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論　文　名	**Development of Novel Drug Delivery System for Poorly Water-soluble Compounds Using Lipocalin-type Prostaglandin D Synthase** （リポカリン型プロスタグランジン D 合成酵素を用いた難水溶性薬剤に対する新規ドラッグデリバリーシステムの開発）
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論文要旨

Introduction

Most newly synthesized compounds during the drug discovery process are generally hydrophobic and thus insoluble in water. Although subsequent chemical modification improves the solubility of these compounds, their potencies seem to decrease in many cases. These hydrophobic compounds have frequently been dropped from development in the preclinical stage. To take advantage of these compounds, special formulations are required to produce an aqueous dispersion.

Lipocalin-type prostaglandin D synthase (L-PGDS) is an enzyme that catalyses the isomerization of prostaglandin (PG)H_2 to PGD_2, the major prostanoid produced in the central nervous system of mammals. L-PGDS is also a member of the lipocalin gene family, which is composed of a group of secretory lipid-transporter proteins, such as retinol-binding protein, β-lactoglobulin, and major urinary protein. A series of biochemical studies of L-PGDS has revealed that this protein can bind a large variety of hydrophobic small molecules (M_w = 200-780) with a dissociation constant (K_d) of 30 nM to 2 μM. The feature defined as ‘broad ligand selectivity’ suggests some mechanisms by which L-PGDS could be used in a drug

delivery system (DDS) for poorly water-soluble compounds.

Here it is shown the feasibility of a novel DDS using L-PGDS as a delivery vehicle for two kinds of poorly water-soluble compounds: diazepam (DZP, M_w = 284.7) and 6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, M_w = 336.3). DZP is a major anxiolytic drug possessing the moiety of benzodiazepine, and NBQX is an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist. In addition, it is exhibited the relationship between the binding affinities of DZP for L-PGDS and the efficacy of the complex *in vivo*.

Chapter 1. The feasibility of DDS for poorly water-soluble drugs using L-PGDS.

In this study the recombinant mouse C65A-substituted L-PGDS mutant, in which a catalytic residue of cysteine was substituted to alanine to get rid of an enzymatic activity of L-PGDS, was used. In order to clarify an affinity and a stoichiometry for the binding of poorly water-soluble compounds to L-PGDS, the calorimetric analysis on DZP/L-PGDS and NBQX/L-PGDS systems was carried out using isothermal titration calolimetry. It was revealed for DZP and NBQX that each L-PGDS held three molecules with K_d = 47.6 and 16.5 μM, respectively. To confirm the relationship of the compound to L-PGDS, the NBQX/L-PGDS complex was analyzed by mass spectrometry (MS) under non-denaturing conditions. By MS, the 1:3 complex of L-PGDS and NBQX was observed. Next, I measured the effect of L-PGDS on the solubility of DZP and NBQX in PBS. L-PGDS of 500 μM increased the solubility of DZP and NBQX 7- and 2-fold, respectively, compared to PBS alone. From this, it is clear that L-PGDS can enhance the solubility of poorly water-soluble compounds without using organic solvent.

As it was found that L-PGDS could bind the poorly water-soluble compounds and increase the solubility of such compounds *in vitro*, I assessed the effect of L-PGDS to the drug potencies of DZP and NBQX *in vivo*. Basically, two delivery strategies were used in the assessment: oral administration of DZP/L-PGDS complex to mice, and intravenous delivery of NBQX/L-PGDS complex to ischemic gerbils. First, I investigated the effect of oral administration of DZP/L-PGDS complex on pentobarbital-induced anesthesia. DZP is known to intensify pentobarbital-induced anesthesia. DZP was suspended in either distilled water containing 0.5% carboxymethylcellulose (CMC), which is widely used as a suspending agent, or L-PGDS solution. As a result, the duration of loss of righting reflex (LORR) in mice treated with DZP/CMC suspension (500 μM DZP) was 50% longer than with CMC, and it was comparable to that of 250 μM DZP/L-PGDS solution. Mice treated with 500 μM DZP/L-PGDS solution showed 1.3-fold longer duration of LORR than those treated with 500 μM DZP/CMC suspension. The metabolism of DZP in plasma and forebrain was then

investigated by LC-MS after oral administration of samples. The result was that the concentrations of DZP active metabolites such as nordiazepam and oxazepam in DZP/L-PGDS solution administered groups were higher than in DZP/CMC suspension administered group. All these results clearly indicate that both the absorption in the gastrointestinal tract and the oral bioavailability of DZP in the presence of L-PGDS are higher than those in the CMC suspension.

Next, it was investigated the applicability of this DDS to intravenous administration of NBQX/L-PGDS complexes. The bilateral common carotid arteries were ligated by clip for 5 min, followed by removal of the clips and reperfusion. PBS in the presence or absence of L-PGDS were saturated with NBQX was administered intravenously (100 μl/gerbil) 3 times at 0, 10, and 25 min after reperfusion. One week after reperfusion, the number of surviving hippocampal CA1 pyramidal cells was counted. The number of pyramidal cells in the control group decreased to 4% in the sham-operated group. The intravenous injection of saturated NBQX in PBS did not produce any discernible effects. In contrast, gerbils administered the same treatment using NBQX/L-PGDS solutions (0.8 mM and 2.0 mM L-PGDS) showed significantly increased fractions of living pyramidal cells (17% and 81%, respectively) compared to the control group.

These results show the feasibility of a novel DDS for poorly water-soluble compounds such as DZP and NBQX using L-PGDS. This feasibility is also supported by the fact that an L-PGDS-mediated drug delivery was more effective than the delivery of these compounds without L-PGDS in both oral and intravenous administration.

Chapter 2. The relationship between the binding affinities of DZP for L-PGDS mutants and the efficacies of these complexes *in vivo*.

In chapter 1, I found that L-PGDS can increase the solubility of DZP and the oral administration of DZP/L-PGDS solution in mice revealed the increased duration of pentobarbital-induced LORR. In this chapter, it was investigated the relationship between the binding affinities of DZP for L-PGDS and the efficacy of intravenous administration of DZP solubilized by L-PGDS in mice.

First, 21 kinds of L-PGDS mutants were made by site-directed mutagenesis at residues which are thought to be involved in binding of DZP by NMR titration experiments of L-PGDS with DZP. Next, the K_d values of L-PGDS mutants and DZP measured by fluorescence quenching of the intrinsic tryptophan residues of L-PGDS, and the solubility of DZP in each mutant solution. As a result, the K_d value of L-PGD and DZP was 83.8 μM, while L-PGDS mutant with the highest binding affinity of DZP (K_d = 2.1 μM) was S81F mutant, in which Ser81 residue was substituted to phenylalanine, and with the lowest binding affinity (K_d = 341 μM) was F83A mutant, in which Phe83 residue was substituted to alanine. In addition, the

solubility of DZP in L-PGDS solution was 361 μM and that in S81F and F83A mutant solution were 365 and 219 μM, respectively, indicating that L-PGDS mutants possessing high binding affinity of DZP showed higher solubilities of DZP in their solutions. Further, I investigated the effect of intravenous administration of DZP/L-PGDS mutant complexes on the duration of the pentobarbital-induced LORR in mice. As a result, the administration of DZP/S81F mutant did not show significantly longer duration of LORR compared with that of control. On the other hand, the administration of DZP/L-PGDS and DZP/F83A mutant solution showed 1.4- and 1.7-fold longer duration of LORR, suggesting that the lower affinity of L-PGDS mutant for DZP, the higher the potency of DZP when DZP/L-PGDS mutant solutions were intravenously-administered. These results indicated that a balance of the binding affinity of L-PGDS and DZP and the solubility of DZP in L-PGDS solution is becoming a really key point.

Conclusions

In this study, it was shown that L-PGDS could improve the solubility of DZP and NBQX in aqueous solutions, and consequently collected evidence which revealed the prospective effect of each compound *in vivo*. In addition, it was identified the relationship between the binding affinity of L-PGDS and DZP and the drug potency of DZP solubilized by L-PGDS. Further investigation using L-PGDS mutants with different binding affinities for other drugs would be able to engineer a tailor-made carrier protein possessing molecular-selective recognition for several hydrophobic drugs using L-PGDS as a template. These results demonstrate that L-PGDS is a beneficial delivery vehicle for poorly water-soluble compounds. This novel DDS will facilitate the pharmaceutical and clinical developments of various water-insoluble compounds.

審査結果の要旨

近年，医薬品に対する安全性が厳しく求められているために，創薬現場では高度なバイオサイエンス技術が要求され，新薬開発コストの急騰を招いている。一方，薬剤が難水溶性であることや，薬剤溶解度向上を図るための化学修飾による薬剤活性の低下により，開発中止になった薬剤候補を，復活させる動きが世界的に始まっている。これらのニーズを実現させるためには大きなブレークスルーが必要であり，その本命としてドラッグデリバリーシステム（DDS）が著しい発展を遂げている。本研究の目的は，生体内輸送蛋白質を用いて難水溶性薬剤に対する新規DDSを開発し，その概念検証を*in vivo*レベルで行うことである。DDS キャリアとして，リポカリン型プロスタグランジン D

合成酵素（L-PGDS）を用い，難水溶性薬剤として，ベンゾジアゼピン系抗不安薬である diazepam（DZP）と AMPA 型グルタミン酸拮抗薬である 6-nitro-7-sulfamoyl-benzo [f]quinoxaline-2,3-dione（NBQX）を用いた。

第 1 章では，L-PGDS と薬剤の相互作用，構造解析，及び *in vivo* における薬剤複合体の効果を調べている。等温滴定型熱量測定により，L-PGDS は DZP，あるいは NBQX を 3 分子結合し，その解離定数（K_d）は，それぞれ 47.6 μM，及び 16.5 μM であることを示した。また，非変性条件下における NBQX/L-PGDS 複合体の質量分析により，L-PGDS は確かに 3 分子の NBQX を結合できることが明らかとなり，熱量測定の結果と一致した。また，X 線小角散乱，及び NMR 測定により，L-PGDS は薬剤をその疎水性ポケット内部に結合し，コンパクトになることが分った。さらに，L-PGDS 存在下における DZP，及び NBQX の濃度は，L-PGDS の濃度依存的に上昇することが判明した。次に，本 DDS の *in vivo* による評価を行うために，ペントバルビタール麻酔下のマウスに DZP/L-PGDS 複合体を経口投与し，DZP の効果を麻酔時間の延長を指標として調べた。その結果，DZP/L-PGDS 複合体を投与したマウスは，対照実験としてカルボキシメチルセルロース（CMC）に懸濁した DZP を投与したマウスと比較して，麻酔時間の有意な延長が観測された。また，液体クロマトグラフィー法により，DZP/L-PGDS 複合体の経口投与後の血中，及び脳内の DZP 活性代謝物の濃度を測定した結果，DZP/CMC 懸濁液を投与したマウスに比べて上昇していた。これらの結果は，CMC と比較して，L-PGDS が DZP のバイオアベイラビリティを上昇させたことを示唆している。さらに，スナネズミ脳虚血モデルにおいて，NBQX/L-PGDS 複合体の静脈内投与による，海馬 CA1 領域の遅発性神経細胞死への影響を調べた。NBQX のみの投与群は，NBQX が難水溶性であるため PBS にほとんど溶解せず，対照群と変わらなかった。しかし，NBQX/L-PGDS 複合体の投与群では，遅発性神経細胞死が有意に抑制された。以上の結果は，難水溶性薬剤に対する本 DDS が，経口投与，及び静脈内投与の両投与法において有用であることを示している。

第 2 章では，難水溶性薬剤/L-PGDS 複合体の静脈内投与における，薬剤と L-PGDS の結合親和性の影響を調べている。まず，部位特異的変異により作製した 21 種類の L-PGDS 変異体と DZP の結合親和性をトリプトファン蛍光消光実験により調べた。また，変異体溶液存在下における DZP 濃度を測定した。その結果，DZP に対する結合親和性が高い変異体ほど，溶液中の濃度が上昇することが判明した。さらに，DZP/変異体複合体のマウス静脈内投与による，ペントバルビタール誘導性麻酔時間への影響を調べた結果，DZP に対する結合親和性が低い複合体ほど，麻酔時間が延長し，DZP の効果が上昇することが判明した。以上の結果は，薬剤の性状に応じたキャリアを作製すること，つまり様々な難水溶性薬剤に応じたテーラメード DDS キャリアの作製が可能であることを示すものである。

本研究は，生体内輸送蛋白質である L-PGDS を用いることにより，十分な薬効を持

つにもかかわらず, 難水溶性のために開発段階で除外されてきた薬剤の臨床使用への可能性を初めて示したものであり, これら薬剤の臨床応用を目指す上で非常に重要な知見であると言える。また，蛋白質を用いた難水溶性薬剤に対する DDS は世界的にも稀有であり，学術的新規性，及び独創性が高い。よって，最終試験の結果とあわせて，博士（応用生命科学）の学位を授与することを適当と認める。