Prevention of Prion Propagation by Dehydrocholesterol Reductase Inhibitors in Cultured Cells and a Therapeutic Trial in Mice

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In prion diseases, the normal cellular form of prion protein (PrP C) is converted into the disease-associated isoforms (PrP Sc) which accumulate in the infected tissues. Although the precise mechanism of this conversion remains unsolved, drugs of various categories have been reported to reduce the accumulation of PrP Sc in prion-infected cultured cells. We here show that AY-9944 (a 7-dehydrocholesterol reductase inhibitor) and U18666A (a 24-dehydrocholesterol reductase inhibitor) prevent PrP Sc from accumulating in prion-infected mouse neuroblastoma cells (ScN2a), with an ED50 of about 0.5 μM and 10 nM, respectively. In order to evaluate the efficacy of these two inhibitors in vivo, C57BL/6J mice inoculated with mouse-adapted scrapie-prion received repetitive intraperitoneal injections of U18666A (10 mg/kg) or a mixture of U18666A (10 mg/kg) and AY-9944 (12 mg/kg). By contrast to the potent anti-prion effects observed in ScN2a cells, the in vivo trial was abortive with neither drug halting the progression of the disease.

Key words prion; U18666A; AY-9944; desmosterol; 7-dehydrocholesterol; lipid raft

Transmissible spongiform encephalopathies, or prion diseases, are fatal neurodegenerative disorders that include sporadic Creutzfeldt-Jakob disease (CJD), variant CJD (vCJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, iatrogenic CJD and kuru in humans.1,2) The diseases cause an intense accumulation of the disease-associated form(s) of prion protein (PrP Sc) in the central nervous system.1,2) PrP Sc is a conformational isoform(s) of host-encoded cellular prion protein (PrP C) and is considered the infectious entity of prion agents.3,4) While PrP C is susceptible to proteolytic digestion by protease K (PK), PrP Sc is partially resistant to PK which leaves undigested C-terminal regions. Since the C-terminal regions contain two potential N-glycosylation sites (Asn 180 and Asn 196 for mouse PrP), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of PK-digested PrP Sc followed by Western-blotting gives three bands, corresponding to non-, mono- and di-glycosylated PrP Sc. Neural cell lines such as neuro2a (N2a)3) and GT14) are known to be prone to prion infection and support the propagation of PrP Sc through continuous conversion of the endogenous PrP C. Sev- eral kinds of compounds, including quinacrine,5—9) are known to inhibit the conversion of PrP C to PrP Sc in ScN2a cells.5—9) However, many of these compounds have not been tested in vivo, or eventually proved ineffective in vivo.5,10) Quinacrine have been applied clinically to treat some forms of CJD patients, but its efficacy in vivo is still being debated.11,12) Thus, anti-prion drugs are still in demand.

PrP C is a glycosylphosphatidylinositol (GPI)-anchored protein present in ‘lipid raft’ domains of plasma membranes; micro-domains where high concentrations of cholesterol and sphingolipids are thought to enforce the liquid-ordered packing of the membranes. Previous reports showed that the accumulation of PrP Sc in prion-infected N2a cells (referred to as ScN2a) was blocked by the inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase9) or squalene synthase.9) Although these studies suggested possible contributions of cholesterol to the propagation of PrP Sc, the inhibitors also led to a decrease in either levels of isoprenoids indispensable for post-translational protein-isoprenylation,13) for N-glycosylation,13) and for GPI-anchoring,13) or levels of the cellular pools of cholesterol precursors which supported cell growth.14) Hence, it could not be ruled out that the anti-prion effects of the above inhibitors were due to a decrease in the amounts of cellular isoprenoids and/or cholesterol precursors. The synthesis of cholesterol in mammals proceeds along two paths after the synthesis of lanosterol; the Kandutsch–Russell pathway in which 7-dehydrocholesterol is the immediate precursor of cholesterol, and the Bloch pathway in which desmosterol is the immediate precursor (Chart 1). In the present study, we show that AY-9944 (1,4-bis-[2-chlorobenzylaminomethyl]-cyclohexene; CAS #: 366-93-8), an inhibitor of 7-dehydrocholesterol reductase (DHCR7, Chart 1),5,10) and U18666A (3-β-[2-diethylaminoethoxy]-androstenone; CAS #: 3039-71-2), an inhibitor of 24-dehydrocholesterol reductase (DHCR24, Chart 1),15,17) prevent the propagation of PrP Sc in ScN2a cells. Furthermore, we have evaluated the efficacies of U18666A and AY-9944 in vivo.

MATERIALS AND METHODS

Cells and Chemicals N2a (from American Type Culture
Carried out using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed using the Total RNA Isolation System (Promega). Reverse-transcribed RNA fractions were prepared from N2a and ScN2a cells by using 1 μg of RNA and an AccessQuick™ RT-PCR system (Promega, Madison, U.S.A.) with the following primers: 5'-AGCGCAAGGCTGATCAGGAC-3’ (dhcr7, exon 3), 5'-TCGGCACTGTTGGGGAACA-3’ (dhcr7, exon 6), 5'-CCGGGAATGGAAGGAACAGG-GCAGTAAAG-3’ (dhcr24, exon 2) and 5'-CACAGGAGGAGGACATAGAAGCAG-3’ (dhcr24, exon 5). The thermal cycle program was as follows: at 45 °C for 45 min for reverse-transcription, then 25 cycles of PCR at 92 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. 

### Anti-prion Effects in ScN2a Cells

ScN2a cells were seeded in 6-well plates with 2 ml of D-MEM supplemented with 4% FCS a day before the experiments.AY-9944 and U18666A were added to the culture medium on Day-1 and the cells were cultured for 4 d without a change of the medium. The medium was discarded on Day-4, 600 μl of lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 0.5% Triton X-100 and 0.25% sodium deoxycholate (pH 7.6)) was added to the cells, and the lysates were centrifuged at 100,000 g for 5 min at 4 °C. Aliquots of 70 μl of the supernatants were kept as undigested samples for determination of total amounts of proteins and for Western-blotting analysis of actin, while aliquots of 420 μl (containing approx. 500 μg of total protein) were digested with 5 μg of PK at 37 °C for 60 min. The digestion was stopped by phenylmethylsulfonyl fluoride (PMSF, final 3 mM), and the digests were centrifuged at 100,000 × g for 60 min at 4 °C by a TLA-55 rotor (Beckman Coulter, Inc., U.S.A.) to precipitate PK-resistant PrPc. The pellets were washed with 500 μl of 10 mM PMSF in methanol and subjected to SDS-PAGE followed by Western-blotting. Detection was carried out with ECL-chemiluminescence reagent (GE Healthcare Bio-Sciences, Uppsala, Sweden).

### Cell Toxicity Assay

The cells were seeded in 96-well plates with 100 μl of D-MEM with 4% FCS a day before the experiments. The drugs in serial dilutions (final 10⁻⁹ to 10⁻³ M) were digested with 5 μg of PK at 37 °C for 60 min. The inhibition of 7-dehydrocholesterol reductase (DHCR7) and 24-dehydrocholesterol reductase (DHCR24) (Chart 1). In the RT-PCR analysis, we obtained products of the expected lengths (0.56 kb for dhcr7 and 0.42 kb for dhcr24, Fig. 1A). The DNA sequence analysis confirmed their identities as the PCR products derived from the dhcr7 and dhcr24 mRNAs. Furthermore, ScN2a cells cultured with 1 μM AY-9944 or 50 nM U18666A for 4 d showed an accumulation of 7-dehydrocholesterol or desmosterol (Fig. 1B). These results indicated that the cells expressed DHCR7 and DHCR24 which were inhibited by the drugs.

### Lipid Analysis

Lipids were extracted from ScN2a cells or the brains according to Bligh and Dyer. The amounts of choline in the extracts were determined with a commercial kit using phospholipase D and choline oxidase (Wako Pure Chemical Industries, Ltd.), and extracts containing equal amounts of choline were applied to silicagel 60 TLC-plates (Merck, Darmstadt, Germany) pre-absorbed with silver nitrate. TLC was developed by chloroform/acetone=85/15 (v/v), then, the sterols were detected by spraying 10% (w/v) copper sulfate in 10% phosphoric acid and heating at 90 °C.

## RESULTS AND DISCUSSION

We first examined whether ScNa2 and N2a cells expressed 7-dehydrocholesterol reductase (DHCR7) and 24-dehydrocholesterol reductase (DHCR24) (Chart 1). In the RT-PCR analysis, we obtained products of the expected lengths (0.56 kb for dhcr7 and 0.42 kb for dhcr24, Fig. 1A). The DNA sequence analysis confirmed their identities as the PCR products derived from the dhcr7 and dhcr24 mRNAs. Furthermore, ScN2a cells cultured with 1 μM AY-9944 or 50 nM U18666A for 4 d showed an accumulation of 7-dehydrocholesterol or desmosterol (Fig. 1B). These results indicated that the cells expressed DHCR7 and DHCR24 which were inhibited by the drugs.
ated by AY-9944 and U18666A, respectively. Based on these results, we explored whether AY-9944 and U18666A would prevent the propagation of PrPSc in ScN2a cells. In the cells cultured with AY-9944 or U18666A for 4 d, the amount of PrPSc decreased in a manner dependent on the concentrations of the drugs (Fig. 2A). The approximate ED50 of AY-9944 was 0.5 mM, while that of U18666A was 10 nM. Under the conditions, the ED50 of quinacrine was about 0.5 mM (data not shown) consistent with previous reports.6,7) The elimination of PrPSc from the cells was unlikely due to facilitation of the release of cell-associated PrPSc into the medium, because no corresponding amount of PrPSc was detectable in the culture medium (data not shown). In addition, the dose–viability curves (Fig. 2B) were substantially the same between ScN2a and N2a cells (data not shown), indicating that the prion infection did not alter the cellular sensitivity to the toxicity of the drugs. Accordingly, the elimination of PrPSc was not likely due to the death of the infected cells caused by the drugs.

AY-9944 and U18666A would accomplish their anti-prion activities by decreasing the amount of cellular cholesterol, or by increasing 7-dehydrocholesterol or desmosterol, or otherwise, via both mechanisms. Considering that desmosterol is significantly less effective than cholesterol in promoting the formation of ordered lipid domains of membranes,22) we presume that the anti-prion activity of U18666A is most likely a result of the partial replacement of cholesterol with desmosterol which alters the physical properties of lipid rafts or the intracellular vesicular transport. The observation that the addition of desmosterol to the culture medium prohibited the propagation of PrPSc in ScN2a cells (unpublished data) supports this notion. While the present study was underway, Klingenstein et al. also found the anti-prion activity of U18666A in cell culture systems.23)

Previous reports showed that the intraperitoneal or subcutaneous administration of tritium-labeled U18666A (10 mg/kg body weight) to juvenile rats resulted in the deposition of U18666A throughout the brain at concentrations ranging from 10^{-5} to 10^{-6} M;7) the concentrations comparable to the ED50 of the anti-prion activity in ScN2a cells (Fig. 2A). Also, the subcutaneous administration of AY-9944 to mice (25 mg/kg every other day for a month) inhibited DHCR7 activity in the brain, resulting in the accumulation of 7-dehydrocholesterol.15) In addition, cholesterol in the brain is synthesized in situ and not transported from the peripheral tissues,24) and the mice with a disruption of the dhcr24 gene were viable.25) These studies persuaded us to examine the anti-prion activities of AY-9944 and U18666A in vivo (Fig. 3A). Because the ED50 of AY-9944 was close to its toxic dose as examined in ScN2a cells (Fig. 2B), we co-injected AY-9944 (12 mg/kg) with U18666A (10 mg/kg) 91 d after the prion-inoculation to minimize the possible toxicity of AY-9944 (Fig. 3A, V). As shown in Fig. 3A, no significant difference in the mean survival period was observed between the drug-treated mice (Fig. 3A, II—V) and the untreated
mice (Fig. 3A, I). The ‘normal’ mice and the ‘un-infected’ mice (see Materials and Methods) were healthy over the entire experimental period. In support of this, the brains of the terminally ill-mice showed similar levels of PrPSc deposition (Fig. 3B). By contrast to the cultured cells (Fig. 1B), the lipid analysis showed that the brain sterol composition was not altered even after prolonged and repetitive use of the drugs (Fig. 3C). We conceive this could be a reason for the lack of the anti-prion effect of AY-9944 and U18666A in vivo. Since the turnover of cholesterol in the adult brain is very slow, the inhibitory effect of AY-9944 and U18666A on DHCR7 and DHCR24 could have been negligible in terms of overall changes in the brain’s sterol composition in such a metabolically static state. Alternatively, although U18666A and AY-9944 injected via an intraperitoneal route delivered rapidly into the brains of young animals, the delivery of the drugs into the brain of adult animals might have been less efficient.

In the present study, we showed that AY-9944 and U18666A blocked the propagation of prion in vitro. The drugs were unable to halt the progression of the disease in mice under our experimental condition, nevertheless, the results should be of importance in integrating our knowledge of potential chemotherapy of prion diseases.

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