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Effect of deoxycholate conjugation on stability of pDNA/polyamidoamine-diethylentriamine (PAM-DET) polyplex against ionic strength

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ABSTRACT

Polyplexes formed from cationic polymer/pDNA have been known to be vulnerable to external ionic strength. To improve polyplex stability against ionic strength, we attempted the chemical conjugation of the hydrophobic deoxycholate (DC) moiety to the polyamidoamine-diethylenetriamine (PAM-DET) dendrimer. Dynamic light scattering studies showed that the tolerance of the resulting PAM-DET-DC against ionic strength is higher than that of PAM-DET. In addition, we confirmed that the stability of polyplex has a strong relationship with the degree of conjugation of the DC moiety to the PAM-DET dendrimer and the charge ratio of PAM-DET-DC. Furthermore, the transfection efficiency of the PAM-DET-DC polyplex is higher than that of PAM-DET but its cytotoxicity remains the same. Therefore, the chemical conjugation of DC is a safe and effective method for increasing the stability of supramolecules formed from electrostatic interaction.

1. Introduction

Supramolecules, such as micelles, liposomes, and nano-particles have received much interest in the field of fundamental, material, and applied science (Yang et al., 2008; Rivest et al., 2007; Eremenko et al., 2002). It has been reported that these supramolecules are formed via several attraction forces including van der Waals forces, hydrophobic interactions, and electrostatic forces (Lin et al., 2001; Herrea et al., 2005; Zhuang et al., 2007; Watkins et al., 1997; Calvo and Wolosiuk, 2004). Among them, hydrophobic interactions have long been studied as the driving force for the formation of the supramolecules. However, hydrophobic interactions have been recognized to be unsuitable for the encapsulation of several water-soluble biological substances, such as DNA, siRNA, and proteins, which have recently been receiving attention for their high potential as therapeutic agents (Mozafari et al., 1998; Kim et al., 2008; Atsushi and Kazunori, 1999, 2003). Instead, electrostatic interactions were suggested as the most suitable driving force for the encapsulation of biological substances to form nanoscale supramolecules. However, the fragility of supramolecular structures formed by electrostatic forces, which is caused by increases in the ionic strength, has been indicated as a weak point that must be overcome. For a wide range of applications, various methods of enhancing the stability of supramolecules formed by electrostatic interactions against ionic strength have been investigated (Neu et al., 2006; Klymchenko et al., 2007; Liu et al., 2008). One of the approaches involves the introduction of another attraction force, i.e., hydrophobic interactions by conjugation of a hydrophobic moiety, to increase the stability (Yang et al., 2008). A polyamidoamine-diethylenetriamine (PAM-DET) dendrimer that was previously synthesized by our group (Jin et al., 2011) exhibits a highly positive charge on its surface and can form nano-sized complexes with negatively charged pDNA through electrostatic interactions. Due to its high transfection efficiency and low cytotoxicity, PAM-DET has a high potential as a gene-delivery carrier but has low stability in environments with high ionic strength because its polyplex is formed via electrostatic interactions.

In this study, we attempt to increase the stability of the pDNA/PAM-DET complex against ionic strength by introducing a hydrophobic moiety, i.e., deoxycholate (DC) (Fig. 1). DC is a well-known, non-toxic, natural fat emulsifier; therefore, deoxycholate conjugation can potentially enhance the stability of the complex without undesirable increases in cytotoxicity. Through a dynamic light scattering (DLS) study, we prove that deoxycholate conjugation enhances the stability of the pDNA/PAM-DET complex. Furthermore, we compare the transfection efficiency and cytotoxicity of deoxycholate-conjugated and non-conjugated PAM-DET to check the possible negative effects of deoxycholate conjugation on transfection efficiency and cytotoxicity. Interestingly, a deoxycholate-conjugated PAM-DET polyplex showed improved

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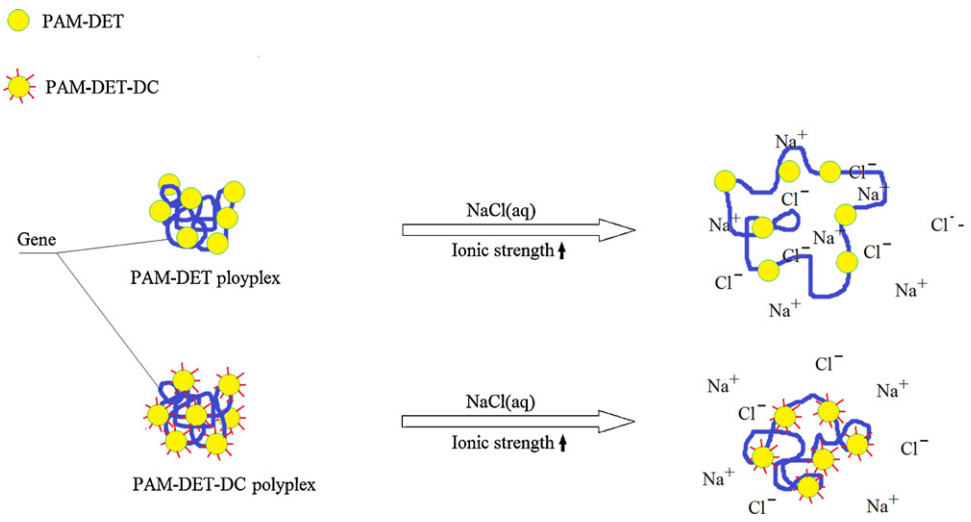

Fig. 1. A conceptual scheme of the structural disruption of two polyplexes (pDNA/PAM-DET and pDNA/PAM-DET-DC) by increased ionic strength.

transfection efficiency while maintaining the low cytotoxicity of the PAM-DET polyplex. These results imply that deoxycholate conjugation is a simple but effective method for enhancing the stability of supramolecules formed by electrostatic interactions without introducing cytotoxicity.

2. Materials and methods

2.1. Materials

Polyamidoamine (PAMAM-NH_2 G3), diethylenetriamine (DETA), deoxycholic acid (DC), dimethylsulfoxide (DMSO), tetrahydrofuran (THF), *N*-hydrooxybenzotriazole (HOBt), *N,N′*-dicyclohexylcarbodiimide (DCC), polyethyleneimine (PEI) 25 kDa, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltriazolium bromide (MTT) and sodium chloride (NaCl) were purchased from Sigma–Aldrich (St. Louis, MO). A micro BCA™ protein assay kit (Pierce, Rockford, IL), luciferase 1000 assay system and reporter lysis buffer (Promega, Madison, WI) were used for the transfection tests. For the transfection and cytotoxicity experiments, Dulbecco's modified Eagle's medium (DMEM), trypsin-ethylenediaminetetraacetic acid (EDTA), antibiotic and fetal bovine serum (FBS), and 1× phosphate buffer solution (PBS) were purchased from GIBCO (Gaithersburg, MD). All chemicals were used without further purification. The firefly luciferase expression plasmid pCN-Luci was constructed by subcloning Photinus luciferase cDNA with the 21 amino acid nuclear localization signal of the Simian vacuolating virus 40 (SV40) large T antigen into pCN.

2.2. Synthesis of PAM-DET-DC

PAM-DET was synthesized as described in a previous paper (Jin et al., 2011). For the conjugation of DC onto the surface of PAM-DET, amide bonds were formed using coupling reagents such as DCC and HOBt (Fig. 2). First, 50 mg of PAM-DET was dissolved in 50 mL of DMSO. Subsequently, DCC, HOBT, and DC were added with a constant equimolar ratio between the reagents; the amount of DC was determined by the target degree of conjugation. Then, the reaction mixture was stirred for 24 h at 50 °C. The reaction mixture was transferred in a dialysis bag and dialyzed in distilled water at 4 °C.

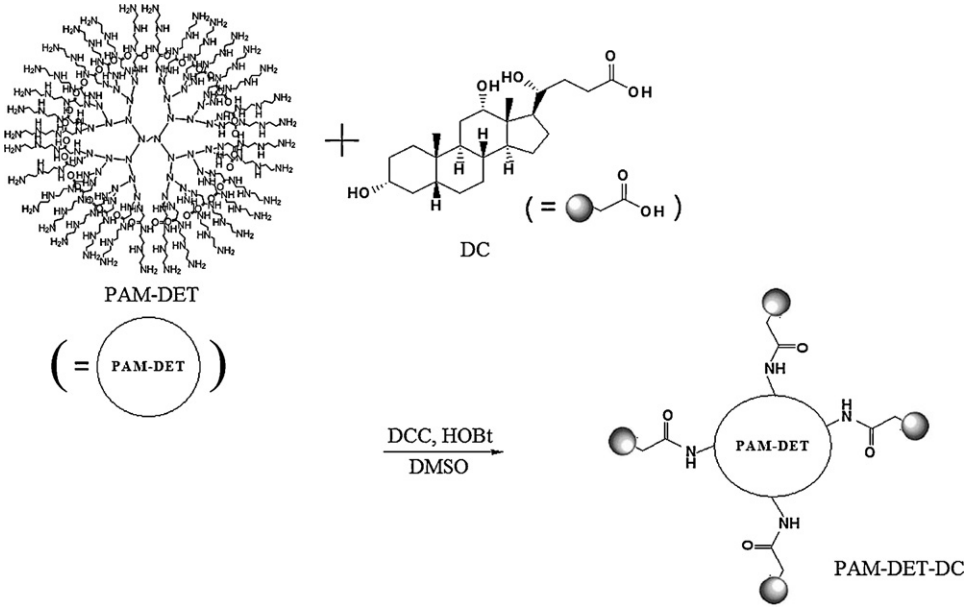

Fig. 2. Synthetic scheme for PAM-DET-DC.

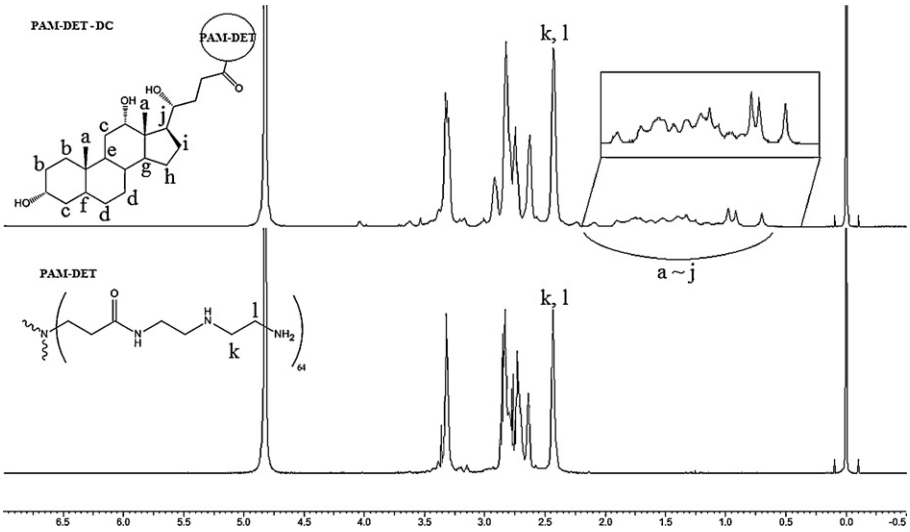

Fig. 3. ^{1}H NMR spectrum of PAM-DET and PAM-DET-DC.

Table 1
The synthetic parameters for PAM-DET-DC. Actual degree of conjugation and molecular weight of PAM-DET-DC were determined based on ^{1}H NMR result.

Entry no.	Target degree of conjugation	Actual degree of conjugation	Name based on actual degree of conjugation	M_n
1	4	2	PAM-DET-DC(2)	17,318
2	8	4	PAM-DET-DC(4)	18,067
3	16	10	PAM-DET-DC(10)	20,314

After 24 h, the purified solution was lyophilized to obtain highly viscous products. The resulting PAM-DET-DC product was analyzed by ^{1}H NMR at 40 °C to calculate the actual degree of conjugation. Each PAM-DET-DC with a different degree of DC conjugation was named based on its actual degree of conjugation (Table 1).

2.3. DLS study

The pDNA/PAM-DET and pDNA/PAM-DET-DC polyplexes were prepared in a 10 mM HEPES buffer (pH 7.4) through incubation for 30 min at 25 °C. 1 mg/mL of pDNA was used for the preparation of all polyplex samples with excess PAM-DET, and PAM-DET-DC (2, 4 and 16) (charge ratio of cationic polymer/pDNA = 16). pDNA/PAM-DET and pDNA/PAM-DET-DC(10) polyplexes with charges of 4, 8, and 16 were also prepared taking into consideration the molecular weight and actual degree of conjugation shown in Table 1. After preparing the polyplex, the concentration of NaCl was adjusted to 50 mM by the addition of 1 M NaCl solution (20×) to the buffer solution. The changes in the sizes of the polyplexes were measured using a Malvern Zeta sizer 3000HAs (Malvern Instrument Ltd., Worcestershire, U.K.) at intervals of 10 min for 1 h.

2.4. Cell culture

Both human cervical cancer HeLa cells and human glioblastoma-astrocytoma cells were grown in DMEM containing 10% FBS and 1% antibiotic. They were incubated in plastic tissue culture cell binder flasks (Corning) at 37 °C in a 5% CO_2 humidified incubator.

2.5. Transfection test

Transfection experiments were performed with both HeLa and U87MG cells to confirm the transfection efficiency of the PAM-DET and PAM-DET-DC polyplexes. Cells were seeded at 30,000 cells/well in 24 well plates in 600 μL of DMEM containing 10% FBS and 1% antibiotic and incubated at 37 °C for 1 day. The cells were treated with a polyplex solution prepared with 2 μg of pDNA and cationic polymer (PAM-DET, PAM-DET-DC(10) and PEI) at different charge ratios in 150 μL of FBS-free DMEM and incubated for 30 min at room temperature. After adding polyplex to each well, the cells were further incubated for 2 days. For assay, the growth medium was removed and the cells were washed with PBS and lysed for 30 min at room temperature with 150 μL of Reporter lysis buffer. The luciferase activity of the transfected cells was measured using an LB 9507 luminometer (Berthold, Germany) with 10 μL of lyate dispensed into the luminometer tube and automatic injection of 50 μL of luciferase assay reagent. All experiments were performed in triplicate.

2.6. Cell viability

To measure the cell viability of the polyplexes, MTT assays were performed. HeLa and U87MG cells were seeded at 10,000 cells/well in 96 well plates in 120 μL of DMEM with 10% FBS and 1% antibiotic and incubated at 37 °C for 1 day prior to the addition of the polyplex. PAM-DET and PAM-DET-DC(10) polyplexes containing 0.2 μg of pDNA at the same charge ratio as that in the transfection experiment in 24 μL of FBS-free DMEM were prepared with 30 min

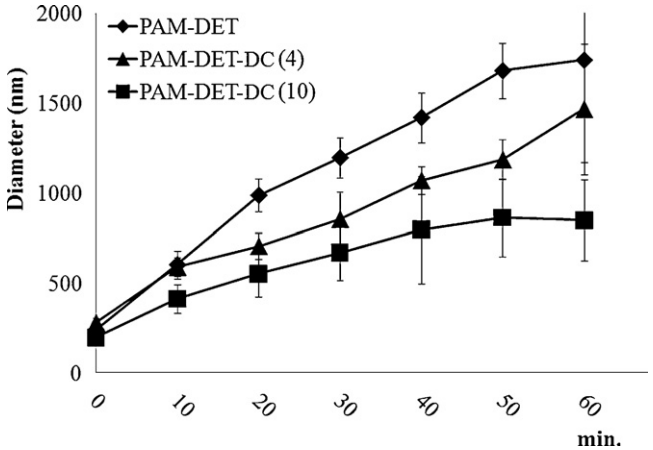

Fig. 4. Time-dependent profile of polyplex size under increased ionic strength (50 mM).

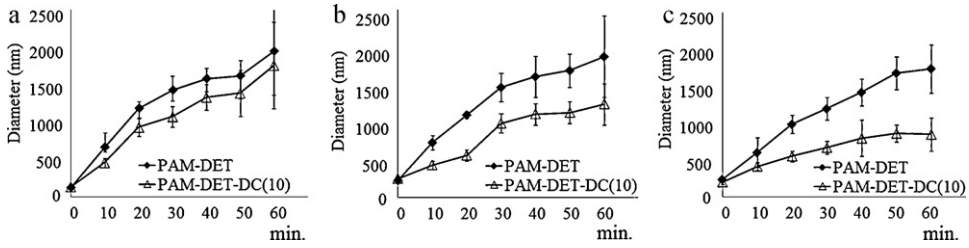

Fig. 5. Time-dependent profile of polyplex size with varying charge ratio (+/−): (a) 4, (b) 8, and (c) 16 in 50 mM $NaCl_{(aq)}$.

of incubation at room temperature. After adding the polyplex, the cells were further incubated for 48 h. Then, the cells were washed with PBS followed by the addition of 26 μL of filtered MTT solution (2 mg/mL in PBS). After incubation at 37 °C for 4 h, the medium was removed from the well and 150 μL of DMSO was added to dissolve the formazan crystal. The absorbance was measured at 570 nm using a microplate reader (Molecular Device Co., Menlo Park, CA) and the cell viability was calculated as a percentage relative to that of the untreated control cells.

3. Result and discussion

3.1. Synthesis and characterization of PAM-DET-deoxycholate (PAM-DET-DC)

For the conjugation of the DC moiety to the surface of PAM-DET, an amide bond was formed between the carboxylic acid group of DC and the primary amine group of PAM-DET (Choi et al., 2004). The synthesis of PAM-DET-DC was confirmed by ^{1}H NMR spectroscopy (Fig. 3). The signal at δ 2.48 is attributed to the protons of the secondary carbons (–CH_2CH_2–CH_2NH_2) in the interior of the PAM-DET molecule, as described in a previous study (Jin et al., 2011; Kim et al., 2007). The group of signals between δ 0.72 and 2.23 is due to the protons of the multi-ring structure in deoxycholate (Hiroki et al., 1999). The ^{1}H NMR study showed successful conjugation of the DC moiety on the PAM-DET surface; the yield of DC conjugation was about 50% (Table 1).

3.2. Dependence of polyplex stability against ionic strength on the degree of DC conjugation

It was reported that polyplex dissociation accompanies the increase of its size to the micrometer level. Therefore, the stability of polyplexes can be determined by observing changes in their size (Rivest et al., 2007). We determined the sizes of the polyplexes over time in an environment with increasing ionic strength (up to 50 mM) by the addition of concentrated NaCl solution. The size-increasing profiles of the polyplexes at 25 °C were obtained by a DLS study. Different patterns of changes in the sizes of the polyplexes formed from PAM-DET, PAM-DET-DC (2, 4, and 10) were found. The pDNA/PAM-DET-DC (2) polyplex showed almost the same change in size as PAM-DET, which implies that there is no contribution of DC to the stability of this polyplex because of the very low conjugation of the hydrophobic moiety. Among all the samples, the pDNA/PAM-DET-DC(10) polyplex showed the least change in size compared with the other polyplexes over 1 h (Fig. 4). This result showed that the degree of conjugation of DC moiety is an important factor for controlling the stability of the polyplex against ionic strength. Accordingly, a high degree of DC conjugation on PAM-DET caused enhanced stability of the polyplex. A similar effect of hydrophobic moiety conjugation on cationic polymers was reported by other groups, thereby supporting our findings (Yang et al., 2008).

3.3. Dependence of polyplex stability against ionic strength on the charge ratio

It is well known that a sufficient amount of cationic polymer in relation to the amount of pDNA is required for the formation of a stable polyplex (Kim et al., 2004). To explore the relationship between the charge ratio and the stability of polyplex in aqueous media with increased ionic strength, the stability of the polyplexes formed from PAM-DET and PAM-DET-DC(10) with various charge ratios were compared. As shown in Fig. 5, the size of the PAM-DET polyplex increased to almost 2 μm after 60 min at relatively low charge ratios (1:4 and 1:8). Upon the addition of NaCl salt, a slower increase in size was observed as the polyplexes formed with higher charge ratios for both PAM-DET and PAM-DET-DC(10). However, it seems that an increase in the charge ratio suppressed the growth of the polyplex formed from PAM-DET-DC(10) more effectively than that of the PAM-DET polyplex. This result is probably due to in part to the increased amount of DC as the charge ratio increased rather than it being solely an effect of the increased charge ratio.

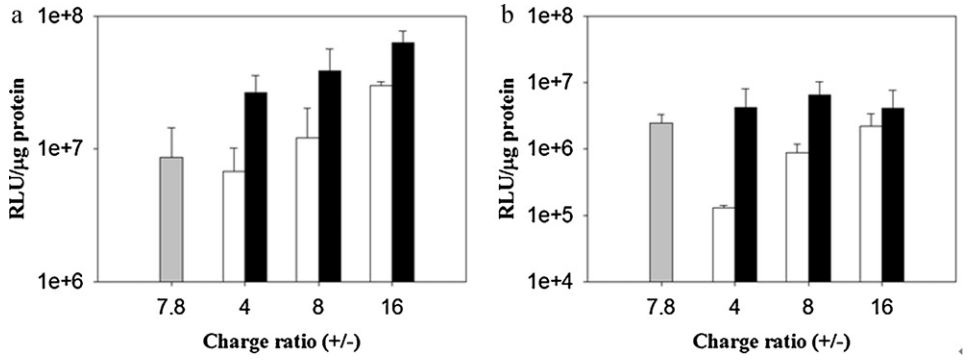

Fig. 6. Transfection efficiency of PEI (gray bars), PAM-DET (white bars) and PAM-DET-DC(10) (black bars) against (a) HeLa and (b) U87MG cells.

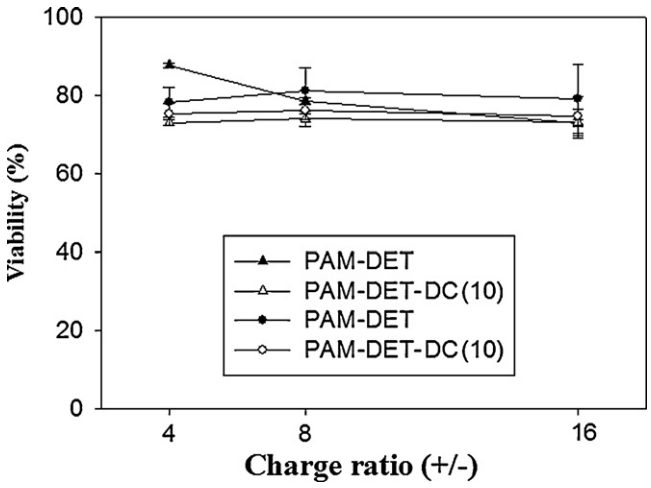

Fig. 7. Cell Viability test of PAM-DET and PAM-DET-DC(10) polyplex varying charge ratio against HeLa (▲ and △) and U87MG cell (● and ○).

3.4. Transfection efficiency and cytotoxicity

To ascertain the negative effects of DC conjugation to PAM-DET, the transfection efficiency and cytotoxicity of PAM-DET-DC were tested. A transfection experiment using PAM-DET and PAM-DET-DC(10) against both HeLa and U87MG cell lines was performed. Interestingly, PAM-DET-DC(10) showed higher transfection efficiency than PAM-DET in both cell lines (Fig. 6). It was reported that increased transfection efficiency is caused by enhanced stability of the polyplex. Similarly, it seems that the enhanced stability of the polyplex due to DC conjugation contributes to an increase of the transfection efficiency. In here, the forte of DC as cell membrane penetration enhancer can also help insertion of the PAM-DET-DC polyplex into the cytosol and partially contribute the positive transfection result as suggested by Rojanasakul et al. (1990). Furthermore, both the PAM-DET and PAM-DET-DC(10) polyplexes showed low cell cytotoxicity for both cell lines (Fig. 7). It is suggested that this low cytotoxicity of PAM-DET-DC is due to the biocompatibility of DC, which is a natural emulsifier derived from a bio-system.

4. Conclusion

We successfully conjugated DC onto the surface of PAM-DET to produce PAM-DET-DC for the formation of polyplexes with enhanced stability against ionic strength. We evaluated its stability by measuring its size: the PAM-DET-DC polyplex showed decreased growth as compared to the PAM-DET polyplex in an environment with increased ionic strength, which implies enhanced stability of PAM-DET-DC against increased ionic strength. Furthermore, DC conjugation caused a slight increase in the transfection efficiency without inducing toxicity. This strongly indicates that the introduction of DC moieties on PAM-DET is a good method to enhance the polyplex stability against ionic strength without diminishing its advantageous properties, such as high transfection efficiency and low cytotoxicity.

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