

FK960 binding proteins, quinone oxidoreductase2 and pyridoxal kinase were identified by LC-MS/MS for drug discovery of Alzheimer's treatment

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Abstract

We established new LC-MS/MS methodology of the target proteome of unknown proteins in cytoplasm of hippocampus for Drug Discovery of Alzheimer's Treatment. Quinone oxidoreductase2 (QR2) of the 25kDa protein and Pyridoxal kinase (PK) of the 37kDa protein are the target proteins of Alzheimer's Disease. FK960 binding to QR2 and PK inhibits respectively over-expression of QR2 disturbing memory formation at the cortex and over-phosphorylation of the microtubule-associated Tau protein accumulated at neurofibrillary tangles in the brains of Alzheimer's disease patients.

Introduction

FK960, [N-(4-acetyl-1-piperazinyl)-p-fluorobenzamide monohydrate], a novel antimentia piperazine derivative, has been shown to reverse scopolamine-induced cognitive deficits in rats in vivo [1], to increase the magnitude of long-term potentiation in guinea pig hippocampus in vitro [2], and improve visual recognition memory in primates [3]. FK960 (100 nM) significantly increased the amplitude of the Excitatory postsynaptic potentials [4]. These studies implicated somatostatinergic, cholinergic, and serotonergic systems. We did not know what kinds of proteins FK960 bind to and act pharmacological effect. For new drug discovery, it is important how to search a small molecule such as FK960 or the small molecule ligands of nuclear receptor (NR) binding promoter DNAs to control transcription of pharmaceutical active proteins such as NF-AT, NF-kB, HDAC, IL-1, TNF and peroxisome [5].

We established new LC-MS/MS methodology of the target proteome of unknown proteins in cytoplasm of hippocampus for Drug Discovery of Alzheimer's Treatment. Quinone oxidoreductase2 (NAD(P)H menadione oxido reductase) of the 25kDa protein and Pyridoxal kinase of the 37kDa protein are the target proteins of Alzheimer's Disease.

Method

Synthesis of FK960-immobilized Sepharose 4B

FK960-immobilized Sepharose 4B was synthesized as shown in Figure 1.

Isolation of FK960 binding proteins in cytoplasm of hippocampus

FK960 binding proteins in cytoplasm of hippocampus was collected from 20 mail Sprague Dawley rats fasted using FK960-immobilized Sepharose 4B incubating with or without FK960 in buffer (50mM

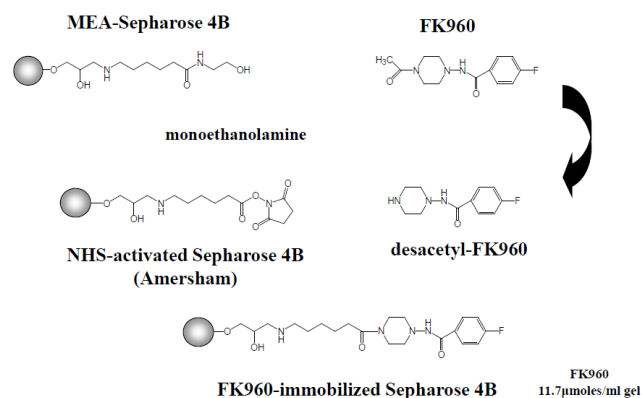


Figure 1. Synthesis of FK960-immobilized Sepharose 4B

Tris-HCl (pH7.6), 20% glycerol, 0.3M NaCl) and then SDS page electrophoresis. FK960 binding proteins bind FK960 more than FK960-immobilized Sepharose 4B and so FK960 specific binding proteins bind with FK960-immobilized Sepharose 4B in the case of incubating without FK960, but in the case of incubating with FK960, FK960 specific

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binding proteins do not bind with FK960-immobilized Sepharose 4B as shown the methodology isolating FK960 binding proteins in Figure 2.

LC-MS/MS analysis of FK960 binding proteins after in gel digestion of SDS page spots

FK960 binding protein bound with FK960-immobilized Sepharose 4B was eluted with FK960 after washing out another protein. The elute, the washout solution and the incubating solution with FK960 were compared the containing proteins using SDS page electrophoresis in both MES buffer and MOPS buffer in Figure 3 and Silver stain (in NuPAGE 4-12% Bis-Tris Gel MOPS buffer) and CBB stain in Figure 4. In the SDS page, the – marked bands contained 37kDa protein and 25kDa protein, but the + marked band did not contain them. 37kDa protein and 25kDa protein stained bands were cut off from the gel, and proteins were reduced by dithiothreitol, alkylated by iodoacetamide (SIGMA, St Louis, MO) and digested by Trypsin that had been modified for peptide sequencing (Roche, Mannheim, Germany). We carried out this in-gel digestion according to a previous report. (The total amount of precipitate was dissolved in 4mL of 6mol/L guanidine, 0.1mol/L tris and 1mmol/L EDTA buffer (pH8.3). After 300μmol DTT treatment to reduce S-S bond, 500μmol iodoacetic acid treatment to protect the produced SH group by carboxymethylation and a micro dialysis with a 6 kDa MW cut-off membrane, trypsin (2% w/w) was added to the solution and incubated at 37°C for 4 hours and lyophilized.

LC-MS/MS analysis of 37kDa protein and 25kDa protein were conducted after in gel digestion of the spot in SDS-page electrophoresis using nanoLC MAGIC 2002 (Magic C18 column (0.75mm x150mm, 3.5μm), Mobile phase ((A) 0.1%(v/v) AcOH in Water, (B) 0.1%(v/v) AcOH in acetonitrile, Gradient (0-5min (B;5%), 5-45min (B;5-45%), 45-55min (B;45-90%), 55-90min (B;90%),90-91min (B;90-5%),91-95 min (B;5%)), Flow rate (50 nL/min)) and ThermoFinnigan ion trap LCQ Deca MS under MS condition of analysis (Sheath gas (75), Auxiliary gas (15), Capillary temperature (300°C), Scan mode (LC/ESI/MS/MS (positive mode, data dependent scan)).

Result

The high sensitive nanoLC-MS/MS measurement was conducted after in-gel digestion of both spots of 37kDa protein and 25kDa protein of SDS page electrophoresis. The following unknown 37kDa protein and 25kDa protein were identified by Bioworks Sequest (Thermo Electron) having high protein coverage, many repeat number of appearance, high quality of mass spectrum and high total score, and using rat and nr fasta data base. Mass Chromatogram (top), mass spectrum (middle) and MS/MS spectrum of 25kDa protein in SDS page electrophoresis are

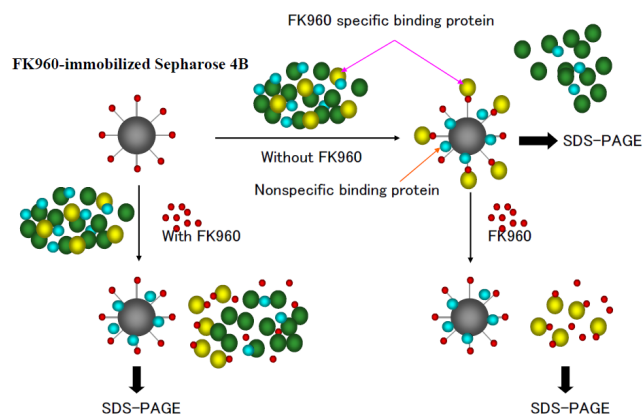


Figure 2. The methodology isolating FK960 binding proteins

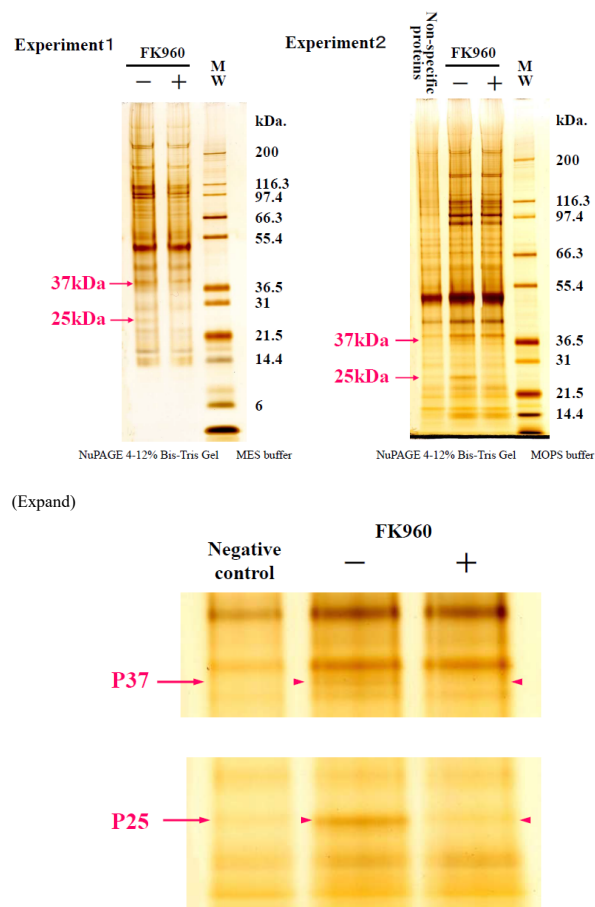


Figure 3. SDS page electrophoresis 1 isolating FK960 binding proteins + and -marks mean incubation with or without FK960

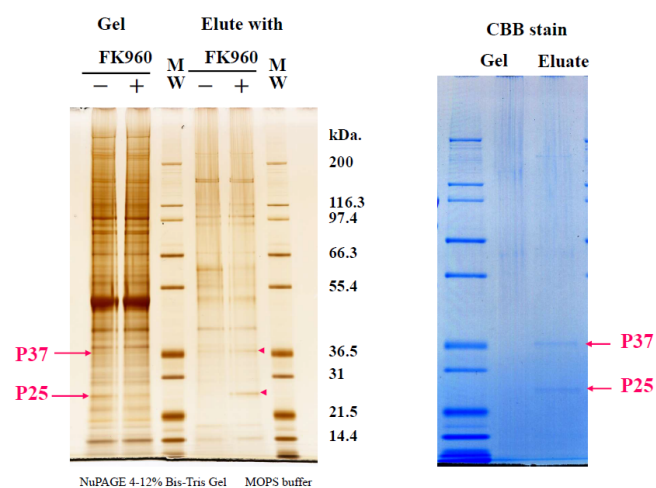
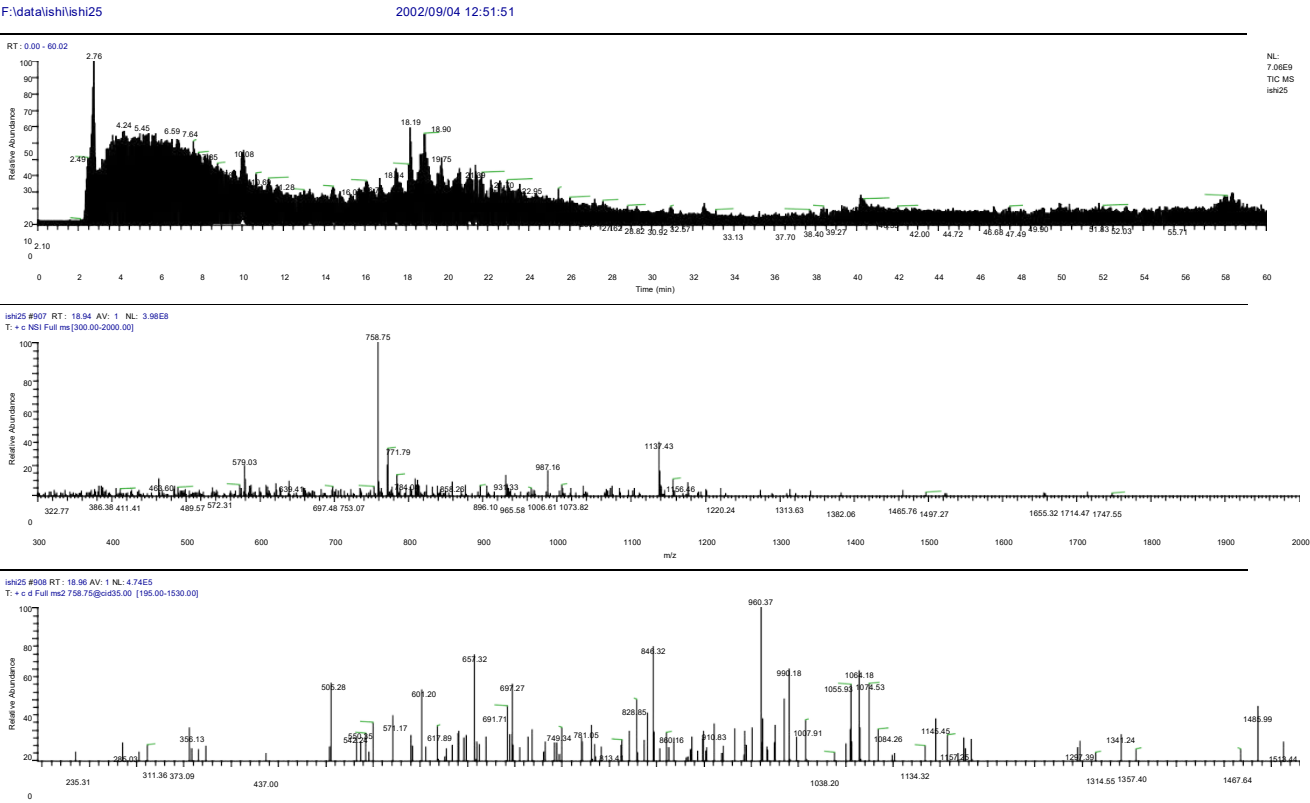


Figure 4. SDS page electrophoresis 2 isolating FK960 binding proteins + and -marks mean incubation with or without FK960

shown in Figure 5. Mass Chromatogram (top), mass spectrum (middle) and MS/MS spectrum of 37kDa protein in SDS page electrophoresis are shown in Figure 6.

The 25kDa protein was assigned Quinone oxidoreductase2 (NAD(P)H menadione oxidoreductase) that had never identified in rat



and so used nr fasta database having protein coverage $52/231=22.5\%$ by amino acid count, many repeat number of appearance, high total score in Figure 7 and high quality of MS/MS spectrum in Figure 8.

The 37kDa protein was assigned Pyridoxal kinase having monoisotopic mass = 34867, Protein Coverage: $108/312 = 34.6\%$ by amino acid count, $11857/34867 = 34.0\%$ by mass, many repeat number

of appearance, high total score in Figure 9 and high quality of MS/MS spectrum in Figure 10.

Discussion

We believe a new mechanism by FK960 binding 25kDa protein assigned Quinone oxido reductase2 (QR2) and FK960 binding 37kDa

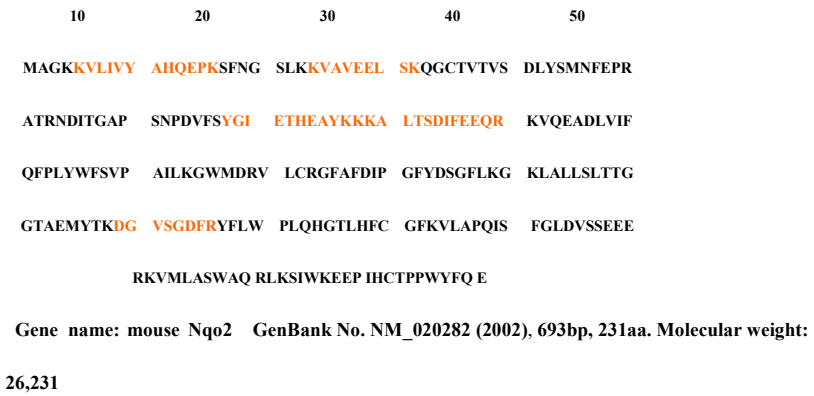


Figure 7. Quinone oxidoreductase2 of the 25kDa protein assigned by Bioworks Sequest soft using nr fasta database

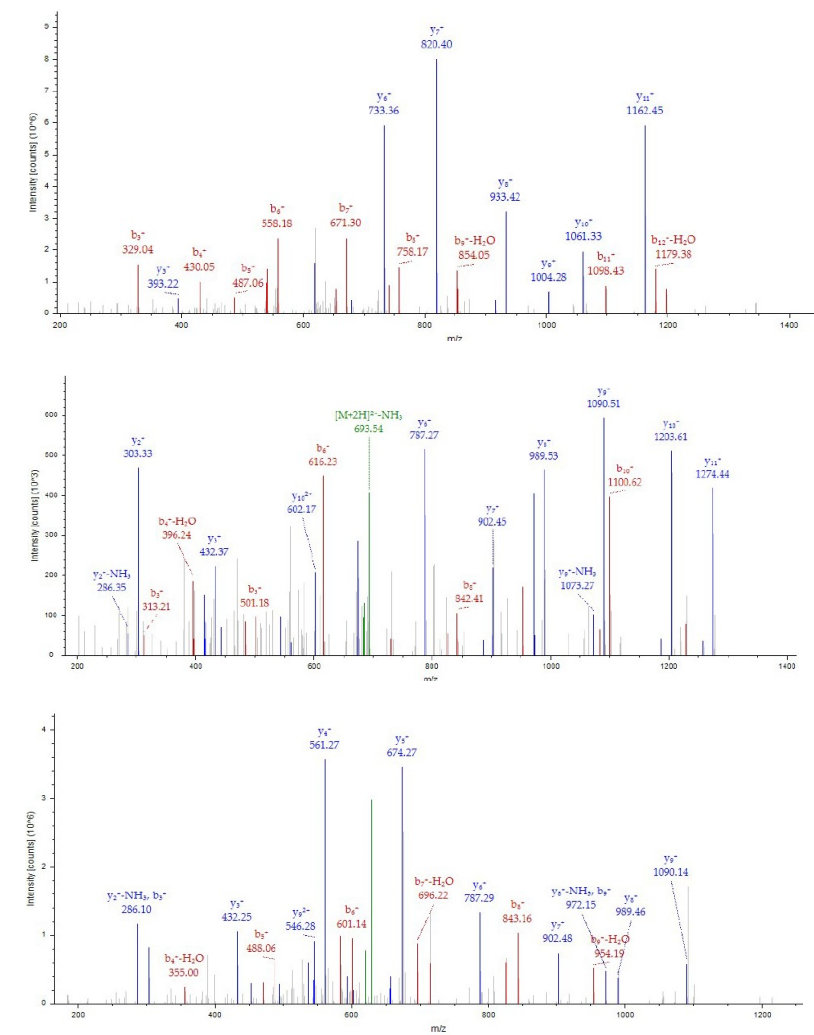


Figure 8. MS/MS spectrum of Quinone oxidoreductase2 of the 25kDa protein assigned by Bioworks Sequest soft using nr fasta database

1020304050

MEGECRVLSIQSHVVRGYVGNRAAMFPLQV LGFEVDAVNS VQFSNHTGYA

HWKGQVLTSQ ELHALYEGLK ANNVNKYDYV LTGYTRDKSF LGMVVDIVQE

LKQQNSRLVY VCDPVMGDKW NGECSMYVPQ DLLPVYREKV VPMADIITFN

QFEAELLSGR KIHSEQEEAFA VMDVLHRMGF DTVVITSSDL PSPKGSDYLM

ALGSQRMKRP DGSTVTQRIK MEMRKVDPVF VGTGDLFAAM LLAWTHKHPD

NLKVACEKTV SAMQHVLRQT IRCAKAEAGE GQKPSPAQLE LRMVQSRKDI

EDPEIVVQAT VL

Gene name: rat Pdxk

GenBank No. AF020346 (1997)

936bp, 312aa.

Molecular weight: 34,885

Figure 9. Pyridoxal kinase of the 37kDa protein assigned by Bioworks Sequest soft using nr fasta database

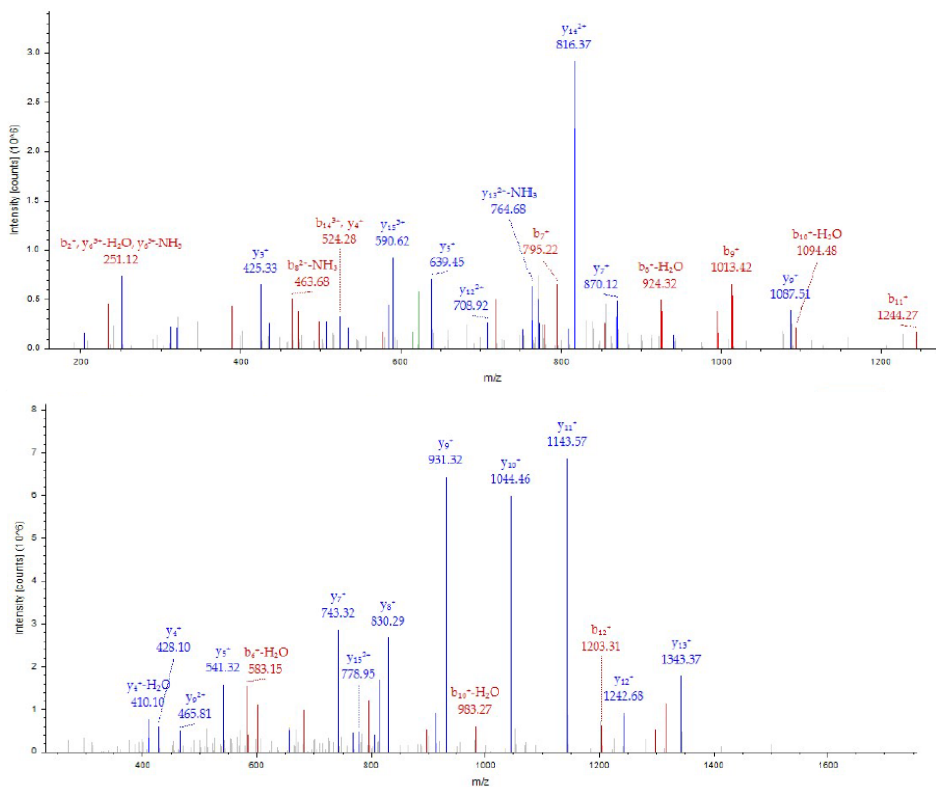


Figure 10. MS/MS spectrum of Pyridoxal kinase of the 37kDa protein assigned by Bioworks Sequest soft using nr fasta database

protein assigned Pyridoxal kinase (PK) to inhibit respectively over-expression of QR2 disturbing memory formation at the cortex [6] and over-phosphorylation of the microtubule-associated Tau [7,8] protein accumulated at neurofibrillary tangles in the brains of Alzheimer's disease patients as described by many scientists [9-12].

We also believe this methodology is useful to find small molecule ligands of nuclear receptor (NR) binding promoter DNAs to control transcription of pharmaceutical active proteins such as NF-AT, NF-kB, HDAC, IL-1, TNF and peroxisome.

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Conflicts of interest

The authors declare no conflict of interest.

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