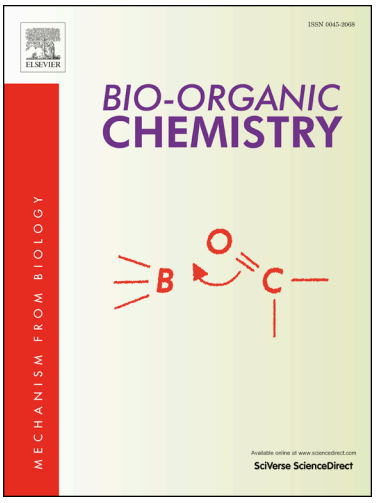

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PII: S0045-2068(18)31417-2
DOI: https://doi.org/10.1016/j.bioorg.2019.02.008
Reference: YBIOO 2783

To appear in: *Bioorganic Chemistry*

Received Date: 5 December 2018
Revised Date: 28 January 2019
Accepted Date: 3 February 2019

Please cite this article as: K. Kucukoglu, H. Inci Gul, P. Taslimi, I. Gulcin, C.T. Supuran, Investigation of Inhibitory Properties of Some Hydrazone Compounds on hCA I, hCA II and AChE Enzymes, *Bioorganic Chemistry* (2019), doi: https://doi.org/10.1016/j.bioorg.2019.02.008

Investigation of Inhibitory Properties of Some Hydrazone Compounds on hCA I, hCA II and AChE Enzymes

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Abstract

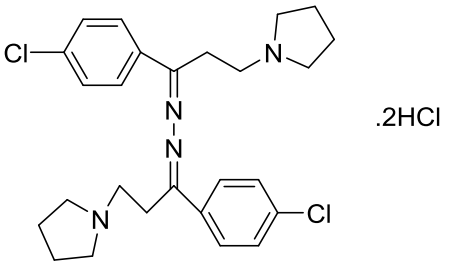
Recently, inhibition of carbonic anhydrase (hCA) and acetylcholinesterase (AChE) have appeared as a promising approach for pharmacological intervention in a variety of disorders such as glaucoma, epilepsy, obesity, cancer, and Alzheimer's disease. Keeping this in mind, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6,** were synthesized to investigate their inhibitory activity against hCA I, hCA II, and AChE enzymes. All compounds in **N, P,** and **R**-series inhibited hCAs (I and II) and AChE more efficiently than the reference compounds acetazolamide (AZA), and tacrine. According to the activity results, the most effective inhibitory compounds were in **R**-series with the K_i values of 203±55-473±67 nM and 200±34-419±94 nM on hCA I, and hCA II, respectively. N,N'-Bis[1-(4-fluorophenyl)-3-(morpholine-4-yl)propylidene]hydrazine dihydrochlorides, **N8,** in **N**-series, N,N'-Bis[1-(4-hydroxyphenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides, **P4,** in **P**-series, and N,N'-bis[1-(4-chlorophenyl)-3-(pyrrolidine-1-yl)propylidene]hydrazine dihydrochlorides, **R5,** in **R**-series were the most powerful compounds against hCA I with the K_i values of 438±65 nM, 344±64 nM, and 203±55 nM, respectively. Similarly, **N8, P4,** and **R5** efficiently inhibited hCA II isoenzyme with the K_i values of 405±60 nM, 327±80 nM, and 200±34 nM, respectively. On the other hand, **P**-series compounds had notable inhibitory effect against AChE than the reference compound tacrine and the K_i values were between 66±20 nM and 128±36 nM. N,N'-Bis[1-(4-fluorophenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides, **P7,** was the most potent compound on AChE with the K_i value of 66±20 nM. The other most promising compounds, N,N'-bis[1-(4-hydroxyphenyl)-3-(morpholine-4-yl)propylidene]hydrazine dihydrochlorides, **N4** in **N**-series and N,N'-bis[1-(4-hydroxyphenyl)-3-(pyrrolidine-1-yl)propylidene]hydrazine dihydrochlorides, **R4** in **R**-series were againts AChE with the K_i values of 119±20 nM, 88±14 nM, respectively.

Keywords: hydrazone; Mannich base; carbonic anhydrase; acetylcholinesterase; enzyme inhibition

Highlights

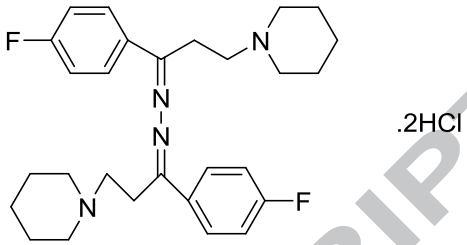
- **N, P, R**-series hydrazone derivatives, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, were tested against hCAs and AChE.
- K_i values were in the range of 203±55 - 738±84 nM against hCA I.
- K_i values were in the range of 200±34 - 710±88 nM against hCA II.
- K_i values were in the range of 66±20 - 308±109 nM against AChE.
- The compounds had potent activity against hCA I, hCA II and AChE.

GRAPHICAL ABSTRACT



R5 (K_i 203±55 nM against hCA I)

(K_i 200±34 nM against hCA II)



P7 (K_i 66±20 nM against AChE)

Lead compounds of the study

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc containing metallo-enzymes and catalyze the hydration reaction of carbon dioxide into bicarbonate in living organisms[1]. There are seven genetically distinct CA families in Bacteria, Archaea, and Eukarya: α-, β-, γ-, δ-, ζ-, η-, and θ-[1-5]. CA isoforms present in various tissues in the cytoplasm, cell membrane, and mitochondria in humans[6] are involved in many physiological and pathological processes such as pH and CO_2 homeostasis, respiration, calcification, bone resorption, electrolyte secretion, biosynthetic reactions (as lipogenesis and gluconeogenesis), tumorigenicity, etc.[7,8] CA inhibitors are used for decades as diuretics[9], antiglaucoma agents[1,10], antiepileptics[11,12]. CA inhibitors have potential as anti-obesity and anti-infective agents[13,14]. More recently, it has been shown that not only CA IX and CA XII but also CA I and CA II isoenzymes have possible roles in tumors as potential targets for cancer therapy[15-17]. Because of involving in these vital processes, CA isozymes have been considerable targets for medicinal chemists[16-28].

Acetylcholinesterase enzyme (AChE, E.C. 3.1.1.7) which is available in all over the peripheral and central neural systems of humans and animals catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline and acetate[29-33]. In accordance with cholinergic hypothesis, imbalances in the cholinergic pathways cause the emerging of neurodegenerative illnesses such as depression, schizophrenia, and Alzheimer's disease (AD)[19,34,35]. AChE inhibitors have been shown to improve cognitive function and these inhibitor compounds including donepezil, tacrine, huperzine A, galanthamine, and rivastigmine have been used as fundamental drugs in AD therapy. Furthermore, in the treatment of glaucoma and Myasthenia gravis AChE inhibitors are used to modulate cholinergic function[32,33].

Hydrazones are a special group of compounds which are synthesized generally by the reaction of a stoichiometric amount of substituted hydrazines/hydrazides and carbonyl compounds such as aldehydes and ketones in suitable solvent under reflux condition[36]. Hydrazones, RR-C=N-R'R'', have two connected nitrogen atoms with different nature. C=N double bond conjugated with a lone electron pair of the terminal nitrogen atom is available in hydrazone molecule. The physical and chemical properties of hydrazones are usually connected to these structural frangments. Nitrogen atoms that are in the hydrazone group have nucleophilic character, moreover the amino type nitrogen is more reactive. In contrast, the carbon atom of hydrazone group has electrophilic and nucleophilic character[37]. Hydrazones and their derivatives have a great importance in chemistry since they are used as intermediates for the syntheses of heterocyclic compounds, which are possible ligands for metal complexes and drug design[38]. Hydrazones can be easily synthesized, crystallized, and have increased hydrolytic stability relative to imines. Because of these favourable properties, hydrazones have been highly studied compounds for a long time. Hydrazones have been reported to have antibacterial[39,40], anticonvulsant[41,42], antitubercular[43], antiplatelet[44], antitumoral[45,46], cytotoxic[47-51] and antiviral[52] activities.

A reactive hydrogen atom, formaldehyde, and secondary amines react together to synthesize aminomethylated compounds, namely Mannich bases, ordinarily[53]. Mannich bases have a great importance in medicinal chemistry and there are some sub Mannich base types such as carbon Mannich bases and nitrogen Mannich bases[54]. Various biological activities had been found in compounds which had Mannich base scaffold as antimicrobial[55-57], antioxidant[58], anti-inflammatory[59,60], antifungal[61,62], cytotoxic and anticancer[23,63-73] and CAs inhibitory[28,74] activities.

In our research laboratory, we designed and synthesized some hydrazone compounds, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N, P,** and **R**-series by

using precursor mono-Mannich bases having 1-aryl-3-heteroaryl-1-propanone structures, and evaluated their cytotoxic activities, and already published (Table 1)[49-51]. Here, we investigated the inhibitory properties of these hydrazone compounds we had presented before against hCA I, hCA II, and AChE (Table 2).

2. Results and discussion

2.1. Chemistry

The synthesis of N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6,** was outlined in Scheme 1. First, corresponding acetophenones were reacted with paraformaldehyde, amine (morpholine HCl; **N**-series, piperidine HCl; **P**-series or pyrrolidine; **R**-series) and HCl (37%) in ethanol. In the second step, mono-Mannich bases obtained were stirred with hydrazine hydrate to give final hydrazone compounds (**N, P,** and **R**-series) in ethanolic acetic acid (%3 w/v). Experimental details, data, and spectral analysis of hydrazones had been presented in our previous studies (Table 1)[49-51].

2.2. Enzyme inhibition results

In this paper, we evaluated the effects of N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6** derivatives on hCA I, hCA II, and AchE enzymes.

α-CAs are made up of 16 isoenzymes and expressed widespread in mammals and humans. These 16 isoenzymes have thioesterase or esterase activity[75]. In some diseases such as cancer, activation or aberrant expression of some isoenzymes of α-CAs is observed so medicinal chemists are interested in the design and development of novel compounds having CAs inhibition properties[76]. Methazolamide, acetazolamide, and dorzolamide which inhibited

hCA are used for the treatment of glaucoma. Furthermore, acetazolamide is the most widespread hCA inhibitor[77]. As AChE inhibitors have been used for the symptomatic treatment of AD, which is characterized by decreased cholinergic transmission, formation of tangles, and amyloid plaques and neuronal loss, they have a great utilization worldwide[78,79]. However, most of the AChE inhibitors available have intense side effects, novel molecules with more powerful and decreased non-desirable effects are urgently needed[80]. In this study, inhibitory effects of N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6** on the activity of hCA I, hCA II, and AChE enzymes were tested under *in vitro* conditions. The following results are presented in Table 2:

Abnormal levels of CA I enzyme in the blood is a marker for hemolytic anemia[81]. All the compounds, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6,** inhibited the slow cytosolic isoform hCA I with K_i values ranging between 203±55 and 738±84 nM. **N**-series compounds showed the inhibitory effect on hCA I with the K_i values of 438±65-738±84 nM, **P**-series compounds inhibited hCA I with the K_i values of 344±64-608±53 nM. 4-Fluoro derivative **N8** was the most powerful compound with the K_i values of 438±65 nM in **N**-series compounds, 4-hydroxy derivative **P4** had the best inhibitory effect on hCA I enzyme with the K_i values of 344±64 nM in **P**-series compounds. The best inhibitory results on hCA I enzyme was found with **R**-series compounds bearing pyrrolidine as a heteroaryl ring with the K_i values of 203±55-473±67 nM. And among **N, P,** and **R**-series compounds the most powerful compound was **R5,** N,N'-bis[1-(4-chlorophenyl)-3-(pyrrolidine-1-yl)propylidene]hydrazine dihydrochlorides, with the K_i values of 203±55 nM. The standard and clinically used drug acetazolamide (AZA) demonstrated a K_i value of 983±119 nM (Table 2). Thus, the investigated compounds had better inhibitory properties compared to AZA.

Additionally, CA II isozyme is often related to some diseases such as glaucoma, osteoporosis, and renal tubular acidosis[76]. All hydrazone compounds tested againts hCA II showed notable inhibitory effects with the K_i values ranging between 200±34 and 710±88 nM. **N, P, R**-series compounds inhibited hCA II with the K_i values of 405±60 nM -710±88 nM for **N**-series, 327±80 nM-483±102 nM for **P**-series, and 200±34 nM-419±94 nM for **R**-series. The most potent compounds among them were **N8, P4,** and **R5** on hCA II isoenzyme. **N8** had the K_i value of 405±60 nM whereas **P4** inhibited hCA II with the K_i value of 327±80 nM. The most effective compound was **R5** that had the K_i value of 200±34 nM against hCA II in all hydrazone compounds. The reference compound AZA had the K_i value of 904±127 nM against hCA II, so all hydrazone compounds tested had better inhibitory profile compared to AZA (Table 2).

Overall, **N, P,** and **R**-series compounds showed excellent inhibitory activity on AChE with the K_i values of 119±20-290±59 nM for **N**-series, 66±20-128±36 nM for **P**-series, and 88±14-308±109 nM for **R**-series. Unlike the inhibitory results on hCA I and hCA II, **P**-series compounds had the most excellent inhibitory effect on AChE. N,N'-Bis[1-(4-hydroxyphenyl)-3-(morpholine-4-yl)propylidene]hydrazine dihydrochlorides, **N4,** had the K_i value of 119±20 nM in **N**-series compounds whereas **R4** which was a 4-hydroxy derivative showed inhibitory effect with the K_i value of 88±14 in **R**-series compounds towards AChE. The most potent compound was N,N'-bis[1-(4-fluorophenyl)-3-(piperidine-1-yl)propylidene]hydrazine dihydrochlorides, namely **P7,** which had a 66±20 nM of the K_i value in three hydrazone series tested. Tacrine, used as a standard AChE inhibitor in this study, inhibited AChE with the K_i value of 358±72 nM. Thus, these results show **N, P, R**-series compounds had better inhibitory profile than the reference compound tacrine. In addition, **P**-series were more selective than the others (Table 2).

3. Conclusion

In this study, some hydrazones synthesized, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, **N1-N11, P1, P4-P8,** and **R1-R6,** tested against hCA I, hCA II, and AChE. All compounds effectively inhibited metabolic enzymes of carbonic anhydrase and acetylcholinesterase. The most potent compounds having inhibitory effect on hCA I and hCA II were **N8, P4,** and **R5.** They inhibited efficiently hCA I with the K_i values of 438±65 nM, 344±64 nM, and 203±55 nM, respectively. And the K_i values of **N8, P4,** and **R5** against hCA II were 405±60 nM, 327±80 nM, and 200±34 nM, respectively. In the inhibitory activity results against AChE, **N4, P7,** and **R4** were the most promising compounds with the K_i values of 119±20 nM, 66±20 nM, and 88±14 nM, respectively. These compounds stand out promising candidates for further studies.

4. Experimental section

4.1. General information

All commercially available reagents were purchased from Merck AG, Fluka AG, Acros Organics, Riedel-de Haën, J. T. Baker or Sigma-Aldrich Chemie and used without further purification. Melting points were measured on an Electrothermal 9100 melting point apparatus (IA9100, Electrothermal, Essex, UK). ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded employing a Varian 400 MHz FT spectrometer (Danbury, USA) for **N**, **P,** and **R-**series hydrazone derivatives, while ^{1}H NMR (60 MHz) spectra were recorded on a Varian EM-360 spectrometer for **Nm, Pm,** and **Rm** compounds (precursor mono-Mannich bases).

*4.2. Synthesis of precursor mono-Mannich bases, 1-aryl-3-(heteroaryl)-1-propanone hydrochlorides, **(N1m-N11m, P1m, P4m-P8m, and R1m-R6m),** (Scheme 1)*

They were reported in our previous studies[49-51].

4.3. Synthesis of hydrazone compounds, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine dihydrochlorides, (**N1-N11, P1, P4-P8, R1-R6,** Scheme 1)

They were reported in our previous studies[49-51].

4.4. Biochemical studies

4.4.1. hCA I and hCA II isoenzymes purification and inhibition studies

To observe the inhibition effects of **N**, **P**, **R**-series hydrazone compounds (**N1-N11, P1, P4-P8,** and **R1-R6**) on hCA I, and II isoforms, these enzymes were purified from fresh human erythrocyte using an affinity chromatography by the procedures of Verpoorte *et al.*[82] as in our previous studies[18-25,27,83,84] and the inhibitory effects were determined by spectrophotometric procedure[16-28]. In this procedure, changes in activity were obtained during 3 min at 22^{o}C. *p*-Nitrophenylacetate (PNA) compound was used as a substrate, and it was converted by both isoforms to *p*-nitrophenolate ions. The quantity of protein was measured according to the previously described Bradford method[85] and bovine serum albumin was used as the standard. After the purification method of the CA isoforms, samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). The change in activity was spectrophotometrically obtained at 348 nm. The IC_{50} values were calculated from activity (%) against compounds inhibition. Three different concentrations were used to calculate K_i values.

4.4.2. AChE activity determination

The inhibitory efficacy of the **N**, **P**, **R**-series hydrazone compounds (**N1-N11, P1, P4-P8,** and **R1-R6**) on AChE activity was tested following the spectrophotometric process of Ellman's test[18,19,24,86]. Acetylthiocholine iodide (AChI) was used as substrates. For the mensuration of the AChE activity, 5,5′-dithio-bis(2-nitro-benzoic)acid compound (DTNB, D8130-1G, Sigma-Aldrich, Steinheim, Germany) was used. Briefly, 50 µl DTNB and 100 µl of Tris–HCl solution (1 M, pH 8.0), 750 ml of sample solution dissolved in distilled water at

disparate concentrations, and 50 µl AChE (5.32×10^{-3} U) solution were incubated and mixed for 15 min at 30°C. Finally, the reaction was started by adding 50 µl of AChI. The enzymatic hydrolysis of this substrate that produces a yellow 5-thio-2-nitrobenzoate anion as the result of the product of thiocholine with DTNB was recorded spectrophotometrically at a wavelength of 412 nm.[24] Tacrine (TAC) was used as a reference compound.

Acknowledgements

This study was supported by the Research Foundation of Atatürk University Erzurum (Turkey).

Conflict of interest

There is no conflict of interest.

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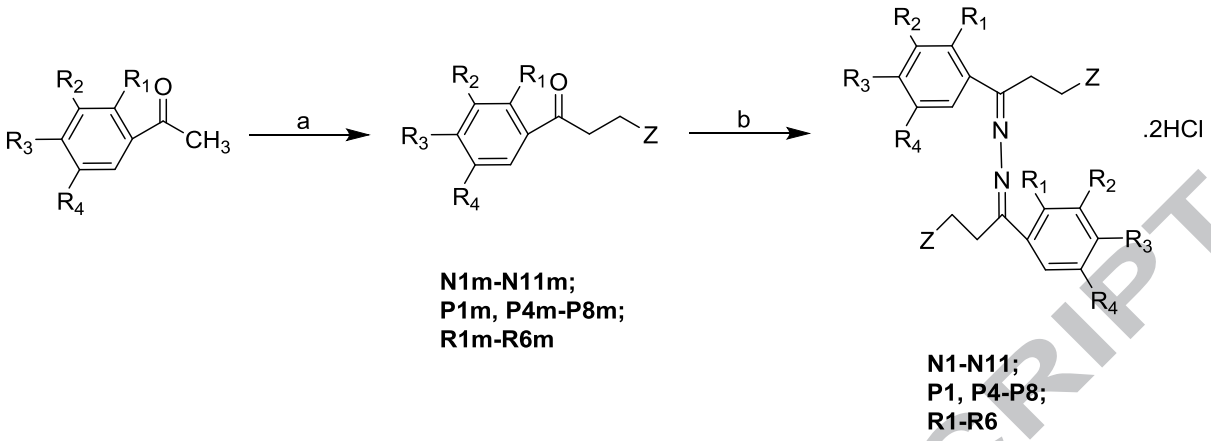

Z : Morpholine-4-yl for **N**-Series, Piperidine-1-yl for **P**-Series, Pyrrolidine-1-yl for **R**-Series

Scheme 1. Synthesis of Hydrazone Compounds, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine Dihydrochlorides **(N1-N11; P1, P4-P8; and R1-R6).** Reagents and conditions: (a) Paraformaldehyde, piperidine HCl/morpholine HCl/Pyrrolidine, HCl (37%) and EtOH, 1-9 h reflux for **N1m-N11m; P1m, P4m-P8m; R1m-R6m;** (b) Ethanolic acetic acid (3% w/v), hydrazine hydrate stirring for 17-26 h exception **R1** for **N1-N11; P1, P4-P8; R2-R6** and 3 h reflux for **R1.**

Table 1. Synthesized Hydrazone Compounds, N,N'-bis[(1-aryl-3-heteroaryl)propylidene]hydrazine Dihydrochlorides **(N1-N11; P1, P4-P8;** and **R1-R6).**

Compound	Substitution on Phenyl Ring	Yield (%)
N1	-	24[50]
N2	4-CH_3	26[50]
N3	4-OCH_3	67[50]
N4	4-OH	56[50]
N5	4-Cl	60[50]
N6	2-OH	16[50]
N7	3-OCH_3	9[50]
N8	4-F	52[50]
N9	4-Br	56[50]
N10	3-OH	86[50]
N11	2-OCH_3	74[50]
P1	-	57[49]
P4	4-OH	64[49]
P5	4-Cl	48[49]
P6	3-OCH_3	88[49]
P7	4-F	12[49]
P8	4-Br	14[49]
R1	-	35[51]
R2	4-CH_3	6[51]
R3	4-OCH_3	34[51]
R4	4-OH	50[51]
R5	4-Cl	24[51]
R6	3-OCH_3	7[51]

Table 2. Enzyme inhibition results of hydrazone compounds, **N1-N11; P1, P4-P8; R1-R6,** against hCA I, hCA II and AChE enzymes.

Compound	**IC_{50} (nM)**						**K_i (nM)**		
	hCA I	**r^2**	**hCA II**	**r^2**	**AChE**	**r^2**	**hCA I**	**hCA II**	**AChE**
N1	704.28	09814	684.73	0.9598	308.84	0.9817	730±100	703±67	206±39
N2	694.18	0.9911	652.04	0.9865	348.03	0.9811	738±84	683±128	200±50
N3	728.40	0.9803	692.84	0.9911	331.83	0.9845	709±110	678±105	248±58
N4	548.18	0.9716	507.83	0.9793	173.18	0.9490	559±78	500±59	119±20
N5	601.73	0.9598	573.84	0.9582	238.37	0.9709	628±93	602±195	186±42
N6	572.06	0.9901	538.91	0.9704	208.74	0.9638	601±104	553±94	149±29
N7	737.03	0.9881	693.84	0.9793	385.01	0.9918	704±203	710±88	290±59
N8	483.08	0.9937	429.05	0.9488	273.98	0.9726	438±65	405±60	209±83
N9	508.36	0.9638	483.27	0.9937	207.38	0.9917	501±90	471±54	146±48
N10	551.04	0.9810	503.98	0.9858	198.97	0.9820	592±148	529±102	130±35
N11	700.88	0.9717	649.83	0.9695	228.16	0.9672	684±111	666±118	154±46
P1	583.77	0.9716	522.64	0.9905	197.73	0.9518	608±53	573±92	105±21
P4	359.63	0.9812	308.94	0.9728	116.30	0.9704	344±64	327±80	84±17
P5	424.63	0.9764	383.64	0.9935	100.43	0.9822	483±102	421±73	68±17

P6	522.54	0.9699	461.53	0.9816	174.62	0.9712	501±93	483±102	128±36
P7	403.42	0.9866	374.15	0.9782	92.53	0.9890	439±60	388±73	66±20
P8	384.51	0.9682	330.62	0.9923	126.93	0.9609	403±111	369±71	100±32
R1	403.72	0.9716	371.53	0.9822	304.82	0.9712	458±83	401±83	243±48
R2	484.72	0.9816	409.64	0.9633	369.26	0.9973	473±67	419±94	308±109
R3	405.17	0.9529	400.63	0.9812	312.55	0.9891	411±134	364±49	251±79
R4	253.17	0.9910	218.26	0.9726	105.82	0.9498	243±43	216±58	88±14
R5	234.92	0.9582	233.83	0.9294	113.84	0.9683	203±55	200±34	100±16
R6	374.92	0.9717	357.12	0.9728	288.02	0.9723	411±99	384±107	227±98
AZA	997.304	0.9889	915.50	0.9719	-	-	983±119	904±127	-
Tacrine	-	-	-	-	443.312	0.9948	-	-	358±72