Effect of Tacrine, Amiridine, Akatinol Memantine, and Triazolam on Phosphorylation, Structure, and Assembly of Microtubules from Brain Microtubular Proteins in Alzheimer Diseases

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In *in vitro* experiments, amiridine in concentration of 100, 200, and 300 μM restored disturbed structure of microtubules assembled from tubulin and microtubule-associated proteins isolated from the brain of patients with Alzheimer disease. Tacrine in a concentration of 100, 250, and 500 μM inhibited phosphorylation of tubulin and microtubule-associated proteins isolated from the brain of patients with Alzheimer disease, while memantine and amiridine in the specified concentrations had no effect on phosphorylation.

Key Words: *Alzheimer disease; microtubules; tacrine; amiridine; memantine*

Effective search for new preparation for the treatment of Alzheimer disease (AD) largely depends on the results of preclinical studies on models most adequately reflecting the picture of intracellular disturbances typical of this condition [9]. This, in turn, poses a problem of choice of the most typical for this disease, methodically simple, and *in vitro* reproducible intracellular disturbance as the test model for these studies. Defect of microtubule (MT) assembly in brain cells and hyperphosphorylation of microtubule-associated proteins (MAP) tau and MAP-2 are the key intracellular disturbance explaining the appearance of other abnormalities typical of AD [3,4,11,13]. previous studies showed that some preparations used for the treatment of AD (sabeluzole, Ginko biloba, tacrine, and cerebrolysin) can modulate the regulation of MT assembly and phosphorylation of tau-protein and MAP-2 [5,7,14,15]. However, these studies were carried out on rat brain. Here we studied the effect of drugs used for the treatment of AD patients on MT assembly and phosphorylation of tau and MAP-2 isolated from the brain of AD patients. Until recently, such investigations were impossible due to difficulties in isolation of tubulin (Tb) and MAP from the brain of AD patients. In our previous study, these problems were solved and now MT can be assembled from Tb and MAP from the brain of AD patients in *in vitro* experiments [2].

The aim of the present work was to evaluate the effect of drugs used for the treatment of AD on phosphorylation of the major structural components of MT, Tb and MAP, and on MT assembly. It should be emphasized that Tb and MAP were isolated from the brain of AD patients.

MATERIALS AND METHODS

Isolation of Tb and MAP from the brain of AD patients and MT assembly were performed by the method of M. L. Shelanski in our modification [2].

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Polymerization was evaluated by the methods of light scatter and electron microscopy as described elsewhere [2]. The obtained microtubular structures were analyzed by electron microscopy with negative contrasting (×20,000, Philips-EM 420 electron microscope). The concentrations of the test drugs in experiments on evaluation of their effects on MT assembly were 100, 200, 300 μM. The concentrations of the test drugs in experiments on evaluation of their effects on Tb and MAP phosphorylation were 100, 250, 500 μM

The following preparations were used: tacrine (Warner-Lambert Company), amiridine (Russian Research Center for the Safety of Bioactive Compounds), and memantine (Merz). Triazolam (Pharmacia&Upjohn) was used as the reference preparation (clinical studies did not prove antialzheimer activity of this drug [13]).

Phosphorylation of Tb and MAP was carried out under the following conditions: 25 μl reaction mixture containing 5 μl Tb and MAP in a concentration of 3 mg/ml, 15 μl ^{32}P-ATP (0.5 MBq) in 50 mM Tris-HCl buffer (pH 6.9) with 2 mM $MgCl_2 \times 6H_2O$, and 5 μl test drug in concentrations of 0.5, 1.25, and 2.5 mM. After 10-min incubation, 5 μl stop-solution containing 10% sodium dodecylsulfate (SDS) and 20% mercaptoethanol was added, the samples were boiled for 5 min, and analyzed by SDS-electrophoresis in 10% gel. The gels were stained with Coomassie R250, dried, and radioautography was carried.

RESULTS

Amiridine and tacrine reduced the rate of polymerization throughout the whole polymerization period (amiridine was more active than tacrine), which was seen from the decrease in light scatter (Figs. 1 and 2). The differences from the control were significant for all concentrations of tacrine and amiridine on minutes 40, 240, 300, and 360 (from $p<0.05$ to $p<0.001$). The effect of triazolam differed from those of tacrine and amiridine (Fig. 3, *c*). Triazolam in all concentrations increased light scatter during the first 20 min of polymerization compared to the control ($p<0.001$). The effect of 100 μM triazolam persisted throughout the polymerization period. The effect of triazolam in concentrations of 200 and 300 μM sharply decreased on minutes 40 and 20, respectively, but the difference from the control remained significant ($p<0.001$).

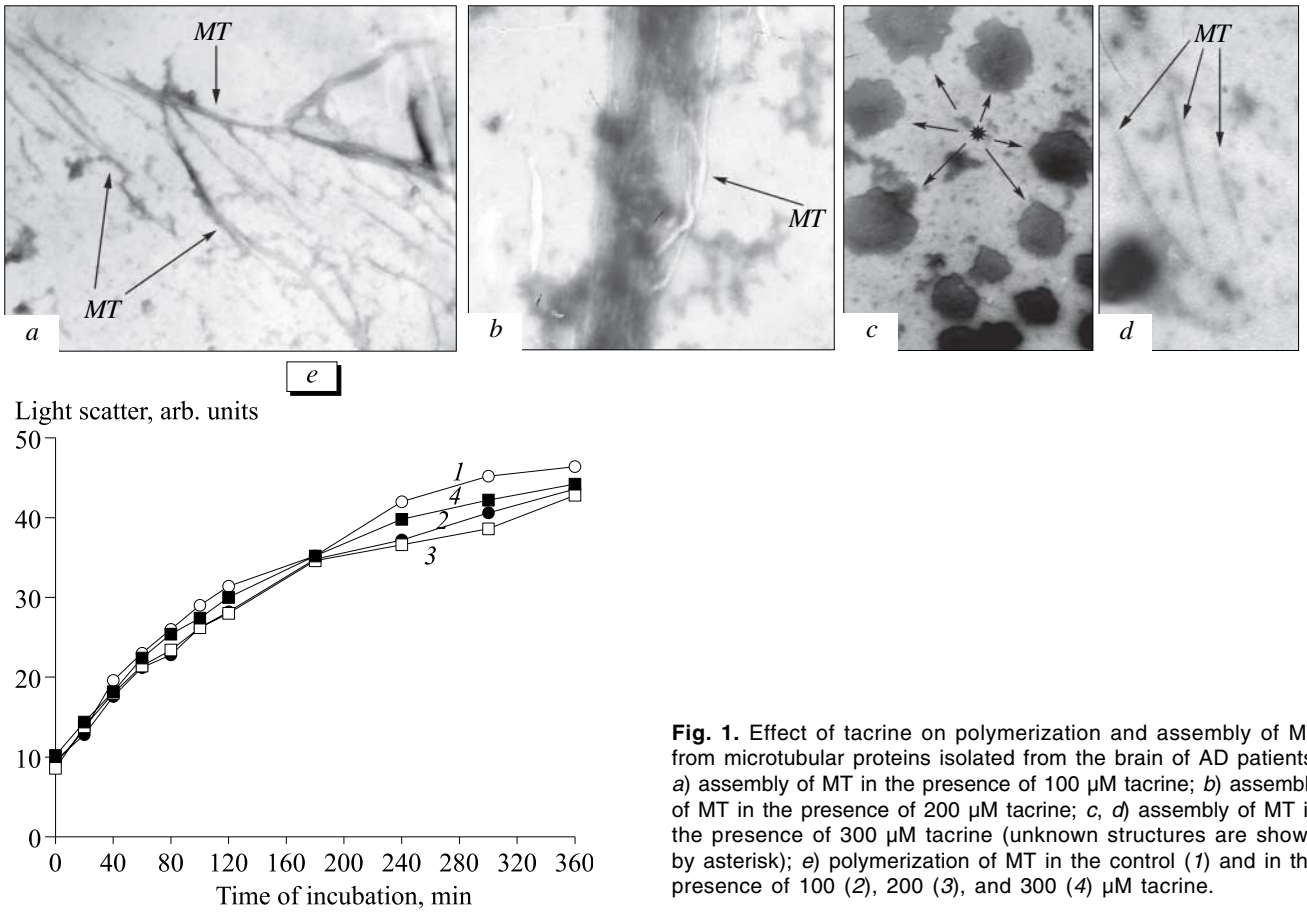
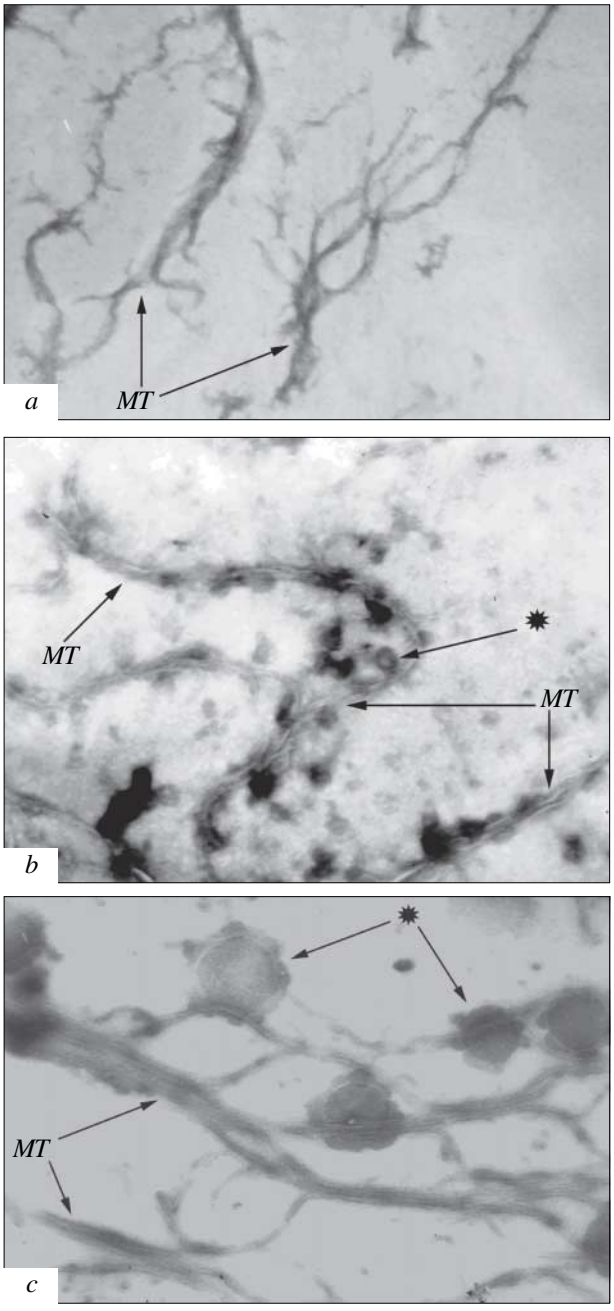


Fig. 1. Effect of tacrine on polymerization and assembly of MT from microtubular proteins isolated from the brain of AD patients. *a*) assembly of MT in the presence of 100 μM tacrine; *b*) assembly of MT in the presence of 200 μM tacrine; *c*, *d*) assembly of MT in the presence of 300 μM tacrine (unknown structures are shown by asterisk); *e*) polymerization of MT in the control (*1*) and in the presence of 100 (*2*), 200 (*3*), and 300 (*4*) μM tacrine.



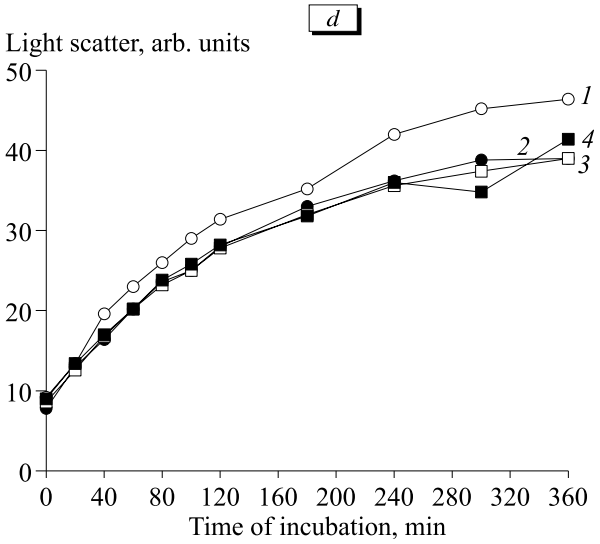


Fig. 2. Effect of amiridine on polymerization and assembly of MT from microtubular proteins isolated from the brain of AD patients. *a*) assembly of MT in the presence of 100 μM amiridine; *b*) assembly of MT in the presence of 200 μM amiridine (unknown ring-shaped structures consisting presumably of tubulin are shown by asterisk); *c*) assembly of MT in the presence of 300 μM amiridine (*unknown structures); *d*) polymerization of MT in the control (*1*) and in the presence of 100 (*2*), 200 (*3*), and 300 (*4*) μM amiridine.

We previously showed that polymerization of Tb and MAP isolated from the brain of AD patients leads to the formation of structurally abnormal MT: coils, bundles, pairwise twisted MT [2]. In the presence of 100 μM tacrine we observed the formation of more regular MT, although they remained more twisted, shortened, deformed, and collected into branched (not parallel) bundles (Fig. 1, *a*). In the presence of 200 μM tacrine, more defective MT forming tangle bundles were seen (Fig. 1, *b*). Moreover, numerous round bodies with a diameter of 200-400 nm appeared; they had electron-dense core and less electron dense shell with uneven borders with a width of 40-50 nm (Fig. 1, *c*). In the presence of 300 μM tacrine, solitary short MT and unknown round bodies were formed (Fig. 1, *d*).

In contrast to tacrine, amiridine can be considered as a preparation promoting the formation of apparently normal MT during polymerization of Tb and MAP isolated from the brain of AD patients. Polymerization of Tb and MAP in the presence of 100 μM amiridine leads to the formation of MT typical of AD (Fig. 2, *a*). Amiridine in a concentration of 200 μM normalized MT structure: numerous long and even MT appeared, but they were collected into twisted, but not parallel bundles. Small

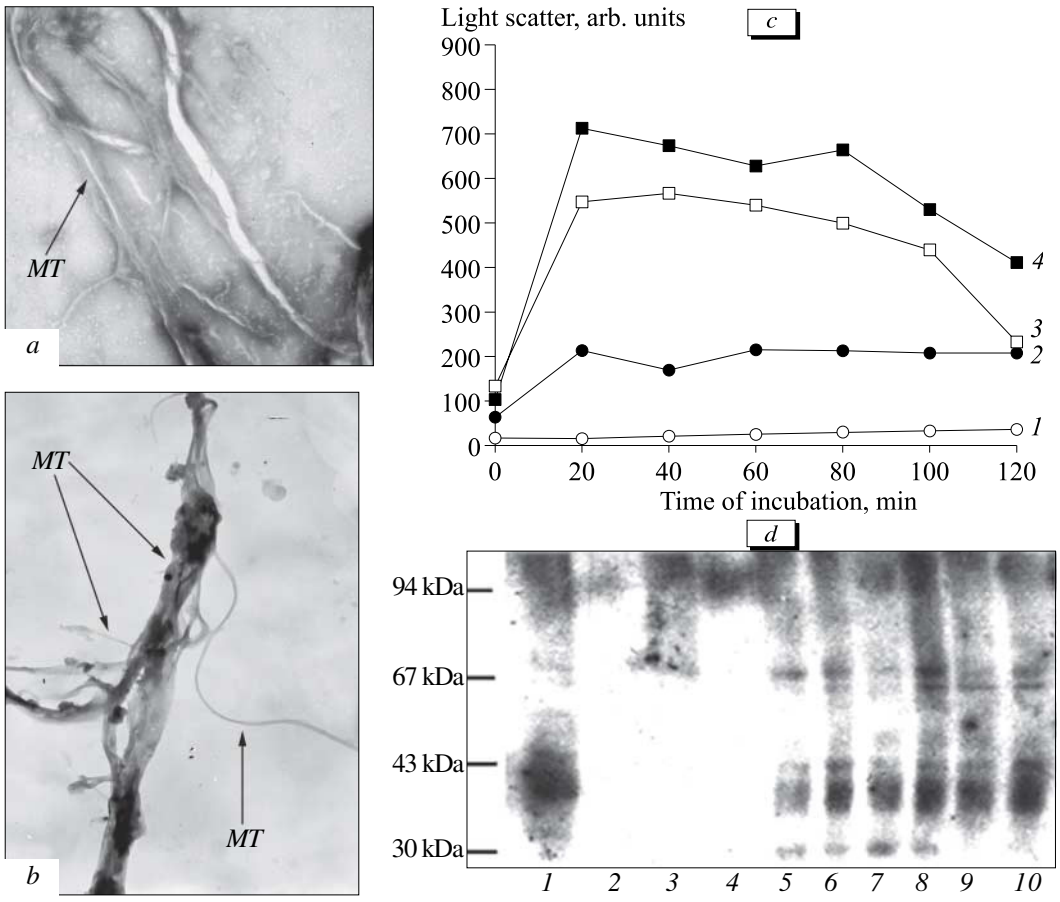


Fig. 3. Effect of triazolam on polymerization and assembly of MT from microtubular proteins isolated from the brain of AD patients. Phosphorylation of microtubular proteins from the brain of AD patients in the presence of tacrine, amiridine, and akatinol memantine. *a*) assembly of MT in the presence of 100 μM triazolam; *b*) assembly of MT in the presence of 300 μM triazolam; *c*) poloymerization of MT in the absence (control, 1) and presence of 100 (*2*), 200 (*3*), and 300 (*4*) μM triazolam; d) phosphorylation in the presence of tacrine, amiridine, and akatinol memantine. *1*) control, *2*) 100 μM tacrine, *3*) 250 μM tacrine, *4*) 500 μM tacrine, *5*) 100 μM amiridine, 6) 250 μM amiridine, *7*) 500 μM amiridine, *8*) 100 μM memantine, *9*) 250 μM memantine, *10*) 500 μM memantine.

ring-shaped structures consisting of Tb were seen around MT and in immediate contact with them (Fig. 2, *b*) [8]. Assembly defects manifested also in the appearance of twisted MT and MT with uneven thickenings. In the presence of 300 μM amiridine, only normal MT (thin, even, collected into parallel bundles) were formed; numerous round granular structures were also seen; they were similar to those observed in the presence of 200 μM tacrine (Fig. 2, *c*).

Triazolam in all tested concentrations disturbed MT assembly and induced the formation of structures differing from microtubules. In the presence of 100 μM triazolam we observed tangled MT bundles containing twisted deformed MT (Fig. 3, *a*); addition of 200 μM triazolam induced the formation of curled plane ribbon-shaped structures (sheet-structures), and triazolam in a concentration of 300 μM aggravated polymerization disturbances and led to the formation of dense tangled bundles in which solitary microtubular structures were discernible (Fig. 3, *b*).

Results of phosphorylation of Tb and MAP isolated from the brain of AD patient in the presence of tacrine, amiridine, and memantine in concentrations of 100, 250, and 500 μM were studied by autoradioautography (Fig. 3, *d*). Tacrine almost completely inhibited phosphorylation of Tb and MAP.

Amiridine and memantine in the specified concentrations had no effect on phosphorylation of Tb and MAP from the brain of AD patients (Fig. 3, *d*). These data suggest that in AD total inhibition of phosphorylation of all microtubular proteins with tacrine is associated with disturbed assembly of MT. The absence of the effect of amiridine on phosphorylation of Tb and MAP from the brain of AD patients together with normalization of MT assembly can attest to a possible mechanism similar to that underlying the effect of taxol or dimethylsulfoxide that stimulate the formation of MT from Tb alone (without MAP) [6,10]. We found that among the test preparations in the specified concentrations,

amiridine is far superior to tacrine in stimulation of MT assembly and similarly to memantine had no effect on phosphorylation of MT proteins. The results of polymerization and electron microscopy of MT did not contradict the data obtained in clinical studies of these preparations. For instance, anti-alzheimer activity of triazolam was not confirmed in clinical studies; amiridine was successfully approved in clinical studies and its daily dose producing maximum therapeutic effect is 2-fold lower than that of tacrine [1]. Moreover, tacrine produced serious side effects limiting its wide use in AD, while amiridine is free from these drawbacks [1].

Thus, drugs used for the treatment of AD patients can modulate the structure of MT formed during polymerization of Tb and MAP from the brain of AD patients. It was demonstrated that amiridine restored impaired structure of MT from brain cells in AD, while tacrine inhibited phosphorylation of Tb and MAP from the brain of AD patients.

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