SHORT COMMUNICATION

Impact of molecular weight on the mechanism of cellular uptake of polyethylene glycols (PEGs) with particular reference to P-glycoprotein

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Abstract  
Polyethylene glycols (PEGs) in general use are polydisperse molecules with molecular weight (MW) distributed around an average value applied in their designation e.g., PEG 4000. Previous research has shown that PEGs can act as P-glycoprotein (P-gp) inhibitors with the potential to affect the absorption and efflux of concomitantly administered drugs. However, questions related to the mechanism of cellular uptake of PEGs and the exact role played by P-gp has not been addressed. In this study, we examined the mechanism of uptake of PEGs by MDCK-mock cells, in particular, the effect of MW and interaction with P-gp by MDCK-hMDR1 and A549 cells. The results show that: (a) the uptake of PEGs by MDCK-hMDR1 cells is enhanced by P-gp inhibitors; (b) PEGs stimulate P-gp ATPase activity but to a

Abbreviations: ACN, acetonitrile; AUC, area under the plasma concentration-time curve; CE, collision energy; C\textsubscript{max}, maximum plasma concentration; CsA, cyclosporine A; DBD, drug-binding domain; DDS, drug delivery system; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; DP, declustering potential; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IS, internal standard; LC–HRMS/MS, liquid chromatography–high resolution tandem mass spectrometry; MW, molecular weight; NBD, nucleotide binding domain; PAC, paclitaxel; PEG, polyethylene glycol; P-gp, P-glycoprotein; VER, verapamil.

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much lesser extent than verapamil; and (c) uptake of PEGs of low MW (<2000 Da) occurs by passive diffusion whereas uptake of PEGs of high MW (>5000 Da) occurs by a combination of passive diffusion and caveolae-mediated endocytosis. These findings suggest that PEGs can engage in P-gp-based drug interactions which we believe should be taken into account when using PEGs as excipients and in PEGylated drugs and drug delivery systems.

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1. Introduction

PEGs in general use are polydisperse molecules applied as excipients to improve solubility and dissolution of pharmaceuticals. They are also used to form conjugates with drug molecules (PEGylation) and to prepare PEGylated drug delivery systems (DDS), such as liposomes and nanoparticles. When PEGs or PEGylated therapeutics are ingested, recipients are inevitably exposed to free PEG. This is generally considered to have little adverse effects due to their tendency to accumulate in tissues and acting as P-gp inhibitors. This paper reports the results of a study into the mechanism of cellular uptake of PEGs with different molecular weight (MW) and the role played by P-gp in the process.

P-gp is a membrane protein that acts as an important mediator of drug efflux from cells through possession of a drug-binding domain (DBD) and a nucleotide binding domain (NBD). P-gp substrates first bind to the DBD after ATP hydrolysis at the NBD by ATPase, which provides the energy to bring about efflux. Some P-gp inhibitors also act in this way but others can act by directly inhibiting ATPase. Given that a molecule must be hydrophobic in order to enter the binding pocket of the DBD, PEGs are generally considered to be too hydrophilic to act as P-gp substrates and, to date, there is no evidence that they can. In addition, while PEGs can act as P-gp inhibitors, it remains unclear whether this is due to their ability to inhibit ATPase or to an indirect mechanism involving the disruption of the cell membrane in which P-gp is embedded.

Using two novel bioanalytical method based on liquid chromatography–high resolution tandem mass spectrometry (LC–HRMS/MS) developed in our laboratory, we investigated the cellular uptake of polydisperse PEGs both as total PEG concentration and as a profile of each individual PEG homolog. This allowed us to determine the effect of MW and illuminate the role of P-gp. We believe the results increase our understanding of the fate and potential toxicity of PEGs when administered as excipients, PEGylated drugs and PEGylated DDS in the future design and clinical use of PEG therapeutics.

2. Materials and methods

2.1. Materials

Materials were supplied as follows: colchicine (purity 98.5%), chlorpromazine (purity > 88%), genistein (purity > 98%), quercetin (purity > 85%) and verapamil (VER, purity > 99 %) (Dalian Meilun Biotechnology Company, Dalian, China); simvastatin (purity >99.8%) for use as internal standard (IS, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China); cyclosporine A (CsA, purity > 98.5%) and methoxy polyethylene glycol (PEG) 750, 2000, 5000 and 20,000 (Sigma-Aldrich, St. Louis, MO, USA); MDCK-hMDR1 and MDCK-mock (P-gp knockdown) cells at passage number 5–15 (Prof. Su Zeng, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China); A549 cells (the American Type Culture Collection, Rockville, MD, USA); Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and trypsin (Gibco, Grand Island, NY, USA); Hanks’ balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and penicillin-streptomycin solution (Dingguo Changsheng Biotechnology Company, Beijing, China); HPLC grade acetonitrile (ACN, Fisher Scientific, Pittsburgh, PA, USA); BCA protein assay kit (Pierce, Rockford, IL, USA); Pgg-GLO™ Assay System (Promega, Madison, WI, USA). All other chemicals were of analytical grade and used as received. Ultrapure water was prepared using the Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Cell culture

Cells were grown in DMEM with 10% FBS, 1% nonessential amino acid solution and 1% penicillin-streptomycin solution in a humidified atmosphere containing 5% CO₂.

2.3. PEG uptake by MDCK-hMDR1, MDCK-mock and A549 cells

All drugs were dissolved in DMSO and subsequently diluted in transport buffer [HBSS containing 10 mmol/L HEPES (pH 7.4)]. PEGs were dissolved and diluted in transport buffer. Cells were seeded on 6-well plates (6 × 10⁴ cells/cm²). Medium was changed every two days and experiments were conducted after 5 days in culture.

To study the effect of P-gp inhibitors on cell uptake of PEGs, MDCK-hMDR1 cells were preincubated (0.5 h) and incubated (2 h) at 37 °C as follows: (1) preincubation with transport buffer followed by incubation with PEG (25 and 50 μmol/L, control); (2) preincubation with VER (200 μmol/L) followed by incubation with VER (200 μmol/L) and PEG (25 and 50 μmol/L) together; and (3) preincubation with CsA (10 μmol/L) followed by incubation with CsA (10 μmol/L) and PEG (25 and 50 μmol/L) together.
To investigate the MW dependence of PEG uptake into MDCK-mock cells, MDCK-mock cells were incubated with PEG 750 (50, 100, 500 and 1000 μmol/L) or PEGs 2000, 5000 or 20,000 (5, 10, 50 and 100 μmol/L) at 37 °C and 4 °C for 2 h. To further investigate the mechanism of uptake of low MW PEGs, A549 cells were incubated at 37 °C with PEG 750 or 2000 (50 μmol/L) for various lengths of time up to 24 h. To further investigate the mechanism of uptake of high MW PEGs, MDCK-mock cells were preincubated (0.5 h) and incubated (2 h) as follows: (1) preincubation with transport buffer followed by incubation with PEG 5000 or 20,000 (100 μmol/L) at 37 °C (positive control); (2) preincubation with transport buffer followed by incubation with PEG 5000 or 20,000 (100 μmol/L) at 4 °C (negative control); (3) preincubation with colchicine (40 μg/mL) followed by incubation with colchicine (40 μg/mL) and PEG at 37 °C; (4) preincubation with chlorpromazine (10 μg/mL) followed by incubation with colchicine (10 μg/mL) and PEG at 37 °C; (5) preincubation with genistein (50 μg/mL) followed by incubation with genistein (50 μg/mL) and PEG at 37 °C; (6) preincubation with quercetin (100 μg/mL) followed by incubation with quercetin (100 μg/mL) and PEG at 37 °C.

After incubations, cells were processed as previously reported. In brief, cells were: (1) rinsed 3 times with ice-cold transport buffer, (2) collected using a cell scraper, (3) sonicated, (4) centrifuged at 9000 g, (5) the protein and intracellular PEG concentration determined. Intracellular PEG concentrations are given in ng/μg protein.

2.4. Determination of PEGs in cell lysates

LC was based on our previously reported method with modifications to improve peak shape. A 50 μL aliquot of sample was mixed with 20 μL IS solution (1 μg/mL in ACN) and 150 μL cold ACN (−20 °C) after which samples were centrifuged at 15,000 rpm (ThermoPico17-A, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min and 50 μL supernatant analyzed. Chromatographic conditions for assay of the different PEGs are listed in Table 1.

HRMS/MS utilized the MS² technique in which PEG precursor ions are dissociated to fragments in the collision cell. The MS parameters for assay of all PEGs were as follows: declustering potential (DP) and collision energy (CE) were 100 V and 30 eV, respectively; nebulizer, heater and curtain gas flow rates (N2) were 5500 V; heater gas temperature was 500 °C. Quantitation was conducted by extracting the product ions of PEGs and IS in the m/z ranges of 133.06–133.10 and 225.16–225.17, respectively. Data acquisition and integration was controlled by Analyst TF version 1.7.1 software. Representative LC–HRMS chromatograms and mass spectra are provided in Supporting Information.

2.5. Effect of PEGs on P-gp ATPase activity

P-gp ATPase activity was estimated using the P-gp-Glo assay system according to the manufacturer’s instructions. Briefly, human P-gp overexpressing membranes were incubated with PEGs (25 and 50 μmol/L), VEP (200 μmol/L), Na2VO4 (100 μmol/L) or buffer alone in a 96-well plate for 5 min. Mg-ATP was added to initiate the reaction (40 min). ATP Detection Reagent (Pgp-GLO™ Assay System, Promega, Madison, WI, USA) was added (20 min) to assay the residual ATP content. ATP consumption was detected as a decrease in luminescence, the less ATM remaining, the higher the P-gp ATPase activity was.

2.6. MW profiling of uptake of PEGs 750 and 2000 by A549 cells

After incubation of A549 cells with PEGs, a 50 μL aliquot of cell lysate was mixed with 200 μL ACN, the mixture centrifuged at

| Table 1 | Analytical conditions for analyzing of total PEG concentration and each individual PEG homolog. |
|---------|-----------------------------------------------------------------------------------|
| Analyte | Chromatography column | Column temperature (°C) | Solvent A | Solvent B | Gradient program | Flow rate (mL/min) |
| PEG 750 | Zorbax 300SB-C18 column | 40 | 0.1% Formic acid in water | ACN | 0–1 min 10% B, 1–3 min 10%–90% B, 3–5.5 min 90% B, 5.5–5.6 min 90%–10% B, 5.6–8 min 10% B | 0.6 |
| PEG 2000 | Zorbax 300SB-C18 column | 40 | 0.1% Formic acid in water | ACN | 0–1 min 20% B, 1–5 min 20%–95% B, 5–7.9 min 95% B, 7.9–8 min 95%–20% B, 8–10 min 20% B | |
| PEG 5000 | PLRP-S column | 55 | 0.1% Formic acid in water | ACN:isopropanol (50:50) | 0–1 min 20% B, 1–4.5 min 20%–80% B, 4.5–6.9 min 80% B, 6.9–7 min 80%–20% B, 7–9 min 20% B, 9–10 min 20% B | |
| MW profiling | Zorbax 300SB-C18 column | 30 | 0.1% Formic acid in water | 0.1% Formic acid ACN | 0–1 min 17% B, 1–10 min 17%–30% B, 10–22 min 30%–33% B, 22–24 min 33%–80% B, 24–26 min 80% B, 26–26.1 min 80%–17% B, 26.1–30 min 17% B | 0.8 |
Impact of MW on the mechanism of cellular uptake of PEGs

3. Results and discussion

3.1. Effect of P-gp inhibitors on PEG uptake by MDCK-hMDR1 cells

Previous studies have shown that free PEGs can act as P-gp inhibitors in vitro. In our preliminary study, PEG was shown to affect the pharmacokinetics (PK) of the P-gp substrate, paclitaxel (PAC), in rat in vivo (Supporting Information Table S2 and Fig. S3). The large interindividual variability in plasma concentration-time curves is probably due to variability in the expression of P-gp and CYP3A, and the low oral absorption of PAC. The maximum plasma concentration (Cmax) and area under the plasma concentration–time curve (AUC) in the PEG pre-treated group is higher than in control. This result is consistent with a previous study showing that PEG 400 can alter the bioavailability of other P-gp substrates. To date there are no reports relating to whether PEGs can act as P-gp substrates yet.

P-gp substrates are expected to accumulate in P-gp over-expressing cells in the presence of P-gp inhibitors. To investigate whether PEGs are effluxed by P-gp, we incubated the P-gp overexpressing MDCK-hMDR1 cell line with PEGs concentrations of around 40 μmol/L (the reported concentration of PEG 2000 in rats after intravenous injection of PEGylated doxorubicin) in the presence of VER and CsA (P-gp substrates and competitive inhibitors). The results shown in Fig. 1 reveal that the uptake of PEGs by MDCK-hMDR1 cells is significantly increased in the presence of VER and to an even greater extent in the presence of CsA. This is consistent with the known greater affinity of CsA for P-gp. The results clearly indicate that PEGs can be effluxed by P-gp.

3.2. Effect of PEGs on P-gp ATPase activity

P-gp-mediated efflux of P-gp substrates leads to activation of P-gp ATPase and increasing consumption of ATP. To seek further evidence that PEGs are P-gp substrates, we applied the P-gp-Glo assay system to determine the consumption of ATP by P-gp in the presence of PEGs. It was found that VER significantly reduced the content of ATP whereas Na3VO4 (a non-competitive inhibitor of P-gp ATPase) significantly increased it. This is consistent with the above results showing that VER stimulates P-gp ATPase directly while Na3VO4 inhibits it indirectly by binding to other sites in P-gp such as the ATP binding site. PEGs, like VER, decreased the content of ATP, albeit to a much lesser extent (Fig. 2), indicating that PEGs, like VER, bind to the P-gp DBD and activate P-gp ATPase. The fact that the efflux of PEGs is inhibited by VER and CsA and that PEGs increase the activity of P-gp ATPase are consistent with previous research suggesting...
that PEGs have the same binding site on P-gp (the DBD) as VER and CsA. The weak stimulation of ATPase activity produced by PEG 20,000 is discussed in Section 3.3.

3.3. PEG uptake by MDCK-mock cells

Whenever a nanoparticle produced by self-assembly of a PEGylated drug encounters a cell, it is rapidly taken up through endocytosis. However, given that PEGs alone do not readily form nanoparticles, this pathway of PEG uptake may not or only apply to PEGs with relatively high MW. Since endocytosis and P-gp efflux require energy, they can be eliminated by incubating cells at low temperature (4°C) unlike passive diffusion which is not affected by temperature. However, given the possibility that P-gp could influence the comparison of PEG uptake at 4°C and at 37°C, we used P-gp knockdown MDCK-mock cells to avoid this potential complication.

The intracellular concentrations of PEG 750 after incubation with 5 or 10 μmol/L were too low to be detected, and therefore required higher concentrations (50, 100, 500 and 1000 μmol/L) than used for PEGs 2000, 5000 and 20,000 (5, 10, 50 and 100 μmol/L). The concentration and temperature dependence of the uptakes of PEGs are shown in Fig. 3 which also shows the proportion of PEGs entering cells by passive diffusion (the ratio of uptake at 4°C to that at 37°C). The results show that the uptakes of PEGs 750 and 2000 increase linearly with concentration and are not affected by temperature indicating they occur by passive diffusion. This result is surprising given that PEGs are amphiphilic polymers that are generally considered to be with limited cellular uptake. PEGs 5000 and 20,000 behaved similarly at concentrations <10 μmol/L, but, at concentrations >50 μmol/L, uptake was greater at 37°C than at 4°C. Furthermore, when the concentration increased from 50 to 100 μmol/L, the proportion of PEGs 5000 and 20,000 entering cells by passive diffusion decreased from 84.6% to 54.0% and 63% to 47%, respectively. This is speculated to arise because PEGs 5000 and 20,000 tend to aggregate at higher concentrations causing a higher proportion of their uptake to occur by endocytosis. Aggregation may also explain the weak stimulation of ATPase activity produced by PEG 20,000 which is much weaker at 4°C than at 37°C.
3.4. MW profiling of PEG uptake by A549 cells

Because PEGylated drugs are mainly used to treat cancer, we chose A549 non-small cell lung cancer cells to examine the MW profiles of PEG 750 and 2000 as a function of duration of incubation. The results for the different time intervals in Fig. 4 show that PEG 2000 enters cells more slowly than PEG 750 as its homologs are only detected after 1 h. Fig. 4 also shows that uptake of the lower MW homologs in PEG 750 (the PEG with the lower average MW) is more rapid than uptake of the higher MW homologs such that, after 0.25 h, the MW profile is skewed towards lower MW. In contrast, uptake of PEG 2000 is much slower and, even after 1 h, gives a profile that shows no evidence of being skewed to low MW. However, by 24 h, both profiles resemble those of the corresponding standard solutions.

3.5. Uptake of high MW PEGs by endocytosis

The results described above reveal that low MW PEGs (750 and 2000) enter cells by passive diffusion with no contribution from endocytosis whereas high MW PEGs enter cells by a combination of passive diffusion and endocytosis. To further investigate the mechanism of uptake of high MW PEGs by endocytosis, MDCK-mock cells were incubated with PEGs 5000 and 20,000 in the presence of the endocytosis inhibitors, colchicine (an inhibitor of microinocytosis), chlorpromazine (an inhibitor of clathrin-mediated endocytosis), genistein (an inhibitor of caveolae-mediated endocytosis) and quercetin (an inhibitor of caveolae- and clathrin-independent endocytosis). MDCK-mock cells were used because all the inhibitors of endocytosis (colchicine, chlorpromazine, genistein and quercetin) are also inhibitors of P-gp and therefore avoid this complication in studying the role of endocytosis in the uptake of PEGs. As shown in Fig. 5, the uptakes of PEGs 5000 and 20,000 are significantly lower at 4°C than at 37°C, (2) alone (negative control) at 4°C, containing (3) colchicine (40 μg/mL), (4) chlorpromazine (10 μg/mL), (5) genistein (50 μg/mL) or (6) quercetin (100 μg/mL) for 0.5 h. After removal of medium, cells were incubated in corresponding solutions containing PEGs (100 μmol/L) for 2 h. Total intracellular PEG concentration (ng/mg protein) was determined by LC–Q-Q-TOF MS using the MS<sup>3</sup> technique and expressed relative to the positive control set at 100%. Data are means ± SD of three independent experiments. *P < 0.01 vs. positive control.

4. Conclusions

PEGs are polydisperse polymers widely used as excipients and in PEGylated drug conjugates and DDS. Despite their hydrophilic nature, there is some evidence that PEGs can access cells and interact with P-gp, but how this occurs is unknown. The results of this study show that PEGs are indeed taken up by cells and act as P-gp substrates. PEGs with relatively low MW (average 750 and 2000 Da) cross cell membranes by passive diffusion whereas those with higher MW (average 5000 and 20,000 Da) enter cells by passive diffusion at low concentration and by a combination of passive diffusion and caveolae-mediated endocytosis at higher concentration. In terms of the intracellular MW profile of PEGs entering by passive diffusion, lower MW homologs are taken up more rapidly. We maintain these results have important implications in the pharmaceutical and clinical use of PEGs.
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Author contributions

Jingkai Gu and Huimin Sun participated in research design. Tingting Wang, Yingjie Guo, Tianming Ren and Lei Yin conducted experiments. Tingting Wang and Yang Paul Fawcett wrote or contributed to the writing of the manuscript.

Conflicts of interest

The authors declare there are no conflicts of interest. This work did not involve studies with human subjects.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsbs.2020.02.001.

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