

Enzyme Inhibition Studies of Antipyrine and Aminopyrine

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Abstract

The study investigated the enzyme inhibition effects of antipyrine and aminopyrine against two human carbonic anhydrase isoforms (hCA I and hCA II), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The effectiveness of these compounds as possible carbonic anhydrase (CA) and cholinesterase (ChE) inhibitors is evident from the nanomolar range IC₅₀ and K_i values obtained. The results demonstrated that aminopyrine was a more effective inhibitor compared with antipyrine. Moreover, molecular docking results obtained from the online docking server SwissDock supported the inhibition activity results.

Keywords: Aminopyrine, Antipyrine, Acetylcholinesterase, Butyrylcholinesterase, Carbonic Anhydrase, Pyrazolone

Introduction

Pyrazolone is the name given to the heterocyclic five membered ring which consists of two nitrogen atoms and a ketonic group. Since the end of the 19th century, there has been a continuous interest in pyrazolone derivatives and many research groups have reported its various physical and chemical properties. Furthermore, compounds possessing the pyrazolone skeleton have attracted much attention due to their wide range of biological activities, which includes

antitumor, antibacterial, antifungal, anti-inflammatory, antitubercular, hypoglycemic, and analgesic (MARKOVIC et al. 2011; RAGAVAN et al. 2009; SONI et al. 2011; GUNASEKARAN et al. 2011; DAS et al. 2008; SRIVALLI et al. 2011). The pyrazolone derivatives antipyrine (**1**) and aminopyrine (**2**) are well known anti-inflammatory, analgesic and antipyretic drugs (Figure 1). These heterocyclic compounds are also important intermediates in organic and medicinal chemistry. Even though these drugs have been meticulously studied, their carbonic anhydrase (CA), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition activities have not been reported.

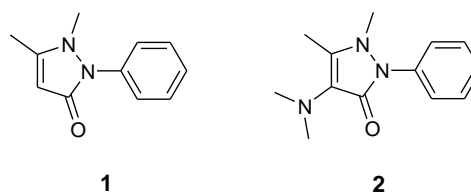


Figure 1. Structures of Antipyrine (**1**) and Aminopyrine (**2**).

Carbonic anhydrases (CAs) are enzymes responsible for life. The sixteen CA isoforms found in humans are involved in numerous physiological and pathological processes, which make them important targets for the design of inhibitors and activators to treat a wide range of disorders (SUPURAN 2008, SUPURAN 2016). Treatment of edema, altitude sickness, obesity, cancer, epileptic seizures, neuropathic pain, and glaucoma has been achieved by inhibition of CA (SUPURAN 2007). Activation of CA has recently been reported to enhance cognition, which is a significant discovery for Alzheimer's disease (AD) research (SUPURAN 2018). Although a wide range of carbonic anhydrase inhibitors (CAIs) are available, they remain non-isoform selective which results in unfavorable side

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effects. In light of this, various compounds are being designed and screened for their enzyme inhibition potential.

Cholinesterases (ChEs) are enzymes which hydrolyze the neurotransmitter acetylcholine (ACh). Two main types of ChEs are found in vertebrates namely AChE and BuChE, which differ in their tissue distribution, substrate specificity, and kinetic properties (AUGUSTINSSON 1948; STEPANKOVA et al. 2008). In neurological disorders such as AD the levels of ACh in the brain is substantially minimized, which leads to impaired communication between the nerve cells and thus a significant loss of cognitive and behavioral function. Cholinesterase inhibitors (ChEIs) prevent the rapid breakdown of ACh, allowing more of the neurotransmitter to be available for neurotransmission, which temporarily improves the symptoms of AD.

The objective of the present study was to quantitatively evaluate the inhibitory activity of antipyrine and aminopyrine against the human CA isoforms I and II (hCA I and hCA II), AChE and BuChE. In addition, molecular docking studies were performed to investigate the possible interactions between the two test compounds with the four enzymes.

Materials and Methods

Chemicals and Enzymes

Antipyrine, aminopyrine and all the compounds needed for the inhibition assays were purchased from Sigma Aldrich and used as received without further purification. AChE lyophilisate from the electric eel and BuChE lyophilisate from equine serum was obtained from Sigma Aldrich and stored at -20 °C. All solvents were purchased from Merck and freshly distilled. Distilled water was used for all experiments.

CA I and CA II inhibition assay

Purification of the CA isozymes were achieved by the method reported in a previous study (STELLENBOOM 2019). The esterase activity was determined spectrophotometrically (Thermo Scientific Evolution 200 Series UV-VIS Spectrophotometer)

according to the method reported by Verpoorte and co-workers (VERPOORTE et al. 1976). The test compound (1 mg) was dissolved in dimethyl sulfoxide (1 mL) and diluted with distilled water. In a quartz cuvette the following was added: 200 μ L buffer (0.5 M, pH 7.4: Tris-SO₄ buffer), 395 μ L 4-nitrophenylacetate (3 mM), 350 μ L water and 5 μ L enzyme solution. The test compound was added in 10 μ L increments (10-60 μ L). A control (no inhibitor) and blank (no enzyme) measurement was recorded. Acetazolamide was used as a positive control. The concentration of the inhibitor needed to reduce the enzyme activity by half (IC₅₀) was calculated from activity (%) vs [inhibitor] graphs. The inhibition constants (K_i) were calculated from the Cheng-Prussoff equation (CHENG et al. 1973).

AChE and BuChE inhibition assay

A slightly modified version of Ellman's spectrophotometric method was utilized to determine the inhibitory potential of the test compounds against AChE and BuChE (ELLMAN et al. 1961, ZILBEYAZ et al. 2018). The test compound (1 mg) was dissolved in dimethyl sulfoxide (1 mL) and diluted with distilled water. In a quartz cuvette the following was added: 200 μ L buffer (AChE assay: 1 M Tris-HCL buffer, pH:8.0; BuChE assay: 1 M phosphate buffer, pH:8.0), 100 μ L 0.5 mM 5-mercapto-2-nitrobenzoic acid (DTNB), 100 μ L 10 mM acetylthiocholine or butyrylthiocholine (substrate), 590 μ L water and 10 μ L enzyme. The test compound was added in 10 μ L increments (10-60 μ L). A control (no inhibitor) and blank (no enzyme) measurement was recorded. Rivastigmine was used as a positive control. The IC₅₀ values for the test compounds were calculated graphically and the K_i values were determined.

Molecular Docking

Molecular docking calculations were achieved using the online docking server SwissDock (EADock DSS, Swiss Institution of Bioinformatics, Switzerland, www.swissdock.ch). SwissDock is a protein ligand docking web service which effectively predicts the molecular interactions that may arise between a protein and ligand (small molecule). The crystal structures of CA I (1AZM), CA II (12CA), AChE (1AMN), and BuChE (1XLU) were taken in PDB format, while the

inhibitors (antipyrene and aminopyrine) were taken in ZINC format, which is required for calculations (GROSDIDIER et al. 2011). The docking study provided the docking binding energy values ($\Delta G =$ kcal/mol) of each ligand to each of the four protein models. The results of the modeled and docked structures were viewed using the UCSF Chimera package (PETTERSEN et al. 2004).

Results and Discussion

Drugs containing the pyrazolone skeleton are widely used in clinical practice. In the old days antipyrene and aminopyrine were used as analgesic, antipyretic and anti-inflammatory drugs. Today pyrazolone

derivatives such as sulfinpyrazone (a uricosuric for gout therapy) and phenylbutazone (a drug with analgesic, antipyretic and anti-inflammatory properties used in rheumatoid arthritis therapy) are available. Research groups are continuously designing pyrazolone derivatives in the search for potential bioactive compounds and it caught our attention that not much research has been carried out on the CA and ChEs inhibition potential of pyrazolones derivatives or the parent drugs antipyrene and aminopyrine. We thus decided to investigate the ability of antipyrene and aminopyrine to inhibit CA and ChEs. The results (IC_{50} and K_i values) for the study are summarized in Table 1 and Table 2. Acetazolamide and rivastigmine were used as control drugs.

Table 1. Inhibition activity of antipyrene **1** and aminopyrine **2** against hCA I and hCA II.

Compound	IC_{50} (nM)	IC_{50} (nM)	K_i (nM)	K_i (nM)
	hCA I	hCA II	hCA I	hCA II
1	36.70	42.20	19.11	19.81
2	31.20	29.90	16.25	14.04
Acetazolamide	32.11	51.01	16.20	24.10

Results are reported as means of three independent experiments. Errors are in the range of $\pm 2\%$ of the reported values.

hCA I and hCA II inhibitory activity

The CA inhibition screening revealed that both **1** and **2** exhibited strong inhibition of both cytosolic enzymes (Table 1). IC_{50} values of 36.70 and 31.20 nm were obtained for **1** and **2**, respectively against hCA I. In the case of hCA II, IC_{50} values of 42.20 and 29.90 nm were obtained for **1** and **2**, respectively. The results indicated that **2** was approximately 1.3-fold stronger at inhibiting hCA I and hCA II compared with **1**. The more effective inhibition observed in **2** may be attributed to the tertiary amine moiety at position C4 of the heterocyclic scaffold. This is not surprising as it has been shown that the presence of a tertiary amine group improves

the inhibition activity of inhibitors and thus various structure modifications of pyrazolones might generate more effective inhibitors. Compared with acetazolamide ($IC_{50} = 32.11$ nm) the inhibitory effectiveness of compounds **1** ($IC_{50} = 36.70$ nm) and **2** ($IC_{50} = 31.20$ nm) against hCA I was not adequately impressive. On the other hand, compounds **1** ($IC_{50} = 42.20$ nm) and **2** ($IC_{50} = 29.90$ nm) were 1.21-fold and 1.71-fold more effective at inhibiting hCA II compared with the control drug acetazolamide ($IC_{50} = 51.01$ nm), respectively. K_i values in the range 14.04 and 19.81 nM were calculated for the CA isoforms.

Table 2. Inhibition activity of antipyrene **1** and aminopyrine **2** against AChE and BuChE.

Compound	IC_{50} (nM)	IC_{50} (nM)	K_i (nM)	K_i (nM)
	AChE	BuChE	AChE	BuChE
1	96.10	38.40	46.14	17.07
2	36.20	35.98	17.38	15.99
Rivastigmine	60.21	14.02	29.00	6.01

Results are reported as means of three independent experiments. Errors are in the range of $\pm 2\%$ of the reported values.

AChE and BuChE inhibitory activity

The inhibiting abilities of compounds **1** and **2** against AChE and BuChE were evaluated by a slightly modified version of the method reported by Ellman and co-workers (ELLMAN et al. 1961). Data from Table 2 shows that the presence of the tertiary amine group once again affected the inhibition in a positive way, since **2** was a better AChE and BuChE inhibitor compared with **1**. Compounds **1** ($IC_{50} = 38.40$ nm) and **2** ($IC_{50} = 35.98$ nm) showed a similar inhibition profile

against BuChE, whereas compound **2** was 2.7-fold more potent than compound **1** against AChE ($IC_{50} = 96.10$ nm for **1** and $IC_{50} = 36.20$ nm for **2**). Compared with rivastigmine, compound **1** was a less active inhibitor of both ChEs. Similarly, **2** was less active at inhibiting BuChE compared with the control drug. Compound **2**, however, was approximately 1.7-fold more active compared with rivastigmine ($IC_{50} = 60.21$ nm) against AChE. K_i values in the range 15.99 and 46.14 nM were calculated for the ChEs.

Table 3. Estimated binding free energies of antipyryne **1** and aminopyryne **2** with hCA I, hCA II AChE and BuChE.

Compound	ΔG (kcal/mol)	ΔG (kcal/mol)	ΔG (kcal/mol)	ΔG (kcal/mol)
	hCA I	hCA II	AChE	BuChE
1	-6.70	-6.38	-6.11	-6.99
2	-7.01	-6.97	-6.48	-7.29

Molecular docking studies

In silico docking studies were performed with SwissDock to identify the potential interactions of compounds **1** and **2** with the amino acid residues within the CA I, CA II, AChE and BuChE active sites. The estimated binding energy values (ΔG) for these interactions are summarized in Table 3. The docking results revealed similar binding energy values for **1** and

2 with the various enzymes and as anticipated, these results support the experimental enzymatic inhibition results reported in this paper. The lowest free energy of binding was observed for compound **2** with hCA II and BuChE, which equaled to -6.97 and -7.29 kcal/mol, respectively. Figure 2 illustrates the best possible binding poses of compound **2** in the active sites of hCA II and BuChE.

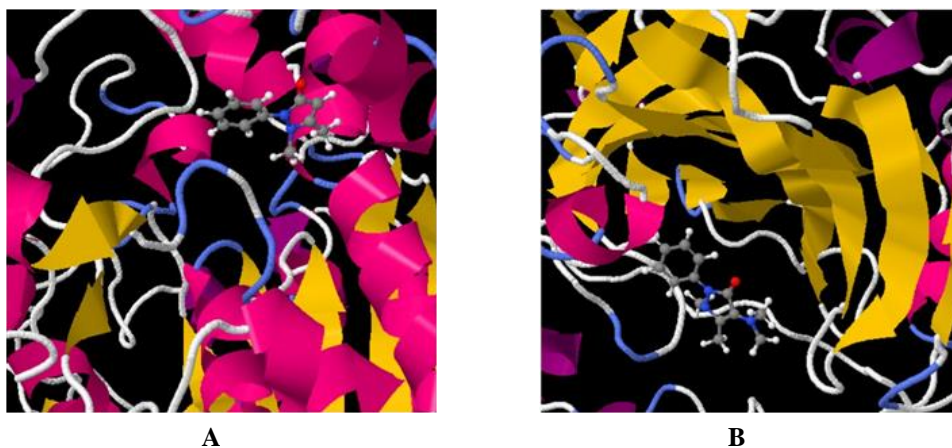


Figure 2. Possible interactions of (A) Aminopyryne with hCA II and (B) Aminopyryne with BuChE.

Conclusion

In summary, antipyryne and aminopyryne were evaluated for their ability to inhibit hCA I, hCA II, AChE, and BuChE, from which nanomolar IC_{50} and K_i values were obtained. The results demonstrated that aminopyryne was a better inhibitor of these enzymes

compared with antipyryne. Our data suggest that the tertiary amine group may enhance the inhibition ability of pyrazolones. The finding of this study is encouraging and these results may be taken into account for the development of improved CA and ChE inhibitors, which is urgently needed. Lastly, a comprehensive structure-activity relationship study

will be undertaken to further shed light on pyrazolone compounds as potential inhibitors of CA or ChEs.

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