Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 361-366
Reprints available directly from the publisher
Photocopying permitted by license only

SPIN-LABELLING STUDIES OF THE INTERACTION OF 9-AMINO-1,2,3,4-TETRAHYDROACRIDINE (THA), A PROPOSED DRUG FOR THE TREATMENT OF ALZHEIMER'S DISEASE, WITH ERYTHROCYTE MEMBRANES

D. ALLAN BUTTERFIELD* and DONNA A. PALMIERI

Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky 40506-0055, USA

ESR spin labels specific for skeletal proteins or cell-surface sialic acid have been used to monitor the interaction of 9-amino-1,2,3,4-tetrahydroacridine (THA) and its structural analogs with human erythrocyte membranes. The results suggest that THA significantly increases skeletal protein-protein interactions and may secondarily alter the physical state of the opposite side of the membrane. The fully aromatic analog of THA, 9-aminoacridine, showed even more pronounced effects on skeletal proteins than did THA. These results are discussed in relation to possible interaction sites of THA in erythrocyte ghosts and to potential mechanisms by which THA reportedly increases mental function of victims of Alzheimer's disease.

KEY WORDS: THA, ESR, spin labelling, Alzheimer's disease, erythrocyte membrane.

INTRODUCTION

Alzheimer's disease (AD) is the major dementing disorder of the elderly in the United States that typically affects between one and three million people annually.[1] Clinically, AD is characterized by a gradual loss of mental function, principally memory. Pathologically, brain tissue from AD patients possesses high concentrations of senile plaques and neurofibrillary tangles.[2,3] In addition to these aspects of AD, several neurochemical abnormalities, including cortical cholinergic deficits, are associated with this disorder. From post-mortem studies, it was demonstrated that the activity of choline acetyltransferase (CAT), an anabolic enzyme for the neurotransmitter acetylcholine (ACh), is diminished by as much as 90% of controls.[4] This enzyme is the primary pathway for the production of ACh, and the loss of CAT activity could account for the decreased ACh levels in the cortex.[3]

Recent clinical treatments for AD have primarily focused on increasing cortical ACh concentrations by the administration of acetylcholinesterase (AChE) inhibitors. Following this strategy, Summers, *et al.* reported that the oral administration of the anti-cholinesterase 9-amino-1,2,3,4-tetrahydroacridine (THA) (Compound # 1, Figure 1) induced marked improvements in the mental capacity and response of AD

*Correspondence: D. Allan Butterfield, Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, Kentucky 40506-0055, USA.

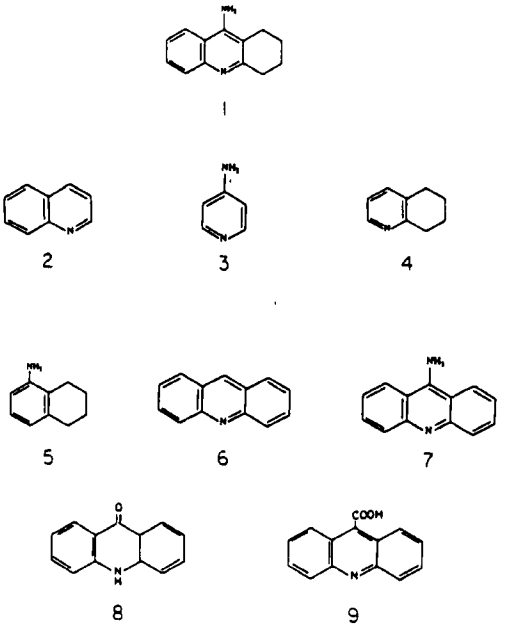

FIGURE 1 Structural formulae of compounds used in this study.

patients.[5] However, the use of other anti- cholinesterases, such as physostigmine, elicited only marginal improvement.[2] Therefore, it has been postulated that the purported increase in mental function generated by THA must be by mechanisms other than simple AChE inhibition.

In the following report, we have investigated the interaction of THA with human erythrocyte (RBC) membrane skeletal proteins and cell-surface carbohydrates via electron spin resonance (ESR) spin-labelling methods. There is an extensive homology between erythrocyte membrane proteins, including spectrin, the principal skeletal protein, and neuronal membrane proteins.[6–10] Added insight into the molecular mechanism(s) of THA with both erythrocyte and neurons is invisaged.

METHODS

Blood was collected from healthy volunteers by venipuncture into heparinized tubes, immediately placed on ice, and processed within 30 minutes of acquisition. Intact erythrocytes or ghosts (isolated RBC membranes) were prepared and spin labeled as previously described.[11,12]

Membrane proteins were labeled through the use of the protein-specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6).[11,13] This probe primarily binds to sulfhydryl groups of cysteine residues via covalent interactions. It has been demonstrated that 70–90% of the MAL-6 is bound to spectrin and the cytoplasmic portion of Band 3 using our labeling procedure.[11,14,15] Using antibodies to MAL-6, Barber *et al.*[15] showed that most of the spin label was bound to spectrin. The parameter used to characterize a spectrum of MAL-6 bound to membrane proteins

is the W/S ratio. The W/S ratio is a convenient and sensitive monitor of membrane-protein conformational changes.[11] We recently showed that decreased (increased) skeletal protein-protein interactions led to increased (decreased) values of the W/S ratio of MAL-6.[16] This labeling procedure and data analysis have also been confirmed by others to be highly reproducible.[17]

Sialic acid, a negatively-charged carbohydrate moiety at physiological pH, was selectively and covalently labeled with 2,2,6,6-[$^2H_{12}$] tetramethyl-4-amino [$^2H_5$, ^{15}N] piperidine-1-oxyl (^{15}N-Tempamine-[d_{17}]) under conditions of reductive amination.[18] Nearly seventy percent of the RBC membrane sialic acid is located on the external portion of the transmembrane protein, glycophorin A.[19] The parameter used to characterize this spectrum, the apparent rotational correlation time, τ_c, can be conceptualized as the time required for a spin-labeled sialic acid residue to rotate through an angle of one radian in space.[12] A decrease in the value of τ_c reflects increased rotational rates of the spin-labeled carbohydrate residues.

ESR spectra were obtained employing a Varian E-109 X-band ESR spectrometer with computerized data acquisition and analysis capabilities. All spectrometer parameters and other data collection information were employed as summarized elsewhere.[13] Membrane protein content and AChE activity were assessed by literature methods.[20,21] Alterations in spectrin aggregation were performed as previously described.[22]

RESULTS

The effect of THA on the erythrocyte membrane protein skeletal network, located on the cytoplasmic face of the bilayer leaflet, was examined with the protein-specific spin label, (MAL-6), as previously described.[11,13] Approximately 90% of the ESR signal intensity of MAL-6 is derived from the spin label bound to skeletal proteins, principally spectrin.[11,15]

THA, in a concentration-dependent manner, significantly increased skeletal protein-protein interactions as evidenced by a decrease in the W/S ratio, a parameter used to characterize the ESR spectrum of MAL-6 covalently bound to membrane proteins (Table 1). At concentrations greater than 1.5 mM, the W/S ratio is so low as to be essentially immeasurable. As shown in previous publications from our laboratory, a decrease in the W/S ratio is indicative of decreased protein segmental motion and increased protein–protein interactions.[11,13,16] However, other anti-cholinesterases, such as $NaAsO_2$,[23] did not affect this parameter (data not shown).

TABLE 1
Effects of THA on Erythrocyte Membrane Components

	Control (N)[a]	THA (N)[a]	p[b]
Protein (1.5 mM THA) (W/S Ratio)			
Sialic Acid Present	4.88 ± 0.33 (6)	2.24 ± 0.11 (6)	< 0.000001
Sialic Acid Absent	4.76 ± 0.12 (4)	2.10 ± 0.13 (4)	< 0.000001
Sialic Acid (5.0 mM THA) (τ_c in nsec)	0.488 ± 0.025 (6)	0.188 ± 0.015 (6)	< 0.000001

[a]Mean ± Std. Dev. (# of samples) are presented.
[b]p-value calculated by a two-tailed Student's t-test.

We have previously demonstrated that alterations in the physical state of one side of the membrane can induce changes in the physical state of the opposite side of the membrane.[13,16] Employing terminal sialic acid spin-labeling methods,[12,18] the effects of THA on this cell-surface carbohydrate were investigated. The addition of THA significantly decreased τ_c (Table 1), suggesting that the anti-cholinesterase induces faster motion of labeled cell-surface sialic acid residues. However, the onset of this effect occurs at higher concentrations than the observed protein results, implying that this alteration in sialic acid motion may be secondary to the protein effect.

To investigate this latter hypothesis, sialic acid was enzymatically cleaved with neuraminidase and ghosts were subsequently labeled with MAL-6. Upon addition of THA to both sialic acid-depleted and sialic acid-rich membranes, no differences in the W/S ratios were observed (Table 1), suggesting that the primary interaction site of THA is with the membrane skeleton.

In order to gain further insight into the mechanism of interaction of THA with the skeleton, the effects of THA on membranes with altered spectrin aggregation were performed. Spectrin normally exists in the tetrameric state, but can be chemically modified to produce membranes with elevated dimer concentrations.[22] Membranes with spectrin in the tetrameric or dimeric states were labeled with MAL-6, and the effects of THA addition were monitored. The magnitude of the decrease in the W/S ratio were statistically identical, implying that the interaction of THA with the skeleton is independent of the state of spectrin aggregation. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Triton shells of control and THA-treated membranes suggest that THA does not crosslink spectrin with the bilayer framework. ESR studies have also shown that the interaction of THA with the membrane is reversible and weakly electrostatic.

In an attempt to correlate the structure of THA with its interaction with the membrane, various analogs of this molecule (Figure 1) were added to MAL-6-labeled ghosts. Of these compounds, only 9-aminoacridine (compound #7, Figure 1) significantly decreased the W/S ratio. This decrease in protein segmental motion was more pronounced for 9-aminoacridine than THA (Figure 2).

DISCUSSION

THA, a potential drug in the treatment of AD, drastically and significantly increased skeletal protein–protein interactions. Changes in cell-surface sialic acid motion were also observed; however, nearly three times the concentration of THA necessary to induce protein changes were required to elicit these alterations in sialic acid motion. These results suggest that the increase in the rotational motion of carbohydrate residues may be secondary to the protein effect induced by THA. The principal interaction site of THA is most likely with the membrane skeleton as implied from the neuraminidase studies (Table 1).

Other anti-cholinesterases, such as $NaAsO_2$, do not seem to significantly affect the skeletal proteins. This observation is consistent with the original premise that the mechanism of action of THA in AD can not be accounted for by simply inhibiting AChE.

The structure-function studies suggest that the positive charge at the 9-position of THA and the planarity of the molecule are important in the mechanism of membrane interaction. It seems that the presence of a positively-charged functional group in the

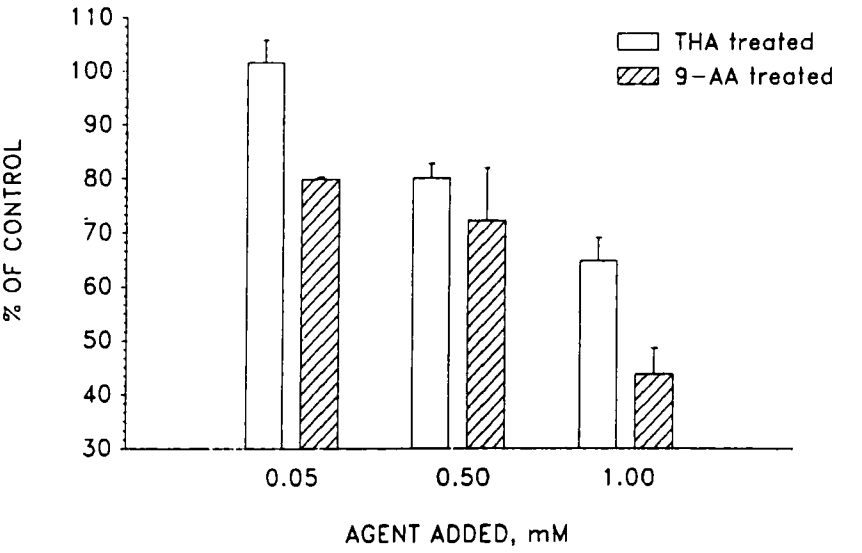

FIGURE 2 Comparison of the effects of THA and 9-amino-acridine on the physical state of membrane skeletal proteins in human erythrocyte ghosts as monitored by the W/S ratio of MAL-6. The data points illustrated for the THA-treated samples were the average of 4, 6, and 6 trials for 0.05, 0.5, and 1.0 mM samples, respectively. Statistical significance between THA and 9-AA was demonstrated for the 0.05 mM ($p < 0.005$) and 1.0 mM ($p < 0.0001$) cases, but not for the intermediate 0.50 mM samples.

9-position of the parent molecule, as well as the number of rings and planarity (aromaticity) may play important roles in the mechanism.

One possible mechanism by which THA may associate with spectrin is via intercalation into the highly negatively charged and highly α-helical framework of this protein. Acridines are known to insert into the grooves of DNA α-helices.[24] The more pronounced effect of 9-aminoacridine on the W/S ratio (Figure 2) lends support to this hypothesis because its structure is more planar, and therefore, probably more capable of inserting into the helical grooves of spectrin. The positive charge that appears to be necessary for the interaction of THA with the membrane may serve as a guide to the anionic protein, spectrin. This explanation is merely speculative at present.

In other studies in our laboratory, it was suggested that there was a defect in the skeletal network of proteins in erythrocytes from AD patients.[25] The erythrocyte skeletal proteins are reported to be highly similar or identical to those of neurons,[6-10] and the neuronal homologue of spectrin, fodrin, has been implicated in a mechanism of memory.[26] If THA interacts with nervous tissue as it does with RBC's and if the skeletal defect observed in AD erythrocytes is present in AD brain tissue, then it is conceivable that THA acts to correct this membrane defect. This may then lead to the purported increase of mental function in AD patients after the administration of THA. Studies to test this idea are in progress.

Acknowledgments

This research was supported, in part, by a grant from the National Science Foundation (RII-86-10671).

References

1. Katzman, R. *Banbury Report No. 15: Biological Aspects of Alzheimer's Disease*. Cold Spring Harbor Press, Cold Spring Harbor, (1983).

2. Terry, R.D. and Katzman, R. Senile dementia of the Alzheimer type. *Neur. Prog.*, **14**, 497–506, (1983).
3. Katzman, R. Alzheimer's disease: Advances and opportunities. *J. Amer. Ger. Soc.*, **35**, 69–73, (1987).
4. Bartus, R.T., Dean, R.L., Beer, B. and Lippa, A.S. The cholinergic hypothesis of geriatric memory dysfunction. *Science*, **217**, 408–417, (1982).
5. Summers, W.K., Majovski, L.V., Marsh, G.M., Tachiki, K. and Kling, A. Oral administration of tetrahydroamino-acridine in long-term treatment of senile dementia, Alzheimer type. *New Engl. J. Med.*, **315**, 1241–1245, (1986).
6. Anderson, D.J. New clues to protein localization in neurons. *Trends Neurosci.*, **7**, 355–357, (1984).
7. Geiger, B. Proteins related to the red cell cytoskeleton in non-erythroid cells. *Trends Biochem. Sci.*, **7**, 388–389, (1982).
8. Bennett, V. The membrane skeleton of human erythrocytes and its implication for more complex cells. *Ann. Rev. Biochem.*, **54**, 273–304, (1985).
9. Cohen, C.M. The molecular organization of the red cell membrane skeleton. *Sem. Hematol.*, **2**, 141–158, (1983).
10. Bennett, V. The spectrin-actin junction of erythrocyte membrane skeletons. *Biochim. Biophys. Acta*, **988**, 107–121, (1989).
11. Butterfield, D.A. Spin-labeling in disease. *Biol. Magn. Res.*, **4**, 1–78, (1982).
12. Feix, J.B. and Butterfield, D.A. Selective spin labeling of sialic acid residues of glycoproteins and glycolipids in erythrocyte membranes: A novel method to study cell-surface interactions. *FEBS Lett.*, **115**, 185–188, (1980).
13. Wyse, J.W. and Butterfield, D.A. Electron spin resonance and biochemical studies of the interaction of the polyamine, spermine, with the skeletal network of proteins in human erythrocyte membranes. *Biochim. Biophys. Acta*, **941**, 141–149, (1988).
14. Fung, L.W.M. and Simpson, M.J. Topology of a protein spin label in erythrocyte membranes. *FEBS Lett.*, **108**, 269–273, (1979).
15. Barber, M.J., Solomonson, L.P. and Eichler, D.C. Spin-labeled erythrocyte membranes: Direct identification of the nitroxide-conjugated proteins. *Biochem. Biophys. Res. Comm.*, **127**, 793–798, (1985).
16. Farmer, B.T., Harmon, T.M. and Butterfield, D.A. ESR studies of the erythrocyte membrane skeletal protein network: Influence of the state of aggregation of spectrin on the physical state of membrane proteins, bilayer lipids, and cell-surface carbohydrates. *Biochim. Biophys. Acta*, **821**, 420–430, (1985).
17. Barber, M.J., Rosen, G.M. and Rauchkman, E.J. Studies of the mobility of maleimide spin labels within the erythrocyte membrane. *Biochim. Biophys. Acta*, **732**, 126–132, (1983).
18. Farmer, B.T. and Butterfield, D.A. 2,2,6,6-Tetra-methyl-4-amino (d_{17}) piperdine-^{15}-N-1-oxyl as a spin label for cell-surface carbohydrates. *Analyt. Lett.*, **18**, 555–561, (1985).
19. Marchesi, V.T. Functional proteins of the human red blood cell membrane. *Sem. Hematol.*, **16**, 3–20, (1979).
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with Folin-Phenol reagent. *J. Biol. Chem.*, **193**, 265–275, (1951).
21. Ellman, G.L., Courtney, K.O., Valentino, A. and Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharamcol.*, **7**, 88–95, (1961).
22. Liu, S.C. and Palek, J. Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeletons. *Nature*, **285**, 586–588, (1980).
23. Page, J.D. and Wilson, I.B. The inhibition of acetylcholinesterase by arsenite and fluoride. *Arch. Biochem. Biophys.*, **226**, 492–497, (1983).
24. Robertson, I., Denny, W.A. and Baguley, B.C. Inhibition of bacteriophage-T4 yield by 9-anilino-acridine: Comparison with in vivo anti-tumor activity. *Eur. J. Cancer*, **16**, 1133–1139, (1980).
25. Markesbery, W.R., Leung, P.K. and Butterfield, D.A. Spin label and biochemical studies of erythrocyte membranes in Alzheimer's disease. *J. Neurol. Sci.*, **45**, 323–330, (1980).
26. Lynch, G. and Baudry, M. The biochemistry of memory: A new and specific hypothesis. *Science*, **224**, 1057–1063, (1984).

Accepted by Prof. E.G. Janzen

Note Added in Proof: Consistent with the results presented here, we recently demonstrated that 9-amino-acridine was more potent that THA in altering the morphology of red blood cell ghost membranes [Palmieri, Jacob, and Butterfield, Biochem. Biophys. Res. Commun. 163, 1351–1355 (1989)]. Spectrin and the skeletal network of proteins, the principal binding sites of MAL-6, are the major determinants of red cell morphology.