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AVALIAÇÃO DA CAPACIDADE ANTIOXIDANTE *IN VITRO* DE NOVOS COMPOSTOS MONO E DISSELENETO

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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AVALIAÇÃO DA CAPACIDADE ANTIOXIDANTE *IN VITRO* DE NOVOS COMPOSTOS MONO E DISSELENETO

Sílvio Terra Stefanello

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

Orientador: Prof Dr Félix Alexandre Antunes Soares

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elaborada por
Sílvio Terra Stefanello

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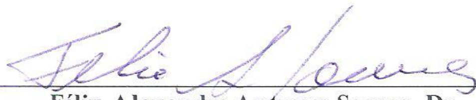

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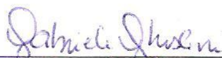

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"A vitória mais bela que se pode alcançar é vencer a si mesmo."
(Santo Inácio de Loyola)

RESUMO

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

AVALIAÇÃO DA CAPACIDADE ANTIOXIDANTE *IN VITRO* DE NOVOS COMPOSTOS MONO E DISSELENETO

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A ação antioxidante dos compostos orgânicos de selênio, como o ebselen e o disseleneto de difenila (DPDS), está intimamente envolvida com a capacidade de formação do grupamento selenol. Neste estudo foi avaliado o perfil antioxidante *in vitro* de novos compostos mono e disseleneto, onde foi comparado se a formação do p-metil-selenol pelos compostos 1-fenil-3-(p-tolilselenil)propano-2-amina (C1) e o 1,2-dip-tolildisseleneto (C4), assim como a formação do grupamento o-metoxi-selenol pelos compostos 1-(2-metoxifenilselenil)-3-fenilpropano-2-amina (C2) e 1,2-bis(2-metoxifenil)disseleneto (C3) pode estar associados com os efeitos antioxidantes apresentados. Os novos compostos mono e disseleneto foram avaliados quanto a capacidade de redução dos níveis de peroxidação lipídica induzida por Fe(II) e nitroprussiato de sódio em homogenatos de cérebro e fígado de ratos, assim como também foi avaliada a capacidade antioxidante através do ensaio da redução do fosfomolibdênio e do radical DPPH. Além disso, foram quantificados os efeitos dos compostos quanto à atividade das enzimas antioxidantes, tioredoxina redutase (TrxR) e glutationa peroxidase (GPx). O efeito oxidante, dos novos compostos, foi investigado através do ensaio da tiol oxidase e da viabilidade celular de leucócitos isolados. Decorrente dos resultados obtidos foi possível evidenciar que ambos os compostos apresentaram uma redução significativa da peroxidação lipídica quando induzidas por diferentes pro oxidantes assim como, uma capacidade antioxidante total semelhante a equivalentes de acido ascórbico. Da mesma forma, os compostos não apresentaram efeito tiol oxidase, assim como não apresentaram uma diminuição da viabilidade celular de leucócitos. Os compostos C1 e C2 não apresentaram atividade mimética a enzima GPx assim como, também não serviram de substrato para a enzima TrxR, provavelmente devido a presença do grupamento amino nas estruturas químicas destas moléculas o que incapacitou a formação dos respectivos grupamentos selenois. No entanto, os compostos análogos ao DPDS apresentaram atividades miméticas a GPx, assim como também apresentaram uma aumento na atividade da TrxR provavelmente devido a formação dos selenois (p-metil-selenol e o-metoxi-selenol).

Palavras-chave: Organocalcogênios. Selenol. Antioxidante. TrxR. GPx.

ABSTRACT

Thesis of Master's Degree
Graduation Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

EVALUATION OF *IN VITRO* ANTIOXIDANT CAPACITY OF NEW MONO AND DISELENIDE COMPOUNDS

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Place and Date of the presentation: Santa Maria, July 26, 2013

The antioxidant action of organic selenium compounds, as well as ebselen and diphenyl diselenide (DPDS), is closely connected to its ability of generating the selenol group. (In) this study it was evaluated the in vitro antioxidant effect of new mono and diselenide compounds, where it was compared whether the formation of p-methyl-selenol from compounds 1-phenyl-3-(p-tolylselanyl)propan-2-amine (C1) and 1,2-dip-tolyldiselenide (C4) and o-methoxy-selenol from compounds 1-(2-methoxyphenylselanyl)-3-phenylpropan-2-amine (C2) and 1,2-bis(2-methoxyphenyl) diselenide (C3) may be involved with their antioxidant effects. The mono and diselenide compounds were tested in their Fe(II) and sodium nitroprusside (SNP)-induced lipid peroxidation in rat brain and liver homogenates and also in their antioxidant ability in phosphomolybdenum test-reductionand and DPPH radical. Besides, the effects of the compounds in the antioxidant enzymes thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) were quantified. The new compounds' oxidant effects were investigated through the thiol oxidase assay and the cellular viability of isolated leukocytes. The results demonstrated that the compounds obtained a significant reduce on the lipid peroxidation induced by different pro-oxidants, as well as an antioxidant effect similarly when compared to ascorbic acid equivalents. In the same manner, the compounds did not present thiol oxidase activity. Furthermore, they did not preset any decrease on the cellular viability of leucocytes. The compounds C1 and C2 did not show mimetic activity of GPx enzyme or had a substrate effect on TrxR enzyme, probably due the amino group presence on their chemical structures which must have inhibited the selenol formation. However, DPDS' analog-compounds presented a mimetic activity of GPx, as well as they showed an increase in the TrxR activity, presumably due the formation of the selenol groups (p-methyl-selenol and o-methoxy-selenol).

Keywords: Organochalcogen. Selenol. Antioxidant. TrxR. GPx.

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Tabela 4 - Calculated I_{max} values of compounds on lipid peroxidation in rat liver.

LISTA DE ABREVIATURAS

C1 - 1-fenil-3-(p-tolilselenil)propano-2-amina

C2 - 1-(2-metoxifenilselenil)-3-fenilpropano-2-amina

C3 - 1,2-bis(2-metoxifenil)disseleneto

C4 - 1,2-dip-tolildisseleneto

CAT - catalase

DPDS - disseleneto de difenila

ER - espécies reativas

ERNs - espécies reativas de nitrogênio

EROs - espécies reativas de oxigênio

GPx - glutationa peroxidase

GSH - glutationa

H_2O - água

H_2O_2 - peróxido de hidrogênio

$HNOO^-$ - peroxinitrito

N_2O - óxido nitroso

$NO^\bullet$ - óxido nítrico

$O2^{\bullet-}$ - ânion superóxido

$OH^\bullet$ - radical hidroxila

Se - selênio

Selenol - R-SeH

SOD - superóxido dismutase

Tiol - R-SH

Trx - tioredoxina

TrxR - tioredoxina redutase

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1- INTRODUÇÃO

O funcionamento normal do metabolismo celular, como por exemplo, a respiração mitocondrial nos organismos aeróbicos, assim como a exposição a fatores ambientais como radiação, infecção e poluição entre outros, pode gerar metabolitos altamente reativos, conhecidos como espécies reativas (ER) (POYTON; BALL; CASTELLO, 2009; PUNTEL et al., 2013; ZADRA et al., 2012). As ER são capazes de desencadear estresse oxidativo justamente devido as suas propriedades oxidantes podendo danificar macromoléculas biológicas tais como ácidos nucléicos, proteínas, lipídios e carboidratos (HALLIWELL; GUTTERIDGE, 2007; MARKESBERY, 2007; VALKO et al., 2007).

Nas últimas décadas, diversos estudos científicos relacionaram o dano celular gerado pelo estresse oxidativo como sendo um dos principais desencadeadores de doenças tais como o Alzheimer, Parkinson, aterosclerose, diabetes mellitus, câncer, assim como a possibilidade de tais oxidantes estarem envolvidos no envelhecimento precoce (BENZI; MORETTI, 1995; DUGAN; QUICK, 2005; JI et al., 2003; KEANE et al., 2011; PELICANO; CARNEY; HUANG, 2004).

Dentre os agentes oxidantes mais importantes envolvidos em processos patológicos estão às espécies reativas de oxigênio (EROs) como o ânion superóxido ($O2^{\bullet-}$), o radical hidroxila ($OH^{\bullet}$) e o peróxido de hidrogênio (H_2O_2) e as espécies reativas de nitrogênio (ERNs) que incluem o óxido nítrico ($NO^{\bullet}$), o óxido nitroso (N_2O) e o peroxinitrito ($HNOO^-$) (GILLHAM et al., 1997; SIES, 1997).

O estresse oxidativo é a representação fisiológica de um desequilíbrio entre os agentes oxidantes e as defesas antioxidantes, podendo ser assim caracterizado tanto pela superprodução de ER no organismo quanto pela diminuição das defesas antioxidantes (GRIFFITHS et al., 2002; REUTER et al., 2010).

O sistema de defesa antioxidante possui a capacidade de inibir a formação ou neutralizar as ER e pode ser dividido de acordo com a sua origem endógena ou exógena (REUTER et al., 2010). O sistema de defesa endógeno representa a maior parcela antioxidante encontrada nos mamíferos e pode ser decorrente de origem enzimática, representadas pelas enzimas glutationa peroxidase (GPx), catalase (CAT), a superóxido dismutase (SOD) e a tioredoxina redutase (TrxR) ou não enzimática, como a glutationa (GSH), a vitamina C e ácido úrico entres outros (FLOHE; GUNZLER; SCHOCK,

1973; FRIDOVICH, 1999; LU; HOLMGREN, 2009; RAHMAN, 2007). Além disso, o organismo ainda dispõe do sistema de defesa antioxidante exógeno que é representado por constituintes de origem natural ou de origem sintética (CAROCHO; FERREIRA, 2013; HASANI-RANJBAR; LARIJANI; ABDOLLAHI, 2009).

Dentre os elementos naturais usualmente estudados devidos as suas propriedades antioxidantes, destaca-se o selênio (Se). O Se faz parte do grupo 16 ou grupo dos calcogênios da tabela periódica, juntamente com os elementos oxigênio, enxofre, telúrio e polônio (NOGUEIRA; ROCHA, 2011). Sendo esta, a principal razão pela qual o elemento Se é capaz de compartilhar com o enxofre algumas propriedades físicas e químicas tão importantes para o balanço redox através da formação dos grupamentos R-SeH (selenol) e R-SH (tiol) (SUZUKI et al., 2008; URSINI; BINDOLI, 1987).

O Se é um elemento traço considerado nutricionalmente essencial aos mamíferos (OLDFIELD, 1987). Sendo estipulada pela Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos a necessidade de uma ingestão diária de 40 - 70 µg para homens e de 45 - 55 µg para mulheres, conforme a idade (KIELISZEK; BŁAŻEJAK, 2013).

Além disso, este elemento quanto presente sob forma do aminoácido selenocisteina desempenha um importante papel antioxidante nos sistemas biológicos justamente por fazer parte do sítio ativo de enzimas como a GPx e TrxR (FLOHE et al., 1973; LU; HOLMGREN, 2009; NOGUEIRA; ROCHA, 2010; ROTRUCK et al., 1973).

Sendo assim, a síntese de compostos orgânicos contendo o elemento selênio obteve um amplo enfoque acadêmico justamente por representar uma nova estratégia terapêutica a ser utilizada no tratamento de doenças relacionadas ao estresse oxidativo (ARTEEL; SIES, 2001; NOGUEIRA; ROCHA, 2011).

O principal interesse por compostos orgânicos contendo selênio se dá pelo fato que estes poderiam imitar a química fisiológica redox dos grupos selenol/selenolato (NOGUEIRA; ZENI; ROCHA, 2004). O grupamento selenolato é mais nucleófilo do que o seu análogo tiolato o que permite após uma reação com GSH a formação do grupamento selenol capaz de um poder redutor maior do que o presente nos grupamentos tiois (NOGUEIRA; ROCHA, 2011).

Dentre os compostos orgânicos de selênio que foram estudados quanto à capacidade antioxidante, destacaram-se o ebselen e o disseleneto de difenila (DPDS).

O ebselen (2-fenil-1,2-benzisoselenazol-3[2H]-ona) (Figura 1) foi relatado na literatura como sendo o primeiro composto orgânico de selênio a apresentar atividade mimética a GPx (MAIORINO et al., 1988; WENDEL et al., 1984). O mecanismo pelo qual o ebselen é capaz de desempenhar a sua atividade redutora de H_2O_2 entre outros hidroperóxidos, ocorre através da reação do composto com um grupamento tiol para produzir o selenenil sulfeto. A produção do grupamento selenol ocorre quando o selenenil sulfeto reage com outro equivalente de GSH. O selenol devido a sua alta nucleofilicidade é capaz de reagir com o H_2O_2 ou hidroperóxidos orgânicos para formar água (H_2O) e ácido selenênico, o qual ao produzir uma nova molécula de H_2O, e capaz de regenerar o ebselen (Figura 2) (NOGUEIRA; ROCHA, 2011; NOGUEIRA; ZENI; ROCHA, 2004).

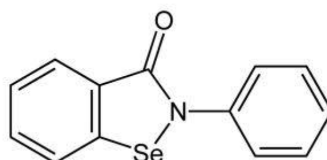


Figura 1: Estrutura química do ebselen.

Além disso, evidências indicam que as propriedades antioxidantes do ebselen também estão relacionadas devido às reações com o sistema da tioredoxina (Trx) (NOGUEIRA; ROCHA, 2010; ZHAO; HOLMGREN, 2002). A redução do ebselen pela TrxR na presença de NADPH ou através da Trx reduzida, são capazes de formar um grupamento selenol (NOGUEIRA; ROCHA, 2011; ZHAO; MASAYASU; HOLMGREN, 2002). Sendo que, o selenol formado a partir do ebselen ao ser oxidado, passa a servir como substrato para TrxR de mamíferos gerando como produto final um selenol ativo (NOGUEIRA; ZENI; ROCHA, 2004).

O DPDS (Figura 3) é o mais simples dos diaril disseleneto e é muito utilizado como composto intermediário para a síntese de novos compostos orgânicos de selênio (NOGUEIRA; ROCHA, 2010; PAULMIER, 1986; SALMAN et al., 2012).

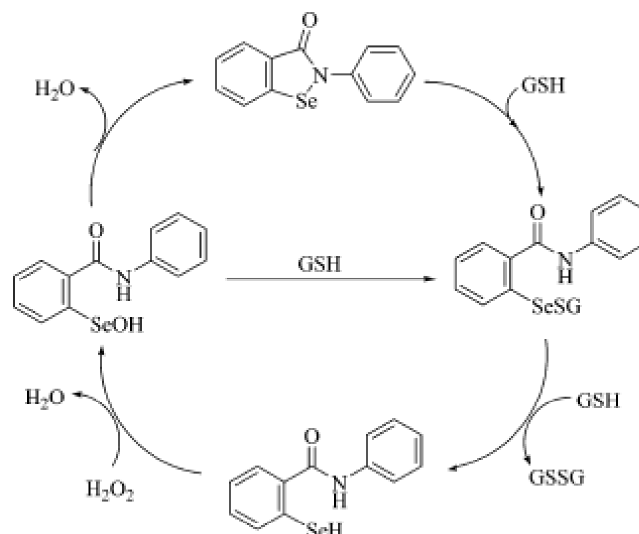


Figura 2: Mecanismo redox de hidroperóxidos pelo ebselen. Fonte: NOGUEIRA; ZENI; ROCHA, 2004.

Estudos demonstram que o DPDS possui atividade anti-inflamatória, cardioprotetora, hepatoprotetora, anti-úlcera, anti-carcinogênica e antidepressiva (BORGES et al., 2005; BRANDAO et al., 2009; DE BEM et al., 2009; DE VARGAS BARBOSA et al., 2008; GHISLENI et al., 2008; NOGUEIRA et al., 2003; SAVEGNAGO et al., 2008). Além disso, sabe-se que o DPDS apresenta tanto uma maior atividade tiol-peroxidase, quanto uma menor toxicidade em roedores quando comparado com os resultados encontrados para o ebselen (MEOTTI et al., 2003; NOGUEIRA; ZENI; ROCHA, 2004).

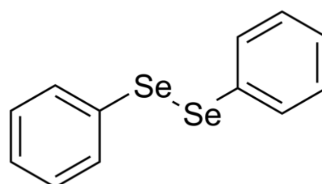


Figura 3: Estrutura química do disseleneto de difenila

A ação antioxidante apresentada pelo DPDS também se encontra intimamente ligada com a capacidade de formação dos grupamentos selenóis, podendo assim apresentar tanto a atividade mimética a GPx (Figura 4) quanto servir de substrato da TrxR (DE FREITAS; ROCHA, 2011; NOGUEIRA; ROCHA, 2010).

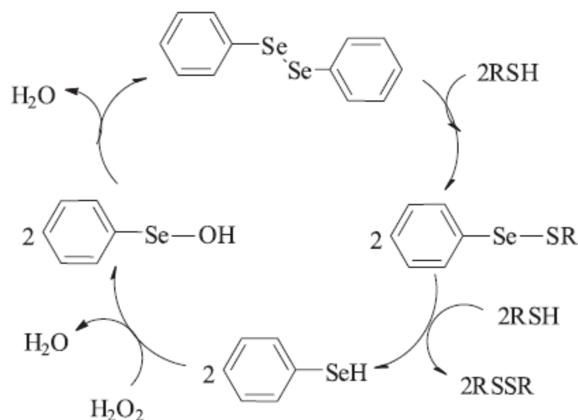


Figura 4: Mecanismo de ação antioxidante do disseleneto de difenila. Fonte: NOGUEIRA; ROCHA, 2010.

Decorrente da reconhecida ação antioxidante dos compostos ebselen e DPDS, inúmeros grupos de pesquisa criaram análogos destes compostos, principalmente na intenção de melhorar os seus efeitos antioxidantes assim como diminuir a toxicidade (BHABAK; MUGESH, 2010; MUGESH; DU MONT; SIES, 2001; WILSON et al., 1989).

Nesse sentido, este estudo buscou evidenciar a capacidade antioxidante *in vitro* de novos compostos orgânicos de selênio, sendo eles, o 1-fenil-3-(p-tolilselenil)propano-2-amina (C1), o 1-(2-metoxifenilselenil)-3-fenilpropano-2-amina (C2), o 1,2-bis(2-metoxifenil)disseleneto (C3) e o 1,2-dip-tolildisseleneto (C4) (Figura 5). Estes compostos são pertencentes a duas classes diferentes, sendo o C1 e C2 denominados como monosselenetos ou β-selenoaminas, e o C3 e C4 como disselenetos ou análogos do DPDS.

Sabe-se que a presença de um grupamento amino próximo ao elemento selênio possui a capacidade de aumentar os níveis antioxidantes da molécula justamente pela obtenção de um grupamento selenol mais estável (HASSAN et al., 2012). Da mesma forma estudos na literatura, já demonstraram que inserção de grupamentos metoxi e metila na molécula do DPDS, apresentam um maior efeito antioxidante associado a uma menor toxicidade quando comparados com o composto clássico (PINTON et al., 2013; WILHELM; BORTOLATTO; NOGUEIRA, 2012).

Neste estudo, os compostos C1 e C4 receberam a inserção de um grupamento metila na posição *para* do fenil, enquanto que os compostos C2 e C3 receberam a inserção de um grupamento metoxi na posição *orto*. Dessa forma, é possível estimar

através da estrutura química dos compostos que o C1 e o C4 podem vir a formar um grupamento p-metil-selenol, enquanto que o C2 e o C3 podem formar o grupamento o-metoxi-selenol.

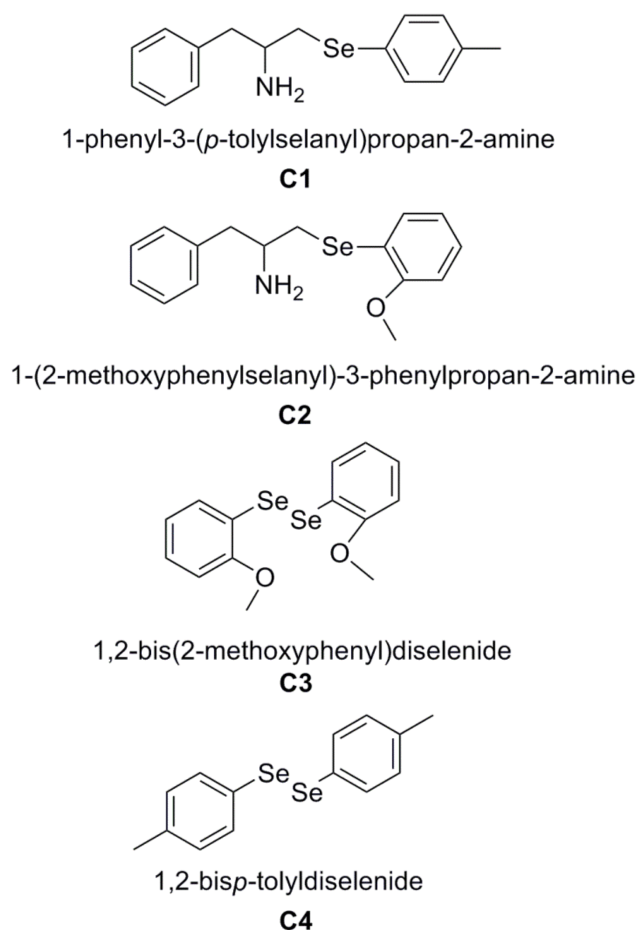


Figura 5: Estrutura química e nomenclatura das β-selenoaminas (C1 e C2) e dos compostos análogos do disseleneto de difenila (C3 e C4). Fonte: STEFANELLO et al., 2013.

Sendo assim, este estudo teve como proposito principal a realização de técnicas *in vitro* utilizando novos compostos mono e disseleneto, para elucidar se as modificações realizadas nas estruturas das moléculas clássicas de compostos orgânicos de selênio como o ebselen e DPDS, apresentavam melhores resultados antioxidantes assim como se reduziam os efeitos oxidantes já previamente descritos na literatura.

2 OBJETIVOS

2.1 Objetivo geral

Verificar a capacidade antioxidante de novos compostos orgânicos de selênio, atribuindo os resultados quanto à inserção dos grupamentos metila e metoxi na estrutura química da molécula, assim como, a possibilidade de formação dos grupamentos selenóis.

2.2 Objetivos específicos

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

- Avaliar a atividade dos novos compostos orgânicos de selênio frente a peroxidação lipídica induzida por ferro (FeII) e nitroprussiato de sódio em cérebro e fígado de ratos;
- Avaliar a atividade antioxidante total dos compostos através do ensaio do fosfomolibdênio;
- Avaliar a capacidade “scavenger” dos compostos através do ensaio empregando o radical DPPH;
- Avaliar se os compostos apresentavam a capacidade de quelar o ion Fe(II);
- Avaliar a toxicidade dos compostos através dos ensaios da tiol oxidase, assim como a viabilidade celular realizada em leucócitos isolados;
- Avaliar se os compostos apresentaram atividade mimética as enzimas TrxR e GPx;
- Elucidar se a estrutura química da molécula pode estar envolvida na atividade antioxidante.

3 RESULTADOS

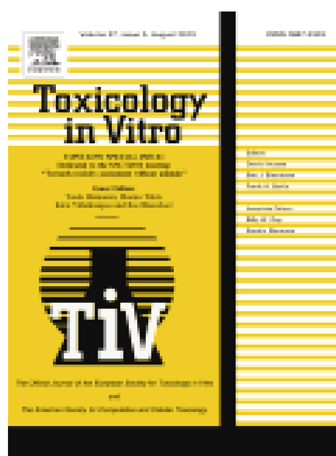
Os resultados que fazem parte dessa dissertação estão apresentados na forma de um artigo científico. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas do artigo estão dispostos de acordo com a recomendação do periódico científico no qual foi publicado.

3.1 Artigo

Avaliação do efeito antioxidante *in vitro* de novos mono e disselenetos

EVALUATION OF IN VITRO ANTIOXIDANT EFFECT OF NEW MONO AND DISELENIDES

Sílvio Terra Stefanello, Alessandro S. Prestes, Tade Ogunmoyole, Syed M. Salman, Ricardo S. Schwab, Caroline R. Brender, Luciano Dornelles, João B.T. Rocha, Félix A.A. Soares



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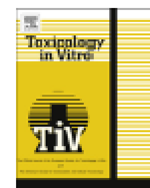

Evaluation of in vitro antioxidant effect of new mono and diselenides

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ABSTRACT

This study was designed to examine the antioxidant activity in vitro of novel mono- and diselenide compounds. We compared whether the formation of p-methyl-selenol from compounds 1-phenyl-3-(p-tolylselanyl)propan-2-amine (C1) and 1,2-dip-tolyldiselenide (C4) and o-methoxy-selenol from compounds 1-(2-methoxyphenylselanyl)-3-phenylpropan-2-amine (C2) and 1,2-bis(2-methoxyphenyl) diselenide (C3) may be involved in their antioxidant effects. The compounds were tested against Fe(II) and sodium nitroprusside (SNP)-induced lipid peroxidation in rat brain and liver homogenates. Likewise, the antioxidant capacity of the compounds was assessed by their ability to decolorize the DPPH radical as well as the Fe(II) chelating assay through the reduction of molybdenum(VI) (Mo6+) to molybdenum(V) (Mo5+). This colorimetric assay was also used to quantify thiol peroxidase (GPx) and oxidase activity and thioredoxin reductase (TrxR) activity. The results showed that the novel selenide compounds inhibit the thiobarbituric acid reactive species (TBARS) induced by different pro-oxidants, but the monoselenides effects were significant only at concentrations higher than the concentrations of the diselenides. Similarly, the total antioxidant activity was higher in the diselenides. Moreover, GPx and TrxR activity was only observed for the diselenides, which indicates that these compounds are more stable selenol molecules than monoselenides.

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1. Introduction

Oxygen metabolism, which typically occurs in aerobic organisms, allows energy formation mediated by the mitochondrial electron transfer system (Puntel et al., 2013). However, oxygen metabolism also leads to the production of small quantities of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Mugesh et al., 2001). Additionally, an aerobe is able to produce reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$) and nitric oxide ($^{\cdot}NO$), which are also as strong biological oxidants (Nathan and Ding, 2010). Accordingly, the imbalance between ROS/RNS formation and the enzymatic/non-enzymatic antioxidant system is associated with many diseases, such as Alzheimer's, myocardial infarction, atherosclerosis, and Parkinson's, and in other pathological conditions, including senescence (Ji et al., 2003; Salmon et al., 2010; Schon and Przedborski, 2011).

Similarly, several research groups developed techniques to create potential antioxidant molecules using chalcogen elements in their structure, and thus, some of these new compounds were characterized as strong free radical scavengers. (Gutteridge and Halliwell, 1992). For example, the organoselenium compounds have shown mimetic glutathione peroxidase-like activity (GPx) and also act as substrates of thioredoxin reductase (TrxR). Therefore, these compounds might represent novel therapeutic targets for diseases caused by oxidative stress (Arteel and Sies, 2001).

The antioxidant effects of organoselenium compounds, such as ebselen and diphenyl diselenide (DPDS), have been shown to be due to their ability to generate a selenol/selenolate chemical form (Nogueira and Rocha, 2010). The selenolate group is a stronger nucleophile than its thiolate analog, which confers stronger reducing power to a given selenol group than the analog thiol group (Nogueira and Rocha, 2011).

However, although the selenol groups are less abundant than thiols and are found only in a small number of selenoproteins, they exhibit a stronger nucleophilicity than their sulfur analogs (Lu et al., 2009). In brief, the presence of selenium (Se) in selenocysteine reduces the enzymatic pK_a, compared to the sulfhydryl enzyme, and therefore leads to Se ionization, forming a selenol group (Gutteridge and Halliwell, 1992).

According to the proposed mechanism, the selenol complex (enzyme-SeH) could react with hydrogen peroxide or other hydroperoxides to produce selenic acid (enzyme-SeOH), which is capable

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when reacting with glutathione (GSH) to reclaim the selenol and form water (Nogueira and Rocha, 2010).

Previous studies reported that the DPDS antioxidant effect was better than that of ebselen, especially in the GPx-like action, and was mainly due to the formation of two selenol structures after interaction with reducing thiol groups (Nogueira et al., 2004). However, the instability of the selenol complex makes it difficult to detect any antioxidant effects during in vitro studies (Bhabak and Mugesh, 2010). Therefore, the emergence of classic, structural organoselenium compound analogs can promote the stability of the selenol (Balkrishna et al., 2011). Indeed, the structural inclusion of a basic amino acid nitrogen near the selenium can increase the antioxidant capacity to create a more stable selenol molecule (Hassan et al., 2012).

Consequently, this study evaluates two different classes of organoselenium compounds, monoselenides (β-selenoamines) and diselenides (analogs of DPDS), using various antioxidant assays. The β-selenoamine chemical structure includes amino groups (C1 and C2) and the diselenides consist of methyl or methoxy group modifications (C3 and C4, respectively) (Fig. 1).

The aim of this study was to evaluate the antioxidant capacity using in vitro models of the compounds cited above and to associate the effects with the capacity of these molecules to form a more stable selenol once the theoretical compounds C1 and C4 generate p-methyl-selenol and compounds C2 and C3 form o-methoxy-selenol.

2. Materials and methods

2.1. Animals

Male, adult Wistar rats (200–250 g) from our own breeding colony were used. The animals were maintained on a 12-h light: 12-h dark cycle, at a room temperature of 22–24 °C and with free access to food and water. The animals were treated according to standard guidelines of the Committee on Care and Use of Experimental Animal Resources.

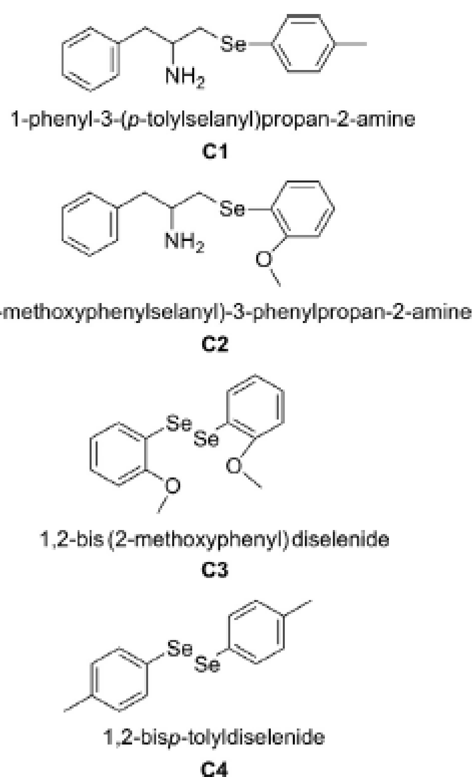

Fig. 1. Chemical structure and nomenclature of the β-selenoamines (C1 and C2) and analogs of diphenyl diselenide (C3 and C4).

2.2. Chemicals

Thiobarbituric acid (TBA), malonldialdehyde (MDA), diphenyl-2'picrylhydrazyl (DPPH), adenine dinucleotide phosphate (NADPH), benzenethiol, Tris–HCl, sodium dodecyl sulfate (SDS), ethylene diamine tetra acetic acid (EDTA) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Fe(II) sulfate, sodium nitroprusside (SNP), ascorbic acid, hydrogen peroxide, acetic acid, 5.5'-dithiobis(2-nitrobenzoate) (DTNB), NaCl, KCl, Na_2HPO_4, KH_2PO_4 and ethanol were obtained from Merck (Rio de Janeiro, RJ, Brazil).

2.3. Compounds

The mono- and diselenides were prepared following previously described methods (Salman et al., 2012), and the purity of the products was accessed by hydrogen and carbon nuclear magnetic resonance and gas chromatography. The compounds tested were 1-phenyl-3-(p-tolylselanyl)propan-2-amine (C1), 1-(2-methoxyphenylselanyl)-3-phenylpropan-2-amine (C2), 1,2-bis(2-methoxyphenyl)diselenide (C3), and 1,2-bisp-tolyldiselenide (C4). All the compounds are dissolved in DMSO.

2.4. Tissue preparation

Animals were sacrificed by decapitation. The brain and liver tissues were removed and immediately placed on ice. The tissues were homogenized in Tris–HCl 10 mM and centrifuged for 10 min at 2000 rpm. The supernatant fraction (S1) was collected immediately for the assays.

2.5. Sample preparation

Heparinized venous blood previously obtained from healthy volunteer donors from the Hospital of Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil. The study protocol was reviewed and approved by the appropriate institutional review board following the Guidelines of the Committee of UFSM (0089.0.243.000-07). The erythrocytes were separated by centrifugation (480g for 10 min at room temperature) and the plasma was aspirated. The cell pellet was washed three times with phosphate buffer-saline (6.1 mM and pH 7.4, containing 150 mM NaCl). The leukocytes were separate and utilized in the cell viability analysis.

2.6. Purification of hepatic TrxR

The rat livers were homogenized in buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 – pH 7.3) and centrifuged at 13,000g for 30 min at 4 °C. The supernatant fraction was collected for TrxR isolation and dialyzed against buffered saline for 24 h to remove low molecular weight thiols. The dialysate was heated at 55 °C for 10 min, cooled, and centrifuged at 13,000g for 30 min (Wagner et al., 2010). The supernatant was used for the TrxR assay.

2.7. TBARS assay

The capacity to prevent end products of lipid peroxidation was determined in tissue samples as previously described (Ohkawa et al., 1979). Aliquots of brain and liver supernatants (100 µL of S1) were incubated for 60 min with freshly prepared Fe(II) (10 µM) or SNP (5 µM) in the absence or presence of different concentrations of the compounds C1–C4 (6.25, 12.5, 25, 50 µM) in a

medium containing Tris–HCl buffer 10 mM pH 7.4. The reaction was stopped by the addition of SDS (final concentration of 1.35%), and lipid peroxidation products were measured by the addition of acetic acid/HCl buffer, pH 3.4 and 0.6% TBA, pH 6.0. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm.

2.8. DPPH· radical scavenging method

The radical scavenging activities of the compounds were determined as previously described (Brand-Williams et al., 1995). Each compound was tested at 6.25, 12.5, 25, 50, 100, 200, and 400 μM in 10% DMSO. Seven different concentrations of ascorbic acid (6.25; 12.5; 25; 50; 100; 200; 400 μM) were used as positive controls. DPPH· (diluted in ethanol) was added to final concentration of 0.3 mM and allowed to react at room temperature for 30 min in dark conditions. The absorbance was measured at 518 nm using Spectra Max Plate Reader® M2 (Molecular Devices), Sunnyvale, California, USA.

2.9. Total antioxidant capacity assay

The total antioxidant potential of the mono- and diselenides was evaluated by the phosphomolybdenum method as previously described (Prieto et al., 1999). A sample solution aliquot in ethanol (0.3 ml) was combined in a vial with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate, 3 ml). The compounds were tested a concentration of 400 μM. The vials were capped and incubated in a water bath at 95 °C for 90 min. After cooling the mixture to room temperature, the absorbance was measured at 695 nm against a blank control.

2.10. Thiol-peroxidase-like activity assay

The GPx catalytic activity of mono- and diselenides was evaluated utilizing 10 mM benzenethiol (PhSH) as a substrate, as previously described (Iwaoka and Tomoda, 1994). The H_2O_2 reduction was monitored at 305 nm for 150 s. The compounds were tested at concentrations of 200 and 400 μM. DMSO was used as a negative control (vehicle).

2.11. Thiol-oxidase activity assay

Thiol oxidase activity of 200 and 400 μM concentrations of the compounds (C1–C4) was determined in a medium containing 10 mM Tris/HCl buffer (pH 7.4) and 1 mM glutathione or PhSH. An aliquot of 100 μL was removed at different time points (0, 30, 60 and 120 min) and added to a solution containing 0.5 mM DTNB and 10 mM Tris/HCl buffer (in the absence of thiol oxidation a maximum of 100 nmol of –SH/ml can be found). The absorbance of each sample was measured at 412 nm (Ellman, 1959).

2.12. NADPH oxidation by TrxR using the selenide compounds as substrates

The reduction of mono- and diselenides (15 μM) by rat hepatic TrxR was performed by a modification of the method previously described by Holmgren and Bjornstedt (1995). TrxR was mixed with a medium containing 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, in the presence or absence of selenide compounds and then, the reaction was started by adding NADPH (final concentration 120 μM).

2.13. Fe(II)-chelating assay

The Fe(II)-chelating ability of compounds was determined using a modified method of Puntel et al. (2005). Freshly prepared 500 μmol/L Fe(II) (150 μL) was added to a reaction mixture containing 168 μL of 0.1 mol/L Tris–HCl (pH 7.4), 218 μL saline and the compounds (100 μM). The reaction mixture was incubated for 5 min prior to the addition of 13 μL of 0.25% 1,10-phenanthroline (w/v). The absorbance was then measured at 510 nm in a spectrophotometer.

2.14. Cell viability analysis

The percentages of viable and nonviable leukocytes in samples incubated (90 min) with the compounds (100 μM) were determined by Trypan blue following the method of Mischell and Shiigi (1980). Cell viability was calculated as the number of living cells divided by the total number of cells multiplied by 100 (Mischell and Shiigi, 1980).

2.15. Protein quantification

The protein concentration was estimated by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

2.16. Statistical analysis

Individual dependent variable data were analyzed statistically by one-way (TBARS, DPPH levels, phosphomolybdenum, Fe^{2+}-chelating ability and cell viability) or two-way (thiol peroxidase, thiol oxidase and TrxR activity) analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $p < 0.05$. Data are expressed as means ± SEM and each experimental procedure was performed in at least 4 individual experiments with 3 replicates each. The compound concentration that causes 50% inhibition (IC_{50}) and the maximal inhibition of compounds (I_{max}) was determined by linear regression analysis from 4 individual experiments, using Graph Pad Prism software.

3. Results

3.1. Effect of compounds on lipid peroxidation induced by Fe(II) and SNP in rat brain

We induced lipid peroxidation in rat brain (Fig. 2) homogenates with Fe(II) (10 μM) and SNP (5 μM), and the antioxidant effect of selenium compounds on these homogenates was investigated. C1 had a protective effect against lipid peroxidation at the concentration range (25–50 μM), while the other compounds (C2, C3 and C4) demonstrated a significant effect from the lowest concentration tested (Fig. 2A). In SNP-induced rat brain homogenates, the monoselenides presented a significant antioxidant effect at the concentration range (12.5–50 μM) for C1 and (25–50 μM) for C2, while the diselenides showed a significant effect at 6.25 μM (Fig. 2B).

The IC_{50} values of the compounds followed the order C4 < C3 < C2 < C1 against Fe(II)-induced lipid peroxidation (Table 1). For SNP-induced lipid peroxidation, the IC_{50} values of the compounds followed the order C4 < C3 < C2 < C1 (Table 1).

The I_{max} values of the compounds against Fe(II)-induced lipid peroxidation was 87%, 92%, 93% and 96% respectively of C1 to C4 (Table 3). For SNP-induced lipid peroxidation, the I_{max} values of the compounds was 83%, 90%, 91% and 92% respectively of C1 to C4 (Table 3).

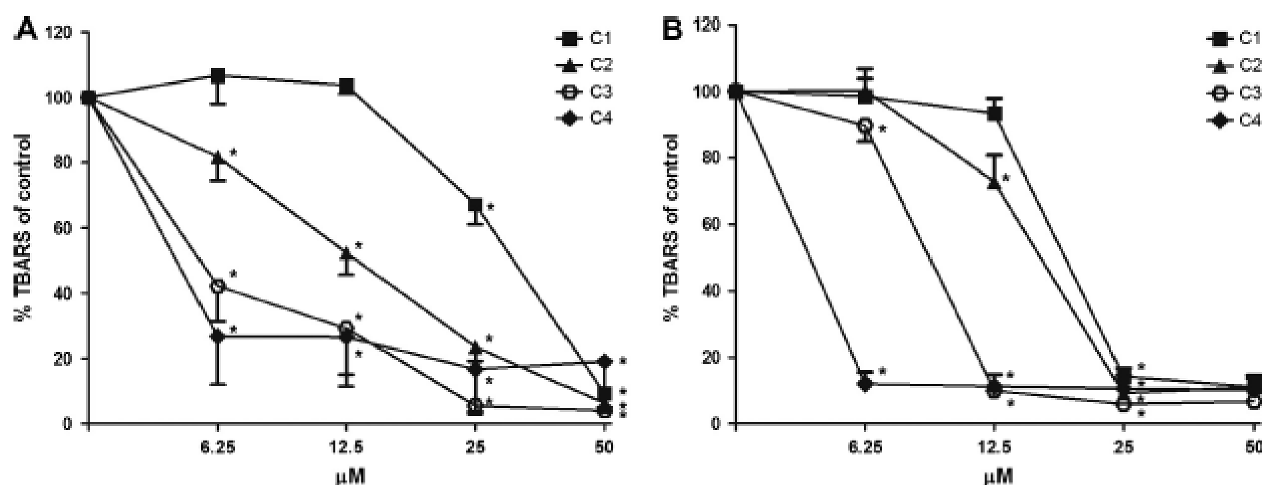

Fig. 2. Effect of the selenium compounds on TBARS production in the brain. The compounds were tested at final concentrations of 6.25, 12.5, 25 and 50 μM, and the results are expressed as a percentage (means ± SEM) relative to the Fe(II)-induced (A) or SNP-induced (B) control. Data is shown as means ± SEM of 4 individual experiments (3 replicates each). * Represents a significant difference when compared with the induced group by Duncan's multiple range test.

Table 1
Calculated IC_{50} values of compounds on lipid peroxidation in rat brain.

Compound	IC_{50} (μM) for Fe(II)	IC_{50} (μM) for SNP
C1	27.67 ± 1.28	20.12 ± 0.44
C2	13.78 ± 2.28	18.77 ± 0.14
C3	5.90 ± 1.32	9.01 ± 0.12
C4	4.73 ± 1.17	3.56 ± 0.15

Data are expressed as mean ± S.E.M and are calculated for fourth independent assays.

Table 2
Calculated IC_{50} values of compounds on lipid peroxidation in rat liver.

Compound	IC_{50} (μM) for Fe(II)	IC_{50} (μM) for SNP
C1	33.71 ± 1.72	38.70 ± 0.93
C2	18.73 ± 1.25	17.26 ± 0.41
C3	28.93 ± 2.05	14.18 ± 0.23
C4	18.23 ± 1.12	3.38 ± 0.21

Data are expressed as mean ± S.E.M and are calculated for fourth independent assays.

3.2. *Effect of compounds on lipid peroxidation induced by Fe(II) and SNP in rat liver*

Rat liver homogenates were induced with Fe(II) or SNP to cause lipid peroxidation, and the effect of selenium compounds on this lipid peroxidation was investigated (Fig. 3). Both the monoselenides and the diselenides decreased the lipid peroxidation induced by Fe(II) at the concentration range (25–50 μM) (Fig. 3A). However, during SNP-induced lipid peroxidation (Fig. 3B), the C1 compound presented a significant effect only at the highest concentration tested, while compounds C2 and C3 had significant effects at the concentration range (25–50 μM). The C4 compound was effective in reducing the lipid peroxidation at the lowest concentration tested.

The IC_{50} values of the compounds followed the order: C4 < C2 < C3 < C1 against Fe(II)-induced lipid peroxidation (Table 2). For SNP-induced lipid peroxidation, the IC_{50} values of the compounds followed the order: C4 < C3 < C2 < C1 (Table 2).

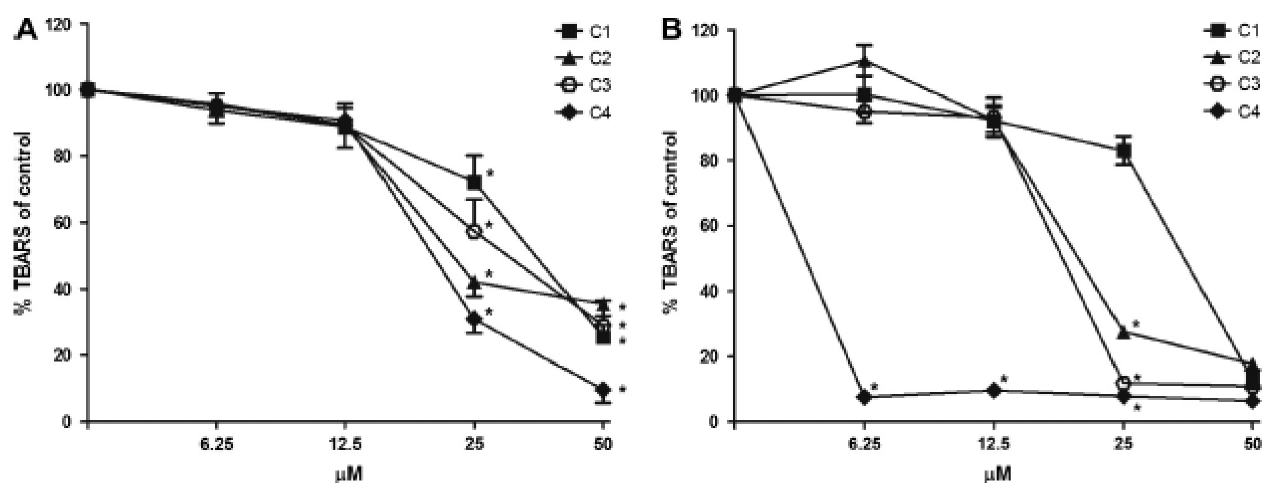

Fig. 3. Effect of the selenium compounds on TBARS production in the liver. The compounds were tested at final concentrations of 6.25, 12.5, 25 and 50 μM, and the results are expressed as a percentage (means ± SEM relative to the Fe(II)-induced (A) or SNP-induced (B) control. Data is shown as means ± SEM of 4 individual experiments (3 replicates each). * Represents a significant difference when compared with the induced group by Duncan's multiple range test.

Table 3
Calculated I_{max} values of compounds on lipid peroxidation in rat brain.

Compound	I_{max} (%) for Fe(II)	I_{max} (%) for SNP
C1	87.11 ± 5.1	83.03 ± 2.1
C2	92.66 ± 2.9	90.99 ± 1.1
C3	93.03 ± 3.2	91.01 ± 2.3
C4	96.37 ± 2.3	92.32 ± 2.6

Data are expressed as mean ± S.E.M and are calculated for fourth independent assays.

Table 4
Calculated Imax values of compounds on lipid peroxidation in rat liver.

Compound	I_{max} (%) for Fe(II)	I_{max} (%) for SNP
C1	67.18 ± 1.9	69.94 ± 3.4
C2	81.97 ± 1.7	79.63 ± 3.1
C3	72.29 ± 2.2	89.33 ± 2.8
C4	90.39 ± 2.1	93.21 ± 1.6

Data are expressed as mean ± S.E.M and are calculated for fourth independent assays.

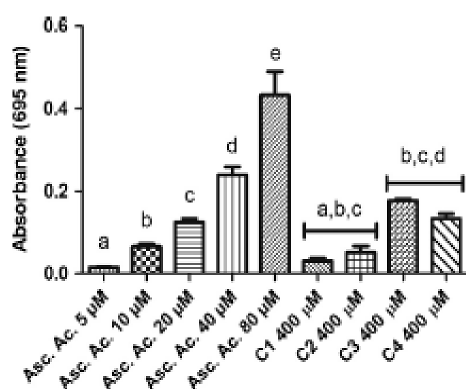

Fig. 4. Total antioxidant activity of the monoselenides and diselenides were measured by the phosphomolybdenum assay. The compounds (400 µM) were incubated with 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate for 90 min: (a–e) represent the effect of ascorbic acid. Values are expressed as absorbance means ± SEM of 4 individual experiments (3 replicates each).

The I_{max} values of the compounds against Fe(II)-induced lipid peroxidation was 67%, 81%, 72% and 90% respectively of C1 to C4 (Table 4). For SNP-induced lipid peroxidation, the I_{max} values of the compounds was 69%, 79%, 89% and 93% respectively of C1 to C4 (Table 4).

3.3. Fe(II)-chelating property, free radical scavenging (DPPH•), thiol-oxidase activity and cell viability

The organoselenium compounds did not show any significant effects in tests involving Fe(II)-chelating properties, free radical scavenging, thiol-oxidase activities and cellular viability (data not shown).

3.4. Total antioxidant activity

The curve of ascorbic acid was determine utilizing the concentration 5, 10, 20, 40 and 80 µM represented at Fig. 4 as the letters a–e. The diselenides at 400 µM showed total antioxidant activity similar to ascorbic acid at 10, 20 and 40 µM. Similarly, the monoselenides at 400 µM demonstrated an antioxidant effect equivalent to that of ascorbic acid at 5, 10 and 20 µM.

3.5. Determination of thiol-peroxidase-like activity

Fig. 5 demonstrates the GPx activity of the organoselenium compounds. The compounds C1 (Fig. 5A) and C2 (Fig. 5B) did not present any significant GPx activity when compared with the control group. DMSO alone had no significant effect on the GPx activity. However, our data reveals that DPDS, C3 (Fig. 5C) and C4 analogs (Fig. 5D) at both concentrations tested demonstrated GPx-like activity.

3.6. NADPH oxidation by TrxR using C1–C4 as substrates

The monoselenides did not show TrxR activity, while the diselenides demonstrated a significant difference compared to the control group. As shown in Fig. 6, C3 and C4 demonstrated 13 and 7 times higher TrxR activity, respectively, than the control.

4. Discussion

The present study aimed to investigate and clarify the antioxidant properties of novel mono- and diselenides compounds. Oxidative stress is involved in various metabolic disorders and in the normal process of aging (Giles et al., 2012; Mugesh et al., 2001). Additionally, antioxidant therapy has been used in an attempt to repair these harmful effects (Nogueira and Rocha, 2011; Zadra et al., 2012). In this context, lipid peroxidation products MDA and 4-hydroxynonenal have been shown to play significant roles in brain and liver toxicities and can serve as markers of oxidative damage (Chen et al., 2005). Prestes reported that monoselenides, which possess an amino group near the selenium, exhibited decreased MDA formation compared to that found for DPDS (Prestes et al., 2012). The novel mono- and diselenides compounds examined in our study demonstrated antioxidant activity against Fe (II)- and SNP-induced lipid peroxidation in rat brain and liver homogenates. We also showed that the β-selenoamines had a similar antioxidant effect as the diselenides in rat liver homogenates following Fe(II)-induced oxidation. The antioxidant effect on lipid peroxidation demonstrated by the diselenide compounds was more pronounced than that of the monoselenide compounds. These results support the assumption that the presence of the amino group decreases selenol formation.

Additionally, using a total antioxidant activity assay, we demonstrated that the diselenides presented a greater antioxidant activity than the monoselenides when compared with equivalents of ascorbic acid. The presence of an amino group in the structure of organoselenium compounds was shown to reduce their antioxidant activity (Sabir et al., 2012). Conversely, the inclusion of a methyl and a methoxy group in the diselenides C3 and C4 does not interfere in the antioxidant activity and most likely maintains the formation of the two selenol structures.

Similarly, the effect of antioxidant compounds on DPPH radical scavenging is involved with their capacity to donate a hydrogen atom. Ogunmoyole et al. reported that DPDS had no significant effect on ability to decolorize the DPPH•, and Prestes et al. reported that β-selenoamines had negligible antioxidant properties in the DPPH assay (Ogunmoyole et al., 2009; Prestes et al., 2012). Thus, in the present study, we also demonstrated that the novel mono- and diselenides did not present any scavenger effects on DPPH radicals, suggesting that the antioxidant mechanism of action of mono- and diselenides may not be related to their ability to donate an electron or hydrogen radical.

Similarly, reducing power is related to the mechanism by which antioxidant agents transfer an electron or hydrogen atom to oxidants or free radicals (Ogunmoyole et al., 2009). Thus, it is possible to assert that the compounds tested in the Fe(II)-chelating assay

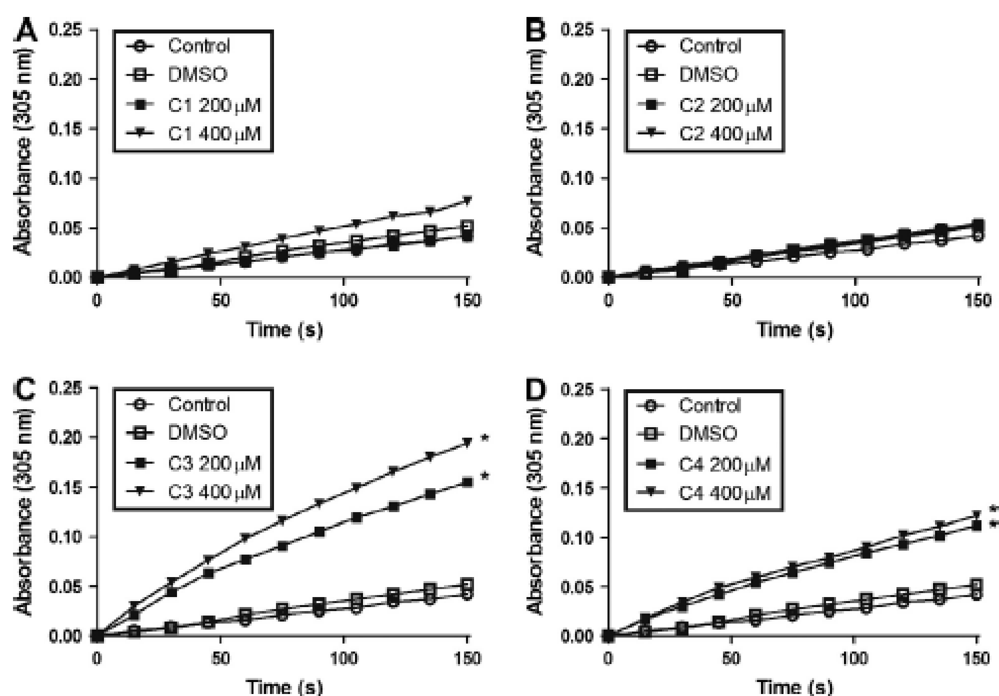

Fig. 5. Determination of thiol-peroxidase-like activity of the organoselenium compounds C1 (A), C2 (B), C3 (C) and C4 (D) at 200 and 400 μM. * Significant difference when compared with the control group by Duncan's multiple range test. Standard error values were omitted for the sake of clarity, and they were <5% of the respective means for 5 individual experiments (3 replicates each).

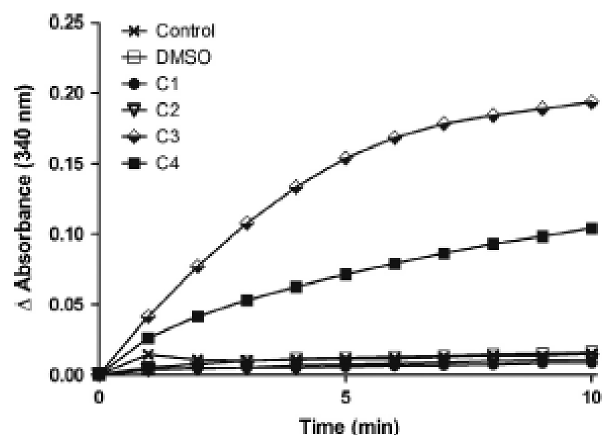

Fig. 6. TrxR activity of the compounds. * Significant difference when compared with the control group by Duncan's multiple range test. Standard error values were omitted for the sake of clarity, and they were <5% of the respective means for 5 individual experiments (3 replicates each).

did not generate significant results due to their inability to donate electron or hydrogen atoms.

Studies in the literature report that organoselenium compounds can cause several toxic effects. These effects are associated with the catalytic oxidation of thiol groups from GSH or from different proteins or enzymes (Meotti et al., 2003; Nogueira et al., 2003a,b). Thus, thiol group oxidation might cause enzyme activity inhibition and can contribute to cellular toxicity (Nogueira and Rocha, 2010). Santos suggested that organochalcogens exhibit hemolytic and genotoxic actions in blood cells, which are most likely linked to their thiol oxidase activity and preferential interaction with sulfhydryl groups critical to enzyme function (Santos et al., 2009). However, when we tested the novel mono- and diselenides, we did not observe any toxic effects in the cellular viability of human leukocytes. Similarly, the compounds examined in this study showed no significant difference in the thiol oxidase activity when compared with the basal group. In agreement with these observations, we propose that the novel mono- and diselenides compounds have a low toxic potential because they did not demonstrate any genotoxic effects and did not interact with sulfhydryl groups in the same way as the classic organochalcogens.

Furthermore, we showed that the novel diselenides demonstrated mimetic GPx-like activity as well as increased TrxR activity when analyzed in vitro. The GPx enzyme neutralizes the toxic or signaling effects of hydrogen and lipid peroxides (Arthur, 2000), which is consistent with the fact that the novel diselenides, by having GPx-like activity, also had a significant inhibitory effect on lipid peroxidation in brain and liver homogenates.

Similarly, TrxR exhibits a broad substrate specificity and can therefore reduce many low molecular weight compounds, including hydrogen peroxide and lipid hydroperoxides (Li et al., 2008). Thus, according to the results obtained for the diselenides, it is possible that increased TrxR activity can be associated with a lipid peroxidation inhibitory effect.

Therefore, we hypothesize that the effects presented in this study for the C3 and C4 compounds, the GPx mimetic effect, and the increased TrxR activity should most likely be attributed to the formation of selenol groups, such as p-methyl-selenol and o-methoxy-selenol.

However, the presence of the basic amino acid inclusion in the monoselenides did not allow the formation of selenol groups, which explains the lack of GPx and TrxR activity. Therefore, the monoselenide effects obtained in the TBARS assay as well as the total antioxidant capacity may simply be due to the nucleophilicity of the amino group near the selenium (Hassan et al., 2012).

In conclusion, structural additions made in classical organoselenium compounds allow the elucidation of antioxidant mechanisms involved in these compounds, enabling the discovery of new drugs. We observed that the inclusion of the amino group in the monosel-

enides resulted in an antioxidant effect, but this effect was not as significant as that observed for the diselenides, which most likely have a higher antioxidant effect due to the formation of selenol groups, as well as their mimetic GPx activity and their elevated TrxR activity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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4 CONCLUSÃO

Os resultados apresentados nesta dissertação permitem sugerir por qual mecanismo os novos compostos mono e disselenetos apresentaram suas atividades antioxidantes.

Os monosselenetos (C1 e C2) apresentaram uma atividade antioxidante significativa nos ensaios do TBARS e da capacidade antioxidante total, provavelmente devido à nucleofilicidade decorrente da presença do grupamento amino próximo ao elemento selênio na estrutura química destes compostos. No entanto, os monosselenetos não apresentaram qualquer atividade GPx e TrxR, justamente porque o grupamento amino pode ter interferido na formação do grupamento selenol.

Os resultados obtidos com compostos análogos ao DPDS (C3 e C4) também foram significativos para o ensaio do TBARS e da capacidade antioxidante total. Da mesma forma, que ambos apresentaram atividade mimética as enzimas TrxR e GPx supostamente devido a formação dos grupamentos p-metil-selenol e o-metoxi-selenol.

5 PERSPECTIVAS

Tendo em vista os resultados obtidos com esse trabalho, as perspectivas para trabalhos posteriores são:

- Investigar as propriedades destes novos compostos orgânicos de selênio em mitocôndrias isoladas de fígado de ratos;
- Avaliar o efeito comportamental e bioquímico de animais submetidos ao tratamento com estes compostos, com a finalidade de identificar a toxicidade.
- Realizar estudos de avaliação destes compostos sobre a atuação como reversores do dano hepático causado por paracetamol e tioacetamida.
- Realizar estudos toxicológicos com modelos alternativos tais como Caenorhabdits elegans.

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