ORIGINAL ARTICLE

SYNERGISTIC EFFECS OT THE DOXEPIN-CANDESARTAN COMBINATION ON THE THERMOALGESIC SENSIBILITY IN MICE

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Abstract

Given the multitude of neuromodulators involved in the generation and transmission of pain, two therapeutic agents with different action mechanisms (an antidepressant - doxepin and an angiotensin-AT1 receptor blocker - candesartan) have been associated in this study. None of the substances is used exclusively as analgesic, but increasingly as adjuvants. The hot-plate test at 52.5°C was used as a study method of the thermal stimulation nociception. For the analysis of the interaction, the additive composite method was used. The study was performed on Swiss mice weighing 18 - 22 grams, which received oral administration of the drugs, either single or combined work-ups. The data obtained demonstrated a synergic type interaction (Zadd = 7.74 ± 0.92, Zmix = 1.82 ± 0.36, p1 = 0.931, p2 = 0.069, = 0.5, γ = 0.282, T_c = 6.20, T_t = 3.79, F_c = 15.30, F_t = 5.14, $p < 0.001$) between doxepin and candesartan. Both substances exhibited antinociceptive effects *per se,* and the fixed-ratio combination proved to be synergistic.

Rezumat

Având în vedere multitudinea de neuromediatori implicaţi în procesul de generare şi transmitere a durerii, în studiul de faţă au fost asociate două substanţe terapeutice cu mecanisme de acţiune diferită (un antidepresiv – doxepinul şi un blocant de receptori AT1 ai angiotensinei – candesartan). Nici una dintre substanţe nu este folosită în mod exclusiv ca analgezic, dar din ce în ce mai mult ca şi adjuvante. S-a folosit testul *hot-plate*, la 52,5□, ca metodă de studiu a nocicepţiei prin stimul termic. Pentru analiza interacţiunii s-a utilizat metoda dreptei aditive compuse. Studiul s-a realizat pe şoareci Swiss în greutate de 18 - 22 g, care au primit pe cale orală secvenţe de doze din substanţele de lucru singure şi în asociere. Datele obţinute au demonstrat o interacţiune de tip sinergic (Zadd = 7,74 ± 0,92, Zmix = 1,82 ± 0,36, p1 = 0,931; p2 = 0,069, = 0,5, γ = 0,282, T_c = 6,20, T_t = 3,79, F_c = 15,30, F_t = 5,14, $p < 0,001$) între doxepin şi candsartan. Ambele substanţe au prezentat efecte antinociceptive *per se*, iar asocierea în proporţie fixă s-a dovedit a fi sinergică.

Keywords: candesartan, doxepin, synergism, pain, hot-plate test

Introduction

Pain is an increasingly studied phenomenon, as more and more types of pain are individualized, and knowledge of pain mediation, transmission path-ways, and perceptual mechanisms at the central and peripheral level are developing. Classical analgesics have not been abandoned, but lately other substances, different from them, started being used either as single agents or as adjuncts in the treatment of pain. Cerebral angiotensin was identified as having a role in pain perception many decades ago [9], and in the years that followed the receptors for angiotensin were identified in a multitude of brain structures involved in nociception (such as the anterior cingulate cortex, prefrontal cortex, thalamus, periaqueductal gray matter, amygdala, *nucleus accumbens* and spinal cord) [1, 4, 10]. Also, besides pain perception and processing, a multitude of structures that are involved in modulating sensory information, learning, memory, stress, emotional and behavioural responses also exhibit AT1 and AT2 receptors in variable amounts and distribution [1, 14, 30]. It has long been known that angiotensin II is a pro-oxidant and pro-inflammatory mediator, as well as a cognitive function inhibitor, therefore there are a multitude of studies that have investigated the effect of converting enzyme inhibitors and, more recently, of the AT1 receptor blockers (ARBs) on the above-mentioned parameters [5, 26]. It has been shown that telmisartan reduces hyper-algesia in neuropathic pain models [5], while the

intrathecal administration of losartan reduces pain in formalin pain models [16]. Candesartan was less studied on pain models, and was therefore chosen as a representative of its group of drugs for this study.
Doxepin is an antidepressant belonging to the family of tri-cyclic antidepressants, which acts by inhibiting noradrenaline and serotonin re-uptake at the pre-synaptic end, thus increasing the effect of adrenergic and central serotoninergic mediation [32]. There are also described antihistaminic and anticholinergic effects [31]. Its main indications are depression, anxiety disorders, sleep disturbances and pruritus. In the last decade, tricyclic antidepressants have become an essential element in the treatment of neuropathic pain, especially tertiary amines, which include doxepin [23]. Interestingly, the effects on the pain seem to be independent of the anti-depressant ones and appear to be obtained at lower doses than those used for the antidepressant therapy [25]. Unfortunately, although effective and stable, their effects are complicated by a number of dangerous side effects such as weight gain, orthostatic hypo-tension, cardiovascular effects and potentially fatal over dosage [11].
For these reasons, we have taken into study these substances in order to identify their therapeutic potential in analgesia and to see if their effects are mutually reinforcing (synergistic).

Materials and Methods

Animals
In the present study, were used white Swiss mice, provided by the "Cantacuzino" Institute, Bucharest, Romania, male, weighing 20 - 25 g. Groups of 6 - 10 animals were housed in 25/35 cm and respectively 30/40 cm Plexiglas cages in a room with controlled temperature (21.00°C ± 2.00°C) and a light/dark cycle (07.00 am/07.00 pm) of 12 hours. The animals received standard food, and water *ad libitum*, and 10 days before the experiments were performed their behaviour was observed. Three hours before each test, access to food was stopped. All experiments were performed beginning with 10.00 am.
Experimental model
The experimental protocols used in this study were in strict conformity with the specific regulations approved by UMF "Grigore T. Popa" Iași, Romania, the international bioethical regulations (European Communities Council Directive of 24 November 1986 86/609/EEC) and the regulations of the International Association For the Study of Pain [33].
The investigated substances, alone or in combination, were suspended in mucilage of sodium carboxymethyl cellulose (0.1% CMC-Na). Their administration was performed orally, using dose sequences in geometric progression according to the study protocol. The following substances were administered in this study: Doxepin (DOX) (Sigma) and Candesartan (CND).
For the nociceptive testing with thermal stimulus we used the method of Woolfe and Mac Donald modified by Eddy and Laborit [10], also adapted in our laboratory. The test mouse was placed in the cylindrical chamber of the hot plate (Model 7280 UGO Basile) on the heated surface at 52.5°C ± 0.1°C. The pain latency period was measured, with a 30 second cut-off. The response for testing the pain threshold at the thermal stimulus consists in licking and/or shaking the hind paw or the tendency to jump in order to leave the enclosure. The animals were tested for the thermal stimulus 45 minutes before being treated, eliminating from the experiment the animals that did not respond in 15 s. After treatment, retesting was performed at 30, 60, 90, 120 minutes, and the latency period of the pain reaction was measured.
Data analysis
Percentage inhibition of the response to the nociceptive stimulus was expressed according to the formula:

$$\% \text{ inhibition} = (Tx - T0)/(Tm - T0) \times 100,$$

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.
The evaluation of the interaction type was made using the method of the composite additive line (gradual effects), comparing the experimentally-obtained line with the composite additive line in a variance analysis of the relationship log dose-effect [28]. The obtained interaction index suggests the interaction type. ($\gamma < 1$ - synergism, $\gamma = 1$ - addition, $\gamma > 1$ – sub-additivity (does not exclude antagonism) [29].

Results and Discussion

The following working groups were used: DOX1, DOX2, DOX3 which received doxepin 5.00 - 20.00 mg/kg b.w. orally, CND1, CND2, CND3 which received candesartan 0.50 - 2.00 mg/kg b.w. orally, and DOX - CND 1 - 4 groups receiving candesartan doxepin 0.96 - 7.74 mg/kg b.w. orally. Doses were administered in geometric progression with a ratio of 2.
This administration algorithm is required to establish the 50% effective doses (ED_{50}) value of the investigated substances on the thermal stimulation nociception model. The obtained values (Table I) allow the determination of proportions in binary combinations, the determination of the Zadd value (Table II) of each combination and the generation of the regression lines.

Table I

Values of ED_{50} in mg/kg b.w. of studied substance, administered alone, for the hot plate test

	Doxepin[2]	Candesartan[3]
ED_{50} (SEM)[1] mg/kg b.w./orally	14.55 (4.13) (Y = -4.55 + 46.90*X) R = 0.923	1.07 (0.23) (Y = 47.97 + 65.72*X) R = 0.935

[1]SEM (standard error of the average); [2]Value obtained by the exposure to thermal stimulation 60 minutes after the administration of the substance; [3]Value obtained by exposure to thermal stimulation 90 minutes after the administration of the substance

Table II

Values of Zadd and Zmix of the drugs administrated in fixed-ratio combinations for the hot-plate test

Combination (ratios)	Total dose mg/kg b.w./orally	Maximal possible effect (MPE)[1] %	DE_{50} combination (SEM) mg/kg b.w./orally	
			Zadd (SEM)	Zmix (SEM)
Doxepin/Candesartan (0.931/0.069)	7.74	63.24	7.74 (0.92) Y = 1.24 + 54.83*X R = 0.936	2.13 (0,36)[2] Y = 41.19 + 26.68*X R = 0.959
	3.87	57.37		
	1.93	52.92		
	0.96	37.94		
γ = 0.282 52.86 % antinociception (inhibition)				

[1]Value obtained by the exposure to thermal stimulation 60 minutes after the administration of the substance; [2]Synergism

From the data analysis, the values were set: DE_{50} = 14.55 ± 4.13 mg/kg b.w. for doxepin, DE_{50} = 1.07 ± 0.23 mg/kg b.w. for candesartan, proportions of the two substances in combination (f = 0.5, p1 = 0.069) yielded a Zadd value = 7.74 ± 0.92 mg/kg b.w., and a Zmix value = 2.13 ± 0.361 mg/kg b.w., γ = 0.282. Our results showed a synergistic interaction of the candesartan - doxepin combination demonstrated by the left - hand shift of the regression line of the association of the two substances compared to the additive line for the dose sequence and their proportion (Figure 1). The maximum effect was obtained 60 minutes after treatment and was maintained for 90 minutes.

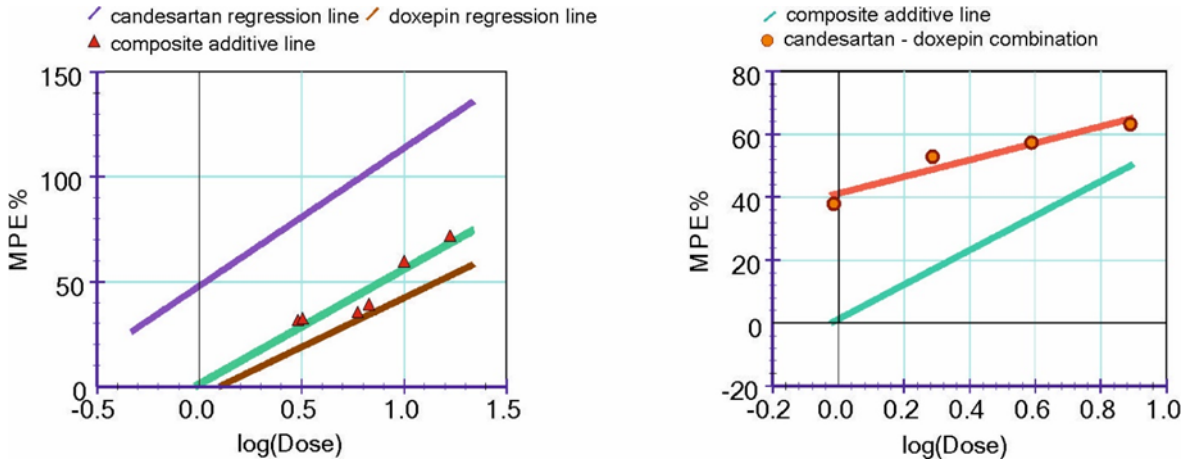

Figure 1.

Analysis of the regression lines of fixed-ratio combination between candesartan and doxepin

The statistical parameters of the regression analysis revealed the significance of these results (F_c = 15.30, F_t = 5.14, t_c = 6.29, t_t = 3.79).

The experimental results have demonstrated a range of analgesic activity for both drugs (demonstrated by the existence of ED_{50} for each of them), but the subsequent testing has shown that the effect of the combination of these drugs is greater than the effect mathematically calculated from the dose sequences which demonstrated the synergy. These results may undergo additional pharmacodynamic mechanisms linking the action mechanisms of these drugs.

AT1 receptors are present on the nociceptive cells of the dorsal spinal nerve root ganglion (DRG) and are involved in the perception of pain [20]. Following the sciatic nerve ligation, the AT1 receptor was overexpressed against the control in 43% of the small neurons and 62% of the large neurons of the DRG. The total increase in AT1-immunoreactive neurons was 38% after 7 days of ligation. All these

results demonstrate that angiotensin II is involved in the pathological mechanisms of pain perception [19]. The ventrolateral caudal *medulla* is one of the structures that modulate pain perception, and there are significant percentages of immunoreactivity neurons for AT1 that are projected into the posterior horn. Angiotensin II administration produced hyperalgesia in both tail-flick and formalin tests, hyperalgesia significantly attenuated by concomitant systemic or local administration of losartan [15]. On the other hand, other studies have shown that the use of losartan reverses the circadian rhythm of pain perception, increasing the perception of pain in the nocturnal period (3 am) and reducing it during the diurnal period [21]. On a neuropathic pain model induced with streptozotocin, Ogata *et al.* [18] have demonstrated that tactile allodynia is inhibited by the intrathecal administration of losartan, but not by PD123319 (AT2-specific blocker), which demonstrates the exclusive involvement, at least at the spinal-medullar level, of the AT1 receptors.

As a practical consideration, the doses of candesartan used in the study were inferior to the antihypertensive ones [3, 17].

With regard to doxepin, a series of isolated results demonstrate that it has interesting and yet unexplained effects, such as analgesia in local applications [2, 6, 27]. The presented studies investigated the effect of doxepin and N-methyl-doxepin compared to bupivacaine, and also their effects as Na^+ channel blockers on a voltage-clamp model. The study demonstrated that doxepin is a Na^+ channel blocker, stronger than bupivacaine. These effects could be responsible for beneficial anti-pruritic actions, post-herpetic neuralgia, or radiation radiotherapy [7, 13, 24].

Secondly, their central effects are the inhibition of noradrenaline and serotonin reuptake, and its active metabolite is desmethyl-doxepin (nordoxepin). An isolated report also shows the inhibitory effects of doxepin on the neuronal Na^+/K^+ ATP-ase in the foetal and adult brain [22]. This phenomenon could have effects of reducing the neuronal membrane resting potential, with yet undetermined effects on the neuronal reactivity. In addition, some authors have proposed anti-inflammatory effects [8], especially by reducing the release of proinflammatory cytokines, such as IL-1β, IL6, TNF-α and IFN-γ [12].

Conclusions

In conclusion, the synergistic effects on the thermoalgic perception of the co-administration of candesartan as a blocker of the AT1 receptor and doxepin as a drug with multiple peripheral and central neuronal effects could be explained by the following assumptions: i) the pro-algesic effect of angiotensin II is known in the gastropyloric receptor (GRP) neurons, ascending medullary pathways, and in the ventromedial medullary propagation for the pain sensations. Blockage of angiotensin receptors at these levels by the oral administration of candesartan produces a reduction in pain perception and explains the prolongation of hot-plate response times; ii) the local anaesthetic effect of doxepin (by blocking the Na^+ channels), mentioned above, can reduce the intensity of perception in the Na^+ channels from the free nerve endings and the thermal receptors, which adds to the demonstrated analgesic effect; iii) the central effects at the level of serotoninergic and adrenergic mediation may also be responsible for reducing the perceptual and emotional impact of painful stimulation with the increase of the analgesic effect.

In addition, a variety of other effects can be added, such as variations in the neuronal membrane polarization and release of doxepin-inducing neuropathic inflammatory mediators, which could explain the synergism of the combination.

References

1. Buda V., Andor M., Cristescu C., Voicu M., Suciu L., Muntean C., Cretu O., Bâibâţă D.E., Gheorghiu C.M., Tomescu M.C., The influence of perindopril on PTX3 plasma levels in hypertensive patients with endothelial dysfunction. *Farmacia*, 2016; 64(3): 382-389.
2. Blackwell B., Evans W., Gensler F., Local anaesthetic effect of doxepin. *Lancet*, 1972; 1(7747): 443.
3. Callera Glaucia E., Antunes Tayze T., Correa Jose W., Moorman D., Gutsol A., He Y., Cat A., Nguyen D., Briones Ana M., Montezano Augusto C., Burns Kevin D., Touyz Rhian M., Differential renal effects of candesartan at high and ultra-high doses in diabetic mice–potential role of the ACE2/AT2R/Mas axis. *Biosci. Rep.*, 2016; 36(5): e00398.
4. Ciobica A., Bild W., Hritcu L., Haulica I., Brain renin-angiotensin system in cognitive function: pre-clinical findings and implications for prevention and treatment of dementia. *Acta Neurol. Belg.*, 2009; 109(3): 171-180.
5. Ciotu I.C., Lupuliasa D., Zbârcea C.E., Negreş S., The effect of nimodipine on a rat model of paclitaxel – induced peripheral neuropathy. *Farmacia*, 2016; 64(4): 493-497.
6. Dworsky Z.D., Bennett R., Kim J.M., Kuo D.J., Severe medication-induced peripheral neuropathy treated with topical doxepin cream in a paediatric patient with leukaemia. *BMJ Case Rep.*, 2017; 2017.
7. Hadley G.R., Gayle J.A., Ripoll J., Jones M.R., Argoff C.E., Kaye R.J., Kaye A.D., Post-herpetic Neuralgia: a Review. Curr. *Pain Headache Rep.*, 2016; 20(3): 17.
8. Hajhashemi V., Minaiyan M., Banafshe H.R., Mesdaghinia A., Abed A., The anti-inflammatory effects of venlafaxine in the rat model of carrageenan-induced paw edema. *Ir. J. Basic. Med. Sci.*, 2015; 18(7): 654-658.

9. Haulica I., Neamtu C., Stratone A., Petrescu G., Branisteanu D., Rosca V., Slatineanu S., Evidence for the involvement of cerebral renin-angiotensin system (RAS) in stress analgesia. *Pain*, 1986; 27(2): 237-245.
10. Horiuchi M., Brain renin-angiotensin-aldosterone system and cognitive function. *Nihon Rinsho*, 2014; 72(4): 641-647.
11. Jann M.W., Slade J.H., Antidepressant agents for the treatment of chronic pain and depression. *Pharmacotherapy*, 2007; 27(11): 1571-1587.
12. Janssen D.G., Caniato R.N., Verster J.C., Baune B.T., A psychoneuroimmunological review on cytokines involved in antidepressant treatment response. *Hum. Psychopharmacol.*, 2010; 25(3): 201-215.
13. Jayakrishnan R., Chang K., Ugurluer G., Miller R.C., Sio T.T., Doxepin for Radiation Therapy-Induced Mucositis Pain in the Treatment of Oral Cancers. *Oncol. Rev.*, 2015; 9(1): 290.
14. Llorens-Cortes C., Mendelsohn F.A., Organisation and functional role of the brain angiotensin system. *J. Renin Angiotensin Aldosterone Syst.*, 2002; 3(Suppl 1): S39-48.
15. Marques-Lopes J., Pinto M., Pinho D., Morato M., Patinha D., Albino-Teixeira A., Tavares I., Micro-injection of angiotensin II in the caudal ventro-lateral medulla induces hyperalgesia. *Neurosci.*, 2009; 158(4): 1301-1310.
16. Nemoto W., Ogata Y., Nakagawasai O., Yaoita F., Tanado T., Tan-No K., The intrathecal administration of losartan, an AT1 receptor antagonist, produces an antinociceptive effect through the inhibiton of p38 MAPK phosphorylation in the mouse formalin test. *Neurosci. Lett.*, 2015; 585: 17-22.
17. Nixon R.M., Müller E., Lowy A., Falvey H., Valsartan *vs.* other angiotensin II receptor blockers in the treatment of hypertension: a meta-analytical approach. *Int. J. Clin. Pract.*, 2009; 63(5): 766-775.
18. Ogata Y., Nemoto W., Nakagawasai O., Yamagata R., Tadano T., Tan-No K., Involvement of Spinal Angiotensin II System in Streptozotocin-Induced Diabetic Neuropathic Pain in Mice. *Mol. Pharmacol.*, 2016; 90(3): 205-213.
19. Oroszova Z., Hricova L., Stropkovska A., Lukacova N., Pavel J., The characterization of AT1 expression in the dorsal root *Ganglia* after chronic constriction injury. *Cell. Mol. Neurobiol.*, 2017; 37(3): 545-554.
20. Pavel J., Tang H., Brimijoin S., Moughamian A., Nishioku T., Benicky J., Saavedra J.M., Expression and transport of Angiotensin II AT1 receptors in spinal cord, dorsal root ganglia and sciatic nerve of the rat. *Brain Res.*, 2008; 1246: 111-122.
21. Pechlivanova D.M., Markova P.P., Stoynev A.G., Effect of the AT(1) receptor antagonist losartan on diurnal variation in pain threshold in spontaneously hypertensive rats. *Meth. Find Exp. Clin. Pharmacol.*, 2010; 32(9): 663-668.
22. Saha U.K., Sengupta T., Sirkar A., Sengupta D., Biochemical studies on the *in vitro* effect of doxepin on Mg^{2+} & Na^{+}, K^{+})-ATPases of human foetal & adult brain. *Ind. J. Med. Res.*, 1989; 90: 27-31.
23. Sansone R.A., Sansone L.A., Pain, pain, go away: antidepressants and pain management. *Psych. (Edgmont)*, 2008; 5(12): 16-19.
24. Sanz R., Calpena A.C., Mallandrich M., Gimeno A., Halbaut L., Clares B., Development of a buccal doxepin platform for pain in oral mucositis derived from head and neck cancer treatment. *Eur. J. Pharm. Biopharm.*, 2017; 117: 203-211.
25. Singh H., Becker P.M., Novel therapeutic usage of low-dose doxepin hydrochloride. *Expert. Opin. Investig Drugs*, 2007; 16(8): 1295-1305.
26. Smith M.T., Muralidharan A., Targeting angiotensin II type 2 receptor pathways to treat neuropathic pain and inflammatory pain. *Exp. Opin. Ther. Targets*, 2015; 19(1): 25-35.
27. Sudoh Y., Cahoon E.E., De Girolami U., Wang G.K., Local anesthetic properties of a novel derivative, N-methyl doxepin, *versus* doxepin and bupivacaine. *Anesth. Analg.*, 2004; 98(3): 672-676.
28. Tallarida R.J., Drug synergism: its detection and applications. *J. Pharmacol. Exp. Ther.*, 2001; 298(3): 865-872.
29. Tallarida R.J., The interaction index: a measure of drug synergism. *Pain*, 2002; 98(1-2): 163-168.
30. von Bohlen und Halbach O., Albrecht D., The CNS renin-angiotensin system. *Cell. Tissue Res.*, 2006; 326(2): 599-616.
31. Weber J., Siddiqui M.A., Wagstaff A.J., McCormack P.L., Low-dose doxepin: in the treatment of insomnia. *CNS Drugs*, 2010; 24(8): 713-720.
32. Wichniak A., Wierzbicka A., Walecka M., Jernajczyk W., Effects of Antidepressants on Sleep. *Curr. Psych. Rep.*, 2017; 19(9): 63.
33. Zimmermann M., Ethical considerations in relation to pain in animal experimentation. *Acta Physiol. Scand. Suppl.*, 1986; 554: 221-233.