Tubulin/microtubules as novel clozapine targets

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Abstract

Aim: Clozapine is currently the only effective drug for treatment-resistant schizophrenia; nonetheless, its pharmacological mechanism remains unclear, and its administration is limited because of severe adverse effects. By comparing the binding proteins of clozapine and its derivative olanzapine, which is safer but less effective than clozapine, we attempted to clarify the mechanism of action specific to clozapine.

Methods: First, using the polyproline rod conjugates attached with clozapine or olanzapine, clozapine-binding proteins in extracts from the cerebra of 7-week-old ICR mice were isolated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify proteins. Second, the effect of clozapine on tubulin polymerization was determined turbidimetrically. Finally, the cellular effects of clozapine were observed in HeLa cells by immunofluorescence microscopy.

Results: Alpha and β tubulins were the most abundant clozapine-binding proteins. We also found that clozapine directly binds with α and β tubulin heterodimers to inhibit their polymerization to form microtubules and disturbs the microtubule network, causing mitotic arrest in HeLa cells.

Conclusion: These results suggest that α and β tubulin heterodimers are targeted by the clozapine and the microtubules are involved in the etiology of schizophrenia.

Keywords
clozapine, microtubule, olanzapine, schizophrenia, tubulin
1 | INTRODUCTION

Clozapine is the first atypical antipsychotic drug\(^1\). In general, clozapine is considered effective for treatment-resistant schizophrenia that is difficult to treat with other antipsychotics\(^2\); it is effective for both positive and negative symptoms\(^3,4\). In addition, the incidence of psychiatric adverse effects often observed with antipsychotic medications, such as extrapyramidal symptoms and hyperprolactinemia, is reduced with clozapine compared with first-generation antipsychotics\(^1\). However, clozapine often causes severe non-neural adverse effects, especially agranulocytosis, and its use requires special caution\(^5\). As an improvement over clozapine, the drug olanzapine, a derivative of clozapine, was developed to reduce the incidence of adverse effects such as agranulocytosis\(^5,6\). Nonetheless, olanzapine is thought to be a less effective antipsychotic than clozapine in treatment-resistant schizophrenia\(^2,7\). Clozapine acts on a broader range of monoamine receptors including dopamine D2 receptor and serotonin 5HT2A receptor and has a higher affinity for many monoamine receptors than for D2. Olanzapine partially overlaps with clozapine in its binding properties to monoamine receptors, but is a more potent antagonist of D2 and 5HT2A than clozapine and is prescribed at lower clinical doses. To date, the differences in the pharmacological actions of these two drugs have been explained primarily by their different effects on monoamine receptors; however, little attention has been paid to other drug-binding proteins. In this study, we aimed to determine new target proteins that contribute to the superior clinical efficacy of clozapine by identifying and comparing proteins that bind to clozapine and olanzapine.

The polyproline rod approach is a powerful technique for isolating target proteins of small molecules from biological samples\(^8,9\). In this study, we used this method to isolate clozapine-binding proteins from mouse brains. Among these proteins, we further investigated the biochemical effects of clozapine on \(\alpha\) and \(\beta\) tubulin heterodimers, which were the most abundant clozapine-binding proteins. We found that clozapine directly binds with the tubulin heterodimer, inhibits its polymerization to microtubules, and disturbs the microtubule network to cause mitotic arrest in HeLa cells.

2 | METHODS

2.1 | Reagents

All reagents were of analytical grade unless specified otherwise. Clozapine was purchased from FUJIFILM Wako Pure Chemical Corporation (038-22741, Tokyo Japan) and Toronto Research Chemicals (587500, Toronto, Canada).

2.2 | Animals

We used mice in this experiment because (i) they are mammals and have high homology of proteins with humans, (ii) the whole genome has already been analyzed and proteins can be identified from peptide sequences, and (iii) fresh brains can be prepared. The animal experimental protocol used in this study was approved by the Animal Care and Experimentation Committee of Fukushima Medical University (No. 24008) and performed in accordance with the ARRIVE guidelines and all relevant regulations. The cerebra of 7-week-old ICR mice (Kumagai-shigeyasu Co., Ltd, Sendai, Japan) were used.

2.3 | Synthesis of clozapine and olanzapine conjugates

Clozapine and olanzapine were demethylated using chloroformic acid 1-chloro-ethyl ester; elongation with ethyl 4-bromobutyrate, followed by hydrolyzation provided the corresponding carboxylic acids. Each compound was coupled with polyproline rods using a standard solid-phase protocol\(^8\). The conjugates were purified using reversed-phase high-performance liquid chromatography and characterized using mass spectrometry with the following spectral specification:

- Clozapine conjugate: calculated for C\(_{174}\)H\(_{274}\)ClN\(_{37}\)O\(_{38}\)S, required mass 3559.8; found (electrospray ionization [ESI]-MS) 3559.9.
- Olanzapine conjugate: calculated for C\(_{173}\)H\(_{275}\)N\(_{37}\)O\(_{38}\)S\(_{2}\), required mass 3545.4; found (ESI-MS) 3544.9.

2.4 | Protein extraction

Three cerebra per column were used. The cerebra were homogenized in 2.5 volumes of ice-cold phosphate-buffered saline (PBS)\([137\ mM\ sodium\ chloride (NaCl), 2.7\ mM\ potassium\ chloride\ (KCl), 1.5\ mM\ potassium\ dihydrogen\ phosphate\ (KH\(_{2}\)PO\(_{4}\)),\ and\ 8\ mM\ sodium\ hydrogen\ phosphate\ (Na\(_{2}\)HPO\(_{4}\)),\ pH\ 7.3\] and centrifuged at 10,000 \(\times\) \(g\) for 10 min at 4\(^\circ\)C. The supernatant was retrieved as the PBS-soluble fraction, and the precipitate was suspended in PBS containing 0.1% Nonidet P-40 (NP-40) to extract PBS insoluble components such as receptor proteins and gently mixed for 2 h at 4\(^\circ\)C. After centrifugation at 10,000 \(\times\) \(g\) for 10 min at 4\(^\circ\)C, the supernatant was collected as the NP-40 soluble fraction.

2.5 | Isolation of binding proteins

Clozapine and olanzapine conjugates were mixed with NeutrAvidin agarose beads (29201, Thermo, Tokyo, Japan) for 16 h and transferred into the spin column (7326008, Bio-Rad, Tokyo, Japan). Then, the 1 ml of soluble fractions was added to each column and incubated with clozapine and olanzapine conjugates on ice for 6 h, mixed every 30 min, and then allowed to flow out of the columns. The soluble fractions were similarly added to the columns six times. After that, the columns were washed with PBS twice for 3 h each on ice. Subsequently, the lysis buffer (PBS containing HRV 3C protease;
71493-3, Novagen, Tokyo, Japan) was added, followed by incubation for 16 h to cut the specific amino acid sequence (LEVLFQGP) of polyproline rod conjugates. The proteins that bound the polyproline rod conjugates were eluted from the columns and concentrated using trichloroacetic acid-acetonet precipitation, followed by separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained using silver staining (AE-1360, Atto, Tokyo, Japan). The bands of clozapine/olanzapine-binding proteins, for which no corresponding band was found in the control lane, were retrieved and sequenced.

2.6 | Peptide sequencing and Protein identification

The disulfide bonds of proteins in gel band were reduced with DTT, and all cysteine residues were alkylated with iodoacetamide. Then, the samples were in-gel digested using trypsin. The peptides in the digests were extracted with acetonitrile and dried completely. The dried samples were dissolved in the sample solution (2% acetonitrile, 97.5% water, 0.5% formic acid) and analyzed by NanoLC-ESI-MS/MS system. A high-performance liquid chromatography (HPLC) system equipped with a reversed-phase C18 column was connected online with an ion trap mass spectrometer (LCQ DECA XP PLUS, Thermo). The samples eluted from the HPLC column were directly ionized by the electrospray ionization (ESI) process and entered into the mass spectrometer. MS/MS data are acquired using the low-energy collision-induced dissociation (CID) method. The mass spectrometry data were used to search against the database using ProtQuest's ProtQuest software package (ProtTech Inc., Phoenixville, PA, USA).

2.7 | Microtubule polymerization assay

Purified porcine tubulin (HTS03-A, Cytoskeleton, CO, USA) was prepared at a concentration of 1 mg/ml in reassembly buffer [RB: 100 mM piperazine-N,N′-bis(2-ethanesulfonic acid), pH 6.8, 10 mM magnesium chloride, 1 mM ethylene glycol tetracetic acid, and 1 mM GTP] containing 20% dimethyl sulfoxide (DMSO) and was mixed with (5, 10, or 20 μM) or without clozapine and kept on ice for 5 min. Then, the mixtures were transferred into prewarmed cuvette at 35°C of a spectrophotometer (SmartSpec Plus, Bio-Rad). Microtubule polymerization was measured turbidimetrically by reading the absorbance at 350 nm.

2.8 | Cellular effects of clozapine

Human cervical epithelioid carcinoma (HeLa) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ in air. The cells were grown on glass coverslips (Matsunami, Osaka, Japan) and treated with clozapine at the indicated concentrations for 3 hours. Then, the cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with PBS containing 0.1% Triton X-100. For the observation of microtubule architecture, the cells were incubated with anti-α tubulin antibody (clone DM1A, 3873S, Cell Signaling, Danvers, MA, USA) for 1 h at room temperature (approximately 20 °C). For the staining of mitotic cells, the cells were incubated with anti-mitotic protein antibody (clone MPM-2, Ab14581, Abcam, Cambridge, England) 11. Next, the cells were treated with DyLight 488 conjugated anti-Mouse IgG (20308; Rockland, Limerick, PA, USA) and 4',6-diamidino-2-phenylindole (DAPI) as reported previously12. The cells were observed using a fluorescence microscope (BX51, Olympus, Tokyo, Japan). All images were taken under the same conditions in each experiment. For morphological comparison of clozapine-treated and nontreated cells, images were taken under a 60× objective lens (numerical aperture (N.A.) = 1.42). Immunofluorescence images of cells using MPM2 antibody (Figure 4A) were also taken with the same lens. The percentage of mitotic cells was calculated by dividing the number of mitotic cells/total cells in the camera fields. The number of mitotic cells was determined by counting the positive cells with anti-mitotic protein antibody in the image taken with a 10× (N.A. = 0.3) objective lens, and the number of total cells in the same field was determined by counting the nuclei stained with DAPI. For cell counting, ImageJ software (Version 1.53a, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) was used. Six coverslips were prepared for the control and treatment groups, respectively, and around four sites were counted for each coverslip. The average number of counted cells for each coverslip was 1416 cells. The same experiment was performed twice, with the same result obtained each time.

2.9 | Statistics

The statistical comparison of the percentages of mitotic cells in clozapine-treated and control HeLa samples was performed with the Student’s t test using Sigmaplot (Version 14, Systat Software, San Jose, CA, USA).

3 | RESULTS

3.1 | Identification of clozapine-binding proteins

To identify the novel target proteins of clozapine, we conducted a binding analysis of clozapine and proteins in mouse brain lysate using clozapine conjugates linked with polyproline rods (Figure 1A). By inserting a long, rigid polyproline helix between the drug and bio- tin, it is expected to maintain the distance between the drug-binding protein and biotin-avidin complex, preventing steric hindrance and consequently improving the affinity purification capability. The polyproline rod conjugate alone was used as the negative control, and the rod conjugate with olanzapine was also tested. The preparation of brain lysate and binding experiment were performed in both PBS and PBS with NP-40. The experiment was independently
repeated three times. A representative result of silver staining after SDS-PAGE is shown in Figure 1B. Specific SDS-PAGE bands of each conjugate were analyzed using LC-MS/MS. The identified proteins from three independent trial (Figure 1C), namely ACAT1, ACO2, ACTB, ACTG1, ALDH2, APOA1, APOE, ARF1, ATP5A1, ATP5B, ATP6V1B2, ATP6V1C1, CD81, CFL1, DPYSL2, DYNCL1L1, EC11, EEF2, ENO1, ENO2, FSCN1, GAPDH, GD11, GFAP, HSD17B10, HSP90AB1, HSPA5, HSPA8, IDH3B, MDH2, NCDN, NSF, PGK1, PPP2R1A, PSMD2, RAB3A, RAB6A, RAB6B, TPI1, TUBA1A, TUBA1B, TUBB2A, TUBB3, TUBB4B, TUBB5, and VCP, are listed.
in Table 1. These proteins were classified into cytoskeletal, membrane and vesicle trafficking, carbohydrate and lipid metabolic enzymes, F and V type ATPase, and heat shock proteins (Table 1). The major 50 kDa band of clozapine-binding proteins observed in both PBS-soluble and NP-40-soluble fractions comprised α (TUBA1A and TUBA1B) and β (TUBB2A, TUBB3, TUBB4B, and TUBB5) subunits of tubulin (Figure 1A and 1B arrowheads). Because α and β tubulin subunits form heterodimers under physiological conditions, it is considered that clozapine binds to either or both of the α and β tubulin subunits, and the α and β heterodimers were recovered together. Since tubulin was identified as the major clozapine-binding protein by using polyproline rod conjugates, subsequent experiments were conducted to investigate the interaction of unconjugated clozapine with purified tubulin heterodimers.

3.2 Clozapine inhibits tubulin polymerization

The effect of clozapine on the polymerization of tubulin was examined using purified heterodimers. Tubulin polymerization induced by DMSO was inhibited by clozapine in a concentration-dependent manner (Figure 2).

The effect of clozapine on the polymerization of tubulin was examined using purified heterodimers. Tubulin polymerization induced by DMSO was inhibited by clozapine in a concentration-dependent manner (Figure 2).

The effects of clozapine on tubulin were observed in HeLa cells, a commonly used cell line for morphological and functional studies, by examining the microtubule morphology using immunofluorescence microscopy (Figure 3). Treatment with 50 μM clozapine for 3 h reduced the integrity of the microtubule structures throughout the cytoplasm in almost all cells. In addition, the cells became round, and indentation of the nucleus was observed (arrow). The above changes were more remarkable in cells treated with 100 μM clozapine for 3 h, and membrane blebs containing tubulin were also observed (arrowheads). As anti-microtubule agents cause mitotic cell cycle arrest, we verified whether clozapine induces mitotic arrest in HeLa cells (Figure 4). The anti-mitotic protein antibody (MPM-2) strongly reacts with HeLa cells in mitosis, especially in metaphase to telophase (Figure 4A) but not in interphase, and the percentage of cells positive with the antibody were compared between clozapine-treated and nontreated group. Treatment with 50 μM clozapine for 5 h increased the number of cells in the mitotic phase 1.7-fold, and the difference was statistically significant [t (10) = 5.67, P < 0.001] (Figure 4B).

4 DISCUSSION

In this study, we aimed to determine new target proteins that contribute to the superior clinical efficacy of clozapine over olanzapine by identifying and comparing proteins that bind to clozapine and olanzapine. We identified α and β tubulin heterodimers as novel clozapine-binding proteins. In addition, we observed that clozapine inhibits tubulin polymerization and affects the microtubule network in HeLa cells.

Ten isoforms for the α subunit and nine for the β subunit of tubulin are expressed in human neural tissues14. They undergo various post-translational modifications15 and are regulated by various microtubule-binding proteins represented by traditional microtubule-associated proteins (MAPs)15 to form microtubules. As a main component of the cytoskeleton, microtubules play a central role in various cell functions16. In the nerve tissue, they are involved in the morphogenesis and maintenance of neurons and axonal transport17. Recently, it has been reported that microtubules may be involved in psychiatric disorders, including schizophrenia18. Gene set enrichment analysis using the term “microtubule regulation and disease” in the MetaCore database (Thomson Reuters) showed high enrichment for schizophrenia and schizophrenia spectrum disorders, and genes with altered mRNA expression in these disorders included α and β tubulin isoforms (eg, TUBA1A, TUBA8, and TUBB2B), MAPs (eg, MAP1A, MAP1B, MAP2, MAP6, FEZ1, DPYSL2, PCM1, and APC), and microtubule-based motor proteins (eg, KIF2A, KIF21A, NDE1, and NDEL1)19. A detailed review found that the mRNA and protein expression levels of tubulin isoforms and their post-translational modifications differ between disease and control groups for both humans and animal models.
| Protein Symbol | Common name | Clozapine binding | Olanzapine binding | Function | First trial | Second trial | Third trial |
|----------------|-------------|-------------------|-------------------|----------|-------------|--------------|-------------|
| ACAT1          | acetyl-Coenzyme A acetyltransferase 1 | ✓ | | metabolism | 23 | C-6 |
| ACO2           | aconitate 2, mitochondrial | | ✓ | metabolism | 9 | | |
| ACTB           | actin, beta | ✓ | ✓ | cytoskeleton | 17, 23 | O-6, O-7, C-3, C-6 |
| ACTG1          | actin, gamma, cytoplasmic 1. | ✓ | | cytoskeleton | 1, 2 | | |
| ALDH2          | aldehyde dehydrogenase 2, mitochondrial, | | ✓ | metabolism | 1 | 1 | |
| APOA1          | Apolipoprotein A1 | ✓ | | metabolism | 5 | 5 | |
| APOE           | apolipoprotein E | ✓ | | metabolism | 3 | 3 | |
| ARF1           | ADP-ribosylation factor 1 | ✓ | | membrane trafficking | | C-10 | |
| ATP5A1         | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, | ✓ | | ATP synthesis | 1 | 1, 2 | C-5 |
| ATP5B          | ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit | ✓ | | ATP synthesis | 1, 2 | | C-5 |
| ATP6V1B2       | ATPase, H+ transporting, lysosomal V1 subunit B2, | ✓ | | ATP synthesis | C-3, C-5 | | |
| ATP6V1C1       | ATPase, H+ transporting, lysosomal V1 subunit C1 | ✓ | | ATP synthesis | C-6 | | |
| CD81           | CD81 antigen, Tapa1, Tapa-1, Tspan28 | ✓ | | cell-cell recognition | 6 | | |
| CFL1           | cofiïn 1, non-muscle, | ✓ | | cytoskeleton | | C-10 | |
| DYNCL1L1       | dynein cytoplasmic 1 light intermediate chain 1, | ✓ | | cytoskeleton | 15 | C-4 | |
| ECI1           | enoyl-Coenzyme A delta isomerase 1, | ✓ | | metabolism | 5 | 5 | |
| EEF2           | eukaryotic translation elongation factor 2, | ✓ | | protein synthesis | | C-2 | |
| ENO1           | enolase 1, alpha non-neuron | ✓ | | metabolism | 1 | | |
| ENO2           | enolase 2, gamma neuronal, | ✓ | | metabolism | 1 | C-5 | |
| FSCN1          | fascin actin-bundling protein 1,fascin-1 | ✓ | | cytoskeleton | 1 | | |
| GAPDH          | glyceraldehyde-3-phosphate dehydrogenase | ✓ | | metabolism | 4 | 3, 4, 9, 20 | C-7, C-8 |
| GDI1           | guanosine diphosphate (GDP) dissociation inhibitor 1 | ✓ | ✓ | membrane trafficking | | O-5 | |
| GFAP           | glial fibrillary acidic protein, | ✓ | | cytoskeleton | 1, 3 | | |
| HSD17B10       | hydroxysteroid (17-beta) dehydrogenase 10, Ads9, ERAB, | ✓ | | metabolism | | C-7, C-8 | |
| HSP90AB1       | heat shock protein 90 alpha (cytosolic), class B member 1 | ✓ | | molecular chaperone | | C-2 | |
| HSPA5          | heat shock protein 5, 78kDa, Hsce70, | ✓ | | molecular chaperone | | | 11 |
| Protein Symbol | Common name | Clozapine binding | Olanzapine binding | Function | First trial | Second trial | Third trial |
|----------------|-------------|-------------------|--------------------|----------|-------------|--------------|------------|
| HSPA8          | heat shock protein 8, Hsc70, | ✓ | ✓ | molecular chaperone | 11 | R-5, O-1, O-2, C-6 |
| IDH3B          | isocitrate dehydrogenase 3 (NAD+ beta) | ✓ | | metabolism | 23 |           |
| MDH2           | malate dehydrogenase 2, NAD (mitochondrial), | ✓ | | metabolism | 3 |           |
| NCDN           | neurochondrin, norbin | ✓ | | signal transduction | C-2, C-3 |           |
| NSF            | N-ethylmaleimide sensitive fusion protein, SKD2 | ✓ | | membrane trafficking | 13 | C-3 |
| PGK1           | phosphoglycerate kinase 1 | ✓ | | metabolism | O-6 |           |
| PPP2R1A        | protein phosphatase 2, regulatory subunit A, alpha, | ✓ | | protein phosphatase | C-4 |           |
| PSMD2          | proteasome (prosome, macropain) 26S subunit, non-ATPase, 2, | ✓ | | protein degradation | C-2 |           |
| RAB3A          | RAB3A | ✓ | | membrane trafficking | C-8 |           |
| RAB6A          | RAB6A | ✓ | | membrane trafficking | 6 |           |
| RAB6B          | RAB6B | ✓ | | membrane trafficking | 6 |           |
| TPI1           | triosephosphate isomerase 1 | ✓ | | metabolism | C-8 |           |
| TUBA1A         | tubulin, alpha 1A, | ✓ | | cytoskeleton | 1, 2, 3, 4, 6 | 15, 20 | R-5, C-2 |
| TUBA1B         | tubulin, alpha 1B, | ✓ | ✓ | cytoskeleton | 1, 2, 3, 8, 9, 11, 13, 17, 23 | O-5, C-3, C-4, C-6, C-7, C-10 |
| TUBB2A         | tubulin, beta 2A | ✓ | | cytoskeleton | 1, 2 |           |
| TUBB3          | tubulin, beta 3 | ✓ | | cytoskeleton | 4 | C-6 |
| TUBB4B         | tubulin, beta 4B | ✓ | | cytoskeleton | 3 | 1, 2, 3, 20 | C-2, C-5 |
| TUBB5          | tubulin, beta 5 | ✓ | ✓ | cytoskeleton | O-5, C-3, C-7 |           |
| VCP            | valosin-containing protein | ✓ | | apoptosis | C-2 |           |
of schizophrenia. In addition, the qualitative and quantitative changes of microtubule-binding proteins, such as MAP2, DPYS2L2 (also called CRMP2), and DISC1, in schizophrenia and related psychiatric diseases have been described. A comparison of microtubules in neuronal cells in patients with schizophrenia and control subjects was performed using olfactory neuronal precursors, and alterations in microtubules were observed. Neuronal precursors derived from patients with schizophrenia not treated with antipsychotics have a higher ratio of polymerized tubulin than controls. In contrast, the cells from patients treated with antipsychotics have a ratio of polymerized tubulin similar to that of the controls. This finding is supported by another study in which the microtubule network in cultured olfactory epithelial-derived cells was more stable in the presence of the microtubule-destabilizing agent nocodazole in patients with schizophrenia than in healthy controls. However, in the above study, all patients with schizophrenia received antipsychotics. From these findings, it is assumed that microtubules are over-stabilized in the neuronal cells of patients with schizophrenia and that antipsychotic drugs partially re-establish these over-stabilized microtubules. Microtubules are highly dynamic structures, particularly in neuronal growth cones and dendritic spines. Therefore, the dynamic properties (i.e., “dynamic instability”) of microtubules are considered important for neural plasticity. We propose that clozapine acts on over-stabilized microtubules in the brains of patients with schizophrenia to recover the “dynamic instability” of microtubules. If this is the case, it may be possible to improve the therapeutic effects of pharmacological treatment of schizophrenia using microtubule-destabilizing agents in addition to antipsychotics. Currently, davunetide, a microtubule stabilizer, is in clinical trials for the treatment of schizophrenia; however, no microtubule destabilizer has been investigated to date. Microtubule destabilizers, such as colchicine and nocodazole, are commonly used in the treatment of cancer and gout and expertise on their safe usage has accumulated; thus, they can now be repositioned for the treatment of schizophrenia.

The finding that clozapine induces mitotic arrest in HeLa cells suggests the possible involvement of microtubule impairment in the pathogenic mechanism of clozapine-induced agranulocytosis. Clozapine has been reported to arrest the cell cycle at G1/S transition in cultured cells by acting as an agonist of histamine H4 receptors. The high expression of histamine receptors in granulocytes supports the hypersensitivity of granulocytes. Therefore, agranulocytosis caused by clozapine may induce both histamine receptor-mediated cell cycle arrest in the G1/S phase and microtubule-targeted mitotic arrest. The effects of clozapine on microtubules may partially contribute to the development of agranulocytosis.

In the present study, the concentration of clozapine required to inhibit the polymerization of tubulin heterodimers in vitro was ≥5 μM and that for the depolymerization of microtubules in HeLa cells was ≥50 μM. However, the target blood concentration of the drug for treatment is approximately 1–1.7 μM and may occasionally exceed 5 μM in clinical practice. These clinical and experimental concentrations are relatively close; nonetheless, the experimental requirements, especially for affecting intracellular microtubule structure, are still higher. Therefore, it is necessary to consider the accumulation of clozapine in the target tissue and the activity of clozapine.
metabolites, such as clozapine N-oxide\textsuperscript{26}, to explain the clinical significance of the effect of clozapine on tubulin.

The clozapine-binding proteins identified in this study include proteins that have been reported to interact with microtubules or tubulin. Therefore, it is necessary to verify whether they are genuine clozapine-binding proteins or proteins retrieved together with tubulin. Among these microtubule-binding proteins, DPYSL2 has recently been noted for its potential involvement in schizophrenia\textsuperscript{27,28}, which needs to be investigated in detail.

There are certain limitations to this study. First, because clozapine and olanzapine conjugates were used instead of the drug itself in the binding experiment for screening, it is possible that some of the original clozapine-binding proteins were not recovered. Second, as a long washing process (6 h) was conducted to isolate clozapine-binding proteins, the proteins with fast dissociation rate constants ($k_{\text{diss}}$) may have been lost during this process. For these two reasons, the known clozapine-binding proteins, especially monoamine receptors, may not have been recovered as clozapine-binding proteins. Therefore, we cannot confirm that clozapine-binding proteins were comprehensively recovered in this study.

In summary, clozapine-binding proteins were investigated in the mouse brain by using the polyproline rod method. In total, 44 proteins were identified as clozapine-binding proteins, and the most abundant were $\alpha$ and $\beta$ tubulins. Subsequent biochemical analysis confirmed that clozapine directly binds with tubulin heterodimers and inhibits their polymerization. Moreover, clozapine inhibited tubulin polymerization in HeLa cells. Further research on microtubules as therapeutic targets for schizophrenia may lead to the development of new treatment options.

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CONFLICT OF INTEREST
All authors declare that they did not receive financial or other support from organizations that may be interested in the work submitted. There are no other relationships or activities that may have influenced the submitted work.

AUTHOR CONTRIBUTIONS
M.H., Y.K., J.M., A.W., S.N., M.S., and H.Y. designed the study. M.H. and T.K. performed the experiments. M.H. wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the supplementary material of this article (Data S1).

APPROVAL OF THE RESEARCH PROTOCOL BY AN INSTITUTIONAL REVIEWER BOARD
Not applicable.

INFORMED CONSENT
Not applicable.

REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL
Not applicable.

ANIMAL STUDIES
The animal experimental protocol used in this study was approved by the Animal Care and Experimentation Committee of Fukushima Medical University (No. 24008) and performed in accordance with the ARRIVE guidelines and all relevant regulations.
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ADDITIONAL SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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