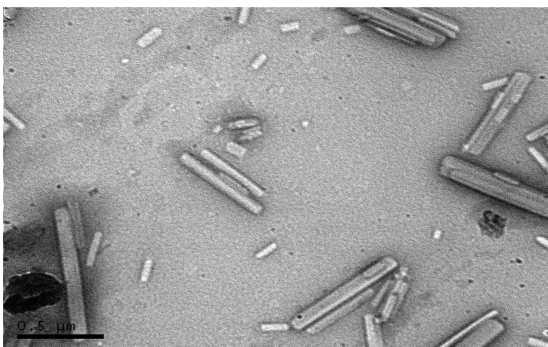
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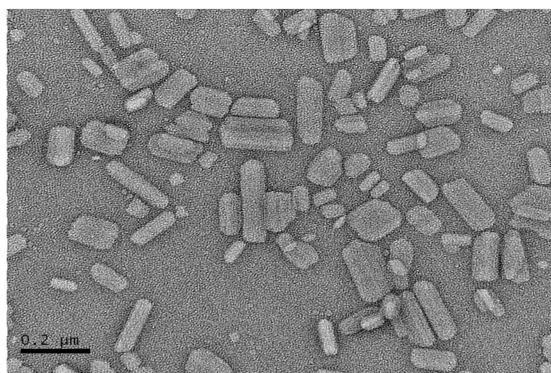
(B)

Figure 1.
Transmission electron microscope images of PTX/Brij 78 nanocrystals with the ratio of 1:2 (A) and 1:5 (B) (w:w).

(A)

(B)

Figure 2.
H460/taxR cell viability after being treated with PTX/Brij or PTX/TPGS nanocrystals with
ratios of 1:5, 1:3 and 1:2 (w:w) for 48 h, and free PTX at a concentration of 5 µM (n=6). (A)
PTX/TPGS, PTX/Brij nanocrystals and free PTX. * indicates significant difference
compared to PTX (in DMSO) at $p < 0.05$. (B) Free TPGS or Brij. Free Brij or TPGS were
prepared by the method of SNC without PTX. * indicates significant difference compared to
DMSO at $p < 0.05$.
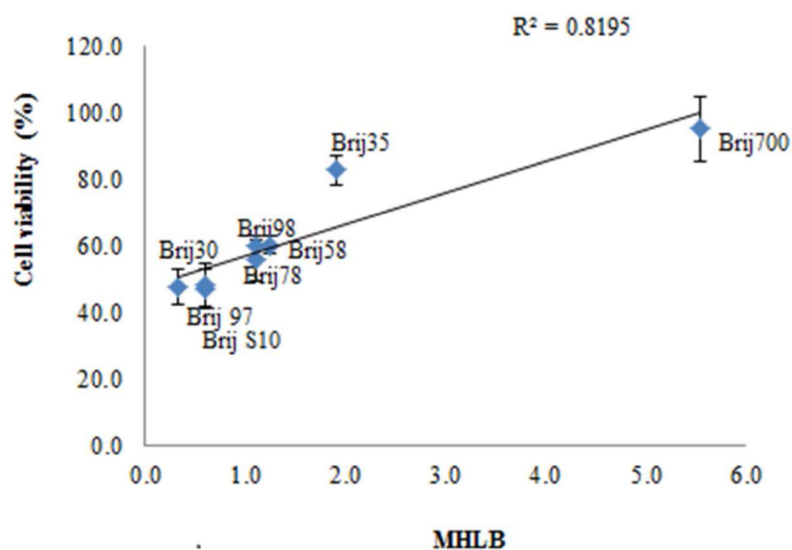
(A)
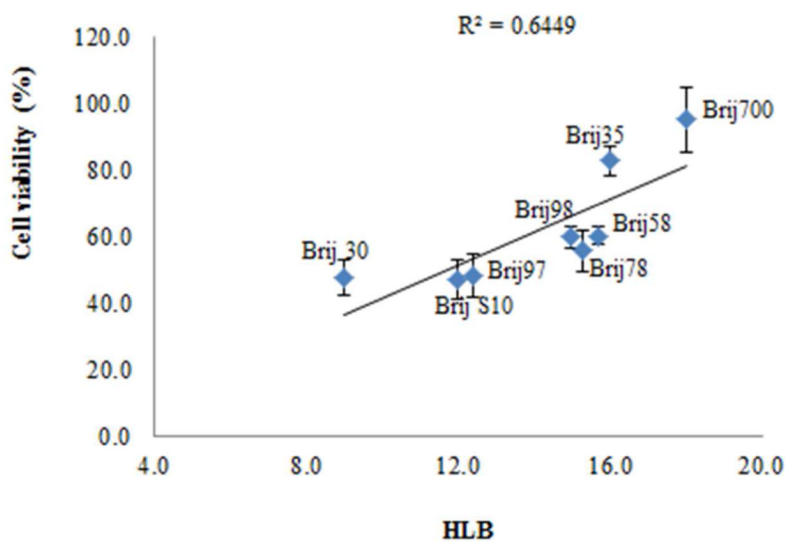
(B)

Figure 3.
The relationship between the MHLB (A) or HLB (B) of Brij molecules and cell viability of
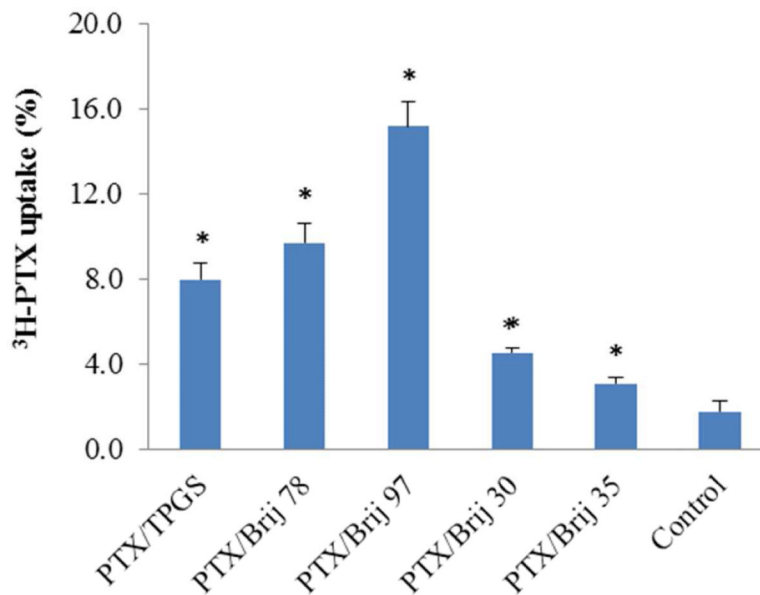H460/taxR cells after treatment with PTX/Brijs NCs. *Points*, mean; *bars*, SD (n=6).
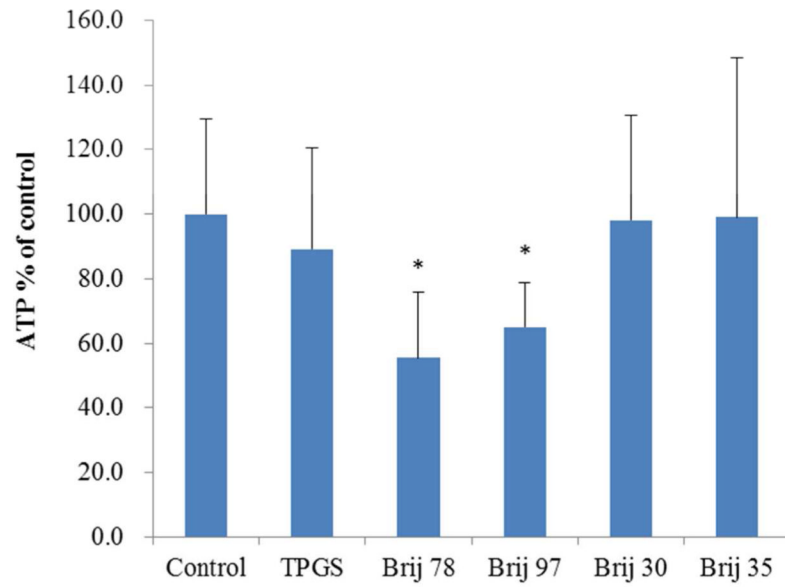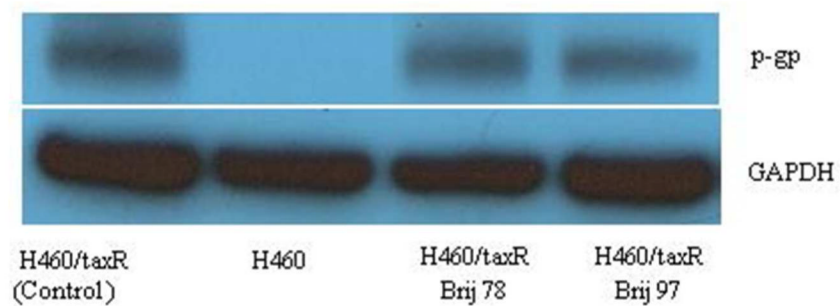
Figure 4.
The effects of Brij and TPGS on cellular uptake of ^{3}H-PTX in H460/taxR cells. H460/taxR cells were treated with free PTX or PTX/Brijs nanocrystals (5 µM PTX containing 32 nCi ^{3}H-PTX per well) and incubated at 37 °C for 5 h. Cellular uptake efficiency of ^{3}H-PTX (%) was calculated by the amount of intracellular ^{3}H-PTX/added ^{3}H-PTX per well × 100. * indicates significant difference compared to the control at $p < 0.05$. Data are represented as the mean ± SD (n=3).
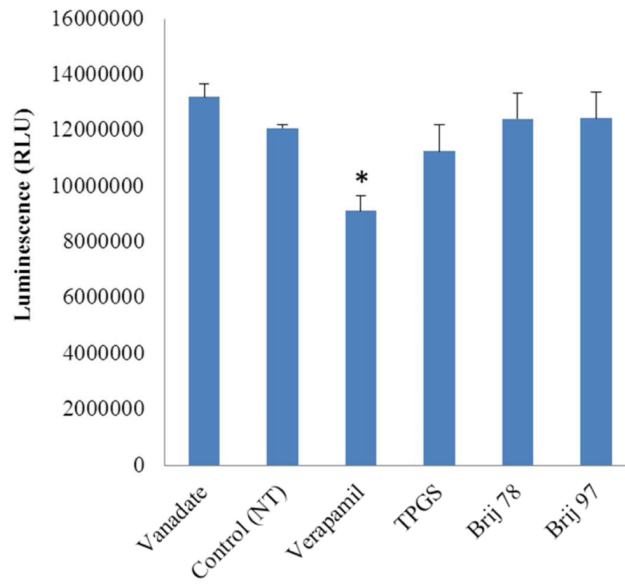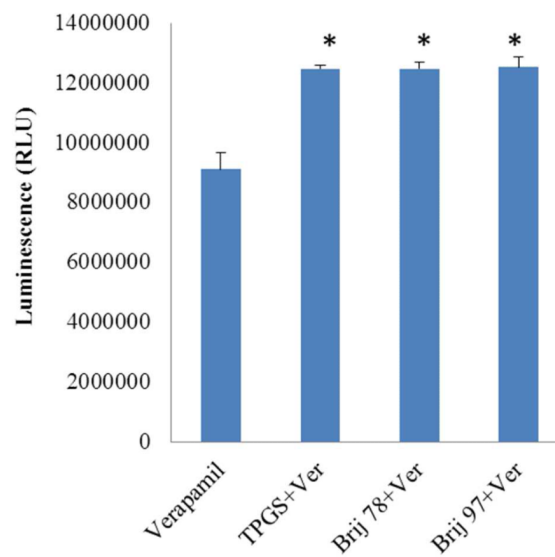
(A)

(B)

Figure 5.
(A) The effects of Brij and TPGS on intracellular ATP levels in H460/taxR cells. Data are represented as the mean ± SD (n=5). * indicates significant difference compared to the control at $p < 0.05$. (B) The effects of Brij and TPGS on P-gp expression by western blotting.

(A)

(B)

Figure 6.
(A) The effects of Brij and TPGS on P-gp ATPase in H460/taxR cells. * indicates significant difference from the control at $p < 0.05$. (B) The effects of Brij and TPGS on Verapamil induced ATPase activity. * indicates significant difference from verapamil at $p < 0.05$. Brij and TPGS at 50 μM. Data are represented as the mean ± SD (n=5).

Table 1

Structures, composition and HLB of the Brij non-ionic surfactants[19]

Trade name	Structures	M_n	HLB	n	m	MHLB n/m	CMC (mM)
Brij 72:	$C_{18}H_{37}(OCH_2CH_2)_2OH$	358.6	4.9	2	18	0.111	3E-04
Brij 52	$C_{16}H_{33}(OCH_2CH_2)_2OH$	330	5	2	16	0.125	19.88
Brij 30	$C_{12}H_{25}(OCH_2CH_2)_4OH$	362	9	4	12	0.33	0.004
Brij S10	$C_{18}H_{37}(OCH_2CH_2)_{10}OH$	711	12	10	18	0.6	0.003
Brij 97	$C_{18}H_{35}(OCH_2CH_2)_{10}OH$	709	12.4	10	18	0.6	0.94
Brij 78	$C_{18}H_{37}(OCH_2CH_2)_{20}OH$	1151	15.3	20	18	1.11	0.006
Brij 98	$C_{18}H_{35}(OCH_2CH_2)_{20}OH$	1149.5	15	20	18	1.11	0.265
Brij 58	$C_{16}H_{33}(OCH_2CH_2)_{20}OH$	1124	15.7	20	16	1.25	0.007
Brij 35	$C_{12}H_{25}(OCH_2CH_2)_{23}OH$	1198	16	23	12	1.92	0.06
Brij 700	$C_{18}H_{37}(OCH_2CH_2)_{100}OH$	4670	18	100	18	5.55	0.02

The physiochemical information of the surfactants was obtained from the manufacturer.

M_n: molecular weight of Brij molecules; HLB: hydrophilic-lipophilic balance of Brij molecules; n: number of hydrophilic repeating units; m: number of hydrophobic repeating units; MHLB (n/m): the ratio of n to m.

Table 2

The particle size and distribution of PTX/Brij nanocrystals (PTX/Brij=1:5, w/w)

	Size (nm)	PDI	Zeta (mV)
PTX/Brij 72	n/a	n/a	n/a
PTX/Brij 52	n/a	n/a	n/a
PTX/Brij 30	104.4	0.256	-24.3
PTX/Brij S10	142.7	0.194	-9.7
PTX/Brij 97	244.7	0.513	-15.7
PTX/Brij 78	144.4	0.258	-11.7
PTX/Brij 58	228.7	0.216	-12.9
PTX/Brij 98	79.12	0.209	-12.5
PTX/Brij 35	66.84	0.187	-23.7
PTX/Brij 700	77.70	0.229	-8.47

n/a: not available