ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Human Erythrocyte Acetylcholinesterase Inhibition by Porphyrin Compounds

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Manuscript Info

Manuscript History:

Received: 12 January 2014
Final Accepted: 23 February 2014
Published Online: March 2014

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Abstract

Study of erythrocyte membrane Acetylcholinesterase (AChE) inhibition is a biomarker of toxicity of chemical compounds. Three Porphyrin derivatives, Tetraphenylporphinesulfonate (TPPS), 5,10,15,20-Tetrakis (4 sulfonatophenyl) porphyrinato Iron(III) Chloride (FeTPPS) and 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinato Iron(III) nitrosyl Chloride (FeNOTPPS), werechemically synthesized and tested against human erythrocyte AChE by enzymatic assays. The results show that FeNOTPPS forms the most stable complex with AChE. The activity of AChE dropped by almost 20% in 10^{-10} M FeNOTPPS solution. The differential effects of Porphyrin compounds can be accounted for by fluidity changes in erythrocyte cell membrane.

Introduction:

Actylcholinesterase (AchE) is an integral part of erythrocytes' cell membrane.[1] This enzyme is externally oriented on the erythrocyte membrane with its active sites exposed outward (Figure 1(a)).[2,3] It is also allosteric in nature and is very sensitive to both physical and chemical changes that occur in its membrane's micro-environments.[4]AChE induces the hydrolysis of Acetylcholine into Choline and Acetic Acid and increases the concentration of endogenous Acetylcholine in the vicinity of Choline Receptors (Figure 1(b)).[5,6]Changes in Actylcholinesterase concentration has been recognized in several human disorders such as Alzheimer's diseases, diabetes, autoimmune hemolytic anemia, menstruation, pregnancy, protein malnutrition, splenomegaly, addiction and uremia.[7,8]Therefore, prevention of over expression of AchE is considered an ideal therapeutic strategy to achieve effective management of such conditions. Chronic overexpression of AChE is a consequence of exposure to anti-Cholinesterase drugs or poisons.High levels of neuronal AChE can cause muscle exhaustion due to small changes in synaptic transmission, muscle function and morphology.

The physiological functions of erythrocyte AchE are still totally unknown. However, it has been extensively investigated as a valid model system for substituting the brain enzyme in 'in vitro' studies.[9]It has been suggested that AChE can be an excellent marker for RBC aging in man.[10]Acetylcholinesterase from human erythrocyte membrane has been purified and well characterized.[11,12,13] The importance of erythrocyte membrane AChE lies in the fact that many of its properties have been found similar to the purified enzyme obtained from brain tissue. However, for a given inhibitor, it is difficult to know how closely AChE inhibition in erythrocytes reflects that in the nervous system because access to blood is always easier than access to the brain.

Porphyrins are a class of macrocyclic compounds which play an important role in the metabolism of living organisms. They have an18-π electron system that makes them aromatic**.** The Porphyrin molecule contains four pyrrole rings linked via methine bridges. Porphyrin chelates with Mg(II), Cd(II), Zn(II) and Fe(III) and can combine with another ligand to form penta-coordinated complexes with square-pyramidal geometry.[14]In this study, Porphyrin derivatives, TPPS, FeTPPS and FeNOTPPS, were synthesized and used to investigate inhibition of Acetylcholinesterase (AChE) of erythrocyte membranes using enzymatic assays.

Materials and Methods:

All chemicals were obtained from commercial sources and used as received. Commercially boughtpyrrole, benzaldehyde and acetone were distilled prior to using in experimental preparations. Column chromatography was performed using silica gel (100-200 mesh). Stock solution (2.5 M) of BF_3etheratesolution was prepared in CH_2Cl_2. ^1H NMR (400 MHz) were recorded in $CDCl_3$. TheUV-Vis. spectra were recorded on Cintra 10 UV-Vis. spectrophotometer.

Synthesis of Porphyrin Compounds:

Approximately 500 ml of dry dichloromethane was taken in a three-necked round bottle flask and one of the necks was closed with a rubber septum. The central neck was fitted with a water condenser and from the other neck argon was passed in DCM for about 10 min to remove the dissolved oxygen. After the neck was closed with stopper, argon was allowed to pass through the top of the condenser throughout the reaction. One equivalent each (3:4.4mL) of pyrrole and benzaldehyde were added into DCM through the neck closed with a rubber septum. The solution was stirred for about 15 minutes. The setup was covered with black paper to avoid photochemical reaction. Then 1mL boron trifloro-etherate was added rapidly using the syringe and the reaction mixture was stirred for about an hour. A calculated amount of p-chloranil was added as an oxidant and then refluxing was continued for an hour. The solvent was then evaporated and the product was separated using column chromatography using initially 1: 2 ratio of DCM and petroleum ether and gradually changing the ratio to 1: 1 (Scheme 1).

TPPS was synthesized according to the published literature procedure (Scheme 1).[15] Pure TPP and concentrated H_2SO_4 were ground into a homogenous paste using mortar and pastel. The paste was transferred to a 250ml flask and 50 ml of concentrated H_2SO_4 was added. The mixture was heated in a steam bath for 4 hours and allowed to stand at room temperature for 48 hours. The filtrate was cautiously diluted with two volumes of distilled water and salt was precipitated with addition of acetone. The resulting bright green precipitate is the HSO_4^- salt of the para-sulfonated diacid of TPP since earlier method to filter the mixture after steam bath heating resulted in a low yield. The diacid was washed several times with acetone and then dissolved in distilled water and NaOH and was added to the solution of H_2TPPS to obtain Na_4TPPS.

FeTPPS was prepared by using published method [15] but a modification was again made during the purification of the final product from excess of un-reacted ferrous sulfate and/or its hydrolyzed/oxidized products. Instead of following the purification process with cation-exchange method that led to demetallation and loss of yield, the classical method of the removal of iron using phosphate salt was adopted. The time consuming method of using cation-exchange resins was avoided and the product FeTPPS was obtained in a much better yield and in the pure form. Excess sodium sulfate and chloride present as impurities were removed by dissolving the crude product in methanol and precipitating FeTPPS from solution by acetone. Using this method, it was possible to improve the yield of the pure product to 90% (Scheme 2).

FeNOTPPS was prepared by using FeTPPS (Scheme 3).The solution was made in 10 mL degassed water and kept at low temperature. In this solution, nitric oxide gas was bubbled (which was produced by the reaction of solution of $FeSO_4$ in $H_2SO_4$and $NaNO_2$ for about 30 min. The reaction progress was checked with UV-Vis spectroscopy. The completion of the reaction was confirmed by the shift in Söret band in UV-Vis spectrum from 394 to 420 nm.

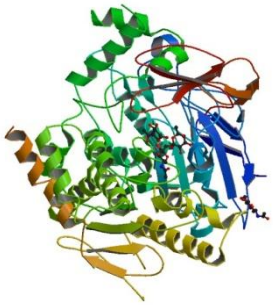
Assays for AChE activity:

Erythrocyte hemolysate was prepared as described by Beutler.[16] Packed RBCs were suspended in 0.154 M NaCl and β-Mercaptoethanol-EDTA as a stabilizing solution was added and the hemolysate was frozen overnight. The hemolysate was thawed before the experimental procedure. The membrane bound AChE activity in the human red blood cell was analyzed following the method ofEllman et al.[17]and Beutler.[16] The reaction mixture composed of 1mol/l TrisHCl, 5mmol/l EDTA with 0.5mmol/l 5,5′–di-thiobis (2-nitro-benzoic acid) (DTNB) solution. The reaction was initiated by adding 0.01 mol/lAcetylthiocholine Iodide and followed by a measurement at 412 nm. Hemoglobin was measured in red blood cell hemolysate as described by Beutler.[16]

Results:

All the three compounds, TPPS, FeTPPS and FeNOTPPS were successfully synthesized (Schemes 1, 2 and 3). FeTPPS was prepared by using a modified version of the published method.[15] Instead of using the literature-described purification process of cation-exchange chromatography that can lead to demetallation and loss of yield,the classical method of the removal of iron using phosphate salt was adopted. Using this method, the yield of the pure product was increased to 90% (Scheme 2).

The in vitro effect of TPPS, FeTPPS and FeNOTPPS on AChE activity was examined by enzymatic assays. The results show that FeNOTPPS forms the most stable complex with AChE. The activity of AChE dropped by almost 20% when concentration of FeNOTPPS reached 10^{-10} M (Table 1).Whereas, when concentration of FeTPPS reached 10^{-10} M it dropped by only 15% and did not drop at all when concentration of TPPS reached 10^{-10} M.



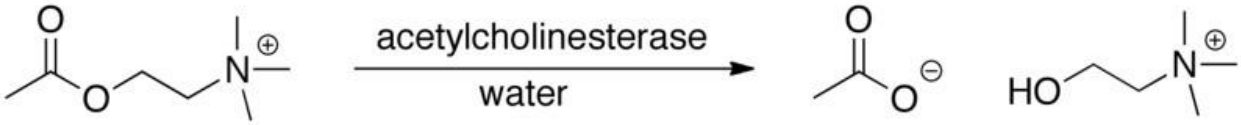
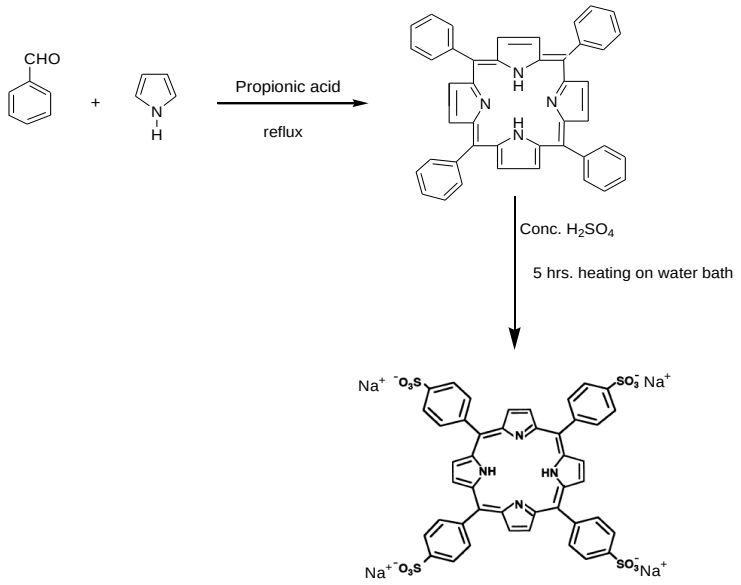


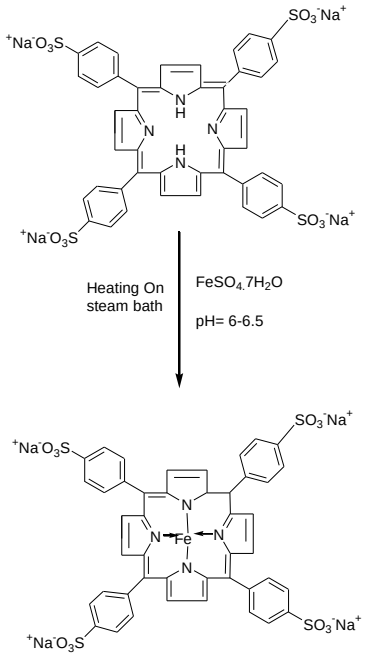
Figure 1(a): Ribbon diagram of human Acetylcholinesterase complexed with the snake-venom toxin, fasciculin-II. (b): Acetylcholinesterase-catalyzed hydrolysis of acetylcholine to produce acetate and choline.

Supplementary Material

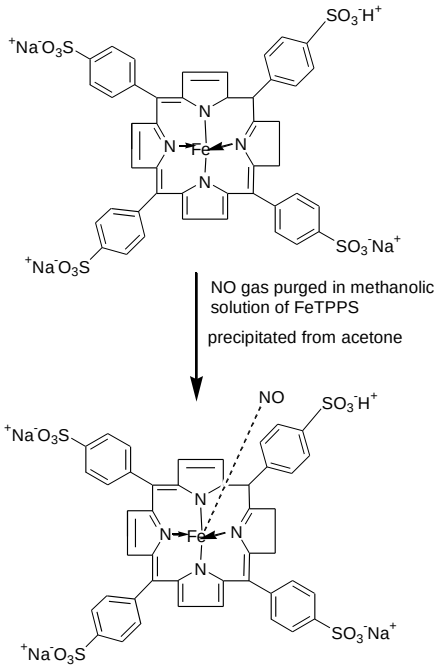
Chemical Characterization: $C_{44}H_{30}N_4$; C, 85.90; H, 4.92; N, 9.12; ^{1}H NMR (400 MHz) ($CDCl_3$) δ -2.62 (br s, 2H, NH), 8.1-8.2 (dd, 8H, pyrrole), 7.6-7.7(s, 20H, phenyl); UV-vis 417, 513, 546, 589, 642 nm.



Scheme 1: Synthesis of TPP and TPPS.



Scheme 2: Synthesis of FeTPPS.



Scheme 3: Synthesis of FeNOTPPS.

Table 1: Human erythrocyte membrane Acetylcholinesterase activity after *in vitro* treatment with synthetic compounds: TPPS, FeTPPS and FeNOTPPS.

Compound		Enzyme Activity[a]
	Normal	32.00 ± 2.39
	Concentration in M	
TPPS (Na_4TPPS)	10^{-10}	34.00 ± 2.11
	10^{-9}	22.37 ± 3.14
	10^{-8}	20.89 ± 4.37
	10^{-7}	18.00 ± 1.74
	10^{-6}	16.02 ± 1.76
	10^{-5}	13.80 ± 3.71
	10^{-4}	11.80 ± 1.48
	10^{-3}	09.88 ± 2.14
FeTPPS	10^{-10}	27.68 ± 2.66
	10^{-9}	25.68 ± 2.92
	10^{-8}	23.42 ± 0.63
	10^{-7}	20.20 ± 2.64
	10^{-6}	18.70 ± 1.07
	10^{-5}	16.20 ± 3.62
	10^{-4}	13.90 ± 1.54
	10^{-3}	11.48 ± 1.07
FeNOTPPS	10^{-10}	26.40 ± 2.08
	10^{-9}	24.30 ± 1.73
	10^{-8}	22.50 ± 1.61
	10^{-7}	19.40 ± 2.55
	10^{-6}	17.70 ± 1.87
	10^{-5}	15.00 ± 4.04
	10^{-4}	13.90 ± 3.64
	10^{-3}	11.20 ± 2.23

[a]Acetylcholinesterase activity is expressed in terms of μ mole of acetylthiocholine iodide hydrolyzed/minute/gram of hemoglobin at 37°C. Each value is the mean of at least 3-4 independent measurements. Values are expressed as mean +/- S.D

Discussion:

Due to toxic effects of many AChE inhibitors, current research is focused on developing new AChE inhibitors or modifying pre-existing ones. This study revealed inhibition of AChE by three Porphyrin derivatives, TPPS, FeTPPS and FeNOTPPS, through *in vitro* experiments. The antagonistic profile of these compounds was estimated using enzymatic assays. It is known from a previous study that Monosulfonate Tetraphenyl Porphyrin (TPPS) forms a 1:1 complex with AChE from electric eel leading to a loss in absorbance at 402 nm in UV-Vis spectrum and the appearance of a new absorbance band centered at 442 nm.[18]

The physiological function of erythrocyte AChE is not known, but the enzyme is the same as that involved in synaptic transmission and its measurement is used to understand the effects on the nervous system. The relation between inhibition of erythrocyte and nervous tissue AChE depends on the pharmacokinetics of inhibitors. Usually erythrocyte AChE inhibition is over-estimated than as compared to the nervous system. Factors such as spontaneous reactivation and aging of inhibited enzyme should also be considered in assessing AChE inhibition. Other factors, such as timing of measurement, add complexity because erythrocyte AChE inhibition persists longer than the one in

the nervous tissues. Among other mechanisms, there is increasing evidence that AChE activity is related to the well known change in cation transport in patients with hypertension. [21]

The enzymatic assays provided evidence about the strength of binding of TPPS, FeTPPS and FeNOTPPS to AChE. The results show that FeNOTPPS forms the most stable complex with AChE.This can be accounted for by fluidity changes in the erythrocyte cell membrane. FeNOTPPS is the most hydrophobic among all these three molecules. It is more stable in the erythrocyte cell membrane and hydrophobic interior of AChE than in the external solution. AChE has an assymetric orientation on the outer surface of the erythrocytes' cell membrane.[22]The allosteric properties of the enzyme depend on the fatty acid composition of the red cell membrane.[23]AChEof erythrocyte membrane is very sensitive to changes in the organization, composition and lipid fluidity of the erythrocyte membrane.[24,25,26]

It has been reported that AChE activity is influenced by membrane surface phenomena.[27] A decrease in the erythrocyte membrane Sialic acid content has been reported during aging.[28] Sialic acid constitutes the principal charged component of the membrane. Its loss from the erythrocyte surface during aging may be expected to reduce the total negative charge on the surface of the erythrocytes, and consequently their membrane potential.[29]

Conclusion:

It has been found by enzymatic assays that TPPS, FeTPPS and FeNOTPPS are potent inhibitors of Acetylcholinesterase (AChE) activity. FeNOTPPS is a nanomolar inhibitor *in vitro* and inhibits AChE activity more effectively than both FeTPPS and TPPS. These Porphyrin derivatives represent a new class of compounds which are promising candidates for therapeutics involving AChE inhibition.

References:

1. Metz J, Bradlow BA, Lewis SM, Dacie JV. The acetylcholinesterase activity of the erythrocytes in paroxysmal nocturnal haemoglobinuria in relation to the severity of the disease.Br J Haematol 1960 6:372–80.

2. Low MG, Finean B. Non-lytic release of Ach. E from erythrocytes by a phosphatidy inositol specific phosphatase C. FEBS Lett 1977 82:143–6.

3. Stock TL. The organization of protein in the human red blood cell membrane J Cell Biol l974 62(1):1–19.

4. Mabood SF. Kinetic characteristic of Ach E. of erythrocyte of warm and cold blooded species of vertebrates. Pak J SciInd Res 1981:24:127–34.

5. Khan H, Ali Khan M, Hussain I. (2007) Enzyme inhibition activities of the extracts from rhizomes of Gloriosasuperba Linn (Colchicaceae). J EnzInhib Med Chem 22, 722-725.

6. Khan H, Tariq SA, Khan MA, Inayat-Ur-Rehman, Ghaffar R, Saifullah. (2011) Cholinesterase and lipoxygenaseinhibition of whole plant Withaniasomnifera.Afri J Pharm Pharmacol 5, 2272-2275.

7. Rozzini L, ViciniChilovi B, Bertoletti E, Trabucchi M, Padovani A. (2007) Acetylcholinesteraseinhibitors and depressive symptoms in patients with mild to moderate Alzheimer's disease.Aging ClinExp Res 19, 220-223.

8. Niazi ID, Tariq SA, Ali A. (2011) Effect of doxorubicin and daunorubicin on the activity of acetylcholinesterase in acute lymphoblastic leukamia. J Abbott Med Coll 23, 45-47.

9. Sramek JJ, Cutler NR. RBC cholinesterase inhibition: a useful surrogate marker for cholinesterase inhibitor activity in Alzheimer disease therapy? Alzheimer Dis AssocDisord. 2000 14(4):216-27.

10. RashmiJha, Syed Ibrahim Rizvi Age-Dependent Declinein ErythrocyteAcetylcholinesterase Activity: Correlationwith Oxidative StressBiomed Pap Med FacUnivPalacky Olomouc Czech Repub. 2009, 153(3):195-198.

11. Ott, P. and Brodbeck, U. (1978) Multiple molecular forms of acetylcholinesterase from human erythrocyte membranes. Eur. J. Biochem. 88, 119-125.

12. Dutta-Choudhury, T.A. and Rosenberry, T.L. (1984) Human erythrocyte acetylcholinesterase is an amphiphatic protein whose short membrane-binding-domain is removed by papain. J. Biol. Chem. 259, 5653-5660.

13. Rosenberry, T.L. and Scoggin, D.M. (1984) Structure of human erythrocyte acetylcholinesterase. J. Biol. Chem. 259, 5643-5652.
14. Falk, JE Porphyrins and Metalloporphyrins, Elsevier, New York, 1975.
15. E.B. Fleischer, J.M. Palmer, T.S. Srivastava, A. Chatterjee, J. Am. Chem. Soc. 93 (1971) 3162.

16. Beutler E. Red cell metabolism: A manual of Biochemical methods. 3rd edition.Grune and Stratton Orlando; 1984.

17. Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. A new and rapid colorimeteric determination of acetylcholinesterase activity.BiochemPharmacol 1961; 7: 88–95.

18. Brandy J. White, H. James Harmon Biosensors and Bioelectronics 17(6–7) 2002, 463–469.

19. Lindvig, P. E.; Greig, M. E.; Peterson, S. W.: Studies on permeability. V.: The effect of acetylcholine andphysiostigmine on lhe permeability of human erythrocytes tosodium and potassium. Arch. Biochem. Biophys., 1951; 30:241-250.

20. VanderKloot, W. O.: Cholinesterase and sodium transportby frog muscle. Nature, 1956; 178: 366-367.

21. Postnov, Y. V.; Orlov, 5. N.: Ccli membrane alterationas a source of primary hypertension. J. Hyper., 1984; 2: 1-6.

22. Frohlich, E. D.; Pfaffer, N. A.: Adrenergic mechanisms in human and SHR hypertension. Clin. Sci. Molec. Med., 1975; 48: 225s-238s.

23. Koelle, G. B.: Cytological distribution and physiological function of cholinesterase. In: GB KoelIe (ed), Handbook of Experimental Pharmacology, vol.v15, Springer VerIag, Berlin
1963, pp. 701-740.

24. Ullah H, Khan ZM, Mabood SF. (1990) Kinetic alteration in MichaelisMentin parameters in human erythrocyticacetylcholinesterase in splenomegally. J ChemSoc Pak 12, 27-29.

25. Ullah H, Rab F, Mabood SF. (1993) Charcterisation of an assay for kinetic studies of acetylcholineesterase. J Postgrad Med Inst 7, 42-45.

26. Klajnert B, Sadowska M, Bryszewska M. The effect of polyamidoaminedendrimers on human erythrocyte membrane acetylcholinesterase activity.Bioelectrochem 2004; 65: 23-6.

27. Livne A, Bar-Yaakow 0. Sensitivity of erythrocyte acethylcholinesterase to inhibition by linolenoyl sorbitol.Dependence on a transmembrane potential BiochimBiophysActa 1976; 419: 358-64.

28. Mazzanti L, Rabini RA, Salvolini E, Tesei M, Martarelli D, Venerando B, et al. Sialic Acid, Diabetes, and Aging: A Study on the Erythrocyte Membrane. Metabolism 1997; 46 (1): 59-61.

29. Kempf C, Klausner RD, Weinstein JN, Van Renswoude J, Picus M, Blumenthal R. Voltage-dependent trans-bilayer orientation of melittin. J BiolChem 1982 257: 2469-76.