EBioMedicine 9 (2016) 238–249

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Structure-Based Drug Discovery for Prion Disease Using a Novel Binding Simulation

Daisuke Ishibashi [a,*,1], Takehiro Nakagaki [a,1], Takeshi Ishikawa [a], Ryuichiro Atarashi [b], Ken Watanabe [a], Felipe A. Cruz [c], Tsuyoshi Hamada [c], Noriyuki Nishida [a]

[a] Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Japan
[b] Division of Microbiology, Department of Infectious Diseases, Faculty of Medicine, University of Miyazaki, Japan
[c] Nagasaki Advanced Computing Center, Nagasaki University, Japan

ARTICLE INFO

Article history:
Received 28 April 2016
Received in revised form 25 May 2016
Accepted 6 June 2016
Available online 8 June 2016

Keywords:
Prion
Drug discovery
In silico screening
Small chemical compounds
Conformational disorders

ABSTRACT

The accumulation of abnormal prion protein (PrP^{Sc}) converted from the normal cellular isoform of PrP (PrP^{C}) is assumed to induce pathogenesis in prion diseases. Therefore, drug discovery studies for these diseases have focused on the protein conversion process. We used a structure-based drug discovery algorithm (termed Nagasaki University Docking Engine: NUDE) that ran on an intensive supercomputer with a graphic-processing unit to identify several compounds with anti-prion effects. Among the candidates showing a high-binding score, the compounds exhibited direct interaction with recombinant PrP *in vitro*, and drastically reduced PrP^{Sc} and protein-aggresomes in the prion-infected cells. The fragment molecular orbital calculation showed that the van der Waals interaction played a key role in PrP^{C} binding as the intermolecular interaction mode. Furthermore, PrP^{Sc} accumulation and microgliosis were significantly reduced in the brains of treated mice, suggesting that the drug candidates provided protection from prion disease, although further *in vivo* tests are needed to confirm these findings. This NUDE-based structure-based drug discovery for normal protein structures is likely useful for the development of drugs to treat other conformational disorders, such as Alzheimer's disease.

1. Introduction

Conformational diseases, which include Alzheimer's disease and Parkinson's disease, are the result of the accumulation of intracellular dysfunctional proteins, such as amyloid-beta and alpha-synuclein (Jucker and Walker, 2011). Abnormal prion protein (PrP^{Sc}) has also been shown to be a pathogenic protein, which is formed by conformational changes to the native cellular prion protein (PrP^{C}) (Weissmann et al., 2002). The molecular mechanisms of conversion remain poorly understood, although drug discovery studies have focused on the conversion process from PrP^{C} and PrP^{Sc}. A variety of drugs have been reported to reduce PrP^{Sc} levels by halting the conversion process as described below: acridines including quinacrine (Vogtherr et al., 2003); anti-PrP antibodies including D18 (Peretz et al., 2001), 6H4 (Enari et al., 2001) and ICSM38 (White et al., 2003); polyanions including pentosane polysulfate (PPS) (Doh-ura et al., 2004; Priola and Caughey, 1994); dextran sulfate (Caughey and Raymond, 1993) and HM2602 (Adjou et al., 2003); the polyene antibiotics including amphotericin B (Mange et al., 2000) and filipin (Marella et al., 2002); the others including suramin (Gilch et al., 2001), Congo-Red (Caughey and Race, 1992), Cpd B (Kawasaki et al., 2007), GN8 (Kuwata et al., 2007) and luminescent-conjugated polythiopherenes (LCPs) (Herrmann et al., 2015). Other studies have focused on the intracellular proteolytic system, such as autophagy of insoluble proteins, because the PrP^{Sc} complex and the PrP oligomer may have toxic effects on the cell (Aguzzi and Calella, 2009). *In vitro* and *in vivo* studies using compounds such as lithium (Heiseke et al., 2009), trehalose (Aguib et al., 2009), rapamycin (Ishibashi et al., 2015), tamoxifen (Marzo et al., 2013), FK506 (Nakagaki et al., 2013), IU-1 (Homma et al., 2015), have reported anti-prion effects. Among them, PPS, Cpd B, LCPs, and FK506 significantly prolong survival periods in mice inoculated with RML or FK-1 prion strains (Doh-ura et al., 2004; Herrmann et al., 2015; Kawasaki et al., 2007; Nakagaki et al., 2013). Recently, it especially has been reported that Anle138b has potent and broad spectrum activity for different protein aggregation disease models (Wagner et al., 2013). Studies have continued to identify suitable compounds for treating the diseases, although none have provided any evidence of benefits against human prion disease, even though some were tested in clinical trials (Tsuboi et al., 2009; Haik et al., 2014).

The structure-based drug discovery (SBDD) using computer simulation was recently facilitated to develop effective chemical compounds.

* Corresponding author at: Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
E-mail address: dishi@nagasaki-u.ac.jp (D. Ishibashi).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ebiom.2016.06.010

This novel approach is based on virtual screening for drug discovery and has successfully identified compounds for treating several diseases, such as nelfinavir (Kaldor et al., 1997) and amprenavir (Highleyman, 1999) for AIDS; zanamivir for influenza (McCauley, 1999); celecoxib (Stratton and Alberts, 2002) and rofecoxib (Mardini and FitzGerald, 2001) as cyclooxygenase 2 inhibitors; antibacterial agents (Simmons et al., 2010); Ras inhibitor for human cancer (Shima et al., 2013). SBDD has also been used in prion disease, showing that Cp-60, −62 compounds that mimic the dominant negative PrP^C mutant inhibit PrP^{Sc} formation (Perrier et al., 2000) and that GN8 strongly stabilises normal conformation by binding to a specific region in PrP^C, which suppresses PrP^{Sc} production and prolongs survival of prion-infected mice (Kuwata et al., 2007). Furthermore, other small compounds that target the same position as the interaction between GN8 and PrP^C have been discovered by virtual screening which used original docking simulation, and those compounds reduced PrP^{Sc} levels in RML prion-infected cells (Hyeon et al., 2015). In this study, we performed original docking simulations, termed Nagasaki University Docking Engine (NUDE) for PrP^C conformation and small compounds in a large chemical compound database using the DEGIMA supercomputer system. Binding interactions were analysed using the fragment molecular orbital (FMO) method to identify novel anti-prion drugs. Following virtual screening, we tested the ability of candidate compounds to bind to PrP^C using surface plasmon resonance (SPR) analysis. The thermal shift assay (TSA) was used to determine whether the compounds influenced thermal change-dependent PrP^C stabilisation. We also evaluated the anti-prion effect of compounds using persistently prion-infected cells and mice, which revealed novel therapeutic candidates.

2. Materials and methods

2.1. Regents and antibodies

All candidate compounds (NPR-015, −050, −053, −056 and −065) identified by *in silico* calculation were purchased from ASINEX (Supplementary Fig. S8). GN8 was gifted from Prof. Kuwata (Gifu University) and served as the positive anti-prion control drug (Kuwata et al., 2007). These compounds were completely dissolved in 100% dimethyl sulfoxide (DMSO) and adjusted to 10 mM as stock solution. Stock solutions of compound were diluted in sterile water, culture medium or phosphate buffered saline (PBS) to perform the several assay in this study. Antibodies specific for PrP (Santa Cruz Biotechnology, M20; SPI-Bio, SAF61), Iba-1 (WAKO, 019-19741 for IHC and 016-20001 for WB) and β-actin (MBL) were purchased from the indicated vendors. Horseradish peroxidase-conjugated anti-goat (Jackson ImmunoResearch) and anti-mouse (GE Healthcare Life Sciences) IgG antibodies were used for immunoblotting.

2.2. In silico screening

To obtain potential candidate anti-prion compounds, we performed a docking simulation using an original chemical compound library that included approximately 210,000 compounds. The three-dimensional structure of each compound was produced by Open Babel software (O'Boyle et al., 2011), which was followed by energy minimisation using GAFF force field (Wang et al., 2004). In our docking simulation, a structure of the globular domain of human PrP^C (124–230th amino acid residues) was used as a receptor, whose atomic coordinates were prepared from the Nuclear Magnetic Resonance (NMR) structure (Biljan et al., 2012) (Protein Data Band code: 2LSB). A cubic space ($64 \times 64 \times 64$ Å) was used as the search region for the docking simulation, and binding with the whole surface of the target protein was examined. We employed an original docking simulation program designed as a Graphics Processing Unit (GPU). In this study, the DEGIMA (DEstination for Gpu Intensive MAchine) supercomputer from the Nagasaki Advanced Computing Center, which was constructed with more than 100 GPUs, was used for the docking simulation.

2.3. Fragment molecular orbital calculation

To analyse binding conformations obtained from the docking simulation, FMO calculations (Kitaura et al., 1999) were performed. To prepare reliable atomic coordinates for the FMO calculations, hydrogen atoms were added to the docking structure and the *N*-terminal of the G124 was capped by —$COCH_3$. An energy minimisation with classical force fields (AMBER99SB (Hornak et al., 2006) and GAFF (Wang et al., 2004)) was performed using the AMBER 10 program (Case, 2008), and the energy minimised structure was used for the FMO calculations. Amino acid residues and compounds were treated as a single fragment, except for C179 and C214, which were merged into one fragment, because they have a disulfide bond. Interaction energies were calculated at the Hartree–Fock (HF) level and second-order Møller–Plesset perturbation (MP2) level with resolution of the identity approximation (Ishikawa and Kuwata, 2009) using cc-pVDZ basis set (Dunning, 1989). In this study, the FMO calculations were performed using the PAICS program (Ishikawa et al., 2009).

2.4. Surface plasmon resonance (SPR) analysis

Interactions between recombinant PrP and compounds were evaluated using a Biacore T200 system (GE Healthcare) as previously described (Nakagaki et al., 2013). Human or mouse PrP (23 − 231) was synthesised by *Escherichia coli* in a protein expression system using the pET vector, and the protein was purified by imidazole in a NTA column as previously described (Atarashi et al., 2011). The recombinant PrP solutions were diluted to 10 μg/mL with running buffer (10 mM HEPES, pH 7.4 containing 150 mM NaCl, 0.05% Tween20 [Sigma Aldrich] and 5% DMSO), and the ligands were immobilised on a CM5 sensor chip (GE, BR-100,530) using an amine coupling kit (GE, BR-1000-50). Immobilisation of PrP was performed with an average of 2000 RUs. Compounds of various concentrations were diluted with the same running buffer and evaluated by injecting them sequentially for 2 min at a flow rate of 30 mL/min, after which the running buffer alone was injected for a further 20 min at the same flow rate to wash out the bound compounds. Data were corrected using a blank sensor chip as a control. Each compound was dissolved in DMSO and diluted to 5% with running buffer (Nakagaki et al., 2013).

2.5. Cell cultures

Mouse neuroblastoma Neuro 2a cells were obtained from the American Type Culture Collection (CCL 131). N2a-58 cells are established from N2a cells overexpressing PrP^C and integrating mouse *prnp* gene in Neuro 2a cells. N2a-FK cells established from N2a-58 cells were infected with a mouse-adapted Gerstmann–Sträussler–Scheinker strain, Fukuoka-1, as previously described (Ishibashi et al., 2015, Ishibashi et al., 2011). N2a-22L cells were established from N2a-58 cells infected with a mouse-adapted scrapie 22L strain as previously described (Homma et al., 2015, Homma et al., 2014a; Nishida et al., 2000). The above cells were grown at 37 °C in 5% CO_2 in Dulbecco's-modified Eagle's medium (Wako) containing 4500 mg/mL glucose, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Nakarai Tesque). Cell viability was determined by counting the living cells with Luna automatic cell counter instrument (Logos Biosystems) and cell form was visualised by a microscope.

2.6. Immunobloting

Immunoblotting was performed as previously described (Homma et al., 2014b). For PrP^{Sc} detection, the lysates were digested with 20 μg/mL proteinase K (PK; Nakarai Tesuque) at 37 °C for 30 min. After addition of SDS-sample buffer, the samples were applied to 15% SDS-PAGE gel and were subsequently transferred to a PVDF membrane. To detect PrP^{Sc}, M20 served as the primary antibody (1:1000) and anti-goat IgG-HRP