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RIFAMPICIN PREVENTS THE AGGREGATION AND NEUROTOXICITY OF AMYLOID β PROTEIN *IN VITRO*

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SUMMARY: The aggregation and cerebral deposition of amyloid β protein (Aβ), which is a major component of senile plaques in Alzheimer's disease (AD) brains, is believed to be involved in the pathogenesis of AD. Inhibition of Aβ aggregation would seem to be a promising strategy for the treatment of AD. Here, we show that rifampicin, which is an antibiotic widely used in the treatment of tuberculosis and leprosy, inhibited the aggregation and fibril formation of synthetic Aβ1-40 peptide in a dose-dependent manner at reasonable concentrations. Furthermore, rifampicin was found to prevent Aβ1-40-induced neurotoxicity on rat pheochromocytoma PC12 cells. Rifampicin may have therapeutic potential as an agent for inhibiting the initial step of amyloid formation in AD.

Amyloid β protein (Aβ) is a 39- to 43-amino acid peptide that is a major component of cerebral amyloid deposits in Alzheimer's disease (AD). Several studies using synthetic peptides have shown that Aβ has a tendency for self-aggregation (1,2) and that aggregated Aβ is toxic to neuronal cells (3,4). Since Aβ was found to be physiologically produced as a soluble form in normal individuals as well as in AD patients (5,6), the aggregation of soluble Aβ into insoluble amyloid fibrils is believed to be a crucial step in the pathogenesis of AD. Therefore, prevention of this process would seem to be a promising strategy for the treatment of this disease.

In the screening for effective drugs against Aβ aggregation, we noticed a report that non-demented elderly leprosy patients showed an unusual absence of senile plaques in their brains compared to age-matched controls (7). Although the finding itself is still a matter of controversy (8), this report hinted us that some drug being used for leprosy might prevent Aβ aggregation, resulting in the absence of senile plaques. To investigate the validity of this idea, we focused on two well-known anti-leprosy drugs, dapsone (diaminodiphenylsulfone, DDS) and rifampicin. These two drugs and the related agents were tested for inhibitory actions against the aggregation of synthetic Aβ1-40 peptide by the thioflavin T (ThT) binding assay and electron microscopy. Here, we show that

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rifampicin and its derivatives prevented the aggregation and fibril formation of Aβ. In addition, it is shown that rifampicin protected neuronal cells from Aβ-induced cytotoxicity.

MATERIALS AND METHODS

Aggregation studies: The test drugs were obtained from Sigma, but rifamycin S was prepared in our laboratory by oxidation of rifamycin SV with MnO_2 in CH_2Cl_2 at room temperature. The structure of rifamycin S was confirmed by ^{13}C-NMR (9). The aggregation of Aβ was measured by the ThT binding assay, in which the fluorescence intensity reflects the degree of aggregation. ThT characteristically stains amyloid-like deposits (10) and exhibits enhanced fluorescence emission at 482 nm and a new excitation peak at 450 nm when added to a suspension of aggregated β-sheeted protein (11). Although rifampicin and its derivatives show absorbance at 482 nm, significant interference with the fluorescence of ThT bound to aggregated Aβ was not observed under the conditions we used (data not shown). None of the tested drugs showed fluorescence at 482 nm (data not shown). All test drugs were dissolved in DMSO (Wako Pure Chemical Industries, Ltd.; Japan) to a concentration of 10 mg/ml. Rifampicin was further diluted with DMSO to 1 mg/ml and 100 μg/ml for a dose-response experiment. The Aβ1-40 peptide (Bachem Feinchemikalien AG; Switzerland) was dissolved in double deionized water and centrifuged at 10,000 × g for 5 min to remove insoluble matter. The supernatant was filtered through a Millex-GV 0.22-μm filter (Millipore), and the peptide concentration of the filtered solution was determined by absorbance at 215-225 nm (12). The peptide solution was diluted to a concentration of 200 μg/ml and dispensed into Eppendorf tubes (100 μl/tube). Two μl of each test drug was added to tubes in triplicate. Then the peptide solutions were mixed with an equal volume of 0.2 M sodium phosphate buffer (pH 7.4), so that both the final peptide concentration and the final drug concentration were 100 μg/ml. The peptide solutions containing each test drug were incubated at room temperature. At various times of incubation, the peptide aggregation was measured by the ThT binding assay, as described previously (11). Briefly, 10 μl of the peptide solution was added to 1 ml of 3 μM ThT (Aldrich) in 50 mM sodium phosphate buffer (pH 6.0) and mixed by vortexing. The fluorescence of the mixture was immediately measured at λex = 450 nm, 5 nm bandpass; λem = 482 nm, 10 nm bandpass, using a JASCO FP-770 spectrofluorometer (JASCO; Japan).

Electron microscopy: For electron microscopic observation, the peptide solutions containing 1% DMSO, 100 μg/ml rifampicin or 100 μg/ml dapsone were removed from each tube. The aggregated peptides in the solutions were adsorbed onto 200-mesh Formvar-coated copper grids and negative-stained with 2% uranyl acetate. The specimens were viewed for fibrils with a JEM-100CX electron microscope (JEOL; Japan) at 80 kV.

Cytotoxicity assays: Cytotoxic effect of Aβ was assessed by measuring cellular redox activity with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).The PC12 cells were cultured in RPMI 1640 containing 10% horse serum and 5% semi-fetal calf serum. The cells were seeded into 96-well culture plates at a density of 5,000 cells/200 μl/well. On the next day, the culture medium was changed with fresh medium without D-glucose (Gibco) but containing mouse 2.5S-NGF (Sigma) at a concentration of 50 ng/ml, and the cells were differentiated into neuronal cells during culture for 6 days. On day 3 of the NGF-treatment, the medium was changed with fresh medium containing NGF. The Aβ1-40 peptide for this assay was synthesized, purified and lyophilized in our laboratory as described previously (13). The peptide solution was prepared at a concentration of 50 μg/ml in 0.1 M sodium phosphate buffer (pH 7.4) as described above, and preincubated at 37 °C for 7 days in the presence or absence of rifampicin at a drug concentration of 10 μg/ml. Then the peptide solution was diluted with PBS to concentrations of 10, 1 and 0.1 μg/ml. After the NGF-treatment of the cells, the medium was changed again, and 20 μl of the diluted peptide solutions was added to

the cell culture to a final concentration of 1, 0.1 or 0.01 μg/ml. After 2-day incubation, the MTT assay was performed as described previously (14).

RESULTS AND DISCUSSION

As shown in Fig. 1, rifampicin was found to prevent Aβ1-40 aggregation in a dose-dependent manner. Rifampicin completely inhibited peptide aggregation at a concentration of 100 μg/ml, while dapsone, another anti-leprosy drug, failed to prevent peptide aggregation even at 100 μg/ml. At a drug concentration of 10 μg/ml, at which the molar ratio of rifampicin (M.W. 823) versus Aβ1-40 peptide (M.W. 4,330) is about 1 : 2, rifampicin was effective until day 3, but the inhibitory action disappeared by day 6. These results were confirmed by electron microscopic observation (Fig. 2). The peptide solution containing 1% dimethyl sulfoxide (DMSO), which was used as the solvent for the test drugs, showed apparent amyloid-like fibrils. On the other hand, very few fibrils were observed in the peptide solution containing 100 μg/ml rifampicin. Dapsone at 100 μg/ml failed to prevent the fibril formation of Aβ1-40, although the diameters of the fibrils were slightly smaller than the control.

It is, at least *in vitro*, being established that one of the biological actions of Aβ is neurotoxicity and is related to its aggregation state (3,4). Thus, we examined the effect of

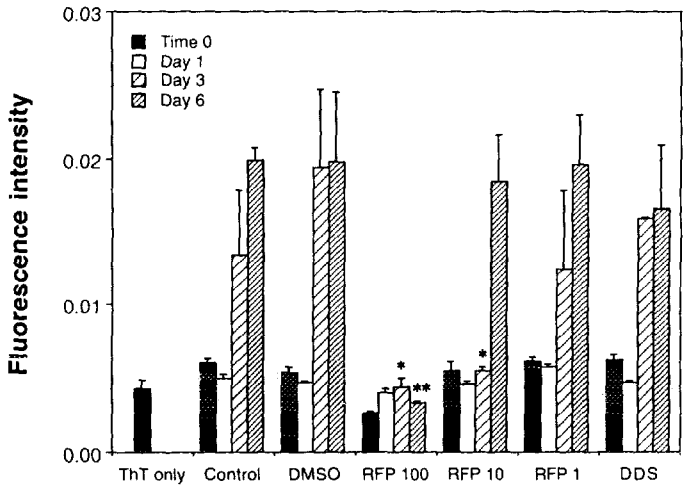

Fig. 1. Effects of rifampicin and dapsone on the aggregation of synthetic Aβ1-40 peptide. Both the peptide control without any test drug (Control) and the peptide solution containing 1% dimethyl sulfoxide (DMSO) showed similar results, starting to aggregate on day 3. Rifampicin at a concentration of 100 μg/ml (RFP 100) completely inhibited the peptide aggregation. At 10 μg/ml (RFP 10), rifampicin was effective until day 3, but the peptide had aggregated to the same degree as the control on day 6. The peptide solution containing 1 μg/ml of rifampicin (RFP 1) showed no significant difference in aggregation from the control. Dapsone at 100 μg/ml (DDS) had no effect. Values represent the mean ± S.D., n = 3. * $p < 0.05$, ** $p < 0.01$ compared to the DMSO control of the same day by Student's t-test.

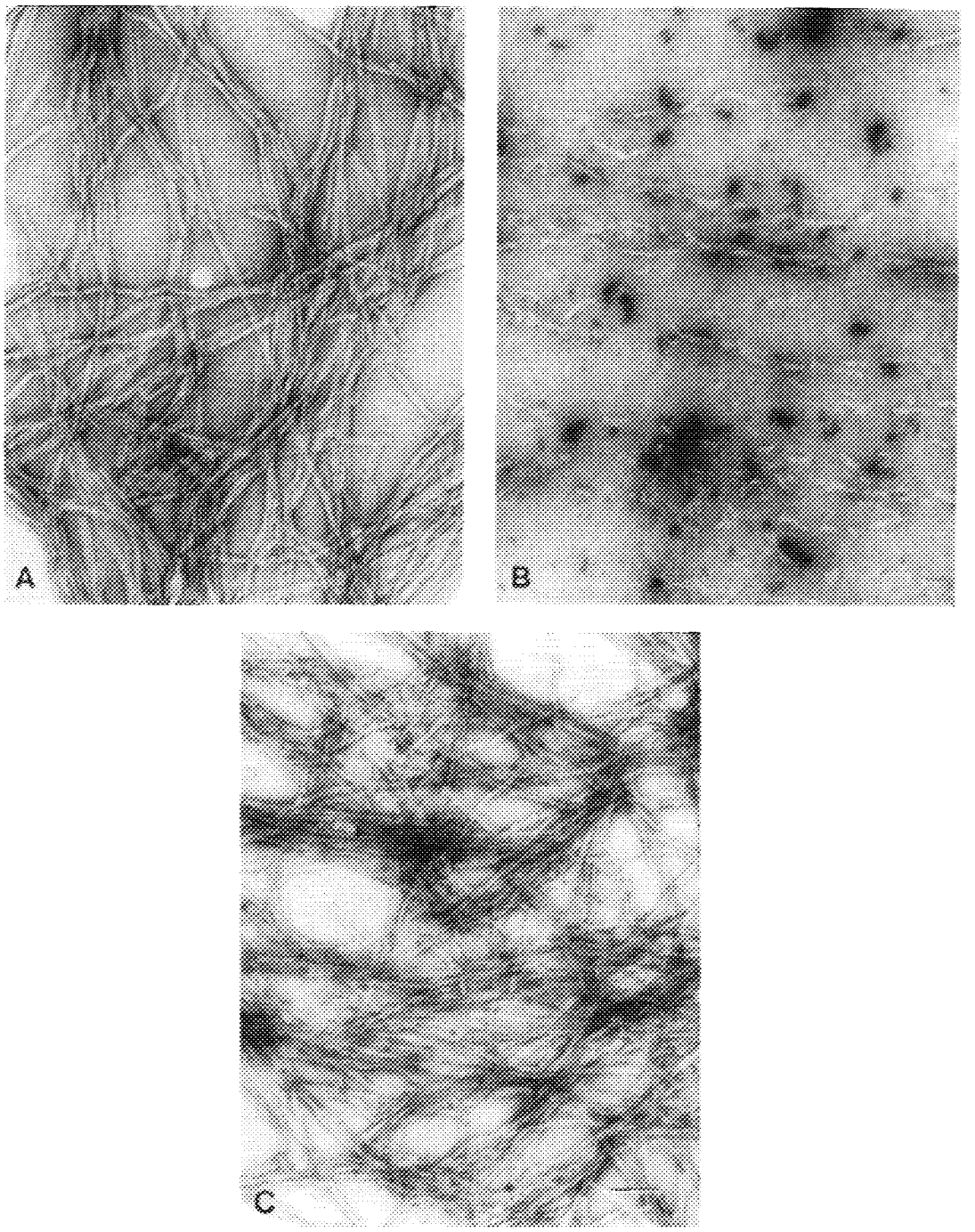

Fig. 2. Effects of rifampicin and dapsone on the fibril formation of synthetic Aβ1-40 peptide. Electron micrographs of Aβ1-40 peptide with test drugs were taken on day 8. The peptide solution containing 1% DMSO showed apparent fibrils of Aβ1-40 (A). Rifampicin at 100 μg/ml prevented the fibril formation (B), whereas dapsone at 100 μg/ml did not (C). The scale bar is 0.1 μm.

rifampicin on the toxicity of Aβ1-40 using cultured rat pheochromocytoma PC12 cells. Freshly prepared Aβ1-40 solution did not show toxicity while aged Aβ solution clearly showed toxicity at concentrations above 0.1 μg/ml (Fig. 3). This Aβ-induced toxic effect was significantly reduced when the peptide was aged in the presence of rifampicin. Since rifampicin alone did not show any toxic or trophic effect on PC12 cells up to 10 μg/ml under the conditions we used (data not shown), these results collectively lead us to a conclusion that the neurotoxicity of Aβ could be prevented by rifamycins probably by inhibition of the peptide aggregation.

The molecular mechanism of the inhibitory action by rifampicin against Aβ aggregation remains to be studied. Recently, a new model for Aβ aggregation and neurotoxicity has been proposed (15,16). According to the papers, Aβ1-40 spontaneously fragments into free radical peptides in aqueous solution and these free radicals could react with one another to generate covalently bonded aggregates and could also attack nerve cell membranes. In support of this hypothesis, several antioxidants have been shown to protect cells from Aβ toxicity, suggesting that at least one pathway to Aβ cytotoxicity results in free radical damage (17,18). Chemical structure of rifampicin possesses a naphthohydroquinone ring which is obviously involved in playing a free radical scavenger role. To confirm this idea, we examined the effect of rifampicin analogues such as rifamycin SV, rifamycin B and rifamycin S (Fig. 4) on Aβ1–40 aggregation. At

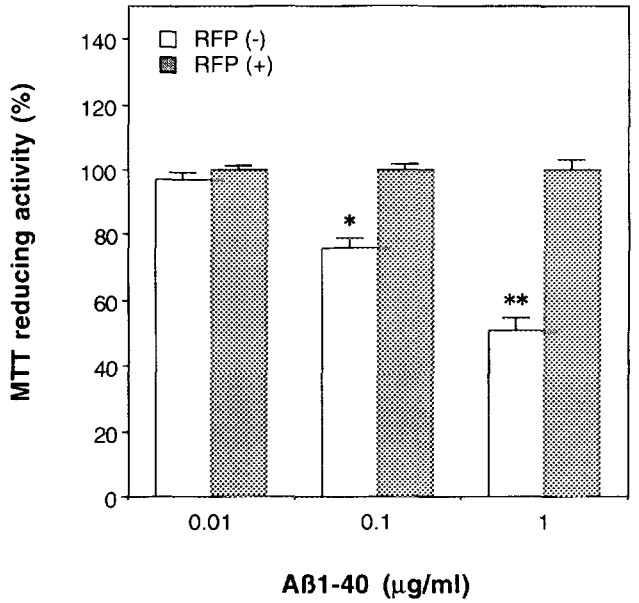

Fig. 3. Effect of rifampicin on the neurotoxicity induced by Aβ1-40 peptide on PC12 cells. Aged Aβ1-40 caused cell damage in a dose-dependent manner. Rifampicin prevented Aβ1-40-induced neurotoxicity when added to the peptide solution before aging of peptide. Values represent the mean ± S.D., n = 3. * $p < 0.01$, ** $p < 0.001$ compared to the control without Aβ1-40 peptide by Student's t-test.

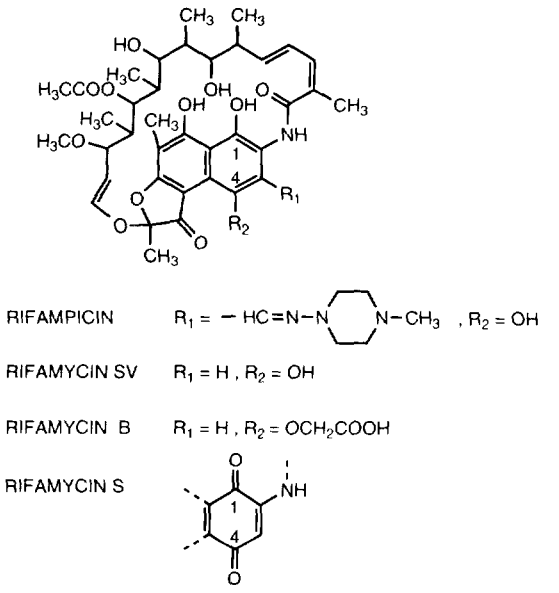

Fig. 4. Structures of rifamycins.

the concentration of 100 μg/ml of drugs, their inhibitory actions were expectedly confirmed (Table 1). These results suggest that the inhibitory action of rifampicin against Aβ aggregation is related to a common structure of rifamycins, probably a

Table 1. Effects of test drugs on the aggregation of synthetic Aβ1-40 peptide in the ThT binding assay. All test drugs were dissolved in DMSO and added to Aβ1-40 peptide solutions as described in Materials and Methods. The final peptide concentration was 100 μg/ml. Fluorescence values represent the mean of three determinations performed on day 6.

Agents	Dose (μg/ml)	Fluorescence (482 nm)	Aggregation (% of DMSO control)
Experiment 1			
ThT only	—	0.0046	0
DMSO	(1%)	0.0325	100
rifampicin	100	0.0031	–4.6
rifamycin SV	100	0.0051	1.5
rifamycin B	100	0.0039	–2.2
erythromycin	100	0.0463	128
indomethacin	100	0.0356	95
Experiment 2			
ThT only	—	0.0046	0
DMSO	(1%)	0.0265	100
rifamycin S	100	0.0047	0.5
chalcomycin	100	0.0265	100
aspirin	100	0.0256	96
phenylbutazone	100	0.0245	91

naphthohydroquinone (or naphthoquinone) ring. Alternatively, rifampicin may bind to Aβ by hydrophobic interactions between its lipophilic ansa chain and the hydrophobic region of the peptide, blocking associations between peptide molecules. The present conclusion may partly explain why a drug treatment of coenzyme Q_{10} (quinone derivative), iron and vitamin B_6 was effective on patients with familial AD (19).

In clinical medicine, rifampicin is prescribed so as to maintain an average peak serum concentration of 10 μg/ml (20). Since it has been reported that about 13% of rifampicin in the blood penetrates into the brain through the blood-brain barrier in rats (21), approximately 1 μg/ml of rifampicin could penetrate into patients' brains. As rifampicin was found to function at 10 to 100 μg/ml, its effective concentration was still higher than that in the cerebrospinal fluid of the medicated patients. However, it may be reasonable if we consider the physiological concentration of Aβ in the cerebrospinal fluid (approximately 2.5 ng/ml) (5). Because of 1) its inhibition of *in vitro* aggregation of Aβ, 2) its prevention of Aβ-induced neurotoxicity and 3) a report suggesting its possible effect on senile plaque formation in leprosy patients (7), we postulate that a rifamycin family has therapeutic potential as an agent for inhibiting the initial step of amyloid formation, and this may provide a new strategy for the prevention and/or therapy of AD.

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