TOXICOLOGICAL MECHANISMS OF TRAMADOL AND TAPENTADOL: BIOMEDICAL AND FORENSIC INSIGHTS FROM *IN VIVO* STUDIES

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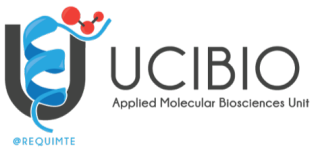

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Aos meus Pais e Irmãos.

To my Parents and Siblings.

AUTHOR'S DECLARATION

Under the terms of the Decree-Law nº 74/2006, of March 24th, it is hereby declared that the following original articles were prepared in the scope of this dissertation.

PUBLICATIONS

Articles in international peer-reviewed journals

Theoretical Background

I. **Barbosa J**, Faria J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative metabolism of tramadol and tapentadol: a toxicological perspective. **Drug Metabolism Reviews** 2016; 48(4): 577-592. DOI: https://doi.org/10.1080/03602532.2016.1229788

Original Research

II. **Barbosa J**, Faria J, Leal S, Afonso LP, Lobo J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Acute administration of tramadol and tapentadol at effective analgesic and maximum tolerated doses causes hepato- and nephrotoxic effects in Wistar rats. **Toxicology** 2017; 389: 118-129. DOI: https://doi.org/10.1016/j.tox.2017.07.001

III. **Barbosa J**, Faria J, Garcez F, Leal S, Afonso LP, Nascimento AV, Moreira R, Queirós O, Carvalho F, Dinis-Oliveira RJ. Repeated Administration of Clinical Doses of Tramadol and Tapentadol Causes Hepato- and Nephrotoxic Effects in Wistar Rats. **Pharmaceuticals (Basel)** 2020; 13(7): 149. DOI: https://doi.org/10.3390/ph13070149

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Original Research

I. **Barbosa J**, Faria J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative toxicity of tramadol and tapentadol in Wistar rats. **Toxicology Letters** 2016; 258S: S103. DOI: https://doi.org/10.1016/j.toxlet.2016.06.1436

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Oral communications

I. **Barbosa J**, Faria J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Acute administration of tramadol and tapentadol induces dose-dependent toxicity in Wistar rats, affecting both metabolizing and target organs. XLVII Reunião Anual da Sociedade Portuguesa de Farmacologia (SPF), 2-4 February 2017, Coimbra, Portugal.

II. **Barbosa J**, Faria J, Carvalho F, Queirós O, Moreira R, Dinis-Oliveira RJ. Acute administration of tramadol and tapentadol induces dose-dependent toxicity in Wistar rats. VII Workshop do Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde (IINFACTS), 20 July 2017, Paredes, Portugal.

III. **Barbosa J**, Faria J, Leal S, Nascimento AV, Afonso LP, Queirós O, Moreira R, Pereira FC, Carvalho F, Dinis-Oliveira RJ. Sub-chronic exposure to therapeutic doses of tramadol and tapentadol causes hepatotoxicity in Wistar rats. XLIX Reunião Anual da Sociedade Portuguesa de Farmacologia (SPF), 6-8 February 2019, Porto, Portugal.

IV. **Barbosa J**, Faria J, Leal S, Nascimento AV, Afonso LP, Moreira R, Pereira FC, Queirós O, Carvalho F, Dinis-Oliveira RJ. O tramadol e o tapentadol causam hepato e nefrotoxicidade em ratos Wistar expostos subcronicamente a doses terapêuticas. 3ª Reunião Internacional da Rede Académica das Ciências da Saúde da Lusofonia, 28 and 29 September 2020, Online event organized by the Rede Académica das Ciências da Saúde da Lusofonia (RACS), Portugal.

V. **Barbosa J**, Faria J, Leal S, Nascimento AV, Afonso LP, Queirós O, Moreira R, Pereira FC, Carvalho F, Dinis-Oliveira RJ. Repeated administration of clinically relevant doses of tramadol and tapentadol causes hepatorenal toxicity in Wistar rats. SPF Meeting 2021 – LI Reunião Anual da Sociedade Portuguesa de Farmacologia (SPF), 17-19 February 2021, Online event organized by the Faculdade de Farmácia da Universidade de Lisboa (FFUL), Portugal.

Poster communications

I. **Barbosa J**, Faria J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Comparative toxicity of tramadol and tapentadol in Wistar rats. I Encontro Anual do Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto (FFUP), 19 July 2016, Porto, Portugal.

II. **Barbosa J**, Faria J, Carvalho F, Queirós O, Moreira R, Dinis-Oliveira RJ. Comparative toxicity of tramadol and tapentadol in Wistar rats. EUROTOX 2016, 4-7 September 2016, Seville, Spain.

III. **Barbosa J**, Faria J, Queirós O, Moreira R, Carvalho F, Dinis-Oliveira RJ. Toxicological effects and pharmacokinetics of tramadol and tapentadol in Wistar rats. UCIBIO External Advisory Board Visit, 21 April 2017, Lisbon, Portugal.

IV. **Barbosa J**, Faria J, Leal S, Nascimento AV, Afonso LP, Queirós O, Moreira R, Pereira FC, Carvalho F, Dinis-Oliveira RJ. Sub-chronic exposure to therapeutic doses of tramadol and tapentadol causes hepatotoxicity in Wistar rats. XIII Jornadas Científicas do Instituto Universitário de Ciências da Saúde (IUCS)/IV Congresso da Associação Portuguesa de Ciências Forenses (APCF), 11 and 12 April 2019, Porto, Portugal.

Under the terms of the referred Decree-Law, the author declares that she afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

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ABSTRACT

Prescription opioids are a cornerstone in the treatment of moderate to severe forms of pain, both in acute and chronic settings. Tramadol and tapentadol are fully synthetic opioid analgesics with structural and mechanistic similarities, combining μ-opioid receptor (MOR) agonism with monoamine reuptake inhibition. Such dual synergistic mechanism of action optimizes their therapeutic effects and safety profile, substantiating their widespread prescription and use. While tramadol inhibits serotonin (5-HT) and noradrenaline (NA) reuptake, tapentadol acts mainly through NA reuptake inhibition. Tapentadol was introduced into the market as an alternative to its predecessor tramadol since, unlike it, does not require hepatic metabolic activation to induce full analgesia, presents a more linear pharmacokinetics and minimizes 5-HT syndrome liability.

In spite of their efficacy, reports of adverse reactions, fatal and non-fatal intoxications, abuse and dependence have been growing along with their use. However, information regarding the molecular, metabolic and histopathological rationale underlying their toxicity is limited, particularly for tapentadol, owing to its shorter market history. Accordingly, the general aim of this thesis was to comparatively study the toxicological effects deriving from the single and repeated exposure to clinically relevant doses of tramadol and tapentadol, using an *in vivo* rodent model.

In view of the paramount role of the liver and the kidneys in tramadol and tapentadol metabolism and excretion, these organs are particularly liable to toxicological injury. In this context, the hepatorenal effects of an acute exposure to therapeutic doses of both opioids were assessed in Wistar rats injected, through intraperitoneal (i.p.) route, with 10, 25 and 50 mg/kg tramadol and tapentadol doses, in 1 mL-units, using normal saline solution (0.9% (*w/v*) NaCl) as vehicle. These doses correspond to a standard, analgesic dose, an intermediate dose and the maximum recommended daily dose, respectively. The control group was administered with the same volume of the vehicle alone. Twenty-four hours after the administration, animals were sacrificed and serum, urine, liver and kidney homogenates were used for biochemical determinations, while liver and kidney samples were processed for histological analysis through hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining procedures. A decrease in lipid peroxidation (LPO), assessed as thiobarbituric acid reactive substances (TBARS), was detected in liver and kidney homogenates, mainly at the intermediate and highest doses of both opioids; in turn, an increase in protein oxidation, determined as protein carbonyl groups, was observed for tapentadol treatment only. Acute exposure to the highest tramadol and tapentadol dose led to increased serum alanine aminotransferase (ALT) activity, decreased serum urea, increased serum total cholesterol and high-density lipoprotein (HDL) cholesterol, while tapentadol treatment caused a rise in serum low-density lipoprotein (LDL) cholesterol and triglyceride contents. Serum butyrylcholinesterase (BuChE) activity decreased upon exposure to all opioid doses. Collectively, these results indicate liver damage upon exposure to both drugs. The analysis of urine samples revealed a decrease in the glomerular filtration rate (GFR) and in urea output, as well as proteinuria, at almost all doses tested, suggesting renal

impairment. In turn, histopathological findings included mononuclear cell infiltrates, hepatic sinusoidal dilatation, microsteatosis, glycogen depletion and tubular disorganization for both opioids; vascular congestion/erythrocyte extravasation and focal, unicellular necrosis/acidophilic bodies were exclusive to tapentadol treatment. In summary, the acute administration of clinical doses of both opioids led to hepato- and nephrotoxicity, with tapentadol causing toxicological damage to a greater extent.

Since prescription opioids are frequently used on a subacute to chronic basis, similar *in vivo* assays have been conducted to mimic an extended exposure, thus analyzing its impact on tramadol and tapentadol toxicological profile. To this purpose, for 14 consecutive days, Wistar rats received single daily i.p. injections of the same doses used in the acute exposure assays. An additional control group was injected with the drug vehicle (0.9% (*w/v*) NaCl). Serum and urine samples were collected, and organs were surgically excised for biochemical and histopathological studies. Besides liver and kidneys, lung, heart and brain cortex, opioid target organs, were also analyzed. Increased LPO was found in liver, kidney, lung and brain cortex homogenates, whereas an increment in protein oxidation was detected in liver, kidney and brain cortex. Decreased liver total antioxidant capacity and serum myeloperoxidase (MPO) activity were also shown, although with no impact on the systemic antioxidant status. Serum inflammation biomarkers, such as C reactive protein (CRP) and tumor necrosis factor-α (TNF-α), increased upon opioid treatment, suggesting systemic inflammation. Liver function tests revealed hepatobiliary impairment, as shown through increased serum ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) activities. Liver synthesis of albumin, urea, BuChE and complement components 3 (C3) and 4 (C4) was also affected by the repeated exposure to both opioids, in view of the decrease in their serum levels. Elevated serum cystatin C, decreased urine creatinine output and increased urine microalbumin levels were detected upon tapentadol treatment, while increased serum amylase and urine *N*-acetyl-β-D-glucosaminidase (NAG) activities were observed for both opioids, indicating detrimental effects on kidney function. Cardiac cell integrity was also affected, as shown by the increase in serum creatine kinase muscle brain (CK-MB) isoform, lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) activities, though with no impact on ventricular function. In turn, results from the analysis of brain cortex homogenates, namely augmented tissue lactate upon exposure to both opioids, as well as increased tissue LDH and creatine kinase (CK) activities upon tapentadol treatment, suggest alterations in brain cortex metabolism. In addition, changes were detected in the lipid profile, as shown through increased serum triglycerides, total cholesterol and LDL cholesterol, as well as in iron metabolism, as deduced from the increase in serum iron, ferritin, haptoglobin and heme oxygenase 1 (HO-1) and from the decrease in serum transferrin, hepcidin and β2-microglobulin (B2M). Gene expression of a panel of liver, kidney, lung, heart and brain toxicity biomarkers was also analyzed through quantitative real-time polymerase chain reaction (qRT-PCR) in the corresponding tissues, showing a good correlation with the remaining inflammatory, oxidative stress, metabolic, organ function and histological findings. H&E staining evidenced sinusoidal

dilatation and microsteatosis in liver sections, glomerular and tubular disorganization and increased Bowman's spaces in kidney sections, alveolar collapse and destruction in lung sections, altered cardiomyocytes and loss of striation in heart sections, as well as neuronal degeneration and accumulation of glial and microglial cells in brain cortex sections. Inflammatory infiltrates were also observed in liver, kidney and heart tissue specimens. As in acute exposure assays, vascular congestion/erythrocyte extravasation was exclusive to tapentadol treatment in liver sections. Masson's trichrome staining, in turn, revealed traces of fibrous tissue between hepatocytes and cardiomyocytes, as well as signs of cardiac perivascular fibrosis, upon treatment with both opioids.

Taken together, these results show that tramadol and tapentadol are not devoid of toxicological risk, even at therapeutic doses, causing systemic and metabolizing and target organ-specific effects. Although some of these effects were exclusive or more pronounced upon tapentadol exposure, both in single and repeated administration settings, the extension of the exposure minimizes the differences between their toxicological profiles and requires comparatively lower doses to elicit toxicological injury. The present studies may be complemented with additional assays, by broadening the exposure period and dose range, as well as by encompassing combined drug exposure experiments, in order to get a more comprehensive insight into the molecular, tissue and metabolic alterations associated with prescription opioid use.

Collectively, the results of the present thesis contribute to the interpretation of adverse reactions and intoxications resulting from both the medical and non-medical use of tramadol and tapentadol. They emphasize the need to carefully consider and monitor their use, especially when dealing with situations of misuse and subacute to chronic pain management.

Keywords: Prescription opioids, tapentadol, tramadol, cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, pneumotoxicity, acute exposure, subacute exposure, *in vivo* assays, toxicity assays.

RESUMO

Os opioides de prescrição são um pilar fundamental no tratamento de formas moderadas a severas de dor, tanto em contexto agudo como crónico. O tramadol e o tapentadol são analgésicos opioides sintéticos com semelhanças estruturais e mecanísticas, combinando o agonismo dos recetores μ-opioides (MOR) com a inibição da recaptação de monoaminas. Este mecanismo de ação dual e sinérgico otimiza os seus efeitos terapêuticos e perfil de segurança, justificando a sua prescrição e uso generalizados. Enquanto o tramadol inibe a recaptação de serotonina (5-HT) e de noradrenalina (NA), o tapentadol atua predominantemente através da inibição da recaptação de NA. O tapentadol foi introduzido no mercado como uma alternativa ao seu antecessor tramadol, uma vez que, ao contrário deste, não requer ativação metabólica hepática para induzir analgesia plena, apresenta uma farmacocinética mais linear e minimiza a suscetibilidade de síndrome serotoninérgica.

Apesar da sua eficácia terapêutica, os relatos de reações adversas, intoxicações fatais e não fatais, abuso e dependência têm vindo a acumular-se em paralelo com o seu uso. Contudo, a informação relativa à base molecular, metabólica e histopatológica subjacente à sua toxicidade é limitada, em particular para o tapentadol, devido ao seu mais curto historial de comercialização. Assim, o objetivo geral desta tese foi o de estudar comparativamente os efeitos toxicológicos decorrentes da exposição única e repetida a doses clinicamente relevantes de tramadol e tapentadol, usando um modelo de roedor *in vivo*.

Considerando o papel crucial do fígado e dos rins no metabolismo e excreção do tramadol e do tapentadol, estes órgãos são particularmente suscetíveis a dano toxicológico. Neste contexto, os efeitos hepatorrenais de uma exposição aguda a doses terapêuticas de ambos os opioides foram avaliados em ratos Wistar injetados, por via intraperitoneal (i.p.), com doses de 10, 25 e 50 mg/kg de tramadol e tapentadol, em unidades de 1 mL, usando soro fisiológico (NaCl 0,9% (*m/v*)) como veículo. Estas doses correspondem a uma dose analgésica padrão, a uma dose intermédia e à dose máxima diária recomendada, respetivamente. Ao grupo controlo, foi administrado unicamente o veículo, no mesmo volume. Vinte e quatro horas após a administração, os animais foram sacrificados e utilizaram-se amostras de soro, urina e homogeneizados de fígado e rim para determinações bioquímicas, processando-se amostras de fígado e rim para análise histológica através das colorações de hematoxilina e eosina (H&E) e ácido periódico de Schiff (PAS). Detetou-se uma diminuição da peroxidação lipídica (LPO), avaliada através das substâncias reativas ao ácido tiobarbitúrico (TBARS), nos homogeneizados de fígado e rim, principalmente nas doses intermédia e mais elevada de ambos os opioides; por sua vez, observou-se um aumento da oxidação proteica, aferida pelo teor de grupos carbonilo proteicos, exclusivamente aquando do tratamento com tapentadol. A exposição aguda à dose mais elevada de tramadol e tapentadol conduziu a um aumento da atividade sérica da alanina aminotransferase (ALT), a uma diminuição da ureia sérica, ao aumento do colesterol sérico total e do colesterol associado às lipoproteínas de alta densidade (HDL), enquanto o tratamento com tapentadol provocou um aumento dos teores séricos de colesterol associado às lipoproteínas de baixa densidade (LDL) e de triglicéridos. A atividade

sérica da butirilcolinesterase (BuChE) diminuiu após exposição a todas as doses de opioide. No seu conjunto, estes resultados apontam para dano hepático após exposição a ambos os fármacos. A análise de amostras de urina revelou uma diminuição da taxa de filtração glomerular (GFR) e da excreção de ureia, assim como proteinúria, em quase todas as doses testadas, sugerindo comprometimento renal. Por sua vez, os achados histopatológicos incluíram infiltrados de células mononucleares, dilatação dos sinusoides hepáticos, microesteatose, depleção de glicogénio e desorganização tubular para ambos os opioides; a congestão vascular/extravasamento de eritrócitos e a necrose focal, unicelular/corpos acidófilos revelaram-se exclusivos do tratamento com tapentadol. Em suma, a administração aguda de doses clínicas de ambos os opioides conduziu a hepato e nefrotoxicidade, tendo o tapentadol causado dano toxicológico em maior extensão.

Uma vez que os opioides de prescrição são frequentemente usados de forma subaguda a crónica, realizaram-se estudos *in vivo* similares para mimetizar a exposição prolongada, analisando o seu impacto no perfil toxicológico do tramadol e do tapentadol. Para este efeito, durante 14 dias consecutivos, ratos Wistar receberam injeções i.p. únicas e diárias das doses usadas nos ensaios de exposição aguda. Injetaram-se os animais de um grupo controlo adicional com o veículo dos fármacos (NaCl 0,9% (*m/v*)). Recolheram-se amostras de soro e urina e removeram-se cirurgicamente órgãos para estudos bioquímicos e histopatológicos. Além do fígado e do rim, analisou-se igualmente o pulmão, coração e córtex cerebral, enquanto órgãos-alvo dos opioides. Detetou-se um aumento da LPO nos homogeneizados de fígado, rim, pulmão e córtex cerebral, assim como um incremento da oxidação proteica no fígado, rim e córtex cerebral. Demonstrou-se, igualmente, a diminuição da capacidade antioxidante total do fígado e da atividade sérica da mieloperoxidase (MPO), embora sem impacto no estado antioxidante sistémico. Biomarcadores séricos de inflamação, como a proteína C reativa (CRP) e o fator de necrose tumoral-α (TNF-α), aumentaram mediante o tratamento com opioide, sugerindo inflamação sistémica. Os testes de função hepática revelaram dano hepatobiliar, demonstrado através do aumento da atividade sérica da ALT, aspartato aminotransferase (AST), fosfatase alcalina (ALP) e γ-glutamiltranspeptidase (GGT). A síntese hepática de albumina, ureia, BuChE e componentes do sistema complemento 3 (C3) e 4 (C4) foi igualmente afetada pela exposição repetida a ambos os opioides, dada a diminuição dos seus níveis séricos. Após tratamento com tapentadol, observou-se elevação da cistatina C sérica, diminuição da excreção de creatinina e aumento dos níveis urinários de microalbumina, a par de níveis aumentados da atividade sérica da amilase e urinária da *N*-acetil-β-D-glucosaminidase (NAG) para ambos os opioides, indicando efeitos prejudiciais na função renal. A integridade das células cardíacas foi igualmente afetada, tal como demonstrado pelo aumento das atividades séricas da isoforma músculo/cérebro da creatina cinase (CK-MB), lactato desidrogenase (LDH) e α-hidroxibutirato desidrogenase (α-HBDH), ainda que sem impacto na função ventricular. Por sua vez, os resultados da análise de homogeneizados de córtex cerebral, nomeadamente o aumento do lactato tecidular após exposição a ambos os opioides, bem como da atividade tecidular da LDH e da creatina cinase (CK) após tratamento

com tapentadol, sugerem alterações no metabolismo deste tecido. Adicionalmente, foram detetadas alterações no perfil lipídico, manifestadas através de níveis séricos aumentados de triglicéridos, colesterol total e colesterol associado às LDL, assim como no metabolismo do ferro, como pode deduzir-se a partir do aumento nos níveis séricos de ferro, ferritina, haptoglobina e heme oxigenase 1 (HO-1) e do decréscimo dos de transferrina, hepcidina e β2-microglobulina (B2M). Foi também analisada, nos tecidos correspondentes, a expressão génica de um painel de biomarcadores de toxicidade hepática, renal, pulmonar, cardíaca e cerebral, através da reação em cadeia da polimerase quantitativa em tempo real (qRT-PCR), revelando uma boa correlação com os restantes resultados relativos a inflamação, stress oxidativo, metabolismo, função orgânica e histologia. A coloração de H&E evidenciou dilatação dos sinusoides e microesteatose nas secções de fígado, desorganização glomerular e tubular e espaços de Bowman aumentados nas secções de rim, colapso e destruição alveolar nas secções de pulmão, cardiomiócitos alterados e perda de estriação nas secções de coração, bem como degeneração neuronal e acumulação de células da glia e microglia nas secções de córtex cerebral. Observaram-se também infiltrados inflamatórios nas secções de fígado, rim e coração. À semelhança dos ensaios de exposição aguda, a congestão vascular/extravasamento de eritrócitos revelou-se exclusiva do tratamento com tapentadol nas secções de fígado. A coloração de tricrómio de Masson, por sua vez, evidenciou vestígios de tecido fibroso entre os hepatócitos e cardiomiócitos, assim como sinais de fibrose cardíaca perivascular, após tratamento com ambos os opioides.

Coletivamente, estes resultados demonstram que o tramadol e o tapentadol não são isentos de risco toxicológico, mesmo em doses terapêuticas, causando efeitos sistémicos e específicos de órgãos metabolizadores e órgãos-alvo. Embora alguns destes efeitos tenham sido exclusivos ou mais pronunciados após a exposição ao tapentadol, tanto em contexto de administração única como de administração repetida, a extensão da exposição minimiza as diferenças entre os seus perfis toxicológicos e requer doses comparativamente mais baixas para produzir dano toxicológico. Os presentes estudos poderão ser complementados com ensaios adicionais, alargando o período de exposição e a gama de doses, assim como contemplando experiências de exposição combinada a fármacos, de modo a conseguir-se uma perspetiva mais abrangente das alterações moleculares, tecidulares e metabólicas associadas ao uso de opioides de prescrição.

No seu conjunto, os resultados da presente tese contribuem para a interpretação de reações adversas e intoxicações decorrentes quer do uso médico, quer do uso não médico de tramadol e tapentadol. Enfatizam a necessidade de considerar e monitorizar cuidadosamente o seu uso, em especial aquando de situações de abuso e de tratamento da dor subaguda a crónica.

Palavras-chave: Opioides de prescrição, tapentadol, tramadol, cardiotoxicidade, hepatotoxicidade, nefrotoxicidade, neurotoxicidade, pneumotoxicidade, exposição aguda, exposição subaguda, ensaios *in vivo*, ensaios de toxicidade.

LIST OF ABBREVIATIONS AND ACRONYMS

18S rRNA, 18S ribosomal ribonucleic acid

5-HT, 5-hydroxytriptamine/serotonin

8-OHdG, 8-hydroxy-2'-deoxyguanosine

8-OHG, 8-hydroxyguanosine

AChE, acetylcholinesterase

ADH, anti-diuretic hormone

AED, animal equivalent dose

Aldoa, fructose-bisphosphate aldolase A

ALP, alkaline phosphatase

ALT, alanine aminotransferase

Angptl4, angiopoietin-like 4

ANOVA, analysis of variance

AOPs, adverse outcome pathways

Apex1, apurinic/apyrimidinic endonuclease 1

APP, acute phase protein

AST, aspartate aminotransferase

B2M, β2-microglobulin

BCA, bicinchoninic acid

BDNF, brain-derived neurotrophic factor

BNP, brain natriuretic peptide

BuChE, butyrylcholinesterase

BUN, blood urea nitrogen

C3, complement component 3

C4, complement component 4

CAT, catalase

Cav-1, caveolin-1

CC16, Clara cell protein-16

CCL5, chemokine (C-C motif) ligand 5

Cd36, cluster of differentiation 36/fatty acid translocase

cDNA, complementary deoxyribonucleic acid

CK, creatine kinase

CK-MB, creatine kinase muscle brain isoform

CNS, central nervous system

CPP, conditioned place preference

CRP, C reactive protein

Ct, threshold cycle

CYP, cytochrome

CYP1A2, cytochrome P450 isoenzyme 1A2

CYP2A6, cytochrome P450 isoenzyme 2A6

CYP2B6, cytochrome P450 isoenzyme 2B6
CYP2C19, cytochrome P450 isoenzyme 2C19
CYP2C9, cytochrome P450 isoenzyme 2C9
CYP2D6, cytochrome P450 isoenzyme 2D6
CYP2E1, cytochrome P450 isoenzyme 2E1
CYP3A, cytochrome P450 subfamily 3A
CYP3A4, cytochrome P450 isoenzyme 3A4
CYP450, cytochrome P450
DDD, defined daily doses
DID, defined daily doses per 1,000 inhabitants per year
DNA, deoxyribonucleic acid
DNPH, 2,4-dinitrophenylhydrazine
DPWG, Royal Dutch Pharmacists Association – Pharmacogenetics Working Group
ECL, enhanced chemiluminescence
ECM, extracellular matrix
EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine
EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid
EIA, enzyme immunoassay
ELISA, enzyme-linked immunosorbent assay
EM, extensive metabolizer
EMCDDA, European Monitoring Center for Drugs and Drug Addiction
ER, extended-release
ERDF, European Regional Development Fund
EU, European Union
GABA, γ-aminobutyric acid
Gamt, guanidinoacetate *N*-methyltransferase
GC/MS, gas chromatography/mass spectrometry
GFAP, glial fibrillary acidic protein
GFR, glomerular filtration rate
GGT, γ-glutamyl transpeptidase
GPx, glutathione peroxidase
GS, glutamine synthetase
GSH, reduced glutathione
GST, glutathione-*S*-transferase
α-HBDH, α-hydroxybutyrate dehydrogenase
HDL, high-density lipoprotein
H&E, hematoxylin and eosin
HFE, human hemochromatosis protein
Hmox1, heme oxygenase 1 gene
HO-1, heme oxygenase 1

IFN-γ, interferon gamma

IgG, immunoglobulin G

IL-10, interleukin-10

IL-17A, interleukin-17A

IL-1B, interleukin-1B

IL-2, interleukin-2

IL-6, interleukin-6

IM, intermediate metabolizer

INFARMED, Autoridade Nacional do Medicamento e Produtos de Saúde, I.P. / Portuguese National Authority of Medicines and Health Products

iNOS, inducible nitric oxide synthase

i.p., intraperitoneal

IR, immediate-release

K_i, inhibition constant

K_m, body surface area correction factor

LD_{50}, median lethal dose

LDH, lactate dehydrogenase

LDL, low-density lipoprotein

Lpl, lipoprotein lipase

LPO, lipid peroxidation

M1, *O*-desmethyltramadol

M10, dehydrated tramadol

M11, 2-formyl-1-(3-methoxyphenyl)cyclohexanol

M12, tramadol glucuronide

M13, *O*-desmethyltramadol glucuronide

M14, *O*,*N*,*N*-tridesmethyltramadol glucuronide

M15, *O*,*N*-didesmethyltramadol glucuronide

M16, 4-hydroxy-cyclohexyltramadol glucuronide

M17, 4-hydroxy-cyclohexyl-*N*-desmethyltramadol glucuronide

M18, 4-hydroxy-cyclohexyl-*N*,*N*-didesmethyltramadol glucuronide

M19, tramadol sulfonate

M2, *N*-desmethyltramadol

M20, *O*-desmethyltramadol sulfonate

M21, *O*,*N*,*N*-tridesmethyltramadol sulfonate

M22, *O*,*N*-didesmethyltramadol sulfonate

M23, 4-hydroxy-cyclohexyltramadol sulfonate

M3, *N*,*N*-didesmethyltramadol

M31, tramadol *N*-oxide

M32, 4-hydroxy-*O*-desmethyltramadol

M33, dehydrated tramadol *N*-oxide

M4, *O*,*N*,*N*-tridesmethyltramadol
M5, *O*,*N*-didesmethyltramadol
M6, 4-hydroxy-cyclohexyltramadol
M7, 4-hydroxy-cyclohexyl-*N*-desmethyltramadol
M8, 4-hydroxy-cyclohexyl-*N*,*N*-didesmethyltramadol
M9, 4-oxo-cyclohexyltramadol
MAOIs, monoamine oxidase inhibitors
MCP-1, monocyte chemoattractant protein-1
MCT2, multidrug resistance-associated protein 2
MDA, malondialdehyde
MHC, major histocompatibility complex
MMP, matrix metalloproteinase
MMP-7, matrix metalloproteinase-7
MMP-9, matrix metalloproteinase-9
MOR, μ-opioid receptor
MPO, myeloperoxidase
mRNA, messenger ribonucleic acid
NA, noradrenaline
NAG, *N*-acetyl-β-D-glucosaminidase
NE, norepinephrine
NK, natural killer
NOP, nociceptin/orphanin FQ peptide
Nphs2, podocin
NTC, non-template control
OCT1, organic cation transporter 1
OD, optical density
p, *p*-value/calculated probability
PAS, periodic acid-Schiff
pH, potential of hydrogen
Plau/UPA, plasminogen activator, urokinase
PM, poor metabolizer
PTX-3, pentraxin 3
PubMed, United States National Library of Medicine Public/Publisher MEDLINE
qRT-PCR, quantitative real-time polymerase chain reaction
RADARS, Researched Abuse, Diversion and Addiction-Related Surveillance
RIPA, radioimmunoprecipitation assay buffer
RiSSC, Research Center on Security and Crime
RNA, ribonucleic acid
ROS, reactive oxygen species
RTC, ribonucleic acid template control

S100β, S100 calcium binding protein B
SD, standard deviation
SDS, sodium dodecyl sulphate
SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNCA, α-synuclein gene
SNPs, single-nucleotide polymorphisms
SNRIs, selective noradrenaline reuptake inhibitors
SOD, superoxide dismutase
SP-A, pulmonary surfactant protein A
SP-D, pulmonary surfactant protein D
SSRIs, selective serotonin reuptake inhibitors
SULT, sulfotransferase
SULT1, sulfotransferase family 1
SULT1A, sulfotransferase subfamily 1A
SULT1A1, sulfotransferase isoenzyme 1A1
SULT1A2, sulfotransferase isoenzyme 1A2
SULT1A3, sulfotransferase isoenzyme 1A3
SULT1C2, sulfotransferase isoenzyme 1C2
SULT2, sulfotransferase family 2
SULT2A1, sulfotransferase isoenzyme 2A1
SULT2A3, sulfotransferase isoenzyme 2A3
SULT2B1, sulfotransferase isoenzyme 2B1
SULT4, sulfotransferase family 4
SULT6, sulfotransferase family 6
Tap, tapentadol
TBARS, thiobarbituric acid reactive substances
TBST, Tris-buffered saline Tween-20
TCAs, tricyclic antidepressants
TGF-β1, transforming growth factor-β1
TGF-β2, transforming growth factor-β2
TIMP, tissue inhibitor of metalloproteinase
TIMP-1, tissue inhibitor of metalloproteinase-1
TNF-α, tumor necrosis factor-α
Tram, tramadol
Tris, tris(hydroxymethyl)aminomethane
UGT1A10, uridine diphosphate-glucuronosyltransferase 1A10
UGT1A7, uridine diphosphate-glucuronosyltransferase 1A7
UGT1A8, uridine diphosphate-glucuronosyltransferase 1A8
UGT1A9, uridine diphosphate-glucuronosyltransferase 1A9
UGT2B15, uridine diphosphate-glucuronosyltransferase 2B15

UGT2B7, uridine diphosphate-glucuronosyltransferase 2B7

UGTs, uridine diphosphate-glucuronosyltransferases

UK, United Kingdom

UM, ultra-rapid metabolizer

USA, United States of America

U.S. FDA, United States Food and Drug Administration

VLDL, very low-density lipoprotein

WHO, World Health Organization

OUTLINE OF THE THESIS

The present thesis is organized into four major parts, which are briefly summarized as follows:

PART I

1. GENERAL INTRODUCTION

This section, presented as a review article, aims to contextualize the state of the art, as well as to provide a general perspective on tramadol and tapentadol metabolic, pharmacological, and toxicological profiles.

2. GENERAL AND SPECIFIC OBJECTIVES OF THE DISSERTATION

The general and specific objectives of the thesis are specified.

PART II

1. ORIGINAL RESEARCH

Part II is structured into three chapters, corresponding to the original articles that the candidate first-authored in the scope of this thesis. Information regarding the journal and date of publication is provided for each article.

PART III

This section is organized into three main points:

1. INTEGRATED OVERVIEW OF THE STUDIES PERFORMED

The studies conducted are discussed in an integrated manner.

2. CONCLUSIONS

The main conclusions deriving from the experimental studies are enumerated.

3. DIRECTIONS FOR FUTURE RESEARCH

Opportunities for complementary works are presented.

PART IV

1. REFERENCES

The references cited in PART III are provided.

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PART I

1. GENERAL INTRODUCTION

REVIEW ARTICLE

Comparative metabolism of tramadol and tapentadol: a toxicological perspective

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REVIEW ARTICLE

Comparative metabolism of tramadol and tapentadol: a toxicological perspective

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ABSTRACT

Tramadol and tapentadol are centrally acting, synthetic opioid analgesics used in the treatment of moderate to severe pain. Main metabolic patterns for these drugs in humans are well characterized. Tramadol is mainly metabolized by cytochrome P450 CYP2D6 to *O*-desmethyltramadol (M1), its main active metabolite. M1 and tapentadol undergo mainly glucuronidation reactions. On the other hand, the pharmacokinetics of tramadol and tapentadol are dependent on multiple factors, such as the route of administration, genetic variability in pharmacokinetic components and concurrent consumption of other drugs. This review aims to comparatively discuss the metabolomics of tramadol and tapentadol, namely by presenting all their known metabolites. An exhaustive literature search was performed using textual and structural queries for tramadol and tapentadol, and associated known metabolizing enzymes and metabolites. A thorough knowledge about tramadol and tapentadol metabolomics is expected to provide additional insights to better understand the interindividual variability in their pharmacokinetics and dose-responsiveness, and contribute to the establishment of personalized therapeutic approaches, minimizing side effects and optimizing analgesic efficacy.

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Metabolomics; opioids; pharmacogenetics; pharmacokinetics; tapentadol; tramadol

Introduction

Opioids are currently a gold standard for the treatment of moderate to severe pain. They consist of natural, semisynthetic and endogenous compounds that act as agonists or antagonists of μ-, κ-, or δ-opioid receptors, being completely antagonized by naloxone, having morphine-like effects and affecting the modulation of pain within the central nervous system (CNS), peripheral neurons, ectodermal cells, neuroendocrine and immune systems (DePriest et al., 2015; Harrison et al., 1998; Kosten & George, 2002). Activation of μ-opioid receptors (MOR) results both in analgesic effects – decreased nociception, reduced proliferation of the action potential, and decreased release of inflammatory peptides at nerve terminals – and in side effects – CNS depression, drowsiness, nausea, vomiting, motor incoordination, mood changes, miosis, dependence and addiction, among others (DePriest et al., 2015; Harrison et al., 1998; Kosten & George, 2002).

Although MOR agonists are effective against acute pain, their effectiveness against chronic pain of neuropathic or inflammatory nature is lower and they may present an unsatisfactory therapeutic window (Giorgi, 2012; Pergolizzi et al., 2012; Power, 2011; Singh et al., 2013). Inherent drawbacks such as the potential risk of adverse events like nausea, constipation and respiratory depression, addiction, tolerance and dependence lead to some reluctance to their use. In turn, suboptimal use is responsible for poor outcomes and low compliance (Giorgi, 2012; Pergolizzi et al., 2012; Power, 2011; Singh et al., 2013). In this context, the combination of MOR agonists with monoamine reuptake inhibitors arises as a valuable strategy to improve opioid therapeutic range and compliance. By reducing MOR activation requirements, the synergistic combination of both mechanisms of action enhances analgesia and mitigates the side effects of MOR agonists (Giorgi, 2012; Pergolizzi et al., 2012; Power, 2011;

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Singh et al., 2013; Tzschentke et al., 2014; Vadivelu et al., 2010).

The therapeutic use of tramadol corresponded to the first development addressing this problem. This atypical, racemic opioid combines MOR activation and 5-hydroxytryptamine (5-HT) and noradrenaline (NA) reuptake (Giorgi et al., 2012a; Grond & Sablotzki, 2004; Hui-Chen et al., 2004; Leppert, 2011; Raffa, 2008; Raffa et al., 2012). However, its pharmacological properties, namely its racemic and prodrug nature, are responsible for a complex pharmacokinetic profile, dependent on metabolic activation by the CYP2D6 polymorphic enzyme and with a variable contribution of both mechanisms of action over time (Duthie, 1998; Gillen et al., 2000; Grond & Sablotzki, 2004; Lai et al., 1996; Leppert, 2011).

Tapentadol, in turn, was introduced in the market as allegedly being a significant advance over tramadol. It is a next generation, structurally related opioid with a dual mechanism of action that provides similar analgesic efficacy to that of a pure MOR agonist, but with an improved side-effect profile. It first received United States Food and Drug Administration (U.S. FDA) approval in November 2008, having been placed into the schedule II category of the Controlled Substances Act in May 2009 (Bourland et al., 2010; Coulter et al., 2010; Dousa et al., 2013). At the end of 2011, it was also approved by the European Medicines Agency and has since been marketed as extended-release (ER) tablets for chronic pain treatment (Giorgi, 2012; Giorgi et al., 2012b). Besides having minimal 5-HT reuptake effects, tapentadol is non-racemic and does not require metabolic activation (Bourland et al., 2010; Hartrick & Rozek, 2011; Raffa et al., 2012). The combination of the most relevant mechanisms of action (MOR activation and NA reuptake inhibition) into a single molecule decreases the risk of side effects and improves tolerability (Giorgi et al., 2012a; Hartrick & Rozek, 2011; Meske et al., 2014; Raffa et al., 2012; Steigerwald et al., 2013; Tzschentke et al., 2014).

In spite of their safety, several adverse events, including fatal intoxications, are associated with their use (Kemp et al., 2013; Larson et al., 2012; Pilgrim et al., 2010, 2011). In particular, the mechanisms underlying their acute and chronic toxicity, as well as their abuse and dependence liability, are far from being completely understood at the molecular level. Metabolomics, as an interdisciplinary area that comprehensively analyses the full set of metabolites in a living system at a given moment, arises as a promising approach to elucidate the biochemical changes deriving from the exposure to any xenobiotic, including opioids (Dinis-Oliveira, 2014, 2015, 2016a; Li et al., 2016; Mannelli et al., 2009; Ramirez et al., 2013).

Tramadol is mainly metabolized by the cytochrome P450 (CYP450) enzyme system through demethylation and oxidation, followed by conjugation (i.e. sulfonate and glucuronic acid) in the liver (Grond & Sablotzki, 2004; Leppert, 2011; Smith, 2009; Wu et al., 2002). Phase I reactions (mainly *O*- and *N*-demethylation) generate 14 metabolites (Leppert, 2011; Smith, 2009; Wu et al., 2001, 2002). Then, further metabolism by phase II reactions (mainly conjugation of *O*- and *N*-demethylated compounds), produces more 12 metabolites (Leppert, 2011; Smith, 2009; Wu et al., 2001, 2002).

Tapentadol is extensively metabolized via phase II pathways, particularly by conjugation with glucuronic acid and sulfonate (minor pathway) to inactive metabolites (Bourland et al., 2010; Hartrick & Rozek, 2011; Raffa et al., 2012; Tzschentke et al., 2007). Hepatic conjugation occurs via uridine diphosphate-glucuronosyltransferases (UGTs) 1A9 and 2B7 enzymes. In parallel, tapentadol also undergoes, to a lesser degree, phase I oxidative reactions via CYP2C9, CYP2C19 and CYP2D6 (DePriest et al., 2015; Terlinden et al., 2007).

With this review, we aim to provide an update on the knowledge available about tramadol and tapentadol metabolism. Biotransformation reactions, metabolizing enzymes and metabolites known to date are depicted and compared. A thorough comprehension of their metabolomics provides insights into their pharmacokinetic profile and represents a valuable tool for the identification of potential events leading to toxicological effects that remain to be clarified at the molecular level. Such approach also facilitates the interconnection between genetic variability in metabolizing enzymes and the corresponding metabolic effects, which, in turn, are directly addressed by metabolomics.

Metabolomics applied to opioids

Metabolomics, also known as metabolic profiling, or metabonomics, is one of the newest areas within the field of the "omics" technology (besides genomics, transcriptomics and proteomics), combining biochemistry, analytical chemistry, bioinformatics and statistics (Bouhifd et al., 2013; Dinis-Oliveira, 2014, 2015, 2016a; Li et al., 2016). It is mainly focused on the high-throughput quantitative and qualitative profiling of molecules with less than 2000 Da, including biomarkers, xenobiotics and their metabolites, thereby providing a global picture of the metabolome, that is, the complete set of metabolites – both by-products and end-products of

anabolic and catabolic pathways – within a cell at a given metabolic, physiological or pathological state (Dinis-Oliveira, 2014, 2015, 2016a; Li et al., 2016; Mannelli et al., 2009; Ramirez et al., 2013). Highly specific and dynamic, the metabolome fills the gap between the genotype and the phenotype, even if it is transient, and lays the ground for the emergence of "phenomics" as a potential new transdisciplinary area (Han et al., 2015; Mannelli et al., 2009). It elucidates the biological effects resulting not only from endogenous pathological conditions, but also from the exposure to exogenous compounds (Li et al., 2016; Zaitsu et al., 2016).

Accordingly, metabolomics has a wide scope of application, providing the opportunity to unveil how genetics, age, sex, body composition, health conditions and/or environmental factors affect metabolism, contributing to the establishment of a "metabolic fingerprint" and to predict the individual therapeutic response (Li et al., 2016; Zhang et al., 2015). Besides its undeniable applications in the clinical, pharmacological and environmental settings (Li et al., 2016; Zhang et al., 2015), metabolomics has a great potential in toxicology. In recent years, clinical and forensic toxicology has been using metabolomics approaches to study drugs of abuse, as a way to monitor their metabolism and the corresponding biochemical alterations, metabolic effects, acute and chronic toxicities, and the extent to which they occur (Dinis-Oliveira, 2016c; Li et al., 2016; Ramirez et al., 2013; Zaitsu et al., 2016). Indeed, metabolomics offers unprecedented means to characterize pathways and signatures of toxicity, encompassing patho-biochemistry, systems biology and molecular biology aspects (Bouhifd et al., 2013). The ultimate goal of metabolomics, within the scope of toxicology, is the interconnection between xenobiotic exposure and the corresponding biochemical alterations (Bouhifd et al., 2013). Since it deals with the outcome of a toxic insult, it overcomes genomics and transcriptomics inherent complexity and allows a more direct correlation between xenobiotics and their phenotypic effects, having been used for *in vitro*, *in vivo* and clinical studies (Bouhifd et al., 2013; Ramirez et al., 2013).

Substance use, abuse, misuse and addiction represents a prominent public health concern and implies significant costs for most societies. The neurobiological complexity of drug addiction makes it difficult to comprehensively follow the underlying molecular changes and emphasizes the need for approaches besides pharmacogenomics to fully understand them (Dinis-Oliveira, 2014; Han et al., 2015). In this sense, metabolomics is expected to underline differences between addicted and healthy subjects, guiding preventive approaches and tailoring drug therapy – a context in which the concepts of "pharmacometabolomics" and "toxicometabolomics" have emerged (Bouhifd et al., 2013; Dinis-Oliveira, 2014).

The use of opioid analgesics is widespread. Once used for acute and cancer pain only, opioid consumption has been rising since the 1990s and is currently aimed at chronic pain management, with possible accidental addiction and overdose (DePriest et al., 2015), as well as recreational use (Dinis-Oliveira, 2014). Paradoxically, although opioids are a cornerstone in chronic pain relief, their long-term effectiveness and safety are not fully established, as they have been associated with oxidative damage, biochemical and energy metabolism alterations (Mannelli et al., 2009), and identified as the cause of several fatal intoxications (DePriest et al., 2015), with tramadol and tapentadol themselves being involved in several of these adverse outcomes (Kemp et al., 2013; Larson et al., 2012; Pilgrim et al., 2010; 2011). Since the response to opioids is dependent on factors as diverse as drug potency, dosage, chemical properties, pharmacokinetics, as well on individual parameters such as diet, weight, health status, concomitant medications and genetic background, its analysis demands for a careful clinical monitoring (DePriest et al., 2015). In this context, pharmacogenomics and metabolomics arise as complementary approaches regarding patient assessment, therapeutic individualization and toxicological monitoring (Dinis-Oliveira, 2014; DePriest et al., 2015; Li et al., 2016).

Concerning opioid metabolomics, few studies have been performed so far (Hu et al., 2012; Mannelli et al., 2009; Meng et al., 2012; Zaitsu et al., 2014; Zheng et al., 2013). The challenge is to identify the metabolites that, considering their half-lives, are more suited as indicators of the therapeutic outcome and even of opioid use and abuse. For instance, 6-acetylmorphine, a heroin metabolite, is used to detect heroin abuse in practice due to its higher half-life, when compared with its parent compound. Endogenous compounds may also represent valuable exposure surrogates, in that they would unveil hidden effects. Indeed, tryptophan, 5-HT and 5-hydroxyindoleacetate have been used as long-term heroin addiction biomarkers; however, the limitations of the methodology used, gas chromatography-time of flight/mass spectrometry, have curtailed the analysis of high molecular weight compounds (Dinis-Oliveira, 2014). In other metabolomics studies, repeated morphine administration was shown to induce alterations in neurotransmitter levels and in amino acid and energy metabolisms in animal models (Hu et al., 2012; Zaitsu et al., 2014; Zheng et al., 2013). Moreover, metabolome profiles were found to be different among morphine,

methamphetamine and cocaine models, showing a strong correlation between metabolome changes and the drugs inducing them, as well as a reversal of those changes upon drug deprivation (Meng et al., 2012; Zheng et al., 2013). In turn, Manelli et al. (2009), in a study with subjects undergoing short-term methadone detoxification, showed alterations in oxidation-reduction activity and purine metabolism in plasma samples, through liquid chromatography electrochemical array detection.

In this context, and given tramadol and tapentadol increasing use and abuse, the present work aims to comprehensively review data about their metabolic reactions, metabolizing enzymes and respective metabolites, summarizing relevant data from the existing works on this subject and laying the foundations for subsequent studies on their metabolomics.

Methodology

An exhaustive literature search was conducted, using textual and structural queries for tramadol, tapentadol, respective metabolites and related metabolizing enzymes and transporters in U.S. National Library of Medicine (PubMed), with no time period restriction. The publications retrieved were additionally scanned as referrals to others. English references concerning human and non-human studies, as well as *in vitro* and *in vivo* approaches, were considered.

Pharmacokinetics of tramadol and tapentadol

Tramadol and tapentadol are two synthetic, centrally acting opioids that have been developed to improve analgesic efficiency and therapeutic safety, providing pain relief through opioid agonist action and by blocking monoamine reuptake. In this sense, their mechanism of action results from the combination of opioid and non-opioid mechanisms, for which they have enhanced analgesic effects and an improved tolerability profile (Giorgi, 2012).

Tramadol (1*RS*, 2*RS*)-2-[(dimethylamino)methyl]-1–(3-methoxyphenyl)-cyclo-hexanol consists of a racemate of two enantiomers, (−)-tramadol and (+)-tramadol, each one having a different activity profile (Grond & Sablot − ki, 2004; Raffa et al., 2012). Both enantiomers are required for an efficient analgesia (Giorgi et al., 2012a; Hui-Chen et al., 2004; KuKanich & Papich, 2004; Leppert, 2011; Raffa et al., 2012), provided by MOR agonist activity, as well as by 5-HT and NE reuptake inhibition, which leads to a neurotransmitter increase in the synaptic cleft and to the inhibition of pain perception (Giorgi et al., 2012a; Grond & Sablotzki, 2004; Hui-Chen et al., 2004; Leppert, 2011; Raffa, 2008; Raffa et al., 2012). (−)-Tramadol is 10 times more potent than (+)-tramadol in the inhibition of NE reuptake, while (+)-tramadol is four times more potent than (−)-tramadol in the inhibition of 5-HT reuptake (Duthie, 1998; Grond & Sablotzki, 2004; Leppert, 2011). Opioid agonist activity is predominantly provided by the (+)-enantiomer of its metabolite *O*-desmethyltramadol (M1) and, to a lesser extent, by the parental compound ((+)-tramadol) (Duthie, 1998; Gillen et al., 2000; Grond & Sablotzki, 2004; Lai et al., 1996; Leppert, 2011). In fact, (+)-M1 shows up to 200-fold higher affinity for μ-receptors than tramadol (Grond & Sablotzki, 2004; Raffa, 2008). In turn, (−)-M1 is responsible for NE reuptake inhibition (Duthie, 1998; Grond & Sablotzki, 2004; Leppert, 2011). Besides M1, the only known active tramadol metabolite is *O*,*N*-didesmethyltramadol (M5), which exhibits lower MOR affinity than (+)-M1 (30-fold less), but higher than (−)-M1 (2.4-fold) and (+)-tramadol (24-fold) (DePriest et al., 2015; Leppert, 2011; Mehvar et al., 2007). However, given the increased polarity of M5 with respect to M1, it may be expected to penetrate the blood–brain barrier to a lesser extent, which may anticipate a lower *in vivo* contribution to analgesia (Gillen et al., 2000).

Tapentadol (3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol) is a non-racemic molecule that does not require metabolic activation via the CYP450 system (Bourland et al., 2010; Hartrick & Rozek, 2011; Raffa et al., 2012). Tapentadol is responsible for moderate MOR agonist activity, high NE reuptake inhibition and minimal 5-HT effect, having no analgesic active metabolites (Hartrick & Rozek, 2011; Meske et al., 2014; Tzschentke et al., 2007). In contrast to tramadol, tapentadol effect on the inhibition of 5-HT reuptake is less pronounced, which reduces the risk of 5-HT syndrome (Giorgi et al., 2012a; Hartrick & Rozek, 2011; Meske et al., 2014; Raffa et al., 2012; Steigerwald et al., 2013; Tzschentke et al., 2014). Tapentadol was the first compound to present this mechanism of action in a single molecule, leading to the creation of a new class of centrally acting analgesics, the MOR Agonists, NE Reuptake Inhibitors (Giorgi et al., 2012a; Kress, 2010; Schroder et al., 2011; Tzschentke et al., 2007).

Administration, absorption and distribution

Tramadol is available in a variety of pharmaceutical formulations for oral, sublingual, intranasal, subcutaneous, rectal, intravenous and intramuscular administration. It is available in immediate-release (IR) formulations, and in ER formulations (Grond & Sablotzki, 2004). The recommended daily dose is in the 100–400 mg range, and

the maximum dose should not exceed 400 mg per day (Grond & Sablotzki, 2004). Normal-release forms may be given every 4–6 h and the ER forms should be given every 12–24 h (Grond & Sablotzki, 2004). Tramadol is subjected to 20–30% first-pass metabolism (Grond & Sablotzki, 2004; Scott & Perry, 2000). After oral, rectal and intramuscular administration, tramadol is almost completely absorbed, with bioavailability being estimated as 68–84% following a single dose, and plasma protein binding of approximately 20% (DePriest et al., 2015; Grond & Sablotzki, 2004; Mahdy et al., 2012a; Mahdy et al., 2012b), with a rapid distribution in the body (Grond & Sablotzki, 2004; Lee et al., 1993). The plasmatic peak is dependent on the route of administration: 1–2 h in oral administration, 3 h in rectal administration and 30–45 mins in intramuscular administration (Grond & Sablotzki, 2004). The analgesic effect is dose-dependent, and serum concentrations considered effective are in the 0.1–0.3 mg/L range (Grond & Sablotzki, 2004).

Tramadol is eliminated mainly in the urine (about 90% of the dose), but also in the feces (Grond & Sablotzki, 2004; Wu et al., 2001, 2002). Upon oral administration of tramadol, about 10–30% of the parental drug is excreted in its intact form, whilst the main metabolites found in urine are M1 (16%), M5 (15%) and their conjugates, as well as *N*-desmethyltramadol (M2) (2%) (Grond & Sablotzki, 2004; Paar et al., 1997; Wu et al., 2002). The elimination half-life of racemic tramadol is approximately 6 h, irrespectively of the route of administration, and about 8 h for M1 (Grond and Sablotzki, 2004; Paar et al., 1997). However, half-lives may be prolonged in people with decreased liver or kidney function (Grond & Sablotzki, 2004). In fact, several studies suggest that co-morbidities, as well as age, should be considered when analyzing tramadol pharmacokinetics. M1 volume of distribution was found to be higher in the elderly and in renal insufficiency patients, who also showed impaired opioid clearance and longer elimination half-life (Skinner-Robertson et al., 2015).

In turn, tapentadol is available in oral IR and ER formulations. The maximum dose should not exceed 600–700 mg per day (Singh et al., 2013; Tzschentke et al., 2007). Data concerning tapentadol disposition in humans is limited. Oral absorption is fast and complete, with maximum serum concentration being typically reached within less than 2 h (Wade & Spruill, 2009). Upon oral administration and under fasting conditions, tapentadol undergoes extensive first-pass metabolism, with an estimated bioavailability of 32% (Coulter et al., 2010; DePriest et al., 2015; Hartrick & Rodriguez Hernandez, 2012; Hartrick & Rozek, 2011). In serum samples, the concentration of conjugated tapentadol was found to be 24-fold higher than that of the parent drug (Terlinden et al., 2007). Only 20% of the drug is bound to plasma proteins (Gohler et al., 2013; Hartrick & Rozek, 2011; Singh et al., 2013; Tzschentke et al., 2007; WHO, 2012). The plasmatic peak occurs in 1.25 h and 3–6 h for the long acting formulations (Tzschentke et al., 2007).

Tapentadol follows a first-order elimination kinetics under a wide range of conditions (Hartrick & Rozek, 2011; Singh et al., 2013). The elimination half-life is ∼4 h for IR formulations and 5–6 h for ER formulations, which allows the achievement of steady-state concentrations at 25–30 h when it is orally administered every 6 h (Hartrick & Rozek, 2011; Wade & Spruill, 2009). It is eliminated mainly through urine (more than 95% within the first 24 h), with the remainder being eliminated through the feces (Singh et al., 2013). Full excretion is reported after ∼5 days (Terlinden et al., 2007; Wade & Spruill, 2009).

Metabolism

Tramadol is extensively metabolized by six metabolic pathways: *O*-demethylation, *N*-demethylation, cyclohexyl oxidation, oxidative *N*-demethylation, dehydration and conjugation (Grond & Sablotzki, 2004; Wu et al., 2002). *O*-demethylation, *N*-demethylation and cyclohexyl oxidation are the major routes, leading to 7 *O*-desmethyl/*N*-desmethyl and hydroxycyclohexyl metabolites (Wu et al., 2002).

Figure 1 summarizes tramadol metabolism, which encompasses at least 14 metabolites from phase I (M1 to M11 and M31 to M33) and 12 metabolites from phase II (7 glucuronides – M12 to 18 – and 5 sulfonates – M19 to 23), totaling 26 metabolites (DePriest et al., 2015; Grond & Sablotzki, 2004; Wu et al., 2002).

The main CYP450 enzymes involved in *O*- and *N*-demethylation are CYP2D6 and CYP3A4 (Grond & Sablotzki, 2004; Leppert, 2011; Wu et al., 2002). The CYP2D6 enzyme metabolizes tramadol to M1 (Leppert, 2011; Raffa, 2008; Smith, 2009; Subrahmanyam et al., 2001), the main active metabolite. In this context, the Organic Cation Transporter 1 (OCT1) has been implied in M1 re-uptake into hepatocytes (Tzvetkov et al., 2011). Similarly, the Multidrug Resistance-associated Protein 2 (MRP2) has been suggested as the responsible for the elimination of the glucuronidated M1 metabolites (Tzvetkov et al., 2011).

The parent compound, tramadol, undergoes both CYP3A4- and CYP2B6-mediated metabolism to M2 (Leppert, 2011; Subrahmanyam et al., 2001). Moreover, *in vitro* and *in vivo* evidence suggests that tramadol

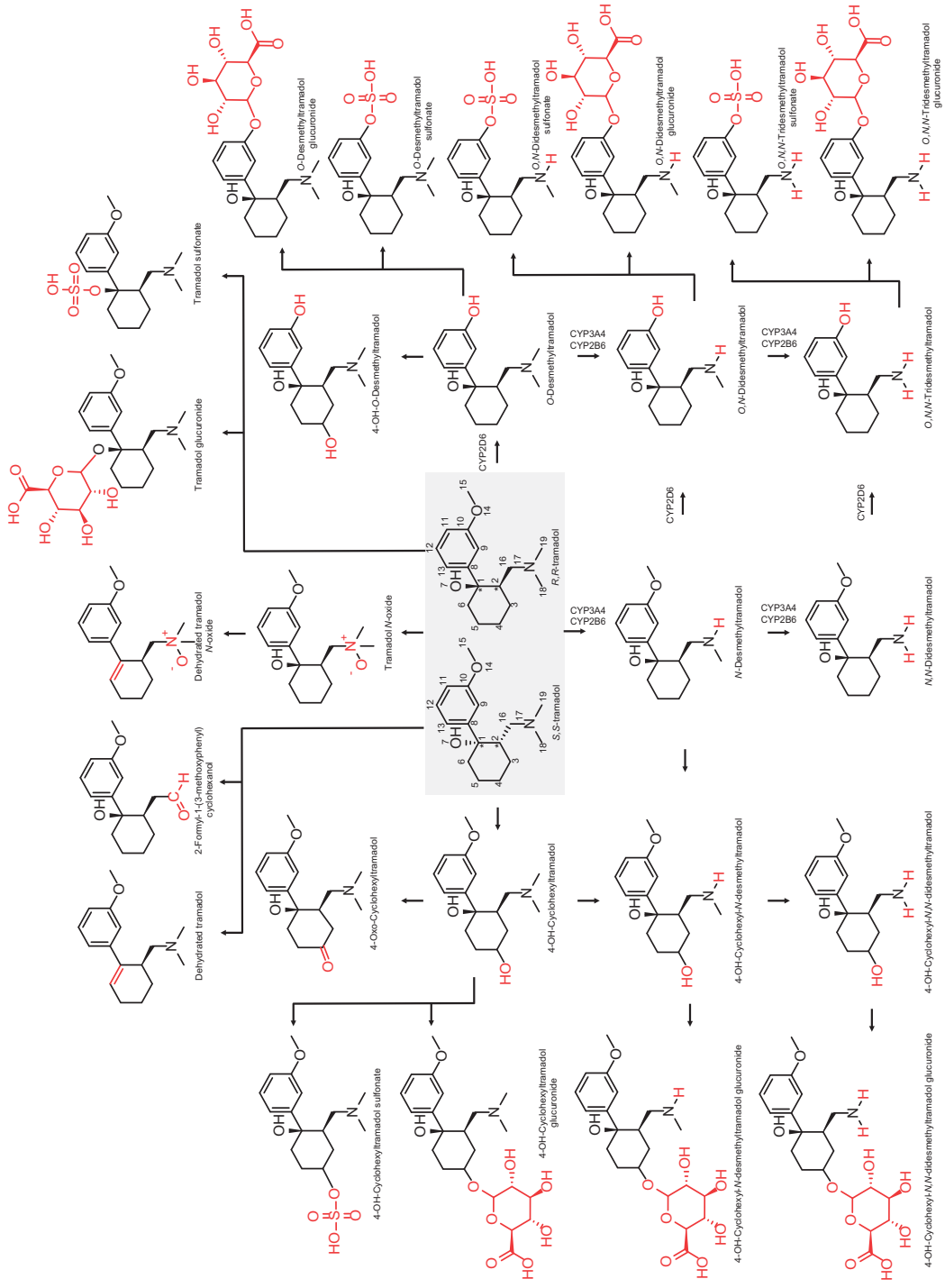

Figure 1. Tramadol metabolism. *O*-desmethyltramadol: M1; *N*-desmethyltramadol: M2; *N,N*-didesmethyltramadol: M3; *O,N,N*-tridesmethyltramadol: M4; *O,N*-didesmethyltramadol: M5; 4-OH-cyclohexyltramadol: M6; 4-OH-cyclohexyl-*N*-desmethyltramadol: M7; 4-OH-cyclohexyl-*N,N*-didesmethyltramadol: M8; 4-oxo-cyclohexyltramadol: M9; dehydrated tramadol: M10; 2-formyl-1–(3-methoxyphenyl)cyclohexanol: M11; tramadol glucuronide: M12; M1 glucuronide: M13; M4 glucuronide: M14; M5 glucuronide: M15; M6 glucuronide: M16; M7 glucuronide: M17; M8 glucuronide: M18; tramadol sulfonate: M19; M1 sulfonate: M20; M4 sulfonate: M21; M5 sulfonate: M22; M6 sulfonate: M23; tramadol *N*-oxide: M31; 4-OH-M1: M32; dehydrated M31: M33.

metabolism is stereoselective: while (−)-tramadol is mostly *O*-demethylated, (+)-tramadol is predominantly *N*-demethylated (Campanero et al., 2004; DePriest et al., 2015; Grond & Sablotzki, 2004; Paar et al., 1992; Unruh et al., 1995).

M1 and M2 are metabolized to M5 through CYP3A4/CYP2B6-mediated *N*-demethylation and CYP2D6-mediated *O*-demethylation, respectively (Leppert, 2011; Paar et al., 1997; Subrahmanyam et al., 2001). M2 can be converted into *N*,*N*-didesmethyltramadol (M3) by CYP2B6 and CYP3A4 (DePriest et al., 2015; Leppert, 2011; Subrahmanyam et al., 2001). Both M5 and M3 are subsequently metabolized to *O*,*N*,*N*-tridesmethyltramadol (M4), by CYP3A4/CYP2B6 and CYP2D6, respectively (DePriest et al., 2015; Leppert, 2011; Subrahmanyam et al., 2001). In accordance to the abovementioned, the formation of *O*-demethylated metabolites is CYP2D6-mediated, whereas *N*-demethylated metabolite formation is catalyzed by CYP3A4 and CYP2B6. The M10 metabolite is dehydrated tramadol and the M11 metabolite is an oxidative *N*-dealkylated product of tramadol (Wu et al., 2002).

The M6–9 metabolites are produced through cyclohexyl oxidation, being further conjugated with glucuronic acid and sulfonate prior to urinary excretion (Wu et al., 2002), resulting in M6 glucuronide (M16), M7 glucuronide (M17) and M8 glucuronide (M18) (Wu et al., 2002). Other conjugation reactions with glucuronic acid give rise to tramadol glucuronide (M12), M1 glucuronide (M13), M4 glucuronide (M14) and M5 glucuronide (M15) (Wu et al., 2002).

UGT isoform 2B7 is the main enzyme responsible for the conjugation reactions of both M1 enantiomers, with slight preference for the 1*S*,2*S*-enantiomer (Gong et al., 2014; Lehtonen et al., 2010; Tzvetkov et al., 2011). Additionally, UGT1A8 has also been implied in M1 and M5 conjugation reactions (Gong et al., 2014; Lassen et al., 2015; Lehtonen et al., 2010). In accordance, Lehtonen *et al.* (Lehtonen et al., 2010), by screening the glucuronidation activity in a panel of human UGTs, showed that UGT2B7 and UGT1A8 are the most active isoforms in M1 conjugation reactions. Furthermore, UGT1A7 through UGT1A10 showed to be strictly stereoselective for the 1*R*,2*R*-enantiomer. In turn, UGT2B15 led to similar, but not strict, enantioselectivity (Lehtonen et al., 2010).

Conjugation reactions with sulfonates, catalyzed by sulfotransferases (SULTs), result in tramadol sulfonate (M19), M1 sulfonate (M20), M4 sulfonate (M21), M5 sulfonate (M22) and M6 sulfonate (M23) (Wu et al., 2002). In turn, M31 is tramadol *N*-oxide, while M32 is hydroxyl-M1 and M33 is M31 dehydrate (Wu et al., 2002).

The M1-M5 metabolites are major compounds detected in urine upon oral administration (Grond & Sablotzki, 2004; Wu et al., 2002), whereas M31 to M33 were only detected in hepatic microsomes (Wu et al., 2002). Therefore, only 23 metabolites were profiled in urine, following oral administration of 100 mg tramadol (Grond & Sablotzki, 2004; Wu et al., 2002). 7 glucuronides (M12 to 18) and 5 sulfonates (M19 to 23) are produced through phase II conjugation reactions (Wu et al., 2002). Not all 26 metabolites identified in humans have been found in rats or dogs: M12, M19 to M23 and M33 have not been detected (Grond & Sablotzki, 2004; Wu et al., 2001, 2002). On the other hand, M25 to M29 metabolites (phase I metabolites) and M24 and M30 (phase II metabolites) have not been identified in humans, but have been identified in dogs or rats (Grond & Sablotzki, 2004; Wu et al., 2001).

Tapentadol is primarily metabolized in the liver via phase II conjugation, through the UGT enzymes UGT1A9 and UGT2B7 (70%), with glucuronidation being the predominant pathway and sulfonation of the phenolic hydroxyl group playing a minor role (Bourland et al., 2010; Coulter et al., 2010; DePriest et al., 2015; Hartrick & Rozek, 2011; Kemp et al., 2013; Kneip et al., 2008; Singh et al., 2013; WHO, 2012). Indeed, tapentadol glucuronide totals 55% of the metabolites, while tapentadol sulfonate represents 15% (Bourland et al., 2010; Terlinden et al., 2010). Tapentadol is also subject to phase I oxidative processes to a lesser extent (15%), leading to *N*-desmethyltapentadol (nortapentadol, 13%) via CYP2C9 and CYP2C19, and to hydroxytapentadol (2%) via CYP2D6 hydroxylation of the aromatic ring (Bourland et al., 2010; Coulter et al., 2010; DePriest et al., 2015; Singh et al., 2013; Terlinden et al., 2010). These metabolites are subsequently conjugated (DePriest et al., 2015; Singh et al., 2013). The remaining 3% is excreted as unchanged tapentadol (Bourland et al., 2010). Figure 2 provides a comprehensive summary of tapentadol biotransformation, with all known metabolizing enzymes and respective metabolites.

Unlike tramadol, all tapentadol metabolites are considered to have no pharmacological activity (DePriest et al., 2015; WHO, 2012), as *in vitro* studies have shown that they either do not bind or have low binding affinity for the MOR (Singh et al., 2013; Terlinden et al., 2007).

The potential for drug-drug interactions involving tapentadol is low. It was found not to induce CYP1A2, CYP2C9, CYP3A4 and other numerous CYP enzymes, except for CYP2D6, which is only minimally induced (Hartrick & Rozek, 2011; Kneip et al., 2008; Pergolizzi et al., 2012; Wade & Spruill, 2009). Since protein binding is low, displacement reactions with co-administered drugs are also unlikely to occur (Hartrick & Rozek, 2011;

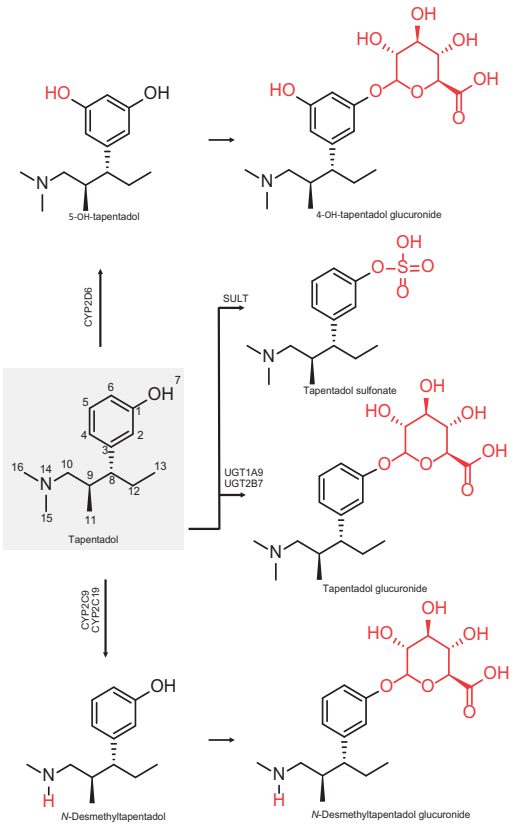

Figure 2. Tapentadol metabolism. *N*-desmethyltapentadol is also known as nortapentadol.

Kneip et al., 2008; Pergolizzi et al., 2012). Unlike genetic polymorphisms in the *CYP2D6* gene in the case of tramadol (addressed in the "Pharmacogenetics and drug–drug interactions" section), those in UGT enzyme-encoding genes do not compromise their activity to the point that they lead to a poor metabolizer (PM) phenotype (Terlinden et al., 2007). Moreover, UGTs are high-capacity enzymes and have no known potent inhibitors, another reason underlying a low drug-drug interaction potential (Giorgi et al., 2012a; Terlinden et al., 2007). Altogether, these factors contribute to a more straightforward pharmacokinetics and explain why tapentadol is claimed to be a significant improvement over tramadol.

Pharmacogenetics and drug-drug interactions

Since tramadol requires metabolic activation, catalyzed by the CYP450, genetic polymorphisms may be responsible for variations in its pharmacokinetics, and hence in its efficacy and potential toxicity (Garcia-Quetglas et al., 2007; Paar et al., 1997). Given CYP2D6 role on tramadol conversion into M1, polymorphic variability in this gene is particularly relevant for its metabolism and analgesic efficiency (Giorgi, 2012; Gong et al., 2014; Grond & Sablotzki, 2004; Leppert, 2011; Lotsch et al., 2004; Paar et al., 1997; Stamer & Stuber, 2007; Zhou, 2009a,b). Currently, at least 74 human CYP2D6 variant alleles (*2 to *75) are known (Gong et al., 2014; Lassen et al., 2015). CYP2D6 ultra-rapid metabolizers (UMs) are associated with cases of toxicity, whereas the analgesic efficacy of tramadol is less pronounced in PMs (Cavallari et al., 2011; DePriest et al., 2015; Giorgi, 2012; Gong et al., 2014; Paar et al., 1997; Stamer & Stuber, 2007), whose frequency in Caucasian populations is as high as 7–10% (Lassen et al., 2015). CYP2D6 PM subjects achieve higher plasma tramadol levels and lower M1 and M2 concentrations than extensive metabolizers (EMs), who experience more adverse events due to epinephrine elevation (Garcia-Quetglas et al., 2007). Accordingly, PM subjects present increased tramadol and M1 half-lives and time to peak plasma concentrations (Lassen et al., 2015). CYP2D6 PMs recurrently show higher tramadol/M1 ratios than EMs, clearly reflecting lower tramadol *O*-demethylation (Pedersen et al., 2006). Accordingly, CYP2D6 polymorphisms leading to a PM phenotype were associated with less analgesic efficacy (Stamer et al., 2007). In parallel, a decrease in tramadol *O*-demethylation leads to an increase in its *N*-demethylation; consequently, the tramadol/M2 ratio decreases (Lassen et al., 2015). In this context, CYP2D6 genetic variants have been screened in *postmortem* samples and positively correlated with altered tramadol/metabolite ratios (Levo et al., 2003). Inter-individual genomic characterization is therefore important in order to optimize therapeutic strategies and prevent adverse effects.

Besides being polymorphic, CYP2D6 can be inhibited by different drugs, for which the concomitant therapy may affect the analgesic effect of tramadol due to drug-drug interactions (Grond & Sablotzki, 2004; Paar et al., 1997; Raffa et al., 2012). Thus, it is possible that standard doses of tramadol do not always yield satisfactory analgesia (Grond & Sablotzki, 2004; Paar et al., 1997; Raffa et al., 2012). In studies with CYP2D6 EMs, CYP2D6 inhibition with paroxetine led to a decrease in some, but not all, opioid effects (Laugesen et al., 2005; Nielsen et al., 2010). In contrast, escitalopram, with low CYP2D6 inhibitory effect, was found not to cause changes in tramadol-mediated analgesia (Noehr-Jensen et al., 2009). Concerning CYP2D6 inhibitors, their concurrent use with tramadol has been reported to decrease some of its analgesic effects, although with some variability (Overholser & Foster, 2011).

These observations on the CYP2D6 genotype and drug-drug interactions have already been translated

into clinical practice in some countries. For instance, the U.S. FDA emphasizes that tramadol levels are approximately 20% higher in PM subjects than in EMs, while M1 levels are 40% lower (Gong et al., 2014). It also draws attention to the fact that the pharmacological impact of tramadol co-administration with CYP2D6 inhibitors, such as fluoxetine and amitriptyline, is not fully known, as far as safety and efficacy are concerned (Gong et al., 2014). Accordingly, FDA-approved drug labels include remarks on CYP2D6 genotypes and on interactions with CYP2D6 or CYP3A4 inhibitors such as fluoxetine and its metabolite norfluoxetine, as well as with paroxetine and quinidine, specifically alerting patients to the increased risk of seizures and 5-HT syndrome when selective 5-HT reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs) are taken together with tramadol (Gong et al., 2014). In turn, the Royal Dutch Pharmacists Association – Pharmacogenetics Working Group (DPWG) has also established therapeutic dose recommendations for tramadol based on CYP2D6 genotypes (Gong et al., 2014). For CYP2D6 PM and intermediate metabolizer (IM) genotypes, they recommend the choice of an alternative drug (excluding oxycodone or codeine, which also involve CYP2D6 in their metabolism) and/or being alert to symptoms of insufficient analgesia. For UM genotypes, a 30%-decrease in tramadol dose and an increased attention to adverse drug reactions are recommended, as well as the preference for an alternative drug, such as paracetamol, nonsteroidal anti-inflammatory drugs or morphine (Gong et al., 2014).

It should be noted that, besides inhibiting CYP2D6, the combination of tramadol with SSRIs such as fluoxetine, paroxetine and, to a lesser degree, sertraline, may cause 5-HT syndrome because SSRIs increase 5-HT levels in the CNS (Gillman, 2005; Leppert, 2011). Although tramadol alone is very unlikely to cause 5-HT syndrome, its combination with serotonergic drugs acting by different mechanisms is capable of raising intra-synaptic 5-HT to life-threatening levels. The most frequent combination precipitating life-threatening 5-HT toxicity is that of a MAOI drug, such as linezolid and moclobemide, with any SSRI (Gillman, 2005; Leppert, 2011). For this reason, they should not be co-administered with tramadol (Gillman, 2005; Leppert, 2011).

Although data regarding CYP3A inhibitors is sparse, their concomitant use should be avoided, as they are expected to increase exposure to tramadol (Overholser & Foster, 2011). Accordingly, cimetidine, a combined CYP2D6 and CYP3A inhibitor, was shown to moderately increase exposure to tramadol, although with no clinical relevance (Grond & Sablotzki, 2004). Conversely, CYP3A inducers may be expected to reduce tramadol exposure and to limit tramadol-associated analgesia, for which their concomitant use should be avoided (Overholser & Foster, 2011). In accordance, studies with carbamazepine showed that co-administration with tramadol reduces exposure to this opioid (Grond & Sablotzki, 2004). Since tramadol use is associated with severe adverse effects such as 5-HT syndrome and seizures, inhibition of its metabolism either via CYP2D6 or CYP3A may raise safety concerns (Overholser & Foster, 2011).

CYP2B6, in turn, has 53 allelic variants, of which *5 and *6 are the most frequent (Lassen et al., 2015). Most studies on the clinical impact of CYP2B6 genetic variability focus on methadone. Inhibition studies demonstrated its contribution for methadone metabolism and disposition, since the use of inhibitors was shown to reduce methadone *N*-demethylation and clearance and to alter plasma methadone concentrations (Kharasch & Stubbert, 2013). Methadone metabolism was found to be slower in CYP2B6*6 homozygous carriers (Dennis et al., 2014; Kharasch et al., 2015), who have lower dose requirements (Levran et al., 2013). Accordingly, *in vitro* studies have shown that the *6 polymorphism leads to a decreased CYP2B6 catalytic activity, with lower methadone *N*-demethylation and intrinsic clearance levels, when compared with wild-type CYP2B6*1 (Gadel et al., 2013, 2015). In contrast, CYP2B6*4 carriers experience higher methadone metabolism and clearance (Kharasch et al., 2015).

UGT2B7 is not very polymorphic and, to date, no polymorphisms have been reported as clinically relevant concerning tramadol metabolism (Lassen et al., 2015). Also, studies have failed to show any correlation between UGT2B7 inhibition or induction and tramadol or M1 concentrations (Lassen et al., 2015).

As far as M1 transporters are concerned, it is worth mentioning that OCT1 polymorphisms, such as amino acid substitutions Arg61Cys, Cys88Arg, Gly401Ser, Gly465Arg and a deletion of Met420, are responsible for reduced or absent OCT1 activity in approximately 10% of the Caucasian population (Tzvetkov et al., 2011). In this context, Tzvetkov and coworkers have shown that, although tramadol plasma concentrations are independent of the OCT1 genotype, M1 re-uptake was significantly increased by OCT1 overexpression, an effect that was reversed when OCT1 inhibitors or loss-of-function variants were tested (Tzvetkov et al., 2011).

Concerning tapentadol, there are, to our knowledge, no studies correlating different metabolizer phenotypes with inter-individual variability in the response to this opioid. Nevertheless, there are some studies with opioids whose metabolism involves tapentadol-metabolizing enzymes, allowing a similar pharmacokinetic impact to be expected for tapentadol. To date, 28

CYP2C19 variants have been described. CYP2C19*2 is the primary allele responsible for a PM phenotype among Asians and Caucasians; CYP2C19*2 through *8 are loss-of-function alleles (Cavallari et al., 2011). In turn, CYP2C9*2 and *3 polymorphisms are common in Caucasians and lead to a decreased enzyme activity (Cavallari et al., 2011; Wang et al., 2013). CYP2C19 gene dose has been shown to be a sensitive indicator of the *R*-methadone/methadone ratio (Wang et al., 2013). While 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP), a main methadone metabolite, was found to be increased in CYP2C19*2 allele carriers, when compared with the wild-type carriers (Carlquist et al., 2015), in other studies, CYP2C9 and CYP2C19 genotypes were not found to influence methadone plasma levels (Crettol et al., 2006, 2005; Dinis-Oliveira, 2016b; Mouly et al., 2015). Similarly, there was no difference in the elimination of tilidine, a synthetic opioid, in CYP2C19 PM and UM subjects (Grun et al., 2012).

Given UGT2B7 increased relevance in tapentadol metabolism, pharmacogenetic variations on this enzyme may be expected to affect its metabolism. Indeed, increased UGT2B7 activity has been associated with a decrease in active opioid exposure (Eissing et al., 2012). UGT2B7 –900G > A significantly changes the morphine-3-glucuronide/morphine ratio, with –900G/G carriers presenting higher morphine concentrations and lower morphine-3-glucuronide levels than the -900A/A ones (Matic et al., 2014). UGT2B7 –842G > A polymorphism has been significantly associated with nausea in a convenience sample receiving hydromorphone therapy (Xia et al., 2015), as well as with higher buprenorphine glucuronidation in human liver microsomes (Rouguieg et al., 2010). The presence of the –842G allele has been correlated with lower morphine and codeine glucuronidation in other studies (Darbari et al., 2008; Joly et al., 2012). Other works failed to find any correlation between the UGT2B7 genotype and methadone plasma levels (Crettol et al., 2006). In turn, the UGT2B7*2 polymorphism is frequent in Caucasians (about 30%) and leads to contradictory results regarding enzyme activity. It has been associated with higher morphine-6-glucuronide levels in patients receiving morphine (Madadi et al., 2013), while lower morphine-3-glucuronide levels have been found in UGT2B7*2 carriers (Mouly et al., 2015; Oliveira et al., 2014). On the other hand, another study has shown the UGT2B7*2 polymorphism not to be determinant of morphine or codeine glucuronidation in human liver microsomes (Court et al., 2003).

In vitro studies have shown that tapentadol does not induce nor inhibit the seven major CYP isoforms involved in drug metabolism (CYP2D6, CYP3A4, CYP1A2, CYP2A6, CYP2C9, CYP2C19, and CYP2E1). Although a slight degree of inhibition was found for CYP2D6, the inhibition constant (K_i) values found for both competitive and noncompetitive inhibition were much higher than the expectable tapentadol plasma concentrations, for which CYP2D6 inhibition by tapentadol is very unlikely to have clinical relevance (Afilalo et al., 2010; Kneip et al., 2008; Sanchez Del Aguila et al., 2015; Wade & Spruill, 2009). Regarding interactions with co-administered drugs, a study by Smit et al. (2010) showed that no clinically relevant interactions occur between tapentadol and paracetamol, naproxen and acetylsalicylic acid. It should be noted that these commonly used non-opioid analgesics are also metabolized via UGT pathways (Afilalo et al., 2010). Similarly, no changes in pharmacokinetic properties have been detected upon tapentadol and metoclopramide, probenecid and omeprazole co-administration (Sanchez Del Aguila et al., 2015).

The impact of pharmacogenetic variability on SULTs is considerably less studied (Gamage et al., 2006). Additionally, heterogeneity in SULT families among different animal species has made extrapolation of the results more difficult (Gamage et al., 2006; Jancova et al., 2010). In humans, four SULT families – SULT1, SULT2, SULT4 and SULT6 –, with a widespread tissue distribution, have been identified and found to encompass at least thirteen members (Gamage et al., 2006; Jancova et al., 2010; Nimmagadda et al., 2006). Human SULT-encoding cDNA cloning has helped to characterize isoform substrate specificity (Burchell & Coughtrie, 1997; Nimmagadda et al., 2006). Cytosolic SULTs catalyze the sulfonation of xenobiotics and small endobiotics such as steroids, bile acids and neurotransmitters (Jancova et al., 2010). SULTs share most of their substrates with UGTs (Gamage et al., 2006). Nevertheless, while the former are considered a high-affinity, low-capacity system, the latter represent a low-affinity, high-capacity one. For this reason, SULTs may have a more prominent role in the metabolism of low-level chemicals, such as those from environmental or food provenience (Gamage et al., 2006). Most interindividual variability in SULT activity derives from polymorphisms in the coding regions of *SULT* genes (Gamage et al., 2006). Although single nucleotide polymorphisms (SNPs) in SULT-encoding genes are uncommon in the population, some are more frequent, particularly those affecting SULT isoform 1A1 – the one with highest liver expression –, leading to missense mutations (Daniels & Kadlubar, 2013; Jancova et al., 2010). Alleles *2 and *3 have lower sulfonation capability than the wild-type *1, for which their carriers are more susceptible to SULT1A inhibition; their frequency varies according to the ethnicity (Eagle, 2012; Jancova et al., 2010).

SULT1A1 polymorphisms have also been correlated with cancer risk and altered sensitivity to some antitumor agents (Daniels & Kadlubar, 2013; Jancova et al., 2010). Genetic polymorphisms are also known for SULT1A2, 1A3, 1C2, 2A1, 2A3 and 2B1 (Jancova et al., 2010; Nimmagadda et al., 2006). Also, naturally-occurring phenols and polyphenols have been found to interact with these enzymes (Eagle, 2012). Importantly, both SULT1A1 and SULT1A3 are widely distributed within the developing human fetal brain, with neurotransmitter systems such as GABA, cholinergic, glutaminergic and δ-opioid receptors being modulated by sulfonated neuro-steroids. Thus, genetic polymorphisms involving these genes may be relevant for opioid effects (Gamage et al., 2006).

Concluding remarks

Tramadol and tapentadol are widely prescribed synthetic opioid analgesics. Both are metabolized in the liver and display a dual mechanism of action as MOR agonists and NE reuptake inhibitors, making them important therapeutic options in the treatment of a broad range of acute and chronic pain situations. Unlike tapentadol, which has minimal serotonergic activity, tramadol presents some liability to cause 5-HT syndrome, particularly when administered together with SSRIs (Gillman, 2005; Leppert, 2011).

Tramadol is a prodrug that mainly undergoes CYP450-mediated *O*- and *N*-demethylation (Grond & Sablotzki, 2004; Raffa et al., 2012). In turn, tapentadol, a more recent drug, is a centrally-acting, synthetic opioid with a distinctive mechanism of action as a moderate MOR agonist and strong NE reuptake inhibitor, with negligible 5-HT reuptake inhibitory activity (Coulter et al., 2010; DePriest et al., 2015; Dousa et al., 2013; Giorgi, 2012; Pergolizzi et al., 2012). Both tapentadol mechanisms of action interact synergistically to increase its effectiveness in a broad spectrum of acute, chronic, nociceptive and neuropathic pain conditions (Giorgi et al., 2012b; Pergolizzi et al., 2012; Schroder et al., 2011; Tzschentke et al., 2007, 2014). In fact, while its activity as a MOR agonist contributes mostly to analgesia in acute situations, monoamine reuptake inhibition is more effective in the treatment of chronic pain (Tzschentke et al., 2007). Besides clinical versatility, this mechanistic synergy confers tapentadol an "opioid-sparing" effect since, when compared with other opioids, it requires lower doses to provide equivalent analgesia (Giorgi, 2012; Giorgi et al., 2012b; Schroder et al., 2011; Tzschentke et al., 2014; Vadivelu et al., 2010). This, in turn, also implies a lower incidence and intensity of side effects (Giorgi, 2012; Giorgi et al., 2012b). Mechanistically, tapentadol is also advantageous in that its analgesic properties reside in a single enantiomer and are independent of metabolic activation (Giorgi, 2012; Giorgi et al., 2012b; Power, 2011; Schroder et al., 2011). Also, the relative contributions of the MOR activation and NE reuptake inhibition are invariable throughout its biotransformation (Giorgi, 2012; Giorgi et al., 2012b). Additionally, tapentadol undergoes mainly glucuronidation reactions. While CYP450 enzymes are remarkably polymorphic, with a significant potential impact on tramadol metabolism, safety and efficacy, UGTs reportedly have no genetic variability leading to a PM phenotype. In fact, currently only the CYP2D6 phenotype seems to be clinically relevant for the treatment with tramadol, with PM subjects experiencing low analgesia and UMs undergoing severe adverse effects (Lassen et al., 2015). Thus, comparatively with tramadol, tapentadol reduces inter-individual variability due to genetic polymorphisms and presents a lower likelihood of drug–drug interactions. However, the "overall advantage" of tapentadol over tramadol is not so clear, since several studies emphasize the pros of using tramadol. In fact, tramadol provides a lower risk of respiratory depression, drowsiness and lethargy, as well as a lower abuse potential (Tsutaoka et al., 2015). Furthermore, it is inexpensive, with a wide diversity of generic formulations being available. Also, a study by Mercier and coworkers showed that both opioids present similar benefit-risk profiles in patients with nonmalignant pain, with tramadol providing a slightly higher efficacy in pain reduction (Mercier et al., 2014). Importantly, tapentadol has a higher potential to induce physical/psychological dependence (similar to schedule II opioids) (Guay, 2009). *In vitro* studies also show that tapentadol exerts more pronounced toxic effects than tramadol, when in equimolar concentrations (Faria et al., 2016). Additionally, since the availability of literature concerning tapentadol is considerably lower, information in terms of efficacy/tolerability, clinical data in specific patient groups, and details about drug-drug interaction potential regarding glucuronidation and quantitation of the risk of 5-HT syndrome is also missing.

Another issue in which literature is limited concerns the veterinary use of tapentadol. While tramadol is a licensed drug in veterinary clinical practice, tapentadol is not, for which information on its use and safety in animals is more scarce (Giorgi, 2012). Regarding tramadol, its clinical efficacy in animals may be different from the observed in humans since some species metabolize it to inactive metabolites (Giorgi, 2012). Thus, the comparability between animal and human tramadol efficacy is still controversial (Giorgi, 2012). In turn, tapentadol

bioavailability may be higher in cats and in other animal species with lower glucuronic acid levels (Giorgi, 2012; Lee et al., 2013). Nevertheless, although further studies are needed in order to ascertain tapentadol clinical utility in veterinary settings, the studies done so far have led to encouraging pharmacokinetic and pharmacodynamic parameters, suggesting that it may become an attractive alternative for animal antinociception (Giorgi, 2012; Giorgi et al., 2012a; Kogel et al., 2014).

Given tramadol and tapentadol increasing consumption, and since their use is associated with adverse effects as severe as 5-HT syndrome (in the case of tramadol), seizures and respiratory depression, detailed knowledge about their metabolism and influencing factors is invaluable. With this review, we have emphasized the potential offered by the combination of areas as metabolomics and pharmacogenetics in this context.

Future perspectives

Data on molecular alterations resulting from acute and chronic exposure to opioids, including tramadol and tapentadol, is lacking. In this context, a detailed comprehension of their metabolism allows the anticipation of their metabolome, thereby facilitating the detection of alterations that may underlie pathophysiological and toxic effects. In this work, we reviewed all known tramadol and tapentadol metabolites. Such knowledge, used from a metabolomics perspective, is expected to provide a global picture of their metabolism, also shedding light into their mechanisms of toxicity and variability of response.

It is increasingly clear that, in order to fully understand living organisms in health and disease, we must envisage them as complex networks. In this sense, only areas as integrative and comprehensive as metabolomics can entirely elucidate the biochemical changes accompanying a given pathophysiological state. Cumulative knowledge on the nature of metabolic, biochemical and physiological changes associated with drug exposure and the development of chronic toxicity and drug dependence is far from the ideal (Dinis-Oliveira, 2016c; Li et al., 2016; Ramirez et al., 2013; Zaitsu et al., 2016). Metabolomics offers the potential to fill this gap, by allowing the identification of new toxicity biomarkers, new metabolites and alternative/complementary metabolic pathways, new pharmacodynamic targets, as well as the organization of metabolites into time- and dose-dependent frameworks (Bouhifd et al., 2013; Ramirez et al., 2013). It stands out from other "omics" approaches for the very direct mechanistic insights it provides, not only clarifying drug mechanisms of action, but also mechanisms of toxicity, thus allowing the cause of death to be more accurately determined or predicted. Indeed, the ability to build models for toxicity prediction is one of the most prominent possibilities offered by metabolomics (Dinis-Oliveira, 2014; Kell & Goodacre, 2014; Zaitsu et al., 2016). To date, metabolomics studies have shown that opioid abuse leads to oxidative stress and changes in energy metabolism (Hu et al., 2012; Mannelli et al., 2009; Meng et al., 2012; Zaitsu et al., 2014; Zheng et al., 2013), which is a clear indicator of the potential of this kind of studies for the elucidation of opioid toxicology. To our knowledge, no metabolomics studies specifically addressing tramadol and tapentadol effects have been published to date, for which this emerges as a relevant research topic.

Moreover, it should be noted that current metabolomics approaches rely on technologies that do not often present high sensitivity in the detection of heavier molecules ($>$ 800 Da), in a molecular weight range that includes phospholipids, polysaccharides, neurotransmitters and hormones, biomolecules that are relevant when toxicological effects are concerned (Bouhifd et al., 2013; Dinis-Oliveira, 2014; Kell & Goodacre, 2014; Li et al., 2016; McClay et al., 2013; Zaitsu et al., 2016). Therefore, additional studies are needed in order to ascertain the potential contribution of techniques such as liquid chromatography-mass spectrometry, capillary electrophoresis-mass spectrometry and nuclear magnetic resonance to this field. Besides, since each "omics" area only captures a static snapshot of the dynamic complexity of a biological system at a given moment, it is difficult to link the causes and effects of drug exposure exclusively through metabolomics. Hence, a "transomics" approach will certainly be the wisest approach in toxicological studies in the short run.

Our review has also emphasized the need to clarify the role of genetic polymorphisms in metabolizing enzymes other than those in CYP2D6 in order to fully understand inter-individual variability in the response to both tramadol and tapentadol. In particular, the impact of CYP2B6, CYP2C9, CYP2C19, UGT2B7 and SULT polymorphic variations on tramadol and tapentadol metabolism should be further studied.

Combined metabolomic and pharmacogenetic approaches will provide further comparative insights into their pharmacokinetic profiles, helping to clarify if, from a toxicological perspective, tapentadol is, as claimed, an actual significant upgrade over tramadol.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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PART I

2. GENERAL AND SPECIFIC OBJECTIVES OF THE DISSERTATION

The general aim of the studies reported in this thesis was to evaluate the toxicological effects deriving from the acute and repeated exposure to tramadol and tapentadol, from a comparative perspective, using *in vivo* models.

Although both opioids are known for their clinical efficacy and safety, their widespread prescription and relatively easy access propitiate their misuse and are associated with several adverse reactions, most of which are poorly documented at the molecular, biochemical, metabolic and histological levels. Tapentadol, claimed to be an improved version of tramadol, has a considerably shorter market history. For this reason, data available on its safety are scarcer, thus hindering a true comparison with other opioids, including tramadol.

Therefore, the original research performed in the scope of the present thesis aimed to provide further knowledge in the field of opioid toxicology, by focusing on the evaluation of tramadol and tapentadol toxicity. To this purpose, it enables a comparative perspective on their effects on metabolizing and target organs, upon single and repeated administration, dissecting them from a molecular, metabolic and histopathological point of view.

The comparative analysis of the results is expected to contribute to clarify adverse reactions and intoxications, as well as to promote well-reasoned and judicious prescription and use of both drugs, thus having forensic and clinical applications.

Such overall objectives were sustained by the specific goals met by the original research works, which are presented in the form of the three chapters of this thesis, as outlined below:

CHAPTER I

In order to evaluate the putative toxicological effects of an acute exposure to tramadol and tapentadol on metabolizing organs, single-exposure assays were performed with male Wistar rats. Serum, urine, liver and kidney samples were collected upon a 24-hour exposure to clinically relevant doses, and assayed for the following aspects:

1. A comparative analysis of lipid and protein oxidative status in liver and kidney homogenates;
2. The assessment of metabolic alterations, from serum parameters;
3. The analysis of liver and kidney function tests, from serum and urinary parameters;
4. The identification of histopathological alterations in liver and kidney tissue sections, from histological staining procedures.

CHAPTER II

To broaden the view provided by CHAPTER I, tramadol and tapentadol hepatorenal effects were complementarily explored through similar *in vivo* assays, using the same therapeutic doses, but following a 14 day-period of single daily administration. Again, analyses were performed in serum, urine, liver and kidney samples. The methodologies undertaken aimed at the evaluation of potential:

1. Oxidative stress and antioxidant capacity alterations taking place in the liver and kidney, from tissue biomarkers and gene expression assays;
2. Liver function changes, as deduced from serum biomarkers and tissue gene expression analysis;
3. Metabolic changes, including alterations in iron metabolism, from serum parameters and tissue gene expression analysis;
4. Kidney function changes, as deduced from serum and urine analytes, as well as from tissue gene expression results;
5. Histopathological alterations in liver and kidney tissue sections, from histological staining procedures and tissue gene expression assays.

CHAPTER III

In order to complement the insights offered by CHAPTER II into the effects of a repeated exposure to tramadol and tapentadol therapeutic doses on metabolizing organs, these were further studied in target organs. To this purpose, serum, lung, heart and brain cortex tissue samples from Wistar rats submitted to a 14-day period of single daily tramadol and tapentadol injections were analyzed. The methodological approach aimed at the assessment of putative:

1. General and organ-specific oxidative status alterations, from serum and tissue oxidative stress biomarkers, as well as from tissue gene expression results;
2. Alterations in general and organ-specific inflammatory and immunological biomarkers, as deduced from serum parameters and tissue gene expression analysis;
3. Changes in cardiac cell integrity, from serum biomarkers and tissue gene expression results;
4. Changes in brain cortex metabolism, from serum and tissue parameters, as well as from tissue gene expression results;
5. Histopathological alterations in lung, heart and brain cortex tissue sections, from histological staining methods and tissue gene expression assays.

PART II

1. ORIGINAL RESEARCH

CHAPTER I

Acute administration of tramadol and tapentadol at effective analgesic and maximum tolerated doses causes hepato- and nephrotoxic effects in Wistar rats

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Acute administration of tramadol and tapentadol at effective analgesic and maximum tolerated doses causes hepato- and nephrotoxic effects in Wistar rats

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ABSTRACT

Tramadol and tapentadol are two atypical synthetic opioid analgesics, with monoamine reuptake inhibition properties. Mainly aimed at the treatment of moderate to severe pain, these drugs are extensively prescribed for multiple clinical applications. Along with the increase in their use, there has been an increment in their abuse, and consequently in the reported number of adverse reactions and intoxications. However, little is known about their mechanisms of toxicity. In this study, we have analyzed the *in vivo* toxicological effects in liver and kidney resulting from an acute exposure of a rodent animal model to both opioids. Male Wistar rats were intraperitoneally administered with 10, 25 and 50 mg/kg tramadol and tapentadol, corresponding to a low, effective analgesic dose, an intermediate dose and the maximum recommended daily dose, respectively, for 24 h. Toxicological effects were assessed in terms of oxidative stress, biochemical and metabolic parameters and histopathology, using serum and urine samples, liver and kidney homogenates and tissue specimens. The acute exposure to tapentadol caused a dose-dependent increase in protein oxidation in liver and kidney. Additionally, exposure to both opioids led to hepatic commitment, as shown by increased serum lipid levels, decreased urea concentration, increased alanine aminotransferase and decreased butyrylcholinesterase activities. It also led to renal impairment, as reflected by proteinuria and decreased glomerular filtration rate. Histopathological findings included sinusoidal dilatation, microsteatosis, vacuolization, cell infiltrates and cell degeneration, indicating metabolic changes, inflammation and cell damage. In conclusion, a single effective analgesic dose or the maximum recommended daily dose of both opioids leads to hepatotoxicity and nephrotoxicity, with tapentadol inducing comparatively more toxicity. Whether these effects reflect risks during the therapeutic use or human overdoses requires focused attention by the medical community.

1. Introduction

Tramadol and tapentadol are centrally acting, synthetic opioid analgesics used in the treatment of moderate to severe pain (Hartrick and Rozek, 2011; Lee et al., 1993; Pinho et al., 2013; Zhou, 2009). Their clinical importance and widespread prescription are mostly due to their low incidence of opioid-type side effects, when compared with classical opioids (Hartrick and Rozek, 2011; Lee et al., 1993). Indeed, they lead to lower rates of symptoms such as nausea, constipation, motor incoordination, dependence and addiction, which are typically associated with opioid use (Barbosa et al., 2016; DePriest et al., 2015; Harrison et al., 1998; Kosten and George, 2002; Lee et al., 1993). By combining μ-opioid receptor (MOR) activation with 5-hydroxytryptamine (5-HT) and noradrenaline (NA) reuptake inhibition, tramadol and tapentadol

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are considered non-classical opioids; this dual mechanism of action allows them to provide enhanced analgesia, a better therapeutic window, improved compliance and tolerability and makes them valuable options for the treatment of chronic neuropathic and inflammatory pain (Barbosa et al., 2016; Giorgi, 2012; Lee et al., 1993; Pergolizzi et al., 2012; Power, 2011; Singh et al., 2013a; Tzschentke et al., 2014; Vadivelu et al., 2010).

Both drugs are metabolized in the liver (Barbosa et al., 2016; DePriest et al., 2015; Leppert, 2011; Zhou, 2009). Tramadol (1*RS*, 2*RS*)-2-[(dimethylamino)methyl]-1–(3-methoxyphenyl)-cyclo-hexanol) is a racemate of two enantiomers, (−)-tramadol and (+)-tramadol, each with a distinct activity profile (Barbosa et al., 2016; Grond and Sablotzki, 2004; Raffa et al., 2012). It is mainly metabolized by cytochrome P450 (CYP450) through demethylation and oxidation, followed by conjugation (Barbosa et al., 2016; DePriest et al., 2015; Leppert, 2011). The CYP2D6 isoenzyme converts it into *O*-desmethyltramadol (M1), its main active metabolite, with higher pharmacological activity than the parent compound (Barbosa et al., 2016; Costa et al., 2013; DePriest et al., 2015; Leppert, 2011; Pinho et al., 2013; Zhou, 2009). M1 enantiomers collectively provide MOR agonist activity and 5-HT and NA reuptake inhibition (Barbosa et al., 2016; Leppert, 2011; Raffa et al., 2012). In turn, tapentadol (3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol) is a non-racemic drug that has been more recently introduced in the market. It undergoes extensive phase II reactions, namely conjugation with glucuronic acid and sulfonate, and has no analgesic active metabolites (Barbosa et al., 2016; DePriest et al., 2015; Hartrick and Rozek, 2011; Kneip et al., 2008; Raffa et al., 2012; Tzschentke et al., 2014). Since it requires no metabolic activation and provides moderate MOR agonist activity, prominent NA reuptake inhibition and minimal 5-HT effect, it considerably decreases serotonin syndrome liability and is claimed to be safer and advantageous over tramadol (Barbosa et al., 2016; DePriest et al., 2015; Hartrick and Rozek, 2011; Kneip et al., 2008; Raffa et al., 2012; Tzschentke et al., 2014).

In spite of their increased tolerability, adverse reactions have also been reported for tramadol and tapentadol, including respiratory depression, serotonin syndrome (Beakley et al., 2015; Pilgrim et al., 2011; Sansone and Sansone, 2009) and fatal intoxications (Cantrell et al., 2016; Costa et al., 2013; Franco et al., 2014; Kemp et al., 2013; Larson et al., 2012; Pilgrim et al., 2011; Pinho et al., 2013; Tjaderborn et al., 2007). Paradoxically, the molecular and biochemical rationale underlying such effects and the toxicodynamics of both opioids is far from being completely understood.

Since both drugs are metabolized in the liver and their metabolites are predominantly excreted by the kidneys, these organs may be considered primary toxicity targets. Indeed, besides leading to neuronal damage (Ezzeldin et al., 2014; Faria et al., 2017, 2016; Ghoneim et al., 2014), tramadol has been found to induce hepatotoxicity and nephrotoxicity both upon acute and chronic exposures. Liver and kidney function parameters, such as aminotransferase activity, creatinine and blood urea nitrogen (BUN) (Atici et al., 2005), oxidative stress markers, as increased malondialdehyde (MDA) levels and decreased expression of antioxidant enzymes (Atici et al., 2005; Ghoneim et al., 2014), and histopathological alterations, such as dilated hepatic sinusoids, apoptosis, necrosis, degeneration, vacuolization, centrolobular congestion, and mononuclear cell infiltration (Atici et al., 2005; Ezzeldin et al., 2014), were reported in rodent animal models. A recent study by our research group has shown that tramadol and tapentadol cause brain, heart and lung toxicity (Faria et al., 2017), but there are no similar studies regarding their acute *in vivo* toxicity in metabolizing organs, particularly concerning tapentadol.

Given the increasing and dangerous trend towards the abuse and misuse of prescription opioids, including tramadol and tapentadol (Tjaderborn et al., 2007; West et al., 2015), and considering the particular lack of information concerning tapentadol toxicity, the clarification of their *in vivo* effects at different doses gains considerable relevance. Therefore, in the present work, we performed an *in vivo* study, using male Wistar rats, to comparatively analyze oxidative stress, biochemical and histological alterations, at the liver and kidney levels, deriving from an acute exposure to a broad range of tramadol and tapentadol doses, corresponding to a typical effective analgesic dose, an intermediate dose and the maximum recommended daily dose. The pertinence of the analysis of the acute effects arising from tramadol and tapentadol administration is underlined by their frequent use in malignant and non-malignant, post-traumatic, obstetric, renal, biliary colic, and postoperative pain (Grond and Sablotzki, 2004; Hartrick and Rozek, 2011; Leppert, 2009; Paris et al., 2013).

2. Materials and methods

2.1. Chemicals

In this study, tramadol hydrochloride (Sigma-Aldrich) and tapentadol hydrochloride (Deltaclon) were dissolved and diluted in saline (0.9% (w/v) NaCl) for subsequent administration. Sodium thiopental was purchased from B. Braun Portugal. All other chemicals were commercial preparations of the best degree of purity available.

2.2. Experimental model

42 male Wistar rats, aged 8 weeks and weighing 250–275 g, were provided by IBMC − i3S animal facility. All animals were housed in plastic cages, under controlled standard laboratory conditions (22 ± 2 °C, 50–60% humidity, 12/12 h light/dark cycles, with *ad libitum* access to tap water and rat chow (standard rodent formula, Mucedola), for a quarantine period of at least one week before the experimental assays.

All procedures were performed as to give the proper animal care, to reduce suffering and stress. Experimental animal procedures were in agreement with the European Council Directive (2010/63/EU) guidelines that where transposed into Portuguese law (Decree-Law n.°113/2013, August 7th). Additionally, the experiments were conducted with the approval of the Ethical Committee of CESPU, Institute of Research and Advanced Training in Health Sciences and Technologies (IINFACTS), Gandra, PRD, Portugal (process no. TETT-CESPU 2014, TETT-CESPU 2016C, and PI4AC 2017) and complied with the guidelines of the Committee and National Council of Ethics for the Life Sciences (CNECV).

2.3. Experimental design and drug treatment

Wistar rats were randomly assigned to 7 groups, composed of 6 animals each. On the assay day, rats were weighed for dose adjustment. Drug doses were delivered in 1 mL-units of normal saline (0.9% (w/v) NaCl). Group 1, the control group, was intraperitoneally (i.p.) injected with 1 mL saline; groups 2, 3 and 4 were i.p. injected with 10, 25 and 50 mg/kg tramadol, respectively, while groups 5, 6 and 7 were i.p. injected with 10, 25 and 50 mg/kg tapentadol, respectively. Opioid doses for rat administration were determined considering their LD_{50} for rats (Matthiesen et al., 1998), concentrations reported in intoxications (Kemp et al., 2013), as well as tramadol and tapentadol maximum recommended daily doses in humans (Barbosa et al., 2016; Matthiesen et al., 1998; Singh et al., 2013a; Tzschentke et al., 2007). Unless otherwise deemed by the physician, a 60-kg patient should receive 50–100 mg tramadol (1.5 mg/kg/day) three to four times daily. The maximum recommended daily dose is 400 mg (Matthiesen et al., 1998). In turn, tapentadol dose should not exceed 600–700 mg daily (Singh et al., 2013a; Tzschentke et al., 2007). Rat doses were calculated using the correction factor (K_m) for the conversion of the human dose into the animal equivalent dose (AED), through the following formula for an average 60 kg-weighing human: AED (mg/kg) = Human dose (mg/kg) × K_m ratio (K_m = 6.2) (Nair and Jacob, 2016; Reagan-Shaw et al.,

2008). For instance, the administration of 100 mg tramadol or tapentadol to a 60 kg-adult corresponds to a 1.67 mg/kg dose, which, when multiplied by the 6.2 factor, is equal to 10.35 mg/kg (rat dose). In line with this, the 10 mg/kg dose is equivalent to a single, effective analgesic 100 mg dose for a 60 kg-weighing human adult, below the maximum recommended daily dose, while 25 mg/kg and 50 mg/kg correspond to an intermediate and the maximum recommended daily dose, respectively.

Immediately after administration, rats were placed in metabolic cages, being given *ad libitum* access to tap water, but no food, until their sacrifice, and kept under monitoring. The remaining environmental conditions were maintained. Animals were sacrificed 24 h after drug administration.

2.4. Collection and processing of biological samples

Urine samples were collected from each animal, into an ice-cold container, throughout the 24 h-exposure period. Samples were centrifuged at 3000*g*, 4 °C, for 10 min, to remove any debris. Animals were sacrificed through anesthetic procedures (i.p. injection with 60 mg/kg sodium thiopental). Blood samples were drawn through cardiac puncture, with a hypodermic heparinized needle. Serum samples were obtained through blood centrifugation at 3000*g*, 4 °C, for 10 min, aliquoted and stored (−80 °C) for further biochemical analysis.

Livers and kidneys were surgically removed, dried with gauze, weighed on an analytical balance and further processed. One portion of each organ was homogenized in an Ultra-Turrax® homogenizer, in 1:4 (w/v) ice-cold 50 mM phosphate buffer (KH_2PO_4 + $Na_2HPO_4.H_2O$), pH 7.4. The corresponding supernatants were obtained through centrifugation at 4000*g*, 4 °C, for 10 min. Aliquots were stored at −80 °C, for subsequent biochemical analysis.

2.4.1. Quantification of oxidative stress parameters

Perchloric acid was added to aliquots of the liver and kidney homogenates to a final concentration of 5% (w/v), to precipitate proteins. Samples were centrifuged at 13000*g*, 4 °C, for 10 min, and supernatants were stored at −80 °C for subsequent quantification of lipid peroxidation (LPO). This was assessed through the thiobarbituric acid-reactive substances (TBARS) methodology described by Buege et al. (Buege and Aust, 1978), with the results being expressed as nanomoles of MDA equivalents per milligram of protein. In turn, pellets were also stored at −80 °C, for the quantification of protein carbonyl groups (ketones and aldehydes), which was performed according to Levine et al. (Levine et al., 1994). Results were expressed as nanomoles of 2,4-dinitrophenylhydrazine (DNPH) incorporated per milligram of protein.

2.4.2. Quantification of biochemical parameters in serum and urine samples

Biochemical parameters were determined directly, using the Prestige 24i automated analyzer (Cormay, Tokyo Boeki), according to the manufacturer's instructions, as previously described (Costa et al., 2015). Calibrations were performed for each parameter, with two appropriate calibrators, in order to plot a standard curve with 5 points. A quality control was also included. Quantifications were performed using undiluted samples. Alkaline phosphatase (ALP), alanine aminotransferase (ALT), butyrylcholinesterase (BuChE), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides and urea were quantified in serum samples, while creatinine, proteins and urea were determined in urine samples. The glomerular filtration rate (GFR) was calculated as the ratio between urine and serum creatinine concentrations, multiplied by the urine flow. Enzyme activities were determined as U/L, while biochemical parameters were retrieved as mg/dL. Results were then normalized against the control, non-treated group, i.p. injected with normal saline.

2.4.3. Liver and kidney histopathological analysis

One portion of liver and kidney tissue from each animal was collected and fixed in 4% (w/v) formaldehyde, for 24 h at room temperature, for subsequent histological analysis. It was then submitted to standard dehydration and paraffin wax-embedding procedures, as previously described (Dinis-Oliveira et al., 2006a,b). Three μm-sections were cut in a microtome (Shandon™ Finesse™ 325, Thermo Scientific) and adhered to glass slides. Hematoxylin and eosin (H & E)- and periodic acid-Schiff (PAS)-stained slides were obtained for liver simples, while kidney samples were processed for H & E staining only. Slides were prepared in accordance to standard methods and analyzed under phase contrast microscopy, using 100× and 600× magnifications (Nikon Eclipse TE2000-U microscope, equipped with a DXM1200F digital camera and controlled by Nikon ACT-1 software). Multiple fields were analyzed, and images were taken from representative ones.

2.5. Statistical analysis

Results were expressed as means ± SD. Statistical data analysis was performed as an Analysis of Variance (ANOVA). Post-hoc analysis was performed through Dunnett's multiple comparisons test. Probability values of $p < 0.05$ were considered as statistically significant. Graphic plotting and all statistical tests were performed using GraphPad Prism® version 7.0a.

3. Results

3.1. The acute administration of tramadol and tapentadol has a differential impact on liver and kidney oxidative stress

In order to have an insight over the mechanisms of toxicity elicited by an acute exposure to both opioids, oxidative stress was assessed in terms of LPO and protein carbonyl group levels in liver and kidney homogenates. Results are expressed in Fig. 1.

Interestingly, TBARS levels (LPO biomarkers), decreased upon a 24-h exposure to both tramadol and tapentadol, falling to as much as about 30% of the control values in liver samples, at 50 mg/kg tramadol and 25 and 50 mg/kg tapentadol, to about 60% of the control values in kidney, at 25 mg/kg and 50 mg/kg tramadol, and to 50% at all tapentadol doses. This suggests that an acute exposure to higher doses of tramadol and tapentadol not only does not lead to LPO, but may also have a protective effect at this level. On the other hand, protein carbonyl groups increased 2.0-fold for the two highest tapentadol doses tested, in the case of liver samples, and 4.0-fold for the highest tapentadol dose, for kidney samples. Therefore, only tapentadol seems to have a significant impact as a protein oxidative stress inducer in both liver and kidney. Taken together, these results show that an acute, 24-h exposure to tramadol and tapentadol has a differential effect on oxidative stress in the liver and kidney: while they seem to have a protective effect on lipids, tapentadol induces oxidation at the protein level, in both organs.

3.2. The acute administration of tramadol and tapentadol leads to liver and kidney dysfunction

Alterations in liver and kidney function 24 h after the acute administration of tramadol and tapentadol were studied by quantifying several biochemical biomarkers in serum and urine samples collected from the experimental groups, as depicted in Fig. 2.

Regarding the analysis of serum samples, enzyme activity determination did not show significant alterations concerning ALP (data not shown). Although it evidenced a trend towards the increase in ALT activity along with the dose used, this was significant only upon exposure to the highest dose of both opioids. Nevertheless, BuChE activity showed a remarkable, accentuated decrease upon exposure to tramadol and tapentadol, at all doses studied. Indeed, BuChE activity

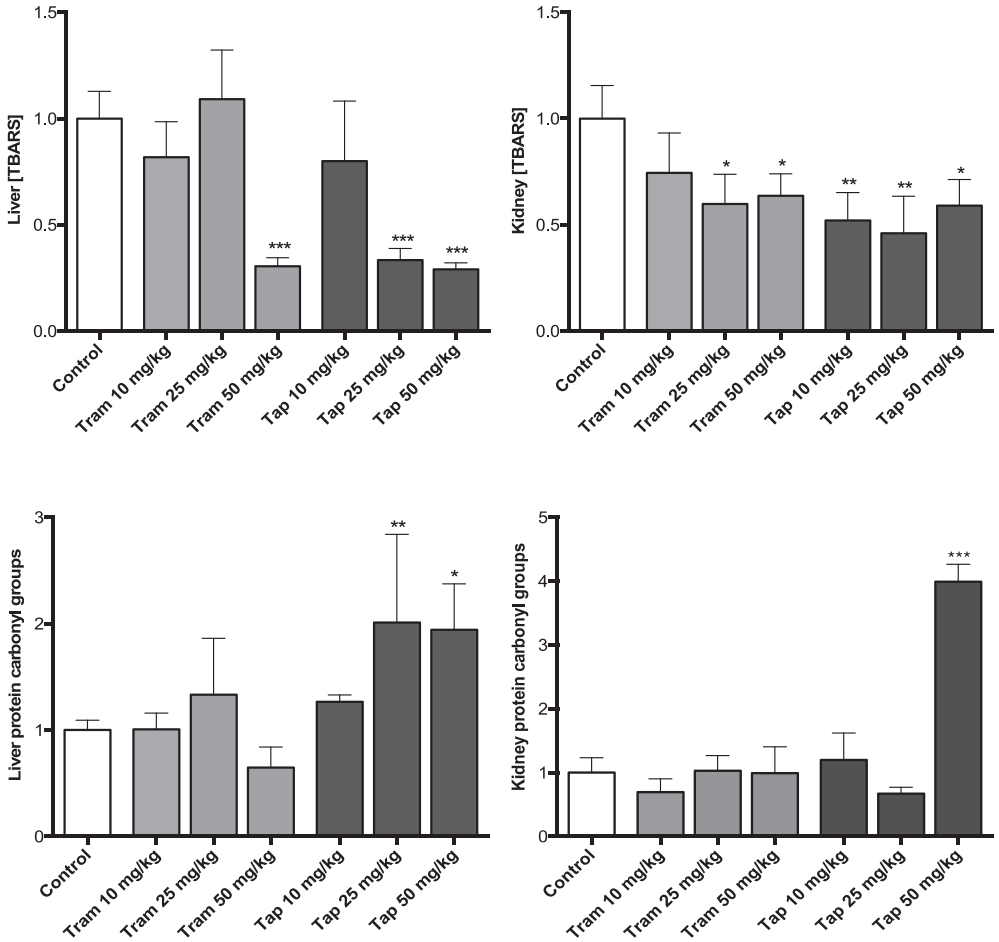

Fig. 1. – Liver and kidney lipid and protein oxidative stress levels, as assayed through the determination of thiobarbituric acid-reactive substances and protein carbonyl groups, respectively, in Wistar rat tissue homogenates prepared upon 24 h of tramadol (Tram) or tapentadol (Tap) exposure. Thiobarbituric acid-reactive substance and protein carbonyl group levels were normalized against the total protein content, as well as against that obtained in the control group, injected with normal saline and established as 1. Results are expressed by means ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

significantly decreased to less than 10% of the control, irrespectively of the opioid and dose administered. Serum urea levels denoted a dose-dependent decrease for both opioids, though this was statistically significant only upon exposure to 50 mg/kg tramadol and tapentadol, where urea levels reached about 62% of the control groups.

In turn, the analysis of serum lipid levels revealed a general, dose-dependent increasing trend in the rats exposed to tramadol and tapentadol. Total cholesterol showed a statistically significant increase upon exposure to 25 and 50 mg/kg tramadol (1.7-fold) and 50 mg/kg tapentadol (1.3-fold), while triglycerides denoted a significant increase (about 1.8-fold) upon exposure to 25 and 50 mg/kg tapentadol only. HDL cholesterol levels were found to be increased at the highest dose tested (50 mg/kg) for both tramadol and tapentadol (1.9- and 1.7-fold increase, respectively), whilst LDL cholesterol was significantly increased after exposure to 10 and 50 mg/kg tapentadol.

The analysis of urine samples (Fig. 3) showed signs of renal dysfunction. Indeed, there was a consistent decrease in the GFR for both opioids, with its levels ranging from 47 to 69% of the control, at all doses tested. Additionally, proteinuria was detected in all groups, when compared to the control; protein concentration in urine increased along with opioid dose, being particularly elevated at the highest dose administered, 50 mg/kg. In this situation, urinary protein loss achieved 3.0- and 3.5-fold the control values for tramadol and tapentadol, respectively. Consistently with serum urea results, there was a dose-dependent decrease in urinary urea levels, which was found to be more pronounced in the case of tapentadol exposure, having the control group as a reference. Upon administration of 50 mg/kg tapentadol, the decrease in urea levels reached 59% of the control.

3.3. The acute administration of tramadol and tapentadol leads to glycogen depletion, steatosis, inflammation and vacuolization in liver and kidney

In order to screen for histological alterations deriving from the acute administration of tramadol and tapentadol, liver and kidney specimens collected from Wistar rats were processed using H & E and PAS staining methods. While the first is a standard staining method for nucleic acids and eosinophilic structures, the latter specifically evidences carbohydrates (such as glycogen) and carbohydrate-rich structures. Opioid exposure effects at the tissue level were assessed having the results of the control, non-treated rats, as a reference. The analysis through phase contrast microscopy showed that both opioids lead to histopathological alterations at all doses tested.

In Fig. 4, the typical aspect of a control liver section, with normal architecture, hepatocyte trabeculae radially deriving from central

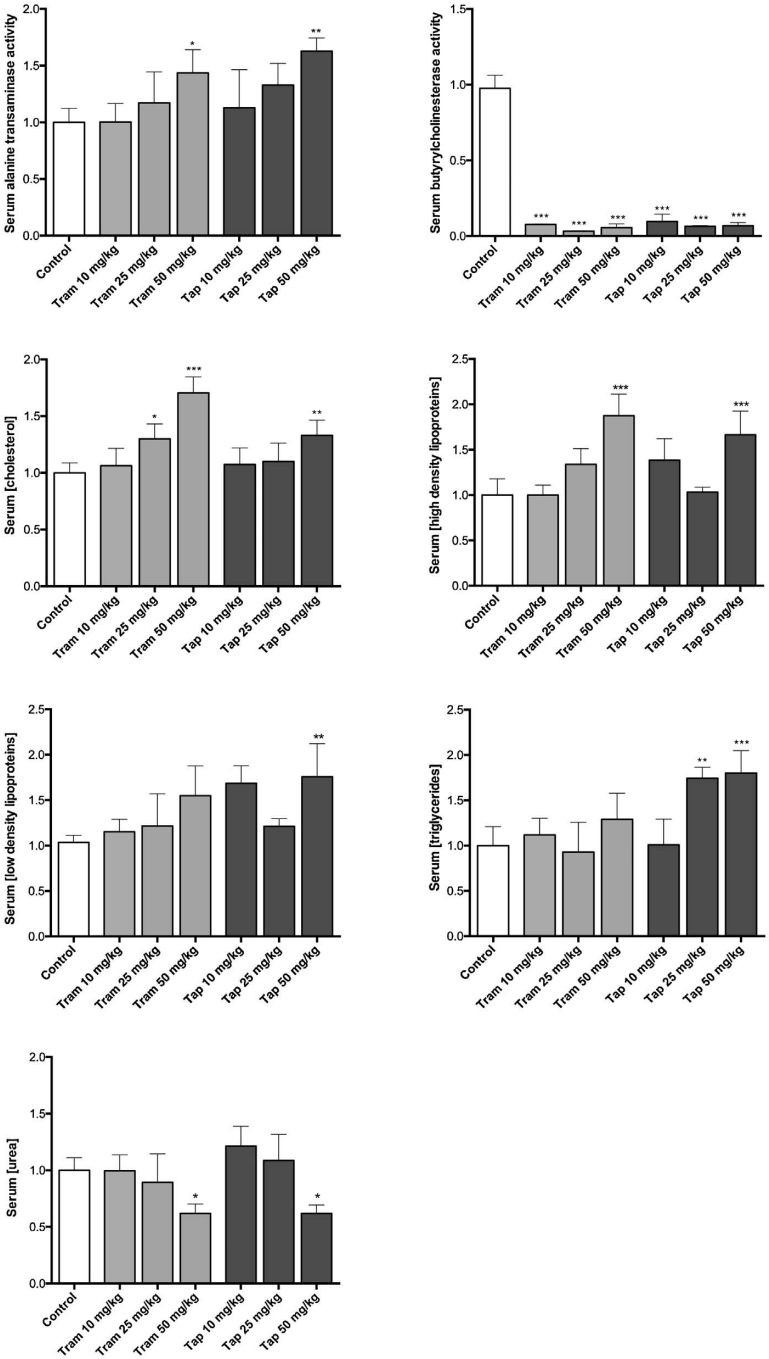

Fig. 2. – Serum alanine aminotransferase and butyrylcholinesterase activities, serum cholesterol, high-density lipoprotein, low-density lipoprotein, triglyceride and urea concentrations, in Wistar rats exposed for 24 h to tramadol (Tram) or tapentadol (Tap). Enzyme activities were determined as U/L, while biochemical parameters were retrieved as mg/dL. Results were subsequently normalized against the control group, injected with normal saline, established as 1. Results are expressed by means ± SD. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

veins, separated by sinusoids, and acidophilic stippled cytoplasms, is shown. In turn, Fig. 5 evidences generalized pink spots corresponding to glycogen granules, particularly abundant near the portal system (Thoolen et al., 2010), in the control group. PAS staining evidenced glycogen depletion upon exposure to 10, 25 and 50 mg/kg tramadol (Fig. 5), as seen by the fainted pink staining. Both PAS and H & E staining revealed microvesicular steatosis, as reflected by the cytoplasmic bubbles that predominantly concentrate in the cells surrounding the blood vessels, a finding that was increasingly evident along with the dose (Figs. 4 and 5). Sinusoidal dilatation was also consistently present (Figs. 4 and 5). Besides leading to these phenomena, exposure to 50 mg/kg tramadol was also associated with atypical, disorganized cell masses. In addition, both staining methods substantiate the presence of infiltrates of inflammatory, sometimes

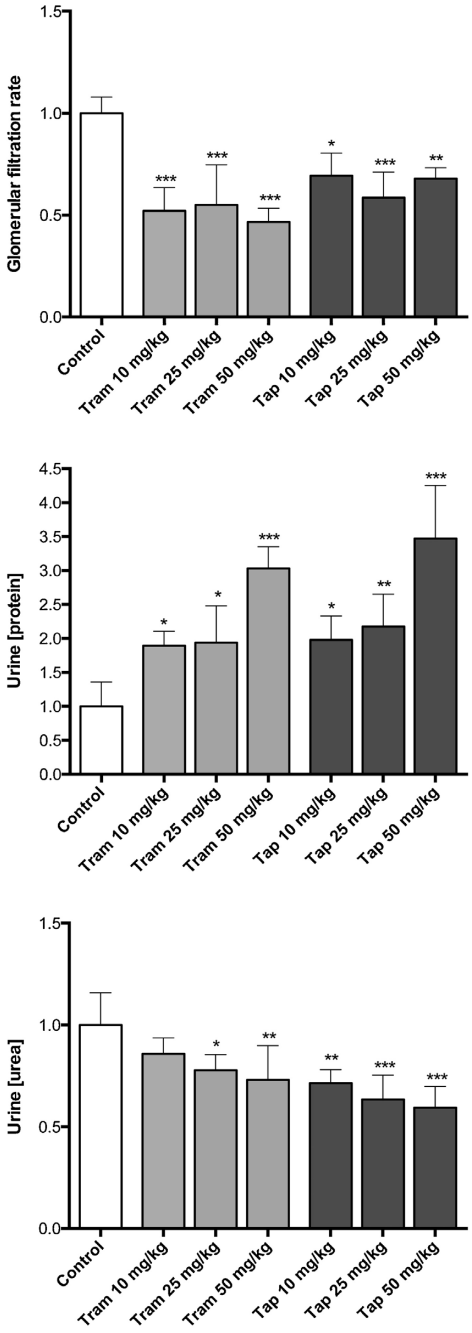

Fig. 3. – Glomerular filtration rate, urine protein and urine urea concentrations, in Wistar rats exposed for 24 h to tramadol (Tram) or tapentadol (Tap). Concentrations, determined as mg/dL, were subsequently normalized against that obtained in the control group, injected with normal saline, established as 1. Results are expressed by means ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

binucleated cells, as well as focal, unicellular necrosis/acidophilic bodies (in the tapentadol group), denoting cell death (Figs. 4 and 5) (Thoolen et al., 2010).

Histological analysis of tapentadol-exposed livers showed weaker glycogen staining and microvesicular steatosis at all doses tested (Figs. 4 and 5). Inflammatory cell infiltrates were observed near blood vessels mainly upon H & E staining (Fig. 4). At 25 mg/kg tapentadol, microvesicular steatosis, blood vessel dilatation and congestion were identified (Fig. 5), whereas erythrocyte extravasation was particularly evident in H & E slides (Fig. 4). In line with these results, microvesicular steatosis, glycogen depletion and inflammatory cell infiltrates were also observed upon 50 mg/kg tapentadol exposure (Figs. 4 and 5).

Fig. 6 depicts the histological analysis of kidney specimens. Control slides evidenced normal glomeruli, composed of glomerular capillaries surrounded by visceral and parietal layers of Bowman's capsule, separated by narrow Bowman's spaces, while sections from tramadol and tapentadol-exposed kidneys evidenced tubular disorganization at all doses studied. Inflammatory mononuclear cell infiltrates were observed even at lower and intermediate doses for both opioids. Signs of cell vacuolization and dotted, fainted cytoplasmic staining became increasingly evident along with the dose.

4. Discussion

Tramadol and tapentadol are synthetic opioids employed in the treatment of moderate to severe pain. In the present study, we have acutely exposed Wistar rats to tramadol and tapentadol doses corresponding to a typical analgesic dose, an intermediate dose and the maximum recommended daily dose, to assess liver and kidney toxic injury, using oxidative stress, biochemical and histopathological markers.

To assess putative damage in metabolizing organs, several biochemical parameters regarding liver and kidney functions were determined in serum and urine samples. Serum ALT activity is a well-known liver function test, since this enzyme is hepatospecific (Tang et al., 2016). Indeed, several studies reported increased activities of enzymes such as ALT, aspartate aminotransferase (AST), ALP and lactate dehydrogenase (LDH) following exposure to opioids, including morphine and tramadol (Albarakai and Alsbery, 2016; Ali et al., 2015; Atici et al., 2005; Hafez et al., 2015; Salahshoor et al., 2016; Saleem et al., 2014). Aminotransferase activities have been found to be markedly increased in a case of death due to tramadol intoxication alone, in a context of multiple organ dysfunction (Wang et al., 2009). Nevertheless, we have detected only a slight, significant increase for ALT activity following exposure to the highest tramadol and tapentadol dose. Previous studies by our research group have shown that, under the same experimental conditions, the increase in AST activity is comparatively higher (Faria et al., 2017), for which it may be reasoned that the extent of muscle injury, as far as cell membrane integrity is concerned, is greater than that of liver. No significant differences between tramadol or tapentadol groups and the control group were found regarding ALP activity (data not shown). Since oxidative stress on hepatocyte membrane lipids has been suggested as the mechanism responsible for enzyme leakage and increase in the serum (Ali et al., 2015; Nehru and Anand, 2005; Salahshoor et al., 2016), and we have found no signs of LPO in liver, this may explain why ALT only had a subtle serum elevation under our experimental conditions. Additionally, apart from the studies from Hafez and Youssef and respective co-workers, who tested tramadol acute toxicity by administering rats a single 300 mg/kg dose (Hafez et al., 2015; Youssef and Zidan, 2015), most of the available studies refer to chronic, repeated opioid administration assays. As for ALP, Hafez and colleagues detected a highly significant increase of activity only for animals injected with 25 and 50 mg/kg/day tramadol for two weeks, but not for acutely exposed rats (Hafez et al., 2015). Therefore, we may infer that higher doses and longer exposure periods may be required for membrane LPO and cell lysis to occur and, consequently, to increase serum hepatocyte injury biomarkers.

In turn, serum BuChE activity levels remarkably decreased upon exposure to both tramadol and tapentadol, irrespectively of the dose

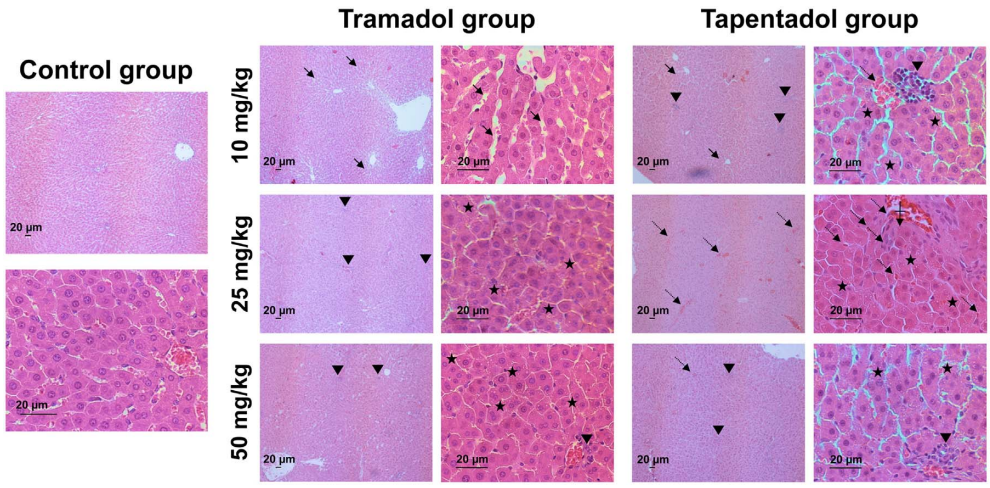

Fig. 4. – Microscopy analysis, upon hematoxylin & eosin staining, of liver tissue samples from Wistar rats following a single exposure to normal saline (control group) and different tramadol and tapentadol doses. Sinusoidal dilatation is observed (arrows), as well as microsteatosis (stars) and mononuclear inflammatory cell infiltrates (inverted triangles). In the tapentadol group, vascular congestion/erythrocyte extravasation (dotted arrows) and evidences of focal, unicellular necrosis/acidophilic bodies (vertical, crossed arrow) are also present. Photos were taken with 100× and 600× magnifications. Scale bar, 20 µm.

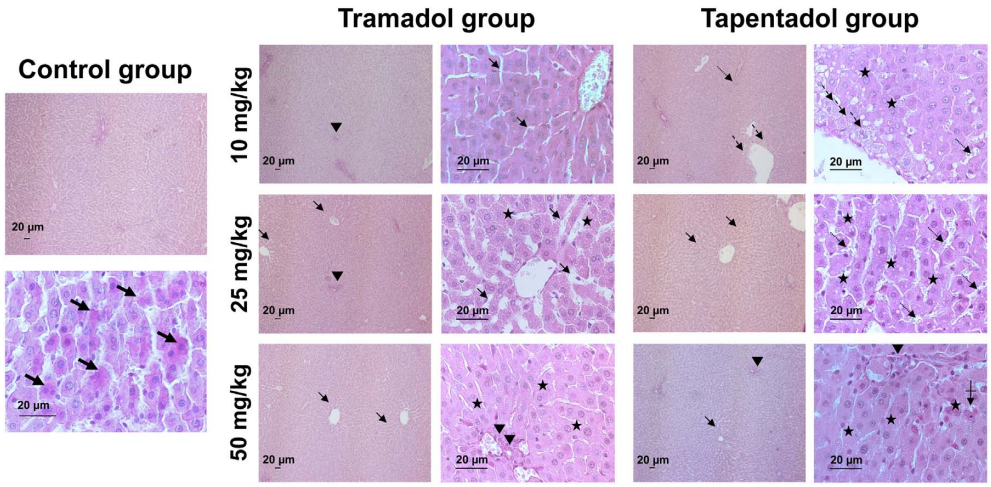

Fig. 5. – Microscopy analysis, upon periodic acid-Schiff staining, of liver tissue samples from Wistar rats following a single exposure to normal saline (control group) and different tramadol and tapentadol doses. Glycogen granules appear as purple areas (thick arrows). Sinusoidal dilatation is observed (thin arrows), as well as microsteatosis (stars) and mononuclear inflammatory cell infiltrates (inverted triangles). In tramadol and tapentadol groups, glycogen staining is weaker than the observed for the control. In the tapentadol group, vascular congestion/erythrocyte extravasation (dotted arrows) and evidences of focal, unicellular necrosis/acidophilic bodies (vertical, crossed arrow) are also present. Photos were taken with 100× and 600× magnifications. Scale bar, 20 µm.

considered. As such, it was seemingly the most sensitive marker of hepatic toxicity used in the study. Although it also hydrolyzes acetylcholine, BuChE preferentially hydrolyzes longer chain-chemicals containing choline ester bonds, such as succinylcholine and butyrylcholine (Abdel-Salam et al., 2016; Jasiecki et al., 2016). It may hydrolyze heroin and act as a detoxifying enzyme for natural compounds (Abdel-Salam et al., 2016; Jasiecki et al., 2016). On one hand, as for other serum proteins, such as albumin and coagulation factors, BuChE is synthetized by the liver and secreted into the blood, reflecting the synthetic function of this organ (Meng et al., 2013; Ramachandran et al., 2014; Tang et al., 2016). In case of liver damage, BuChE production and consequent serum activity decrease, for which it is a sensitive indicator of inflammatory injury in liver parenchyma cells (Meng et al., 2013; Ramachandran et al., 2014; Tang et al., 2016). On the other hand, morphine and morphinans, as well as various other opioids, have been reported to inhibit BuChE activity in *in vitro* assays, which is particularly relevant considering that they are frequently co-administered with other drugs (Bailey and Briggs, 2005; Galli et al., 1996; Sim and Chua, 1986). Consistently, BuChE activity decreased by 17.6-36.5% upon 10–20 mg/kg subcutaneous daily administration of tramadol for 6 weeks, and has been suggested as a marker of tramadol abuse (Abdel-Salam et al., 2016). This inhibitory effect has been found not to be confined to a specific structural category and to be accentuated for concentrations above the analgesic ones (Galli et al., 1996). Thus, the notable decrease in BuChE activity following exposure to tramadol and tapentadol might result from a combination of its defective synthesis by the liver, reflecting opioid-induced hepatotoxicity, and its direct inhibition by these drugs. Additionally, inhibition of acetylcholinesterase (AChE) may be inferred from BuChE inhibition. Since it leads to acetylcholine accumulation and promotes cholinergic crisis, such inhibition

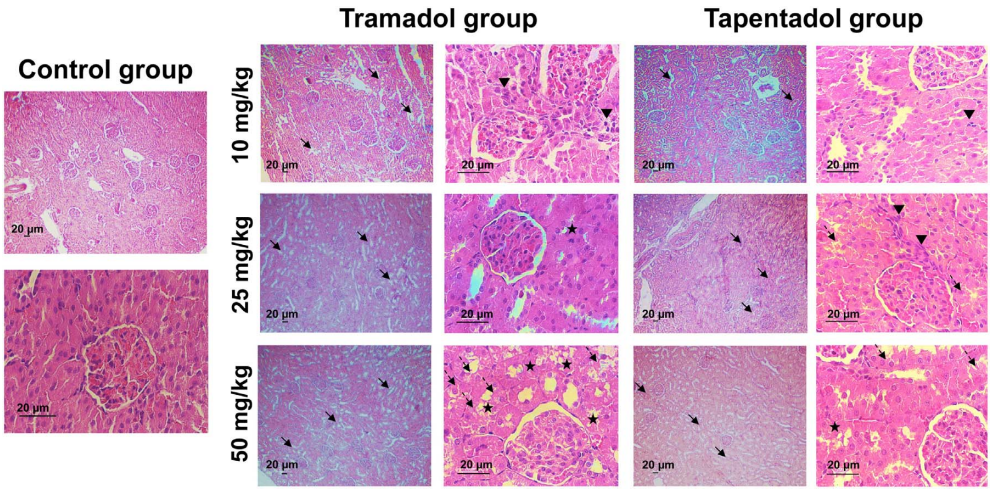

Fig. 6. – Microscopy analysis, upon hematoxylin & eosin staining, of kidney tissue samples from Wistar rats following a single exposure to normal saline (control group) and different tramadol and tapentadol doses. Inflammatory mononuclear cell infiltrates are observed (inverted triangles), as well as a disorganized tubular structure (arrows). A significant number of cells shows vacuolization (stars), while others evidence a weaker, dotted cytoplasmic staining (dashed arrows). Photos were taken with 100× and 600× magnifications. Scale bar, 20 μm.

might lead to continuous muscle, gland and central nervous system stimulation, inducing bradycardia, hypoventilation, paralysis, peripheral neuropathy, and other neurological symptoms (Nam et al., 2016). This supports neuronal damage, which has previously been demonstrated by our research group (Faria et al., 2016). Also, it should be emphasized that the BuChE gene is considerably polymorphic, with more than 100 variants being described, many of them having implications in the metabolism of choline- and non-choline esters such as aspirin, cocaine, organophosphate pesticides and nerve agents (Jasiecki et al., 2016). Thus, such genetic polymorphisms might account, to some extent, for interindividual variability in tramadol and tapentadol response, an issue that deserves further studies.

Consistently with the hypothetical impairment of liver metabolic functions, serum urea levels decreased upon opioid exposure, most notably for the 50 mg/kg dose. This decrease in urea production is subsequently reflected in its lower urinary levels. Since urea is exclusively synthetized in the liver through the urea cycle, from the ammonium ion deriving from amino acid catabolism, these results suggest that the acute exposure to tramadol and tapentadol induces adverse effects in the liver, compromising its metabolic and synthetic ability.

Serum lipid levels were found to be increased among animals exposed to tramadol and tapentadol. Exposure to high tramadol and tapentadol doses led to an increase in total and HDL cholesterol levels, while triglycerides and LDL cholesterol increased upon exposure to tapentadol only. These results oppose those of El-Gaafarawi and colleagues, who reported a significant decrease in total cholesterol and triglyceride levels, as well as a decrease in lipid-derived hormones, upon administration of both 40 and 80 mg/kg/day tramadol, for one month (El-Gaafarawi, 2006). Again, it should be considered that these results, unlike ours, were obtained upon a long-term exposure. It may thus be hypothesized that tramadol and tapentadol exposure causes an increase in lipid levels in an acute context, possibly by compromising liver ability to recycle circulating lipids, with this increase being followed by a decrease in the medium and long term, reflecting the progressive commitment of its synthetic ability. Another possibility is that lipid biosynthesis is acutely upregulated following tramadol and tapentadol exposure, further supporting metabolic derangement as the dominant effect under such conditions. Tapentadol has a more pronounced effect in this context, since triglycerides and LDL cholesterol significantly increased upon exposure to this opioid only.

Opioid exposure has also been consistently associated with increased oxidative stress in several organs, resulting both from an increase in oxidative damage to biomolecules, most notably lipids and proteins, and from a decrease in the activity of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) (Abdel-Zaher et al., 2011; Atici et al., 2005; Awadalla and Salah-Eldin, 2015, 2016; El-Gaafarawi, 2006; Elkhateeb et al., 2015; Ghoneim et al., 2014; Nafea et al., 2016; Samarghandian et al., 2014; Zhang et al., 2004). One study reported a decrease in reduced glutathione (GSH) content and in SOD and CAT activities, as well as an increase in MDA levels, in liver and kidney samples from Wistar rats orally receiving 40 mg/kg tramadol for 20 consecutive days (Awadalla and Salah-Eldin, 2015). Other studies have found the same trend in liver upon repeated intraperitoneal administration of increasing morphine doses, for up to 30 days (Samarghandian et al., 2014; Zhang et al., 2004), additionally showing a decrease in GST activity (Samarghandian et al., 2014) and an increase in DNA damage, as assayed by 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels (Zhang et al., 2004). Intriguingly, in our study, we have found that, although tapentadol induces protein oxidation in a dose-dependent manner in both liver and kidney, tramadol and tapentadol seem to have a protective effect from LPO in these organs. Paradoxically, due to their high polyunsaturated fatty acid content, biological membranes are a main target of oxidative damage, with LPO being an indirect marker of oxidant-induced cell injury (Lurie et al., 1995). Consistently with this, serum, liver and kidney MDA levels have been found to increase in a dose-dependent manner in rats receiving daily oral doses of tramadol for one month (El-Gaafarawi, 2006; Elkhateeb et al., 2015). It should be noted, however, that most of the available studies concern chronic, instead of acute, exposure assays. Therefore, we may anticipate that, in acute settings, these opioids have a differential effect in terms of oxidative stress to membranes. Interestingly, Atici and colleagues (Atici et al., 2005), in a comparative, long-term study concerning the administration of increasing doses of morphine and tramadol, reported a significant increase in mean serum MDA levels for the morphine group only, with no differences being found between the tramadol and control groups. Moreover, we have previously demonstrated that an acute exposure of Wistar rats to similar tramadol and tapentadol doses also led to protein oxidation, but not to LPO, in heart and lung tissues (Faria et al., 2017). A combination of time- and structural/mechanistic-dependent aspects may then explain the differential impact of tramadol and tapentadol on lipid and protein oxidation. Additionally, since

DNPH reacts with aldehydes and ketones whether they are incorporated into proteins or not (Levine et al., 1994; Weber et al., 2015), it may be hypothesized that tapentadol impact on protein may have been overestimated. Alternatively, tapentadol itself and/or its metabolites may react with DNPH. Taking these hypotheses into account, the quantification of protein sulfhydryl groups and/or the histological examination of protein oxidation could represent confirmatory approaches. In addition, an alternative perspective over the significance of LPO downregulation may be considered. Sub-lethal levels of LPO products induce cellular adaptive responses and enhance tolerance against subsequent oxidative stress, through control of gene expression (*e.g.*, upregulation of antioxidant compounds and enzymes) and modulation of intracellular signaling (Niki, 2009). As such, a decrease in their levels might have promoted pathological dysregulation, instead of reflecting a protective mechanism.

The acute effects of tramadol and tapentadol exposure on the liver have also been assessed at the histological level. Indeed, liver steatosis, congestion and enlargement are described in fatal intoxication case reports involving tramadol and tapentadol intake (Clarkson et al., 2004; Franco et al., 2014; Mannocchi et al., 2013). Acute liver failure, including one case of fulminant hepatic necrosis, as well as nonfatal hepatobiliary dysfunction, have also been reported for tramadol (Loughrey et al., 2003; Randall and Crane, 2014). In our study, microvesicular steatosis, hepatocyte vacuolization and sinusoidal dilatation were particularly evident and dose-dependent findings for both tramadol- and tapentadol-exposed Wistar rats (Thoolen et al., 2010). These results are in line with those reported by other studies, which mostly refer to chronic administration of various tramadol doses through different routes (Ali et al., 2015; Atici et al., 2005; Awadalla and Salah-Eldin, 2015; Elkhateeb et al., 2015; Hafez et al., 2015; Kaoud et al., 2013; Rabei, 2011; Samaka et al., 2012). Consistently with some of these studies (Albarakai and Alsbery, 2016; Awadalla and Salah-Eldin, 2015; Elkhateeb et al., 2015; Rabei, 2011; Samaka et al., 2012), inflammatory mononuclear cell infiltrates have also been observed, often with a perivascular location. Exposure to higher tramadol doses has led to disorganized cell masses, which have also been described by other authors, who reported hydropic degeneration and tissue architecture disruption upon tramadol and morphine repeated administration (Atici et al., 2005; Rabei, 2011; Salahshoor et al., 2016). Tapentadol exposure led to erythrocyte extravasation, a finding that was less evident upon tramadol treatment.

In turn, PAS staining, which is specific for the detection of polysaccharide-rich structures, evidenced glycogen depletion following exposure to both opioids, at all doses tested. Glycogen depletion may be, at least in part, a result of fasting-induced glycogenolysis. However, even upon a 24-h fasting, glycogen granules were observed in control rats, showing that additional glycogen depletion occurred for tramadol- and tapentadol-exposed rats. Weak PAS reactivity had already been reported by Awadalla and co-workers (Awadalla and Salah-Eldin, 2015), after oral administration of 40 mg/kg tramadol for 20 consecutive days. Thus, glycogenolysis might be activated as a compensatory mechanism for metabolic alterations resulting from acute opioid exposure. This observation, together with the absence of pronounced signs of cell death, suggests that the derangement of metabolic pathways, rather than the direct activation cell death mechanisms, is an important contributor to the toxicological impact of an acute exposure to tramadol and tapentadol, as far as metabolizing organs are concerned.

Besides the assessment of liver function biomarkers, biochemical parameters regarding renal function were determined in serum and urine samples. The GFR, which reflects the ratio between urinary and serum creatinine concentrations, has been found to be decreased at all tramadol and tapentadol doses studied. Together with the evidence of dose-dependent proteinuria for both opioids, these data suggest that their acute administration is sufficient to induce kidney injury. Considering the commitment of renal function and the decreased GFR, the kidneys may represent sites of tramadol/tapentadol and metabolite accumulation. This might account for their increased sensitivity to toxicological damage when compared to the liver. In fact, creatinine, BUN and potassium were found to be increased in a context of tramadol intoxication, following a 6-month period of tramadol abuse (Wang et al., 2009), reflecting renal commitment. Such parallelism between our study and reported clinical findings for tramadol further supports the validity of the comparison with tapentadol. Nephrotoxicity is, indeed, one of the possible direct or indirect adverse effects of the exposure to drugs, including opioid analgesics (Alinejad et al., 2016; Singh et al., 2013b). Various mechanisms have been suggested for such acute kidney injury, encompassing rhabdomyolysis − for which opiate toxicity is one of the most common causes (Alinejad et al., 2016; Mercadante and Arcuri, 2004; Singh et al., 2013b; Talaie et al., 2008, 2007) −, secondary amyloidosis (Crowe et al., 2000; Mercadante and Arcuri, 2004; Singh et al., 2013b) and decreased renal perfusion (Alinejad et al., 2016; Crowe et al., 2000; Mercadante and Arcuri, 2004). High doses of μ agonists, such as morphine, heroin and methadone, decrease systemic arterial blood pressure, increasing anti-diuretic hormone (ADH) secretion and increasing central sympathetic outflow, thereby decreasing renal perfusion (Alinejad et al., 2016; Crowe et al., 2000; Mercadante and Arcuri, 2004). This leads to renal function commitment, manifested as a transient decrease in urinary flow rate and, consequently, in GFR (Alinejad et al., 2016; Crowe et al., 2000; Mercadante and Arcuri, 2004). Although it is known that NA might lead to renal hypoperfusion, it is still poorly explored whether its accumulation due to tramadol intake affects kidney hemodynamics and function (Mercadante and Arcuri, 2004). Considering the similarity of mechanisms of action, the same effects might be anticipated for tapentadol. Accordingly, several studies have reported increased creatinine and BUN levels upon exposure to opioids, due to a decrease in their renal depuration (Ali et al., 2015; Atici et al., 2005; El Fatoh et al., 2014; El-Gaafarawi, 2006; Elkhateeb et al., 2015; Hafez et al., 2015; Saleem et al., 2014; Youssef and Zidan, 2015). Again, most of these studies refer to chronic exposure assays. For instance, Elkhateeb, El-Gaafarawi and respective colleagues found creatinine and BUN to be increased upon a one-month exposure to tramadol doses ranging from 30 to 80 mg/kg/day (El-Gaafarawi, 2006; Elkhateeb et al., 2015). Interestingly, Atici and co-workers (Atici et al., 2005) reported a significant increase in creatinine and BUN in Wistar rats intraperitoneally injected with increasing morphine doses for the same period, compared to rats similarly administered with increasing tramadol doses, with no significant differences being found between tramadol and control groups. This suggests that the extent of nephrotoxicity might be dependent on the opioid used. In turn, Hafez, Youssef and respective colleagues have compared tramadol chronic and acute administration, both intramuscular and by gavage, showing that BUN levels increase in both situations (Hafez et al., 2015; Youssef and Zidan, 2015). The dose selected for acute treatment in these studies (300 mg/kg) corresponds to the LD_{50} for the route of administration used. In this regard, our study provides evidence supporting that tramadol- and tapentadol-induced nephrotoxicity occurs not only for higher doses, but also for lower, effective analgesic doses, even in acute settings.

Histological examination of tramadol- and tapentadol-exposed kidney specimens have also confirmed acute effects, evidencing signs of tubular disorganization. Several studies, mostly addressing chronic exposure, report degenerated renal tubules upon tramadol treatment (Ali et al., 2015; Awadalla and Salah-Eldin, 2016; Elkhateeb et al., 2015; Ezzeldin et al., 2014; Singh et al., 2013b; Youssef and Zidan, 2015). One study reports multifocal tubular epithelial degeneration following both a chronic exposure to 50 mg/kg/day for two weeks and a single acute exposure to 300 mg/kg tramadol, the LD_{50} for intramuscular administration (Hafez et al., 2015). Various studies described interstitial cellular infiltrates, including lymphocytes, macrophages and serum cells, surrounding degenerated renal tubules, mainly upon repeated tramadol administration (Atici et al., 2005; Awadalla

and Salah-Eldin, 2015; Elkhateeb et al., 2015; Hafez et al., 2015; Youssef and Zidan, 2015). In fact, pulmonary edema, associated with opioid use and abuse, may lead to hypoxia, to which the kidneys are particularly sensitive and respond through inflammatory cell infiltration (Alinejad et al., 2016). In our study, inflammatory mononuclear cell infiltrates were also observed, though with little prominence when compared to other findings. In parallel, cell vacuolization and weak cytoplasmic staining became increasingly evident along with the dose. Vacuolization of endothelium cells of renal tubules has been reported in studies of chronic morphine and tramadol administration (Ali et al., 2015; Atici et al., 2005). Large vacuolated areas, due to substantial deposition of lipid-like material in all glomerular cells and infiltrating macrophages, associated with nephrotic syndrome, acute renal failure, and hypertension, have also been correlated with renal damage induced by opioid addiction (Mercadante and Arcuri, 2004). Autolytic changes were also reported on kidney microscopic examination following tapentadol intoxication (Franco et al., 2014). Taken together, our histological analysis substantiates that many findings that had previously been only reported for chronic opioid administration do also occur in liver and kidneys upon an acute tramadol and tapentadol exposure. It also complements a previous study from our research team, which evidenced morphological alterations, cell death and inflammatory infiltrates in lung, heart and brain cortex samples (Faria et al., 2017), by showing that these alterations are not confined to target organs, but do also occur in the metabolizing ones. Likewise, these results further highlight the toxicological potential of tapentadol, which had already been emphasized by the same study (Faria et al., 2017).

Concerning the differential impact of tramadol and tapentadol on some of the parameters analyzed, and considering the i.p. route of administration used, it should be noted that the doses used in our work were not pharmacologically equivalent. Although the i.p. route bypasses the intestine, it resembles oral administration in that the primary route of absorption is into the mesenteric vessels draining into the portal vein, for which i.p.-administered substances actually undergo hepatic metabolism before reaching systemic circulation (Lukas et al., 1971). In this context, the bioavailability of both opioids should be taken into account. While tramadol bioavailability is reported as 68–84% upon oral administration, that of tapentadol is approximately 32% (Barbosa et al., 2016; Grond and Sablotzki, 2004). Considering such lower bioavailability, tapentadol dose could be increased to obtain pharmacologically equivalent doses to tramadol. Nevertheless, even at pharmacologically lower doses, toxicological damage was more evident for tapentadol, for which it is reasonable to anticipate that more pronounced effects would be obtained if such pharmacological equivalence was considered. Furthermore, differences in tramadol and tapentadol affinity for receptors and transporters, as well as in the pharmacokinetics and pharmacodynamics of their metabolites, cannot be ruled out and may also account for their differential impact in some of the parameters analyzed.

It should also be emphasized that tramadol and tapentadol are opioid receptor partial agonists, and such extent of interaction must be considered while comparing our results with those deriving from studies with full agonists such as morphine and heroin.

Lastly, since our results show that tramadol and tapentadol acute exposure causes toxicological injury, even if minor, it may be reasoned that repeated daily intake may lead to cumulative damage and, thus, to more severe effects on metabolizing organs.

5. Conclusions

Despite widespread prescription of opioid drugs, increasing abuse and concomitant reports of adverse reactions and fatalities, knowledge about their toxicological effects, at the cellular and metabolic level, is lacking, especially for opioid receptor partial agonists such as those addressed in this work. Data on this subject add information to the interpretation of adverse drug reactions and fatal intoxications, particularly in the case of tapentadol, for which less literature is available. In fact, while animal and human data on tramadol toxicity accumulated along with its prolonged history of use, our study represents an added value concerning data on tapentadol, considering the more limited clinical and experimental reports with this drug.

Given their paramount role in opioid metabolism and excretion, liver and kidneys are potential toxicity targets. Our data demonstrate that a single exposure to tramadol and tapentadol, both at effective analgesic doses and at maximum recommended daily doses, elicits toxicological damage in these organs. Tapentadol increases protein oxidation in liver and kidney. Increased serum lipid levels and decreased urea concentration and BuChE activity denote hepatic commitment. In turn, proteinuria and decreased GFR reflect renal injury. At similar doses, tapentadol led to more pronounced effects concerning liver and kidney protein oxidation, serum LDL cholesterol and triglyceride levels. Histopathological analysis of liver and kidney specimens also confirmed findings hitherto mainly reported for chronic use situations, including sinusoidal dilatation, microsteatosis, vacuolization, inflammatory cell infiltrates, loss of tissue architecture, and erythrocyte extravasation in the specific case of tapentadol. Taken as a whole, the results suggest that acute toxicological injury involves mainly metabolic alterations, rather than evident cell death.

This is, to our knowledge, the first study addressing *in vivo* tapentadol acute toxicity in liver and kidney. Our results evidence that even a single effective dose of tramadol or tapentadol may cause hepatotoxicity and nephrotoxicity to some extent. As such, our findings emphasize the need to carefully consider and tailor the prescription of these drugs, as well as to monitor drug response, even in acute settings. Additionally, it was also evidenced that, although tapentadol may represent a better alternative due to its simpler pharmacokinetics and regarding the treatment of specific pain states, its use should be particularly pondered when considering patients with hepatic and/or renal insufficiency.

Further *in vivo* assays, concerning chronic administration and the use of a wider dose range, would complement the present study. Likewise, combined exposure assays to tramadol/tapentadol and other frequently co-administered drugs − *e.g.*, selective serotonin reuptake inhibitors, tricyclic antidepressants and monoamine oxidase inhibitors −, sharing common metabolic and excretion pathways with these opioids, would shed light into possible toxicity exacerbation due to drug–drug interaction and consequent accumulation.

Disclosure statement

Authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties. No writing assistance was utilized in the production of this manuscript.

Conflict of interest

None.

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CHAPTER II

Repeated administration of clinical doses of tramadol and tapentadol causes hepato- and nephrotoxic effects in Wistar rats

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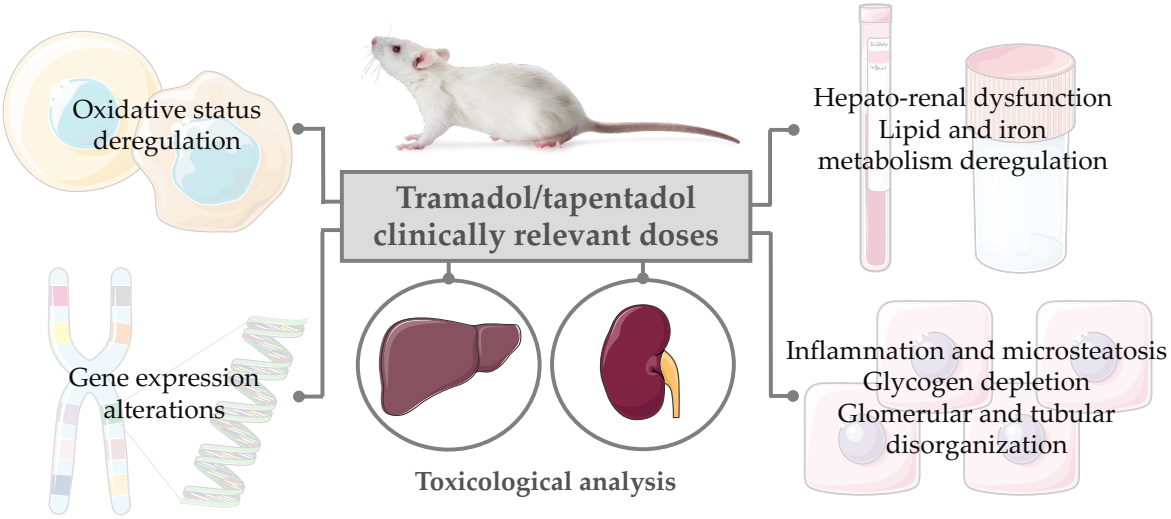
Graphical Abstract



Systemic and organ-specific (liver and kidney) effects of the repeated exposure of Wistar rats to clinically relevant doses of tramadol and tapentadol, as assessed through oxidative stress, molecular, biochemical and histological analysis

pharmaceuticals

MDPI

Article

Repeated Administration of Clinical Doses of Tramadol and Tapentadol Causes Hepato- and Nephrotoxic Effects in Wistar Rats

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Abstract: Tramadol and tapentadol are fully synthetic and extensively used analgesic opioids, presenting enhanced therapeutic and safety profiles as compared with their peers. However, reports of adverse reactions, intoxications and fatalities have been increasing. Information regarding the molecular, biochemical, and histological alterations underlying their toxicological potential is missing, particularly for tapentadol, owing to its more recent market authorization. Considering the paramount importance of liver and kidney for the metabolism and excretion of both opioids, these organs are especially susceptible to toxicological damage. In the present study, we aimed to characterize the putative hepatic and renal deleterious effects of repeated exposure to therapeutic doses of tramadol and tapentadol, using an in vivo animal model. Male Wistar rats were randomly divided into six experimental groups, composed of six animals each, which received daily single intraperitoneal injections of 10, 25 or 50 mg/kg tramadol or tapentadol (a low, standard analgesic dose, an intermediate dose and the maximum recommended daily dose, respectively). An additional control group was injected with normal saline. Following 14 consecutive days of administration, serum, urine and liver and kidney tissue samples were processed for biochemical, metabolic and histological analysis. Repeated administration of therapeutic doses of both opioids led to: (i) increased lipid and protein oxidation in liver and kidney, as well as to decreased total liver antioxidant capacity; (ii) decreased serum albumin, urea, butyrylcholinesterase and complement C3 and C4 levels, denoting liver synthesis impairment; (iii) elevated serum activity of liver enzymes, such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and γ-glutamyl transpeptidase, as well as lipid profile alterations, also reflecting hepatobiliary commitment; (iv) derangement of iron metabolism, as shown through increases in serum iron, ferritin, haptoglobin and heme oxygenase-1 levels. In turn, elevated serum cystatin C, decreased urine creatinine output and increased urine

microalbumin levels were detected upon exposure to tapentadol only, while increased serum amylase and urine *N*-acetyl-β-D-glucosaminidase activities were observed for both opioids. Collectively, these results are compatible with kidney injury. Changes were also found in the expression levels of liver- and kidney-specific toxicity biomarker genes, upon exposure to tramadol and tapentadol, correlating well with alterations in lipid profile, iron metabolism and glomerular and tubular function. Histopathological analysis evidenced sinusoidal dilatation, microsteatosis, mononuclear cell infiltrates, glomerular and tubular disorganization, and increased Bowman's spaces. Although some findings are more pronounced upon tapentadol exposure, our study shows that, when compared with acute exposure, prolonged administration of both opioids smooths the differences between their toxicological effects, and that these occur at lower doses within the therapeutic range.

Keywords: tramadol; tapentadol; prescription opioids; hepatotoxicity; nephrotoxicity; in vivo studies

1. Introduction

Opioid drugs that produce morphine-like effects by interacting with opioid receptors are a cornerstone of moderate to severe, malignant and non-malignant pain treatment, both in acute and chronic settings [1–5]. Their widespread prescription, abuse, misuse and related mortality has increased in developed countries, contributing to an "opioid crisis" in the United States of America and reinforcing the scrutiny over their benefit-risk balance [6–11]. In other continents, including Asia, Northern and Western Europe, albeit the situation is not as dramatic, it is still raising serious public health issues and awareness [3–7,9,12–18].

Tramadol ((1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol) and tapentadol (3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol) are fully synthetic analgesic opioids that synergistically combine mu-opioid receptor (MOR) agonism with monoamine reuptake inhibition, justifying their classification as "atypical opioids" [1,2,11,19–26]. Such dual mechanism of action optimizes analgesia and minimizes opioid-typical side effects, such as drowsiness, nausea, vomiting, constipation, motor incoordination and respiratory depression [1,2,11,19,27], explaining their indications for the treatment of post-surgical, musculoskeletal, inflammatory, cancer and neuropathic pain, as well as mixed pain states [1,19,27–32]. Also, owing to the synergistic combination of their mechanisms of action, these opioids allow the dose administered to be reduced without compromising analgesic efficacy, thus reducing the potential for abuse and addiction [11,33].

Tramadol is commercially available as a racemate; while (+)-tramadol provides for serotonin (5-HT) reuptake inhibition, (−)-tramadol accounts for noradrenaline (NA) uptake inhibition [1,2,11,19,32,34–37]. It undergoes extensive hepatic metabolism, mainly through O- and N-demethylation and conjugation reactions, yielding at least 14 phase I and 12 phase II metabolites [1,2,11,35,37–39]. Ninety percent of racemic tramadol elimination is ensured by the kidneys, with an elimination half-life of 5–6 h [1,2,19,35,38].

Tapentadol has been developed from the structures of tramadol, *O*-desmethyltramadol and morphine, having been more recently made available on the market [1,40,41]. It acts mainly on NA reuptake inhibition and has minimal 5-HT activity, thus minimizing serotonin syndrome liability [1,2,25,27,30,32,33,36,39,42–45]. It is metabolized mainly through phase II glucuronidation and sulphonation reactions [1,2,25,36,39,43–45]. Kidneys are also the major elimination route for tapentadol, accounting for 99% of its excretion; its elimination half-life is about 4 or 5–6 h (for immediate and prolonged release formulations, respectively) [1,2,38,39,44,46]. Its mu-load, i.e., the contribution of the opioid component to the adverse effect magnitude, in relation to pure MOR agonists at equianalgesia, has been estimated as ≤40% [30,43]. It is argued to be an upgrade of comparable opioids, particularly tramadol, whose drawbacks have inspired tapentadol design [1,2]. However, its shorter market history

limits the amount of clinical and toxicological information on its use, hindering a true comparison between both opioids [1,2,32,42].

Although tramadol and tapentadol are claimed to have better safety profiles than their opioid peers, several adverse events have been reported, including nausea, vomiting, dizziness, seizures, dyspnea, respiratory depression [1,2,14,47–51], and even fatal cases [10,42,51–68]. In the VigiBase™ World Health Organization (WHO) Global Database of Individual Case Safety Reports concerning 5-HT toxicity, tramadol ranks 1st and tapentadol ranks 3rd (with 647 and 115 cases out of 1641, respectively) as the only suspected cause or amongst other drugs, and 1st and 2nd (with 62 and 42 cases out of 147, respectively) as the only suspected cause [34,69]. In addition, in spite of their theoretically lower potential for abuse and dependence, cases of misuse, dependence and addiction have been reported [1,2,10,11,14,60,70]. Therefore, while their public health burden is reported to be low, it is not absent [10,33].

Considering the roles of liver and kidney on tramadol and tapentadol metabolism and excretion, these organs are particularly liable toxicity targets. A case cross-over study addressing the period of 2004–2013 identifies an association between tramadol use and increased mortality risk, with renal and hepatic disease representing prominent risk factors [51]. Accordingly, in vivo studies document the hepato- and nephrotoxicity of various opioids, particularly tramadol, morphine, and heroin. Such studies, mainly performed in rodents, encompass several routes of administration (e.g., oral, intraperitoneal (i.p.), intramuscular, subcutaneous), exposure periods ranging from acute to chronic, and doses ranging from therapeutic ones to overdoses. All have shown liver and kidney commitment, evidenced through increased liver enzyme activities, blood urea nitrogen (BUN), creatinine [71–87], tissue oxidative markers (e.g., increased liver and kidney malondialdehyde (MDA) levels) [75,76,80,82,84,88–90], as well as through decreased antioxidant activity (e.g., decreased catalase, superoxide dismutase and glutathione peroxidase activities, decreased glutathione levels) [76,80,82–84,88–91]. Hepato- and nephrotoxicity were also observed at the histological level. Liver histological findings include centrilobular congestion, cytolysis and sinusoidal dilatation [71,73,74,76–79,81,84,85,87,88,92–96], while kidney histopathology comprises endothelial cell swelling, atrophied glomeruli with collapsed tufts, wide Bowman's spaces and interstitial nephritis; in turn, inflammatory cell infiltration, vacuolization, degeneration, focal necrosis, hemorrhage and fibrosis have been reported for both organs [71,74,76–78,81,84,85,88,95].

In line with this, previous studies by our group have shown toxicological damage, using in vitro and in vivo approaches, following an acute exposure to tramadol and tapentadol [97–99]. In particular, hepato- and nephrotoxicity were found upon Wistar rat exposure to therapeutic doses [98]. Nevertheless, to our knowledge, no similar comparative studies concerning short-term, repeated therapeutic dose administrations, are available. In this context, in the present study, we aimed to characterize the putative hepato- and nephrotoxic effects resulting from the repeated administration of clinical doses of tramadol and tapentadol at the molecular, biochemical, and histological levels, at a subacute time point that precedes most of those assayed in comparable studies. Also, we aimed to ascertain whether these effects are intensified along with the exposure, as compared to our acute context results. The importance of this work is further underlined by opioid use on a frequently subacute, sub-chronic and chronic basis, as well as by the gap of toxicological information on tapentadol.

2. Results

2.1. Repeated Exposure to Tramadol and Tapentadol Causes Oxidative Stress and Differentially Changes the Antioxidant Status of Liver and Kidney

To characterize the effect of tramadol and tapentadol repeated administration of therapeutic doses in liver and kidney oxidative stress, thiobarbituric acid reactive substances (TBARS) and carbonyl groups, biomarkers of lipid and protein oxidative stress, respectively, were quantified in tissue homogenates. Additionally, the total antioxidant capacity was determined in the same samples, through spectrophotometry. Results are depicted in Figure 1.

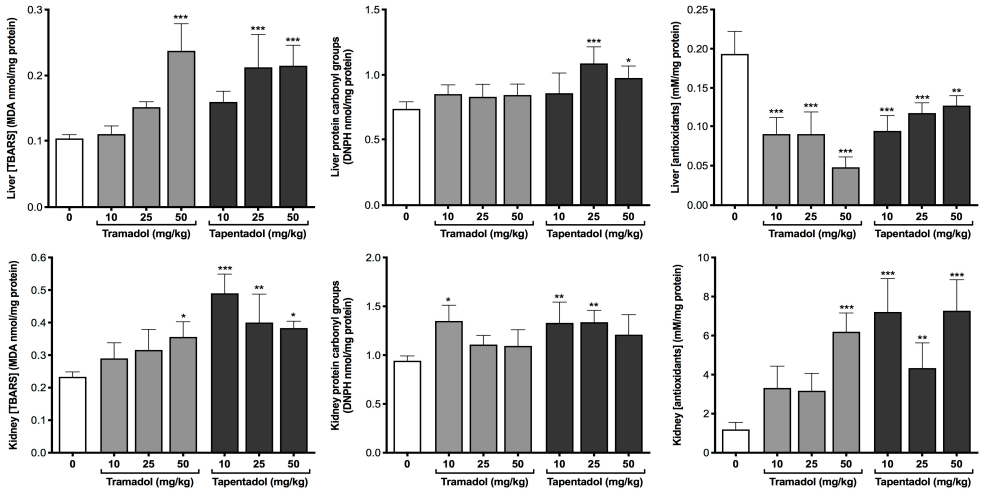

Figure 1. Liver and kidney oxidative stress analysis, assayed as thiobarbituric acid reactive substances (TBARS), protein carbonyl groups and total antioxidant capacity (Trolox equivalents), in Wistar rat tissue homogenates prepared upon daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results were normalized against total protein content and are expressed by means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. MDA: malondialdehyde; DNPH: 2,4-dinitrophenylhydrazine.

Both opioids led to increased TBARS levels; while tramadol caused a significant increase at 50 mg/kg only, in both liver and kidney, tapentadol led to the same effect at 25 and 50 mg/kg in liver, and at all doses in kidney. Protein carbonyl groups increased at the intermediate and highest tapentadol doses only (liver), while in kidney such increase was observed at the lowest tramadol and lowest and intermediate tapentadol doses. In turn, the total antioxidant capacity is significantly lower in liver at all doses of both opioids, but augmented at the highest tramadol dose and at all tapentadol doses in kidney. Thus, it might be hypothesized that liver and kidney respond differently to oxidative insult and that it has a differential impact on the antioxidant status of these organs.

2.2. Repeated Exposure to Tramadol and Tapentadol Compromises Liver and Kidney Metabolic and Excretion Functions

A battery of biochemical and immunological parameters was quantified in serum and urine samples to get an insight into the putative metabolic and inflammatory effects of the repeated exposure to tramadol and tapentadol clinical doses. Serum results are represented in Figures 2–5, while urinary determinations appear in Figure 5 only.

Figure 2 concerns liver enzymes—alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT) and butyrylcholinesterase (BuChE)—and immunological parameters of hepatic origin—α-1-acid glycoprotein and complement component 3 (C3) and 4 (C4) proteins. The activity of all liver enzymes, except for BuChE, was found to be significantly increased at almost all doses of both tramadol and tapentadol, with ALT activity rising around 3-fold, AST 2-fold, and ALP 1.6-fold, on average, above the control. GGT activity increased roughly 2.5-fold at 10 and 25 mg/kg tramadol, while tapentadol led to an approximate average increase of 2.0-fold, irrespectively of the dose. Although α-1-acid glycoprotein concentrations did not change in a statistically significant manner, BuChE activity and complement C4 levels decreased to about 46% and 63% of the control values, respectively, at all opioid doses. Complement C3 levels decreased significantly to about 72% of the control at the highest tramadol and tapentadol dose.

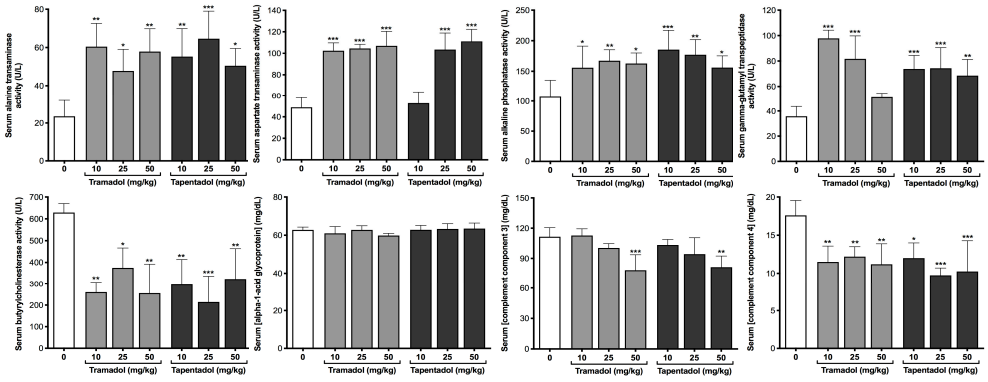

Figure 2. Concentrations of serum biochemical parameters, concerning liver synthetic function and liver function tests, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

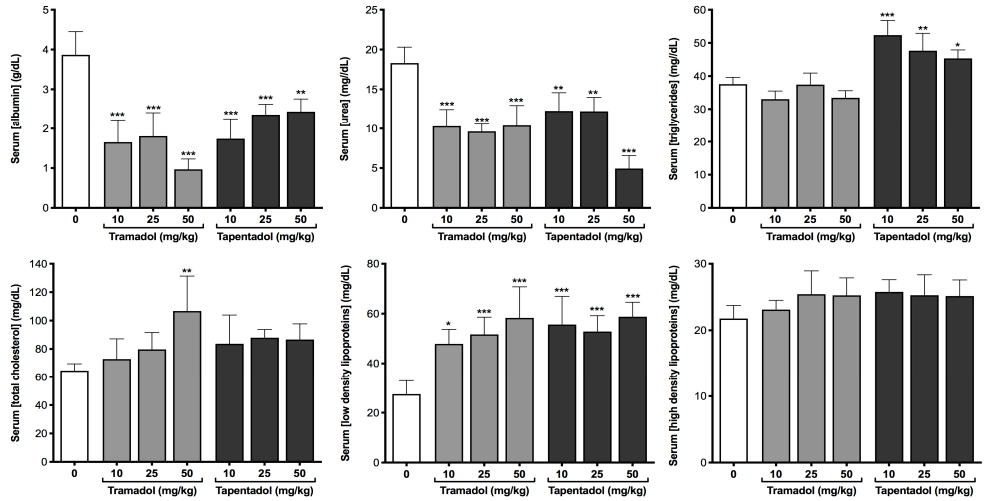

Figure 3. Concentrations of serum biochemical parameters, concerning liver synthetic function and lipid profile, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 3 data illustrates liver synthetic function and lipid profile. Although serum total proteins had no statistically significant changes (results not shown), serum albumin and urea levels are markedly decreased in all experimental groups; while albumin concentration decreases to about 25% and 63% of the control values at 50 mg/kg tramadol and tapentadol, respectively, urea decreases to about 60% at all opioid doses except at 50 mg/kg tapentadol, where it reaches 27% of the control values. In turn, serum lipid parameters also denote alterations in the lipid profile. While only tapentadol leads to significant increases in triglyceride levels, total cholesterol increased solely at the highest tramadol dose. Low-density lipoprotein (LDL) cholesterol increased upon repeated administration of all opioid doses, whilst no statistically significant differences were found between control and experimental

groups for high-density lipoprotein (HDL) cholesterol. Together with those from Figure 2, these data support liver damage at different levels.

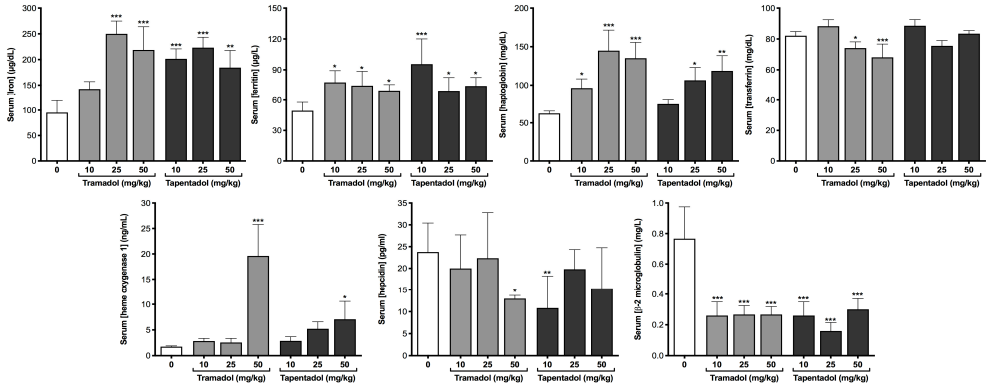

Figure 4. Concentrations of serum biochemical parameters, concerning iron metabolism, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

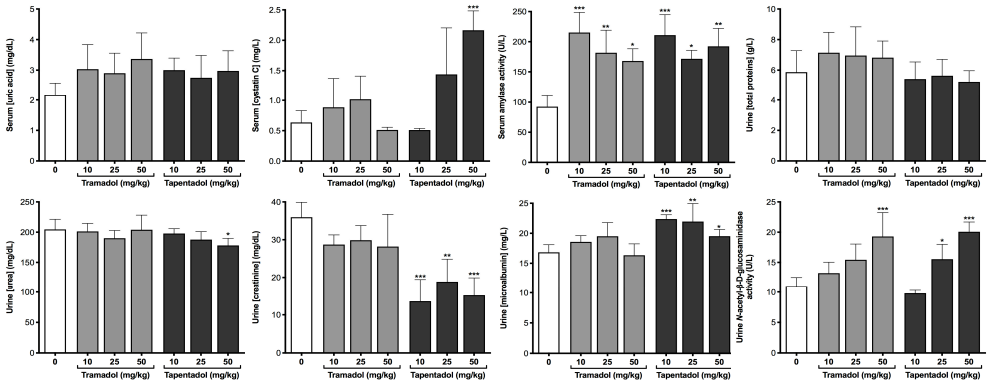

Figure 5. Concentrations of serum and urine biochemical parameters, concerning kidney function, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Collectively, the parameters in Figure 4 reflect changes in iron metabolism. The average 2.3-fold increase in serum iron concentrations, observed at almost all opioid doses, was accompanied by increases in ferritin (1.6-fold, on average), in haptoglobin (to a maximum of 2.3-fold), and heme oxygenase 1 (HO-1, whose activity increased 11.2-fold and 4.0-fold upon exposure to 50 mg/kg tramadol and tapentadol, respectively). In addition, serum transferrin levels were found to decrease at 25 and 50 mg/kg tramadol doses, while serum hepcidin concentration significantly decreased at tramadol and tapentadol highest and lowest dose, respectively. β-2-Microglobulin (B2M) markedly decreased to about 33% of the control values, irrespectively of the opioid and dose considered.

In turn, Figure 5 shows the results concerning kidney function biomarkers. While there were no statistically significant increases in serum uric acid concentrations, serum cystatin C levels were significantly elevated at 50 mg/kg tapentadol, and amylase activity nearly doubled at all doses of both opioids. Figure 5 also encompasses data obtained from the analysis of Wistar rat urine samples.

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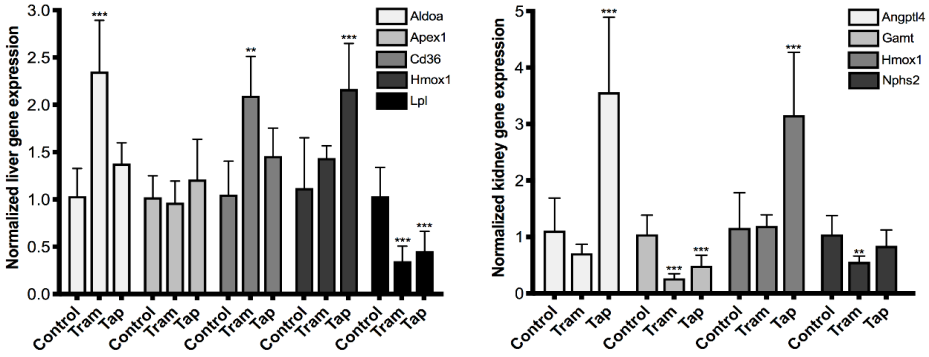
While there were no statistically significant changes in total protein concentration between control and experimental groups, urea levels significantly decreased at 50 mg/kg tapentadol, paralleling the more pronounced decrease found in serum samples from this group (Figure 3). All tapentadol doses led to a decrease in creatinine urinary elimination (with the values reaching 44% of the control) and an increase in urine microalbumin levels. In turn, tramadol highest dose and tapentadol intermediate and highest doses caused an increase in *N*-acetyl-β-D-glucosaminidase (NAG) activity (1.8-fold average increase at 50 mg/kg opioid).

Taken as a whole, Figure 5 substantiates that there are renal changes following repeated administration of tramadol and tapentadol therapeutic doses.

2.3. Repeated Exposure to Tramadol and Tapentadol Leads to Changes in the Gene Expression of Liver and Kidney Toxicity Biomarkers

Aiming at the characterization of the putative hepato-renal impact of the repeated exposure to tramadol and tapentadol clinical doses, a small-scale gene expression profiling was performed through quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), for a selection of toxicity biomarkers (Figure 6). RNA was isolated from liver and kidney specimens from Wistar rats exposed to 50 mg/kg tramadol and tapentadol, and gene expression levels were compared to those of the control (non-treated) group.



(**a**) (**b**)

Figure 6. Normalized gene expression levels of liver (**a**) and kidney (**b**) toxicity biomarkers, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 50 mg/kg tramadol (Tram) or tapentadol (Tap), for 14 consecutive days. Expression levels were normalized against the respective 18S ribosomal RNA (18S rRNA) gene expression, and then against the respective controls (administered with normal saline), set as 1. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$. Aldoa: fructose-bisphosphate aldolase A; Angptl4: angiopoietin-like 4; Apex1: apurinic/apyrimidinic endonuclease 1; Cd36: cluster of differentiation 36/fatty acid translocase; Gamt: guanidinoacetate *N*-methyltransferase; Hmox1: heme oxygenase 1; Lpl: lipoprotein lipase; Nphs2: podocin.

Regarding the liver toxicity biomarker panel (Figure 6a), tramadol led to increases in fructose-bisphosphate aldolase A (Aldoa, 2.3-fold) and cluster of differentiation 36/fatty acid translocase (Cd36, 2.0-fold) gene expression, while tapentadol also approximately doubled that of heme oxygenase 1 (Hmox1). Lipoprotein lipase (Lpl) gene expression was found to be reduced upon exposure to both opioids (reaching 34% and 44% of the control for tramadol and tapentadol, respectively), whilst no significant changes were detected in apurinic/apyrimidinic endonuclease 1 (Apex1) expression. As far as the kidney panel is concerned (Figure 6b), angiopoietin-like 4 (Angptl4) and Hmox1 expression roughly triplicated upon tapentadol exposure. In turn, guanidinoacetate *N*-methyltransferase (Gamt)

gene expression decreased after exposure to tramadol and tapentadol (achieving 26% and 47% of the control, respectively), while only tramadol led to a significant decrease in podocin (Nphs2) expression (reaching 53% of the control). Therefore, the expression of almost all genes under study was found to be altered upon exposure to at least one of the opioids, showing that tramadol- and tapentadol-induced hepato- and nephrotoxicity also have gene expression implications.

2.4. Repeated Exposure to Tramadol and Tapentadol Leads to Glycogen Depletion, Microsteatosis and Inflammation in Liver and Kidney, and to Fibrous Tissue Deposition between Hepatocytes

The in vivo effects of repeated tramadol and tapentadol administration were also studied at the histopathological level, by comparing liver and kidney specimens from Wistar rats exposed to 10, 25 and 50 mg/kg tramadol or tapentadol with those from controls, injected with saline. Liver tissue samples were stained with hematoxylin & eosin (H&E, Figure 7), periodic acid-Schiff (PAS, Figure 8) and Masson's trichrome (Figure 9) procedures. Kidney tissue samples were stained with H&E (Figure 10). H&E staining evidences cell nuclei as blue, extracellular matrix and cytoplasm as pink and other cell structures as different shades and combinations of these colors, providing an overview of the tissue's structure. PAS staining, in turn, detects polysaccharides and mucosubstances, while Masson's trichrome is a three-color protocol that stains nuclei dark red/purple, cytoplasm red/pink and connective tissue blue.

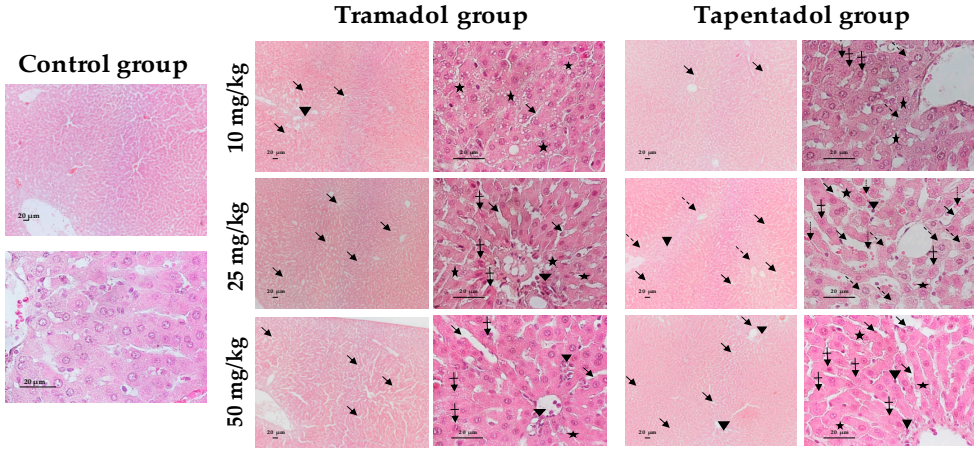


Figure 7. Liver sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group) for 14 consecutive days, upon hematoxylin & eosin (H&E) staining. Mononuclear inflammatory infiltrates (inverted triangles), sinusoidal dilatation (arrows), vacuolization/microsteatosis (stars), fragmented nuclei/loss of definition of nuclear membranes (vertical, crossed arrows), hypopigmented areas (dashed arrows) and vascular congestion/erythrocyte extravasation (vertical, dotted arrows) are observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

The controls showed the typical liver tissue architecture, with polyhedral hepatocytes arranged in cords, separated by sinusoids and radiating from the central vein to the portal areas, with a granular eosinophilic cytoplasm [100]. However, all three staining methods evidenced the presence of histological alterations in liver sections from the experimental groups, such as sinusoidal dilatation and vacuolization, which was valued as microsteatosis (Figures 7–9). Sinusoidal dilatation became increasingly patent along with tramadol dose, whilst it was found beyond perivascular regions, denoting more extensive damage, on tapentadol slides (Figures 7 and 8).

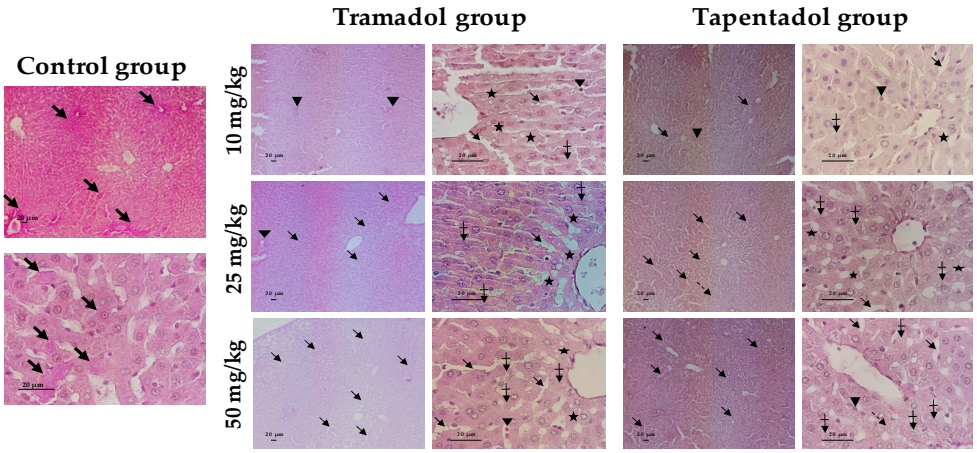


Figure 8. Liver sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group) for 14 consecutive days, upon periodic acid-Schiff (PAS) staining. Glycogen granules appear as purple areas (thick arrows). Mononuclear inflammatory infiltrates (inverted triangles), sinusoidal dilatation (arrows), vacuolization/microsteatosis (stars), fragmented nuclei/loss of definition of nuclear membranes (vertical, crossed arrows) and hypopigmented areas (dashed arrows) are observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

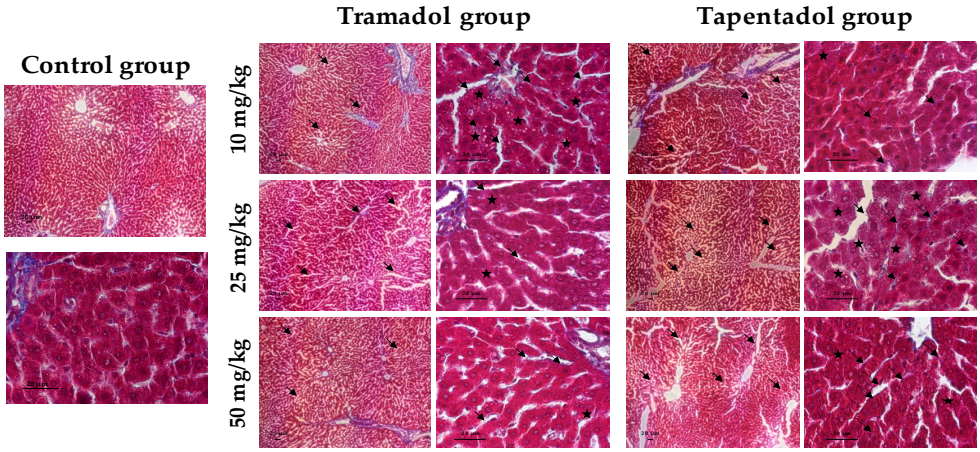


Figure 9. Liver sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group) for 14 consecutive days, upon Masson's trichrome staining. Sinusoidal dilatation (arrows) and vacuolization (stars) are observed. Traces of fibrous tissue (dotted arrows) are found between hepatocytes. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

Mononuclear cell infiltrates were observed at all tramadol doses, although for tapentadol they became more evident along with the dose (Figures 7 and 8). Some cells displayed poorly contoured nuclei, often with a fragmented appearance, upon exposure to both opioids (Figures 7 and 8). Signs of vascular congestion/erythrocyte extravasation were apparent in H&E sections from the

tapentadol group (Figure 7), which also produced hypopigmented areas through H&E and PAS methods (Figures 7 and 8).

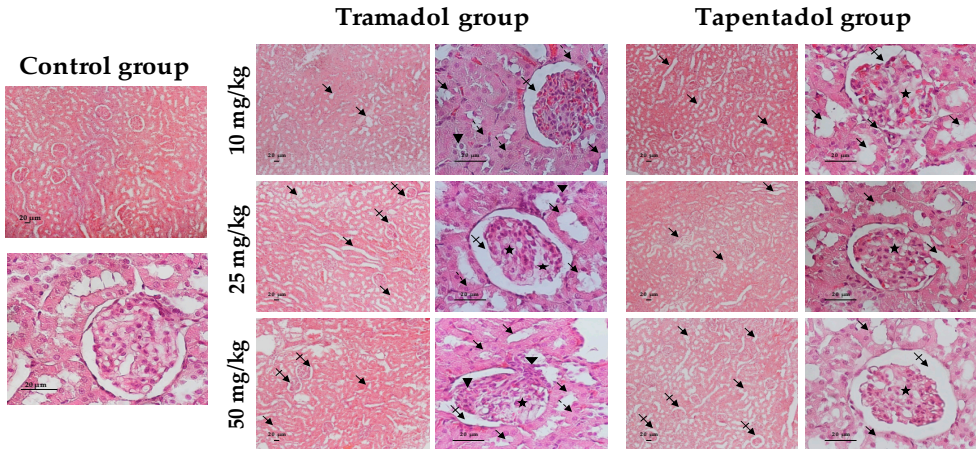


Figure 10. Kidney sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group) for 14 consecutive days, upon hematoxylin & eosin (H&E) staining. Inflammatory mononuclear cell infiltrates (inverted triangles), increased Bowman's spaces (crossed arrows), disorganized and vacuolized glomeruli (stars), swollen cells (dashed arrows), and disorganized and poorly contoured tubules (arrows) are observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

All tramadol and tapentadol doses led to glycogen depletion, as inferred from the weaker purple staining in the experimental groups, when compared with the controls (Figure 8).

In turn, Masson's trichrome staining allowed the identification of fibrous tissue between hepatocytes at all doses of both opioids, though more abundant and thicker for tapentadol, whose dose increments seemingly intensified this effect (Figure 9).

As far as kidney sections are concerned (Figure 10), the control shows the expected histology, with glomeruli composed of capillary tufts lying within the Bowman's capsule, from which they are separated by narrow Bowman's spaces, and a network of proximal and distal tubules. The microscopic analysis of experimental group slides reveals that both opioids led to tubule disorganization at all doses studied. Glomeruli also appeared disorganized and vacuolated at all tapentadol doses, although for tramadol such observation became more obvious at 25 and 50 mg/kg; in contrast, increased Bowman's spaces were seen at all tramadol doses, but were more evident at 50 mg/kg tapentadol only. In addition, tramadol exposure was associated with the presence of inflammatory cell infiltrates and swollen cells.

Thus, a combined analysis of the results from the three staining methods shows the presence of histological signs compatible with toxicological damage at all doses of both opioids. Whether these signs are dose-dependent or -independent, it varies according to the opioid and finding considered.

3. Discussion

Tramadol and tapentadol are two prescription opioids widely used in the treatment of moderate to severe forms of pain. Their generalized prescription is greatly due to their therapeutic efficiency and safety, owing to their synergistic and atypical mechanism of action. Nevertheless, adverse events and fatalities have been reported and, given their common use on a repeated and chronic basis, concerns about dependence liability and abuse potential have been rising. Considering that liver and kidney are central players in tramadol and tapentadol pharmacokinetics, we aimed to study their putative hepato- and nephrotoxic effects, in an in vivo model submitted to repeated administration of therapeutic doses.

This is, to our knowledge, the first study addressing tramadol and tapentadol comparative toxicity upon repeated administration. The effects of an acute exposure of Wistar rats to the same doses were already reported by our own research team [98,99]. Since hepato- and nephrotoxicity have already been demonstrated on such acute settings [98], and considering that tramadol and tapentadol are often consumed for longer periods, the present study not only broadens the picture provided by our acute exposure assays, but also more closely reflects the real consumption scenario for both opioids.

It should be stressed that, in spite of their chemical resemblance, there are differences between tramadol and tapentadol regarding receptor and transporter affinity, as well as their pharmacokinetics, metabolite profiles and pharmacodynamics, which may also account, to some extent, for different results [1,2,98,99]. The route of tramadol and tapentadol administration used in our study also deserves an additional important remark. Despite bypassing the intestine, i.p. injection resembles oral administration from a pharmacokinetic point of view, since drugs are absorbed into the mesenteric vessels draining into the portal vein [101]. Therefore, they may undergo hepatic metabolism before reaching systemic circulation. In this sense, given that the two opioids have different bioavailabilities (68–84% and 32% for tramadol and tapentadol, respectively, upon oral administration [1,2,35]), the doses used in our study, although mathematically equal, were not pharmacologically equivalent. From this perspective, to ensure pharmacological equivalence, tapentadol doses should be increased. Such approach would further accentuate differences in the results obtained for some of the parameters discussed below.

3.1. Repeated Exposure to Tramadol and Tapentadol Induces Hepato-Renal Oxidative Stress, Affecting Liver and Kidney Cell Integrity and Function

The association between opioid exposure and oxidative stress is well documented. Multiple studies report increased MDA levels in liver, kidney and serum upon opioid repeated administration, such as those from Awadalla, El-Gaafarawi, Elkhateeb, Ibrahim and their respective colleagues, who orally administered rats with 30 to 150 mg/kg tramadol, for 20 to 30 days [75,76,84,88,89], as well as similar studies with morphine [80,82] and heroin [90]. These studies have also associated tramadol exposure with decreased levels of antioxidant defenses, such as reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase in liver and kidney tissues [76,84,88], as well as in serum [89]. Studies concerning repeated administration of morphine in mice have also led to similar results in liver [82,91].

In the present study, an increase in TBARS and protein carbonyl groups was found in liver and kidney homogenates, following repeated exposure to clinical doses of both opioids, particularly for tapentadol (Figure 1). While the same trend was found for protein carbonyl groups in our previous acute exposure assays [98], TBARS results were different, as their liver and kidney levels were decreased upon acute exposure [98], but increased upon repeated exposure. This suggests that the protective effect against lipid peroxidation (LPO), hypothesized for acute exposure settings [98], is lost upon repeated administration. Also, TBARS and protein carbonyl groups data may be paralleled with total antioxidant capacity results (Figure 1). It might be argued that, while hepatocytes experience increased oxidative stress as a result of a decreased antioxidant capacity (as seen through increased TBARS and protein carbonyl groups), kidney cells increase their antioxidant capacity as a response to opioid-induced oxidative protein damage, thus possibly explaining carbonyl group results, whose increase is not statistically significant for the highest doses (Figure 1). Taken together, our results show that, as for similar studies, the induction of lipid and protein oxidative stress is a toxicity mechanism associated with in vivo repeated administration of tramadol and tapentadol, even at therapeutic doses.

3.2. Repeated Exposure to Tramadol and Tapentadol Causes Cumulative Hepatocellular and Hepatobiliary Damage

In an attempt to further characterize the hepatic effects of tramadol exposure, several studies have also reported increased serum ALT, AST, ALP and GGT activities following repeated administration of

rodents with doses ranging from 3 to 200 mg/kg, through different routes [71–79,81,84]. Analogous assays with morphine have led to similar results [80,82,87]. In accordance, ALT, AST, ALP and lactate dehydrogenase activities were found to be elevated among tramadol abusers [102]. In a case report concerning fatal hepatic failure following accidental tramadol overdose, ALT and AST activities increased by more than 30-fold in relation to the reference range, while GGT was close to the upper reference limit [59]. In line with this, we found serum ALT and AST activities to be increased at almost all doses of both tramadol and tapentadol (Figure 2), with the increase in ALT, a more sensitive and hepatospecific enzyme than AST [103–105], being higher. Such results are compatible with membrane leakage, which may be promoted by oxidation of the lipid membrane components [98,106], from which TBARS are biomarkers. ALP and GGT activities were also found to be increased; since their synthesis increases and their excretion is blocked in case of intra or extrahepatic obstruction, both are cholestasis biomarkers [103,104]. Consequently, tramadol- and tapentadol-induced hepatotoxicity involve hepatocellular and hepatobiliary injury. Regarding ALT activity, the increase obtained upon a 14-day administration, at all doses, approximately doubled that of a single administration of 50 mg/kg tramadol or tapentadol [98]; similarly, while ALP activity did not change significantly as a result of an acute treatment [98], it increased at almost all opioid doses following repeated administration. Therefore, we might anticipate that hepatocyte and hepatobiliary damage is cumulative.

3.3. Repeated Exposure to Tramadol and Tapentadol Compromises Liver Synthesis

As seen in our acute exposure assays, serum BuChE activity decreased at all tramadol and tapentadol doses when administered repeatedly (Figure 2). BuChe has been described as a sensitive marker of liver parenchyma cell inflammation and damage in patients with chronic hepatitis, with lower serum levels indicating higher severity of liver fibrosis [104,107]. However, as previously discussed [98], decreased BuChE activity might result from opioid-induced inhibition, besides defective BuChE hepatic synthesis [98,104,107]. Since our liver histopathological analysis evidences fibrous tissue deposition, but no signs of marked fibrosis (Figure 9), reduced BuChE activity may reflect both phenomena and ultimately indicate the potential for progression to fibrosis.

The metabolic impact of the exposure to both opioids has also been studied. While serum α-1-acid glycoprotein levels did not change significantly (Figure 2), serum complement C3 and C4 (Figure 2), albumin and urea (Figure 3) concentrations decreased upon exposure to tramadol and tapentadol, at almost all doses. In the case of urea, its urinary output is also lower at 50 mg/kg tapentadol, probably because of its decreased production (Figure 5). Urea concentrations had already been found to be diminished in our previous acute administration assays [98], though serum levels had decreased significantly for the 50 mg/kg tramadol/tapentadol only. In this context, the quantification of serum ammonia would provide additional information. In turn, albumin levels were found to be decreased in a tramadol-induced fatal overdose with liver failure [59]. Also, decreased serum albumin and total proteins were reported in opium-addicted diabetic males [108], as well as upon repeated intramuscular administration of 40 mg/kg tramadol [86]. Such results show that liver synthetic function is impaired, since these analytes are exclusively or primarily produced by this organ [103]. Indeed, liver disease is associated with hypocomplementemia: it is due to decreased C3 and C4 synthesis in fulminant hepatic failure, whilst in chronic active hepatitis it results from the formation of immune complexes and consequent complement activation [109]. A possible explanation for the fact that α-1-acid glycoprotein was the only protein whose levels did not change is its considerably longer half-life (164.8 h in rats) [110,111], when compared with those of complement C3 and C4 (46-70 h in humans) [112], albumin (2.6 h in rats) [113] and urea (5 h in rats) [114]. Therefore, due to its longer half-life, α-1-acid glycoprotein is not as useful as complement proteins as a biomarker to evaluate acute or subacute toxic exposures.

3.4. Repeated Exposure to Tramadol and Tapentadol Affects Lipid Profile, Correlating with Hepatobiliary Commitment and Lipid Deposition

The lipid profile is also altered, with increased triglyceride levels at all tapentadol doses, increased total cholesterol at 50 mg/kg tramadol, and increased LDL cholesterol at all doses from both opioids (Figure 3). No significant changes were identified regarding HDL cholesterol (Figure 3). When compared with our previous acute exposure results [98], the increase in triglyceride and LDL cholesterol levels is now extensible to more doses, suggesting that the derangement in lipid metabolism is also cumulative. While human studies are inconsistent, animal assays with opium, morphine, heroin and tramadol have proven to be more conclusive towards a deleterious impact of opioid use on lipid profile and dyslipidemia [115–117]. Although El-Gaarafawi, Youssef and Othman and respective colleagues have reported decreased serum cholesterol, triglycerides and lipid-derived hormones [75,81,90], Ezzeldin and co-workers have reported increased cholesterol [77]. Also, while assays with healthy, hypercholesterolemic and diabetic rodents, mostly comprising oral opium administration for 1 to 3 months, have shown no major effects on serum lipid parameters [118–120], others have reported increased serum triglycerides, total and LDL cholesterol, and decreased HDL cholesterol [121–124]. In this context, various mechanisms have been proposed to explain the action of opium consumption on blood and tissue lipids [115,116]. Short-term effects may be justified by increased lipolysis in adipose tissue, increased lipogenesis in liver [125] and decreased biliary cholesterol excretion [121], the latter being corroborated by our ALP and GGT results. In turn, long-term outcomes may derive from liver damage and insufficient lipid turnover [82,91], decreased hepatic LDL clearance and increased hepatic triglyceride synthesis [126], among others [115]. Overall, these mechanisms explain the most frequent serum lipid findings in animal studies – unchanged or increased triglycerides, total and LDL cholesterol, as well as unchanged or decreased HDL cholesterol [119,127]—which are substantiated in our own study. Interestingly, since cholesterol has a prominent role on the central nervous system and on synaptic plasticity [128], a relationship with drug addiction might be remotely implied and remains a subject for further scrutiny.

3.5. Repeated Exposure to Tramadol and Tapentadol Affects Iron Metabolism, Correlating with Oxidative Stress, Cellular Damage, Inflammation and Steatosis

Tramadol and tapentadol repeated administration also impacted iron metabolism, as increased serum iron levels were also found in most opioid experimental groups (Figure 4). In line with our results, a comparative study between non-insulin-dependent diabetes mellitus opium-addicts and non-addicts showed increased iron levels in addicted males [108]. Indeed, iron is implicated in dopamine synthesis and monoamine metabolism, having been shown to accumulate in specific brain regions in chronic cocaine use [129,130]. It is noteworthy that free iron may generate reactive oxygen species (ROS), such as the powerful hydroxyl radical, via Fenton chemistry—thereby worsening inflammation—and is profibrogenic [131,132]. Alterations in serum iron levels prompted the investigation of iron metabolism-related parameters (Figure 4). Serum ferritin, haptoglobin and HO-1 levels increased upon tramadol and tapentadol treatment; transferrin decreased upon tramadol exposure, while hepcidin decreased for tramadol highest dose and tapentadol lowest dose. In turn, B2M concentrations decreased at all opioid doses (Figure 4).

Ferritin is a positive acute phase protein (APP) [133], whose synthesis increases in case of oxidative stress and inflammation, or due to increased iron uptake by hepatocytes [134,135]. Since it is a safe form of iron storage, its serum form is argued to arise from damaged cells, thus representing a cellular damage marker [135,136]. Serum ferritin levels correlate with serum markers of hydroxyl radical formation, including MDA [136]. In this context, it has been hypothesized that, unlike its intracellular form, serum ferritin releases iron, which induces hydroxyl radical formation and consequent oxidative stress [136]. Therefore, increased serum ferritin levels are consistent with elevated serum iron concentrations, as well as with our results regarding oxidative stress and hepatocyte damage.

Hepcidin, in turn, is a hormone that binds ferroportin and elicits its internalization and degradation, preventing iron release from macrophages, hepatocytes and enterocytes [131,135,137,138]. Though hepcidin levels did not change significantly at all opioid doses, its decrease at tramadol and tapentadol highest and lowest doses, respectively, might also account, at least in part, for increased serum iron availability.

Serum B2M is a small protein that non-covalently binds to the other polypeptide chain to form major histocompatibility complex (MHC) class I or MHC I-like structures, including human hemochromatosis protein (HFE) [139–142]. Since it is filtered in the glomeruli and massively reabsorbed in the proximal tubules, low serum and high urine concentrations indicate renal tubular disease [139–145]. Although this condition could be hypothesized in view of decreased serum B2M at all opioid doses, increases in its urine levels were not statistically significant (results not shown). However, an association between B2M, hepcidin and iron circulating levels might be postulated. B2M interacts with HFE in order to allow its surface expression; this, in turn, interacts with hepcidin, which prevents intracellular iron release. Thus, B2M influences iron uptake and efflux mediated by HFE and hepcidin, respectively [138]. Indeed, B2M-deficient mice present iron overload and hemochromatosis, whose pathogenesis likely involves other B2M-interacting protein(s) [138,139,146,147]. Therefore, the decreases in B2M and hepcidin levels might be correlated and, eventually, lead to both serum and liver iron accumulation. High hepatic iron content has been suggested as a steatosis causative agent, given iron involvement in oxidative stress and LPO, with consequent lipid biosynthesis and accumulation [147].

Transferrin, an iron transport protein [135], was found to decrease upon repeated administration of Wistar rats with 25 and 50 mg/kg tramadol (Figure 4). It is a negative APP [135], suggesting that tramadol treatment might be particularly inflammatory. Indeed, transferrin is lower in patients with cirrhosis, fatty liver disease and impaired synthetic function; low transferrin and high ferritin, a combination that, in the present study, is seen for tramadol, may indicate inflammation [148]. Toxic nontransferrin-bound iron is uptaken by hepatocytes, causing their overload; hepatocellular impairment then follows, decreasing hepcidin production and leading to uncontrolled iron release from cells [148], which is compatible with our results. Thus, it is arguable whether increased serum iron levels are a driver or a consequence of liver disease [148].

Serum HO-1 levels were found to be increased upon exposure to 50 mg/kg tramadol and tapentadol (Figure 4), while its gene expression in liver and kidney significantly increased upon exposure to 50 mg/kg tapentadol only (Figure 6). HO-1 is an inducible isoform of heme oxygenase whose expression is increased by several stimuli, including drugs, cytokines and ROS [131,149–155]. HO-1 catalyzes the conversion of heme into biliverdin, carbon monoxide and iron, which collectively provide its antioxidant, antiapoptotic, anti-inflammatory, anti-fibrotic and tissue repair properties [131,132,149–151,156–159]. Oxidized LDLs have been suggested to induce HO-1 expression in endothelial cells, smooth muscle cells and macrophages [149]. Although we did not specifically quantify oxidized LDLs, we have shown increased serum LDL cholesterol and increased LPO in liver and kidney cells, for which we might also hypothesize LDL oxidation–and, thus, a correlation with HO-1 induction. Interestingly, and similarly to our results, HO-1 expression has been shown to be increased in non-alcoholic steatohepatitis and to reflect the severity of the disease, with a significant correlation with ferritin and LPO [131,149]. Hence, in our study, HO-1 overexpression might be a response to increased oxidative stress (including eventual LDL oxidation), an attempt to curtain fibrosis, correlated with hepatic lipid deposition and ferritin increase and, ultimately, with some extent of liver and kidney disease. Indeed, lack of HO-1 induction has been associated with oxidative damage and hepatic and renal iron accumulation, as well as with chronic inflammatory states [131,156,157]. Its up-regulation has been reported in experimental models of hepatic porphyria, fibrosis, cirrhosis, among other liver injury situations [151,160], as well as in several renal disorders, including acute kidney failure, acute glomerulonephritis and other glomerular, tubular, interstitial and vascular diseases, having been suggested as a candidate disease biomarker [157,158].

Haptoglobin, a glycoprotein mostly synthetized in the liver, stoichiometrically combines with hemoglobin, participating in its turnover and clearance by the mononuclear phagocyte system, also mainly in the liver; thus, it contributes to iron homeostasis and prevents its oxidative activity [133,161–163]. Increased haptoglobin levels are found in patients with obstructive biliary disease, where a correlation between its levels and ALP has been identified, suggesting that a higher level in obstruction might be related to biliary retention [161,162]. Haptoglobin also reduces hemoglobin loss through glomeruli, preventing renal iron loading during aging and following acute plasma heme-protein overload [163]. In addition, since it is a major or moderate APP (depending on the species), showing anti-inflammatory properties and binding to integrins on leukocytes, its increase is also a response to inflammation [133]. Therefore, in our study, increased haptoglobin levels might be due to a combined status of biliary obstruction (already suggested by augmented GGT, ALP, total cholesterol and LDL cholesterol) and inflammation, as well as to a possible attempt to minimize renal iron overload.

3.6. Repeated Exposure to Tramadol and Tapentadol Compromises Kidney Glomerular and Tubular Functions

In turn, nephrotoxicity is reported as a consequence of opioid exposure [164]. Rhabdomyolysis, secondary amyloidosis, membranous nephropathy, nephrotic syndrome, acute glomerulonephritis, focal and segmental glomerulosclerosis due to deposition of immune complexes, progressive chronic renal failure and tubular epithelial cell degeneration have been observed in chronic heroin, morphine and methadone users [164–167]. Moreover, there is an association between cholestasis—suggested by some of our results—kidney tubular changes and nephrotoxicity, though the exact underlying mechanisms are not known [166]. Elevated levels of opioid agonists may exert deleterious effects through oxidative stress, nitric oxide (NO) overproduction, apoptosis and vascular endothelial dysfunction [166]. ROS induce LPO in renal arterial endothelium, mesangial and renal tubular cells, causing renal failure [166].

In the present study, the alterations in serum uric acid were not statistically significant, as well as those in urinary total protein levels (Figure 5), the latter opposing the evidences of proteinuria seen in our acute exposure studies [98]. Nevertheless, all other renal function biomarkers assayed are compatible with kidney damage.

Serum cystatin C, regarded as a more accurate and sensitive marker of early kidney dysfunction than serum creatinine, increased at tapentadol highest dose. This might reflect a lower glomerular filtration rate [98,142,168–170]. Although urinary levels did not change significantly (results not shown), its mere detection in urine samples reflects proximal tubular injury, since cystatin C is reabsorbed and catabolized by tubular cells, with no tubular secretion [142,170].

Figure 5 also evidences that microalbuminuria (i.e., moderate increases in urine albumin) occurs at all tapentadol doses. Albuminuria may derive from increased glomerular permeability due to endothelial cell, basement membrane or podocyte dysfunction, as well as to inhibited proximal tubule reabsorption [171]. Given albumin role as a fatty acid transporter and that proteinuric kidneys preferentially lose albumin with low fatty acid content, there is a progressive retention of albumin with high fatty acid content, leading to serum fatty acid accumulation and their limited uptake by skeletal muscle, heart and adipose tissue [172,173]. This correlates well with increased serum triglycerides—since they are composed of fatty acids—which were compatibly observed at all tapentadol doses (Figure 3).

Urinary creatinine levels also decreased at all tapentadol doses, which may reflect decreased glomerular filtration; indeed, the degree of urinary creatinine decline has been associated with faster renal disease progression and poorer outcomes [168,174]. Conversely, several in vivo studies, concerning oral and intramuscular tramadol acute and chronic administration to rats, mice and rabbits, at doses ranging from 10 to 300 mg/kg, have led to increased serum creatinine concentrations [72,74–79,81,83]. The same trend was found among tramadol abusers [102].

Serum amylase activity is also elevated at all opioid doses, which might be associated with renal impairment, since amylase enters urine primarily via glomerular filtration, with partial tubular reabsorption [175–177]. Indeed, altered amylase clearance might arise from increased glomerular permeability and tubular dysfunction, both in acute and chronic kidney disease [176,177]. Hyperamylasemia may occur in other conditions, such as acute pancreatitis, which was not investigated in the present work; however, the elevation seen in renal insufficiency is rarely greater than 2 times the upper reference limit [178,179], which is compatible with the depicted in Figure 5. Liver disease might also account for increased serum amylase levels, since a large proportion of the circulating enzyme is cleared by the mononuclear phagocyte system and subsequent removal through bile [180,181]. In this context, a combination of renal impairment with biliary obstruction, whose presence has already been suggested by our results, may contribute to elevated serum amylase.

Urinary NAG activity increased at 50 mg/kg tramadol and 25 and 50 mg/kg tapentadol. NAG is a lysosomal enzyme of the proximal tubule epithelial cells; due to its large molecular weight, it is not filtered through the glomerulus, and is neither absorbed nor secreted by renal tubules. Unlike other renal function biomarkers that are filtered through the glomerulus, increased urine levels of NAG, deriving exclusively from tubule cells, specifically reflect proximal tubule dysfunction [142,182–186]. NAG has been suggested as a more sensitive biomarker of early nephropathy than albuminuria [185,186]. Interestingly, increased urinary NAG activity, as well as renal morphologic changes, were found in cholestatic rats and reversed by naltrexone treatment, suggesting the involvement of endogenous opioids in cholestatic nephrotoxicity [183]. Since our data are compatible with biliary obstruction, the hypothesis of exogenous opioid-induced cholestatic nephrotoxicity could be considered.

3.7. Repeated Exposure to Tramadol and Tapentadol Alters Hepato-Renal Toxicity Biomarker Gene Expression, Correlating with Metabolic Changes, Cell Toxicity and Glomerular Dysfunction

Concerning liver expression of hepatotoxicity biomarker genes (Figure 6a), Aldoa (encoding for fructose-bisphosphate aldolase A, a glycolytic enzyme) significantly increased upon tramadol exposure, as previously seen in serum from patients with fulminant hepatitis [187] and drug-induced liver injury [188], as well as in liver tissue from animal models acutely and sub-acutely exposed to different xenobiotics [189–192]. Aldoa upregulation has also been reported in cirrhotic and hepatocellular carcinoma livers [193,194], confirming the high glycolytic phenotype as a typical feature of both precancerous and cancerous lesions. Enhanced glucose oxidation (and inherent glycogen mobilization) may represent a metabolic response to tramadol-induced stress.

Apex1, in turn, encodes for apurinic/apyrimidinic endonuclease 1, an enzyme involved in base excision repair and a regulator of gene expression as a redox co-activator of different transcription factors [195]. Apex1 up-regulation was observed in liver tissue from drug-treated rodents, since its expression is induced by ROS as a defense mechanism against genomic instability [160,191,192,195]. Since Apex1 gene expression did not change significantly in our study, it might be hypothesized that, in the conditions that were assayed, genotoxicity is not a predominant hepatotoxicity mechanism, or that repair mechanisms are not yet being recruited. Additional studies are needed in order to confirm these hypotheses.

Cd36 encodes for cluster of differentiation 36/fatty acid translocase, showing ability to bind oxidized LDL, long chain fatty acids, phospholipids and collagen [196–198]. Increased expression in hepatocytes is associated with augmented fatty acid uptake, triglyceride accumulation and, thus, hepatic fibrogenesis, steatosis and non-alcoholic fatty liver disease [196–198]. Furthermore, in several mouse strains, it has been identified as the gene having highest correlation with fatty liver, and its disruption has been shown to protect against systemic inflammation and insulin resistance [196,198]. Thus, Cd36 overexpression upon exposure to 50 mg/kg tramadol might be correlated with the high total and LDL-cholesterol serum levels, as well as with a higher profibrogenic potential.

Lpl, in turn, encodes for lipoprotein lipase, an endothelium-anchored enzyme that catalyzes the hydrolysis of triglycerides from chylomicrons and very low density lipoproteins (VLDL) into free

fatty acids, enabling their uptake by extrahepatic tissues [199–201]. The decrease in Lpl expression, as seen upon exposure to 50 mg/kg tramadol and tapentadol, might therefore be associated with higher serum triglyceride levels—which is observed in tapentadol groups (Figure 3)—and higher serum cholesterol levels—as seen in the 50 mg/kg tramadol group (Figure 3)—as these lipids are transported in the form of lipoproteins. Indeed, lipid disorders frequently accompany liver disease, with increased hepatic secretion of VLDL particles due to increased concentration of free fatty acids and glucose, and decreased VLDL clearance due to reduced activity of lipoprotein lipase [201].

Regarding the nephrotoxicity biomarker gene panel, Angptl4 kidney expression was found to be upregulated in tapentadol-treated rats (Figure 6b). Angptl4, angiopoietin-like 4 protein, is secreted from podocytes, having been implicated in processes as diverse as glucose and energy homeostasis, angiogenesis and vascular permeability, inflammation, tumorigenesis, cell differentiation, wound healing and redox regulation [202–204]. It induces morphological and clinical manifestations of human minimal change disease and is being increasingly recognized as a contributor to proteinuria in experimental diabetic nephropathy [152,172,173,205]. However, one of its most studied roles is as a regulator of lipid metabolism, having been shown to modulate both intracellular and extracellular lipolysis [206], and linked to lipoprotein lipase inhibition and hypertriglyceridemia in nephrotic syndrome [173,202,204,206–208], which correlates well with the increased serum triglyceride levels (Figure 3) and decreased liver Lpl expression (Figure 6a) observed in tapentadol groups.

Gamt, in turn, encodes for guanidinoacetate *N*-methyltransferase, the enzyme that catalyzes the last step of creatine biosynthesis [209]. Its gene expression has been shown to be downregulated in kidneys from tramadol- and tapentadol-administered rats (Figure 6b). In line with our results, Gamt inhibition and down-regulation have been reported following drug-induced nephrotoxicity and suggested as a result of toxicity progression and biochemical feedback mechanisms to compensate for altered creatinine clearance, since creatinine is a product of creatine [153,154,209,210]. Lower Gamt activity leads to decreased creatine synthesis and precursor buildup; while the former ultimately compromises the creatine/phosphocreatine energy buffer system, the latter has been associated with cell toxicity through a number of mechanisms [211].

Nphs2 encodes for podocin, a slit diaphragm protein that acts as a structural scaffold in podocyte foot processes and interacts with other slit diaphragm proteins to facilitate anti-apoptotic signaling events. It is essential for the establishment and maintenance of the glomerular filtration barrier [212–216], having been found to be downregulated in lupus nephritis, pediatric nephrotic syndrome and focal segmental glomerulosclerosis [217]. Indeed, loss of podocin, as well as inactivating mutations on its gene, are associated with glomerular lesions (including mesangial proliferation), glomerulosclerosis, albuminuria, hypercholesterolemia, hypertension, and renal failure, which characterize nephrotic syndrome [212–215,218]. Thus, since Nphs2 gene expression is significantly decreased in tramadol-treated rats (Figure 6b), glomerular injury might be anticipated. Such hypothesis is consistent with the results concerning other glomerular function biomarkers, such as serum amylase (Figure 5).

3.8. Repeated Exposure to Tramadol and Tapentadol Causes Liver and Kidney Histopathological Changes, Correlating with Metabolic and Gene Expression Alterations

The hepatic and renal effects of the repeated administration of tramadol and tapentadol clinical doses were also studied at the histological level, reinforcing the results from our previous acute exposure assays to the same doses [98]. In addition, such results also have forensic significance, since acute liver failure, extensive fulminant necrosis, marked steatosis, congestion and enlargement have been reported upon lethal intoxication with both tramadol [59,219–221] and tapentadol [54].

Regarding liver, sinusoidal dilatation was a recurrent finding at all opioid doses, being more profuse on tapentadol groups (Figures 7–9). Such results had already been reported in acute to sub-chronic rat exposure assays to tramadol doses ranging from 12.5 to 300 mg/kg [71,77,78,92–94]. Mononuclear cell inflammatory infiltrates were another seemingly dose-dependent finding for

both opioids (Figures 7 and 8), which is also consistent with similar exposure studies, mostly sub-chronic and chronic [76,78,85,88,92–95]. Signs of cellular degeneration, including nuclei fragmentation and poor definition, were increasingly apparent along with tramadol dose, while they were observed at all tapentadol doses, on whose slides hypopigmented areas could also be seen (Figures 7 and 8). Indeed, related cellular and tissue alterations, comprising necrosis, apoptosis, hydropic degeneration, karyolitic and pyknotic nuclei, cytolysis, tissue disorganization and loss of architecture, were reported in analogous studies using mainly tramadol, but also heroin, nalbuphine and morphine [71,73,76–79,81,84,85,87,88,90,92–95]. In turn, vascular congestion, with erythrocyte extravasation, was unique to tapentadol exposure (Figure 7), as seen in our previous acute exposure assays [98]. In this context, there are reports of congestion, dilated blood vessels, hemorrhage and stagnant blood upon exposure to 3 to 300 mg/kg tramadol—but also in studies concerning opioids such as morphine, heroin and nalbuphine—for periods ranging from acute to chronic [71,73,76–79,84,85,87,88,92–95]. Hepatocyte vacuolization and microsteatosis were also consistent observations (Figures 7–9), again in line with comparable studies [74,76,79,84,85,92–94]. As already discussed, such evidence might be correlated with the derangement of lipid metabolism, increased iron levels and elevated Cd36 gene expression. In turn, PAS staining evidenced lower liver glycogen accumulation in experimental groups (Figure 8), in line with the observed in our previous acute exposure studies [98] and upon a 20-day period of daily oral administration of 40 mg/kg tramadol to rats [88]. Though glycogen depletion may indeed be due to the 24h-fasting that preceded rat sacrifice, the controls still present denser glycogen masses, showing that glycogenolysis might be a compensatory mechanism to cope with opioid-induced metabolic stress [98]. Enhanced glycolysis, corroborated by Aldoa gene overexpression in tramadol-treated rats (Figure 6a), might be a downstream event. Finally, Masson's trichrome staining revealed fibrous tissue accumulation between hepatocytes, which was particularly evident on liver specimens from tapentadol-exposed rats (Figure 9). On one hand, such observation may be interpreted as a sign of revascularization, a possible response to liver injury, and is supported by studies concerning mostly sub-chronic exposure to tramadol therapeutic and supratherapeutic doses [76,77,84,88,94,95]. On the other hand, increased collagen fibers were suggested to be the result of ROS deleterious action either on collagen itself or on enzymes involved in its metabolism [222], which may represent an additional explanation. Moreover, hepatic microsteatosis and fibrosis might be correlated with increased liver iron content [131,132,147], which is also hypothesized in this paper.

Interestingly, histopathological studies performed upon exposure to tramadol doses up to 300 mg/kg and for periods up to 150 days do also report bile duct proliferation and hyperplasia—which are mainly associated with biliary disorders [223,224]—as well as cholestatic hepatitis [76,78,94,95]. Also, non-fatal cases of tramadol poisoning report hepatobiliary dysfunction [59]. Although, in our study, this was not a valuable finding from the histopathological point of view, our biochemical results—increased GGT, ALP, total cholesterol, LDL cholesterol and haptoglobin—are consistent with biliary obstruction. Thus, it might be hypothesized that dose and/or exposure time increments lead to the accumulation of histological evidence of biliary disease.

Concerning kidney histopathological study, disorganized and poorly contoured tubules, as well as increased Bowman's spaces, were omnipresent findings on all opioid group slides, and cell swelling was observed at all tramadol doses (Figure 10). Such observations are in line with those from similar studies, which report tubular endothelial cell degeneration, vacuolization, swelling and even necrosis [71,74,76–78,81,88,95]. A case report of a fatal intoxication by tapentadol does also mention kidney cell autolytic changes [54]. In turn, while glomerular disorganization and vacuolization were patent at all tapentadol doses, they became increasingly evident along with tramadol dose (Figure 10). In this context, several analogous studies report glomerular atrophy, with collapsed tufts [76,81,88,95]. It is also noteworthy that mononuclear cell infiltrates were observed on tramadol slides only, irrespectively of the dose considered. Studies concerning tramadol oral, intramuscular and i.p. administration to rats and sheep, at doses ranging from 5 to 300 mg/kg, refer similar

findings [71,76,78,81,88,95]. Some of these studies also report hemorrhage, congestion, inter-tubular blood vessel dilatation and thickening, and even renal cast formation/mineralization in corticomedullary tubules [71,76,77,81,95,96], although we did not find relevant signs of them. In addition, Elkhateeb and co-workers reported an increase in collagen fibers in rat kidney samples upon a 30-day exposure period to 30 mg/kg tramadol [76], for which it would be interesting to assess whether a shorter, 14-day exposure period to a similar dose (25 mg/kg) and/or to a higher dose (50 mg/kg) produces similar results. However, we did not perform Masson's trichrome staining with kidney specimens; thus, that may only be hypothesized.

Taken together, the results of the present work offer additional insights to our previous studies addressing liver, kidney, heart, lung and brain cortex toxicity following an acute exposure to the same tramadol and tapentadol doses [98,99]. Our biochemical and histological analysis shows that hepatic and renal alterations, at the metabolic and histopathological levels, occur and accumulate subsequently to longer periods of administration than that previously assayed, but shorter than those implemented in most comparable repeated administration studies, and for lower tramadol and tapentadol doses.

4. Materials and Methods

4.1. Chemicals

Tramadol hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA), while tapentadol hydrochloride was provided by Deltaclon (Madrid, Spain). Both compounds were dissolved and diluted in saline (0.9 g/L (*w/v*) NaCl) immediately prior to administration. Sodium thiopental was obtained from B. Braun Medical (Queluz de Baixo, Portugal). All other chemicals were commercial preparations of the highest available degree of purity.

4.2. Experimental Models and Animal Handling

42 male Wistar rats, aged 8 weeks and weighing 250–300 g, were provided by the i3S animal facility (Porto, Portugal). All animals were housed in acrylic cages with wood chips and paper towels as enrichment items, under controlled standard laboratory conditions (22 ± 2 °C, 50–60% humidity, 12/12 h light/dark cycles). Rats were given *ad libitum* access to tap water and rat chow (standard short and middle period maintenance formula for rodents, reference 4RF21, Mucedola/Ultragene (Milan, Italy), as well as a quarantine period of at least one week before experimental assays.

Animal experimentation complied with the European Council Directive (2010/63/EU) guidelines, transposed into the Portuguese law (Decree-Law no. 113/2013, 7th August). All assays were also approved by the Ethics Committee of CESPU, Institute of Research and Advanced Training in Health Sciences and Technologies (IINFACTS), Gandra, PRD, Portugal (processes no. PI4AC 2017, PI4AC 2018 and PI-3RL 2019), and complied with the National Ethics Council for the Life Sciences (CNECV) guidelines.

4.3. Experimental Design and Drug Treatment

Wistar rats were randomly assigned to seven groups, composed of six animals each. The sample size/number of animals per group was determined through the G*Power software, version 3.1.9.6 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), assuming a significance level of 0.05, an 80% power and effect size values adjusted accordingly with the biochemical parameters to be analyzed (based on literature and on the previous experience of the team in similar analyses).

Drug treatment consisted of daily i.p. injections of 1 mL-units, using saline (0.9% (*w/v*) NaCl) as vehicle, at the same time every day, for 14 consecutive days. Group 1 (control group) received saline administrations, groups 2, 3 and 4 received 10, 25 and 50 mg/kg tramadol, respectively, while groups 5, 6 and 7 received 10, 25 and 50 mg/kg tapentadol, respectively.

Rat doses were determined by converting the human dose into the animal equivalent dose (AED), using a body surface area correction factor (K_m) of 6.2 and the following formula, assuming an

average 60 kg-human: AED (mg/kg) = human dose (mg/kg) × K_m ratio [225,226]. In order to establish opioid doses for rat administration, their median lethal dose (LD_{50}) for rats [227], concentrations reported in intoxications [56], and tramadol and tapentadol maximum recommended daily doses for humans [2,24,227,228] were considered. Except for specific pathological conditions or other clinically relevant situations, the standard tramadol dose for a 60-kg patient is 50–100 mg (1.67 mg/kg/day) three to four times a day, totaling a maximum recommended daily dose of 400 mg [1,227]. In turn, tapentadol maximum recommended daily dose is reported as 600–700 mg/day [1,24,228]. The 1.67 mg/kg/day standard, corresponding to a 100 mg-dose, is thus equivalent to 10.35 mg/kg (when multiplied by 6.2). Accordingly, 10 mg/kg corresponds to an effective, analgesic 100 mg-dose; 25 and 50 mg/kg are equivalent to an intermediate and the maximum recommended daily dose, respectively, considering a 60 kg-adult [98,99].

Immediately after the last administration, rats were placed in metabolic cages and given unlimited access to tap water, but no food, for the remaining 24 h. Animals were kept under monitoring throughout this period, upon which they were sacrificed.

4.4. Collection and Processing of Biological Samples

Urine samples were collected from each animal, into an ice-cold container, during the last 24 h-exposure period. Samples were processed through centrifugation at 3000× *g*, 4 °C, for 10 min, to remove any debris. Animals were sacrificed by means of anesthetic procedures (i.p. injection with 60 mg/kg sodium thiopental, using saline as vehicle). Blood samples were drawn with a hypodermic heparinized needle, through cardiac puncture, and further submitted to centrifugation at 3000× *g*, 4 °C, for 10 min, to obtain serum. Samples were then aliquoted and stored (−80 °C) for further biochemical analysis.

Livers and kidneys were surgically collected, dried with gauze, weighed on an analytical balance, and further processed. One portion of each organ was homogenized in an Ultra-Turrax® (IKA®, Staufen, Germany) in 1:4 (*w*/*v*) ice-cold 50 mM phosphate buffer (KH_2PO_4 + $Na_2HPO_4{\cdot}H_2O$), pH 7.4. The respective supernatants were obtained through centrifugation at 4000× *g*, 4 °C, for 10 min. The aliquots thus obtained, as well as the remaining intact portions of the organs, were stored at −80 °C, regarding subsequent analysis.

4.4.1. Quantification of Oxidative Stress Parameters

Oxidative stress was assessed, in liver and kidney homogenates, as the degree of LPO and protein oxidation, through the quantification of TBARS and protein carbonyl groups (ketones and aldehydes), respectively. The total antioxidant capacity was also determined in the same samples.

Total protein content was determined through the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's microplate procedure.

Perchloric acid was added to liver and kidney homogenates to a final concentration of 5% (*w*/*v*), to precipitate proteins. Samples were centrifuged at 13,000× *g*, 4 °C, for 10 min, with both pellets and supernatants being stored at −80 °C for subsequent analysis. LPO quantification was performed in supernatants, through the TBARS method reported by Buege et al. [229]. Results were expressed in terms of nanomoles of MDA equivalents per milligram of protein.

In turn, carbonyl groups were quantified in protein pellets, according to Levine et al. [230]. Results were expressed as nanomoles of DNPH incorporated per milligram of protein.

The total antioxidant capacity was determined with the Total Antioxidant Capacity Assay Kit (Sigma-Aldrich), following the manufacturer's instructions. Liver homogenates were diluted 20-fold, while kidney samples were used directly. Results were expressed in terms of mM of antioxidants (Trolox equivalents) per milligram of protein.

4.4.2. Quantification of Biochemical Parameters in Serum and Urine Samples

Albumin, ALP, ALT, amylase, AST, α-1-acid glycoprotein, BuChE, total cholesterol, HDL cholesterol, LDL cholesterol, complement C3 and C4, GGT, iron, ferritin, haptoglobin, transferrin, total proteins, triglycerides and uric acid were quantified in serum samples, while urine proteins, creatinine, microalbumin and NAG were determined in urine samples. Cystatin C, B2M and urea were determined both in serum and urine samples. Unless otherwise stated, biochemical parameters were quantified in an automated analyzer (Prestige 24i, Tokyo Boeki, Tokyo, Japan), according to the manufacturer's instructions, as previously described [97–99,231], and using undiluted samples. Calibrations were appropriately performed for each parameter, with two appropriate calibrators, in order to plot 5-point standard curves. Quality controls were also included. All automated analyzer reagents were supplied by Cormay PZ (Warsaw, Poland), except for those concerning B2M, which were purchased from Spinreact (Barcelona, Spain).

NAG activity was quantified with the NAG assay (Diazyme, Poway, CA, USA), according to the manufacturer's directions. Urine proteins were determined through the microplate procedure of Pierce™ BCA Protein Assay Kit (Thermo Scientific), upon removal of interfering substances according to Yalamati and co-authors [232] and a 6-fold sample dilution in 0.5 N NaOH.

Enzyme activities were determined as U/L, while biochemical parameters were retrieved as mg/dL, except for albumin (g/dL), cystatin C, B2M and microalbumin (mg/L), ferritin (μg/L), iron (μg/dL) and serum and urine proteins (g/L).

In turn, HO-1 and hepcidin were determined in serum samples, through enzyme-linked immunosorbent assay (ELISA), using the HO-1 (rat) ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) and the Rat Hepcidin (Hepc) ELISA kit (Abbexa, Cambridge, UK), respectively, according to the manufacturers' specifications. For HO-1 quantification, samples were diluted 10-fold with sample diluent, while undiluted samples were used for hepcidin analysis. ELISA results were retrieved as ng/mL (HO-1) or pg/mL (hepcidin).

4.4.3. Gene Expression Analysis through qRT-PCR

Total RNA was isolated from liver and kidney samples using the NZYol reagent (NZYTech, Lisbon, Portugal), according to the manufacturer's instructions concerning tissue samples. RNA integrity was assessed through 1.4% (*w*/*v*) agarose gel electrophoresis. RNA purity, regarding protein and organic compound contamination, was determined as the optical density (OD) $OD_{260\ nm}/OD_{280\ nm}$ and $OD_{260\ nm}/OD_{230\ nm}$ ratios, respectively (NanoDrop 2000 spectrophotometer, Thermo Scientific). Samples with $OD_{260\ nm}/OD_{280\ nm}$ and $OD_{260\ nm}/OD_{230\ nm}$ ratios ≥ 1.8 were selected for complementary DNA (cDNA) synthesis. 800 ng total RNA were converted into cDNA using the NZY First Strand cDNA Synthesis kit (NZYTech), according to the supplier's instructions.

Gene expression was analyzed using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's directions. Each cDNA sample was diluted 10-fold in ultrapure water and analyzed in duplicate, totaling 12 replicates for each condition. Cd36, Aldoa, Apex1, Lpl, Angptl4, Hmox1, Nphs2 and Gamt genes were analyzed. 18S ribosomal RNA (18S rRNA) was used as housekeeping gene, for loading control purposes. Each amplification mixture totaled 25 μL, comprising 12.5 μL 2× iQ™ SYBR® Green Supermix (Bio-Rad), 2 μL diluted cDNA, forward and reverse primers to a final concentration of 100 nM each, and 10 μL RNase-free water. The primers used for amplification (STABvida, Caparica, Portugal) are described in Table 1.

Table 1. Primer nucleotide sequences [233–241] and number of amplification cycles used for gene expression analysis of hepato- and nephrotoxicity biomarkers through quantitative Real-Time PCR (qRT-PCR).

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	No. of Amplification Cycles	Reference
Cd36 (Cluster of differentiation 36/fatty acid translocase)	AGGAAGTGGCAAAGAATAGCAG	ACAGACAGTGAAGGCTCAAAGA	37	[233]
Aldoa (Fructose-bisphosphate aldolase A)	ATGCCCCACCCATACCCAGCACT	AGCAGCAGTTGGCGGTAGAAGCG	37	[234]
Apex1 (Apurinic/apyrimidinic endonuclease 1)	GAATGTGGATGGGCTTCGA	AAGATGTCTGGTGCTTCTTCCTTT	41	[235]
Lpl (Lipoprotein lipase)	CTTAAGTGGAAGAACGACTCCTACT	GTCATGGCATTTCACAAACACTGCA	41	[236]
Angptl4 (Angiopoietin-like 4)	GCCGCTACTATCCACTAC	CCTGTTGCTCTGACTGTT	45	[237]
Hmox1 (Heme oxygenase 1)	ACAGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG	45	[238]
Nphs2 (Podocin)	TGGAAGCTGAGGCACAAAGA	AGAATCTCAGCCGCCATCCT	38	[239]
Gamt (Guanidinoacetate *N*-methyltransferase)	ACTCATGCTTTCCGTTTGCT	AGGCACCTGAGTCTCCTCAA	38	[240]
18S rRNA (18S ribosomal RNA)	TTCGGAACTGAGGCCATGATT	TTTCGCTCTGGTCCGTCTTG	In line with that of the target gene	[241]

RNA template controls (RTC) and non-template controls (NTC) were included in each run. The qRT-PCR program was run in a C1000™ Thermal Cycler equipped with a CFX96™ Real-Time System, both from Bio-Rad Laboratories. The amplification program comprised an initial denaturation step at 95.0 °C for 3 min, and then 37–45 amplification cycles composed of a denaturation step at 94.0 °C for 20 s, an annealing step at 55.0 °C for 30 s, an extension step at 72.0 °C for 30 s and a plate read step. The number of amplification cycles used for the analysis of each gene is specified in Table 1.

A melt curve was finally acquired between 65.0 and 95.0 °C, with 0.5 °C increments at every 5 s, followed by plate reads. Results were retrieved using the Bio-Rad CFX Manager software, version 3.1 (Bio-Rad Laboratories), and normalized against those of the control group. Relative changes in gene expression were determined through the Δ(ΔCt) algorithm.

4.4.4. Liver and Kidney Histopathological Analysis

One portion of liver and kidney tissue from each animal was collected and fixed in 4% (*w/v*) formaldehyde, for 24 h at room temperature, for subsequent histological analysis. It was then submitted to standard dehydration and paraffin wax-embedding procedures, as previously described [242,243]. Three µm-sections were cut in a microtome (Shandon™ Finesse™ 325, Thermo Scientific) and adhered to glass slides. H&E, PAS and Masson's trichrome staining procedures were performed with liver simples, while kidney samples were processed for H&E staining only. Slides were prepared through standard methods and observed under phase contrast microscopy, using 100× and 600× magnifications (Eclipse TE2000-U microscope, Nikon, Melville, NY, USA), coupled to a DXM1200F digital camera and controlled by Nikon ACT-1 software, version 2.70). Multiple microscope fields of observation were analyzed, and images were taken from representative ones.

4.5. Statistical Analysis

Results were expressed as means ± SD. Statistical data analysis was performed as an Analysis of Variance (ANOVA). Post-hoc analysis consisted of Dunnett's multiple comparisons test. Probability values of $p < 0.05$ were considered as statistically significant. Graphic plotting and all statistical tests were performed using GraphPad Prism® version 8.3.1 (GraphPad Software, LLC, San Diego, CA, USA). In all determinations, results were compared with those of the control group, injected with saline.

5. Conclusions

The increase in opioid prescription, use and abuse is accompanied by an increase in the number of adverse event reports. Although tramadol and tapentadol are known for their safety, having been designed to specifically address the drawbacks of their opioid peers, several adverse events and fatalities are being reported in the literature. Paradoxically, such phenomena are poorly documented at the molecular, biochemical, cellular, and histological levels. In this sense, our study attempts to fill some gaps regarding the mechanistic rationale underlying tramadol and tapentadol organ-specific toxicity. The novelty of the information applies most particularly to tapentadol, for which, owing to its shorter market history, there is fewer data available. In addition, our studies also represent an added value. In fact, while most toxicological information concerns full opioid receptor agonists, often at a supratherapeutic or overdose range, we provide comprehensive and comparative results for two partial agonists, administered at therapeutic doses. In this context, this is, to the best of our knowledge, the first in vivo study comparatively addressing tramadol and tapentadol toxicity upon repeated administration of clinically relevant doses. Furthermore, we have broadened the spectrum of parameters in relation to that studied in our previous acute assays, adding more biochemical/metabolic biomarkers, and including gene expression assays and additional histological staining methods.

In the present work, we demonstrate that a 14-day period of daily single administration of tramadol and tapentadol therapeutic doses induces hepato- and nephrotoxicity, as substantiated by changes in a panel of several biochemical, metabolic and histological parameters. Although some of the reported findings are exclusive to or more intense for tapentadol—a trend that had already

been identified in our previous acute studies—the extension of the exposure tended to smooth the differences between the results from both opioids. Alterations proven to be more specific or more pronounced at the highest doses of one opioid, in our acute studies, were now shown to appear upon repeated administration of both opioids, and at lower doses. Oxidative stress biomarkers were augmented in both liver and kidney tissues, and liver synthetic function indicators, such as albumin, urea, BuChE and complement C3 and C4, were decreased upon exposure to both opioids. Alterations in the lipid profile, as well as in liver function tests such as ALT, AST, ALP and GGT, are strongly suggestive of hepatic dysfunction under the conditions assayed. Iron metabolism was also found to be deranged following exposure to both tramadol and tapentadol, as seen from the alterations in a panel including ferritin, haptoglobin and HO-1, among other related parameters. In turn, kidney function is also seemingly committed, and most prominently upon tapentadol treatment, as deduced from serum and urine alterations in parameters such as cystatin C, creatinine, microalbumin and NAG activity. Liver histopathological analysis revealed the presence of sinusoidal dilatation, inflammatory cell infiltrates, microsteatosis, glycogen depletion and cell degeneration. Accumulation of fibrous tissue was more evident following tapentadol treatment, to which erythrocyte extravasation was exclusive. Kidney histopathological findings comprised tubular and glomerular disorganization, as well as increased Bowman's spaces, for both opioids, while mononuclear cell infiltrates and cell swelling were more apparent upon tramadol exposure. Gene expression assays have also identified quantitative changes in almost all liver and kidney toxicity biomarkers studied, upon exposure to either one or both opioids. Likewise, gene expression results correlate well with metabolic and histopathological results concerning, for instance, lipid and iron metabolism derangement, liver microsteatosis and kidney glomerular and tubular dysfunction. Therefore, rather than evident signs of cell death, repeated administration of tramadol or tapentadol at therapeutic doses elicits hepato- and nephrotoxicity mainly at the biochemical, metabolic and tissue organization levels.

Such results require reinforced attention from the scientific and clinical point of view, emphasizing the need for careful consideration of the maximum recommended daily doses, as well as for liver and kidney function monitoring when prescribing tramadol and tapentadol. Although tapentadol presents several advantages over tramadol, such as a more linear pharmacokinetics and properties that make it a better option for specific types of pain, it seemingly does not offer significant extra safety, as far as our endpoint results are concerned. Hence, the use of both tramadol and tapentadol should be carefully deliberated and monitored in patients with liver and/or kidney disease, particularly when more prolonged, subacute to chronic contexts of use are considered.

Additional studies, broadening the dose range assayed and extending the administration period, would further complement and clarify the results hereby presented, since they would shed light on the effects of chronic tramadol and tapentadol use. Immunohistochemistry assays, using appropriate toxicity/inflammation markers (e.g., tumor necrosis factor α (TNF-α), inducible NO synthase (iNOS), caveolin-1 (Cav-1) and pentraxin 3 (PTX3)), would also complement biochemical and histopathological analyses. Combined administration of tramadol/tapentadol with drugs that are often concomitantly used with them, such as selective serotonin reuptake inhibitors, tricyclic antidepressants, and monoamine oxidase inhibitors, would also be informative. Indeed, they would elucidate whether toxicological results are exacerbated by eventual drug-drug interactions and subsequent accumulation. The use of metabolites and/or opioid antagonists could also be considered in the experimental design. Also, to account for sex-dependent differences in drug metabolism, and considering that opioids are used in the treatment of sex-independent forms of pain, future studies should include female animals. Behavioral studies would also enlighten about abuse and dependence potential under comparable experimental settings.

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CHAPTER III

Repeated administration of clinically relevant doses of the prescription opioids tramadol and tapentadol causes lung, cardiac, and brain toxicity in Wistar rats

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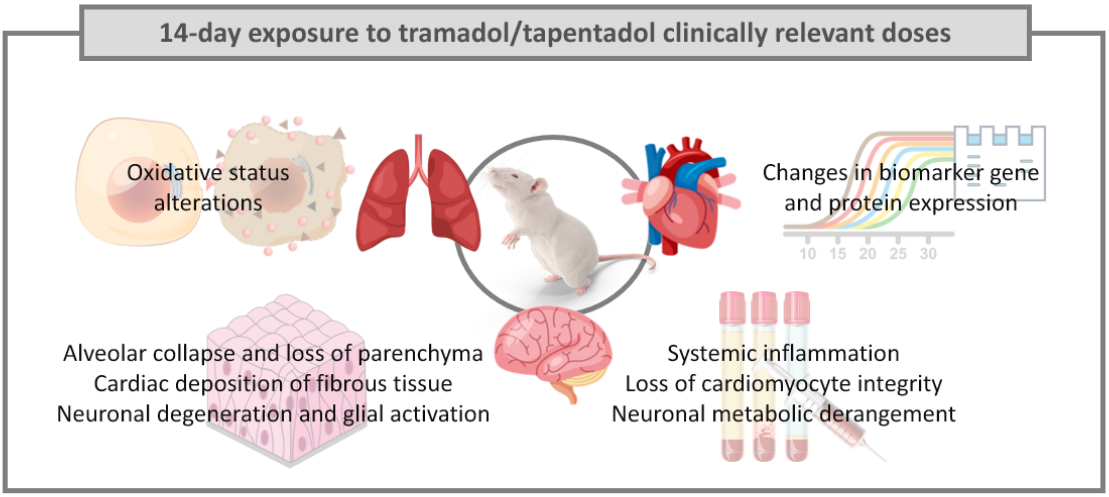
Graphical Abstract



Systemic and organ-specific (lung, heart and brain cortex) effects of the repeated exposure of Wistar rats to clinically relevant doses of tramadol and tapentadol, as assessed through oxidative stress, molecular, biochemical and histological analysis

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Article

Repeated Administration of Clinically Relevant Doses of the Prescription Opioids Tramadol and Tapentadol Causes Lung, Cardiac, and Brain Toxicity in Wistar Rats

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Abstract: Tramadol and tapentadol, two structurally related synthetic opioid analgesics, are widely prescribed due to the enhanced therapeutic profiles resulting from the synergistic combination between μ-opioid receptor (MOR) activation and monoamine reuptake inhibition. However, the number of adverse reactions has been growing along with their increasing use and misuse. The potential toxicological mechanisms for these drugs are not completely understood, especially for tapentadol, owing to its shorter market history. Therefore, in the present study, we aimed to comparatively assess the putative lung, cardiac, and brain cortex toxicological damage elicited by the repeated exposure to therapeutic doses of both prescription opioids. To this purpose, male Wistar rats were intraperitoneally injected with single daily doses of 10, 25, and 50 mg/kg tramadol or tapentadol, corresponding to a standard analgesic dose, an intermediate dose, and the maximum recommended daily dose, respectively, for 14 consecutive days. Such treatment was found to lead mainly to lipid peroxidation and inflammation in lung and brain cortex tissues, as shown through augmented thiobarbituric acid reactive substances (TBARS), as well as to increased serum inflammation biomarkers, such as C reactive protein (CRP) and tumor necrosis factor-α (TNF-α). Cardiomyocyte integrity was also shown to be affected, since both opioids incremented serum lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) activities, while tapentadol was associated with increased serum creatine kinase muscle brain (CK-MB) isoform activity. In turn, the analysis of metabolic parameters in brain cortex tissue revealed increased lactate concentration upon exposure to both drugs, as well as augmented LDH and creatine kinase (CK) activities following tapentadol treatment. In addition, pneumo- and cardiotoxicity biomarkers were quantified at the gene level, while neurotoxicity biomarkers were quantified both at the gene and protein levels; changes in their expression correlate with the oxidative stress, inflammatory, metabolic, and histopathological changes that were detected. Hematoxylin and eosin (H & E) staining revealed

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several histopathological alterations, including alveolar collapse and destruction in lung sections, inflammatory infiltrates, altered cardiomyocytes and loss of striation in heart sections, degenerated neurons, and accumulation of glial and microglial cells in brain cortex sections. In turn, Masson's trichrome staining confirmed fibrous tissue deposition in cardiac tissue. Taken as a whole, these results show that the repeated administration of both prescription opioids extends the dose range for which toxicological injury is observed to lower therapeutic doses. They also reinforce previous assumptions that tramadol and tapentadol are not devoid of toxicological risk even at clinical doses.

Keywords: tramadol; tapentadol; prescription opioids; pneumotoxicity; cardiotoxicity; neurotoxicity; in vivo studies

1. Introduction

Opioids currently represent a mainstay option for the treatment of moderate to severe forms of pain. In this context, tramadol and tapentadol, synthetic and structurally related opioids, are widely prescribed in acute and chronic settings, finding application in the treatment of several clinical conditions, such as postoperative, musculoskeletal, neuropathic, cancer and mixed pain states [1–12]. However, the misuse and abuse of prescription opioids, such as tramadol and tapentadol, is increasing due to their easy access, leading to addiction and toxicity cases. Thus, understanding the toxicology of prescription opioids is a challenge for modern societies.

Tramadol (1*RS*, 2*RS*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclo-hexanol) is a racemic opioid [13,14], whose analgesic efficiency is dependent on its metabolization to *O*-desmethyltramadol (M1) via cytochrome P450 (CYP450) [13–17]. In turn, tapentadol, 3-[(1*R*,2*R*)-3-(dimethylamino)-1-ethyl-2-methylpropyl]phenol, is a single active molecule. Both opioids combine μ-opioid receptor (MOR) activation and serotonin (5-HT) and noradrenaline (NA) reuptake inhibition [13,14], although tapentadol shows minimal 5-HT reuptake inhibition properties [14,17–23]. Interestingly, tapentadol noradrenergic component is associated with anti-apoptotic and pro-neurogenic effects, counteracting MOR-mediated deleterious effects. This protective effect, along with the increasing contribution of the noradrenergic component in persistent neuropathic states, supports its use in neuropathic pain treatment [1,4–6,24–27].

Although tramadol and tapentadol are safe and effective in pain relief, they have already been associated with many cases of addiction and toxicity, some of which fatal [1,9,28–53]. Such observations emphasize the importance of understanding the mechanisms underlying their toxicity. Our group has already studied the effects of an acute exposure to clinically relevant doses of tramadol and tapentadol [54–56]. We previously reported biochemical alterations in serum and urine samples from in vivo models, as well as in a neuronal cell model, having found oxidative status and histological alterations in brain cortex, lung, heart, liver and kidney tissues [54–56]. Our results showed that, in acute contexts, tapentadol causes more pronounced toxic damage [54–56]. In a more recent study by our group, we showed that repeated administration of clinically relevant doses of tramadol and tapentadol smooths the differences between the toxicological profiles of both opioids, and that hepatorenal damage occurs at lower doses, when compared with acute exposure [57]. Several other studies with animal models were performed with high tramadol doses, in particular the median lethal dose (LD_{50}). In the rat model, tramadol LD_{50} was already associated with brain congestion, edema, gliosis, microglial and oligodendrocyte proliferation and inflammatory cell infiltrates [58], while its repeated administration at doses ranging from 30 to 168 mg/kg induced several brain and lung histological alterations [58–62]. Besides histological changes, chronic tramadol administration in rodents was also associated with increased reactive oxygen species (ROS) and mitochondrial alterations in tissues such as brain and lung [58,59,63]. In fact, treatment with antioxidants is

suggested as a strategy to decrease tramadol-induced tissue damage [64]; prolonged dose interval or dose reductions are also suggested during chronic treatment [65].

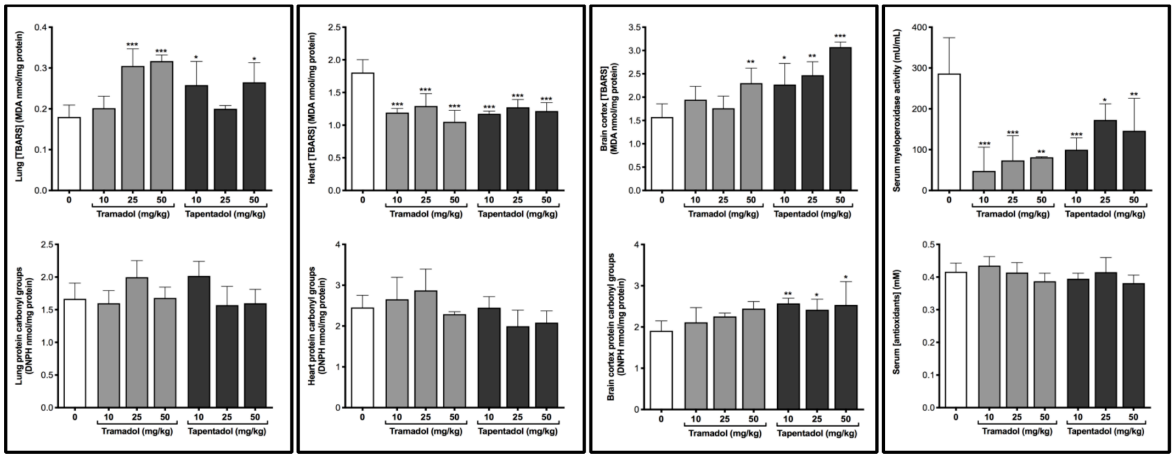
Concerning tapentadol toxicity, Channell and Schug reported many adverse events, including neurological, respiratory, and cardiac function impairment [29]. However, few studies were performed to understand the mechanisms associated with tapentadol toxicity, as underlined in their systematic review [29], since it is a more recent drug. In addition, there are few comparative studies on the long-term effects of clinical doses of tramadol or tapentadol [57], particularly in target tissues such as brain, heart, and lung.

Hence, the present work aimed to evaluate the in vivo toxicological effects of the repeated administration of clinically relevant doses of tramadol or tapentadol, through the comparative analysis of brain, cardiac and lung toxicity. Our study combines molecular, biochemical, and histological approaches and, thus, contributes to a more complete and comprehensive understanding of tramadol and tapentadol toxicological profile.

2. Results

2.1. Repeated Exposure to Tramadol and Tapentadol Causes Oxidative Stress in Lung and Brain Cortex

Wistar rats were used as a model to study the effect of repeated administration of tramadol and tapentadol in lung, heart, and brain cortex. In order to evaluate the effects on oxidative status and putative oxidative damage, thiobarbituric acid reactive substances (TBARS), protein carbonyl groups, myeloperoxidase (MPO) activity and total antioxidant capacity were quantified in tissue and serum samples (Figure 1).



(**a**) (**b**) (**c**) (**d**)

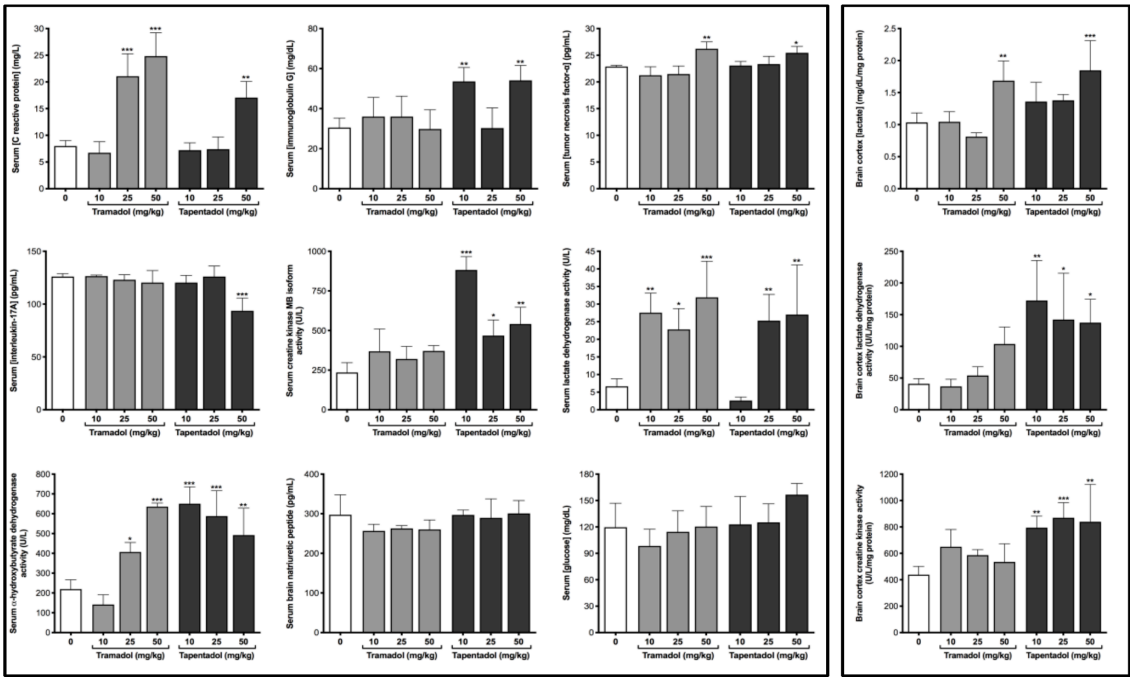
Figure 1. Oxidative stress analysis, assayed as thiobarbituric acid reactive substances (TBARS) and protein carbonyl groups, in Wistar rat lung (**a**), heart (**b**) and brain cortex (**c**) tissue homogenates, as well as serum myeloperoxidase (MPO) activity and total antioxidant capacity (Trolox equivalents) (**d**). Both tissue homogenates and serum samples were processed upon repeated daily intraperitoneal (i.p.) administration of 10, 25, or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. TBARS and protein carbonyl group results were normalized against total protein content. Results are expressed by means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. DNPH: 2,4-dinitrophenylhydrazine; MDA: malondialdehyde.

A significant increase in lung TBARS levels was observed after exposure to 25 and 50 mg/kg tramadol (rising around 1.7-fold), and 10 and 50 mg/kg tapentadol (rising around 1.5-fold) (Figure 1a). In turn, in heart tissue, TBARS levels decreased to about 67% of the control, on average, at all doses of both opioids (Figure 1b). Analysis of brain cortex homogenates showed that the highest tramadol dose, 50 mg/kg, causes a significant 1.5-fold increase in TBARS levels, while this happened for all tapentadol

doses (around 1.7-fold, on average) (Figure 1c). No significant differences were observed for protein carbonyl groups in any of the organs studied, except for brain cortex at all tapentadol doses, for which they increased about 1.3-fold, on average (Figure 1c). These results suggest that, among the tissues under analysis, brain cortex is more susceptible to oxidative damage, particularly after tapentadol exposure. Regarding serum MPO activity, a significant decrease was observed after exposure to both opioids, and at all doses tested, with the values reaching about 36% of the control, on average (Figure 1d). Nonetheless, the exposure to tramadol or tapentadol did not lead to alterations in serum total antioxidant capacity (Figure 1d).

2.2. Repeated Exposure to Tramadol and Tapentadol Causes Alterations in Immunological and Inflammatory Biomarkers

Aiming to evaluate the effects of the repeated administration of therapeutic doses of tramadol and tapentadol on the immunological and inflammatory status, some serum biomarkers were tested, as shown in Figure 2a.



(**a**) (**b**)

Figure 2. Concentrations of serum immunological, inflammatory, cardiac and metabolic biomarkers (**a**), as well as tissue biochemical parameters concerning brain cortex metabolism (**b**), upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25, or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Exposure to 25 and 50 mg/kg tramadol led to an increase in C reactive protein (CRP) levels (2.9-fold, on average); the highest tramadol dose also caused a significant increase in tumor necrosis factor-α (TNF-α) levels (1.2-fold). 50 mg/kg tapentadol led to an increase in CRP (2.1-fold) and TNF-α (1.1-fold). In turn, immunoglobulin G (IgG) levels increased about 1.8-fold, on average, at tapentadol lowest and highest doses. Although no effects were detected on interleukin-17A (IL-17A) levels after tramadol exposure, they significantly decreased at 50 mg/kg tapentadol, reaching 74% of the control values.

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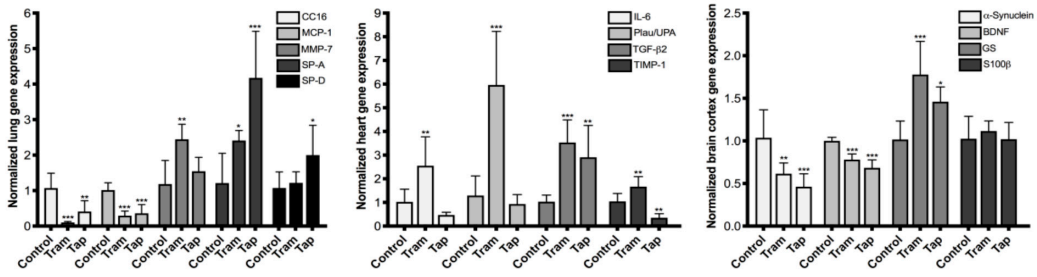
2.3. Repeated Exposure to Tramadol and Tapentadol Compromises Cardiac Cell Integrity and Brain Cortex Metabolism

Several serum biomarkers were analyzed in order to evaluate cardiac cell integrity and function, as shown in Figure 2a. While creatine kinase muscle brain (CK-MB) isoform activity did not change significantly upon tramadol treatment, lactate dehydrogenase (LDH) activity significantly increased at all its doses, rising around 4.1-fold, on average, above the control. However, α-hydroxybutyrate dehydrogenase (α-HBDH) activity increased only when the intermediate and highest doses of tramadol (25 and 50 mg/kg) were administered, to a maximum of 2.9-fold. In turn, 25 and 50 mg/kg tapentadol doses led to an approximate increase of 3.9-fold in LDH activity. At all doses tested, tapentadol caused an increase in CK-MB (to a maximum of 3.8-fold) and α-HBDH (2.6-fold, on average) activities. Though serum brain natriuretic peptide (BNP) levels did not change significantly after exposure to any of the opioid doses tested, when taken together, these data suggest that tramadol and tapentadol cause cardiac damage.

The analysis of biochemical parameters related to brain cortex metabolism (Figure 2b) showed 50 mg/kg tramadol and tapentadol to cause a significant increase (1.6- and 1.8-fold, respectively) in tissue lactate levels. Contrarily to tapentadol, which caused an increase in brain LDH and creatine kinase (CK) activities (3.7-fold and 1.9-fold, on average, respectively), irrespectively of the dose, tramadol led to no statistically significant differences in these enzymes. Serum glucose concentrations also did not change in a significant manner (Figure 2a). These results collectively suggest that tapentadol causes higher brain metabolic alterations.

2.4. Repeated Exposure to Tramadol and Tapentadol Leads to Changes in the Expression of Lung, Heart and Brain Toxicity Biomarkers

Potential toxic effects arising from the repeated administration of clinically relevant doses of tramadol and tapentadol were investigated at the molecular level, through the quantification of toxicity biomarker genes and proteins in lung, heart and brain cortex tissue samples. To this purpose, total RNA from tissues collected from animals exposed to 50 mg/kg tramadol or tapentadol were used in gene expression assays (Figure 3). In turn, brain cortex extracts from animals treated with all opioid doses were used in protein expression assays of neuronal and astrocytic markers (Figure 4).



(**a**) (**b**) (**c**)

Figure 3. Normalized gene expression levels of lung (**a**), heart (**b**) and brain cortex (**c**) toxicity biomarkers, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 50 mg/kg tramadol (Tram) or tapentadol (Tap), for 14 consecutive days. Expression levels were normalized against the respective 18S ribosomal RNA (18S rRNA) gene expression, and then against the respective controls (administered with normal saline), set as 1. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. BDNF: brain-derived neurotrophic factor; CC16: Clara cell protein-16; GS: glutamine synthetase; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1; MMP-7: matrix metalloproteinase-7; Plau/UPA: plasminogen activator, urokinase; S100β: S100 calcium binding protein B; SP-A: pulmonary surfactant protein A; SP-D: pulmonary surfactant protein D; TGF-β2: transforming growth factor-β2; TIMP-1: tissue inhibitor of metalloproteinase-1.

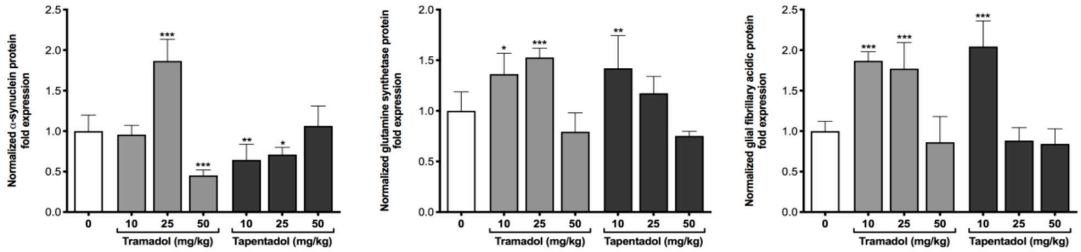


Figure 4. Normalized protein expression levels of neuronal marker α-synuclein and astrocytic markers glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP), in protein extracts from brain cortex tissue, upon Wistar rat repeated daily intraperitoneal (i.p.) administration of 10, 25 or 50 mg/kg tramadol or tapentadol, for 14 consecutive days. Expression levels were normalized against total protein content, using α-tubulin as loading control, and then against the respective controls (administered with normal saline), set as 1. Results are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Five pulmonary toxicity biomarkers were analyzed, as shown in Figure 3a. Tramadol and tapentadol caused a significant decrease in the expression of Clara cell protein-16 (CC16, reaching 10% and 41% of the control, respectively) and monocyte chemoattractant protein-1 (MCP-1, achieving 29% and 36% of the control, respectively). On the other hand, tramadol led to a significant increase in the expression of matrix metalloproteinase-7 (MMP-7, 2.4-fold), tapentadol caused an increase in the expression of pulmonary surfactant protein D (SP-D, 2.0-fold), and both increased the expression of pulmonary surfactant protein A (SP-A, whose gene expression increased 2.4-fold and 4.2-fold upon exposure to 50 mg/kg tramadol and tapentadol, respectively). Concerning cardiac biomarkers (Figure 3b), tramadol caused an increase in the expression of interleukin-6 (IL-6, 2.5-fold) and plasminogen activator, urokinase (Plau/UPA, 6.0-fold); tramadol and tapentadol increased transforming growth factor-β2 (TGF-β2) expression (3.5-fold and 2.9-fold, respectively). Regarding tissue inhibitor of metalloproteinase-1 (TIMP-1) expression, tramadol caused an increase (1.7-fold), unlike tapentadol, which induced a considerable reduction (achieving 35% of the control). The results from brain biomarker gene analysis (Figure 3c) showed that the exposure to tramadol and tapentadol causes a decrease in the expression of α-synuclein (to about 62% and 46% of the control values, respectively) and brain-derived neurotrophic factor (BDNF, reaching 78% and 68% of the control, respectively), as well as an increase in glutamine synthetase (GS) expression (1.8-fold and 1.5-fold, respectively). Nonetheless, no significant differences in S100 calcium binding protein B (S100β) levels were found after tramadol or tapentadol treatment.

As shown in Figure 4, the protein expression levels of a neuronal marker (α-synuclein) and two astrocytic markers (GS and glial fibrillary acidic protein (GFAP)) are altered upon treatment with both opioids. The 25 mg/kg tramadol dose increased the levels of neuronal marker α-synuclein by 1.9-fold; in contrast, the 50 mg/kg dose induced its decrease (achieving 45% of the control levels). The exposure to 10 and 25 mg/kg tramadol caused a significant increase in GS and GFAP protein levels (average 1.4- and 1.8-fold, respectively). The treatment with 10 mg/kg tapentadol caused a significant increase in GS and GFAP protein levels (1.4- and 2.0-fold, respectively), while 10 and 25 mg/kg tapentadol caused a decrease in the neuronal marker α-synuclein (to about 64% and 71% of the control values, respectively).

Taken together, such results demonstrate that the repeated administration of tramadol and tapentadol clinically relevant doses impacts lung, heart, and brain cortex physiology and metabolism at the gene and protein levels.

2.5. Repeated Exposure to Tramadol and Tapentadol Leads to Lung Alveolar Collapse, Cardiac Inflammation and Fibrosis and Neuronal Degeneration

Putative histopathological alterations induced by the treatment with tramadol or tapentadol therapeutic doses were also investigated in lung (Figure 5), heart (Figures 6 and 7), and brain cortex (Figure 8) tissues.

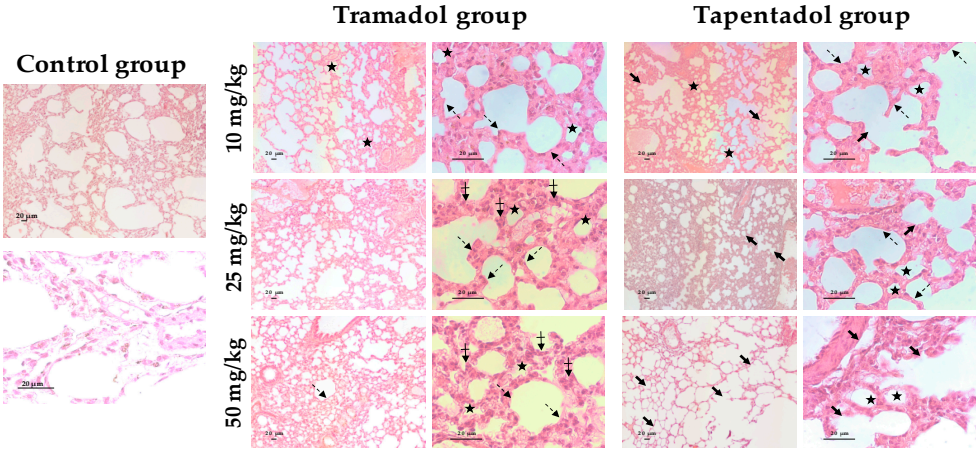


Figure 5. Photomicrographs of lung sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group), for 14 consecutive days, upon hematoxylin and eosin (H & E) staining. Alveolar collapse (stars), alveolar wall thickening and hyperpigmentation (dashed arrows) and disorganized cells (vertical, crossed arrows), as well as alveolar destruction and loss of parenchyma (thick arrows), are observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

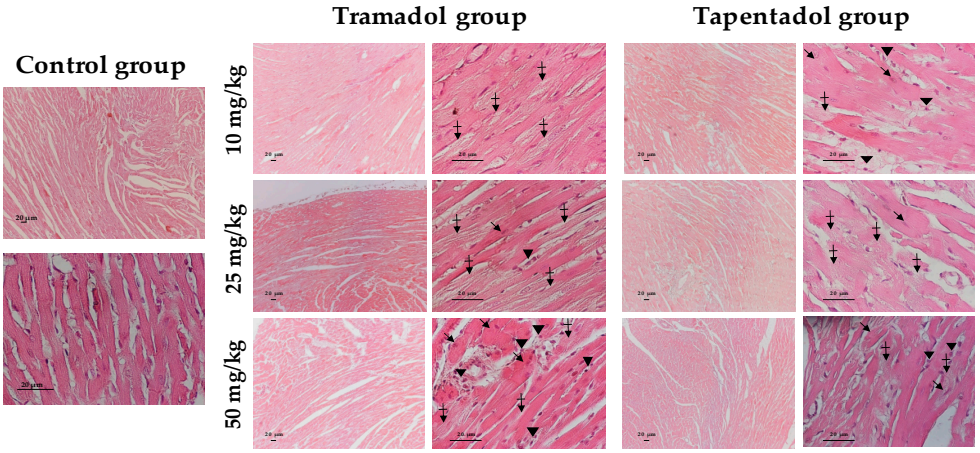


Figure 6. Photomicrographs of heart sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group), for 14 consecutive days, upon hematoxylin and eosin (H & E) staining. A dotted staining (vertical, crossed arrows), possibly denoting fibrous tissue deposition, is observed among cardiomyocytes. Mononuclear inflammatory cells (inverted triangles) and altered cardiomyocytes (arrows), as well as loss of striation, are also observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

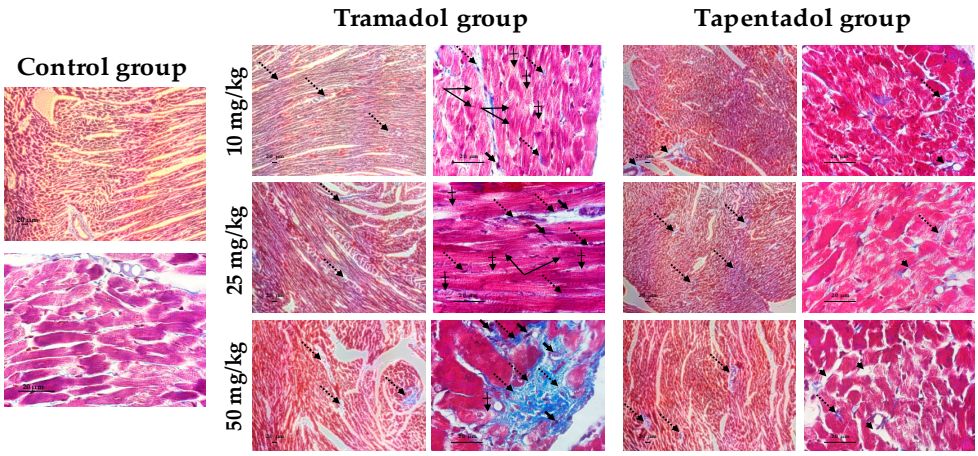


Figure 7. Photomicrographs of heart sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group), for 14 consecutive days, upon Masson's trichrome staining. Fibrous tissue (dotted arrows) and purple cell infiltrates (thick arrows), possibly corresponding to fibroblasts, as well as a dotted staining (vertical, crossed arrows), are observed among cardiomyocytes. Cardiomyocyte fiber filaments are disorganized and show heterogeneous pigmentation (double arrows). Increased perivascular spaces (arrow heads) are also observed. Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

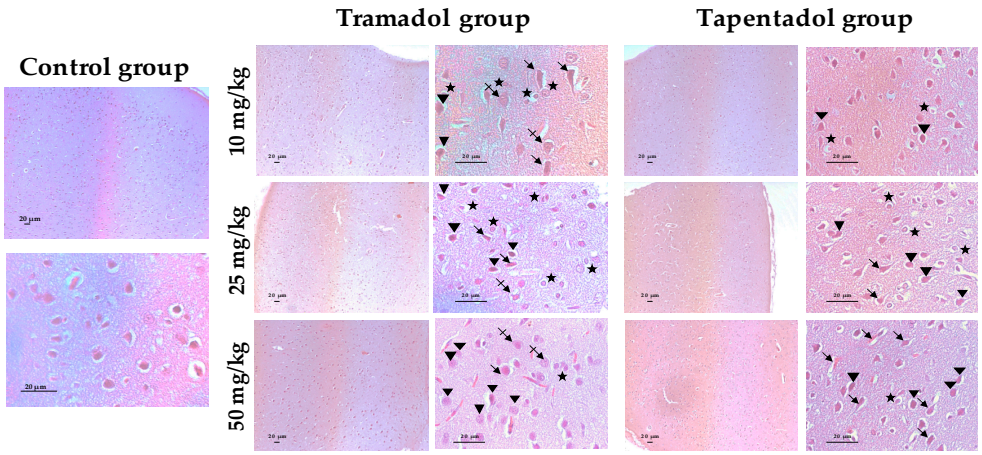


Figure 8. Photomicrographs of brain cortex sections of Wistar rats intraperitoneally injected with different tramadol and tapentadol doses or saline (control group), for 14 consecutive days, upon hematoxylin and eosin (H & E) staining. Glial and microglial cells are observed (stars), as well as swollen neurons (crossed arrows), irregularly-shaped neurons (inverted triangles) and degenerated neurons (long arrows). Photographs were taken with 100× and 600× magnifications. Scale bar, 20 μm.

The histological study of lung tissue samples, upon hematoxylin and eosin (H & E) staining (Figure 5), showed alveolar collapse and wall thickening, as well as hyperpigmentation, to be consequences of the exposure to all tramadol and tapentadol doses. At higher tramadol doses (25 and 50 mg/kg), disorganized cells were observed. Additionally, after tapentadol treatment, alveolar destruction and loss of parenchyma were observed, leading to a "holey" pattern, even in the vicinity of great vessels.

Figure 6 evidences heart tissue damage caused by tramadol and tapentadol, as seen through H & E staining. Interestingly, treatment with tramadol comparatively led to more pronounced injury along with dose increase. A dotted staining between cardiomyocytes, possibly reflecting cardiomyocyte substitution by fibrous tissue, is observed as a consequence of tramadol treatment. Furthermore, at higher doses (25 and 50 mg/kg), mononuclear inflammatory cells and altered cardiomyocytes were detected, as well as loss of striation. At the highest dose (50 mg/kg), a lower pigmentation was observed in vessel vicinity, suggesting a context of perivascular fibrosis. In turn, tapentadol caused similar alterations at all doses, including the dotted staining between cardiomyocytes, eventually suggesting fibrous tissue deposition; inflammatory cell infiltrates, altered cardiomyocytes and loss of striation were also found.

In order to clarify the potential signs of fibrosis suggested by H & E staining, Masson's trichrome staining was performed with heart tissue samples (Figure 7).

Indeed, the presence of fibrous tissue between cardiomyocytes was confirmed after tramadol and tapentadol treatment, being evident at tramadol doses as low as 10 mg/kg; such observations increased along with dose increment, with the 50 mg/kg dose leading to marked perivascular fibrosis. Besides these findings, cardiomyocyte fiber filaments are disorganized and show heterogeneous pigmentation. In animals treated with tapentadol, fibrous tissue was observed between cardiomyocytes, possibly delimiting newly formed capillaries and, thereby, suggesting revascularization. Despite being found at all tapentadol doses, a more evident increase in perivascular space was observed at 50 mg/kg.

Brain cortex histological analysis through H & E staining is shown in Figure 8. Both tramadol and tapentadol exposure cause glial activation with microglial proliferation and are associated with degenerated and irregularly-shaped neurons. Such histopathological changes accumulated along with tapentadol dose. Moreover, tramadol treatment led to swollen neurons.

Thus, clinically relevant doses of both tramadol and tapentadol lead to histopathological damage in all tissues under analysis.

3. Discussion

Prescription opioids are not exempt from toxicological risks, especially when used for prolonged periods. In the present study, we aimed to analyze putative lung, heart, and brain cortex detrimental effects deriving from the repeated administration of clinically relevant doses of tramadol and tapentadol to Wistar rats. By addressing, in parallel, tramadol and tapentadol subacute effects on target organs, we complement the subacute study regarding the effects on metabolizing organs, as well as our previous acute exposure studies. In fact, since these opioids are often consumed on a subacute to chronic basis, our repeated exposure-based experimental design also provides a realistic approximation to their actual consumption scenario.

Comparison of tramadol and tapentadol safety profiles is justified by their structural and mechanistic similarities. However, when comparing their toxicological effects, differences in their pharmacokinetic and pharmacodynamic properties, including metabolic pathways, metabolite profiles, receptor, and transporter affinities [1,9,55–57], should be kept in mind. Additionally, intraperitoneally-injected drugs bypass the intestine, but are absorbed into the mesenteric vessels draining into the portal vein, thereby giving room for hepatic metabolism to occur before reaching systemic circulation [66]. Hence, there are pharmacokinetic similarities between intraperitoneal (i.p.) and oral administration, which is a common route of administration, and the only one in which tapentadol is currently available [1,9]. Thus, although the doses used in our study are equal in absolute terms, they are not pharmacologically equivalent. In fact, the two opioids present different oral bioavailabilities (68–84% for tramadol and 32% for tapentadol [1,9,13]). In line with this, tapentadol doses should be increased to achieve pharmacological equivalence to tramadol, which would furthermore exacerbate differences between the results. All these remarks should be taken into account while comparatively addressing both toxicological profiles.

3.1. Repeated Administration of Tramadol and Tapentadol Leads Mainly to Lipid Peroxidation in Lung and Brain Cortex Tissues, but Has Seemingly a Protective Effect in Cardiac Tissue

Although their dose determines their overall effect on the oxidation status, opioids are known to induce oxidative stress, with multiple studies reporting increased serum and tissue oxidative stress biomarkers and decreased antioxidant defense mechanisms. Decreased brain glutathione, glutathione peroxidase, and superoxide dismutase (SOD) activities, as well as increased brain malondialdehyde (MDA), nitric oxide (NO), inducible nitric oxide synthase (iNOS), and 8-hidroxydeoxyguanosine levels, have been described in mice and rat models repeatedly administered with 20 to 168 mg/kg tramadol, through different routes [60,62,67–71]. Furthermore, under the conditions assayed in the present study, we have previously shown increased TBARS—a surrogate of lipid peroxidation (LPO)—and protein carbonyl groups—indicative of protein oxidation—in liver and kidney homogenates from Wistar rats exposed to both tramadol and tapentadol [57].

In single-exposure assays to 10, 25 and 50 mg/kg tramadol and tapentadol, we found almost no significant alterations in TBARS levels in lung, heart, and brain cortex homogenates [55]. In turn, the protein carbonyl group contents increased in lung and heart tissues at the intermediate and highest doses, whilst they significantly decreased in brain cortex upon tramadol treatment [55]. Repeated administration changed such scenario, since, following exposure to both opioids, TBARS concentrations increased in lung and brain cortex, while they decreased in heart homogenates (Figure 1). Protein carbonyl groups did not change significantly, except for brain cortex from animals exposed to tapentadol, where they increased (Figure 1c).

Therefore, it might be hypothesized that prolonged administration changes the oxidation status in an opioid- and organ-specific manner. Indeed, LPO was now induced in lung and brain cortex, but there seems to be a protective effect in heart tissue. Consistently with this, 20 mg/kg tramadol prevented a rise in cardiac tissue MDA levels in a rat ischemia-reperfusion model [72]. The authors of the study suggest that tramadol reduces oxidative stress by scavenging peroxyl radicals and increasing antioxidant capacity [72]. In this regard, serum MPO results should also be taken into account. MPO is a member of the superfamily of heme peroxidases that is mainly expressed in polymorphonuclear neutrophils and monocytes, which contribute to the generation of reactive species that elicit inflammation and LPO [73]. Several lines of evidence support an association between MPO (and its product hypochlorous acid (HOCl)) and cardiovascular disease, given that, among other effects, it generates dysfunctional lipoproteins and atherosclerotic plaque instability [73]. Hence, reduced MPO activity might be correlated with cardiac tissue protection from LPO. In fact, MPO activity was found to be reduced in lung tissue, relative to the controls, after intravenous administration of 20 mg/kg tramadol in a rat model of ischemia-reperfusion [74]. Interestingly, morphine has been reported as an MPO inhibitor [75], for which a similar effect might be anticipated for other structurally related opioids. Still, tissue quantification of MPO activity would add information on this aspect, since an increase in its levels was reported in rat brain tissue following a 9-week daily treatment with 22.5 to 90 mg/kg tramadol [68].

As far as protein carbonyl groups are concerned, the protective effect observed in brain samples in an acute context is lost upon repeated tapentadol administration, while the deleterious effects in lung and heart appear to fade for both opioids.

While tramadol effects on brain cortex oxidative stress are minor, they are more substantial upon tapentadol treatment. In fact, due to its much greater potency at the MOR, comparable or higher NA transporter inhibition and significantly lower 5-HT transporter inhibition, tapentadol has a greater central nervous system (CNS) functional activity than tramadol, being 2 to 5 times more potent across different animal models of pain [14]. In addition, in vitro and in vivo studies suggest that tramadol is actively transported across the blood-brain barrier, at least in part, by proton-coupled organic cation antiporter [76]. Disproportionally less of the stronger opioid metabolite M1 crosses the blood-brain barrier than its weaker opioid parent, with the disparity increasing as tramadol dose is increased [14].

In turn, tapentadol readily crosses the blood-brain barrier, following its concentration gradient, with no known active transport mechanism [77]. Altogether, these pharmacokinetic and pharmacodynamic differences may contribute to explain the greater impact of tapentadol on the CNS and, more specifically, on brain cortex oxidative stress. The reasons underlying tapentadol higher potency and efficacy may simultaneously underlie its deleterious effects in target organs.

Taken together, the results indicate that the extension of the exposure period leads to a shift towards the intensification of lipid oxidative stress mechanisms, from which the cardiac tissue seems to be spared. The results obtained with brain samples might be correlated with the high rates of brain oxygen consumption, which make it particularly prone to oxidative damage. Nonetheless, such local, organ-specific alterations do not impact the systemic antioxidant status, since no significant alterations were found in the serum concentration of antioxidants. It should be noted, however, that, although total antioxidant capacity assays predominantly measure low molecular weight, chain breaking antioxidants such as urate, ascorbate, bilirubin, and α-tocopherol, they do not measure important antioxidant components such as SOD, glutathione peroxidase, and catalases [78,79]. While the serum levels of the former, in opioid-treated rats, might be comparable to those of the controls—as deduced from the absence of statistically significant differences among groups, the activities of the latter might be decreased, both in serum and in tissues. This possibly explains increased tissue lipid and protein oxidative stress and is supported by several studies reporting decreased antioxidant enzyme activity upon tramadol exposure [60,67–69,71].

3.2. Repeated Administration of Tramadol and Tapentadol Leads to Inflammation, with Possible Compensatory Recruitment of Anti-Inflammatory Pathways

Opioids are suggested to suppress immune competency in pain-free subjects, even at subanalgesic doses [80]. In fact, tramadol has been reported to have anti-inflammatory properties [81] and to lead to less immunomodulatory effects when compared with pure MOR agonists, which are known for suppressing natural killer (NK) cell activity and T lymphocyte proliferation [80]. Nonetheless, diverse histopathological studies, some of which by our own group [55–57], describe its ability, as well as that of tapentadol, to cause tissue inflammation in acute and subacute contexts. In order to understand if a subacute exposure to tramadol and tapentadol clinically relevant doses causes immunological and inflammatory alterations, some serum biomarkers were analyzed (Figure 2a).

CRP is an acute-phase protein, synthesized by the liver, whose plasma levels increase in response to inflammation. Our results show that serum levels of CRP increase after tramadol and tapentadol administration, which is compatible with an inflammatory condition. Consistently, patients who received 100 mg tramadol every 8 h experienced a 123%-increase over their CRP baseline, 72 h after removal of an impacted lower third molar [81]. Moreover, following the administration of the highest dose in the present study (50 mg/kg), we also detected an increase in TNF-α, a cytokine involved in both physiological and pathological processes. Due to its participation in essential cellular pathways associated with inflammation, apoptosis, and necrosis, it is used as a systemic marker for tissue injury and systemic inflammation [68]. A significant increase in serum concentrations of pro-inflammatory cytokines TNF-α and interleukin-1B (IL-1B) had already been associated with chronic administration of therapeutic (22.5 mg/kg/day) and high tramadol doses (30, 60, and 90 mg/kg/day) [68]. Increased serum IgG levels were also found upon exposure to tapentadol. Both pro- and anti-inflammatory roles have been associated with IgG, the most abundant antibody in human serum and an indicator of the immune status [82]. In particular, raised IgG serum concentrations are found in interstitial lung disease, characterized by chronic inflammation and irritation of the alveolar walls (alveolitis) and adjacent supporting tissue (interstitium), which may progress to fibrosis [83], and are thus compatible with the histopathological alterations observed in lung slides (Figure 5). Interestingly, elevated serum IgG levels were found at 10 and 50 mg/kg tapentadol, but not at 25 mg/kg. We hypothesize that both the lowest and highest

tapentadol doses lead to an inflammatory state, characterized by an imbalance between pro- and anti-inflammatory stimuli, and reflected in increased serum IgG levels. While, at 10 mg/kg, there is an activation of the immune system, with consequent recruitment of anti-inflammatory components, these are overtaken by more potent and/or abundant pro-inflammatory stimuli at 50 mg/kg. IgG levels are similar at 10 and 50 mg/kg because, although the concentrations of pro- and anti-inflammatory components may differ in both conditions, they vary proportionally. In turn, at 25 mg/kg, the intermediate dose, there might be an anti-inflammatory compensation of pro-inflammatory stimuli, explaining comparable IgG levels between the controls and this condition. Therefore, a combination of time- and dose-dependent effects is suggested to underlie seemingly inconsistent IgG results. The analysis of pro- and anti-inflammatory balance along the exposure time, instead of endpoint results, would add information on this hypothesis.

On the other hand, our results showed that tapentadol highest dose causes a decrease in IL-17A, a pro-inflammatory cytokine that acts in concert with TNF-α to induce the production of many other cytokines, chemokines and prostaglandins. In fact, alterations in inflammatory parameters were already reported as a tramadol effect, after treatment for 15 consecutive days (45 mg/kg during the first week and 90 mg/kg during the second week); serum interferon gamma (IFN-γ) decreased, while alterations in interleukin-10 (IL-10) serum levels were not detected [84]. Consistently with this, rats intraperitoneally injected with 1 mg/kg tramadol showed decreased IL-6 and unchanged interleukin-2 (IL-2) levels, although 10 and 20 mg/kg doses reversed alterations in IL-6 [85]. It was previously suggested that 5-HT reuptake inhibition could be involved in the immune effects of tramadol [85]. In accordance, histopathological examination of different tissues, such as lung, heart and brain, after H & E staining, showed that tramadol was associated with less inflammatory cell infiltrates than tapentadol [55]. In this sense, considering tramadol analgesic potency and lower immunosuppressive effects, it was suggested as a better alternative for pain treatment than classical opioids, since it may have an immune enhancing effect and, thus, be especially considered in conditions where immunosuppression is contraindicated [80,85–87].

Our study provides seemingly contradictory evidence on tramadol and tapentadol potential for inflammatory modulation—increased CRP and TNF-α for both opioids but decreased IL-17A and increased IgG for tapentadol, along with variable degrees of histological evidence of inflammation. Although it might be hypothesized that anti-inflammatory pathways are being recruited to compensate for inflammatory injury, it should be emphasized that more studies are needed to better understand tramadol and tapentadol role in the modulation of the immunological and inflammatory profiles.

3.3. Repeated Administration of Tramadol and Tapentadol Leads to Cardiac Muscle Cell Damage, though with No Impact on Ventricular Function

Electrocardiographic changes are one of the side effects associated with tramadol use, and clinical, hematological, and toxicological findings, such as troponin and myoglobin elevation, suggest myocardial damage upon intoxication with this opioid [48,88,89]. Cardiac troponin I was significantly elevated in rats receiving 12.5–300 mg/kg tramadol per day for two weeks [90]. Acute doses of tramadol and tapentadol were also shown to be cardiotoxic at the same doses used in the present study [55]. Thus, a series of serum biomarkers was assessed in order to study the putative impact of the repeated administration of tramadol and tapentadol on cardiac muscle cell integrity and function (Figure 2a). While serum BNP levels remained unchanged upon opioid treatment, CK-MB activity was found to increase upon exposure to tapentadol, while those of LDH and α-HBDH increased upon exposure to both opioids.

BNP is produced in cardiac ventricles, serving as a quantitative marker of heart failure; it proportionally reflects ventricular systolic and diastolic dysfunction, as well as acute hemodynamic change [91,92]. The lack of alterations in this biomarker suggests that tramadol and tapentadol do not impair these aspects of cardiac function.

Isoenzymes CK-MB, $LDH_{1–2}$, and α-HBDH are found mainly in heart muscle, for which they are used as cardiac biomarkers [93], often with a good correlation with oxidative stress and inflammatory parameters [94]. α-HBDH is considered to represent LDH_1 activity alone or both LDH_1 and LDH_2 activities [93]; consistently, in our study, the changes in LDH and α-HBDH were in the same direction and detected upon both tramadol and tapentadol treatment. Since LDH is an unspecific cell lysis biomarker, LDH_{1-2} also occur in rat kidneys in considerable amounts and nephrotoxicity has been reported under these experimental conditions [57], CK-MB arises as the most sensitive indicator of myocardial damage [93]. Accordingly, serum CK and CK-MB activities, as well as cardiac troponin I, were elevated in a case of multiple organ dysfunction after tramadol overdose [48].

Hence, the analysis of cardiac biomarkers as a whole is indicative of myocardial injury following repeated administration of both opioids.

3.4. Repeated Administration of Tramadol and Tapentadol Modifies Brain Cortex Metabolism, with Tapentadol Causing a Higher Degree of Metabolic Modulation

Aiming to investigate whether consecutive administration of therapeutic doses of tramadol and tapentadol impacts brain cortex metabolic profile, we have quantified metabolic parameters in the corresponding homogenates (Figure 2b).

The results are in agreement with those from previous studies by our own group, where we used the same animal model and opioid doses, but in an acute treatment context [55]. Although no significant changes were detected in serum glucose levels, brain cortex lactate contents were found to be elevated at the highest dose for both opioids. These changes were matched by an increase in the activity of LDH, the catalyst of lactate production, at all tapentadol doses. This is an extension of the effect observed in acute settings, where a significant elevation was limited to 50 mg/kg tapentadol [55]. We have previously shown that the exposure to tramadol and tapentadol affects the expression of energy metabolism enzymes [54], leading to a possible bioenergetic crisis that is supported by other studies [95–97] and reflected in lactate overproduction and decreased ATP synthesis. Interestingly, supratherapeutic tramadol doses have been found to partially inhibit the activities of respiratory chain complexes I, III, and IV, correlating with increased oxidative stress and explaining clinical and histopathological effects such as seizures and apoptosis [97]. Increased lactate levels were also observed in rat spinal cord dorsal horn upon acute and chronic morphine administration [98]. In parallel, astrocytic glycolysis and lactate production are closely associated with the astrocytic reuptake of glutamate and with neuronal oxidative metabolism, which is fueled by lactate [99]. This prompted us to further investigate the expression of astrocytic markers, namely GS, given lactate role in glutamine/glutamate cycling.

In turn, CK catalyzes the reversible phosphorylation of creatine to phosphocreatine, a highly diffusible energy carrier [100]. Brain CK is reported to locally fuel ATPases by providing phosphocreatine, as well as to maintain local ATP buffering under limited oxygenation and/or nutrient supply, where mitochondrial function and phosphocreatine regeneration is partially or totally impaired [100]. Increased expression of brain CK may therefore be regarded as an adaptation to opioid-induced stress and mitochondrial dysfunction.

Since LDH and CK activities were significantly augmented upon tapentadol exposure only, it might be deduced that this opioid causes greater brain metabolic modulation.

3.5. Repeated Exposure to Tramadol and Tapentadol Alters the Expression of Lung, Heart and Brain Toxicity Biomarkers at the Gene and Protein Levels, Correlating with Oxidative Stress, Inflammation, Metabolic and Histological Parameters

To ascertain the potential impact of tramadol and tapentadol repeated administration on gene and protein expression levels, a panel of toxicity biomarkers was assayed in lung, heart, and brain cortex samples from Wistar rats exposed to 50 mg/kg opioid, the highest dose under study. Alterations were found for most of these biomarkers (Figure 3), with their nature and extent being similar for most of the genes studied. We hypothesize the excep-

tions to be due to differences in tramadol and tapentadol structure, chemical properties and mechanisms of action. These account for different pharmacokinetics and pharmacodynamics and, consequently, for different potency and effects on target organs [14], which possibly include gene expression. Nevertheless, it should be underlined that changes in mRNA transcript levels do not necessarily translate into protein expression, since there might be posttranscriptional and posttranslational events affecting mRNA and protein stability.

Regarding the lung toxicity biomarker panel (Figure 3a), CC16 gene expression levels were found to be decreased upon both tramadol and tapentadol exposure. CC16, a protein with anti-inflammatory and immunomodulatory activities, is the major secretory product of the Clara cells, which play an important role in bronchial epithelial repair mechanisms [101,102]. Clara cells have the highest levels of CYP450 in the lung and are the main site of xenobiotic detoxification, rendering them particularly sensitive to injury, due to the production of toxic metabolites [101,103]. In this sense, tramadol bioactivation by CYP450 should not be overlooked, since there is evidence of pulmonary expression of isoenzymes such as CYP2B6 and CYP3A4 [104], supporting the possibility that tramadol metabolites may contribute to its pneumotoxicity. Clara cell destruction leads to decreased CC16 production, for which bronchoalveolar lavage or serum CC16 has been reported as a sensitive indicator of bronchial or epithelial injury. Accordingly, its decrease has been described in smokers and in occupational groups with an history of chronic exposure to several air pollutants [101,105], as well as in subjects with respiratory disease [102,103]. In mice models, reduced CC16 levels are associated with pulmonary inflammation and injury, alveolar septal cell apoptosis, airway mucus metaplasia, emphysema, and small airway remodeling [103,105]. Cigarette smoke-exposed *CC16*$^{-/-}$ mice show increased lung levels of pro-inflammatory mediators chemokine (C-C motif) ligand 5 (CCL5) and matrix metalloproteinase-9 (MMP-9) and pro-fibrotic mediator transforming growth factor-β1 (TGF-β1), but lower levels of anti-inflammatory IL-10 than their wild-type counterparts [103]. Thus, pulmonary inflammation, dysfunction, and remodeling might be indicative of possible effects of repeated exposure to 50 mg/kg tramadol and tapentadol.

MCP-1 expression was also found to be downregulated upon exposure to both opioids. Although MCP-1 is a potent profibrotic chemokine, its plasma concentration has been found to be reduced in patients with a higher grade of pulmonary toxicity 1 h after radiotherapy [106,107], as well as upon exposure to some drugs and pollutants [108,109]. Since it has been implicated in alveolar tissue repair and, thus, in the resolution of inflammation, reduced MCP-1 expression might contribute, at least in part, to alveolar collapse and structural changes observed through histopathological analysis (Figure 5). Interestingly, in vitro TGF-β and TNF-α co-treatment decreased MCP-1 gene and protein expression in endothelial cells [110]. Since their levels have been found to be increased in our study—in heart tissue, at the gene level, for TGF-β (Figure 3b), and in serum samples, at the protein level, for TNF-α (Figure 2a), a similar correlation with MCP-1 under-expression might be hypothesized. Furthermore, heme oxygenase-1 (HO-1) induction, which we have previously shown to occur in the experimental conditions assayed in the present work [57], has been associated with a decrease in ROS and MCP-1 [111], thus providing an additional possible explanatory mechanism.

Matrix metalloproteinases are a family of endopeptidases involved in extracellular matrix (ECM) degradation and remodeling, being implicated in innate immunity, tissue repair, and homeostasis, but also in inflammation, modulation of bioactive compounds, apoptosis, and in the progression of several diseases, including xenobiotic-induced interstitial lung disease [112–114]. MMP-7, also known as matrilysin, is highly overexpressed in human idiopathic pulmonary fibrosis, participating in neutrophil transepithelial efflux and in fibrotic response [112,114,115]. In fact, it has been suggested as a reliable prognostic biomarker for lung disease [114,115]. Thus, MMP-7 overexpression upon treatment with 50 mg/kg tramadol might be correlated with the histopathological alterations observed for this condition (Figure 5), representing a possible predictor of lung function decline and disease progression.

In turn, besides being part of the first line of immune defense in the lung, surfactant proteins SP-A and SP-D, mainly secreted by type II pneumocytes and Clara cells, control inflammation and fibrosis and participate in the organization, stability, and metabolism of lung parenchyma [116,117]. Importantly, they contribute to the structural and functional integrity of pulmonary surfactant, thus avoiding alveolar collapse by reducing the surface tension at the air/liquid interface [118]. Their serum concentrations increase in pulmonary alveolar proteinosis, idiopathic pulmonary fibrosis, interstitial pneumonia with collagen vascular diseases, asthma, and respiratory distress syndrome [119,120], having been proposed as good differential diagnosis and prognosis biomarkers for idiopathic pulmonary fibrosis [117]. Interestingly, they were suggested to protect lungs from xenobiotic-induced oxidative injury, as deduced from correlations with TBARS levels in rat lung [116]. A similar correlation may be done in our study, considering the increase in lung TBARS contents upon treatment with both opioids (Figure 1a), SP-A gene overexpression upon tramadol treatment, and SP-A and SP-D gene overexpression upon tapentadol treatment. Therefore, gene expression results may reflect increased oxidative stress in opioid-treated rat lungs, as well as susceptibility to pulmonary disease.

IL-6 gene expression levels were quantified within the scope of the analysis of cardiotoxicity biomarker genes (Figure 3b), having increased upon tramadol treatment. IL-6 is a pleiotropic cytokine that connects innate and adaptive immunity and plays different roles throughout time. Although, in the short term, IL-6 initiates acute phase response and wound healing, directs immune cell activation and trafficking, having a pro-inflammatory and protective effect, it becomes pathogenic in a chronic context [121]. Chronically elevated IL-6 concentrations are associated with chronic inflammation, fibrotic disorders, myocardial hypertrophy, reduced contractility, remodeling and, ultimately, heart failure [121]. Increased IL-6 gene expression upon tramadol treatment might thus correlate with the observed higher degree of histopathological alterations, including inflammation and fibrosis (Figure 6). In addition, IL-6 abnormalities lead to dyslipidemia and cardiac lipotoxicity, although it is unclear whether excess or deficiency is responsible [122]. Given that serum lipid alterations were identified in the experimental conditions under study [57], such hypothesis should not be disregarded.

Plau/UPA is a serine protease that is suggested to play a role in cardiac fibrosis, since its absence seems to impair fibroblast ability to migrate into infarcted tissue, synthetize collagen and form fibrotic scars [123]. However, besides being active at sites of tissue remodeling and inflammation, Plau/UPA was paradoxically proven to protect heart tissue from oxidative damage, by promoting DNA repair [124]. Therefore, increased expression of Plau/UPA in cardiac tissue upon repeated administration of 50 mg/kg tramadol might simultaneously be associated with the histological evidence of fibrosis observed for this condition (Figure 7) and with an attempt to curtain opioid-induced oxidative damage. Interestingly, although we did not specifically measure DNA oxidation biomarkers, our results indicate that the heart tissue is the least affected by oxidative injury (Figure 1b), probably reflecting the activation of antioxidant defense mechanisms.

TGF-β superfamily members are central players in cell proliferation, differentiation and migration of different components of the cardiovascular system, being involved in cardiac hypertrophy, fibrosis, contractility, metabolism, angiogenesis, repair, remodeling, and regeneration [125,126]. Specifically, TGF-β2 is upregulated and undergoes de novo synthesis promptly after infarction and ischemic injury [125,126]. Several studies indicate that, in an infarcted myocardium, TGF-β family members regulate immune function by modulating chemotaxis, chemokine synthesis, immune cell differentiation and activation [126]. In addition, they have anti- or pro-apoptotic actions, enhance cardiomyocyte performance, promote myofibroblast conversion, stimulate ECM protein synthesis and have matrix-preserving effects, by inhibiting collagenase and increasing tissue inhibitor of metalloproteinase (TIMP) levels. Fibroblast activation or conversion into myofibroblasts drives ECM accumulation and pathological fibrosis [126]. Additionally, TGF-β stimulation was also shown to lead to endothelial-to-mesenchymal transition, which contributes to cap-

illary rarefaction, tissue ischemia and consequent fibrotic myofibroblast deposition [127]. These roles are on the borderline between inflammation and repair, which might be a reasonable scenario upon treatment with both tramadol and tapentadol, considering that, in our study, TGF-β2 gene expression increased in both situations. Furthermore, upon tramadol exposure, TGF-β2 higher overexpression, combined with that of TIMP-1, is in line with the histological evidence of fibrosis and structural changes observed for this condition (Figure 7).

In turn, TIMPs maintain the homeostatic balance of myocardial ECM by inhibiting activated matrix metalloproteinases (MMPs) and their ECM-degrading function [128,129]. Elevated tissue and plasma TIMP-1 levels have been correlated with myocardial fibrosis and diastolic dysfunction, both in human patients and in animal models, through both MMP activation-dependent and -independent mechanisms [128,129]. Thus, there might be an association between TIMP-1 gene overexpression and the evidence of cardiac fibrosis observed at 50 mg/kg tramadol (Figure 7). Moreover, TIMP-1 gene overexpression in heart tissue, following tramadol repeated administration, may be correlated with that of IL-6, since this pro-inflammatory cytokine may directly upregulate TIMP-1 expression, suggesting common regulatory pathways [130].

Neurotoxicity biomarkers have been quantified at the gene (at the highest opioid dose) and protein (at all doses tested) levels (Figures 3c and 4, respectively). As previously noted, though a correlation is expected, changes in mRNA levels might not be reflected in protein expression, due to posttranscriptional and posttranslational effects modulating mRNA and protein stability.

α-Synuclein, one of the biomarkers assayed, is associated with synaptic vesicular trafficking, transmission, and plasticity [131,132]. Aggregates of misfolded, toxic forms are reported in synucleinopathies, having been associated with alterations in structural cell components, multiple cellular pathways, protein clearance mechanisms and mitochondrial function [131]. This pre-synaptic protein has also been shown to negatively regulate dopaminergic neurotransmission, since it decreases the expression and inhibits the activity of enzymes involved in dopamine synthesis, affects the activity of dopamine transporters and the capacity of refilling and storage of pre-synaptic dopamine-containing vesicles [132,133]. Such observations are highly suggestive of α-synuclein participation in opioid-elicited effects on the dopaminergic reward pathway [132]. Indeed, besides leading to cellular stress and toxicity, increases in α-synuclein levels have been associated with predisposition to addiction to different drugs of abuse, such as cocaine and alcohol [132–134]. Although α-synuclein mRNA levels decreased upon exposure to 50 mg/kg tramadol and tapentadol (Figure 3c), such alterations were reflected at the protein level for 50 mg/kg tramadol, 10 and 25 mg/kg tapentadol only (Figure 4). In fact, its protein levels increased at 25 mg/kg tramadol and were unchanged at 50 mg/kg tapentadol (Figure 4). The same trend was observed in mice brains upon chronic morphine treatment and 48 h of withdrawal; while downregulation of α-synuclein mRNA was observed in the basolateral amygdala, dorsal striatum, nucleus accumbens, and ventral tegmental area, its protein levels were significantly increased in the amygdala and striatum/accumbens. The authors of the study argue that opposite changes in gene and protein levels might take place in different populations of projection neurons whose somata locate in distinct brain areas [132]. In addition, posttranslational mechanisms, such as phosphorylation and ubiquitination, influence α-synuclein degradation rate and stability, further possibly explaining differences between mRNA and protein levels [132]. Other opioid exposure-studies reiterate such inconsistencies, as α-synuclein protein levels decrease in human serum [133], but increase in brain paranigral nucleus and substantia nigra ventral part after chronic heroin use [134]. In turn, α-synuclein protein levels increase in neuroblastoma cells after chronic exposure to morphine [135], as well as in rat forebrain cortex upon a 10-day exposure to the same drug [136], but decrease in rat hippocampus under the same conditions [137]. Therefore, a combination of brain area-specific phenomena and posttranslational mechanisms reg-

ulating protein stability might account for discrepancies between α-synuclein gene and protein expression levels.

BDNF is a small neurotrophin that is also involved in pain transmission, neuroinflammation, neuromodulation, memory, learning, addiction behavior, and opioid analgesic tolerance [138–140]. Upregulation of BDNF and its receptor has been suggested as an important neuroadaptation, being implicated in synaptic plasticity and neuronal survival [139]. Indeed, BDNF gene and protein overexpression has been reported in lumbar spinal cord samples from morphine-tolerant mice [138] and in hippocampal samples from rats repeatedly administered with the same opioid, but not upon acute exposure [141]. Increased BDNF serum concentrations were also reported for heroin-dependent male patients undergoing methadone maintenance treatment [140]. However, in line with our results, a decrease in BDNF-encoding mRNA levels was detected in Wistar rat brain cortical areas upon repeated daily i.p. injection of 20 mg/kg tramadol for 21 days, while no changes were identified in hippocampus, both in short- and long-term contexts [139,142]. The authors theorize that, unlike antidepressant drugs, for which neurotrophic effects have been postulated, tramadol does not induce such kind of neuroadaptation [139]. Likewise, acute and repeated i.p. injections of 1–10 mg/kg tapentadol to rats did not lead to changes in BDNF transcript levels in ganglia and central tissues [143]. The influence on BDNF levels might thus depend on the opioid used, on the brain region under analysis, and on the exposure regimen.

Opioid brain signaling and information processing were found to induce the activation of glial cells, especially astrocytes, by direct MOR stimulation in astrocyte membranes [144,145]. GS is highly expressed in astrocytes, for which it serves as an astrocytic marker [146]. It is a key regulatory enzyme in brain glutamate and glutamine dynamics, which, in turn, is involved in opioid addiction and dependence. Tight control of glutamate extracellular levels is crucial, not only for nociception neurotransmission, but also to avoid neuronal over-excitation and excitotoxicity [147]. Glucose taken by astrocytes is metabolized via glycolysis into lactate, thereby producing ATP to meet energy requirements, mostly for glutamate reuptake from the synaptic cleft [99,146]. Glutamate may also be synthetized from α-ketoglutarate, a Krebs cycle intermediate, through transamination via mitochondrial aspartate aminotransferase. Glutamate is then condensed with toxic ammonia, via GS, to form non-toxic glutamine [99,146]. This, in turn, is transported to presynaptic terminals, where it is converted into glutamate in excitatory synapses and γ-aminobutyric acid (GABA) in inhibitory synapses [99,146]. Astrocytic glucose consumption and lactate production appear to be largely coupled by the astrocytic reuptake of glutamate released at excitatory synapses, with the lactate produced by astrocytic glycolysis serving as a substrate for neuronal oxidative metabolism [99]. Studies on GS expression yield contradictory results. While some proteomic analyses report a decrease in GS levels following morphine administration, others—including one study with rat cerebral cortex synaptosomes—report an increase [148]. When GS activity was measured instead of its protein levels, no changes were found [148]. Muscoli and co-authors added that, although GS total protein levels did not change after morphine repeated administration, the levels of its nitrated, inactivated form increased, which might represent a contributory mechanism for antinociceptive tolerance [149]. In turn, GS activity increased in different brain regions upon Wistar rat daily injection with 31 mg/kg tramadol for 3 consecutive days, peaking on day 3 or 6 post-administration [150]. Opioid peptides also led to an increase in GS activity in cell lines with astrocytic phenotype [151]. The authors conclude that astrocytes respond to opioids and argue that GS increased activity contributes to brain glutamate mobilization and compartmentation and, consequently, to prevent its pathological effects [150–152]. Such argument may explain our own results, in view of GS increased expression at low and intermediate opioid doses (as determined through Western blotting, Figure 4) and at the highest opioid dose (as determined through quantitative Real-Time PCR (qRT-PCR), Figure 3c). Furthermore, we found lactate and LDH levels to be increased in brain cortex

homogenates (Figure 2b), which might be correlated with our GS gene expression results, in view of the interdependence between lactate and glutamate metabolism.

S100β, another astrocytic marker, plays a key role in neuroinflammation by activating signaling cascades that lead to the production and secretion of inflammatory cytokines. Its levels increase in hippocampal tissue, cerebrospinal fluid and serum during anoxic brain damage and pathophysiological situations, acting as a neuroapoptotic factor [153,154]. Serum S100β levels increased in pediatric patients following general anesthesia by a combination of fentanyl with non-opioid drugs; only the total dose of fentanyl was significantly correlated with the difference between post-exposure and baseline S100β levels [154]. In turn, acute administration of remifentanil also led to increased serum S100β levels in rats, which was associated with cognitive dysfunction [155]. However, Kuklin and co-authors found no changes in S100β serum levels between control and morphine-treated Wistar rats subsequently subjected to asphyxia cardiac arrest [156]. In line with this study, we have found no statistically significant differences between control and opioid-exposed groups, as far as S100β gene expression is concerned (Figure 3c). We hypothesize that the extent of brain injury, as assessed through this biomarker, is lower than that caused by other opioids.

In the present study, the protein content of GFAP, another astrocyte activation biomarker, was also found to be increased in brain cortex extracts upon exposure to the lowest and intermediate opioid doses (Figure 4). GFAP hippocampal immunoreactivity increased in juvenile and adult mice treated with 40 mg tramadol/kg/day for 1 month, which, along with astrocytic swelling, was reported as astrogliosis [144]. Morphine exposure has been reported to lead to similar effects in different brain areas, including ventral tegmental area, nucleus accumbens, striatum, and frontal cortex [145,157,158]. Several studies support the role of GFAP upregulation in opioid dependence and tolerance [145,157,158]. Indeed, chronic drug abuse-induced astrogliosis is considered an innate immunity response to neurotoxicity and brain damage, which may lead to alterations in synaptogenesis and neurogenesis, apoptosis and/or necrosis [144,158]. Thus, in our study, increased GFAP expression is compatible with the signs of glial proliferation and hypertrophy observed in histopathological examination; these, in turn, are a response to opioid-induced injury (Figure 8). Given the roles of GS and GFAP, their increased protein expression for the lowest opioid doses might be hypothesized as an attempt to reduce neurotoxicological injury, which is lost at the highest opioid doses, due to damage accumulation.

Interestingly, there seems to be a dissociation between glial and metabolic markers, since, while tramadol induces glial alterations, as determined through qRT-PCR, Western blotting, and histological analysis (Figures 3c, 4 and 8 respectively), it does not appear to significantly affect brain metabolism, apart from its effect on lactate concentrations (Figure 2b).

3.6. Repeated Exposure to Tramadol and Tapentadol Leads to Histopathological Damage in Lung, Heart and Brain Cortex Tissues from the Lowest Therapeutic Dose

In addition to analyzing the effects of a repeated exposure to clinical doses of tramadol and tapentadol at the molecular, biochemical, and metabolic levels, we have also studied their impact on lung, cardiac, and brain cortex histopathology. In fact, several histopathological alterations were documented by our own group in liver and kidney, following acute and repeated administration of the therapeutic doses of tramadol and tapentadol used in the current study [56,57], whilst lung, heart, and brain cortex tissue alterations had been described in an acute context [55].

Pulmonary fibrosis, congestion, edema, emphysema, and endoalveolar hemorrhage are reported as autopsy findings in fatal poisonings by tramadol or M1, alone or in combination with other drugs [36,39,40,43–46,48,88,159–161], as well as by tapentadol [33]. Furthermore, lung histopathological alterations were reported in animal models following acute and chronic administration of therapeutic and supratherapeutic doses of both opioids [55,58,59]. Interstitial alterations comprise pulmonary congestion, hemorrhage, fibrin deposition, inflammatory infiltrates, edema and fibrosis, while alveolar changes include alveolar wall and septa thickening and destruction (emphysema) to varying extents, as

well as intra-alveolar edema and hemorrhage [58,59]. In our previous acute administration assays, interstitial congestion and hemorrhage were dose-dependent for tramadol, while they were observed at all tapentadol doses; in turn, alveolar collapse became evident at the highest doses [55]. The results of the present study point predominantly to alveolar alterations—alveolar wall thickening and collapse, cellular hyperpigmentation and disorganization, which are now dose-independent. Such findings are compatible with CC16, MCP-1 and MMP-7 gene expression results (Figure 3a). It is noteworthy that alveolar destruction and loss of parenchyma are more evident for tapentadol, corroborating our previous postulate that this opioid causes lung damage to a greater extent [55].

With respect to cardiac tissue, our study has evidenced altered cardiomyocytes, fiber filament disorganization and heterogeneous pigmentation, loss of striation, inflammatory infiltrates, and fibrous tissue deposition both through H & E and Masson's trichrome staining methods (Figures 6 and 7, respectively). Such results are in line with those from our previous acute exposure studies [55]; however, in contrast to these, histopathological findings are now evident even at the lowest dose for tramadol and are more profuse for both opioids. In particular, while fibrous tissue deposition was not even suspected upon single exposure, it is now supported by both staining methods, as well as by the gene expression changes of the cardiotoxicity biomarkers assayed (Figure 3b). The signs of fibrosis are more evident after tramadol treatment, which also led to more intense alterations in the expression of cardiac markers. The origin of myocardial fibrosis in opiate use is still unclear [162]. Nevertheless, similarly to our results, heroin users have been reported to present up to a 5-fold increase in the number of inflammatory cells in the myocardium, suggesting a general activation of the cellular immune system and pointing to post-inflammatory focal interstitial fibrosis [161,162]. Hypereosinophilic bundles, congestion, hemorrhage, and leukocytic infiltration were also reported in cardiac tissue from rabbits submitted to short- and long-term passive opium smoking; minimum evidence of myonecrosis was reported for long-term exposure only [163]. Regarding postmortem investigation evidence, cardiomegaly is declared in autopsy reports of tramadol and tapentadol fatal poisoning cases [33,39,40,159].

In relation to brain cortex histological analysis, the continuous exposure to clinical doses of tramadol and tapentadol led mainly to neuron swelling and degeneration. Though they are observed in all conditions, these alterations accumulate along with tapentadol dose, whereas they are more profuse and diverse at all tramadol doses; neurons with irregular morphology are also more evident upon exposure to this opioid. Glial and microglial cells are now observable at all doses, consistently with the increase in astrocytic markers GS and GFAP (Figures 3c and 4), while they were more evident for intermediate and highest doses in single-exposure assays [55]. Such observations strengthen our previous hypothesis that tapentadol might not be so comparatively advantageous in the treatment of neuropathic pain, despite having a lower inhibitory effect on hippocampal neurogenesis [27]. These results are also in line with those from similar studies, mostly concerning rat consecutive administration with tramadol doses ranging from 25 to 200 mg/kg, for periods up to 60 days. Such studies report disorganized cortical layers and hypercellularity [59–62], as well as degenerated, vacuolated neurons, irregular in shape, with pyknotic and vacuolated nuclei, often with heterogeneous pigmentation and evident signs of apoptosis [58–62,70,164–166]. Neuronal degeneration and histological changes have been correlated with glucose metabolism alterations and with the bioenergetic crisis discussed in Section 3.4 [55,60]. Cellular infiltrates are also mentioned in these studies [58,60,164], as well as gliosis, satellitosis, and microglial and oligodendrocyte proliferation [58,62,144,164]. Vascular dilatation and congestion, hemorrhage, and brain edema are also cited [58–62,70,164,166]. Indeed, severe brain edema and hypoxic brain damage are reported in opiate-related deaths, including those from tramadol [39,40,45,161].

Collectively, our results show that lung, heart, and brain cortex toxicological damage occurs at the biochemical, metabolic, and histological levels upon exposure to clinical doses of tramadol and tapentadol (Figure 9).

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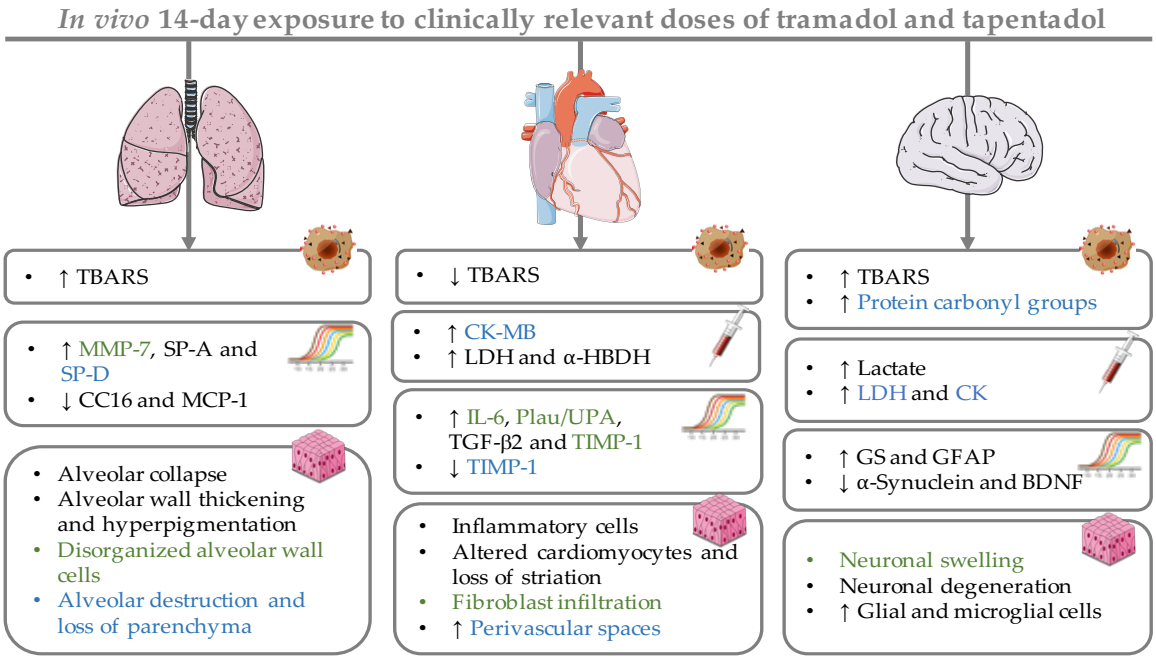

Figure 9. Schematic representation of the pulmonary, cardiac, and brain cortex effects of a 14-day exposure of Wistar rats to clinically relevant doses of tramadol or tapentadol, assessed at the molecular, oxidative stress, metabolic and histological levels. α-HBDH: α-hydroxybutyrate dehydrogenase; BDNF: brain-derived neurotrophic factor; CC16: Clara cell protein-16; CK-MB: creatine kinase muscle brain isoform; CK: creatine kinase; GFAP: glial fibrillary acidic protein; GS: glutamine synthetase; IL-6: interleukin-6; LDH: lactate dehydrogenase; MCP-1: monocyte chemoattractant protein-1; MMP-7: matrix metalloproteinase-7; Plau/UPA: plasminogen activator, urokinase; SP-A: pulmonary surfactant protein A; SP-D: pulmonary surfactant protein D; TBARS: thiobarbituric acid reactive substances; TGF-β2: transforming growth factor-β2; TIMP-1: tissue inhibitor of metalloproteinase-1.

As seen in our study addressing hepatorenal toxicity following consecutive opioid administration [57], lower therapeutic doses are able to induce injury if administered repeatedly. Damage accumulates along lengthier exposure periods than those we have previously assayed [55,56], but shorter than those employed in most peer studies. Figure 10 summarizes tramadol and tapentadol mechanisms of action, as well as the common and exclusive toxicological effects found in our study. Overall, tapentadol appears to induce alterations in more oxidative stress, cardiac, and brain cortex metabolism biomarkers, while tramadol seems to have more histopathological impact (Figures 9 and 10).

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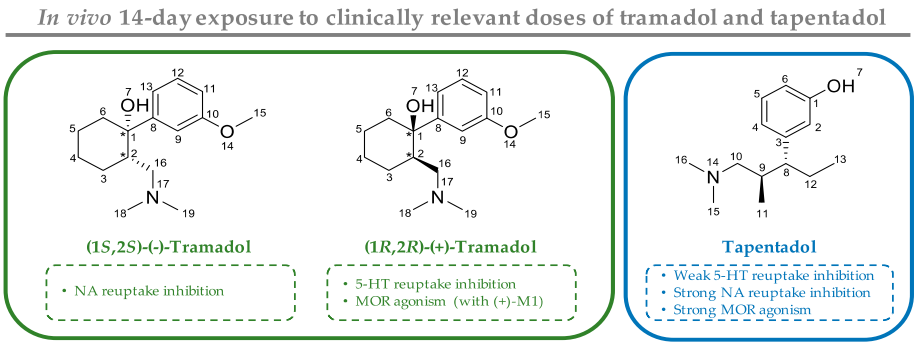

Toxicological mechanisms and common effects	Tramadol/Tapentadol exclusive effects
Systemic inflammation ↑ Serum CRP and TNF-α	↑ Serum IgG ↓ Serum IL-17A
Oxidative status alterations ↑ Lung and brain cortex TBARS ↓ Serum MPO	↑ Brain cortex protein carbonyl groups
Decreased cardiac cell integrity ↑ Serum LDH and α-HBDH	↑ Serum CK-MB
Brain cortex metabolic derangement ↑ Brain cortex lactate	↑ Brain cortex LDH and CK
Altered gene/protein expression ↑ SP-A, TGF-β2, GS and GFAP ↓ CC16, MCP-1, α-Synuclein and BDNF	↑ MMP-7, IL-6, Plau/UPA, TIMP-1
	↑ SP-D ↓ TIMP-1
Histopathological alterations Alveolar collapse and wall thickening Cardiac inflammatory infiltrates, loss of striation Neuronal degeneration, ↑ glial and microglial cells	Disorganized alveolar cells Cardiac fibroblast infiltration Neuronal swelling
	Alveolar destruction and loss of parenchyma ↑ Cardiac perivascular spaces

Figure 10. Summary of the toxicological mechanisms associated with a 14-day exposure of Wistar rats to clinically relevant doses of tramadol or tapentadol. 5-HT: serotonin; α-HBDH: α-hydroxybutyrate dehydrogenase; BDNF: brain-derived neurotrophic factor; CC16: Clara cell protein-16; CK-MB: creatine kinase muscle brain isoform; CK: creatine kinase; CRP: C reactive protein; GFAP: glial fibrillary acidic protein; GS: glutamine synthetase; IgG: immunoglobulin G; IL-17A: interleukin-17A; IL-6: interleukin-6; LDH: lactate dehydrogenase; M1: *O*-desmethyltramadol; MCP-1: monocyte chemoattractant protein-1; MMP-7: matrix metalloproteinase-7; MOR: μ-opioid receptor; MPO: myeloperoxidase; NA: noradrenaline; Plau/UPA: plasminogen activator, urokinase; SP-A: pulmonary surfactant protein A; SP-D: pulmonary surfactant protein D; TBARS: thiobarbituric acid reactive substances; TGF-β2: transforming growth factor-β2; TIMP-1: tissue inhibitor of metalloproteinase-1; TNF-α: tumor necrosis factor-α.

4. Materials and Methods

4.1. Chemicals

Tramadol and tapentadol hydrochloride salts were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Deltaclon (Madrid, Spain), respectively, having been dissolved and diluted in saline (0.9 g/L (*w*/*v*) NaCl) for administration. Sodium thiopental was supplied by B. Braun Medical (Queluz de Baixo, Portugal). All other chemicals were commercial preparations of the highest available degree of purity.

4.2. Experimental Models and Animal Handling

In this experimental study, 42 male Wistar rats, aged 8 weeks and weighing 250–300 g, were provided by the i3S animal facility (Porto, Portugal). Animals were housed in acrylic cages, in an environment enriched with wood chips and paper towels, and maintained under controlled conditions (22 ± 2 °C, 50–60% humidity, 12/12 h light/dark cycles). They were given unlimited access to tap water and rat chow (standard short and middle period maintenance formula for rodents, reference 4RF21, Mucedola/Ultragene (Milan, Italy)), and kept under a quarantine period of at least one week before experimental assays.

Animal experimentation was conducted in conformity with the European Council Directive (2010/63/EU) guidelines, transposed into the Portuguese law (Decree-Law no. 113/7 August 2013). Experimentation approval was also obtained from the Ethics Committee of CESPU, Institute of Research and Advanced Training in Health Sciences and Technologies (IINFACTS), Gandra, PRD, Portugal (processes no. PI4AC 2017, PI4AC 2018 and PI-3RL 2019), and complied with the National Ethics Council for the Life Sciences (CNECV) guidelines.

4.3. Experimental Design and Drug Treatment

Following acclimatization, rats were randomized into 7 groups of 6 animals each. The sample size and number of animals per group were established through the G*Power software, version 3.1.9.6 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), assuming a significance level of 0.05, an 80% power and effect size values adjusted in accordance with the biochemical parameters under analysis, based on literature and on the previous experience of the team.

Drugs were delivered daily, via single 1 mL-i.p. injections, using saline solution (0.9% (*w*/*v*) NaCl) as vehicle. Administrations were conducted at the same time each day, throughout 14 consecutive days. Each group was injected with a specific dose of each opioid—10, 25 or 50 mg/kg tramadol or tapentadol, whereas the control group received saline solution administrations.

Human therapeutic doses were converted into the animal equivalent doses (AED) by assuming a body surface area correction factor (K_m) of 6.2 and the following formula, for a 60 kg-human: AED (mg/kg) = Human dose (mg/kg) × K_m ratio [167–169]. In line with that described in our previous studies, 10 mg/kg is equivalent to an effective, analgesic dose, whilst 25 and 50 mg/kg are equivalent to an intermediate and the maximum recommended daily dose, respectively [55–57].

Immediately upon the last administration, rats were transferred to metabolic cages and allowed free access to tap water, but no food, for the remaining 24 h. Animals were monitored along this period, and then they were sacrificed through anesthetic procedures (i.p. injection with 60 mg/kg sodium thiopental, dissolved in saline solution).

4.4. Collection and Processing of Biological Samples

Blood samples were collected with a hypodermic heparinized needle, through cardiac puncture. Serum was obtained through centrifugation at 3000× *g*, 4 °C, for 10 min. Samples were aliquoted and stored (−80 °C) for biochemical analysis.

Lungs, heart and brain cortex were surgically removed from each animal, dried with gauze and weighed on an analytical balance. A portion of each organ was homogenized in an Ultra-Turrax® (IKA®, Staufen, Germany), in 1:4 (*w*/*v*) ice-cold 50 mM phosphate buffer (KH_2PO_4 + Na_2HPO_4 H_2O), pH 7.4. Homogenates were submitted to centrifugation at 4000× *g*, 4 °C, for 10 min. Supernatants were aliquoted and stored at −80 °C, along with the remaining intact portions of the organs.

4.4.1. Quantification of Oxidative Stress Parameters

Oxidative stress was assessed for LPO and protein oxidation, in lung, heart and brain cortex homogenates. TBARS and protein carbonyl groups (ketones and aldehydes) were used as LPO and protein oxidation biomarkers, respectively. Results were normalized

against total protein content, which was determined through the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's microplate procedure, and using 10-fold diluted homogenates.

Perchloric acid was added to each homogenate to a final concentration of 5% (*w*/*v*), to precipitate proteins. Acidified samples were centrifuged at 13,000× *g*, 4 °C, for 10 min; pellets and supernatants were stored at −80 °C. LPO quantification was performed in the supernatants, according to Buege et al. [170]. Results were expressed as nanomoles of MDA equivalents per milligram of protein. In turn, protein pellets were used for carbonyl group quantification, following the method reported by Levine et al. [171]. Results were expressed as 2,4-dinitrophenylhydrazine (DNPH) nanomoles incorporated per milligram of protein.

MPO activity was assayed in undiluted serum samples with the MPO Colorimetric Activity Assay Kit (Sigma-Aldrich), following the manufacturer's recommendations. Results were expressed in terms of mU/mL.

In turn, the total antioxidant capacity was determined in undiluted serum samples, through the Total Antioxidant Capacity Assay Kit (Sigma-Aldrich), according to the manufacturer's directions. Results were expressed in terms of mM of antioxidants (Trolox equivalents).

4.4.2. Quantification of Biochemical/Immunological Parameters in Serum Samples and in Brain Cortex Homogenates

CRP, CK-MB isoform, glucose, α-HBDH and IgG were quantified in undiluted serum samples, while lactate and CK were determined in undiluted brain cortex homogenates. In turn, LDH activity was quantified both in serum and brain cortex homogenates. Biochemical/immunological analytes were quantified in an automated analyzer (Prestige 24i, Tokyo Boeki, Tokyo, Japan), following the manufacturer's instructions, as previously reported [54–57,172], and using undiluted samples. Calibration was conducted for each parameter, by using two appropriate calibrators and plotting 5-point standard curves. Quality controls were also included. All automated analyzer reagents were supplied by Cormay PZ (Warsaw, Poland).

CK, CK-MB, α-HBDH, and LDH enzyme activities were determined as U/L. In turn, biochemical/immunological parameters were retrieved as mg/dL, except for CRP (mg/L). Results from homogenate determinations were further normalized against total protein content and are thus expressed as mg/dL/mg protein (lactate) or U/L/mg protein (CK and LDH).

TNF-α and IL-17A were determined in serum samples, through enzyme-linked immunosorbent assay (ELISA), using the ELISA MAX™ Deluxe Set Rat TNF-α and ELISA MAX™ Deluxe Set Rat IL-17A (BioLegend, San Diego, CA, USA), respectively. BNP was also determined, through enzyme immunoassay (EIA), in serum samples, using the Brain Natriuretic Peptide EIA Kit (Sigma-Aldrich). All determinations were performed in conformity with the manufacturers' specifications. For IL-17A and BNP quantification, samples were diluted 2-fold with assay diluent, whereas undiluted samples were used for TNF-α analysis. Immunoassay results were expressed as pg/mL.

4.4.3. Gene Expression Analysis Through qRT-PCR

Total RNA was extracted from lung, heart, and brain cortex samples using the NZYol reagent (NZYTech, Lisbon, Portugal), following the manufacturer's instructions for tissues. RNA integrity was confirmed through 1.4% (*w*/*v*) agarose gel electrophoresis, while its degree of protein and organic compound contamination was determined as the optical density (OD) $OD_{260\,nm}/OD_{280\,nm}$ and $OD_{260\,nm}/OD_{230\,nm}$ ratios, respectively (NanoDrop 2000 spectrophotometer, Thermo Scientific). Samples presenting $OD_{260\,nm}/OD_{280\,nm}$ and $OD_{260\,nm}/OD_{230\,nm}$ ratios $\geq$ 1.8 were used for complementary DNA (cDNA) synthesis, which was performed from 800 ng total RNA, using the NZY First Strand cDNA Synthesis kit (NZYTech), according to the manufacturer's directions.

Gene expression was analyzed using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), following the supplier's instructions. Each cDNA sample was diluted 10-fold in ultrapure water and analyzed in duplicate, thereby totaling 12 replicates for each experimental condition. CC16, MCP-1, MMP-7, SP-A, SP-D, IL-6, Plau/UPA, TGF-β2, TIMP-1, α-synuclein (SNCA), GS, BDNF, and S100β genes were analyzed. 18S ribosomal RNA (18S rRNA) housekeeping gene was used as a loading control.

Each amplification mixture was composed of 12.5 µL 2× iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 2 µL diluted cDNA (equivalent to 8 ng cDNA), forward and reverse primers (STABvida, Caparica, Portugal) to a final concentration of 100 nM each, and 10 µL RNase-free water, thus totaling a final volume of 25 µL. Primer sequences are specified in Table 1. RNA template controls (RTC) and non-template controls (NTC) were included in each run.

Table 1. Primer nucleotide sequences and specifications of the amplification programs used for quantitative Real-Time PCR (qRT-PCR) gene expression analysis of lung, cardiac, and neurotoxicity biomarker genes.

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Annealing Temperature (°C)	No. of Amplification Cycles	Reference
CC16 (Clara cell protein-16)	CATCAGCCCACATCTACAGAC	GGGCTTTAGCGTAGAATATCT	55	35	[173]
MCP-1 (Monocyte chemoattractant protein-1)	CCCACTCACCTGCTGCTACTC	AGAAGTGCTTGAGGTGGTTGTG	55	40	[174]
MMP-7 (Matrix metalloproteinase-7)	TCGGCGGAGATGCTCACT	TGGCAACAAACAGGAAGTTCAC	50	40	[175]
SP-A (Pulmonary surfactant protein A)	TACCAGAGCAGGAGGCAACA	CAATACTTGCAATGGCCTCGTT	55	35	[176]
SP-D (Pulmonary surfactant protein D)	AAATCTTCAGGGCGGCAAA	GGCCTGCCTGCACATCTC	55	40	[176]
IL-6 (Interleukin-6)	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC	55	40	[177]
Plau/UPA (Plasminogen activator, urokinase)	TCACTGGCTTCGGACAAGAGA	CCAATGTGGGACTGAATCCAG	55	40	[178]
TGF-β2 (Transforming growth factor-β2)	TTCAGAATCGTCCGCTTCGAT	TTGTTCAGCCACTCTGGCCTT	50	41	[179]
TIMP-1 (Tissue inhibitor of metalloproteinase-1)	TCTGGCATCCTCTTGTTGCTAT	CCACAGCGTCGAATCCTT	50	41	[180]
SNCA (α-Synuclein)	TGCTGTGGATATTGTTGTGG	AGGTGCGTAGTCTCATGCTC	55	35	[181]
BDNF (Brain-derived neurotrophic factor)	AAACGTCCACGGACAAGGCA	TTCTGGTCCTCATCCAGCAGC	55	37	[182]
GS (Glutamine synthetase)	CCACTGTCCCTGGGCTTAGTTTA	AGTGACATGCTAGTCCCACCAA	55	37	[183]
S100β (S100 calcium binding protein B)	GGGTGACAAGCACAAGCTGAA	AGCGTCTCCATCACTTTGTCCA	55	35	[184]
18S rRNA (18S ribosomal RNA)	TTCGGAACTGAGGCCATGATT	TTTCGCTCTGGTCCGTCTTG	In line with that of the target gene		[185]

The qRT-PCR program was run in a C1000™ Thermal Cycler, equipped with a CFX96™ Real-Time System (Bio-Rad Laboratories). It comprised an initial denaturation step at 95.0 °C for 3 min, 35–41 amplification cycles composed of a denaturation step at 94.0 °C for 20 s, an annealing step for 30 s, an extension step at 72.0 °C for 30 s and a plate read step. The number of amplification cycles and the annealing temperatures used in the analysis of each gene are listed in Table 1. A melt curve was then acquired between 65.0 °C and 95.0 °C, with 0.5 °C increments at every 5 s, followed by plate reads.

Results were analyzed with the Bio-Rad CFX Manager software, version 3.1 (Bio-Rad Laboratories), and normalized against those of the control group. Relative changes in gene expression were quantified using the Δ(ΔCt) algorithm.

4.4.4. Brain Cortex Protein Expression Analysis through Western Blotting

Brain cortex α-synuclein, GS and GFAP expression was also assessed at the protein level, by means of Western blotting assays. Brain cortex samples from each animal were homogenized in 1:5 (*w*/*v*) ice-cold RIPA lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA, 1% (*v*/*v*) Triton X-100, 0.5% (*w*/*v*) sodium deoxycholate, 0.1% (*w*/*v*) SDS), supplemented at 1:100 with protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 1.5 mM pepstatin A; Sigma-Aldrich), according to the supplier's instructions. Lysates were incubated for 15 min on ice, and then centrifuged (15,000× *g*, 15 min, 4 °C) to remove cell debris. Supernatants were stored at −80 °C until further use. Total protein content was determined through the Pierce™ BCA Protein Assay Kit (Thermo Scientific), according to the manufacturer's microplate procedure, and using 10-fold diluted protein extracts. 20 μg protein were loaded and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)—12% (GFAP and GS) or 15% (α-synuclein)—and transferred to a nitrocellulose membrane (110 mA, 75 min). Membranes were stained with Ponceau S to confirm sample transfer. They were then blocked with 5% (*w*/*v*) non-fat dry milk in TBST (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (*v*/*v*) Tween 20) and probed with anti-GFAP mouse antibody (G-A-5) (1:2000, Merck Millipore, Burlington, MA, USA), anti-GS mouse antibody, clone GS-6 (1:500, Merck Millipore) or anti-α-synuclein mouse antibody (4D6) (1:500, Abcam, Cambridge, UK), diluted in 1% (*w*/*v*) non-fat dry milk in TBST, overnight at 4 °C. Then, membranes were incubated for 1 h, at room temperature, with appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich), diluted 1:1500 in 1% (*w*/*v*) non-fat dry milk in TBST. To confirm equal protein loading, membranes were reprobed with anti-α-tubulin rabbit antibody (1:200, Abcam). Bands were visualized by treating the immunoblots through the enhanced chemiluminescence (ECL) method (Thermo Scientific) and scanned in a Gel Doc™ XR densitometer (Bio-Rad Laboratories). Densitometric analysis was performed with The Discovery Series™ Quantity One® 1-D analysis software, version 4.6.5 (Bio-Rad Laboratories). Band intensities were normalized against those from α-tubulin and then against the control.

4.4.5. Lung, Heart, and Brain Cortex Histopathological Analysis

One portion of lung, heart, and brain cortex tissue from each animal was fixed in 4% (*w*/*v*) formaldehyde for 24 h at room temperature, regarding histological analysis. It then underwent routine dehydration and paraffin wax-embedding procedures, as previously reported [186,187]. Three μm-thick sections were obtained in a Shandon™ Finesse™ 325 microtome (Thermo Scientific) and adhered to glass slides. H & E and Masson's trichrome staining procedures were performed with heart samples, while lung and brain cortex samples were processed for H & E staining only. Slides were analyzed under phase contrast microscopy, in an Eclipse TE2000-U microscope (Nikon, Melville, NY, USA), coupled to a DXM1200F digital camera and controlled by the ACT-1 software, version 2.70 (Nikon). Multiple microscope fields of observation were analyzed, and the most representative ones were photographed using 100× and 600× magnifications.

4.5. Statistical Analysis

Statistical analysis was performed by Analysis of Variance (ANOVA), followed by Dunnett's multiple comparisons test as post-hoc analysis. Data are presented as means ± SD and probability values of $p < 0.05$ were considered as statistically significant. Graphic plotting and all statistical tests were performed using GraphPad Prism® version 8.3.1 (GraphPad Software, LLC, San Diego, CA, USA). In all quantifications, results were compared with those from the control animals, injected with saline solution.

5. Conclusions

Opioid abuse and misuse are a current trend and a worldwide concern. In spite of being designed to circumvent the mechanistic, pharmacokinetic and pharmacodynamic flaws of their predecessors, synthetic prescription opioids such as tramadol and tapentadol imply some degree of toxicological risk, especially if misused or used for prolonged periods. The study hereby reported successfully attempted to explore the molecular, metabolic and cellular mechanisms underlying the toxicological effects from a repeated exposure to two common prescription opioids. The repeated administration of therapeutic doses, instead of supratherapeutic ones or overdoses, enables an approximation to their real consumption conditions, often in clinical settings and on a subacute to chronic basis.

Our results evidence that the repeated exposure to tramadol and tapentadol clinically relevant doses elicits lung and brain cortex lipid peroxidation, as seen through increased tissue TBARS levels, along with a generalized inflammatory status, as deduced from augmented serum CRP and TNF-α. The results are also compatible with damage to cardiac tissue integrity, in view of elevated CK-MB, LDH, and α-HBDH activities, though such alterations are not reflected in ventricular dysfunction, as no changes were detected in serum BNP levels. In turn, consistently with previous studies, the brain cortex seems to undergo a shift towards anaerobic metabolism, given the increase in tissue lactate contents and in LDH and CK activities, which might be associated with neuronal degeneration. Histopathological evidence comprises findings as diverse as alveolar collapse and destruction, cardiomyocyte and cardiac fiber alterations, inflammatory infiltrates, fibrous tissue deposition between cardiomyocytes, neuronal degeneration, and glial and microglial cell accumulation. Changes in the expression levels of toxicity biomarker genes and proteins correlate well with the alterations detected in oxidative stress, inflammation, metabolic and histopathological parameters in all tissues under analysis.

The present study provides additional insights to our previous single-exposure assays focusing on acute liver, kidney, heart, lung, and brain cortex toxicity caused by the same opioid doses. Furthermore, it demonstrates that, instead of being restricted to metabolizing organs, the toxicological effects deriving from a repeated exposure do also occur in target tissues. Indeed, this is, to the best of our knowledge, the first study comparatively focusing on lung, cardiac and brain cortex toxicity following consecutive administration of tramadol and tapentadol. Our results not only add information to the interpretation of adverse events, but should also draw the attention of the scientific and medical communities to the need to carefully prescribe and use tramadol and tapentadol, particularly in the presence of cardiopulmonary and/or neuropathic concomitant disease, and/or if lengthy usage periods are required.

Although tapentadol is considered an upgrade over tramadol, in view of its more linear pharmacokinetics, independence from CYP450 bioactivation and lower impact on the inhibition of hippocampal neurogenesis, we have demonstrated that it also causes some degree of neurotoxicity, underlining the need to carefully deliberate its use, even in a context of neuropathic pain treatment.

The assays herewith presented could be complemented by further studies. Behavioral studies would clarify if the present experimental conditions imply some degree of dependence and abuse potential, which remain particularly elusive for tapentadol. Moreover, immunohistochemistry would shed light on the expression of specific tissue and cell toxicity and damage biomarkers, thereby broadening the information obtained through

histological analysis. Regarding brain toxicity, molecular, metabolic, and histological analyses could be performed with samples from brain regions besides the cortex, such as the hippocampus, to ascertain whether the effects are region-specific. The dose range and the exposure period assayed could also be expanded in order to unveil eventual dose- and time-dependent outcomes, as well as to mimic overdose and chronic use situations. To clarify the putative toxicological role of active metabolites such as M1, these could be directly administered instead of the parental compounds. Likewise, the use of opioid antagonists could also elucidate the contribution of opioid receptor agonism to toxicity. Since opioids are frequently used together with other medications, combined drug exposure assays could be performed, for instance, with selective 5-HT reuptake inhibitors, tricyclic antidepressants, and monoamine oxidase inhibitors. Such an approach would clarify the possibility of toxicity exacerbation due to drug–drug interactions.

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PART III

1. INTEGRATED OVERVIEW OF THE STUDIES PERFORMED

Opioids are the most potent and effective analgesics available, representing a valuable option for the treatment of acute and chronic forms of pain. Nonetheless, their continued use potentially leads to dependence and addiction, with prescription opioid misuse and abuse representing emergent and highly recognized public health and socioeconomic concerns. For this reason, along with a growing demand for adverse reaction monitoring and regulation of prescription practices, there is a continuous challenge for the development of safer alternative drugs, with high analgesic efficacy and low abuse and addiction potential (Mendes-Morais et al., 2020).

Pain perception derives from the combination of multiple mechanisms that determine the way how a noxious stimulus is converted into a painful sensation. Its transmission is conducted along afferent, “ascending” pathways, and modulated by efferent, “descending” pathways (Pergolizzi et al., 2018b). While opioids modulate pain solely via the ascending pathways, noradrenergic and serotonergic projections represent an important component of descending pain circuits, besides being involved in pain chronification (Caraci et al., 2019; Pergolizzi et al., 2018b). In this sense, innovative opioid analgesics, adding strategic nonopioid mechanisms of action to μ-opioid receptor (MOR) agonism, are a prerequisite for enhancing response rates and tolerability, improving the therapeutic range and chronic pain management, and minimizing side effects. Tramadol and tapentadol, MOR agonists and monoamine reuptake inhibitors, thus classified as “atypical opioids” and “multigesic agents”, illustrate such concept (Dickenson and Kress, 2019; Pergolizzi et al., 2018a). Indeed, a review of data captured by the Researched Abuse, Diversion and Addiction-Related Surveillance (RADARS®) System Poison Center Program, regarding seven opioid analgesics, revealed tramadol and tapentadol to have led to the two lowest rates of serious adverse events per 100 kg dispensed, from 2010 through 2016 (Murphy et al., 2018).

Owing to such optimized therapeutic and safety profiles, they are widely prescribed, but also more readily accessible and prone to misuse, abuse, diversion and addiction, which lead to increased morbidity and mortality (Cicero and Ellis, 2017; Mendes-Morais et al., 2020). A systematic review reported rates of opioid misuse and addiction to average between 21-29% and 12-18%, respectively, among patients with chronic pain (Vowles et al., 2015). This emphasizes the biomedical and forensic relevance of the study of both the mechanisms underlying and resulting from such phenomena, including those with toxicological implications. In fact, tramadol was the most commonly seized non-heroin opioid in Europe throughout 2018, with the line between legal and illegal drug markets being increasingly thinner (EMCDDA, 2020). It is believed that, in some regions, the market for recreational tramadol use has originally appeared as a result of the increased demand based on the supply available for medical use. A new supply-driven phenomenon then further expanded the market with illicitly manufactured products (United Nations, 2020).

Besides the well-known North American “opioid crisis”, Middle East, South Asia and North, Central and West Africa countries currently face a so-called “tramadol crisis”. This opioid is being diverted from the legal market and trafficked in higher dosages than those used medically,

which leads to an increase of people with tramadol use disorder entering treatment (United Nations, 2020). Several factors favor such phenomenon: ease of manufacturing, easy accessibility and low-cost production increase the profitability of tramadol illicit market; the absence of international regulation on the drug encourages its large-scale manufacture; the interchangeability within the pharmaceutical and illicit drug markets makes it more difficult to address its misuse (United Nations, 2020). Besides being used in a context of self-medication for pain relief, tramadol is misused by young people and some categories of workers to boost their energy, to be able to endure more working hours, or to enhance sexual performance, perceived euphoria and attentiveness (United Nations, 2020).

The European Drug Report 2020, drafted by the European Monitoring Center for Drugs and Drug Addiction (EMCDDA), supports the existence of an evolving threat of non-medical use of pharmaceutical and synthetic opioids in several member states of the European Union (EU). As for 2018, it reports 1.3 million high-risk opioid users and 660,000 opioid users receiving substitution treatment. In the same year, opioids were found in 82% of fatal overdoses, with methadone, buprenorphine, fentanyl (and its derivatives) and tramadol representing a significant share in some European countries (EMCDDA, 2020). In 2017, at least 300 deaths were reported, in the EU, with tramadol being either present or implicated (United Nations, 2020).

The national scenario seemingly follows the European trends. According to the Portuguese Country Drug Report 2019, there were 33,290 high-risk opioid users (5.2‰ of the adult population) and 16,888 opioid substitution clients in 2015 (EMCDDA, 2019). A slight decrease to 28,287 opioid users (4.5‰ of the adult population) was projected for 2018 (Carapinha and Lavado, 2020). Complementary data provided by the National Institute of Forensic Medicine estimates opioids – including heroin, morphine, codeine and tramadol – to have been detected in 44% of overdose deaths in 2019. The number of overdose deaths, considering the period from 2011 onwards, peaked in 2018 and 2019, with the number of opiate overdoses doubling between 2017 and 2018 (SICAD, 2020). In 2019, opiates and methadone were detected in 31% and 14%, respectively, of all deaths involving at least one illicit substance or its metabolites, but assigned to other causes (SICAD, 2020). Among other reasons, these statistics are relevant in the sense that, since there is less stigma around prescription opioids than around recreational/illicit opioids, and since prescription rates are increasing, the former arise as strong alternatives for diversion by opioid users and addicts (Basu et al., 2020; Chan et al., 2021; Cicero and Ellis, 2017).

A cross-sectional nationwide epidemiological study reported chronic pain to affect 36.7% of the Portuguese adult general population in 2007/2008 (Azevedo et al., 2012), marginally exceeding estimations of 25-35% in several other countries (Mendes-Morais et al., 2020). However, the frequency of prescription opioid use was reported to be low within the same period, alerting to a possible pain undertreatment scenario on a national scale (Azevedo et al., 2013). Yet, the rates of opioid prescription have steadily increased since then (Caldeira et al., 2021; Veiga et al., 2019). A contributing factor may have been the legislative changes made,

in 2008, to the reimbursements of opioid analgesics for moderate to severe chronic pain, in an attempt to match the consumption patterns of other countries. These changes, which encompassed tapentadol, eased prescription regulation and established a reimbursement rise from 37% to a current value of 90% (Ministério da Saúde, 2016a; b). Indeed, the number of opioid analgesic packages consumed in Portugal had a 141% increment between 2010 and 2018, increasing from 1,532,044 to 3,685,992 (Guedes, 2019).

According to the Portuguese National Authority of Medicines and Health Products (INFARMED), tramadol, in combination with paracetamol, ranks eighth in the list of active substances with greater use in ambulatory care settings, representing 2,206,354 packages sold (corresponding to an homologous variation of + 34,626 packages) and 1.4% weight in the market (corresponding to an homologous variation of + 1.6%) between January and December 2020 (INFARMED, 2021a). Between January and March 2021, the same active substance combination kept its position in the ranking, with 550,952 packages sold (INFARMED, 2021b). A recent, observational, retrospective descriptive analysis focusing on the Health Administrative Region of Lisbon and Tagus Valley, which covers more than one-third of the Portuguese population, showed that, between 2013 and 2017, opioid drug prescription increased 1.67-fold in ambulatory care settings (Caldeira et al., 2021). Tramadol (with + 1,070 thousand defined daily doses (DDD) and + 0.80 defined daily doses per 1,000 inhabitants per year (DID)) and tapentadol (with + 828 thousand DDD and + 0.62 DID) showed the greatest absolute increases in prescription within this period; tramadol ranked first in the list of the top opioid drugs used in Portugal in 2017 (3.5 million DDD; 2.63 DID), while tapentadol ranked third (828 thousand DDD; 0.62 DID), behind buprenorphine (Caldeira et al., 2021). The authors underline that such numbers reflect the increasing relevance of tapentadol in the national pain management arsenal, as well as a “positive” reaction to concerns of inertia for pain control (Caldeira et al., 2021). Conversely, it is argued that these statistics warrant monitoring and highlight the need to reinforce good prescription practices, since there is no evidence that they are matched by an increase in the incidence of the conditions for which opioids can be considered as a therapeutic option (Caldeira et al., 2021).

Given tramadol and tapentadol national and international emerging notoriety in pain treatment, as well as the concomitant increase in their misuse, abuse and addiction, the work presented in this thesis aimed to comparatively study the toxicological effects associated with the *in vivo* exposure to clinically relevant doses of both opioids, at the molecular, biochemical and histopathological levels. The increasingly thinner line between the medical and non-medical use of prescription opioids substantiates the biomedical and forensic scope of the present dissertation. Main results are summarized in Figure 1. Tapentadol-related findings are particularly relevant, in view of its more recent market authorization, which limits the amount of data available on its safety.

The work presented in CHAPTERS I and II explored the hepatorenal effects of the single (1-day) and repeated (14-day) exposure of Wistar rats to 10, 25 and 50 mg/kg tramadol or tapentadol – a standard analgesic dose, an intermediate dose and the maximum recommended

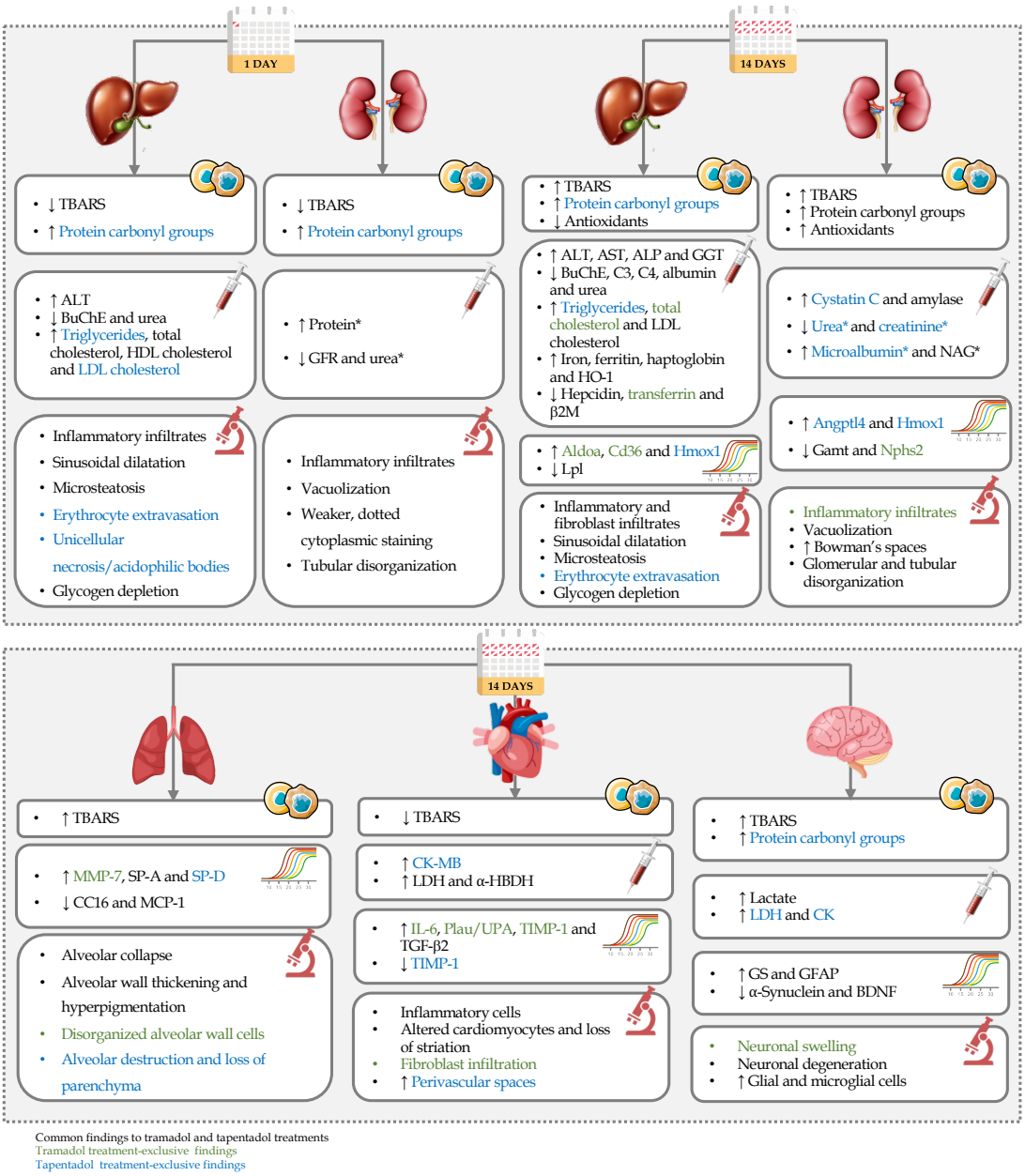

Figure 1. Schematic representation of the hepatic, renal, pulmonary, cardiac and brain cortex effects of the *in vivo* exposure to clinically relevant doses of tramadol or tapentadol. Wistar rats were administered with 10, 25 or 50 mg/kg tramadol or tapentadol, through single (1 day) or repeated (14 consecutive days) daily intraperitoneal injection. Controls were injected with saline solution (0.9% (*w/v*) NaCl) throughout the same periods. Parameters marked with asterisk (*) were determined in urine samples, while the remaining biochemical analytes were quantified in serum samples. Gene/protein expression and oxidative stress studies were performed with the corresponding tissues and tissue homogenates, respectively. Aldoa: fructose-bisphosphate aldolase A; ALP: alkaline phosphatase; ALT: alanine aminotransferase; Angptl4: angiopoietin-like 4; AST: aspartate aminotransferase; β2M: β2-microglobulin; BDNF: brain-derived neurotrophic factor; BuChE: butyrylcholinesterase; C3: complement component 3; C4: complement component 4; CC16: Clara cell protein-16; Cd36: cluster of differentiation 36/fatty acid translocase; CK: creatine kinase; CK-MB: creatine kinase muscle brain isoform; Gamt: guanidinoacetate *N*-methyltransferase; GFAP: glial fibrillary acidic protein; GFR: glomerular filtration rate; GGT: γ-glutamyl transpeptidase; GS: glutamine synthetase; α-HBDH: α-hydroxybutyrate dehydrogenase; HDL: high-density lipoprotein; Hmox1: heme oxygenase 1 gene; HO-1: heme oxygenase 1; IL-6: interleukin-6; LDH: lactate dehydrogenase; LDL: low-density lipoprotein; Lpl: lipoprotein lipase; MCP-1: monocyte chemoattractant protein-1; MMP-7: matrix metalloproteinase-7; NAG: *N*-acetyl-β-D-glucosaminidase; Nphs2: podocin; Plau/UPA: plasminogen activator, urokinase; SP-A: pulmonary surfactant protein A; SP-D: pulmonary surfactant protein D; TBARS: thiobarbituric acid reactive substances; TGF-β2: transforming growth factor-β2; TIMP-1: tissue inhibitor of metalloproteinase-1. Adapted from (Barbosa et al., 2021).

daily dose, respectively (Barbosa et al., 2020; Barbosa et al., 2017) (Figure 1, upper panel). Using thiobarbituric acid reactive substances (TBARS) as biomarkers, it was shown that, while the acute exposure seemingly leads to a protective effect from lipid peroxidation (LPO) in liver and kidney, the repeated exposure reverses such effect. In turn, increments in protein oxidative stress, as assessed through protein carbonyl group quantification, were observed in both organs, particularly upon tapentadol treatment (Barbosa et al., 2020; Barbosa et al., 2017). In the context of repeated exposure, the total antioxidant capacity was shown to be decreased in liver homogenates, but increased in kidney samples (Barbosa et al., 2020). It should be noted, however, that further oxidative stress-related parameters – e.g., superoxide dismutase (SOD) activity and glutathione levels – should be assayed to better characterize the antioxidant status. Liver function tests were performed in both studies, revealing augmented alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) serum activities upon opioid treatment, which denotes hepatobiliary damage. Decreased serum concentrations of biochemical analytes with exclusive or predominant liver synthesis, such as albumin, urea, complement components 3 (C3) and 4 (C4) and butyrylcholinesterase (BuChE), denoted the impairment of this function in the experimental groups. In turn, lipid profile derangement was observed in both acute and subacute exposure assays, through increased total cholesterol, high-density lipoprotein (HDL) and/or low-density lipoprotein (LDL) cholesterol and triglyceride levels, the last of which upon tapentadol treatment only (Barbosa et al., 2020; Barbosa et al., 2017). Increased serum iron levels, detected upon opioid treatment for 14 consecutive days, prompted the screening of iron metabolism-related parameters. Increased serum ferritin, haptoglobin and heme oxygenase 1 (HO-1), as well as decreased serum transferrin, hepcidin and β2-microglobulin (B2M), were detected, possibly correlating with oxidative stress, cell damage, inflammation and steatosis (Barbosa et al., 2020). In turn, kidney function was also assessed in single and repeated treatment settings. The acute exposure to both prescription opioids was associated with diminished glomerular filtration rate (GFR) and urea excretion, as well as with proteinuria (Barbosa et al., 2017). In the repeated exposure study, treatment with tapentadol led to more pronounced alterations in renal function, causing augmented serum cystatin C, microalbuminuria and decreased urea and creatinine urine excretion. Urine *N*-acetyl-β-D-glucosaminidase (NAG) activity was increased upon repeated administration of the highest dose of both opioids (Barbosa et al., 2020). Altogether, these findings suggest glomerular and tubular function impairment to occur as a result of the acute and subacute exposure to therapeutic doses of both prescription drugs. In the repeated administration context, a panel of hepatorenal toxicity biomarker genes was also quantified, through quantitative real-time polymerase chain reaction (qRT-PCR), in liver and kidney tissue samples, with the results being consistent with the metabolic alterations, cell toxicity and glomerular dysfunction suggested by the remaining parameters assayed (Barbosa et al., 2020) (Figure 1, upper panel). Finally, both studies comprised the histological analysis of liver and kidney sections. Liver histopathological findings encompassed sinusoidal dilatation, microsteatosis, atypical nuclei, inflammatory

infiltrates and glycogen depletion, after single and repeated administration of tramadol and tapentadol. Vascular congestion and erythrocyte extravasation were exclusive to tapentadol treatment in both studies and, though not exclusive, fibrous tissue deposition was also more noticeable for this opioid in the repeated exposure scenario. The histopathological study of kidney samples from animals exposed to both drugs revealed glomerular and tubular disorganization, increased Bowman's spaces and mononuclear cell infiltrates (Barbosa et al., 2020; Barbosa et al., 2017). Such alterations were associable with changes in metabolic and gene expression parameters (Barbosa et al., 2020).

In turn, CHAPTER III presents the results of the study of the lung, cardiac and brain toxicological effects deriving from the repeated exposure of Wistar rats to the same tramadol and tapentadol doses, using the same experimental approach detailed in CHAPTER II (Barbosa et al., 2021) (Figure 1, lower panel). The research team had previously analyzed the effects of these opioid doses, but in a context of acute exposure (Faria et al., 2017). While, in single-exposure assays, almost no significant alterations in TBARS levels were found in lung, heart and brain cortex homogenates, they increased in lung and brain cortex following repeated administration. Protein carbonyl groups increased in lung and heart tissues upon the acute opioid treatment, whilst only the repeated exposure to tapentadol triggered significant levels of protein oxidation, and in brain cortex only (Barbosa et al., 2021; Faria et al., 2017). Following repeated drug treatment, though serum myeloperoxidase (MPO) activity was found to be decreased at all opioid doses, the total antioxidant capacity remained unchanged (Barbosa et al., 2021). Myocardial damage was detected upon repeated opioid treatment, through elevated serum lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (α-HBDH) activities, while tapentadol exposure led to increased serum creatine kinase muscle brain (CK-MB) isoform levels. No significant changes were found for serum brain natriuretic peptide (BNP) levels, denoting no impact on ventricular function (Barbosa et al., 2021). Such results are in line with those of the previous acute exposure assays, which led to increased serum AST/ALT ratio, LDH and CK-MB activities in the experimental groups (Faria et al., 2017). Alterations were also found in metabolic parameters measured in brain cortex homogenates. In both studies, brain cortex lactate levels increased upon exposure to both opioids, and LDH activity increased upon exposure to tapentadol only; in turn, the increase in creatine kinase (CK) activity was exclusive to the repeated treatment with tapentadol (Barbosa et al., 2021; Faria et al., 2017). Inflammation biomarkers were also analyzed in the repeated exposure study, showing serum C reactive protein (CRP) and tumor necrosis factor-α (TNF-α) to be increased in the animals from the experimental groups; serum immunoglobulin G (IgG) was further increased in tapentadol-treated rats (Barbosa et al., 2021). Histopathological analysis, performed in the repeated administration settings, confirmed acute exposure findings, namely alveolar collapse and destruction in lung sections, altered cardiomyocytes, loss of striation and inflammatory infiltrates in heart sections, and neuronal degeneration and glial cell accumulation in brain cortex sections (Barbosa et al., 2021; Faria et al., 2017). In the repeated exposure study, Masson's trichrome staining was further performed with heart sections, revealing fibrous tissue

deposition, particularly after tramadol treatment (Barbosa et al., 2021). As presented in CHAPTER II, some lung, cardiac and brain toxicity biomarker genes were quantified in the corresponding tissues; neuronal and astrocytic markers were additionally quantified at the protein level, correlating well with oxidative stress, inflammation, metabolic and histological parameters (Barbosa et al., 2021) (Figure 1, lower panel).

Taken as a whole, the results from these studies demonstrate that tramadol and tapentadol trigger adverse outcome pathways (AOPs) in metabolizing and target organs, even when administered at therapeutic doses, and even in a context of acute exposure. The extension of the exposure period lowers the dose at which the toxicological effects are observed and eliminates the dose-response relationship observed, at least for some parameters (e.g., serum ALT activity, serum urea levels and some histopathological findings), in single administration studies. Toxicity mechanisms include oxidative stress, inflammatory, metabolic and histopathological processes, which are extensible to all tissues under analysis, and occur upon treatment with both opioids.

Oxidative stress induction is recognized as a consequence of the exposure to several opioids, prompting pre- or co-treatment with different antioxidants to be advocated as a strategy to mitigate oxidative damage. The protective effects, as deduced from improvements in biochemical, histological and histochemical parameters, have been documented in different tissues, suggesting that such strategy might be translated into clinical practice (Baghishani et al., 2018; Ibrahim et al., 2020; Kader et al., 2021; Sheweita et al., 2018). Oxidative damage is closely associated with cellular and histological abnormalities, which had been reported in different *in vivo* studies addressing the effects of tramadol on metabolizing and target tissues (Ali et al., 2020; Atici et al., 2005; Awadalla and Salah-Eldin, 2016; Baghishani et al., 2018; Elkhateeb et al., 2015; Ezzeldin et al., 2014; Ghoneim et al., 2014; Hussein et al., 2020; Ibrahim et al., 2020; Sheweita et al., 2018; Youssef and Azza, 2016). However, while most of these studies invested in prolonged, chronic exposure periods to tramadol, the results of the present thesis, together with previous observations from the research team, demonstrate that histopathological findings are extensible to tapentadol treatment, and detectable upon single and briefer repeated administration of therapeutic doses (Barbosa et al., 2021; Barbosa et al., 2020; Barbosa et al., 2017; Faria et al., 2017).

In line with the results featured in this thesis, brain metabolic alterations have also been reported in other opioid exposure assays. Increased brain lactate levels and LDH activity reflect a shift towards anaerobic metabolism that had previously been described in *in vitro* and *in vivo* studies with tramadol, tapentadol and morphine. These effects have been correlated with a bioenergetic crisis, driven by alterations in the expression of energy metabolism enzymes, partial inhibition of respiratory chain complexes and mitochondrial dysfunction (Faria et al., 2016; Mehdizadeh et al., 2017; Mohamed and Mahmoud, 2019; Mohamed et al., 2015; Perez-Alvarez et al., 2010; Sharma et al., 2003; Zhuo et al., 2012). Given their positive charge at physiological pH, tramadol and tapentadol accumulate in negatively charged compartments such as mitochondria, therefore exacerbating deleterious mitochondrial effects (Cunha-Oliveira

et al., 2008; Faria et al., 2016). Consequent apoptosis, induction of pro-inflammatory markers, striatal and cerebellar atrophy, hippocampal degeneration, astrogliosis, microgliosis and histological abnormalities have been associated with cognitive, spatial learning, memory, motor coordination and neuromuscular activity impairment, in contexts of chronic tramadol treatment (Aghajanpour et al., 2020; Baghishani et al., 2018; Ezi et al., 2021; Mehdizadeh et al., 2017; Mohamed and Mahmoud, 2019; Soltani et al., 2020). Decreased intracellular signaling, deregulation of signaling cascades involved in neurodegenerative diseases and energy metabolism, neurotransmitter and pro-oxidant imbalance have been proposed as possible causes for increased dark neurons, neuroplasticity abnormalities, apoptosis, neuronal death and the consequent cognitive and central nervous system (CNS) impairment following tramadol exposure (Aghajanpour et al., 2020; Baghishani et al., 2018; Ezi et al., 2021; Mohamed and Mahmoud, 2019). In this respect, the metabolomic analysis of mice cerebrum upon chronic administration of 20 or 50 mg/kg tramadol identified several potential toxicity biomarkers, including up-regulated γ-aminobutyric acid (GABA) and down-regulated succinate semialdehyde (Xia et al., 2020). Compared with the low-dose tramadol group, there were twenty-nine potential biomarkers in the high-dose tramadol group, mainly related to the pentose phosphate pathway and glycerophospholipid metabolism. The authors postulate that long-term tramadol abuse may lead to oxidative damage, inflammation, and disruption of the GABA neurotransmitter system (Xia et al., 2020). This, in turn, is interconnected with the glutamate/glutamine cycle, whose alterations, approached in CHAPTER III, were suggested to contribute to tramadol and tapentadol neurotoxicity (Barbosa et al., 2021). Overall, the effects reported in CHAPTER III indicate that such neurotoxicological phenomena do also occur upon shorter periods of repeated administration of both tramadol and tapentadol.

As detailed in CHAPTER II, metabolic alterations were not confined to the brain, since serum lipid and iron metabolism parameters were also shown to be affected, following 14 consecutive days of daily intraperitoneal (i.p.) injection of tramadol and tapentadol (Barbosa et al., 2020). The metabolic impact of tramadol use had been previously approached. It has been associated with hypoglycemia, both at therapeutic doses and overdoses, possibly correlating with enhanced glucose consumption and decreased gluconeogenesis, mainly mediated by MOR activation (Nakhaee et al., 2020). Although we were unable to find significant alterations in rat serum glucose levels, upon acute and repeated opioid exposure (Barbosa et al., 2021; Faria et al., 2017), we reported hepatic glycogen depletion under both circumstances (Barbosa et al., 2020; Barbosa et al., 2017). Also in the scope of the analysis of metabolic alterations, mice treated with 50 mg/kg tramadol for 5 weeks were recently shown to undergo changes in their proteomic and metabolomic profiles, as compared with the controls (Jiang et al., 2021). Proteomic analysis revealed thirty-one differentially expressed serum proteins, mainly including enzyme inhibitor-associated proteins, mitochondria-related proteins and cytoskeleton proteins, which were predominantly associated with protein digestion and absorption pathways. In turn, metabolome analysis showed differentially expressed metabolites to be mainly involved in protein ingestion and absorption, fatty acid biosynthesis, steroid hormone biosynthesis and bile

secretion (Jiang et al., 2021). Thus, multiple metabolic pathways are affected by tramadol and tapentadol treatment. The results substantiate the importance of further studies analyzing metabolic alterations from a large-scale, integrative point of view.

In parallel, serotonin (5-HT) toxicity must not be disregarded for both prescription opioids, especially when these are combined with other serotonergic drugs, such as selective serotonin reuptake inhibitors (SSRIs), selective noradrenaline reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) (Krcevski-Skvarc et al., 2021). While (+)-tramadol inhibits 5-HT reuptake, tapentadol has a weaker serotonergic activity, for which it is associated with lower 5-HT syndrome liability (Baldo and Rose, 2020; Barbosa et al., 2016; Faria et al., 2018). However, in the VigiBase™ World Health Organization (WHO) Global Database of Individual Case Safety Reports, tramadol and tapentadol rank first and third as the only suspected cause of 5-HT toxicity or among other drugs, and first and second, respectively, as the only suspected cause (Baldo and Rose, 2020). In this context, tapentadol has been reported to induce an increase in spinal 5-HT levels via activation of opioid receptors in brain regions with serotonergic projections to the dorsal horn. This might contribute to reconcile WHO data with the assumption that tapentadol is not a significant cause of 5-HT toxicity, in view of its lower 5-HT transporter affinity (Benade et al., 2017).

Cytochrome P450 (CYP450) isoenzyme 2D6 (CYP2D6) crucial contribution to tramadol metabolism has a significant impact on its pharmacokinetics, owing to the diversity of drugs metabolized by the enzyme and to the polymorphic nature of its encoding gene. In fact, metabolizer phenotypes directly influence the concentrations of tramadol and its metabolites and, consequently, analgesic efficacy and toxicity (Lassen et al., 2015; St Sauver et al., 2017). In an era of personalized medicine, this underlines the importance of individually tailoring tramadol therapy, besides adding another complexity layer to the interpretation of clinical and forensic data concerning its use. In this sense, given its substantially lower dependence on CYP450 metabolism, tapentadol pharmacokinetics is far more linear (Barbosa et al., 2016; Faria et al., 2018). Indeed, uridine diphosphate-glucuronosyltransferases (UGTs), main catalysts of tapentadol metabolism, are high-capacity enzymes, entailing lower drug-drug interaction liability, and UGT genetic polymorphisms are rarer, with fewer clinical implications (Barbosa et al., 2016). Nevertheless, the pharmacological effect and toxicological potential of tapentadol have been recently shown to increase upon its co-administration with sorafenib (Karbownik et al., 2020), warranting further studies on the subject.

Although the extension of the exposure period smooths the differences between the toxicity profiles of both opioids, tapentadol was shown to modulate a higher number of parameters to a greater extent (Barbosa et al., 2021; Barbosa et al., 2020) (Figure 1). Previous *in vitro* studies by the research team, using an undifferentiated neuroblastoma cell line, had also reported tapentadol to elicit greater acute toxicological damage than tramadol, when in equimolar concentrations (Faria et al., 2016). It should be highlighted that, despite their similarities, tramadol and tapentadol have different three-dimensional molecular shapes, receptor and transporter affinities, pharmacokinetic and pharmacodynamic properties (Barbosa

et al., 2016; Faria et al., 2018; Raffa, 2014). Interestingly, tapentadol design was inspired by tramadol drawbacks, such as 5-HT syndrome liability and dependence on variable CYP2D6-mediated bioactivation, for which the former is regarded as an upgrade over the latter (Barbosa et al., 2016). Indeed, post-marketing surveillance data shows that, in the United States of America (USA), diversion, misuse, abuse and its endorsement, overdose and street demand are lower for tapentadol than for other prescription opioids (Cepeda et al., 2014; Cepeda et al., 2013; Dart et al., 2014; Dart et al., 2012; Dart et al., 2021; Dart et al., 2016; Faria et al., 2018; Freynhagen et al., 2021; McNaughton et al., 2015; Pergolizzi et al., 2018b; Vosburg et al., 2020). Notwithstanding these observations, it should be noted that a direct comparison between tapentadol and its peers might be misleading, since the duration of its post-marketing surveillance is considerably shorter, thus limiting the availability of systematic data, and the monitoring and restrictions on its use, as well as the supply of tamper-resistant and abuse-deterrent formulations, are lower in other countries (Mukherjee et al., 2020). In fact, evidence has been accumulating on the easiness of tapentadol tablet tampering, repurposing into injection and misuse, which are associated with abuse and fatal intoxications (Kemp et al., 2013; Khaja et al., 2017; Mukherjee et al., 2020).

In addition, when sold illicitly, tapentadol is cheaper than its comparators (Ansley and Sethi, 2020; Dart et al., 2016). Besides being purchased by vulnerable groups of patients struggling with addictive disorders, it is readily substituting prescription opioids that have been brought into stricter regulation (Basu et al., 2018; Basu et al., 2020). Some studies on opioid demand patterns show that spatial and temporal trends of online interest in tapentadol have been paralleling those of tramadol, which are on the rise (Mukherjee et al., 2020; Wightman et al., 2017). Remarkably, tramadol ranked first and tapentadol ranked fourth (with 32.5% and 12% of all opioid offers, respectively) in the Research Center on Security and Crime (RiSSC) list of opioids available for sale, between December 2015 and February 2016, on Alphabay, an online darknet market, providing a picture of the illegal supply for both prescription opioids (Mignone and Novara, 2017). Online acquisition is predicted to find its place as an alternative for patients with opioid use disorder, who are less likely to buy medication through in-person purchasing or via “doctor shopping” (Ansley and Sethi, 2020).

Albeit less than for other potent opioids, tapentadol prominent MOR agonism is associated with mood alterations and euphoria and, thus, with dependence and abuse potential (Basu et al., 2018; Kathiresan et al., 2019). Such potential to induce physical and psychological dependence, supported by rewarding, reinforcing and conditioned place preference (CPP) effects comparable to those of morphine, underpinned its classification as a Schedule II substance in the USA (Guay, 2009). A study with occasional opioid users showed that, unlike tramadol, therapeutic doses of tapentadol led to a profile of exclusively positive subject-rated effects, comparable to that of hydromorphone, but with faster onset and offset, which may contribute to a greater frequency of use (Guay, 2009; Stoops et al., 2013). An integrative descriptive analysis of post-marketing safety data on the use of tapentadol in a broad range of pain conditions revealed the most common side effects to be opioid-typical, such as nausea,

dizziness, vomiting and constipation (Stollenwerk et al., 2018). However, the administration of higher than recommended doses resulted mostly in dose-dependent clinical signs concerning the CNS, psychiatric and perceptual abnormalities, including fearfulness, sedation or excited behavior, recumbency and hunched posture, impaired respiratory function, convulsions (in fewer cases), restless legs syndrome, insomnia, memory and attention impairment, withdrawal syndrome and suicidal depression (Stollenwerk et al., 2018). In line with this, a retrospective analysis of tramadol and tapentadol toxicities, based on reported single-medication exposure cases, had found tapentadol to be associated with a significantly greater risk of severe outcomes, with higher rates of respiratory depression, coma, drowsiness/lethargy, slurred speech, hallucination/delusion and confusion, while tramadol was associated with higher rates of seizures and vomiting (Tsutaoka et al., 2015). In this context, it should be underlined that, besides their chemical and mechanistic differences, there is no active transport mechanism known for tapentadol and it freely crosses the blood-brain barrier, while tramadol is actively transported (Kitamura et al., 2014; Raffa et al., 2012). This may explain both tapentadol higher CNS functional activity and its CNS-related detrimental effects, including those concerning dependence, addiction and abuse potential, which deserve further studies.

The results reported upon a 14-day period of daily administration of therapeutic doses of tramadol or tapentadol (Barbosa et al., 2021; Barbosa et al., 2020) demonstrate hepatobiliary, renal, pulmonary, cardiac and brain detrimental effects to be cumulative, when compared with those observed upon a single administration (Barbosa et al., 2017; Faria et al., 2017). The relevance of such findings is underlined by the frequent use of prescription opioids on a subacute to chronic basis. It might thus be anticipated that tramadol and tapentadol continued use, misuse or abuse intensifies such multiorgan outcomes, which might be particularly exacerbated when supratherapeutic doses are concerned. Consistently with this, a recent review lists the gastrointestinal, CNS, cardiovascular, respiratory, endocrine, musculoskeletal and renal systems to be the main affected by tramadol poisoning (Nakhaee et al., 2021). Reports of fatal and non-fatal intoxications have been accumulating for both opioids, mostly pursuant to misuse, abuse, intentional or accidental overdose and polysubstance use; many of them involve multiorgan dysfunction and failure (Barbera et al., 2013; Cantrell et al., 2016; Clarkson et al., 2004; De Backer et al., 2010; De Decker et al., 2008; Franco et al., 2014; Iravani et al., 2010; Kemp et al., 2013; Khaja et al., 2017; Nedahl et al., 2021; Pilgrim et al., 2010; 2011; Randall and Crane, 2014; Shadnia et al., 2008; Tjaderborn et al., 2007; Wang et al., 2009).

Given that toxicological outcomes have been shown to occur in liver, kidney, lung, heart and brain cortex, even in acute and short repeated exposure settings, the need to carefully deliberate and monitor tramadol and tapentadol use is further emphasized. In fact, cardiovascular, respiratory, hepatic and renal diseases were associated with a higher mortality risk in tramadol users (Jeong et al., 2019). In accordance with this, cautionary recommendations have been made on tramadol and tapentadol use in patients with gastrointestinal, kidney, liver, respiratory, cardiovascular and neurodegenerative diseases,

among other special populations (Coluzzi et al., 2020; Dwyer et al., 2014; Hartrick and Rozek, 2011; Krcevski-Skvarc et al., 2021; O'Brien et al., 2017; Vachhani et al., 2014). Current guidelines advise to sensibly weigh the risks and benefits of opioid prescription; limit opioid use to situations in which non-pharmacological and nonopioid approaches are ineffective, not tolerated or contraindicated; start treatment with the lowest effective dose, titrate doses gradually and continue treatment for the briefest period possible. Regular reassessment of the risk-benefit ratio is recommended; if required, changes to therapy may include dose reduction, prolonged dose interval, opioid rotation or discontinuation (Freynhagen et al., 2021; Hauser et al., 2021; Krcevski-Skvarc et al., 2021; Nafziger and Barkin, 2018; O'Brien et al., 2017).

The present thesis demonstrates that, in spite of their enhanced multimodal mechanism of action, tramadol and tapentadol still pose toxicological risks, even when administered for brief periods and at clinically relevant doses. The need to raise awareness for tramadol and tapentadol toxicological potential is highlighted. The results reinforce the common assumption that, as part of the pain treatment armamentarium, opioids remain as two-edged swords – with high analgesic efficacy when used appropriately, but harmful if misused, used abusively or with no suitable monitoring. Inherently, the search for novel opioids is an ongoing process; cebranopadol, a mixed MOR and nociceptin/orphanin FQ peptide (NOP) receptor agonist under development (Tzschentke et al., 2019), illustrates such quest.

In the last analysis, understanding the molecular and cellular rationale behind toxicological damage aids in the engineering of new, safer drugs. At the same time, it enables the identification of toxicity biomarkers and, potentially, the molecular screening of at-risk patients (Muriel et al., 2019; Wu et al., 2018). Prescription regulation and monitoring by health authorities arise as emerging demands, and the relevance of continuous medical education on the correct use of prescription opioids, individually and dynamically tailored to the patient, is ultimately emphasized.

PART III

2. CONCLUSIONS

The work performed within the scope of the present thesis has shown that multiple alterations occur upon single and repeated exposure to clinically relevant doses of tramadol and tapentadol, in an *in vivo* experimental model. Main conclusions are summarized below:

1. Besides causing acute hepatorenal toxicity, tramadol and tapentadol induce cumulative liver, kidney, lung, heart and brain cortex toxicological damage when repeatedly administered to Wistar rats;
2. Single exposure to tramadol and tapentadol decreases LPO in the liver and kidney, while tapentadol treatment increases protein oxidation in both organs;
3. Repeated treatment with both opioids increases LPO in liver, kidney, lung and brain cortex, increases protein oxidation in liver, kidney and brain cortex, decreases liver antioxidant capacity and serum MPO activity, but has no effect on the systemic antioxidant status;
4. Repeated exposure to tramadol and tapentadol induces systemic inflammation, as shown by increased serum CRP and TNF-α levels;
5. Single and repeated administration of both opioids impair hepatobiliary integrity and function, as deduced from increased serum ALT, AST, ALP and GGT activities, as well as from decreased serum concentrations of albumin, urea, complement C3 and C4 and BuChE activity;
6. *In vivo* acute and repeated exposure to tramadol and tapentadol have deleterious effects on renal function, as shown through decreased GFR, urinary output of urea and creatinine, increased serum cystatin C, proteinuria, microalbuminuria and urinary NAG activity;
7. Repeated administration of tramadol and tapentadol affects cardiac muscle cell integrity, as supported by increased serum LDH, CK-MB and α-HBDH activities, but does not impact ventricular function;
8. Repeated treatment with tramadol and tapentadol modulates brain cortex metabolism, as shown through augmented tissue lactate, LDH and CK activities;
9. Acute and repeated treatment with therapeutic doses of both opioids modulates the lipid profile, incrementing total cholesterol, LDL and HDL cholesterol and triglyceride serum contents;
10. Repeated administration of clinically relevant doses of both opioids impacts iron metabolism, as inferred from increased serum iron, ferritin, haptoglobin and HO-1, and decreased serum transferrin, hepcidin and B2M, correlating with oxidative stress, cell damage, inflammation and steatosis;
11. Repeated exposure to clinical doses of tramadol and tapentadol induces expression alterations in liver, kidney, lung, heart and brain cortex toxicity biomarker genes, as well as in neuronal and astrocytic biomarker proteins, providing additional correlational insights into the observations regarding inflammation, metabolic derangement, organ dysfunction and histopathology;

12. Histopathological findings from acute and repeated exposure assays include: fibrous tissue deposition, sinusoidal dilatation, microsteatosis, glycogen depletion and vascular congestion/erythrocyte extravasation in liver tissue; glomerular and tubular disorganization and increased Bowman's spaces in kidney tissue; alveolar collapse, thickening and parenchyma destruction in lung tissue; cardiomyocyte alterations, loss of striation and perivascular fibrosis in heart tissue; neuronal degeneration and glial and microglial cell accumulation in brain cortex tissue. Inflammatory infiltrates are observed in liver, kidney and heart tissue sections;
13. Tapentadol tends to induce more pronounced alterations in more parameters, both in single and in repeated administration settings. Nevertheless, the extension of the exposure period from 1 day to 14 days minimizes the differences between tramadol and tapentadol toxicological profiles, eliminates dose dependence for some parameters and decreases the therapeutic dose required to trigger toxicological injury.

PART III

3. DIRECTIONS FOR FUTURE RESEARCH

In spite of having shed light into the molecular, metabolic and histopathological consequences of the exposure to therapeutic doses of tramadol and tapentadol, the data presented in this thesis should be complemented in order to provide a wider perspective on the mechanistic rationale behind their toxicity.

The dose range and exposure periods tested in the studies of the original research articles composing this thesis could be extended, so as to mimic overdose and chronic use situations. The quantification of tramadol, tapentadol and their metabolites in tissues and body fluids (e.g., through gas chromatography/mass spectrometry (GS/MS)) would provide additional insights into their biodistribution, and eventual organ-specific accumulation and dose/effect relationships, thus clarifying their toxicokinetics and toxicodynamics. Correlations with clinical and *post-mortem* findings from human users would ultimately be enabled. In this sense, access to and analysis of samples from tramadol and tapentadol users would bridge the gap between the present *in vivo* studies and the real human consumption scenario, directly illustrating the scope of applicability of this thesis.

Since opioids are frequently co-administered with other drugs, *in vitro* and *in vivo* combined exposure studies with drugs as SSRIs, MAOIs and TCAs would be useful in screening for eventual toxicity exacerbation due to drug accumulation. Similarly, the use of opioid antagonists, such as naloxone, would enlighten on the contribution of the μ-opioid component to the final outcomes under study. In fact, the combined exposure to enzyme (namely CYP450) inhibitors and inducers, receptor antagonists and anti-inflammatory agents would additionally enable the construction and study of AOPs and their networks.

Although apurinic/apyrimidinic endonuclease 1 (Apex1) gene expression levels did not change significantly in our studies, organ-specific genotoxicity could be further investigated; comet assays, as well as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) quantification, could serve this purpose. Oxidative stress studies could also be complemented with the determination of additional enzymatic and non-enzymatic antioxidant defense systems, protein sulfhydryl groups or immunohistochemical examination of DNA, lipid and protein oxidation.

In order to investigate fibrosis in organs besides the liver and heart, Masson's trichrome staining could be performed with other tissues. Other histological staining procedures, as well as immunohistochemistry assays for pro-fibrotic markers, could also be considered to supplement histopathological analysis.

The potential impact of UGT polymorphisms on tapentadol pharmacokinetics and toxicity, as well as its liability for drug-drug interactions, could be further investigated through *in vitro* and *in vivo* approaches.

Microarray gene expression platforms, proteomic and metabolomic *in vivo* studies could also be used to identify new gene and protein toxicity biomarkers associated with single and repeated exposure to tramadol, tapentadol and, eventually, related opioids. Serum and tissue homogenates could be screened, paving the way for the identification of eventual common and differential toxicity pathways.

Eventual reinforcement properties of therapeutic doses of both opioids – namely addiction, reward and dependence potential – could also be ascertained through animal behavioral studies, such as CPP assays.

The ultimate goal of the extension of the studies presented in this thesis would be to contribute to individualize prescription according to the patient's clinical profile and to minimize adverse reactions, as well as to raise awareness of tramadol and tapentadol toxicological potential among medical and scientific communities.

PART IV

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