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AVALIAÇÃO *IN VITRO* E *IN VIVO* DA TOXICIDADE DO COMPOSTO 2,2'-DISSELENETO DE DITIENILA EM RATOS

DISSERTAÇÃO DE MESTRADO

Pietro Maria Chagas

Santa Maria, RS, Brasil
2013

AVALIAÇÃO *IN VITRO* E *IN VIVO* DA TOXICIDADE DO COMPOSTO 2,2'-DISSELENETO DE DITIENILA EM RATOS

Pietro Maria Chagas

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do grau de
Mestre em Bioquímica Toxicológica

Orientadora: Prof.ª Dr.ª Cristina Wayne Nogueira

Santa Maria, RS, Brasil
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Universidade Federal de Santa Maria
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A Comissão Examinadora, abaixo assinada, aprova a Dissertação de Mestrado

AVALIAÇÃO *IN VITRO* E *IN VIVO* DA TOXICIDADE DO COMPOSTO 2,2'-DISSELENETO DE DITIENILA EM RATOS

elaborada por
Pietro Maria Chagas

como requisito parcial para obtenção do grau de
Mestre em Bioquímica Toxicológica

COMISSÃO EXAMINADORA:

Cristina Wayne Nogueira, Dr.ª
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Santa Maria, 5 de agosto de 2013.

Dedico esta dissertação em memória de

Francielli Vieira e Bruna Occai,

Meu desejo era que pudéssemos ter terminado essa etapa juntos,

Sabemos que o tempo passa, infelizmente ele não volta,

mas ainda que o tempo passe, não levará a lembrança do sorriso, o exemplo de dedicação, e a saudade que ficará guardada em nossos corações...

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"Lembrai do tempo que levastes para chegar aqui,
de todas as vitórias e lágrimas,
de todos os sorrisos e fracassos.

Lembrai dos sonhos realizados,
das frustrações,
das decepções colhidas.

Lembrai de tudo o que passou.
Ganhastes mais força,
mais sabedoria
e finalmente podes olhar para o que há diante de ti
e perceber que apenas chegastes ao começo.
– Seja bem vindo ao começo!"

(Começo – Augusto Branco)

RESUMO

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

AVALIAÇÃO *IN VITRO* E *IN VIVO* DA TOXICIDADE DO COMPOSTO 2,2'-DISSELENETO DE DITIENILA EM RATOS

AUTOR: PIETRO MARIA CHAGAS
ORIENTADORA: CRISTINA WAYNE NOGUEIRA
Data e Local da Defesa: Santa Maria, 2013

O composto 2,2'-disseleneto de ditienila (DSDT), um composto orgânico de selênio com grupamento tiofeno, fora comprovado como um promissor antioxidante *in vitro* e *in vivo*, assim como um agente antifúngico e antimicrobiano. Entretanto, sua toxicidade ainda não fora avaliada, representando um importante ponto a ser investigado. O objetivo deste estudo foi avaliar se o DSDT apresenta potencial toxicidade *in vitro* ou *in vivo*. Para este fim, a atividade de enzimas sulfidrílicas, como δ-aminolevulato desidratase (δ-ALA-D) e Na^+, K^+-ATPase fora testada para predizer a toxicidade *in vitro* do DSDT em homogeneizado de cérebro de ratos, bem como a sua atividade tipo-tiol oxidase. Em outra seção de experimentos, o DSDT foi administrado em ratos (50 ou 100 mg/kg; oralmente) com o intuito de determinar parâmetros toxicológicos *in vivo*. Amostras de plasma foram retiradas para dosagem dos parâmetros bioquímicos: atividade das enzimas alanina (ALT) e aspartato (AST) aminotrasferase e níveis de ureia e creatinina. Além disso, em homogeneizado de cérebro foram dosadas a atividade das enzimas δ-ALA-D e Na^+, K^+-ATPase, assim como os níveis de peroxidação lipídica e as defesas antioxidantes (atividade das enzimas catalase e superóxido dismutase e níveis de ácido ascórbico e glutationa reduzida). O composto DSDT inibiu *in vitro,* tanto a atividade da δ-ALA-D quanto da Na^+, K^+-ATPase (IC_{50} 2μM e 17μM, respectivamente). O efeito inibitório do DSDT sobre a atividade das enzimas δ-ALA-D e Na^+, K^+-ATPase foi restaurado pelo ditiol ditiotreitol. Adicionalmente, DSDT (5-25μM) apresentou atividade do tipo-tiol oxidase. *In vivo*, o DSDT (50 e 100 mg/kg) causou uma diminuição no consumo de comida e água e perda de peso corporal, evidenciando toxicidade sistêmica, causando inclusive morte de ratos. Quando administrado na dose de 100 mg/kg, DSDT diminui os níveis de ureia e aumentou a atividade plasmática da ALT e da AST. Os níveis de peroxidação lipídica encontraram-se aumentados em ambas as doses administradas. Na maior dose, o DSDT inibiu a atividade da δ-ALA-D. Em contrapartida, nem a atividade da Na^+, K^+-ATPase nem as defesas antioxidantes foram alteradas no cérebro de ratos expostos ao DSDT. Em conclusão, a interação com grupos tióis de enzimas sulfidrílicas parece mediar o efeito inibitório do DSDT em relação a atividade da δ-ALA-D e da Na^+, K^+-ATPase *in vitro*. Além disso, nas doses administradas, o DSDT induz toxicidade cerebral e sistêmica em ratos. Embora outros estudos sejam necessários para fornecer mais informações sobre este composto em específico, estes dados contribuem para o conhecimento sobre a toxicologia do DSDT, um composto com propriedades farmacológicas.

Palavras-chave: 2,2′-disseleneto de ditienila. Compostos Orgânicos de Selênio. δ-ALA-D. Na^+, K^+-ATPase. Tióis. Toxicidade.

ABSTRACT

Dissertation of Master's Degree
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EVALUATION *IN VITRO* AND *IN VIVO* OF THE TOXICITY OF THE COMPOUND 2,2'-DITHIENYL DISELENIDE IN RATS

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Date and Place of the Defense: Santa Maria, 2013

The compound 2,2'-dithienyl diselenide (DTDS), an organoselenium compound with thiophene moieties, has been proven to be a promising antioxidant *in vitro* and *in vivo*, as well as an antifungal and antimicrobial agent. However, its toxicity, an important point to be investigated, has not been evaluated. The objective of this study was to evaluate whether DTDS has potential toxicity *in vitro* or *in vivo*. For this reason, sulfhydryl enzyme activities, such as δ-aminolevulinic acid dehydratase (δ-ALA-D) and Na^+, K^+-ATPase were assessed to predict *in vitro* DTDS toxicity in rat brain homogenate, in addition to its thiol oxidase-like activity. In other section of experiments, DTDS was administered to rats (50 or 100 mg/kg; per orally) in order to determine toxicological parameters *in vivo*. Plasma samples were collected in order to measure the biochemical parameters: alanine (ALT) and aspartate (AST) aminotransferase activities and urea and creatinine levels. Besides, in brain homogenates, it was determined the activity of the enzymes δ-ALA-D and Na^+, K^+-ATPase, as well as lipid peroxidation levels and antioxidant defenses (catalase and superoxide dismutase activities and ascorbic acid and reduced glutathione levels). The compound DTDS inhibited *in vitro* both δ-ALA-D and Na^+, K^+-ATPase activities (IC_{50} 2 μM and 17 μM, respectively). The DTDS inhibitory effect on δ-ALA-D and Na^+, K^+-ATPase activities was restored by dithiol dithiothreitol. In addition, DTDS (5-25 μM) showed a thiol oxidase-like activity. *In vivo*, DTDS (50 and 100 mg/kg) caused a decrease in food and water intakes and the loss of body weight, indicating systemic toxicity, even causing death of the animals. At a dose of 100 mg/kg, DTDS decreased urea levels and increased plasma alanine and aspartate aminotransferase activities. Lipid peroxidation was increased in both administered doses. Moreover, in the highest dose, DTDS inhibited δ-ALA-D activity. By contrast, neither Na^+, K^+-ATPase activity nor antioxidant defenses were altered in the brain of rats exposed to DTDS. In conclusion, the interaction with thiol groups of sulfhydryl enzymes seems to mediate the inhibitory effect of DTDS against δ-ALA-D and Na^+, K^+-ATPase activities *in vitro*. Furthermore, in the administered doses, DTDS causes cerebral and systemic toxicity in rats. Although other studies are necessary to give more information about this specific compound, our findings contribute to the knowledge on the toxicology of DTDS, a compound with pharmacological properties.

Keywords: 2,2′-dithienyl diselenide. Organoselenium. δ-ALA-D. Na^+, K^+-ATPase. Sulfhydryl. Toxicity.

LISTA DE FIGURAS

INTRODUÇÃO

LISTA DE ABREVIATURAS

ANVISA – Agência Nacional de Vigilância Sanitária

ATP – trifosfato de adenosina

BHE – barreira hemato-encefálica

DHAR – deidroascorbato redutase

DIO – iodotironina deiodinases

DNA – ácido desoxirribonucleico

DSDT – disseleneto de ditienila

DTT – ditiotreitol

EDTA – ácido etilenodiamino tetra-acético

ERNs – espécies reativas de nitrogênio

EROs – espécies reativas de oxigênio

FAO – Organização das Nações Unidas para e Agricultura e Alimentação

GPx – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa reduzida

GST – glutationa-S-transferase

IC_{50} – concentração responsável pela inibição de 50% da atividade enzimática

IDR – ingestão diária recomendada

OMS – Organização Mundial da Saúde

PBG – porfobilinogênio

Pi – fosfato inorgânico

SOD – superóxido dismutase

SPS2 – selenofosfato sintetase 2

TrxR – tiorredoxina redutase

δ-ALA – ácido δ-aminolevulínico

δ-ALA-D – δ-aminolevulinato desidratase

SUMÁRIO

1 INTRODUÇÃO

1.1 O Elemento Químico Selênio

O elemento químico selênio (Se) foi descoberto em 1817 pelo químico sueco Jöns Jacob Berzelius, sendo este elemento não-metal pertencente à família dos calcogênios da tabela periódica, mesmo grupo a qual pertence também os elementos oxigênio (O), enxofre (S) e Telúrio (Te) (WIESER e COPLEN, 2011). O Se pode apresentar-se em diversos estados de oxidação, incluindo o selênio elementar (Se^{0}), assim como selenito (Se^{+6}), selenato (Se^{+4}) e seleneto (Se^{-2}) (SAGER, 2006). Em relação à forma como são encontrados na natureza, os compostos que contêm átomos de selênio são geralmente separados em dois grupos: compostos inorgânicos (*e. g.*, selenato e selenito) e orgânicos (*e. g.*, selenometionina e selenocisteína), sendo a forma orgânica reportada como mais biodisponível e menos tóxica (KIM e MAHAN, 2001; YOUNG et al., 1982). O Se é bem estabelecido como um mineral traço essencial e de fundamental importância para a saúde humana, estando presente em no mínimo 25 proteínas (KRYUKOV et al., 2003). Diferentemente de outros elementos, os quais interagem com proteínas na forma de cofatores, o Se é incorporado a cadeias polipeptídicas como parte do aminoácido selenocisteína (PAPP et al., 2007).

Quantidades adequadas de Se são necessárias para a manutenção da saúde, por este motivo, tanto a Organização Mundial da Saúde (OMS) quanto a Agência Nacional de Vigilância Sanitária (ANVISA) aconselham uma ingestão diária recomendada (IDR) de 34-35 µg em adultos (BRASIL, 2005; FAO/WHO, 2002). Concentrações insuficientes na dieta, ou seja, IDR menor que 10 µg/dia, como em regiões de solo pobre em Se, estão relacionadas ao aparecimento da doença de Keshan, a qual apresenta sintomas como fadiga e perda de apetite, insuficiência cardíaca, cardiomegalia e falência cardíaca congestiva (LI et al., 1985). Por outro lado, concentrações elevadas também podem levar ao aparecimento de toxicidade, embora os sinais e sintomas de superexposição ao Se não sejam tão bem definidos. Os aspectos clínicos mais comuns são perda de cabelo, mudanças estruturais na queratina do cabelo e unhas, desenvolvimento de pele icteroide e distúrbios gastrointestinais, sintomas relacionados a um consumo de alimentos com alto teor de Se, cultivados em solos seleníferos, podendo chegar a mais de 900 µg/dia (FAO/WHO, 2002). Embora não haja um limite diário máximo bem estabelecido, A

OMS recomenda que o consumo diário não ultrapasse níveis maiores do que 400 µg/dia, com o intuito de prover uma margem de segurança adequada (FAO/WHO, 2002). Como principais fontes de Se em alimentos, podemos citar castanha-do-pará, cebola, cebola, cogumelos, brócolis, cereais, pescados, carnes e ovos (DUMONT et al., 2006).

A importância do Se para os sistemas biológicos deve-se ao fato de este desempenhar importantes funções em diversas vias metabólicas, incluindo síntese de ácido desoxirribonucleico (DNA), funções tireoidiana e imunológica e principalmente no sistema de defesa antioxidante, podendo este papel ser desempenhado de forma não-enzimática ou incorporado a famílias de selenoenzimas (RAYMAN, 2000). De forma não-enzimática, o Se exerce funções principalmente como antioxidante e anti-mutagênico, na forma de compostos de baixo peso molecular, como o ácido metil-selênico, metil-selenocisteína e selenometionina (PAPP et al., 2007). Os efeitos do Se no organismo são concentração dependente, variando de essencial e antioxidante em concentrações na faixa de nano-micromolar a potencialmente pró-oxidante em concentrações acima do necessário para a síntese máxima de selenoproteínas. Em concentrações muito elevadas, o Se pode acumular-se e reagir com tióis intracelulares, dando origem a estresse oxidativo e dano a componentes celulares (VINCETI et al., 2001). Relacionado à sua função enzimática, sabe-se que o resíduo de selenocisteína está presente em enzimas como a glutationa peroxidase (GPx), a tiorredoxina redutase (TrxR), iodotironina deiodinases (DIO) e selenofosfato sintetase 2 (SPS2), desempenhando as mais diversas funções (PAPP et al., 2007).

1.2 Compostos Orgânicos de Selênio

Compostos orgânicos de selênio sintéticos têm despertado interesse científico nas últimas décadas devido as suas propriedades bioquímicas e farmacológicas, visto que muitos estudos demonstram o potencial terapêutico destes compostos em relação a inúmeras patologias humanas (NOGUEIRA et al., 2004; SORIANO-GARCIA, 2004). Esta classe de compostos é reportada como potentes antioxidantes em modelos de estresse oxidativo *in vitro* e *in vivo* (ACKER et al., 2009a; MEOTTI et al., 2004; POSSER et al., 2008; PRIGOL et al., 2008). A atividade antioxidante destes compostos tem sido relacionada à sua capacidade de mimetizar as enzimas

GPx, deidroascorbato redutase (DHAR) e glutationa-S-transferase (GST), assim como agir como substrato para a enzima TrxR (LUCHESE e NOGUEIRA, 2010; NOGUEIRA et al., 2004; SAUSEN DE FREITAS et al., 2010). Estudos demonstram que o $(PhSe)_2$ e seus derivados parecem ter como um dos principais órgãos alvo o cérebro, uma vez que apresentam natureza lipofílica, são capazes de atravessar a barreira hemato-encefálica (BHE) (NOGUEIRA et al., 2004; PRIGOL et al., 2013). Esta propriedade dos disselenetos de diarila de penetrar a BHE parece ser de extrema importância para seus efeitos farmacológicos, uma vez que já foram reportados por possuírem atividades do tipo-antidepressiva (ACKER et al., 2009b; SAVEGNAGO et al., 2008), ansiolítica (BRUNING et al., 2009b; SAVEGNAGO et al., 2008) e nootrópica (BORTOLATTO et al., 2012; SOUZA et al., 2010).

Estes compostos, entretanto, podem reagir com grupamentos tiólicos, como cisteína, ditiotreitol (DTT) e glutationa reduzida (GSH), produzindo selenocisteína, selenois e dissulfetos (WALTER et al., 1972). Resíduos de cisteína de proteínas endógenas podem também ser oxidados, e causar, no caso de enzimas sulfidrílicas, um decréscimo na atividade catalítica (NOGUEIRA et al., 2003). Os compostos orgânicos de selênio, ebselen (Figura 1A) e disseleneto de difenila $(PhSe)_2$ (Figura 1B), são exemplos de compostos que embora possuam atividades farmacológicas, podem inibir enzimas sulfidrílicas, além de interferirem no conteúdo de tióis endógenos, sendo tóxicos, em altas doses, podendo causar injúria oxidativa no cérebro e fígado (NOGUEIRA e ROCHA, 2010). Como anteriormente mencionado, os disselenetos de diarila atravessam a BHE, podendo causar efeitos tóxicos cerebrais em altas doses, relacionados a convulsões e o aumento do estresse oxidativo no tecido cerebral (PRIGOL et al., 2007).

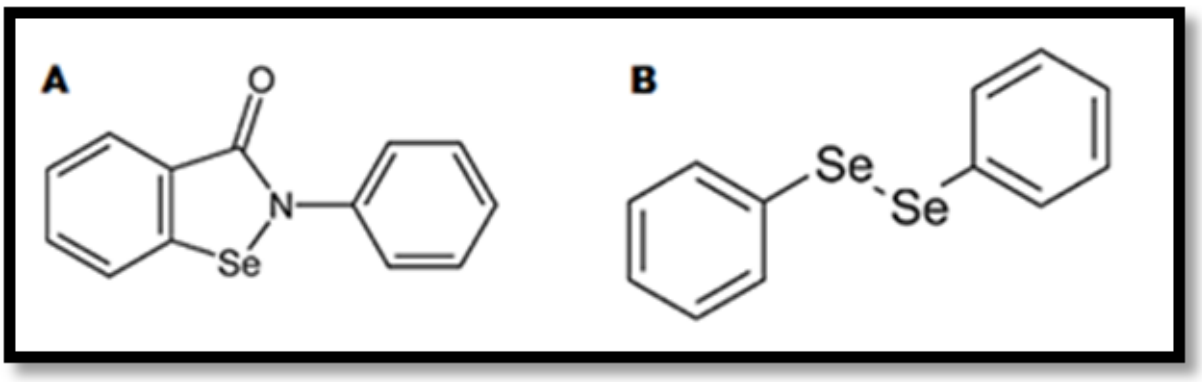

Figura 1 – Estrutura dos compostos (A) ebselen, (B) disseleneto de difenila.

1.3 Aspectos Toxicológicos de Compostos Orgânicos de Selênio

1.3.1 Inibição de Enzimas Sulfidrílicas

Como anteriormente mencionando, os compostos orgânicos de selênio são capazes de inibir enzimas sulfidrílicas, sendo a δ-aminolevulinato desidratase (δ-ALA-D, E.C. 4.2.1.24) um importante exemplo dessa classe, também conhecida como porfobilinogênio sintase, reponsável por catalisar a condensação de duas moléculas de ácido δ-aminolevulínico (δ-ALA; Figura 2) com o intuito de formar o monopirrol porfobilinogênio (PBG) (JAFFE, 1995). Esta enzima é um ponto chave na síntese de compostos tetrapirrólicos, como o grupamento heme, presente em macromoléculas como hemoglobina, mioglobina e citocromos, sendo essencial para o metabolismo aeróbico animal (SHEMIN e RUSSELL, 1953).

Em seu sítio ativo, a δ-ALA-D apresenta resíduos de cisteína vicinais, os quais estão envolvidos na complexação de íons Zn^{2+}, sendo a presença dos grupamentos tiólicos destes aminoácidos o principal motivo pelo qual esta enzima é particularmente sensível à oxidação, uma vez que esta necessita ter seu sítio ativo no estado reduzido para catalisar a formação do PBG (EMANUELLI et al., 1998; MARKHAM et al., 1993). O mesmo Zn^{2+} também está envolvido na estabilização dos grupamentos tióis e sua retirada por agentes quelantes ou que compitam com o Zn^{2+} pelo sítio ativo, podem acelerar o processo de auto-oxidação (BEBER et al., 1998; EMANUELLI et al., 1998).

Sabe-se que a atividade sanguínea diminuída da enzima δ-ALA-D é considerada um importante biomarcador clínico de exposição ao Pb^{2+}, embora esta enzima também sofra modulação por outros metais, como Hg^{2+} e Cu^{2+}, assim como outras situações também relacionadas a processos de estresse oxidativo (ROCHA et al., 2012). Estudos demonstram, tanto *in vitro* quanto *in vivo*, que a atividade da enzima δ-ALA-D é um importante alvo molecular dos efeitos tóxicos de organocalcogênios, sendo esta inibição dependente principalmente da oxidação dos resíduos de cisteína anteriormente citados (BARBOSA, 1998; BRUNING et al., 2009a). Outro ponto interessante a ser considerado é o fato de que compostos orgânicos de selênio podem interagir com metais, entre eles o Zn^{2+}, formando complexos e consequentemente contribuindo para a inibição da atividade enzimática da δ-ALA-D (BRANDÃO et al., 2008; BRUNING et al., 2009a; SANTOS et al., 2005).

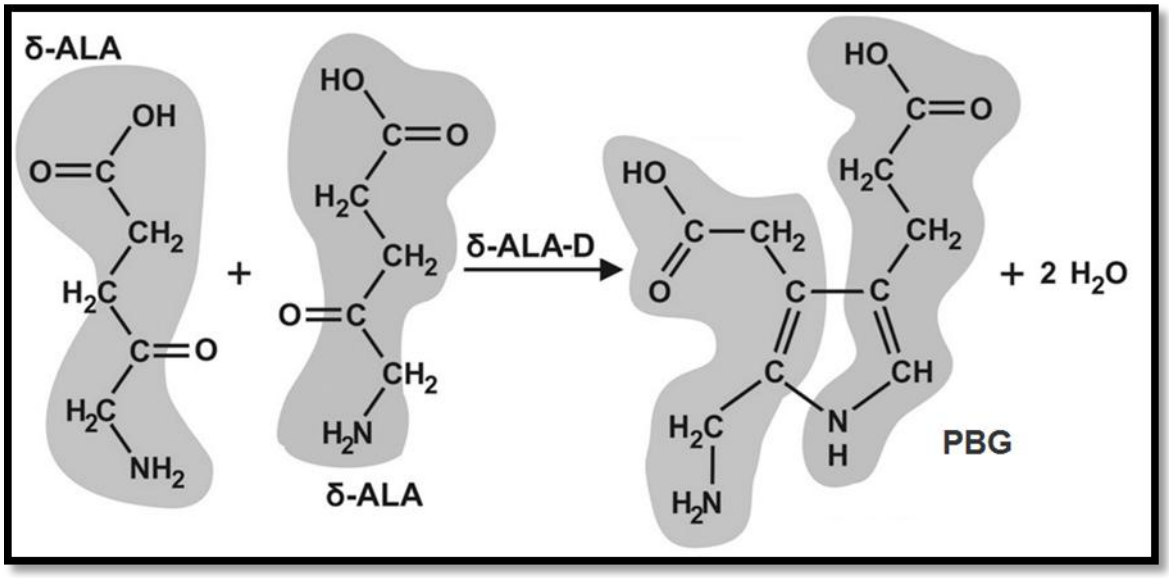

Figura 2 – Reação catalisada pela enzima δ-ALA-D: Síntese do PBG através da condensação de duas moléculas de δ-ALA. Adaptado de ROCHA et al., 2012.

Outra importante enzima sulfidrílica é a Na^+, K^+-ATPase (E.C. 3.6.3.9) também conhecida como ATP fosfoidrolase, a qual é uma enzima integral de membrana responsável pelo transporte ativo de íons Na^+ e K^+ no sistema nervoso central (Figura 3), utilizando energia proveniente da hidrólise da ligação do fosfato (Pi) terminal do trifosfato de adenosina (ATP) (KAPLAN, 2002; SATO et al., 1995). Esta enzima existe em diversas isoformas no cérebro e consome a maior parte do ATP disponível através do transporte de três cargas positivas de Na^+ para o meio extracelular e duas de K^+ para o meio intracelular, configurando um antiporte eletrogênico (BERTORELLO e KATZ, 1995). A reação catalisada por esta enzima regula as concentrações intracelulares dos íons Na^+ e K^+, consequentemente seus gradientes através da membrana plasmática, o que é extremamente necessário para as funções vitais como cotransporte de membranas, regulação do volume celular e excitabilidade da célula (LEES, 1991). A inativação da Na^+, K^+-ATPase pode levar a despolarização parcial da membrana, permitindo um influxo excessivo de Ca^{2+} para dentro do neurônio (VELDHUIS et al., 2003). O aumento dos níveis de Ca^{2+} extracelulares pode induzir a liberação de diversos neurotransmissores, dentre eles o glutamato, o qual está envolvido em processos de excitotoxicidade, o que pode levar a morte neuronal (WESTERINK et al., 1989). Similar a δ-ALA-D, esta enzima também é altamente susceptível ao estresse oxidativo e agentes oxidantes, que

podem oxidar grupos tiois cruciais para a função desta enzima (MATSUOKA et al., 1990).

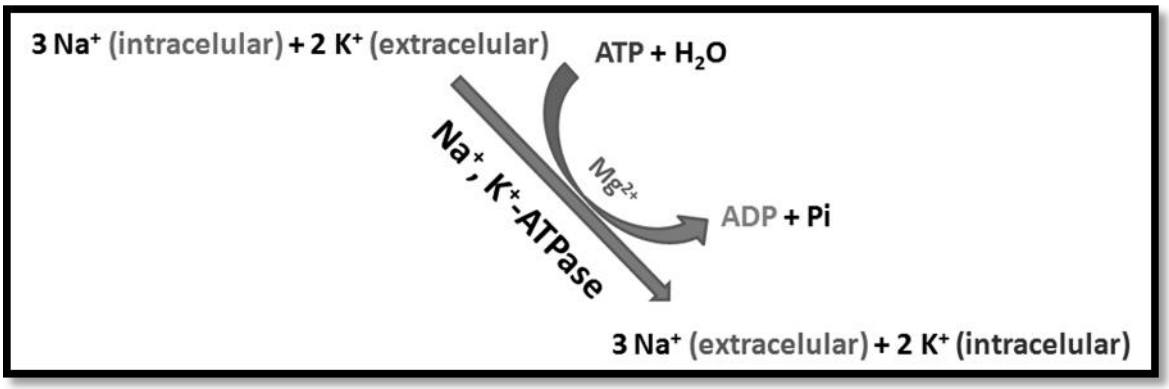

Figura 3 – Reação catalisada pela enzima Na^+, K^+-ATPase: transporte ativo de íons Na^+ e K^+, utilizando energia proveniente da hidrólise da ligação do Pi terminal do ATP.

Devido à reversão da atividade inibitória por compostos contendo grupamentos tiólicos, fora proposto um possível mecanismo pelo qual os disselenetos de diarila inibem enzimas sulfidrílicas (NOGUEIRA et al., 2004; ROCHA et al., 2012). O primeiro passo da oxidação da inativação envolve o ataque nucleofílico dos tióis do sítio ativo da enzima, acontecendo a reação da enzima (E-Cys-S) com o disseleneto (RSe-SeR) para formar o intermediário (E-Cys-S-SeR). Consequentemente, o outro resíduo de cisteína, devido a proximidade espacial com o resíduo mais reativo, ataca a ligação S-Se do intermediário E-Cys-S-SeR, resultando na oxidação e consequente inativação da enzima e na formação de um selenol (RSeH) o qual regenera o respectivo disseleneto (RSe-SeR) (Figura 4).

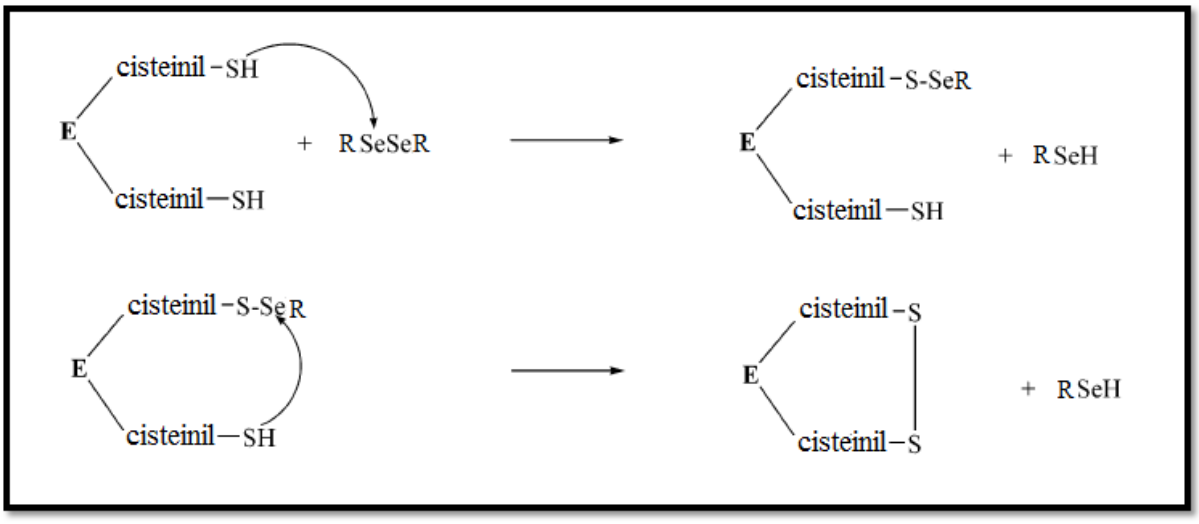

Figura 4 – Esquema do mecanismo proposto para a inibição dos disselenetos de diarila sobre a atividade de enzimas sulfidrílicas.

1.3.2 Indução de Processos Oxidativos em Altas Doses

Em suma os efeitos tóxicos relacionados aos compostos orgânicos de selênio são geralmente relacionados à depleção de tióis endógenos e inibição de enzimas sulfidrílicas, o que pode levar a processos de estresse oxidativo. Como anteriormente mencionado, o Se, de forma dose/concentração-dependente pode desempenhar papéis tanto anti- quanto pró-oxidantes (NOGUEIRA e ROCHA, 2011; VINCETI et al., 2001). Sabe-se que níveis basais de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) são produzidos fisiologicamente por diversos mecanismos, incluindo redução parcial do O_2 via cadeia transportadora de elétrons, desaminação oxidativa de aminas biogênicas, e por células polimorfonucleares como parte da resposta imune com o intuito de defesa do organismo (CADENAS e DAVIES, 2000; FIALKOW et al., 2007). Dentre as EROs, podemos citar o oxigênio singlete ($^1O_2$), o ânion superóxido ($O_2^{\cdot-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\cdot}OH$), por outro lado as ERNs compreendem o óxido nítrico (NO), o peroxinitrito ($ONOO^-$) entre outros, derivados da de ERNs com EROs (FIALKOW et al., 2007). As EROs e ERNs são geralmente detoxificadas por mecanismos antioxidantes celulares enzimáticos e não-enzimáticos, assim mantendo o estado redox natural da célula (HALLIWELL e GUTTERIDGE, 1990).

O organismo apresenta uma série de defesas antioxidantes, defesas que podem ser produzidas de forma endógena ou obtidas através da dieta, evitando o efeito deletério de espécies reativas, diminuindo sua produção, neutralizando-as ou promovendo a reparação dos danos causados por elas (CADENAS e DAVIES, 2000). As defesas antioxidantes não-enzimáticas compreendem o ácido ascórbico, o tocoferol, carotenoides, flavonoides, ácido úrico e o GSH (BREWER, 2011). Dentre as defesas antioxidantes enzimáticas, podemos mencionar a superóxido dismutase (SOD), catalase (CAT) e as enzimas do ciclo da GSH: glutationa redutase (GR), GST e a selenoenzima GPx (HARRIS, 1992; SUN, 1990).

Entretanto, em situações em que haja um desequilíbrio entre a produção de espécies reativas e a capacidade antioxidante natural do organismo, esta situação é considerada como estresse oxidativo. Diversas macromoléculas biológicas podem sofrer oxidação devido ao aumento de EROs e ERNs, ou outros compostos pró-oxidantes, dentre estas macromoléculas estão compreendidas as proteínas, lipídios e ácidos nucleicos, ocasionando diversos danos celulares (PACIFICI e DAVIES,

1991). Já é reportado que doses elevadas do $(PhSe)_2$, protótipo dos disselenetos de diarila podem aumentar parâmetros de estresse oxidativo como peroxidação lipídica e diminuição da atividade de enzimas antioxidantes principalmente no tecido cerebral (PRIGOL et al., 2007). Corroborando com estes dados, sabe-se que o cérebro é um tecido especialmente sensível ao dano oxidativo, devido ao fato deste órgão apresentar concentrações relativamente baixas de enzimas antioxidantes, grande quantidade de ácidos graxos insaturados e catecolaminas, substratos suscetíveis ao ataque de espécies reativas, além de apresentar elevadas quantidades de Fe^{2+} e aminoácidos excitatórios (IKONOMIDOU e KAINDL, 2011).

1.4 O Composto 2,2'-disseleneto de Ditienila

O 2,2'-disseleneto de ditienila (DSDT; Figura 5), um derivado tiofênico do $(PhSe)_2$, é um composto primariamente empregado na síntese de oligo(seleno-2,5-tienilenos) através da 2-tienilsselenilação de tiofenos (TIECCO et al., 2000). Estudos também demonstram o papel biológico e farmacológico de compostos contendo tiofeno, sabe-se que estes podem atuar como agentes antioxidantes, antinociceptivos e quimioterápicos (MEOTTI et al., 2003; MISHRA et al., 2011; YANAGIMOTO et al., 2002). Pouco se sabe sobre sua toxicidade, mas entre os poucos relatos existentes, os efeitos tóxicos relacionados a estes compostos parecem ser ausentes ou relacionados a altas doses ou metabolização (MERCER et al., 2009; OLIVEIRA et al., 2009).

O DSDT, em particular, possui comprovada atividade antioxidante em homogeneizado de cérebro de ratos *in vitro*, protegendo da peroxidação lipídica induzida por indutores como Fe^{2}+/EDTA (ácido etilenodiamino tetra-acético), malonato e nitroprussiato de sódio (BORTOLATTO et al., 2013). Assim como apresenta efeito anticonvulsivante no modelo *in vivo* de estado epilético induzido por ácido caínico em ratos (BORTOLATTO et al., 2011). Outra importante atividade biológica relacionada a este composto, mas relacionada ao seu potencial pró-oxidante dependente da concentração, é a sua ação antifúngica contra *Candida albicans* e antimicrobiana frente a diversas bactérias gram-positivas (PESARICO et al., 2013).

Entretanto, ainda não há estudos relatando sua possível toxicidade, e devido à ação promissora desse composto e a toxicidade relacionada a altas doses de organocalcogênios, este é um importante ponto a ser investigado.

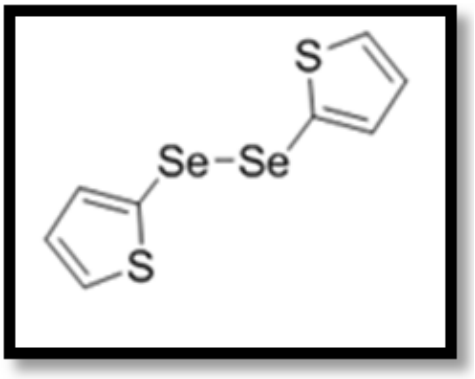

Figura 5 – Estrutura do composto 2,2'-disseleneto de ditienila.

2 OBJETIVOS

2.1 Objetivos gerais

Uma vez que a toxicidade de compostos orgânicos de selênio está relacionada tipicamente à oxidação de tióis endógenos, o objetivo do estudo foi avaliar a possível atividade inibitória do DSDT frente a enzimas sulfidrílicas para predizer sua toxicidade *in vitro*. Baseado nos dados obtidos *in vitro*, investigou-se também a potencial toxicidade aguda do DSDT *in vivo*.

2.2 Objetivos específicos

Considerando os aspectos mencionados, os objetivos específicos deste estudo compreendem:

- Avaliar o possível efeito inibitório do DSDT frente à atividade de enzimas sulfidrílicas, como δ-ALA-D e Na^+, K^+-ATPase, em homogeneizado de cérebro de ratos;
- Verificar a influência do ditiol DTT sobre a inibição das enzimas sulfidrílicas causada pelo DSDT, assim como do Zn^{2+} sobre a inibição da δ-ALA-D;
- Determinar a potencial atividade do tipo-tiol oxidase do DSDT;
- Investigar o efeito *in vivo* de administrações orais únicas do DSDT sobre marcadores de toxicidade hepática, renal e cerebral em ratos.

3 DESENVOLVIMENTO

Os resultados que fazem parte dessa dissertação estão apresentados na forma de um artigo científico. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas do artigo estão dispostos de acordo com a recomendação do periódico científico no qual está publicado.

3.1 Artigo

Altas doses do 2,2'-diseleneto de ditienila causam toxicidade sistêmica em ratos: Um estudo *in vitro* e *in vivo*

HIGH DOSES OF 2,2′-DITHIENYL DISELENIDE CAUSE SYSTEMIC TOXICITY IN RATS: AN *IN VITRO* AND *IN VIVO* STUDY

Pietro Maria Chagas, Cristiani Folharini Bortolatto, Ethel Antunes Wilhelm, Cristina Wayne Nogueira

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High doses of 2,2′-dithienyl diselenide cause systemic toxicity in rats: an *in vitro* and *in vivo* study

Pietro Maria Chagas, Cristiani Folharini Bortolatto, Ethel Antunes Wilhelm and Cristina Wayne Nogueira*

ABSTRACT: Organoselenium compounds have important pharmacological properties. However, these compounds can cause toxicity, typically related to oxidation of endogenous thiols. The aim of this study was to investigate whether 2,2′-dithienyl diselenide (DTDS) has potential toxicity *in vitro* and *in vivo*. Therefore, sulfhydryl-containing enzyme activities, δ-aminolevulinic acid dehydratase (δ-ALA-D) and Na^+–K^+-ATPase were used to predict DTDS toxicity in rat brain homogenate *in vitro*. In *in vivo* experiments, a DTDS administration (50 or 100 mg kg^{-1}, p.o.) to rats was performed and toxicological parameters were determined. DTDS inhibited δ-ALA-D (IC_{50} 2 μM) and Na^+–K^+-ATPase (IC_{50} 17 μM) activities *in vitro*. The inhibitory effect of DTDS on δ-ALA-D and Na^+–K^+-ATPase activities was restored by dithiothreitol. DTDS (5–25 μM) elicited a thiol oxidase-like activity. *In vivo*, DTDS (50 and 100 mg kg^{-1}) caused systemic toxicity, evidenced by a decrease in water and food intakes and body weight gain, as well as the death of rats. DTDS at the dose of 100 mg kg^{-1} increased plasma alanine and aspartate aminotransferase activities and decreased urea levels. At 50 and 100 mg kg^{-1}, it increased lipid peroxidation levels. At the highest dose, DTDS inhibited δ-ALA-D activity. By contrast, Na^+–K^+-ATPase activity and antioxidant defense were not altered in the brains of rats exposed to DTDS. In conclusion, interaction with the cisteinyl residues seems to mediate the inhibitory effect of DTDS on sulfhydryl-containing enzymes *in vitro*. In addition, high oral doses of DTDS induce toxicity in rats. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: 2,2′-dithienyl diselenide; organoselenium; δ-ALA-D; Na^+–K^+-ATPase; sulfhydryl; toxicity

INTRODUCTION

Selenium is considered an essential trace element for human health; a component of selenoenzymes such as glutathione peroxidase and thioredoxin reductase, it plays an important role in maintaining the intracellular redox state and DNA synthesis (Rayman, 2000). Selenium compounds are generally separated into two forms: inorganic (selenate and selenite) and organic (selenomethionine and selenocysteine; Watanabe *et al.*, 1997), the latter form (*i.e.* organic) being reported as more bioavailable and less toxic (Young *et al.*, 1982; Kim and Mahan, 2001).

Organoselenium compounds, including the synthetic forms, have important biological properties; for instance, they act as potent antioxidants in *in vitro* and *in vivo* models of oxidative stress (Acker *et al.*, 2009; Prigol *et al.*, 2009). The antioxidant activity of these compounds has been shown to be related to their ability to mimic native glutathione peroxidase, dehydroascobate reductase and glutathione *S*-transferase as well as to be a substrate for the mammalian thioredoxin reductase (Nogueira *et al.*, 2004; Freitas *et al.*, 2010; Luchese and Nogueira, 2010).

On the other hand, organoselenium compounds can react with thiols, such as cysteine, dithiothreitol (DTT) and reduced glutathione (GSH), producing selenocysteine, selenols and disulfides (Walter *et al.*, 1972). Reduced cysteinyl residues from proteins can also be oxidized by simple diselenides, which can cause, in the case of enzymes, a decrease in catalytic activity (Nogueira *et al.*, 2003). δ-Aminolevulinic acid dehydratase (δ-ALA-D) and Na^+–K^+-ATPase are sulfhydryl enzymes sensitive to oxidizing agents (Folmer *et al.*, 2004; Nogueira *et al.*, 2004). In fact, δ-ALA-D and Na^+–K^+-ATPase activities are inhibited by high doses of organoselenium in rodents (Prigol *et al.*, 2007).

Ebselen and diphenyl diselenide $(PhSe)_2$ are examples of organoselenium compounds that have pharmacological activity and inhibit sulfhydryl-containing enzymes, besides interfering in the endogenous low-molecular thiol content; they are toxic at high doses, causing oxidative injury, especially in the brain and liver (Nogueira *et al.*, 2004; Nogueira and Rocha, 2010). Another organoselenium compound, 2,2′-dithienyl diselenide (DTDS), a thiophene diselenide derivative, has been proven to be a promising antioxidant in rat brain homogenate *in vitro* (Bortolatto *et al.*, unpublished observations). In this way, the antioxidant property of DTDS contributes to its neuroprotective effect demonstrated by attenuating kainate-induced status epilepticus in rats and the subsequent hippocampal damage (Bortolatto *et al.*, 2011). Based on these considerations, the toxicology of DTDS represents an important point to be investigated.

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Journal of AppliedToxicology

Since the toxicity of organoselenium compounds is related to the oxidation of endogenous thiols (Nogueira and Rocha 2010), we used sulfhydryl-containing enzymes, δ-ALA-D and Na^+–K^+-ATPase, to predict the *in vitro* toxicity of DTDS. Based on the *in vitro* data, we investigated the *in vivo* effect of a single oral administration of DTDS on markers of systemic toxicity in rats.

MATERIALS AND METHODS

Chemicals

DTDS (Fig. 1) was prepared according to the literature method (Tiecco *et al.*, 2000). Analysis of ^{1}HNMR and ^{13}CNMR spectra showed that the obtained compound presented spectroscopic data in full agreement with its assigned structure. The chemical purity of this compound (99.9%) was determined by GC/MS. DTDS was dissolved in dimethylsulfoxide (DMSO) and canola oil for *in vitro* and *in vivo* experiments, respectively.

δ-Aminolevulinic acid (δ-ALA), GSH, β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) tetrasodium salt, DTT, ouabain and ATP were obtained from Sigma (St Louis, MO, USA). All other chemicals were obtained from analytical grade and standard commercial suppliers.

Animals

Male adult albino Wistar rats (200–300 g) from our own breeding colony were used. The animals were kept in a separate animal room, with a 12 h light/dark cycle (lights on at 7:00 a.m.), at a room temperature of 22 ± 2 °C, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

In Vitro Experiments

In vitro experiments were carried out in order to investigate the potential inhibitory effect of DTDS on δ-ALA-D and Na^+–K^+-ATPase activities in rat brain homogenates. In addition, further *in vitro* assays were carried out to prove whether or not the oxidation of sulfhydryl groups is involved in the inhibitory effect of DTDS. The *in vitro* concentrations were chosen randomly.

Tissue Preparation

Animals were killed by decapitation; brains were quickly removed, placed on ice and homogenized in 50 mM Tris/HCl pH 7.4, 1:5 and 1:10 (w/v) for δ-ALA-D and Na^+–K^+-ATPase assays, respectively. The homogenate was centrifuged at 2400 *g* for 10 min at 4 °C to yield a low-speed supernatant fraction (S_1).

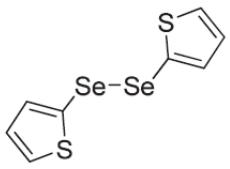

Figure 1. Chemical structure of 2,2′-dithienyl diselenide.

δ-ALA-D Activity

δ-ALA-D activity was assayed according to the method described by Sassa (1982), with some modifications. S_1 (200 µl) was pre-incubated for 10 min at 37 °C in the presence of DTDS at different concentrations (0.1–5 µM) or DMSO in the control tube. The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8 and incubated for 3 h at 37 °C. The incubation was stopped by adding 10% trichloroacetic acid solution (TCA) with 10 mM $HgCl_2$. The reaction product (porphobilinogen) was measured at 555 nm using modified Ehrlich's reagent. The values are expressed as nmol Porphobilinogen (PBG) mg^{-1} protein h^{-1}.

Effect of DTT and $ZnCl_2$ as Restoring Agents for δ-ALA-D Inhibition Caused by DTDS

The potential restorative effect of DTT (3 mM; Barbosa *et al.*, 1998) and $ZnCl_2$ (100 µM; Emanuelli *et al.*, 1998) on δ-ALA-D inhibition caused by DTDS was carried out to investigate if oxidation of sulfhydryl groups is involved in this inhibitory effect. DTDS (the half maximal inhibitory concentration, IC_{50}) was pre-incubated with S_1 for 10 min at 37 °C. After this time, the reaction was started by the addition of substrate in the presence of 3 mM DTT or 100 µM $ZnCl_2$ and performed as already described in the previous section.

Na^+–K^+-ATPase Activity

The reaction mixture for the Na^+–K^+-ATPase activity assay contained 3 mM $MgCl_2$, 125 mM NaCl, 20 mM KCl, and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 µl. S_1 (50 µl) was pre-incubated at 37 °C for 10 min in the presence of DTDS (1.75–25 µM) or DMSO. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM and incubated at 37 °C for 30 min. To obtain the ouabain-sensitive activity, other samples were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+–K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (P_i) was measured by the method of Fiske and Subbarow (1925). The values are expressed as nmol Pi mg^{-1} protein h^{-1}.

Effect of DTT as Restoring Agent for Na^+–K^+-ATPase Inhibition Caused by DTDS

Aiming to study the effect of DTT on reversing Na^+–K^+-ATPase inhibition, DTDS, at IC_{50} concentration, was pre-incubated with S_1 at 37 °C for 10 min. After this time, the reaction was started by the addition of DTT (3 mM) and ATP (3 mM) and performed as already described in the previous section.

Thiol Oxidase-like Activity

The catalytic effect of DTDS on the GSH oxidation was assessed using the rate of oxidation (Ellman, 1959). The rate of GSH oxidation was determined in the presence of 50 mM Tris–HCl, pH 7.5, and DTDS (1–25 µM) or DMSO. Incubation at 37 °C was initiated by the addition of GSH (1.0 mM). At 0, 30, 60 and 120 min, aliquots of the reaction mixture (200 µl) were checked for the amount of GSH at 412 nm. The values are expressed as percentage of control.

Journal of AppliedToxicology

In Vivo Experiments

Based on *in vitro* data and in order to obtain better understanding about the toxicity of this organoselenium compound, some markers of toxicity were assessed in whole brain homogenate and plasma of rat. The doses for the *in vivo* experiments were selected based on our previous study which demonstrated the anticonvulsant action of DTDS in kainate-induced status epilepticus in rats (Bortolatto *et al.*, 2011).

Protocol of Acute Exposure

Rats were divided into three groups of 12 animals each and treated with a single oral dose of DTDS: (a) control (vehicle: canola oil, 1 ml kg^{-1} of body weight); (b) 50 mg kg^{-1} of DTDS (1 ml kg^{-1} of body weight); and (c) 100 mg kg^{-1} of DTDS (1 ml kg^{-1} of body weight). After drug administration, animals were observed up to 72 h to determine the lethal potential of DTDS. The individual body weight gain was recorded and calculated according to the formula [baseline body weight (obtained before the beginning of treatment) − body weight at the end of the experiment]. Water and food consumptions were measured daily in rats exposed to DTDS. The average weight of water and food consumed was calculated according to the formula [water and food intake (g)/number of animals per cage].

Tissue Preparation

After 72 h, rats were anesthetized with ketamine chloridrate and xylazine (5:1; 0.1 ml per 100 g), then blood was collected by heart puncture in tubes containing heparin. Plasma was obtained by centrifugation at 2000 *g* for 10 min (hemolyzed plasma was discarded). Animals were killed by decapitation and S_1 was obtained as described above.

Biochemical Parameters

Hepatic and renal functions were determined by using alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, urea and creatinine levels. All assays were carried out using commercial kits (Labtest, Diagnostica S.A., Minas Gerais, Brazil).

δ-ALA-D and Na^+–K^+-ATPase Activities

δ-ALA-D and Na^+–K^+-ATPase activity assays were performed as described above, except for the absence of the pre-incubation step.

Lipid Peroxidation Levels

S_1 (200 µl) was added to the reaction mixture containing 500 µl 0.8% thiobarbituric acid (TBA), 200 µl sodium dodecyl sulfate (SDS) and 500 µl acetic acid (pH 3.4), and incubated at 95 °C for 2 h. TBA reactive species (TBARS) were determined as described by Ohkawa *et al.* (1979), using malondialdehyde (MDA, an end product of lipid peroxidation) as an external standard.

GSH Levels

Levels of GSH were determined fluorometrically following Hissin and Hilf's (1976) method using *o*-phthalaldehyde (OPA) as fluorophore. Briefly, samples were homogenized in 0.1 M $HClO_4$. Homogenates were centrifuged at 2000 *g* for 10 min and supernatants were separated for measurement of GSH. An aliquot of 100 µl of supernatants was incubated with 100 µl of OPA (0.1% in methanol) and 1.8 ml of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in dark. Fluorescence was measured at excitation wavelength of 350 nm and at emission wavelength of 420 nm. GSH levels were expressed as nmoles per gram of tissue.

Catalase Activity

Enzymatic reaction was initiated by adding an aliquot of 20 µl of the S_1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.5. The enzymatic activity was measured at 240 nm and expressed as units (U) per milligram protein (1 U decomposes 1 µmole of H_2O_2 per minute at pH 7 at 25 °C; Aebi, 1984).

Superoxide dismutase activity

S_1 was diluted 1:10 (v/v) for determination of superoxide dismutase (SOD) activity on the test day. Aliquots of supernatant were added in a Na_2CO_3 buffer 50 mM pH 10.3. Enzymatic reaction was started by adding epinephrine. The color reaction was spectrophotometrically measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C (Misra and Fridovich, 1972). The enzymatic activity was expressed as units per milligram[1] protein.

Ascorbic acid Levels

Ascorbic acid (AA) level determination was performed as described by Jacques-Silva *et al.* (2001) with some modifications. Briefly, S_1 was precipitated in 10% TCA solution. An aliquot of sample (300 µl), at a final volume of 575 µl of the solution, was incubated for 3 h at 38 °C and then 500 µl H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg ml^{-1} dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg ml^{-1}) at 520 nm. The content of ascorbic acid is related to tissue amount (µmol AA g^{-1} tissue).

Protein Quantification

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin (1 mg ml^{-1}) as the standard.

Statistical Analysis

Statistical analysis of data was performed using a one-way analysis of variance (ANOVA), followed by the Newman–Keuls's multiple range test when appropriate, except for thiol oxidase-like activity, which was statistically analyzed using two-way repeated-measures ANOVA. IC_{50} was calculated by linear regression from individual experiments using a statistical program (GraphPad software, San Diego, CA, USA). The IC_{50} values were reported as means accompanied by their 95% confidence limits. Maximal inhibition (I_{max}) values were calculated at the most effective concentration used. All data for the experiments are expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant.

Journal of AppliedToxicology

RESULTS

In Vitro Experiments

Effect of DTDS on δ-ALA-D activity

DTDS at concentrations equal or greater than 1 μM significantly inhibited δ-ALA-D activity from rat brain homogenate (Fig. 2). The IC_{50} value was 2 μM (1.78–2.36) and I_{MAX} was 91 ± 3%.

Effect of DTT and $ZnCl_2$ as restoring agents for δ-ALA-D inhibition caused by DTDS

The inhibitory effect of DTDS on δ-ALA-D activity was completely restored by the addition of DTT (Fig. 3A), but not by $ZnCl_2$, in the reaction medium (Fig. 3B).

Effect of DTDS on Na^+–K^+-ATPase Activity

DTDS at the concentration of 25 μM significantly inhibited Na^+–K^+-ATPase activity from rat brain homogenate (Fig. 4). The IC_{50} value was 17 μM (13.72–21.85) and I_{MAX} was 76 ± 2%.

Effect of DTT as restoring agent for Na^+–K^+-ATPase inhibition caused by DTDS

The inhibitory effect of DTDS on Na^+–K^+-ATPase activity was completely restored by the addition of DTT in the reaction medium (Fig. 5).

Thiol oxidase-like activity

DTDS showed a time- and concentration-dependent thiol oxidase-like activity. The rate of thiol oxidation was significantly increased and the percentage of reduced thiol in relation to the control was decreased by DTDS as a function of time. At 30 min, DTDS, at the concentration of 25 μM, significantly reduced the initial thiol content. At 60 and 120 min, the concentration of 5 μM was effective in significantly reducing the thiol content (Fig. 6).

In vivo Experiments

Lethality

A single oral administration of DTDS, at doses of 50 and 100 mg kg^{-1}, caused the death of 25 and 50% of rats, respectively. Both doses of DTDS significantly reduced the body weight gain, food and water intakes (Table 1).

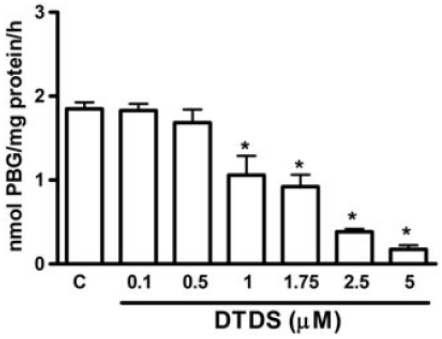

Figure 2. Effect of 2,2′-dithienyl diselenide on δ-aminolevulinic acid dehydratase activity in rat brain homogenates *in vitro*. Data are reported as means ± SEM for three to five experiments performed in duplicate, on different days, using different animals. * $P < 0.05$ compared with the control (one-way ANOVA/Newman–Keuls).

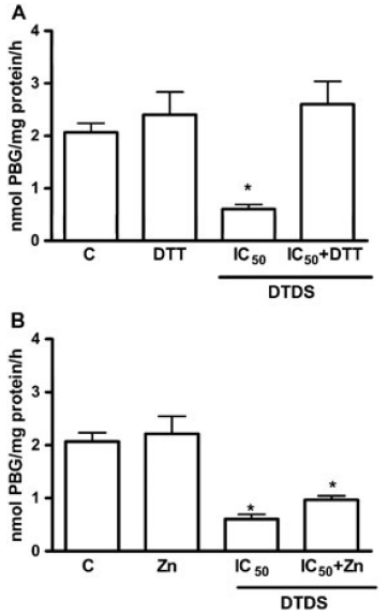

Figure 3. Effect of dithiothreitol (DTT) (a) and $ZnCl_2$ (b) as restoring agents for δ-aminolevulinic acid dehydratase inhibition caused by 2,2′-dithienyl diselenide (DTDS; IC_{50} = 2 μM). Data are reported as means ± SEM for three to five experiments performed in duplicate, on different days, using different animals. * $P < 0.05$ compared with the control; # $P < 0.05$ compared with the IC_{50} of DTDS (one-way ANOVA/Newman–Keuls).

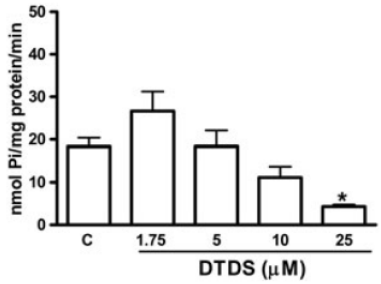

Figure 4. Effect of 2,2′-dithienyl diselenide on Na^+–K^+-ATPase activity in rat brain homogenates *in vitro*. Data are reported as means ± SEM for three to five experiments performed in duplicate, on different days, using different animals. * $P < 0.05$ compared with the control (one-way ANOVA/Newman–Keuls).

Biochemical parameters

Oral administration of DTDS at doses of 50 and 100 mg kg^{-1} increased AST activity and decreased urea levels in plasma. By contrast, neither dose of DTDS significantly altered creatinine levels. Only the highest dose of DTDS significantly increased ALT activity (Table 2) in plasma of rats.

Journal of AppliedToxicology

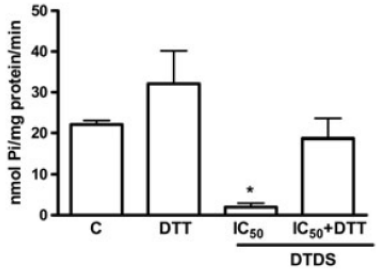

Figure 5. Effect of 2,2′-dithienyl diselenide as a restoring agent for Na^+–K^+-ATPase inhibition caused by 2,2′-dithienyl diselenide (DTDS; $IC_{50} = 17\ \mu M$). Data are reported as means ± SEM for three to five experiments performed in duplicate, on different days, using different animals. * $P < 0.05$ compared with the control; # $P < 0.05$ compared with the IC_{50} of DTDS (one-way ANOVA/Newman–Keuls).

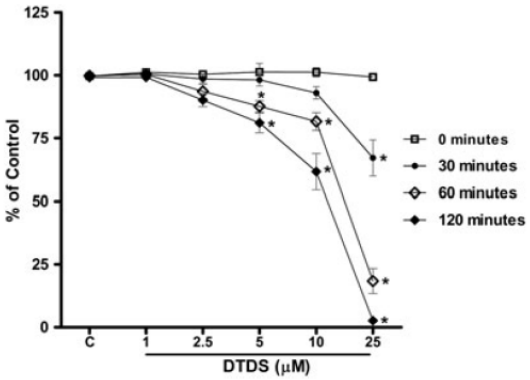

Figure 6. Rate of thiol oxidation by 2,2′-dithienyl diselenide (DTDS) as a function of time: (A) 0 min before the reaction be started; (B) 30 min; (C) 60 min and (D) 120 min. Data are reported as means ± SEM for three to five experiments performed in duplicate, on different days. * $P < 0.05$ compared with the vehicle (two-way ANOVA repeated-measures ANOVA).

Lipid peroxidation levels

DTDS, at both doses, significantly increased TBARS levels in rat brain homogenates when compared with those of the control group (Table 3).

δ-ALA-D activity

DTDS at the dose of 100 mg kg^{-1}, but not at 50 mg kg^{-1}, significantly inhibited δ-ALA-D activity when compared with that of the control group (Table 3).

Na^+–K^+-ATPase activity

Na^+–K^+-ATPase activity was not modified in rats that received DTDS compared with those of the control group (Table 3).

CAT and SOD activities

As demonstrated in Table 4, no significant differences were found in catalase (CAT) and SOD activities from brains of rats exposed to DTDS compared with those of the control group.

GSH and AA levels

No significant differences were found in GSH and AA levels from brains of rats exposed to DTDS compared with those of the control group (Table 4).

DISCUSSION

The *in vitro* results obtained in this study indicate that DTDS inhibited both δ-ALA-D and Na^+–K^+-ATPase activities. Furthermore, DTDS had thiol oxidase-like activity, which represents the ability of a compound to oxidize thiol groups. In addition, the *in vivo* data showed that a single oral administration of DTDS, at doses of 50 and 100 mg kg^{-1}, caused systemic toxicity and the death of rats. DTDS at both doses increased cerebral lipid peroxidation levels and, at the highest dose, inhibited cerebral δ-ALA-D activity. These changes could be described as potentially indicative of DTDS neurotoxicity in exposed animals.

δ-ALA-D, a sulfhydryl- and zinc-containing enzyme, catalyzes the asymmetrical condensation of two molecules of δ-ALA to produce PBG an intermediary in heme biosynthesis (Jaffe, 1995). δ-ALA-D is extremely susceptible to oxidizing agents and must be in the reduced state to catalyze the substrate formation (Emanuelli *et al.*, 2000). DTDS, at low micromolar range, inhibited δ-ALA-D activity, which was restored by the addition of a dithiol agent, DTT, in the reaction medium, indicating that δ-ALA-D inhibition is mediated by the oxidation of essential sulfhydryl groups of the enzyme. Interactions with sulfhydryl groups are also related to δ-ALA-D inhibition caused by other organochacogen compounds (Brüning *et al.*, 2009). Moreover, it has been reported that the oxidation of sulfhydryl groups of δ-ALA-D leads to the release of essential zinc (Tsukamoto *et al.*, 1979). Another interesting point to be considered here is the fact that organoselenium compounds could interact with metals, forming complexes. Consequently, the formation of a complex with the essential zinc of the enzyme and DTDS would contribute to δ-ALA-D inhibition (Santos *et al.*, 2005; Brandão *et al.*, 2008; Brüning *et al.*, 2009). Thus, we tried to recover δ-ALA-D activity by adding $ZnCl_2$ to the reaction medium, but the effect of $ZnCl_2$ on δ-ALA-D activity inhibited by DTDS was insignificant, demonstrated by the absence of protection against this inhibition. Hence, the release of essential zinc seems not to be involved in the mechanism of δ-ALA-D inhibition by DTDS.

Na^+–K^+-ATPase is a sulfhydryl containing enzyme responsible for the active transport of sodium and potassium ions in the central nervous system (Sato *et al.*, 1995). Therefore, this enzyme is required for vital functions such as membrane cotransports, cell volume regulation and membrane excitability (Lees, 1991). Similar to δ-ALA-D, Na^+–K^+-ATPase is also highly susceptible to oxidative stress and oxidizing agents, which could oxidize crucial sulfhydryl groups (Matsuoka *et al.*, 1990). DTT is also effective in reverting Na^+–K^+-ATPase inhibition caused by DTDS, further indicating that DTDS oxidized sulfhydryl residues of the enzyme. In accordance with these data, ebselen and $(PhSe)_2$, organoselenium compounds, inhibit Na^+–K^+-ATPase through the oxidation of thiol groups (Borges *et al.*, 2005).

The thiol oxidase-like activity showed by DTDS corroborates with the idea that the compound inhibits sulfhydryl enzymes via the oxidation of thiol groups contained in both δ-ALA-D and Na^+–K^+-ATPase. The mechanism by which DTDS inhibits sulfhydryl- containing enzymes is probably similar to that proposed by our research group to explain the inhibition of

Journal of AppliedToxicology

Table 1. Effect of oral administration of DTDS on the body weight gain, water and food intakes in rats

Group	Number of deaths	Weight gain (g per animal)	Water intake (g per animal)	Food intake (g per animal)
Control	0/12	7.1 ± 3.1	33.3 ± 0.7	51.2 ± 0.8
50 mg kg^{-1}	3/12	−42.8 ± 4.6*	8.4 ± 1.1*	13.2 ± 1.0*
100 mg kg^{-1}	6/12	−45.3 ± 3.9*	6.9 ± 1.5*	9.4 ± 1.7*

Data are reported as means ± SEM of 12 animals. * $P < 0.05$ compared with the control group (one-way ANOVA/Newman–Keuls). DTDS, 2,2′-dithienyl diselenide.

Table 2. Effect of oral administration of DTDS on biochemical parameters related to hepatic and renal toxicity

Group	ALT (U l^{-1})	AST (U l^{-1})	Urea (mg dl^{-1})	Creatinine (mg dl^{-1})
Control	47.5 ± 1.8	120.4 ± 7.4	25.8 ± 1.3	0.50 ± 0.08
50 mg kg^{-1}	58.6 ± 5.8	207.9 ± 40.3*	21.3 ± 1.1*	0.47 ± 0.05
100 mg kg^{-1}	70.4 ± 10.7*	340.9 ± 39.5*	17.9 ± 1.0*	0.54 ± 0.06

Data are reported as mean ± SEM of 12 animals. * $P < 0.05$ compared with the control group (one-way ANOVA/Newman–Keuls). ALT, alanine aminotransferase; AST, aspartate aminotransferase; DTDS, 2,2′-dithienyl diselenide.

Table 3. Effect of oral administration of DTDS on TBARS levels, δ-ALA-D and Na^+–K^+-ATPAse activities in rat brain

Group	TBARS (nmol MDA/g tissue)	δ-ALA-D activity (nmol PBG mg^{-1} protein h^{-1})	Na^+–K^+-ATPase activity (nmol Pi mg^{-1} protein/min)
Control	9.8 ± 0.5	1.21 ± 0.03	28.2 ± 2.7
50 mg kg^{-1}	12.1 ± 0.6*	1.14 ± 0.04	30.4 ± 2.0
100 mg kg^{-1}	11.5 ± 0.4*	1.04 ± 0.04*	28.7 ± 3.8

Data are reported as mean ± SEM of 12 animals. * $P < 0.05$ compared with the control group (one-way ANOVA/Newman–Keuls). δ-ALA-D, δ-aminolevulinic acid dehydratase; DTDS, 2,2′-dithienyl diselenide; TBARS, thiobarbituric acid reactive species.

Table 4. Effect of oral administration of DTDS on SOD and CAT activities and GSH and AA levels in rat brain

Group	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	GSH (nmol CDNB min^{-1} mg^{-1} protein)	AA (μmol g^{-1} tissue)
Control	13.9 ± 1.7	7.6 ± 0.5	1.21 ± 0.04	206.4 ± 3.5
50 mg kg^{-1}	18.3 ± 2.1	8.6 ± 0.3	1.25 ± 0.09	200.1 ± 5.3
100 mg kg^{-1}	15.5 ± 0.9	8.3 ± 0.7	1.44 ± 0.09	198.4 ± 2.9

Data are reported as mean ± SEM of 12 animals. * $P < 0.05$ compared with the control group (one-way ANOVA/Newman–Keuls). AA, Ascorbic acid; CAT, catalase; DTDS, 2,2′-dithienyl diselenide; GSH, reduced glutathione; SOD, superoxide dismutase.

δ-ALA-D activity by other diselenides (Nogueira *et al.*, 2004). The first step of oxidation of thiol groups involves the reaction of the enzyme (E–Cys–SH) with the diselenide (RSe–SeR) to generate the intermediate E–Cys–S–SeR passing through a thiol nucleophilic attack in the selenium atom of the diselenide. Subsequently, the other cysteinyl residue, owing to its close spatial proximity to the more reactive residue, attacks the sulfur–selenium bond of the intermediate (E–Cys–S–SeR), producing the oxidized enzyme (inactive) and selenophenol (RSeH), which regenerates the respective diselenide.

Organochalcogens have been recently reported to be effective in inhibiting different sulfhydryl-containing enzymes, for instance lactate dehydrogenase (Kade and Rocha, 2010) and squalene monooxygenase (Gupta and Porter, 2002), this being one of the mechanisms by which this class of compounds causes toxicity in mammals (Barbosa *et al.*, 1998).

Since organoselenium compounds have interesting pharmacological properties, for example chemopreventive (Sanmartín *et al.*, 2009), antidepressant-like, anxiolytic-like, anti-hypercholesterolemic (Nogueira and Rocha, 2010) and anticonvulsant (Bortolatto *et al.*,

Journal of AppliedToxicology

2011), the toxicology of organic forms of selenium is an essential point to be investigated.

Based on the *in vitro* results, we extend the study in order to better understand the effects of a single oral administration of DTDS in rats. It was demonstrated that DTDS caused a reduction in the body weight gain, food and water intakes, with the death of a significant fraction of animals, parameters widely used to determine acute or chronic toxicity (Che *et al.*, 2011; Belcher *et al.*, 2011). These results are in accordance with studies of our research group, which demonstrated that treatment with organoselenium compounds, even at doses that do not cause toxic effects, reduces food and water intakes and body weight gain, probably owing to an anorexigenic effect of this class of compounds (Meotti *et al.*, 2008; Savegnago *et al.*, 2009).

Moreover, a single oral administration of DTDS in rats promoted an increase in activities of ALT and AST and a decrease in plasma urea levels, suggesting a systemic toxicity caused by this compound. The increase in ALT and AST activities typically characterizes an increase in the permeability of hepatocytes that is caused specially in hepatic injury induced by toxins (Rao and Mehendale, 1991). On the one hand, the decrease in urea levels, found in rats treated with DTDS, could be related to the acute liver failure induced by toxic agents (Lum and Leal-Khourl, 1989). In fact, a decrease in the urea formation could represent a potential hazard to animals; whereas the urea cycle is the main pathway to detoxify ammonia, this decrease can be related to a condition known as hyperammonemia, characterized by signs like weight loss, loss of appetite, irreversible brain damage and even death (Häberle, 2011). On the other hand, it is important to consider that the observed clinical signs could simply be the result of acute toxicity to DTDS and have nothing to do with hyperammonemia. By contrast, the kidney seems not to be affected by DTDS, since creatinine levels were not altered at the administered doses.

There are data reported in the literature indicating that lipophilic organoselenium compounds can cross the blood–brain barrier, making the brain a potential target to toxicity of them (Rosa *et al.*, 2007; Nogueira *et al.*, 2003). The *ex vivo* δ-ALA-D inhibition by DTDS exposure could be related to oxidation of thiol groups, as demonstrated by *in vitro* experiments. The inhibition of cerebral δ-ALA-D activity by organoselenium compounds may result in accumulation of δ-ALA, a metabolite with excitotoxic and convulsive properties (Emanuelli *et al.*, 2000), leading to alterations of oxidative status. Cerebral Na^+–K^+-ATPase activity, unlike δ-ALA-D, was not altered in DTDS-exposed animals. This result indicates that, as seen in the *in vitro* experiments, δ-ALA-D is more sensitive to DTDS and that the administered doses were not sufficient to inhibit Na^+–K^+-ATPase activity.

Although, an increase in lipid peroxidation levels were demonstrated in animals that received DTDS, no change was found in endogenous antioxidant defenses, represented by SOD and CAT and the nonprotein antioxidants, AA and GSH. The exact mechanism by which DTDS increased lipid peroxidation levels is not completely understood; however; we believe that the inhibition of δ-ALA-D activity resulting from DTDS exposure could contribute to this effect.

In conclusion, DTDS inhibited cerebral δ-ALA-D and Na^+–K^+-ATPase activities *in vitro* by interacting with cysteinyl residues of the enzymes. A single oral administration of high doses of DTDS to rats caused systemic toxicity, resulting in death. Although other studies are necessary to give more information about this specific compound, our findings contribute to the knowledge on the toxicology of DTDS, a compound with pharmacological properties.

CONFLICT OF INTEREST

The authors declare that they have no personal or competing financial interests.

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4 CONCLUSÃO

Os resultados apresentados nesta dissertação nos permitem concluir que:

✓ O DSDT inibe as enzimas sulfidrílicas δ-ALA-D e Na^+, K^+-ATPase;

✓ O composto ditiol DTT reverte a inibição enzimática causada pelo DSDT, indicando uma possível interação do composto com resíduos de cisteína presentes no sítio ativo destas enzimas;

✓ A ação do tipo-tiol oxidase do DTDS corrobora com a ideia de que o composto inibe enzimas sulfidrílicas através da oxidação de grupos tióis;

✓ Nas doses avaliadas, 50 e 100 mg/kg pela via oral, o DSDT apresentou sinais de toxicidade tanto cerebral quanto sistêmica, sendo o mecanismo de ação semelhante ao observado nos experimentos *in vitro*;

✓ Embora mais estudos sejam necessários, principalmente em relação a sua toxicidade sub-crônica e crônica, os dados contribuem para a compreensão da toxicologia do DSDT, composto com propriedades farmacológicas promissoras, visando que as doses/concentrações usadas possam ser ajustadas conforme uma janela terapêutica confiável.

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