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TESE DE DOUTORADO

Avaliação *in vitro* e *in vivo* de 2-(4-(metiltio(metilsulfonil))fenil)-3-substituído tiazolidin-4-onas como agentes multialvo para a doença de Alzheimer: uma nova abordagem terapêutica.

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Orientador: Prof. Dr. Wilson João Cunico Filho
Co-orientadora: Profª. Drª. Roselia Maria Spanevello

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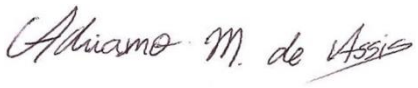
Banca examinadora:

Prof. Dr. Wilson Cunico (Orientador) – Doutor em Química pela Universidade Federal de Santa Maria.



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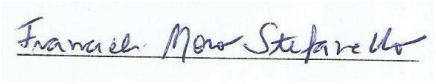

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RESUMO

SILVA, Daniel Schuch da. Avaliação in vitro e in vivo de 2-(4-(metiltio(metilsulfonil))fenil)-3-substituído tiazolidin-4-onas como agentes multialvo para a doença de Alzheimer: uma nova abordagem terapêutica. 2020. 101f. Tese (Doutorado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas.

A incidência de demência aumenta com o avanço da idade e leva a um declínio nas funções cognitivas e memória. A causa mais comum de demência é a neurodegenerativa progressiva doença de Alzheimer (DA). A fisiopatologia dessa doença é complexa e sua exata etiologia ainda não é clara, porém sabe-se do envolvimento do sistema colinérgico no desenvolvimento dos sintomas característicos da DA. Atualmente são poucas as opções terapêuticas disponíveis e as mesmas não são capazes de impedir a progressão da doença, levando apenas ao alívio dos sintomas. Esses medicamentos possuem importantes efeitos adversos e possuem uma maior efetividade em estágios mais leves, perdendo eficácia em estágios mais avançados da doença. Tendo em vista a complexidade da DA e as poucas opções terapêuticas para o manejo do quadro dos pacientes, este estudo teve como objetivo avaliar o efeito de diferentes 1,3-tiazolidin-4-onas derivadas do 4-(metiltio)benzaldeído e do 4-(metilsulfonil)benzaldeído em estudos experimentais *in vitro* sobre a atividade da acetilcolinesterase (AChE) e *in vivo* em modelo de amnésia induzido pela escolopamina. Inicialmente foi avaliado o potencial dos compostos na inibição da AChE em córtex, hipocampo, e linfócitos de ratos, e quanto ao potencial de ligação, encaixe tridimensional e tipo de inibição gerada a partir da interação com a enzima. Essa triagem *in vitro* serviu de base para aprofundar o conhecimento e descrever os potenciais efeitos das tiazolidinonas. Três dos compostos testados destacaram-se nos valores de IC_{50} para a inibição da AChE: os compostos **1b** (13.81 μM e 3.13 μM para o córtex e hipocampo, respectivamente), **1c** (55.36 μM e 44.33 μM) e **2b** (3.11 μM em ambas estruturas). Os três compostos também foram capazes de inibir a AChE em linfócitos. Os resultados revelaram que o substituinte derivado da *N*-(3-aminopropil)piperidina (**b**) se demonstrou importante para a inibição da AChE. O docking molecular revelou um encaixe tridimensional semelhante ao donepezil e a própria ACh, e os estudos de cinética enzimática demonstraram um tipo de inibição mista para os três compostos. Devido ao fato de o composto **1b** ter sido capaz de inibir ambas as isoformas G1 e G4 da AChE, este foi selecionado para a sequência dos estudos *in vivo*. Assim, o 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one (codificado **DS12**) foi levado a novos estudos para avaliar o seu efeito preventivo frente ao modelo de déficit de memória induzido pela escopolamina. Assim, o pré-tratamento de 7 dias com **DS12** (5 ou 10 mg/kg) revelou-se capaz de evitar o declínio da memória causado pela escopolamina (1 mg/kg), através da esquiva inibitória, e de prevenir as alterações neuroquímicas nos animais causadas pela escopolamina: aumento dos níveis de espécies reativas, como as EROs; redução da atividade das enzimas antioxidantes catalase (CAT) e superóxido dismutase (SOD); aumento da atividade da AChE em córtex, hipocampo e em linfócitos, e da BuChE em soro; e redução da atividade da Na^+/K^+-ATPase. Ressalta-se, portanto, que o **DS12** preveniu as alterações sem revelar alterações em marcadores hepáticos e renais. Dessa forma, o **DS12** surge como um importante composto multi-alvo para evitar alterações neurobioquímicas em modelos experimentais de DA.

Palavras-chave

1,3-tiazolidin-4-onas; escopolamina; estresse oxidativo; acetilcolinesterase; demência.

ABSTRACT

SILVA, Daniel Schuch da. Avaliação in vitro e in vivo de 2-(4-(metiltio(metilsulfonil))fenil)-3-substituído tiazolidin-4-onas como agentes multialvo para a doença de Alzheimer: uma nova abordagem terapêutica. 2020. 101f. Tese (Doutorado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas.

Incidence of dementia increases with age and leads to a decline in cognitive functions and memory. The most common cause of dementia is a progressive neurodegenerative Alzheimer's disease (AD). The pathophysiology is complex and its exact etiology is still unclear, but it is known the involvement of the cholinergic system in the development of characteristic symptoms of AD. Currently, few therapeutic options are available and these do not prevent disease progression, causing only symptom relief. These drugs have significant side effects and are more effective in early stages, losing effectiveness in more advanced stages of the disease. Due to the complexity and the few therapeutic options for the management of patients, this research aimed to evaluate the effect of different 1,3-thiazolidin-4-ones derived from 4-(methylthio)benzaldehyde and 4-(methylsulfonyl)benzaldehyde in experimental *in vitro* studies on the acetylcholinesterase (AChE) activity and through *in vivo* amnesia model induced by scolopamine. The potential of compounds to inhibit AChE was evaluated in cerebral cortex, hippocampus, and lymphocytes of rats, as well as the binding potential, the three-dimensional fit and the type of inhibition generated from the interaction with the enzyme. This screening stimulated further studies by our research group, to deepen knowledge and describe the potential effects of thiazolidinones. Three of tested compounds stood out in IC_{50} values for AChE inhibition in rat brain: **1b** (13.81 μM and 3.13 μM in cerebral cortex and hippocampus, respectively), **1c** (55.36 μM and 44.33 μM) and **2b** (3.11 μM in both structures). These compounds were also able to inhibit AChE in lymphocytes. The results revealed that the substituent derived from *N*-(3-aminopropyl)piperidine (**b**) demonstrated important for AChE inhibition. Molecular docking revealed a three-dimensional fit like donepezil and ACh, and studies of enzymatic kinetics demonstrated a mixed inhibition type for these three compounds. Due to the fact of compound **1b** was capable to inhibit both G1 and G4 AChE isoforms, it was selected for the sequence of *in vivo* trails. Therefore, 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one (encoded **DS12**) has been taken into further studies to assess its preventive effect against to scopolamine-induced memory deficit model. 7-day pretreatment with **DS12** (5 or 10 mg/kg) proved to be able to prevent the memory decline caused by scopolamine (1 mg/kg), through inhibitory avoidance test, and to prevent neurochemical changes caused by scopolamine: increased levels of reactive species, such as ROS; reduced activity of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD); increased AChE activity in cerebral cortex, hippocampus and lymphocytes, and of BuChE activity in serum; and reduction of Na^+/K^+-ATPase activity. Therefore, it is noteworthy that **DS12** prevented the effects of scopolamine without demonstrating changes in hepatic and renal markers. Thus, **DS12** emerges as a multi-target compound to prevent neurobio-chemical changes in experimental models of AD.

Keywords

1,3-thiazolidin-4-ones; scopolamine; oxidative stress; acetylcholinesterase; dementia.

LISTA DE FIGURAS

Obs: As demais figuras e tabelas desta tese fazem parte do artigo e/ou do manuscrito com numerações sequenciais e, portanto, não estão elencadas nesta lista.

LISTA DE ABREVIATURAS E SIGLAS

ACh	Acetilcolina
AChE	Acetilcolinesterase
BuChE	Butirilcolinesterase
CAS	Sítio ativo catalítico
CAT	Catalase
CEEA	Comitê de Ética em Experimentação Animal
ChE	Colinesterase
CoIQ	Subunidade de colágeno denominada Q
DA	Doença de Alzheimer
DS	Solúvel em detergente
ERO	Espécies reativas de oxigênio
GSH	Glutationa reduzida
GSH-Px	Glutationa peroxidase
IL-6	Interleucina 6
LPS	Lipopolissacarídeo
LCR	Líquido cefalorraquidiano
NMDA	*N*-metil-D-aspartato
PAS	Sítio periférico aniônico
PET	Emissão de pósitrons
SNC	Sistema Nervoso Central
SOD	Superóxido dismutase
SS	Solúvel em sal
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TNF-α	Fator de necrose tumoral α

SUMÁRIO

1. INTRODUÇÃO

O aumento da idade pode levar a uma síndrome chamada demência, que causa um declínio nas funções cognitivas, incluindo memória, linguagem, comportamento e desempenho de atividades diárias básicas. A causa mais comum de demência é a doença neurodegenerativa progressiva conhecida como doença de Alzheimer (DA), que tem como principal fator de risco idade avançada. A DA é responsável por mais de 80% dos casos diagnosticados de demência, representando cerca de 46,8 milhões de pessoas diagnosticadas em todo o mundo, e os números têm aumentado ao longo dos anos. Além disso, a proporção de mortes relacionadas à DA tem aumentado, destacando-se uma elevação de 89% entre 2000 e 2014 nos Estados Unidos (Bai et al, 2019; Denver & McClean, 2018; Weller & Budson, 2018).

A fisiopatologia da DA é complexa e várias hipóteses tentam explicar a patogênese da doença, no entanto, a etiologia exata não é clara (Bai et al, 2019; Joe & Rigman, 2019; Kumar et al, 2016). A hipótese colinérgica propõem que a perda de memória e a disfunção cognitiva, principais sintomas da DA, são causadas pelo baixo nível de acetilcolina (ACh). Nesse sentido, as colinesterases têm um importante papel regulador na neurotransmissão colinérgica. Sabe-se que a acetilcolinesterase (AChE) hidrolisa rapidamente a ACh e desempenha o papel principal de enzima reguladora nas sinapses colinérgicas, porém, a butirilcolinesterase (BuChE) serve como um co-regulador da transmissão colinérgica e, enquanto os níveis de AChE no cérebro diminuem com a progressão da DA, os níveis de BuChE permanecem constantes ou se elevam expressivamente acima dos níveis normais (Bai et al, 2019; Kumar et al, 2018; Xie et al, 2013).

Vários estudos relataram que o comprometimento cognitivo na doença de Alzheimer está correlacionado com danos aos neurônios colinérgicos (Ferreira-Vieira et al, 2016; Mohammad et al, 2017; Mufson et al, 2008). Em um contexto farmacológico, a inibição da atividade cerebral da AChE tem sido o principal alvo terapêutico para aumentar os níveis de ACh nas sinapses, levando à melhora dos déficits cognitivos na doença de Alzheimer (Du et al, 2018; Ferris & Farlow, 2013; Kumar et al, 2018).

A terapia atualmente utilizada para pacientes com DA se baseia em duas classes de medicamentos, os anticolinesterásicos e a memantina, antagonista do receptor NMDA. Os inibidores da AChE donepezil, rivastigmina e galantamina são recomendados para pacientes com DA nos diferentes estágios, porém a eficácia desses é maior em estágios

mais leves, reduzindo com a progressão da doença. Ainda, esses medicamentos apresentam importantes efeitos adversos e representam um custo elevado aos pacientes (Renn et al 2018; Khoury et al 2018; Weller & Budson, 2018).

Tendo em vista as restritas opções terapêuticas, que não são capazes de gerar resultados significativos em estágios avançados, é de extrema importância o estudo de novas classes de compostos na busca de melhores terapias, com menos efeitos colaterais, e que possam beneficiar os portadores de doenças neurodegenerativas como a DA. Nos últimos anos, houve um grande interesse no desenvolvimento de novos inibidores das colinesterases, e as 1,3-tiazolidin-4-onas, importante grupo de compostos heterocíclicos, já demonstrou possuir um amplo espectro de atividades biológicas (Manjal et al, 2017; Tripathi et al, 2014).

Iyer e colaboradores (2015) demonstraram uma potencial afinidade entre algumas tiazolidinonas e a AChE através de análises de docking molecular. Sadashiva e colaboradores (2009) revelaram ação de 1,3-tiazolidin-4-onas sob receptores colinérgicos corticais, demonstrando a capacidade desses compostos em reduzir o déficit de memória induzido pela escopolamina em ratos. Além disso, Berwaldt e colaboradores (2019) revelaram o potencial inibitório da AChE por 1,3-benzotiazin-4-onas em córtex cerebral e hipocampo de ratos, compostos estes estruturalmente semelhantes às tiazolidinonas.

Um modelo amplamente utilizado para estudar o efeito de novos compostos em doenças relacionadas à demência, como a DA, é o modelo de déficit de memória induzido pela escopolamina (Ishola et al, 2019; Malviya et al, 2009; Sadashiva et al, 2009; Tang, 2019). A escopolamina é um antagonista do receptor muscarínico usado em vários estudos neurocomportamentais por sua capacidade de prejudicar a memória e o aprendizado. Entre os parâmetros celulares e moleculares alterados pelo tratamento com escopolamina, estão o aumento do estresse oxidativo, disfunção mitocondrial e a neuroinflamação, bem como a diminuição dos níveis de ACh e o comprometimento das defesas antioxidantes, alterações essas relacionadas a fisiopatologia da DA (Tang, 2019).

Diante do exposto, o objetivo deste estudo é investigar o potencial inibitório de uma série de 1,3-tiazolidin-4-onas frente a atividade da AChE em cérebro e em linfócitos de ratos. Além disso, determinar grupamentos importantes para a atividade biológica, verificar a afinidade dos compostos e o encaixe dos mesmos na enzima através de modelagem molecular, e verificar o tipo de inibição gerada por análise de cinética enzimática. Em seguida, este estudo se propõe a avaliar o efeito antiamnésico do composto de maior potencial nos ensaios *in vitro* frente ao modelo de déficit de memória

induzido pela escopolamina em ratos, e avaliar marcadores bioquímicos para determinar toxicidade sistêmica, efeitos sobre o estresse oxidativo, sobre o sistema colinérgico e sobre a atividade da Na^+/K^+-ATPase.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o efeito de diferentes 1,3-tiazolidin-4-onas derivadas do 4-(metiltio)benzaldeído e do 4-(metilsulfonil)benzaldeído em modelos experimentais *in vitro* sobre a atividade da acetilcolinesterase (AChE) e *in vivo* em modelo de amnésia induzido pela escolopamina.

2.2. OBJETIVOS ESPECÍFICOS

a) Investigação do efeito de uma série de 1,3-tiazolidin-4-onas frente a inibição da AChE a partir de testes *in vitro* em córtex, hipocampo e linfócitos de ratos.

- Avaliar o potencial de diferentes 1,3-tiazolidin-4-onas na inibição da AChE em córtex e hipocampo de ratos adultos.
- Avaliar o potencial das 1,3-tiazolidionas quanto a inibição da AChE periférica em linfócitos de ratos.
- Verificar a capacidade de inibição nas isoformas G1 e G4 da AChE pelas tiazolidinonas.
- Analisar a conformação tridimensional e a energia livre de ligação envolvida na interação das tiazolidinonas testadas com a enzima AChE a partir de análises de docking molecular.
- Verificar o tipo de inibição gerada pelas 1,3-tiazolidin-4-onas na enzima AChE a partir de análises de cinética enzimática.

b) Investigação do efeito protetor da 2-(4-(metiltio)fenil)-3-(3-(piperidin-1-il)propil)tiazolidin-4-ona (**DS12**) frente alterações nos parâmetros comportamentais, neuroquímicos e bioquímicos em modelo animal de déficit de memória induzido pela escopolamina.

- Avaliar o potencial da **DS12** na prevenção do déficit de memória induzido pela escopolamina em ratos, através do teste comportamental da esquiva inibitória.
- Verificar o efeito protetor da **DS12** em relação a disfunção das enzimas AChE e Na^+/K^+-ATPase induzida pela escopolamina em córtex e hipocampo de ratos.

- Verificar o potencial de prevenção da **DS12** frente as alterações das colinesterases AChE e BuChE periféricas em linfócitos e soro de ratos, respectivamente, a partir da indução com escopolamina.

- Analisar o efeito preventivo da **DS12** sobre as defesas antioxidantes e as espécies reativas em relação ao estímulo desses marcados causados pela escopolamina em córtex e hipocampo de ratos.

- Analisar o efeito da **DS12** frente à parâmetros séricos para determinar potenciais efeitos tóxicos renais e hepáticos.

3. REVISÃO DA LITERATURA

3.1. DOENÇA DE ALZHEIMER (DA)

A DA é uma das doenças neurodegenerativas progressivas mais comuns e é a maior causa de quadros de demência na sociedade. A demência é caracterizada pelo declínio progressivo de dois ou mais domínios cognitivos, levando a uma diminuição da capacidade de desenvolver atividades diárias (Kumar et al, 2015; Kumar et al, 2016; Weller & Budson, 2018).

A DA é responsável por mais de 80% dos casos diagnosticados de demência, além disso, as taxas de mortes relacionadas à DA tem se elevado ao longo dos anos. Estimativas sugerem que 47 milhões de pessoas no mundo haviam sido acometidas por esta doença e a perspectiva é que esse número aumente até 2050 para aproximadamente 131 milhões de pessoas (Denver & McClean, 2018). Nesse sentido, é de extrema importância o desenvolvimento de novas terapias que possam retardar ou impedir a progressão da doença (Denver & McClean, 2018; Weller & Budson, 2018; Xie et al, 2013).

Tendo em vista que a qualidade da assistência terapêutica tem evoluído ao longo dos anos, a expectativa de vida da população mundial tem aumentado, trazendo consigo o aumento da prevalência de doenças associadas ao envelhecimento, como a DA que possui como importante fator de risco o avanço da idade (Denver & McClean, 2018). Sem dúvida, a DA é um dos grandes problemas de saúde pública e um dos maiores desafios da geriatria, pois o indivíduo portador dessa patologia apresenta dificuldade ou total incapacidade de realizar atividades diárias, comprometendo assim, não só a sua qualidade de vida, mas também a de seus familiares (Inouye & Oliveira, 2004).

Assim, a marca dessa doença está na perda de memória e no déficit cognitivo, causados por mal funcionamento e morte gradual neuronal (Kumar et al, 2016). Por conta da neurodegeneração progressiva e do forte comprometimento cognitivo, a DA apresenta importantes sintomas neuropsiquiátricos, como depressão, confusão, alucinações e mesmo sintomas parkinsonianos. Os sintomas primários são a perda de memória em curto prazo, apatia e desorientação de tempo e espaço, sendo que com o passar dos anos os sintomas pioram com a deteriozação das funções cerebrais levando a incapacidade de aprendizado, perda de peso, total dependência e morte (Inouye & Oliveira, 2004). Devido a fisiopatologia complexa e a etiologia multifatorial envolvida, a busca para um tratamento adequado se torna extremamente difícil (Roldán-Peña et al, 2017).

Existem algumas classificações que dividem a DA em diferentes fases, sendo que uma dessas divisões é feita em três principais fases, sendo elas: I) fase leve onde a manifestação é detectada pelo declínio da memória de curto e médio prazo; II) fase moderada: onde ocorre piora da habilidades de comunicação, principalmente a fala, com concomitante perda de memória; III) fase grave ou tardia: ocorre comprometimento em atividades como caminhar e ingerir alimentos, bem como no reconhecimento de amigos e parentes próximos (Klimova et al, 2015).

Do ponto de vista neuropatológico a DA tem sido caracterizada por uma série de alterações genéticas, neuroquímicas e neurofisiológicas (Cummings et al, 1998; Wen et al, 2013). Nesse contexto, dentre os fatores envolvidos na fisiopatologia da DA citam-se: a) o rápido acúmulo extracelular de placas β-amilóides; b) formação de emaranhados neurofibrilares intracelulares, originados pela hiperfosforilação da proteína tau; c) baixos níveis de ACh devido a diminuição do número de neurônios colinérgicos nas diferentes áreas do cérebro. Além desses, fatores como o estresse oxidativo e o desbalanço nos níveis dos biometais também estão relacionados (Kumar et al, 2016; Martini et al, 2018; Xie et al, 2013).

Apesar do conhecimento que se tem a respeito da fisiopatologia da doença, o diagnóstico definitivo de DA ainda requer avaliação post-mortem do tecido cerebral. No entanto, já existem biomarcadores de líquido cefalorraquidiano (LCR) e de emissão de pósitrons (PET) que, combinados com a avaliação clínica, podem auxiliar o diagnóstico em pacientes vivos (Weller & Budson, 2018).

Como os pacientes com Alzheimer possuem muitos filamentos de proteína tau, tornou-se evidente o envolvimento desses na progressão da doença. Entende-se que as placas β-amiloides e as anormalidades da proteína tau, como sua fosforilação, estão envolvidas no processo inflamatório e na morte neuronal, levando ao déficit cognitivo, no entanto, o mecanismo que leva a este desfecho ainda não é totalmente compreendido (Denver & McClean, 2018; Kumar et al, 2016). Vale ressaltar que as placas amiloides são anormalidades precoces e invariáveis, enquanto os emaranhados neurofibrilares se acumulam de maneira a se correlacionar diretamente com a disfunção cognitiva (Francis et al, 2010; Kumar et al 2015).

Portanto, várias hipóteses têm sido propostas e estudadas para explicar o mecanismo de desenvolvimento da DA. De particular interesse, alterações na sinalização colinérgica, as quais incluem redução nos níveis de ACh (Schliebs & Arendt, 2006); redução da atividade da enzima colina acetiltransferase (Oda, 1999) e alterações no número e

sensibilidade de receptores nicotínicos e muscarínicos cerebrais (Aubert et al., 1992) tem sido descritas na literatura em relação a essa patologia.

3.2. SISTEMA COLINÉRGICO

A chamada hipótese colinérgica descreve que a deterioração cognitiva e o déficit de memória, principais sintomas da doença, são causados pelo baixo nível do neutrotransmissor ACh no sistema nervoso central. Assim, é de suma importância a manutenção dos níveis desse neurotransmissor com o intuito de retardar a progressão e aliviar os sintomas do paciente (Xie et al., 2013).

O sistema colinérgico possui um papel crucial no sistema nervoso central (SNC), pois está envolvido em vários processos fisiológicos como aprendizagem, memória, processamento da informação sensorial, organização cortical do movimento, controle do fluxo sanguíneo cerebral e diferenciação pós-sináptica (Silman & Sussman, 2005; Parfitt et al, 2012). Esse sistema é composto pelo neurotransmissor ACh, os receptores colinérgicos nos quais a ACh exerce seus efeitos (nicotínicos e muscarínicos) e as enzimas colina acetiltransferase e AChE (Silmann & Sussman, 2005).

A ACh é um neurotransmissor excitatório do SNC sendo sintetizada nos terminais nervosos a partir da acetil-coenzima A e da colina em uma reação catalisada pela enzima colina acetiltransferase. Após o potencial de ação, a ACh é liberada na fenda sináptica ligando-se a receptores nicotínicos e muscarínicos (Purves et al., 2005). A ação da ACh é finalizada pela sua hidrólise pela AChE com liberação de acetato e colina (Das et al, 2005; Polachini et al, 2014).

A AChE possui um papel regulatório clássico na neurotransmissão colinérgica, pois é a principal responsável pela hidrólise rápida da ACh, modulando a concentração deste transmissor na fenda sináptica e, regulando assim, a sinalização induzida por essa molécula (Costa et al, 2012; Purves et al., 2005). Existem dois tipos de colinesterases (ChE) responsáveis pela hidrólise da ACh: a AChE e a BuChE. Uma vez que a AChE é responsável por aproximadamente 80% da hidrólise da ACh em cérebros normais, essa recebe maior atenção do que a BuChE (Xie et al., 2013). Salienta-se que a atividade de ambas as enzimas se encontram aumentadas em doenças inflamatórias como a DA (Costa et al, 2012; Polachini et al, 2014).

A AChE é uma glicoproteína encontrada nos neurônios colinérgicos e em concentrações elevadas na junção neuromuscular e em células como eritrócitos e linfócitos (Massoulié et al, 1993). A estrutura tridimensional da AChE foi determinada por cristalografia de raios-X pela análise de diversos complexos ligante-enzima, o que possibilitou a identificação de dois principais sítios de ligação: o sítio ativo catalítico (CAS), formado por tríade catalítica Ser-His-Glu, e um sítio periférico aniônico (PAS), conectado por um profundo bolsão hidrofóbico (Fang et al, 2014). A tacrina foi o primeiro inibidor da AChE liberado pela FDA e, devido aos seus importantes efeitos adversos, como a alta toxicidade hepática, foi descontinuado do mercado. Esse fármaco interage apenas com o sítio CAS da AChE, portanto, após a descoberta desses efeitos adversos, as pesquisas farmacêuticas voltaram o seu interesse para possibilidades terapêuticas voltadas para a inibição enzimática a partir da interação simultânea com ambos os sítios CAS e PAS. O donepezil, outro fármaco inibidor da AChE, é hoje o único fármaco aprovado para o tratamento da DA que possui interação com ambos os sítios de ligação (Fang et al, 2014).

O sítio CAS, sítio ativo da AChE, possui uma tríade catalítica composta por resíduos de aminoácidos de serina-203, histidina-447 e glutamato-334. Essa enzima é classificada como uma serina hidrolase e seu mecanismo catalítico assemelha-se ao de outras hidrolases, onde o grupamento hidroxila da serina torna-se altamente nucleofílico por um sistema de reposição de cargas que envolvem o grupamento carboxila do glutamato, o imidazol da histidina e a hidroxila da serina (Sussman et al., 1991).

Dependendo de sua conformação, a AChE existe de duas formas: assimétrica e globular. A primeira, consiste de quatro tetrâmeros (A4, A8 e A12) catalíticos ligados de forma covalente a uma subunidade de colágeno denominada Q (CoIQ). Na segunda (forma globular), cada subunidade catalítica é formada por monômeros (G1), dímeros (G2) e tetrâmeros (G4) (Das et al, 2005).É importante salientar que no tecido nervoso a AChE está presente em duas isoformas: a forma G1 é a forma citosólica enquanto que a G4 é a forma aderida à membrana plasmática, ligada a glicofosfolipídeos ou em uma sequência de aminoácidos hidrofóbicos, assim, encontram-se distribuídas em diferentes compartimentos celulares. A fração solúvel em detergente (DS) da AChE contém predominantemente a isoforma G4, enquanto a fração solúvel em sal (SS) contém a isoforma G1. Destaca-se, ainda, que em cérebro de mamíferos, a forma G4 (DS) representa em torno de 60-90% da AChE total, dependendo da região cerebral (Das et al, 2005; Martini et al, 2018). Assim, de maneira geral, deve-se destacar que a isoforma DS encontra-se seletivamente aumentada na DA, provavelmente relacionada às sinapses colinérgicas,

tornando sua inibição mais importante para a melhora dos sintomas cognitivos do que a isoforma SS (Das et al, 2005; Martini et al, 2018; Siek et al, 1990).

Além da sua propriedade catalítica, a AChE tem potentes efeitos na adesão celular, extensão neurítica e diferenciação pós-sináptica (Paraoanu & Layer, 2008; Bigbee et al., 2000), sendo por isso considerada o marcador bioquímico mais importante da sinalização colinérgica no SNC. Essa enzima constitui-se em um importante alvo terapêutico para melhorar a deficiência colinérgica associada com doenças neurodegenerativas (Martini et al, 2018).

Além da AChE, é importante que seja destacada a importância da BuChE. A BuChE tem sua ação intimamente relacionada à AChE, no entanto, sabe-se que enquanto os níveis da AChE diminuem com o progresso da DA, os níveis da BuChE se mantêm ou se elevam de maneira expressiva (Bai et al, 2019; Kumar et al, 2018).

Diversos estudos têm sugerido que a inibição da BuChE pode contornar os efeitos adversos causados pela toxicidade colinérgica a partir do uso de inibidores clássicos da AChE. Assim, o efeito duplo de inibição tanto da AChE quanto da BuChE poderia representar uma melhor estratégia terapêutica para o tratamento da DA (Bai et al, 2019; Bartorelli et al, 2005; Sun et al, 2019).

Nesse sentido, podemos citar o caso do fármaco rivastigmina que é o único medicamento inibidor colinesterásico que possui essa dupla inibição. Sugere-se que essa inibição dupla possua importantes vantagens tanto na melhoria dos sintomas relacionados à DA, principalmente nos benefícios comportamentais, como também na manutenção dos efeitos mesmo com o progresso da doença, onde há um grande declínio na atividade da AChE. Essas características fazem desse fármaco uma melhor opção terapêutica em fases mais avançadas da doença. Ainda, a rivastigmina também possui a vantagem de sofrer baixa metabolização hepática, sendo metabolizada principalmente pelas colinesterases (Bartorelli et al, 2005).

3.3. MODELO DE DÉFICIT DE MEMÓRIA INDUZIDO PELA ESCOPOLAMINA

Dentre os modelos capazes de possibilitar o estudo de novos compostos frente as alterações causadas pelas doenças relacionadas à demência, como a DA, destaca-se o amplamente utilizado modelo de déficit de memória induzido por escopolamina (Ishola et

al, 2019; Malviya et al, 2009; Sadashiva et al, 2009). A escopolamina é um antagonista dos receptores muscarínicos usada em diversos estudos por causar disfunção cognitiva, principalmente déficit de memória, e prejudicar o processo de aprendizagem. Ainda, está bem estabelecido na literatura que esse composto aumenta a atividade das colinesterases e reduz os níveis de ACh, levando a uma disfunção do sistema colinérgico semelhante à encontrada na DA (Chen and Yeong, 2020; Tang, 2019).

Além da disfunção colinérgica, dentre as alterações celulares e moleculares causadas pela escopolamina, estão o aumento do estresse oxidativo, redução das defesas antioxidantes, a disfunção mitocondrial, o aumento da deposição β-amilóide e a neuroinflamação, todas estas alterações similares às que ocorrem na DA (Chen and Yeong, 2020; Tang, 2019).

Ainda, dentre as alterações causadas pela escopolamina, destaca-se a redução da atividade da enzima Na^+/K^+-ATPase. Apesar das alterações neurobioquímicas relacionadas à patogênese na DA ainda não serem claras, já existem evidências de que o Na^+/K^+-ATPase possui um potente efeito neuroprotetor e desempenha um papel fundamental na DA. A isoforma α da Na^+/K^+-ATPase é importante no processo de aprendizado e na memória, e especificamente a isoforma α3, expressa exclusivamente em neurônios, é conhecida por estar envolvida na consolidação da memória, particularmente quando há danos no hipocampo (Holm et al, 2016; Zhang et al, 2013). Nesse sentido, a redução da atividade dessa enzima pela escopolamina pode estar relacionada à amnésia causada a ratos em testes comportamentais (Luchese et al, 2020; Silva et al, 2017).

Além disso, sugere-se que a redução na atividade de Na^+/K^+-ATPase pode ser causada pelo aumento do estresse oxidativo. A Na^+/K^+-ATPase é um importante alvo das espécies reativas a nível neuronal. Nesse sentido, sabe-se que o acúmulo de espécies reativas de oxigênio (ERO), induzida pelo tratamento com escopolamina, é capaz de suprimir a atividade da ATPase. Ainda, o aumento do estresse oxidativo e a diminuição da atividade da Na^+/K^+-ATPase levam a prejuízos nas funções cerebrais, principalmente memória e cognição. Portanto, conclui-se que Na^+/K^+-ATPase está diretamente relacionada à patogênese da DA e está ligada ao aumento do estresse oxidativo presente nessa doença. (Fan et al, 2005; Zhang et al, 2013).

Vale destacar que os efeitos da escopolamina frente a elevação dos níveis das espécies reativas estão relacionados à disfunção dos mecanismos de defesa antioxidante (Tang, 2019). Diferentes modelos usando escopolamina demonstraram sua capacidade de diminuir a atividade da catalase (CAT), da superóxido dismutase (SOD) e da glutationa

peroxidase (GSH-Px), importantes enzimas que são parte do sistema de defesa antioxidante celular, e reduzir a liberação de glutationa reduzida (GSH) em diferentes estruturas cerebrais, especialmente em hipocampo e córtex cerebral (Ajami et al, 2012; Fan et al, 2005; Hancianu et al, 2013; Qu et al, 2017).

Essa supressão das defesas antioxidantes pode estar associada ao acúmulo de radicais livres, principalmente EROs, nas células cerebrais. Sugere-se, ainda, que o aumento da produção de EROs, que é induzida pela escopolamina, pode estar relacionada à disfunção mitocondrial e apoptose dos neurônios corticais e hipocampais, outras características marcantes da DA. Ainda, em relação ao efeito da escopolamina no estresse oxidativo, é bem estabelecido que essa é capaz de elevar os níveis de EROs, nitritos e de substâncias reativas ao ácido tiobarbitúrico (TBARS), além de diminuir a atividade das enzimas de defesa antioxidante, efeito esse supracitado (Tang, 2019).

Além disso, espécies reativas de oxigênio e nitrogênio em altos níveis podem causar modificações oxidativas de diferentes biomoléculas, como elevada peroxidação lipídica e oxidação de proteínas e ácidos nucleicos. Essas alterações estão presentes na DA, principalmente no hipocampo e no córtex cerebral, estruturas que apresentam as principais lesões neuropatológicas da doença (Agostinho et al, 2010; Tang, 2019).

3.4. TERAPIAS FARMACOLÓGICAS ATUAIS PARA A DA

Devido as alterações descritas no sistema colinérgico na DA, as estratégias terapêuticas têm sido amplamente focadas em melhorar a hipofunção colinérgica. Entre as várias abordagens testadas, a inibição da AChE é a única considerada eficaz. Sendo assim, os inibidores dessa enzima vêm sendo usados para aumentar os níveis de ACh e assim melhorar os déficits cognitivos característicos da DA (Anand & Sing 2013, Ferris & Farlow, 2013).

Hoje em dia duas classes terapêuticas estão disponíveis e são as escolhas para o tratamento dos pacientes com DA. Uma das classes é a dos inibidores da colinesterase, como o donepezil, rivastigmina e galantamina, que são recomendados para pacientes com DA, com quadros clínicos de demência leve, moderada ou grave, bem como demência por doença de Parkinson. Além da classe dos anticolinesterásicos, é aprovada ainda a memantina, um antagonista não-competitivo do receptor *N*-metil-D-aspartato (NMDA) e

agonista da dopamina, sendo utilizado em pacientes com DA moderada a grave (Weller & Budson, 2018).

Esses inibidores da AChE são amplamente utilizados nos diferentes estágios da DA, incluindo estágios avançados da doença, porém atuam de maneira mais eficiente em estágios mais leves. Adicionando-se ainda o fato de as opções terapêuticas para o tratamento da doença serem muito restritas, os inibidores da AChE são tipicamente utilizados como farmacoterapia de primeira linha para o tratamento sintomático da DA (Renn et al 2018; Khoury et al 2018).

No entanto, esses medicamentos anticolinesterásicos apresentam efeitos colaterais indesejados como náuseas, vômitos, diarreia, bradicardia e tontura além de custos elevados aos pacientes (Minett & Bortolucci, 2000). Além dessas classes de medicamentos citadas, a hipótese da cascata amilóide reforçou a busca por novas classes de medicamentos para a DA e auxiliou o progresso da pesquisa na área. No entanto, devido a frequentes fracassos em ensaios clínicos desses medicamentos com o intuito de tratar a DA e não apenas os seus sintomas, as empresas farmacêuticas frearam o desenvolvimentos de pesquisas relacionadas a terapias com base na hipótese da cascata amiloide. Essa atitude gera uma importante disparidade entre o desenvolvimento de medicamentos e a elevada prevalência da doença (Denver & McClean, 2018). Assim, o estudo de novas classes de compostos na busca de melhores terapias, com menos efeitos colaterais, e que possam beneficiar os portadores de doenças neurodegenerativas como a DA, é de suma importância.

3.5. IMPORTÂNCIA DAS 1,3-TIAZOLIDIN-4-ONAS

Levando em conta o supracitado, nos últimos anos tem se demonstrado interesse no desenvolvimento de novas classes de compostos de origem sintética, capazes de inibir a AChE e com potencial atividade para o tratamento da DA. Dentre os compostos heterociclos com potencial farmacológico, destacam-se as tiazolidinonas, heterociclos de cinco membros contendo um átomo de enxofre (posição 1), um átomo de nitrogênio (posição 3) e uma carbonila que pode se apresentar nas posições 2, 4 ou 5. As 1,3-tiazolidin-4-onas (**Figura 1**), grupo de interesse com a carbonila na posição 4 do anel, podem ser funcionalizadas apresentando diferentes substituintes nas posições 2, 3 e 5 do anel (Tripathi et al, 2014).

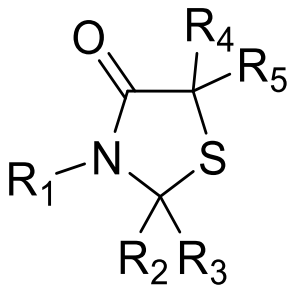

Figura 1. Estrutura geral das 1,3-tiazolidin-4-onas

Este núcleo heterocíclico vem sendo amplamente estudado devido a vasta aplicabilidade na área da química medicinal e devido às diferentes atividades biológicas que tem demonstrado (Tripathi et al, 2014). São encontrados na literatura diversos trabalhos que relatam as diversas aplicações das tiazolidin-4-onas na química medicinal, tais como a atividade antitumoral (Silva et al, 2016; Silveira et al, 2017), anticonvulsivante (Ahmed et al., 2014), anti-inflamatória (Hu et al, 2013; Maccari et al., 2014), antidiabética (Raza et al., 2013), antimicrobiana (Desai et al., 2014), antiviral (Masoud et al., 2013), entre outras. Além disso, existem fármacos como o etozoline, anti-hipertensivo, a pioglitazona, antidiabético, e o ralitoline, anticonvulsivante, que contém em sua estrutura o anel tiazolidinônico (**Figura 2**) (Jain et al., 2012).

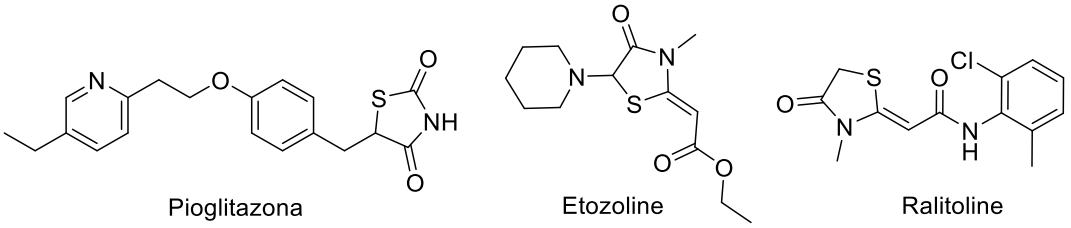

Figura 2. Estrutura de fármacos contendo o anel tiazolidinônico.

As tiazolidinonas têm sido reportadas por possuir potente ação anti-inflamatória. Diferentes derivados apresentaram resultados significativos em modelos de inflamação aguda, como o edema de pata e de pleurisia induzidos por carragenina em ratos (Tripathi et al, 2014). Hu et al. (2013) demonstraram atividade significativa *in vitro* de diversas tiazolidinonas na concentração de 10 μM, para a inibição da liberação de citocinas pró-inflamatórias como o TNF-α e a IL-6 estimulada com lipopolissacarídeo (LPS) em células RAW 264.7 de macrófagos de ratos. Seis compostos foram capazes de reduzir fortemente a expressão de TNF-α, e nove compostos reduziram a expressão de IL-6, sendo que cinco inibiram a expressão de ambas citocinas. Alguns compostos apresentaram resultados melhores que o controle positivo curcumina no ensaio.

Embora estudos *in vitro* e/ou *in vivo* para avaliação do potencial inibitório da AChE por tiazolidin-4-onas sejam pouco explorados na literatura, ensaios de docking molecular já abordaram o assunto. Iyer et al. (2015), em estudo exploratório de identificação de alvos terapêuticos para derivados tiazolidinônicos, encontraram resultados demonstrando uma potencial afinidade de ligação entre esses derivados e a AChE, indício da atividade desses compostos frente a esse alvo.

Sadashiva e colaboradores (2009) revelaram através de análises *in vitro* a afinidade de 1,3-tiazolidin-4-onas derivadas da arecolina a receptores muscarínicos do tipo M1 em homogeneizado de córtex de ratos. Esse estudo ainda demonstrou o potencial *in vivo* das tiazolidinonas em reverter a perda de memória e o déficit de aprendizado através de modelos comportamentais após realização do modelo de déficit de memória induzido pela escopolamina em ratos. Assim, os autores sugeriram que o estudo foi capaz de revelar o efeito agonista muscarínico M1 de tiazolidinonas derivadas da arecolina, o que demonstra o potencial dessa classe em um diferente alvo do sistema colinérgico no SNC.

Além disso, Berwaldt e colaboradores (2019) demonstraram uma inibição expressiva da AChE com 1,3-benzotiazin-4-onas derivadas da *N*-(3-aminopropil)piperidina em córtex cerebral e hipocampo de ratos. Esses compostos ainda revelaram um bom perfil de nos testes de citotoxicidade frente a células MCR-5, não apresentando toxicidade na concentração de 100 μM. Essas 1,3-tiazin-4-onas são estruturalmente semelhantes às 1,3-tiazolidin-4-onas, apresentando um anel heterocíclico de 6 membros, enquanto as tiazolidinonas apresentam um anel de 5 membros, com os mesmos heteroátomos nas mesmas posições do anel.

Nesse contexto, a síntese de novas tiazolidinonas e a avaliação do seu potencial biológico são essenciais para elevar o conhecimento sobre essa classe. Para isso, a química orgânica dispõe de diferentes rotas sintéticas para a síntese desses compostos. A rota sintética principal das 1,3-tiazolidin-4-onas se utiliza de três componentes: uma amina primária, um aldeído ou cetona e o ácido mercaptoacético, podendo esta reação ocorrer tanto em uma como em duas etapas. Independente da condição reacional, ocorre inicialmente a formação de um intermediário imina seguido por uma ciclocondensação intramolecular (Tripathi et al, 2014).

Conforme ilustrado na **Figura 3**, as condições de reação podem ser subdividas em: a) multicomponente *one pot*, com a adição do ácido mercaptoacético após um determinado tempo de reação; b) multicomponente, onde se adiciona todos os reagentes ao

mesmo tempo no início do processo reacional; c) duas etapas, onde ocorre o isolamento do intermediário imina e posterior adição do ácido mercaptoacético. (Masteloto et al, 2015; Neves et al, 2015; Tripathi et al, 2014).

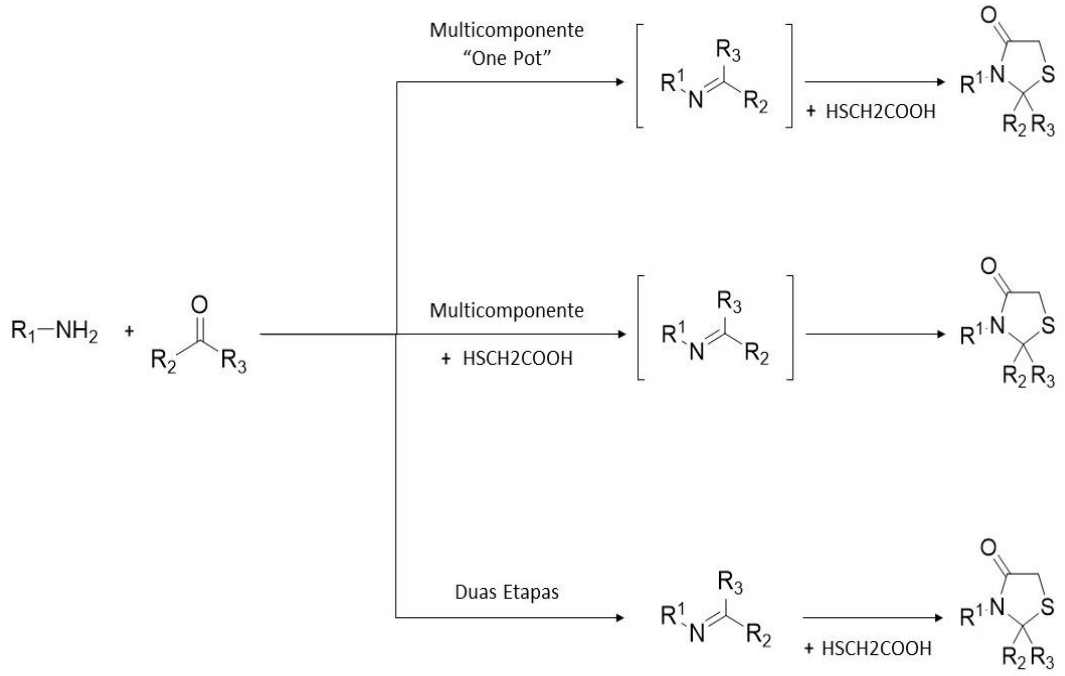

Figura 3. Métodos sintéticos das 1,3-tiazolidin-4-onas.

De maneira geral, o mecanismo de formação do anel tiazolidinônico envolve uma reação de adição seguida de substituição à carbonila. Uma representação esquemática da reação pode ser vista na **Figura 4**, proposta por Masteloto et al. (2015), onde ocorre inicialmente um ataque do par de elétrons do nitrogênio da amina à carbonila do aldeído ou cetona, deslocando os elétrons da dupla ligação para o oxigênio, ocorrendo sua protonação através de um equilíbrio, com formação do intermediário imina e de uma molécula de água. Uma molécula de ácido mercaptoacético promove a protonação do nitrogênio imínico e o par de elétrons do enxofre de outra molécula de ácido mercaptoacético ataca o carbono imínico parcialmente positivo, deslocando os elétrons da ligação dupla para o nitrogênio, formando um intermediário tetraédrico. Assim, o par de elétrons do nitrogênio ataca a carbonila do ácido mercaptoacético, em uma ciclização intramolecular, ocorrendo a formação do anel tiazolidinônico e a liberação de mais uma molécula de água. Finalmente, ocorre a desprotonação da carbonila do anel tiazolidinônico (Masteloto et al, 2015).

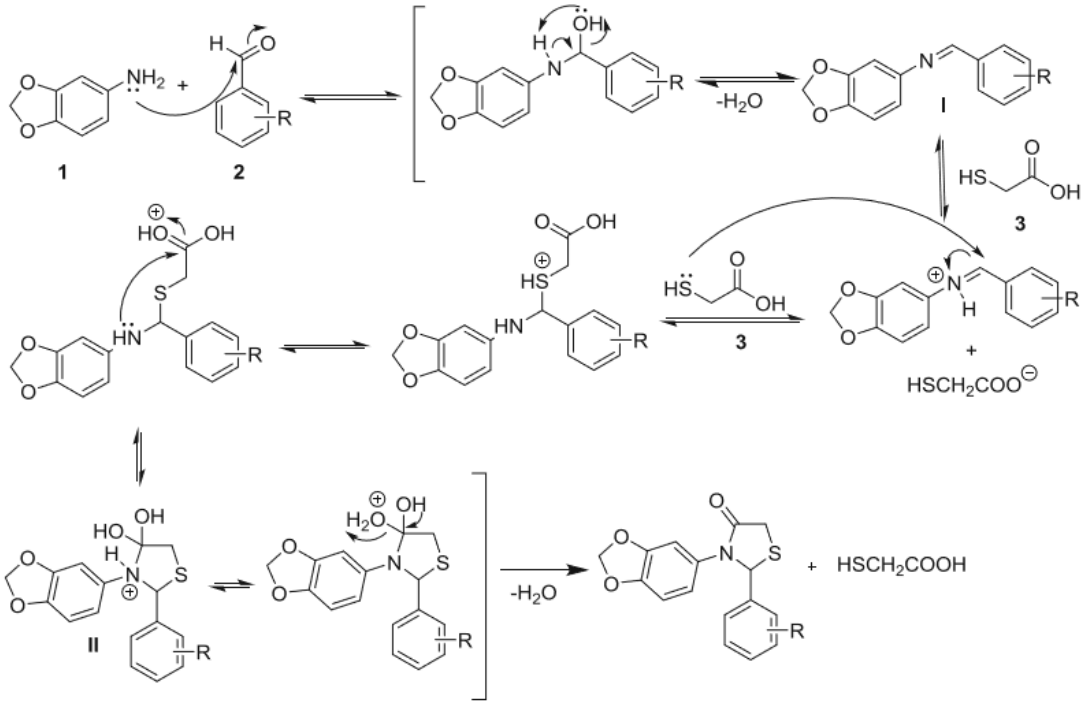

Figura 4. Representação esquemática de mecanismo reacional proposto por Masteloto et al, 2015 (Retirado de Masteloto et al, 2015).

Como há um equilíbrio no estágio de formação do intermediário imina, é essencial que o sistema reacional disponha de uma maneira de eliminar as moléculas de água formadas no processo, evitando a reação de voltar aos reagentes de partida. O aparelho *Dean-Stark* aparece como a opção simples e eficiente para a remoção da mistura azeotrópica formada pelas moléculas de água e o solvente, deslocando assim a reação no sentido da formação dos produtos (Kunzler et al., 2013 e Neuenfeldt et al., 2011).

Diferentes métodos sintéticos podem ser utilizados para a síntese da 1,3-tiazolidin-4-onas, contudo, a mais utilizada é a de aquecimento térmico convencional (Tripathi et al, 2014). Silva e colaboradores (2016) demonstraram a síntese de 1,3-tiazolidin-4-onas derivadas do 4-(metiltio)benzaldeído e do 4-(metilsulfonil)benzaldeído a partir do método de aquecimento térmico convencional, tendo obtido 14 compostos com rendimentos de moderados a bons. Foram utilizadas metodologia multicomponente para alguns compostos e multicomponente *one-pot* para outros, em refluxo de tolueno, utilizando um aparelho *Dean-Stark* para remoção da água por destilação azeotrópica.

4. JUSTIFICATIVA DA TESE

Neste contexto, tendo em vista a complexidade da fisiopatologia da DA, a falta de novas opções terapêuticas para o manejo do quadro do paciente, e considerando o potencial das 1,3-tiazolidin-4-onas na área da química medicinal, esta tese teve por objetivo avaliar o efeito de diferentes 1,3-tiazolidin-4-onas derivadas do 4-(metiltio)benzaldeído e do 4-(metilsulfonil)benzaldeído em estudos experimentais *in vitro* sobre a atividade da AChE e *in vivo* em modelo de amnésia induzido pela escolopamina. A **Figura 5** demonstra os testes de triagem a serem realizados com a série de tiazolidinonas sintetizadas para o estudo.

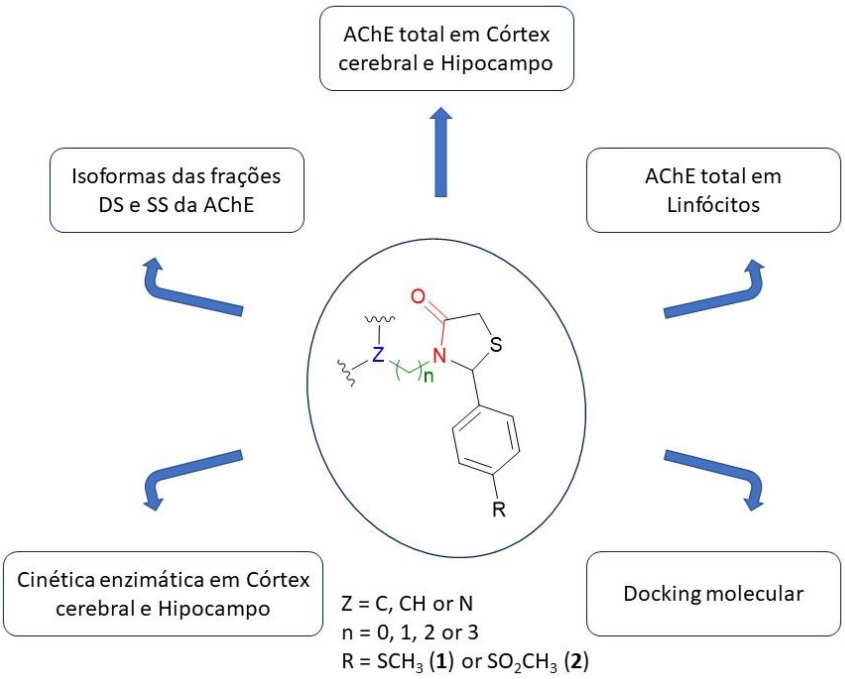

Figura 5. Resumo gráfico da estrutura geral dos compostos e dos principais testes realizados para avaliação do potencial biológico das 1,3-tiazolidin-4-onas sintetizadas.

O melhor composto nessa triagem foi selecionado para estudos *in vivo*, com o intuito de avaliar o potencial preventivo do tratamento com a tiazolidinona através do modelo de déficit de memória induzido pela escopolamina. O estudo em modelo animal permitiu avaliar a prevenção de alterações em parâmetros comportamentais e neurobioquímicos, bem como avaliar toxicidade através de marcadores de função hepática e renal.

5. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo e um manuscrito. As seções materiais e métodos, resultados, discussão e referências encontram-se nos próprios artigos e representam a íntegra desse estudo.

O artigo e o manuscrito estão estruturados de acordo com as revistas as quais foram publicados ou submetidos.

5.1. ARTIGO

In Vitro Effects of 2-{4-[Methylthio(methylsulfonyl)]phenyl}- 3-substitutedthiazolidin-4-ones on the Acetylcholinesterase Activity in Rat Brain and Lymphocytes: Isoform Selectivity, Kinetic Analysis, and Molecular Docking

Publicado no periódico Neurochemical Research

https://doi.org/10.1007/s11064-019-02929-8

Em anexo a licença para uso do artigo publicado na tese (**ANEXO A**). A aprovação junto ao comitê de ética em experimentação animal (CEEA), autorizando a realização da pesquisa desenvolvida neste artigo, encontra-se em anexo a esta tese (**ANEXO B**).

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BRIEF COMMUNICATION

In Vitro Effects of 2-{4-[Methylthio(methylsulfonyl)] phenyl}-3-substitutedthiazolidin-4-ones on the Acetylcholinesterase Activity in Rat Brain and Lymphocytes: Isoform Selectivity, Kinetic Analysis, and Molecular Docking

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Abstract

This work evaluated the in vitro effect of thiazolidin-4-ones on the activity of AChE (total and isoforms) isolated from the cerebral cortex, hippocampus, and lymphocytes. Kinetic parameters were evaluated and molecular docking was performed. Our results showed that thiazolidinones derived from 4-(methylthio)benzaldehyde (**1**) and from 4-(methylsulfonyl)benzaldehyde (**2**) were capable of inhibiting the AChE activity in vitro. Three compounds, two with a propylpiperidine (**1b** and **2b**) moiety and one with a 3-(diethylamino)propyl (**1c**) moiety showed IC_{50} values of 13.81 µM, and 3.13 µM (**1b**), 55.36 µM and 44.33 µM (**1c**) for cerebral cortex and hippocampus, respectively, and 3.11 µM for both (**2b**). Enzyme kinetics revealed that the type of AChE inhibition was mixed. Compound **1b** inhibited the G1 and G4 AChE isoforms, while compounds **1c** and **2b** selectively inhibited the G4 isoform. Molecular docking showed a possible three-dimensional fit into the enzyme. Our findings showed that these thiazolidin-4-ones, especially those containing the propylpiperidine core, have a potential cholinesterase inhibitory activity and can be considered good candidates for future Alzheimer's therapy.

Keywords 1,3-Thiazolidin-4-ones · Acetylcholinesterase · AChE isoforms · Hippocampus · Cerebral cortex

Introduction

The neurotransmitter acetylcholine is involved in critical physiological processes, such as attention, learning, memory, stress response, wakefulness, sleep, and sensory information [1–3]. Acetylcholinesterase (AChE) is an important regulatory enzyme that rapidly hydrolyzes acetylcholine, thereby determining the duration and efficacy of cholinergic neurotransmission [4]. This enzyme is present mostly in the central nervous system; however, it can also be found in blood cells such as lymphocytes [5].

AChE is a serine hydrolase with an active site composed of a catalytic triad of amino acid residues serine (Ser-200), histidine (His-440), and glutamate (Glu-327) [6]. Specific molecular forms of AChE can be distinguished based on molecular weight, charge, and solubility [4]. In the brain, the most abundant AChE isoforms are the salt soluble (SS) cytosolic G1 (4S) and detergent soluble (DS) membrane bound G4 (10S) [7].

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Several studies have reported that cognitive impairment in Alzheimer's disease is correlated with damage to cholinergic neurons [8–10]. In a pharmacological context, inhibition of brain AChE activity has been the major therapeutic target for increasing acetylcholine levels in the synapses, leading to improvement of cognitive deficits in Alzheimer's disease [11, 12].

In recent years, there has been a great interest in developing novel AChE inhibitors of natural and synthetic origin. Furthermore, 1,3-Thiazolidin-4-one is an important class of heterocyclic compounds containing sulfur and nitrogen atoms. This class possesses a broad spectrum of biological activities such as anti-inflammatory, antimicrobial, antifungal, anticonvulsant, antioxidant, and antitumor activities [13]. Previous study by our research group demonstrated the antiglioma effect of thiazolidine-4-ones [14]. In particular, these compounds show structural similarity with neurotransmitter acetylcholine (Fig. 1): a basic nitrogen that could be protonated (blue), a chain of two or three methylenes (green), and an amide carbonyl moiety (red). Therefore, it is expected that these compounds interact with and inhibit AChE in line with our previous studies on benzothiazinone compounds [15]. The natural substrate-based strategy was successfully applied to the discovery of inhibitors propranolol and cimetidine [16–18].

Given the above background, the aim of this study was to investigate in vitro the potential AChE inhibitory effect of thiazolidin-4-ones: (a) on the total activity of AChE in rat cerebral cortex, hippocampus, and lymphocytes, (b) on the activities of molecular AChE isoforms, and (c) on the kinetic parameters. In addition, a molecular docking study was also performed to evaluate the affinity of thiazolidin-4-ones for AChE.

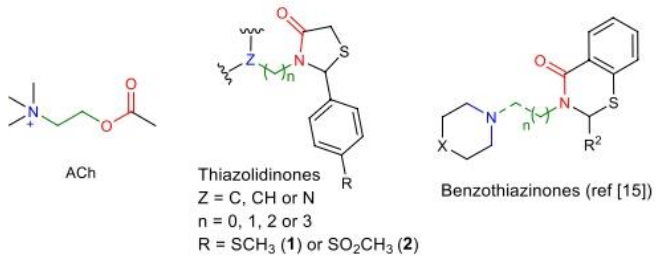

Fig. 1 Structural similarity between acetylcholine (ACh), benzothiazinones [15] and thiazolidin-4-ones **1** and **2**

Experimental Methods

Synthesis of the 1,3-Thiazolidin-4-one Compounds

The compounds were synthetized as described previously by our research group [14]. The 1,3-thiazolidin-4-ones **1** were obtained from 4-(methylthio)benzaldehyde and **2** from 4-(methylsulfonyl)benzaldehyde, where lower case letters represent different amine cores (**a**–**g**), as shown in Fig. 2.

Animals and Ethical Approval

Wistar male rats (60-day-old, 300–400 g) were obtained from the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. The Committee of Ethics and Animal Experimentation of the institution approved all animal procedures (protocol number CEEA 9219).

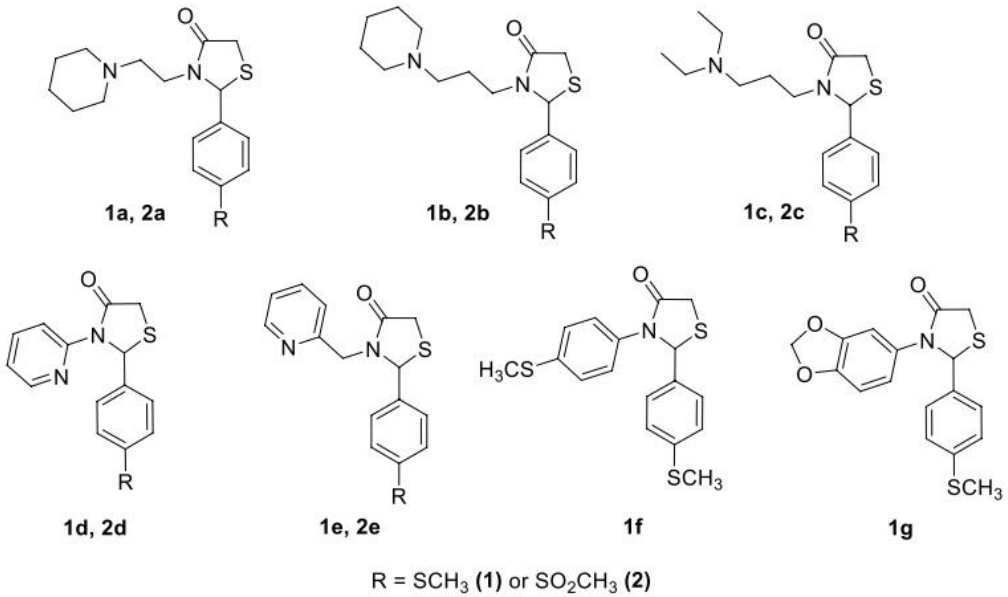

Fig. 2 Structure of 1,3-thiazolidin-4-ones **1a**–**g** and **2a**–**e**

Effect of 1,3-Thiazolidin-4-ones on the Total Brain AChE Activity

Ten rats were anesthetized and subjected to euthanasia. The brain was removed, and cerebral cortex and hippocampus were collected. The brain structures were homogenized in a solution of 10 mM Tris–HCl (pH 7.4) and centrifuged at 1300×*g* for 10 min at 4 °C. The pellet was discarded, and the supernatant was used for the assay of total AChE activity.

The AChE activity was determined according to the protocol described by Ellman et al. [19]. This method is based on the formation of a yellow anion, 4,4-dithio-bis-nitrobenzoic acid. The reaction was measured by monitoring absorbance at 412 nm for 2 min with intervals of 30 s at 27 °C. The 1,3-thiazolidin-4-ones were solubilized in methanol and used in the AChE assays at final concentrations of 0.1, 0.5, 1, 5, 10, 25, 50 and 100 µM. Cerebral cortex or hippocampus homogenates were pre-incubated with 1,3-thiazolidin-4-ones for 2 min. Water and vehicle (methanol) were used as controls. The reaction was initiated by adding acetylcholine iodide as a substrate. AChE activity was expressed in µmoles of AcSCh/h/mg of protein. Protein concentrations were determined by Bradford method [20] using bovine serum albumin as a standard.

Effect of 1,3-Thiazolidin-4-ones on the Activities of Brain AChE Isoforms

Cerebral cortex and hippocampus were homogenized in cold 30 mmol/L sodium phosphate buffer (pH 7.0) and protease inhibitor, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (10 mM; 1/10, w/v). The homogenates were centrifuged at 100,000×*g* at 4 °C for 60 min. The supernatant was collected and stored at 4 °C, which consisted of the salt soluble (SS or G1) isoform. The pellet was resuspended in 1% Triton X-100 (10% w/v in 30 mmol/L of sodium phosphate buffer, pH 7.0) and incubated at 4 °C for 60 min. These samples were then centrifuged at 100,000×*g* at 4 °C for 60 min. The supernatant was collected and stored at 4 °C, which consisted of the detergent soluble (DS or G4) isoform.

The AChE activity was measured for G1 and G4 isoforms by the method described by Ellman et al. [19] and Das et al. [7] with minor modifications, such as the final concentration (1 mmol/L) of substrate, acetylthiocholine iodide and chromophore, 5′,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (1 mM), which resulted in a better kinetic profile. The enzyme sample (0.5 µg of protein) was pre-incubated for 2 min at 25 °C in the presence of increasing concentrations of the compounds (0.1, 0.5, 1, 10, 25, 50 and 100 µM). The enzymatic reaction was initiated by adding the substrate acetylthiocholine iodide. The enzyme activity was determined at 412 nm for 2 min with intervals of 30 s. AChE isoform activities were expressed in µmoles/min/mg of protein.

Kinetic Parameters of AChE Inhibition by 1,3-Thiazolidin-4-ones in Brain Structures

The kinetics of the interactions between the compounds and AChE were determined using the Lineweaver–Burk double reciprocal plot by plotting 1/V against 1/S analyzed over a range of acetylthiocholine concentrations (10, 50, 100, 500 and 1000 µM) either in the absence or presence of 1,3-thiazolidin-4-ones (5, 10, 20 and 30 µM). Km values were obtained by two different estimates, 1/V versus 1/S and V versus V/S.

Effect of 1,3-Thiazolidin-4-ones on the Total AChE Activity in Lymphocytes

Blood was collected in tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. Total blood was diluted in equal volume of saline and lymphocyte separation was performed with Ficoll density gradients as previously described [21]. AChE activity was measured as described by Ellman et al. [19] with few modifications suggested by Fitzgerald and Costa [22]. The reaction mixture was composed of acetylthiocholine (1 mM), 5,5′-dithio-bis-2-nitrobenzoic acid (0.1 mM), phosphate buffer (pH 8.0; 0.1 M), intact lymphocytes suspended in saline solution and 1,3-thiazolidin-4-ones dissolved in methanol at final concentrations of 50, 100 and 250 µM. The absorbance was read on a spectrophotometer at 412 nm before and after incubation with 1,3-thiazolidin-4-ones for 30 min at 27 °C. The AChE activity was expressed as µmol/h/mg of protein.

Molecular Docking

Molecular docking was performed using AutoDock Vina 1.1.2 [23] and AutoDock Tools [24] for getting insights into the conformation of the compounds when bound to AChE. The docking simulation was prepared using a framework for visual screening as proposed by Seus et al. [25]. The dockings were executed with an exhaustiveness of 128. The receptor used was the crystal structure of the human AChE (PDB: 4EY7) and the ligands used were compounds **1a–g** and **2a–e** in addition to acetylcholine and donepezil. The protein structure was rotated 25° around Z-axis for better alignment with the grid box and only chain A was considered. The grid box was determined around the binding site, where donepezil was bound. It was centered at the coordinates − 20 X, − 50 Y and 20 Z with the length of 22 Å on all axes.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer's test. Differences between the groups were considered significant when $P < 0.05$. All data were expressed as mean ± standard error (SEM). The concentration of compound that caused 50% of enzyme inhibition (IC_{50} value) was determined by nonlinear regression analysis and was measured using GraphPad Prism 5.

Results and Discussion

Total Acetylcholinesterase Activity in Cerebral Cortex and Hippocampus

Figures 3 and 5 show the effects of the compounds **1a–g** on the total AChE activity in brain structures. The compound **1a** inhibited the AChE activity in cerebral cortex at the concentrations of 100 µM (52%) and 250 µM (76%), and in hippocampus, at the concentrations of 5, 10, and 25 µM (around 30%), 50 and 100 µM (around 50%), and 250 µM (75%) when compared to the control group ($P < 0.05$) (Fig. 3). The compounds **1d–g** did not alter the in vitro AChE activity in both cerebral cortex and hippocampus at any of the concentrations tested (Fig. 3).

The compounds **1b** and **1c** showed more potent inhibitory effect on the AChE activity (Fig. 5). Compound **1b** decreased the AChE activity in cerebral cortex at the concentrations of 1, 5 and, 10 µM (between 40 and 60%), 25 and 50 µM (75%), 100 µM (83%), and 250 µM (91%). In hippocampus, compound **1b** inhibited the AChE activity at the concentrations of 1 µM (25%), 5 and 10 µM (50%), 25 µM (65%), 50 µM (72%), 100 and 250 µM (80%) when compared to the control group ($P < 0.05$) (Fig. 5). The IC_{50} values were 13.81 µM and 3.13 µM for cerebral cortex and hippocampus, respectively (Fig. 5).

The compound **1c** inhibited the AChE activity in cerebral cortex at the concentrations of 25 µM (40%), 50 µM (55%), 100 µM (66%), and 250 µM (80%) in relation to the control ($P < 0.05$). In hippocampus, the enzyme activity was inhibited by the concentrations of 1 µM (20%), 5 and 10 µM (35%), 25 µM (46%), 50 µM (58%), 100 µM (72%), and 250 µM (80%) ($P < 0.05$). The IC_{50} values of the compound **1c** were 55.36 µM and 44.33 µM for cerebral cortex and hippocampus, respectively (Fig. 5).

Figures 4 and 5 show the effects of the compounds **2a–g** on the total AChE activity. In cerebral cortex, compound **2a** inhibited the enzyme activity only at the highest concentration (56%), while in hippocampus, the enzyme activity was inhibited at the concentrations of 50, 100 (30%), and 250 µM (63%) when compared to the control group ($P < 0.05$) (Fig. 4). In cerebral cortex, compound **2c** decreased the AChE activity at the concentrations of 25 and 50 µM (around 50%), 100 µM (68%), and 250 µM (82%), while compound **2d** inhibited the AChE activity only at the highest concentration tested [250 µM (38%)] ($P < 0.05$). Compound **2e** did not show any inhibitory effect on AChE activity in cerebral cortex (Fig. 4). In hippocampus, the AChE activity was inhibited by compound **2c** at the concentrations of 5, 10 and 25 µM (around 30%), 50 µM (54%), and 100 and 250 µM (70%). In contrast, compound **2e** inhibited the AChE activity only at the higher concentrations tested [100 and 250 µM (40%)] in relation to the control groups ($P < 0.05$). Compound **2d** did not show inhibitory activity in hippocampus (Fig. 4).

The major inhibitory potential of thiazolidinones **2** was observed for compound **2b**. In cerebral cortex, compound **2b** inhibited the AChE activity at the concentrations of 1 µM (38%), 5, 10, 25 µM (around 60%), 50 and 100 µM (around 80%), and 250 µM (93%), and in hippocampus, at the concentrations of 0.5 and 1 µM (20%), 5 µM (46%), 10 µM (43%), 25, 50, 100 and 250 µM (around 70–80%) when compared to the control group ($P < 0.05$). The IC_{50} value of the compound **2b** was 3.11 µM for both cerebral cortex and hippocampus (Fig. 5).

Our findings showed that compounds derived from 4-(methylthio)benzaldehyde (**1**) and 4-(methylsulfonyl)benzaldehyde (**2**) have similar effects on the AChE activity in brain structures. However, the substituent 2-(4-methylsulfonyl)phenyl was able to exhibit inhibitory effect on the AChE activity in the brain structures for all class **2** compounds evaluated. However, we did not get similar results for class **1** compounds.

On the other hand, it is clear that the amine core is important for inhibitory effect. Analyzing the results, it was verified that among all the amine cores studied, the compounds having ethylpiperidine (**a**), propylpiperidine (**b**) and 3-(diethylamino)propyl (**c**) moieties stood out, considering that the other compounds (**d–g**) did not exhibit significant inhibitory potential. Thus, it can be concluded that these substituents are important for the inhibitory activity, with emphasis on thiazolidinones derived from *N*-(3-aminopropyl)piperidine (**b**). These results are in agreement with our previous studies on benzothiazinones which showed the best result with a compound having the propylpiperidine group (IC_{50} of 8.5 µM in cerebral cortex) [15]. It is important to note that ACh has an ethylene chain between its quaternary nitrogen and the oxygen atom of the ester group, while compounds **b** and **c** have a propylene chain between their nitrogens. Three compounds were chosen for further study, two with propylpiperidine moiety (**1b** and **2b**), and one with 3-(diethylamino) propyl moiety (**1c**). In addition, it is important to consider that compounds **1b**, **1c** and **2b** inhibited in vitro the AChE

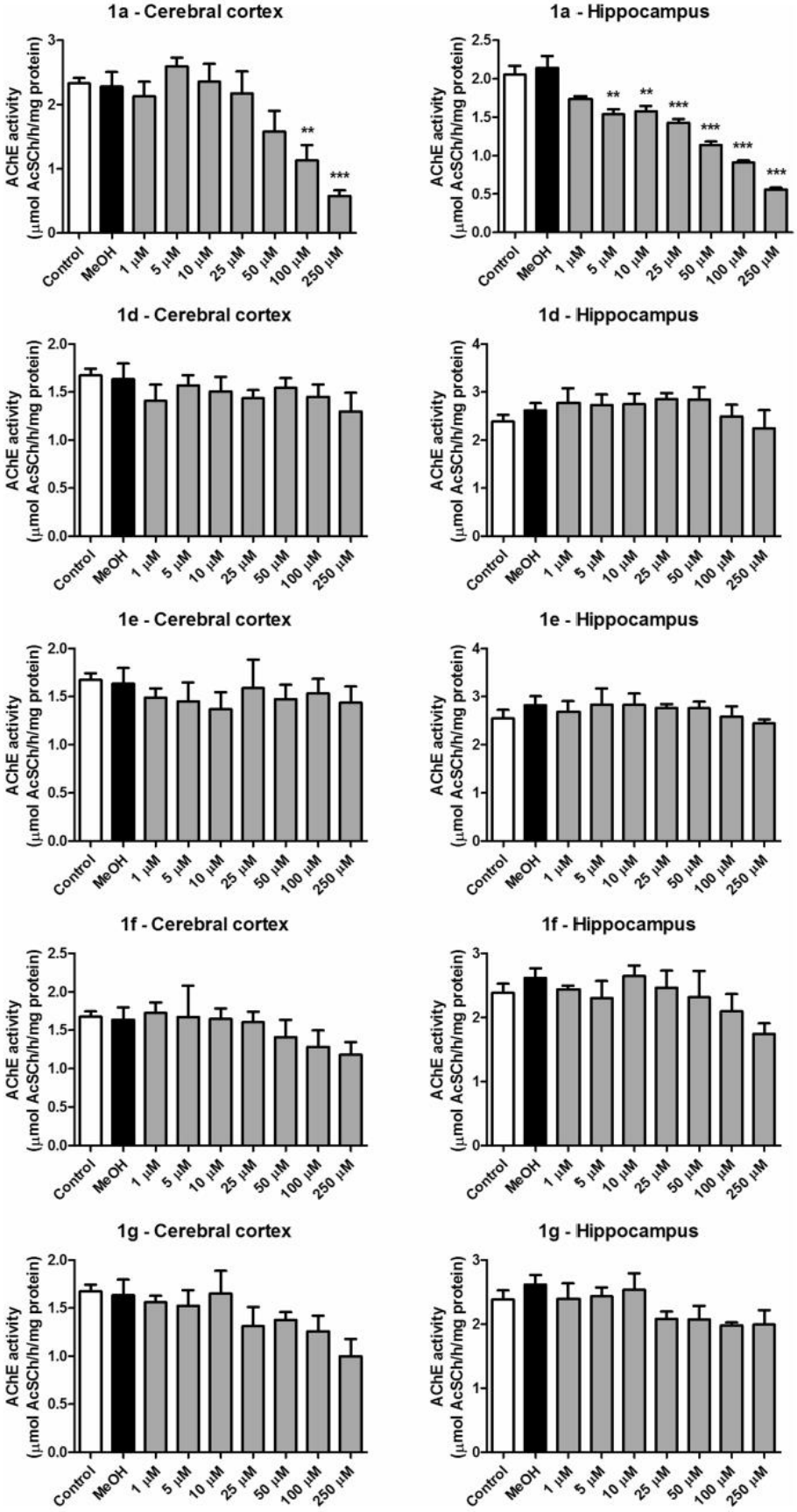

Fig. 3 In vitro effects of the compounds **1a** and **1d–g** on the acetylcholinesterase (AChE) activity in rat cerebral cortex and hippocampus. Results are expressed in µmol AcSCh/h/mg of protein. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group (water)

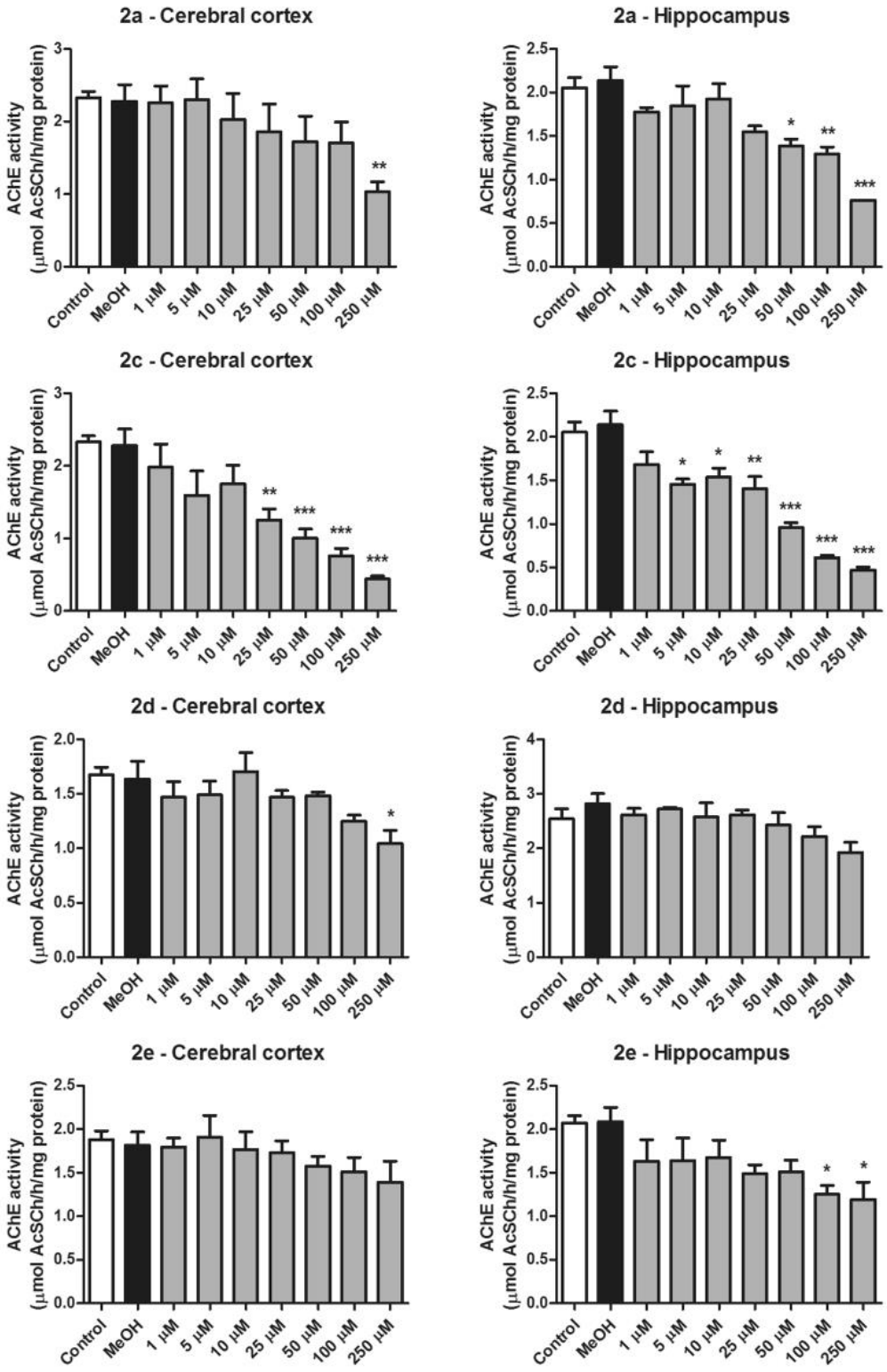

Fig. 4 In vitro effects of the compounds **2a** and **2a–c** on the acetylcholinesterase (AChE) activity in rat cerebral cortex and hippocampus. Results are expressed in µmol AcSCh/h/mg of protein. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to the control group (water)

activity in cerebral cortex and hippocampus, brain structures related to memory and cognition [26]. In addition, Silva et al. [14] revealed that none of these compounds showed toxicity at 100 µM in astrocyte cultures.

Kinetic Parameters

Considering that the compounds **1b**, **1c** and **2b** showed the major cholinesterase inhibitory effect, we next evaluated the

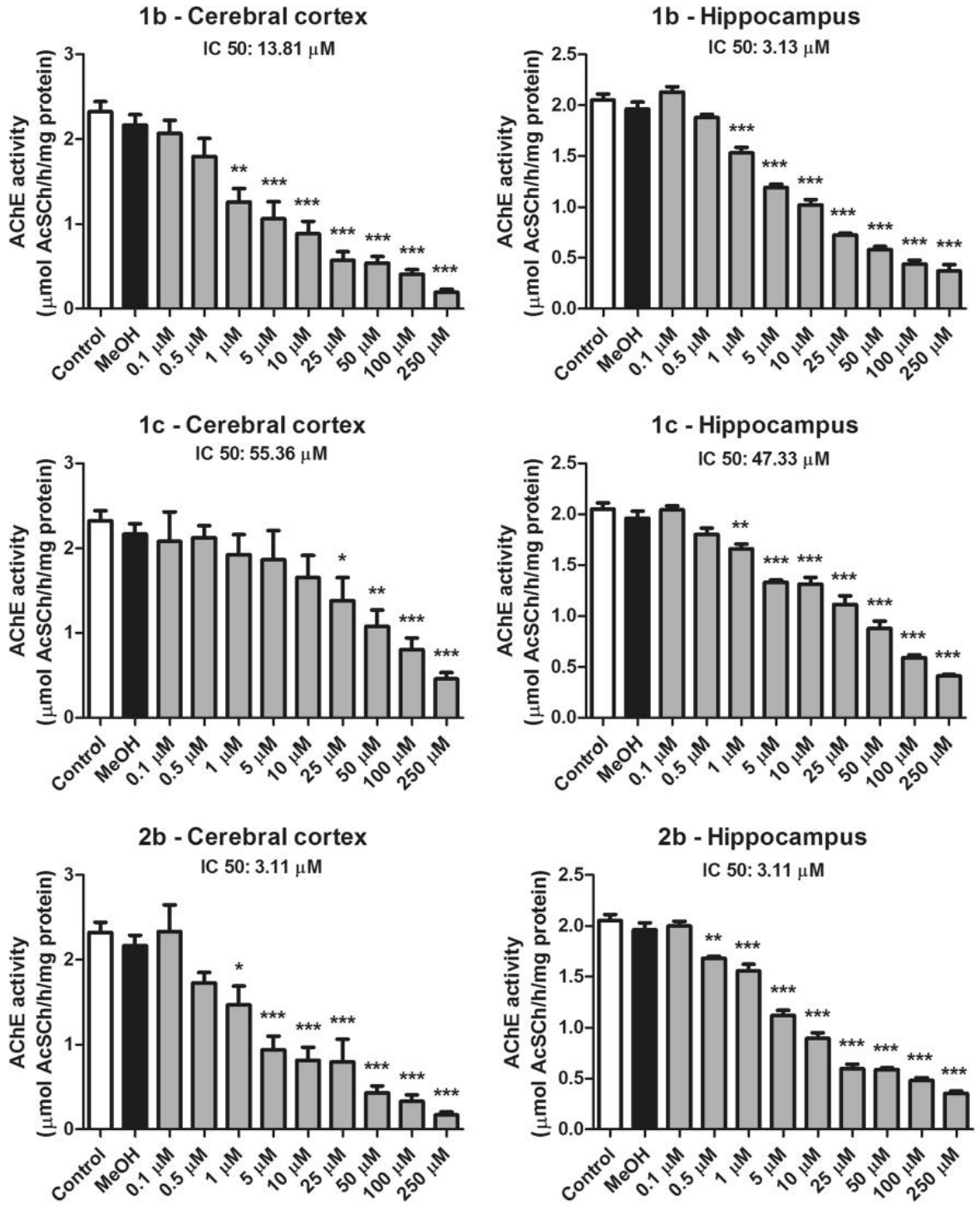

Fig. 5 In vitro effects of the compounds **1b**, **1c** and **2b** on the acetylcholinesterase (AChE) activity in rat cerebral cortex and hippocampus. Results are expressed in μmol AcSCh/h/mg of protein. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to the control group (water)

type of AChE inhibition involved. Analysis of kinetic data indicated that the inhibition caused by compounds **1b**, **1c** and **2b** was mixed in both cerebral cortex and hippocampus. The decrease observed in the Vmax values and elevation in the Km values confirmed the type of inhibition (Table 1 and Fig. 6). Mixed inhibition indicates that compounds can bind both free enzymes as well as enzyme–substrate complex. A mixed inhibitor binds to enzyme at a site, which is different

Table 1 Km and Vmax values for thiazolidinones **1b**, **1c** and **2b**

Compounds	Cerebral cortex Vmax	Cerebral cortex Km	Hippocampus Vmax	Hippocampus Km
1b—0 μM	9.462	0.391	9.681	0.569
1b—5 μM	8.834	0.504	7.646	0.580
1b—10 μM	7.866	0.758	6.079	0.611
1b—20 μM	6.747	0.804	5.914	0.844
1b—30 μM	6.340	1.087	5.175	1.026
1c—0 μM	4.582	0.091	7.778	0.100
1c—5 μM	4.361	0.107	7.278	0.116
1c—10 μM	3.446	0.142	5.040	0.138
1c—20 μM	2.439	0.170	3.786	0.179
1c—30 μM	2.001	0.216	3.227	0.255
2b—0 μM	1.290	0.109	9.681	0.569
2b—5 μM	1.106	0.234	7.626	0.820
2b—10 μM	0.956	0.270	7.491	1.370
2b—20 μM	0.813	0.291	6.431	1.750
2b—30 μM	0.755	0.440	5.134	2.354

from the enzyme active site. Standard drug donepezil also exhibits this type of inhibition [27].

Activity of the Detergent-Soluble (DS) and Salt-Soluble (SS) Fractions Acetylcholinesterase

The effects of compounds **1b**, **1c** and **2b** on SS (G1) and DS (G4) AChE fractions in cerebral cortex are shown in Fig. 7. Compound **1b** inhibited the activity of AChE DS fraction at the concentrations of 10 μM (51%), 25 μM (64%), 50 μM (77%), and 100 μM (85%) when compared to the control group ($P < 0.05$ Fig. 7). The activity of AChE DS fraction was also inhibited by **1c**, which showed reduction in the enzyme activity at the concentrations of 25 μM (65%), 50 μM (75%), and 100 μM (81%) when compared to the control group. Compound **2b** reduced the activity of AChE DS fraction at the concentrations of 25 μM (64%), 50 μM (75%), and 100 μM (86%). In contrast, the activity of AChE SS fraction was only inhibited by **1b** at 100 μM (64%). Compounds **1c** and **2b** did not show inhibitory effect on the AChE SS fraction in cerebral cortex (Fig. 7).

The compounds **1b** and **2b** were able to inhibit the activity of AChE DS fraction in hippocampus at the concentrations of 10 μM (57% and 47% respectively), 25 μM (around 63%), 50 μM (around 75%) and 100 μM (around 80%) when compared to the control group ($P < 0.05$) (Fig. 8). Compound **1c** reduced the activity of AChE DS fraction in hippocampus at the concentrations of 50 μM (64%) and 100 μM (64%) ($P < 0.05$). The activity of the SS fraction in hippocampus could only be inhibited by compound **1b** at the concentrations of 25 μM (71%), 50 μM (74%), and 100 μM (69%) when compared to the control group. Compounds **1c** and **2b** did not alter the activity of the SS fraction in hippocampus ($P > 0.05$) (Fig. 8).

AChE inhibitors are used to increase the acetylcholine level and thus improve the memory decline in AD patients. In brain, the most abundant AChE form found is membrane-associated form G4. However, a selective reduction of G4 has been observed in Alzheimer's disease, possibly as a consequence of neurodegeneration [28]. Previous studies have demonstrated that drugs used as AChE inhibitors, such as tacrine, donepezil, rivastigmine and galantamine can selectively inhibit particular molecular forms of AChE (G1 and G4) [29, 30] and that this selectivity varies between brain regions [31]. These differences between AChE inhibitors in relation to AChE isoform selectivity and brain region specificity can affect the therapeutic efficacy as well as adverse effects of these drugs.

In the present study, we showed that compound **1b** inhibits G1 and G4 isoforms in both the brain structures tested. Considering G1 isoform is relatively unaltered in Alzheimer's brain, the effect of compound **1b** may be important in therapeutic applications in order to increase the available acetylcholine in the remaining cholinergic neurons in AD.

Acetylcholinesterase Activity in Lymphocytes

Table 2 shows the effects of compounds on the AChE activity in lymphocytes. Compound **1b** inhibited the AChE activity at 50 μM (70%), 100 μM (80%) and 250 μM (90%) when compared to the control group ($P < 0.05$) (Table 2). Similar results were observed for compound **1c** ($P < 0.05$). Compound **1a** inhibited the AChE activity in lymphocytes only at the higher concentrations evaluated [100 μM (40%) and 250 μM (70%)]. However, the compounds **1d**–**g** did not

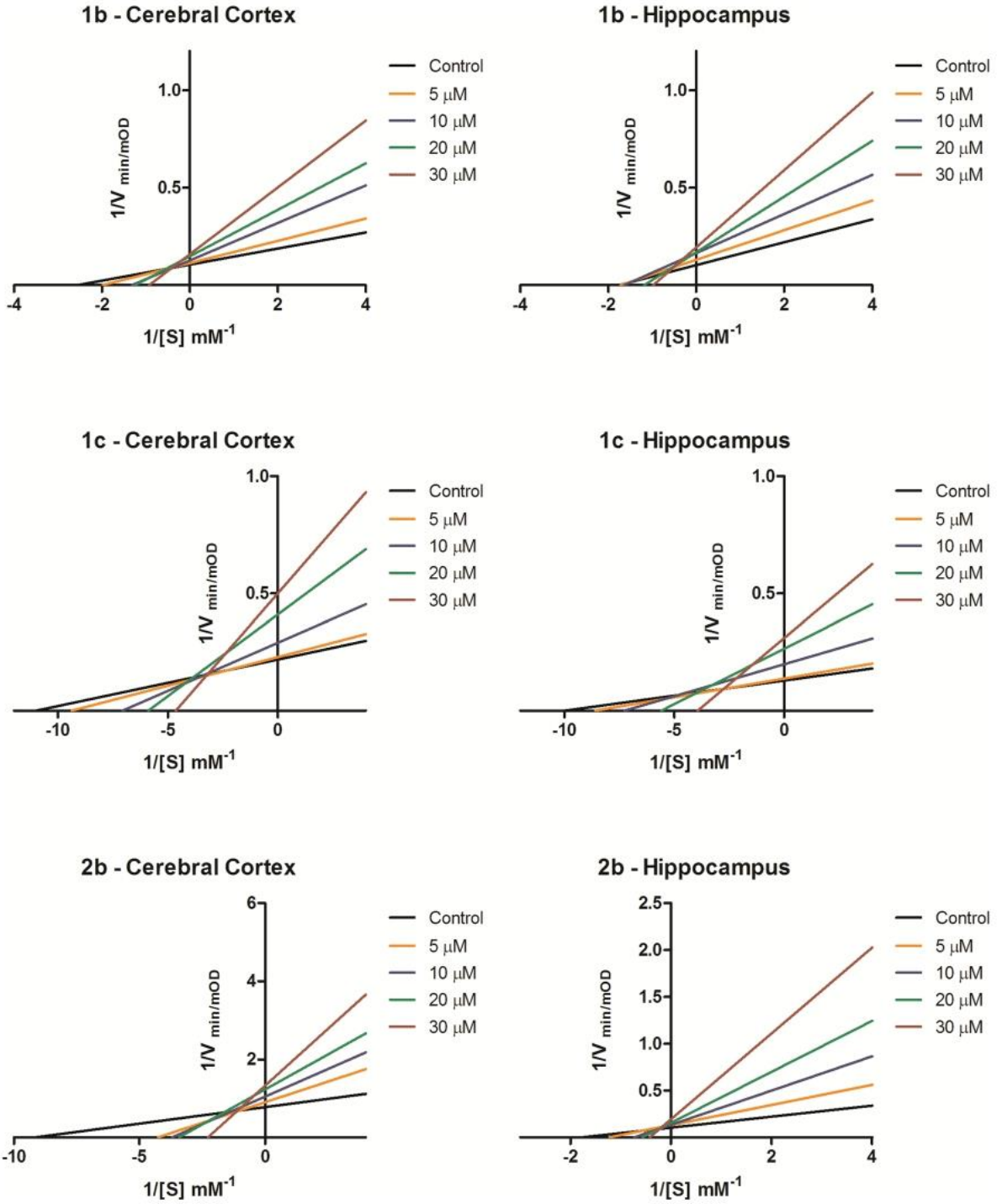

Fig. 6 Lineweaver–Burk representation of inhibition of AChE activity in cerebral cortex and hippocampus by compounds **1b**, **1c** and **2b**. Acetylcholine was used as a substrate. Double reciprocal plot was constructed by plotting 1/V against 1/S analyzed over a range of substrate concentrations (10–1000 μM) in the absence or presence of indicated compounds (5–30 μM). The plot represents the mean of three independent experiments (n = 5)

alter in vitro the AChE activity. The enzyme activity was inhibited by compound **2a** at the concentrations of 50, 100, and 250 μM (30–70%). The compounds **2b** and **2c** showed similar results. These compounds reduced the AChE activity in lymphocytes at the concentrations of 50 μM (around 60%), 100 μM (around 70%), and 250 μM (around 80%) when compared to the control group ($P < 0.05$). The compounds **2d** and **2e** did not alter the AChE activity.

Previous studies have discussed that AChE inhibitors may also be modulating the immune responses. Consistent

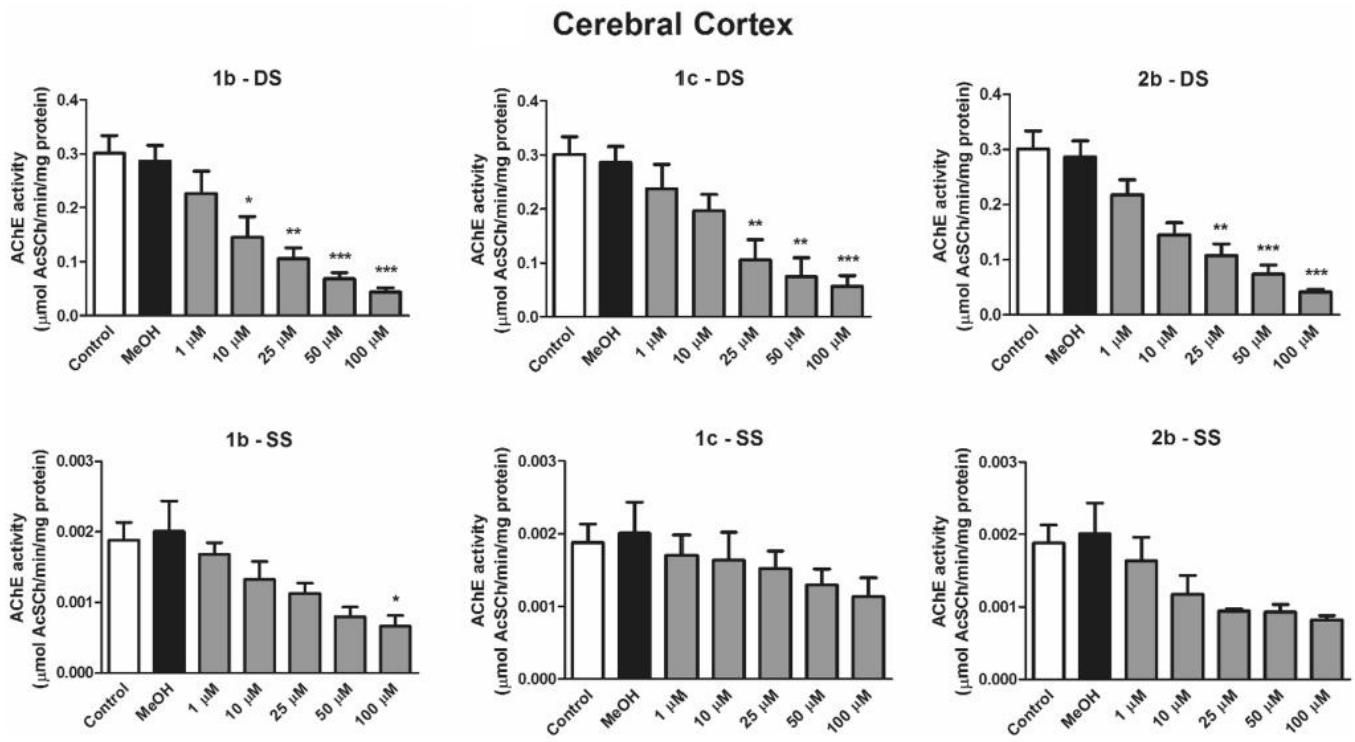

Fig. 7 In vitro effects of the compounds **1b**, **1c** and **2b** on the activities of detergent-soluble (DS) and salt-soluble (SS) fractions of acetylcholinesterase (AChE) isolated from rat cerebral cortex. Results are expressed in µmol AcSCh/min/mg of protein. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to the control group (water)

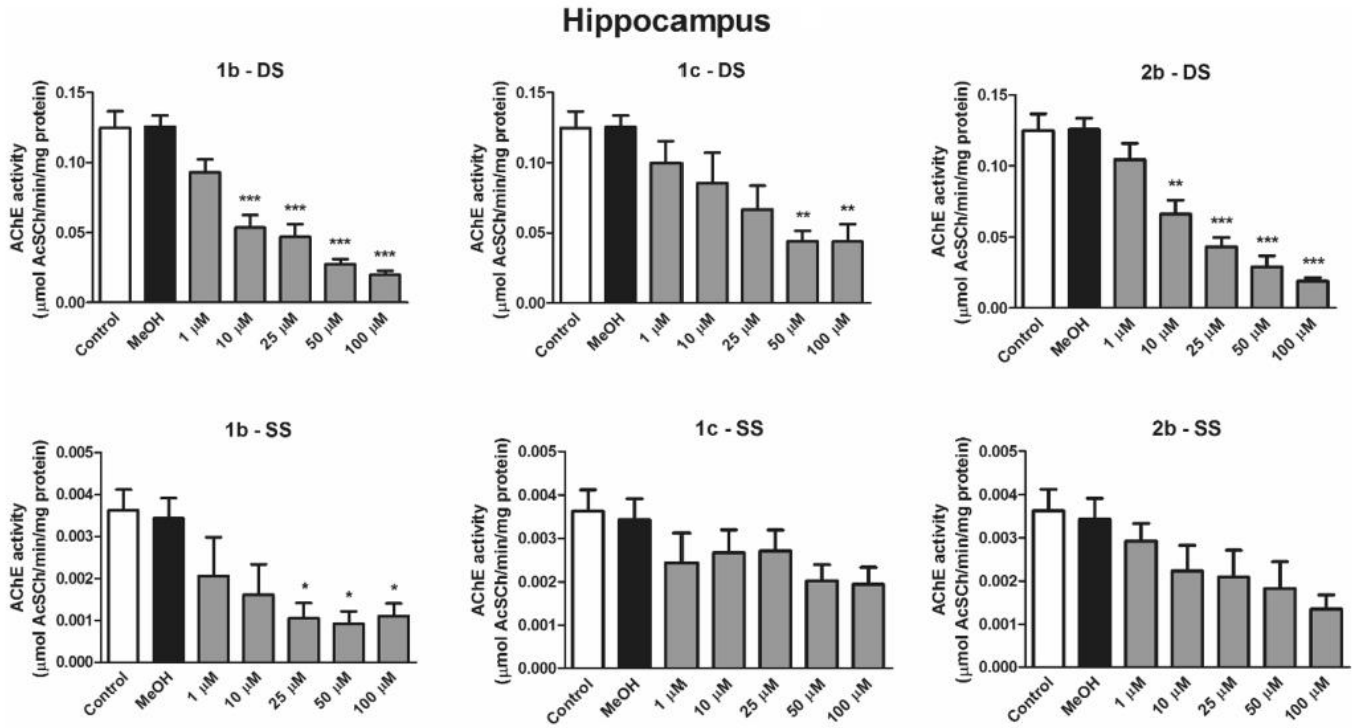

Fig. 8 In vitro effects of the compounds **1b**, **1c** and **2b** on the activities of detergent-soluble (DS) and salt-soluble (SS) fractions of acetylcholinesterase (AChE) isolated from rat hippocampus. Results are expressed in µmol AcSCh/min/mg of protein. Values are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to the control group (water)

Table 2 Effects of compounds **1a–g** and **2a–e** (50 µM, 100 µM and 250 µM) on the acetylcholinesterase activity in rat lymphocytes

Compound	AChE activity in lymphocytes (µmol AcSCh/h/mg of protein)				
	Control	MeOH	50 µM	100 µM	250 µM
1a	30.3 ± 2.12	30.74 ± 2.43	25.67 ± 0.83	19.95 ± 1.91**	10.97 ± 1.08***
1b	30.3 ± 2.12	30.74 ± 2.43	10.33 ± 1.95***	6.15 ± 1.46***	3.51 ± 0.39***
1c	30.3 ± 2.12	30.74 ± 2.43	10.48 ± 0.59***	5.12 ± 0.44***	2.74 ± 0.24***
1d	21.51 ± 1.90	20.98 ± 2.11	16.31 ± 2.04	16.49 ± 1.52	15.33 ± 0.57
1e	21.51 ± 1.90	20.98 ± 2.11	16.85 ± 1.48	15.64 ± 1.36	14.95 ± 1.55
1f	38.2 ± 1.98	37.99 ± 1.85	38.70 ± 1.75	42.65 ± 2.72	48.77 ± 0.48
1g	37.26 ± 1.87	37.99 ± 1.85	30.20 ± 1.79	31.40 ± 1.77	39.17 ± 2.84
2a	21.51 ± 1.90	20.98 ± 2.11	14.13 ± 0.82*	11.03 ± 0.82**	6.94 ± 0.31***
2b	30.3 ± 2.12	30.74 ± 2.43	13.18 ± 2.16***	9.95 ± 1.38***	4.54 ± 0.44***
2c	30.3 ± 2.12	30.74 ± 2.43	14.87 ± 1.32***	10.82 ± 0.80***	6.39 ± 0.53***
2d	37.26 ± 1.87	37.99 ± 1.85	38.60 ± 1.54	37.41 ± 1.47	37.40 ± 1.04
2e	21.51 ± 1.90	20.98 ± 2.11	20.09 ± 3.12	17.09 ± 0.41	15.26 ± 0.68

Results are expressed in µmol AcSCh/h/mg of protein. Values represent mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the control group

with this hypothesis, AChE inhibitors increased the acetylcholine levels, a molecule with anti-inflammatory function. Available acetylcholine interacts with nicotinic receptors expressed on lymphocyte surfaces and thus inhibits the secretion of cytokines associated with inflammation [32, 33]. Corroborating with the results obtained in brain structures, the compounds **1b**, **1c** and **2b** showed potent AChE inhibitory activity in lymphocytes. Our findings suggest that these compounds can increase the cholinergic transmission by interacting with both central and peripheral AChE. Therefore, these compounds may be used as potential therapeutic agents to improve memory deficits and neuroinflammation.

Molecular Docking Analyses

Molecular docking showed that nearly all thiazolidinones had the same three-dimensional conformation with respect to the main groups in the molecule. Molecular modeling data demonstrated that all tested molecules had similar free energy of binding (FEB). These values were lower than that for donepezil (DNZ) (FEB: compounds **1** from − 7.6 to − 9.2 kcal/mol, compounds **2** from − 7.5 to − 9.1 kcal/mol, and DNZ = − 11.8 kcal/mol). Thus, all compounds showed potential binding with the active site of AChE. These results are in agreement with the findings of Iyer et al. [34]. They performed molecular docking study to determine the affinities of thiazolidin-4-ones for several targets, including AChE, and demonstrated that thiazolidin-4-ones had good binding affinities for the active site of AChE.

Interestingly, compounds **1b**, **1c** and **2b** showed good three-dimensional fit with AChE similar to donepezil and ACh. It was observed that the amide nitrogen of the thiazolidinone ring was close to the piperidinic nitrogen of donepezil and the quaternary nitrogen of the ACh. In addition, the basic nitrogen atoms of compounds (piperidine of **1b** and **2b** or diethylamino of **1c**) were close to the ACh ester group binding site. Therefore, the compounds **1b**, **1c** and **2b** share a common binding site with both donepezil and ACh as shown in Fig. 9.

Conclusion

Our results show the potential of 1,3-thiazolidinones as cholinesterase inhibitors, highlighting the importance of the propylpiperidine (**b**) moiety. Thiazolidinones **1b** and **2b** seem to be promising compounds against memory decline in AD, which is associated with cholinergic dysfunctions in brain. Molecular docking suggests a good overlap of thiazolidinones with the active site of AChE, similar to ACh

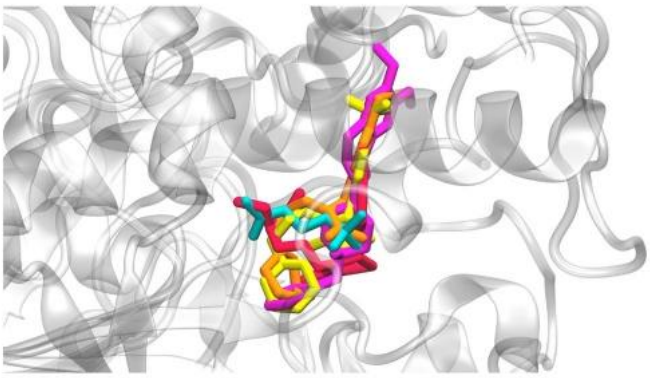

Fig. 9 Three-dimensional arrangement of molecular docking between compounds **1b** (orange), **1c** (red), **2b** (yellow), donepezil (purple) and ACh (cyan) with AChE enzyme

and donepezil. Enzyme kinetics suggested mixed AChE inhibition, which is also exhibited by donepezil. In addition, the ability of these compounds to inhibit the AChE activity in lymphocytes suggests a potential role in modulating cholinergic signaling associated with inflammation process. These preliminary results provide novel insights into the role of thiazolidinone compounds. Further in vivo studies are needed to establish the physiological importance of these compounds.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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5.2. MANUSCRITO

O manuscrito foi submetido à revista Chemico-Biological Interactions.

A aprovação junto ao comitê de ética em experimentação animal (CEEA) autorizando a realização da pesquisa desenvolvida neste manuscrito encontra-se em anexo a esta tese (**ANEXO C**). O comprovante de submissão pode ser conferido no **ANEXO D**. O guia para autores com as normas para publicação da revista Chemico-Biological Interactions encontra-se no **ANEXO E**.

Multitarget effect of 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one in a scopolamine-induced amnesic rat model

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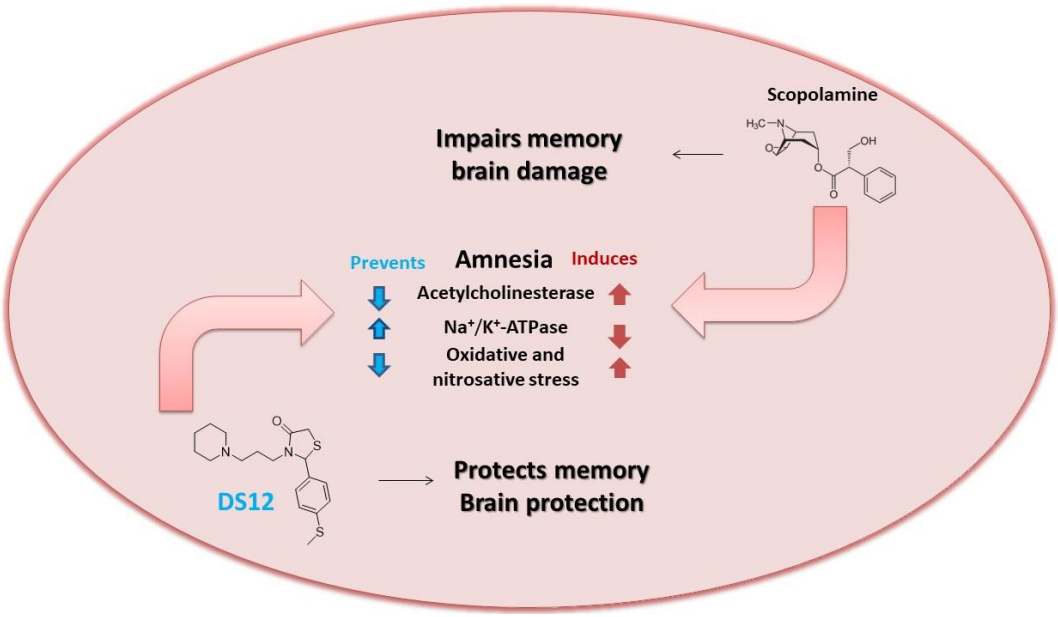
Graphical abstract



Highlights

1. DS12 prevented memory deficits induced by scopolamine.
2. DS12 prevented brain acetylcholinesterase alterations induced by scopolamine.
3. DS12 prevented alterations in cholinesterase activity in serum and lymphocytes.
4. DS12 prevented brain oxidative damage induced by scopolamine.
5. Inhibition of Na^+/K^+-ATPase activity in an amnesic model was prevented by DS12.

Abstract

Cholinergic system dysfunction, oxidative damage, and alterations in ion pump activity have been associated with memory loss and cognitive deficits in Alzheimer's disease (AD). 1,3-thiazolidin-4-ones have emerged as a class of compounds with potential therapeutic effects due to their potent anticholinesterase activity. Accordingly, this study investigated the effect of the 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one **(DS12**) compound on memory, cholinergic and oxidative stress parameters, ion pump activity, and serum biochemical markers in a scopolamine-induced memory deficit model. Male Wistar rats were divided into four groups: I- Control; II- Scopolamine; III- DS12 (5 mg/kg) + scopolamine; and IV- DS12 (10 mg/kg) + scopolamine. The animals from groups III and IV received DS12 diluted in canola oil and administered for 7 days by gavage. On the last day of treatment, scopolamine (1 mg/kg) was administered intraperitoneally (i.p.) 30 min after training in an inhibitory avoidance apparatus. Twenty-four hours after scopolamine administration, the animals were subjected to an inhibitory avoidance test and were thereafter euthanized. Scopolamine induced memory deficits, increased acetylcholinesterase activity and oxidative damage, and decreased Na^+/K^+-ATPase activity in cerebral cortex and hippocampus. Pretreatment with DS12 prevented these brain alterations. Scopolamine also induced an increase in acetylcholinesterase activity in lymphocytes and whereas butyrylcholinesterase in serum and treatment with DS12 prevented these changes. In animals treated with DS12, no changes were observed in renal and hepatic parameters when compared to the control group. In conclusion, DS12 emerged as an important multitarget compound capable of preventing neurochemical changes associated with memory deficits.

Keywords: 1,3-thiazolidin-4-ones; scopolamine; oxidative stress; acetylcholinesterase; dementia

1. Introduction

Dementia incidence increases with age and causes a decline in cognitive functions, including memory, language, behavior, and the performance of basic daily activities. The most common cause of dementia is Alzheimer's disease (AD), a progressive neurodegenerative disease. AD is responsible for more than 80% of diagnosed cases of dementia, representing about 46.8 million people worldwide, a number that has been increasing over the years. In addition, death rates related to AD have also increased [1-3].

The pathophysiology of AD is complex, and several mechanistic models have been proposed to explain the pathogenesis of the disease; however, the exact etiology is unclear [1,4,5]. Cholinergic hypotheses propose that memory loss and cognitive dysfunction, the main symptoms of AD, are caused by low levels of acetylcholine (ACh). In this sense, cholinesterases have a regulatory role in cholinergic neurotransmission. It is known that acetylcholinesterase (AChE) rapidly hydrolyzes ACh and performs the important role of a regulatory enzyme at cholinergic synapses. Butyrylcholinesterase (BuChE) is closely related to AChE; however, while AChE levels in the brain decrease with disease progression, BuChE levels remain at least the same [1,6,7].

Another important feature involved in the development of AD is oxidative stress, which occurs in early stages [1,8]. The altered mitochondrial membrane potential and the deposition of amyloid beta aggregates (Aβ) are involved in the production of reactive oxygen species (ROS), and it is known that there is an imbalance between ROS production and antioxidant defense in AD. Thus, oxidative stress and free radical generation could lead to neuronal damage and death [6,9].

Currently, there are few therapeutic options available for the treatment of AD. Furthermore, current treatments only lead to symptom relief and are unable to treat or impede the progress of the disease [2,4,6]. Therefore, it is important to investigate new classes of compounds in search of better therapies that can benefit patients with neurodegenerative diseases such as AD.

Scopolamine-induced memory deficit is a widely used model to study dementia-related illnesses, such as AD [8,10-12]. Scopolamine is an antagonist of muscarinic receptors, and is used in several neurobehavioral studies due to its ability to impair memory and learning. Alterations in cellular and molecular parameters induced by scopolamine treatment include an increase in oxidative stress, mitochondrial dysfunction, and

neuroinflammation as well as a reduction in ACh levels, increase in cholinesterase activity, and impairments in antioxidant defenses. Therefore, this is an important model in the study of antiamnesic effects of novel compounds [8].

In recent years, 1,3-thiazolidin-4-ones have emerged as a class of compounds with AChE inhibitory activity, as shown by our research group [13]. We have also demonstrated the potential of AChE's inhibition of benzothiazin-4-ones, a seven-membered ring analog of thiazolidinone [14]. Silva and collaborators [13] demonstrated the ability of 1,3-thiazolidin-4-ones, synthesized from 4-(methylthio)benzaldehyde and 4-(methylsulfonyl)benzaldehyde, as well as of mercaptoacetic acid and different amine cores, to act as cholinesterase inhibitors *in vitro*, and highlighted the affinity of these compounds to active sites of AChE through molecular modeling. These previous studies from our laboratory have demonstrated that the compound 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one (DS12) (**Figure 1**) was capable of inhibiting the *in vitro* AChE activity in the brain and lymphocytes of rats. In addition, DS12 also inhibited different isoforms of AChE in brain structures involved in memory processes, such as cerebral cortex and hippocampus [13].

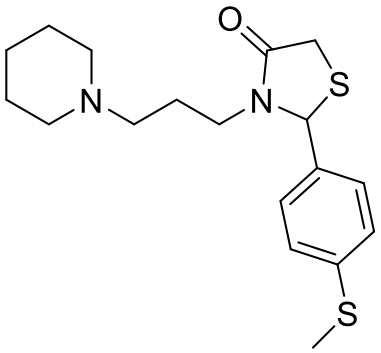


Figure 1. Structure of **DS12**.

Considering the promising results described by *in vitro* studies of DS12 and the need to obtain new therapeutic targets for treating memory deficits, the aim of this study was to investigate the antiamnesic effect of DS12 in a scopolamine-induced memory deficit model in rats. The protective effect of DS12 on cholinergic and brain oxidative stress parameters, ion pump activity, and serum biochemical markers were also evaluated.

2. Materials and methods

2.1 Synthesis of 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one (DS12)

DS12 was synthesized according to the method described by Silva and colleagues [15], and was accurately identified by gas chromatography-mass spectrometry (GC-MS).

2.2 Animals and ethical approval

All animal experimental protocols were approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, RS, Brazil (protocol number: CEEA 46528-2018). Male Wistar rats (60 days old) were obtained from the Central Animal House of the Federal University of Pelotas. The animals were maintained at a constant temperature (22 ± 1°C) with a 12/12 h light/dark cycle, humidity (50% – 65%), and with food and water available ad libitum. All animals were arbitrarily assigned before starting treatment, without specific tools, to achieve randomization in the animal facility room. The use of the animals was in accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities (DBCA), National Council of Control of Animal Experimentation (CONCEA), and with the NIH Guide for Care and Use of Laboratory Animals.

2.4 Scopolamine protocol and treatment with DS12

The animals were divided into four groups (10 animals per group): I- Control; II- Scopolamine; III- **DS12** (5 mg/kg) + scopolamine; and IV- **DS12** (10 mg/kg) + scopolamine. The animals from groups III and IV received DS12 diluted in canola oil for 7 days by oral administration. Groups I and II received the same volume of canola oil. On the last day of treatment, the animals received DS12 30 min before training in an inhibitory avoidance apparatus. Scopolamine (1 mg/kg) was dissolved in saline and injected intraperitoneally (i.p.) 30 min after training in the inhibitory avoidance apparatus, as previously described [14,16,17]. Twenty-four hours after scopolamine administration, the animals were subjected to the inhibitory avoidance test and were subsequently euthanized (**Figure 2**).

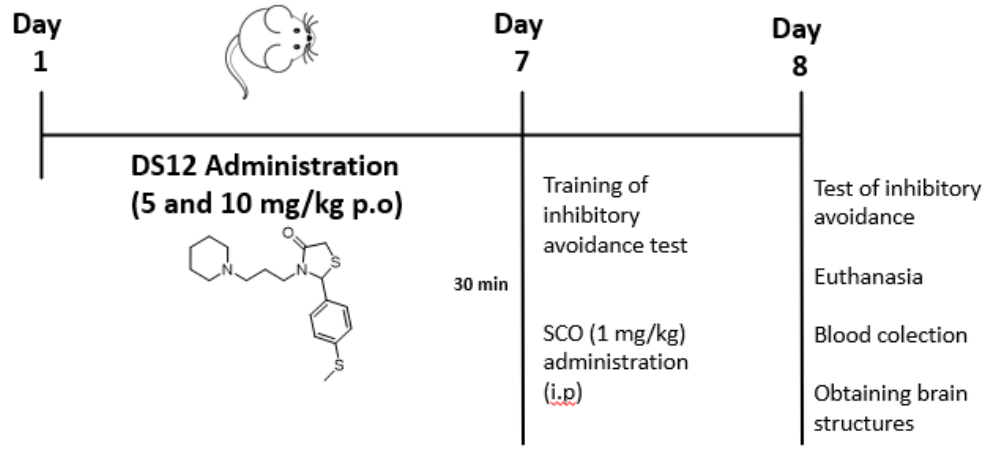


Figure 2: Schematic of the experimental design.

2.5 Behavioral procedure

2.5.1 Inhibitory avoidance test

The rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 × 25 × 35 cm box with a grid floor whose right portion was covered with a 7 × 25 cm platform, 2.5 cm high. The rats were placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 0.5 mA shock was applied to the grid for 3 s. Twenty-four hours after the training, the animals were tested in a step-down inhibitory avoidance task. The test step-down latency was taken as a measure of retention, and a cutoff time of 300 s was established. After this behavioral test, the animals were anesthetized with isoflurane and euthanized, and their brains and blood were collected. The hippocampus, cerebral cortex, and serum were obtained and used for biochemical determinations.

2.6 Brain Biochemical Analyses

2.6.1 Acetylcholinesterase activity

Brain samples (hippocampus and cerebral cortex) were placed in a solution of Tris-HCl (10 mM, pH 7.4), homogenized, and centrifuged at 1800 rpm for 10 min at 4°C. The supernatant was used for the AChE assay, as previously described by Ellman et al. (1961) [18]. The reaction mixture comprised 10 mM 5,5-dithiobis-(2-nitrobenzoic acid), 10 mM phosphate buffer (pH 7.5), 15 µL supernatant, and 0.8 mM acetylthiocholine. The absorbance at 412 nm was read on a spectrophotometer at 30-s intervals for 2 min at 27°C. AChE activity was expressed as µmol AcSCh/h/mg protein.

2.6.2 Na^+/K^+-ATPase activity

The hippocampus and cerebral cortex were homogenized (1/10 w/v) in 0.32 mM sucrose containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at 1000×g for 10 min, and the supernatants were removed for further analysis. The reaction mixture for this assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, and 40 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of ATP to a final concentration of 3 mM. Control samples were generated under the same conditions with the addition of 1 mM ouabain. Na^+/K^+-ATPase activity was calculated by measuring the difference in absorbance between the study samples and controls, as described by Wyse et al. (2007) [19]. The level of released inorganic phosphate (Pi) was measured as described by Chan et al. (1986) [20]. Specific enzyme activity was expressed as nmol Pi released/min/mg protein.

2.6.3 Oxidative stress parameters

Brain samples (hippocampus and cerebral cortex) were homogenized (1/10 w/v) using 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenates were centrifuged at 2500×g for 10 min at 4°C. The supernatants were collected and used for further analyses.

2.6.3.1 Reactive oxygen species (ROS) assay

The oxidation of DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) was measured. Briefly, DCF fluorescence intensity emission was recorded at excitation wavelengths of 525 and 488 nm 30 min after the addition of DCFH-DA to the medium. ROS formation was expressed as μmol DCF/mg protein [21].

2.6.3.2 Nitrite quantification

Nitrite was measured by the Griess reaction [22]. In brief, 50 μL homogenate was incubated with 50 μL 1% sulfanilamide and 50 μL 0.3% N-1-naphthylethylenediamine dihydrochloride at room temperature (RT) for 10 min. Nitrite was measured at 540 nm using sodium nitrite as a standard. Results are expressed as μM nitrite/mg protein.

2.6.3.3 Total sulfhydryl content assay

Supernatants were added to PBS buffer (pH 7.4) containing EDTA. The reaction was initiated by the addition of DTNB. One hour after incubation in the dark, DTNB reduced by thiol groups was oxidized (disulfide) and a yellow derivative (TNB) was generated, whose absorbance at 412 nm was measured. The results were reported as nmol TNB/mg protein [23].

2.6.3.4 Thiobarbituric acid reactive substances (TBARS) assay

The supernatants were mixed with 10% trichloroacetic acid and centrifuged. The supernatant was collected and mixed with TBA (0.67%) and incubated in a dry block at 100°C for 30 min. TBARS levels were determined by measuring absorbance at 535 nm and reported as nmol of TBARS/mg protein [24].

2.6.3.5 Superoxide dismutase (SOD) activity

This assay is based on the inhibition of superoxide-dependent adrenaline autooxidation to adrenochrome. The intermediate in this reaction is superoxide, which is scavenged by SOD, and the absorbance is measured at 480 nm on a spectrophotometer. SOD activity was measured as previously described by Misra and Fridovich (1972) [25] and reported as units/mg protein.

2.6.3.6 Catalase (CAT) activity

The decomposition of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored at 240 nm for 180 s at 37°C, as previously reported by Aebi (1984) [26]. CAT activity was reported as units/mg protein.

2.6.4 Enzymatic assays in lymphocytes

2.6.4.1 Isolation of lymphocytes from whole blood

Blood was collected in tubes containing the anticoagulant EDTA. Total blood was diluted in an equal volume of saline, and the separation of lymphocytes was realized with Ficoll-Histopaque density gradients [27]. The methodology described by Böyum (1968) [27] was employed for separating mononuclear cells, whereas experiments based on the study performed by Jaques et al. [28] demonstrated that there is a high incidence of lymphocytes, about 95%, in these samples, and that the amount of monocytes is practically insignificant.

2.6.4.2 Acetylcholinesterase activity

AChE activity was determined as described by Ellman et al. (1961) [18], modified by Fitzgerald and Costa (1993) [29]. The reaction mixture was composed of 1.0 mM acetylthiocholine, 0.1 mM 5,5′-dithio-bis-2-nitrobenzoic acid, and 0.1 M phosphate buffer (pH 8.0), and intact lymphocytes suspended in saline solution were added to the reaction. The absorbance was read at 412 nm. All samples were run in triplicate and the activity of lymphocyte AChE was expressed as μmol/h/mg of protein.

2.6.5 Serum preparation and biochemical analysis

2.6.5.1 Serum preparation

Blood samples were collected in tubes without an anticoagulant and subsequently centrifuged at 2500×g for 15 min at room temperature. The clot was removed and the resulting serum was used for biochemical analysis.

2.6.5.2 Butyrilcholinesterase assay

BuChE activity was determined by a modification of the method of Ellman et al. (1961) [18]. The method is based on the formation of 5,5-dithiobis-acid nitrobenzoic acid measured at 412 nm. The reaction was initiated by adding butyrylthiocholine iodide (BuSCh). BuChE activity was expressed in μmol BuSCh/h/mg of protein.

2.6.5.2 Hepatic and renal marker assays

Hepatic and renal functions were evaluated using the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, and urea, regardless of fasting. The effect of the 7-day pretreatment with DS12 on these parameters was evaluated only at the highest concentration tested. Urea analyses were performed using a Labtest commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil) and analyses of ALT, AST, and creatinine were carried out on commercial Cobas kits on the Cobas C501 automated equipment (Roche/Hitachi, Roche Diagnostica Brazil Ltda., São Paulo, Brazil).

2.6.6 Protein determination

Protein concentration was measured as previously described by Lowry et al. (1951) [30] or according to the method previously described by Bradford (1976) [31].

2.7 Statistical analysis

Results from the inhibitory avoidance test assumed a non-parametric distribution. Thus, only these data were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test. In addition, these data are expressed as median ± interquartile range.

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests using GraphPad Prism 5.0 (Intuitive Software for Science, São Diego, CA, USA). $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1 DS12 prevents memory loss induced by scopolamine

The results from experiments examining inhibitory avoidance are demonstrated in **Figure 3**. The administration of scopolamine reduced the latency time to the platform ($H = 22.67$; $P < 0.001$), indicating memory deficits. In contrast, pretreatment with **DS12** at both doses (5 and 10 mg/kg) prevented this effect ($P < 0.05$ and $P < 0.001$, respectively).

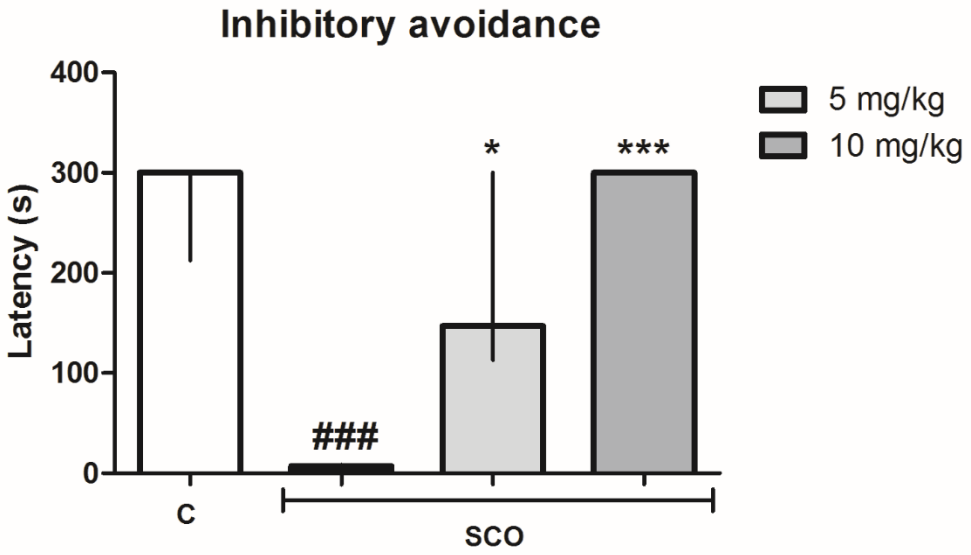


Figure 3: Effect of **DS12** (5 and 10 mg/kg) and scopolamine (SCO 1 mg/kg) on the latency time in the inhibitory avoidance test. Bars represent the median ± interquartile range. Statistical analysis was performed by the Kruskal–Wallis test followed by Dunn's post hoc test for multiple comparison. ### $P < 0.001$ represents a significant difference compared to the control group (C). * $P < 0.05$, ***$P < 0.001$ represents a significant difference compared to the SCO group.

3.2 DS12 prevents changes in AChE and Na^+/K^+-ATPase activity induced by scopolamine

Figure 4 shows that AChE activity was increased by scopolamine in the cerebral cortex ($F_{(3, 22)} = 15.48$, $P < 0.001$); however, pretreatment with **DS12** at both doses (5 and 10 mg/kg) was able to prevent this change ($P < 0.001$; Fig. 4A). Similar results were found in the hippocampus, in which scopolamine enhanced AChE activity ($F_{(3, 22)} = 9.90$, $P < 0.001$), whereas **DS12** at a concentration of 10 mg/kg was able to prevent this change ($P < 0.01$; Fig. 4B).

Regarding Na^+/K^+-ATPase, as shown in **Figure 4**, we observed a decrease in enzymatic activity in cerebral cortex ($F_{(3, 20)} = 22.30$, $P < 0.05$; Fig. 4C) and hippocampus ($F_{(3, 21)} = 5.08$, $P < 0.05$; Fig. 4D). Pretreatment with **DS12** at both doses prevented this alteration in the Na+/K+-ATPase in the cerebral cortex and hippocampus.

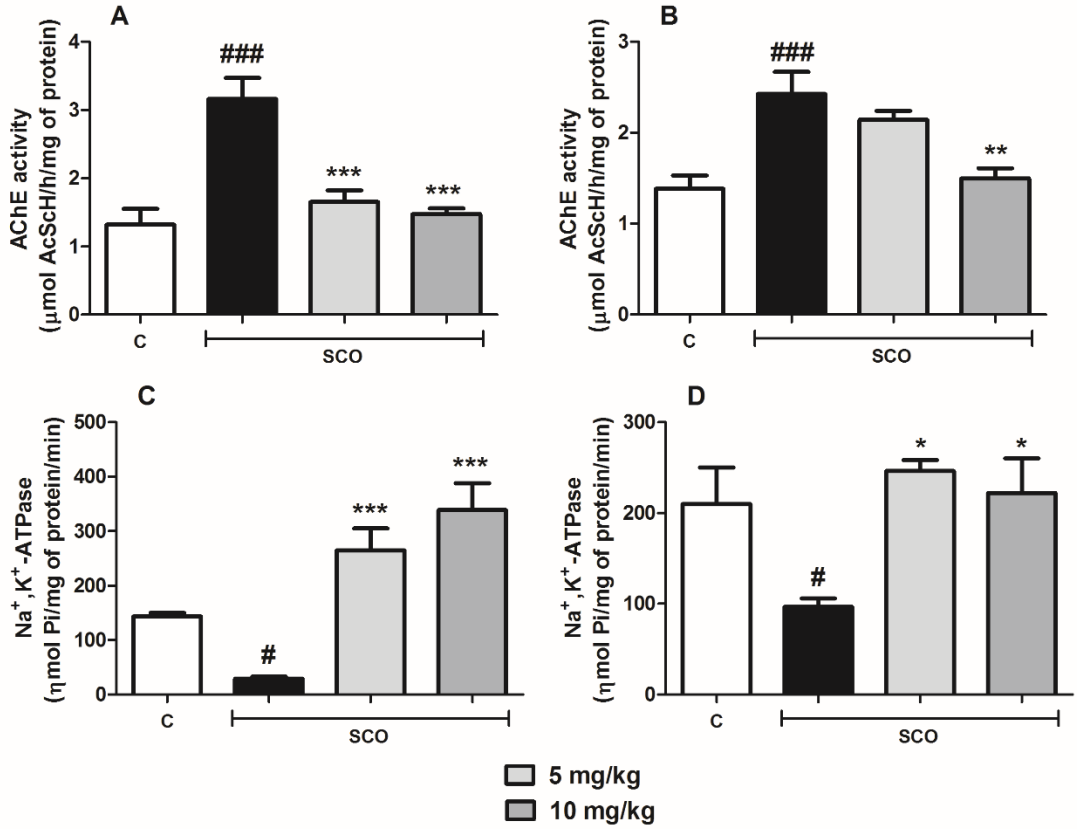


Figure 4: Effect of **DS12** (5 and 10 mg/kg) and scopolamine (SCO 1 mg/kg) on acetylcholinesterase (**A**: cerebral cortex; **B**: hippocampus) and Na^+/K^+-ATPase (**C**: cerebral cortex; **D**: hippocampus) activity in adult rats. Bars represent the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for

multiple comparison. $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.001$ represents a significant difference compared to the control group (C). ***** $P < 0.05$, ******$P < 0.01$, *******$P < 0.001$ represents a significant difference compared to the SCO group.

3.3 DS12 protects against scopolamine-induced increase in peripheral cholinesterase activity

As shown in **Figure 5**, pretreatment with both doses (5 and 10 mg/kg) of **DS12** prevented the increase in AChE activity in lymphocytes induced by scopolamine ($F_{(3, 16)} = 6.48$, $P < 0.05$). Similar results were demonstrated for BuChE activity in serum. **DS12** pretreatment at 10 mg/kg prevented the enhancement of BuChE activity caused by scopolamine ($F_{(3, 36)} = 14.24$, $P < 0.001$).

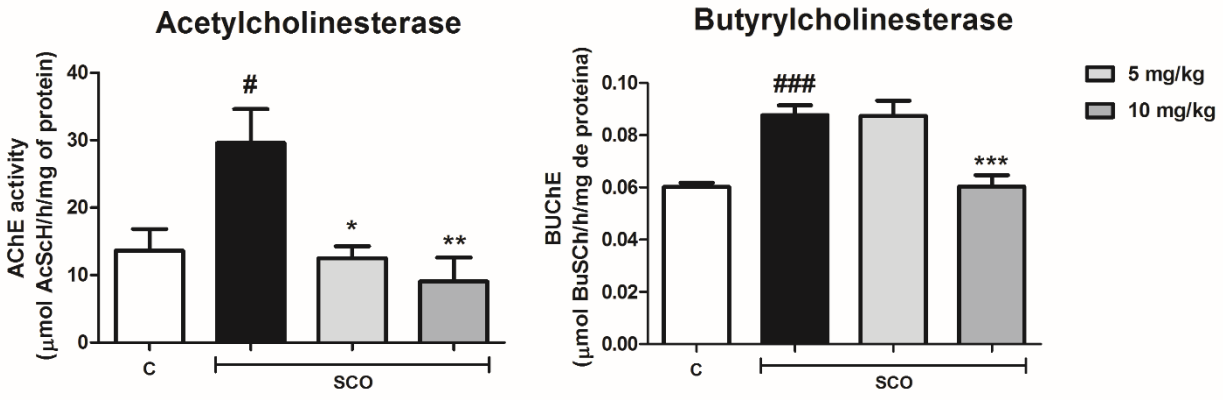


Figure 5: Effect of **DS12** (5 and 10 mg/kg) and scopolamine (SCO 1 mg/kg) on the activity of acetylcholinesterase in lymphocytes and butyrylcholinesterase in the serum of adult rats. Bars represent the mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. $^{\#}$ $P < 0.05$, $^{\#\#\#}$ $P < 0.001$ represents a significant difference compared to the control group (C). ***** $P < 0.05$, ****** $P < 0.01$, ******* $P < 0.001$ represents a significant difference compared to the SCO group.

3.4 DS12 protects the cerebral cortex and hippocampus from oxidative stress caused by scopolamine

Figure 6 shows the effects of scopolamine and **DS12** on oxidative stress parameters in cerebral cortex. ROS ($F_{(3, 25)} = 9.97$, $P < 0.01$) and TBARS ($F_{(3, 16)} = 5.13$, $P < 0.05$) levels were significantly increased in the scopolamine group; however, pretreatment with 10 mg/kg of **DS12** was able to prevent these changes. Similar results were found for nitrite levels, which were high in the scopolamine group ($F_{(3, 18)} = 13.41$, $P < 0.001$). On

the other hand, the administration of 5 and 10 mg/kg of **DS12** prevented this increase. No significant differences were found in SH content between any of the experimental groups. Regarding the antioxidant enzymes, there was a decrease in SOD ($F_{(3, 13)} = 10.66$, $P < 0.001$) and CAT ($F_{(3, 15)} = 13.23$, $P < 0.001$) activity in the scopolamine group; however, pretreatment with **DS12** at 5 and 10 mg/kg was able to prevent this change in both SOD and CAT activity.

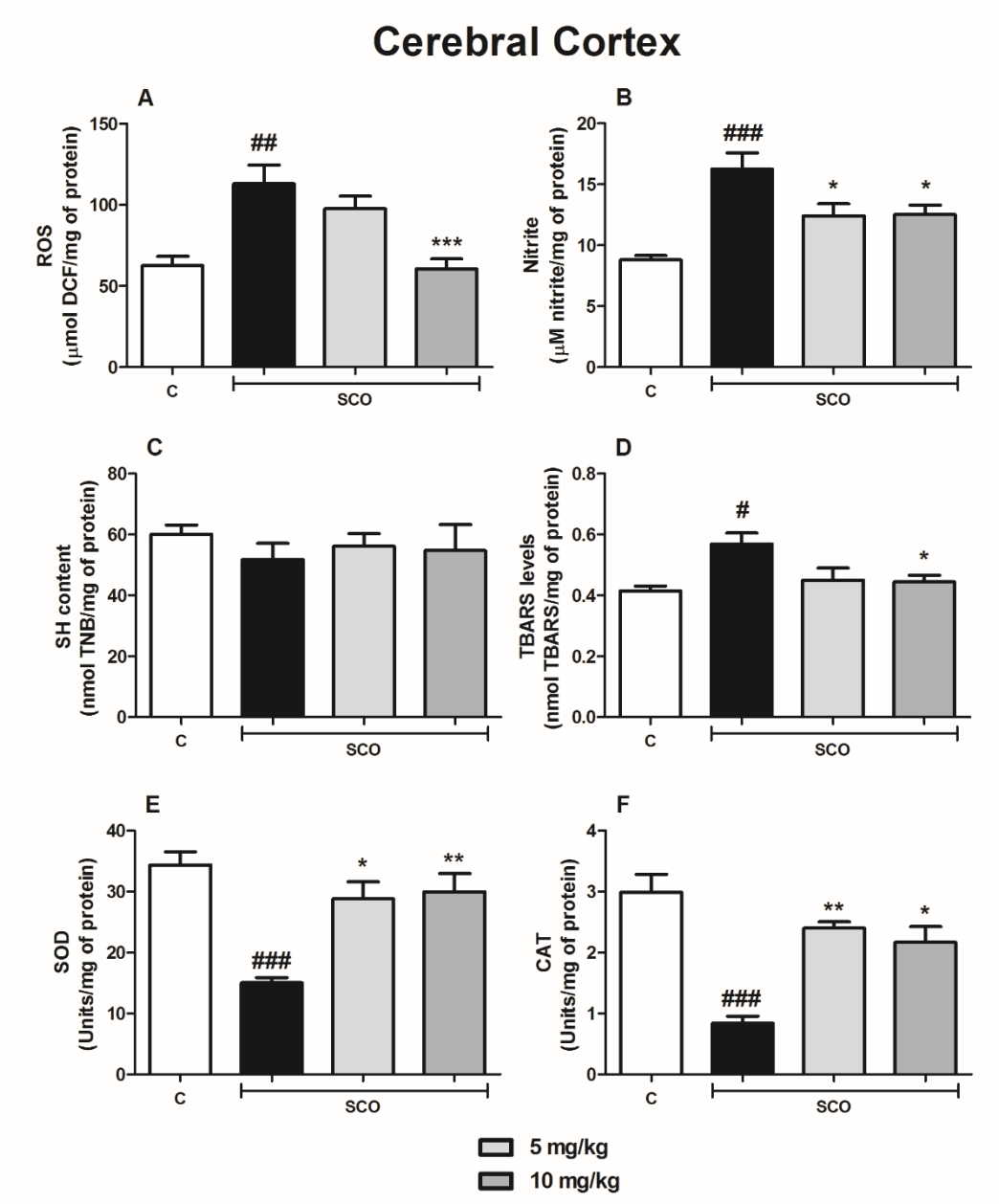


Figure 6: Effect of **DS12** (5 and 10 mg/kg) and scopolamine (1 mg/kg) on levels of reactive oxygen species (**A**), nitrite (**B**), total thiol content (**C**), thiobarbituric acid reactive substances (**D**) and activity of the superoxide dismutase (**E**), and catalase (**F**) enzymes in cerebral cortex of adult rats. Bars represent the mean ± SEM. Statistical analysis was

performed by one-way ANOVA followed by Tukey post hoc test for multiple comparison. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ represents a significant difference compared to the control group. ***** $P < 0.05$, ******$P < 0.01$, *******$P < 0.001$ represents a significant difference compared to the SCO group.

In the hippocampus, it was observed (**Figure 7**) that **DS12** at 10 mg/kg was able to prevent the increase in ROS production caused by scopolamine ($F_{(3, 25)} = 6.85$, $P < 0.05$). In addition, pretreatment with the same dose of the compound prevented the reduction in CAT activity caused by scopolamine administration ($F_{(3, 15)} = 4.10$, $P < 0.05$). No changes were found in nitrite ($F_{(3, 15)} = 0.42$, $P > 0.05$), SH ($F_{(3, 15)} = 2.46$, $P > 0.05$), and TBARS ($F_{(3, 15)} = 2.44$, $P > 0.05$) levels and in SOD activity.

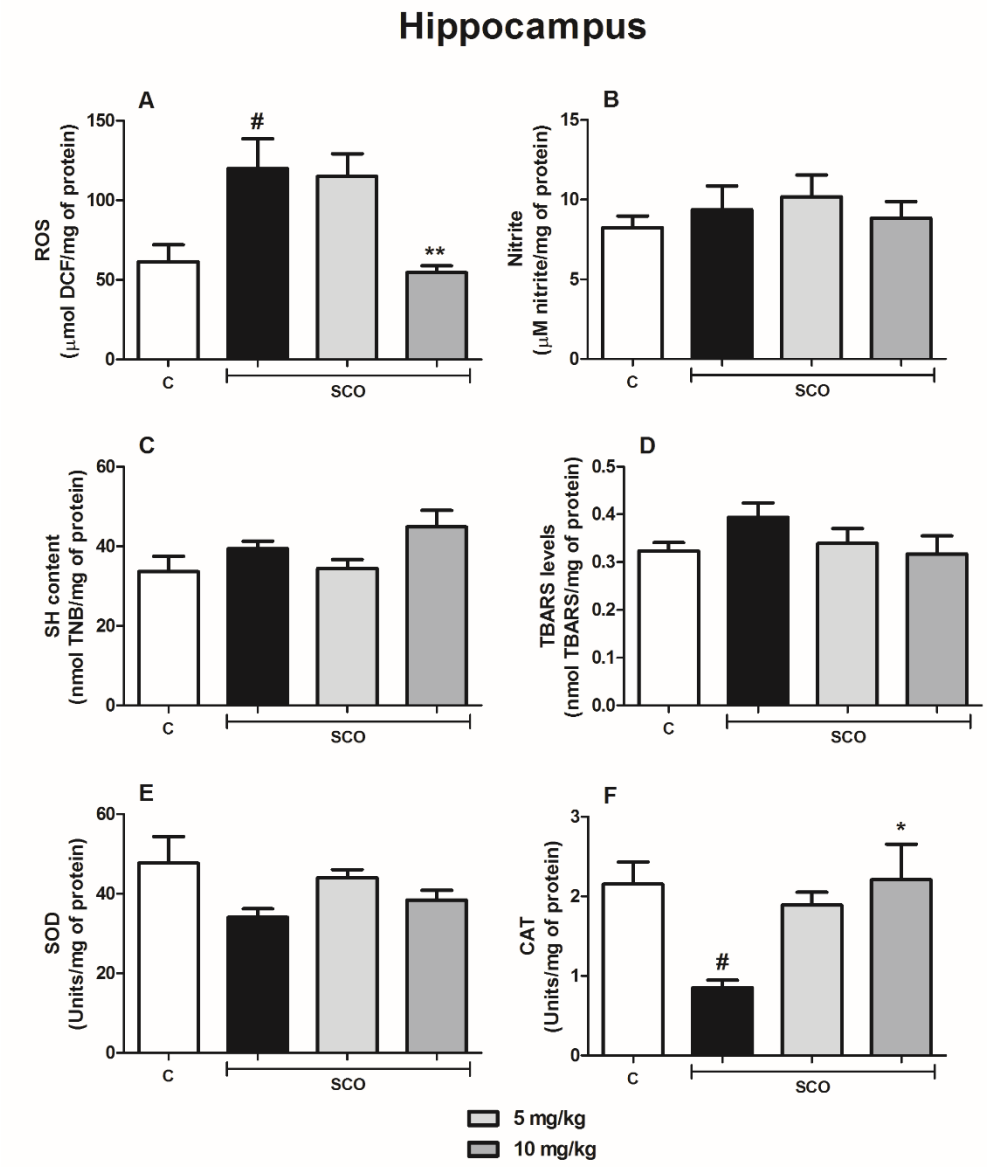


Figure 7: Effect of **DS12** (5 and 10 mg/kg) and scopolamine (SCO 1 mg/kg) on levels of reactive oxygen species (**A**), nitrite (**B**), total thiol content (**C**), thiobarbituric acid reactive

substances (**D**), and activity of the superoxide dismutase (**E**) and catalase (**F**) enzymes in the hippocampus of adult rats. Bars represent the mean ± SEM. $^{\#}P < 0.05$ represents a significant difference compared to the control group (C). ***** $P < 0.05$, ****** $P < 0.01$ represents a significant difference compared to the SCO group.

3.5 DS12 treatment did not alter hepatic and renal markers and glucose levels

As shown in **Table 1**, scopolamine (1 mg/kg) did not cause any changes in renal and hepatic parameters compared to the control group. Furthermore, a 7-day treatment with DS12 at 10 mg/kg did not change any biochemical parameters analyzed in the serum.

Table 1: Effect of scopolamine (1 mg/kg) and 7-day pretreatment with **DS12** (10 mg/kg) on liver and kidney parameters in the serum of adult rats.

Biochemical markers	Control	SCO	SCO + DS12 (10 mg/kg)
Urea	537 ± 40	619 ± 52	498 ± 46
Creatinine	0.29 ± 0.026	0.27 ± 0.018	0.29 ± 0.013
AST	122 ± 17.0	135 ± 9.6	110 ± 6.3
ALT	28 ± 2.0	28 ± 0.7	28 ± 1.1

Values are represented as mean ± SEM. Urea and creatinine values are expressed as mg/dL, and AST and ALT are expressed as U/L. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

4. Discussion

The present study shows the potential effect of **DS12** in preventing neurochemical alterations caused by scopolamine in an adult rat model. The results demonstrate that scopolamine induces memory impairment and causes changes like those found in AD such as oxidative stress and cholinergic dysfunction [8].

Pretreatment for seven days with **DS12** (5 and 10 mg/kg) was capable of preventing memory loss induced by scopolamine (**Figure 3**). Inhibitory avoidance is a learning test that assesses memory consolidation [32] and is a widely used behavioral test to validate neurotoxic models including those that simulate AD-related neurochemical changes [33-35].

Preston and Eichenbaum (2013) [36] highlighted the importance of the prefrontal cortex and the hippocampus as two key brain structures in coding new information, and in consolidating and recovering memory. The aversive memory deficit demonstrated by our results caused by scopolamine can be associated with neurochemical changes in both cerebral cortex and hippocampus [8,36,37]. In this sense, **DS12** was able to protect against the increase in AChE activity induced by scopolamine in both brain structures (**Figure 4**).

Scopolamine is an antagonist of the muscarinic receptor and it is well established that it increases the activity of cholinesterases and reduces the levels of acetylcholine [8]. Silva et al. (2020) [13] demonstrated the inhibitory potential of some 1,3-thiazolidin-4-ones, particularly DS12, on AChE. The authors, through *in vitro* analyses, showed the ability of DS12 to inhibit AChE in cerebral cortex and hippocampus, in lymphocytes, and in both isoforms G1 and G4. Furthermore, molecular modeling analyses showed binding potential to the active site of the enzyme.

Accordingly, it is important to note that the results observed in graphs A and B of **Figure 4** suggest that **DS12** can cross the blood-brain barrier and protect changes caused by neurotoxic compounds such as scopolamine. Still, **Figure 5** demonstrates that **DS12** also prevents the increase of AChE activity in lymphocytes and that of BuChE activity in serum. The results related to AChE activity in lymphocytes corroborate the findings of Silva et al. (2020) [13], which revealed the potential for inhibition of both central and peripheral enzymes. In addition, the authors suggested that the inhibition of AChE increases the level of ACh, which can play an important role as an anti-inflammatory agent acting on nicotinic receptors expressed in lymphocytes.

Regarding the results of BuChE in serum, it is important to note that **DS12** also prevented the increase in BuChE activity caused by scopolamine. This finding is noteworthy because in AD, while AChE activity decreases progressively, BuChE levels remain the same or increase to above normal levels. Data from the literature suggest that the inhibition of BuChE may circumvent cholinergic side effects such as toxicity caused by classical inhibitors of AChE; thus the dual inhibition of these enzymes could provide a better therapeutic strategy for the treatment of AD [1,38,39].

Graphs C and D of **Figure 4** show that scopolamine reduced Na^+/K^+-ATPase activity, and that **DS12** was able to prevent this induction in the cerebral cortex and hippocampus. Although the pathogenesis-related neurochemical changes in AD are not yet clear, there is already evidence that Na^+/K^+-ATPase has potent neuroprotective

properties, and that it plays a key role in AD. The α isoform of Na^+/K^+-ATPase plays an important role in learning and memory, and the α3 isoform, which is exclusively expressed in neurons, is known to disrupt memory consolidation particularly through hippocampal damage [40,41]. Accordingly, the reduction in enzyme activity by scopolamine may be related to the amnesia observed in rats in the behavioral test, which has previously been suggested by other authors using the same model [42,43].

In addition, the reduction in Na^+/K^+-ATPase activity can be associated with the increase in oxidative stress, as shown in **Figures 6 and 7**. Na^+/K^+-ATPase is an important target of oxidative stress at the neuronal level. Moreover, Fan et al. (2005) [44] demonstrated that ROS accumulation generated by treatment with scopolamine suppressed ATPase activity. However, increased oxidative stress and decreased Na^+/K^+-ATPase activity led to impaired brain functions, especially memory and cognition. Therefore, it can be concluded that Na^+/K^+-ATPase alterations are directly associated with memory deficits and that **DS12** protects these changes caused by scopolamine. [41].

As described previously, oxidative stress in the brain is associated with cognitive impairment induced by scopolamine. Importantly, it is known that scopolamine can impair antioxidant defense mechanisms, such as CAT and SOD [8]. Different models using scopolamine have demonstrated its ability to decrease the activity of CAT, SOD, and GSH-Px, as well as to release GSH in both the hippocampus and cerebral cortex [44-47]. This suppression of antioxidant defenses may be associated with accumulation of free radicals, especially ROS, in brain cells. Thus, scopolamine has also been reported to increase ROS production, which may be related to mitochondrial dysfunction and apoptosis of hippocampal and cortical neurons [8].

Furthermore, oxygen and nitrogen species at high levels cause oxidative modifications of different biomolecules, such as elevated lipid peroxidation and protein and nucleic acid oxidation. These changes are present in AD, mainly in hippocampus and cerebral cortex, regions of the brain that present with the main neuropathological lesions observed in AD [48]. These mechanisms may also be related to the memory decline observed in this study and it was demonstrated that **DS12** can prevent these alterations, suggesting a possible antioxidant role of this compound.

Interestingly, our findings also revealed that a 7-day pretreatment with **DS12** at 10 mg/kg did not alter creatinine or urea blood levels, and neither did it affect AST or ALT activity, suggesting that treatment with this compound did not cause systemic

damage or renal and hepatic toxicity (**Table 1**). In addition, Silva et al. (2016) [15] reported that **DS12** did not show toxicity at 100 µM in astrocytes in primary culture.

Therefore, the most notable finding of this study is the multitarget potential of **DS12** against different neurochemical changes in the model induced by scopolamine without providing any toxicological effect. It should also be noted that this experimental model reproduces important well-established changes in the pathophysiology of AD.

5. Conclusion

Together, the results suggest that **DS12** is an important compound for the prevention of neurochemical changes induced in an experimental model of memory deficit. Our findings suggest that this compound is capable of modulating the neurochemical pathways involved in memory, such as those involving the cholinergic system, ion pump activity, and oxidative stress. In addition, **DS12** also has an effect on the peripheral cholinergic system. In conclusion, based on our results, **DS12** emerged as a multitarget compound with antioxidant, anti-inflammatory, and anticholinesterase effects. However, further studies are necessary to evaluate the therapeutic potential of this compound in diseases associated with memory dysfunction.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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6. CONCLUSÃO

Em conclusão, os resultados encontrados mostram o potencial das 1,3-tiazolidin-4-onas como inibidores das colinesterases. O estudo de modelagem molecular demonstrou uma sobreposição das tiazolidinonas com o sítio ativo da AChE, semelhante à ACh e donepezil. A cinética enzimática sugeriu uma inibição mista, que também é exibida pelo donepezil. Além disso, a capacidade desses compostos de inibir a atividade da AChE nos linfócitos sugere um papel potencial na modulação da sinalização colinérgica associada ao processo de inflamação.

O composto **DS12** emerge como um importante composto multialvo para prevenir alterações comportamentais e neurobioquímicas nos modelos experimentais da DA. No pré-tratamento de sete dias, o composto foi capaz de proteger a disfunção de memória causada pela escopolamina, além de prevenir o aumento da atividade das colinesterases (AChE e BuChE), a diminuição da atividade da Na^+/K^+-ATPase, o aumento das espécies reativas, em especial EROs, e a diminuição das defesas antioxidantes (CAT e SOD) em córtex e hipocampo de ratos, sem demonstrar alterações em marcadores hepáticos e renais.

Nesse sentido, destaca-se a importância do surgimento da classe das 1,3-thiazolidin-4-onas, especialmente o composto **DS12**, como uma nova abordagem terapêutica para a DA. Para melhor elucidar essa questão, a perspectiva é o seguimento e o aprofundamento dos estudos avaliando o potencial da **DS12**. Novos estudos buscando avaliar a interação da **DS12** com diferentes alvos, bem como dar seguimento nestes através de diferentes modelos experimentais de Alzheimer já foram elaborados e almejam entender melhor o potencial deste composto como uma possível alternativa terapêutica. Além disso, novas possibilidades sintéticas da classe das 1,3-tiazolidin-4-onas seguem sendo estudadas e pesquisas de novos análogos com atividade no sistema colinérgico estão sendo planejadas para avaliar o papel desta classe nas patologias envolvendo comprometimento deste sistema.

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8. ANEXOS

8.1. ANEXO A – LICENÇA PARA USO DO ARTIGO NA TESE

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8.2. ANEXO B – APROVAÇÃO DO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL – ARTIGO.

Pelotas, 22 de janeiro de 2014

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professora Roselia Maria Spanevello

Centro de Ciências Químicas, Farmacêuticas e de Alimentos

Senhora Professora:

A *CEEA* analisou o projeto intitulado: **"Avaliação do efeito de benzilpiperazinas e tiazolidinonas de origem sintética na atividade da acetilcolinesterase de ratos submetidos a um modelo experimental de déficit de memória",** processo n°23110.009220/2013-80, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro nº **CEEA 9220**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA

Ciente em: 28/01/2014

Assinatura da Professora Responsável:

Roselia Maria Spanevello

8.3. ANEXO C – APROVAÇÃO DO COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL – MANUSCRITO.

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº 4/2019/CEEA/REITORIA
PROCESSO Nº 23110.046528/2018-11

Certificado

Certificamos que a proposta intitulada **"Potencial anticolinesterásico de heterociclos contendo átomos de nitrogênio e enxofre: estudos in vitro e in vivo"** processo número 23110.046528/2018-11, de responsabilidade de Roselia Maria Spanevello- que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua complementação pela Comissão de Ética em Experimentação Animal, em reunião de 10/12/2018.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	15/01/2019 a 15/11/2021
Espécie/linhagem/raça	*Rattus norvegicus*/Wistar
N° de animais	366
Idade	20 com 1-3 dias e 336 com 60 dias
Sexo	Machos
Origem	Biotério Central-UFPel

Código para cadastro **CEEA 46528-2018**

8.4. ANEXO D – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO NA REVISTA CHEMICO-BIOLOGICAL INTERACTIONS.

Gmail **Daniel Schuch <danielschuch08@gmail.com>**

Fw: Successfully received: submission Multitarget effect of 2-(4-(methylthio)phenyl)-3-(3-(piperidin-1-yl)propyl)thiazolidin-4-one in scopolamine-induced amnesic model in rats: a new therapeutically approach for Chemico-Biological Interactions

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8.5. ANEXO E – GUIA PARA AUTORES DA REVISTA CHEMICO-BIOLOGICAL INTERACTIONS.

ELSEVIER

CHEMICO-BIOLOGICAL INTERACTIONS

A journal of molecular, cellular and biochemical toxicology

AUTHOR INFORMATION PACK

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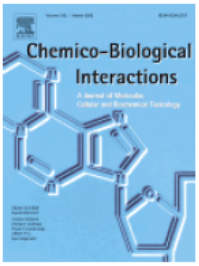

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DESCRIPTION

Chemico-Biological Interactions publishes research reports and review articles that examine the molecular, cellular, and/or biochemical basis of toxicologically relevant outcomes. Special emphasis is placed on **toxicological mechanisms** associated with interactions between **chemicals** and **biological systems**. Outcomes may include all traditional endpoints caused by synthetic or naturally occurring chemicals, both *in vivo* and *in vitro*. Endpoints of interest include, but are not limited to **carcinogenesis**, **mutagenesis**, **respiratory toxicology**, **neurotoxicology**, **reproductive** and **developmental toxicology**, and **immunotoxicology**.

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