難水溶性薬剤結合に伴うリポカリン型プロスタグランジンD合成酵素（L-PGDS）の構造変化
Conformational Changes Induced in Lipocalin-type Prostaglandin D Synthase (L-PGDS) by Binding of Lipophilic Drugs

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Introduction

Lipocalin-type prostaglandin D synthase (L-PGDS) is abundantly expressed in the central nervous system of various mammals [1], and is a unique dual functional protein acting as both a PGD_2-synthesizing enzyme and an extracellular transporter protein for lipophilic ligands [2]. So far, the features responsible for the lipid-transporter protein has been focused especially, and the biochemical properties of L-PGDS has been studied. The results of such studies revealed that L-PGDS could bind to a large variety of lipophilic ligands, such as retinoids, thyroids, biliverdin, and bilirubin with high affinities *in vitro* [3-5]. Thus, it was concluded that L-PGDS has a broad selectivity toward small lipophilic ligands [6].

Some drugs are lipophilic and rather insoluble in water. Although chemical modification increases the solubility of these drugs, it follows that the drug action is reduced. Then, these drugs are dropped out from the development. For the delivery of these drugs, special formulations are required to make their aqueous dispersion using surfactant. However many surfactants themselves tend to increase the systemic toxicity of the drug formulation. Therefore, there is increasing interest for developing a novel drug delivery system (DDS).

The present study aimed to show a concept of DDS for lipophilic drugs using L-PGDS as a delivery vehicle.

[1] Urade, Y., and Hayaishi, O. (2000) *Biochim. Biophys. Acta* **1482**, 259-271
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[3] Tanaka, T., *et. al.* (1997) *J. Biol. Chem.* **272**, 15789-15795
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[5] Inui, T., *et. al.* (1999) *Biochem. Biophys. Res. Commun.* **266**, 641-646
[6] Inui, T., *et al.* (2003) *J. Biol. Chem.* **278,** 2845-2852.

Results

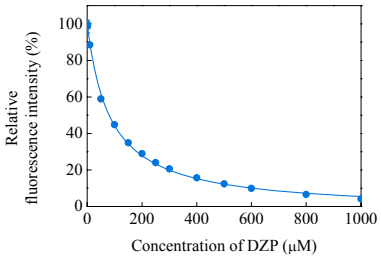


Fig. 4. Tryptophan fluorescence quenching of L-PGDS by DZP. The relative fluorescence intensities of L-PGDS in the presence of various concentrations of DZP were obtained. The final protein concentration were 1.5 μM. It was shown that DZP caused a concentration-dependent decrease in the intrinsic fluorescence of L-PGDS.

Table 1. The K_d values of L-PGDS for DZP and NBQX.

Drug	DZP	NBQX
K_d (μM)	84 ± 4	5.6 ± 0.5

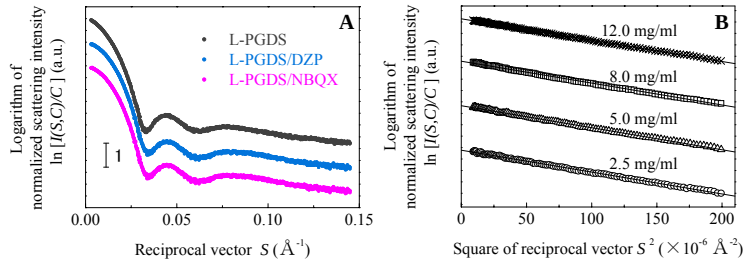
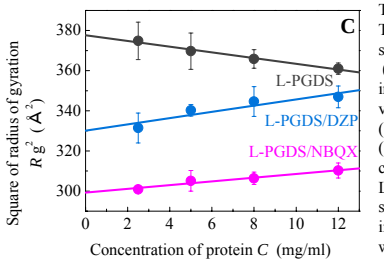


Fig. 5. (A) SAXS profiles of L-PGDS, L-PGDS/DZP, and L-PGDS/NBQX. The logarithm of scattering intensity is shown as a function of reciprocal vector (S). They have been shifted along the ordinate for clarity. These curves revealed that L-PGDS has a globular shape in the presence or absence of drugs.
(B) Guinier plots of L-PGDS. Logarithms of intensities were plotted against squared scattering vector length of L-PGDS at concentration of 2.5 mg/ml (○), 5.0 mg/ml (△), 8.0 mg/ml (□) and 12.0 mg/ml (×). They have been shifted along the ordinate for clarity. The Guinier plot at different concentrations of L-PGDS showed a single linear regression line without significant upward curvature even at the low S^2 region, indicating that the sample solution is mono-dispersed without aggregation in the solution at the highest concentration of 12.0 mg/ml.



(C) Concentration-dependence of R_g^2 of L-PGDS with different drugs. From the linear extrapolations of R_g to infinite dilution, which eliminated interparticle interferences, $R_g(0)$ was calculated to be 19.39 ± 0.01 Å for L-PGDS, 18.25 ± 0.08 Å for L-PGDS/DZP, 17.36 ± 0.03 Å for L-PGDS/NBQX.

Table 2. Solubility (μM) of lipophilic small ligands.

	DZP	NBQX	BR
PBS	151	516	0.3
BSA (500 μM)	542	1556	8
L-PGDS (500 μM)	1052	1658	170

Materials & Methods

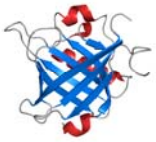


Fig. 1. The X-ray crystal structure of L-PGDS (C65A) (PDB ID: 2CZT). The tertiary structure of mouse recombinant L-PGDS showed an 8-stranded β barrel structure [1].

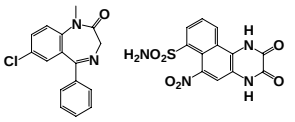


Fig. 2. The structure of diazepam (DZP, MW = 285, left) and NBQX (MW = 336, right).

In this study, C65A-substituted recombinant mouse L-PGDS expressed in *Escherichia coli* BL21 (DE3) was used. As lipophilic drugs, diazepam (DZP) which was benzodiazepine anxiolytic and NBQX which was AMPA receptor antagonist were used.

Fluorescence quenching assays

Various concentration of drugs were added to L-PGDS in 5 mM Tris/HCl, pH 8.0 with 5% dimethyl sulfoxide, to give a final protein concentration of 1.5 μM. After incubation at 25 °C for 30 min, the intrinsic tryptophan fluorescence was measured by an excitation wavelength at 282 nm and emission wavelength at 334 nm. The dissociation constant (K_d) value for binding between L-PGDS and drugs were calculated by the method of Cogan [7].

[7] Cogan, U., *et al.* (1976) *Eur J Biochem* **65**, 71-8.

Small angle X-ray scattering (SAXS)

Samples of L-PGDS, L-PGDS/DZP and L-PGDS/NBQX were concentrated and adjusted to concentrations (2.5 mg/ml, 5.0 mg/ml, 8.0 mg/ml and 12.0 mg/ml in 5mM Tris/HCl, pH 8.0). All SAXS measurements were performed at BL40B2 in SPring-8 (Hyogo, Japan), under following conditions; X-ray wavelength: 1.000 Å, camera distance: 1,000 mm, detector: RIGAKU RXIS IV^{++} (Image Plate), temperature: 25 °C. To avoid systematic errors, we measured sample and buffer solutions alternately.

Solubility measurement of lipophilic small ligands

Bilirubin (BR), DZP and NBQX were saturated in PBS, 500 μM bovine serum albumin (BSA), or 500 μM L-PGDS, respectively. Each solution was passed through a filter to remove the insoluble ligand. The concentration of ligands were determined by spectroscopically based on their respective molar absorption coefficients, i.e.,ε_{453} in methanol for BR = 61,700 M^{-1} cm^{-1}, ε_{317} in DMSO for DZP = 1,910 M^{-1} cm^{-1}, ε_{392} in DMSO for NBQX = 10,210 M^{-1} cm^{-1}.

Loss of righting reflex test in mice

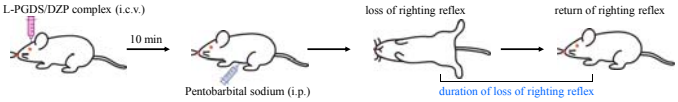


Fig. 3. Experimental procedure of loss of righting reflex.

Male ddY mice (6 weeks old) were used. DZP of 10 mM was mixed with L-PGDS (25, 100, and 250 mM in 5 mM Tris/HCl, pH 8.0) and passed through a filter to remove DZP left insoluble. Mice were given an intracerebroventricular (i.c.v) injection of each sample (5 μl) ten minutes before they were given an intraperitoneal (i.p) injection of pentobarbital sodium (35 mg kg^{-1}). Duration of Pentobarbital-induced loss of righting reflex was defined as the time between loss of righting reflex and return of the righting reflex.

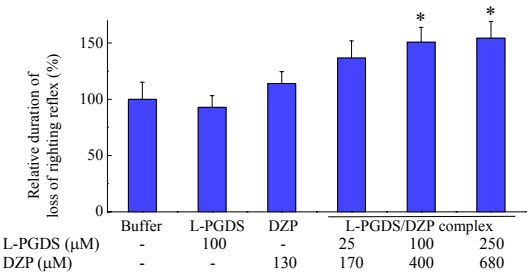


Fig. 6. Effects of i.c.v.-treatment with L-PGDS/DZP complex on the duration of the pentobarbital-induced loss of righting reflex in mice. Mice treated with complex showed a longer duration of loss of righting reflex in a concentration-dependent manner compared to mice treated with only DZP. Each column represents the mean ± S.E. of 6 - 8 mice. * P<0.05 *vs* respective buffer-treated group.

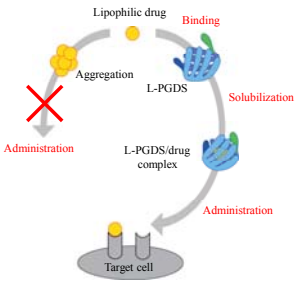


Fig. 7. A concept of DDS by using L-PGDS. L-PGDS binds to the lipophilic drug with poor water solubility, and carries it to specific receptor on target cells. The drug is transferred from L-PGDS to the target receptor by the difference of binding affinities between L-PGDS for drug and receptor for drug.

Conclusions

- The R_g values were estimated to be 19.4 Å for L-PGDS, 18.3 Å for L-PGDS/DZP, and 17.4 Å for L-PGDS/NBQX complexes, indicating that L-PGDS became compact after binding of these drugs by the induced compact packing (Fig. 5). Such structural flexibility of the L-PGDS molecule was responsible for the broad ligand selectivity of L-PGDS.
- L-PGDS increased the solubility of lipophilic ligands. (Table 2).
- Mice treated with L-PGDS/DZP complex (i.c.v.) showed a longer duration of loss of righting reflex in a concentration-dependent manner compared to mice treated with only DZP (Fig. 4) and it was succeeded to validate the concept of a DDS for lipophilic drugs by using L-PGDS.