Demonstrating the antinociceptive action of renin-angiotensin system modulators in mice

Demonstrarea acțiunii antinociceptive la șoarece a unor modulatori ai sistemului renină-angiotensină

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Abstract

Research done in the present study belongs to a wider area of experimental determinations on nociception models, when using laboratory animals. Their aim is to determine the ED50 value (Efficient dose 50) in conditions of inflammatory (chemical stimulus) and non-inflammatory (thermic stimulus) antinociception of some renin-angiotensin system modulators: captopril, ramipril, candesartan. The experimental determinations were realized accordingly to bioethical regulations concerning laboratory animals. The study has used Swiss mice, weighing between 20-30g, being held in constant temperature (21°C ± 2°C) and a dark/light cycle of 12 hours (7.00 AM / 7.00 PM). The researched substances are administered as CMC-Na 0.1% suspensions in geometrical progression doses. The following nociception models have been used: abdominal constrictive response test, hot plate test, formalin test. The abdominal constrictive response test has been evaluated as quantal, the hot plate test and the formalin test have been interpreted as gradual. The regression line, the correlation coefficient and the interval of trust for each substance and studied model have been analyzed. The obtained ED_{50} values are compared to each other to evaluate the potency of the substances for each nociception model. The obtained data is used for realizing fixed-ratio antinociceptive combinations.

Rezumat

Cercetările din prezentul studiu fac parte dintr-o arie mai largă de determinări experimentale pe modele de nocicepție la animalele de laborator. Acestea au drept scop determinarea valorii DE_{50} (doza eficace 50) în condițiile antinocicepției inflamatorii (stimul chimic) și non-inflamatorii (stimul termic) a unor modulatori ai sistemului renină-angiotensină: captopril, ramipril, candesartan. Determinările experimentale s-au efectuat în acord cu reglementările de bioetică referitoare la animalele de laborator. Studiul s-a realizat pe șoareci Swiss cu greutatea 20-30g menținuți în condiții de temperatură constantă (21°C ±2°C) și un ciclu de lumină/întuneric de 12 ore (7.00AM / 7.00PM). Substanțele de cercetat sunt administrate sub formă de suspensii în CMC-Na 0.1% în secvențe de doze administrate în progresie geometrică. Modelele de nocicepție utilizate sunt: testul răspunsului constrictiv abdominal, testul plăcii încălzite, testul la formalină. Testul răspunsului constrictiv abdominal a fost evaluat ca fiind cuantal, testul plăcii încălzite și testul la formalină au fost interpretate ca fiind gradate. Se analizează dreapta de regresie, coeficientul de corelație și intervalul de încredere pentru fiecare compus și model studiat. Valorile ED50 obținute se compară între ele pentru a evalua potența compușilor caracteristică fiecărui model de nocicepție. Datele obținute sunt utilizate pentru realizarea unor combinații antinociceptive în proporție fixă.

Introduction

The angiotensin converting enzyme inhibitors and the angiotensin receptor AT1 blockers are frequently used as medication for the treatment of arterial hypertension in chronic treatment.

Because the renin-angiotensin system is one of the systems with important physiological and pathophysiological implications, influencing it through the use of pharmacological agents can determine behavioral effects. The nociceptive test has

been done in dose sequences that do not influence the normal arterial tension.

Although analgesic therapy is based mainly on two big groups of analgesics (opioids and non-opioids) prescribed on a large scale, they do not cover all the pain syndromes.

Researchers are investigating a series of substances that, even though they are not pure analgesics, they can improve analgesia by association with classical analgesics.

These substances belong to different therapeutical groups that influence the central or peripheral nervous system and they are named co-analgesics and para-analgesics.

Generically, they are named adjuvants. The adjuvant group includes antidepressants, some muscular relaxing drugs, a series of anxiolytics, anticonvulsants. In this study, we propose to determine the ED_{50} value of the antinociceptive action of substances that influence the renin-angiotensin system for associating them with other medical substances in fixed proportions.

Materials and Method

The experimental protocol is composed of white male Swiss adult mice, weighing 20-25 g, 25-30 g and 20-30 g, divided randomly in groups of study.

The animals have been bought from Bucharest Cantacuzino Institute, their transportation being done accordingly to current legislation.

The husbandry conditions (transit, habitation) have been made according to the specific conditions for each test, within de Experimental laboratory of Pharmacodynamics from the department of Pharmacodynamics and Clinical Pharmacy. The animals were placed in special cages with adequate size, adapted with a water container and support for food, which assure water and food *ad libitum.* The room has a constant temperature (21°C ± 2°C) and a light/dark cycle of 12 hours (7.00AM / 7.00PM). The experience animals were grouped in 6-15 animals /group.

The behavior of the experience animals was observed for 15 days before the experiment, in the acclimatization period, considering the fact that pain involves behavior reactions. This involved observation of the appetite, water consume, digestive transit, neurological signs, etc.

Used substances:

- Captopril, ramipril, candesartan (substances that influence the renin-angiotensin system)
- Inflammatory/nociceptive agents: formaldehyde solution, Zymosan A. suspension

Nociceptive stimulus:

The inflammatory/nociceptive agents have been administered i.p. or s.c. in the plantar region. The thermal stimulus has been applied using Hot Plate UGO BASILE model 7280. The administration of the irritating and the inflammatory agents has been done with single-use syringes with appropriate scales and needles, subcutaneous in the plantar region. For these substances, the vehicle that is used is physiological serum.

The testing from this study have been realized in accordance with international bioethical regulations, internal USAMV, regulation and statutes of the International Association for the Study of Pain, referring to the experimental protocols that involve laboratory animals for the study of pain (Zimmermann, 1986).

Nociceptive testing methods

The battery of tests is comprised of the following: formalin test, abdominal constrictive response test, hot plate test. These tests have been selected for verifying the antinociceptive action in inflammatory and non-inflammatory conditions of research-*substances with a primary action which is not analgesic.*

Abdominal constrictive response test

The test uses the method of Siegmund and col. 1957, Koster's technique and col. 1959 modified (Domer, 1971; Tallarida et al., 2003; Turner and Hebborn, 1965).

The method consist of intraperitoneal injection on mice or rats of an irritating solution (acetic acid) or suspension (Zymosan A), or other compounds that can produce a characteristic response named abdominal constrictive response.

Ribeiro and his collaborators have shown that in mice, the Zymosan A induced abdominal constrictive response is TNF-alfa, Interleukin 1-beta and Interleukin-8 mediated (Cunha et al., 1999; Pettipher et al., 1997).

In the present study, the number of abdominal constrictive responses is recorded for 12 minutes after the administration of Zymosan A suspension, 40mg/kgbw. The interpretation of the response is quantal, characterized by the presence or the absence of the constrictive response (Bild et al., 2009).

It is considered inhibitory effect of the studied substances the percent of inhibition obtained as an absence of response from the total number of animals from the work group.

$$\%\ \text{(antinociception) inhibition} = \frac{no.non-responders}{no.total\ animals} \times 100$$

Hot plate test

In the present study, we used the method of Woolfe and McDonald 1944, modified by Eddy and Laborit 1953 (Le Bars et al., 2001).

The experimental temperature is 52.5 +/- 0.1°C and 55.00 +/- 0.1°C. The latency period of the pain is measured, with a 30 second cut-off time. The response consists of licking, posterior paw shaking, jumping for leaving the enclosure.

The animals are preliminary tested before being treated with the researched substance. After the treatment, the animals are being tested with thermic stimulus at 30, 60, 90, 120 minutes.

The experimental model is interpreted as being gradual. For this test, the data is presented as a percentage of the maximum possible effect, for lowering the incidence of errors that can occur because of inter individual variability.

$$\%\ \text{(antinociception) inhibition} = \frac{T_x - T_0}{T_m - T_0} \times 100$$

Where: T0 - latency of the response measured prior to administration of the study substance, Tx - latency at different time intervals following administration of the test substance, Tm - cut-off time.

Formaline test in mice

The test allows the evaluation of the analgesic action in inflammatory conditions and consists in administering the nociceptive agent, a formaldehyde solution with a concentration of 1-5%. Then, the latency is recorded until the appearance of the pain reaction for 5 minutes from the injection of the formaldehyde solution and for 10 minutes after 20 minutes from the administration moment. (Hunskaar et al., 1985; Rosland et al., 1990).

The response is biphasic, the first phase characterizing mostly the nociception and the second phase characterizes the inflammation. Nociception is probably related to the direct effect of the formaldehyde on the sensory receptors, because formaldehyde binds itself to the free amino groups and can alter proteins. The inflammatory reaction appears because of the release of pro inflammatory mediators such as histamine, bradykinin, serotonin, prostaglandins from the injured cells (Muir and Anderson, 1976).

Histological examination reveals the aspect of an acute inflammatory reaction that begins at approximately 1 hour after injection and can last up to 480 hours (Rosland et al., 1990)

Analgesia evaluation is done using a behavior motor test (licking-biting the paw) that is not influenced by a possible block of the propagation ways of the painful sensation.

The specific answer (licking-biting the paw) is easy to notice and to quantify especially in mice (Hunskaar et al., 1985). The formalin test uses a long-time nociceptive stimulus that is similar to clinical pain (Dubuisson and Dennis, 1977).

The formalin test consists of the intra-plantar administration of a solution of formaldehyde and recording the response for 5 minutes from the moment of administering the irritating agent and 10 minutes starting from

the 20th minute after administering the irritating agent.

Based on the two evaluating possibilities, in this study, the formalin test is a screening test. It is considered antinociceptive effect the percentage report compared to the control subject

$$\% \text{ inhibition (antinociception)} = \frac{M-T}{M} \times 100$$

Where:
M – value of the inhibition level of the control group
T – value of the inhibition level of the treated group

For evaluating the analgesic action, based on the diversity of the pro nociceptive mediation and the nervous structures involved, for each model the ED_{50} dose will be determined for each substance. This is based on the analysis of a regression line, which can demonstrate the ED_{50} value.

Results and Discussion

The formalin test

Groups of 6 animals/lot, weighing between 20-25 g have been treated with studied substances in sequences of doses in geometrical progression, with a *ratio* of 2 as follows:

- Ramipril 0.250–2mg/kg orally in CMC-Na 0.1% suspension
- Captopril 0.780-6.250 mg/kg orally in CMC-Na 0.1% suspension
- Candesartan 0.250-2.00 mg/kg orally in CMC-Na 0.1% suspension

60 minutes after the last administration, the animals were treated s.c. in the plantar region as follows: right paw with 20 µL of saline solution of formaldehyde with a concentration of 5% and left paw with 20 µL with physiological serum.

The experiment lasts 30 minutes. The animal behavior has been recorded for 5 minutes (starting from the administration of the nociceptive agent) and for 10 minutes (from the 20th minute of the administration of the nociceptive agent) (Hiramatsu et al., 1990;).

After the regression analysis, the following data were obtained:

Table 1

ED50 values of the substances used for the formalin test

Group	Formalin 5 % Evaluation time 5 minutes	Formalin 5% Evaluation time 10 minutes from the 20th minute
	ED_{50} value mg/kgbw	
ramipril	ED50 = 0.449 +/- 0.079 Y = 62.616 + 36.282*X, R = 0.936 True Confidence Limits (0.022, 0.892)	na
captopril	ED50 = 1.040 +/- 0.279 Y = 49.477 + 30.955*X, R = 0.944 True Confidence Limits (1091.2, 2.390)	ED50 = 0.841 +/- 0.277 Y = 53.838 + 51.104*X, R = 0.936 True Confidence Limits, (1339.7, 2.047)
candesartan	ED50 = 0.318 +/- 0.063 Y = 71.634 + 43.494*X, R = 0.969, True Confidence Limits (0.017, 0.588)	na*

**na (not available)*

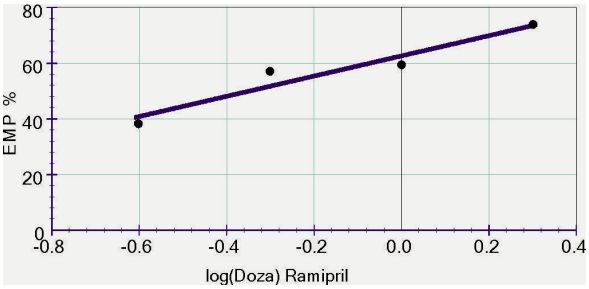

Fig. 1. – Analysis of the regression line of ramipril for the 1st phase of the formalin test

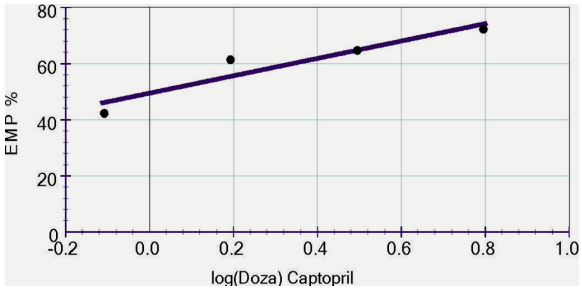

Fig. 2. – Analysis of the regression line of captopril for the 1st phase of the formalin test

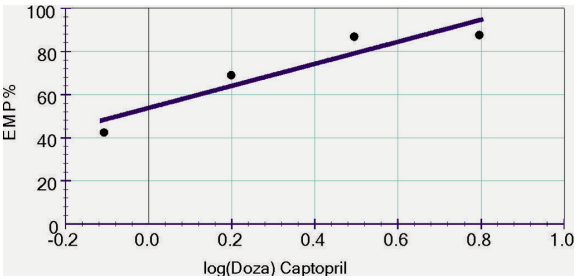

Fig. 3. – Analysis of the regression line of captopril for the 1st phase of the formalin test

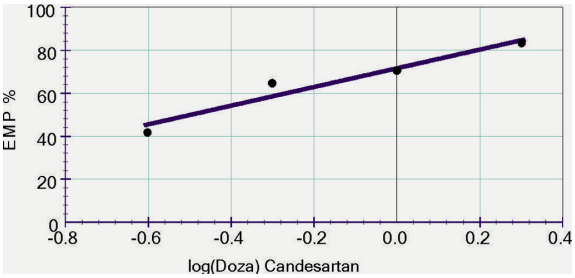

Fig. 4. - Analysis of the regression line of candesartan for the 1st phase of the formalin test

All the substances have shown their action mostly on the 1st phase, demonstrating an antinociceptive behavior similar to those of non-opioid analgesics.

Captopril, on the other hand, has demonstrated the antinociceptive action for both phases of the formalin test, demonstrating both non-opioid analgesic behavior and antinociceptive behavior in inflammatory conditions (table 1).

Abdominal constrictive response test

Groups of 6 animals/lot weighing between 20-30 g have been treated with the studied substances in sequential doses in geometrical progression ratio 2 as follows:

- Ramipril 0.500-4.00 mg/kg orally in CMC-Na 0.1% suspension
- Captopril 3.25-25 mg/kg orally in CMC-Na 0.1% suspension
- Candesartan 0.250-2.00 mg/kg orally in CMC-Na 0.1% suspension
- Doxepine 2.5-20.00 mg/kg orally in CMC-Na 0.1% suspension

60 minutes after the treatment Zymosan A 40 mg/kgbw has been intraperitoneally administered.

The experiment lasted 12 minutes.

The behavior of the animals characterized by the presence or absence of the abdominal constrictive response has been recorded. Following the regression analysis (Fig. 5-7), the following data has been obtained (table 2):

Table 2

ED50 values of the substances used in the experiment for the abdominal constrictive response test induced with Zymosan A

Group	Zymosan A 40mg/kgbw (Evaluation time 12 minutes)
	ED50 mg/kg value
ramipril	ED50 = 2.177 +/- 0.679 Y = 4.228 + 2.285*X, R = 0.921
captopril	ED50 = 6.208 +/- 2.282 Y = 3.774 + 1.547*X, R = 0.999
candesartan	ED50 = 1.371 +/- 0.407 Y = 4.626 + 2.729*X, R = 0.976

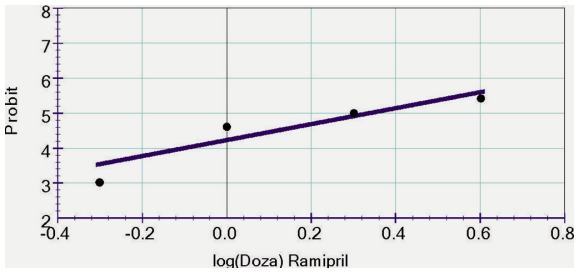

Fig. 5. – Analysis of the regression line of ramipril for the abdominal constrictive response test

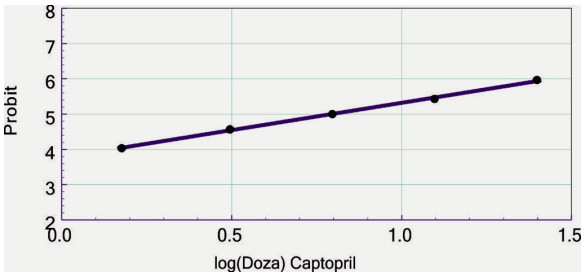

Fig. 6. – Analysis of the regression line of captopril for the abdominal constrictive response test

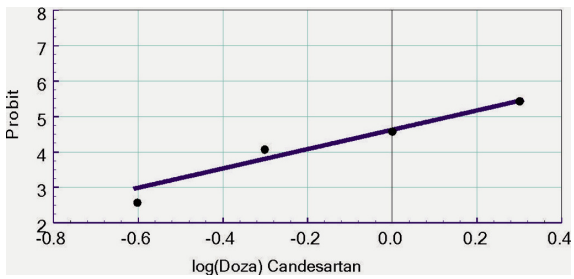

Fig. 7. – The analysis of the regression line of captopril for the abdominal constrictive response test

All of the substances used for this study have demonstrated their antinociceptive action

for the abdominal constrictive response test induced with Zymosan A.

Hot plate test

For this test the animal groups have been selected by randomization in groups of 10 animals / group Swiss male white mice, weighing between 20-30g, the habitat conditions being described in the materials and method chapter.

The animals were initially tested to verify the pain perception threshold.

Groups of 8-10 animals / group weighing between 20-30g have been given the following research substances, in sequential doses in geometrical progression with a ratio of 2 as follows:

- Ramipril 0.250-2.00 mg/kg orally in CMC-Na 0.1% suspension
- Captopril 3.125-12.5 mg/kg orally in CMC-Na 0.1% suspension
- Candesartan 0.50-2.00 mg/kg orally in CMC-Na 0.1% suspension

The animals were tested on a period of 120 minutes as in the following protocol: 30, 60, 90 at a temperature of 52.5 ± 0.2°C.

The latency to pain for 30 seconds (cut-off) has been noted, each animal being his own control. Antinociception was calculated as in the description from the materials and method chapter, the antinociceptive effect being expressed in percentage (EMP%)

After the regression analysis (Fig. 8-10), the following data was obtained (table 3)

Table 3

ED50 values of the substances used for the hot plate test

Group	Hot plate test (working temperature 52.5°C)
	ED_{50} mg/kg value
ramipril	ED_{50} = 0.666 +/- 0.156 *(90 minutes determination time)* Y = 56.579 + 37.315*X , R = 0.919, True Confidence Limits (2.573, 0.231)
captopril	ED50 = 6.907 +/- 0.804 *(60 minutes determination time)* Y = -1.578 + 61.456*X., R = 0.980, True Confidence Limits (11.327, 3.329)
candesartan	ED_{50} = 1.074 +/- 0.233 *(90 minutes determination time)* Y = 47.970 + 65.724*X, R = 0.935, True Confidence Limits (1.777, 0.559)

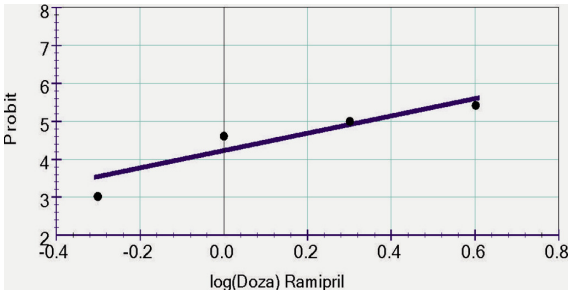

Fig. 8. – Analysis of the regression line of ramipril for the hot plate test

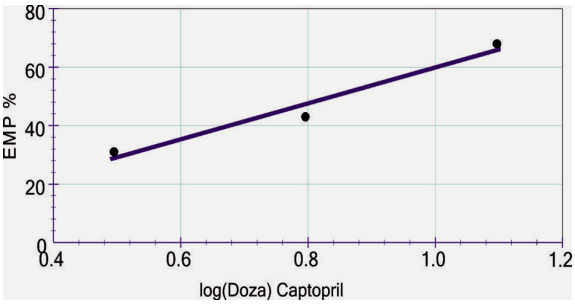

Fig. 9. – Analysis of the regression line of captopril for the hot plate test

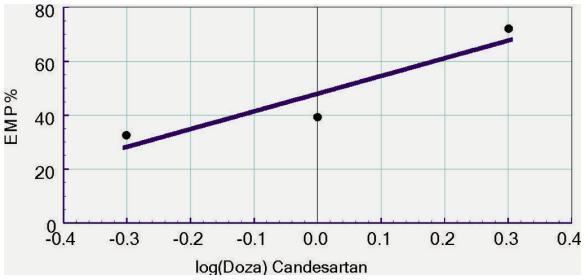

Fig. 10. – Analysis of the regression line of candesartan for the hot plate test

For the 52.5°C, all the substances from the study have shown antinociceptive action as follows: ramipril at 90 minutes evaluation time, captopril at 60 minutes evaluation time and candesartan at 90 minutes evaluation time.

Conclusions

From the data analysis the following are concluded:

- All the substances used in this study have demonstrated the antinociceptive action for the used nociception models
- For the formalin test the 1st phase of antinociceptive evaluation, the potency decreases in the following order: candesartan, ramipril, captopril
- For the Zymosan A induced test, the potency decreases in the following order: ramipril candesartan, captopril
- For the hot plate test, the potency decreases in the following order: ramipril, candesartan, captopril.

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