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BIOQUÍMICA TOXICOLÓGICA**

**CARACTERIZAÇÃO DO ESTERÓIDE α -ESPINASTEROL COMO UM
NOVO ANTAGONISTA DO RECEPTOR TRPV1 COM EFEITO
ANTINOCICEPTIVO**

DISSERTAÇÃO DE MESTRADO

Gabriela Trevisan dos Santos

Santa Maria, RS, Brasil

2011

**CARACTERIZAÇÃO DO ESTERÓIDE α -ESPINASTEROL COMO UM
NOVO ANTAGONISTA DO RECEPTOR TRPV1 COM EFEITO
ANTINOCICEPTIVO**

Por

Gabriela Trevisan dos Santos

Dissertação apresentada ao curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Juliano Ferreira

Santa Maria, RS, Brasil

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**Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica**

A comissão examinadora, abaixo assinada,
Aprova a Dissertação de Mestrado

**CARACTERIZAÇÃO DO ESTERÓIDE α -ESPINASTEROL COMO UM
NOVO ANTAGONISTA DO RECEPTOR TRPV1 COM EFEITO
ANTINOCICEPTIVO**

elaborada por

Gabriela Trevisan dos Santos

Como requisito parcial para obtenção do grau de
Mestre em Ciências Biológicas: Bioquímica Toxicológica

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Santa Maria, 1 de Setembro de 2011

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"Vós, investigadores, não deveis confiar em autores que, apenas pelo emprego da imaginação, se fazem intérpretes entre a natureza e o homem, mas somente naqueles que exercitaram seu intelecto com os resultados de experimentos."

Leonardo da Vinci

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

CARACTERIZAÇÃO DO ESTERÓIDE α -ESPINASTEROL COMO UM NOVO ANTAGONISTA DO RECEPTOR TRPV1 COM EFEITO ANTINOCICEPTIVO

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Orientador: Juliano Ferreira

Data e Local da defesa: Santa Maria, 1 de Setembro de 2011.

O receptor de potencial transitório vanilóide 1 (TRPV1) é relevante para a percepção de estímulos nocivos e tem sido estudado como um alvo terapêutico para o desenvolvimento de novos analgésicos. O objetivo deste estudo foi desenvolver uma triagem *in vivo* e *in vitro* para caracterizar novos antagonistas do receptor TRPV1 isolados das folhas de *Vernonia Tweedieana* Baker, uma planta medicinal, com atividade antinociceptiva em camundongos. Todas as frações e o extrato hidroalcolico apresentaram efeito antinociceptivo no teste da capsaicina, sendo que a fração diclorometano (Dcm) também mostrou efeito antiedematogênico. Entre os compostos isolados da fração Dcm, apenas o α -espinasterol reduziu a nocicepção e o edema induzidos pela injeção intraplantar de capsaicina. Além disso, o α -espinasterol foi capaz de deslocar o radioligante [³H]-resiniferatoxina, e também de diminuir o influxo de cálcio estimulado pela capsaicina. A fração Dcm e o composto α -espinasterol apresentaram efeito anti-hiperalgésico na nocicepção induzida por estímulo térmico, mas não induzida por estímulo mecânico em animais sem injúria. Porém, o composto α -espinasterol não apresentou atividade antinociceptiva em animais pré-tratados sistemicamente com resiniferatoxina. Este composto e a fração Dcm foram capazes de reduzir a hiperalgisia mecânica e térmica, e também o edema induzidos por adjuvante completo de Freund. A fração Dcm e o α -espinasterol não foram capazes de induzir alteração na temperatura corporal ou atividade locomotora. Também, o α -espinasterol mostrou boa absorção por via oral, e alta penetração no cérebro e na medula espinhal de camundongos. Assim, o α -espinasterol, isolado da fração Dcm, age como um antagonista do receptor TRPV1 com eficaz efeito antinociceptivo, sem indução de efeitos adversos.

Palavras-chave: Esteróide; TRPV1; capsaicina; vanilóide; antinocicepção; *Vernonia*.

ABSTRACT

Dissertation of Master's Degree
Graduating Program in Biological Sciences (Toxicological Biochemistry)
Federal University of Santa Maria, RS, Brazil

CHARACTERIZATION OF THE PLANT STEROID α -SPINASTEROL AS A NOVEL TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 ANTAGONIST WITH ANTINOCICEPTIVE EFFECT

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Advisor: Juliano Ferreira

Place and date: Santa Maria, September 1st, 2011.

The transient receptor potential vanilloid 1 (TRPV1) is relevant to the perception of noxious information and has been studied as a therapeutic target for the development of new analgesics. The goal of this study was to perform *in vivo* and *in vitro* screens to identify novel, efficacious, and safe TRPV1 antagonists isolated from leaves of the medicinal plant *Vernonia tweedieana* Baker. All of the fractions and the hydroalcoholic extract produced antinociception in mice during the capsaicin test, but the dichloromethane fraction (Dcm) also had antioedematogenic effect. Among the compounds isolated from the Dcm fraction, only α -spinasterol reduced the nociception and oedema induced by capsaicin injection. Moreover, α -spinasterol demonstrated good oral absorption and high penetration into the brain and spinal cord of mice. Besides, α -spinasterol was able to displace [³H]-resiniferatoxin (RTX) binding and diminish calcium (Ca²⁺) influx mediated by capsaicin. Orally administration of the Dcm fraction and α -spinasterol also produced antinociceptive effect in the noxious heat-induced nociception test; however, they did not change the mechanical threshold of naive mice. The treatment with α -spinasterol did not produce antinociceptive effect in mice systemically pre-treated with RTX. In addition, α -spinasterol and the Dcm fraction also reduced the oedema, mechanical and heat hyperalgesia elicited by complete Freund's adjuvant (CFA) paw injection. The Dcm fraction and α -spinasterol did not affect body temperature or locomotor activity. In conclusion, α -spinasterol is an efficacious and safe antagonist of the TRPV1 receptor with antinociceptive effect.

Key words: Steroid; TRPV1; capsaicin; vanilloid; antinociception; *Vernonia*.

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LISTA DE ABREVIATURAS

Act	Acetato de etila ou ethyl acetate
ATP	Adenosina trifosfato
BSA	Albumina sérica bovina ou bovine serum albumin
But	Butanólica ou butanolic
CFA	Adjuvante completo de Freund ou complete Freund's adjuvant
CGRP	Peptídeo relacionado ao gene da calcitonina
CI ₅₀	Concentração inibitória 50%
COX	Ciclooxigenase
Dcm	Diclorometano ou dichloromethane
DMSO	Dimetilsulfóxido ou dimethyl sulfoxide
cDNA	DNA complementar
GDR	Gânglio da raiz dorsal
HE	Hydroalcoholic ou hidroalcolico
12-HPETE	Ácido 12-hidroperoxi-eicosatetraenico
IC ₅₀	Inhibitory concentration 50%
ID ₅₀	Inhibitory dose 50%
I _{max}	Maximal inhibition
i.pl.	Intraplantar injection
LTB ₄	Leucotrieno B ₄
NGF	Fator de crescimento do nervo
NADA	N-araquidonoil dopamina
OEA	Oleiletanolamida
p.o.	Via oral ou oral via
GT	Gânglio trigeminal
TRP	Receptores de potencial transitório
TRML	Receptores de potencial transitório mucolipina
TRPA	Receptores de potencial transitório anquirina
TRPC	Receptor de potencial transitório canônico
TRPM	Receptores de potencial transitório melastatina
TRPN	Receptores de potencial transitório não sensível a estímulo mecânico
TRPP	Receptores de potencial transitório policistina
TRPV	Receptores de potencial transitório vanilóide
RTX	Resiniferatoxina
SNC	Sistema Nervoso Central

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1. APRESENTAÇÃO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo, o qual se encontra no item Artigo. As seções Materiais e métodos e Resultados, encontram-se no próprio artigo.

Os itens Discussão, Conclusões e Referências bibliográficas encontram-se no final desta dissertação.

As Referências bibliográficas referem-se somente as citações que aparecem nos itens Introdução, Revisão de literatura e Discussão desta dissertação.

O artigo está estruturado de acordo com as normas da revista científica *Biochemical Pharmacology* a qual será submetido.

O artigo que foi anexado a esta dissertação e já foi publicado encontra-se no apêndice no final desta dissertação.

2. INTRODUÇÃO

O receptor de potencial transitório vanilóide do tipo 1 (TRPV1) é um membro da superfamília de canais iônicos denominada de receptores de potencial transitório (TRP) (Caterina et al., 1997). Este receptor é constituído por seis domínios transmembrana, formando, então, um canal catiônico não seletivo expresso principalmente no sistema nervoso; no entanto, também é encontrado em tecidos não neuronais (Jara-Oseguera et al., 2008). O receptor TRPV1 é geralmente expresso em fibras sensoriais C e A δ , sendo assim importante para a detecção de estímulos nocivos (Szalassi et al., 2007). Este canal pode ser ativado por substâncias exógenas como a capsaicina (composto pungente da pimenta vermelha) e resiniferatoxina (isolada da *Euphorbia resinifera*), por agentes inflamatórios endógenos, e também por calor nocivo (>43°C) (Jara-Oseguera et al., 2008; Schumacher, 2010).

Sendo o receptor TRPV1 um relevante sensor de estímulos nocivos, que pode ser sensibilizado por diversos mecanismos, incluindo por mediadores inflamatórios, este canal é relacionado a diferentes patologias dolorosas (dor inflamatória, visceral, neuropática e relacionada ao câncer) (Jara-Oseguera et al., 2008; Adcock, 2009; Patapoutian et al., 2009). Dessa maneira, este receptor tem sido estudado como um alvo potencial para o desenvolvimento de novos analgésicos, sendo que já existem diversos antagonistas em fase clínica de estudo (Levine e Alessandri-Haber, 2007; Wong e Gavva, 2009).

Os antagonistas seletivos do receptor TRPV1 induzem efeitos anti-hiperalgésicos em modelos de nocicepção em animais e em humanos (Rami et al., 2006; Chizh et al., 2007; Lehto et al., 2008). Entretanto, um importante efeito adverso relacionado aos antagonistas do receptor TRPV1 é o desenvolvimento de hipertermia severa, como descrito para o composto AMG-517 (Gavva et al., 2008). Assim, a identificação de novos antagonistas do receptor TRPV1 que não promovam o desenvolvimento de hipertermia seria interessante, sendo estes alvos promissores como novos agentes analgésicos (Wong e Gavva, 2009).

Uma das fontes que poderia ser explorada para a descoberta de novos antagonistas do receptor TRPV1 seriam os produtos naturais. É descrito que os compostos isolados de plantas são considerados como um alvo relevante para a descoberta de novas drogas, incluindo compostos analgésicos (Calixto et al., 2000;

Calixto et al., 2005; Koehn e Carter, 2005). Além disso, diversos compostos derivados de produtos naturais que são capazes de modular o receptor TRPV1, como a capsaicina, podem ser usados no tratamento da dor e de sintomas relacionados às doenças respiratórias (Corson e Crews, 2007; Adcock, 2009; Schumacher, 2010). E também, compostos isolados de plantas já foram caracterizados como potentes antagonistas do receptor TRPV1 com atividade antinociceptiva (Neacsu et al., 2010; Rossato et al., 2011). Dessa forma, o desenvolvimento de novos antagonistas do receptor TRPV1, a partir de produtos naturais, pode ser uma fonte útil para a descoberta de analgésicos mais eficazes e seguros.

A espécie *Vernonia tweedieana* Baker, popularmente conhecida no Brasil como “assa-peixe”, é uma planta herbácea distribuída nas planícies do Paraguai, Argentina e sul do Brasil (Zanon et al., 2008) e suas folhas são usadas na medicina tradicional para o tratamento de doenças respiratórias (Zanon et al., 2008). Sabe-se que diversas espécies do gênero *Vernonia* já foram identificadas como tendo atividade antinociceptiva e antiinflamatória (Frutuoso et al., 1994; Gupta et al., 2003; Iwalewa, 2003; Mazumder et al., 2003; Njan et al., 2008; Risso et al., 2010).

A análise química prévia da fração diclorometano extraída do extrato bruto hidroalcolico das folhas de *Vernonia tweedieana* levou ao isolamento de α -amirina, β -amirina, lupeol, estigmasterol e α -espinasterol (Figura 1) (Zanon et al., 2008). Como parte do objetivo do grupo que desenvolve a presente pesquisa é encontrar novos compostos isolados de produtos naturais que possam agir como antagonistas do receptor TRPV1 sem indução de hipertermia, foi realizada uma triagem *in vivo* e *in vitro* para identificar novos ligantes do receptor TRPV1 isolados a partir da planta *V. tweedieana* com atividade antinociceptiva.

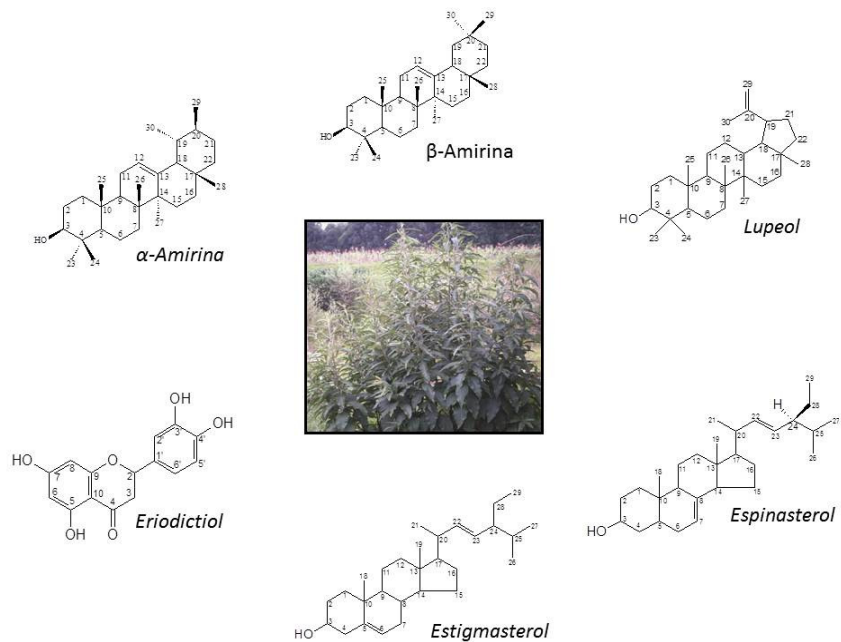


Figura 1. Imagem ilustrando a planta *Vernonia Tweediana Baker* e os compostos isolados da fração diclorometano proveniente das folhas.

3. OBJETIVOS

3.1. Objetivo geral

Desenvolver uma triagem *in vivo* e *in vitro* para caracterizar novos antagonistas do receptor TRPV1 isolados das folhas de *Vernonia Tweedieana* Baker com atividade antinociceptiva.

3.2. Objetivos específicos

- Averiguar, a partir de triagem *in vivo*, se o extrato bruto, frações e compostos isolados das folhas de *Vernonia Tweedieana* Baker previniriam a nocicepção e o edema induzidos pela administração intraplantar de capsaicina em camundongos;

- Investigar, a partir de triagem *in vitro*, se a fração diclorometano e compostos isolados desta fração seriam capazes de deslocar a união específica da [³H]-resiniferatoxina em frações de membrana de medula espinhal de camundongos;

- Avaliar se o composto α -espinasterol inibiria o influxo de cálcio estimulado por capsaicina em fração rica de sinaptossomas *in vitro*;

- Averiguar se o α -espinasterol e a fração diclorometano apresentariam efeito antinociceptivo após a aplicação de estímulo nocivo térmico e mecânico;

- Avaliar se o pré-tratamento sistêmico com resiniferatoxina previniria o efeito antinociceptivo do composto α -espinasterol após a aplicação de estímulo nocivo térmico;

- Verificar se o composto α -espinasterol e a fração diclorometano apresentariam efeito anti-hiperalgésico e antiedematogênico em um modelo de dor inflamatória;

- Determinar a concentração plasmática, cerebral e espinhal do composto α -espinasterol em camundongos através de dosagem por cromatografia líquida de alta eficiência;

- Avaliar se o composto α -espinasterol e a fração diclorometano promoveriam alterações na atividade locomotora e na temperatura corporal em camundongos.

4. REVISÃO BIBLIOGRÁFICA

4.1. Dor e nocicepção

A dor é um importante problema de saúde, sendo uma razão muito comum para a procura por cuidados médicos e uso de fármacos. Além disso, é estimado que 80% da população mundial tem acesso limitado a medicamentos para tratamento da dor moderada a severa (Bassols et al., 2002; Fishman, 2007; Lohman et al., 2010). Assim, tratamento correto da dor ainda é considerado um desafio terapêutico, tanto em países desenvolvidos como em desenvolvimento, e a falha deste tratamento poderia ser interpretada como uma revogação de um direito humano (Brennan et al., 2006; Lohman et al., 2010).

A dor gera um profundo impacto na qualidade de vida dos pacientes, levando a conseqüências tanto em nível fisiológico (aumento de complicações pós-operatórias, desenvolvimento de dor crônica), psicológico (aparecimento de transtornos psiquiátricos como depressão e ansiedade), social (menor capacidade de trabalho levando ao desemprego e menor renda familiar e interação social) e econômico (diminuição da capacidade de trabalho, gastos com cuidados médicos e medicamentos) (Brennan et al., 2006). Assim, o tratamento adequado da dor principalmente crônica, levaria a uma melhora na qualidade de vida dos pacientes.

De acordo com a Associação Internacional para o Estudo da Dor (IASP), a dor pode ser definida como “uma experiência sensorial e emocional desagradável associada à lesão tecidual real ou potencial, ou ainda descrita em termos que sugerem tal lesão”. Sendo assim, a sua percepção é complexa e não envolve apenas a transmissão de um estímulo nocivo, mas também processos emocionais e cognitivos, a dor então pode ser dita como um fenômeno subjetivo. O termo nocicepção deve ser entendido como os processos neurais de codificação e processamento de estímulos nocivos, os quais podem se estabelecer sem que ocorra dor ou vice-versa, mas geralmente aparecem associados a patologias dolorosas (Loeser e Treede, 2008).

Para que um determinado estímulo nociceptivo desencadeie uma resposta dolorosa é necessário que nociceptores localizados na periferia sejam ativados por estímulos mecânicos, térmicos ou químicos. Os nociceptores são terminações livres periféricas de axônios provenientes de neurônios nociceptivos, que apresentam seu corpo celular nos gânglios da raiz dorsal (GRD) para o corpo, no gânglio nodoso e

vagal para as vísceras e no gânglio do trigêmio (GT) para a face. Estes neurônios possuem terminações tanto periféricas (órgão inervado) como centrais (medula espinhal e tronco cerebral). Existem dois tipos principais de neurônios nociceptivos: fibras do tipo A δ , que são neurônios mielinizados de médio diâmetro, e fibras C, que são de pequeno diâmetro e não mielinizadas; estas diferem tanto no perfil de receptores expressos na membrana, na velocidade de condução e também na classe de estímulo doloroso conduzido.

Quando um determinado estímulo é capaz de ativar um nociceptor, gerando um potencial de ação, este é transmitido ao corno dorsal da medula (principalmente lâminas I, II e V) onde ocorre a liberação de neurotransmissores excitatórios (principalmente glutamato e substância P). Uma vez realizado este processo, é possível que ocorra a geração de uma resposta reflexa a esta estimulação nociva, com o intuito de afastar o membro afetado da fonte de estímulo, ativando, então, um neurônio motor reflexo. Além disso, ocorrerá ativação de um neurônio de segunda ordem sensorial e este sinal é, então, enviado a centros supra-espinhais, como tálamo, amígdala e córtex frontal, onde estes estímulos serão devidamente reconhecidos e discriminados como dolorosos (Meyer et al., 2008; Basbaum e Nilius, 2009).

A sensação de dor alerta para uma real ou provável lesão, e desencadeia respostas apropriadas para proteger o organismo. Dessa forma, a dor eminentemente aguda (nociceptiva) possui grande valor adaptativo relacionado à sobrevivência do organismo lesado (Basbaum e Nilius, 2009; Woolf, 2010). Normalmente, após a resolução da lesão, a dor deixa de existir, entretanto, se a lesão persistir ou caso ocorra modificação de vias de modulação da dor, a dor que antes funcionaria apenas como um sinal de alerta ao organismo se torna um sintoma debilitante e crônico, como na dor patológica (Scholz e Woolf, 2002; Woolf, 2010).

Dessa maneira, as alterações na condução dolorosa podem levar à hipersensibilidade, o que pode ser visto como uma extensão do processo natural de cura onde a área sensível é assim resguardada, como ocorre na dor inflamatória, ou ainda pode ser observado em patologias crônicas dolorosas onde a dor persiste mesmo após a cura da injúria tecidual. Assim, pode ser observado nos pacientes o aparecimento de dor a estímulos antes inócuos, como um leve toque, (dor a

estímulos não nociceptivos denominada alodínia), ou ainda, ocorre a percepção exarcebada da dor a estímulos dolorosos (hiperalgesia) e também aparecimento de dor espontânea.

Estes sintomas clínicos podem ser mantidos a nível molecular tanto por mecanismos periféricos como centrais e estão associados ao processo de sensibilização. Este fenômeno pode causar um aumento na resposta de neurônios nociceptivos a estímulos supra-limiáres, ou ainda, resposta a estímulos abaixo do limiar de ativação destes. E podem, também, ocorrer descargas espontâneas e aumento do campo receptivo dos nociceptores (Loeser e Treede, 2008; Basbaum e Nilius, 2009; Woolf, 2010).

Existem diferentes alvos moleculares que estão envolvidos na transdução de estímulos dolorosos de acordo com patologia dolorosa estudada, e podem assim ser explorados como elementos importantes para a descoberta de novos analgésicos. Dentre estes, um relevante alvo para a descoberta de novos analgésicos é o receptor de potencial transitório do tipo 1 (TRPV1) (Scholz e Woolf, 2002; Burgess e Williams, 2010).

4.2. Receptores de potencial transitório – TRP

Os canais iônicos são de importância primordial para a sobrevivência e função adequada das células (Minke, 2010). A superfamília de receptores de potencial transitório (TRP) é uma das maiores subfamílias de canais iônicos conhecida, possuindo uma grande variedade de funções biológicas e mecanismos de ativação descritos. Estes receptores representam uma nova família de canais iônicos permeáveis a cátions, sendo que mais de 50 membros já foram caracterizados apresentando uma ampla distribuição filogenética (Vriens et al., 2009; Minke, 2010).

A primeira descoberta relativa à superfamília TRP ocorreu em 1969 quando Cosens e Manning identificaram uma variedade mutante espontânea de *Drosophila melanogaster*. Devido a seu fenótipo comportamental, estas ficavam cegas quando colocadas sobre intensa iluminação. Analisando seu sistema visual, foi observado que a mosca com fenótipo mutante apresentava respostas no eletroretinograma

transitórias à estimulação luminosa intensa e prolongada, enquanto que o fenótipo normal apresentava uma resposta contínua. Assim, a denominação de receptor de potencial transitório (TRP) foi designada ao fotoreceptor desta variante mutante devido à resposta transitória a partir de estímulo com luz intensa (Minke et al., 1975). Posteriormente, TRP foi a nomenclatura adotada para designar a superfamília de canais iônicos de potencial transitório (Montell et al., 2002).

Em 1985, Montell e colaboradores isolaram pela primeira vez a porção de DNA que continha o gene *trp*, e após, este gene foi clonado e sequenciado, assim a proteína relacionada ao gene *trp* foi descrita como contendo 1275 aminoácidos e oito segmentos transmembrana (Montell e Rubin, 1989; Wong et al., 1989). Em seguida, foi observado que o receptor TRP era um canal permeável a cálcio e que a inibição destes canais usando La^{3+} criava um fenótipo igual a mutação observada em *Drosophila melanogaster*, e que o mesmo não era expresso no tipo mutante (Suss Toby et al., 1991, Hardie e Minke, 1992).

É descrito que além do receptor TRP outra proteína designada como *TRP-like* (TRPL) também é importante para a detecção do estímulo luminoso e é encontrada no sistema visual da espécie *Drosophila*. Este receptor TRPL possui homologia com o canal TRP, e apresenta dois locais de ligação para a calmodulina na região carboxi terminal, e, além disso, também possui repetições do tipo anquirina na região amino terminal (Phillips et al., 1992). Foi, ainda, observado que esta proteína (TRPL) apresentava homologia com a região do sensor de voltagem de alguns canais de cálcio, indicando que este gene codificaria subunidades de um canal permeável a cálcio (Phillips et al., 1992, para revisão Minke, 2010).

A primeira descrição de que proteínas relacionadas à família TRP poderiam ser encontradas em sistemas biológicos diferentes dos encontrados em fotoreceptores da espécie *Drosophila* foi no estudo de Petersen e colaboradores (1995), no qual foram identificadas sequências parciais homólogas à proteína TRP em oócitos de *Xenopus* e também em cérebro camundongos. Posteriormente, a sequência completa de uma proteína homóloga ao TRP foi identificada em humanos, e este foi denominado TRPC1 (canônico) (Wes et al., 1995; Zhu et al., 1995). Subsequentemente, diversos grupos de pesquisa clonaram e sequenciaram

diversas outras subfamílias dos canais TRP, independente do receptor TRP e TRPL observado na *Drosophila*.

Os receptores da superfamília TRP são essencialmente classificados de acordo com a sequência primária de aminoácidos, pois a classificação a partir de seletividade ou afinidade por ligantes seria complicada devido às propriedades heterogêneas de ativação e aos diversos mecanismos de regulação. Já foram identificados 28 canais da classe TRP em mamíferos, e estes podem ser classificados em 6 subfamílias denominadas: TRPV (vanilóide), TRPC (canônico), TRPM (melastatina), TRPP (policistina), TRPML (mucolipina) e TRPA (anquirina). A família TRPN (não sensível a estímulo mecânico) não pode ser encontrada em mamíferos (Figura 2) (Vriens et al., 2009, Minke, 2010; Wu et al., 2010).

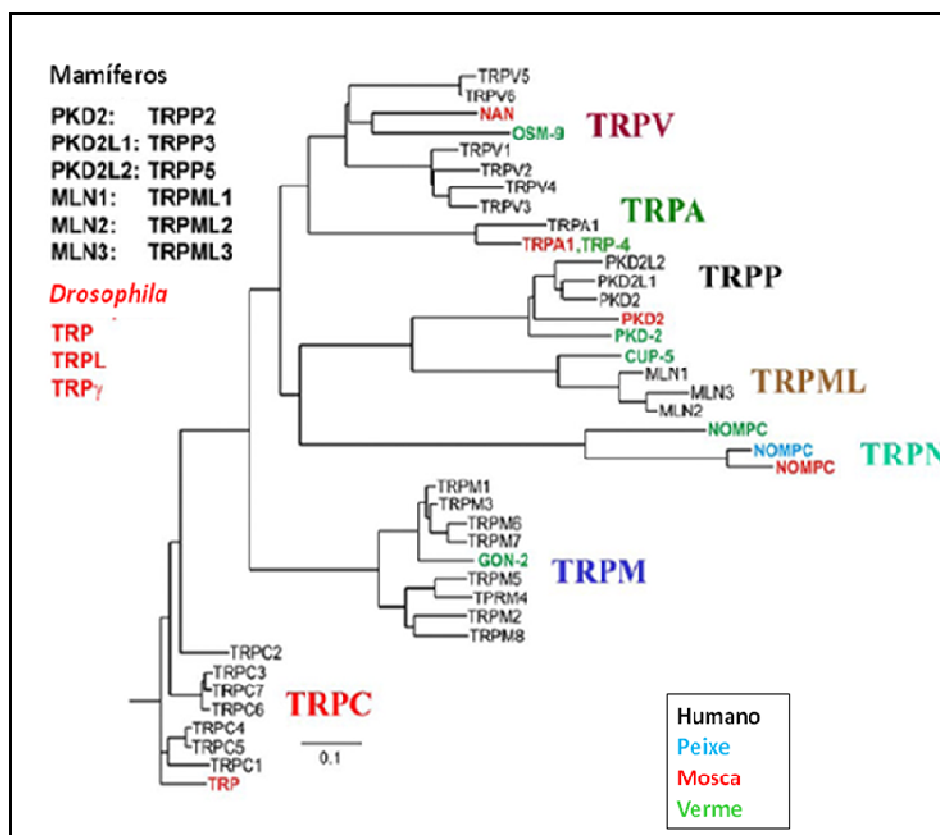


Figura 2. Divisão filogenética das subfamílias dos canais TRP. Adaptado de Minke et al., (2010).

A estrutura molecular destes canais compreende seis domínios transmembrana (S1-S6), sendo que a região formadora do poro está localizada entre os segmentos S5 e S6, ainda as regiões amino e carboxi terminais estão localizadas intracelularmente possuindo comprimentos variáveis (Figura 3). São necessárias quatro subunidades para formar um canal funcional heterólogo ou homólogo. Estes canais, mesmo tendo relações estruturais com os canais sensíveis à voltagem e mesmo que alguns subtipos ainda possuam ativação voltagem dependentes, não possuem um sensor de voltagem na sua estrutura (Montell, 2005; Minke, 2010).

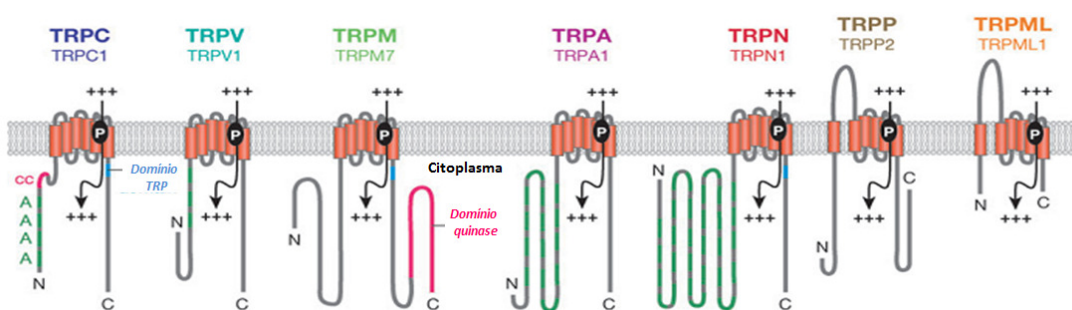


Figura 3. Representação espacial da estrutura das diferentes famílias de receptores TRP. Adaptado de Montell (2005).

A região carboxi-terminal apresenta variação dentre os diferentes tipos de canais TRP, sendo que nas subfamílias TRPC, TRPN e TRPM existe uma região conservada de 23 a 25 aminoácidos, denominada domínio TRP, sendo utilizada como marcador para o rastreamento destes canais. Além disso, alguns canais ainda apresentam sítio de ligação para calmodulina, como no receptor TRPV1, ou domínios enzimáticos, como nos receptores TRPMs na região carboxi-terminal (Zhu, 2005, Venkatachalam e Montell, 2007). A região amino-terminal apresenta-se formada por uma série de repetições de anquirina na maioria dos canais TRPs, sendo que os canais TRPMs não apresentam este tipo de repetição. Estas repetições formam uma superfície estável e rígida para o acoplamento de diversas proteínas ao receptor, e ainda servem como âncoras para o trânsito do receptor de

vesículas até a membrana plasmática, e também para a sua estabilização com a mesma e o citoesqueleto celular (Montell, 2005; Venkatachalam e Montell, 2007). Os canais TRP são não seletivos ao influxo de cátions, com exceção dos canais TRPM4 e TRPM5 que são seletivos a permeação de cátions monovalentes e os canais TRPV4 e TRPV5 que são seletivos ao influxo de cálcio (Clapham, 2003).

A maioria dos receptores TRPs são polimodais, ativados ou regulados por diversos mecanismos, sendo então descritos como sensores fisiológicos a estímulos externos e internos, sejam estímulos térmicos, químicos (variações de pH e osmolaridade), sonoros, mecânicos, luminosos ou ainda ativação por ligantes (Clapham, 2003; Nilius et al., 2007; Venkatachalam e Montell, 2007; Minke, 2010). Além disso, é descrito o envolvimento dos canais TRPs em vários outros processos fisiológicos e também em funções celulares como adesão, crescimento e diferenciação celular (Minke, 2010).

Devido ao envolvimento dos canais TRP em diversos processos fisiológicos, são descritas inúmeras doenças em humanos relacionadas à deficiência e disfunção destes canais (Levine e Alessandrini-Haber, 2007; Nilius et al., 2007).

4.3. Receptor de potencial transitório vanilóide 1 – TRPV1

4.3.1. Caracterização histórica

Os produtos naturais contendo compostos capazes de ativar o receptor TRPV1 são utilizados há milhares de anos por diversas civilizações (Calixto et al., 2005; Schumacher, 2010). Hoje, já é descrito que a capsaicina (presente em espécies de *Capsicum*) e a resiniferatoxina (presente na *Euphorbia resinifera*), substâncias pungentes isoladas de plantas, são capazes de ativar este receptor expresso em fibras sensoriais e promover o influxo de cátions (principalmente cálcio) (Calixto et al., 2005). A capsaicina é o princípio pungente da pimenta vermelha (*Capsicum* sp.), e esta planta já era utilizada desde 7.000 a.c., porém teve seu uso disseminado na Europa para a culinária e também para uso medicinal a partir de 1400 d.c. (Calixto et al., 2005; Schumacher, 2010).

Existem aproximadamente 20 espécies de *Capsicum* (Solanaceae), sendo que apenas cinco espécies são cultiváveis: *Capsicum annuum*, *Capsicum*

frutescens, *Capsicum chinese*, *Capsicum pendulum* e *Capsicum pubescens* (Szallasi e Blumberg, 1999). O uso popular deste gênero é principalmente para o tratamento de dor, o reumatismo e o aumento do apetite, sendo que é também descrito o uso da mesma para tosse e bronquite (Szallasi e Blumberg, 1999; Corson e Crews, 2007).

Classicamente, sabe-se que o extrato da pimenta vermelha, quando aplicado topicamente na pele, provoca vermelhidão, edema e dor, muitas vezes descrita como queimação. Além desses aspectos negativos, aplicações repetidas desse extrato levavam à perda de sensibilidade dolorosa, o que muitas vezes culminou na aplicação do extrato como tratamento para diferentes tipos de dores, principalmente de origem neuropática (Calixto et al., 2005; Schumacher, 2010). O princípio pungente deste extrato foi apenas isolado em 1846 por Thresh e denominado capsaicina após a elucidação da estrutura química deste composto, descrita como trans-8-metil-N-vanilil-6-noneamida (Nelson, 1919, Para revisão do histórico de uso da capsaicina ver Calixto et al., 2005) (Figura 4).

Porém, mesmo com a descrição de que a capsaicina e os extratos da pimenta vermelha poderiam ser utilizados para o alívio da dor (Turnbull, 1850), apenas em 1949 os estudos relativos a esta substância e seu provável receptor foram retomados (Jancsó e Jancsó-Gabor, 1949). Assim, pesquisas demonstraram as respostas fisiológicas causadas pela administração de capsaicina na pele de humanos, como o desenvolvimento de dor em queimação e vasodilatação. Esse fenômeno foi seguido pelo desenvolvimento rápido de um estado refratário, denominado dessensibilização, onde a pele tratada apresentava-se relativamente resistente, não somente à capsaicina, mas também a outros agentes químicos irritantes e ao calor nocivo (Jancsó e Jancsó-Gabor, 1949; Jancsó, 1955). Também, foi descrito que a administração de capsaicina era capaz de causar a degeneração de fibras sensoriais específicas (fibras de pequeno diâmetro) quando administrada a animais adultos e em neonatos, e isso levava a um efeito analgésico (Jancsó et al., 1977; Holzer, 1991). Então a dor, a dessensibilização e a degeneração causada pela capsaicina foram atribuídas à estimulação de uma classe específica de fibras sensoriais que também eram ativadas por calor nocivo e irritantes químicos (Jancsó et al., 1977; Holzer, 1991).

Já que a capsaicina apresentava seletividade por determinadas classes de neurônios sensoriais, estes deveriam expressar um receptor específico sensível a

esta substância (Szolcsányi e Jancsó-Gábor, 1975). A partir dessa proposta, vários experimentos foram realizados para a descoberta do receptor sensível à capsaicina, usando diferentes radioligantes, entre eles dihidrocapsaicina, um análogo estrutural hidrossolúvel da capsaicina (Szebeni et al., 1978; James et al., 1988). Apesar disso, estes estudos não obtiveram sucesso devido à baixa potência da capsaicina e seus análogos, e também devido a sua hidrofobicidade (Szallasi e Blumberg, 1999).

Os estudos relacionados ao sítio de ligação para a capsaicina foram impulsionados pela descoberta da resiniferatoxina (RTX), um diterpeno extremamente irritante isolado do látex seco da planta *Euphorbia resinífera*, um cacto nativo do Marrocos (Euphorbiaceae) (Hergenhahn et al., 1975). Até então, esperava-se que a RTX agisse como outros ésteres de forbol através da ativação da proteína quinase C, entretanto este composto não apresentava as mesmas características químicas e efeitos biológicos, pois causava irritação da pele, não era, no entanto, promotor de tumor (Driedger e Blumberg, 1980a, 1980b).

Szallasi e Blumberg (1989) caracterizaram os efeitos descritos a partir da aplicação de resiniferatoxina (RTX) como sendo devido à ativação de um mecanismo de ação semelhante ao da capsaicina, pois este composto também apresentava um grupo homovanilínico, presente também na molécula da capsaicina (Figura 4). Devido à presença do grupo homovanilínico, estes compostos foram denominados como substâncias vanilóides. Concomitantemente, a RTX era capaz de induzir alterações fisiológicas semelhantes às observadas após a aplicação de capsaicina, mas com maior potência (hipotermia, dor, inflamação neurogênica e desenvolvimento de desensibilização) (Szallasi e Blumberg, 1989).

Assim, foram realizados experimentos de ligação específica utilizando a molécula de RTX radioativa, mostrando, então, pela primeira vez, sítios de ligação de alta afinidade, saturáveis e específicos para [H^3]-RTX em gânglios da raiz dorsal (Szallasi e Blumberg, 1990b). Esse dado permitiu mapear a distribuição dos sítios de ligação para [H^3]-RTX em diferentes tecidos, como medula espinhal, GRD, epitélio urogenital, mucosa nasal e do cólon de diferentes espécies, como ratos, camundongos e humanos (Szallasi e Blumberg, 1991; Szallasi e Blumberg, 1993). Essas evidências levaram a propor que a capsaicina e a RTX estariam agindo sobre o mesmo tipo de receptor designado como receptor vanilóide (VR) (Calixto et al., 2005).

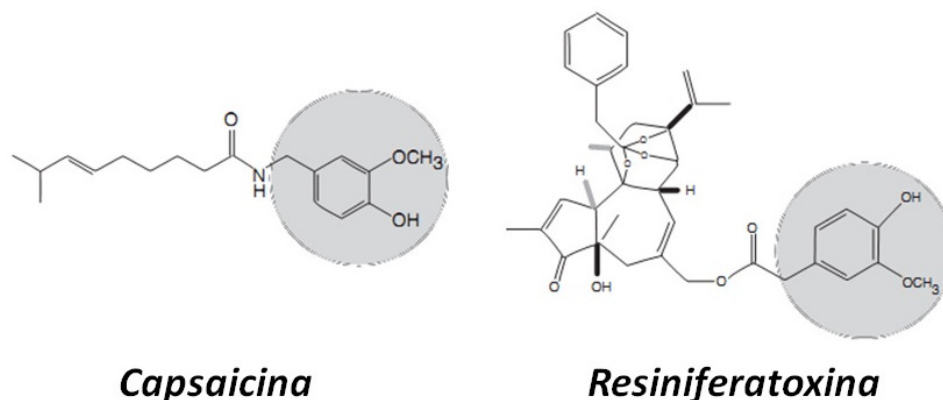


Figura 4. Estruturas químicas da capsaicina e resiniferatoxina ilustrando o grupamento homovanilínico essencial para a atividade biológica. Adaptado de Schumacher (2010).

Estudos eletrofisiológicos e bioquímicos já haviam caracterizado que a capsaicina era capaz de promover a ativação de nociceptores causando um aumento de cálcio intracelular, mas o mecanismo molecular responsável por este fenômeno ainda era desconhecido (Caterina et al., 1997). Com o isolamento do clone de DNA complementar (DNAc) que era relacionado com o canal iônico ativado pela capsaicina em 1997, a base molecular do receptor vanilóide VR1 (TRPV1) foi então solucionada (Caterina et al., 1997). Para isso, células não neuronais eucarióticas (HEK293) foram transfectadas com diferentes grupos de DNAc provenientes de uma biblioteca de DNAc de neurônios sensoriais de ratos, e, utilizando ensaios de imagem para Ca^{2+} intracelular, foram selecionadas os grupos transfectados que eram responsivos a capsaicina. Após, os grupos que respondiam ao estímulo da capsaicina eram subdivididos em grupos menores e novamente transfectados em células eucarióticas, até o isolamento de um único DNAc responsável pela proteína com afinidade pela capsaicina (VR1). Este gene codificava uma proteína composta por 838 aminoácidos em ratos com uma estrutura semelhante ao dos receptores TRP (Caterina et al., 1997).

Assim, o receptor TRPV1 foi caracterizado como sendo um canal iônico não seletivo para cátions, com preferência a cálcio e não discriminação entre cátions monovalentes, expresso principalmente no GRD e GT (Caterina et al., 1997; Tominaga et al., 1998). Este canal é ativado por calor nocivo ($>43^{\circ}\text{C}$), relacionando

este tipo de ativação com a sensação de queimação elucidada pela administração de capsaicina, e, além disso, este receptor também é ativado por pH abaixo do fisiológico (Caterina et al., 1997; Tominaga et al., 1998). Dessa forma, o receptor TRPV1 parece ser um integrador molecular de estímulos químicos e físicos que causam dor (Szallasi et al., 2007).

A partir desse ponto, o até então chamado receptor vanilóide (VR) passou a integrar a família dos TRPs, formando um novo subtipo – vanilóide, sendo assim denominado TRPV1. A subfamília TRPV (vanilóide) foi então nomeada após a descrição do receptor vanilóide 1 (Caterina et al., 1997), sendo então composta por seis tipos de proteínas em mamíferos (TRPV1–6) (Wu et al., 2010).

4.3.2. Aspectos gerais do receptor TRPV1

Como descrito anteriormente, o receptor TRPV1 é um canal iônico permeável a cátions, principalmente cálcio. Após a ativação deste receptor o aumento de cálcio e sódio intracelular pode contribuir para a geração de potenciais de ação e também para mecanismos relacionados à liberação de neurotransmissores (Basbaum e Nilius, 2009). A expressão deste canal é descrita como sendo preferencialmente em neurônios sensoriais, e nos gânglios da raiz dorsal, do trigêmio, nodoso e vagal, e também no sistema nervoso central (SNC). Além disso, o receptor TRPV1 também pode ser encontrado em tecidos não neuronais como em queratinócitos, músculo liso, bexiga, rim, pulmão e fígado (Szallasi et al., 2007; Jara-Oseguera et al., 2008; Schumacher, 2010).

Outro aspecto importante oriundo dos estudos com a capsaicina foi a descoberta de que a estimulação de subtipos de fibras aferentes primárias pode causar a liberação de substâncias vasoativas, especialmente neuropeptídeos (Holzer, 1988). As fibras C e A δ periféricas que expressam TRPV1 são normalmente peptidérgicas, assim, contém diferentes peptídios, como substância P e o peptídeo relacionado ao gene da calcitonina (CGRP) (Szallasi et al., 2007; Basbaum e Nilius, 2009). A ação destes mediadores em células alvos através da interação com seus receptores específicos está envolvida com vários processos fisiopatológicos, como a regulação da produção de muco pelo estômago e a indução de sinais clássicos da inflamação (um fenômeno conhecido como inflamação neurogênica) (Holzer, 1988; Richardson e Vasko, 2002).

O canal TRPV1 é formado por uma proteína de membrana que possui seis domínios transmembrana e um prolongamento hidrofóbico formador do poro, localizado entre as regiões cinco e seis, com as caudas carboxi e amino-terminais localizadas intracelularmente, esta última contendo seis repetições de anquirina. Similarmente aos demais receptores TRP, os receptores TRPV1 provavelmente formam homotetrâmeros ou heterotetrâmeros funcionais, onde todos os monômeros contribuem para a formação do poro e seletividade do canal. A permeação de íons pelo canal é controlada alostericamente através de interações entre as diferentes subunidades e por ativação do canal (Szallasi et al., 2007; Jara-Oseguera et al., 2008; Schumacher, 2010).

Já foram descritos vários mecanismos de ativação do receptor TRPV1, tanto por substâncias endógenas como exógenas. Dentre as substâncias exógenas isoladas de produtos naturais, pode-se citar a capsaicina e a resiferatoxina, além de vários outros compostos, como a alicina (presente no alho) e a cânfora (presente na *Cinnamomum camphora*). Além destes ligantes, outros já foram descritos, e são responsáveis pelas características sensoriais apresentadas por diferentes tipos de alimentos e resinas (Calixto et al., 2005; Jara-Oseguera et al., 2008).

Fisiologicamente, este receptor é responsável pelo reconhecimento de várias modalidades de estímulos nocivos, como aumento de temperatura ($>43^{\circ}\text{C}$) e diminuição do pH (< 5.2) (Caterina et al., 1997; Jordt et al., 2000; Welch et al., 2000). Porém, não era claro como as alterações de temperatura eram capazes de promover a ativação do receptor TRPV1, assim foi proposto que a região distal da cauda carboxi terminal é responsável pelo reconhecimento deste tipo de estímulo (Vlachova et al., 2003; Brauchi et al., 2006). A ativação do receptor TRPV1 a temperaturas relacionadas ao calor nocivo em humanos ($>43^{\circ}\text{C}$) indica que esse poderia ser o transdutor endógeno deste tipo de estímulo. As evidências que suportam esta hipótese são: o receptor TRPV1 é expresso na maioria das fibras sensoriais sensíveis ao calor, as correntes evocadas pelo calor e pela capsaicina são idênticas. Além disso, as respostas de nocicepção moduladas pelo receptor TRPV1 são exarcebadas na presença de agentes pró-nociceptivos e inflamatórios, sendo que estes causam hiperalgesia em modelos animais. E também animais que não possuem o receptor TRPV1 não apresentam resposta nociceptiva a capsaicina, e demonstram respostas nociceptivas diminuídas ao calor nocivo (Basbaum e Nilius, 2009). São descritas outras variantes do receptor TRPV1 que apresentam ativação

por temperaturas de menor intensidade, como por exemplo, em morcegos hematófagos e no peixe-zebra (Gracheva et al., 2011).

Este receptor também pode ser ativado por diferentes substâncias endógenas, além de ser também sensibilizado por estas através de vias de segundos mensageiros e também por modificação da atividade do receptor (fosforilação) (Jara-Oseguera et al., 2008), contribuindo assim para o desenvolvimento do fenômeno descrito como sensibilização periférica, ou seja, aumento da resposta e uma diminuição do limiar de ativação das fibras nociceptivas periféricas (Szallasi et al., 2007; Loeser e Treede, 2008). Dessa maneira, o canal TRPV1 pode ser ativado e sensibilizado por lipídios como a anandamida, ácido 12-hidroperoxi-eicosatetraenóico (12-HPETE), N-araquidonoil dopamina (NADA), oleiletanolamida (OEA), leucotrieno B4 (LTB4) e também por óxido nítrico (Jara-Oseguera et al., 2008). Além disso, é descrito que outras substâncias endógenas como trifosfato de adenosina (ATP), bradicinina e prostaglandinas são capazes de sensibilizar o receptor TRPV1 e também ativar através de vias intracelulares (Jara-Oseguera et al., 2008; Schumacher, 2010). É também descrito que o fator de crescimento do nervo (NGF) é capaz de ativar e sensibilizar o receptor TRPV1, e também aumentar a expressão do mesmo nos terminais sinápticos (Szallasi et al., 2007; Schumacher, 2010) (Figura 5). Desse modo, este receptor após ser sensibilizado em situações inflamatórias podendo então ser ativado em temperaturas corporais normais (Szallasi et al., 2007; Jara-Oseguera et al., 2008).

O receptor TRPV1 apresenta 18 resíduos de cisteína em sua seqüência primária, o que levou diferentes grupos a pesquisar possíveis modulações redox de sua atividade (Caterina et al., 1997). Neste âmbito, já foi descrito que agentes redutores como a glutathione e o ditioneitol (DTT) são capazes de promover uma diminuição do limiar de ativação do receptor à temperatura (Vyklícky et al., 2002; Susankova et al., 2006). Também, mutações sítio-dirigido para a Cys621 identificaram um sítio de modulação redox extracelular, principalmente para agentes redutores. Apesar disso, já foi demonstrado que alguns agentes oxidantes, como a cloramina-T, e agentes alquilantes são também capazes de promover uma diminuição no limiar de ativação térmico do receptor TRPV1 (Susankova et al., 2006). A partir desses dados, é possível supor que o receptor TRPV1 também se apresente como um sensor do estado redox da célula, explicando a sua participação em diferentes patologias, como a isquemia e a inflamação, nas quais já foi

evidenciado o desenvolvimento de estresse oxidativo (Mirshafiey et al., 2008; Lakhan et al., 2009).

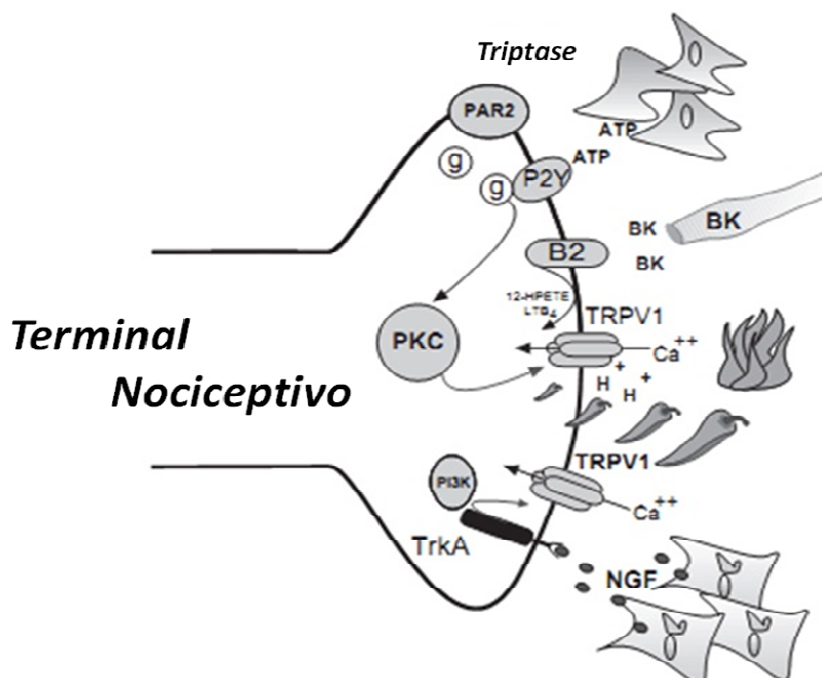


Figura 5. Terminal nociceptivo expressando o receptor TRPV1. E ilustrando as diversas formas de ativação deste receptor (capsaicina, calor nocivo e prótons), além de mediadores inflamatórios capazes de ativar ou sensibilizar o receptor, como bradicinina, ATP, triptase e NGF. Adaptado de Schumacher (2010).

4.3.3. Envolvimento do receptor TRPV1 em patologias dolorosas

O envolvimento do receptor TRPV1 em diversas doenças já foi descrito, sendo que o seu principal foco de estudo atualmente é em patologias dolorosas (Schumacher 2010). O receptor TRPV1 é um dos principais receptores expressos em neurônios sensoriais nociceptivos, dessa forma tem importância na transdução de estímulos nociceptivos (Szallasi et al., 2007). Assim, diversas evidências levam a crer que o desenvolvimento de fármacos que possam agir como antagonistas ou agonistas do receptor TRPV1 em humanos possam ser utilizados em diversos tipos de síndromes dolorosas (Levine e Alessandrini-Haser et al., 2007; Patapoutian et al., 2009; Wong e Gavva, 2010).

Os agonistas do receptor TRPV1 causam analgesia por uma série de mecanismos propostos como desensibilização, pela disfunção do nociceptor, depleção de neuropeptídeos, e destruição dos terminais sinápticos que expressam o TRPV1 (Schumacher et al., 2010). Assim, a aplicação de capsaicina causa desensibilização dos canais TRPV1, ocasionando diminuição da nocicepção em modelos animais (Szallasi e Blumberg, 1999) e em humanos (Knotkova et al., 2008).

Ainda hoje, vários estudos mostram o uso de cremes contendo capsaicina para o alívio da dor em patologias como neuralgia pós herpética, também em pacientes com neuropatia associada à síndrome do complexo regional e ao câncer (Schumacher, 2010). Porém, alguns fatores limitam o tratamento como a necessidade de várias aplicações de capsaicina e também a dor inicial, dessa forma, alternativas como o uso de anestésicos locais e formulações de liberação prolongada de capsaicina tem sido usadas para aumentar a aderência ao tratamento pelos pacientes (Schumacher, 2010).

O receptor TRPV1 pode ser sensibilizado por substâncias inflamatórias e participa no desenvolvimento e na manutenção do fenômeno de inflamação neurogênica através da liberação de CGRP e SP na periferia (Szallasi et al., 2007). Diversas condições patológicas já foram descritas como tendo um componente inflamatório, como enxaqueca, asma, síndrome do intestino irritável, cistite e osteoartrite (Szalassi et al., 2007; Schumacher, 2010). É também descrito que a expressão do receptor TRPV1 pode ser aumentada durante condições inflamatórias com o desenvolvimento de hiperalgesia, tanto em modelos animais como em diversas doenças, como na síndrome do intestino irritável, na vulvodínia, no refluxo gastroesofágico e na mastalgia (Szallasi et al., 2007; Schumacher, 2010).

Os antagonistas do receptor TRPV1 são eficientes na diminuição da nocicepção em modelos animais relacionados a diversos tipos de estímulos dolorosos como a inflamação, a osteoartrite e a neuropatia (Gavva et al., 2005b; Honore et al., 2005; Rami et al., 2006; Walker et al., 2003). Também, o receptor TRPV1 parece estar envolvido com a dor relacionada ao câncer, sendo que ocorre aumento da expressão deste canal nesta patologia em modelos animais (Ghilardi et al., 2005; Menendez et al., 2006; Niiyama et al., 2007). Os antagonistas com capacidade de atravessar a barreira hematoencefálica mostraram melhor perfil anti-hiperalgésico que um análogo que age apenas periféricamente, assim a expressão

do receptor TRPV1 em estruturas supra-espinhais parece ser importante para o efeito antinociceptivo (Cui et al., 2006).

Desta forma, tanto agonistas como antagonistas do receptor TRPV1 tem sido investigados para o tratamento de diversas patologias dolorosas, sendo que muitos já estão em fase clínica de estudo (Wong e Gavva, 2009). Entretanto, testes em humanos e animais mostraram que os antagonistas do receptor TRPV1 são capazes de aumentar a temperatura corporal, sendo que este é o principal efeito adverso observado após testes clínicos com estes compostos (Gavva et al., 2008; Romanovsky et al., 2009). Então, compostos que possam agir como antagonistas do receptor TRPV1 e diminuir a nocicepção em modelos animais, bem como não levarem ao desenvolvimento de hipertermia, parecem ser bons protótipos de novas drogas analgésicas.

4.4. Importância dos produtos naturais para a descoberta de novos analgésicos

Os produtos naturais, especialmente aqueles derivados de plantas e microorganismos, possuem relevância histórica como fonte de descoberta de novas substâncias ativas com atividade farmacológica. Assim, as plantas são ainda consideradas fonte de investigação de novos fármacos em potencial. As substâncias ativas obtidas das plantas são usadas como protótipos para o desenvolvimento de novos medicamentos, além de proporcionar a identificação de uma nova possibilidade de tratamento terapêutico. Então, os produtos naturais são considerados como sendo importantes alvos para a descoberta de novos analgésicos, além de terem sido importantes para a descoberta de vias de modulação da dor e de receptores envolvidos nestas vias e analgésicos ainda hoje usados na clínica (Calixto et al., 2000; Yunes et al., 2005; Newman e Cragg, 2007).

O ópio extraído da cápsula da planta *Papaver Somniferum* (Papaveraceae) tem sido utilizado como analgésico desde 3000 a.c., e o seu cultivo é descrito antes de 3400 a.c. na Mesopotâmia (Calixto et al., 2000; Trescot et al., 2008). O termo ópio se refere à mistura de alcalóides do látex extraído da cápsula da papoula; deste é isolado o alcalóide morfina além de várias outras substâncias como a codeína e a tebaína (Calixto et al., 2000). A morfina é ainda hoje um dos analgésicos mais usados na clínica para o tratamento de uma enorme quantidade de patologias como

a dor relacionada ao câncer (Plante e VanItallie, 2010). Além disso, este composto auxiliou no estudo das vias relacionadas à dor (Yunes et al., 2005; Trescot et al., 2008). A morfina age como agonista de receptores opióides, principalmente μ opióide e possui diversos efeitos adversos, sendo os principais descritos como constipação, hipotermia, retenção urinária e, além disso, desenvolvimento de tolerância, o que limita seu uso clínico (Trescot et al., 2008).

Diversos outros agentes analgésicos, como o ácido acetilsalicílico, protótipo da classe descrita como analgésicos não esteroidais (AINES), foi desenvolvido a partir de produtos naturais. Este composto foi sintetizado a partir do ácido salicílico extraído de diversas espécies de *Salix* (Salicaceae), como *Salix alba* e *Salix fragilis*. O ácido acetilsalicílico possui efeito analgésico, antipirético e antiinflamatório e age inibindo a enzima ciclooxigenase (COX) e então a produção de prostaglandinas (Calixto et al., 2000; Yunes et al., 2005). A analgesia produzida pelos AINES é limitada por efeitos adversos como irritação gastrointestinal, alterações na função renal e também por problemas cardiovasculares (Fernandez et al., 1995; Lichtenberger et al., 1995; Suleyman et al., 2007).

Alem disso, é observado que as opções de intervenções terapêuticas seriam escassas se não fosse a descoberta dessas substâncias ativas provenientes de plantas medicinais, na qual 80% dos princípios ativos de medicamentos até 1996 foram diretamente ou derivados de compostos naturais (Harvey, 2007). E uma revisão de novas drogas introduzidas no mercado entre 1981 e 2002, 28% de 868 novos medicamentos ou eram produtos naturais ou diretamente derivados, e 24% foram criados a partir de mudanças químicas de estruturas derivadas de produtos naturais (Newman et al., 2003). Estes dados enfatizam a importância dos compostos isolados de produtos naturais como alternativas para a busca por novos compostos bioativos (McChesney et al., 2007).

Os estudos com compostos derivados de produtos naturais levaram também ao isolamento e à descoberta de novos antagonistas do receptor TRPV1, como o composto eriodiciol, que foi eficaz em diminuir a hiperalgesia térmica induzida em um modelo de dor inflamatória (Rossato et al., 2011). Outro composto o MCS-18 foi delineado em estudos *in vitro* como sendo um potente antagonista do receptor

TRPV1 (Neacsu et al., 2010). Assim, compostos isolados de plantas parecem ser relevantes como fontes de novos antagonistas do receptor TRPV1.

O gênero *Vernonia* (Asteraceae) contém mais de 500 espécies de plantas distribuídas em áreas subtropicais e tropicais do mundo, especialmente na África e na América do Sul. As espécies deste gênero são usadas na medicina tradicional para o tratamento de diversas patologias como malária, febre, doenças gástricas e respiratórias e processos inflamatórios (Awe et al., 1998; Abosi e Raseroka, 2003; Iwalewa et al., 2003; Tona et al., 2004; Barbastefano et al., 2007; Bardón et al., 2007; Zanon et al., 2008). Os principais constituintes químicos relacionados com o gênero *Vernonia* são triterpenos, esteróides, ligninas, lactonas sesquiterpênicas, flavonóides, alcalóides e taninos (Zanon et al., 2008). Recentemente, foi demonstrado que os extratos de *Vernonia cinerea* possuem atividade analgésica, antipirética e antiinflamatória (Gupta et al., 2003; Iwalewa, 2003; Mazumder et al., 2003). Além disso, foram também descritas atividades antinociceptiva e antiulcerogênica para a *Vernonia condensata* Baker (Frutuoso et al., 1994; Risso et al., 2010). Estudos também mostram atividade antinociceptiva após a administração do extrato aquoso das folhas de *Vernonia amygdalina* (Njan et al., 2008).

A espécie *Vernonia tweedieana* Baker, popularmente conhecida no Brasil como “assa-peixe” é uma planta herbácea distribuída nas planícies do Paraguai, Argentina e sul do Brasil (Zanon et al., 2008). As folhas desta planta são usadas na medicina tradicional para o tratamento de doenças respiratórias, principalmente como expectorante (Zanon et al., 2008). Diversos compostos já foram isolados da fração diclorometano das folhas de *Vernonia tweedieana* como a mistura de α -amirina e β -amirina, lupeol, estigmasterol and α -espinasterol (Zanon et al., 2008). Existem estudos mostrando que o α -espinasterol possui propriedades farmacológicas como atividade antiinflamatória, antioxidante, anti-carcinogênica, antiproliferativa, antimutagênica e antiulcerogênica (Boller et al., 2010; Coballase-Urrutia et al., 2010; Jeong et al., 2010; Klein et al., 2010; Ravikumar et al., 2010; Villasenõr and Domingo, 2000). Além disso, estudos demonstraram o efeito antinociceptivo deste composto (Meotti et al. 2006; Ribas et al., 2008; Freitas et al., 2009). Entretanto, o mecanismo de ação pelo qual este composto apresenta atividade antinociceptiva ainda não foi descrito.

Dessa maneira a planta *Vernonia tweedieana* foi utilizada para o desenvolvimento de uma triagem *in vivo* e *in vitro* para caracterizar novos antagonistas do receptor TRPV1 com atividade antinociceptiva.

Characterisation of the plant steroid α -spinasterol as a novel transient receptor potential vanilloid 1 antagonist with antinociceptive properties

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ABSTRACT

The transient receptor potential vanilloid 1 (TRPV1) is relevant to the perception of noxious information and has been studied as a therapeutic target for the development of new analgesics. The goal of this study was to perform *in vivo* and *in vitro* screens to identify novel, efficacious, and safe TRPV1 antagonists isolated from leaves of the medicinal plant *Vernonia tweedieana* Baker. All of the fractions and the hydroalcoholic extract produced antinociception in mice during the capsaicin test, but the dichloromethane fraction (Dcm) also had antioedematogenic effect. Among the compounds isolated from the Dcm fraction, only α -spinasterol reduced the nociception and oedema induced by capsaicin injection. Moreover, α -spinasterol demonstrated good oral absorption and high penetration into the brain and spinal cord of mice. Besides, α -spinasterol was able to displace [³H]-resiniferatoxin (RTX) binding and diminish calcium (Ca^{2+}) influx mediated by capsaicin. Orally administration of the Dcm fraction and α -spinasterol also produced antinociceptive effect in the noxious heat-induced nociception test; however, they did not change the mechanical threshold of naive mice. The treatment with α -spinasterol did not produce antinociceptive effect in mice systemically pre-treated with RTX. In addition, α -spinasterol and the Dcm fraction also reduced the oedema, mechanical and heat hyperalgesia elicited by complete Freund's adjuvant (CFA) paw injection. The Dcm fraction and α -spinasterol did not affect body temperature or locomotor activity. In conclusion, α -spinasterol is an efficacious and safe antagonist of the TRPV1 receptor with antinociceptive effect.

Key words: Steroid; TRPV1; capsaicin; vanilloid; antinociception; *Vernonia*.

1. INTRODUCTION

The transient receptor potential vanilloid type 1 (TRPV1 receptor) is a member of the TRP family [1]. TRPV1 is expressed mainly in the nervous system and is composed of six transmembrane domains that form a nonselective cation channel (principally for calcium) [2]. Expression is particularly abundant in C and A δ nociceptive fibres, where it plays a key role in the detection of noxious painful stimuli [3]. It is activated by exogenous substances, such as capsaicin (the active component of chili peppers), resiniferatoxin (RTX, isolated from *Euphorbia resinifera*), endogenous inflammatory agents, extracellular protons, bioactive lipids, and noxious heat (>43°C) [4,2].

Because the TRPV1 receptor plays an important role in the detection and integration of noxious stimuli and is sensitised by a number of different stimuli and mechanisms, including various inflammatory mediators, it is involved in inflammatory, visceral, and cancer pain [5,6,2]. For these reasons, TRPV1 has been studied as a relevant therapeutic target for the development of novel analgesics [7,8]. Antagonists of TRPV1 have been reported to produce an antihyperalgesic effect in animal pain models and in human diseases [9,10,11]. However, some of these antagonists are implicated in the development of severe hyperthermia [12]. Thus, novel TRPV1 antagonists without a hyperthermic effect should be explored as new therapeutic agents for the treatment of pain [7].

Natural products have been an important source for new drugs, including analgesic molecules [13,14]. Products isolated from plants that are able to modulate the TRPV1 receptor, as capsaicin, could be used to treat pain, and symptoms of respiratory illness [4,15,16]. The Vernonia species are herbaceous plants found all

over the world and used as analgesics [18,19,20]. Moreover, some studies have shown the antinociceptive and anti-inflammatory effects of *Vernonia* species [18,19,20]. The species *Vernonia tweedieana* Baker is widely distributed on the plains of Paraguay, Argentina, and southern Brazil, its leaves are used in Brazilian folk medicine to treat respiratory diseases [17].

As a part of our on-going research to identify phytochemicals isolated from natural sources that can act as novel TRPV1 antagonists, we performed *in vivo* and *in vitro* screens to identify novel, efficacious, and safe TRPV1 antagonists contained in *V. tweedieana*.

2. METHODS

2.1. Plant material, extraction and isolation procedures

Vernonia tweedieana Baker leaves were collected in March 2004 in Ijuí, Rio Grande do Sul, southern Brazil. A voucher herbarium specimen was deposited at the herbarium of the Federal University of Santa Maria, Brazil under number SMDB 9536. The leaves were dried in a stove with circulating air at 40°C and pulverised in a mill. Dried and powdered leaves (1.900 g) were extracted by maceration with 65% ethanol in water at room temperature for 7 days. The extract was filtered, and the ethanol was removed under reduced pressure. The hydroalcoholic extract was successively fractionated using solvents with increasing polarity: dichloromethane, ethyl acetate and *n*-butanol (4 x 200 ml for each solvent). The extract and fractions were concentrated with a rotary evaporator at a temperature not exceeding 40°C to yield the dichloromethane (Dcm, 30.0 g, 1.58%), ethyl acetate (Act, 25.62 g, 1.35%), and *n*-butanol-soluble (But, 55.85 g, 2.94%) fractions. A mixture of α - and β -amyrin, lupeol, stigmasterol, and α -spinasterol were isolated from the Dcm fraction at a concentration of 0.001% (w/w) each as previously described [17].

2.2. Animals

Male albino Swiss mice (25-35 g) bred in-house were used. Animals were housed in a controlled environment (22±2°C) with a 12 hour light/12 hour dark cycle (lights on 6:00 am) and fed standard lab chow and tap water *ad libitum*. The animals were acclimated to the experimental room for at least 1 hour before the experiments.

Each animal was used only once. The experiments reported in this study were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [21].

2.3. *In vivo* screening of TRPV1 antagonists

2.3.1. Capsaicin-induced spontaneous nociception and oedema test

The capsaicin test was used to screen new TRPV1 compounds because it is capable of inducing nociception in experimental animals and humans. It has the advantage of being a simple and short method to measure nociception, and nociception intensity (and animal discomfort) can be reduced by using sub-maximal doses of capsaicin [22,23,24]. The intraplantar (i.pl.) capsaicin test was carried out as previously described [22]. After the acclimation period, 20 μ l of capsaicin (1 nmol/paw) was subcutaneously injected i.pl. under the dorsal surface of the right hind paw (intraplantar, i.pl.). The animals were individually observed for 5 minutes after capsaicin injection. The amount of time spent licking or biting the injected paw was timed with a chronometer and was used as a measure of nociception. Vehicle (0.15% ethanol in saline, 0.9% NaCl) was prepared and used as a control for the capsaicin experiments. Treatment with vehicle did not evoke nociception behaviour (data not show).

We also evaluated oedema that developed 15 minutes after capsaicin injection (1 nmol/paw, i.pl.), as previously described [25]. Oedema formation was described as

Δ = basal paw thickness-test paw thickness. Capsaicin-induced oedema and nociception were observed in the same group of animals.

2.3.2. *In vivo* treatment

To verify the possible antinociceptive and antioedematogenic effects of *V. tweedieana* hydroalcoholic extract and fractions, mice received oral (p.o.) administration of the hydroalcoholic extract (HE, 100 mg/kg), the dichloromethane (Dcm, 100 mg/kg), ethyl acetate (Act, 100 mg/kg), and butanolic (But, 100 mg/kg) fractions or vehicle (10 ml/kg) 1 hour before capsaicin injection. The extract and fractions were dissolved in a solution of 5% Tween 80, 20% polyethylene glycol, and 75% saline (0.9% NaCl).

We evaluated the possible antinociceptive and antioedematogenic effect of isolated compounds from the Dcm fraction (mixture of α - and β -amyrin, lupeol, stigmasterol, and α -spinasterol) in a separate experiment. Compounds (0.3 μ mol/kg, p.o.) were administered 1 hour before capsaicin injection. The doses were chosen based on their concentration (0.001%) in the Dcm fraction. The animals were treated with α -spinasterol (0.03-1 μ mol/kg, p.o.) or vehicle 1 hour before capsaicin injection. Afterwards, we generated a dose-response curve for α -spinasterol. Control animals received a similar volume of vehicle solution (10 ml/kg, p.o.). To determine the time-course curve, α -spinasterol (0.3 μ mol/kg, p.o.) was administered in a separate group of animals, and its antinociceptive effect was assessed at different times after administration (0.5, 1, 2, and 4 hours). The selective TRPV1 receptor antagonist SB-366791 (3 μ mol/kg, p.o.) was used as a positive control, and its antinociceptive and antioedematogenic effects were evaluated 0.5, 1, 2, and 4 hours after the

capsaicin test. The isolated compounds and SB-366791 were dissolved in a solution of 5% Tween 80, 20% polyethylene glycol and 75% saline (0.9% NaCl). The SB-366791 dosage was based on previous reports of its antinociceptive effect [26].

2.5. *In vitro* screening of TRPV1 antagonists

2.5.1. [³H]-RTX binding assay

To determine if the Dcm fraction or its individual components were capable of binding the TRPV1 receptor, we performed the [³H]-RTX binding assay as previously described [27,28]. Briefly, mouse spinal cords were homogenised in buffer A (5 mM KCl, 5.8 mM NaCl, 2 mM MgCl₂, 0.75 mM CaCl₂, and 137 mM sucrose, pH 7.4) with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanosulfonic acid) and centrifuged for 10 minutes at 1 000 x *g* at 4 °C, and the supernatant was further centrifuged for 30 minutes at 35 000 x *g* at 4 °C. The resulting pellets were resuspended in buffer A and frozen until analysis. The binding mixture containing buffer A (plus 0.25 mg/ml of bovine serum albumin), membranes (0.5 mg/ml of protein), and [³H]-RTX (2 nM) in the presence or absence of the isolated compounds (10 μM), Dcm fraction (0.1 mg/ml) or vehicle (0.1% dimethyl sulfoxide, DMSO) had a final volume of 500 μl. The α-spinasterol was also tested in different concentrations (3-100 μM). For the measurement of nonspecific binding, 100 μM of nonradioactive RTX was included in different tubes. The reaction was initiated by incubating the tubes at 37 °C for 60 minutes and stopped by transferring the tubes to an ice bath and adding 100 μg of bovine α₁-acid glycoprotein to allow the detection of specific binding. Finally, [³H]-RTX in the bound and free membranes were separated by centrifugation for 30

minutes at 35 000 x *g* at 4°C. Radioactivity in the pellet was quantified by scintillation. The pellets were suspended in 1 ml scintillation fluid, and the radioactivity was counted in a scintillator apparatus. Specific binding was calculated as the difference of the total and nonspecific binding, and the results are reported as % of specific binding. Total protein was measured with Coomassie Blue dye, and bovine serum albumin (BSA) was used as a standard [29].

2.5.2. Calcium (Ca²⁺) Influx Assay

After evaluating the ability of selected compounds to bind the TRPV1 receptor, we estimated their capacity to affect capsaicin-induced calcium influx in synaptosomes [27]. Mouse spinal cords were homogenised in assay buffer (50 mM phosphate buffer and 320 mM sucrose, pH 7.4) and centrifuged for 5 minutes at 1 000 x *g* at 4°C. Then the supernatant was centrifuged for 20 minutes at 10 000 x *g* at 4°C. The final pellet was resuspended in Krebs-Ringer buffer (Ca²⁺-free) at a final protein concentration of 1 mg/ml and incubated with Fura 2-AM (10 µM) for 30 minutes at 37°C. After, the samples were centrifugated for 30 seconds at 12 000 x *g*, and the final pellet was resuspended in 1.5 ml Krebs-Ringer medium (Ca²⁺-free). To start the reaction, 15 µl of 0.1 M CaCl₂ was added to each sample, and after 10 minutes, different concentrations of α-spinasterol (10, 30, 100, and 300 µM) or vehicle (0.1% DMSO) were added, followed by the addition of 15 µl capsaicin (20 µM). Ca²⁺ influx was measured by monitoring the fluorescence at 382 nm (excitation) and 505 nm (emission) in a spectrofluorimeter (RF-5301 PC, Shimadzu). Background fluorescence was determined using an equivalent sample of synaptosomes that were not loaded with Fura 2-AM. Calibration was performed by

recording the maximum and minimum fluorescence values after adding 15 μ l of 10% (w/s) Triton-X 100 at the end of each experiment. The results are expressed as the percentage of the maximum response obtained with Triton-X 100 and then were compared with the influx of capsaicin [27].

2.6. Assessment of the antinociceptive action of α -spinasterol and the Dcm fraction in different pain models

2.6.1. Heat stimulus-induced nociception

We evaluated the possible antinociceptive effect of Dcm fraction and α -spinasterol in the paw reaction test to a heat stimulus [27,30]. The selective TRPV1 receptor antagonist SB-366791 was used as a positive control. A radiant light beam from a 60 W light bulb was directed onto the right hind paw (99% of the total intensity was used). The time between the onset of the stimulus and paw withdrawal was measured and used as an index of the thermal nociceptive threshold. The baseline latency was determined before the test, and a maximum latency of 30 seconds was imposed to prevent tissue damage. After the administration of vehicle (10 ml/kg, p.o.), Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.) or SB-366791 (3 μ mol/kg, p.o.), the paw reaction latency was measured at 0.5, 1, 2, and 4 hours.

2.6.2. Mechanical stimulus-induced nociception

The mechanical threshold was measured using von Frey filaments in the up-down paradigm as described previously [27,31]. First, mice were acclimated (1 hour)

in individual clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the plantar surface of the hind paws. Then, the paw was touched with a series of 7 von Frey hairs in logarithmic increments of force (0.02, 0.07, 0.16, 0.4, 1.4, 4.0, and 10.0 g). The von Frey hairs were applied perpendicular to the plantar surface with sufficient force to cause slight buckling against the paw, and held for approximately 2-4 seconds. Absence of paw lifting after 5 seconds led to the use of the next filament with increased weight, whereas paw lifting indicated a positive response and led to the use of the next weaker filament. Stimuli were presented at intervals of several seconds, allowing the mice to recover from the behavioural response to the previous stimulus. This paradigm continued until six measurements were collected or until four consecutive positive or negative responses occurred. The 50% mechanical paw withdraw threshold (PWT) response was then calculated from these scores as described previously [24, 28]. The PWT was measured before and 0.5, 1, 2, and 4 hours after the administration of vehicle (10 ml/kg, p.o.), Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.) or SB-366791 (3 μ mol/kg, p.o.).

2.6.3. Complete Freund's adjuvant (CFA)-induced inflammatory nociception

To induce the development of inflammatory heat and mechanical hyperalgesia, and oedema, mice were lightly anaesthetised with halothane, and CFA (20 μ l, 1 mg/ml of the heat killed *Mycobacterium tuberculosis*) was injected i.pl.) [27]. The assessment of the mechanical threshold, PWT to a heat stimulus and oedema were performed as described above. Basal values were observed before the injection of CFA, and the development of heat hyperalgesia, mechanical hyperalgesia, and oedema was observed 48 hours after CFA injection. The same measures were assessed again at 0.5, 1, 2, and 4 hours after the administration of vehicle (10 ml/kg,

p.o.), the Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.) or SB-366791 (3 μ mol/kg, p.o.).

2.7. Desensitisation of TRPV1 positive fibres

To further explore the role of TRPV1 positive fibres in the antinociceptive effect of α -spinasterol (0.3 μ mol/kg, p.o.), animals were submitted to a systemic desensitisation protocol with RTX as previously described [32]. Animals were anaesthetised using a mixture of ketamine (90 mg/kg) and xylazine (3 mg/kg), before systemic administration of RTX (50 μ g/kg, s.c.) or vehicle (0.5% ethanol, 0.5% Tween-80 in saline). The baseline latency was determined before RTX injection and seven days after the test; a maximum latency of 30 seconds was imposed to prevent tissue damage. Then, the animals were treated with vehicle (10 ml/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.), and SB-366791 (3 μ mol/kg, p.o.), and the latency was assessed at 0.5, 1, 2, and 4 hours after treatment. The time between the onset of the heat stimulus and the PWT was automatically measured and taken as an index of the thermal nociceptive threshold as described above.

To determine the effect of systemic RTX treatment with RTX on the TRPV1 receptor expression in sensory nerves, we performed a Western blot analysis on RTX-and vehicle-treated animals [33]. The sciatic nerves were quickly isolated and homogenised in lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM NaF, 10 μ g/ml aprotinin, 10 mM β -glycerophosphate, 1 mM phenylmethanesulphonyl fluoride, 1 mM DL-dithiothreitol (DTT) and 2 mM sodium orthovanadate. After centrifugation (3 000 x *g* for 30 minutes at 4°C), the supernatant was collected. The protein content was

determined using BSA as a standard [29]. Sciatic nerve protein (30 μ g) was mixed with loading buffer (200 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 2.75 mM β -mercaptoethanol and 0.04% bromophenol blue) and boiled for 10 minutes. The proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes, according to the manufacturer's instructions (Perkin Elmer, USA). The proteins on the PVDF membrane were stained with a solution of 0.5% actin and 1% glacial acetic acid in water, and this served as the loading control [34]. After staining, the membranes were dried, scanned and quantified. Membranes were processed using the SNAP i.d. system (Millipore, USA), blocked with 1% BSA and 0.05% Tween 20 in Tris-borate saline (TBS-T) and then incubated for 10 minutes with an anti-TRPV1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:150 in TBS-T. Blots were washed three times with TBS-T, followed by incubation with an alkaline phosphatase-coupled secondary antibody (1:3000, anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 minutes. Protein bands were visualised with a 5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium system (BCIP/NBT; Millipore). The membranes were dried, scanned and quantified with the Scion Image PC version of NIH Image.

2.8. Side Effect Evaluation

In order to evaluate possible non-specific muscle-relaxant or sedative effects of the extract, mice were evaluated for motor impairment. First, we examined spontaneous motor coordination at the open-field test [27]. The apparatus consisted of a wooden box measuring 40 \times 60 \times 50 cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed with all paws was counted in a

5 minute session. Mice were treated with Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.) or vehicle (10 ml/kg, p.o.) 1 hour before the test.

Forced motor activity was also evaluated using the rotarod test [27]. Briefly, 24 hours before the experiments, all animals were trained on the rotarod (3.7 cm in diameter, 8 rpm) until they could remain in the apparatus for 60 seconds without falling. On the day of the experiment, animals were injected with Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg, p.o.) or vehicle (10 ml/kg, p.o.) and subjected to the test 1 hour after administration. The number of falls and latency to first fall from the apparatus was recorded up to 240 seconds.

Severe hyperthermia is a previously reported side effect of TRPV1 receptor antagonists. To further evaluate whether Dcm fraction (100 mg/kg, p.o.) or α -spinasterol (0.3 μ mol/kg, p.o.) administration increased body temperature, the rectal temperature was determined before and 1 hour after drug administration as previously described [27]. The difference between the pre-injection and post-injection values was calculated ($\Delta^{\circ}\text{C}$). The TRPV1 antagonist AMG-9810 (30 μ mol /kg, p.o.) was used as a positive control [27].

2.9. Determination of the α -spinasterol pharmacokinetic profile

High performance liquid chromatography (HPLC-diode array detection) was performed with the Prominence Autosampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to the DGU 20A5 degasser with the CBM 20A integrator, DAD (photodiode) SPD-M20A UV-VIS detector and the LC solution 1.22 SP1 software.

Reverse phase chromatographic analyses were carried out under isocratic conditions using a C₁₈ column (4.6 mm x 250 mm) packed with 5 µm diameter particles. The mobile phase was methanol: 0.5% aqueous H₃PO₄ (88:12, v/v), following the method described by Zou and Chen (2008) with slight modifications [35]. To the study of pharmacokinetic profile of α-spinasterol (0.3 µmol/kg, p.o.), mouse plasma, brain, and spinal cord samples were collected 0.5, 1, 2, and 4 hours after drug administration in mice [36,37]. The samples of brain and spinal cord were homogenised in 100 mM Na-K phosphate buffer (pH 7.4), and the protein samples were precipitated with acetone. After centrifugation (10 000 x g, 10 minutes, 4°C), the supernatant was separated and used for analysis. The mean plasma-to-brain and plasma-to-spinal cord ratios were calculated from drug concentrations in each tissue 1 hour after drug administration. The chromatography peak was confirmed by comparing its retention time with that of a reference standard and by DAD spectra (190 to 400 nm). The flow rate was 0.8 ml/minute, the injection volume was 40 µl and the wavelength was 210 nm. All of the samples and the mobile phase were filtered through a 0.45 µm membrane filter (Millipore) and then degassed in an ultrasonic bath prior to use. A stock solution of spinasterol standard was prepared in the HPLC mobile phase at a concentration range of 0.018-0.606 nmol/ml. The calibration curve for spinasterol was $Y = 461482x + 4284.1$ ($r = 0.9994$). All chromatography operations were carried out in triplicate at ambient temperature.

2.10. Drugs and Reagents

Capsaicin was purchased from Sigma, dissolved in 90% ethanol and 10% Tween 80, and diluted to the appropriate concentration in PBS (137 mmol/l NaCl, 2.7

mmol/l KCl, and 10 mmol/l phosphate buffer). Methanol and phosphoric acid were purchased from Merck (Darmstadt, Germany). BSA, bovine α_1 -acid glycoprotein, CFA, Fura 2-AM, HEPES, Triton-X 100, resiniferatoxin, SB-366791, and AMG-9810 were purchased from Sigma Chemical Co. (St. Louis, USA). [3 H]-RTX was purchased from Perkin Elmer (Boston, USA) and diluted in assay buffer immediately before use.

2.11. Statistical Analysis

The results are presented as the mean \pm S.E.M., except for the ID₅₀ (the dose of compound that inhibits the nociceptive effect by 50% relative to the control value) and IC₅₀ (the concentration of compound that inhibited Ca²⁺ influx or specific binding by 50% relative to the control value) values, which are reported as geometric means accompanied by respective 95% confidence limits. The ID₅₀ and IC₅₀ values were determined by non-linear regression analyses with a sigmoid dose-response equation using the GraphPad Software 5.0 (GraphPad, USA). The percentages of maximal inhibition (I_{max}) are reported as the mean \pm S.E.M. of inhibition obtained in each individual experiment in relation to the control values (vehicle for the *in vivo* results, 100% specific binding for the [3 H]-RTX binding assay, 100% response obtained with Triton-X 100 for the Ca²⁺ influx assay). The significance level was set at $p < 0.05$. Data were analysed using Student's *t*-test, one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) post-hoc test or two-way ANOVA followed by Bonferroni post-hoc test.

3. RESULTS

3.1. *In vivo* screening: evaluation of antinociceptive and antioedematogenic effects of the hydroalcoholic extract, fractions and isolated compounds from *V. tweedieana*

The hydroalcoholic extract, fractions and isolated compounds from *V. tweedieana* had antinociceptive and antioedematogenic effects in the capsaicin test. Treatment with the HE extract (100 mg/kg) and the Dcm (100 mg/kg), Act (100 mg/kg), and But (100 mg/kg) fractions produced an antinociceptive response in the capsaicin test (administered 1 hour before the test) of 51 ± 4 , 67 ± 5 , 44 ± 4 , and $33\pm 9\%$ inhibition, respectively, compared with the vehicle-treated group (Figure 1A). Statistical analysis of the time to nociception after capsaicin injection (one-way ANOVA, followed by SNK test) revealed that the Dcm fraction produced a greater antinociceptive effect compared to the other fractions or the HE extract. In addition, only the Dcm fraction showed an antioedematogenic effect, with $66\pm 9\%$ inhibition (Figure 1B).

Among the compounds isolated from the Dcm fraction, α -spinasterol had the greatest effect on capsaicin-induced nociception 1 hour after treatment ($58\pm 4\%$ inhibition; Figure 2A). Stigmasterol also decreased capsaicin-induced nociception ($40\pm 7\%$ inhibition). However, it did not reduce the associated oedema, whereas α -spinasterol produced an antioedematogenic effect (inhibition of $66\pm 10\%$; Figure 2B). The mixture of α - and β -amyrin and lupeol had no effect on nociception or oedema at the dose tested.

Because α -spinasterol produced antinociceptive and antioedematogenic effects in the capsaicin test, this compound was used in further experiments. The dose-response curve showed that α -spinasterol had maximal antinociceptive and antioedematogenic effects of $64\pm 6\%$ and $70\pm 4\%$, respectively, at $1\ \mu\text{mol/kg}$ (p.o.), 1 hour after treatment. The ID_{50} values for the antinociceptive and antioedematogenic effects were $0.055\ \mu\text{mol/kg}$ (with a range of $0.039\text{--}0.077\ \mu\text{mol/kg}$) and $0.11\ \mu\text{mol/kg}$ (with a range of $0.074\text{--}0.16\ \mu\text{mol/kg}$), respectively (Figure 3A and 3B). The results presented in Figure 3C show that α -spinasterol ($0.3\ \mu\text{mol/kg}$, p.o.) markedly inhibited the capsaicin-induced nociceptive response from 0.5 to 4 hours after administration, with $60\pm 6\%$ inhibition (1 hour after treatment). The antioedematogenic effect of α -spinasterol occurred from 0.5 to 2 hours after treatment, with $62\pm 11\%$ inhibition 1 hour after treatment (Figure 3D).

SB-366791 ($3\ \mu\text{mol/kg}$, p.o., used as a positive control) produced an antinociceptive effect from 0.5 to 2 hours after treatment, with $68\pm 5\%$ inhibition 1 hour after treatment (Figure 4A). It also had an antioedematogenic effect during the capsaicin test ($74\pm 6\%$ inhibition 1 hour after treatment) from 0.5 to 1 hours after treatment (Figure 4B).

3.2. *In vitro* screening: characterisation of α -spinasterol as a TRPV1 antagonist

First, we assessed the possible binding of the isolated compounds and the Dcm fraction to the TRPV1 receptor in a binding assay using [^3H]-RTX, an ultrapotent TRPV1 receptor agonist. We observed that the Dcm fraction ($1\ \text{mg/ml}$) and α -spinasterol ($10\ \mu\text{M}$) displaced the specific binding of [^3H]-RTX from spinal cord membranes by $49\pm 2\%$ and $67\pm 11\%$, respectively (Figure 5A). On the other hand,

stigmasterol, the mixture of α - and β -amyrin and lupeol (10 μ M) isolated from the Dcm fraction did not alter the specific binding of [3 H]-RTX (Figure 5A). The IC₅₀ value for α -spinasterol was 1.4 μ M (with a range of 0.7-2.6 μ M), and the maximal inhibition was 64 \pm 3% for 10 μ M (Figure 5B). Because α -spinasterol was able to bind to TRPV1 receptor, we investigated whether this molecule could alter capsaicin-mediated Ca²⁺ influx. In the presence of α -spinasterol, there was a substantial reduction in the response to capsaicin, with a maximal inhibition of 62 \pm 10% and an IC₅₀ of 40 μ M (with a range of 23-68 μ M) (Figure 5C).

3.3. Antinociceptive effects of α -spinasterol and Dcm fraction assessed with different pain models

3.3.1. Evaluation of the Dcm fraction and α -spinasterol antinociceptive effects on noxious heat stimulus-induced nociception

Administration of α -spinasterol (0.3 μ mol/kg, p.o.) produced antinociception in the noxious heat induced-nociception test from 0.5 to 2 hours after administration of 26 \pm 6% inhibition (1 hour after administration). Treatment with the Dcm fraction (100 mg/kg, p.o) showed an antinociceptive effect from 1 to 2 hours of 19 \pm 2% inhibition (1 hour after administration), and SB-366791 (3 μ mol/kg, p.o., used as a positive control) produced an antinociceptive effect from 0.5 to 1 hours of 20 \pm 6% inhibition (1 hour after administration) (Figure 6A).

3.3.2. Evaluation of the Dcm fraction and α -spinasterol antinociceptive effects on mechanical stimulus-induced nociception

Administration of the Dcm fraction (100 mg/kg, p.o), α -spinasterol (0.3 μ mol/kg, p.o.), and SB-366791 (3 μ mol/kg, p.o., used as a positive control) did not affect the mechanical threshold of uninjured animals in any of the observed time points (Figure 6B).

3.3.3. Participation of TRPV1 positive fibres in the antinociceptive effect of α -spinasterol on noxious heat stimulus-induced nociception

Mice were pre-treated with RTX to evaluate the role of positive TRPV1 fibres in the antinociceptive effect of α -spinasterol. Systemic pre-treatment with RTX significantly reduced the immunoreactivity of the TRPV1 protein in the sciatic nerve 7 days after injection ($72\pm 10\%$ compared to the control group), confirming a reduction in TRPV1-positive sensory fibres (Fig. 7A). As expected, RTX pre-treatment increased the latency to paw withdrawal after noxious heat stimuli (Figure 7B). The antinociceptive effects of α -spinasterol (0.3 μ mol/kg, p.o.), and SB-366791 (3 μ mol/kg, p.o.) were abolished by RTX pre-treatment, confirming the participation of TRPV1-positive fibres in the antinociceptive effect (Figure 7B).

3.3.4. Antihyperalgesic and antioedematogenic effects of the Dcm fraction and α -spinasterol in the CFA model

Mice injected with CFA developed mechanical hyperalgesia characterised by a significant reduction in PWT when von Frey filaments were applied to the injected

paw (PWT diminished from 2.60 ± 0.44 g at baseline to 0.15 ± 0.04 g 48 hours after CFA administration; $p < 0.001$, Student's *t*-test). CFA injection also caused heat hyperalgesia (latency diminished from 12.33 ± 1.02 seconds at baseline to 8.72 ± 0.90 seconds 48 hours after CFA administration; $p < 0.05$, Student's *t*-test) and oedema (paw thickness increased from 2.91 ± 0.07 mm at baseline to 4.01 ± 0.17 mm 48 hours after CFA administration; $p < 0.001$, Student's *t*-test).

Treatment with the Dcm fraction (100 mg/kg, p.o) and α -spinasterol (0.3 μ mol/kg, p.o.) produced large reductions in CFA-induced mechanical hyperalgesia from 0.5 to 2 h after treatment, with $82 \pm 14\%$ and $96 \pm 15\%$ inhibition, respectively (1 hour after treatment; Figure 8A). The antihyperalgesic effect of α -spinasterol was also observed for CFA-induced heat hyperalgesia from 0.5 to 2 hours after treatment (100% inhibition observed at 1 hour after treatment); the Dcm fraction also had an antinociceptive effect from 0.5 to 1 hours in the same test (100% inhibition at 1 hour after treatment; Figure 8B). The Dcm fraction and α -spinasterol also reduced CFA-induced oedema; this effect was observed from 0.5 to 2 hours after Dcm fraction treatment and from 0.5 to 4 hours after α -spinasterol treatment, with $38 \pm 10\%$ and $35 \pm 6\%$ inhibition observed 2 hours after treatment, respectively (Figure 8C). However, SB-366791 (3 μ mol/kg, p.o.) only reduced CFA-induced heat hyperalgesia from 0.5 to 1 hour (100% inhibition at 1 hour after treatment), without affecting mechanical hyperalgesia or oedema (Figure 8A and 8C).

3.4. Side effect evaluation

We also evaluated the possible actions of Dcm fraction (100 mg/kg, p.o) and α -spinasterol (0.3 μ mol/kg, p.o.) on motor performance and body temperature.

Neither treatment altered forced or spontaneous locomotion assessed by rotarod and open-field tests (Table 1). Moreover, the Dcm fraction (100 mg/kg, p.o) and α -spinasterol (0.3 μ mol/kg, p.o.) did not change body temperature 1 hour after drug administration ($\Delta^{\circ}\text{C}$ of 0.24 ± 0.21 , 0.34 ± 0.16 , -0.18 ± 0.25 for vehicle, the Dcm fraction and α -spinasterol treatment groups; $p>0.05$, one-way ANOVA followed by SNK test). Conversely, AMG-9810 (30 μ mol /kg, p.o.) induced a significant increase in rectal temperature ($\Delta^{\circ}\text{C}$ of 1.03 ± 0.05 ; $p<0.05$, Student's *t*-test).

3.5. Pharmacokinetic characterisation

The plasma concentration of α -spinasterol reached a maximal concentration (C_{max}) of 0.25 ± 0.003 nmol/ml 1 hour after oral administration of 0.3 μ mol/kg (Figure 11). We were able to detect α -spinasterol in plasma from 0.5 up to 4 hours after administration. We also detected α -spinasterol in the brain and spinal cord with C_{max} of 1.63 ± 0.12 and 5.8 ± 0.88 nmol/g, respectively, 1 hour after oral administration (Figure 9). The temporal profiles of drug levels in the brain and spinal cord were almost identical to that in plasma. The plasma-to-brain and plasma-to-spinal cord ratios were 6.5 and 23.4 for the C_{max} at 1 hour after treatment, respectively.

4. DISCUSSION

The TRPV1 receptor is a relevant target for the development of novel analgesic drugs and has been studied in a wide range of diseases, including inflammatory pain, migraine, cancer and osteoarthritic-related pain [2,4,6,7]. In this study, we performed *in vivo* and *in vitro* screens to identify novel TRPV1 antagonists derived from a medicinal plant. We observed that the hydroalcoholic extract and fractions of *V. tweediana* have antinociceptive effects in the capsaicin test. Moreover, the Dcm fraction had an antioedematogenic effect in the same model. Among the compounds isolated from the Dcm fraction, α -spinasterol and stigmasterol showed antinociceptive effects during the capsaicin test. However, only α -spinasterol had an antioedematogenic effect in the dose tested. α -spinasterol also diminished capsaicin-induced Ca^{2+} influx in synaptosomes and displaced [^3H]-RTX binding in spinal cord membranes. Also, α -spinasterol had antinociceptive effects in heat and inflammatory pain models without causing motor alteration or hypothermia.

The *in vivo* capsaicin test was used to screen for antinociceptive and antioedematogenic effects. This is a particularly relevant model to screen new TRPV1 compounds because capsaicin is a selective TRPV1 agonist capable of inducing a acute nociception in experimental animals and pain in humans [19,20,21]. As expected for SB-366791, TRPV1 receptor antagonism reduced both nociception and oedema produced by i.pl. capsaicin injection. On the other hand, we previously demonstrated that non-steroidal anti-inflammatory drugs inhibited nociception but not oedema induced by capsaicin [38]. Thus, we selected a fraction of *V. tweediana* and its isolated compounds that could reduce both capsaicin-induced effects. Only the Dcm fraction and α -spinasterol reduced oedema formation and spontaneous nociception *in vivo*. Our results are in accordance with previous findings that

demonstrated that α -spinasterol exerted antinociceptive effects in some pain models [39,40,41]. However, the mechanisms underlying the antinociceptive effect of α -spinasterol remain to be elucidated.

Next, we confirmed our *in vivo* findings with *in vitro* experiments to assess the ability of the Dcm fraction and α -spinasterol to bind and antagonise the TRPV1 receptor. Of the compounds tested, only these two displaced the specific binding of [³H]-RTX from spinal cord membranes. Our results clearly indicate that α -spinasterol is a TRPV1 ligand from *V. tweedieana*. We then used a capsaicin-mediated Ca²⁺ influx assay to determine whether α -spinasterol binding led to functional modulation of the TRPV1 receptor. This compound inhibited Ca²⁺ influx in the same potency range as observed in the binding assay. This demonstrates that α -spinasterol acts as a TRPV1 receptor antagonist. The fact that the steroid α -spinasterol binds the TRPV1 receptor is not unexpected because other steroid compounds, such as pregnenolone sulfate and dehydroepiandrosterone, also act as TRPV1 antagonists [42,43]. Moreover, the low micromolar concentration of α -spinasterol necessary to bind and inhibit TRPV1 receptors can be reached with oral administration because it is well distributed in the nervous system, where TRPV1 is expressed.

TRPV1 is a polymodal receptor expressed mainly in sensory neurons, and it is important for the perception of acute noxious information [8]. Several reports indicated that mice lacking TRPV1 have diminished responses to acute thermal but not mechanical stimuli [1,44]. Accordingly, we observed that α -spinasterol inhibited nociceptive behaviour induced by acute noxious heat, but it did not change the mechanical pain threshold of naive animals. In fact, a recent study showed that TRPV1 receptors are mainly expressed in sensory fibres that transduce heat pain and express μ -opioid receptor [45]. However, α -spinasterol action in heat-evoked

nociception is not opioid receptor-dependent because its antinociceptive action was not reversed by naloxone (data not shown). To confirm that the effect of α -spinasterol is dependent on TRPV1 *in vivo*, we depleted TRPV1 positive fibres using a systemic RTX treatment [32]. As expected, RTX-injected animals have reduced TRPV1 receptor expression and an increase in response latency to nociceptive heat stimuli. Moreover, the antinociceptive effect of both α -spinasterol and the selective TRPV1 antagonist SB-366791 was abolished in mice pre-treated with RTX. Collectively, these results suggest that the TRPV1 receptor is essential for the antinociceptive action of α -spinasterol *in vivo*.

The importance of the TRPV1 receptor in the pain related to inflammatory process is recognised [5]. In preclinical trials, TRPV1 antagonists reduced inflammatory related mechanical and thermal hyperalgesia, while animals lacking the TRPV1 protein are deficient in pain inflammatory signals and are unable to develop thermal hyperalgesia and pain inflammatory signals [46,47,48,49]. In addition, pain related to inflammatory conditions is associated with increased TRPV1 receptor expression [50,51,52]. Our study demonstrates that α -spinasterol may actively inhibit the oedema, mechanical, and heat hyperalgesia observed in the CFA-induced model of inflammatory pain. The fact that α -spinasterol did not alter mechanical nociception in naive animals but reduced mechanical hyperalgesia in inflamed mice may be explained by the increase of TRPV1 receptor expression in mechanically activated myelinated A-fibres after CFA injection [53,54]. The antinociceptive effect of α -spinasterol in CFA-induced mechanical hyperalgesia could also be related to the capacity of this molecule to access the central nervous system (CNS). This is supported by evidence showing that TRPV1 receptor antagonists that access the CNS have better antihyperalgesic effects than peripheral antagonists, mainly against

mechanical hyperalgesia [55]. Indeed, α -spinasterol is well distributed in the CNS with plasma-to-brain and plasma-to-spinal cord ratios of 6.5 and 23.4, respectively.

Many drugs elicit false-positive responses in nociception tests because they cause sedation, motor activity impairment, or body temperature alteration [56]. The induction of severe hyperthermia by some TRPV1 antagonists, such as AMG-517 (or its analogue of AMG-9810), has hampered the development of these drugs as analgesics [12]. Neither the Dcm fraction nor α -spinasterol induced locomotor alteration or hyperthermia in mice. This indicates that α -spinasterol might have better features than other known TRPV1 antagonists. Finally, we found that orally administered α -spinasterol had good absorption with the peak concentrations in the plasma, brain and spinal cord comparable to the time of maximal antinociception. These findings are relevant because oral administration is more clinically viable; it is low-cost, safe, and easy to administer [57].

In conclusion, α -spinasterol showed antinociceptive and antioedematogenic effects in distinct pain models, particularly hyperalgesia, a common complaint of chronic pain patients [58]. The plant steroid α -spinasterol has been characterised as a novel, safe, and orally effective TRPV1 receptor antagonist, and it may be an attractive future target for pain therapy.

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6. REFERENCES

- [1] Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816-24.
- [2] Jara-Oseguera A, Simon SA, Rosenbaum T. TRPV1: on the road to pain relief. *Curr Mol Pharmacol* 2008;1:255-69.
- [3] Szallasi A, Cortright DN, Blum CA, Eid SR. The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept. *Nat Rev Drug Discov* 2007;6:357-72.
- [4] Schumacher MA. Transient receptor potential channels in pain and inflammation: therapeutic opportunities. *Pain Pract* 2010;10:185-200.
- [5] Patapoutian A, Tate S, Woolf CJ. Transient receptor potential channels: targeting pain at the source. *Nat Rev Drug Discov* 2009;8:55-68.
- [6] Adcock JJ. TRPV1 receptors in sensitisation of cough and pain reflexes. *Pulm Pharmacol Ther* 2009;22:65-70.
- [7] Wong GY, Gavva NR. Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks. *Brain Res Rev* 2009;60:267-77.
- [8] Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. *Biochim Biophys Acta* 2007;1772:989-1003.
- [9] Rami HK, Thompson M, Stemp G, Fell S, Jerman JC, Stevens AJ, et al. Discovery of SB-705498: a potent, selective and orally bioavailable TRPV1 antagonist suitable for clinical development. *Bioorg Med Chem Lett* 2006;16:3287-91.

- [10] Lehto SG, Tamir R, Deng H, Klionsky L, Kuang R, Le A, et al. Antihyperalgesic effects of (R,E)-N-(2-hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluoromethyl)phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. *J Pharmacol Exp Ther* 2008;326:218-29.
- [11] Chizh BA, O'Donnell MB, Napolitano A, Wang J, Brooke AC, Aylott MC, et al. The effects of the TRPV1 antagonist SB-705498 on TRPV1 receptor-mediated activity and inflammatory hyperalgesia in humans. *Pain* 2007;132:132-41.
- [12] Gavva NR, Treanor JJ, Garami A, Fang L, Surapaneni S, Akrami A, et al. Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* 2008;136:202-10.
- [13] Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nat Rev Drug Discov* 2005;4:206-20.
- [14] Calixto JB, Beirith A, Ferreira J, Santos AR, Filho VC, Yunes RA. Naturally occurring antinociceptive substances from plants. *Phytother Res* 2000;14:401-18.
- [15] Adcock JJ. TRPV1 receptors in sensitisation of cough and pain reflexes. *Pulm Pharmacol Ther* 2009;22:65-70.
- [16] Corson TW, Crews CM. Molecular understanding and modern application of traditional medicines: triumphs and trials. *Cell* 2007;130:769-74.
- [17] Zanon RB, Pereira DF, Boschetti, TK, Santos M, Athayde ML. Phytoconstituents isolated from dichloromethane fraction of *Vernonia tweediana* Baker leaves. *Rev Bras Farmacogn* 2008;18:226-29.

- [18] Iwalewa EO, Iwalewa OJ, Adeboye JO. Analgesic, antipyretic, anti-inflammatory effects of methanol, chloroform and ether extracts of *Vernonia cinerea* less leaf. *J Ethnopharmacol* 2003;86:229-34.
- [19] Njan AA, Adzu B, Agaba AG, Byarugaba D, Díaz-Llera S, Bangsberg DR. The analgesic and antiplasmodial activities and toxicology of *Vernonia amygdalina*. *J Med Food* 2008;11:574-81.
- [20] Risso WE, Scarminio IS, Moreira EG. Antinociceptive and acute toxicity evaluation of *Vernonia condensata* Baker leaves extracted with different solvents and their mixtures. *Indian J Exp Biol* 2010;48:811-6.
- [21] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109-10.
- [22] Sakurada T, Katsumata K, Tan-No K, Sakurada S, Kisara K. The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. *Neuropharmacology* 1992;31:1279-1285.
- [23] Walker K, Fox AJ, Urban LA. Animal models for pain research. *Mol Med Today* 1999;5:319-21.
- [24] Simone DA, Baumann TK, Lamotte RH. Dose-dependent pain and mechanical hyperalgesia in human after intradermal injection of capsaicin. *Pain* 1989;38:99-107.
- [25] Milano J, Rossato MF, Oliveira SM, Drewes C, Machado P, Beck P, et al. Antinociceptive action of 4-methyl-5-trifluoromethyl-5-hydroxy-4, 5-dihydro-1H-pyrazole methyl ester in models of inflammatory pain in mice. *Life Sci* 2008b;83:739-46.

- [26] Niiyama Y, Kawamata T, Yamamoto J, Furuse S, Namiki A. SB366791, a TRPV1 antagonist, potentiates analgesic effects of systemic morphine in a murine model of bone cancer pain. *Br J Anaesth* 2009;102:251-58.
- [27] Rossato MF, Trevisan G, Walker CI, Klafke JZ, de Oliveira AP, Villarinho JG, et al. Eriodictyol: a flavonoid antagonist of the TRPV1 receptor with antioxidant activity. *Biochem Pharmacol* 2011;81:544-51.
- [28] Szallasi A, Biro T, Modarres S, Garlaschelli L, Petersen M, Klusch A, et al. Dialdehyde sesquiterpenes and other terpenoids as vanilloids. *Eur J Pharmacol* 1998;356:81–9.
- [29] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;72:248–54.
- [30] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32: 77–88.
- [31] Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 1980;20:441–62.
- [32] Hsieh YL, Chiang H, Tseng TJ, Hsieh ST. Enhancement of cutaneous nerve regeneration by 4-methylcatechol in resiniferatoxin-induced neuropathy. *J Neuropathol Exp* 2008;67:93-104.
- [33] Andre E, Ferreira J, Malheiros A, Yunes RA, Calixto JB. Evidence for the involvement of vanilloid receptor in the antinociception produced by the dialdehydes unsaturated sesquiterpenes polygodial and drimaniol in rats. *Neuropharmacology* 2004;46:590–7.

- [34] Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 2010;401:318-20.
- [35] Zou S, Chen W. Determination of oleanolic and ursolic acids in different parts of *Prilla frutescens* by High-Performance Liquid Chromatography. *Journal of the Brazilian Chemical Society* 2008;19:1429-32.
- [36] Watabiki T, Kiso T, Tsukamoto M, Aoki T, Matsuoka N. Intrathecal Administration of AS1928370, a Transient Receptor Potential Vanilloid Antagonist, Attenuates Mechanical Allodynia in a Mouse Model of Neuropathic Pain. *Biol Pharm Bull* 2011;34:1105-8.
- [37] Zhao YY, Qin XY, Zhang Y, Lin RC, Sun WJ, Li XY. Quantitative HPLC method and pharmacokinetic studies of ergosta-4,6,8(14),22-tetraen-3-one, a natural product with diuretic activity from *Polyporus umbellatus*. *Biomed Chromatogr* 2010;24:1120-4.
- [38] Oliveira SM, Gewehr C, Dalmolin GD, Cechinel CA, Wentz A, Lourega RV, Sehnem RC, Zanatta N, Martins MA, Rubin MA, Bonacorso HG, Ferreira J. Antinociceptive effect of a novel tosylpyrazole compound in mice. *Basic Clin Pharmacol Toxicol* 2009;104:122-9.
- [39] Freitas CS, Baggio CH, Dos Santos AC, Mayer B, Twardowschy A, Luiz AP, et al. Antinociceptive properties of the hydroalcoholic extract, fractions and compounds obtained from the aerial parts of *Baccharis illinita* DC in mice. *Basic Clin Pharmacol Toxicol* 2009;104:285-92.
- [40] Meotti FC, Ardenghi JV, Pretto JB, Souza MM, d' Avila Moura J, Junior AC, et al. Antinociceptive properties of coumarins, steroid and dihydrostyryl-2-

- pyrones from *Polygala sabulosa* (Polygalaceae) in mice. *J Pharm Pharmacol* 2006;58:107-12.
- [41] Ribas CM, Meotti FC, Nascimento FP, Jacques AV, Dafre AL, Rodrigues AL, et al. Antinociceptive effect of the *Polygala sabulosa* hydroalcoholic extract in mice: evidence for the involvement of glutamatergic receptors and cytokine pathways. *Basic Clin Pharmacol Toxicol* 2008;103:43-7.
- [42] Chen SC, Chang TJ, Wu FS. Competitive inhibition of the capsaicin receptor-mediated current by dehydroepiandrosterone in rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 2004;311:529-36.
- [43] Chen SC, Wu FS. Mechanism underlying inhibition of the capsaicin receptor-mediated current by pregnenolone sulfate in rat dorsal root ganglion neurons. *Brain Res.* 2004;1027:196-200.
- [44] Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, et al. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 2000;405:183-7.
- [45] Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, et al. Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 2009;137:1148-59.
- [46] Walker KM, Urban L, Medhurst SJ, Patel S, Panesar M, Fox AJ, et al. The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 2003;304:56–62.
- [47] Gavva NR, Tamir R, Qu Y, Klionsky L, Zhang TJ, Immke D, et al. AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-

- yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. *J Pharmacol Exp Ther* 2005;313:474-84.
- [48] Barton NJ, McQueen DS, Thomson D, Gauldie SD, Wilson AW, Salter DM, Chessell IP. Attenuation of experimental arthritis in TRPV1R knockout mice. *Exp Mol Pathol* 2006;81:166-170.
- [49] Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM, et al. A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. *J Pharmacol Exp Ther* 2005;314:410–21.
- [50] Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, et al. NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. *Eur J Neurosci* 2004;20:2303–10.
- [51] Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heatgated ion channels. *Embo J* 2005;24:4211–23.
- [52] Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 2002;36:57–68.
- [53] Amaya F, Oh-hashii K, Naruse Y, Iijima N, Ueda M, Shimosato G, et al. Local inflammation increases vanilloid receptor 1 expression within distinct subgroups of DRG neurons. *Brain Res* 2003;963:190-6.
- [54] McGaraughty S, Chu KL, Brown BS, Zhu CZ, Zhong C, Joshi SK, et al. Contributions of central and peripheral TRPV1 receptors to mechanically

evoked and spontaneous firing of spinal neurons in inflamed rats. *J Neurophysiol* 2008;100:3158-66.

[55] Cui M, Honore P, Zhong C, Gauvin D, Mikusa J, Hernandez G, et al. TRPV1 receptors in the CNS play a key role in broad-spectrum analgesia of TRPV1 antagonists. *J Neurosci* 2006;26:9385-93.

[56] Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. *J Pharmacol Exp Ther* 2006;319:507-14.

[57] Buxton ILO. Pharmacokinetics and pharmacodynamics: the dynamics of drug absorption, distribution, action and elimination. In: Brunton L, Lazo JS and Parker KLP, editors. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, The McGraw-Hill: 2006, pp. 1-40.

[58] Schaible HG, Richter F, Ebersberger A, Boettger MK, Vanegas H, Natura G, et al. Joint pain. *Exp Brain Res* 2009;196:153-62.

7. STATEMENT OF CONFLICTS OF INTEREST

None.

8. FIGURE AND LEGENDS

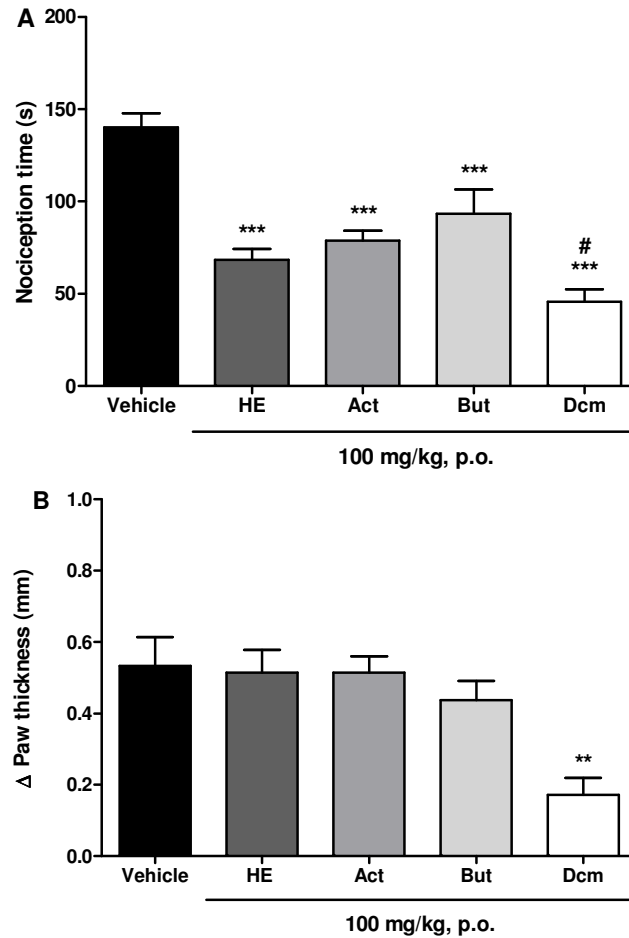


Figure 1. Evaluation of (A) antinociceptive and (B) antioedematogenic effects of the hydroalcoholic (HE), dichloromethane (Dcm), ethyl acetate (Act), or butanolic (But) fractions during the capsaicin test in mice, 1 hour after administration of drugs. Nociception was observed for 5 minutes after i.pl. capsaicin injection (1 nmol/paw), and oedema was observed 15 minutes after capsaicin injection. Data are reported as the mean \pm S.E.M. (n=7-9). ** p <0.01, *** p <0.001, when compared with vehicle group. # p <0.05, when compared with Dcm fraction group (one-way ANOVA followed by SNK post-hoc test).

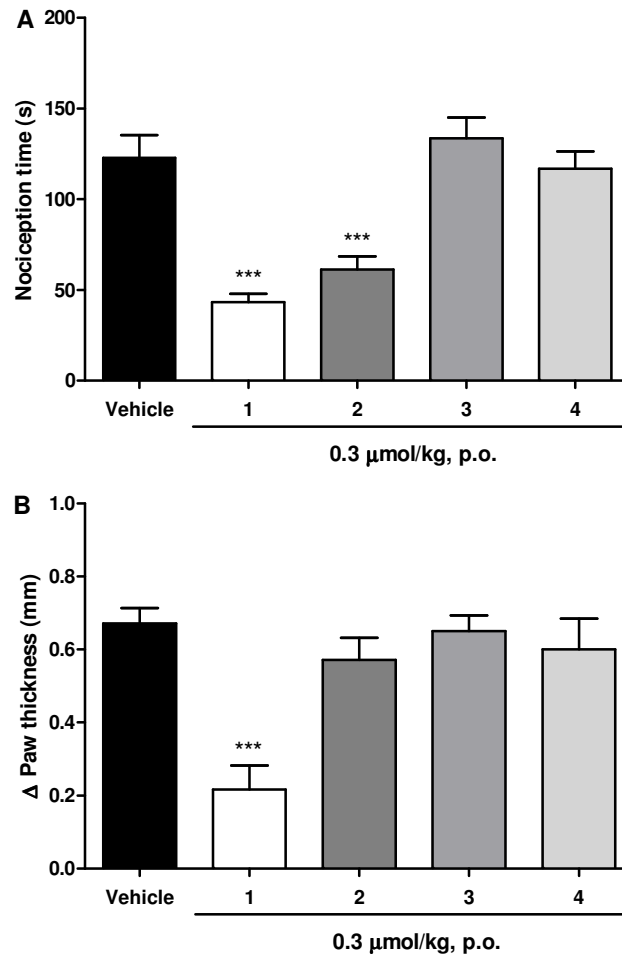


Figure 2. Evaluation of (A) antinociceptive and (B) antioedematogenic effects of α -spinasterol (1), stigmasterol (2), mixture of α - and β -amyrin (3), and lupeol (4) ($0.3 \mu\text{mol/kg}$; p.o.) during the capsaicin test in mice ($n=6-7$), 1 hour after administration of drugs. Nociception was observed for 5 minutes after i.pl. capsaicin injection (1 nmol/paw), and oedema was observed 15 minutes after capsaicin injection. Data are reported as the mean \pm S.E.M. *** $p < 0.001$, when compared with vehicle group (one-way ANOVA followed by SNK post-hoc test).

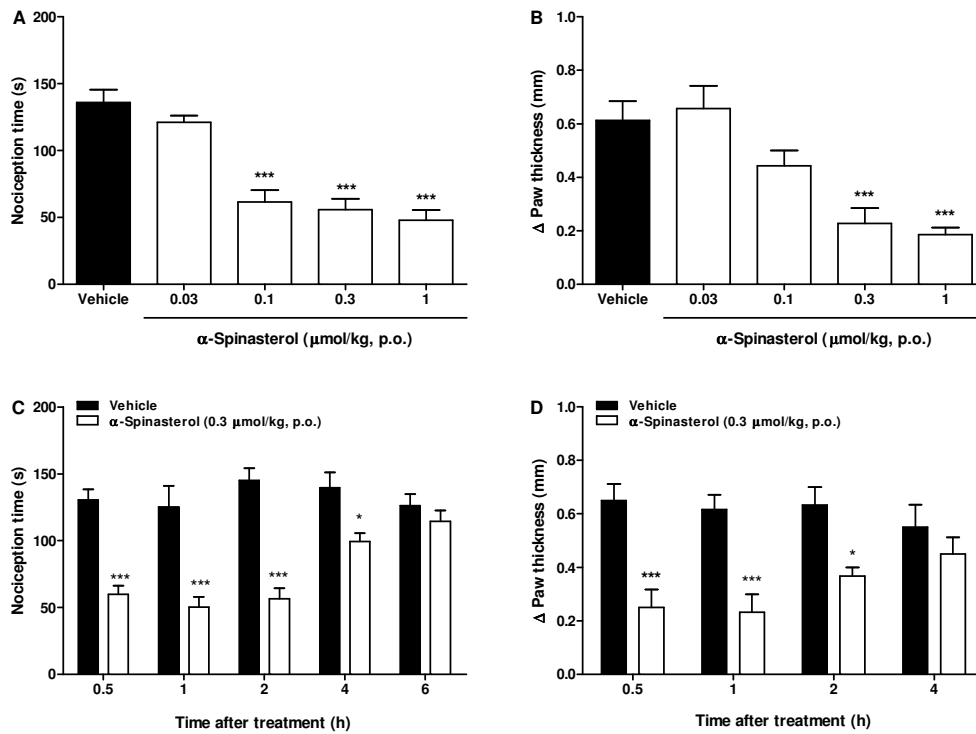


Figure 3. (A) Dose-response curve of the antinociceptive effect of α -spinasterol administered 1 hour before the capsaicin test (n=7-8). (B) Evaluation of the antioedematogenic effect of different doses of α -spinasterol administered 1 hour before the capsaicin test (n=7-8). (C) Time-course of the antinociceptive effect of α -spinasterol (0.3 μ mol/kg; p.o.) during the capsaicin test in mice (n=6). (D) Time-course of evaluation of the antioedematogenic effect of α -spinasterol (0.3 μ mol/kg; p.o.) in the capsaicin test (n=6). Nociception was observed for 5 minutes after i.pl. capsaicin injection (1 nmol/paw), and oedema was observed 15 minutes after capsaicin injection. Data are expressed as the mean \pm S.E.M. * p <0.05, *** p <0.001, when compared with vehicle group (one-way ANOVA followed by SNK post-hoc test or two-way ANOVA followed by Bonferroni post-hoc test).

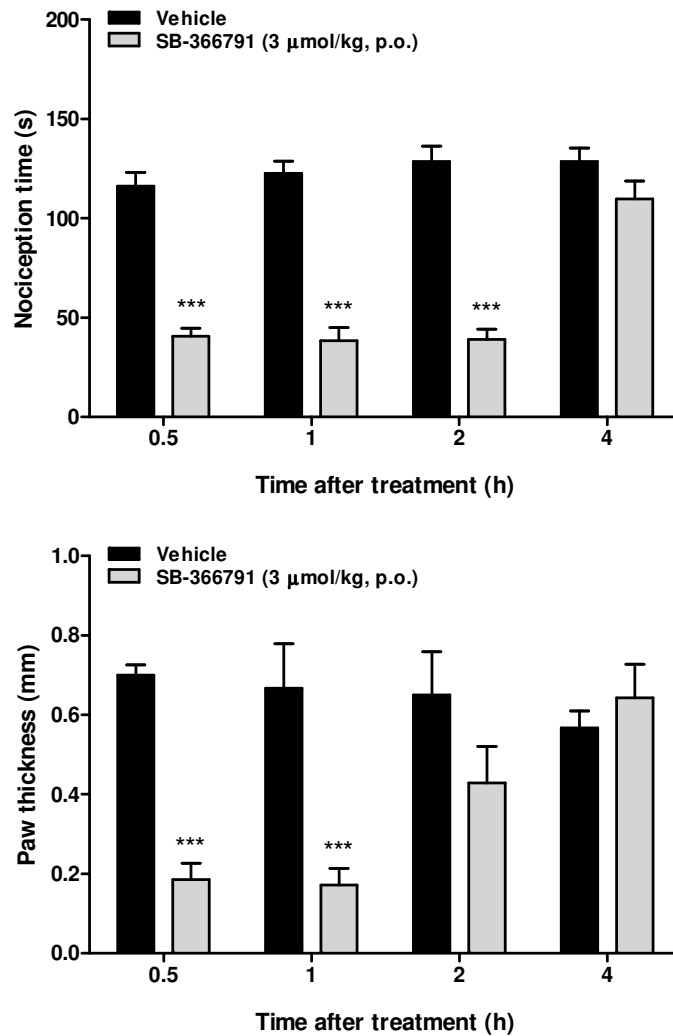


Figure 4. (A) Time-course of the antinociceptive effect of SB-366791 (3 μmol/kg; p.o.) during the capsaicin test in mice (n=6-7). (B) Evaluation of the antioedematogenic effect of SB-366791 (3 μmol/kg; p.o.) at different time points during the capsaicin test (n=6-7). Nociception was observed for 5 minutes after i.pl. capsaicin injection (1 nmol/paw), and oedema was observed 15 minutes after capsaicin injection. Data are expressed as the mean±S.E.M. *** $p < 0.001$, compared with vehicle (two-way ANOVA followed by Bonferroni post-hoc test).

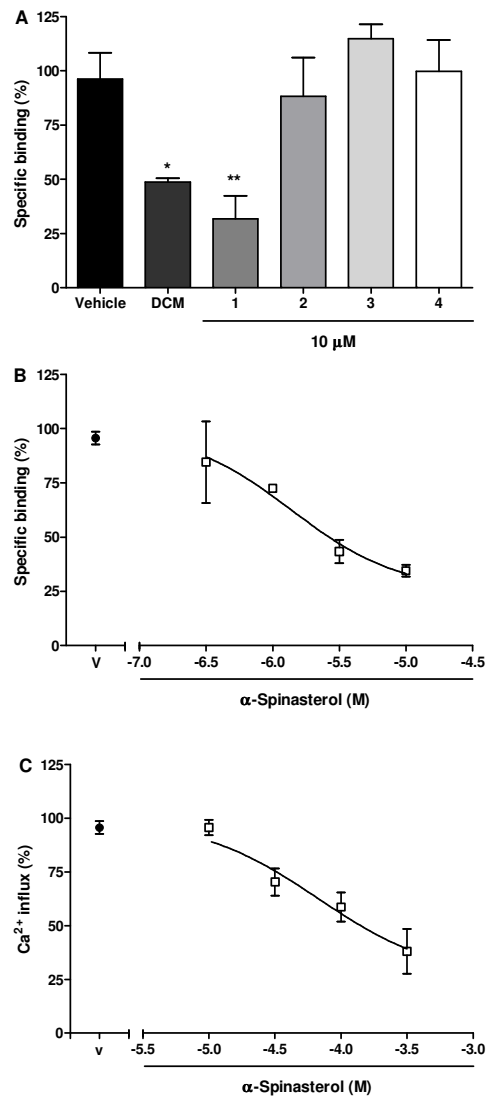


Figure 5. (A) Evaluation of the abilities of the Dcm fraction (Dcm), α-spinasterol (1), stigmasterol (2), mixture of α- and β-amyrin (3), and lupeol (4) isolated from the Dcm fraction to displace [³H]-RTX binding (n=3-5). (B) Affinity of different concentrations of α-spinasterol to displace [³H]-RTX binding, the IC₅₀ value of α-spinasterol was 1.4 μM (with a range of 0.7-2.6 μM) (n=3). (C) Inhibitory effect of α-spinasterol of Ca²⁺ influx elicited by capsaicin (20 μM) in mouse spinal cord synaptosomes; the IC₅₀ value of α-spinasterol was 40 μM (with a range of 23-68 μM) (n=3-5). Data are expressed as the mean+S.E.M, *p<0.05, **p<0.01, compared with vehicle (one-way ANOVA followed by SNK post-hoc test). The IC₅₀ value was calculated using non-linear regression.

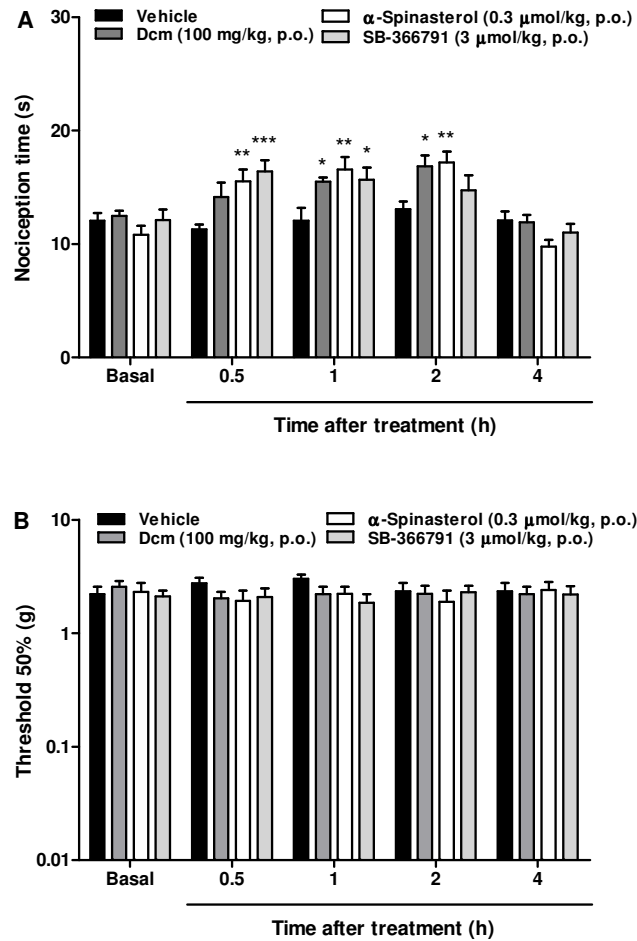


Figure 6. (A) Assessment of the antinociceptive effect of the Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg; p.o.), and SB-366791 (3 μ mol/kg; p.o.) during the noxious heat stimulus-induced nociception test in mice ($n=7-8$) several time points after administration of the drugs. Nociception time was observed as the paw withdrawal latency to a noxious heat stimulus. Data are reported as the mean \pm S.E.M. (B) Effect of the Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg; p.o.), and SB-366791 (3 μ mol/kg; p.o.) on nociception induced by mechanical stimulus ($n=6$). Data are expressed as the mean \pm S.E.M. of painful hypersensitivity (determined with von Frey filaments, expressed as PWT in g). * $p<0.05$, ** $P<0.01$, *** $P<0.001$, compared with basal values (two-way ANOVA followed by Bonferroni post-hoc test).

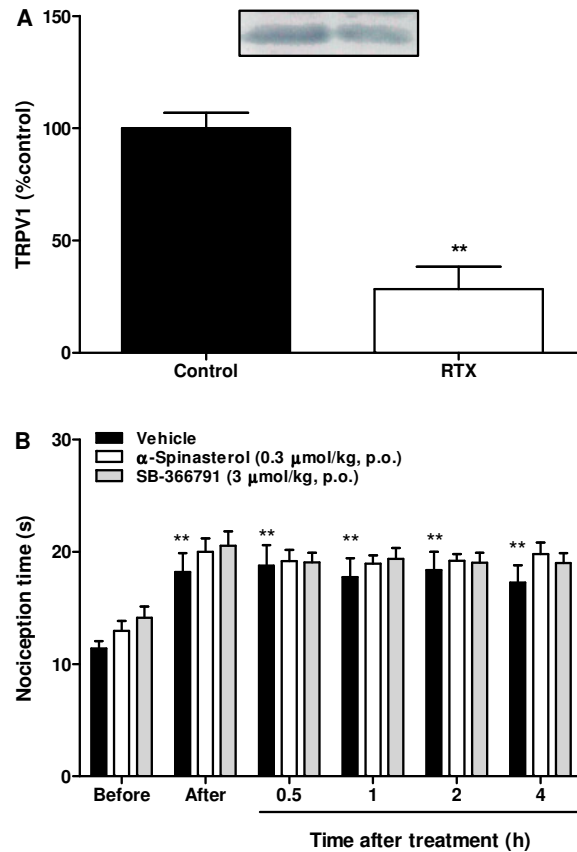


Figure 7. Effect of systemic RTX treatment (50 μ g/kg, s.c.) on the antinociceptive effect of α -spinasterol. (A) Western blot (inset) showing TRPV1 immunoreactivity in sciatic nerves after treatment with RTX or vehicle (n=3). Western blot results are expressed as arbitrary density units. Asterisks denote the significance levels. (B) Effect of RTX desensitisation (50 μ g/kg, s.c.) on α -spinasterol (0.3 μ mol/kg; p.o.) and SB-366791 (3 μ mol/kg; p.o.) antinociceptive effects in noxious heat-induced nociception in mice (n=6-7). The basal latency was assessed before and 7 days after RTX treatment. Data are expressed as the mean \pm S.E.M. ** p <0.001, compared with vehicle (A, Student's t -test) or compared with basal values before RTX treatment (two-way ANOVA followed by Bonferroni post-hoc test).

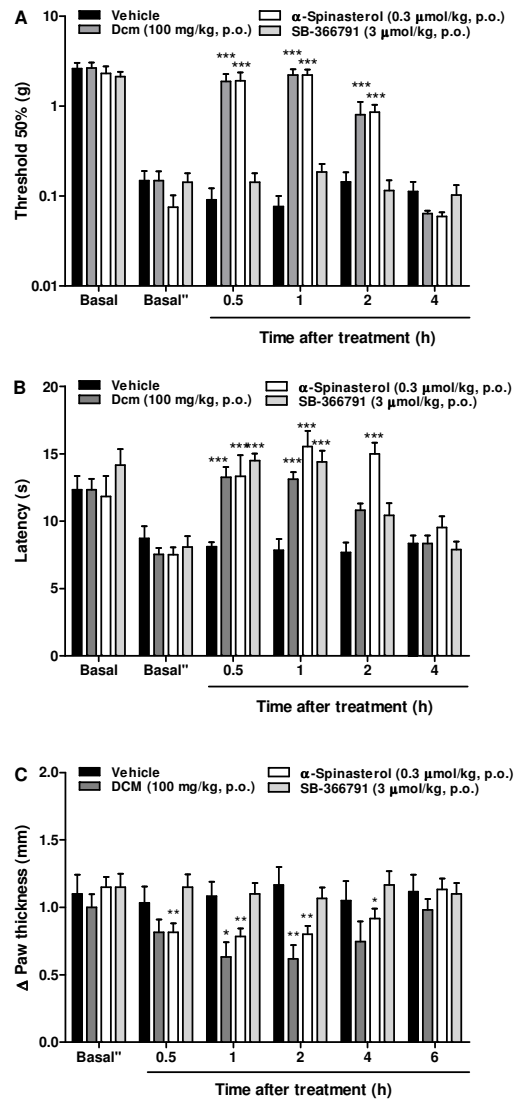


Figure 8. Effect of the Dcm fraction, α -spinasterol, and SB-366791 on CFA-induced inflammatory nociception. The (A) mechanical hyperalgesia, (B) heat hyperalgesia, and (C) oedema were observed 48 hours after the injection of CFA (Basal''). The antinociceptive and antioedematogenic effects were evaluated at different time points (0.5, 1, 2, 4, and 6 hours) after treatment with the Dcm fraction (100 mg/kg, p.o.), α -spinasterol (0.3 μ mol/kg; p.o.) and SB-366791 (3 μ mol/kg; p.o.). Basal' values were observed 48 hours before Data are expressed as the mean \pm S.E.M. of mechanical painful hypersensitivity (determined with von Frey filaments, expressed as PWT in g), nociception time expressed as PWT to a noxious heat stimulus, or variation in paw thickness. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with basal' or basal'' values (two-way ANOVA followed by Bonferroni post-hoc test).

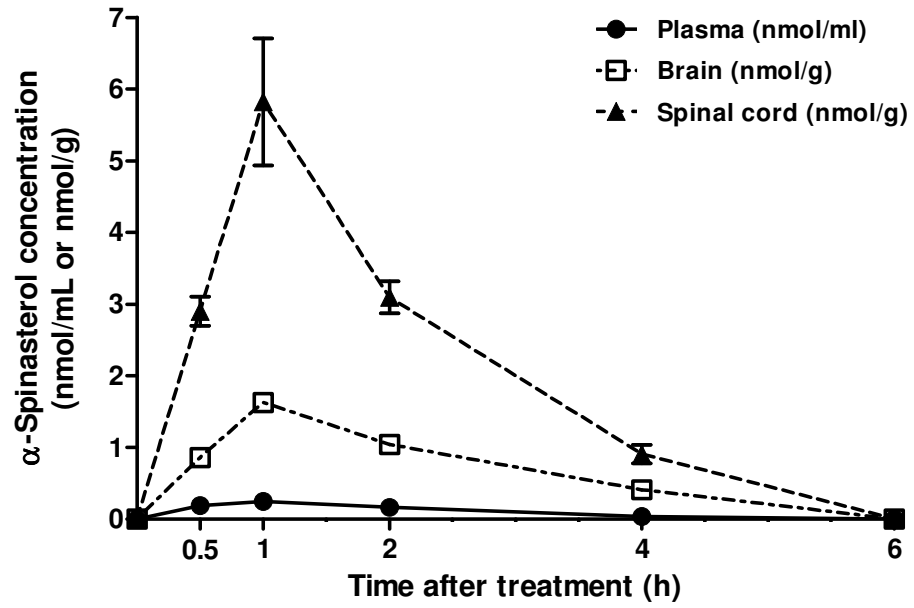


Figure 9. Pharmacokinetic profile of α -spinasterol in mice. The concentrations of α -spinasterol in the plasma (nmol/ml), brain (nmol/g), and spinal cord (nmol/g) were measured 0.5, 1, 2, and 4 hours after administration of α -spinasterol ($0.3 \mu\text{mol/kg}$; p.o.). Data are presented as the mean \pm S.E.M. ($n=3-4$).

Table 1. Effect of Dcm fraction (100 mg/kg, p.o) and α -spinasterol (0.3 μ mol/kg; p.o.) or vehicle on spontaneous and forced locomotor activity in mice 1 hour after treatment.

Treatment (p.o.)	Open Field		Rotarod	
	Crossing	Rearing	First Fall	N° Fall
Vehicle	49 \pm 7	22 \pm 4	63 \pm 15	3.0 \pm 0.6
Dcm fraction (100 mg/kg)	41 \pm 5	16 \pm 4	55 \pm 16	4.1 \pm 0.5
α -Spinasterol (0.3 μ mol/kg)	56 \pm 4	16 \pm 2	54 \pm 15	1.8 \pm 1.2

No significant differences were observed between groups (Student "t" test). Results are expressed as the mean \pm S.E.M (n = 5-7).

6. DISCUSSÃO

O receptor TRPV1 tem sido considerado como um alvo promissor para a busca de novas drogas analgésicas devido à relevância deste canal em vias de modulação da dor e a eficácia dos novos antagonistas do receptor TRPV1 (Wong e Gavva, 2009). Assim, este canal tem sido estudado para o tratamento da dor relacionada a um grande número de doenças, como dor inflamatória, enxaqueca, dor relacionada ao câncer e também dor relacionada à osteoartrite (Jara-Oseguera et al., 2008; Adcock, 2009; Wong e Gavva, 2009; Schumacher, 2010).

Neste estudo, foi realizada uma triagem *in vivo* e em *in vitro* para identificar novos antagonistas do receptor TRPV1 derivados das folhas da planta *V. tweedieana*. Dessa maneira, observa-se que o extrato hidroalcolico e frações de *V. tweedieana* apresentaram efeito antinociceptivo no teste da capsaicina. Além disso, a fração Dcm possui ação antiedematogênica no mesmo modelo. Entre os compostos isolados testados da fração Dcm, o α -espinasterol e o estigmasterol apresentaram efeito antinociceptivo no teste da capsaicina, porém apenas o composto α -espinasterol apresentou efeito antiedematogênico na mesma dose testada. O α -espinasterol também foi capaz de diminuir o influxo de cálcio induzido pela capsaicina em sinaptossomas de medula espinhal de camundongos, e este composto e a fração Dcm também deslocaram a [³H]-RTX dos sítios de ligação específica para o receptor TRPV1. Ambos também mostraram efeito anti-hiperalgésico no modelo de nocicepção por calor nocivo, e também no modelo de dor inflamatória induzida por adjuvante (CFA). Além disso, o composto α -espinasterol não foi capaz de alterar a atividade locomotora, e de induzir hipertermia um efeito adverso observado comumente para os compostos antagonistas do receptor TRPV1. Todas estas evidências suportam a idéia que o composto α -espinasterol age como um antagonista do receptor TRPV1.

Para a identificação de novos antagonistas do receptor TRPV1, o teste da capsaicina em animais foi utilizado como modelo de triagem *in vivo*. Assim, primeiramente foram identificados se o extrato hidroalcolico e as frações de *V. tweedieana* possuíam atividade antinociceptiva e antiedematogênica no teste da capsaicina. Esse teste foi usado para a triagem de novos compostos antagonistas do receptor TRPV1, porque a capsaicina é um agonista seletivo do TRPV1 e é capaz de induzir nocicepção em animais de experimentação e também dor em humanos (o que permite uma transposição do ensaio pré-clínico para o clínico).

Além disso, este é um método simples e de curta duração para a medida da nocicepção, cuja intensidade da nocicepção pode ser reduzida usando doses submáximas de capsaicina (Simone et al., 1989; Sakurada et al., 1992; Walker et al., 1995), o que é importante para reduzir o desconforto dos animais de experimentação. Como observado para o composto SB-366791, usado como controle positivo, os antagonistas do receptor TRPV1 são capazes de reduzir a dor e a formação de edema induzidos pela administração intraplantar de capsaicina. Todas as frações testadas foram capazes de reduzir a nocicepção no teste da capsaicina, porém apenas a fração Dcm reduziu a formação de edema no mesmo teste. Assim, compostos isolados desta fração foram testados no teste da capsaicina e apenas o α -espinasterol apresentou efeito antinociceptivo e antiedematogênico.

O composto α -espinasterol isolado da fração Dcm foi então utilizado em uma triagem *in vitro* para observar a capacidade deste composto de agir como um antagonista do receptor TRPV1. Existem evidências mostrando que α -espinasterol possui diversas atividades biológicas como ação antioxidante, anti-carcinogênica, antiproliferativa, antimutagênica e antiulcerogênica (Coballase-Urrutia et al., 2010; Jeong et al., 2010; Klein et al., 2010; Ravikumar et al., 2010; Villasenõr e Domingo, et al., 2000). Além de atividade antiinflamatória inibindo o edema de orelha induzido por TPA, e induzindo a expressão da enzima heme oxygenase-1 em cultura de células (Boller et al., 2010; Jeong et al., 2010). Além disso, já foi demonstrado que o composto α -espinasterol apresenta atividade antinociceptiva no modelo de dor induzida pela administração de ácido acético em camundongos (Meotti et al. 2006; Freitas et al., 2009), e também inibiu a resposta nociceptiva induzida por glutamato em camundongos (Ribas et al., 2008). Porém, o mecanismo pelo qual este composto possui atividade antinociceptiva ainda não foi relatado.

Assim, para investigar se os compostos isolados da fração diclorometano apresentavam capacidade de interagir com receptor TRPV1, foi realizado o ensaio de união específica utilizando [3 H]-RTX. Nesse, apenas o α -espinasterol e a fração Dcm foram capazes de deslocar o radioativo com um valor de CI_{50} de aproximadamente 1.4 μ M, assim com maior potência quando comparado a outros compostos como o ligante clássico do receptor, a capsaicina (aproximadamente 3.2 μ M) (Szallasi et al., 1999).

Uma vez observado esse fato, o próximo passo foi averiguar se esta ligação ao receptor promoveria alguma alteração funcional sobre o TRPV1. Para isso realizou-se o ensaio de influxo de cálcio estimulado por capsaicina. Nesse experimento, observou-se que o α -espinasterol foi capaz de promover uma diminuição no influxo estimulado por capsaicina, com menor potência que interage com o receptor. Essa menor potência pode ser explicada pela amplificação de sinal que ocorre no ensaio funcional. E esse resultado indicou que o α -espinasterol é capaz de se ligar ao receptor TRPV1 e atuar como um antagonista sobre o mesmo.

O receptor TRPV1 é um receptor polimodal expresso principalmente em neurônios sensoriais, que é importante para a percepção de estímulos nocivos, (Levine e Alessandri-Haber, 2007). Vários estudos indicam que camundongos que não expressam o receptor TRPV1 não são capazes de evocar resposta nociceptiva após a injeção de capsaicina e possuem resposta diminuída ao calor nocivo (Caterina et al., 1997; Davis et al., 2000). Nesse estudo, foi observado que o α -espinasterol foi capaz de inibir a resposta nociceptiva induzida por calor nocivo, mas não modificou o limiar mecânico de animais sem injúria. Esses resultados estão de acordo com o fato que os receptores TRPV1 são principalmente envolvidos com a transdução da dor por calor nocivo do que por estímulos mecânicos como descrito anteriormente (Scherrer et al., 2009). Esses resultados mostram como este composto age de maneira similar aos antagonistas do receptor TRPV1.

Muitas substâncias podem agir como agonistas do receptor TRPV1, como prótons, calor nocivo ($>43^{\circ}\text{C}$), substâncias inflamatórias e vanilóides como a capsaicina e resiniferatoxina (Jara-Oseguera et al., 2008; Schumacher, 2010). A administração de capsaicina e resiniferatoxina provoca dor em queimação, como descrito em humanos e em animais de experimentação (Schumacher, 2010). Além disso, a capsaicina e a resiniferatoxina possuem atividade analgésica, como relatado em pacientes com dor de origem neuropática, e formulações contendo estas substâncias ainda são usadas para o estudo de novos fármacos para a dor (Schumacher, 2010). O efeito dessas substâncias pode ser explicado por uma combinação de mecanismos, principalmente pela destruição dos terminais de fibras que expressam o receptor TRPV1 que estão envolvidas com a condução de estímulos nocivos (Schumacher, 2010).

Neste estudo, foi realizado um protocolo onde a resiniferatoxina foi utilizada para a depleção das fibras que expressam o receptor TRPV1, para observar se o efeito antinociceptivo do composto α -espinasterol no teste de nocicepção induzido por calor nocivo poderia ser diminuído. O composto α -espinasterol e o SB-366791 não apresentaram efeito antinociceptivo neste teste em animais pré-tratados sistemicamente com resiniferatoxina, o que está de acordo com o fato que as fibras que expressam o receptor TRPV1 são necessárias para este efeito neste teste de nocicepção.

O receptor TRPV1 foi descrito como um importante alvo para a pesquisa de novos analgésicos (Jara-Oseguera et al., 2008; Adcock, 2009; Wong e Gavva, 2009; Schumacher, 2010). Em modelos pré-clínicos de dor inflamatória tem sido relatado que antagonistas do receptor TRPV1 são capazes de reduzir tanto a hiperalgisia mecânica como térmica, enquanto que animais que não expressam o receptor TRPV1 não são capazes de desenvolver hiperalgisia térmica e sinais inflamatórios em diferentes tipos de modelos de dor inflamatória (Davis et al., 2000; Walker et al., 2003; Gavva et al., 2005; Honore et al., 2005; Barton et al., 2006). Uma vez que o composto α -espinasterol, como confirmado neste estudo, pode inibir a hiperalgisia mecânica e térmica, além de reduzir a formação de edema induzida no modelo de dor inflamatória por adjuvante (CFA), estes resultados estão de acordo com o observado para os antagonistas do receptor TRPV1.

Além disso, é observado que a dor relacionada a condições inflamatórias é associada a um aumento da expressão do receptor TRPV1 (Ji et al., 2000; Amaya et al., 2004; Zhang et al., 2005). Após a injeção de CFA é relatado um aumento da expressão do receptor TRPV1 em fibras mielinizadas do tipo A, estes dados podem estar associados com o efeito anti-hiperalgésico mecânico pós a injeção de CFA em animais (Amaya et al., 2003; McGaraughty et al., 2008). O efeito antinociceptivo do α -espinasterol na hiperalgisia mecânica induzida por CFA pode estar também relacionado com a capacidade deste composto de agir sobre o SNC. Como descrito anteriormente, antagonistas do receptor TRPV1 com capacidade de agir centralmente apresentaram melhor efeito anti-hiperalgésico, principalmente em relação à hiperalgisia mecânica (Cui et al., 2006). De fato, isso parece ocorrer, pois α -espinasterol possui um bom coeficiente de penetração no SNC. Assim, o composto α -espinasterol mostrou um melhor perfil quando comparado com o SB-

366791, pois esta molécula não foi capaz de reduzir a hiperalgesia mecânica e o edema induzidos por CFA.

A fração Dcm e o composto α -espinasterol não foram capazes de provocar alteração na atividade locomotora dos animais. Como descrito anteriormente, muitas drogas que induzem alteração da atividade locomotora podem induzir resultados falso positivos em testes de nocicepção por induzir sedação, alteração da temperatura corporal ou redução na atividade locomotora (Negus et al., 2006). A indução de severa hipertermia pelo antagonista do receptor TRPV1 em humanos, o composto AMG517 (análogo do AMG9810) impediu o desenvolvimento desta nova molécula como uma droga analgésica promissora (Gavva et al., 2008). A administração do composto AMG517 em humanos foi relatada como causando hipertermia severa e persistente (39-40 °C), em doses baixas, e também concentrações plasmáticas, sem analgesia relacionada aos mesmos níveis plasmáticos da droga, e esse efeito parece ser exclusivamente mediado pelo receptor TRPV1 (Gavva et al., 2008). Foi observado, ainda, que o composto AMG9810 causou efeito hipertérmico nos animais, o que não foi observado para o composto α -espinasterol, indicando que esta molécula parece ter melhores características do que outros antagonistas do receptor TRPV1 já estudados. Já foram descritos distintos perfis de antagonistas do receptor TRPV1, e o bloqueio da corrente por prótons parece ser um mecanismo que pode contribuir para o desenvolvimento de hipertermia (Wong and Gavva, 2009; Garami et al., 2010). Então, o composto α -espinasterol pode não bloquear este tipo de corrente, porém novos experimentos devem ser realizados para verificar este efeito.

Concluindo, o composto α -espinasterol apresentou potente efeito antinociceptivo e antiedematogênico em diversos modelos de dor, sendo também eficaz no tratamento da hiperalgesia, um sintoma frequente de pacientes com dor crônica (Schaible et al., 2009). Dessa maneira, o composto esteróide α -espinasterol derivado da planta *Vernonia tweedieana* foi caracterizado como um novo, eficaz, e disponível por via oral antagonista do receptor TRPV1 e pode então representar uma nova molécula para a terapia da dor.

7. CONCLUSÕES

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir que:

- 7.1. O extrato hidroalcolico, as frações e os compostos isolados das folhas de *Vernonia tweedieana* Baker apresentaram efeito antinociceptivo no teste da capsaicina, sendo que apenas a fração diclorometano e o α -espinasterol apresentaram efeito antiedematogênico. Desse modo, a fração diclorometano e o α -espinasterol mostraram um perfil semelhante ao de antagonistas do receptor TRPV1 nesse teste, reduzindo a nocicepção e o edema induzidos pela administração intraplantar de capsaicina;
- 7.2. Apenas o composto α -espinasterol e a fração diclorometano foram capazes de deslocar a ligação específica da [³H]-resiniferatoxina em fração de membrana de medula espinhal de camundongos. O α -espinasterol também diminui o influxo de cálcio induzido pela capsacina em fração rica de sinaptossomas de medula espinhal de camundongos, comportando-se desta forma como um antagonista do receptor TRPV1;
- 7.3. A fração diclorometano e o composto α -espinasterol apresentaram efeito antinociceptivo na nocicepção induzida por estímulo térmico, mas não induzida por estímulo mecânico, comportando dessa forma semelhante aos antagonistas do receptor TRPV1;
- 7.4. O composto α -espinasterol não apresentou atividade antinociceptiva em animais depletados de fibras sensoriais TRPV1 positivas, mostrando que estas fibras seriam importantes para o efeito antinociceptivo desse composto nesse modelo de nocicepção;
- 7.5. A fração diclorometano e o composto α -espinasterol apresentaram efeito anti-hiperalgésico e antiedematogênico no modelo de dor inflamatória induzida pela administração de adjuvante completo de Freund, sendo este efeito relevante, pois o desenvolvimento de hiperalgesia é um dos principais sintomas clínicos observados em patologias dolorosas crônicas;
- 7.6. O composto α -espinasterol mostrou boa absorção por via oral, e alta penetração no cérebro e medula espinhal de camundongos, sugerindo que este composto assim como outros antagonistas do receptor TRPV1 que são capazes de agir no SNC central mostram melhor perfil anti-hiperalgésico;

7.7. Tanto a fração diclorometano como o composto α -espinasterol não causaram alterações na atividade locomotora e na temperatura corporal dos animais, assim este composto apresenta eficaz efeito antinociceptivo sem o desenvolvimento de hipertermia que é o principal efeito adverso observado para antagonistas do receptor TRPV1.

Todas estas evidências suportam a idéia que o composto α -espinasterol age como um antagonista do receptor TRPV1 possuindo efeito antinociceptivo.

7. REFERÊNCIAS BIBLIOGRÁFICAS

- Abosi AO, Raseroka BH. In vivo antimalarial activity of *Vernonia amygdalina*. Br J Biomed Sci 2003; 60: 89-91.
- Adcock JJ. TRPV1 receptors in sensitisation of cough and pain reflexes. Pulm Pharmacol Ther 2009; 22: 65-70.
- Amaya F, Oh-hashii K, Naruse Y, Iijima N, Ueda M, Shimosato G, et al. Local inflammation increases vanilloid receptor 1 expression within distinct subgroups of DRG neurons. Brain Res 2003;963:190-6.
- Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, Suzuki H, Tanaka M. NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. Eur J Neurosci 2004; 20: 2303-10.
- Awe SO, Olajide O, Makinde JM. Cathartic effect of the leaf extract of *Vernonia amygdalina*. Phytother Res 1998; 12: 57-58.
- Barbastefano V, Cola M, Luiz-Ferreira A, Farias-Silva E, Hiruma-Lima CA, Rinaldo D, Vilegas W, Souza-Brito AR. *Vernonia polyanthes* as a new source of antiulcer drugs. Fitoterapia 2007; 78: 545-51.
- Bardón A, Borkosky S, Ybarra MI, Montanaro S, Cartagena E. Bioactive plants from Argentina and Bolivia. Fitoterapia 2007; 78: 227-31.
- Barton NJ, McQueen DS, Thomson D, Gauldie SD, Wilson AW, Salter DM, Chessell IP. Attenuation of experimental arthritis in TRPV1R knockout mice. Exp Mol Pathol 2006; 81: 166-70.
- Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell 2009; 139: 267-84.
- Bassols A, Bosch F, Baños JE. How does the general population treat their pain? A survey in Catalonia, Spain. J Pain Symptom Manage 2002; 23: 318-28.
- Boller S, Soldi C, Marques MC, Santos EP, Cabrini DA, Pizzolatti MG, Zamprônio AR, Otuki MF. Anti-inflammatory effect of crude extract and isolated compounds from *Baccharis illinita* DC in acute skin inflammation. J Ethnopharmacol 2010; 130: 262-6.

- Brauchi S, Orta G, Salazar M, Rosenmann E, Latorre R. A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *J Neurosci* 2006; 26: 4835-40.
- Brennan F, Carr DB, Cousins M. Pain management: a fundamental human right. *Anesth Analg* 2007; 105: 205-21.
- Burgess G, Williams D. The discovery and development of analgesics: new mechanisms, new modalities. *J Clin Invest* 2010; 120: 3753-9.
- Calixto JB, Beirith A, Ferreira J, Santos AR, Filho VC, Yunes RA. Naturally occurring antinociceptive substances from plants. *Phytother Res* 2000; 14: 401-18.
- Calixto JB, Kassuya CA, André E, Ferreira J. Contribution of natural products to the discovery of the transient receptor potential (TRP) channels family and their functions. *Pharmacol Ther* 2005; 106: 179-208.
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997; 389: 816-24.
- Chizh BA, O'Donnell MB, Napolitano A, Wang J, Brooke AC, Aylott MC, Bullman JN, Gray EJ, Lai RY, Williams PM, Appleby JM. The effects of the TRPV1 antagonist SB-705498 on TRPV1 receptor-mediated activity and inflammatory hyperalgesia in humans. *Pain* 2007; 132: 132-41.
- Clapham DE. TRP channels as cellular sensors. *Nature* 2003; 426: 517-24.
- Coballase-Urrutia E, Pedraza-Chaverri J, Camacho-Carranza R, Cárdenas-Rodríguez N, Huerta-Gertrudis B, Medina-Campos ON, Mendoza-Cruz M, Delgado-Lamas G, Espinosa-Aguirre JJ. Antioxidant activity of *Heterotheca inuloides* extracts and of some of its metabolites. *Toxicology* 2010 30; 276: 41-8.
- Corson TW, Crews CM. Molecular understanding and modern application of traditional medicines: triumphs and trials. *Cell* 2007; 130: 769-74.
- Cosens DJ, Manning A. Abnormal electroretinogram from a *Drosophila* mutant. *Nature* 1969; 224: 285-87.

- Cui M, Honore P, Zhong C, Gauvin D, Mikusa J, Hernandez G, Chandran P, Gomtsyan A, Brown B, Bayburt EK, Marsh K, Bianchi B, McDonald H, Niforatos W, Neelands TR, Moreland RB, Decker MW, Lee CH, Sullivan JP, Faltynek CR. TRPV1 receptors in the CNS play a key role in broad-spectrum analgesia of TRPV1 antagonists. *J Neurosci* 2006; 26: 9385-93.
- Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 2000; 405: 183-87.
- Driedger PE, Blumberg PM. Different biological targets for resiniferatoxin and phorbol 12-myristate 13-acetate. *Cancer Res* 1980a; 40: 1400-04.
- Driedger PE, Blumberg PM. Specific binding of phorbol ester tumor promoters. *Proc Natl Acad Sci USA* 1980b; 77: 567-71.
- Fernandez AG, Salcedo C, Palacios JM. Aspirin, salicylate and gastrointestinal injury. *Nat Med* 1995; 1: 602-3.
- Fishman SM. Recognizing pain management as a human right: a first step. *Anesth Analg* 2007; 105: 8-9.
- Freitas CS, Baggio CH, Dos Santos AC, Mayer B, Twardowschy A, Luiz AP, Marcon R, Soldi C, Pizzolatti MG, Dos Santos EP, Marques MC, Santos AR. Antinociceptive properties of the hydroalcoholic extract, fractions and compounds obtained from the aerial parts of *Baccharis illinita* DC in mice. *Basic Clin Pharmacol Toxicol* 2009; 104: 285-92.
- Frutuoso VS, Gurjão MR, Cordeiro RS, Martins MA. Analgesic and anti-ulcerogenic effects of a polar extract from leaves of *Vernonia condensata*. *Planta Med* 1994; 60: 21-5.
- Garami A, Shimansky YP, Pakai E, Oliveira DL, Gavva NR, Romanovsky AA. Contributions of different modes of TRPV1 activation to TRPV1 antagonist-induced hyperthermia. *J Neurosci* 2010;30:1435-40.

- Gavva NR, Tamir R, Qu Y, Klionsky L, Zhang TJ, Immke D, Wang J, Zhu D, Vanderah TW, Porreca F, Doherty EM, Norman MH, Wild KD, Bannon AW, Louis JC, Treanor JJ. AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. *J Pharmacol Exp Ther* 2005b; 313: 474-84.
- Gavva, N.R., Treanor, J.J., Garami, A., Fang, L., Surapaneni, S., Akrami, A., Alvarez, F., Bak, A., Darling, M., Gore, A., Jang, G.R., Kesslak, J.P., Ni, L., Norman, M.H., Palluconi, G., Rose, M.J., Salfi, M., Tan, E., Romanovsky, A.A., Banfield, C., Davar, G., Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* 2008; 136, 202-10.
- Ghilardi JR, Rohrich H, Lindsay TH, Sevcik MA, Schwei MJ, Kubota K, Halvorson KG, Poblete J, Chaplan SR, Dubin AE, Carruthers NI, Swanson D, Kuskowski M, Flores CM, Julius D, Mantyh PW. Selective blockade of the capsaicin receptor TRPV1 attenuates bone cancer pain. *J Neurosci* 2005; 25: 3126-31.
- Gracheva EO, Cordero-Morales JF, González-Carcacía JA, Ingolia NT, Manno C, Aranguren CI, Weissman JS, Julius D. Ganglion-specific splicing of TRPV1 underlies infrared sensation in vampire bats. *Nature* 2011; 476: 88-91.
- Gupta M, Mazumder UK, Manikandan L, Bhattacharya S, Haldar PK, Roy S. Evaluation of antipyretic potential of *Vernonia cinerea* extract in rats. *Phytother Res* 2003; 17: 804-6.
- Hardie RC, Minke B. The *trp* gene is essential for a light-activated Ca²⁺ channel in *Drosophila* photoreceptors. *Neuron* 1992; 8: 643-51.
- Harvey AL. Natural products as a screening resource. *Curr Opin Chem Biol* 2007; 11: 480-4.
- Hergenbahn M, Adolf W, Hecker E. Resiniferatoxin and other esters of novel polyfunctional diterpenes from *Euphorbia resinifera* and *unispina*. *Tetrahedron Lett* 1975; 19: 1595-98.

- Holzer, P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 1988; 24: 739-68.
- Holzer P. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Review* 1991; 43, 143-201.
- Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM, Gomtsyan A, El Kouhen R, Lee CH, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF. A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. *J Pharmacol Exp Ther* 2005; 314: 410-21.
- Iwalewa EO, Iwalewa OJ, Adeboye JO. Analgesic, antipyretic, anti-inflammatory effects of methanol, chloroform and ether extracts of *Vernonia cinerea* less leaf. *J Ethnopharmacol* 2003; 86: 229-34.
- James IF, Walpole CSJ, Hixon J, Wood JN, Wrigglesworth R. Long-lasting agonist activity produced by a capsaicin-like photoaffinity probe. *Mol Pharmacol* 1988; 33: 643-49.
- Jancsó N, Jancsó-Gabor A. Desensitization of sensory nerve endings (in Hungarian). *Kiserl Orvostud* 1949; 2: 15.
- Jancsó N. Stoffanreicherung in Retikuloendothelial und in der Niere. Budapest: Aladémiai Kiadó, Speicherung. 1955.
- Jancsó G, Kiraly E, Jancsó-Gabor A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* 1977; 270: 741-43.
- Jara-Oseguera A, Simon SA, Rosenbaum T. TRPV1: on the road to pain relief. *Curr Mol Pharmacol* 2008; 1: 255-69.
- Jeong GS, Li B, Lee DS, Kim KH, Lee IK, Lee KR, Kim YC. Cytoprotective and anti-inflammatory effects of spinasterol via the induction of heme oxygenase-1 in

- murine hippocampal and microglial cell lines. *Int Immunopharmacol* 2010; 10: 1587-94.
- Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 2002; 36: 57–68.
- Jordt SE, Tominaga M, Julius D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci U S A* 2000; 97: 8134-39.
- Klein LC Jr, Gandolfi RB, Santin JR, Lemos M, Cechinel Filho V, de Andrade SF. Antiulcerogenic activity of extract, fractions, and some compounds obtained from *Polygala cyparissias* St. Hillaire & Moquin (Polygalaceae). *Naunyn Schmiedebergs Arch Pharmacol* 2010; 381: 121-6.
- Knotkova H, Pappagallo M, Szallasi A. Capsaicin (TRPV1 Agonist) therapy for pain relief: farewell or revival? *Clin J Pain* 2008; 24: 142-54.
- Koehn FE, Carter GT. The evolving role of natural products in drug discovery. *Nat Rev Drug Discov* 2005; 4: 206-20.
- Lakhan SE, Kirchgessner A, Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med* 2009; 7: 97.
- Lehto SG, Tamir R, Deng H, Klionsky L, Kuang R, Le A, Lee D, Louis JC, Magal E, Manning BH, Rubino J, Surapaneni S, Tamayo N, Wang T, Wang J, Wang J, Wang W, Youngblood B, Zhang M, Zhu D, Norman MH, Gavva NR. Antihyperalgesic effects of (R,E)-N-(2-hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluoromethyl)phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. *J Pharmacol Exp Ther* 2008; 326: 218-29.
- Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. *Biochim Biophys Acta* 2007; 1772: 989-1003.

- Lichtenberger LM, Wang ZM, Romero JJ, Ulloa C, Perez JC, Giraud MN, Barreto JC. Non-steroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury. *Nat Med* 1995; 1: 154-8.
- Loeser JD, Treede RD. The Kyoto protocol of IASP Basic Pain Terminology. *Pain* 2008; 137: 473-7.
- Lohman D, Schleifer R, Amon JJ. Access to pain treatment as a human right. *BMC Med* 2010; 8, 8.
- Mazumder UK, Gupta M, Manikandan L, Bhattacharya S, Haldar PK, Roy S. Evaluation of anti-inflammatory activity of *Vernonia cinerea* Less. extract in rats. *Phytomedicine* 2003; 10: 185-8.
- McChesney JD, Venkataraman SK, Henri JT. Plant natural products: back to the future or into extinction? *Phytochemistry* 2007; 68: 2015-22.
- McGaraughty S, Chu KL, Brown BS, Zhu CZ, Zhong C, Joshi SK, et al. Contributions of central and peripheral TRPV1 receptors to mechanically evoked and spontaneous firing of spinal neurons in inflamed rats. *J Neurophysiol* 2008; 100: 3158-66.
- Menendez L, Juarez L, Garcia E, Garcia-Suarez O, Hidalgo A, Baamonde A. Analgesic effects of capsazepine and resiniferatoxin on bone cancer pain in mice. *Neurosci Lett* 2006; 393: 70-3.
- Meotti FC, Ardenghi JV, Pretto JB, Souza MM, d' Avila Moura J, Junior AC, Soldi C, Pizzolatti MG, Santos AR. Antinociceptive properties of coumarins, steroid and dihydrostyryl-2-pyrones from *Polygala sabulosa* (Polygalaceae) in mice. *J Pharm Pharmacol* 2006; 58: 107-12.
- Meyer RA, Ringkamp M, Campbell JN, Raja SN. Peripheral mechanisms of cutaneous nociception. Wall and Melzack's Textbook of Pain, S.B. McMahon and M. Koltzenburg, eds. (Philadelphia: Elsevier), p. 3-34, 2008.
- Minke B, Wu C, Pak WL. Induction of photoreceptor voltage noise in the dark in *Drosophila* mutant. *Nature* 1975; 258: 84-87.

- Minke B. The history of the *Drosophila* TRP channel: the birth of a new channel superfamily. *J Neurogenet* 2010; 24: 216-33.
- Mirshafiey A, Mohsenzadegan M. The role of reactive oxygen species in immunopathogenesis of rheumatoid arthritis. *Iran J Allergy Asthma Immunol* 2008; 7: 195-202.
- Montell C, Jones K, Hafen E, Rubin G. Rescue of the *Drosophila* phototransduction mutation *trp* by germline transformation. *Science* 1985; 230: 1040-43.
- Montell C, Rubin GM. Molecular characterization of the *Drosophila trp* locus: a putative integral membrane protein required for phototransduction. *Neuron* 1989; 2: 1313-23.
- Montell C, Birnbaumer L, Flockerzi V, Bindels RJ, Bruford EA, Caterina MJ, et al. A unified nomenclature for the superfamily of TRP cation channels. *Mol Cell* 2002; 9: 229–231.
- Montell C. The TRP superfamily of cation channels. *Sci STKE* 2005; 272, re3.
- Neacsu C, Ciobanu C, Barbu I, Toader O, Szegli G, Kerek F, Babes A. Substance MCS-18 isolated from *Helleborus purpurascens* is a potent antagonist of the capsaicin receptor, TRPV1, in rat cultured sensory neurons. *Physiol Res* 2010; 59: 289-98.
- Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. *J Pharmacol Exp Ther* 2006;319:507-14.
- Nelson, E. K. The constitution of capsaicin-the pungent principle of capsicum I. *J Am Chem Soc* 1919; 41: 1115-19.
- Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. *J Nat Prod* 2003; 66: 1022-37.
- Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 2007; 70: 461-77.

- Niiyama Y, Kawamata T, Yamamoto J, Omote K, Namiki A. Bone cancer increases transient receptor potential vanilloid subfamily 1 expression within distinct subpopulations of dorsal root ganglion neurons. *Neuroscience* 2007; 148: 560-72.
- Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. *Physiol Rev* 2007; 87: 165-217.
- Njan AA, Adzu B, Agaba AG, Byarugaba D, Díaz-Llera S, Bangsberg DR. The analgesic and antiplasmodial activities and toxicology of *Vernonia amygdalina*. *J Med Food* 2008; 11: 574-81.
- Patapoutian A, Tate S, Woolf CJ. Transient receptor potential channels: targeting pain at the source. *Nat Rev Drug Discov* 2009; 8: 55-68.
- Petersen CC, Berridge MJ, Borgese MF, Bennett DL. Putative capacitative calcium entry channels: expression of *Drosophila* *trp* and evidence for the existence of vertebrate homologues. *Biochem J* 1995; 311: 41-44.
- Phillips AM, Bull A, Kelly LE. Identification of a *Drosophila* gene encoding a calmodulin-binding protein with homology to the *trp* phototransduction gene. *Neuron* 1992; 8: 631-42.
- Plante GE, VanItallie TB. Opioids for cancer pain: the challenge of optimizing treatment. *Metabolism* 2010; 59: S47-52.
- Rami HK, Thompson M, Stemp G, Fell S, Jerman JC, Stevens AJ, Smart D, Sargent B, Sanderson D, Randall AD, Gunthorpe MJ, Davis JB. Discovery of SB-705498: a potent, selective and orally bioavailable TRPV1 antagonist suitable for clinical development. *Bioorg Med Chem Lett* 2006; 16: 3287-91.
- Ravikumar YS, Mahadevan KM, Manjunatha H, Satyanarayana ND. Antiproliferative, apoptotic and antimutagenic activity of isolated compounds from *Polyalthia cerasoides* seeds. *Phytomedicine* 2010; 17: 513-8.
- Ribas CM, Meotti FC, Nascimento FP, Jacques AV, Dafre AL, Rodrigues AL, Farina M, Soldi C, Mendes BG, Pizzolatti MG, Santos AR. Antinociceptive effect of the *Polygala sabulosa* hydroalcoholic extract in mice: evidence for the involvement

- of glutamatergic receptors and cytokine pathways. *Basic Clin Pharmacol Toxicol* 2008; 103: 43-7.
- Richardson JD, Vasko MR. Cellular mechanisms of neurogenic inflammation. *J Pharmacol Exp Ther* 2002; 302: 839-845.
- Risso WE, Scarminio IS, Moreira EG. Antinociceptive and acute toxicity evaluation of *Vernonia condensata* Baker leaves extracted with different solvents and their mixtures. *Indian J Exp Biol* 2010; 48: 811-6.
- Romanovsky AA, Almeida MC, Garami A, Steiner AA, Norman MH, Morrison SF, Nakamura K, Burmeister JJ, Nucci TB. The transient receptor potential vanilloid-1 channel in thermoregulation: a thermosensor it is not. *Pharmacol Rev* 2009; 61: 228-61.
- Rossato MF, Trevisan G, Walker CI, Klafke JZ, de Oliveira AP, Villarinho JG, Zanon RB, Royes LF, Athayde ML, Gomez MV, Ferreira J. Eriodictyol: a flavonoid antagonist of the TRPV1 receptor with antioxidant activity. *Biochem Pharmacol* 2011; 81: 544-51.
- Sakurada T, Katsumata K, Tan-No K, Sakurada S, Kisara K. The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. *Neuropharmacology* 1992;31:1279-1285.
- Schaible HG, Richter F, Ebersberger A, Boettger MK, Vanegas H, Natura G, et al. Joint pain. *Exp Brain Res* 2009;196:153-62.
- Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, et al. Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 2009; 137: 1148-59.
- Scholz J, Woolf CJ. Can we conquer pain? *Nat Neurosci* 2002; 5: 1062-7.
- Schumacher MA. Transient receptor potential channels in pain and inflammation: therapeutic opportunities. *Pain Pract* 2010; 10: 185-200.

- Simone DA, Baumann TK, Lamotte RH. Dose-dependent pain and mechanical hyperalgesia in human after intradermal injection of capsaicin. *Pain* 1989;38:99-107.
- Suleyman H, Demircan B, Karagoz Y. Anti-inflammatory and side effects of cyclooxygenase inhibitors. *Pharmacol Rep* 2007; 59: 247-58.
- Susankova K, Tousova K, Vyklicky L, Teisinger J, Vlachova V. Reducing and oxidizing agents sensitize heat-activated vanilloid receptor (TRPV1) current. *Mol Pharmacol* 2006; 70: 383-94.
- Suss Toby E, Selinger Z, Minke B. Lanthanum reduces the excitation efficiency in fly photoreceptors. *J Gen Physiol* 1991; 98: 849-68.
- Szallasi A, Blumberg PM. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience* 1989; 30: 515-20.
- Szallasi A, Blumberg PM. Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res* 1990b; 524: 106-11.
- Szallasi A, Blumberg PM. Characterization of vanilloid receptors in the dorsal horn of pig spinal cord. *Brain Res* 1991; 547: 335-38.
- Szallasi A, Blumberg PM. [³H] Resiniferatoxin binding by the vanilloid receptor: species-related differences, effects of temperature and sulfhydryl reagents. *Naunyn-Schmiedeberg's Arch Pharmacol* 1993; 347: 84-91.
- Szallasi A, Blumberg PM. Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol Rev* 1999; 51: 159- 212.
- Szallasi A, Szabó T, Bíró T, Modarres S, Blumberg PM, Krause JE, et al. Resiniferatoxin-type phorboid vanilloids display capsaicin-like selectivity at native vanilloid receptors on rat DRG neurons and at the cloned vanilloid receptor VR1. *Br J Pharmacol* 1999;128: 428-34.
- Szallasi A, Cortright DN, Blum CA, Eid SR. The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept. *Nat Rev Drug Discov* 2007; 6: 357-72.

- Szebeni A, Jancsó G, Wolleman M. Capsaicin receptor binding. *Acta Physiol Acad Sci Hung* 1978; 51: 13.
- Szolcsányi J, Jancsó-Gábor A. Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain producing potency of pungent agents. *Arzneim-Forsch* 1975; 25, 1877-81.
- Thresh LT. Isolation of capsaicin. *Pharm J* 1846; 6: 941-49.
- Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998; 21: 531-43.
- Tona L, Cimanga RK, Mesia K, Musuamba CT, De Bruyne T, Apers S, Hernans N, Van Miert S, Pieters L, Totté J, Vlietinck AJ. In vitro antiplasmodial activity of extracts and fractions from seven medicinal plants used in the Democratic Republic of Congo. *J Ethnopharmacol* 2004; 93: 27-32.
- Trescot AM, Datta S, Lee M, Hansen H. Opioid pharmacology. *Pain Physician* 2008; 11: S133-53.
- Turnbull, A. Tincture of capsaicin as a remedy for chilblains and toothache. *Dublin Free Press* 1850; 1: 95-96.
- Venkatachalam K, Montell C. TRP channels. *Annu Rev Biochem* 2007; 76: 387-417.
- Vlachova V, Teisinger J, Susankova K, Lyfenko A, Ettrich R, Vyklicky L. Functional role of Cterminal cytoplasmic tail of rat vanilloid receptor 1. *J Neurosci* 2003; 23: 1340-50.
- Villaseñor IM, Domingo AP. Anticarcinogenicity potential of spinasterol isolated from squash flowers. *Teratog Carcinog Mutagen* 2000; 20: 99-105.
- Vriens J, Appendino G, Nilius B. Pharmacology of vanilloid transient receptor potential cation channels. *Mol Pharmacol* 2009; 75: 1262-79.
- Vyklicky L, Lyfenko A, Susankova K, Teisinger J, Vlachova V. Reducing agent dithiothreitol facilitates activity of the capsaicin receptor VR-1. *Neuroscience* 2002; 111: 435-441.

- Walker K, Fox AJ, Urban LA. Animal models for pain research. *Mol Med Today* 1999; 5: 319-21.
- Walker KM, Urban L, Medhurst SJ, Patel S, Panesar M, Fox AJ, McIntyre P. The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 2003; 304: 56-62.
- Welch JM, Simon SA, Reinhart PH. The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from the activation by either acid or heat. *Proc Natl Acad Sci U S A* 2000; 97: 13889-94.
- Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C. TRPC1, a human homolog of a *Drosophila* store-operated channel. *Proc Natl Acad Sci, U S A* 1995; 92: 9652-56.
- Wong F, Schaefer EL, Roop BC, LaMendola JN, Johnson Seaton D, Shao D. Proper function of the *Drosophila trp* gene product during pupal development is important for normal visual transduction in the adult. *Neuron* 1989; 3: 81-94.
- Wong GY, Gavva NR. Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks. *Brain Res Rev* 2009; 60: 267-77.
- Woolf CJ. What is this thing called pain? *J Clin Invest* 2010; 120: 3742-4.
- Wu LJ, Sweet TB, Clapham DE. International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol Rev* 2010; 62: 381-404.
- Zanon RB, Pereira DF, Boschetti, TK, Santos M, Athayde ML. Phytoconstituents isolated from dichloromethane fraction of *Vernonia tweedieana* Baker leaves. *Rev Bras Farmacogn* 2008; 18: 226-29.
- Yunes RA, Cechinel FV, Ferreira J, Calixto JB. The use of natural products as sources of new analgesic drugs. *Studies in Natural Products Chemistry*, 30 (Bioactive Natural Products (Part K)). (Elsevier B.V.) p. 191-212, 2005.
- Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat gated ion channels. *Embo J* 2005; 24: 4211-23.

Zhu X, Chu PB, Peyton M, Birnbaumer L. Molecular cloning of a widely expressed human homologue for the *Drosophila trp* gene. FEBS Lett 1995; 373: 193-98.

Zhu MX. Multiple roles of calmodulin and other Ca(2+)-binding proteins in the functional regulation of TRP channels. Pflugers Arch 2005; 451: 105-15.

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Full Papers

Antinociceptive Effects of 14-Membered Cyclopeptide Alkaloids

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The analgesic potential of six 14-membered-ring cyclopeptide alkaloids, namely, franganine (1), discarine B (2), scutianines B (3), C (4), and D (5), and adouetine X (6), have been investigated. Among the compounds tested, only franganine (1) and adouetine X (6) produced antinociceptive effects in a mouse model of acute pain, without inducing undesirable side effects. Furthermore, compound 6 also exhibited a pronounced analgesic effect in a chronic neuropathic pain model in mice. It has been found that adouetine X (6) can decrease the activities of Ca^{2+} -ATPase and Na^+/K^+ -ATPase in vitro. Thus, the present findings have demonstrated that adouetine X (6) is a promising analgesic agent.

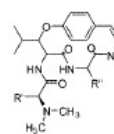
Cyclopeptide alkaloids are defined as polyamidic basic compounds of plant origin embodying a *p*- or *m*-ansa structure.¹ These compounds are found mainly in the Rhamnaceae and have been classified by different methods. However, the most common classification method is according to the size of the macrocycle, as either 13-, 14-, or 15-membered cyclic ether rings.² Some studies have showed that these compounds may function as ionophores in plants and may be involved in the process of nutrient and metal absorption, but the real physiological role of cyclopeptide alkaloids in plants is still unknown.^{1,2} Studies of their biological properties have been hampered due to the low amounts present in the plants of origin of these compounds and a lack of practical synthetic methods.¹ Thus, few biological activities have been reported for this group of substances, including antibacterial, antifungal, antiplasmodial, sedative, and immunostimulant activities.¹

The 14-membered ring class has the most representative compounds and is the largest subgroup of cyclopeptide alkaloids.² This class includes almost all the scutianine-, discarine-, and sanjoine-related compounds. Scutianines B (3) and D (5) and discarine B (2), but not scutianine C (4) and franganine (1), showed modest antibacterial activity.^{3,4} Sanjoine A exhibited sedative, hypnotic, and analgesic properties, and it is reported to be an effective inhibitor of calmodulin-induced activation of Ca^{2+} -ATPase and a modulator of the γ -amino butyric acid (GABA) system.^{5–8}

The search for new analgesic compounds with few undesirable effects and good efficacy in chronic painful conditions is potentially very important. Neuropathy-associated pain has become a major clinical problem, so the search for new drug molecules to alleviate this intractable pain is now an interesting strategy.⁹ Therefore, in the present study, we have investigated the potential antinociceptive effect of six 14-membered cyclopeptide alkaloids in mice as well as their possible mechanism of action and ability to develop undesirable effects.

Results and Discussion

The tail-flick test is a simple pain model that uses an acute noxious stimulus to screen new analgesic drugs, usually substances that act on the central nervous system, such as opioid agonists.¹⁰



	R'	R''
Franganine (1)	$\text{CH}_2\text{CH}(\text{CH}_3)_2$	$\text{CH}_2\text{CH}(\text{CH}_3)_2$
Discarine B (2)	$\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$	$\text{CH}_2\text{C}_6\text{H}_5\text{N}$
Scutianine B (3)	$\text{CH}_2\text{C}_6\text{H}_5$	$\text{CH}_2\text{C}_6\text{H}_5$
Scutianine C (4)	$\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$	$\text{CH}_2\text{C}_6\text{H}_5$
Scutianine D (5)	$\text{CH}_2\text{C}_6\text{H}_5$	$\text{CH}(\text{OH})\text{C}_6\text{H}_5$
Adouetine X (6)	$\text{CH}_2\text{CH}(\text{CH}_3)_2$	$\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$

Since only small amounts of cyclopeptide alkaloids are available for in vivo studies, restricting the possibility of systemic administration, we injected cyclopeptide alkaloids by the intrathecal (i.t.) route because it requires less compound than systemic routes. In addition, i.t. administration is used clinically to deliver analgesic drugs, and the spinal cord is an important modulator in pain pathways.¹¹ The i.t. injection of adouetine X (6), franganine (1), scutianine B (3), and discarine B (2) (10 nmol/site) produced antinociception in the mice tail-flick test, 15 and 60 min after administration (Figure 1). Scutianine D (5) or vehicle did not change tail-flick latency at any time tested. On the other hand, i.t. administration of scutianine C (4) (10 nmol/site) produced hyperalgesia in the tail-flick test, 15 and 60 min after injection (Figure 1). Among the cyclopeptide alkaloids tested, adouetine X (6) was more effective in producing antinociception and increased latencies in the tail-flick test by about 70%. The opposite action of some cyclopeptide alkaloids in the tail-flick test (i.e., induction of analgesia or hyperalgesia) is in accordance with Lee and co-workers, who demonstrated that the 13-membered cyclopeptide alkaloids paliurines A and F acted as sedatives in mice and the closely related alkaloid nummularine H acted as a stimulant under identical conditions, indicating the significant effect of minor structural modifications on the pharmacological actions of cyclopeptide alkaloids.¹² In addition, cyclopeptide alkaloids could interact with some putative neurotransmitter

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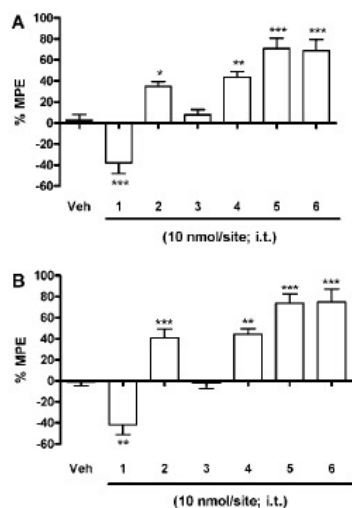


Figure 1. Effects of cyclopeptide alkaloid administration (10 nmol/site; i.t.) in the tail-flick test. The tail-flick response (% MPE) was measured 15 min (A) and 60 min (B) after the injection in mice ($n = 7-8$). Data are expressed as means \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one way ANOVA followed by SNK post hoc test.

systems of the central nervous system (CNS) that modulate nociception in an opposite manner.

As the interaction with glutamate receptors seems to contribute to mechanisms underlying antinociceptive or hyperalgesic actions of some compounds,¹³ and glutamate is the major excitatory transmitter in the CNS, we investigated the role of glutamate receptors in the antinociceptive action of cyclopeptide alkaloids. In the spinal cord, agonists of glutamate receptors produce nociception, while antagonists might produce analgesia.¹³ However, the cyclopeptide alkaloids tested cannot change the specific binding of [³H]-L-glutamate to cerebral plasma membranes in mice, showing that the interaction with glutamate receptors is not necessary for the actions of these compounds in nociception (Table 1). Furthermore, another important neurotransmitter system in the CNS is the GABAergic system, which has opposite actions compared to the glutamatergic system. The action of γ -aminobutyric acid (GABA) is an important factor for modulation of pain. In this way, GABA receptor agonists and antagonists usually function as analgesic or hyperalgesic drugs, respectively.¹⁴ It is known that the 14-membered cyclopeptide alkaloid sanjoinine A stimulates the GABAergic system.⁷ However, the involvement of GABA receptors in the antinociceptive or hyperalgesic actions of the cyclopeptide alkaloids tested must be further determined.

Certain drugs can provide false-positive responses in the tail-flick test because they may induce sedation, motor activity impairment, or body temperature alteration.¹⁰ Knowing that some cyclopeptide alkaloids can induce a sedative effect,¹² we assessed the undesirable effects of discarine B (2), scutianines B (3), C (4), and D (5), franganine (1), and adouetine X (6) in mice. The open-field test is sensitive to compounds that cause sedation or motor impairment.¹⁵ Discarine B (2) and scutianine C (4) (10 nmol/site) decreased the number of crossings, while scutianine B (3) (10 nmol/

site) was able to decrease the number of rearings and crossings in the open-field test. However, franganine (1), scutianine D (5), and adouetine X (6) did not change the motor activity in mice 15 min after injection (Table 2). Moreover, adouetine X (6) did not alter motor activity 60 or 120 min after its intrathecal administration (Table 3). Intrathecal administration of discarine B (2) (10 nmol/site) produced a hypothermic effect in mice 15 and 60 min after its administration. No other cyclopeptide alkaloid tested was able to alter mice body temperature (Table 2). Taken together, these data suggest that for some cyclopeptide alkaloids [namely, discarine B (2) and scutianines B (3) and C (4)], the analgesic effects observed in the tail-flick test might be false positives since they also alter mice motor activity in the open-field test or body temperature. On the other hand, franganine (1) and adouetine X (6) seem to produce a pure analgesic effect, as they did not change the spontaneous locomotion or body temperature.

Thus, since adouetine X (6) showed no undesirable effects and shows good efficacy at producing antinociception, the antinociceptive action of this compound was further evaluated. The ID_{50} value assessed for adouetine X (6) was 2.47 (2.25–2.69) nmol/site at 15 min in the tail-flick test in mice. The time–response curve of the antinociceptive effect of adouetine X (6) (10 nmol/site) showed that the antinociceptive effect of this compound started at 0.25 h and lasted up to 1 h after treatment (Figure 2). Next, we investigated the effect of adouetine X (6) in neuropathic pain. Injury to a peripheral nerve often results in a chronic neuropathic pain condition that is characterized by abnormal painful hypersensitivity, such as allodynia (pain responses to non-noxious stimuli).⁹ This type of chronic pain differs substantially from acute pain not only in terms of the persistence of pain but also with regard to the maladaptive changes at various levels of the nervous system.¹⁶ Thus, the available analgesic drugs often have limited therapeutic value in the management of neuropathic pain, and they may even represent a risk to the patient due to their common side effects.⁹ Therefore, the development of safe and efficacious drugs to treat chronic pain is a priority. We observed that partial ligation of the sciatic nerve in mice induced a significant allodynia (characterized as a decrease in mechanical threshold in response to von Frey hair filaments) 7 days after the nerve injury compared to the sham-operated group. Adouetine X (6) (10 nmol/site; i.t.) was markedly effective in reducing this mechanical allodynia induced by nerve injury in the operated group from 0.5 to 1 h after treatment (Figure 3). Moreover, adouetine X (6) did not alter the detection of normal mechanical stimuli assessed in sham-operated mice (Figure 3). These findings are certainly relevant, considering that chronic pain is difficult to treat since it is quite resistant to most of the analgesic drugs currently available on the market.⁹

Finally, we attempted to find a target responsible for the antinociceptive action of adouetine X (6). Previous findings have demonstrated that the 14-membered cyclopeptide alkaloids sanjoinine-A and sanjoinine-G2, along with the synthetic derivatives, sanjoinine AH-1 and sanjoinine-A dialdehyde, effectively inhibit calmodulin-induced activation of Ca^{2+} -ATPase.^{5,6} Thus we investigated the possible effect of adouetine X (6) in distinct Mg^{2+} -dependent ATPase activities (Ca^{2+} -ATPase or Na^{+}/K^{+} -ATPase). Adouetine X (6) was able to decrease the Ca^{2+} -ATPase activity, with inhibition values of $68.5 \pm 9.9\%$ and $91.8 \pm 6.5\%$ for concentrations of 3 and 10 μM , respectively. Adouetine X (6), at a concentration of 10 μM , but not 3 μM , also reduced the activity of Na^{+}/K^{+} -ATPase, with $92.5 \pm 5.6\%$ inhibition (Figure 4). Thus, adouetine X (6) seems to be a potent inhibitor, since the classical Na^{+}/K^{+} -ATPase inhibitor ouabain reduces about 30% of the enzyme

Table 1. Effects of Cyclopeptide Alkaloids Tested (1–6) in the Displacement of [³H]-L-Glutamate Binding to Mice Brain Membranes^a

vehicle	1	2	3	4	5	6
107.1 \pm 0.1	122.7 \pm 8.3	102.2 \pm 1.8	101.1 \pm 3.2	94.8 \pm 3.2	94.4 \pm 14.6	111.8 \pm 16.2

^aThe results are presented as means \pm SEM of at least two separate displacements.

Table 2. Effect of Treatments with Cyclopeptide Alkaloids (10 nmol/site; i.t.) after 5 min on Spontaneous Activity (number of crossings and rearings) in the Open-Field Test and after 20 or 60 min on Rectal Temperature (°C) in Mice^a

compound	crossings	rearings	rectal temperature (20 min)	rectal temperature (60 min)
vehicle	120.0 ± 8.0	19.7 ± 1.9	0.16 ± 0.1	-0.31 ± 0.09
scutianine C (4)	74 ± 4.9*	17.6 ± 2.7	-0.23 ± 0.11	-0.40 ± 0.15
scutianine D (5)	120.8 ± 14.4	31.6 ± 7.3	-0.08 ± 0.22	-0.26 ± 0.12
scutianine B (3)	78.0 ± 9.4*	10 ± 1.9*	0.16 ± 0.44	-0.45 ± 0.49
discarine B (2)	70.0 ± 10.1*	13.5 ± 3.2	-0.37 ± 0.09*	-0.94 ± 0.19*
franganine (1)	113.3 ± 10.8	19.2 ± 3.0	-0.2 ± 0.12	-0.11 ± 0.13
adouetine X (6)	106.7 ± 10.1	18.17 ± 2.1	-0.08 ± 0.12	-0.20 ± 0.11

^a Values represent means ± SEM for 7 or 8 animals in each group. **p* < 0.05; Student's *t* test.

Table 3. Effect of Treatment with Adouetine X (6) (10 nmol/site; i.t.) after 60 and 120 min on Spontaneous Activity (number of crossings and rearings) in the Open-Field Test in Mice^a

time (min)	crossings		rearings	
	vehicle	adouetine X (6)	vehicle	adouetine X (6)
60	51.8 ± 5.3	39.8 ± 3.1	7.0 ± 0.7	6.0 ± 1.8
120	22.1 ± 5.2	31.3 ± 4.2	3.8 ± 1.0	4.5 ± 2.1

^a Values represent means ± SEM for 6 animals in each group.

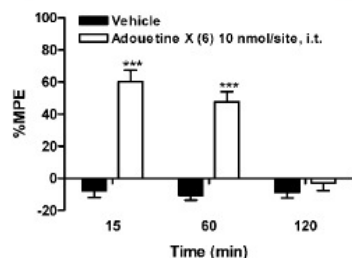


Figure 2. Time course of the antinociceptive effect of adouetine X (6) after intrathecal administration (10 nmol/site) in the tail-flick test in mice (*n* = 7 or 8). Data are expressed as means ± SEM (% MPE). ****p* < 0.001, two-way ANOVA followed by Bonferroni post hoc test.

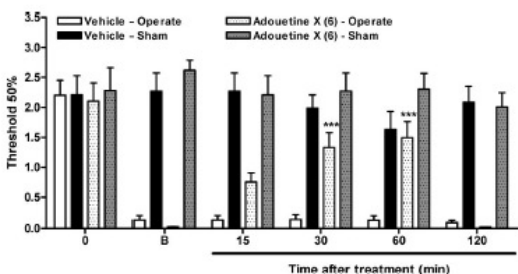


Figure 3. Mechanical sensitivity to von Frey hair filament stimulation of the right hindpaw in sham-operated and operated animals in partial ligation of the sciatic nerve (PLSN). Mice were treated with adouetine X (6) (10 nmol/site, i.t.) or vehicle at different intervals of time after drug treatment. Data are expressed as means ± SEM (% MPE). Significantly different from control values: ****p* < 0.001 (two-way analysis of variance followed by Bonferroni post hoc test).

activity at 10 μM in synaptosomes.¹⁷ It has been demonstrated that pro-nociceptive mediators can stimulate plasma membrane Ca²⁺-ATPase, keeping the intracellular Ca²⁺ concentration low and increasing the excitability of sensory neurons that conduct the nociceptive stimulus to the CNS.¹⁸ Thus, the inhibition of plasma membrane Ca²⁺-ATPase in sensory neurons could produce analgesia. Additionally, i.t. injection of Na⁺/K⁺ ATPase inhibitors, such

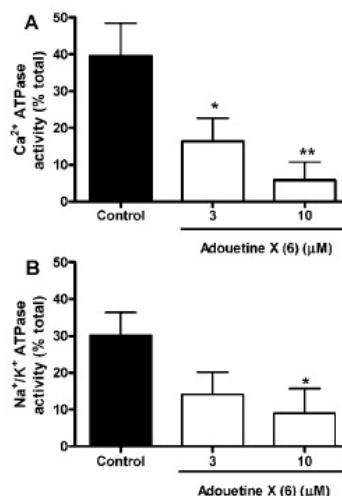


Figure 4. Ca²⁺-ATPase (A) and Na⁺/K⁺-ATPase (B) activity in the presence of 3 or 10 μM adouetine X (6). Values represent means ± SEM (% total activity) of determinations made in six separate experiments. Significantly different from control values: ***p* < 0.01, **p* < 0.05 (one-way ANOVA followed by SNK post hoc test).

as ouabain, can produce an antinociceptive effect in the tail-flick test in rodents.¹⁹ Moreover, adouetine X (6) could act indirectly on ATPases through the inhibition of Ca²⁺/calmodulin-dependent protein kinase. In fact, plasma membrane Ca²⁺-ATPase and Na⁺/K⁺ ATPase are modulated by Ca²⁺/calmodulin-dependent protein kinase,^{20,21} and some cyclopeptide alkaloids can cause inhibition of Ca²⁺/calmodulin-dependent protein kinase II.⁵ A putative action of adouetine X (6) on Ca²⁺/calmodulin-dependent protein kinase could also explain its antinociceptive effect on neuropathic pain. In fact an increased activity of Ca²⁺/calmodulin-dependent protein kinase II was observed in the spinal cord after peripheral nerve injury. Moreover the intrathecal administration of an inhibitor of calcium calmodulin-dependent kinase II attenuated the development of mechanical allodynia in rats.^{22,23} Further studies must be carried out to assess the effect of adouetine X (6) on Ca²⁺/calmodulin-dependent protein kinase activity.

In summary, we have found that adouetine X (6) showed an analgesic effect in acute and chronic neuropathic pain models, without inducing detectable undesirable side effects. The effect of adouetine X (6) seems to be related to the inhibition of Ca²⁺-ATPase and Na⁺/K⁺ ATPase. These findings suggest that the cyclopeptide alkaloid adouetine X (6) is an interesting prototype analgesic drug.

Experimental Section

General Experimental Procedures. Melting points were determined in an MQAPF-301 melting point apparatus and are uncorrected. Optical rotations were taken on a Perkin-Elmer 341 digital polarimeter. NMR

spectra were acquired on a Bruker DPX-400 operating at 400 and 100 MHz, for ^1H and ^{13}C , respectively. Chemical shifts are given in ppm using TMS as an internal standard. Mass spectra MS: 70 eV. FAB-MS were obtained on a VG analytical 70±150-S mass spectrometer equipped with a FAB ion source from a 3-nitrobenzyl alcohol matrix.

Plant Materials. The root bark of *Discaria americana* Gill. & Hook. (Rhamnaceae) and *Scutia buxifolia* Reissek (Rhamnaceae) was collected in São Sepé, Brazil (29°45'30" S, 54°20'33" W) in December 2000 and January 2003 and authenticated by Prof. Renato Záchia and Prof. Adelino Alvarez Filho, Department of Botany, Universidade Federal de Santa Maria, RS Brazil, where the specimen samples (SMDB 2688, SMD-B146) were deposited, respectively. The roots of *Melochia chamaedrys* A. St.-Hil. (Sterculiaceae) were collected in February–May 2001 in the state of Rio Grande do Sul, Brazil, and authenticated by Prof. Thais S. C. Dorow, Department of Botany, Universidade Federal de Santa Maria, RS, Brazil, where a specimen sample (SMDB9262) was deposited at the herbarium of the Federal University of Santa Maria.

Extraction and Isolation. The alkaloids franganine (1) and discarine B (2) were isolated from the roots of *D. americana* (Rhamnaceae), whereas scutianines B (3), C (4), and D (5) were isolated from the roots of *S. buxifolia* (Rhamnaceae), and adouetine X (6) was isolated from the roots of *M. chamaedrys* (Sterculiaceae), as described previously by Morel et al.,^{24–26} respectively.

Animals. Adult male Swiss mice (25–35 g; $n = 6$ or 7) were used in all experiments. The animals were housed in groups of 20 to a cage at controlled temperature (22 ± 1 °C) with a 12 h light/dark cycle and with standard laboratory chow and tap water ad libitum. Each animal was used only once. The experiments reported in this study were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals.²⁷ The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of treatment using the tested compounds.

Treatment. Test compounds were injected intrathecally (i.t.) into the spinal column between L5 and L6 of unanesthetized mice, as reported previously.²⁸ The dose used for the animal treatment was 10 nmol/site (5 μL) for all compounds, and the vehicle used was 1% DMSO (dimethylsulfoxide) plus 1% Tween 80 in PBS. The dose of 10 nmol/site was used because it was the largest amount of compound that could be diluted. The rectal temperature, tail-flick reaction time, and locomotor activity were measured in each group of animals, as described earlier.²⁹

Locomotor Activity. To evaluate possible nonspecific muscle relaxant or sedative effects of the test compounds, the spontaneous locomotor performance was measured in the open-field test.¹⁵ The apparatus was a rectangular arena (28 × 18 × 12 cm) with the floor divided into 18 equal squares. The number of areas crossed with all paws and the number of rearing responses were recorded. Mice were placed in an open-field 5 min after injection of the test compounds (10 nmol/site), and the spontaneous activity was measured during the next 10 min period.

Rectal Temperature. Since many drugs can change body temperature, the rectal temperature was measured to evaluate if the test compounds were capable of causing any variation in this parameter.³⁰ Baseline temperatures were recorded before test drug administration, measuring approximately 37–38 °C for each mouse. The body temperature was measured again 15 and 60 min after the administration of each test compound (10 nmol/site) was tested and $\Delta^\circ\text{C}$ was used to calculate the change in body temperature ($\Delta^\circ\text{C} = \text{test temperature} - \text{baseline temperature}$).

Tail-Flick Test. In order to evaluate the possible antinociceptive (analgesic) effect of the compounds tested, the tail-flick reaction time to a heat stimulus was used. The warm-water tail immersion test was performed according to a method described previously,³¹ using a water bath with the temperature maintained at 48 °C. Before vehicle or test compound administration (10 nmol/site; i.t.), the baseline latency period (6–7 s) was determined. At 15 and 60 min after treatment with control or test compounds, tail-flick latency was reassessed, and differences in relation to the baseline values were calculated. An 18 s maximum latency was used to avoid tissue damage. Antinociception was expressed as percentage of maximum possible effect (MPE), which was calculated as $\% \text{MPE} = [(\text{test latency control latency}) / (10 - \text{control latency})] \times 100$. Percent MPE was calculated for each mouse using at least six mice per test compound.

Determination of Time Course and Potency for Antinociception Caused by Adouetine X (6). To evaluate the time course of the antinociceptive effect of adouetine X (6), a dose of 10 nmol/site (i.t.) was used. The baseline latency (6–7 s) was measured before the injection of the compound. At 0.25, 1, and 2 h after the administration of adouetine X (6), the latency was reassessed and the MPE (%) was calculated for each time. In addition, the rectal temperature and locomotor activity were observed at 0.25, 1, and 2 h after the administration of adouetine X (6) in the same animals.

The ID_{50} value for adouetine X (6) was measured by the up-and-down method in the tail-flick test.³² The up-and-down method is a procedure that has been confirmed to reduce the number of animals needed to determine LD_{50} values without compromising reliability. Subjects were evaluated sequentially such that the results of each animal determined the dose that the subsequent animal received. The first animal was given a dose of drug that was close to the expected ED_{50} and then evaluated in the tail-flick test. If the %MPE value exceeded 50%, then the dose for the next animal was incrementally decreased (e.g., decreased by log dose of 0.301). Alternatively, if the tail-flick latency did not exceed the 50% MPE criterion, then the dose was incrementally increased (e.g., increased by log dose of 0.301). The first dose tested was 10 nmol/site. If the animal showed a MPE higher than 50%, the dose was decreased logarithmically. However, when the MPE was lower than 50%, the dose was increased. Six animals were used to determine the ID_{50} value for this compound. The ID_{50} value was calculated from appropriate tables given by Dixon.³³ A confidence limit (C.L.) of 95% was determined by the following equation: $95\% \text{ C.L.} = \text{dose increment} \times (\sqrt{2/n}) \times 1.96$. In addition, tree vehicle-treated subjects were evaluated to control for handling and stress factors.

Partial Sciatic Nerve Ligation. A partial nerve ligation of the sciatic nerve (PLSN) was produced by tightly ligating a common sciatic nerve of the left hindpaw under deep anesthesia.³⁴ Mice were anaesthetized intraperitoneally (i.p.) using a mixture of ketamine (90 mg/kg) and xylazine (3 mg/kg). The same surgical procedure was used for sham mice except for nerve ligation. The experiments were conducted 1 week after the surgery. The operated and sham animals were treated with adouetine X (6) (10 nmol/site) or vehicle. The mechanical allodynia was evaluated at different time intervals (0.25, 0.5, 1, and 2 h) as a reduction in mechanical threshold to withdrawal using von Frey hair filaments, as described before.³⁵

[^3H]-Glutamate Binding Assay. Cerebral cortices obtained from mice were used for membrane preparation, carried out as described before.³⁶ Sodium-independent [^3H]-L-glutamate binding to cerebral plasma membranes was investigated as described previously.³⁷ Briefly, membranes were incubated in a 0.5 mL reaction mixture containing 50 mM Tris-acetate buffer (pH 7.4), 40 nM [^3H]-L-glutamate, and 10 μM of each test compound (dissolved in water and 1% DMSO). Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by filtration using GF/B glass microfiber filters. Dried filters were transferred to Eppendorf tubes containing scintillation fluid, and the radioactivity was determined with a Packard scintillation spectrometer at 40–45% efficiency. Specific binding was calculated as the difference between total binding and nonspecific binding, which was measured in the presence of a 10 000-fold excess (4 mM) of unlabeled L-glutamate. The assays were performed in triplicate, and the results represent the combined data from three individual experiments. [^3H]-L-Glutamate was purchased from Amersham (São Paulo, Brazil). Protein concentration was measured using bovine serum albumin as standard.³⁸

ATPase Activity Assay. The ATPase activity was estimated by a method adapted from Mark et al.³⁹ and Figuera et al.⁴⁰ The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of the synaptosomal fraction. Synaptosomes were isolated from mice brains by a method adapted from Rodrigues et al.⁴¹ Protein concentration was assayed by the method described previously,³⁸ using bovine serum albumin as a standard. The method allows the quantification of distinct Mg^{2+} -dependent ATPase activities (Na^+/K^+ -ATPase or Ca^{2+} -ATPase activity) in the same sample. Activities were measured in triplicate in covered 96-well microtiter plates at 37 °C. Fifty microliters of assay buffer (5 mM HEPES, 80 mM NaCl, 15 mM KCl, 3 mM MgCl_2 , and 0.1 mM EGTA, pH 7.1) was placed in each plate after 50 μL of another assay buffer (5 mM HEPES and 320 mM sacrose) containing 1 μg of membrane protein was added to each well. The Na^+/K^+ -ATPase activity was determined by subtracting the ouabain (0.2 mM)-sensitive activity

from the overall Mg^{2+} -ATPase activity level, and the percentage of activity in relation to the total was calculated from that observed for the control and adouetine X (6) (3 and 10 μ M). The Ca^{2+} -ATPase activity was determined by subtracting the activity measured in the presence of Ca^{2+} and ouabain from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA) and the presence of ouabain from that observed for the control and adouetine X (6) (3 and 10 μ M). The plate was preincubated at 37 °C for 10 min, and the assay was started with the addition of 50 μ L of adenosine triphosphate (ATP) to a final concentration of 3 mM, making the final reaction volume 160 μ L. After 60 min, the reaction was terminated by the addition of 50 μ L of 5.7% ammonium molybdate in 6 N H_2SO_4 plus 10 μ L of 8% acid ascorbic. The level of inorganic phosphate present, quantified by the colorimetric method of Fiske and Subbarow,⁴² was used as a measure of ATPase activity. Plates were read on a Fisher Biotech Microkinetics Reader BT 2000 at 630 nm. The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that was included in the assay procedure. Ouabain, adenosine triphosphate, and ammonium molybdate ascorbic acid were purchased from Sigma (St. Louis, MO). Values reported represent the mean and SD of at least six separate experiments.

Statistical Analysis. The results of antinociceptive activity and the side effects were expressed as means \pm SEM, which are reported as geometric means accompanied by their respective 95% confidence limits. Data were analyzed by Student's *t* test and one-way or two-way analysis of variance (ANOVA). Post hoc tests (Student–Newman–Keuls, SNK) were carried out when appropriate. The level of significance was set at $p < 0.05$.

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Supporting Information Available: Physical and NMR spectroscopic data for the cyclopeptide alkaloids used in this study. This information is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- Hesham, R.; El-Seedi, H. R.; Zahra, M. H.; Goransson, U.; Verpoorte, R. *Phytochem. Rev.* **2007**, *6*, 143–165.
- Joullié, M. M.; Richard, D. *J. Chem. Commun.* **2004**, *21*, 2011–2015.
- Morel, A. F.; Maldaner, G.; Ilha, V.; Missau, F.; Silva, U. F.; Dalcol, I. I. *Phytochemistry* **2005**, *66*, 2571–2576.
- Giacomelli, S. R.; Maldaner, G.; Gonzaga, W. A.; Garcia, C. M.; da Silva, U. F.; Dalcol, I. I.; Morel, A. F. *Phytochemistry* **2004**, *65*, 933–937.
- Han, Y. N.; Hwang, K. H.; Han, B. H. *Arch. Pharm. Res.* **2005**, *28*, 159–163.
- Hwang, K. H.; Han, Y. N.; Han, B. H. *Arch. Pharm. Res.* **2001**, *24*, 202–206.
- Ma, Y.; Han, H.; Eun, J. S.; Kim, H. C.; Hong, J. T.; Oh, K. W. *Biol. Pharm. Bull.* **2007**, *30*, 1748–1753.
- Park, C. W.; Kim, Y. S.; Han, B. H.; Park, J. W.; Jang, I. J.; Choi, J. Y.; Chung, D. B.; Lee, Y. S.; Kim, M. S. *Kor. J. Toxicol.* **1996**, *12*, 181–194.
- Woolf, C. J.; Mannion, R. J. *Lancet* **1999**, *353*, 1959–1964.
- Negus, S. S.; Vanderah, T. W.; Brandt, M. R.; Bilsky, E. J.; Becerra, L.; Borsook, D. *J. Pharmacol. Exp. Ther.* **2006**, *319*, 507–514.
- Millan, M. J. *Prog. Neurobiol.* **2002**, *66*, 355–474.
- Lee, S. S.; Su, W. C.; Liu, K. C. *Phytochemistry* **2001**, *58*, 1271–1276.
- Bleakman, D.; Alt, A.; Nisenbaum, E. S. *Semin. Cell. Dev. Biol.* **2006**, *17*, 592–604.
- Dickenson, A. H.; Chapman, V.; Green, G. M. *Gen. Pharmacol.* **1997**, *28*, 633–638.
- Archer, J. *Anim. Behav.* **1973**, *21*, 205–235.
- Besson, J. M. *Lancet* **1999**, *353*, 1610–1615.
- Brodsky, J. L.; Guidotti, G. *Am. J. Physiol.* **1990**, *258*, C803–11.
- Usachev, Y. M.; DeMarco, S. J.; Campbell, C.; Strehler, E. E.; Thayer, S. A. *Neuron* **2002**, *33*, 113–122.
- Zeng, W.; Chen, X.; Dohi, S. *J. Pain* **2007**, *8*, 983–988.
- Werth, J. L.; Usachev, Y. M.; Thayer, S. A. *J. Neurosci.* **1996**, *16*, 1008–1015.
- Netticadan, T.; Temas, R. M.; Kawabata, K.; Dhalla, N. S. *Circ. Res.* **2000**, *86*, 596–605.
- Dai, Y.; Wang, H.; Ogawa, A.; Yamanaka, H.; Obata, K.; Tokunaga, A.; Noguchi, K. *Eur. J. Neurosci.* **2005**, *21*, 2467–2474.
- Luo, F.; Yang, C.; Chen, Y.; Shukla, P.; Tang, L.; Wang, L. X.; Wang, Z. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 267–275.
- Morel, A. F.; Giacomelli, S. R.; Maldaner, G.; Gonzaga, W. A.; Garcia, C. M.; Silva, U. F.; Dalcol, I. I. *Phytochemistry* **2004**, *65*, 933–937.
- Morel, A. F.; Maldaner, G.; Ilha, V.; Bissau, F.; Silva, U. F.; Dalcol, I. I. *Phytochemistry* **2005**, *66*, 2571–2576.
- Morel, A. F.; Dias, G. C. D.; Gressler, V.; Hoenzel, S. C. S. M.; Silva, U. F.; Dalcol, I. I. *Phytochemistry* **2007**, *68*, 668–672.
- Zimmermann, M. *Pain* **1983**, *16*, 109–110.
- Hylden, J. K. L.; Wilcox, G. L. *Eur. J. Pharmacol.* **1980**, *67*, 313–316.
- Compton, D.; Aceto, M.; Lowe, J.; Martin, B. *J. Pharmacol. Exp. Ther.* **1996**, *277*, 586–594.
- Otuki, M. F.; Lima, F. V.; Malheiros, A.; Cechinel-Filho, V.; Delle Monache, F.; Yunes, R. A.; Calixto, J. B. *Life Sci.* **2001**, *69*, 2225–2236.
- D'Amour, F. E.; Smith, D. L. *J. Pharmacol. Exp. Ther.* **1941**, *72*, 74–79.
- Lichtman, A. H. *J. Pharmacol. Toxicol. Methods* **1998**, *40*, 80–85.
- Dixon, W. J. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 441–462.
- Ferreira, J.; Beirith, A.; Mori, M. A.; Arajo, R. C.; Bader, M.; Pesquero, J. B.; Calixto, J. B. *J. Neurosci.* **2005**, *25*, 2405–2412.
- Kassuya, C. A.; Ferreira, J.; Claudino, R. F.; Calixto, J. B. *Br. J. Pharmacol.* **2007**, *150*, 727–737.
- Emanuelli, T.; Antunes, V. F.; Souza, D. O. *Biochem. Mol. Biol. Int.* **1998**, *44*, 1265–1272.
- Sinhorin, V. D.; Carpes, M. J.; Roehrs, C.; Zimmer, M. F.; Sauzem, P. D.; Rubin, M. A.; Correia, C. R.; Mello, C. F. *Pharmacol., Biochem. Behav.* **2003**, *76*, 295–299.
- Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
- Mark, R. J.; Hensley, K.; Butterfield, D. A.; Mattson, M. P. *J. Neurosci.* **1995**, *15*, 6239–6249.
- Figuera, M. R.; Royes, L. F.; Furian, A. F.; Oliveira, M. S.; Fiorenza, N. G.; Frussa-Filho, R.; Petry, J. C.; Coelho, R. C.; Mello, C. F. *Neurobiol. Dis.* **2006**, *22*, 611–623.
- Rodrigues, R. J.; Almeida, T.; Richardson, P. J.; Oliveira, C. R.; Cunha, R. A. *J. Neurosci.* **2005**, *25*, 6286–6295.
- Fiske, C. H.; Subbarow, Y. *J. Biol. Chem.* **1925**, *66*, 375–400.

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