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To cite this article: Turan Bayram, Oktay Arslan, Halil Ibrahim Ugras, Umit Cakir & Ozen Ozensoy (2007) Purification of carbonic anhydrase from dog erythrocytes and investigation of *in vitro* inhibition by various compounds, Journal of Enzyme Inhibition and Medicinal Chemistry, 22:6, 739-744, DOI: [10.1080/14756360601114312](https://doi.org/10.1080/14756360601114312)

To link to this article: <https://doi.org/10.1080/14756360601114312>



Published online: 04 Oct 2008.



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Purification of carbonic anhydrase from dog erythrocytes and investigation of *in vitro* inhibition by various compounds

TURAN BAYRAM, OKTAY ARSLAN, HALIL IBRAHIM UGRAS, UMIT CAKIR, & OZEN OZENSOY

Balikesir University, Science & Art Faculty, Department of Chemistry, Cagis Yerleskesi / Kampus, 10100, Balikesir, Turkey

(Accepted in final form 22 September 2006)

Abstract

The enzyme carbonic anhydrase (E.C. 4.2.1.1) has a stimulatory effect on glaucoma, an eye disease that has a risk to dogs, which are models for the human eye disease, that is similar to that in humans.

In this study, some sulfonamide derivatives, 2-(3-cyclohexene-1-carbamido)-1,3,4-thiadiazole-5-sulfonamide (CCTS), 4-(3-cyclohexene-1-carbamido) methyl-benzenesulfonamide (CCBS), 2-(9-octadecenoylamido)-1,3,4-thiadiazole-5-sulfonamide (ODTS), 2-(4,7,10-trioxa-tetradecanoylamido)-1,3,4-thiadiazole-5-sulfonamide (TDTS), and 2-(8-methoxycoumarine-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS), as well as some anionic compounds (perchlorate and chloride) and existing medicines (dorzolamide-HCl, gentamicine sulphate, tropicamide, and procaine-HCl) were assayed for their inhibition of dog carbonic anhydrase (dCA), which was purified from erythrocytes on an affinity gel of L-tyrosine-sulfonamide-Sepharose 4B. ODTS showed the highest potency amongst the synthetic compounds with IC_{50} value 1.18×10^{-5} M. Amongst the medicines tested, only dorzolamide showed inhibition with IC_{50} value 5.05×10^{-4} M. Procaine and tropicamide actually showed an activatory effect, whereas gentamicine sulfate had no significant effect. The inhibitory effects of anionic compounds such as perchlorate and chloride were also investigated; whereas perchlorate showed inhibition, chloride did not.

Keywords: Carbonic anhydrase, dog, affinity chromatography, inhibition, sulfonamides, glaucoma, CA

Introduction

The term “glaucoma” has been simply defined as the process of ocular tissue destruction caused by a sustained elevation of the intraocular pressure (IOP) above its normal physiological limits [1]. It is the specific effect of that elevated pressure upon the composite parts of the optic nerve that renders glaucoma an emergency. The existence of “normal tension” and “low tension” glaucomas in man has blurred this simple definition, for these diagnoses have their origin in the clinical similarities of the optic nerve degeneration seen with elevated IOP and distinct, non-pressure related factors such as disc ischaemia or retinal excitotoxicity. It has even be argued that the rise in IOP seen in primary open-angle glaucoma in

man is an effect rather than the cause, with only the effect being assessed and treated by current therapies. Open-angle glaucoma has limited incidence in domesticated animal species, and we are rarely in a position to diagnose its early presence and thus inhibit ganglion cell degeneration early in the process. There is evidence to indicate that abnormality in ganglion cell function exists in beagle dogs with inherited primary open-angle glaucoma, before the elevation in IOP occurs. There is thus a strong temptation to use this evidence to suggest that the IOP changes themselves are purely a secondary feature to another, as yet ill-defined, disease process. Approximately one-half of one per cent of all dogs in the United States develop this problem. It is much rarer in cats. In animals and man, IOP increases because the

Correspondence: Dr. Ozen Ozensoy, Balikesir University, Science and Art Fac., Dept. of Chem., Cagis Yerl./Kampus, 10100 Balikesir, Turkey. Tel: +90 266 6121263 / 1064. Fax: +90 266 6121215. E-mail: ozensoy@balikesir.edu.tr.

normal channels through which fluid leaves the eye become obstructed [2].

Two forms of glaucoma are recognized: primary and secondary. Primary glaucoma is due to an inherited abnormal angle at the point where the iris meets the cornea. This abnormal angle obstructs the exit of fluid from the eye. Because it is genetic, the second eye often becomes affected within 6–12 months of the first. Middle-aged and older dogs, as well as female dogs, are most at risk of primary glaucoma. While human primary glaucoma is termed open angle glaucoma (beagle dogs also get this), canine primary glaucoma is usually closed angle. Dog breeds commonly affected with closed angle glaucoma are Spaniels, Toy Poodles, Boston Terriers, Dalmatians, Basset Hounds and Huskies. Persian cats are the most susceptible cat breeds to glaucoma. Glaucoma is not a disease that can be cured. Primary glaucoma has a genetic component; pets with this condition should not be bred. Secondary glaucoma usually affects only one eye. This is the most common form in cats. Secondary glaucoma occurs due to inflammation of structures within the eye and there are many causes for such inflammation; infectious or autoimmune disease, trauma or cancer. Scarring (synechia) and distortion at the corneal/iris angle impedes fluid exit; fluid pressure then increases as it does in primary glaucoma. Secondary glaucoma can also occur when the lens is jarred loose from its attachments or when it moves due to degenerative changes in its attachments [3].

An early sign of glaucoma is enlargement of the blood vessels of the sclera, or whites of the eyes, along with impaired vision or blindness. At an early stage, blindness may be reversible. Once blindness is 3–5 days old it is usually irreversible. These eyes often show a bluish, diffuse cloudiness of the cornea and the pupils of these eyes are dilated (mydriasis) because light stops at a damaged retina and cannot reach the optic centers of the brain [4].

The carbonic anhydrase (CA) enzyme (E.C. 4.2.1.1) is found in many tissues of the body including the eye and catalyzes the reversible reaction between the hydration of carbon dioxide and the dehydration of carbonic acid, that is vital in pH homeostasis and CO₂ transport. In humans, CA exists as a number of isoenzymes, the most active being CA-II, found primarily in red blood cells (RBCs), but also in other tissues. Inhibition of CA in the ciliary processes of the eye decreases aqueous humor secretion, presumably by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport. The result is a therapeutic reduction in IOP [5].

In this study, our goal was to investigate the inhibitory effects upon dog carbonic anhydrase (dCA) activity of various compounds originally synthesized by our group as potential CA inhibitors [6], in comparison with some well-known drugs used in the treatment of glaucoma as well as some common anion inhibitors.

More potent inhibitors may be useful in the development of new approaches in glaucoma treatment.

Materials and methods

Chemicals

L-tyrosine and *p*-aminobenzenesulfonamide were from Merck Chem. Co (Milan/Italy). 2-(3-cyclohexene-1-carbamido)-1,3,4-thiadiazole-5-sulfonamide (CCTS); 4-(3-cyclohexene-1-carbamido) methylbenzenesulfonamide (CCBS); 2-(9-octadecenoylamido)-1,3,4-thiadiazole-5-sulfonamide (ODTS); 2-(4,7,10-trioxa-tetradecanoylamido)-1,3,4-thiadiazole-5-sulfonamide (TDTS); and 2-(8-methoxycoumarine-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS) were prepared as previously described [6]. All other chemicals were obtained from Sigma Chem. Co. (Milan / Italy) and were of analytical grade.

Preparation of hemolysate

Blood samples (25 mL), taken from 5 healthy dogs weighing approximately 15 kg each, were anticoagulated with ACD (Acid-citrate-dextrose), centrifuged at 1848 × g for 20 min at 4°C and the supernatant was removed. The packed RBCs were washed three times with 0.9% NaCl and then hemolysed in cold water. The ghosts and any intact cells were removed by centrifugation at 18924 × g for 25 min at 4°C, and the pH of the hemolysate was adjusted to 8.5 with solid Tris-base. The 25 mL hemolysate was applied to an affinity column containing L-tyrosine-sulfonamide-Sepharose-4B [7,8] equilibrated with 25 mM Tris-HCl / 0.1 M Na₂SO₄ (pH 8.5). The affinity gel was washed with 50 mL of 25 mM Tris-HCl / 22 mM Na₂SO₄ (pH 8.5). The dCA was then eluted with 0.1 M NaCH₃COO / 0.5 M NaClO₄ (pH 5.6); fractions of 3 mL were collected and their absorbance was measured at 280 nm.

Protein content determination

A quantitative protein determination was done on the pooled peak of eluted dCA using the Coomassie Brilliant Blue G-250 method [9].

Enzyme assay

Carbonic anhydrase activity was measured by the Maren method [10], which is based on determination of the time required for the pH of a standard solution to decrease from 10.0 to 7.4 due to CO₂ hydration. The assay solution was 0.5 M Na₂CO₃ / 0.1 M NaHCO₃ (pH 10.0) and Phenol Red was added as the pH indicator. One unit of CA activity is defined as that amount of the enzyme that reduces by 50% the time of CO₂ hydration measured in the absence of enzyme.

Inhibition studies

The inhibition of dCA activity by various sulfonamides (CCTS, CCBS, ODTS, TDTS, MCTS), drugs (dorzolamide, procain, tropicamide and gentamicine sulphate) and simple anions (perchlorate and chloride) were studied by determining their effects on the dCA-catalyzed CO_2 hydration rate at 1°C . CO_2 hydration rates were determined at five different inhibitor concentrations using an initial substrate concentration of 70 mM. Regression analysis was carried out on the graph of (percent inhibition) vs (inhibitor concentration). The inhibitor concentration which reduced enzyme activity by 50% (IC_{50}), was then determined by interpolation.

Results and discussion

Inhibitors of carbonic anhydrase play an important role in ophthalmology, where they are used to reduce elevated IOP; this includes dorzolamide (Trusopt) and brinzolamide (Azopt) which are commonly used for glaucoma treatment [11,12]. In this study, our aim was to determine the inhibitory effects of some new compounds (i.e. CCTS, CCBS, ODTS, TDTS and MCTS) compared with Trusopt and a number of other drugs that are used in glaucoma treatment or generally in ophthalmology, i.e. Benoxinate (local anaesthesia) [13], Tropamid (anticholinergy, midriatic) [14], Gentagut (antibacterial) [15]. All compounds were tested against the purified dog carbonic anhydrase enzyme.

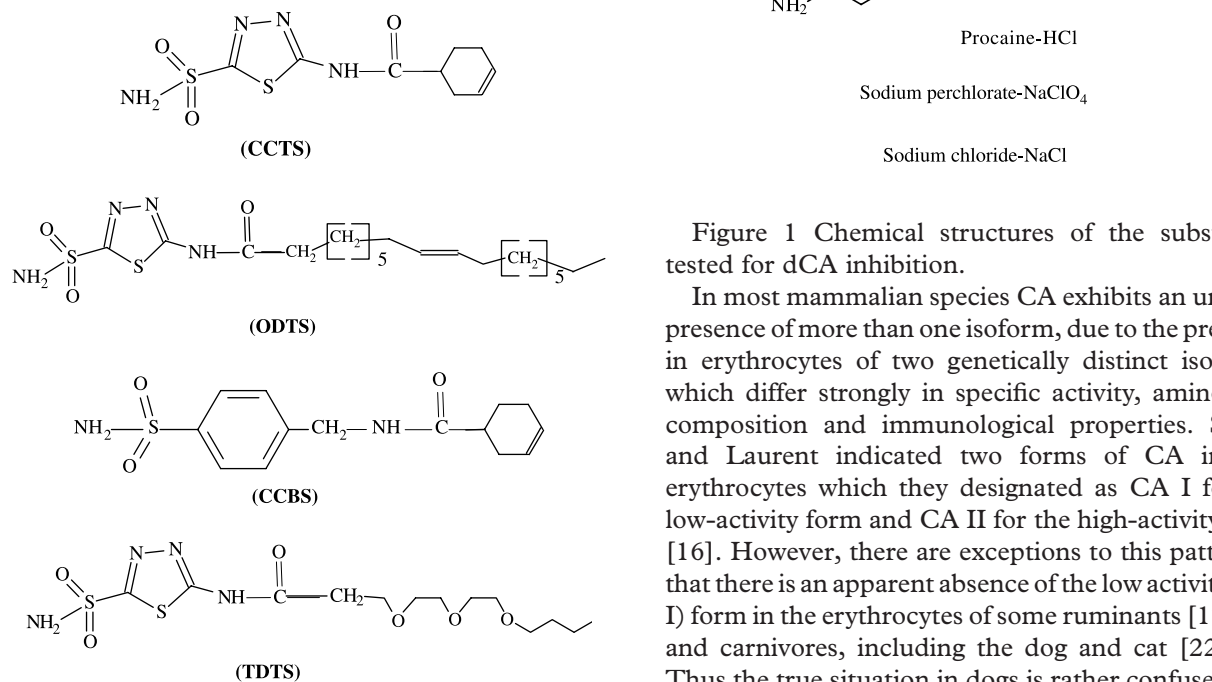


Figure 1 Chemical structures of the substances tested for dCA inhibition.

In most mammalian species CA exhibits an unusual presence of more than one isoform, due to the presence in erythrocytes of two genetically distinct isozymes which differ strongly in specific activity, amino acid composition and immunological properties. Sciaky and Laurent indicated two forms of CA in dog erythrocytes which they designated as CA I for the low-activity form and CA II for the high-activity form [16]. However, there are exceptions to this pattern in that there is an apparent absence of the low activity (CA I) form in the erythrocytes of some ruminants [17–21] and carnivores, including the dog and cat [22–24]. Thus the true situation in dogs is rather confused.

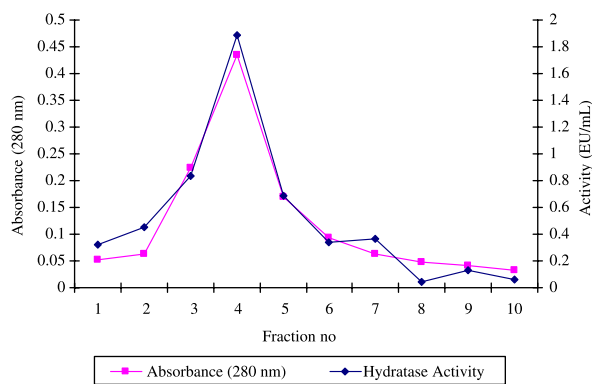


Figure 1. The CO₂-hydratase activity and protein concentration (280 nm absorbance) of dCA fractions eluted from affinity column with 0,1 M NaCH₃COO / 0,5 M NaClO₄ (pH5.6).

We have previously demonstrated the effectiveness of the immobilized ligand L-tyrosine-sulfonamide for the single-step affinity purification of CA from the erythrocytes of various freshwater and seawater fish [7,8]. In the present study, dCA was effectively purified from dog erythrocytes by one-step chromatography on L-tyrosine-sulfonamide coupled to Sepharose-4B (Figure 1). A 452-fold purification with a yield of 29.7% was achieved (Table I), and the mass and purity of the enzyme was assessed by SDS-PAGE (Figure 2). The purified CA appeared as a single species with a mass of 29 kDa and apparent high activity, i.e. probably corresponding to CA II (Figure 2).

We determined the inhibition constants for all the test compounds with respect to the CO₂-hydratase activity of CA, which is the primary physiological function of this enzyme. The IC₅₀ values are presented in Table II. All the five new sulfonamide compounds demonstrated inhibitory activities against dCA. Comparing them, it is clear that the most potent inhibitor of dCA was ODTS with an IC₅₀ value of 1.18×10^{-5} M. In previous literature, Cakir and his colleagues had determined the IC₅₀ values for ODTS acting upon the human CA isozymes, hCA-I and hCA-II, as being 0.61×10^{-5} M and 0.52×10^{-5} M, respectively [6]. In that same study, the most potent inhibitor against the hCA-I and hCA-II isozymes was TDTS. But in the present work, a slightly poorer IC₅₀ value of 1.59×10^{-5} M was obtained with dCA. The other three sulfonamide derivatives tested, i.e. CCTS, CCBS and MCTS, were even less effective, yielding

IC₅₀ values with dCA of 2.57×10^{-5} M, 2.40×10^{-5} M and 2.53×10^{-5} M, respectively (Table II). Cakir's study had reported corresponding IC₅₀ values for the same compounds acting upon hCA-I as being 0.43×10^{-5} , 0.55×10^{-5} and 0.47×10^{-5} M; and upon hCA-II as being 0.29×10^{-5} , $0,17 \times 10^{-5}$ and 0.96×10^{-5} M, respectively [6].

Trusopt, Benoxinate, Tropamid and the antibacterial drug Gentagut were also tested for their inhibitory effects upon dCA activity. Interestingly, the effective compounds that comprise these various drugs had varied effects upon dCA activity.

The classical CA inhibitors such as acetazolamide, dorzolamide and brinzolamide are used for glaucoma treatment [25]. Here we showed that dorzolamide inhibited dCA activity with an IC₅₀ of 5.05×10^{-4} M, which is poorer than all five of the new sulfonamide compounds tested in parallel in this study. Previously this drug showed inhibitory activities against hCA-I, hCA-II and hCA-IV isozymes with IC₅₀ values of 6×10^{-7} M, 1.8×10^{-10} M and 6.9×10^{-9} M, respectively [26]. However, Supuran's group obtained IC₅₀ values for dorzolamide-HCl against hCA-I and hCA-II isozymes of 5.0×10^{-5} and 9×10^{-9} , respectively [27].

Surprisingly, both Benoxinate and Tropamid exhibited potentiation of dCA activity with increasing concentration, the effect being most marked with the former compound. In contrast, gentamicine showed a weak biphasic response. An initial weak inhibitory response at low concentrations (no IC₅₀ was attained) was partially reversed at higher concentrations.

It is known that certain anions are potent inhibitors of carbonic anhydrase enzymes [28]. For N₃⁻ ion at pH 5.8, K_i values were reported as being 1.5×10^{-5} M and 0.2×10^{-3} M for hCA-I and hCA-II, respectively. Also for the SCN⁻ ion at pH 6.0, K_i values were 1.8×10^{-5} M and 0.3×10^{-3} M, and for the I⁻ ion at pH 7.5, they were 0.7×10^{-6} M and 8.7×10^{-3} M for hCA-I and hCA-II, respectively. Their mode of binding to the enzyme is known to be completely different from that of the sulfonamides. This effect is most marked against hCA-II versus hCA-I at low pH.. For the Cl⁻ anion at pH 6.5, K_i values of 0.004 M and 0.27 M, respectively, have been obtained; at pH 7.5 the corresponding values are 0.018 M and 0.73 M respectively [28]. Other anions have also been shown to be inhibitory including perchlorate [28]. Supuran and his group have determined IC₅₀ values of

Table I. Purification of dCA by affinity chromatography.

Enzyme	Fraction	Activity (EU)	Total Activity (U/mL)	Total Protein (mg/ml)	Specific Activity (U/mg)	%Yield	Purification fold
DCA	Hemolysate (25 mL)	34.9	872.3	93.8	0.372	100	1.0
	Enzyme (10 mL)	25.9	259.0	0.154	168.18	29.7	452.1

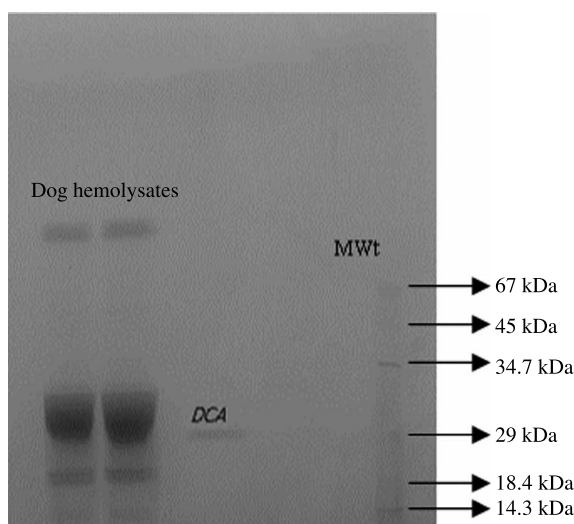


Figure 2. SDS-PAGE of dCA on a 10% polyacrylamide reducing gel. Protein standards are: lysozyme (14.3 kDa), β -galactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), pepsin (34.7 kDa), ovalbumin (45 kDa), albumin bovine (BSA, 67 kDa).

0.69×10^{-3} and 1.26×10^{-3} for perchlorate acting upon hCA-I and hCA-II, respectively [29]. In the present study a similar IC_{50} of 1.07×10^{-3} M was determined for perchlorate acting upon dCA. By comparison, chloride anion was only weakly inhibitory at the lowest concentrations and activity broadly recovered with increasing concentrations.

In conclusion, we show in this study that dog CA can be readily purified by a one-step affinity chromatography procedure for subsequent use in the evaluation of potential CA inhibitors. The susceptibilities of dCA to inhibition by the classical CA inhibitor, dorzolamide, the perchlorate anion, and some new sulfonamide compounds are comparable to the human CA isoforms. All the five new sulfonamide compounds tested show lower IC_{50} values than dorzolamide, with the most active, ODTS, being over 40-fold more potent. Interestingly, two other drugs used in ophthalmology apparently exhibit the

Table II. IC_{50} values for various compounds that inhibited dCA, ranked in decreasing effectiveness.

Inhibitor name	IC_{50} (M)		
	HCA I	hCA II	dCA
ODTS	0.61×10^{-5}	0.52×10^{-5}	1.18×10^{-5}
TDTS	0.24×10^{-5}	0.17×10^{-5}	1.59×10^{-5}
CCBS	0.55×10^{-5}	0.17×10^{-5}	2.40×10^{-5}
MCTS	0.47×10^{-5}	0.96×10^{-5}	2.53×10^{-5}
CCTS	0.43×10^{-5}	0.29×10^{-5}	2.57×10^{-5}
Dorzolamide – HCl	5.0×10^{-5}	9×10^{-9}	5.05×10^{-4}
Perchlorate	0.69×10^{-3}	1.26×10^{-3}	1.07×10^{-3}

ability, at the concentrations tested, to potentiate CA activity in these assays, by an, as yet, unknown mechanism. Hopefully, the levels of inhibition seen with the new sulfonamides will stimulate further drug development with the aim of generating more therapeutically-effective CA inhibitors for potential use in the treatment of glaucoma.

Acknowledgements

The authors would like to thank Balikesir University Research Center of Applied Sciences (BURCAS / Balikesir, Turkey) for providing the research facilities and also to Dr. Malcolm LYON, Senior Research Fellow in the Paterson Institute for Cancer Research University of Manchester for his invaluable contribution to this paper.

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