Journal of Toxicology and Environmental Health, Part A: Current Issues
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uteh20

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Published online: 01 Nov 2011.


To link to this article: http://dx.doi.org/10.1080/15287394.2011.618978

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ALKYLATING ANTITUMOR DRUG MECHLORETHAMINE CONCEALS A STRUCTURED PrP DOMAIN AND INHIBITS IN VITRO PRION AMPLIFICATION

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Prion diseases are a group of incurable transmissible neurodegenerative disorders. The key molecular event in the pathogenesis of prion diseases is the conversion of the cellular prion protein (PrP\(^\text{C}\)) into its pathological isoform (PrP\(^\text{Sc}\)), accompanied by a conformational transition of \(\alpha\)-helix into \(\beta\)-sheet structure involving the structured \(\alpha\)-helix 1 domain from residues 144–154 of the protein (PrP\(_{144-154}\)). Blocking the accessibility of PrP\(_{144-152}\) with anti-PrP antibody 6H4 was found to prevent PrP conversion and even to cure prion infection in cell models (Enari et al. 2001). Previously, Yuan et al. (2005) demonstrated that the reduction and alkylation of PrP induced concealment of the 6H4 epitope. This study examined the ability of mechlorethamine (MCT), an alkylating antitumor drug, to conceal the 6H4 epitope and block PrP conversion in the presence of a reducing reagent. Mechlorethamine treatment significantly decreased in vitro amplification of PrP\(^\text{Sc}\) in the highly efficient protein misfolding cyclic amplification system. Our findings suggest that MCT may serve as a potential therapeutic agent for prion diseases.

Prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative disorders that affect both animals and humans (Prusiner 1998). Animal prion diseases include scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, and bovine spongiform encephalopathy (BSE) in cattle. Human prion diseases, whose etiologies can be familial, sporadic, or acquired by infection, include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease, fatal insomnia, and kuru (Gambetti et al. 2003). However, despite having distinct etiologies, all prion diseases are associated with prions or pathogenic prion protein conformers (PrP\(^\text{Sc}\)) that derive from a cellular prion protein (PrP\(^\text{C}\)) by a structural transition (Prusiner 1998).

Thus far, prion diseases are incurable, although intensive efforts have been undertaken in the search for therapeutic agents. The pathogenesis of these diseases also remains poorly understood. However, it is well known that the presence of PrP\(^\text{C}\) and PrP\(^\text{Sc}\) is a prerequisite. Specifically, PrP\(^\text{C}\) constitutes the substrate of PrP\(^\text{Sc}\) propagation. In mice whose PrP gene was knocked out, PrP\(^\text{Sc}\) inoculated into these mice was nontoxic and noninfectious (Büeler et al. 1993; Manson et al. 1994; Sailer et al. 1994; Sakaguchi et al. 1995). Therefore, one of the current strategies for...
the development of prion disease therapies aims to reduce levels of PrPc (Trevitt and Collinge 2006). For instance, depleting PrPc from neurons of prion-infected mice in which the PrP gene could be turned off not only inhibited progression of clinical disease but also reversed spongiform degeneration, early cognitive deficits, and neurophysiological dysfunction (Mallucci et al. 2003). In addition, the recruiting of PrPc by PrPsc is believed to involve a physical interaction between the two conformers (Prusiner 1998; Telling et al. 1995). As a result, Heppner et al. (2001) proposed that masking PrPc at critical sites of prion replication may prevent prion infection.

The 3F4 epitope is located in the flexible N-terminal region between residues 106 and 110 (Kascsak et al. 1987; Zou et al. 2010). The 6H4 epitope located in the α-helix 1 region, which possesses high helix propensity between residues 144 and 152 (Liu et al. 1999), is believed to be converted into a β-sheet structure during PrPsc formation (Govaerts et al. 2004; Prusiner 1998). Most of the anti-C epitope is located in α-helix 3 from residues 220 to 231. Thus, the 6H4 epitope is located between the 3F4 and anti-C epitopes. Anti-PrP antibody 6H4, occupying most of the α-helix 1 region ranging from residues 144 to 152, blocked PrP conversion and even cured prion infection in a cell model (Enari et al. 2001). Moreover, transgenic expression of the µ-chain of 6H4 completely prevented transgenic mice from developing scrapie when they were inoculated with prion (Heppner et al. 2001). Therefore, the availability of the 6H4 epitope in the PrPc molecule is critical for prion propagation. Blocking this region may inhibit PrPsc from recruiting PrPc.

Reduction and alkylation of either PrPc or PrPsc by dithiothreitol (DTT) and N-ethylmaleimide (NEM) were reported to inhibit PrP conversion in a cell-free system (Herrmann and Caughey 1998) and in the highly efficient protein misfolding cyclic amplification (PMCA) system (Lucassen et al. 2003). Yuan et al. (2005) demonstrated that reduction and alkylation of the PrPc molecule by tributylphosphine (TBP) and iodoacetamide specifically rendered the 6H4 epitope in α-helix 1 inaccessible not only in the solid phase but also in the liquid phase, whereas the accessibility of epitopes in the N- and C-terminal PrP domains remained unchanged. Moreover, data also showed that concealment of the 6H4 epitope was associated with reduced and alkylated Cys residues, although these are separated by 25 and 60 residues, respectively.

Thus, it was of interest to examine (1) whether there might be any Food and Drug Administration (FDA)-approved alkylating drugs that block this critical PrPsc binding site in PrPc by concealment and (2) whether these drugs inhibit PrPsc formation. The effects of three alkylating antitumor drugs including mechloethamine (MCT), doxorubicin, and streptozotocin (STZ) were examined. Mechloethamine belongs to nitrogen mustard with alkylating properties (Simon et al. 2000). Doxorubicin, an anthracycline antibiotic, acts through DNA intercalation, alterations of membrane function, and free radical formation (King and Perry 2001). Streptozotocin is a nitrosourea antibiotic that seems capable of functioning as both an alkylating and a carbamylating agent (King and Perry 2001). The effects of these three drugs were examined on the 6H4 epitope and ability to inhibit PrPsc generation.

MATERIALS AND METHODS

Materials
Phenylmethylsulfonyl fluoride (PMSF), proteinase K (PK), MCT, doxorubicin, and STZ were purchased from Sigma Chemical Co. (St. Louis, MO). Tributylphosphine (TBP) and dithiothreitol (DTT) were from Bio-Rad (Richmond, CA). The rabbit antiserum called anti-C antibody recognizes an epitope of human PrP residues 220–231 (RESQAYYQRGSS) (Monari et al. 1994). Mouse monoclonal antibodies 3F4, which recognizes an epitope of human PrP residues 93–109 (GGTHSQWNKPSKPKTNM), were
purchased from Covance (Emeryville, CA). Mouse monoclonal antibody 6H4, which recognizes an epitope of human PrP residues 144–152 (DYEDRYRE) (Korth et al. 1997), was purchased from Prionics AG (Zurich, Switzerland). Recombinant human full-length PrP23-231 and N-terminally truncated form PrP90-231 were kindly provided by Dr. Witold Surewicz (Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse or donkey anti-rabbit secondary antibody was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Human brain tissues were collected at autopsy and were kept frozen at the National Prion Disease Pathology Surveillance Center (Cleveland, OH) at −80°C until use.

**Preparation of Brain Homogenates**

Brain homogenates (10% w/v) from normal human or scrapie-infected or uninfected hamster brains were prepared in lysis buffer (100 mM NaCl, 10 mM ethylenediamine tetraacetic acid [EDTA], 0.5% Nonidet P 40 (NP-40), 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.5). To prepare the brain homogenates for PMCA, conversion buffer (0.1% sodium dodecyl sulfate [SDS], 0.1% Triton X-100, 1 × PBS, pH 7.0) instead of lysis buffer was used. After homogenization on ice, samples were centrifuged at 1000 × g for 10 min to remove cellular debris.

**Reduction and Alkylation of PrP**

Brain homogenates (10%) or recombinant PrP were boiled in equal volume of 2 × sample buffer (6% SDS, 5% β-mercaptoethanol [β-ME], 4 mM EDTA, 20% glycerol, 125 mM Tris-HCl, pH 6.8). The samples were incubated with a fivefold volume of prechilled methanol at −20°C for 2 h, followed by a centrifugation at 16,000 × g for 20 min at 4°C. Pellets were resuspended in 20 μl TBP buffer (5 mM TBP, 8 M urea, 2% CHAPS, 20 mM Tris-HCl, pH 8.0) for 1 h at room temperature and then incubated in the dark with alkylation antitumor drugs for a designated time period. The samples were incubated with prechilled methanol and centrifuged at 16,000 × g for 20 min at 4°C. Finally, the pellets were resuspended in 20 μl samples buffer and subjected to SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting with various anti-PrP antibodies.

**Protein Misfolding Cyclic Amplification (PMCA)**

The protein misfolding cyclic amplification (PMCA) and serial PMCA were performed as described with slight modifications (Castilla et al. 2005; Saborio et al. 2001). In brief, 99 μl of brain homogenate from uninfected hamsters was incubated with designated amounts of alkylation antitumor drugs for 30 min at room temperature, followed by addition of DTT. Subsequently, 1 μl brain homogenate from scrapie-infected hamster (263K) was added. After addition of designated amounts of alkylation drugs and DTT, all samples were prepared to have equal amounts of volumes by the addition of conversion buffer (0.1% SDS, 0.1% Triton X-100, 1 × PBS, pH 7). After an aliquot was frozen at −80°C as a sample control without PMCA, the remaining mixture was transferred to a 0.2-μl polymerase chain reaction (PCR) tube that was placed on a microplate horn filled with water. The sample was subjected to PMCA, consisting of cycles of 30 min of incubation at 37°C followed by a 400-s pulse of sonication at 60% potency for 18 h in a sonicator (Misonix model 3000, Farmingdale, NY). To detect the amplified PrPSc, a 20-μl PMCA-treated or untreated sample was treated with PK at 100 μg/ml for 70 min at 45°C. The reaction was terminated by adding PMSF at a final concentration of 5 mM and an equal amount of SDS sample buffer, then boiling at 100°C for 10 min. A 10-μl sample was subjected to SDS-PAGE and Western blotting with 6D11. The serial PMCA without PrPSc template was used to determine whether the MCT-treated PrP from normal hamster brain homogenate can form PK-resistant PrPSc after several rounds of PMCA in the absence of PrPSc.
Normal hamster brain homogenates were incubated with 0.5 mg/ml MCT and 1.5 mg/ml DTT at a final concentration for 30 min at room temperature before serial PMCA.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting**

Samples were mixed with an equal volume of 2 × SDS sample buffer and boiled for 10 min. Proteins were separated using 15% Tris-HCl precast gels (Bio-Rad). Electrophoresis of the proteins from the gels to the polyvinylidene difluoride (PVDF) membranes was performed at 70 V for 2 h. The membranes were blocked with 5% nonfat milk in TBS-T buffer overnight at 4°C or 1 h at 37°C prior to incubation with antibodies. Membrane-bound proteins were probed with anti-C antibody at 1:3000, 6H4 at 1:5000, or 3F4 antibody at 1:40,000. The blot was then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit or sheep anti-mouse antibody at 1:3000. The PrP bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

**Statistical Analysis**

Statistical significance of differences in PrP intensity was evaluated using Student’s t-test. A difference was considered statistically significant if the p value was <.05.

**RESULTS**

**MCT Induced Region-Specific Concealment of the 6H4 Epitope**

Previously, Yuan et al. (2005) demonstrated that alkylation of PrP induced concealment of the 6H4 epitope in the presence of reducing reagents. Using epitope mapping, the effect of the three drugs was initially investigated on accessibilities of epitopes of anti-PrP antibodies with Western blot analysis, which may reflect structural changes of PrP. Three anti-PrP antibodies against different regions of the protein were used in this study.

After treatment with different amounts of doxorubicin, MCT, or STZ (from 0 to 2 mg/ml) in the presence of the reducing reagent TBP, normal human brain homogenates were subjected to SDS-PAGE and Western blot analysis probing with 3F4, 6H4, and anti-C antibody (Figure 1). On the blot probed with either 6H4 or anti-C antibody, PrP was detectable in the samples treated with amounts of each one of the three drugs (Figures 1A and 1C). In contrast, PrP was completely nondetectable in the samples treated with MCT from 0.5 to 2 mg/ml, but was noted in the untreated samples or in samples treated with either doxorubicin or STZ (Figure 1B). Therefore, although the three drugs possess alkylating activity, only MCT specifically blocked accessibility of the 6H4 epitope.

**Concealment of the 6H4 Epitope by MCT Is Concentration- and Time-Dependent**

Whether blockage of the 6H4 epitope induced by MCT is dependent on amounts of the drug used and incubation time of treatment was subsequently determined. The brain homogenates were treated with different...
concentrations of MCT from 0 to 0.8 mg/ml in the presence of TBP prior to SDS-PAGE and Western blotting with either 6H4 or anti-C antibody (Figure 2). The amount of PrP detected with 6H4 was decreased significantly with increasing amounts of MCT, whereas no significant changes in the quantity of PrP detected with anti-C antibody were observed (Figures 2A and 2B). Thus, the accessibility of the 6H4 epitope was inhibited by incubation with MCT in a concentration-dependent manner. Based upon three independent experiments, the half-inhibition rate IC50 of the 6H4 epitope accessibility by MCT was approximately 0.15 mg/ml (Figures 2A and 2C).

The effect of incubation time on the accessibility of 6H4 by MCT was also evaluated. The brain homogenates were incubated with MCT at 0.5 mg/ml in the presence of TBP for 0, 10, or 20 min prior to SDS-PAGE and Western blotting with 6H4 or anti-C antibody (Figures 2D and 2E). Compared to the samples without incubation, the amounts of PrP detected with 6H4 were significantly decreased after 10 min of incubation. No PrP was found in the samples treated with MCT for 20 min. In contrast, no significant changes in the levels of PrP were observed with the anti-C antibody (Figure 2E). Thus, the accessibility of the 6H4 antibody was inhibited by MCT in a time-dependent manner.

**MCT May Induce PrP\(^{C}\) Aggregation**

Exposure of cultured human epidermal keratinocytes to MCT induced keratin aggregation (Dillman et al. 2003). However, it remains unclear whether there is a direct effect of MCT on pure PrP. To address this issue, recombinant full-length (PrP23-231) and N-terminally truncated short (PrP90-231) forms of human PrP were exposed to MCT in the presence or absence of TBP prior to SDS-PAGE or Western blotting with 3F4, 6H4, or anti-C antibody (Figure 3).

For the untreated recombinant full-length human PrP23-231, all three antibodies detected a major PrP band migrating at approximately 23 kD and a faint band migrating at approximately 46 kD (Figure 3A through 3C).
According to the molecular mass of recombinant PrP23-231, the former may be the monomer of the protein, whereas the latter might be the dimer. On the anti-C blot, there was an additional band migrating at approximately 16 kD that corresponds to the N-terminally truncated PrP90-231 (Figure 3C). After treatment with TBP, there were no significant differences in the blots probed with the three antibodies compared to untreated PrP. Only a quantitative decrease in the intensity of the two bands (23 kD and 46 kD) was detected in the three blots. However, in the samples treated with MCT alone, a significant increase in the intensity of the PrP band migrating at approximately 46 kD was detected by all three antibodies (Figure 3A through 3C). Moreover, two additional bands migrating at approximately 67 kD and ∼92 kD, as well as a band migrating above 100 kD, were detected with 3F4 (Figure 3A). Compared to the 3F4 blot, the intensity of the three bands was numerically weaker in the 6H4 and anti-C blots. These bands might represent trimer, tetramer, and higher oligomers of PrP. The rise in the dimeric form and appearance of the additional bands with higher molecular mass suggest that PrP formed aggregates in the presence of MCT. After treatment with both TBP and MCT, more oligomers of PrP were detected by 3F4, in addition to the 23-kD PrP monomer (Figure 3A). In contrast to 3F4 and anti-C, 6H4 noted virtually no PrP bands in the samples treated with both TBP and MCT. Therefore, MCT treatment may cause aggregation of recombinant full-length PrP and inhibit the accessibility of the 6H4 antibody in the presence of TBP. Nevertheless, although it seemed that MCT induced PrP oligomerization, proteinase K (PK)-resistant PrP was not found in the MCT-treated full-length and N-terminally truncated PrP after PK-treatment at 25 µg/ml for 1 h at 37°C (data not shown).

For the untreated and TBP-treated truncated PrP, all three antibodies detected two major bands: a band with higher intensity migrating at approximately 16 kD, and another band with lower intensity migrating at approximately 31–32 kD (Figure 3D through 3F). According to the molecular mass of the
truncated PrP, the latter may be a dimer of the former. After treatment with MCT, an additional band migrating at 47 kDa became detectable by the three antibodies, in addition to an increase in the intensity of the 31–32 kD band (Figure 3D through 3F). The 47-kD band corresponds to the trimer of PrP90-231 according to its molecular mass. In the presence of TBP, MCT treatment enhanced the intensity of PrP oligomers but decreased the intensity of the monomer of PrP90-231 in the 3F4 and anti-C blots (Figure 3D). Moreover, 3F4 and anti-C detected a band migrating at 57 kD and another band migrating above 100 kD (Figure 3D and 3F). In contrast, 6H4 detected only a faint band migrating at approximately 16 kD, corresponding to the monomer of PrP90-231 (Figure 3E). Therefore, although there might be differences in the effect of MCT on the full-length and truncated PrP, MCT consistently and significantly concealed the 6H4 epitope in the two forms of the recombinant PrP.

The epitope of the anti-PrP antibody 6D11 is located in the PrP region that overlaps with the 3F4 epitope (PrP93-109 vs. PrP106-110) (Zou et al. 2010). Moreover, it has extended N-terminal amino acids from residues 105 to 93 except for lacking residue 110 at the C-terminus of the epitope compared to the 3F4 epitope. The 6D11 antibody was used to confirm its accessibility to the area containing the 3F4 epitope. As was the case with the 3F4 epitope, no significant change in the accessibility of the 6D11 epitope was observed after MCT and DTT treatment (Figure 3G), indicating that our antibody–epitope mapping was reliable.

**MCT but Not STZ Inhibit PrPsc Amplification In Vitro**

Herrmann and Caughey (1998) found that reduction and alkylation of PrPC were observed to inhibit the conversion of PrPC into PrPsc in vitro. Whether the alkylating antitumor drugs inhibit PrPsc formation in the presence of reducing agents was thus studied. Brain homogenates from uninfected and 263K-infected hamsters were used as substrates and templates, respectively. Either MCT or STZ at the designated concentrations was incubated with uninfected hamster brain homogenates at room temperature for 30 min prior to adding scrapie-infected hamster brain homogenates for PMCA. The amplified PrPsc was detected with Western blot analysis probed with 6D11 antibody after PK treatment.

PK-resistant PrPsc in the PMCA-treated samples was significantly decreased with increasing concentrations of MCT (Figure 4A and 4C). A decrease in the PrPsc intensity was also noted in the frozen sample, although it was less severe than in PMCA-treated samples. As indicated previously (Saborio et al. 2001), quantitatively less amplification of hamster PrPsc was also observed by incubation of PrPC with PrPsc in the absence of sonication. Given that the normal brain homogenate was incubated with MCT at room temperature for more than 30 min before the aliquoted samples were frozen, it is conceivable that the less quantitative amplification in the aliquoted samples at room temperature might also be inhibited by added MCT. Therefore, the preincubation of the PrP substrate with MCT in frozen samples was the basis for comparison to the sample without MCT. PK-resistant PrPsc was also decreased with increasing in concentrations of MCT in frozen samples. In contrast, no significant alterations in the levels of PrPsc were detected by 6D11 in the samples treated with STZ (Figure 4B and 4C).

Since MCT may induce PrPC aggregation (Figure 3), it was important to determine whether the MCT-induced PrP aggregates are a PMCA-amplifiable feature of PrPsc by PMCA in vitro. To test for this possibility, serial PMCA was conducted that was previously reported to generate spontaneous de novo PrPsc without PrPsc templates (Barria et al. 2009). Normal hamster brain homogenates treated with MCT and DTT were subjected to serial PMCA with continuously refreshing normal brain homogenates. No PK-resistant PrPsc application was observed until 8 rounds of PMCA (data not shown), indicating that no spontaneous de novo PrPsc was generated with MCT-treated PrP.
FIGURE 4. Effect of MCT on PrPSc amplification by PMCA. Normal hamster brain homogenates (substrates) were incubated with MCT or STZ along with DTT at the designated concentrations for 30 min at room temperature prior to addition of the scrapie-infected hamster brain homogenates (templates) for PMCA process. Amplified PK-resistant PrPSc was detected with Western blotting probed with 6D11 after PMCA. (A) MCT treatment. (B) STZ treatment. PMCA, frozen samples without PMCA; +, samples subjected to PMCA. The data shown in (C) represent the average of results from three independent experiments.

DISCUSSION

Several lines of evidence indicate that reducing the availability of PrPC, the precursor of the prion pathogen PrPSc, is one of the most effective strategies for developing prion therapeutics (Trevitt and Colinge 2006; White and Mallucci 2009). Because availability of the 6H4 epitope is important in prion propagation (Enari et al. 2001; Heppner et al. 2001), our previous finding that treatment of PrPC by a combination of alkylation and reduction concealed the 6H4 epitope motivated us to examine drugs that possess alkylation activity. Our current study provides the following four new findings. First, in the presence of reducing agents, treatment of PrP with the alkyllating antitumor drug MCT, but not with doxorubicin and STZ, significantly decreased the accessibility of the 6H4 epitope in concentration- and time-dependent manner. Second, although there might be differences in the effect of MCT on the full-length and truncated PrP, MCT consistently and significantly concealed the 6H4 epitope in both forms of PrP. Third, MCT may induce PrP aggregation. Finally, MCT but not STZ inhibited in vitro PrPSc amplification. Taking these together, our study indicated that inhibition of in vitro PrPSc amplification by MCT may be specifically attributed to concealment of the 6H4 epitope and reduction of availability of PrPC.

Doxorubicin, MCT, and STZ all belong to a group of alkylating anticancer chemotherapeutic drugs. The nitrogen mustard alkyllating agents, including MCT, have the ability to form DNA-DNA cross-links and DNA-protein cross-links (Lawley and Brookes 1965; Osborne and Lawley 1993; Ross et al. 1978; Thomas et al. 1978; Loeber et al. 2009). MCT (also known as chloromethine, mustine, Mustargen, or HN2) is the prototype of nitrogen mustards. MCT is believed to prevent cell duplication by binding to the N7 nitrogen on the DNA base guanine. Mustargen, the brand name for MCT, is usually administered intravenously and is indicated for the palliative treatment of Hodgkin’s disease, lymphosarcoma, chronic myelocytic or chronic lymphocytic leukemia, polycythemia vera, mycosis fungoides, and bronchogenic carcinoma. The mechanism(s) underlying nervous and nonnervous tissue injuries induced by nitrogen mustards are believed to result from covalent alkylation of DNA, forming intra- and interstrand DNA-cross-linked compounds that inhibit cell proliferation (Colvin and Chabner 1990; Hopkins et al. 1991). Because only MCT inhibits in vitro prion amplification and STZ does not, it is most likely that the inhibition of PrPSc amplification by MCT is associated with concealment of the 6H4 epitope for PrPSc binding and not with DNA alkylation.

Our current study with the recombinant full-length and truncated PrP molecules also provides evidence that MCT may interact directly with proteins, in addition to...
its interaction with DNA. Indeed, MCT was observed to induce keratin aggregation in cultured human epidermal keratinocytes (Dillman et al. 2003). Moreover, covalent binding of nitrogen mustard to the Cys residue in human serum albumin was reported previously (Noort et al. 2002; Yeo et al. 2008). As indicated in our previous study with the alkylating agent iodoacetamide (Yuan et al. 2005), after reduction of PrP by TBP or DTT to break the disulfide bond, alkylation by MCT may block two free thiols (Welker et al. 2002), which results in concealment of the 6H4 epitope via a putative rearrangement of the PrP structure. The availability of this region may be necessary during PrP conversion, which is consistent with the observation that occupation of this epitope in PrP by the 6H4 antibody prevented PrP conversion and even cured prion infection in cell and animal models (Enari et al. 2001; Heppner et al. 2001). Interestingly, Lucassen et al (2003) also reported that alkylation and reducing reagents including N-ethylmaleimide, DTT, and β-mercaptoethanol inhibited PrPSc amplification by PMCA. In addition, data demonstrated that MCT may induce PrP aggregation, which may underlie MCT-induced inhibition of PrPSc formation by decreasing the effective PrPc substrates for conversion.

Rose and Dorms (1988) proposed that DTT and MCT exposed Cys residues, and this exposure of Cys residues is commonly believed to trigger protein aggregation. Studies with scrapie-infected neuronal cell lines suggest that the conversion of the monomeric PrPc into aggregated PrPSc occurs after PrPc reaches the cell membrane. Two different aggregation pathways of PrP on cell membranes consisting of varied lipids were proposed (Robinson and Pinheiro 2010). Given our new finding that MCT not only decreases the accessibility of the 6H4 epitope but also induces PrP aggregation, it is possible that these prion disease-like clinical manifestations, in particular ataxia, tremors, memory deficits, and motor dysfunction (Anslow et al. 1947; Graef et al. 1948), may be associated with PrP conformational changes produced by these agents. By performing serial PMCA with MCT-treated normal brain homogenates, our result might rule out a possibility that these manifestations are associated with potential spontaneous de novo PrPSc formation induced by MCT treatment. Moreover, our finding that no PK-resistant PrP was detected in the MCT-treated PrP may also rule out this possibility. In conclusion, data not only indicate a potentially new application of MCT, the conventional antitumor drug, in therapy of prion diseases, but also denote the significance of reevaluating the role of PrP in nervous tissue injury induced by MCT.

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