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PHARMACOLOGICAL STUDIES ON THE DISCOVERY OF NOVEL TRPV1 ANTAGONISTS WITH POTENTIAL USE IN PAIN THERAPY

PH.D. THESIS SUMMARY

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Introduction

Current pain management involves the use of non-pharmacological techniques in combination with pharmacological treatment that includes drugs from the classes of antipyretic analgesics, nonsteroidal anti-inflammatory drugs, opioids, antidepressants and anticonvulsants. However, adverse reactions such as hepatotoxicity, gastrointestinal disorders, increased risk of cardiovascular events, as well as tolerance and drug dependence limit the use of these drugs in the treatment of pain. Moreover, low efficacy has been reported for most existing pharmacological options.

The TRPV1 receptor is a non-selective, ligand-dependent cationic channel expressed centrally in the thalamus, striatum, amygdala or peripherally in the dorsal root ganglion. TRPV1 plays a crucial role in pain modulation, being essential for the recognition and integration of nociceptive chemical and thermal stimuli.

The multiple advantages of in silico drug repurposing studies have supported the significant increase in the use of these models for the discovery of new modulators for different therapeutic targets.

Animal models are also currently used to determine the mechanism of action and characteristics of different types of pain, as well as to assess the analgesic effect of an active substance.

The main objectives of the research were:

- implementation of an in silico drug repurposing model for the discovery of novel TRPV1 antagonists with potential use in pain therapy;
- validation of an animal model of paclitaxel-induced visceral pain and evaluation of the analgesic effects of substances identified using in silico drug repurposing studies on this animal model;
- evaluation of the analgesic and anti-inflammatory effects of the test substances on an animal model of complete Freund's adjuvant-induced osteoarthritis using pharmacological tests, radiological, biochemical and histological determinations;
- evaluation of analgesic effects of substances in an animal model of capsaicin-induced pain using pharmacological tests;
- in vitro evaluation of the activity of compounds resulted from in silico studies.

I. General aspects

1. Chronic pain

1.1. Introduction

Pain is defined as an unpleasant sensory and emotional experience, often correlated with the development of tissue damage [1, 2]. According to the International Association for the Study of Pain, pain that persists for more than 3 months is considered chronic pain [2, 3].

According to literature data, it has been observed that there is a strong correlation between chronic pain and depression, anxiety and insomnia [4, 5]. This major health problem is associated with a high social and economic impact and requires complex management to improve the patient's quality of life.

1.2. Treatment options in chronic pain

The pharmacological management of chronic pain is based on treating the patient's symptoms or pain-related pathology. In this sense, the main goal of therapy is to increase the patient's quality of life [5].

The main classes of drugs used to treat pain are non-steroidal anti-inflammatory drugs, anticonvulsants, antidepressants and opioids, but other drugs may also be used [6].

Non-pharmacological treatment of chronic pain associated with pharmacotherapy plays a key role in reducing pain intensity, increasing mobility, muscle strength and endurance [7, 8].

Moreover, the use of cognitive-behavioral approaches of chronic pain has been correlated with a reduction in pain-related stress and disability in clinical trials involving patients with different types of chronic pain [9-11].

1.3. Biological mechanisms of pain

Chronic pain persists or recurs over time. This may be the result of a pathology (e.g. arthritis or cancer), an initial injury or dysfunction of the nervous system [12].

In chronic pain, sensitization of nociceptive pathways occurs with the development of hyperalgesia or allodynia. This process may be the result of changes in nociceptors, in the release of inflammatory neurotransmitters or mediators and in neural functioning [13].

Inflammation also plays a significant role in chronic pain associated with multiple conditions, including rheumatoid arthritis, osteoarthritis, chronic low back pain, fibromyalgia [14], but also in neuropathic, post-surgical or post-traumatic pain [15].

2. TRPV1 receptor

2.1. Overview

Transient potential ion channels (TRPs) are cation channels expressed in various cell types, including neurons. Multiple physiological and pathological roles have been highlighted for these channels [16, 17].

The TRPV1 channel, also called the capsaicin receptor, is expressed centrally in the thalamus, amygdala and corpus striatum, but also peripherally in the dorsal root ganglia, intestine, skin, cornea and bladder [18-24].

2.2. Structural characteristics of the TRPV1 channel

TRPV1 is structurally characterized as a homotetrameric channel. Each of the four subunits contains six transmembrane domains [25, 26]. Each monomeric chain comprises a total of 838 amino acids, with amino acid residues 433–684 form the transmembrane domains [27-28]. Thus, the transmembrane region comprises six helices forming the voltage sensor-like domain and an inner pore region [29, 30].

TRPV1 has a large cytoplasmic domain consisting of two intracellular terminal sequences [31]: the long N-terminal region formed by multiple ankyrin repeats, responsible for the activation of the channel under the action of agonist substances such as capsaicin, resiniferatoxin or high temperature (50 °C) [32], and the short C-terminal region with a role in the stability and function of the receptor [28, 33-35].

2.3. Endogenous TRPV1 receptor agonists

Endogenous TRPV1 channel ligands are anandamide (AEA), N-arachidonoyl dopamine (NADA), N-oleoylethanolamine (OEA), lysophosphatidic acid, oleyldopamine (OLDA), but also other molecules (12-hydroperoxyeicosatetraenoic acid (12-HPETE)) [36-38].

2.4. Natural and synthetic TRPV1 receptor agonists

TRPV1 receptor activation modulates various biological responses, such as apoptosis and cell proliferation [39], nociception and body temperature [40], glucose homeostasis [41] and regulation of bladder function [38, 42].

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), the established TRPV1 receptor agonist, is a substance of the genus Capsicum, which belongs to the family Solanaceae [43]. Following the formation of the ligand-receptor interaction, the receptor is activated and an influx of calcium occurs, leading to pain sensation. After initial stimulation, capsaicin causes desensitization of the receptor with the development of an analgesic effect [38, 44, 45].

Resiniferatoxin, isolated from Euphorbia resinifera, is a compound used in traditional medicine to treat pain. It is considered a potent TRPV1 agonist, with significantly superior potency compared to capsaicin [46].

Ginger, Zingiber officinale Roscoe, is commonly used in the food industry as a spice and in traditional medicine for its multiple beneficial therapeutic effects [24, 47, 48]. Active substances, such as shogaoli, gingeroli, paradoli and zingeron, isolated from Zingiber officinalis act as TRPV1 channel agonists [49, 50].

2.5. Natural and synthetic TRPV1 receptor antagonists

Interest in the identification and discovery of TRPV1 antagonists has increased significantly following the finding that TRPV1 receptor knockout in experimental animals attenuates inflammation-induced thermal hyperalgesia [51, 52].

TRPV1 modulators change body temperature [53-55].

Natural TRPV1 channel antagonists include grifolin and neogrifolin, isolated from Peperomia galioides, Rhododendrom dauricum and Albatrellus sp. Inhibition of capsaicin-induced Ca2+ influx has been observed for yohimbine [56], voacangin [57], pelitorin [58], monanchomycin B [59] and pulchranins [60].

Bisabol, from Matricaria chamomilla essential oil, possesses a high affinity for the geometric centre of the TRPV1 receptor, acting by blocking it [61].

TRPV1 antagonist properties have also been reported for eucalyptol [62], naringenin [63], cochinchinemin A and B, loureirin B [64], gomisin A [65], eriodictyol [66], quercetin [67] and vitexin [68].

The first synthetic competitive TRPV1 antagonist identified was capsazepine [69, 70].

High-potency TRPV1 antagonists have been obtained by halogenation of agonists, e.g. by introducing iodine into the resiniferatoxin structure [71, 72].

SB-705498 inhibited TRPV1 activity stimulated by capsaicin, acid pH and heat and was the first TRPV1 antagonist used in clinical trials for its analgesic potential [69, 70, 73].

AMG-517 is a selective TRPV1 antagonist, but in clinical trials produced hyperthermia [74, 75].

SB-366791 is a TRPV1 antagonist with superior selectivity to capsazepine and does not produce changes in body temperature, while AMG-9810 inhibits proton, heat and endovanilloid-mediated TRPV1 receptor activation, but showed a poor pharmacokinetic profile in laboratory animals [76-79].

II. Personal contributions

3. Working hypothesis and general objectives

Chronic pain is correlated with disability and significant impairment of the patient's quality of life. Moreover, more and more patients are frequently seeking medical care to treat pain [80, 81].

In recent years, several studies have highlighted the capsaicin receptor, TRPV1, as a therapeutic target for the discovery of new analgesics [69, 82, 83].

Although some TRPV1 antagonists have been evaluated in phase I and II clinical trials for the treatment of chronic pain, the severe hyperthermia observed following administration of these substances has led to discontinuation of clinical trials [84].

Based on the information presented above, the overall objectives of the present work were to identify new TRPV1 antagonists using in silico drug repurposing studies, to validate a paclitaxel-induced visceral pain model and to determine the analgesic and anti-inflammatory effects of the substances in different animal models of pain and then to evaluate their activity on the TRPV1 receptor in vitro.

4. In silico drug repurposing studies to identify new potential TRPV1 modulators

A drug repurposing virtual screening framework was implemented in our study to identify, among approved drugs, new potential ligands that may interact with the TRPV1 receptor, considering that for these substances the pharmaco-toxicological profiles are well known [86].

Materials and methods

A virtual screening framework was implemented with the scope of discovering potentially novel TRPV1 antagonists and agonists/desensitizers, using both ligand-based and structure-based in silico approaches. The implemented framework focused on building a machine learning algorithm (artificial neural network) based on structural scaffolds, flexophores, molecular descriptors and predicted binding affinities [85].

Results and discussions

After initial curation, the datasets contained the chemical structures and activity values of 2377 TRPV1 antagonists (ANT set), 194 agonists (AG set), and 996 experimentally determined inactive molecules (IN set). In order to establish a set of decoy molecules with matching properties with the active molecules, ANT and AG sets were merged and four drug-likeness parameters were calculated with DataWarrior: MW, logP, HBD and HBA. Further, a set of molecules was downloaded from ChEMBL, containing structures with MW values ranging between 226.3 and 796.6 g/mol, logP values between 0.837 and 11.074, 1–12 HBA and 0–4 HBD atoms. Among these structures, 500 diverse molecules, presumably inactive on TRPV1, were retrieved as the decoy set (DCY). Candidates for repurposing were retrieved from DrugBank database and contained a total of 1981 approved drugs.

The first independent variable that was established in the proposed framework was the average activity score (AAS). This average score was calculated as the arithmetic mean of three scores based on the structural features of TRPV1 antagonists and agonists: Bemis–Murcko structural skeletons, plain ring systems and clustering based on flexophore descriptors.

Antagonists had overall higher AAS values than agonists, since antagonists represented a significantly larger population among the biologically active compounds.

The predictive power of the established average activity score was assessed by generating ROC curves and calculating ROC AUC values. The ROC AUC value for antagonist activity scores was 0.963, while the same parameter was 0.986 for predicting agonists, denoting high predictive accuracies in both cases.

ROC curves were generated based on activity classes and molecular descriptors in order to build classification models using cutoff values. We chose to include a minimum of three and a maximum of eight molecular descriptors as independent variables. ROC AUC values were calculated for all descriptors to assess the discriminatory power of each variable. The eight descriptors were chosen based on four criteria: satisfactory ROC AUC values, statistically significant differences between values of active and inactive molecules, correlation coefficients between each pair of descriptors lower than 0.75 and ease of describing the respective molecular property.

Molecular docking results were the third and final independent variable in the proposed repurposing predictive model. Two crystal structures were used in this study: activated TRPV1 bound to agonist RTX and TRPV1 in a closed state bound to antagonist CPZ. Both qualitative and quantitative validations of the docking procedure were performed. First of all, the accuracy of binding mode predictions was assessed by docking the co-crystallized ligands into the binding site and superposing the predicted conformation with the experimentally determined ligand pose. The RMSD values calculated after superposition were 1.1277 Å for CPZ and 1.2564 Å for RTX, showing low deviations from original conformation and satisfying accuracy for pose prediction. Binding energies for the positive controls were −9.13 kcal/mol for CPZ and −11.55 kcal/mol for RTX, respectively.

A second validation of the docking protocol was performed by assessing the capability of the two TRPV1 conformations to discriminate against active and inactive ligands by analyzing the predicted binding energies or ligand efficiencies. Therefore, a selection of TRPV1 agonists (n = 194), antagonists (n = 222), inactive molecules (n = 488) and decoys (n = 500) were docked against the binding sites of active (PDB ID 7MZC) and inactive (PDB ID 5IS0) conformations of TRPV1.

ROC curves were generated to assess the suitability of the docking procedure for discriminating between active and inactive ligands. Notably, the molecular docking experiment showed greater accuracies in predicting true antagonists than true agonists.

After establishing activity scores, the number of satisfied descriptor criteria, and binding affinities and efficacies for antagonists, agonists and inactive molecules, these data were integrated into one global predictive model in order to increase the predictive accuracy by adding weights to each of the aforementioned parameters. Since antagonist, agonist and inactive datasets are rather unbalanced, we generated the machine learning model using only the compounds that were selected for molecular docking, thus creating a more balanced training dataset. The machine learning algorithm that we selected for this task was the multilayer perceptron neural network since it also allows the prediction of multiple classes. The architecture with the most optimal parameters had the following characteristics: six input nodes (average activity scores and satisfied descriptor criteria for both antagonists and agonists, binding energies for antagonists, LELP values for agonists), one hidden layer with four neurons (which, in fact, represents the geometric mean between the number of input and output nodes) activated with tanh function, and the output layer with three nodes corresponding to the probabilities for each of the three classes, generated with the softmax function.

The generated classification model showed higher values for specificity over sensitivity, and thus the algorithm identifies true negatives relatively more accurately than true positives.

The independent variables with the highest importance in predicting the three classes were average activity scores for antagonists and agonists, followed by binding energy and LELP, while the numbers of satisfied molecular descriptor criteria for antagonists and agonists had the lowest weights.

The most promising candidates for repurposing as TRPV1 modulators with pharmacotherapeutic utility in pain relief were chosen based on three criteria: high probability of being active, favorable interactions with relevant residues within the binding site of TRPV1 and acceptable safety profiles. Therefore, three potential antagonists (repaglinide, telmisartan and agomelatine) and one potential agonist (protokylol) were proposed as repositioning candidates.

Binding pose analysis of repaglinide revealed its potential to competitively block the vanilloid binding site of TRPV1. Moreover, our analysis showed that repaglinide shares relevant structural features with certain TRPV1 antagonists. To the best of our knowledge, there are no available preclinical studies that analyzed the analgesic potential of repaglinide. Frequent adverse reactions such as hypoglycemia and weight gain associated with repaglinide treatment

[86] indicate that its use as an analgesic agent might be limited to patients suffering from diabetic neuropathy.

Sisignano et al. used electrophysiological measurements and calcium-imaging experiments to investigate the possibility of interaction between telmisartan and the TRPV1 channel and did not observe an effect of the substance on TRVP1-dependent calcium transients or inward currents [87]. Considering these findings, telmisartan can be considered as a false positive discovered by our algorithm as a potential TRPV1 antagonist. The lack of telmisartan activity on TRPV1 could be explained by the molecular docking results. Unlike other ligands, telmisartan lacks a vanilloid-like head substructure and therefore its conformation cannot fit as well into the vanilloid pocket.

Agomelatine fits satisfyingly into the vanilloid binding pocket, and it shares a high similarity with a TRPV1 antagonist, the methyl radical within the acetamide moiety being replaced with the trifluoromethoxy-methyl substructure in the known antagonist. Agomelatine has an optimal safety profile, with few side effects (such as dizziness, nausea, diarrhea and dry mouth) occurring especially early in the treatment [88].

Protokylol, a β2-adrenergic agonist used as a bronchodilator [89] emerged as a compound with the second highest probability of exerting agonist activity on TRPV1. Two different, overlapping binding sites were observed for protokylol, one specific to vanilloids and competitive antagonists and another for phosphoinositides [90]. Protokylol formed favorable interactions with relevant amino acid residues within both binding sites. Therefore, protokylol could potentially show analgesic activity either by desensitizing TRPV1 through interaction with the vanilloid binding site or by inhibiting the channel through allosteric modulation, similar to phosphoinositides [90].

Due to their optimal predicted binding into TRPV1 active sites and high estimated probabilities of being active ligands, we propose repaglinide and agomelatine as potential TRPV1 antagonists and protokylol as a potential TRPV1 agonist/desensitizer. Further studies are required to experimentally evaluate the interactions between the proposed molecules and TRPV1 and to investigate their activity in various animal models of pain-related disorders.

5. Validation of an animal model of paclitaxel-induced visceral pain and evaluation of the analgesic effects of agomelatine and repaglinide in this model

5.1. Validation of an animal model of paclitaxel-induced visceral pain

The present study was designed to investigate the reproducibility of previous studies and to understand if paclitaxel is suitable for developing an animal method that would allow a consistent assessment of visceral pain-related behavior and the effectiveness of analgesics [91].

Materials and methods

Experimental procedures were performed in accordance with bioethics norms proposed by the NIH Guide for the Care and Use of Laboratory Animals.

For this study, we used 40 rats divided in 5 equal groups (8 animals per group). The treatment was administered as a single intraperitoneal dose as follows: Group C (control group): distilled water 1 mL/kg; group PAC1: paclitaxel 3 mg/kg; group PAC2: paclitaxel 5 mg/kg; group PAC3: paclitaxel 10 mg/kg; and group PAC4: paclitaxel 15 mg/kg.

We used as a starting point the highest dose used in paclitaxel-induced visceral pain studies [92].

Visceral nociception was qualitatively evaluated using a scale of abdominal pain immediately after paclitaxel administration. Tactile and thermal hypersensitivity induced by paclitaxel were determined before administration (baseline sensitivity) at 24 h (day 3) and 48 h (day 4) after the administration.

Results and discussions

Abdominal pain reaction scores were significantly impacted by the paclitaxel administration (**Fig. 5.2.A.,** ANOVA, $F_{(4,35)} = 32,3$, $p < 0,0001$). The most pronounced effect was observed at group PAC4 - 15 mg/kg corp ($p < 0,001$, corecție Bonferroni). Paclitaxel-induced visceral pain is dose dependent. Acute pain was observed in laboratory animals approximately 5–10 min after administration with a maximum intensity of 20–30 min and lasted 40 min for the highest dose of paclitaxel used in the experiment (**Fig. 5.2.B.**) [91].

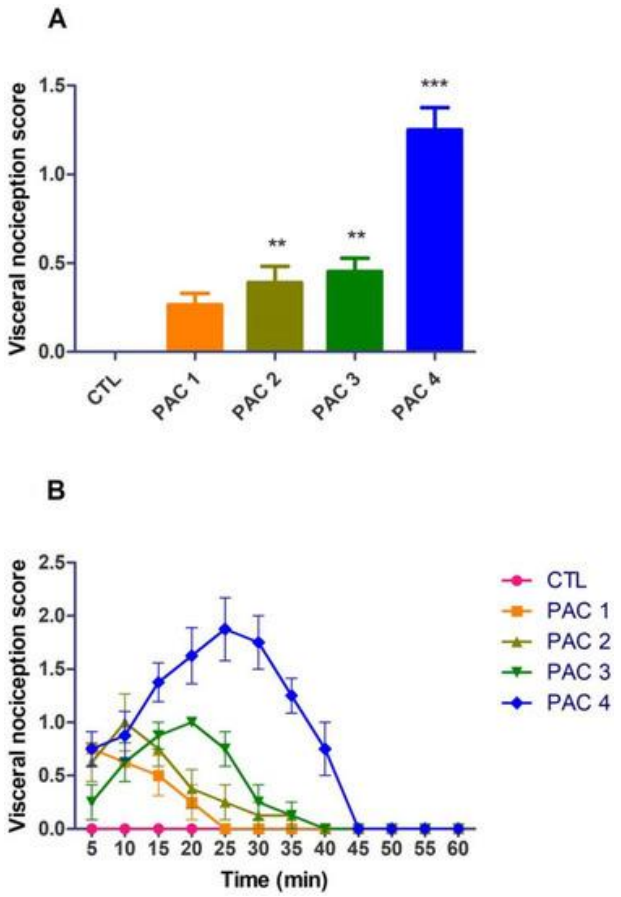

Fig. 5.2. Evaluation of visceral pain. **(A)** Scale of abdominal pain—variation of visceral nociception score between treated groups. Data are presented as mean of visceral nociception score ± S.E.M. ** $p < 0.01$ vs. CTL; *** $p < 0.001$ vs. CTL. **(B)** Evolution of abdominal pain over time after administration of a single dose of paclitaxel.

Variations of tactile sensitivity were significant after 48 h (Fig. ANOVA, $F(4,35) = 3.34$, $p = 0.0203$). A statistically significant decrease in the 50% withdrawal threshold was observed for the PAC3 group (10 mg/kg body) ($p < 0.01$, Bonferroni correction) compared to the control group at 48 h [91].

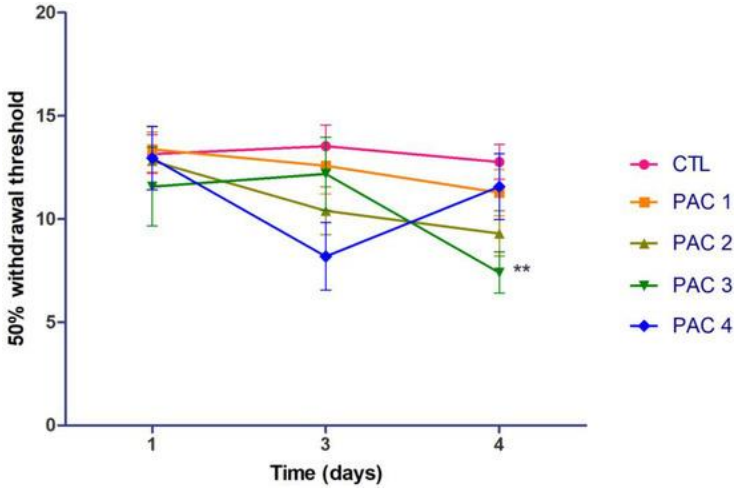

Fig. 5.3. Time-dependent variation of thermal hypersensitivity after administration of a single dose of paclitaxel over time. Data are presented as mean ± S.E.M. of latency. ** $p < 0.01$ vs CTL.

No significant variations in thermal hypersensitivity were observed after paclitaxel administration compared to the control group on day 3 (ANOVA, $F(4,35) = 0.350$, $p = 0.842$) or day 4 (ANOVA, $F(4,35) = 1.43$, $p = 0.244$).

5.2. Evaluation of the analgesic effects of agomelatine and repaglinide in the animal model of paclitaxel-induced visceral pain

Based on the hypothesis that paclitaxel-induced visceral pain is mediated by TRPV1 receptor activation, and that agomelatine and repaglinide might be TRPV1 receptor antagonists, we aimed in the present experiment to evaluate the analgesic effects of these two substances on the animal model of paclitaxel-induced visceral pain.

Materials and methods

Forty female Wistar rats were used in this experiment. The experimental groups were:

- Group CTL: saline 1 mL/kg i.p. + saline 1 mL/kg per os;
- Group PAC: paclitaxel 15 mg/kg i.p. + saline 1 mL/kg per os;
- Group PAC + TRM: paclitaxel 15 mg/kg i.p. + tramadol 80 mg/kg per os;
- Group PAC + AGO: paclitaxel 15 mg/kg i.p. + agomelatine 40 mg/kg per os;
- Group PAC + REPA: paclitaxel 15 mg/kg i.p. + repaglinid 16 mg/kg per os.

The substances used in this experiment were administered as a single dose.

Abdominal pain scale was used for qualitative assessment of visceral nociception.

Results and discussions

Statistically significant variations in visceral nociception were observed in this experiment (ANOVA, $F(4,35) = 12.89$, $p < 0.0001$). Intraperitoneal administration of paclitaxel at a dose of 15 mg/kg produced visceral pain compared to the CTL group ($p < 0.0001$, Bonferroni correction). Only tramadol showed a significant analgesic effect ($p < 0.05$).

6. Evaluation of the effects of agomelatine and repaglinide on pain, inflammation and oxidative stress in an animal model of osteoarthritis

6.1. Evaluation of analgesic and anti-inflammatory effects of agomelatine and repaglinide by pharmacological tests in the animal model of osteoarthritis

In the present study we aimed to determine the effects of agomelatine and repaglinide on pain and inflammation in an animal model of osteoarthritis.

Materials and methods

All experimental procedures performed were in compliance with bioethical guidelines corresponding to the NIH Guidelines for the Care and Use of Laboratory Animals and ARRIVE guidelines.

The experimental groups were:

- Paraffin oil 0.1 mL intraplantar (ipl) and distilled water 1 mL/100 g per os (CTL);
- Complete Freund's adjuvant 0.1 mL ipl and distilled water 1 mL/100 g per os (CFA);
- Complete Freund's adjuvant 0.1 mL ipl and tramadol 80 mg/kg per os (CFA + TRM);
- Complete Freund's adjuvant 0.1 mL ipl and dexamethasone 1 mg/kg per os (CFA + DEXA);
- Complete Freund's adjuvant 0.1 mL ipl and agomelatine 40 mg/kg per os (CFA + AGO);
- Complete Freund's adjuvant 0.1 mL ipl and repaglinid 16 mg/kg per os (CFA + REPA).

Allodynia and mechanical hyperalgesia were assessed using the von Frey filaments and the Randall Selitto test, thermal hyperalgesia with the Hot plate test (53^0 C), and paw edema

with the plethysmometer. Pharmacological tests were performed before administration, at 7 and 14 days after intraplantar administration of complete Freund's adjuvant.

Results and discussions

The assessment of mechanical hyperalgesia showed significant changes on day 7 of the experiment (ANOVA, $F(5, 54) = 5.664$, $p = 0.003$), but not on day 14 (Kruskal-Wallis, $H(5) = 10.38$, $p = 0.065$).

Rats in the CFA group showed mechanical hyperalgesia on day 7. Freund's complete adjuvant significantly decreased the mechanical sensitivity threshold compared to the CTL group. A significant analgesic effect was observed for tramadol, dexamethasone and repaglinide, which produced an increase in the mechanical sensitivity threshold compared to the CFA group.

Following assessment of mechanical allodynia, significant variations in the 50% response threshold were observed after 1 week (Kruskal-Wallis, $H(5) = 19.65$, $p = 0.0015$) and 2 weeks of treatment (Kruskal-Wallis, $H(5) = 21.42$, $p = 0.0007$).

Intraplantar administration of Freund's reagent produced a significant reduction in the 50% paw withdrawal threshold ($p = 0.0007$, Bonferroni-Holm correction) after 7 days compared to the CTL group. The effect after 14 days was lower.

An increase in the 50% response threshold was recorded for all substances used in the experiment, but the variations were significant only for the reference substance (tramadol).

Notably, there were significant changes in thermal hyperalgesia after 2 weeks of treatment, (Kruskal-Wallis, $H(5) = 30.25$; $p < 0.001$). Animals treated with Freund's reagent showed thermal hypersensitivity, with a reduction in pain perception time compared to the CTL group ($p = 0.0324$, Bonferroni-Holm correction). A significant antihyperalgesic effect was observed for reference substances and agomelatine.

Our results are similar to reports from preclinical studies [93, 94].

Hind paw volume showed significant variations at both 7 days (ANOVA, $F(5, 54) = 19.15$, $p < 0.001$) and 14 days (Kruskal-Wallis, $H(5) = 49.75$, $p < 0.001$).

Animals in the CFA group showed local inflammation after 7 days, characterized by hind paw edema. Moreover, on day 14 of the preclinical study, Freund's adjuvant-induced inflammation was maintained.

Anti-inflammatory effect was reported for one substance, dexamethasone.

6.2. Radiological evaluation of the effects of agomelatine and repaglinide on inflammation in the animal model of osteoarthritis

The main objectives of the X-ray imaging evaluation were to confirm the development of osteoarthritis after intraplantar administration of complete Freund's adjuvant and to evaluate the anti-inflammatory effect of the reference substances (tramadol and dexamethasone) and the test substances (agomelatine and repaglinide).

Materials and methods

Male Sprague-Dawley rats used in the experiment presented in **Chapter 6** were selected after 18 days for X-ray imaging evaluation.

X-ray examination was performed at Pet Stuff Veterinary Hospital Bucharest using the Drager machine.

The images obtained were examined and interpreted by a radiologist. Osteoarticular changes were analyzed and semi-quantitatively classified using scores from 0-3 [95-97].

Results and discussions

According to the radiological evaluation performed on day 18 of the experiment, intraplantar administration of Freund's single-dose complete adjuvant induced osteoarthritis, characterized by degenerative bone and joint changes, periosteal reaction and soft tissue edema. The effect recorded was significant compared to the CTL group ($p = 0.001$, Bonferroni correction).

Oral administration of tramadol did not influence osteoarticular changes induced by Freund's adjuvant. Animals in this group showed an osteoarthritis score between 2 and 3.

Dexamethasone, the reference substance with anti-inflammatory effect, showed a protective effect against the action of complete Freund's adjuvant. For the animals in this group a low score was recorded, with minimal osteoarticular changes and oedema. The anti-inflammatory effect of dexamethasone assessed by radiological analysis of the hind paw is in concordance with the result determined using the plethysmometer. Moreover, a reduction in Freund's adjuvant-induced inflammation following dexamethasone administration has also been shown in other preclinical studies [98]. The beneficial effect of dexamethasone is the result of

decreased levels of proinflammatory cytokines involved in the initiation and maintenance of inflammation [99].

No protective effect was observed for tramadol, agomelatine and repaglinide against the action of complete Freund's adjuvant. Variations in osteoarthritis severity score were non-significant compared to the CFA group ($p > 0.05$).

6.3. Histological evaluation of the effects of agomelatine and repaglinide on inflammation in the animal model of osteoarthritis

In this study we aimed to perform histological evaluation of tissue samples obtained from posterior paws of experimental animals tested in **Chapter 6.** in order to validate the induction of osteoarthritis following the administration of complete Freund's adjuvant and to determine a possible protective action of the drugs used.

Materials and methods

Thirty-four male Sprague-Dawley rats were selected for histological determinations (first 2 groups - 5 animals/group, last 4 groups - 6 animals/group).

Experimental animals were euthanized by cervical dislocation. Right hind paws were removed from rats by cutting 2-3 mm above the ankle. After tissue fixation, decalcification was performed. Samples were processed, sectioned and stained according to current protocols [100].

Standard HE staining was used to highlight the degree of inflammation and the presence and distribution of cells in the tissue [100].

Tissue samples were assessed for the establishment of the degree of inflammation, and semi-quantitative analysis was performed using a score with points ranging from 0-3 [97, 101, 102].

The degree of inflammation was determined using the scores by an analyst without knowing details of the sections analysed.

Results and discussions

Intraplantar administration of Freund's complete adjuvant produced profound inflammation, with damage to subcutaneous tissue, striated muscle, deep periarticular tissues and periosteum. The inflammatory infiltrate present in the tissues consisted of lymphocytes, plasma cells, macrophages, polymorphonuclear cells and neutrophils. Epithelioid histiocytes

were involved in the formation of granulomatous inflammation. Significant inflammation of hind paw tissues was evident for the CFA group compared to the CTL group ($p < 0.001$, Bonferroni correction). Our results are similar to literature data. Previous studies have demonstrated similar histopathological changes following Freund's reagent administration [101].

Dexamethasone exhibited anti-inflammatory effect, producing a significant reduction in Freund's adjuvant-induced inflammatory infiltrate ($p = 0.01$). The anti-inflammatory effect of dexamethasone has been demonstrated by histopathological analysis in other preclinical studies, and the results obtained in our experiment are similar to those in the literature [95, 103].

For tramadol, agomelatine and repaglinide no anti-inflammatory effect was observed.

6.4. Biological evaluation of the effects of agomelatine and repaglinide on inflammation and oxidative stress in animal model of osteoarthritis

In this study, we aimed to evaluate the effects of drugs on inflammation and oxidative stress in the animal model of osteoarthritis, described previously in **Chapter 6.**

Materials and methods

Forty-eight male Sprague-Dawley rats were selected for biological determinations. For determination of proinflammatory cytokine and total thiol levels, animals were euthanized by cervical dislocation.

Results and discussions

The results of the research showed for the CFA group a statistically significant increase in IL-6 levels compared to the control group without osteoarthritis. Comparing with the CFA control group, it was observed that the substances tested in this experiment did not significantly change the IL-6 level, with the exception of tramadol which decreased the IL-6 level.

Serum TNF-α concentration was significantly reduced for the repaglinide and dexamethasone-treated groups compared to both the non-osteoarthritic control group and the control group with complete Freund's adjuvant-induced osteoarthritis. Our results showed that repaglinide reduces the levels of the two cytokines without influencing osteoarthritis-specific radiological changes, leading to the conclusion that the substance has no anti-inflammatory effect at the joint level. The reduction in serum TNF-α concentration compared to the CFA

group achieved by dexamethasone administration ($p < 0.0001$) correlates with the reduction in osteoarthritis severity induced by Freund's adjuvant.

The research results showed a significant reduction in glutathione levels in animals with osteoarthritis compared to the non-osteoarthritic control group. Compared to the CFA group, only dexamethasone produced a significant increase in glutathione levels ($p = 0.0056$). The tramadol, agomelatine and repaglinide treated groups induced increases in glutathione, but not significant compared to the osteoarthritic control group.

7. Evaluarea efectelor analgezice ale agomelatinei și repaglinidului într-un model animal de durere indusă cu capsaicină

The main objective of the present study was to evaluate the analgesic effects of agomelatine and repaglinide, two potential TRPV1 antagonists, in an animal model of capsaicin-induced pain.

Materials and methods

The present experiment was performed in accordance with the bioethical guidelines for research on laboratory animals for scientific purposes as described in the NIH Guidelines for the Care and Use of Laboratory Animals and the ARRIVE guidelines.

The experimental groups were:

- Saline 0,1 mL ipl. and saline 0,1 mL per os (CTL);
- Capsaicin 10 µg/0.1 mL ipl. and saline 0,1 mL per os (CAP);
- Capsaicin 10 µg/0.1 mL ipl. and capsazepine 1 µg/mL ipl (CAP + CPZ);
- Capsaicin 10 µg/0.1 mL ipl. and agomelatine 80 mg/kg per os (CAP + AGO);
- Capsaicin 10 µg/0.1 mL ipl. and repaglinid 32 mg/kg per os (CAP + REPA).

In the animal model of pain induced by intraplantar administration of capsaicin, pharmacological tests were performed to determine the analgesic effect of the administered drugs. Thus the capsaicin test was performed and then thermal hyperalgesia was determined using the Hot plate test.

Results and discussions

Significant changes were observed between experimental groups in the capsaicin test (Kruskal-Wallis, $H(4) = 22.40$, $p = 0.0002$). Compared to the CTL group, intraplantar administration of capsaicin produced a statistically significant increase in time correlated with spontaneous nociception ($p < 0.001$, Bonferroni correction). This spontaneous nociception is characterized by lifting, licking or biting of the injected paw and lasts for approximately 5 minutes [104].

Our results were similar to those reported in preclinical studies [104]. Immediately after capsaicin administration, animals in the CAP group showed a significant increase in the time associated with spontaneous nociception compared to the CTL group, followed at 5 min by hyperalgesia to the thermal stimulus, but which disappeared after 30 min. Thermal hyperalgesia after capsaicin administration has also been evaluated in other preclinical studies and the intensity of hypersensitivity was reported to decrease over time, our results are similar to this observation [104, 105].

The analgesic effect of the substances (capsazepine, agomelatine, repaglinide) was significant in the Hot plate test. These substances produced a significant increase in the latency of the pain response compared to the values recorded for the CAP group after 5 minutes. The analgesic action probably occurs as a consequence of TRPV1 receptor blockade.

8. Evaluation of the effects of agomelatine and repaglinide on TRPV1 and TRPA1 channel activity

In the present study we aimed to investigate the effects of two substances, agomelatine and repaglinide, on TRPV1 and TRPA1 channel activity. In order to determine the mechanism of action of the drugs, we evaluated the agonist or antagonist activity of the substances.

Materials and methods

In vitro evaluation was carried out in collaboration with the Centre for Physiology and Pharmacology, Institute of Physiology, Medical University of Vienna, Austria.

Untransfected and transfected HEK293T cells were used to determine calcium levels. Test substances were applied before agonists of the two channels (allyl isothiocyanate for TRPA1 and capsaicin for TRPV1).

Results and discussions

For agomelatine, an increase in intracellular calcium levels is observed at 10 μM concentration, which indicates an agonist-type action on the TRPA1 receptor. For TRPV1 activity, a reduction in intracellular calcium concentration was observed following the application of 100 μM agomelatine. This effect is correlated with a modest antagonist action on the TRPV1 receptor. The lack of selectivity can be explained based on the structural similarity between the two channels [106]. Similar effects on the two channels have also been shown for capsazepine [107, 108].

Variations in TRPA1 and TRPV1 channel activity recorded for repaglinide are minimal. The lack of antagonist activity on the TRPV1 receptor observed in the present experiment shows that repaglinide is a false positive identified in in silico drug repurposing studies.

Conclusions and personal contributions

We performed in silico drug repurposing studies to identify new potential TRPV1 modulators. The proposed predictive model had higher accuracy for classifying TRPV1 agonists than antagonists, with ROC AUC values of 0.980 for predicting agonists, 0.972 for antagonists and 0.952 for inactive molecules. After screening of approved drugs with the validated algorithm, repaglinide (antidiabetic) and agomelatine (antidepressant) were identified as potential TRPV1 antagonists and protokylol (bronchodilator) as agonist.

The research continued with the validation of an animal model of visceral pain induced by single-dose intraperitoneal administration of paclitaxel. We found that paclitaxel induced significant visceral pain followed by mechanical and thermal hypersensitivity. In the animal model of paclitaxel 15 mg/kg-induced visceral pain, we showed that agomelatine and repaglinide reduced the intensity of visceral pain, but the variations were not significant.

Subsequently, the analgesic and anti-inflammatory effects of agomelatine and repaglinide were investigated in an animal model of osteoarthritis. Agomelatine significantly reduced thermal hypersensitivity after 14 days and repaglinide mechanical hypersensitivity after 7 days. The substances tested did not reduce inflammation induced by Freund's adjuvant complete, an effect also evidenced by radiological and histological evaluation. Repaglinide did however produce a significant decrease in serum proinflammatory cytokines and the changes recorded for agomelatine were not significant. Both substances showed an antioxidant effect.

In addition, agomelatine and repaglinide significantly reduced capsaicin-induced thermal hypersensitivity.

Using in vitro studies we showed that agomelatine acts as a TRPA1 agonist and modest TRPV1 antagonist, and the effects of repaglinide are independent of the activity of the two channels.

Based on the results obtained, it can be concluded that agomelatine and repaglinide showed an analgesic effect in the two pain models, but not an anti-inflammatory effect.

Personal contributions to the research are:

- We established an in silico model of drug repurposing using ligand-based, structure-based and machine learning strategies to identify novel TRPV1 receptor modulators. Based on this model, two molecules with potential analgesic effect were identified for the first time: repaglinide and agomelatine, with potential antagonist action on TRPV1 receptors. We also identified protokylol, a potential agonist of these receptors (**Chapter 4**);
- We validated a model of visceral pain induction by intraperitoneal administration of single-dose paclitaxel. To validate this method we performed dose-effect relationship on the intensity of cytostatic-induced visceral pain. We also evaluated the analgesic effect of agomelatine and repaglinide in the animal model of paclitaxel-induced visceral pain. This model can be used for investigations of various compounds with potential analgesic action **(Chapter 5);**
- We induced osteoarthritis by administering complete Freund adjuvant to rats and compared the analgesic and anti-inflammatory effect of two reference substances: tramadol and dexamethasone and test substances: repaglinide and agomelatine (**Chapter 6**);
- We highlighted the analgesic effect of agomelatine repaglinide in osteoarthritis-induced pain, an effect comparable to tramadol (**Chapter 6**);
- We demonstrated that repaglinide, agomelatine, but also tramadol do not have anti-inflammatory effects and do not protect against the progression of osteoarthritis, an effect observed with dexamethasone (**Chapter 6**);
- We demonstrated that repaglinide produces a decrease in the level of proinflammatory cytokines in the blood (**Chapter 6**);
- We concluded that both substances tested reduced oxidative stress as a consequence of the antioxidant effect (**Chapter 6**);

- In the animal model of capsaicin-induced pain, we demonstrated the occurrence of analgesic effects of agomelatine and repaglinide **(Chapter7**);
- We have demonstrated in vitro studies that agomelatine acts as a TRPA1 agonist and modest TRPV1 antagonist, and the analgesic effect of repaglinide is independent of the activity of the two calcium ion-dependent channels (**Chapter 8**).

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List of published scientific papers

1. **Andrei C**, Zanfirescu A, Nițulescu GM, Negreș S. Understanding the Molecular Mechanisms Underlying the Analgesic Effect of Ginger. Nutraceuticals 2022; 2(4):384–403, https://doi.org/10.3390/nutraceuticals2040029. BDI. (Capitolul 2, pag. 25, 31, 33)
2. **Andrei C**, Zanfirescu A, Nițulescu GM, Olaru OT, Negreș S. Natural Active Ingredients and TRPV1 Modulation: Focus on Key Chemical Moieties Involved in Ligand–Target Interaction. Plants 2023; 12(2):339, https://doi.org/10.3390/plants12020339. FI = 4,5. Q1. (Capitolul 2, pag. 26-31, 35, 38)
3. **Andrei C**, Mihai DP, Zanfirescu A, Nitulescu GM, Negres S. In Silico Drug Repurposing Framework Predicts Repaglinide, Agomelatine and Protokylol as TRPV1 Modulators with Analgesic Activity. Pharmaceutics 2022; 14(12):2563, https://doi.org/10.3390/pharmaceutics14122563. FI = 5,4. Q1. (Capitolul 4, pag. 43-70)
4. **Andrei C**, Zanfirescu A, Mihai DP, Negreș S. Paclitaxel—A Valuable Tool for Inducing Visceral Pain in Preclinical Testing? Int J Transl Med 2023; 3(1):108–119, https://doi.org/10.3390/ijtm3010010. BDI. (Capitolul 5, pag. 72-84)