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IN VITRO STUDIES ON THE RELATIONSHIP BETWEEN ANTI HELICOBACTER PYLORI THERAPY AND CARBONIC ANHYDRASE ISOENZYMES II AND IV

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ABSTRACT: H+/K+ ATPase discovery resulted in the re-evaluation of the role of carbonic anhydrase (CA) in acid secretion. In addition to its traditional role, proposed by Davenport as a source of H + ions by catalytic hydration of CO2 in the current biochemical model of acid secretion, CA is involved in segregation of H^+ and OH^- ions in the parietal cell. In our study we observed the effect of anti *H. pylori* therapy on the two isoenzymes of CA involved in acid secretion changes. The study was conducted in terms of two experiments: the dose - response and kinetic studies. The results of this study mainly confirms the implication of CA II and CA IV in the mechanism of gastric acid secretion also explaining the beneficial effects of combination therapy in the treatment of *H. pylori* infection as a result of gastric mucosa CA isoenzymes inhibition.

Keywords: Helicobacter pylori, Carbonic anhydrase

INTRODUCTION:

H^+/K^+ ATPase or acid pump is considered the last step of acid secretion produced by the parietal cell. (Hersey S.J et al., 1995) Regarding acid pump inhibitors, the research started in 1966 and led to the discovery in 1979 of omeprazole (Lindberg P. et al., 1990) to be held today as one of the most effective antiulcer therapeutic agents, like all other proton pump inhibitors. Regarding the mechanism of action of omeprazole in reducing gastric acid secretion, research to date shows that it consists in blocking H^+ - K^+ exchange in the parietal cell.

Research on the implication of another enzyme, carbonic anhydrase in gastric acid secretion, were started in 1952 by Janowitz , Colcher and Hollander (Janowitz H.D et al., 1952) who showed that acetazolamide, the specific inhibitor of CA, inhibits the flow rate of hydrochloric acid up to 97 % when given iv in dogs at a dose of 75-120 mg / kg b.w. who also were receiving i.v. histamine infusion.

In addition to its traditional role, proposed by Davenport (Devenport H.W, 1939) as a source of H^+ ions by catalytic hydration of CO2 in the current biochemical model of acid secretion , carbonic anhydrase is involved in segregation ions H + and OH- parietal cell. (Puşcaş I et al., 1986) Prof.Puşcaş research started in 1971 (Puşcaş I., 1971) and continued until now have shown favorable effects of CA inhibitors in the treatment of peptic ulcers in humans. (Puşcaş I., 1976, 1984, 1987, 1990)

Regarding the physiological role of CA isoenzymes, Prof.Puşcaş research proved that CA II isoform is involved in changes in gastric acid secretion and CA IV - known as the membrane isozyme (Wistrand P.J., 1984) - present in the gastric parietal cell membrane, kidney and lung has organ specificity. Moreover, the major stimuli of gastric acid secretion as: histamine, gastrin and acetylcholine activates CA IV of gastric parietal cell membrane and does not alter the activity of the same isoenzymes of kidney and lung (Puşcaş I., 1981); somatostatin and calcitonin decrease gastric CA IV (Wistrand P.J., 1984) activity and do not influence the activity of CA in the kidney and lung.

Starting from these premises, in this *in vitro* study we observed the effect of anti H. pylori therapy on the two isoenzymes of carbonic anhydrase involved in acid secretion changes.

MATERIALS AND METHODS:

The study was conducted in terms of two experiments: the dose-response and kinetic studies.

Dose-response:

We analyzed the effect of a proton pump inhibitor, esomeprazole, at concentrations between 10^{-8} and 10^{-4} M in the 2 CA isoforms isolated from parietal cells of the gastric mucosa. Then we studied the effect of esomeprazole, metronidazole and amoxicillin, at the same concentrations on the same isoenzymes.

All reagents were weighed and dissolved in double distilled water and stock solutions were made for each at a concentration of 10^{-3} M and brought to a pH of 7.5.

For each substance successive dilutions were performed, obtaining concentrations of 10^{-4} M and 10^{-8} M.

Activity of each subtype was measured at the beginning, namely purified CA II and CA IV isolated from parietal cells of the gastric mucosa.

In the next step, to each of the CA isoform were added increasing concentrations of the substance under investigation, esomeprazole or esomeprazole with anti-*H. pylori* therapy and enzyme activity was measured by calculating the percentage inhibition of each subtype for each substance.

Kinetic studies:

In order to determine the type of mechanism of action of the substances studied on CA isoenzymes we performed kinetic studies for each type of substance on purified isoform II.

The initial rate of CO_2 hydration reaction is measured by stopped-flow method using HEPES para-nitrophenol system. The temperature is maintained at 25^0C, and the initial pH is 7.5. The concentration of

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CA II in the reaction tank is $1.74x10^{-8}$ M and the ionic strength is kept constant with 0.1 M Na_2SO_4.

The following parameters were determined: maximum reaction rate (V_{max}) and Michaelis-Menten kinetic constant (K_m) for both of the purified CA and for the enzyme PPI complex.

For measuring the initial reaction rate the substrate concentration was varied between 5 mM and 30 mM CO_2.

Lineweaver-Burk analysis of the Michaelis-Menten equation was performed using graphics software (Erithacus Software Ltd., UK), and calculation of kinetic parameters was performed using the kinetic software RKBIN IS-1 (Hi-Tech, England).

Measurements of enzyme activity, both for studies of dose response and for kinetic studies have been carried out with a rapid kinetic spectrophotometer model **SF-51 HI-TECH MX** (England) equipped with interface for data processing and kinetic software kinetic.

RESULTS:

The results of these *in vitro* studies are given below for the two types of experiments, namely the dose-response and kinetic studies.

Dose-response

Data from dose-response type experiments performed are shown both tabular and in graphical form in order to highlight the effects of pharmacological agents studied have on CA isoenzymes.

Table 1 shows the gradual decrease of purified CA II and CA IV from the parietal cells of the gastric mucosa isoenzymes activity after associating substances studied. The decrease in activity is dependent on the concentration of inhibitor. The effect is present at 10^{-8} M and becomes maximum at the concentration of 10^{-4} M. The basal activity of the isoenzymes studied is recorded in the table.

Table 1.

Effect esomeprazole and anti - H. pylori therapy on CA isoenzymes. Values are presented as mean ± standard deviation; n = 5 determinations at each concentration; * Significant difference (p <0.05) compared to basal activity of the enzyme (Student's test).

Substance	Conc. (M)	Purified CA II *Basal= 1.00± 0.01*(UE/ml)	Gastric CA IV *Basal = 1.420 ± 0.02* (UE/ml)
Esomeprazole	10^{-8}	0.841 ± 0.01*	1.127 ± 0.01*
	10^{-7}	0.732 ± 0.03 *	0.814 ± 0.02 *
	10^{-6}	0.615 ± 0.03 *	0.683 ± 0.02 *
	10^{-5}	0.468 ± 0.03 *	0.571 ± 0.01 *
	10^{-4}	0.326 ± 0.02 *	0.412.± 0.02 *
Metronidazole	10^{-8}	0.951 ± 0.02	1.412 ± 0.02
	10^{-7}	0.903 ± 0.02	1.401 ± 0.01
	10^{-6}	0.897 ± 0.02	1.397 ± 0.01
	10^{-5}	0.871 ± 0.01 *	1.372 ± 0.01
	10^{-4}	0.868 ± 0.03 *	1.348 ± 0.02*
Esomeprazole +	10^{-8}	0.798 ± 0.01*	1.029 ± 0.01*
	10^{-7}	0.706 ± 0.03 *	0.787 ± 0.02 *
Metronidazole +	10^{-6}	0.551 ± 0.03 *	0.595 ± 0.02 *
Amoxicillin	10^{-5}	0.426 ± 0.03 *	0.445 ± 0.02 *
	10^{-4}	0.258 ± 0.02 *	0.329 ± 0.04 *

As can be seen from the table, the inhibitory effect of esomeprazole on CA isoenzymes is present at a concentration of 10^{-8}M, being statistically significant at all concentrations studied.

Unlike esomeprazole, metronidazole does not change the activity of the two CA isoforms, at concentrations smaller then 10^{-4} M, which achieved 13% inhibition of the purified CA II and 5% of the CA IV.

The association of esomeprazole with metronidazole results in a more pronounced decrease in activity of purified CA II and gastric CA IV, leading to an inhibition of 75% in CA II and 77% in CA IV.

Changes in basal activity of pure CA II isoform produced by the substances studied are plotted in Figure 1. Graphicaly are represented concentrations of 10^{-8}, 10^{-6} and 10^{-4} M to separate substances and their association. Basal activity of the purified enzyme was 1.00 EU / ml.

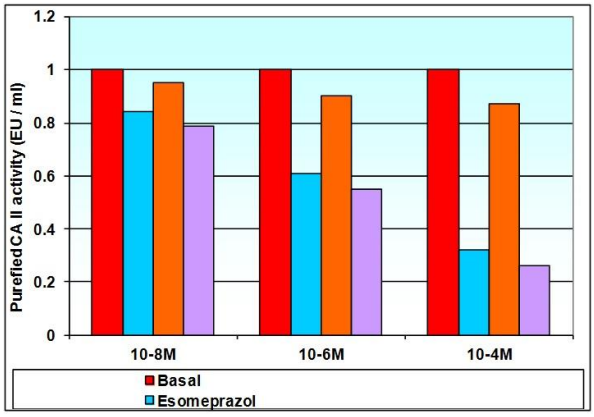

Fig 1. Inhibition produced by esomeprazole and anti *H. pylori* therapy on purified CA II.

Kinetic studies:

Lineweaver-Burk processing of the Michaelis-Menten equation in the system of coordinates (1 / s - 1 / v) (v / s - s) or (v - v / s) in the presence and in the absence of esomeprazole are used to determine the biochemical mechanism of action of this substance on the purified CA II isoform.

Please note that kinetic measurements were performed only on the purified enzyme, namely CA II and studied only the effect of esomeprazole, because the combination of several substances is not conclusive for kinetic studies.

In the case of Lineweaver-Burk representation in the coordinates (1 / s - 1 / v), all the lines obtained at increasing concentrations of esomeprazole intersects the axis 1 / s at a single point.

The representation in the system of axes v/s – s, all the lines intersect in one point located on the axis S.

Representation in the system of axis v - v/s, all relevant lines corresponding to increasing concentrations of esomeprazole are parallel, all having the same slope.

These results clearly indicate that esomeprazole does affect only the Vmax and do not change the Michaelis constant (Km), demonstrating a mechanism of non-competitive inhibition.

In this case, the CA inhibitor binds the zinc ion in the active site of the enzyme, as is the case with simple anions, known as non-competitive inhibitors.

The results of the kinetic studies for purified CA II isoform alone and associated with esomeprazole in concentrations of 10^{-8} M, 10^{-6} M and 10^{-4} M, and the conditions under which the experiments were carried out are given in Table 2. As it is noted the data are expressed as mean ± standard deviation, for each concentration of the activator were carried out five measurements with statistical significance ($p < 0.05$).

Table 2.

Kinetic data for CO_2 hydration reaction catalyzed by purefied CA II in the presence of esomeprazole.

System	Vmax[a] (mM/s)	Km[a] (mM)
CA II	1.116 ± 0.02	8.41 ± 0.20
CA II + Esomeprazole 10^{-8} M	0.783 ± 0.02	8.39 ± 0.16
CA II + Esomeprazole 10^{-6} M	0.698 ± 0.03	8.48 ± 0.11
CA II + Esomeprazole 10^{-4} M	0.565 ± 0.02	8.43 ± 0.04

pH = 7.5; T=25°C; Concentration CA II = $1{,}65 \times 10^{-8}$M
[a] = mean ± standard deviation (n= 5 measurements)

DISCUSSION AND CONCLUSIONS:

The study on the dose-response relationship revealed that esomeprazole used in our *in vitro* experiments produce direct inhibition of carbonic anhydrase isoenzymes II and IV. The effect occurs at the concentration of 10^{-8} M, and gradually increases with increasing concentration of the inhibitor, reaching peak maximum at 10^{-4} M concentration where inhibition of CA II is 67%, and of CA IV is 71%. The studies were not carried out at concentrations higher than 10^{-4} M since at these concentrations the results obtained are not relevant for physiological processes.

Metronidazole alone does not have an effect on the activity of the two isoforms of CA, the only significant effect was present at a concentration of 10^{-4} M, where the inhibitory effect is still modest.

The combination of esomeprazole and amoxicillin with metronidazole produces an increase in the inhibitory effect of esomeprazole, the percentage of inhibition of the two isoforms of CA reached 75% and 77%.Note that amoxicillin alone it does not produces a significant inhibition of CA.

From the kinetic data obtained one can may notice a decrease in Vmax for esomeprazole. The decrease in the rate of reaction also occurs simultaneously with the increase in the concentration of the inhibitor introduced in the reaction medium. It was also observed that the Michaelis-Menten constant value (Km) remained around a constant value in all tests carried out, regardless of the concentration of substrate, enzyme or inhibitor

These data indicate that there was an inhibition of a non–competitive type, which means that the inhibitor of CA binds the substrate at different sites, namely CO_2, therefore, does not compete with the substrate for the active site of the enzyme.

The results of this *in vitro* study mainly confirms the implication of CA II and CA IV in the mechanism of gastric acid secretion also explaining the beneficial effects of combination therapy (esomeprazole - metronidazole – amoxicillin) in the treatment of *H. pylori* infection as a result of gastric mucosa CA isoenzymes inhibition.

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