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EFEITO HEPATOPROTETOR CAUSADO PELO 3-ALQUINIL SELENOFENO CONTRA O DANO OXIDATIVO INDUZIDO POR AGENTES QUÍMICOS EM RATOS

DISSERTAÇÃO DE MESTRADO

Ethel Antunes Wilhelm

Santa Maria, RS, Brasil
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EFEITO HEPATOPROTETOR CAUSADO PELO 3-ALQUINIL SELENOFENO CONTRA O DANO OXIDATIVO INDUZIDO POR AGENTES QUÍMICOS EM RATOS

por

Ethel Antunes Wilhelm

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 parcial para a obtenção do grau de
Mestre em Bioquímica Toxicológica.

Orientadora: Prof[a]. Dr[a]. Lucielli Savegnago
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Santa Maria, RS, Brasil

Universidade Federal de Santa Maria
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EFEITO HEPATOPROTETOR CAUSADO PELO 3-ALQUINIL SELENOFENO CONTRA O DANO OXIDATIVO INDUZIDO POR AGENTES QUÍMICOS EM RATOS

Elaborada por **Ethel Antunes Wilhelm** como requisito parcial para a obtenção do grau de **Mestre em Bioquímica Toxicológica**

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Santa Maria, fevereiro de 2009.

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RESUMO

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

EFEITO HEPATOPROTETOR CAUSADO PELO 3-ALQUINIL SELENOFENO CONTRA O DANO OXIDATIVO INDUZIDO POR AGENTES QUÍMICOS EM RATOS

AUTOR: Ethel Antunes Wilhelm
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DATA E LOCAL DA DEFESA: Santa Maria, fevereiro de 2009.

O fígado apresenta extraordinária pluralidade funcional, destacando-se no controle de produção de energia, defesa imunológica e reserva volêmica. No meio ambiente e ocupacionalmente, o ser humano está exposto a uma variedade de compostos hepatotóxicos, como por exemplo, no uso de tintas e seus derivados (2-nitropropano, 2-NP), reagentes químicos (tetracloreto de carbono, CCl_4) e na exposição ao cigarro (2-NP). Portanto, é interessante o estudo de terapias que previnam ou até mesmo revertam a intoxicação causada por estes compostos. Considerando que as espécies reativas de oxigênio (EROs) apresentam importante papel sobre diversas patologias, em especial nas doenças hepáticas, o uso de terapias antioxidantes deve ser considerada. Neste contexto, destacam-se os compostos heterocíclicos contendo selênio em sua estrutura. Deste modo, neste estudo investigou-se a atividade antioxidante de 3-alquinil selenofenos em modelos de dano oxidativo *in vitro* e *ex vivo* em ratos (Wistar, machos, pesando entre 200 – 300 g). Para esse fim, testou-se uma classe de compostos 3-alquinil selenofeno, com diferentes substituições na estrutura química, com o objetivo de avaliar o perfil antioxidante e seu possível efeito tóxico *in vitro* em ratos. Como resultado, 3-alquinil selenofenos tiveram atividade antioxidante, porém esta atividade foi dependente da presença de um alquino terminal na molécula ou da fácil conversão da molécula a um alquino terminal. Além disso, o possível efeito tóxico dos 3-alquinil selenofenos foi avaliado através da atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D) *in vitro*. Os resultados obtidos demonstraram que nenhum dos 3-alquinil selenofenos testados inibiu a atividade desta enzima, sugerindo que esta classe de compostos não apresentou toxicidade sobre a atividade da δ-ALA-D. A partir destes resultados, selecionou-se o selenofeno **h** (que obteve melhor atividade antioxidante *in vitro*) para a avaliação do seu efeito protetor contra o dano oxidativo induzido por 2-NP e CCl_4 em ratos (*ex vivo*). O selenofeno **h** (25 mg/kg) protegeu contra o aumento dos marcadores de dano hepático (aspartato aminotranferase (AST) e alanina aminotransferase (ALT)) e de estresse oxidativo induzidos pela administração do 2-NP. O 2-NP induziu alterações microscópicas avaliadas por inspeções histopatológicas as quais foram protegidas pelo composto. O selenofeno **h** demonstrou efeito protetor contra o aumento da peroxidação lipídica e inibição da atividade da δ-ALA-D nos animais tratados com 2-NP. Além disso, o selenofeno **h** protegeu contra o dano oxidativo induzido pelo CCl_4 em ratos. Uma única dose de CCl_4 causou significante hepatotoxicidade, evidenciada por elevação da atividade plasmática das enzimas AST e ALT, aumento da incidência de lesões histopatológicas, aumento dos níveis de peroxidação lipídica e da atividade da enzima glutationa-S-transferase (GST), bem como diminuição dos níveis de ácido ascórbico e da atividade das enzimas catalase e δ-ALA-D. A partir dos resultados demonstrados, verificou-se que o selenofeno **h** protegeu contra todas estas alterações, confirmando o seu efeito hepatoprotetor. Considerando os resultados obtidos, pode-se sugerir que o

selenofeno **h**, uma molécula com atividade antioxidante, pode ser uma útil terapia contra o dano oxidativo induzido pelos hepatotoxicantes: 2-NP e CCl_4.

Palavras-chave: dano hepático, selênio, 3-alquinil selenofeno, tetracloreto de carbono, 2-nitropropano.

ABSTRACT

Dissertation of Master's Degree
Federal University of Santa Maria, RS, Brazil

HEPATOPROTECTIVE EFFECT OF 3-ALKYNYL SELENOPHENE AGAINST OXIDATIVE DAMAGE INDUCED BY CHEMICAL INDUCTORS IN RATS

AUTHOR: Ethel Antunes Wilhelm
ADVISOR: Lucielli Savegnago
DATE AND PLACE OF THE DEFENSE: Santa Maria, 2009.

The liver presents extraordinary functional diversity, particularly in the control of energy production, immune defense and volemic reserve. The human being is exposed occupationally and in the environment to a variety of hepatotoxic compounds, such as the use of paints and their derivatives (2-nitropropane, 2-NP), chemical reagents (carbon tetrachloride, CCl_4) and exposure to cigarette (2-NP). Therefore, it is interesting the study of therapies to prevent or even reverse the poisoning caused by these compounds. Considering that reactive oxygen species (ROS) have an important role in various diseases, especially in liver diseases, the use of antioxidant therapies should be considered. In this context, the heterocyclic compounds containing selenium in their structures have attracted the attention of researchers. Thus, this study investigated the antioxidant activity of 3-alkynyl selenophenes in models of oxidative damage *in vitro* and *ex vivo* in rats (Wistar, male, weighing 200-300g). A class of 3-alkynyl selenophene compounds with different substitutions was tested, with the objective to assess their antioxidant profile and their possible toxic effect *in vitro*. As a result, 3-alkynyl selenophenes had antioxidant activity, but this activity was dependent on the presence of terminal alkynes in the molecule or easy conversion to it. The possible toxic effect of 3-alkynyl selenophenes was evaluated through the activity of the enzyme δ-aminolevulinate dehydratase (δ-ALA-D) *in vitro*. The results showed that none of 3-alkynyl selenophenes inhibited the activity of this enzyme, suggesting that this class of compound did not present toxicity on this enzyme. From these results, selenophene **h** (compound that had the best antioxidant activity *in vitro*) was selected for the evaluation of its protective effect against oxidative damage induced by 2-NP and CCl_4 (*ex vivo*). Selenophene **h** (25 mg/kg) protected against the increase of markers of liver damage (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities) and oxidative stress induced by administration of 2-NP in rats. 2-NP induced microscopic changes, evaluated by histopathological inspections, that were protected by this compound. Selenophene **h** showed a protective effect against the increase of lipid peroxidation and inhibition of activity of δ-ALA-D in animals treated with 2-NP. Selenophene **h** protected against oxidative damage induced by CCl_4 in rats. A single dose of CCl_4 caused significant hepatotoxicity, evidenced by elevated plasma enzyme activity of AST and ALT, increased incidence of histopathological lesions, increased lipid peroxidation levels and the activity of Glutathione-S-transferase (GST), decreased levels of ascorbic acid and the activity of catalase and δ-ALA-D. In conclusion, 3-alkynyl selenophene protected from all these changes, confirming its hepatoprotective effect. Considering the results, we suggest that 3-alkynyl selenophene, an antioxidant, may be a useful therapy for the oxidative damage induced by 2-NP or CCl_4.

Keywords: liver damage, selenium, 3-alkynyl selenophene, carbon tetrachloride, 2-nitropropane.

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

MANUSCRITO 1

Figura 2: Photomicrography of segment of the hepatic lobe (A) of an animal control; note the hepatic tissue with normal aspect, (B) of animal treated with selenophene **h** 100 mg/kg; observe the hepatocyte strings, centrillobular vein and sinusoid capillaries with normal aspect, (C) of animal treated with 2-NP; note intense infiltration of inflammatory cells (arrow-head) in the hepatic tissue and the loss of cellular architecture (*), (D) of animal treated with selenophene **h** 25 mg/kg+2NP; note the reduction of infiltration of inflammatory cells (arrow-head) in the hepatic tissue with normal appearance. Hepatocyte strings (arrow); Centrillobular vein (CV); Sinusoid capillaries (sc). Both H.E. 100X. 67

MANUSCRITO 2

Figure 1: Photomicrography of segment of the hepatic lobe (**A**) of a control animal. Note the hepatocyte strings (arrow), the centrilobular vein (CV) and sinusoid capillaries (*) with normal aspect, (**B**) of an animal treated with CCl_4, note intense ballooning degeneration (arrow) and (*) infiltration of inflammatory cells, (**C**) of an animal treated with selenophene **h** (50 mg/kg), note the hepatocyte strings (arrow), the centrilobular vein and some inflammatory cells (*), (**D**) of an animal treated with CCl_4 + 25 mg/kg; observe around the centrilobular vein some hepatocytes with vacuolation (head-arrows), ballooning degeneration (arrow) and the presence of inflammatory cells (*) in the sinusoid capillaries, (**E**) of an animal treated with CCl_4 + 50 mg/kg. Observe the hepatocyte strings (arrow), the centrilobular vein and sinusoid capillaries (*) with normal aspect. H.E. 100X. 92

LISTA DE TABELAS

LISTA DE ESQUEMAS

LISTA DE ABREVIATURAS

δ-ALA-D - delta-aminolevulinato desidratase ou porfobilinogênio sintase

$(PhSe)_2$ – disseleneto de difenila

2-NP - 2-nitropropano

ALT – alanina aminotransferase

ANOVA – análise de variância

AST – aspartato aminotransferase

ATP – Adenosina trifosfato

AZT - azidovudina

CAT – catalase

CYP-450 - sistema P-450

DL_{50} - dose letal para 50 % dos animais

ERNs - espécies reativas de nitrogênio

EROs - espécies reativas de oxigênio

GST – glutationa S-transferase

i.p. – intraperitoneal

MDA - malondialdeído

R-SeH – selenóis

R-SH – tióis

S.D – standard deviation

S.E.M – standard error of the mean

S1 – sobrenadante

TBARS – espécies reativas ao ácido tiobarbitúrico

v.o. – via oral

SUMÁRIO

1. INTRODUÇÃO

O estresse oxidativo corresponde a uma excessiva formação endógena de espécies reativas de oxigênio (EROs) associada a uma diminuição nas defesas antioxidantes. As EROs podem induzir um grande número de alterações nos constituintes celulares, incluindo inativação de enzimas, danos às bases nitrogenadas dos ácidos nucléicos e às proteínas, além de peroxidação dos lipídios de membrana (Ha et al., 2006). O conceito formulado por Sies (1997) define estresse oxidativo como sendo um desequilíbrio entre a produção de agentes oxidantes e antioxidantes, em favor dos oxidantes, com potencial para ocasionar dano celular. Nos últimos anos, evidências têm demonstrado o papel central exercido pelas EROs em um variado número de reações biológicas fundamentais, sugerindo que muitas doenças e processos degenerativos podem estar associados com a superprodução das EROs (Young e Woodside, 2001).

Dentre essas doenças, cabe salientar as hepáticas, as quais são um problema de saúde pública mundial. No meio ambiente e ocupacionalmente, o ser humano está exposto a uma variedade de compostos hepatotóxicos, como por exemplo, no uso de tintas e seus derivados (2-nitropropano, 2-NP), reagentes químicos (tetracloreto de carbono, CCl_4) e na exposição ao cigarro (2-NP). Portanto, é interessante o estudo de terapias que previnam ou até mesmo revertam a toxicidade causada por estes compostos (Henry, 1999; Kalil et al., 2001).

Evidências crescentes relacionam as EROs com a cascata de eventos que regulam o início e a progressão das doenças hepáticas, independentemente do agente que as originou (Loguercio e Frederico, 2003; Vitaglione et al., 2004). Assim, o uso de terapias antioxidantes (Lima et al., 2007), de drogas que interferem no sistema de metabolização (Sistema P-450) do agente hepatotóxico e aumentam a atividade de sistemas enzimáticos de defesa são as opções terapêuticas usadas no estudo da hepatotoxicidade (Weber et al., 2003).

Considerando que as EROs apresentam importante papel sobre diversas patologias, em especial nas doenças hepáticas, é conveniente ressaltar a importância exercida por compostos que apresentam atividade antioxidante. Em vista disso, tem sido alvo de interesse de muitos pesquisadores a busca por novos compostos que possuam

atividade biológica com o mínimo de toxicidade e efeitos adversos (Mugesh et al., 2001; Xu et al., 2006).

Nesse contexto, cabe salientar que 85% dos fármacos disponíveis na medicina moderna são de origem sintética, destes, 62% são compostos heterocíclicos (Barreiro et al., 2001). Os compostos heterocíclicos possuem grande importância, uma vez que muitos processos que sustentam a vida no planeta possuem a participação indispensável destes compostos, os quais estão distribuídos em grande número na natureza. Além disso, de maneira geral, esses compostos apresentam enormes aplicações farmacêuticas, agroquímicas, entre outras (Barreiro et al., 2001).

Um fato que vem reforçar a importância crescente dos compostos heterocíclicos é a notoriedade da aplicação de alguns representantes desta categoria no combate a doenças que invariavelmente levam a morte de milhares de pessoas (Cao et al., 2008; Clercq, 2008).

A azidovudina (Figura 1), mais conhecida como AZT, sem dúvida nenhuma é um dos mais conhecidos anti-HIV (Clercq, 2008), o qual ilustra exemplarmente as afirmações anteriores.

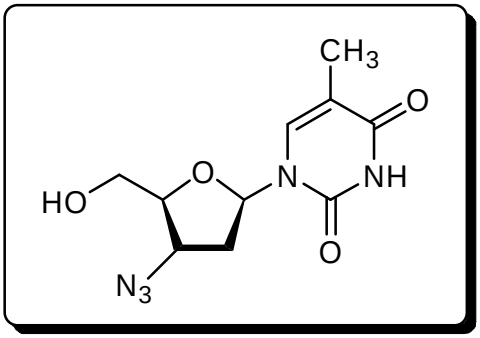

Figura 1. Estrutura química do AZT

Com base na importância crescente da área de síntese de compostos heterocíclicos, um grande número de reações vêm sendo desenvolvidas, e a preparação de novos compostos tem sido objeto de estudos de vários grupos de pesquisa ao redor do mundo (Barluenga et al.,2003; Yue et al., 2006; Arcadi et al., 1999; Alves et al., 2008). Dentre estas inúmeras classes de compostos heterocíclicos que vêm sendo preparadas, os compostos contendo enxofre, selênio e telúrio surgem como uma importante alternativa, que estimula testes bioquímicos ou farmacológicos.

Neste contexto destacam-se os compostos heterocíclicos contendo selênio em sua estrutura (Parnhan e Graf, 1990; Shiah et al., 2007). Entre eles destaca-se o ebselen (2-fenil-1,2-benzilsoselenazol-3(2H)-ona) (Figura 2) o qual exibe atividade catalítica e propriedades antioxidantes similares à glutationa peroxidase (Parnhan e Graf, 1990). Esse composto possui baixa toxicidade, pois ele não libera selênio de sua molécula (Parnhan e Graf, 1987). De fato, Wendel e colaboradores (1984) demonstraram que, em animais deficientes de selênio, a atividade da enzima glutationa peroxidase não aumentava pela suplementação com ebselen.

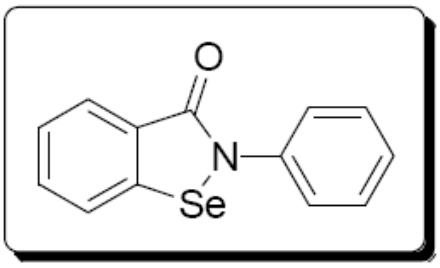

Figura 2: Estrutura química do ebselen

Adicionalmente têm-se o composto denominado D-501036 (Figura 3), um derivado de selenofeno que apresenta atividade anti-tumoral, atuando diretamente na apoptose de células cancerígenas de humanos (Shiah et al., 2007).

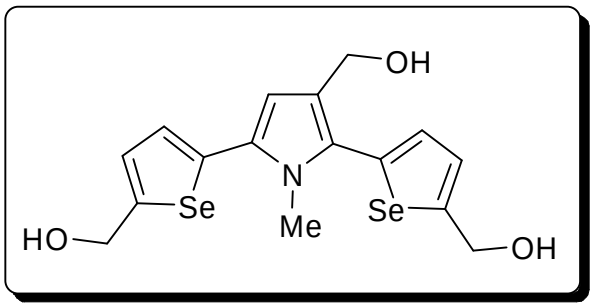

Figura 3: Estrutura química do D-501036

2. OBJETIVOS

Objetivo Geral

Tendo em vista a busca constante por novos fármacos que representem uma alternativa terapêutica no combate e/ou controle de doenças hepáticas e as importantes atividades biológicas já descritas para compostos heterocíclicos contendo selênio, este trabalho visou investigar a atividade antioxidante de 3-alquinil selenofenos em modelos de dano oxidativo *in vitro* e *ex vivo* em ratos.

Objetivos Específicos

Considerando os aspectos já mencionados, os objetivos específicos deste trabalho compreenderam:

- § Selecionar diferentes compostos 3-alquinil selenofenos considerando a atividade antioxidante e o potencial tóxico *in vitro*.
- § Definir a dose do selenofeno **h** (que obteve melhor atividade antioxidante *in vitro*) que não cause toxicidade em ratos *ex vivo*.
- § Investigar o efeito hepatoprotetor do selenofeno **h** sobre o dano oxidativo induzido por 2-NP e CCl_4 em ratos *ex vivo*.

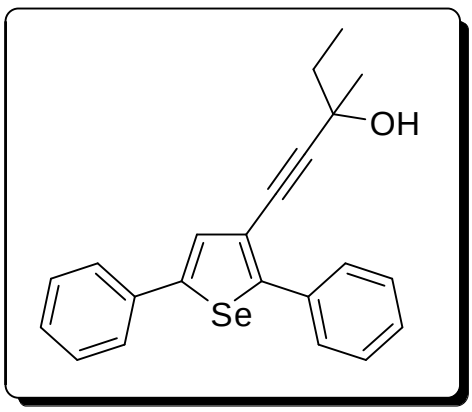

Figure 4: Estrutura química do selenofeno **h**.

3. REVISÃO BIBLIOGRÁFICA

3.1. Fígado

O fígado é um grande órgão, não só no sentido do tamanho como também por ser um órgão de extrema importância para a nossa sobrevivência. O fígado apresenta extraordinária pluralidade funcional, destacando-se no controle de produção de energia, defesa imunológica e reserva volêmica (Kalil et al., 2001). Sua importância no metabolismo e armazenamento de vitaminas, carboidratos, proteínas e lipídeos, bem como na metabolização e excreção de compostos endógenos e exógenos circulantes, torna complexo o estudo de sua fisiologia.

O fígado pesa em torno de 1200g a 1600g no adulto, ou seja, 2% do peso corpóreo e pode executar mais de 500 funções. Localiza-se no quadrante superior direito abdominal. É constituído por milhões de células, chamadas de hepatócitos. A cada célula cabe a produção de diversas substâncias essenciais para o equilíbrio do organismo humano.

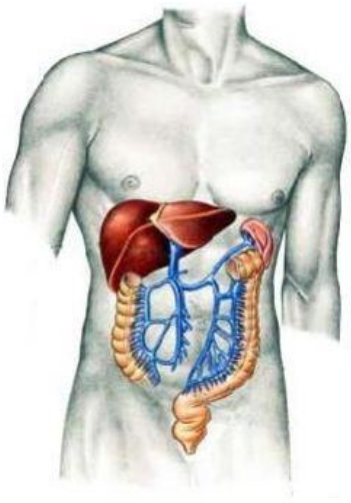

Figura 5.: Localização do fígado no organismo humano.

É um órgão bastante vascularizado, recebendo cerca de 70% do seu sangue proveniente da veia porta e o restante pela artéria hepática. A artéria hepática, uma ramificação da aorta, fornece o sangue com oxigênio ao fígado. A veia porta drena o sangue do sistema digestório (estômago, intestinos, pâncreas e baço) diretamente ao fígado. A importância fisiológica do fluxo sanguíneo portal, é que todas as substâncias provenientes do sistema digestório, com exceção dos lipídeos, passam inicialmente pelo

fígado antes de atingir o sistema circulatório. O fígado possui uma estrutura anatômica única. As células hepáticas estão em contato com a circulação sanguínea de um lado e o canalículo biliar de outro. Deste modo, o hepatócito tem uma grande área de contato tanto com um sistema nutriente proveniente dos sinusóides da veia porta e um sistema de escoamento quanto com o canalículo biliar que transporta as secreções e excreções dos hepatócitos (Motta et al., 2000; Kalil et al., 2001).

Os nutrientes absorvidos pelo intestino chegam ao fígado pela via linfática. No fígado são metabolizados e acumulados. As substâncias tóxicas absorvidas são neutralizadas e eliminadas através da bile. O fígado possui atividade endócrina e exócrina. A fisiologia hepática é altamente especializada no cumprimento de diversas funções conhecidas, tais como: metabólicas, excretoras, secretoras, armazenamento, protetoras, circulatórias e de coagulação sanguínea. Dentre as funções citadas, uma das mais relevantes consiste na função de desintoxicação, através do sistema microssomal de biotransformação de xenobióticos (sistema P-450 ou CYP-450) (Motta et al., 2000; Kalil et al., 2001).

Devido à grande amplitude funcional hepática, este órgão é constantemente exposto a substâncias do meio externo, atuando como órgão alvo de diversos xenobióticos (Motta et al., 2000). Os sinais e sintomas que refletem algum transtorno hepático são: astenia, dor abdominal, náusea, vômito e icterícia. Além destes sintomas, exames laboratoriais são utilizados para confirmar o diagnóstico de doença hepática e sua severidade, sendo que a insuficiência hepática pode levar a falência de outros órgãos como o encéfalo, rins e coração (Kim et al., 1998).

A biotransformação de xenobióticos consiste na conversão de substâncias lipofílicas em substâncias polares, passíveis de excreção. Esse processo de biotransformação é crucial para eliminação de compostos tóxicos. O metabolismo é realizado por enzimas, muitas das quais são específicas e estão localizadas principalmente no retículo endoplasmático. Algumas estão localizadas no citosol e poucas são encontradas em outras organelas como, por exemplo, as mitocôndrias (Timbrell, 1991).

As reações envolvidas na biotransformação dos xenobióticos podem ser agrupadas em duas fases distintas: Fase Pré-Sintética ou Fase I, onde ocorrem reações de oxidação, redução e hidrólise e Fase Sintética, de Conjugação ou Fase II, onde ocorrem reações de conjugação. Na primeira fase, as oxidações são, geralmente, catalisadas por uma classe de enzimas monooxigenases encontradas no retículo

endoplasmático e conhecidas como enzimas microssomais, que são enzimas complexas, inespecíficas (oxidam diferentes tipos de compostos) e que necessitam de NADPH e O_2 para agirem. Durante a oxidação elas exigem uma molécula de oxigênio para cada molécula de fármaco a ser oxidado (um átomo de oxigênio é incorporado ao fármaco ocorrendo a oxidação e outro é, geralmente, combinado com H_2, formando água). A enzima ou sistema enzimático principal na oxidação de xenobióticos é o sistema P-450. Ele recebe os elétrons provenientes de outras fases da reação, se reduz e se liga ao O_2 e ao fármaco, promovendo de fato a oxidação do composto. As reações de fase II são reações de conjugação que envolvem a adição de grupos endógenos aos xenobióticos, os quais geralmente são polares. Os grupos doados nas reações de conjugação incluem derivados de carboidratos, aminoácidos, glutationa e sulfato (Timbrell, 1991; Motta et al., 2000). Esse processo de biotransformação é conhecido como processo de desintoxicação, entretanto em alguns casos podem ser formados metabólitos reativos que são mais tóxicos que os originais. As reações de fase I são as mais comumente envolvidas nesse processo (Timbrell, 1991).

3.1.1. Hepatotoxicidade

As doenças hepáticas são um problema de saúde pública mundial, sendo que a evolução das mesmas inicia-se com a hepatite, esteatose, fibrose, cirrose até o carcinoma hepatocelular (Loguercio e Frederico, 2003; Vitaglione et al., 2004). A confirmação do dano hepático é realizada por meio de exames diagnósticos sorológicos específicos, como a dosagem da atividade enzimática das transaminases (AST e ALT), fosfatase alcalina, γ-glutamil-transferase (Henry et al., 1999). Estas são enzimas presentes em vários tecidos, de função intracelular, e sua presença no sangue é conseqüência de liberação anormal para a circulação. A elevação das transaminases no soro pode ocorrer mesmo após agressão celular mínima. A utilização destes marcadores pode delinear o tipo de dano hepático, sua extensão e o prognóstico da doença hepática. Entretanto, somente as técnicas histopatológicas confirmam e complementam a avaliação do dano, auxiliando também na verificação precoce da efetividade de terapias que protejam e/ou revertam a injúria hepática causada por agentes indutores de dano hepático (Henry et al., 1999).

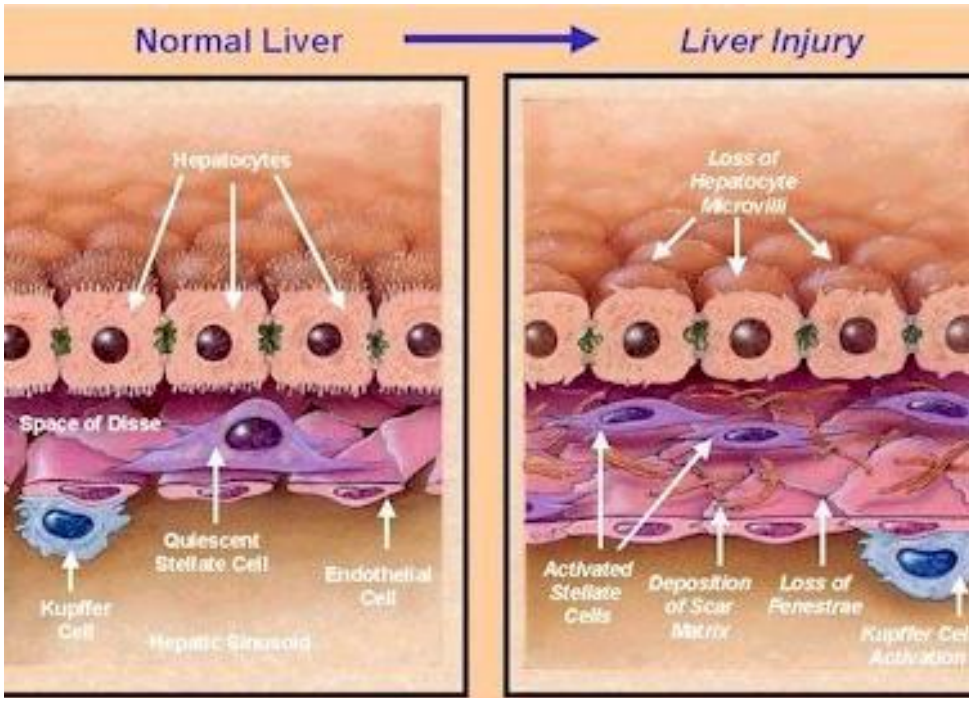

Figura 6: Representação esquemática dos mecanismos da evolução do dano hepático. Space of disse: espaço de Disse; Hepatocytes: hepatócitos; Kupffer cell: célula de Kupffer; Quiescent stellate cell: macrófagos; Endothelial cell: célula endotelial; Hepatic sinusoid: sinusóide hepático; Loss of hepatocyte microvilli: perda das microvisolidades; Activate stellate cells: macrófago ativado; Deposition of scar matrix: depósito de restos celulares; Loss of fenestrae: perda das fenestras; Kupffer cell activation: célula de Kupffer ativada. Adaptado a partir de Gaw et al., 1999.

3.1.2. Indutores químicos de hepatotoxicidade

3.1.2.1. 2-Nitropropano (2-NP)

O 2-NP tem sido amplamente utilizado como um intermediário em reações químicas, como solvente, componente de tintas, vernizes, colas, adesivos, no cigarro e em lavanderias nas lavagens a seco (IARC, 1982). Este composto é altamente hepatotóxico (Zitting et al., 1981), hepatocarcinogênico tanto se inalado (Lewis et al., 1979) como por via oral (Fiala et al., 1987), podendo induzir hepatocarcinoma (Petrelli et al., 1993) e linfomas não-Hodkins ou leucemia em humanos expostos ocupacionalmente a solventes que contenham o 2-NP (Roscher et al., 1990; Robbiano et al., 1991). O 2-NP,

após a administração oral (v.o.) ou intraperitonial (i.p.), muda sua conformação molecular para propano-2-nitronato, com meia-vida de aproximadamente 2 horas, sendo excretado por via pulmonar, renal e fecal (Kohl et al., 1995). O fígado é o orgão alvo da toxicidade do 2-NP, devido ao processo de metabolização pelo sistema P-450 (Ulrich et al., 1978), especificamente pelas isoformas: CYP2B1 e CYP1A2 (Fiala et al., 1987) que favorecem as reações de conjugação mediadas por sulfotransferases e nitroredutases formando os principais metabólitos (N-isopropil hidroxilamina-IPHA e o ácido orto-sulfônico hidroxilamina-HAS) (Figura 7). Estudos demonstraram que esses metabólitos induzem a formação de EROs e de espécies reativas de nitrogênio (ERNs), 8-amino deoxiguanosina, 8-oxiguanosina e malondialdeído (MDA) (Fiala et al., 1989, Fiala et al., 1987; Guo et al., 1990).

O 2-NP também demonstrou ser um substrato para a glutationa S-tranferase (Habig et al., 1974), sendo que estes processos de biotransformação que induzem a formação dos metabólitos descritos anteriormente desequilibram a estrutura das membranas celulares causando peroxidação lipídica (Fiala et al., 1989; Zitting et al., 1981), carcinogênese (Roscher et al., 1990), genotoxicidade (Fiala et al., 1989; Kohl et al., 1995), dano ao DNA (Robbiano et al., 1991) e dano pulmonar e renal (Kim et al., 1998; Guo et al., 1990). As alterações celulares podem ser evidenciadas pela análise histopatológica, a qual demonstra que a administração intraperitoneal de 2-NP induz ao acúmulo de lipídeos no hepatócito, levando a necrose centro-lobular, desgranulação do sistema retículo endotelial e formação de células balonosas, similares às encontradas no tratamento com outros hepatotóxicos (Zitting et al., 1981).

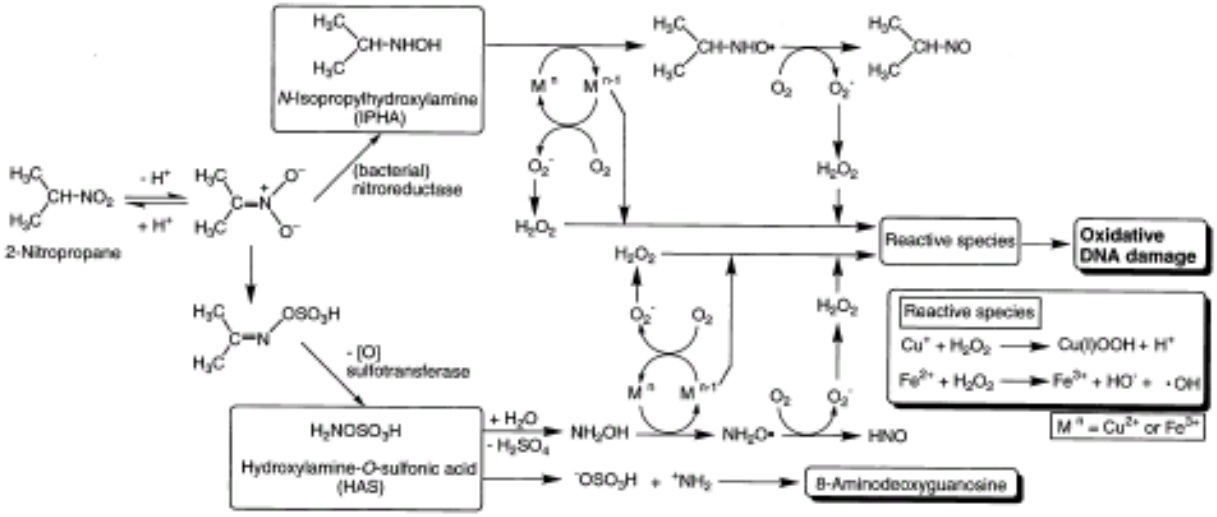

Figura 7: Representação esquemática dos mecanismos de dano oxidativo induzido por 2-NP na presença de metais. Adaptado a partir de Kawanishi et al., 2002.

3.1.2.2. Tetracloreto de carbono (CCl_4)

O CCl_4 também chamado de perclorometano ou tetraclorometano é um líquido volátil, não inflamável com odores distintos e imiscível em água. É um hidrocarboneto halogenado, muito utilizado em processos de síntese orgânica de compostos clorados, particularmente compostos aromáticos halogenados e também na indústria de lavagem a seco, sendo um agente químico altamente tóxico, causando principalmente danos hepáticos e renais (Basu, 2003).

A primeira investigação caracterizando a hepatotoxicidade causada pelo CCl_4 foi descrita por Cameron e Karunarate em 1936. Desde essa data, numerosos estudos têm sido desenvolvidos para uma melhor compreensão dos mecanismos operacionais envolvidos neste processo causado pelo CCl_4 (Brattin et al., 1985; Achliya et al., 2004; Wang et al., 2005). A hepatotoxicidade induzida pelo CCl_4 tem sua gênese em uma reação de desalogenação redutiva catalizada pelo sistema P-450, sendo que o resultado desta biotransformação é a formação de um radical altamente reativo ($CCl_3^{\bullet}$) (Recknagel et al., 1967; 1989; 1991; Lima et al., 2007). Este radical reage com o oxigênio para formar o radical triclorometilperoxil ($CCl_3OO^{\bullet}$)**.** Estes radicais iniciam uma cadeia de

reações que direta ou indiretamente interferem em moléculas celulares importantes (ácidos nucléicos, proteínas, lipídeos e carboidratos) desordenando a fisiologia celular, aumentando a peroxidação lipídica (Recknagel et al., 1967; 1989; Lima et al., 2007), depletando o estoque de glutationa (Recknagel et al., 1989; 1991; Lima et al., 2007) com subsequente dano e/ou morte celular (Weber et al., 2003; Lima et al., 2007) (Figura 8). A bioativação do CCl_4 é predominantemente executada pela isoenzima CYP2E1 (Raucy et al., 1993; Weber et al., 2003), mas em altas doses deste composto, outras isoformas como CYP2B1, CYP2B2 e CYP3A4 são capazes de biotransformar este haloalcano (Weber et al., 2003).

O uso do CCl_4 como intermediário em reações químicas é severamente restrito, devido a sua severa toxicidade (Weber et al., 2003). Entretanto, a utilização deste composto em protocolos experimentais auxilia a elucidar os mecanismos de hepatotoxicidade e suas consequências: inflamação, esteatose, necrose centrolobular, hepatite, fibrose, cirrose e carcinogênese (Lima et al., 2007; Weber et al., 2003; Schatzki, 1963). Além disso, o dano induzido por CCl_4 induz alterações histológicas muito semelhantes às observadas em uma hepatite viral (Weber et al., 2003).

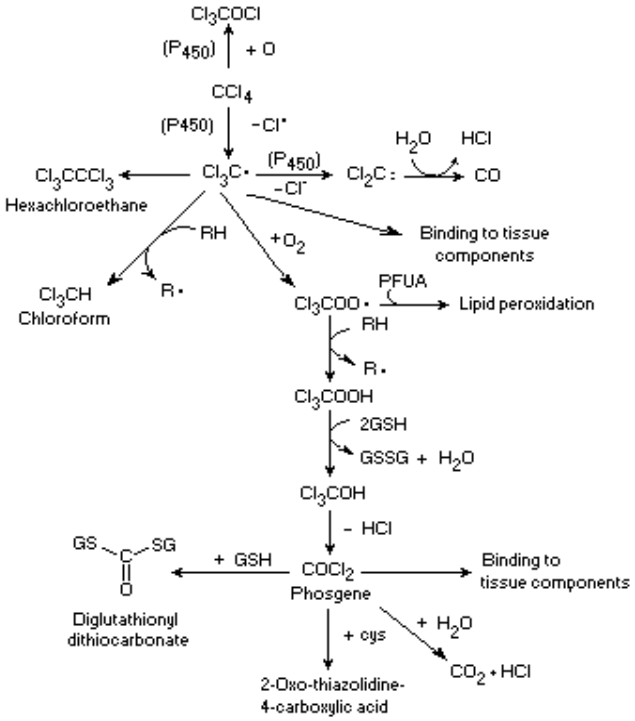

Figura 8: Representação esquemática dos mecanismos de dano oxidativo induzido por tetracloreto de carbono. Adaptado a partir de McGregor e Lang., 1996.

3.2. Estresse Oxidativo

O estresse oxidativo corresponde a uma excessiva formação endógena de EROs associada a uma diminuição nas defesas antioxidantes (Dawson e Dawson, 1996; Halliwel, 1992). As EROs são capazes de gerar estresse oxidativo em conseqüência de suas propriedades oxidantes e reação com os constituintes celulares (Josephy, 1997; Timbrell, 2000). Estas são geradas por uma variedade de processos, podendo atacar uma diversidade de biomoléculas alvo, tais como, DNA, lipídeos e proteínas (Josephy, 1997; Timbrell, 2000).

As membranas biológicas apresentam uma estrutura geral comum. Estas são constituídas de uma bicamada lipídica as quais estão associadas a proteínas. As proteínas presentes na membrana celular são responsáveis pelo transporte de moléculas específicas através da bicamada lipídica. Além disso, essas proteínas podem agir como catalisadoras de reações associadas às membranas, como a síntese de ATP (Alberts et al., 1994). As membranas biológicas são constituídas principalmente por fosfolipídeos, os quais possuem uma cabeça polar e duas caudas hidrofóbicas. Geralmente, as caudas hidrofóbicas são compostas por ácidos graxos, que podem diferir no comprimento e na configuração em que se apresentam, podendo uma das caudas apresentar uma ou mais ligações duplas (insaturações) (Alberts et al., 1994; Halliwell e Gutteridge, 1989). Quando as EROs reagem com esses ácidos graxos insaturados, modificam os lipídeos e a membrana perde suas características arquitetônicas, tornando-se menos firme e menos flexível, criando-se verdadeiras fendas iônicas que alteram sua semipermeabilidade, o que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula, provocando sua ruptura e lise com necrose (Josephy, 1997; Timbrell, 2000).

As principais EROs vinculadas ao estresse oxidativo são: o radical ânion superóxido (O^{-2}), radical hidroxil ($^{\cdot}OH$), peróxido de hidrogênio (H_2O_2), óxido nítrico (NO) e peroxinitrito ($ONOO^{-}$). Estes por sua vez são neutralizados por um elaborado sistema de defesa antioxidante constituído de enzimas tais como a catalase, a superóxido dismutase, a glutationa peroxidase, além de inúmeros sistemas de defesas não-enzimáticas incluindo as vitaminas A, E e C, flavonóides, ubiquinonas e o conteúdo de glutationa reduzida (Alexi et al., 1998; Gianni et al., 2004).

3.2.1. Hepatotoxicidade x Estresse Oxidativo

Recentemente, o estresse oxidativo tem sido sugerido como uma das principais causas de lesão tecidual (Loguercio e Frederico, 2003). Segundo Lee e colaboradores (2001), o estresse oxidativo tem um papel fundamental no início e desenvolvimento das patologias hepáticas. O reconhecimento do envolvimento das EROs em diversas enfermidades tem levado à implementação da terapia antioxidante (Young e Woodside, 2001).

Antioxidantes como α-tocoferol podem bloquear a fibrogênese hepática (Lee et al., 2001). Os antioxidantes são compostos que funcionam como bloqueadores dos processos óxido-redutivos desencadeados pelas EROs. Portanto, funcionam em vários tipos de processos degenerativos. Nutrientes dietéticos com propriedades antioxidantes estão assumindo grande significado no contexto de certas doenças, como a aterosclerose (Bem et al., 2008). Antioxidantes sintéticos têm potencial uso na química, indústria alimentícia e medicina (Packer e Cadenas, 1997). Alguns desses compostos contêm um grupo funcional quimicamente análogo ao de antioxidantes “naturais”, e introduzem novos grupos químicos que aumentam sua amplitude de ação celular ou melhoram sua biodisponibilidade. Por outro lado, outros antioxidantes sintéticos não apresentam analogia estrutural aos naturais, mas exercem alta reatividade para com as EROs e/ou protegem seletivamente alguns tecidos (Packer e Cadenas, 1997).

3.3. Selênio

O selênio (Se) foi descoberto em 1817, pelo químico sueco J. J. Berzelius. O Se é um elemento do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}).

O Se compartilha propriedades químicas e físicas com o enxofre (S). Esta similaridade permite que o Se substitua o S, promovendo interações Se-S nos sistemas biológicos. Por outro lado, as diferenças nas propriedades fisico-químicas entre Se e S constituem a base de seus papéis biológicos específicos (Stadtman,1980). Os selenóis (R-SeH) são as formas correspondentes aos tióis (R-SH), onde ocorre a substituição do átomo de S pelo átomo de Se (Klayman e Günther,1973).

O selênio é um elemento traço essencial, cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz e Foltz, 1957). Nos últimos anos, têm sido descrito que baixos níveis de selênio podem levar à predisposição para o desenvolvimento de algumas doenças, tais como câncer, esclerose, doença cardiovascular, cirrose e diabetes (Navarro-Alarcón e López-Martinez, 2000). Neste contexto, a suplementação de dietas com selênio, tanto para animais quanto para humanos, tem sido aceita pela comunidade científica. Para humanos, a Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propõe uma ingestão diária de 50-200 µg, a qual é considerada segura e saudável para adultos. Por outro lado, sabe-se que a concentração alimentar requerida de selênio é muito próxima da dose que pode ser tóxica (Oldfield, 1987). De fato, estudos demonstraram que altas doses de selênio podem ser citotóxicas, uma vez que possuem a habilidade de oxidar grupos –SH e gerar radicais livres (Barbosa et al. 1998; Nogueira et al. 2004). Este elemento pode ser encontrado nos seguintes alimentos: castanha-do-pará, alho, cebola, brócolis, cogumelos, cereais, pescados, ovos e carnes (Dumont et al., 2006).

Este calcogênio apresenta um grande número de funções biológicas, sendo a mais importante a de antioxidante. Sabe-se que as moléculas contendo selênio, como por exemplo o disseleneto de difenila $(PhSe)_2$, podem ser melhores antioxidantes do que os antioxidantes clássicos (Arteel e Sies, 2001). Já é conhecido que o selênio está presente como resíduo de selenocisteína no sítio ativo das enzimas glutationa peroxidase (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990) e selenoproteína P (Ursini et al., 1990). A atividade redox do selênio tem importância fundamental porque ele faz parte do sítio ativo dessas enzimas.

Nos mamíferos, o selênio parece ser rapidamente absorvido no duodeno, seguido pelo jejuno e íleo. Além do trato gastrointestinal, o selênio pode ser absorvido por tecidos cutâneos e inalação. Estas duas últimas vias de absorção estão relacionadas com a exposição e intoxicação ocupacional por compostos de selênio (Whanger et al., 1976).

Após a absorção, os maiores níveis de selênio estão localizados nos eritrócitos, baço, coração, unha e esmalte de dentes (Martin e Gerlack, 1972). Na intoxicação crônica em animais, o selênio é depositado principalmente nos rins e fígado, seguido pelo pâncreas, baço e pulmões (Wilber, 1980). A primeira evidência de metabolização dos compostos de selênio em animais foi determinada após um longo período de

tratamento com o selenito de sódio. Os animais apresentavam odor gárlico característico, que posteriormente demonstrou-se ter sido causado pelo seleneto de dimetila (Klayman e Gunther, 1973). Este metabólito pode ser resultado do processo de detoxificação do selênio, o qual envolve uma série de metilações dependentes da S-adenosilmetionina (Hoffman e McConnell, 1986).

O selênio pode ser excretado por três vias: urina, fezes e ar expirado. A excreção urinária deste composto pode auxiliar em casos de intoxicações ou de exposições a altos níveis deste elemento (Valentine et al., 1978). Recentemente, foi demonstrado que dentro dos níveis normais de selênio, ou seja, não tóxicos, a principal forma encontrada na urina é como seleno-açúcar. Entretanto, nos casos de doses tóxicas de selênio, o marcador biológico encontrado na urina é o trimetilselenônio (Suzuki et al., 2006). Em indivíduos expostos acidentalmente a altos níveis de selênio, pode ser realizada a detecção do composto volátil seleneto de dimetila (Mozier et al., 1988).

Devido à tentativa crescente de desenvolvimento de compostos que possuam atividades biológicas e aplicações farmacológicas (Parnham e Graf, 1990), têm chamado bastante atenção os compostos orgânicos de selênio (organocalcogênios) com propriedades antioxidantes, que em geral, são inibidores da peroxidação lipídica (Sies, 1993; Kanda et al., 1999).

Durante as últimas décadas, o interesse por esta classe de compostos tem sido intensificado, principalmente devido ao fato de que uma variedade destes compostos possui propriedades farmacológicas (Nogueira et al., 2004). De fato, estudos em animais de laboratório têm demonstrado que estes compostos apresentam propriedades antiúlcera (Savegnago et al., 2006), antiinflamatória e antinociceptiva (Savegnago et al., 2007a), antidepressiva e ansiolítica (Savegnago et al., 2007b), neuroprotetora (Ghisleni et al., 2003), hepatoprotetora (Borges et al., 2005; 2006, Wilhelm et al., 2008), anti-hiperglicêmica (Barbosa et al., 2006) e pode retardar o desenvolvimento de câncer (Barbosa et al., 2008). Além disso, apresenta efeitos antioxidantes em diversos modelos de toxicidade induzida por estresse oxidativo (Meotti et al. 2004; Luchese et al., 2007), incluindo exposições ao cádmio (Santos et al., 2004; 2005; Borges et al., 2008).

3.3.1. Selênio x hepatotoxicidade

A associação da importância do selênio com a hepatoproteção foi demonstrada em meados de 1957, graças a estudos pioneiros desenvolvidos por Schwartz e Foltz, onde foi demonstrado que ratos alimentados com dieta pobre em selênio poderiam desenvolver necrose hepática. Este interessante estudo levou ao reconhecimento que doenças oriundas da privação de nutrientes, poderiam ser causadas por deficiência de selênio na dieta (Oldfield, 1987). Outra pesquisa relevante demonstrou que a administração oral de ebselen, pode inibir as lipoxigenases em um modelo experimental de indução de hepatite pela administração da endotoxina galactosamina (Wendel et al., 1984). De fato o ebselen demonstrou suas propriedades hepatoprotetoras em diversos modelos de dano hepático, tais como os induzidos por paracetamol (Li et al., 1994; Rocha et al., 2005), CCl_4 (Wasser et al., 2001), lipopolissacarídeo e *Propionibacterium acnes* (Koyanagi et al., 2001), etanol (Kono et al., 2001) e isquemia e reperfusão (Ozaki et al., 1997).

Além disso, nosso grupo de pesquisa demonstrou que o disseleneto de difenila apresenta efeito hepatoprotetor contra dano hepático induzido por 2-NP (Borges et al., 2005, 2006; Wilhelm et al., 2008), cádmio (Borges et al., 2008) e mostra-se efetivo contra dano oxidativo induzido por acetaminofen (paracetamol) em ratos (Wilhelm et al., 2009).

3.4. Compostos heterocíclicos

Vários compostos heterocíclicos são fármacos mundialmente consumidos que apresentam atividades farmacológicas diversificadas, tais como, inibidor do HIV (AZT), antitumoral (D-501036); antifúngica (5-(3-buten-1-inil)-2,2'-bitienila); antinflamatória e analgésica (dipirona); antiprotozoária (metronidazol) e antiviral (ribavirina) (Barreiro et al., 2001; Shiah et al., 2007; Juang et al., 2007) (Figura 9).

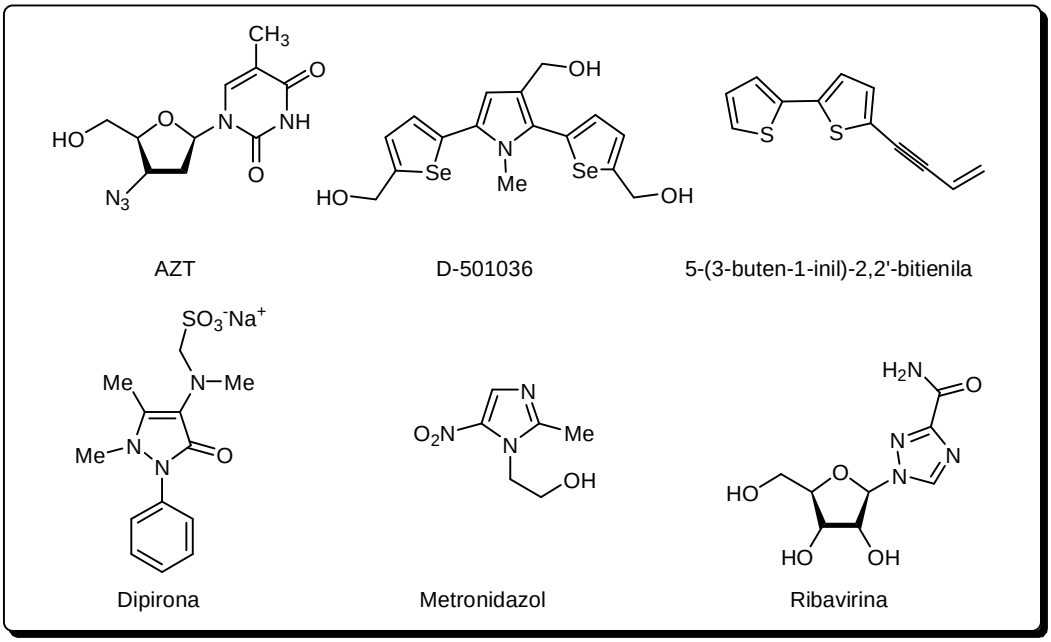

Figura 9. Fármacos contendo unidade heterocíclica

Os heterocíclicos aromáticos de cinco membros contendo átomos de calcogênio pertencem à classe de substâncias denominada genericamente de calcogenofenos, sendo que o mais simples deles é o furano. Também estão inclusos nesta classe o tiofeno, selenofeno e telurofeno (Figura 10). Ainda estão nesta classe os benzo derivados de calcogenofenos, sendo mais comumente encontrados os benzo[b]furanos e benzo[b]tiofenos (Bruice, 2006).

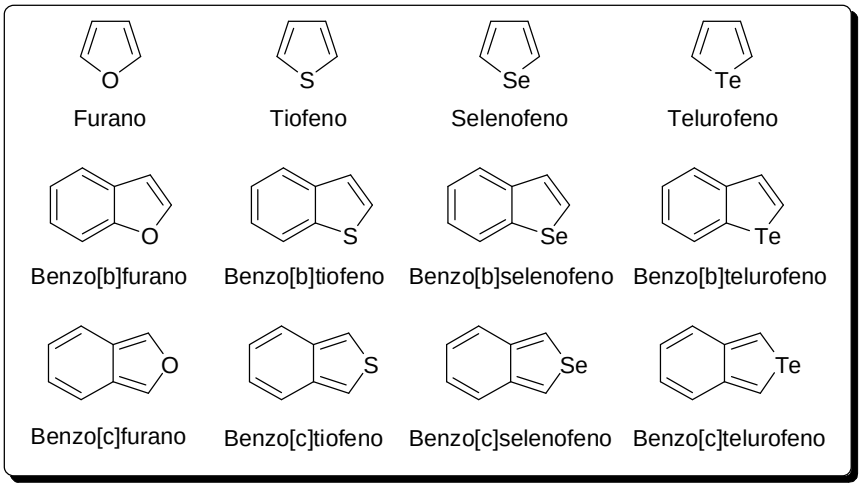

Figura 10. Exemplos de calcogenofenos

Os calcogenofenos são amplamente estudados em vista de seus diversos efeitos biológicos (Chan et al., 1975; Hudson et al., 1989; Gonçalves et al., 2005). Além de sua

atividade atioxidante (Meotti et al., 2004), os calcogenofenos apresentam propriedades antinociceptiva e antiinflamatória (Zeni et al., 2001; Meotti et al., 2003; Gonçales et al., 2005)

Entre os calcogenofenos, furanos, tiofenos e seus derivados têm despertado o interesse de pesquisadores na química orgânica sintética, pois suas ocorrências em produtos naturais que apresentam alguma atividade biológica são relativamente freqüentes, incentivando a procura de metodologias para a síntese destes compostos (Sperry e Wright, 2005; Tachibana et al., 2008; Tran et al., 2008). Os selenofenos, telurofenos e seus derivados vêm recebendo menos atenção da comunidade científica quando comparados com seus análogos tiofenos e furanos. De fato, os telurofenos e selenofenos são escassamente relatados na literatura tanto na área biológica, quanto na área de síntese e reatividade destes compostos. Este fato incentiva estudos que busquem demonstrar possibilidades de síntese bem como a busca de compostos com possíveis atividades biológicas.

3.4.1. Compostos heterocíclicos x Selênio

Uma vez que moléculas contendo selênio podem ser melhores antioxidantes do que os antioxidantes clássicos (Arteel e Sies, 2001), a incorporação do átomo de selênio em moléculas orgânicas permite a preparação de inúmeros compostos, com propriedades farmacológicas bastante amplas.

Neste contexto destacam-se os compostos heterocíclicos contendo selênio em sua estrutura. Como mencionado anteriormente, o ebselen (Figura 2) apresenta importantes atividades biológicas: exibe atividade catalítica e propriedades antioxidantes similares à glutationa peroxidase (Parnhan e Graf, 1990), possui baixa toxicidade (Parnhan e Graf, 1987), reage com grupos tióis, como a glutationa (Ulrich et al., 1996), inibe a peroxidação lipídica (Parnhan e Graf, 1987; Rossato et al., 2002; Nowak et al., 2006), inibe a lipoxigenase (Parnhan e Graf, 1987), bloqueia a produção de ânion superóxido e desempenha um papel protetor contra o peroxinitrito (Masumoto e Sies, 1996). Além disso, o ebselen tem sido usado como antioxidante, como neuroprotetor em culturas de neurônios (Osaki et al., 1997; Takasago et al.,1997; Kondoh et al., 1999; Imai et al., 2001; Porciúncula et al., 2003), no tratamento clínico de pacientes com isquemia aguda (Yamaguchi et al., 1998; Kondoh et al., 1999), em modelos de Parkinson

(Moussaoui et al., 2000) e como antiinflamatório (Parnhan e Graf, 1987; Walther et al., 1999; Haddad et al., 2002; Mugesh et al., 2001).

Outro composto heterocíclico que destaca-se é o D-501036 (Figura 3), um derivado de selenofeno que apresenta atividade anti-tumoral, atuando diretamente na apoptose de células cancerígenas de humanos (Shiah et al., 2007).

4. MANUSCRITOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscritos, os quais se encontram assim organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas encontram-se nos próprios manuscritos. O manuscrito 1 e 2 estão dispostos da mesma forma que foram submetidos para avaliação.

4.1. Selenofeno protege contra dano oxidativo induzido por 2-nitropropano em fígado de ratos.

4.1.1. Manuscrito 1

SELENOPHENE PROTECTS AGAINST OXIDATIVE DAMAGE INDUCED BY 2-NITROPROPANE IN LIVER OF RATS

(Submetido à Journal of Pharmacological Science)

Selenophene Protects Against Oxidative Damage Induced by 2-Nitropropane in Liver of Rats

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Abstract

The aim of this study was the *in vitro* study of the antioxidant function of recently synthesized selenophenes. It was evaluated using iron/EDTA-induced thiobarbituric acid reactive species (TBARS) and δ-aminolevulinate dehydratase (δ-ALA-D) assays. Selenophenes **b**, **c**, **d**, **e**, **f**, and **i** presented poor antioxidant profiles in the TBARS assay (IC_{50} > 400 µM) when compared to **a**, **g** and **h** (IC_{50} = 313, 233 and 237 µM, respectively). Selenophenes **a**, **g** and **h** presented maximal inhibition (Imax) of lipid peroxidation of 75%. The antioxidant activity of selenophenes was dependent of a terminal alkyne bonded directly at 3-position of selenophene. A second objective was to investigate the antioxidant action of selenophene **h**, against oxidative damage induced by 2-nitropropane (2-NP) in liver of rats. Selenophene **h** 25 mg/kg protected against the increase in TBARS levels and in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities induced by 2-NP (100 mg/kg of body weight). Compound **h** 25mg/kg significantly attenuated 2-NP-induced hepatic histopathological alterations. The inhibition of δ-ALA-D activity caused by 2-NP was protected by selenophene **h**. This study proved the antioxidant effect of selenophene **h** at a concentration of 25 mg/kg in a model of oxidative damage induced by 2-NP in rats.

Keywords: selenophene, selenium, antioxidant, oxidative stress, liver.

Introduction

Aerobic life is characterized by a steady formation of pro-oxidants balanced by a similar rate of their consumption by antioxidants. To maintain homeostasis, there is a requirement for the continuous regeneration of antioxidant capacity, and if this is not met, oxidative damage occurs (1). Oxidative stress is characterized by a significantly increased concentration of intracellular oxidizing species, such as reactive oxygen species (ROS) and is often accompanied by the simultaneous loss of antioxidant defense capacity (2). To counteract ROS levels more effectively, exogenous compounds should combine a range of antioxidant activities in one chemically simple molecule (3).

In the last two decades the interest in organoselenium chemistry and biochemistry has increased, mainly due to the fact that these compounds have been described to possess very interesting biological activities (4, 5, 6, 7). Several reports have been published on glutathione peroxidase (GPX)-mimetic compounds, which, like the native enzyme, rely on the redox cycling of selenium (4, 8, 9, 10). In fact, scientists have paid more attention to glutathione peroxidase artificial imitation in view of its instability, poor availability and high molecular weight, which have limited its therapeutic use (11, 12). Ebselen is the best-known mimic of GPX (13). In this context, diphenyl diselenide has also been reported as a good GPX mimic and an antioxidant in different experimental models of oxidative damage (14, 15, 16, 17, 18).

Although the peroxidase-like activity of organoselenium compounds may account for their antioxidant properties, the SH–selenide exchange catalyzed by organochalcogens may contribute to their toxicological properties by oxidizing relevant SH-containing metabolites and proteins without consuming toxic substances such as

peroxides (19). Selenides can react with -SH groups, forming selenosulfide or –SeH and disulfides (19).

Chalcogenophenes, a class of organochalcogen heterocycles containing a five-membered ring in the structure, have drawn the attention of researchers in view of their interesting biological activities (20, 21, 22, 23) including antioxidant properties (24, 25, 26). Among chalcogenophenes, selenophenes play an important role in organic synthesis (27) because of their excellent electrical properties and environmental stability.

Based on the important chemistry and pharmacological properties of organoselenium compounds, the aim of this study was to evaluate the *in vitro* antioxidant activity of recently synthesized selenophenes (27, 28). Considering the results obtained *in vitro*, a second objective of this study was to investigate the antioxidant action of selenophene **h** against the oxidative liver damage induced by 2-nitropropane (2-NP) in rats. 2-NP, a nitroalkane, is known to be an acute hepatotoxicant (29) and a potent hepatocarcinogen in rodents (30, 31). The mechanism by which 2-NP causes toxicity is not completely elucidated, but accumulating evidence suggests that generation of reactive oxygen species via the metabolism of 2-NP-nitronate to acetone and nitrite plays an important role for the carcinogenic effect of 2-NP (32, 33).

Materials and Methods

Chemicals

Selenophenes (**a**-**i**) (Fig. 1) were prepared according to literature methods (27, 28). For *in vitro* experiments, selenophenes were dissolved in dimethylsulphoxide (DMSO). For *ex vivo* experiments, selenophene **h** and 2-nitropropane (2-NP) were dissolved in canola oil.

δ-Aminolevulinic acid (δ-ALA), *p*-dimethylaminobenzaldehyde and 2-nitropropane were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from standard commercial suppliers.

Animals

Male adult Wistar rats, weighing 200-300g, were obtained from a local breeding colony. The animals were kept in separate animal rooms, on a 12 h light/dark cycle, in an air conditioned room (22 ± 2°C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied *ad libitum*. This study was approved by the Ethics and Animal Welfare Committee of Universidade Federal de Santa Maria.

In vitro model

In vitro experiments were carried out to screen selenophenes (**a**-**i**) by using iron/EDTA-induced thiobarbituric acid reactive species (TBARS) levels and δ-aminolevulinate dehydratase (δ-ALA-D) activity. For this end, rats were euthanized and liver tissues were rapidly homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v) and centrifuged at 2,400×*g* for 15 min. The supernatants (S1) were separated and used to determine the effect of different concentrations of selenophenes (**a-i**) in iron/EDTA-induced TBARS and δ-ALA-D activity assays.

Ex vivo model

Considering the *in vitro* results and the reaction conditions, selenophene **h** was chosen to evaluate its antioxidant activity in *ex vivo* experiments. It is important to point out that selenophenes **a**, **g** and **h** had similar IC_{50} and Imax values, but selenophene **h** was the most cheapest selenophene obtained.

In the first set of experiments, selenophene **h** was administered at different doses to rats to find a dose which does not induce toxicity. For these experiments, rats were randomly divided into four groups consisting of five to eight animals each. In group I, canola oil (5 ml/kg of body weight) was administered to rats. In groups II, III and IV rats received selenophene **h** at 25, 50 and 100 mg/kg of body weight, respectively. Selenophene **h** was administered by intragastric gavage as a single oral dose.

In the second set of experiments, selenophene **h** (25 mg/kg) was used to test its antioxidant property against oxidative damage induced by 2-NP in the liver of rats. For these experiments, rats were randomly divided into four groups consisting of five to eight animals each. In group V, rats received two doses of canola oil (5 ml/kg). In group VI, rats received canola oil and 24 h later 2-NP was administered. Animals belonging to group VII were exposed to selenophene **h** and 24 h later received canola oil. In group VIII, rats received selenophene **h** and 24 h later 2-NP was administered. Selenophene **h** (25 mg/kg) and 2-NP (100 mg/kg) were administered to rats as a single oral dose by intragastric gavage. The dosage of 2-NP was based on Borges et al. (16).

Seventy two hours after selenophene **h** administration (one sets of experiments) or twenty-four hours after 2-NP administration (two sets of experiments) all rats were anesthetized for blood collection by heart puncture (hemolyzed plasma was discharged). After this procedure, rats were euthanized and the liver of animals was removed, dissected and kept on ice until the time of assay. The samples of liver were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v), centrifuged at 2,400×*g* for 15 min. The supernatants (S1) were separated and used for biochemical assays. To histological evaluation, at sacrifice, all rats were slightly anesthetized and subjected to a through necropsy evaluation.

Lipid peroxidation

To determine the antioxidant effect of selenophenes (**a-i**), $FeSO_4$ and EDTA were used as classical inductors of lipid peroxidation. An aliquot of 200 µl of homogenate (S1) was added to the reaction mixture containing: 50 µM $FeCl_2$, 100 µM EDTA and selenophenes (**a-i**) at different concentrations (50 - 400 µM). After that, the mixture was pre-incubated for 1 h at 37°C. The reaction product was determined using 500 µl thiobarbituric acid (0.8%), 200 µl SDS (sodium dodecyl sulfate, 8.1%) and 500 µl acetic acid (pH 3.4), after the incubation for 2 h at 95°C. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. TBARS were determined as described by Ohkawa et al. (34) and expressed as nmol equivalents of MDA (malondialdehyde)/mg protein .

In *ex vivo* experiments, an aliquot of S1 (200 µl) from rats belonging to the experimental groups was reacted as described above except for the pre-incubation step.

δ-ALA-D activity

Persuasive evidence has indicated that δ-ALA-D is extremely sensitive to the presence of pro-oxidant agents (35, 36, 37), which oxidize –SH groups essential for the enzyme activity (38, 39). Since this enzyme is very sensitive to organoselenium compounds, δ-ALA-D activity was used as a marker of toxicity.

δ-ALA-D activity was assayed according to the method described by Sassa (40) by measuring the rate of product porphobilinogen (PBG) formation. In *in vitro* experiments, an aliquot of 200 µl of S1 was pre-incubated for 10 min at 37°C in the presence or absence of selenophenes (**a-i**) at different concentrations (50 - 500 µM). The enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.4 mM in a medium containing 100 mM phosphate buffer, pH 6.8 and incubated for 1 h at 37°C.

The reaction product was determined using modified Erlich's reagent at 555 nm. The enzymatic activity was expressed as nmol PBG/mg protein/hour.

In *ex vivo* experiments, an aliquot of S1 (200 μl) from animals belonging to the experimental groups was reacted as described above except for the pre-incubation step.

Ascorbic acid determination

Ascorbic acid determination was performed as described by Jacques-Silva et al. (41). Proteins were precipitated in 10 volumes of a cold 4 % trichloroacetic acid solution. An aliquot of homogenate at a final volume of 1 ml of the solution was incubated for 3 h at 38°C then 1 ml 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 dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount. Ascorbic acid content was expressed as μmol ascorbic acid/g tissue.

Catalase activity

Catalase activity was assayed spectrophotometrically by the method of Aebi (42), which involves monitoring the disappearance of H_2O_2 in the homogenate at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 μl of S1 and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in as UI/mg protein.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity

Plasma enzymes AST and ALT were used as the biochemical markers for the early acute hepatic damage (43), using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

Histopathological analysis

Small pieces of liver tissues from individual rats were fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 4µm and stained with hematoxylin and eosin. To histological evaluation, four animals per group were used.

Protein quantification

Protein concentration was measured by the method of Bradford (44), using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis of *in vitro* data was performed using a one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test when appropriate. IC_{50} (concentration inhibiting 50% of lipid peroxidation) was determined by linear regression from individual experiments using "GraphPad Software". The IC_{50} values were reported as geometric means accompanied by their 95% confidence limits. Maximal inhibition (Imax) values were calculated at the most effective dose used using "GraphPad Software" (GraphPad software, San Diego, CA, USA).

Ex vivo data were analysed by using a one-way analysis of variance (ANOVA) for assays of selenophene **h** toxicity and two-way analysis of variance (ANOVA) (selenophene **h** x 2-NP) for 2-NP induced damage followed by Duncan's Multiple Range Test when appropriate. Main effects are presented only when the second order interaction was non-significant.

All data of *in vitro* and *ex vivo* experiments were expressed as means ± S.D. Values of p<0.05 were considered statistically significant.

Results

Lipid peroxidation

As shown in Table 1, selenophenes **a**, **f**, **g** and **h** reduced iron/EDTA-induced lipid peroxidation at concentrations of 100 µM and greater. Selenophene **b** decreased iron/EDTA-induced lipid peroxidation only at concentrations of 300 and 400 µM. Selenophenes **c**, **d**, **e,** and **i** did not reduce iron/EDTA-induced lipid peroxidation at all concentrations tested.

Selenophenes **b**, **c**, **d**, **e**, **f**, and **i** presented poor antioxidant profiles (IC_{50} > 400µM) when compared to **a**, **g** and **h** that had IC_{50} = 313, 233 and 237 µM, respectively. Selenophenes **a**, **g** and **h** presented maximal inhibition (Imax) of 75%.

Selenophene **h** at 25 mg/kg decreased basal lipid peroxidation levels in rat liver when compared to the control group. At doses of 50 and 100 mg/kg selenophene **h** did not alter basal lipid peroxidation levels (Table 2).

Two-way ANOVA of basal lipid peroxidation levels yielded a significant main effect of 2-NP ($F_{1,20}$ = 33.081; p<0.001) and of selenophene **h** ($F_{1,20}$ = 39.424; p<0.01) (Table 3). Post hoc comparisons showed that 2-NP increased basal TBARS levels (p<0.05) and selenophene **h** significantly decreased the basal levels in liver of rats when compared to the 2-NP group (Table 3).

d- ALA-D activity

Selenophenes **a-i** at different concentrations (50 – 500 µM) did not significantly inhibit hepatic δ-ALA-D activity (data not shown).

δ-ALA-D activity was not altered in the liver of rats which received selenophene **h** 25 and 50 mg/kg. Selenophene **h** 100 mg/kg inhibited the activity of this enzyme when compared to the control group (Table 2).

Two-way ANOVA of δ-ALA-D activity revealed a significant selenophene **h** and 2-NP interaction ($F_{1,22}$ = 15.414; p<0.001). Post hoc comparisons demonstrated that 2-NP inhibited δ-ALA-D activity (p<0.05) and selenophene **h** completely protected δ-ALA-D inhibition induced by 2-NP (p<0.05) (Table 3).

Ascorbic acid

The levels of ascorbic acid were not changed by selenophene **h** (Table 2).

Catalase activity

Administration of selenophene **h** did not change catalase activity in liver (Table 2). A non-significant dose-dependent increase was observed.

2-NP alone did not affect catalase activity (Table 3). Post hoc comparisons showed that selenophene **h** and 2-NP significantly increased hepatic catalase activity (30.66 %)(p<0.05) when compared to the 2-NP group (Table 3) but the enzyme activity was similar to the control group (Table 3).

ALT and AST activities

Selenophene **h** significantly increased plasma ALT and AST activities at 50 and 100 mg/kg when compared to the control group (Table 4). ALT and AST activities were not altered by 25 mg/kg of selenophene **h** when compared to the control group (Table 4).

Two-way ANOVA of plasma ALT activity showed a significant main effect of 2-NP ($F_{1,17}$ = 5.130; p<0.001). Post hoc comparisons revealed that 2-NP increased ALT activity

(234.86 %) *(p*<0.05) compared to the control group. Selenophene **h** significantly reduced 2-NP modulated ALT activity (*p*<0.05) (Table 3).

Two-way ANOVA of plasma AST activity yielded a significant main effect of 2-NP ($F_{1,16}$ = 21.940; *p*<0.001). Post hoc comparisons demonstrated that 2-NP increased AST activity (120.03 %) *(p*<0.05) compared to the control group. In fact, selenophene **h** was effective in completely preventing AST activity increased by 2-NP (*p*<0.05) (Table 3).

Histological examination

The severity of the liver morphological changes induced by 2-NP treatment is shown in Fig. 2C. The liver tissues from rats treated with 2-NP revealed extensive injuries, characterized by intense infiltration of inflammatory cells and loss of cellular architecture (tumefation) (Fig. 2C). Selenophene **h** 25 mg/kg significantly attenuated 2-NP -induced hepatic histopathological alterations. It was observed that selenophene **h** administration at the highest dose (100 mg/kg, p.o.) did not cause appreciable changes in the morphology of liver (Fig. 2B).

Discussion

In this study we reported the antioxidant activity of selenophenes, an important class of organochalcogen compounds. The interest in natural and synthetic antioxidant compounds that could potentially retard the development of diseases has grown considerably in the scientific community in the last decades. Accordingly, data from our research group have demonstrated that organochalcogens presented important pharmacological activities (21, 22, 25, 26, 45).

A closer inspection of the *in vitro* results revealed that the antioxidant activity was sensitive to substitution on the selenophene ring. Furthermore, literature data have indicated that the chemical structure of organochalcogens has an important role in establishing their antioxidant activity (46). Therefore, selenophene derivatives containing R= C_6H_5 - selenophene **c**; R= C_5H_{11} - selenophene **d**; R= $CCH_2OCH_2CH_3$ - selenophene **e** as substitution and with no substitutent on the selenophene ring - selenophene **i** exhibited poor antioxidant activity in the TBARS assay (IC_{50} > 400 µM). In fact, selenophene **i** did not exert antioxidant effects on lipid peroxidation. These results strongly indicated that the substitution on selenophene ring could be responsible for the antioxidant effect exerted by these compounds. Selenophenes **a**, **f**, **g**, and **h** had better antioxidant activity when compared to **b**, **c**, **d**, and **i**. Since compound **f** has a terminal alkyne, and selenophenes **a**, **g**, and **h** are easily converted to terminal alkynes via ketone elimination (47), these results suggest that a terminal alkyne bonded directly at 3-position of selenophene was crucial for the selenophene antioxidant effect. Other data that support this argument is the fact that selenophenes **b**, **c**, **d**, and **i** do not form terminal alkynes. In accordance with this idea, selenophene **i**, without substituent at 3-position, did not show antioxidant activity.

Recent persuasive evidence has indicated that δ-ALA-D activity is a sulfhydryl-containing enzyme extremely sensitive to the presence of pro-oxidant agents (35, 36, 37), which can oxidize its –SH groups during the oxidative stress (39, 48). This enzyme catalyzes the asymmetric condensation of two molecules of δ-ALA to form the monopyrrole porphobilinogen (PBG) (49, 50). In the subsequent steps, PBG is assembled into tetrapyrrole molecules, which constitute the prosthetic groups of physiologically significant proteins such as hemoglobin, cytochromes and enzymes such as catalase.

According to Barbosa and collaborators (51) oxidation of sulfhydryl enzymes is one of the mechanisms by which organochalcogens cause toxicity in mammals. Previous studies from our research group have also reported that organochalcogens inhibited δ-ALA-D activity (35, 52). Considering its sensitivity to organoselenium compounds, δ-ALA-D activity was used as a marker of toxicity (4, 39, 48). Selenophenes **a**-**i** did not inhibit δ-ALA-D activity *in vitro*, suggesting a low toxicity of these compounds.

Based on *in vitro* results, the antioxidant activity of selenophene **h** was assessed against a model of liver oxidative damage in rats. The mechanism by which 2-NP exerts its hepatotoxicity is not clearly understood, but some authors suggested that 2-NP metabolism may increase ROS levels and cause cellular damage (53, 54). In this study, oxidative damage induced by 2-NP was characterized by an increase in TBARS levels and an inhibition of δ-ALA-D activity in liver of rats. To the best of our knowledge this is the first study reporting the inhibition of δ-ALA-D activity by 2-NP exposure, reinforcing the sensitivity of this enzyme to agents which cause oxidative stress (55).

An increase in AST and ALT activities, markers of hepatic toxicity, has been reported after 2-NP exposure (16, 17). Accordingly, in this study the activity of AST and ALT was increased in plasma of rats exposed to 2-NP. The increase in ALT and AST activities was associated to the intense infiltration of inflammatory cells and the loss of cellular architecture of the liver on histopathological analysis. Borges et al. (17) have reported that 2-NP significantly decreased catalase activity in liver of 2-NP-exposed rats. Conversely, 2-NP exerted no significant effect on catalase activity in the experimental protocol used in this study. One explanation for the lack of effect on catalase activity in this study is the differences among these two protocols. In Borges et al. (17) protocol, 2-NP was administered by intraperitoneal route at the dose of 100 mg/kg, in this study rats were exposed to 100 mg/kg of 2-NP by oral route. The main differences between these

two protocols rely on a difference in the drug biovailability, leading to a lower efficacy of the drug administered by intragastric gavage. In agreement, the lower efficacy of the drug administered by intragastric gavage (per oral) when compared to intraperitoneal route has been reported by others (56, 57, 58). The liver is in fact the first target of these compounds when administered by ip or po route, but the difference is that i.p. administration might be generally more sensitive than those with p.o. administration (56, 57, 58).

Selenophene **h** protected the increase in TBARS levels induced by 2-NP administration. The changes related to the oxidative stress, such as oxidative liver damage, were known to be reduced by selenium administration; possibly due to scavenging the intermediates derived from 2-NP metabolism, including nitrogen oxide or their radical derivatives (54, 16, 17).

Another result found in this study was the protective effect of selenophene **h** (25 mg/kg) against the inhibition caused by 2-NP in δ-ALA-D activity. One explanation for this fact is that 25 mg/kg selenophene **h** reduces oxidative stress by acting as an antioxidant compound. The reduction of AST, ALT activities and histopathological alterations in animals treated with 25 mg/kg selenophene **h** is also consistent with the hepatoprotective effect of this organoselenium compound against 2-NP induced toxicity. Selenophene **h** 100 mg/kg did not alter histopathological features of liver.

We believe that the conjugation of terminal alkyne with selenophene ring and the great potential of selenium atom to stabilize radicals are responsible for the protection against 2-NP induced damage in this study. Therefore, it is possible that selenophene **h** exerts its beneficial effects by scavenging OH radicals, acting as an antioxidant. Consistent with these findings, our group of research has reported that diphenyl diselenide, an organoselenium compound, protects against 2-NP induced damage by its

antioxidant activity (16). Several studies have demonstrated that well-known antioxidants display their effects at doses higher than the dose in which compound **h** has antioxidant activity. In fact, Chen et al., (59) demonstrated that pre-treatment with ascorbic acid (500 mg/kg, i.p.) protected against lipopolysaccharide (LPS) – induced damage. Carnosine in a single i.p. dose of 250 mg/kg significantly compensated deficits in the hepatic antioxidant defense system and ameliorated liver damage induced by ischemia/reperfusion (60). The most recognized organoselenium compound with antioxidant activity, ebselen, at the dose of 30 mg/kg/day, for 10 days, protected against hydrophobic bile acid-induced liver injury in rats (61).

One important point to be addressed here is that selenophene **h** at 25 mg/kg may be beneficial however 50 and 100 mg/kg seem to exert very different effects. In fact, at the doses of 50 and 100 mg/kg selenophene **h** alone modulated ALT and AST activities and inhibited δ-ALA-D activity in liver of rats. These might be adverse effects but were dependent on the dose used. Therefore, the paradoxical results found with different doses of selenophene **h** could be attributed to antioxidant/prooxidant activities. Selenophene **h** is active as an antioxidant at low doses but probably as a prooxidant at high doses.

In the light of these observations, the claim that selenophene **h** at 25 mg/kg was an antioxidant compound was based on the following findings: (i) selenophene **h** reduced hepatic damage when administered together with 2-NP demonstrated by AST and ALT activities, histological examination and TBARS levels; (ii) the dose in which this compound displays antioxidant effect did not cause hepatic damage.

In summary, the most relevant additional findings of the present study are that (i) selenophenes **a**, **f**, **g**, and **h** were the most promising antioxidant compounds tested in

TBARS assay *in vitro*; (ii) the antioxidant activity was dependent on the substituents on selenophenes; (iii) selenophene **h** at the dose of 25 mg/kg was less toxic than 50 and 100 mg/kg; (iv) selenophene **h** 25 mg/kg protected against oxidative damage induced by 2-NP in liver of rats.

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Table 1. Effect of selenophenes on iron/EDTA-induced lipid peroxidation *in vitro*.

Selenophenes	Concentration (μM)					
	0	**50**	**100**	**200**	**300**	**400**
a	946.5 ± 24.4	901.1 ± 52.4	821.5 ± 53.1 *	707.9 ± 68.9 *	521.6 ± 57.2 *	256.2 ± 19.0 *
b	910.6 ± 67.5	884.1 ± 88.2	872.6 ± 65.2	776.8 ± 112.6	731.6 ± 97.2 *	612.5 ± 95.5*
c	983.5 ± 37.7	939.6 ± 68.2	938.5 ± 67.7	910.3 ± 54.2	910.2± 63.6	912.6 ± 63.6
d	930.1 ± 7.6	934.7 ± 10.2	920.8 ± 11.6	911.9 ± 29.0	927.1 ± 9.9	916.6 ± 29.2
e	931.9 ± 43.6	942.8 ± 35.9	933.1 ± 42.5	919.2 ± 12.6	895.1 ± 32.8	876.9 ± 17.1
f	1012.4 ± 32.6	978.2 ± 44.8	852.4 ± 46.7 *	719.0 ± 75.1 *	635.8 ± 63.1 *	538.1 ± 42.1 *
g	950.4 ± 95.2	833.5 ± 79.3	733.8 ± 55.4 *	454.3± 58.5 *	320.2 ± 61.8 *	236.6 ± 32.4 *
h	976.0 ± 16.7	845.7 ± 37.1	712.6 ± 54.4 *	559.4 ± 78.1 *	287.2 ± 32.3 *	287.5 ± 34.8 *
i	955.6 ± 59.0	962.7 ± 76.9	959.3 ± 62.4	981.4 ± 38.1	954.15 ± 84.8	1014.4 ± 106.6

Data are reported as mean ± S.D. and expressed as nmol equivalents of MDA (malondialdehyde)/mg protein. (*) Denotes $p < 0.05$ as compared to the Fe + EDTA sample (without selenophene) (one-way ANOVA/Duncan). The basal data were 262.43 ± 28.01 nmol equivalents MDA/mg protein.

Table 2. Effect of selenophene **h** on liver of rats.

Dose (mg/kg)	TBARS[a]	Ascorbic acid[b]	Catalase[c]	d-ALA-D[d]
0	23.5 ± 2.6	235.3 ± 41.9	220.5 ± 20.0	19.6 ± 0.7
25	14.8 ± 4.1 *	222.4 ± 27.3	227.8 ± 4.8	17.2 ± 3.2
50	20.0 ± 1.3	264.4 ± 38.8	229.7 ± 27.0	16.2 ± 2.1
100	22.7 ± 1.9	283.1 ± 40.2	231.4 ± 33.0	14.4 ± 2.2 *

Data are reported as mean ± S.D. (*) Denotes $p < 0.05$ as compared to the sample without selenophene (canola oil), (two-way ANOVA/Duncan), and expressed as [a] nmol equivalents MDA (malondialdehyde)/mg protein, [b]µg ascorbic acid/g tissue, [c] U/mg protein, [d] nmol of porphobilinogen/mg protein/hour.

Table 3. Effect of selenophene **h** in liver and plasma of 2-NP exposed rats.

Tissue	**Liver**			**Plasma**	
Groups	**TBARS[a]**	**Catalase[c]**	**d-ALA-D[d]**	**ALT[e]**	**AST[e]**
Control	22.6 ± 2.6 #	218.5 ± 19.9	18.1 ± 0.6 #	22.9 ± 5.3 #	64.0 ± 16.9 #
2-NP	33.4 ± 5.1 *	191.3 ± 28.8	10.8 ± 2.3 *	72.7 ± 29.3 *	138.2 ± 26.5 *
Sel h + 2-NP	21.8 ± 1.7 #	250.0 ± 42.3 #	16.9 ± 2.6 #	42.5 ± 6.6 *#	55.2 ± 3.8 #

Data are reported as mean ± S.D. (*) Denotes $p < 0.05$ as compared to the control group (canola oil), (two-way ANOVA/Duncan) (#) denotes $p < 0.05$ as compared to the 2-NP group and expressed as [a]nmol equivalents MDA (malondialdehyde)/mg protein, [b]µg ascorbic acid/g tissue, [c]U/mg protein, [d]nmol of porphobilinogen/mg protein/hour and [e]U/ml. *Abbreviations:* **C**- control; **2-NP**- 2-nitropropane (100 mg/Kg); **Sel h + 2-NP –** selenophene **h** (25mg/kg) plus 2-nitropropane (100 mg/kg).

Table 4. Effect of selenophene **h** at different doses on biochemical parameters in rats.

Dose (mg/kg)	AST	ALT
0	62.8 ± 18.3	21.7 ± 4.2
25	54.4 ± 3.7	27.7 ± 6.9
50	105.9 ± 4.7 *	34.6 ± 9.0 *
100	127.5 ± 24.7 *	37.2 ± 6.2 *

Data are reported as mean ± S.D. (*) Denotes $p < 0.05$ as compared to the sample without selenophene (two-way ANOVA/Duncan) and expressed as U/ml.

Legends

Fig. 1 - Chemical structures of selenophenes.

Fig. 2 - Photomicrography of segment of the hepatic lobe (A) of an animal control; note the hepatic tissue with normal aspect, (B) of animal treated with selenophene **h** 100 mg/kg; observe the hepatocyte strings, centrillobular vein and sinusoid capillaries with normal aspect, (C) of animal treated with 2-NP; note intense infiltration of inflammatory cells (arrow-head) in the hepatic tissue and the loss of cellular architecture (*), (D) of animal treated with selenophene **h** 25 mg/kg+2NP; note the reduction of infiltration of inflammatory cells (arrow-head) in the hepatic tissue with normal appearance. Hepatocyte strings (arrow); Centrillobular vein (CV); Sinusoid capillaries (sc). Both H.E. 100X.

Figure 1

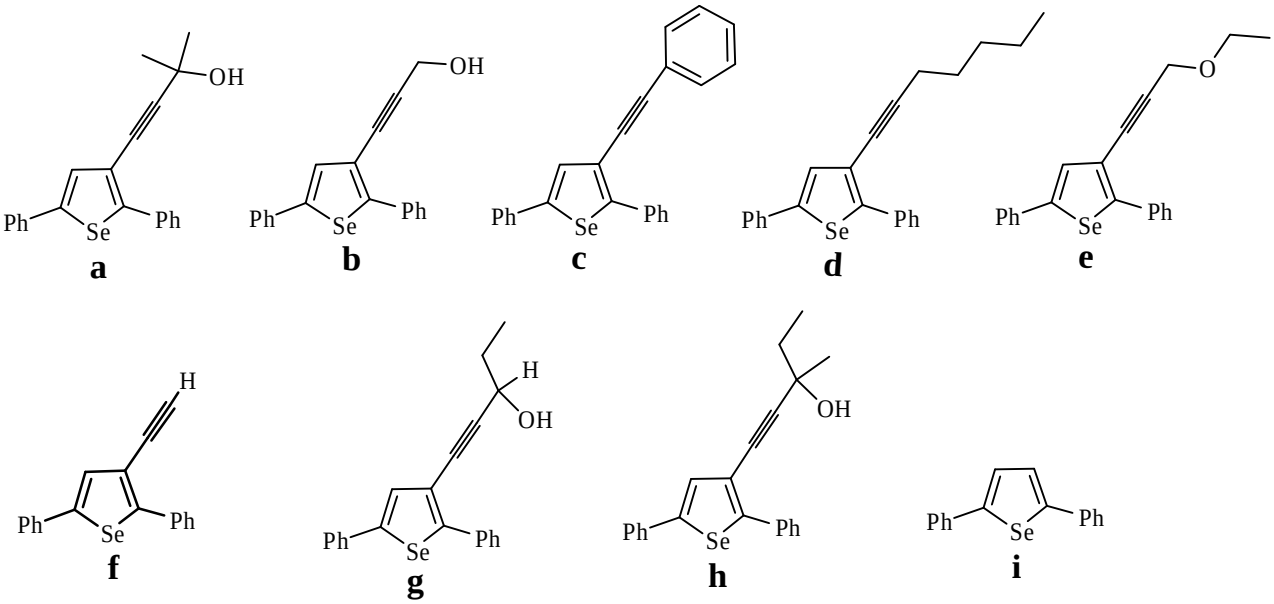
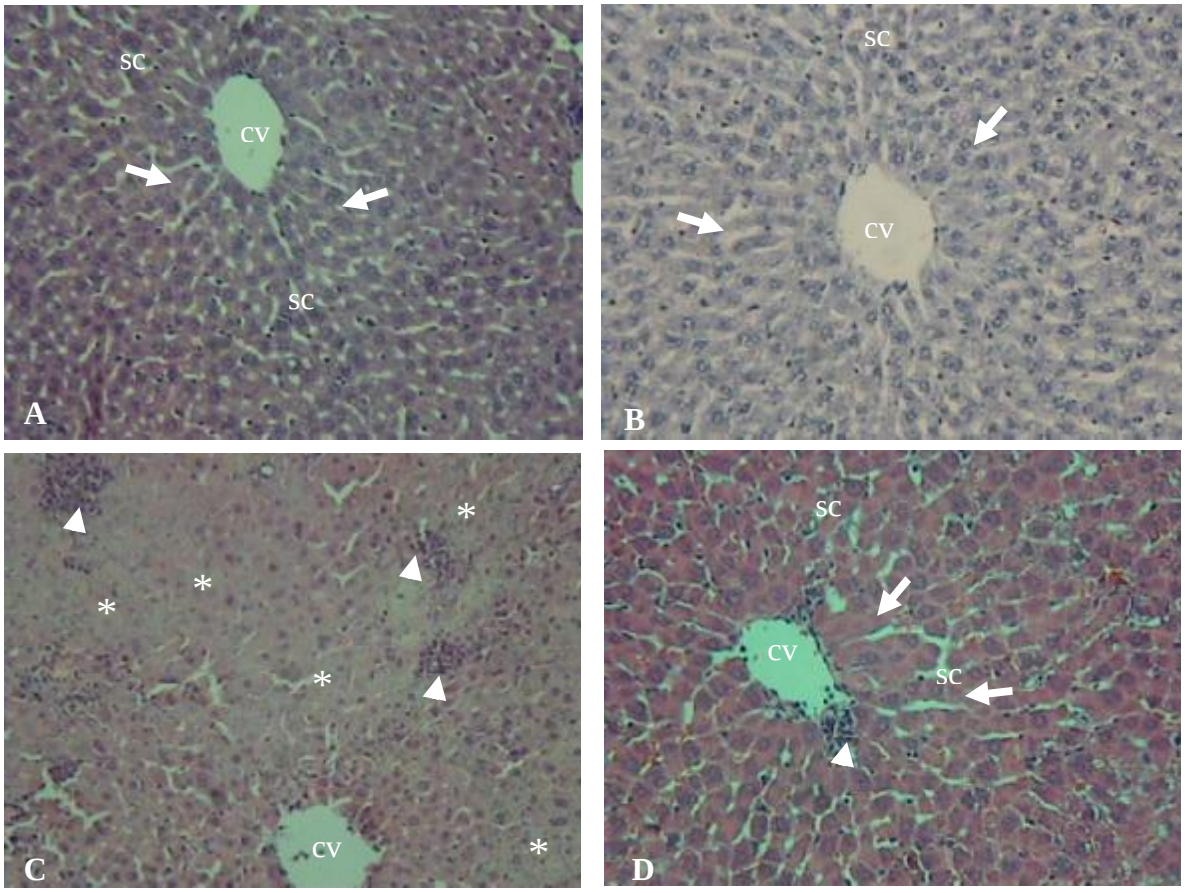


Figure 2



4.2. Efeito hepatoprotetor do 3-alquinil selenofeno contra o dano no fígado induzido pelo tetracloreto de carbono em ratos.

4.2.1. Manuscrito 2

HEPATOPROTECTIVE EFFECT OF 3-ALKYNYL SELENOPHENE AGAINST CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE IN RATS

(Sumetido à BMB Reports)

Hepatoprotective effect of 3-alkynyl selenophene against carbon tetrachloride-induced liver damage in rats

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Abstract

The aim of this study was to investigate the hepatoprotective effect of 1-(2,5-diphenylselenophen-3-yl)-3-methylpent-1-yn-3-ol (selenophene **h**) on acute liver injury induced by carbon tetrachloride in rats. In the first day of treatment, animals received selenophene **h** (25 and 50 mg/kg; per oral, p.o.). In the second day, rats received CCl_4 (1 mg/kg; intraperitoneal, i.p.). Twenty-four hours after CCl_4 administration animals were euthanized and plasma and liver were removed to the biochemical and histological analysis. Selenophene **h** protected against the increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities induced by CCl_4 administration. The histological data showed extensive injury in liver from CCl_4-treated rats, which was attenuated by selenophene **h**. Selenophene **h** ameliorated lipid peroxidation and ascorbic acid levels, catalase, δ-aminolevulinic dehydratase (δ-ALA-D) and glutathione S-transferase (GST) activities altered by CCl_4 .The antioxidant effect of selenophene **h** on acute liver injury induced by CCl_4 in rats was demonstrated.

Keywords: selenium, CCl_4, liver, hepatic damage.

Introduction

The liver regulates many important metabolic functions. Hepatic injury is associated with distortion of these metabolic functions (1). Additionally, it is the key organ of metabolism that is continuously and variedly exposed to xenobiotics because of its strategic placement in the body. The toxins absorbed from the intestinal tract gain access first to the liver resulting in a variety of liver ailments. Thus, liver diseases remain one of the serious health problems. Modern medicines have little to offer for alleviation of hepatic diseases and there are not much drugs available for the treatment of liver disorders (2).

CCl_4 is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. CCl_4 requires bioactivation by phase I cytochrome P450 system in liver to form reactive metabolic trichloromethyl radical (CCl_3•) and peroxy trichloromethyl radical (•$OOCCl_3$). These free radicals can bind with polyunsaturated fatty acid (PUFA) to produce alkoxy (R•) and peroxy radicals (ROO•) (3). These free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cellular protein is considered to be the initial step in a chain of events, eventually leading to membrane lipid peroxidation and finally cell necrosis (4). Consequently, CCl_4 is known to induce reactive oxygen species (ROS) formation, deplete GSH of phase II enzyme, and reduce antioxidant enzymes and substrates to induce oxidative stress that is an important factor in acute and chronic liver injury. (4)

It is believed that ROS may injure cell membranes through lipid peroxidation and modify or damage biomolecules, i.e., proteins, lipids,

carbohydrates and DNA in vitro and in vivo (5). Significant cellular damage occurs when the amount of produced free radicals exceeds the capacity of endogenous cellular antioxidant defense system. According to the free radical theory, blocking or retarding the chain reaction of oxidation is one of the practicable strategies to preventing oxidative stress-induced hepatotoxicity.

The biochemistry and pharmacology of selenium is a subject of intense current interest, particularly from the viewpoint of public health (6). Selenium, long recognized as a dietary antioxidant, is now known to be an essential component of the active sites of several enzymes, including glutathione peroxidase and thioredoxin reductase, which catalyse reactions essential to the protection of cellular components against oxidative damage (7). In addition to their antioxidant property (8), selenium compounds were found to have neuro-protective (9), anti-inflammatory (10) and hepatoprotective (11) properties. Therefore, a number of novel pharmaceutical agents which are selenium-based or which target specific aspects of selenium metabolism are under development. Among these are the orally active antihypertensive agents, anticancer, antiviral, immunosuppressive and antimicrobial agents, and organoselenium compounds, which reduce oxidative tissue damage and edema (12). The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants, has led to design synthetic organoselenium compounds (13).

Besides chalcogenophenes play an important role in organic synthesis (14) they display antioxidant (8), antinociceptive and anti-inflammatory (15, 16) properties. Based on the above considerations the objective of this study was to

investigate the effect of 1-(2,5-diphenylselenophen-3-yl)-3-methylpent-1-yn-3-ol (selenophene **h**), a selenophene, on acute liver injury induced by CCl_4 in rats.

Results

Hepatic profile

A significant main effect of CCl_4 ($F_{1,40}$ = 15.27; p<0.0004) was observed. Post hoc comparisons revealed that CCl_4 increased ALT activity. Selenophene **h** at the dose of 50 mg/kg prevented the increase of ALT activity induced by CCl_4 (Table 1).

Two-way ANOVA of plasma AST activity revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 5.06; p<0.011). Post hoc comparisons revealed that CCl_4 ($F_{1,40}$ = 79.88; p<0.0001) increased AST activity. Selenophene **h** ($F_{2,40}$ = 6.35; p<0.004) prevented significantly AST activity increased by CCl_4. (Table 1).

Selenophene **h** at both doses did not alter plasma ALT and AST activities (Table 1).

Histological examination

Livers from CCl_4-treated rats showed extensive injury, specifically massive centrilobular necrosis, ballooning degeneration and cellular infiltration. Selenophene **h** at both doses prevented pathological alterations caused by CCl_4 administration (Table 2; Figure 1).

Lipid peroxidation

Two-way ANOVA of lipid peroxidation levels revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 5.51; p<0.0077). Post hoc comparisons showed that CCl_4 exposure increased lipid peroxidation levels ($F_{1,40}$ = 14.74; p<0.0004) and selenophene **h** (25 and 50 mg/kg) significantly decreased lipid peroxidation levels in liver of rats when compared to the CCl_4 group (Table 1).

Lipid peroxidation levels were not altered in the liver of rats which received selenophene **h** at both doses (Table 1).

Ascorbic acid

Two-way ANOVA of ascorbic acid levels revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 7.82; p<0.0014). Post hoc comparisons showed that selenophene **h** at the dose of 50 mg/kg significantly increased ascorbic acid levels reduced by CCl_4 ($F_{1,40}$= 10.49; p<0.0024) in liver of rats (Table 1).

Ascorbic acid levels were not altered in the liver of rats which received selenophene **h** at both doses (Table 1).

Catalase activity

Two-way ANOVA of catalase activity revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 22.35; p<0.0001). Post hoc comparisons showed that selenophene **h** at the doses of 25 and 50 mg/kg significantly increased catalase activity reduced by CCl_4 ($F_{1,40}$= 22.35; p<0.0001) in liver of rats (Table 1).

Catalase activity was not altered in the liver of rats which received selenophene **h** at both doses (Table 1).

GST activity

Two-way ANOVA of GST activity revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 5.76; p<0.0063). Post hoc comparisons showed that selenophene **h** at the dose of 25 and 50 mg/kg significantly decrease catalase activity increased by CCl_4 ($F_{1,40}$= 6.16; p<0.0174) in liver of rats (Table 1).

GST activity was not altered in the liver of rats which received selenophene **h** at both doses (Table 1).

δ- ALA-D activity

Two-way ANOVA of δ-ALA-D activity revealed a significant selenophene **h** x CCl_4 interaction ($F_{2,40}$= 10.59; p<0.0002). Post hoc comparisons demonstrated that CCl_4 ($F_{1,40}$= 79.46; p<0.0001) inhibited δ-ALA-D activity and selenophene **h** at the 50 mg/kg ($F_{2,50}$ = 0.55; p<0.5790) protected δ-ALA-D inhibition induced by CCl_4 (Table 1).

δ-ALA-D activity remained unaltered in the liver of rats which received selenophene **h** at the both doses (Table 1).

Discussion

Several research groups have demonstrated that organoselenium compounds have hepatoprotective effect (11, 26). In this study we reported the hepatoprotective effect of selenophene **h**, an organoselenium compound, on acute liver injury induced by CCl_4 in rats. As expected, a single oral dose of CCl_4 showed significant hepatotoxicity, as evidenced by a dramatic elevation in the plasma AST and ALT activities and an increased incidence of

histopathological lesions in liver of rats. In addition, CCl_4 exposure produced oxidative damage, as evidenced by a significant increase in lipid peroxidation levels and GST activity as well as a decrease in ascorbic acid levels, catalase and δ-ALA-D activities, which suggest a role of oxidative stress in CCl_4-induced hepatotoxicity. Pretreatment with selenophene **h** showed a significant protective effect against CCl_4-induced acute oxidative damage and hepatotoxicity in rats.

Preventive action of liver damage induced by CCl_4 has been widely used as an indicator of liver protective activity of drugs in general (27). Since the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (28), CCl_4-mediated hepatotoxicity was chosen as the experimental model. In the present study, CCl_4-expoused rats sustained significant hepatic damage as represented by an increase in the ALT and AST activities that are in accordance with others (29). These results were associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver observed in histopathological evaluation that is the according to Plaa and Charbonneau (30). In this view, the ability of selenophene **h** in normalizing the activity of transaminases and morphological aspects of liver reflects the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4. These findings are in agreement with the commonly accepted view that plasma levels of transaminases return to normal levels with the healing of hepatic parenchyma and regeneration of hepatocytes (31). Therefore, data on the literature have demonstrated that liver damage is a therapeutic target of selenorganic compounds (12).

Oxidative stress is considered to play a prominent causative role in many diseases including liver damage (32). Free radicals are capable of binding to

proteins or lipids, or abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage while playing a significant role in the pathogenesis of diseases (33). Lipid peroxidation, usually measured through its catabolites, such as MDA, is widely accepted to be one of the principal causes of CCl_4-induced liver injury (34). In this study we found an increase in lipid peroxidation levels induced by CCl_4 exposure; this result has been reported by others (29). Selenophene **h** normalizes lipid peroxidation levels, suggesting that the antioxidant activity is important in the protection against CCl_4-induced liver lesion (35). Accordingly, our research group has reported that diphenyl diselenide, a well-known antioxidant and organoselenium compound, has hepatoprotective effect (11, 26).

The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as catalase and non-enzymatic antioxidant defenses as ascorbic acid. Under oxidative stress, some endogenous protective factors such as catalase and ascorbic acid are activated in the defense against oxidative injury. In this study catalase activity and ascorbic acid levels were decreased by CCl_4 exposure in accordance with Jain et al. (36) and Ohta et al. (37). Selenophene **h** was effective in protecting against this decrease, reinforcing the antioxidant activity of this compound, since a reduction in these parameters is associated with the accumulation of highly reactive free radicals (38).

Besides, we observed a decrease in δ-ALA-D activity in rats that received CCl_4 and selenophene **h** was effective in restoring enzyme activity. Since we have reported that δ-ALA-D activity is extremely sensitive to situations

associated with oxidative stress (12), the beneficial effect of selenophene **h** on this enzyme demonstrates the antioxidant capacity of this compound. Actually, CCl_4-induced inhibitory effect on hepatic δ-ALA-D activity can be tentatively related to lipid peroxidation in this study. We can also infer that δ-ALA-D inhibition participates, at least in part, in the toxicity manifestations caused by CCl_4.

In addition, the increase of GST activity can be due to the probable response towards increased ROS generation and/or due the detoxification mechanism. The effect of selenophene **h** in GST activity suggests a compensatory mechanism to counteract lipid peroxidation induced by CCl4 in liver of rats. Many powerful antioxidants, such as Vitamin E, GSH and melatonin, show great ability in protection of experimental liver injuries (39, 40).

In conclusion, the results suggest that the selenophene **h** as an antioxidant seems to be useful in therapy of CCl_4 damage, since it has the capability to alleviate many of the harmful effects of CCl_4.

Experimental Procedure

Chemicals

1-(2,5-Diphenylselenophen-3-yl)-3-methylpent-1-yn-3-ol (selenophene **h**) was prepared according to the literature method (14). Selenophene **h** and CCl_4 were dissolved in canola oil. All chemicals were obtained from standard commercial suppliers.

Animals

Male adult Wistar rats, weighing 200-300g, were obtained from a local breeding colony. The animals were kept in separate animal rooms, on a 12 h light/dark cycle, in an air conditioned room (22 ± 2°C). Commercial diet (GUABI, RS, Brazil) and tap water were supplied *ad libitum*. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil.

Exposure

Five to eight animals per group were usually tested in the experiments. Rats received selenophene **h** (25 or 50 mg/kg of body weight; p.o.) (groups 2 and 3). The control group received only vehicle (canola oil, 5 ml/kg, p.o.) (group 1). Twenty-four hours later, animals received CCl_4 (1 mg/kg of body weight; i.p., 1:1) (17) (groups 4, 5 and 6). The protocol of rat treatments is given below:

Group (1) Canola oil (p.o.) plus canola oil (5 ml/kg, p.o.).

Group (2) Selenophene **h** (25 mg/kg, p.o.) plus canola oil (5 ml/kg, p.o.).

Group (3) Selenophene **h** (50 mg/kg, p.o.) plus canola oil (5 ml/kg, p.o.).

Group (4) Canola oil (5 ml/kg, i.p.) plus CCl_4 (1 mg/kg; i.p.)

Group (5) Selenophene **h** (25 mg/kg, p.o.) plus CCl_4 (1 mg/kg; i.p.)

Group (6) Selenophene **h** (50 mg/kg, p.o.) plus CCl_4 (1 mg/kg; i.p.)

Twenty-four hours after CCl_4 administration all rats were anesthetized for blood collection by heart puncture (hemolyzed plasma was discharged). After this procedure, rats were euthanized and the liver of animals was removed, dissected and kept on ice until the time of assay. The samples of liver were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10, w/v), centrifuged at 2,400×*g* for

15 min. The low-speed supernatants (S_1) were separated and used for biochemical assays.

Hepatic profile

Plasma AST and ALT activities were used as the biochemical markers for the early acute hepatic damage (18), using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). The enzymatic activities were expressed as U/L.

Histopathology

To histological evaluation, all rats were subjected to a through necropsy evaluation. Tissues were saved and fixed in 10% formalin. For light microscopy examination, tissues were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin (HE). Liver sections were evaluated to sings of toxicity characterized by the damage of liver cells around the central vein, centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver cells. Pathological alterations were graded on a numeric score according to their severity. The severity were graded from score 0 to 3, with 0 indicating absent, 1 indicating low injury, 2 indicating moderate injury and 3 indicating intense liver injury.

Lipid peroxidation

An aliquot of S1 (200 µl) from rats belonging to the experimental groups was added to the reaction mixture containing 500 µl thiobarbituric acid (0.8%), 200 µl SDS (sodium dodecyl sulfate, 8.1%) and 500 µl acetic acid (pH 3.4), and

was incubated for 2 hours at 95°C. TBARS were determined as described by Ohkawa et al. (19). Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. The lipid peroxidation was expressed as nmol MDA equivalents/g tissue.

Ascorbic acid determination

Ascorbic acid levels are a non-enzymatic antioxidant defense that is involved in protecting against the injurious effects of ROS. Ascorbic acid levels determination was performed as described by Jacques-Silva et al. (20) with some modifications. Briefly, S_1 was precipitated in 10 % trichloroacetic acid solution. An aliquot of S_1 (300 µl) at a final volume of 575 µl of the solution was incubated for 3 h at 38°C 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 dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount (µmol ascorbic acid/g tissue).

Catalase activity

Catalase is an enzymatic antioxidant defense that is involved in protecting against the injurious effects of ROS. Catalase activity was assayed spectrophotometrically by the method of Aebi (21), which involves monitoring the disappearance of H_2O_2 in the S_1 presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 µl of S_1 and the substrate (H_2O_2) to a

concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in as UI/mg protein.

Glutathione S-transferase (GST) activity

GST is an enzymatic antioxidant defense that protect against ROS-induced injurious. GST activity was assayed through the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig et al. (22). An aliquot of 100 µl of S_1 was added in 0.1 M potassium phosphate buffer, pH 7.4, with CDNB, as substrate, and 50 mM GSH. The enzymatic activity was expressed in µmol/ min/ mg protein.

δ-Aminolevulinic dehydratase (δ-ALA-D) activity

δ-ALA-D, a sulfhydryl- and Zn^{2+}-containing enzyme, is highly sensitive to pro-oxidants and heavy metals (23). δ-ALA-D activity was assayed according to the method of Sassa (24) by measuring the rate of product (porphobilinogen) formation except that 100 mM sodium phosphate buffer pH 6.8 and 2.4 mM δ-ALA were used. An aliquot of 200 µl of S_1 tissue was incubated for 1 h at 37 °C. The reaction product was determined using modified Erlich's reagent at 555 nm. The enzymatic activity was expressed as nmol PBG/mg protein/hour.

Protein Quantification

Protein concentration was measured by the method of Bradford (25), using bovine serum albumin as the standard.

Statistical analysis

Data were analyzed by using a two-way analysis of variance (ANOVA) (selenophene **h** x CCl_4), followed by Duncan's Multiple Range Test when appropriate. Main effects are presented only when the second order interaction was non-significant. All data of experiments were expressed as means ± S.E.M. Values of $p<0.05$ were considered statistically significant.

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Table 1. Effect of selenophene **h** on ALT and AST activities and ascorbic acid and TBARS levels, CAT, GST and δ-ALA-D activities.

	PLASMA		LIVER				
	ALT	**AST**	**ASCORBIC ACID**	**CAT**	**GST**	**δ-ALA-D**	**TBARS**
Control	48.01 ± 3.54	28.02 ± 4.24	224.58 ± 9.05	59.70 ± 5.11	82.18 ± 10.29	8.19 ± 1.03	26.97 ± 0.88
25	36.75 ± 6.66$^{\#}$	21.50 ± 4.21$^{\#}$	213.05 ± 3.85$^{\#}$	55.02 ± 5.50$^{\#}$	108.25 ± 10.60$^{\#}$	7.41 ± 0.91$^{\#}$	30.41 ± 2.97$^{\#}$
50	42.75 ± 5.17$^{\#}$	22.75 ± 4.32$^{\#}$	197.75 ± 9.49$^{\#}$	57.98 ± 2.13$^{\#}$	97.58 ± 9.85$^{\#}$	6.86 ± 0.46$^{\#}$	30.02 ± 2.58$^{\#}$
CCl_4	206.30 ± 8.01*	199.20 ± 15.27*	163.02 ± 6.82*	35.52 ± 2.64*	151.86 ± 7.81*	4.63 ± 0.33*	40.23 ± 1.72*
25 + CCl_4	222.09 ± 30.26*	133.72 ± 18.31$^{\#*}$	174.84 ± 10.01*	47.06 ± 2.80$^{\#*}$	101.61 ± 11.33$^{\#}$	4.91 ± 0.66*	33.75 ± 1.54$^{\#*}$
50 + CCl_4	94.00 ± 9.73$^{\#*}$	94.50 ± 6.24$^{\#*}$	209.42 ± 7.32$^{\#}$	48.68 ± 2.05$^{\#}$	108.75 ± 9.69$^{\#}$	6.10 ± 0.62$^{\#*}$	31.78 ± 1.29$^{\#}$

Data are reported as mean ± S.M. E. (two-way ANOVA/Duncan). AST and ALT activities were expressed as U/L. Ascorbic acid levels, CAT, GST, δ-ALA-D activities and TBARS levels were expressed as μg ascorbic acid/g tissue, U/mg protein, μmol/min mg protein, nmol PBG/mg protein/h, nmol MDA equivalents/g tissue, respectively. Data are expressed as mean±S.E. of 5 - 8 animals per group. * Denoted $p < 0.05$ as compared to the control group (ANOVA and Duncan's Multiple Range Test). $^{\#}$ Denoted $p < 0.05$ as compared to the CCl_4 group (ANOVA and Duncan's Multiple Range Test).

Table 2. Effect of selenophene **h** on histopathological scores in hepatic injury induced by CCl_4 in rats.

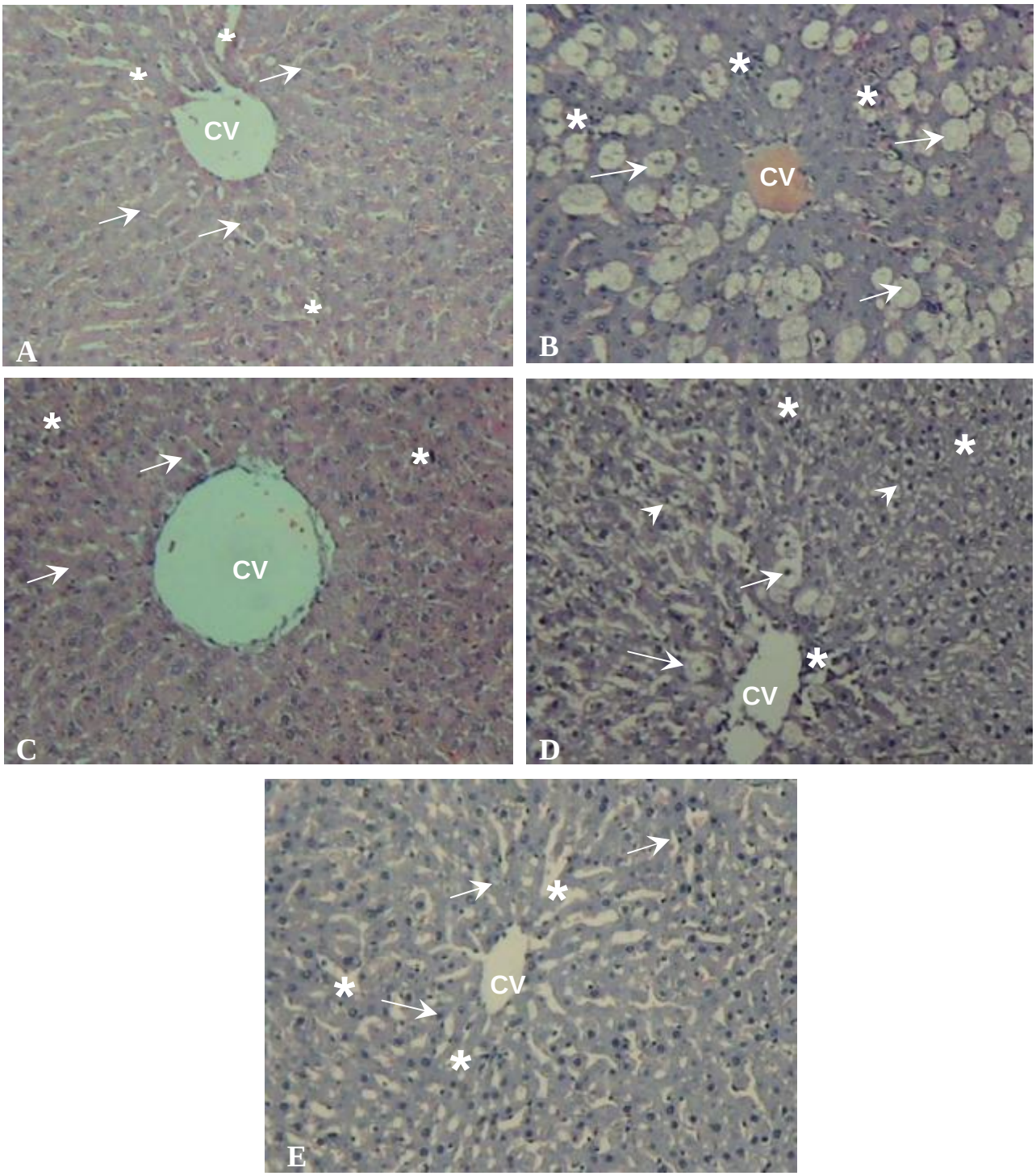
		Control	CCl_4	25	50	25 + CCl_4	50 + CCl_4
Vascular congestion	0	0	0	0	0	0	0
	1	5	0	5	4	2	3
	2	0	4	0	1	3	2
	3	0	1	0	0	0	0
Megalocytosis	0	5	0	5	5	4	5
	1	0	2	0	0	1	0
	2	0	3	0	0	0	0
	3	0	0	0	0	0	0
Cell infiltration	0	0	0	0	0	0	0
	1	5	1	5	5	2	3
	2	0	3	0	0	2	1
	3	0	1	0	0	1	1
Eosinophilic cells	0	5	0	4	4	4	4
	1	0	3	1	1	1	1
	2	0	2	0	0	0	0
	3	0	0	0	0	0	0
Ballooning degeneration	0	5	0	5	5	0	3
	1	0	1	0	0	2	1
	2	0	1	0	0	3	1
	3	0	3	0	0	0	0
Nuclear hiperchromatism	0	1	0	1	5	2	2
	1	4	2	4	0	0	1
	2	0	3	0	0	3	2
	3	0	0	0	0	0	0
Tumefation of hepatocytes	0	0	0	0	0	0	0
	1	5	0	4	4	2	3
	2	0	1	1	1	2	2
	3	0	4	0	0	1	0

Grade designation of the histological findings: (0) absent, (1) low, (2) moderate, (3) intense. Each value is the number of animals with grading changes. Each group consists of 5 rats.

Legends

Figure 1 - Photomicrography of segment of the hepatic lobe (**A**) of a control animal. Note the hepatocyte strings (arrow), the centrilobular vein (CV) and sinusoid capillaries (*) with normal aspect, (**B**) of an animal treated with CCl_4, note intense ballooning degeneration (arrow) and (*) infiltration of inflammatory cells, (**C**) of an animal treated with selenophene **h** (50 mg/kg), note the hepatocyte strings (arrow), the centrilobular vein and some inflammatory cells (*), (**D**) of an animal treated with CCl_4 + selenophene **h** (25 mg/kg); observe around the centrilobular vein some hepatocytes with vacuolation (head-arrows), ballooning degeneration (arrow) and the presence of inflammatory cells (*) in the sinusoid capillaries, (**E**) of an animal treated with CCl_4 + selenophene **h** (50 mg/kg). Observe the hepatocyte strings (arrow), the centrilobular vein and sinusoid capillaries (*) with normal aspect. H.E. 100X.

Figure 1



5. DISCUSSÃO

O interesse por compostos naturais ou sintéticos que possam previnir, reverter ou retardar o desenvolvimento de diversas patologias tem crescido consideravelmente na comunidade científica nas últimas duas décadas. Neste contexto, compostos heterocíclicos têm sido bastante estudados quanto as suas atividades biológicas. De fato, estes compostos apresentam atividades farmacológicas diversificadas, tais como: inibidor do HIV, antitumoral, antifúngica, antinflamatória e analgésica, antiprotozoária, antiviral e antioxidante (Barreiro et al., 2001; Zeni et al., 2001; Meotti et al., 2003, 2004; Gonçales et al., 2005; Shiah et al., 2007; Juang et al., 2007). Dentre estes compostos heterocíclicos destacam-se os que apresentam selênio em sua estrutura como, por exemplo, o ebselen que apresenta importantes atividades biológicas tais como: atividade catalítica e propriedades antioxidantes similares à glutationa peroxidase (Parnhan e Graf, 1990), atividade neuroprotetora (Osaki et al., 1997; Tan et al., 1997; Takasago et al., 1997; Kondoh et al., 1999; Imai et al., 2001; Porciúncula et al., 2003) entre outras. Outro composto heterocíclico que se destaca é o D-501036, um derivado de selenofeno, que apresenta atividade anti-tumoral (Shiah et al., 2007). Entretanto, existem poucos trabalhos na literatura que descrevem a atividade biológica desta classe de compostos.

Baseando-se nestas perspectivas, um dos nossos objetivos foi investigar a atividade antioxidante de 3-alquinil selenofenos em modelos de dano oxidativo *in vitro* e *ex vivo* em ratos. Para isso testamos uma classe de compostos 3-alquinil selenofeno, com diferentes substituições na ligação tripla na posição 3 da molécula do selenofeno, com o objetivo de avaliar o perfil antioxidante e o possível efeito tóxico *in vitro*. Como resultado, encontramos compostos com atividade antioxidante, porém essa atividade foi dependente da substituição na ligação tripla da molécula. De fato, Tiano e colaboradores (2000) demonstraram que a estrutura química de compostos orgânicos de selênio tem um importante papel no estabelecimento da atividade antioxidante.

Os resultados obtidos no **Manuscrito 1** indicaram que os selenofenos que apresentam um alquino terminal em sua estrutura ou que são facilmente convertidos a alquinos terminais via eliminação de cetona (Shostakovskii et al.,

1974) demonstraram uma melhor atividade antioxidante, o que sugere que a presença de um alquino terminal na posição 3 do selenofeno é crucial para este efeito.

Considerando as evidências que indicam que a enzima δ-ALA-D é sensível a agentes pró-oxidantes (Nogueira et al., 2003; Fachinetto et al., 2006; Luchese et al., 2007) e que a oxidação de enzimas sulfidrílicas é um dos mecanismos pelo qual compostos orgânicos de selênio causam toxicidade em mamíferos (Barbosa et al., 1998), o possível efeito tóxico dos 3-alquinil selenofenos foi avaliado através da atividade desta enzima *in vitro*. Os resultados obtidos demonstraram que nenhum dos 3-alquinil selenofenos testados inibiu a atividade desta enzima, sugerindo que esta classe de compostos não apresentou toxicidade sobre a atividade da δ-ALA-D.

Baseado nos resultados *in vitro*, o nosso próximo objetivo foi avaliar a atividade hepatoprotetora do selenofeno **h** (que obteve melhor atividade antioxidante *in vitro*) perante o modelo de indução de dano oxidativo pela administração de 2-NP em ratos (*ex vivo*). A escolha da dose para os experimentos *ex vivo* foi feita através de uma determinação da dose letal 50 (LD_{50}), na qual a dose de 25 mg/kg deste composto não apresentou evidências de toxicidade. Os resultados obtidos indicaram o efeito hepatoprotetor deste composto contra o dano oxidativo induzido pelo 2-NP em ratos. De fato, estudos têm demonstrado que compostos orgânicos de selênio podem atuar como agentes terapêuticos em modelos de hepatotoxicidade (Nogueira et al., 2004; Borges et al., 2005, 2006; Wilhelm et al., 2008).

O selenofeno **h** (25 mg/kg) protegeu contra o aumento dos marcadores de dano hepático (AST e ALT) e do estresse oxidativo induzido pela administração do 2-NP em ratos. Considerando a hepatotoxicidade do 2-NP, este nitroalcano também induziu alterações microscópicas avaliadas por inspeções histopatológicas. De fato, estudos revelaram que as alterações no microambiente celular com destruição da integridade do hepatócito e liberação do conteúdo intracelular para a circulação, antecedem as alterações histopatológicas. Neste estudo, as avaliações histopatológicas demonstraram extensiva injúria, caracterizada por intensa infiltração de células inflamatórias e perda da arquitetura celular nos animais que receberam a administração de 2-

NP. O selenofeno **h** protegeu o fígado desta injúria, enfatizando o efeito hepatoprotetor deste composto.

Uma vez que o selenofeno **h** apresenta atividade antioxidante *in vitro* e demostrou efeito protetor contra o aumento da peroxidação lipídica e a inibição da atividade da enzima δ-ALA-D nos animais tratados com 2-NP, pode-se sugerir que o efeito hepatoprotetor esteja intimamente ligado a atividade antioxidante deste composto, diminuindo o estresse oxidativo possivelmente por inativar os efeitos dos intermediários nocivos oriundos do metabolismo do 2-NP (IPHA e HAS)(Kohl et al., 1995). Tem sido demonstrado que a administração de selênio reduz o estresse oxidativo, tal como o dano oxidativo hepático (Kohl et al., 1995; Borges et al., 2005, 2006).

Sendo assim, os resultados do **Manuscrito 1** demonstraram que o selenofeno **h** apresenta efeito hepatoprotetor na dose de 25 mg/kg contra o dano oxidativo induzido pelo 2-NP em ratos. Baseado nestes resultados, teve-se como objetivo avaliar este efeito frente a outro indutor de hepatotoxicidade. Utilizamos o modelo experimental de dano hepático induzido pela administração de CCl_4, composto conhecido pela sua toxicidade e com mecanismo de ação bem descrito na literatura (Brattin et al., 1985; Basu, 2003; Wang et al., 2005).

Os resultados obtidos no **Manuscrito 2** demonstraram que o selenofeno **h** protege contra o dano oxidativo induzido pelo CCl_4 em ratos. A ação preventiva contra o dano hepático induzido pelo CCl_4 tem sido usada como indicador de atividade protetora do fígado de drogas em geral (Clauson, 1989). Uma única dose de CCl_4 causou significante hepatotoxicidade, evidenciada por elevação da atividade plasmática das enzimas AST e ALT, aumento da incidência de lesões histopatológicas (necrose centrolobular, degeneração balanosa, infiltração celular), aumento dos níveis de peroxidação lipídica e da atividade da GST, bem como, diminuição dos níveis de ácido ascórbico e da atividade das enzimas catalase e δ-ALA-D. Apartir dos resultados demonstrados, verificamos que o selenofeno **h** protegeu de todas estas alterações, confirmando o efeito hepatoprotetor deste composto em ratos.

A habilidade do selenofeno **h** de normalizar a atividade das transaminases e os aspectos morfológicos do fígado reflete a estabilização da membrana plasmática, bem como, o reparo do dano no tecido hepático

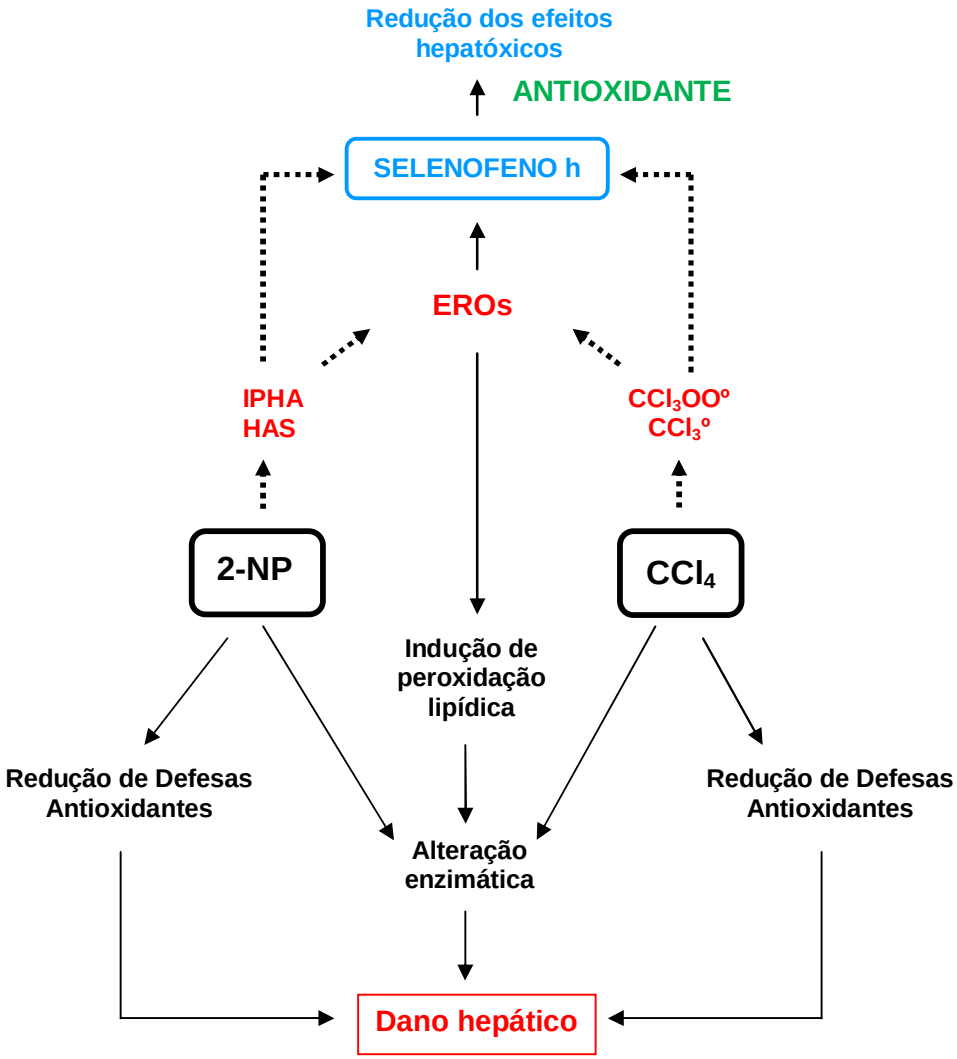
causado pelo CCl_4, dados estes corroborados por Plaa e Charbonneau (1989) que sustentam a idéia de que os níveis plasmáticos das transaminases retornam aos níveis normais com a cicatrização do parênquima hepático e a regeneração dos hepatócitos. De acordo com estes achados, dados da literatura tem demonstrado que o dano hepático é alvo terapêutico de compostos orgânicos de selênio (Nogueira et al., 2004).

Os resultados obtidos com este modelo experimental corroboram com a idéia obtida através dos resultados do **Manuscrito 1** de que o selenofeno **h** exerce efeito hepatoprotetor possivelmente devido a atividade antioxidante, visto que neste modelo observamos uma proteção sobre a diminuição das defesas antioxidantes enzimáticas (catalase, GST) e não enzimáticas (ácido ascórbico) e da atividade da enzima δ-ALA-D causada pela exposição ao CCl_4. De fato, a redução destes parâmetros está associada com o acúmulo de radicais livres altamente reativos ($CCl_3OO^{\cdot}$, $CCl_3^{\cdot}$) (Sheela and Angusti, 1995). Adicionalmente, este composto protegeu contra a peroxidação lipídica induzida por CCl_4.

Em conclusão, os resultados dos **Manuscritos 1 e 2** sugerem que o selenofeno **h**, uma molécula com atividade antioxidante, pode ser uma útil terapia contra o dano oxidativo induzido pelos hepatotoxicantes: 2-NP e CCl_4, considerando que este composto tem a habilidade de proteger dos efeitos nocivos causados por estes. Além disso, podemos inferir que o selenofeno **h** apresenta atividade hepatoprotetora pois demonstrou-se efetivo nos dois modelos experimentais utilizados.

No **Esquema 1**, mostrado a seguir, é possível ter uma visão geral dos efeitos do selenofeno h frente aos compostos hepatotóxicos (2-NP e CCl_4) estudados neste trabalho.

Esquema 1 - Visão geral dos efeitos do selenofeno **h** frente aos compostos hepatotóxicos (2-NP e CCl_4) estudados neste trabalho.



- As linhas cheias (—) indicam os efeitos demonstrados neste trabalho.
- As linhas pontilhadas (---) indicam possíveis efeitos, baseado em estudos da literatura.

6. CONCLUSÕES

Com base nos resultados obtidos, nós podemos concluir que:

- O selenofeno **h** apresentou a melhor atividade antioxidante e não demonstrou toxicidade *in vitro* (**Manuscrito 1**).
- O selenofeno **h** administrado pela via oral na dose de 25 mg/kg não causou toxicidade em ratos (**Manuscrito 1**).
- O selenofeno **h** demonstrou-se efetivo em proteger contra o dano oxidativo induzido pelo 2-NP (**Manuscrito 1**) e CCl_4 (**Manuscrito 2)** em fígado de ratos, sendo a atividade antioxidante deste composto um dos mecanismos envolvidos neste efeito.

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