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Differential *in vitro* inhibition effects of some antibiotics on tumor associated carbonic anhydrase isozymes of hCA-IX and hCA-XII

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Abstract
Hypoxia is a common characteristic of locally advanced solid tumors that has been associated with diminished therapeutic response and malignant progression. Human carbonic anhydrase (hCA) hCA IX and XII isozymes are tumor associated isoforms which contribute to acidification of the tumor environment by catalyzing the hydration of carbon dioxide to bicarbonate and protons.In the present study our goal was to investigate the inhibition effects of 15 different antibiotics belonging to the following classes: Lactams, cephalosporins, macrolides etc., on the tumor associated carbonic anhydrase isozymes hCA-IX, hCA-XII and cytosolic carbonic anhydrase hCA-I and hCA-II.

Keywords: *Hypoxia, carbonic anhydrase, hCA I, hCA II, hCA-IX, hCA-XII, antibiotic, inhibition*

Introduction

Carbonic anhydrase (CA) is an enzyme that assists rapid inter-conversion of carbon dioxide and water into protons and bicarbonate ions. Members of the four different classes share very little sequence or structural similarity, yet they all perform the same function and require a metal ion at the active site. [1]. Membrane-bound CAs, such as hCA IX, have an extracellular active site and can provide the H^+ or HCO_3^- ions formed during catalytic turnover for various physiological/pathological processes, among which is extracellular acidification. Recently, it has been shown that two CA isozymes (hCA IX and hCA XII) are prominently associated with and over-expressed in many tumors, where they are involved in crucial processes connected with cancer progression and response to chemotherapy [2–7].

Over the past decades, many studies have been made to find the role of CA in tumor progression, either as a biomarker or a tumor-associated protein. The expression of CA I and CA II has been most frequently investigated in a variety of tumor cells, cell lines and some carcinoma patients [8–10] but it has been difficult to find a clear-cut relationship between the expression of CA isozymes in normal and malignant cells. However, no evidence of a direct relationship between malignant transformation and CA expression has been presented for CAs I through VII. It appears that only the recently characterized isoform CA IX and CA XII is an exception which their expression was asociated with tumorigenesis [11].

In this paper, our aim is to investigate the effects of antibiotics on tumor associated isozymes of hCA IX and hCA XII as well as on the cytosolic isoforms CA I and II. There were so many studies of CA isozymes inhibition with sulfonamides [1–2] but there aren't so many studies about CA isozymes inhibited especially by antibiotics thus in this present work 15 antibiotics are used as inhibitors for CA isozymes whether to investigate their inhibition abilities.

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Antibiotics are drugs derived wholly or partially from certain microorganisms and are used to treat bacterial infections. They are ineffective against viruses which either kill microorganisms or stop them from reproducing [12]. The great number of diverse antibiotics currently available can be classified in different ways, e.g., by their chemical structure, their microbial origin, or their mode of action. They are also frequently designated by their effective range.

Tetracycline, the most widely used broad-spectrum antibiotics, is effective against both Gram-positive and Gram-negative bacteria, as well as against rickettsias and psittacosis-causing organisms [13].

Beta-lactams are compounds with a beta-lactam ring in their structure. They comprise principally the penicillins and the cephalosporins, but also they include clavulanic acid. The aminoglicoside are products of actinomycetes (soil bacteria) or semi-synthetic derivatives of the natural products [14]. The beta-lactam antibiotics bind to and inhibit enzymes needed for the synthesis of the peptidoglycan wall. While they have little effect on resting bacteria, they are lethal to dividing bacteria as defective walls cannot protect the organism form bursting in hypotonic surroundings.

The structural formulae of effective antibiotics

Many antibiotics have been used in therapies. There are few literature reports related with changing of enzyme activities. It has been reported that some increasing or decreasing were found on human liver enzyme activity levels such as aspartate aminotransferase (AST;SGOT), alanine aminotransferase (ALT; SGPT) and alkaline fosfatase [15–19]. Since the effects of some antibiotics have not been analyzed on CA isozymes which are contained at the highest molar amounts in erythrocytes. The interaction between human erythrocyte lysates and antibiotics was studied, and the effect of intracellular components on the activity and binding of the drugs was determined by Kornguth et. al. [20]. In that study experiments with pure preparations of carbonic anhydrase revealed that the CA isozyme is the major binder of the tetracyclines and that zinc is required for binding did not inhibit enzymatic activity of carbonic anhydrase.

Cefazolin sodium, cephradine, and sulbactam/ cefoperazone and chloramphenicol sodium succinate antibiotics were studied by Coban et al. and in this study the IC_{50} values were indicated as of 9, 16, 19 and 48 mM on hCA I and 6, 10, 15 and 17 mM on hCA II, respectively [21]. But there is no report related with tumor associated carbonic anhydrase isozymes hCA IX and hCA XII with the inhibition of antibiotics. For this purpose in this present study, several kind of antibiotics are used for detecting the inhibition of both tumor associated isozymes and cytosolic human carbonic anhydrases (hCA I and hCA II).

Materials and methods

Materials

The materials including Sepharose 4B, L-tyrosine, sulfonamide, protein assay reagents and other chemicals were obtained from Sigma Chem. Co (Milan / Italy). Medical drugs were provided by a local pharmacy in Turkey/Balikesir.

CA IX and CA XII genes were a gift from Dr. Claudiu T. SUPURAN, Florence University / Italy.

Methods

CA catalytic domain. The protein expression was induced by adding 1 mM isopropyl-b-D-thiogalactopyranoside; the cells were harvested when the OD 600 reached a value of 1.00 and lysed by sonication in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) twice for 30 s each at 4°C. Centrifugation at 30,000 g for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepackedglutathione Sepharose 4B column, extensively washed with buffer, and the fusion (GST-CA XII) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally, the GST part of the fusion protein was cleaved with thrombin. The advantage of this method is that CA XII is purified quite easily and the procedure is quite simple.The amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate. The GST-hCA IX construct previously reported [22] was transfected into *E. coli* strain BL21 for production of the CA IX protein, similar to the procedure already described for hCA XII above [22].

The final CA domains of CA IX and CA-XII was further purified by Sepharose 4B-L-tyrosine-sulfonamide affinity gel [23] with 129.15 and 92.29 fold purifications for CA-IX and CA-XII, respectively. The amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate [11].

Preparations of enzyme solutions. Cytosolic human carbonic anhydrases as trial isozymes were purchased from Sigma Co. Ltd (Milan/Italy) had the concentrations 1×10^{-6} M and 1×10^{-7} M for hCA I and hCA II, respectively [18].

Tumor associated carbonic anhydrase isozymes were further purified by Sepharose 4B-L-tyrosine-sulfonamide affinity gel [20] had the concentrations 4×10^{-6} M for both hCA IX and hCA XII, respectively [18].

CA enzyme assay. SX.18MV-R Applied Photophysics stopped-flow instrument has been used for measuring the initial velocities for the CO_2 hydration reaction catalyzed by different CA isozymes, by following the change in absorbance of a pH indicator. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20°C were used as substrate. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates.

Stock solutions of antibiotics were prepared at a concentration of 1–3 mM (in DMSO/water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, from Lineweaver–Burk plots, as reported earlier, [11] and represent the mean from at least three different determinations.

Results and discussion

β-lactam compounds have been used in the treatment of bacterial infections for nearly 60 years [24]. Following the initial introduction of penicillin, a variety of other classes of b-lactam antibiotics were subsequently identified and used clinically, including cephalosporins, penems, carbapenems, nocardicins, and monobactams. The bacterial targets of these antibiotics are membrane-bound transpeptidases referred to as the penicillin-binding proteins, which are responsible for creating crosslinks within the bacterial cell wall [24].

N-thiolated β-lactams, such as β-lactam 1, induced DNA damage, inhibited DNA replication, and induced tumor cell apoptosis in a timeand concentration-dependent manner. Smith et. al had demonstrated discovered and characterized the apoptosis-inducing properties of a family of novel β-lactam antibiotics against human leukemia, breast, prostate, and head-and-neck cancer cells. They found that one particular lead compound (lactam 1) with an *N*-methylthio group was able toinduce DNA damage and inhibit DNA replication in Jurkat T cells within a 2-h treatment [25].

The carbonic anhydrases (CAs, EC 4.2.1.1) constitute interesting targets for the design of pharmacological agents useful in the treatment or prevention of a variety of disorders such as glaucoma, acid–base disequilibria, epilepsy and other neuromuscular diseases, altitude sickness, edema, and obesity [26–29].

Some of sulfonamide derivatives showed a certain degree of selectivity for inhibition of the tumor associated over the cytosolic isoforms [30], thus human isozymes hCA IX and hCA XII are the targets for the development of novel antitumor therapies [31]. These enzymes catalyze the simplest physiological reaction,CO_2 hydration to bicarbonate and a proton [32]. Acidic extracellular pH is also a typical attribute of the hypoxic tumor microenvironment, with a strong impact on cancer progression and treatment outcome. CA IX and to a smaller extent also CA XII are highly overexpressed in hypoxic tumors [11]. Inhibition of CA IX and hCA XII by potent sulfonamide inhibitors leads to a significant increase of the tumor microenvironment pH of 0.5–1.0 unit. Many such sulfonamides were shown earlier to possess strong *in vitro* and *in vivo* anticancer activity [33]. These findings constitute the proof-of-concept that such enzyme inhibitors may lead to the development of novel antitumor therapies.

For this purpose, 15 different antibiotics are used in appropriate dosages for detecting the enzyme activities at the Stop-Flow Instrument with different concentrations. In the Table I, the IC_{50} values of 4 carbonic anhydrase isozymes are reported. According to these findings, the most potent inhibition is seen by Rifampicin 1.27×10^{-5} mM for hCA IX and for hCA XII 1.41×10^{-5} mM, respectively. Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding its beta subunit, thus preventing transcription of messenger RNA (mRNA) and subsequent translation to proteins [34].

Secondly, Sodium ampicilin is followed the highest IC_{50} values as 1.69×10^{-5}, 1.66×10^{-5} and 1.14×10^{-5} mM for hCA IX, hCA XII and hCA II, respectively. The β-lactamases are a large family of enzymes that hydrolyse the β-lactam ring of different members of the family and sodium ampicilin is one of the members of this class. Ampicillin is closely related to amoxicillin, another type of penicillin.

In this study, there is less inhibition was seen with Cefuroxime axetil for hCA IX and hCA XII isozymes as IC_{50} value of 8.21×10^{-5} and 5.88×10^{-5} mM, respectively. Cefuroxime axetil is the subgroup of β-lactams named as cephalosporin [35]. But the cytosolic carbonic anhydrase isozymes were given more inhibition effects than tumor associated isozymes of hCA IX and hCA II with their IC_{50} values (hCA I; 2.92×10^{-5} mM, hCA II; 2.12×10^{-5} mM). But on the other hand another cephalosporin member which is Cefazoline sodium, showed the greatest inhibition on hCA IX (IC_{50}; 1.54×10^{-5} mM) followed by this isozyme was hCA XII with an IC_{50} value of 2.17×10^{-5} mM. The antibiotic cefazolin sodium is significant decrease of liver glucose 6-phosphate dehydrogenase and human carbonic anhydrase I and II [36,21].

Another β-lactam group which belongs to Amoxicillin and Amoxicillin-potassium cluvanat antibiotics was potent inhibitors for hCA IX and hCA XII, seen in Table I. Thus, it is usually the drug of choice within the class because it is better absorbed, following oral administration, than other beta-lactam antibiotics. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria. Amoxicillin (in either trihydrate or sodium salt forms) may be combined with Clavulanic acid (as potassium clavulanate), a β-lactamase inhibitor, to increase the spectrum of action against Gram-negative organisms, and to overcome bacterial antibiotic resistance mediated through β-lactamase production [37].

Ciprofloxacin, Prurifloxacin and Moxifloxacin antibiotics are quinolone members and in this study the inhibition also was seen with these antibiotics on both four isozymes. Among this tree, hCA IX had the potent inhibition (1.83×10^{-5} mM) with Prurifloxacin and hCA XII had the great inhibition with Moxifloxacin (1.6×10^{-5} mM). Moxifloxacin inhibits bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. Topoisomerases are essential enzymes which play a crucial role in the replication and repair of bacterial DNA. This mechanism is lethal to susceptible bacteria. Moxifloxacin is often referred to as a chemotherapeutic drug because its mode of action has so far not been noted in any natural occurring or semi-synthetic antibiotic [38].

Unsubstituted sulfonamides are classical type inhibitors of CA family, and there are many studies to explain the clear relationship of these compounds [39]. Co-trimoxazole, is an antibiotic combination of trimethoprim and sulfamethoxazole, in the ratio of 1 to 5, used in the treatment of a variety of bacterial infections. Sulfamethoxazole acts as a false-substrate inhibitor of dihydropteroate reductase. Sulfonamides such as sulfamethoxazole are analogues of *p*-aminobenzoic acid (PABA) and are competitive inhibitors of the enzyme; inhibiting the production of dihydropteroic acid. Trimethoprim acts by interfering with the action of bacterial dihydrofolate reductase, inhibiting synthesis of tetrahydrofolic acid [40].

But its inhibition effect was less than the other group antibiotics such as Rifampicin and β-lactams. According to the findings, most inhibition was seen with hCA II and hCA XII, 2.25×10^{-5} mM and 2.07×10^{-5} mM for hCA II and for hCA XII respectively.

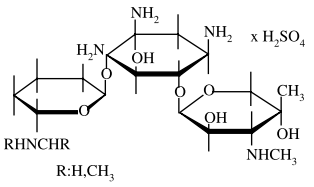
Clindamycin phosphate is a lincosamide [41] and with this drug both hCA II and hCA XII isozymes showed the greatest inhibition 1.89×10^{-5} mM and 2.92×10^{-5} mM for hCA II and hCA XII respectively.

Chloramphenicol is bacteriostatic by inhibiting the enzyme peptidyl transferase, which inhibits ribosomal activity and protein synthesis by preventing the binding of amino acyl-tRNA to the A site on the 50S subunit [42] and it had the greatest inhibition for hCA II and hCA IX with the IC_{50} values of 2.08×10^{-5} mM and 2.06×10^{-5} mM, respectively. In the literature, chloramphenicol is defined as its succinate ester, because pure chloramphenicol does not dissolve in water. This creates a problem: Chloramphenicol succinate ester is an inactive prodrug and must first be hydrolysed to chloramphenicol. While chloramphenicol and the macrolide class of antibiotics function ultimately in the same manner of affecting the 50S ribosomal subunit, chloramphenicol is not a macrolide [43].

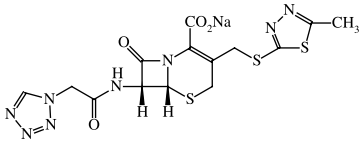
Gentamycin sulfate is aminoglycoside antibiotic and an inhibitor for NADPH oxidase and glucose 6-phosphate dehydrogenase and in this present study as a member of aminoglicoside [36,44] showed also the inhibition for both isozymes, hCA I, hCA II, hCA IX and hCA XII as the IC_{50} values 2.35×10^{-5}; 2.53×10^{-5}; 2.16×10^{-5}; 3.16×10^{-5}, respectively.

Table I. IC_{50} Values of CA Isozymes with certain antibiotics.

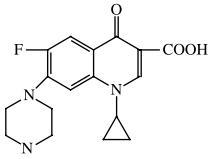
	IC50 Values (mM)			
Antibiotics	hCA I	hCA II	hCA IX	hCA XII
Sodium ampicilin	0.00163	0.00114	0.00169	0.00166
Amoxicillin-potasium cluvanat	0.00496	0.00374	0.00173	0.00268
Amoxicillin	0.00037	0.00416	0.00181	0.00347
Cefazoline sodium	0.00358	0.00308	0.00154	0.00217
Cefuroxime axetil	0.00292	0.00212	0.00821	0.00588
Clatromicin	0.00352	0.00253	0.00255	0.00247
Erythromycin	0.00227	0.00092	0.00123	0.00128
Rifampisin	0.00252	0.00102	0.00127	0.00141
Clindamicine phosphate	0.00341	0.00189	0.00356	0.00292
Gentamicine sulphate	0.00235	0.00253	0.00216	0.00316
Co-trimoxazole	0.00716	0.00225	0.00039	0.00207
Ciprofloxacin	0.00216	0.00297	0.00338	0.00376
Prurifloxacin	0.00286	0.00481	0.00183	0.00287
Moxifloxacin	0.00237	0.00026	0.00027	0.00016
Chloramphenicol succinate	0.00472	0.00208	0.00206	0.00318



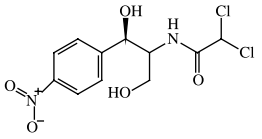
Gentamycin sulfate



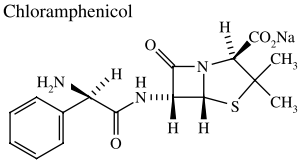
Cefazolin sodium



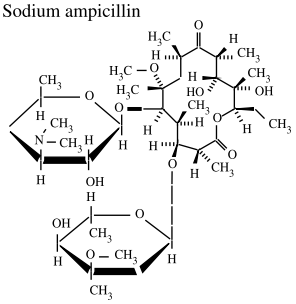
Ciprofloxacin



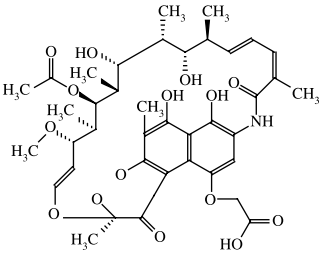
Chloramphenicol



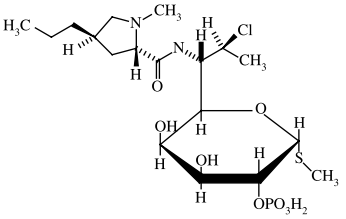
Sodium ampicillin



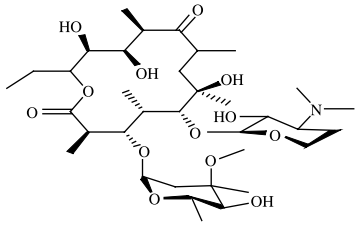
Clarithromycin



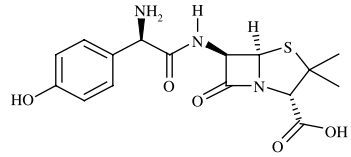
Rifampicin SV



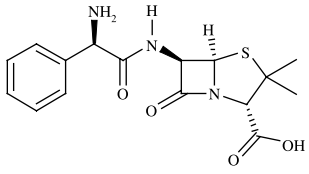
Clindamycin phosphate



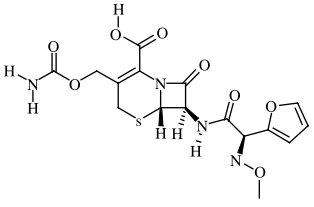
Erythromycin



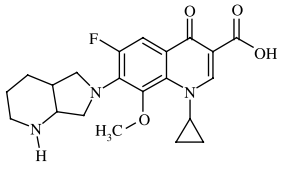
Amoxicillin



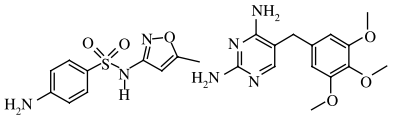
Ampicillin



Cefuroxime axetil



Moxifloxacin



Sulfamethoxazole and Trimethoprim

The Structural formulae of effective antibiotics

A macrolide antibiotic of Clarithromycin is responsible for protein synthesis in the cell like Erythromycin antibiotic. But it didn't have the greatest inhibition as well as Erythromycin which the values are seen in Table I. Clarithromycin prevents bacteria from growing, by interfering with their protein synthesis. Clarithromycin binds to the subunit 50S of the bacterial ribosome, and thus inhibits the translocation of peptides. Clarithromycin has similar antimicrobial spectrum as erythromycin, but is more effective against certain gram-negative bacteria, particularly *Legionella pneumophila*. Clarithromycin has a fairly rapid first-pass hepatic metabolism, i.e it is metabolised by the liver. However, this metabolite, 14-hydroxy clarithromycin is almost twice as active as clarithromycin. Clarithromycin's and its metabolites' main routes of elimination are urinary and biliary excretion [45].

Erythromycin is another macrolide antibiotic which is used in this study, has an antimicrobial spectrum similar to or slightly wider than that of penicillin, and is often used for people who have an allergy to penicillins. Among the antibiotics, Rifampicin and sodium ampicilin, the IC_{50} values are indicated that the most inhibition was seen with Erythromycin in Table I. And hCA II is being most affected with this antibiotic, IC_{50} value, and 9.2×10^{-5} mM. Erythromycin is very rapidly absorbed and diffused into most tissues and phagocytes and most of erythromycin is metabolised by demethylation in the liver. Its main elimination route is in the bile, and a small portion in the urine [43].

Consequently, this paper contributes the inhibition effects with various antibiotics thus it mainly is the first report on tumor associated carbonic anhydrases (hCA IX, hCA XII) and cytosolic carbonic anhydrases. With these results, antibiotics come close to being ideal chemotherapeutic agents in that they are administered orally and have a large population-based safety profile antibiotics might decrease the incidence of tumor recurrence. At the very least, one would expect antibiotics to increase the efficacy of chemotherapeutic agents in preventing tumor recurrence. Clinical trials are needed to test these hypotheses thus our future goal is to investigate the inhibition effects with different chemoterapy drugs on tumor associated isozymes and cytosolic carbonic anhydrases as well.

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