A Candidate Anti-Prion Disease Agent, 2,2′-Biquinoline, Decreases Expression of Prion Protein and mRNA in Prion-Infected Cells

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(Received March 15, 2009; Accepted April 8, 2009; Published online May 14, 2009)

Transmissible spongiform encephalopathies (TSEs) are a family of invariably fatal neurodegenerative diseases. This group includes scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and Creutzfeldt-Jakob disease (CJD) in humans. These diseases are characterized by the accumulation of the abnormal isoform prion protein (PrP^{Sc}), which is a misfolded version of the cellular prion protein (PrP^{C}) and is resistant to enzymatic degradation. Numerous compounds have been reported to inhibit prion replication and PrP^{Sc} accumulation in cell cultures. Among them, we selected 2,2′-biquinoline (BQ) and studied the mechanism of its anti-prion disease activity. Its effect on prion protein (PrP) expression was examined in mouse neuroblastoma (N2a) cells and in prion-infected N2a (ScN2a) cells, using proteinase K (PK) treatment to discriminate between PrP^{C} and PrP^{Sc}. We found that BQ time dependently decreased the total amount of PrP and PrP mRNA expression in infected N2a cells, but not uninfected N2a cells. Our results indicate that the inhibition of PrP^{Sc} production by BQ was due to a decrease in the total amount of PrP.

Key words —— prion, prion protein, cellular isoform of the prion protein, scrapie isoform of the prion protein, 2,2′-biquinoline, anti-prion disease compound

INTRODUCTION

Transmissible spongiform encephalophathies (TSEs) or prion diseases are a group of fatal neurodegenerative disorders,[1] and their development is associated with oxidative damage to the brain, accumulation of aggregated proteins, and neuronal cell loss.[2,3] TSEs are characterized by the generation of a protein called scrapie isoform of the prion protein (PrP^{Sc}), which is a conformational variant of the normal host protein cellular isoform of the prion protein (PrP^{C})[4] and is resistant to enzymatic degradation. It is thought that the conversion of PrP^{C} into PrP^{Sc} is the key event in the pathogenesis of TSEs.

Most PrP^{C} is normally localized on the cell surface, where it is attached to the membrane *via* a *C*-terminal, glycosyl-phosphatidylinositol (GPI) anchor.[5] PrP^{C} is expressed in most tissues, especially in the central nervous system[6] and is associated with several physiologic functions. It has copper-binding ability,[7] it protects against oxidative stress,[8,9] and has been shown to influence cell-signaling mechanisms.[10,11] During prion infection, PrP^{C} on the cell surface acts as a substrate for PrP^{Sc} biosynthesis, and formation of PrP^{Sc} occurs either at the cell membrane or during the endocytic pathway. The coexistence of PrP^{C} and PrP^{Sc} in lipid rafts or caveolae-like domains suggests that cholesterol- and sphingolipid-enriched membrane microdomains are the sites of interaction between PrP^{C} and PrP^{Sc}. Attempts have been made to deduce the function of PrP^{C} using PrP-null mice, but these mice displayed no major anatomic or developmental deficits.[12] Even mouse lines in which the PrP gene is deleted postnatally are phenotypically relatively normal.[13] Thus, expression of PrP^{C} is essential, albeit not sufficient, for prion disease propagation and pathogenesis.

We previously showed that heterocyclic compounds with copper-selective chelating ability, such as 2,2′-biquinoline (BQ) and 2-(2′-pyri-

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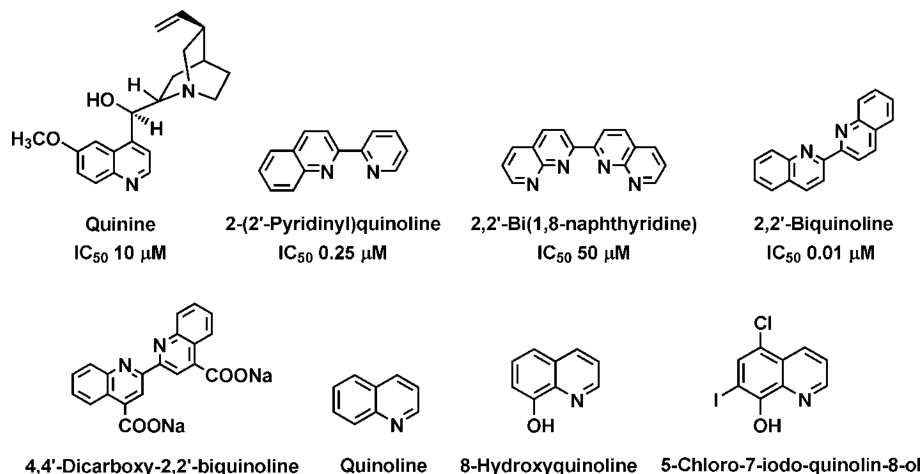

Fig. 1. Chemical Structures of Some Heterocyclic Derivatives
IC_{50}, Concentration of a compound causing 50% inhibition of PK-resistant PrP formation relative to the control.

dinyl)quinoline, are more effective inhibitors of prion disease propagation than nonselective metal-chelating compounds, such as quinoline and 8-hydroxyquinoline (for structures, see Fig. 1).[14] We found that many, but not all, compounds with selective copper-chelating ability exhibit for anti-prion disease activity, such as 4,4′-dicarboxy-2,2′-biquinoline (DCBQ) (Fig. 1). Thus copper-selective chelating ability *per se* may not be essential for anti-prion disease activity. Although the effects of candidate anti-prion disease agents have been investigated *in vitro* and *in vivo*, the mechanisms and sites of action of many of them remain unknown. Several approaches have targeted either of the two PrP isoforms, aiming, for example, to stabilize the PrP^{C} conformation, thereby inhibiting the initial steps of misfolding and aggregation that lead to the formation of PrP^{Sc}. Other strategies were designed to enhance PrP^{Sc} degradation or to prevent interaction between the cellular and the pathogenic molecules, an essential event in prion disease.[15] We were especially interested in the observation that compounds with related structures do not necessarily exert the same effects. In the present study, we studied the mechanism of the anti-prion disease activity of BQ, a candidate anti-prion disease agent.

MATERIALS AND METHODS

Chemicals —— Copper(II) sulfate and BQ were purchased from Wako Pure Chemical Indstries (Osaka, Japan). Quinine, 2-(2′-pyridinyl)quinoline, 2,2′-bi(1,8-naphthyridine), DCBQ, quinoline, 5-chloro-7-iodo-8-hydroxyquinoline, and 8-hydroxyquinoline were purchased from Maybridge Chemical Company (Cornwall, U.K.). They were dissolved in dimethyl sulfoxide (DMSO) just before use.

Cell Lines —— Mouse neuroblastoma (N2a) cells and N2a cells infected with the RML strain of TSE [prion-infected N2a (ScN2a)] were used in this study. N2a cells and ScN2a cells were grown in six-well culture plates in Opti-MEM (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum. When the cells reached confluence, 1/20 of the cells were passaged, and chemicals at various concentrations were added to the medium. The final concentration of DMSO in the medium was less than 0.2%. Treated cultures were allowed to grow to confluence (2 or 3 days).

Immunoblot Analysis —— Briefly, compounds were added at designated concentrations to the medium when cells were passaged at 10% confluency. The cells were allowed to grow to confluence and then lysed with lysis buffer [0.5% sodium deoxycholate, 0.5% Nonidet P-40, phosphate-buffered salin (PBS)]. Lysates were mixed with GLASSFOG (Q-Bio Gene, Irvine, CA, U.S.A.) for 2 min and centrifuged at 15000 rpm for 5 min at 24°C. The pellets were resuspended in sample loading buffer and boiled. Samples were separated using electrophoresis on 15% Tris-glycine-sodium dodecyl sulface (SDS)-polyacrylamide gel and electroblotted. Proteinase K (PK)-resistant PrP was detected using antibody SAF83 (1 : 5000; SPI-Bio, Massy, France), followed by an alkaline phosphatase-conjugated secondary antibody (Promega, Madison,

WI, U.S.A.). Immunoreactive signals were visualized using CDP-Star detection reagent (Amersham Biosciences Buckinghamshire, U.K.) and analyzed densitometrically.

PK Treatment—— Aliquots of post-nuclear lysates were incubated for 30 min at 37°C with 20 μg/ml PK (Funakoshi, Tokyo, Japan); proteolysis was stopped by the addition of protease inhibitor. Samples were precipitated with methanol and analyzed using the immunoblotting assay.

Phosphatidylinositol-specific Phospholipase (PI-PLC) Treatment—— PrP^C analysis in culture medium was immunodetected with Western blotting. To release cell surface PrP^C, cultures were treated with PI-PLC (0.2 units/ml) (Sigma, Maybridge) in opti-MEM serum-free medium at 37°C for 2 hr. The medium was collected and centrifuged at 4°C for 5 min at 7500 rpm to pellet any cells. PI-PLC-released proteins in the medium were precipitated with GLASSFOG and immunoblotted as described above with the appropriate antibody to detect PrP. The cell was lysed with lysis buffer and immunoblotted as described above.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis—— Total RNA was isolated from N2a and ScN2a cells with TRIzol Reagent (Invitrogen). The concentration of RNA was determined through spectrophotometric measurement. cDNA was prepared using 2 μg of RNA sample and a first-strand cDNA synthesis kit (Promega, Mannheim, Germany). PrP forward and reverse primers were 5′-AGG CTA ATAC CCC TGG CAC T-3′ and 5′-CCA AAA CAA AGC CCC AAC TA-3′, respectively, and gave a 0.45-kb product. The housekeeping gene β-actin was adopted as an endogenous control for RNA normalization. β-actin forward and reverse primers were 5′-TGT TAC CAA CTG GGA CGA CA-3′ and 5′-TCT CAG CTG TGG TGG TGA AG-3′, respectively, and gave 0.58-kb product. Cycling conditions consisted of 25 rounds (β-actin) and 30 rounds (PrP) at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. PCR products were examined on 2% agarose gel containing 0.4 μg/ml of ethidium bromide, and the specific bands of PCR products were measured using imaging software (Scion Image, Maryland, MD. U.S.A.). The gel was then visualized under UV light after ethidium bromide staining. The ratio of target PrP/β-actin amplicon was calculated as the relative mRNA level. Differences were analyzed using Student's t-test and were considered statistically significant at $p < 0.05$.

RESULTS

The reported anti-prion activity[14] of heterocyclic derivatives (Fig. 1) in the concentration range from 5 nM to 80 nM may involve various mechanisms. For example, a decrease in PrP^{Sc} can be explained by an increased degradation rate of PrP^{Sc} or by interference with its *de novo* biogenesis. We therefore investigated the effects of various quinolines on PrP expression by incubating N2a cells and ScN2a cells with the compounds for 3 days. Then the amount of PrP in cell lysates treated with PK or protease inhibitor was determined by immunoblotting. In immunoblotting, PK-resistant PrP characteristically shows an abnormal glycoprotein profile, contrasting with the triplet pattern seen in classical scrapie, comprising di-, mono-, and unglycosylated PrP bands. We confirmed that the formation of PK-resistant PrP was inhibited in ScN2a cells treated with effective compounds (Fig. 2A, lanes 1–4), in

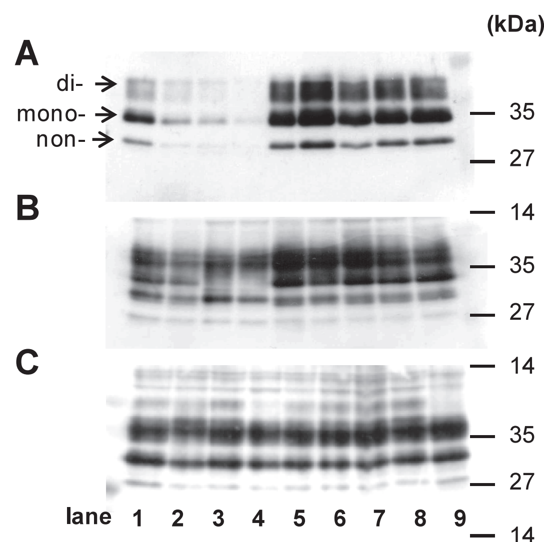

Fig. 2. Western Blotting Analysis of PrP in N2a and ScN2a Cells

The three bands characteristic of non-, mono-, and diglycosylated forms of PrP between 15 and 30 kD were present in cell lysates. SAF83 antibody recognized an epitope in both PrP^C and PrP^{Sc}. After protease K digestion, SAF83 was used to detect intact PrP^{Sc}. Compounds with anti-prion activity (lane 1, quinine 10 μM; lane 2, 2-(2′-pyridinyl)quinoline 0.25 μM; lane 3, 2,2′-bi(1,8-naphthyridine) 50 μM; lane 4, BQ 0.01 μM) were applied at the concentration giving 50% inhibition of PK-resistant PrP formation for 3 days. Uneffective compounds (lane 5, DCBQ; lane 7, quinoline; lane 8, 8-hydroxyquinoline; lane 9, 5-chloro-7-iodo-8-hydroxyquinoline) were applied at 10 μM in DMSO. Lane 6 was treated with DMSO alone. The effects of compounds on PrP^{Sc} were determined by immunoblot analysis of ScN2a cells treated with PK (A). The effects of compounds on PrP were determined using immunoblot analysis of ScN2a treated with a protease inhibitor (B) and uninfected cells (N2a) treated with protease inhibitor (C). The candidate anti-prion compounds, BQ and quinoline derivatives, influenced the amount of PrP^C in prion-infected cells. Bars on the right indicate molecular mass markers.

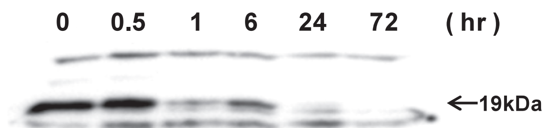

Fig. 3. PrP Expression in ScN2a Cells

ScN2a cells were cultured for 72 hr and BQ (1 μM) was added to the culture medium at the indicated times. BQ reduced the amount of PrP^{C} in prion-infected cells in a time dependent manner.

agreement with our previous findings.[14)]

Anti-Prion Drugs Regulate PrP^{C} Expression in ScN2a Cells

To examine whether the level of PrP^{C} expression correlated with anti-prion activity, we analyzed the total amount of PrP by immunoblotting without PK treatment. The results showed that BQ (Fig. 2B, lane 3) and certain other effective compounds (Fig. 2B, lanes 1–4) decreased the amount of PrP detected in the absence of PK treatment, as well as that detected after PK treatment in ScN2a cells (Fig. 2A). In the uninfected cell line N2a, these compounds had no effect on total PrP content (Fig. 2C). Similar results were obtained with non-quinoline-related copper chelators, cimetidine and D-penicillamine (data not shown). This decrease in PrP content in ScN2a cells after culture with BQ was time dependent. Interestingly, the bands around 19 kDa disappeared much earlier than the others (Fig. 3).

BQ Regulates PrP^{C} mRNA Expression in ScN2a Cells

Since BQ decreased PrP^{C} expression, we next evaluated PrP^{C} mRNA expression in N2a cells and ScN2a cells treated with BQ and an ineffective compound, DCBQ (1 μM). After incubation of the cells for 72 hr, the expression levels of mRNA were quantified using a one-step RT-PCR method. BQ caused a 2-fold decrease in the expression of PrP mRNA only in ScN2a cells (Fig. 4). Furthermore, the decrease was time dependent (Fig. 5). The decrease in PrP mRNA was observed after over 6 hr, whereas the decrease in PrP protein expression was observed within a few hours after the addition of BQ. These findings may indicate that the decrease in PrP mRNA is induced by the change in PrP expression.

BQ Decreases the Amount of Cellular PrP^{C}

PrP^{C} on the cell surface acts a substrate for PrP^{Sc} biosynthesis, and the formation of PrP^{Sc} occurs either at the cell membrane or during the endocytic pathway. The coexistence of PrP^{C} and PrP^{Sc} in lipid rafts or caveolae-like domains suggests that cholesterol- and sphingolipid-enriched membrane microdomains are the sites of interaction between PrP^{C} and PrP^{Sc}. The experiments described above established that BQ induces a decrease in the expression of PrP^{C} and its mRNA in prion-infected cells. The following experiments were designed to localize PrP using PI-PLC, which cleaves PrP^{C} at

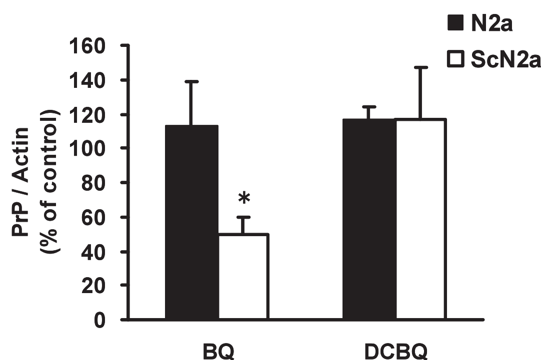

Fig. 4. Effects of BQ and DCBQ on PrP mRNA Contents of N2a and ScN2a Cells

Cells were treated with either BQ (1 μM) or DCBQ (1 μM) for 3 days. The PrP mRNA contents were determined using RT-PCR. Results were calculated as the means ± scanning electron microscope (SEM) of values from 3 experiments and are indicated relative to the DMSO-treated control. $^{*}p < 0.05$ *vs.* DCBQ-treated.

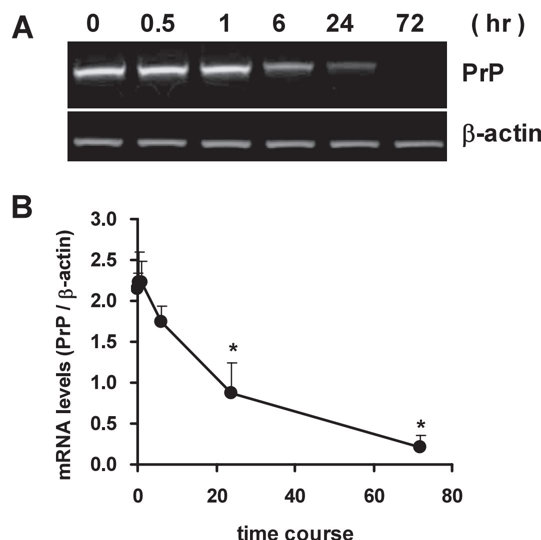

Fig. 5. PrP mRNA Expression in ScN2a Cells

ScN2a cells were cultured for 72 hr and BQ (1 μM) was added to the culture medium at the indicated times. PrP mRNA content was determined using RT-PCR. Results were calculated as the means ±SEM of values from 3 experiments and are indicated relative to the DMSO-treated control. $^{*}p < 0.05$ *vs.* 0 hr treated.

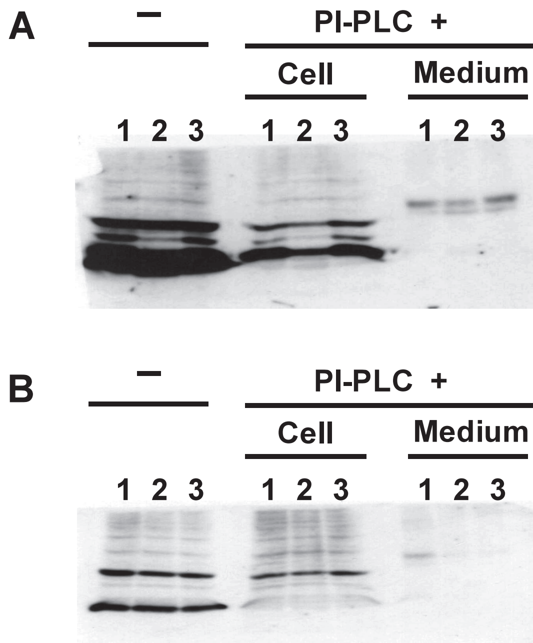

Fig. 6. Release of PrP Following PI-PLC Digestion

Treatment of ScN2a and N2a cells with BQ at 1 μM for 3 days influenced the amount of PrP^C in prion-infected cells. ScN2a and N2a cells were treated with (+) or without (−) 0.2 unit/ml PI-PLC for 2 hr at 37°C. Medium and cells in ScN2a cells (A) and N2a cells (B) were collected and their PrP content was determined. Lane1, DMSO; lane 2, BQ; lane3, DCBQ.

the cell surface. We measured the amount of PrP^C in cellular lysates and media using an immunoblotting procedure. The results shown in Fig. 6 indicate that a decrease in PrP^C expression was evident after BQ treatment only in the cellular lysate of ScN2a cells. No decrease in PrP^C was observed on the cell surface of either cell line or in lysate of uninfected cells. Thus, compounds with anti-prion activity may block the biosynthesis-promoting feedback signals generated by a decrease in PrP^C, the substrate for conversion to PrP^{Sc}, by fixing PrP^C on the cell surface, *i.e.*, blocking or inhibiting internalization. It was reported that misfolding of proteins at the cell surface compromises recycling and activates quality control mechanisms.[16)]

DISCUSSION

Recent interest in prion disease has spurred a great deal of research into the development of therapeutic strategies. Many substances have been found to inhibit PrP^{Sc} formation in cell culture and/or cell-free systems, including amyloid-binding dyes,[17)] sulfated glycosaminoglycans, tetrapyrrole compounds,[18)] cysteine protease inhibitors,[19)] substituted tricyclic derivatives such as chlorpromazine and quinacrine,[19, 20)] branched polyamines,[21)] and peptides.[22)] Some of these have already been examined mouse *in vivo*. Recently, Doh-ura *et al*.[23)] and Murakami-Kubo colleagues[24)] have shown that intraventricular administration of pentosan polysulfate and quinine prolonged the incubation period in a prion-infected transgenic mouse model, even at a late stage of infection. Further *in vivo* studies may lead to the establishment of effective therapeutics for prion diseases. However, to achieve more efficient therapeutics, it is essential to elucidate the mechanisms of action in detail.

In this study, we have investigated the mechanism of the anti-prion activity of BQ, a candidate anti-prion disease agent. Our results indicate that BQ time dependently decreases the total amount of PrP and PrP mRNA expression in prion-infected cells. Expression of PrP^C is essential, albeit not sufficient, for prion propagation and pathogenesis[12, 25—27)] as well as for neuroinvasion after peripheral infection.[28)] The combination of inhibiting PrP biosynthesis and suppressing PrP mRNA expression might be an effective approach to the therapy of prion diseases. Furthermore, our results indicate that the decrease in PrP^C does not occur at the cell surface. Other studies on N2a cells have shown that the removal of PrP^C from the plasma membrane precludes the initiation of infection.[29)] It was also shown that infection was impaired if PrP^C was not expressed when PrP^{Sc} entered the cells. Those reports indicated that there are precise temporal and spatial requirements for PrP^C expression in the early steps of successful prion infection. Our findings suggest that BQ may inhibit PrP biosynthesis or/and PrP transition to the cell surface and decrease the amount of substrate available for conversion to PrP^{Sc}. We could not establish whether BQ acts directly on cell surface PrP^C and PrP^{Sc}. However, the ability of BQ to decrease both prion protein and PrP mRNA expression in prion-infected cells makes it an attractive candidate for further evaluation as an anti-prion disease agent. We believe that the development of therapeutic agents focusing on the mechanisms underlying the expression and localization of PrP^C and PrP^{Sc} is likely to be a fruitful approach.

Acknowledgement We thank Dr. Katsumi Doh-ura (Department of Neurological Science, Tohoku University) for providing cell lines and for important contributions to the experiments.

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