Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: informahealthcare.com/journals/ienz20

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To cite this article: Murat Şentürk, Hacı Ahmet Alıcı, Şükrü Beydemir & Ömer İrfan Küfrevioglu (2012) *In vitro* and *in vivo* effects of some benzodiazepine drugs on human and rabbit erythrocyte carbonic anhydrase enzymes, Journal of Enzyme Inhibition and Medicinal Chemistry, 27:5, 680-684, DOI: 10.3109/14756366.2011.607447

To link to this article: https://doi.org/10.3109/14756366.2011.607447

Journal of Enzyme Inhibition and Medicinal Chemistry, 2012; 27(5): 680–684

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2011.607447

informa
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RESEARCH ARTICLE

In vitro and *in vivo* effects of some benzodiazepine drugs on human and rabbit erythrocyte carbonic anhydrase enzymes

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Abstract

Carbonic anhydrase inhibitors (CAIs) are a class of pharmaceuticals used as antiglaucoma agents, diuretics and antiepileptics. Thus, discovery of novel CAIs has become of great importance in the recent years. In the current study, *in vitro* and *in vivo* inhibition effects of benzodiazepine drugs, diazepam and midazolam, on human erythrocytes carbonic anhydrase I and II isozymes were investigated. After purification of the isoenzymes, *in vitro* inhibition assays were performed and K_i values were determined to be of 141.5 μM and 40.7 μM for hCA I and of 5.11 μM and 0.58 μM against hCA II by the esterase activity assay, respectively. The drugs showed strong inhibitory effects on hCA II, in the same range as the clinically used sulphonamide acetazolamide. For *in vivo* studies, five adult male New Zealand White rabbits (3–4.2 kg) were selected for intravenous administrations of the drugs (2 mg/kg and 0.2 mg/kg body weight, respectively). The enzyme was significantly inhibited by 2 mg/kg diazepam ($p < 0.05$), and 0.2 mg/kg midazolam ($p < 0.05$) for up to 30 min following intravenous administration.

Keywords: Carbonic anhydrase, benzodiazepine, midazolam, diazepam, inhibition

Introduction

Benzodiazepine drugs have been used for many years as intravenous sedation agents. They are widely used in medicine, for their anxiolytic and amnestic properties. Diazepam, a benzodiazepine, has been commonly used for the last 45 years. An intra-lipid preparation of diazepam reduces the irritant effects of the propylene glycol component in instruments used for injectable diazepam. Midazolam has recently been introduced and has several claimed advantages over diazepam, primarily faster onset and recovery as well as enhanced amnestic qualities. The pharmacology of these drugs is well documented[1,2]. Diazepam and midazolam are often administered to patients during general anaesthesia or for sedation of critically ill patients[3,4]. Although these benzodiazepines are characterized by relatively minor alterations of hemodynamic variables, they decrease systemic blood pressure in humans and animals[3–5]. These actions result from various effects on different target tissues, including the central and autonomic nervous systems, vascular smooth muscle and cardiac muscle. Therefore, the direct effects of benzodiazepines on myocardial contractility are difficult to study *in vivo*. The negative inotropic effects of diazepam and midazolam have been reported in rat isolated heart preparations[6].

Carbonic anhydrase (EC 4.2.1.1., CA) has been a well-characterized pH regulatory enzyme in most tissues including erythrocytes[7]. The CA catalyses the reversible hydration of CO_2 to HCO_3^- and H^+. At least 16 CA isozymes have so far been described in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX[8–10]. The first one is primarily found in red blood cells but also in many other secretory tissues of the gastrointestinal tract, kidneys, lungs, eye, central

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(Received 27 May 2011; revised 20 July 2011; accepted 20 July 2011)

nervous system (CNS), etc.[8,10], whereas the second one is a tumour-associated isoform[10–12]. Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion[9,13].

In this paper, we report that midazolam and diazepam show relevant inhibition properties against human CA I, II isoforms. We also report on an *in vivo* study conducted on New Zealand White rabbits treated with the drugs. The CA inhibitors are used for several purposes, in particular for the treatment of glaucoma, epilepsy and as diuretics or antitumour agents/diagnostic tools[7,10]. Also these benzodiazepine drugs are widely used in anaesthesiology and thus we chose these drugs to test. Therefore, this study demonstrates that midazolam and diazepam are active carbonic anhydrous inhibitors (CAIs) and it is required to perform further studies to determine whether or not they might be used as antiglaucoma, antimalaria and anticancer drugs[8,12–16].

Materials and methods

Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. All other chemicals were analytical grade and obtained from Merck. The drugs were obtained from the medical faculty of Atatürk University.

Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

The purification of the enzymes was performed according to the literature methods[10,17–19].

Hydratase activity assay Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson. The CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0 - t_c / t_c)$, where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively[20,21].

Esterase activity assay Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV-VIS) according to the method described by Verpoorte et al.[21]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

In vitro *inhibition study* The inhibitory effects of midazolam and diazepam were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. The hCA I enzyme activities were measured for midazolam (10–49.5 μM) and diazepam (28–130.4 μM) at cuvette concentrations and hCA II enzyme activities were measured for midazolam (0.15–2.75 μM) and diazepam (0.65–10.33 μM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor, Activity (%)-[Inhibitor] graphs were drawn. To determine K_i values, three different inhibitor concentrations were tested. In these experiments, 4-nitrophenyl acetate was used as substrate at five different concentrations (0.15–0.75 mM)[22].

In vivo *inhibitory study* Five adult male New Zealand White rabbits (3–4.2 kg) were selected for intravenous administration of diazepam and midazolam (2 and 0.2 mg/kg body weight, respectively). Blood samples (10 × 0.5 mL) were taken from each rabbit prior to diazepam and midazolam administration as well as at 10, 30 min, 1, 2, 4, 6 and 24 h intervals thereafter. The blood was placed into test tubes containing Ethylenediaminetetraacetic acid (EDTA) (2 mM) and subjected to centrifugation at 2500 × *g* for 15 min at 4°C (HERMLE Z383K). The erythrocyte pellet was washed three times with cold 0.16 M KCl and the supernatant was discarded. One volume of erythrocyte pellet was suspended in five volumes of ice water to give an erythrocyte haemolysate. The CA activity was determined colourimetrically as described above[19,23].

Statistical analysis

Statistical analysis was performed using the Statistica 6.0 statistical package. For determination of enzyme activities in drug-treated rabbits, 2-way analysis of variance (ANOVA) was used. In twin comparisons, the LSD test was performed and *p*-values were obtained. Values are presented as numbers (% activity), mean ± SD. A *p*-value < 0.05 was considered statically significant[24].

Table 1. Summary of purification procedure for human carbonic anhydrase izoenzymes (hCA I and hCA II) by a sepharose-4B-aniline-sulphanilamide affinity column chromatography.

Purification steps		Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate		119	30	14.14	424.2	3570	8.41	100	1
Sepharose-4B-aniline-sulphanilamide affinity column chromatography	hCA I	406	6	0.46	2.76	2436	882.61	68.23	104.95
	hCA II	748	3	0.12	0.36	2244	6233.33	62.86	741.18

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as a standard[25].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure[26].

Results and discussion

There are a few studies existing in the literature on the interactions of anaesthetic drugs and carbonic anhydrase enzymes. The interactions of ceftriaxone, morphine and imipenem with two human isozymes, CA I–II, has only recently been investigated[27], evidencing some low milimolar/micromolar inhibitors as well as the possibility to design isozyme selective CAIs. Indeed, the inhibition profile of various isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the sub-micromolar level for many simple chlorbenzyl-containing molecules[27]. Thus, it seemed reasonable to us to extend the previous studies[27], including this study on benzodiazepine drugs with common clinical applications.

We report here the first study on the inhibitory effects of midazolam and diazepam of type on the esterase activity of hCA I and II. The acetazolamide (AZA) CAI acetazolamide[14] was used as a negative control in our experiments, and for comparison purposes. The previous studies by Coban et al.[18], investigated morphine by using a Verpoorte method, 4-nitrophenyl acetate esterase assay for monitoring CA inhibition[21]. The data in Table 2 show the following regarding inhibition of hCA I and II with midazolam, diazepam and AZA, by an esterase assay[21], with 4-nitrophenyl acetate (4-NPA) as the substrate:

Against the slow cytosolic isozyme hCA I, diazepam behaves as a weak inhibitor, with a K_i value of 141.5 μM. Midazolam showed better inhibitory activity when compared to the previously mentioned diazepam, with a K_i value of 40.7 μM (Table 2). Thus, the nature of the group in *ortho'*- to the *F* moiety strongly influences hCA I inhibitory activity. The AZA is also a medium-weak CAI in this assay and substrate against hCA I (K_i of 36.2 μM). Kinetic investigations (Lineweaver Burk plots, data not shown) indicate that similar to sulphonamides, salicylic acid derivatives, antioxidant phenols, some metal and inorganic anions[10,14,27–35], all the investigated drugs act as non-competitive inhibitors with 4-NPA as substrate, that is they bind in different regions of the active site cavity when compared to the substrate. However, the binding site of 4-NPA is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme[27,33,36].

Table 2. The hCA I and II inhibition data with diazepam, midazolam and acetazolamide[8,23], by an esterase assay with 4-nitrophenyl acetate as substrate.

	Ki (μM)	
Compound	hCA I	hCA II
Diazepam	141.5	5.11
Midazolam	40.7	0.58

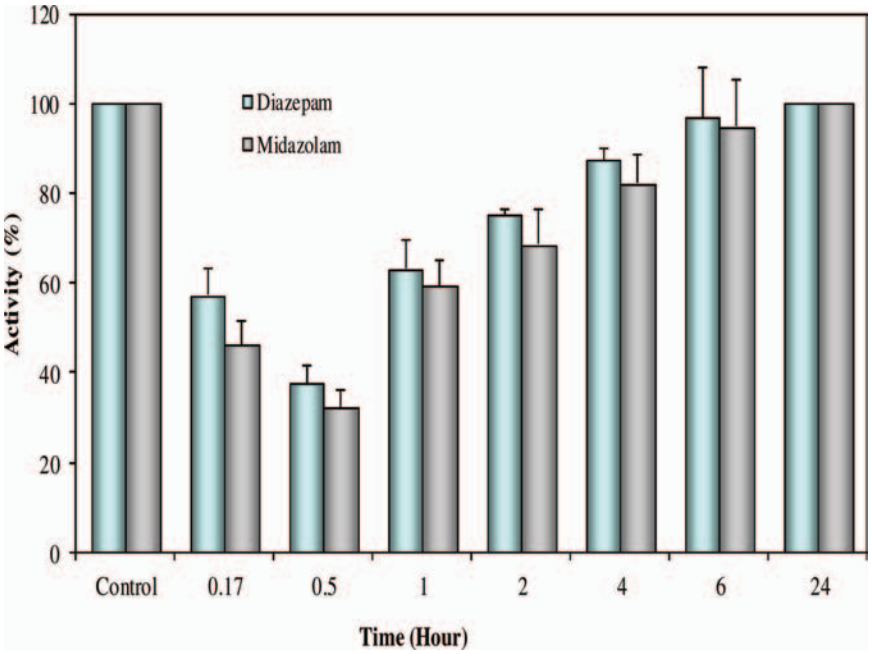

Figure 1. The *in vivo* effects of diazepam and midazolam on rabbit erythrocytes CA activity. Five adult male New Zealand white rabbits (3–4.2 kg) were selected for intravenous administration of diazepam and midazolam (2 and 0.2 mg/kg body weight, respectively). Blood samples (10 × 0.5 ml) were taken from each rabbit prior to diazepam and midazolam administration as well as at 0.17, 0.5, 1, 2, 4, 6 and 24 h intervals thereafter.

A better inhibitory activity has been observed with midazolam and diazepam for the inhibition of the rapid cytosolic isozyme hCA II (Table 2). Diazepam showed moderate hCA II inhibitory activity with a K_i value of 5.11 μM (Table 2), whereas midazolam was quite an effective hCA II inhibitor, with a K_i value of 0.58 μM, (Table 2). Structure-activity relationship is thus quite sharp for these drugs, with *F* moiety in *ortho* and *ortho'* is already a sub-micromolar hCA II inhibitor. It must be stressed that K_is measured using the esterase method are always in the micromolar range because hCA I and II are weak esterases[15–36].

However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile when compared to the sulphonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

Five adult male New Zealand White rabbits (3–4.2 kg) were used for intravenous administration of the drugs (2 mg/kg and 0.2 mg/kg body weight, respectively) in *in vivo* studies. The enzyme was significantly inhibited by 2 mg/kg diazepam ($p < 0.05$), and 0.2 mg/kg midazolam ($p < 0.05$) for up to 30 min following intravenous administration (Figure 1).

Conclusions

Midazolam and diazepam drugs affected the activity of CA isozymes due to the presence of the different functional groups (Cl, F and CH_3) present in their aromatic scaffold. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulphonamides/sulphamates/sulphamides, the benzyl, *p*-chlorobenzyl and *o*-fluorobenzoyl bearing bulky *ortho* moieties in their molecules.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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