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Structure–activity investigation of the alteration of the physical state of the skeletal network of proteins in human erythrocyte membranes induced by 9-amino-1,2,3,4-tetrahydroacridine

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The oral administration of 9-amino-1,2,3,4-tetrahydroacridine (THA) is purported to increase the mental function of Alzheimer's disease patients (Summers et al. (1986) N. Engl. J. Med. 315, 1241–1245). Numerous erythrocyte membrane proteins are known to be identical or highly similar to neuronal proteins. In a previous study (Butterfield and Palmieri ((1990) Free Radical Res. Commun., in press), we showed that THA greatly increased skeletal protein–protein interactions in erythrocyte membranes as monitored by a spin label specifically bound to membrane proteins. In this report, a structure–activity study has been performed to determine which THA structural components are involved in its effect on the physical state of human erythrocyte membrane skeletal proteins. The results imply that both the planarity of the molecule and the amino group at the 9-position of the parent acridine molecule are important in the mechanism of interaction with membrane proteins.

Introduction

The oral administration of 9-amino-1,2,3,4-tetrahydroacridine (THA) (compound **1**, Fig. 1) was reported to induce significant improvement in the cognitive function in patients afflicted with senile dementia of the Alzheimer type [1]. At the present, the molecular basis of Alzheimer's disease (AD) is not known [2,3], but we were, to our knowledge, the first to suggest that this disorder may be associated with a general membrane defect of the skeletal network of proteins [4], an elaborate infrastructure of proteins lining the cytoplasmic face of the membrane [5]. Since there is an extensive homology between erythrocyte, or red blood cell (RBC), proteins including spectrin, the major cyto-

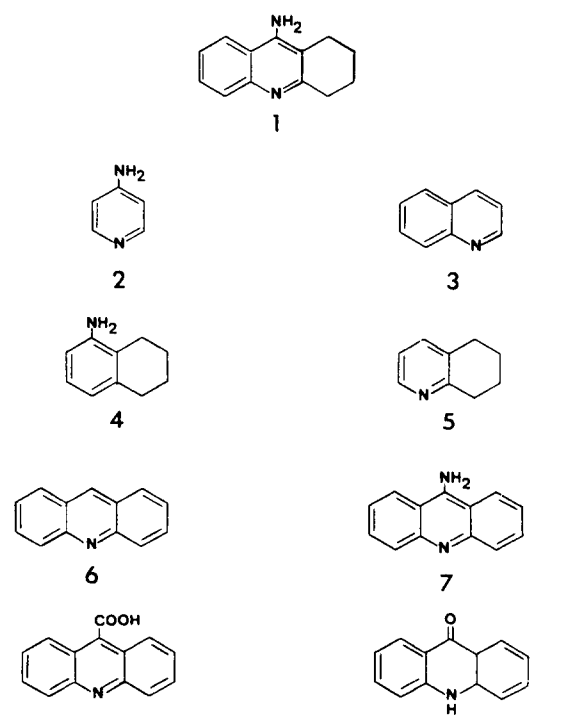

Fig. 1. Structures of compounds used in this study. Nomenclature of these compounds is given in Materials and Methods.

Abbreviations: THA, 9-amino-1,2,3-4-tetrahydroacridine; AD, Alzheimer's disease; RBC, red blood cell; MAL-6, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl; PBS, phosphate-buffered saline; 5P8, 5 mM sodium phosphate (pH 8.0); EPR, electron paramagnetic resonance; AChE, acetylcholinesterase.

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skeletal protein, and neuronal membrane skeletons, it is possible that added insight into the basis of AD may be gained using the erythrocyte as a model system [4–11].

In previous studies [12,13], we investigated the molecular interactions of the anticholinesterase, THA, with the human erythrocyte membrane proteins, cell-surface carbohydrates, and bilayer lipids employing electron paramagnetic resonance (EPR) spin-labeling methods. These studies implied that the primary interaction site of THA with the membrane seems to be the skeletal network of proteins, and that THA addition appears to greatly increase protein–protein interactions in this infrastructure (approx. 50% reduction in the relevant EPR parameter, $P < 0.00001$ [12,13]). In the present studies, the structure–activity relationships of THA that give rise to these changes are probed using THA structural components and analogues.

Materials and Methods

The protein-specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (THA) (**1**), 4-aminopyridine (**2**), quinoline (**3**), 5-aminotetralin (**4**), 5,6,7,8-tetrahydroquinoline (**5**), acridine (**6**), 9-aminoacridine (**7**), 9-acridine carboxylic acid (**8**), and acridone (**9**) were obtained in highest purity from Aldrich. The numbers following the compounds refer to those in Fig. 1.

Blood was collected by venipuncture from healthy individuals into heparinized tubes, placed on ice, and processed within 30 min of acquisition. Intact packed cells were isolated by centrifugation at $600 \times g$ for 5 min at 4°C and three successive washings in phosphate-buffered saline (PBS) (150 mM NaCl/5 mM sodium phosphate (pH 8.0)). After each centrifugation step, the buffy coat was carefully removed by aspiration. Erythrocyte membrane ghosts were obtained by hemolytic lysis in 5P8 (5 mM sodium phosphate (pH 8.0)) and centrifuged at $27000 \times g$ for 10 min at 4°C. Ghost membranes were continually washed with 5P8 until they were free of residual hemoglobin. Membrane protein concentration was estimated by the method of Lowry et al. [14], and acetylcholinesterase (AChE) activity was determined by the assay of Ellman et al. [15].

Isolated erythrocyte membranes were selectively spin-labeled with the maleimide probe, MAL-6, as previously described [8,9,16]. All EPR spectra were recorded on a Varian X-band EPR spectrometer equipped with computerized data acquisition and analysis capabilities, and housed in a room with constant temperature and humidity. Spectra were obtained using a scan width of 100 G, 0.32 G modulation amplitude, and 18 mW power incident on the resonant cavity, which was continually purged with dry N_2 gas.

When possible, stock solutions of THA structural analogues were prepared in 5P8, and pH- and ionic-strength-matched controls were used when appropriate. However, in some cases, ethanol or DMSO was used in order to dissolve some of the analogues; in those cases, solvent controls were used in order to separate solvent effects from those induced by the analogues. At no time did the composition of organic solvent in the sample exceed 4%. Those samples requiring ethanol or DMSO controls are listed in the footnotes of Tables I and II.

Results

MAL-6 is a maleimide spin label that binds primarily to sulfhydryl groups on spectrin, the major cytoskeletal protein, as well as those on the cytoplasmic pole of band 3 [8,9,16–21]. A typical EPR spectrum of MAL-6 covalently bound to erythrocyte membrane proteins is shown in Fig. 2. This spectrum, discussed extensively in previous publications from our laboratory [8,9,16–19] and others [20], shows that there are at least two distinct classes of MAL-6 binding sites discernible by their motion: those that are strongly immobilized (S sites) and those that are only weakly immobilized (W sites). The ratio of the EPR spectral amplitudes of the $M_I = +1$ resonance lines, the W/S ratio, is a convenient and sensitive monitor of conformational changes in membrane proteins, as well as alterations in the state of spectrin aggregation [8,9,16–20]. In general, a decrease in the W/S ratio of MAL-6 bound to erythrocyte ghosts implies decreased segmental motion, or increased protein–protein interactions, of the spin-labeled domains of the proteins [8,9,16–19].

We showed that THA caused a dramatic decrease in the W/S ratio of MAL-6 bound to erythrocyte proteins, even at a concentration of 0.5 mM ($P < 0.00001$), sug-

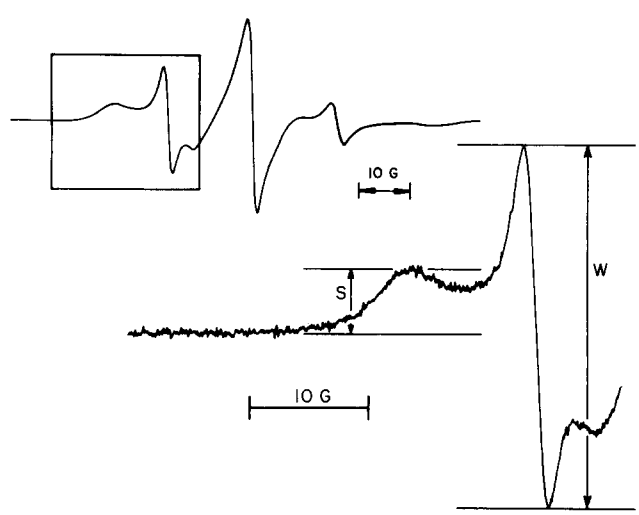

Fig. 2. A typical spectrum of MAL-6 covalently bound to erythrocyte membrane proteins. The spectral region of the $M_I = +1$ lines of MAL-6 (box) is expanded showing the spectral amplitudes of the low-field ($M_I = +1$) strongly (S) and weakly (W) immobilized MAL-6 binding sites.

TABLE I

Effect of THA structural analogues on the physical state of erythrocyte membrane proteins as monitored by the W/S ratio of MAL-6

Analogue [a]	0.5 mM (*N*) [b]	5.0 mM (*N*) [b]	Control
Left-sided modifications			
2	4.61 ± 0.04(2)	4.03 ± 0.27(4) [c]	4.65 ± 0.01(4) [d]
3	4.93 ± 0.18(2)	5.02 ± 0.08(2)	4.77 ± 0.10(2) [e]
Right-sided modifications			
4	4.67 ± 0.18(4)	4.65 ± 0.26(4)	4.88 ± 0.42(6) [e]
5	5.01 ± 0.18(4)	5.14 ± 0.55(4)	4.88 ± 0.42(6) [e]
Entire molecule modifications			
6	4.75 ± 0.17(4)	4.87 ± 0.17(4)	5.07 ± 0.39(4) [e]
7	3.57 ± 0.67(4) [f]	2.14 ± 0.19(4) [e,h]	4.91 ± 0.28(4) [d]

[a] Numbers refer to compound numbers listed in Fig. 1

[b] Values are given as mean *W/S* ratio ± S.D. (number of samples). Unless explicitly noted, all means are statistically insignificant relative to controls as determined by a two-tailed Student's *t*-test. *W/S* values for THA were: Control: 4.88 ± 0.33(6); 0.5 mM THA: 4.13 ± 0.18(6), $P < 0.0001$; 1.5 mM THA: 2.24 ± 0.11(6), $P < 0.00001$ [12].

[c] $P < 0.05$.

[d] 5P8 control.

[e] Ethanol control.

[f] $P < 0.02$.

[g] 1.0 mM compound **7**.

[h] $P < 0.00001$.

gesting that this agent greatly increased skeletal protein-protein interactions [12,13]. No change in the linewidths of the S and W spectra components were observed after THA addition, consistent with a single two-state model in which W sites were converted to S sites by this drug [12,13]. The interaction of THA with erythrocyte membrane proteins was suggested to be weakly electrostatic in nature [12,13]. In order to determine systematically which structural components of THA were required for this large decrease in the *W/S* ratio, structural analogues that are modifications of the left and right sides of THA, as well as those of the entire three-ring structure (Fig. 1) were added to MAL-

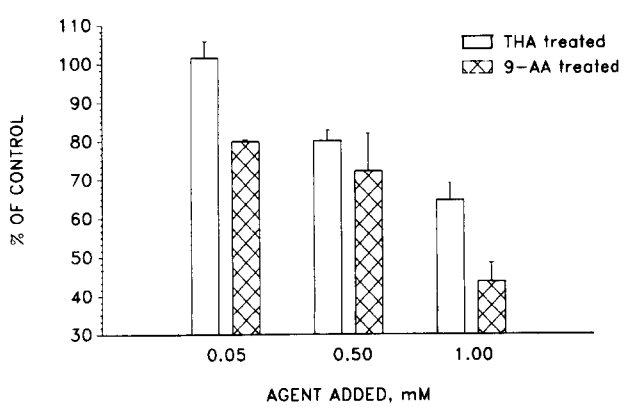

Fig. 3. Comparison of the effects of THA and 9-aminoacridine (9-AA) (compound **7**) on the physical state of human erythrocyte membrane skeletal proteins as monitored by the *W/S* ratio.

TABLE II

Influence of 9-position substitution of acridine on the physical state of erythrocyte membrane proteins as monitored by the W/S ratio of MAL-6

Analogue [a]	0.5 mM (*N*) [b]	2.0 mM (*N*) [b]	Control
7	3.57 ± 0.67(4) [c]	2.14 ± 0.19(4) [d,e]	4.91 ± 0.28(4) [f]
8	4.92 ± 0.11(2)	4.69 ± 0.12(2)	4.93 ± 0.05(2) [f]
9	4.88 ± 0.08(2)	4.89 ± 0.13(2)	5.04 ± 0.11(2) [g]

[a] Numbers refer to compound numbers listed in Fig. 1.

[b] Values are given as mean *W/S* ratio ± S.D. (number of samples). Unless explicitly noted, all means are statistically insignificant relative to controls as determined by a two-tailed Student's *t*-test.

[c] $P < 0.02$.

[d] 1.0 mM compound **7**.

[e] $P < 0.00001$.

[f] 5P8 control.

[g] DMSO control.

6-labeled erythrocyte ghosts. The results of these studies are summarized in Table I.

The addition of the two-ring compounds **3**, **4** and **5**, did not seem to affect skeletal protein–protein interactions. In the presence of high concentrations of **2** (5.0 mM), a slight (15%) decrease in the *W/S* ratio was observed relative to controls, but the effects of this compound could not alone account for the drastic, 46% decrease in the *W/S* ratio induced by a lower concentration of THA (1.5 mM) [12,13].

Since no obvious changes in the *W/S* ratio of MAL-6 were caused by interaction of two-ring analogues of THA, the effects of THA structural analogues resembling the entire heterocycle were studied. The addition of **6**, the parent acridine molecule, did not alter the *W/S* ratio. However, **7**, the aminated analogue of **6**, drastically increased protein–protein interactions, and seemed to have an even more pronounced effect than THA (Fig. 3). Similarly, **7** was the only THA analogue that decreased acetylcholinesterase (AChE) activity similar to the known anti-cholinesterase THA (data not shown). These data suggest that the presence of all three rings of THA and a positive charge at the 9-position of the parent compound are important in the mechanisms of membrane interaction.

To examine further the significance of the positively charged amino group at the 9-position of **7**, analogues having a negatively charged carboxylic acid (compound **8**) and a neutral ketone functionality (compound **9**) substituted for the amino group were investigated (Table II). Neither of these compounds seemed to affect the *W/S* ratio nor decrease AChE activity.

Discussion

The results of this structure–activity study suggest that the site of interaction of THA or compound **7** with the erythrocyte protein, presumably spectrin, the prin-

cipal binding site of MAL-6 [8,9,21], has a specific geometry. Compounds having only two rings or the parent acridine molecule without an amino group at the 9-position were ineffective at altering protein conformation. Comparison of the effects induced by THA and **7** (Fig. 3) suggests that the association of these molecules with spectrin seems to be enhanced with increasing aromatically and planarity. Consistent with this finding we found that **7** caused larger morphological alterations of red cell ghosts than did THA [22], and the protein skeletal network is known to be the primary determinant of cell morphology [5]. There are statistically significant differences between the EPR findings caused by **7** and THA at both 0.05 mM ($P < 0.005$) and 1.0 mM ($P < 0.0001$) added agent. The standard deviation of the results employing 0.50 mM 9-aminoacridine (compound **7**) was unexpectedly high. Consequently, although the same trend is observed, statistical significance could not be demonstrated. However, since differences at the 0.05 mM concentrations were noted, we expect that the differences between THA and **7** are real at 0.50 mM as well.

Upon examination of the results of THA, **6**, **7**, **8** and **9** on the W/S ratio (Table II), it is suggested that the interaction of THA with skeletal proteins required the presence of a positive charge. The positive charge may serve as a guide to the negatively charged and highly α-helical matrices of spectrin, and is consistent with the previous studies which suggested that THA interacted with the skeleton in a weakly electrostatic manner [12,13]. Given the more planar geometry of **7** relative to THA, the more pronounced effect on protein–protein interactions elicited by the former is consistent with the hypothesis of a specific interaction site on the membrane proteins.

Although the molecular basis of the results presented here remains unknown, given the extensive homology between erythrocyte and neuronal membrane skeletal proteins [5–11], continued investigation of the interaction of THA with skeletal proteins in various tissue plasma membranes may lead to a better understanding of the purported therapeutic effect of this drug in AD [1]. Such studies are currently in progress.

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