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RESEARCH ARTICLE

Evaluation of *in vitro* effects of some analgesic drugs on erythrocyte and recombinant carbonic anhydrase I and II

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Abstract
The *in vitro* effects of the injectable form of analgesic drugs, dexketoprofen trometamol, dexamethasone sodium phosphate, metamizole sodium, diclofenac sodium, thiocolchicoside, on the activity of purified human carbonic anhydrase I and II were evaluated. The effect of these drugs on erythrocyte hCA I and hCA II was compared to recombinant hCA I and hCA II expressed in *Ecoli*. IC_{50} values of the drugs that caused inhibition were determined by means of activity percentage diagrams. The IC_{50} concentrations of dexketoprofen trometamol and dexamethasone sodium phosphate on hCA I were 683 μM and 4250 μM and for hCA II 950 μM and 6200 μM respectively. Conversely, the enzyme activity was increased by diflofenac sodium. In addition, thiocolchicoside has not any affect on hCA I and hCA II. The effect of these drugs on erythrocyte hCA I and hCA II were consistent with the inhibition of recombinant enzymes.

Keywords: Carbonic anhydrase, analgesic drugs, inhibition, IC_{50}

Introduction

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyses a very simple but critically important physiological reaction: the involvement of the carbonic anhydrase (CA) enzyme family, which catalyses the physiological hydration of CO_2 to yield bicarbonate and a proton, in many physiological/pathological processes opens up widespread opportunities for the development of diverse, specific inhibitors for clinical application[1–3].

CAs catalyse a simple physiological reaction, the conversion of CO_2 to the bicarbonate ion and protons. The active site of most CAs contains a zinc ion (Zn^{2+}), which is essential for catalysis. The CA reaction is involved in many physiological and pathological processes, including respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis; bone resorption; calcification; and tumorigenicity[4–12].

Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including edema, glaucoma, obesity, cancer, epilepsy and osteoporosis[13–18]. Given the physiological importance of the CA, the metabolic impact of chemicals for crop production should receive greater study. However, there is not much inhibition study available on CA activity.

Non-steroidal anti-inflammatory drugs (NSAIDs) play an important role in the clinical setting, especially at local anaesthetics. Their analgesic effect is based on a diminished prostaglandin synthesis by inhibition of the cyclooxygenase (COX) enzyme in the arachidonic acid metabolism. The discovery of at least two COX isoforms led to the development of selective COX-2-inhibitors (coxibs) that were thought to have an improved

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risk-benefit ratio compared with traditional NSAIDs. Two studies have shown that oral administration of coxibs can result in pain relief after disk surgery[19,20].

Dexketoprofen trometamol (**1**) is a newly developed NSAID belonging to the aryl-propionic acid group. It is a water-soluble salt of the S (+)-enantiomer of the racemic compound ketoprofen[21]. It has been widely demonstrated in preclinical studies that the anti-inflammatory and analgesic effect of ketoprofen is due entirely to the S (+)-enantiomer (dexketoprofen), while the R (–)-enantiomer is devoid of such activity[21]. Animal models of inflammation and analgesia have shown that dexketoprofen is at least twice as potent as the parent compound ketoprofen[22]. In humans, the analgesic efficacy of dexketoprofen trometamol using an oral formulation has been demonstrated in painful conditions such as dental pain and dysmenorrhea[23,24]. Currently, there are few available NSAIDs that can be used parenterally, which is the preferable route of administration in the immediate postoperative period[25].

Dexamethasone sodium phosphate (DSP) (**2**) is the most common corticosteroid used in the treatment of edema paired with brain tumours. As with other corticosteroids, DSP has some adverse effects on the cardiovascular, immune and nervous systems[26,27].

Metamizol sodium (**3**), a pyrazolone derivate, provides additional antipyretic, antispasmodic, and anti-inflammatory effects. It is a very popular non-opioid analgesic in Germany, Spain, and South America, whereas in other countries it has been banned because of its disputed association with potentially life-threatening agranulocytosis[28]. It was introduced for therapeutic use in 1922. It is a highly hydrolysed in metil-amino-antipirine (MAA). Its analgesic effect ranges in peak from 20 to 45 min after the intravenous administration. Its active metabolites are MAA and amino-antipyrine (AA). The half-life of MAA/AA complex is about 2.7 h. The excretion is predominantly renal[29,30]. Metamizol is an effective analgesic known wordwide. Its effectiveness has been shown in several painful situations, such as post-surgery pain, odontalgias and oncologic surgeries. It is commonly used in headaches, in particular migraines[31-,32].

Diclofenac (**4**) is an NSAID with very well-characterised pharmacokinetic and pharmacodynamic properties[34,35]. It is widely used in the treatment of inflammatory diseases and is also administered as an analgesic in osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and acute muscle pain[36]. The favourable properties of diclofenac, such as high anti-inflammatory activity, good absorbability along the gastrointestinal tract, and lack of accumulation, result in a wide application of the substance in the routine pain treatment[37].

Thiocolchicoside (**5**) is a semisynthetic derivative of colchicoside, with selective affinity for gamma-aminobutyric acid and glycinergic receptors. It is used as muscle relaxant agent in the symptomatic treatment of spasms and contractures in muscular, rheumatic, traumatic and neurological disorders[38].

The injectable drugs are often used as a painkiller at emergency clinics and hospitals. Thus, the determination of the effects of these drugs on human CA I and II activity is vital. The aim of this study is to define the effects of dexketoprofen trometamol (**1**), DSP (**2**), metamizol sodium (**3**), diclofenac sodium (**4**), thiocolchicoside (**5**) on erythrocyte CA I and II and thus evaluate the toxicological effects of these drugs *in vitro*. In addition, the effects of these drugs will be further compared to the activity of recombinant hCA I and hCA II enzymes.

Materials and methods

Materials

Sepharose 4B, L-tyrosine, sulfonamide, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck. All drugs were provided by the local pharmacy.

CA enzyme assay

CA activity was measured by the Maren method that is based on determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO_2 hydration. Phenol red was added to the assay medium as the pH indicator, and the buffer was 0.5 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0). One unit of CA activity is defined as the amount of the enzyme that reduces by 50% the time of CO_2 hydration measured in the absence of enzyme. In the inhibition studies, the CO_2 concentration was 70 mM and at last five different inhibitor concentrations were used. IC_{50} values were calculated using computer regression analysis[39]. Human CA I and II were purified from red blood cells according to the method of Ozensoy et al.[40].

Overexpression of recombinant hCA I and hCA II in *E. coli*

Recombinant hCA I and hCA II genes were transformed into BL21(DE3) [B F– dcm ompT hsdS(rB– mB–) gal λ(DE3)] *E. coli* strain containing the T7 promoter region. The cloning strategy of hCA I gene was mentioned before[41] and hCA II plasmid was a gift from David Silverman, USA.

Inoculate with a single colony from a fresh plate of BL21(DE3)/pET31hCA I and BL21(DE3)/pET31hCA II in a sterile 50 mL falcon tube, prepare 10 mL of Luria Broth supplemented with 10 μL 100 mg/mL ampiciline solution. Grow both cultures at 37°C with moderate agitation (120 rpm). Inoculate with 5 mL overnight culture in a sterile 500 mL flask; prepare 200 mL of Luria Broth supplemented with 200 μL of 12.5 mg/mL ampiciline solution. Grow both cultures at 37°C with moderate agitation (300 rpm) for 3–5 h and monitor the growth of the culture by measuring the optical density at 550 nm. When the cell cultures reached an optical density of 0.6–0.8 (at 550 nm), expression of the wild-type or mutant hCA II was induced with 400 μL 0.1 M IPTG (isopropylthiogalactoside) and 250 μL 5 mM $ZnCl_2$, and

incubation was continued for 4–5 h at 30°C. Cells were harvested by centrifugation at 3000 rpm and frozen at –20°C prior to purification of the recombinant hCA I and hCA II proteins.

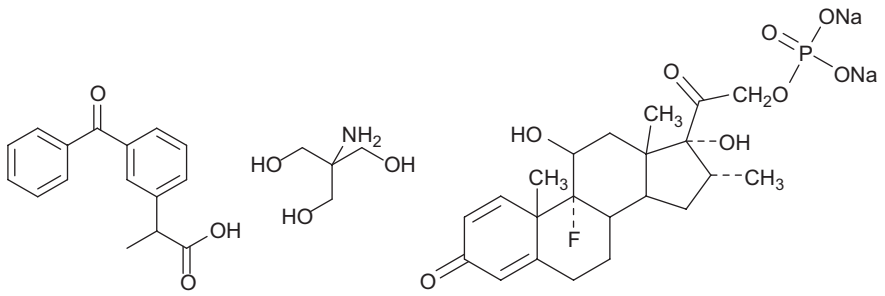
Purification and protein analysis

To purify the protein, *E. coli* cells were collected by centrifugation at 3000 rpm for 10 min at 40°C. The pellet was washed with buffer (50 mM Tris–HCl, pH 7.6) and pellet was resuspended in lysis buffer (20 mM Tris/0.5 mM EDTA/0.5 mM EGTA/pH 8.7). Hundred microlitre of 100 mM PMSF (1 mM final concentration) and 250 µL of a 10 mg/mL solution of lysozyme were added, and the pellet was thawed at room temperature. After 30 min, 1 mL of the 3.0% protamine sulphate solution was added to the cell lysate and centrifuged. Before purification of proteins, cell lysates was dialysed to affinity equilibration buffer for 3 h at 4°C. Every 30 min, the affinity equilibration buffer was changed. The purification step was carried out as above.

In vitro inhibition studies

For the inhibition studies of dexketoprofen trometamol, DSP, metamizol sodium, diclofenac sodium,

Table 1. The IC_{50} values of analgesic drugs.

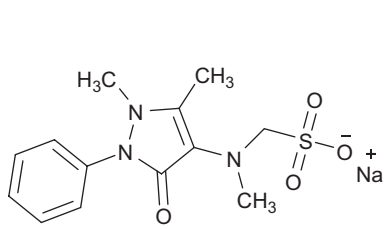


Dexketoprofen trometamol

1

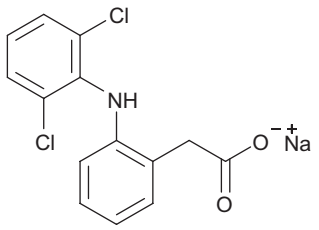
dexamethasone sodium phosphate

2



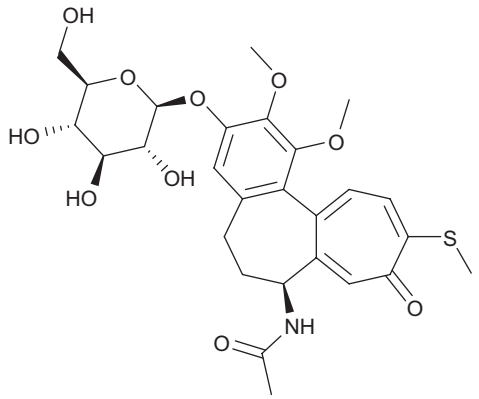
Metamizol sodium

3



Diclofenac sodium

4



Thiocolchicoside

5

Compound	Erythrocyte hCA I	Erythrocyte hCA II	Recombinant hCA I	Recombinant hCA II
1	683 µM	950 µM	1450 µM	1850 µM
2	4250 µM	6200 µM	3765 µM	4380 µM
3	Activated	Activated	395 µM	356 µM
4	Activated	Activated	Activated	Activated
5	Not affected	Not affected	Not affected	Not affected

thiocolchicoside different concentrations of these drugs were added to the enzyme activity. Activity % values of CA for different concentrations of each drug were determined by regression analysis using Microsoft Office 2000 Excel. CA enzyme activity without a drugs solution was accepted as 100% activity. For the drugs having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs.

Results and discussion

In this study, CA I and II isoenzymes from human erythrocytes were purified by a simple one step procedure by using Sephrarose 4B-L-tirozin-sulfanilamide affinity column. The activity of the eluents was determined as described in material and methods. The inhibitory effects of some commonly used analgesic drugs on human cytosolic CA I and II activity were investigated. In order to confirm the effect of the drug on erythrocyte hCA I and hCA II, the activity of recombinant hCA I and hCA II were also investigated against the same drug. The effects of the drugs on that obtained from erythrocyte hCA I and hCA II were compared to that of which recombinant hCA I and hCA II enzymes. Different inhibition effects of the applied drugs were obtained and showed in Table 1. Generally, recombinant hCA I and hCA II exhibit the same manner effect to the drugs tested except for metamizol sodium (**3**). Metamizol sodium (**3**) activates the erythrocyte hCA I and hCA II, whereas it causes the inhibition of the activity of recombinant hCA I and hCA II enzymes. This difference may be resulted from the individual differences of erythrocyte hCA I and hCA II enzymes.

As shown in Table 1, dexketoprofen trometamol (**1**) has been shown to be the strongest inhibitor against the erythrocyte hCA I and hCA II activity. Metamizol sodium (**3**) causes the strongest inhibition on recombinant hCA I and hCA II enzymes. hCA I and hCA II enzyme activity was increased by diflofenac sodium (**4**) in erythrocyte and recombinant ones. In addition, hCA I, hCA II and recombinant hCA I, hCA II enzymes are not affected by thiocolchicoside (**5**).

CA that is a widespread metalloenzyme has previously been purified and characterised from many living organisms including animals[42–44]. The isozymes of CA play important roles in different tissues[45,46]. The similarities of CAs from various sources have been determined from their crystal structures[47]. It is known that CA has been purified many times from different organisms and the effects of various chemicals, pesticides and drugs on its activity have been investigated[48–50].

Puscas et al. reported that indomethacin, *in vitro* and *in vivo*, induces an increase in erythrocyte CA I and CA II activity. Acetazolamide, a specific inhibitor of CA, reduces the activity of CA I and CA II from red cells. Indomethacin completely antagonises CA activity, i.e. abolishes the inhibitory effect of acetazolamide on CA. In humans, an increase or decrease in erythrocyte CA II activity is correlated with an increase or decrease in gastric acid secretion. Indomethacin is not only an activator of CA but also antagonises the effect of acetazolamide, a specific inhibitor of this enzyme. In view of the role of CA in acid-base balance as well as the fact that an increase or decrease in its activity is accompanied by an increase or decrease in intra- and extracellular pH[51].

We have determined the IC_{50} values of 356–4380 μM for the inhibition of recombinant hCA II activity and 683–6200 for the inhibition of hCA II activity. These compounds therefore have weak CA II inhibitory potencies to that of acetazolamide (IC_{50} 25 nM), the well-known hCA II inhibitor[52].

Innocenti et al. reported that the activator bound within the enzyme active site facilitates the shuttling of protons between the Zn (II) ion-coordinated water molecule and the environment, with generation of the nucleophilic zinc hydroxide, catalytically active species of the enzymes[53]. DSP (**2**) has a phosphate group and metamizol sodium has a sulphate group. These groups may to take of protons from the Zn (II) ion-coordinated water molecule and environment, with generation of the nucleophilic zinc hydroxide, catalytically active species of the enzymes.

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Declaration of interest

The authors report no conflicts of interest.

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