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AVALIAÇÃO DA ATIVIDADE DA ISATINA-3-N^4-BENZILTIOSSEMICARBAZONA (IBTC) COMO ANTIOXIDANTE NA PROTEÇÃO DA LDL CONTRA A OXIDAÇÃO

DISSERTAÇÃO DE MESTRADO

Rômulo Pillon Barcelos

Santa Maria, RS, Brasil

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Rômulo Pillon Barcelos

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elaborada por
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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DA ATIVIDADE DA ISATINA-3-N^4-BENZILTIOSSEMICARBAZONA (IBTC) COMO ANTIOXIDANTE NA PROTEÇÃO DA LDL CONTRA A OXIDAÇÃO

AUTOR: RÔMULO PILLON BARCELOS

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Muitos estudos têm revelado que a oxidação da lipoproteína de baixa densidade (LDL) é um fator pró-aterogênico e que o estresse oxidativo tem um papel central na formação de LDL oxidada. As Tiossemicarbazonas são compostos usados em terapias anti-câncer, antiviral e antifúngica em humanos. No entanto, estudos sobre a atividade redox desta classe de moléculas ainda são bastante controversos. O objetivo deste trabalho foi avaliar a atividade antioxidante de um composto derivado de uma tiossemicarbazona: a isatina-3-N^4-benziltiossemicarbazona (IBTC) e seu possível efeito protetor contra a oxidação da LDL *in vitro*. Tais atividades foram avaliadas através da formação de dienos conjugados em LDL e em soro humano induzidos por 2,2-azobis(2-amidinopropano diidroclorido) AAPH e por $CuSO_4$; da perda da fluorescência do triptofano (Trp) em LDL induzida pelo $CuSO_4$; da formação de substâncias reativas ao ácido tiobarbitúrico (TBARS) em LDL, plasma humano e em diferentes tecidos de ratos e do potencial antioxidante total (TRAP) do IBTC. A toxicidade do IBTC foi investigada em fatias da artéria aorta. Os resultados mostraram que a IBTC foi eficaz em reduzir a formação de dienos conjugados induzidos tanto pelo AAPH quanto pelo $CuSO_4$, aumentando de modo concentração-dependente a fase lag. A IBTC também preveniu a perda acentuada da fluorescência do Trp, reduziu a formação de TBARS na LDL no plasma e nos tecidos isolados de ratos e não causou toxicidade em fatias da artéria aorta. Esses dados indicam que a IBTC pode ser considerado um promissor agente antioxidante e exibir propriedades anti-aterogênicas *in vitro*.

Palavras-Chave: LDL; estresse oxidativo, Tiossemicarbazonas; Aterosclerose; Antioxidantes.

ABSTRACT

Master Dissertation
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Federal University of Santa Maria, RS, Brazil

ANTIOXIDANT ACTIVITY EVALUATION OF ISATIN-3-N^4-BENZILTHIOSEMICARBAZONE (IBTC) ON LDL PROTECTION AGAINST OXIDATION

AUTHOR: RÔMULO PILLON BARCELOS
ADVISOR: NILDA BERENICE DE VARGAS BARBOSA
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Several lines of evidence support the hypotheses that the oxidation of low density lipoprotein (LDL) is a proatherogenic factor. Oxidative stress is one of the causes of the overproduction of reactive species that increase the formation of oxidized LDL. Thiosemicarbazones are compounds used in anticancer, antiviral and antifungal therapy; however, their redox activities have been controversial. Thus, we tested, in vitro, the possible antioxidant activity of a thiosemicarbazone derivate, the isatin-3-N^4-benzilthiosemicarbazone (IBTC). We measured the conjugated diene formation in serum and LDL induced by two oxidant agents, 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH) and Cu^{2+}, as well as the loss of tryptophan fluorescence in LDL induced by Cu^{2+}. The total reactive antioxidant potential (TRAP), Thiobarbituric acid reactive substances (TBARS) formation in human LDL and plasma and in different rat tissues was also assessed. The toxicity of IBTC was measured using aortic slices viability assay. The results show that IBTC reduced the formation of conjugated dienes AAPH and Cu^{2+}- induced increased in a dose-dependent manner the lag phase. IBTC also prevented the tryptophan fluorescence loss, and reduced the TBARS formation in LDL, plasma and rat tissues, showing no toxicity to aortic slices. These data indicate that IBTC is a good antioxidant and a promising antiatherogenic agent for further studies in vivo.

Key words: LDL; oxidative stress; Thiosemicarbazones; Atherosclerosis; Antioxidants.

LISTA DE APREVIATURAS

DCV – Doenças Cardiovasculares

DPPH – 1,1-difenil-2-picrilhidrazil

EROS – Espécies reativas de oxigênio

IBTC – Isatina-3-N^4-benziltiossemicarbazona

LDL – Lipoproteína de Baixa Densidade

MPO – Mieloperoxidase

OxLDL – LDL oxidada

SMase – Esfingomielinase

SMC – Células Musculares Lisas

sPLA-2 – Fosfolipase 2 secretada

VLDL – Lipoproteína de Muito Baixa Densidade

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APRESENTAÇÃO

No item **INTRODUÇÃO**, está descrito uma sucinta revisão bibliográfica sobre os temas trabalhados nesta dissertação.

O **DESENVOLVIMENTO** da dissertação está apresentado sob a forma de um artigo publicado na revista Life Sciences o qual se encontra alocado no item **ARTIGO CIENTÍFICO.** As seções Materiais e Métodos, Resultados, Discussão dos Resultados, Conclusão e Referências Bibliográficas, encontram-se no próprio artigo e representam a íntegra deste estudo.

Os itens **CONCLUSÕES e PERSPECTIVAS** são encontrados no final desta dissertação e apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** uma vez que o artigo científico contém as suas próprias referências.

1. INTRODUÇÃO

As doenças cardiovasculares (DCV) são as principais causas de morte na maioria dos países desenvolvidos (Hennekens et al., 1997). Em 2008, as DCV foram responsáveis por mais de 17 milhões de mortes, o que representa 30,5% de todas as mortes no mundo (World Health Organization, 2008), sendo que no Brasil representam 33% (Costa et al., 2000) e na região sul 33,5% (Cardoso et al., 2011) das causas de mortes. Aproximadamente 81% das mortes por doenças cardiovasculares são de pessoas acima de 60 anos.

Dentre as DCV destaca-se a aterosclerose, uma doença multifatorial e crônica que acomete as artérias. Esta doença é caracterizada pelo acúmulo de lipídios e elementos fibrosos nos grandes vasos e geralmente oriunda de uma resposta inflamatória dos vasos sanguíneos ao dano causado pelo início da formação da placa aterosclerótica (Li, 2005; Tracy, 1998).

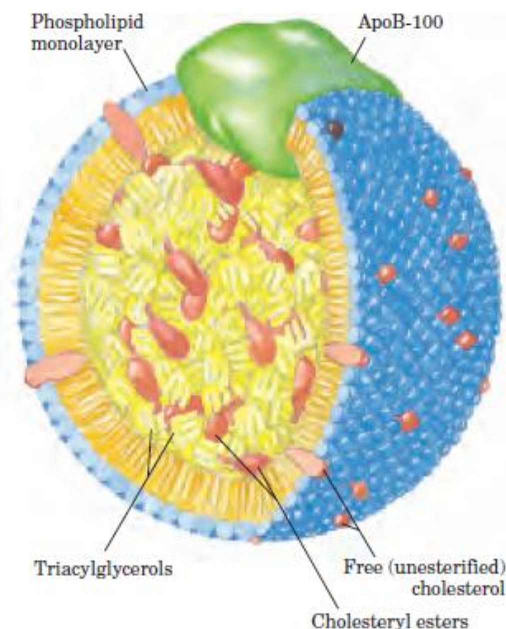

Figura 1: Lipoproteína de baixa densidade (LDL) (Nelson e Cox, 2004).

Dados epidemiológicos têm demonstrado que a aterosclerose é uma doença ligada ao envelhecimento, e que seu aparecimento prematuro pode ser ocasionado por várias condições clínicas, sendo as mais proeminentes o excesso de lipoproteínas de baixa densidade (LDL) (Figura 1), o diabetes mellitus, a hipertensão e o tabagismo (Smith et al., 2004). Muitas evidências sugerem que o processo de formação da placa aterosclerótica inicia-se na infância e progride lentamente até a vida adulta, quando ocorrerão as manifestações clínicas da doença, verificando-se,

portanto, a existência de um longo período assintomático (Františoso e Coates, 2002). Isto se dá principalmente pelo fato do processo aterogênico requerer uma prolongada exposição aos fatores de predisposição. Apenas o último estágio da doença que progride rapidamente e leva às manifestações clínicas. O processo de desenvolvimento da lesão aterosclerótica e os eventos clínicos são distintos (Françoso e Coates, 2002).

A placa aterosclerótica é caracterizada pelo acúmulo de lipídios e elementos fibrosos nas grandes artérias, sendo as mais comumente afetadas e relevantes clinicamente a aorta, a coronária, a carótida e as artérias cerebrais (Matsuura et al., 2006), que por sofrerem um espessamento gradual da camada íntima perdem a elasticidade e apresentam estreitamento do lúmen (Figura 2). Esta condição fisiopatológica reduz o fornecimento de sangue tecidual, e conseqüentemente pode causar angina pectoralis, infarto do miocárdio e acidente vascular cerebral (Libby, 2002; Matsuura et al., 2008).

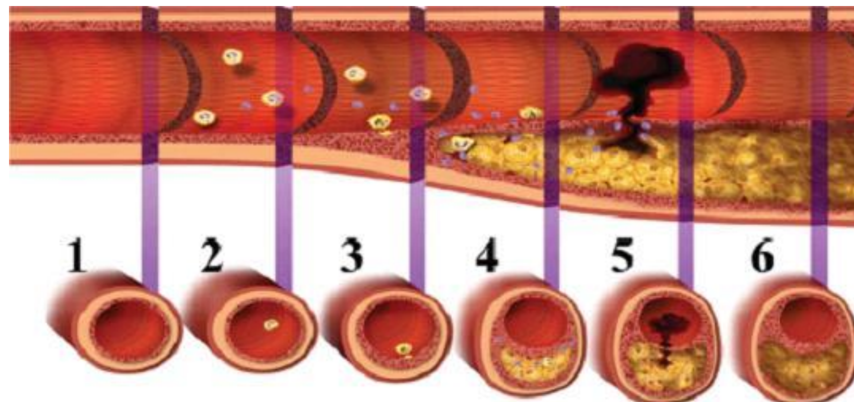

Figura 2: Depósito de gordura e diminuição do lúmen do vaso sanguíneo: 1 – artéria normal, 2 – o acúmulo de lipídios na camada mais interna da artéria. 3 – evolução para estágios iniciais da placa. 4 – formação de uma placa complexa, limitada por uma capa fibrótica. 5 – Ruptura da placa aterosclerótica. 6 – A reabsorção do trombo pode ser seguida por sua organização e aumento do volume da placa, piorando os sintomas pregressos (biompato.blogspot.com).

A aterosclerose se desenvolve gradualmente, principalmente em virtude de depósitos de colesterol, cálcio, colágeno e outros materiais que vão se depositando sobre a parede das artérias, restringindo o fluxo sangüíneo. Muitas vezes uma fissura, laceração ou ruptura de uma placa culmina com a formação de trombos que podem se desprender e ocluir artérias de menor calibre, ocasionando um infarto. A trombose produzida por uma placa é considerada uma das principais causas dos

eventos cardiovasculares que ocorrem de forma súbita e/ou aguda (Libby, 2002; Matsuura et al., 2008).

As paredes das artérias são constituídas de três camadas ou túnicas: a íntima (a camada mais interna), a média (camada intermediária) e a adventícia (a camada mais externa), conforme mostrado na figura 3 abaixo:

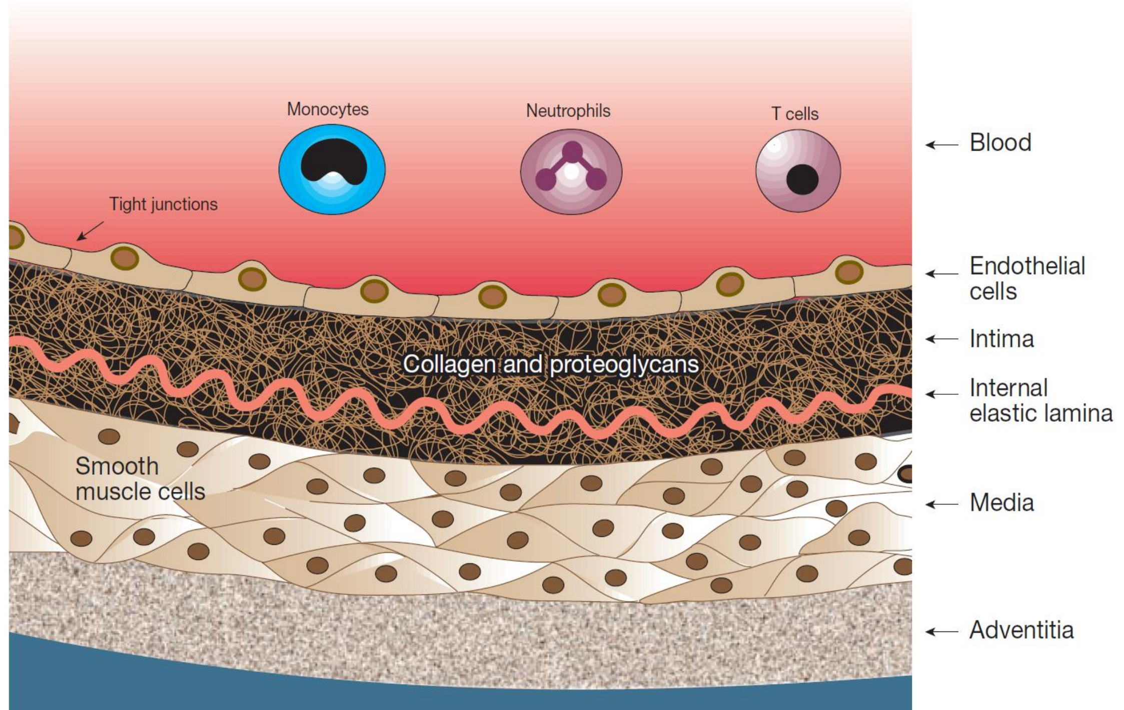

Figura 3: Estrutura de uma artéria de grande calibre. Esta artéria consiste de três camadas morfologicamente distintas. A íntima, a camada mais interna, é limitada por uma monocamada de células endoteliais em contato com a luz do vaso e pela lâmina elástica interna na periferia. A íntima normal é uma região muito delgada (tamanho exagerado nesta figura) e é constituída de matriz extracelular de tecido conectivo principalmente proteoglicanos e colágeno. A média, a camada intermediária, consiste de células musculares lisas (SMC). A adventícia, a camada mais externa, consiste de tecido conectivo com fibroblastos e SMC (Lusis, 2000).

A opinião contemporânea sobre a patogenia da doença considera a aterosclerose uma resposta inflamatória crônica da parede arterial iniciada por algum tipo de lesão ao endotélio. Os seguintes eventos são considerados fundamentais nesta hipótese: 1) desenvolvimento de regiões focais de lesão endotelial crônica, geralmente sutil, com conseqüente disfunção endotelial, como aumento da permeabilidade endotelial e aumento da adesão dos leucócitos; 2) acúmulo de lipoproteínas na parede do vaso, principalmente lipoproteína de baixa densidade (LDL), bem como lipoproteínas de densidade muito baixa (VLDL), e modificação destas lipoproteínas por oxidação; 3) adesão dos monócitos e outros leucócitos circulantes ao endotélio, seguida de migração destas células na íntima e

sua transformação em macrófagos e células espumosas; 4) adesão de plaquetas nas áreas focais ou de leucócitos aderentes; 5) liberação de fatores das plaquetas ativadas, macrófagos ou células vasculares, que induzem a migração das (células musculares lisas (SMC) da média para a íntima; 6) proliferação das SMCs da íntima e elaboração de matriz extracelular, resultando em acúmulo de colágenos e de proteoglicanos; 7) aumento do acúmulo de lipídeos tanto no interior das células (macrófagos e células musculares lisas) quanto no meio extracelular (Schoen e Cotran, 2000).

A hipótese da modificação oxidativa tem sido o foco principal da investigação desta patogênese (Ross e Glomset, 1976; Steinberg et al., 1989). A LDL é o principal transportador de colesterol na corrente sanguínea, e a concentração de colesterol – LDL está diretamente correlacionada com a incidência de DCV (Austin et al., 1988; Campos et al., 1992). O acúmulo de LDL na matriz subendotelial é o evento inicial para o desenvolvimento da aterosclerose. O acúmulo é maior quando os níveis de LDL circulante são maiores, e o transporte e a retenção de LDL estão aumentados em locais predispostos à formação da lesão.

A hipótese da modificação oxidativa da LDL como evento chave no processo aterogênico é sustentada por evidências experimentais, incluindo a presença de produtos de oxidação de lipídios e proteínas em ateromas humanos, bem como o isolamento de LDL oxidada destas lesões (Carpenter et al., 1995; Hazell et al., 1996; Leeuwenburgh et al., 1997; Niu et al., 1999; Steinbrecher e Lougheed, 1992; Suarna et al., 1995; Upston et al., 2002; Ylä-Herttuala et al., 1989) e a presença de anticorpos específicos contra estas lipoproteínas em pacientes com fatores de risco elevados (Hörkkö et al., 2000; Salonen et al., 1992). Os mecanismos pelos quais a LDL é modificada podem variar bastante. As partículas de LDL podem ser oxidadas (oxLDL) via espécies reativas de oxigênio (EROS) produzidas pelas células endoteliais e macrófagos e/ou via ação de enzimas como a mieloperoxidase, a esfingomielinase e a fosfolipase A_2 (Lusis, 2000).

Além disso, a LDL pode ser modificada pelas principais células da parede arterial como as células endoteliais, células musculares lisas e monócitos-macrófagos (Cathcart et al., 1985; Heinecke et al., 1986; Heinecke et al., 1993; Hiramatsu et al., 1987). Os oxidantes na parede arterial são gerados pela ação da NADPH oxidase, xantina oxidase, óxido nítrico sintase, mieloperoxidase e lipoxigenase, enzimas presentes na lesão aterosclerótica (Stocker e Keaney, 2005).

A LDL também pode ser modificada oxidativamente via metais de transição como ferro e cobre. Neste contexto, tem sido demonstrado que produtos de oxidação do Cu^{+2} *in vitro*, incluindo a oxLDL, apresentam atividade pró-oxidante similar àqueles oxidados *in vivo* (Steinberg et al., 1989; Steinbrecher et al., 1984; Ylä-Herttuala et al., 1989). Consequentemente, estes produtos vêm sendo amplamente usados em modelos experimentais de aterosclerose em vários estudos (Jialal e Devaraj, 1996; Rice-Evans et al., 1996). É importante destacar que durante o processo de oxidação um amplo espectro de espécies de LDL oxidadas (oxLDL) podem ser produzidas, uma vez que tanto a parte lipídica quanto a parte protéica desta lipoproteína podem ser oxidadas. Assim, estas espécies exibem diferenças estruturais e funcionais marcantes dependendo do grau da modificação oxidativa (Heinecke, 2001; Steinberg e Witztum, 2002).

Muitos estudos têm revelado que o aumento de oxLDL é um fator pró-aterogênico por desencadear um processo inflamatório (Witztum, 1993). Uma vez oxidada, a LDL pode induzir a ativação de células endoteliais e musculares-lisas; a secreção de mediadores inflamatórios e a expressão de moléculas de adesão (Witztum e Steinberg, 1991), uma seqüência de passos que culminam com o acúmulo de leucócitos no espaço subendotelial. Uma vez na camada íntima, os monócitos tornam-se macrófagos, os quais podem capturar a oxLDL via receptores scanvengers (Libby, 2002). As partículas de oxLDL são reconhecidas pelos receptores scavenger dos macrófagos (CD-36, CD-68 e SR-A) e internalizadas. Os macrófagos continuam capturando a oxLDL e acumulando uma quantidade significativa de lipídios na parede arterial (Osterud e Bjorklid, 2003), os quais induzem a liberação de citocinas pró-inflamatórias pelos macrófagos. Estes eventos também promovem o recrutamento de mais monócitos e o acúmulo de macrófagos carregados de lipídios, que é o tipo de célula mais predominante na lesão aterosclerótica inicial, chamada estria gordurosa (Osterud e Bjorklid, 2003). Assim, forma-se um ciclo vicioso de oxidação, modificação de lipoproteínas e inflamação na artéria pela presença dessas oxLDL. Isto leva a formação das chamadas células espumosas (Parthasarathy et al., 1986). A figura 4 abaixo ilustra o processo de oxidação da LDL e formação das células espumosas (foam cells):

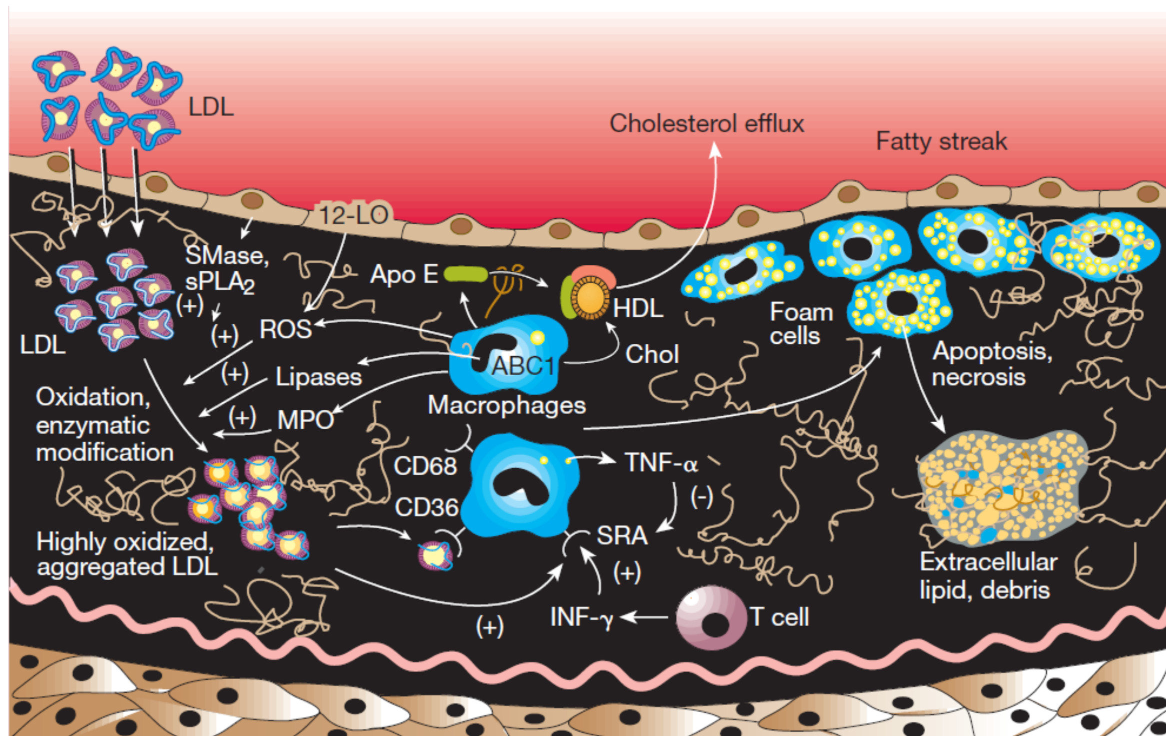

Figura 4: LDL oxidada e a formação de células espumosas (foam cells). Agregados de LDL altamente oxidadas são formados no vaso como resultado da ação de espécies reativas de oxigênio (EROS) e de enzimas como a esfingomielinase (SMase), fosfolipase 2 secretada (sPLA-2), outras lipases e mieloperoxidase (MPO). O agregado de oxLDL é reconhecido pelos receptores removedores dos macrófagos como o SR-A, CD-36 e CD-68. A expressão dos receptores removedores é mediada por citocinas com TNF-α e INF-γ. A morte das células espumosas resulta em uma massa extracelular de lipídeos e outras células necróticas (Lusis, 2000).

Considerando os eventos acima citados, verifica-se que o estresse oxidativo, um desequilíbrio no status oxidante/antioxidante celular, tem um papel importante na patogênese e/ou progressão das DCV (Dhalla et al., 2000). As espécies reativas geradas de forma excessiva podem causar danos oxidativos às biomoléculas e estruturas celulares (Augusto et al., 2002). Para neutralizar a ação das espécies reativas, as células exibem mecanismos de proteção e reparo tanto enzimáticos quanto não enzimáticos (Halliwell, 2006). No entanto, em muitas doenças a produção exacerbada de espécies reativas ultrapassa a capacidade das células de reparo e regeneração (Büyükokuroglu, 2008; Gutteridge, 1994). Neste contexto, vários trabalhos na literatura sugerem que o estresse oxidativo pode ser considerado crítico no processo de aterogênese. Uma das observações obtidas neste sentido inclui o fato de diferentes antioxidantes como, por exemplo, o butil hidroxitolueno e o N,N'-difenil-fenilenodiamina inibirem tanto a oxidação ex vivo da LDL como a aterosclerose em animais (Heinecke, 1998; Stocker, 1999).

Corroborando também esta hipótese, estão os estudos que mostram a presença de anticorpos que reagem com a oxLDL, bem como uma variedade de lipídios oxidados em placas ateroscleróticas tanto em humanos como em animais (Hultén et al., 1996; Praticò et al., 1997). Além disso, o nível de oxLDL na circulação

sanguínea é um conhecido marcador de risco para doenças cardiovasculares em humanos (Berliner et al., 1995; Parthasarathy et al., 2008; Steinberg, 1997; Steinberg, 2009). Há ainda muitas pesquisas demonstrando os efeitos benéficos oriundos do consumo de vegetais e frutas que possuem antioxidantes nas suas composições, sugerindo que antioxidantes naturais podem ter um efeito protetor sobre a aterosclerose e sobre a mortalidade e morbidade causada pelas doenças coronárias (Cherubini et al., 2005; Kardinaal et al., 1993; Rimm et al., 1993; Smith et al., 1989; Stampfer et al., 1993). Além disso, diferentes moléculas sintéticas têm sido desenvolvidas com a finalidade de exibirem um potencial efeito antiaterogênico. Probucol e moléculas análogas têm sido usadas para o tratamento da aterosclerose devido as suas ações sobre o estresse oxidativo e a inflamação (Adameova et al., 2009).

Seguindo este propósito, as tiossemicarbazonas são uma classe de moléculas bem conhecidas como quelantes de íons metálicos tais como o ferro, o cobre e o zinco (DeConti et al., 1972; French e Blanz, 1966; Pandeya et al., 1999). Sua propriedade em quelar íons metálicos depende da presença do sistema de coordenação tridentado N, N, S (doador brando) (Yu et al., 2009) e muitas das suas atividades biológicas têm sido atribuídas à formação de complexos biologicamente ativos (Kalinowski e Richardson, 2005).

As tiossemicarbazonas têm recebido considerável interesse na pesquisa devido à sua ampla utilidade farmacológica como terapias antineoplásica, antibacteriana, antiviral e antifúngica (Beraldo e Gambino, 2004; Sartorelli et al., 1971; Sartorelli e Booth, 1967) e à sua versatilidade como ligantes, o que lhes permitem dar origem a uma grande variedade de modos de coordenação (DeConti et al., 1972; French e Blanz, 1966; Pandeya et al., 1999; Pedrido et al., 2008).

Além disso, complexos metálicos de tiossemicarbazonas têm sido investigados como neutralizadores de radicais (Wada et al., 1994). Trabalhos recentes mostram que determinados compostos derivados de tiossemicarbazonas exibem uma forte atividade scavenger de radicais superóxido, hidroxil e DPPH (Zhong et al., 2010), bem como inibem a peroxidação lipídica induzida por ferro (Prathima et al., 2010). No entanto, a propriedade redox desta classe de moléculas ainda é controversa. De fato, determinadas tiossemicarbazonas são apontadas como potentes antioxidantes (Ghosh et al., 2009; Hrnciarová et al., 1998; Kaur e Ali,

1982), enquanto outras exibem propriedades pró-oxidantes (Byrnes et al., 1990; Karatas et al., 2006).

Tendo em vista que a aterogênese envolve distúrbios oxidativos, o uso de substâncias antioxidantes poderia representar uma terapia eficaz na prevenção de doenças ateroscleróticas (de Lima Portella et al., 2008). No entanto, há muita controvérsia sobre o uso de antioxidantes na prevenção da aterosclerose (Mashima et al., 2001) e isso reflete o limitado entendimento sobre a terapia com antioxidantes e também a necessidade de mais estudos nesta área. Da mesma forma, investigações sobre o possível potencial antioxidante de compostos de tiossemicarbazonas são necessárias, uma vez que existem poucos e divergentes resultados neste sentido.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral deste trabalho é avaliar a capacidade de uma molécula derivada de uma tiossemicarbazona – a isatina-3-N^4-benziltiossemicarbazona (IBTC) – em prevenir modificações oxidativas nas porções lipídicas e protéicas da LDL, mediadas por diferentes oxidantes in vitro.

2.2. Objetivos específicos

- ✓ Avaliar o efeito da IBTC na prevenção da oxidação lipídica e protéica de LDL isolada e de soro/plasma de humanos;
- ✓ Avaliar o efeito da IBTC na prevenção de lipoperoxidação induzida por nitroprussiato de sódio em diferentes tecidos de ratos;
- ✓ Determinar a atividade antioxidante total (TRAP) da IBTC;
- ✓ Verificar a toxicidade da IBTC em fatias da artéria aorta;
- ✓ Verificar a atividade quelante da IBTC frente ao Cu^{+2}.

3. DESENVOLVIMENTO

O desenvolvimento que faz parte desta dissertação está apresentado sob a forma de artigo científico. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no próprio artigo. O artigo encontra-se na formatação de publicação da revista científica Life Sciences.

ARTIGO: DERIVADO DE TIOSSEMICARBAZONA PROTEGE A LDL DA OXIDAÇÃO INDUZIDA POR AAPH E CU^{+2}.

Artigo científico publicado na revista Life Sciences, 2011

THIOSEMICARBAZONE DERIVATE PROTECTS FROM AAPH AND CU^{+2} – INDUCED LDL OXIDATION

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Thiosemicarbazone derivate protects from AAPH and Cu^{2+}-induced LDL oxidation

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ABSTRACT

Aims: Several lines of evidence support the hypotheses that the oxidation of low density lipoprotein (LDL) may play a crucial role in the initiation and progression of atherosclerosis. Oxidative stress is one of the causes of the overproduction of reactive species that increase the formation of oxidized LDL. Thiosemicarbazones are compounds used in anticancer, antiviral and antifungal therapy; however, its redox activity has been controversial. Thus, we tested, in vitro, a possible antioxidant activity of a thiosemicarbazone derivate, the isatin-3-N^4-benzilthiosemicarbazone (IBTC).
Main methods: We measured the conjugated diene formation in serum and LDL as well as the loss of tryptophan fluorescence in LDL induced by two oxidant agents, 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH) and Cu^{2+}. Thiobarbituric acid reactive substances (TBARS) formation in LDL and in different rat tissues was also assessed. The toxicity of IBTC was measured using aortic slices viability assay.
Key findings: Our results show that IBTC significantly reduced the AAPH and Cu^{2+}-induced formation of conjugated dienes, increased in a dose-dependent manner the lag phase and the $t_{1/2}$ of tryptophan fluorescence, and reduced the TBARS formation in LDL, plasma and rat tissues, showing no toxicity to aortic slices.
Significance: These results indicate that IBTC is a good antioxidant and a promising antiatherogenic agent for further studies in vivo.

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Introduction

Cardiovascular disease (CVD) is the leading cause of death in developed countries (Hennekens et al., 1997). Atherosclerosis is a response of blood vessels to injury at the beginning of the formation of an atherosclerotic plaque (Li, 2005; Tracy, 1998). Low-density lipoprotein (LDL) is a major cholesterol carrier in the bloodstream, and the concentration of LDL-cholesterol is directly correlated with the incidence of CVD (Austin et al., 1988; Campos et al., 1992). Numerous studies have revealed that increased oxidized LDL (oxLDL) acts as an atherogenic factor by triggering an inflammatory process. OxLDL can induce cell activation, secretion of inflammatory mediators, and expression of adhesion molecules (Witztum and Steinberg, 1991), that culminates with leukocyte accumulation in the subendothelial space. Notably, recruited inflammatory cells can enhance the formation of oxLDL. Once in the intima, monocytes become tissue macrophages, which can internalize oxLDL (Libby, 2002). This leads to the formation of foam cells (hallmark of early atherogenesis) (Parthasarathy et al., 1986). Furthermore, oxLDL has been shown to enhance atherogenesis by other mechanisms, such as cytotoxicity and stimulation of thrombotic and inflammatory events (Witztum, 1993).

Remarkably, oxidative stress plays an important role in the pathogenesis and/or progression of CVD (Dhalla et al., 2000). Intracellular components can be damaged by reactive species (RS) generation (Augusto et al., 2002). To remove RS, cells display several enzymatic and non-enzymatic protection and repair mechanisms (Halliwell, 2006). Several pathological conditions can be associated with an increase in RS production that surpasses the coping capacity of the cellular antioxidant system (Büyükokuroglu, 2008; Gutteridge and Halliwell, 1994).

Thiosemicarbazones are well-known metal chelators and their biological activities have been attributed to their ability to form biologically active complexes (Kalinowski and Richardson, 2005). Thiosemicarbazones have received considerable interest in research; firstly, due to their wide pharmacological utility (Beraldo and Gambino, 2004; Sartorelli et al., 1971). Secondly, their versatility as ligands allows them to generate a great variety of coordination modes (Pandeya et al., 1999; Pedrido et al., 2008). Moreover, thiosemicarbazone metal complexes have also been investigated as radical scavengers (Wada et al., 1994).

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However, their redox properties have been controversial; some studies report them to be antioxidant compounds (Ghosh et al., 2009; Hrnciarová et al., 1998) while other studies report them to be oxidative compounds (Byrnes et al., 1990; Karatas et al., 2006).

A possible method to prevent atherosclerotic diseases would be through the administration of antioxidants (de Lima Portella et al., 2008). However, there is some controversy regarding the usefulness of antioxidant therapy for the prevention of atherosclerosis (Mashima et al., 2001), and this reflects our limited understanding of the therapeutic use of antioxidants and the need for further studies. To the best of our knowledge, there is little research on the redox activity of thiosemicarbazones, and therefore, their potential as antioxidants must be explored. Thus, this study was conducted to test the thiosemicarbazone isatin-3-N^4-benzilthiosemicarbazone (IBTC) in vitro and ex vivo in order to determine if IBTC might be useful against LDL oxidation and represent a possible therapeutic drug for testing in future atherosclerosis studies.

Materials and methods

Synthesis of isatin-3-N^4-benzilthiosemicarbazone

The synthesis of isatin-3-N^4-benzilthiosemicarbazone (IBTC) is described by (Fonseca et al., 2010). The chemical structure of IBTC is shown in Fig. 1.

Animals

Adult Wistar rats (200–250 g) were obtained from the Federal University of Santa Maria and maintained in an air conditioned room (20–25 °C) under natural lighting conditions with water and food ad libitum. All the experimental procedures performed were conducted according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

LDL isolation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by Luiz da Silva et al. (1998), with few modifications. Briefly, plasma of non-fasted healthy normolipidemic voluntary donors was collected and pooled with EDTA (1 mg/mL). Sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of the EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then, 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on the top of the plasma. Ultracentrifugation was run at 350,000 g for 2 h at 4 °C, in a Himac CP80MX ultracentrifuge. LDL particles were collected by the aspiration of the yellow/orange band at the center of the saline layer and dialyzed exhaustively overnight at 4 °C with 10 mM phosphate buffer (pH 7.4). Protein concentration in LDL solution was determined by Lowry's method (Lowry et al., 1951). The purity of LDL preparation was verified by agarose gel electrophoresis (>98%). Isolated LDL was stored at −20 °C for no longer than 2 weeks.

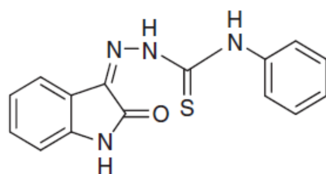


Fig. 1. Chemical structure of IBTC.

LDL oxidation

Conjugated dienes and TBARS formation

LDL samples (50 μg protein/mL) were pre-incubated at 37 °C in a medium containing 10 mM phosphate buffer (pH 7.4) and different IBTC concentrations (see figure legends). After 5 min, the oxidation was initiated by the addition of $CuSO_4$ (5 μM) or 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH) (1 mM). The oxidation was monitored by measuring the increase in absorbance at 234 nm due to conjugated diene (CD) formation as previously described (Esterbauer et al., 1989). Aliquots were also removed at different time points for evaluating thiobarbituric acid reactive substances (TBARS) production as previously described (Ohkawa et al., 1979).

Determination of lag phase and maximum oxidation rate

In the studies of CD formation, there are several parameters which can be obtained from diene vs. time profiles. The value of the lag phase is commonly determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption. Another parameter is the maximum oxidation rate, given by the peak of the first derivative, i.e., change of A234 as a function of time (Gieseg and Esterbauer, 1994). These parameters were calculated for CD formation on LDL, using IBTC and also commonly used antioxidants compounds as Vitamin C, Vitamin E (Duthie and Bellizzi, 1999), Quercetin (Cushnie and Lamb, 2005; Hanasaki et al., 1994) and Resveratrol (Kimura et al., 1983; Pace-Asciak et al., 1996) at the same concentrations as the ones used for IBTC. EC50 for lag phase or maximum CD formation for IBTC and for these antioxidants compounds was obtained in order to compare the antioxidant activity of these compounds versus IBTC.

Measurement of LDL-tryptophan fluorescence

The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the tryptophan (Trp) residues in ApoB-100 (Giessauf et al., 1995). Loss of tryptophan fluorescence is a marker for oxidations at the protein core of LDL (Giessauf et al., 1995; Reyftmann et al., 1990). Tryptophan (Trp) fluorescence was measured in a solution of LDL (50 μg protein/mL) in PBS (10 mM), pH 7.4 at 37 °C, using a Shimadzu espectrofluorometer (excitation at 282 nm and emission at 331 nm) (Giessauf et al., 1995; Reyftmann et al., 1990). The kinetics of LDL oxidation was followed by measuring the decrease of Trp-fluorescence, corresponding to the decomposition of this amino acid, after the addition of 5 μM $CuSO_4$ in the absence or presence of IBTC. The cuvettes had to be removed from the excitation light between the single measurements to avoid photooxidation of the tryptophan residues; fluorescence was measured every 20 min. Data are shown as percentage of the decrease of Trp fluorescence in each sample. The time required for reaching half Trp fluorescence ($t_{1/2}$) was calculated.

Serum oxidation

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1000 g for 15 min. Serum diluted 100-fold was incubated at 37 °C in a medium containing 10 mM phosphate buffer (pH 7.4) and different IBTC concentrations (see figure legends). The oxidation was initiated by the addition of $CuSO_4$ (30 μM) and CD formation was monitored at 245 nm as previously described (Schnitzer et al., 1998).

Plasma oxidation

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing anticoagulant (sodium citrate) and centrifuged at 1000 g for 15 min. Plasma was

removed and immediately used in the oxidation assays. Briefly, plasma samples were diluted 1:4 in 10 mM potassium phosphate buffer, pH 7.4 and incubated at 37 °C with $CuSO_4$ (100 μM) (de Bem et al., 2008) and/or IBTC (1–20 μM). The total volume was 9 mL. At different time points, aliquots (200 μL) were removed to evaluate TBARS levels. Serum TBARS levels were determined based on Ohkawa et al. (1979).

Total reactive antioxidant potential (TRAP) assay

TRAP was determined by measuring the intensity of luminol chemiluminescence induced by AAPH thermolysis in a luminometer BioTek Synergy 2 (Lissi et al., 1992). The reaction mixture contained 300 μL of 10 mM AAPH and 10 μL of 1 mM luminol dissolved in 0.1 M glycine buffer (pH 8.6). Incubation of this mixture produces a nearly constant light intensity at room temperature after stabilization. IBTC was added at different concentrations to determine the TRAP activity. At this point, the intensity of luminescence is practically abolished. As time goes by there is loss of antioxidant capacity of IBTC and the luminescence intensity returns to the initial values. The area under the curve (AUC) was evaluated for each IBTC concentration and compared to vehicle AUC (Dresch et al., 2009).

Lipid peroxidation assay

Lipid peroxidation was determined by measuring TBARS as described by Ohkawa et al. (1979) from liver, kidney and brain homogenates. Aliquots of 200 μL of liver, kidney and brain homogenates were mixed to incubating medium containing Tris–HCl (0.01 mM), IBTC at indicated concentrations (see figure legends) and the oxidant sodium nitroprusside (SNP) 5 μM was added in order to start the reaction, then it was incubated at 37 °C for 60 min. The reaction was stopped by adding 0.5 mL of acetic acid buffer, and lipid peroxidation products were measured by the addition of 0.5 mL of Thiobarbituric Acid (TBA) 0.6%. Tubes were then incubated in boiling water for 60 min before spectrophotometric analysis. TBARS levels were measured at 532 nm using a standard curve of Malondialdehyde (MDA). Considering the variation in the tissues response to SNP and

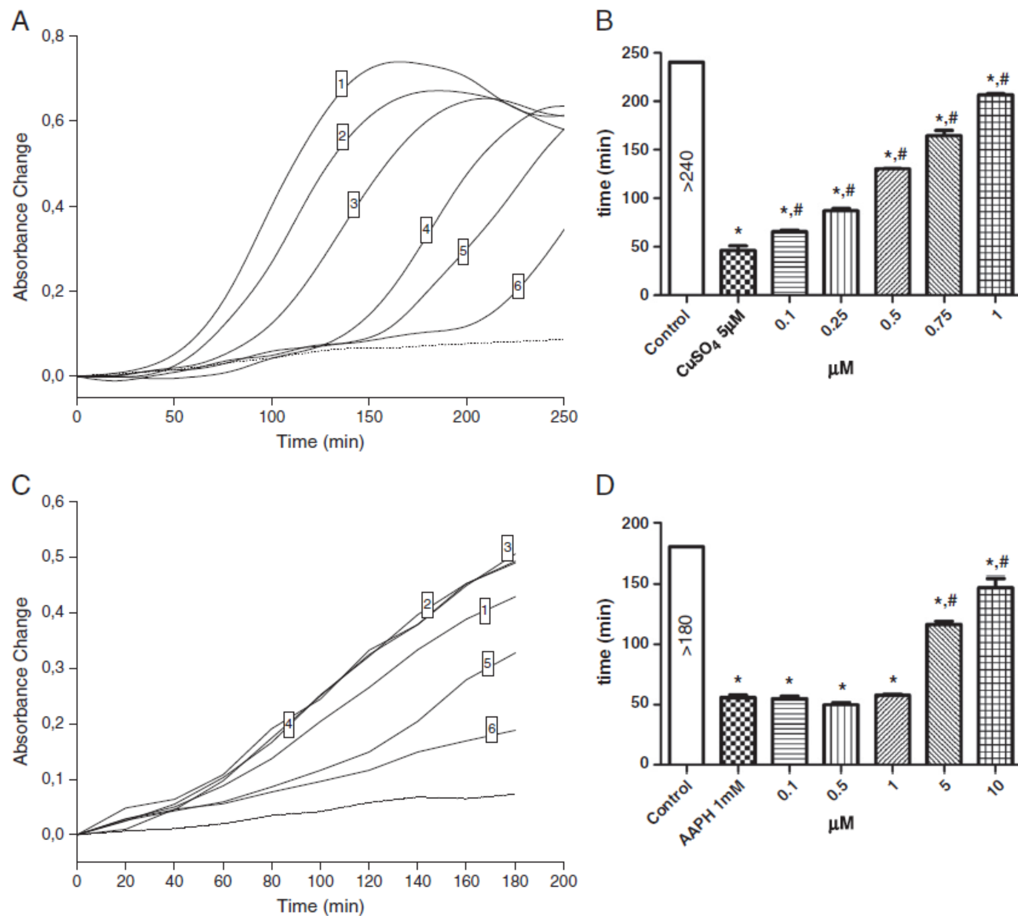


Fig. 2. Effect of IBTC on conjugated dienes formation. LDL was incubated in the presence of $CuSO_4$ (A and B) or AAPH (C and D). [A] Representative figure of the dienes formation. The incubation medium without (1) or with 0.1 μM (2), 0.25 μM (3), 0.5 μM (4), 0.75 μM (5) or 1 μM (6) of IBTC and $CuSO_4$. [B] The effect of IBTC on lag phase values for conjugated dienes formation in isolated human LDL. [C] Representative figure of the dienes formation. The incubation medium without IBTC (1) or with 0.1 μM (2), 0.5 μM (3), 1 μM (4), 5 μM (5) or 10 μM (6) of IBTC and AAPH. [D] Effect of IBTC on lag phase values for conjugated dienes formation in isolated human LDL. The values are expressed as mean ± SEM of four independent experiments in duplicate. Conjugated dienes were measured by determining the absorbance at 234 nm every 20 min. ">180 min" indicates a lag phase higher than the time assay. Dotted line represents the control without $CuSO_4$/AAPH and IBTC. * indicates statistical difference from control group ($p<0.05$). # indicates statistical difference from $CuSO_4$ group ($p<0.05$).

Table 1
Effect of IBTC on the oxidation rate for conjugated dienes formation in human LDL.

Conjugated dienes	
Oxidation rate (Δabs/20 min)	
Control	ND
$CuSO_4$ 5 μM	0.182 ± 0.0131
0.1 μM	0.166 ± 0.049
0.25 μM	0.164 ± 0.0381
0.5 μM	0.157 ± 0.0106
0.75 μM	ND
1 μM	ND

LDL was oxidized in the presence of $CuSO_4$ 5 μM, and different concentrations of IBTC. The values are expressed as mean ± S.D. of four independent experiments in duplicate. ND: not determined.

IBTC, the results are presented as the variation of induced lipid peroxidation in the different groups as compared to the variation in the control group (Δ% Control) as described below:

$$A = B - C \quad (1)$$

This equation indicates the levels of MDA produced by SNP-induction without IBTC in the different tissues tested. Where:

A nmol MDA/mg of protein induced by SNP
B nmol MDA/mg of protein in the presence of SNP (Control induced Group)
C nmol MDA/mg of protein without SNP (General control group)

The A value obtained was considered as the maximum level on MDA produced by SNP for each tissue and group tested without IBTC.

$$D = E - F \quad (2)$$

This equation indicates the levels of MDA produced by SNP in the presence of different IBTC concentrations in the different tissues tested. Where:

D nmol MDA/mg of protein induced by SNP in the presence of IBTC.
E nmol MDA/mg of protein induced by SNP in the presence of IBTC.
F nmol of MDA/mg of protein with IBTC and without SNP.

The D value obtained was considered as the maximum level of MDA produced by SNP for each group tested in the presence of IBTC.

$$\Delta\% \text{ Control} = (D \times 100) / A \quad (3)$$

Table 2
Effect of IBTC on the oxidation rate for conjugated dienes formation in human LDL.

Conjugated dienes	
Oxidation rate (Δabs/20 min)	
Control	ND
AAPH 1 mM	0.0677 ± 0.0038
0.1 μM	0.074 ± 0.0044
0.5 μM	0.0727 ± 0.0031
1 μM	0.0807 ± 0.0067
5 μM	0.0643 ± 0.0025
10 μM	0.034 ± 0.0057*

LDL was oxidized in the presence of AAPH 1 mM, and different concentrations of IBTC. The values are expressed as mean ± S.D. of four independent experiments in duplicate. ND: not determined.
* Indicates statistically difference from AAPH group ($p<0.05$).

Table 3
Effect of IBTC, Vitamin C, Vitamin E, Quercetin and Resveratrol on the lag phase and maximum formation for conjugated dienes formation in human LDL.

	EC_{50} (μM)	
	Lag phase	Max formation
	$CuSO_4$ 5 μM	AAPH 1 mM
IBTC	0.345 ± 0.018	9.687 ± 0.503
Vitamin C	0.303 ± 0.022*	15.9 ± 2.35
Vitamin E	>1	>20
Quercetin	0.279 ± 0.003*	17.8 ± 3.82
Resveratrol	>1	>20

LDL was oxidized in the presence of $CuSO_4$ 5 μM or AAPH 1 mM. The values are expressed as mean ± S.D. for EC50 of four independent experiments in duplicate.
* Indicates statistically difference from IBTC group ($p<0.05$).

Chelating activity of IBTC

The copper chelating capacity of IBTC was assessed by spectral shifts produced when copper ions were incubated with the test compound as described by Turchi et al.(2009). The reaction mixture consisted of 2 mL of phosphate buffer saline (PBS, 10 mM, pH 7.4), 20 μL of IBTC solution (in DMSO, final concentration of 37.5, 75, 150 and 300 μM) and 50 μL of $CuSO_4$ solution (in distilled water, final concentration 150 μM). Spectra (200–600 nm) were recorded immediately after preparing the mixtures and again after 15 min, in the presence and absence of copper ions.

Aortic slices viability

Adult Wistar rats were decapitated, thoracic aorta were quickly dissected and then removed, rinsed and submerged in a petri dish filled with ice-cold isosmotic phosphate buffer (NaCl 124 mM, Na_2HPO_4 10 mM, NaH_2PO_4 5 mM, KH_2PO_4 5 mM, glucose 10 mM, pH 7.4) and cleaned of adherent fat and connective tissue in an ice-bath (de Bem et al., 2008). Slices were normalized by weight and incubated with IBTC concentrations (1–100 μM). After 30 min slices were washed three times with isosmotic phosphate buffer. Immediately after incubation with treatments, slices were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test for 30 min (37 °C) (Cordova et al., 2004). After incubation, aorta slices were washed with isosmotic phosphate buffer three times (pH 7.4).

MTT is converted into a purple formazan after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of DMSO, resulting in a colored compound

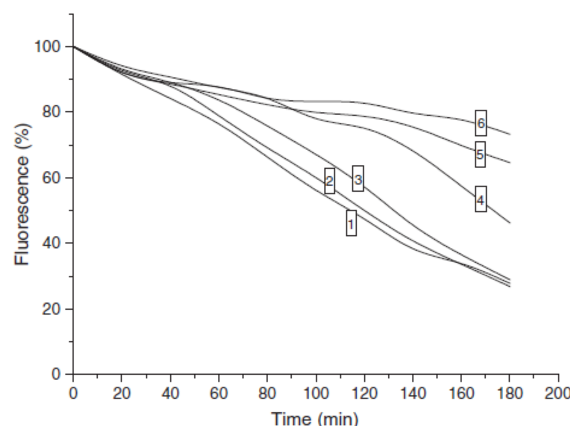


Fig. 3. Effect of different IBTC concentrations on decreased Trp fluorescence mediated by $CuSO_4$. LDL was incubated in PBS with 5 μM $CuSO_4$. The incubation medium without IBTC (1) or with 0.1 μM (2), 0.25 μM (3), 0.5 μM (4), 0.75 μM (5) or 1 μM (6) of IBTC. Fluorescence (Ex/Em = 282/331 nm) was measured at intervals of 20 min at 37 °C.

Table 4
Effect of IBTC on the oxidation $t_{1/2}$ values for Trp fluorescence in human LDL.

	$T_{1/2}$ Trp fluorescence
	Mean ± S.D. (min)
$CuSO_4$ 5 μM	123.565 ± 8.995
0.1 μM	131.217 ± 9.409
0.25 μM	144.593 ± 13.943
0.5 μM	> 180*
0.75 μM	> 180*
1 μM	> 180*

LDL was oxidized in the presence of $CuSO_4$ 5 μM, and different concentrations of IBTC. The values are expressed as mean ± S.D. of four independent experiments in duplicate. ">180 min" indicates a lag phase higher than the time assay.
* Indicates statistically difference from $CuSO_4$ group ($p<0.05$).

whose optical density, following by a centrifugation for 1000 g for 5 min, the supernatants were read at 570 nm with spectrophotometer (Liu et al., 1997). This data are expressed as ABS/mg of tissue.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using a one-way and two-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test when appropriate. In addition, linear regression was performed to identify a possible dose dependent effect. Values of $p<0.05$ were considered significant.

Results

Effect of IBTC on LDL oxidation

Conjugated diene formation in LDL

The formation conjugated dienes (CDs) during Cu^{2+}-induced LDL oxidation was significantly reduced when IBTC was added at all concentrations tested ($p<0.05$, Fig. 2A) (IC50 = 0.46 ± 0.006 μM). This formation is also shown in Fig. 2B, where the IBTC caused a concentration-dependent increase of the lag phase of CDs formation ($r^2=0.967$, $p<0.001$). The oxidation rate was not significantly different at any concentration of IBTC (Table 1).

When AAPH was used as an oxidation reagent, the formation of CDs was significantly reduced only at the two highest concentrations of IBTC (Fig. 2C) (IC50 = 0.60 ± 0.012 μM). IBTC caused a concentration-dependent increase in the lag phase at higher concentrations of −5 μM and 10 μM–($r^2=0.908$, $p<0.001$) (Fig. 2D). The oxidation rate was significantly reduced at 10 μM IBTC ($p<0.05$, Table 2).

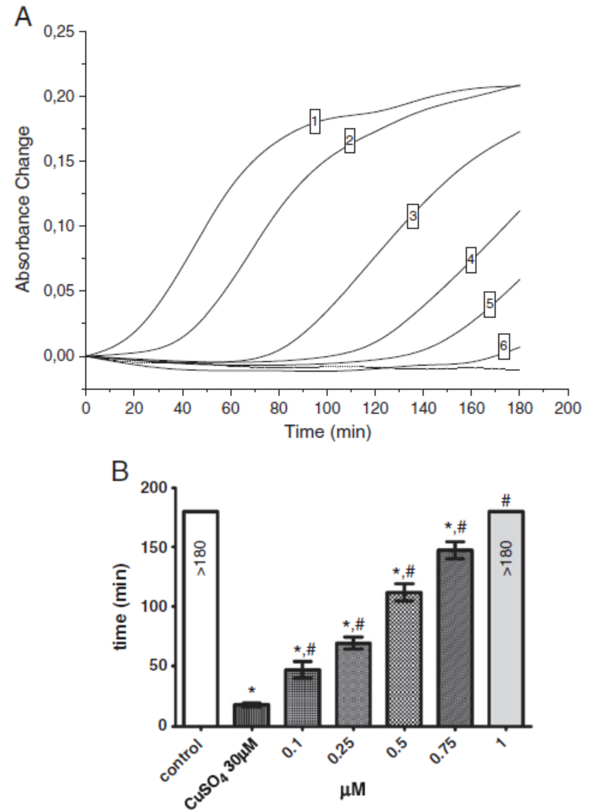


Fig. 5. Effect of IBTC on conjugated dienes formation in serum induced by copper. Serum (diluted 100-fold) was incubated in presence of $CuSO_4$. [A] Representative figure of the dienes formation. The incubation medium without (1) or with 0.1 μM (2), 0.25 μM (3), 0.5 μM (4), 0.75 μM (5) or 1 μM (6) of IBTC. [B] The effect of IBTC on lag phase values for conjugated dienes formation in isolated human LDL. Conjugated dienes were measured by determining the absorbance at 245 nm every 20 min. The values are expressed as mean ± SEM of six independent experiments in duplicate. ">180 min" indicates a lag phase higher than the time assay. Dotted line represents the control without $CuSO_4$ and/or IBTC.* indicates statistical difference from control group ($p<0.05$). # indicates statistical difference from $CuSO_4$ group ($p<0.05$).

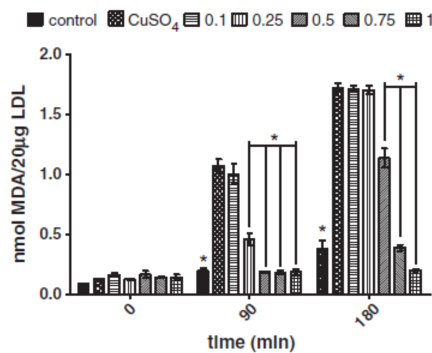


Fig. 4. Effect of IBTC concentrations on time-dependent changes in TBARS production during LDL oxidation induced by $CuSO_4$. LDL was incubated at 37 °C in the presence of 5 μM $CuSO_4$. Data are expressed as mean ± SEM of four experiments in duplicate. The values are expressed by nmol MDA/20 μg of LDL. Numbers in the legends indicate the IBTC concentration in μM. * indicates statistical difference from $CuSO_4$ group ($p<0.001$).

Table 5
Effect of IBTC on the oxidation rate for conjugated dienes formation in human serum.

	Conjugated dienes
	Oxidation rate (Δabs/20 min)
Control	ND
$CuSO_4$ 30 μM	0.0692 ± 0.0176
0.1 μM	0.061 ± 0.0148
0.25 μM	0.0634 ± 0.0197
0.5 μM	0.065 ± 0.0116
0.75 μM	0.06 ± 0.0112
1 μM	ND

LDL was oxidized in the presence of $CuSO_4$ 30 μM, and different concentrations of IBTC. The values are expressed as mean ± S.D. of six independent experiments in duplicate. ND: not determined.

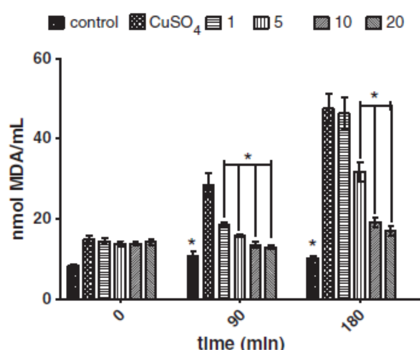


Fig. 6. Effect of IBTC concentrations on time-dependent changes in TBARS production during plasma oxidation. Plasma (diluted 1:4) was incubated at 37 °C in the presence of 100 μM $CuSO_4$. Data are expressed as mean ± SEM of four experiments in duplicate. The values are expressed by nmol MDA/mL. Numbers in legends indicate the IBTC concentration in μM. * indicates statistical difference from $CuSO_4$ group ($p<0.001$).

IBTC was compared with other antioxidants to determine if the effects of IBTC on the EC50 for the CD lag phase and the maximum CD formation were similar to those of naturally occurring popular antioxidants. The EC50 for IBTC was significantly higher than the EC50 values for quercetin or vitamin C, when studied for the CD lag phase in Cu^{2+}-induced LDL oxidation. However, there were no significant differences in EC 50 values among IBTC, vitamin C, and quercetin for the maximum formation of CD in AAPH-induced LDL. The EC50 values for vitamin E and resveratrol could not be determined in our experimental protocol (Table 3).

Effect of IBTC on LDL tryptophan fluorescence

The loss of tryptophan (Trp) fluorescence in LDL during the Cu^{2+}-induced LDL oxidation is shown in Fig. 3. When LDL was incubated with different concentrations of IBTC, there was a significant increase in $t_{1/2}$ of Trp at the three highest IBTC concentrations (0.5, 0.75 and 1 μM, Table 4), prolonging it to >180 min.

Effect of IBTC on TBARS formation in LDL

TBARS formation in LDL is shown in Fig. 4. In the presence of IBTC at concentrations of 0.25, 0.5, 0.75, and 1 μM, TBARS formation was significantly reduced compared to $CuSO_4$ (5 μM) during a 90 min time course of LDL oxidation ($p<0.001$). When the time course of Cu^{2+}-induced LDL oxidation reached 180 min, IBTC at concentrations of 0.5, 0.75, and 1 μM significantly reduced TBARS formation ($p<0.001$).

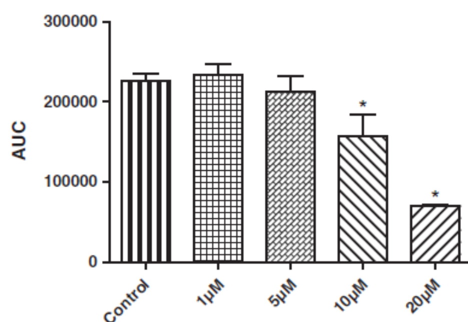


Fig. 7. Effect of IBTC on total radical-trapping antioxidant potential (TRAP). Columns represent mean ± SEM of four experiments in duplicate. The values are expressed by AUC. * indicates statistical difference from control group ($p<0.001$).

Effect of IBTC on blood oxidation

Conjugated diene formation in serum oxidation. Fig. 5A shows the formation of CDs in serum using 30 μM $CuSO_4$ as the oxidation agent. The CD formation was significantly reduced ($p<0.05$) when IBTC was added to all tested concentrations (IC50 = 0.46, ± 0.013 μM). The compound caused an increase in the CD lag phase in a concentration-dependent manner ($r^2=0.958$, $p<0.001$, Fig. 5B). The oxidation rates were the same among different groups (Table 5).

Effect of IBTC on TBARS formation from plasma oxidation

Fig. 6 shows TBARS formation from Cu^{2+}-induced plasma oxidation. IBTC (1, 5, 10, and 20 μM) significantly reduced TBARS formation at 90 min compared to 100 μM $CuSO_4$ ($p<0.001$). At 180 min of Cu^{2+}-induced plasma oxidation, IBTC (5, 10, and 20 μM) significantly reduced TBARS formation ($p<0.001$).

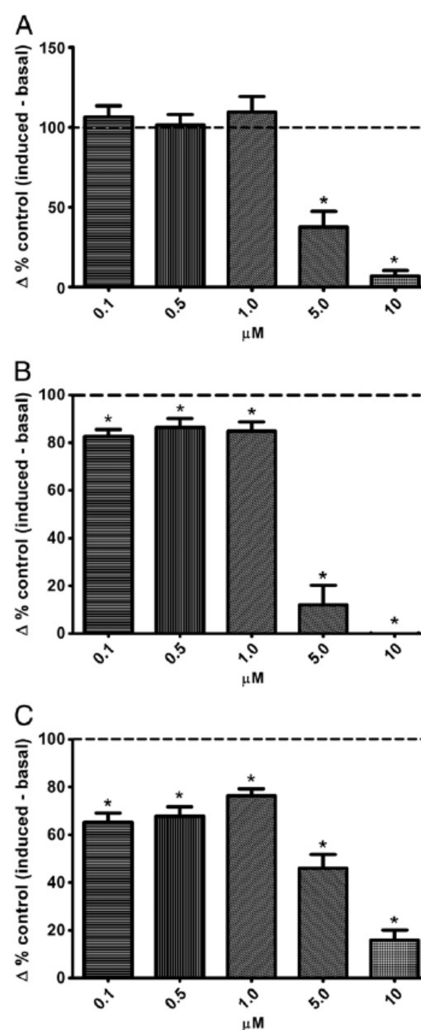


Fig. 8. Effect of IBTC on liver [A] kidney [B] and brain [C] homogenates on TBARS production. 5 μM Sodium nitroprusside (SNP) was used as oxidant to induce TBARS production. TBARS are expressed as Δ% Control (induced − basal). The values are expressed as mean ± SEM of four independent experiments in duplicate. Dotted line represents the control group (100% induced). * indicates statistical difference from control group ($p<0.001$).

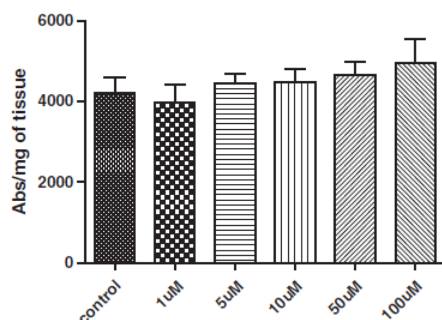


Fig. 9. Aortic slices viability. Aortic slices were incubated in presence of IBTC (1, 5, 10, 50 and 100 μM), and viability was measured by MTT assay. The data are expressed as ABS/mg of tissue. The values are mean ± SEM of six independent experiments in duplicate ($p<0.05$).

Effect of IBTC on TRAP assay and chelating activity

Fig. 7 shows the antioxidant potential of IBTC when AAPH was used as an oxidizing agent. Concentrations of IBTC ranging from 10 to 20 μM showed a significant effect on total radical-trapping antioxidant potential. No chelating activity was detected between IBTC and $CuSO_4$ when they were incubated together (data not shown).

Effect of IBTC on SNP-induced lipid peroxidation in rat tissues

The TBARS formation induced by SNP (5 μM) in liver homogenate is shown in Fig. 8A. IBTC significantly reduced TBARS formation at 5 and 10 μM IBTC ($p<0.001$). In kidney (Fig. 8B) and brain (Fig. 8C) homogenates, all IBTC concentrations significantly reduced TBARS formation induced by 5 μM SNP ($p<0.001$).

Aortic slices viability

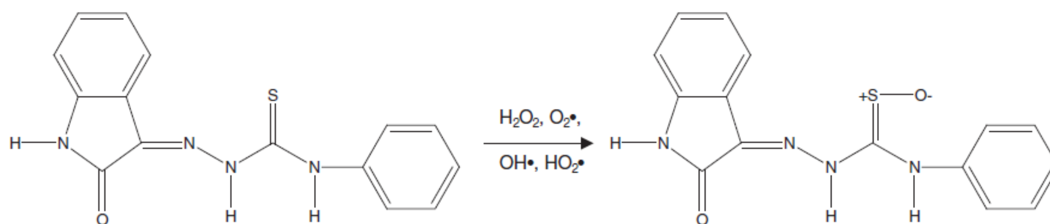
Aortic slices in the presence of IBTC at concentrations of 1 to 100 μM did not show any changes in the MTT assays compared to control, and there are no significant differences among groups (Fig. 9) ($p<0.05$).

Discussion

There are controversial studies concerning the use of antioxidants as an alternative therapy for atherosclerotic prevention (Mashima et al., 2001), and these conflicting findings may reflect our limited understanding about the LDL oxidation process in atherogenesis and about how antioxidants protect from CVD. To investigate a possible atheroprotective property of IBTC, and to provide knowledge regarding IBTC in this therapeutic strategy, we tested IBTC's antioxidant properties in LDL and serum when they were exposed to different oxidants. Our results showed that IBTC was able to reduce the formation of CDs and increase the CD lag phase in concentration-dependent manners when either AAPH or $CuSO_4$ was used as the LDL oxidizing agent. The results were similar when we tested IBTC in human serum under the same conditions. There was an increase in $t_{1/2}$ of Trp fluorescence of Cu^{2+}-oxidized LDL in the presence of IBTC. IBTC produced an interesting result in the TRAP assay, indicating its role as a ROS scavenger. The TBARS formation in LDL and plasma was reduced when IBTC was added to the reaction medium and SNP-induced lipid peroxidation was also reduced when IBTC was present. The assay of aortic slices viability showed that IBTC has no cytotoxicity in aortic slices.

There is evidence suggesting that an elevated plasma copper concentration is associated with existing atherosclerosis and represents a risk factor (Ferns et al., 1997). Catalytically active copper ion has been found in gruel taken from human advanced atherosclerotic lesions, indicating that the interior of these lesions is a highly oxidative environment. Consequently, the use of copper ions to promote peroxidation of LDL under in vitro conditions can be considered a valid model to study events occurring in the atherosclerotic arterial wall (Smith et al., 1992). In addition, we used AAPH, a hydroxyl radical generator, which generates peroxyl radicals at a constant rate in aqueous medium and induces the chain oxidation of human LDL by a free radical mediated mechanism (de Bem et al., 2008). AAPH has also been reported as a good alternative for mimicking oxidative insults that occur to lipid and protein moieties of LDL during the atherogenic process (Atkin et al., 2005). The study of CD formation in LDL oxidized by copper is often used to characterize the atherogenicity of LDL or the therapeutic usefulness of antioxidants (Esterbauer et al., 1989; Matsuura et al., 2008; Stocker and Keaney, 2004; Zolese et al., 2005). The lag phase determination, which represents the intrinsic resistance of LDL to oxidation, is widely used in studies to test the activity of possible antiatherogenic compounds (Gieseg and Esterbauer, 1994; Witztum, 1994). Furthermore, the protective effect of IBTC against LDL oxidation can occur via several mechanisms, such as (A) scavenging of peroxyl radicals, thus breaking the peroxidation chain reaction, (B) chelating free Cu^{2+} to form redox-inactive complexes and thus reducing metal-catalyzed oxidation of LDL, and (C) inhibiting the binding of Cu^{2+} to apolipoproteins and subsequently preventing the modification of amino acid-apoB protein residues (Berrougui et al., 2006).

In our study, we observed that IBTC could prolong, in a concentration-dependent manner, the lag phase in CD formation when $CuSO_4$ or AAPH were used as oxidizing agents. The increase in the lag phase for copper-induced LDL oxidation by antioxidants has already been described (Wen et al., 1999). Vitamin E, an endogenous antioxidant, is known to prevent LDL oxidation by donating the hydrogen atom of its phenolic hydroxyl group to a lipid peroxyl radical (Meydani, 1995; Seppo et al., 2005). Vigna et al. (2003) showed that the increase in the lag phase and the reduction of the lipid peroxide formation are probably related to antioxidant properties or to mechanisms involving ion (Cu^{2+}) chelation. Notably, the reduction of CD formation by IBTC is clearly observed in Fig. 2A and C ($CuSO_4$ and AAPH-induced CD formation, respectively). Considering that TRAP indirectly measures the content of non-enzymatic antioxidant defenses (Lissi et al., 1995), our results support the hypothesis that IBTC has ROS scavenging activity (Fig. 7). Additionally, we clearly demonstrate that IBTC possesses antioxidant activity in levels that could be compared to vitamin C



Scheme 1. Possible IBTC antioxidant mechanism.

and quercetin (Table 3), thus, indicating that IBTC could act as an antioxidant molecule and its potential could be compared to other naturally occurring antioxidants. Moreover, the concentration of IBTC used in this study was much lower than the copper concentration, indicating that Cu^{2+} chelation could not be the likely mechanism of IBTC. In fact, we did not detect Cu^{2+} chelating activity by IBTC in our tests, even when using concentrations higher than those for $CuSO_4$ (data not shown). Based on our results, we believe that IBTC produces its antioxidant activity via free radical scavenging rather than by chelating Cu^{2+}.

Searching for modifications caused by IBTC in the protein moieties of LDL, we observed that IBTC prevented the Cu^{2+}-induced loss of Trp fluorescence in LDL. Intrinsic ApoB-100 fluorescence has been widely used to monitor protein oxidative damage in Cu^{2+}-induced oxidized LDL (de Lima Portella et al., 2008; Giessauf et al., 1995). It has been reported that the fluorescence spectrum of native LDL is associated with Trp residues in apo-B, and Trp fluorescence is a marker for oxidation occurring at the protein core of LDL (Reyftmann et al., 1990). Degeneration of Trp is an early event in Cu^{2+}-mediated LDL oxidation and plays an important role in initiating lipid oxidation in the LDL particle (Giessauf et al., 1995). Apo-B is wrapped around the outer surface of the LDL molecule with hydrophobic segments immersed in the hydrocarbon chain region of the phospholipid monolayer, making these hydrophobic domains more susceptible to oxidative attack (Prassl et al., 1998). Thus, when the mechanism by which LDL is oxidized is considered, some elements, including lipid–apo-B interactions and physicochemical properties of LDL must be considered, because they affect the oxidation behavior and consequently the mechanism involved (Abuja et al., 1999; Prassl et al., 1998). Considering this, our results show that IBTC exhibited a protective effect on AAPH and Cu^{2+}-induced Trp oxidation, corroborating the hypotheses that IBTC may be acting as an antioxidant molecule, thus avoiding the oxidation and degeneration of Trp in apo-B.

Formation of lipid hydroperoxides in LDL is accepted as an early event in LDL oxidation (Girotti, 1998), because these lipid hydroperoxides can up-regulate the uptake of LDL into the cells (Kawamura et al., 1994). Remarkably, we showed that IBTC decreased TBARS formation in human LDL at higher concentrations after 90 min (partially oxidized-LDL) and 180 min (completely oxidized-LDL) of incubation. According to this evidence, IBTC may interfere with LDL oxidation by interacting with ApoB-100, thus avoiding lipid hydroperoxide oxidation. We suggest that the effect exerted by IBTC against lipid peroxidation of human plasma LDL may be of pathophysiologic relevance.

Spranger et al. (1998) suggested that LDL oxidation in vivo can be more adequately tested in vitro using the whole plasma/serum, because LDL in vivo occurs in the interstitial space of the arterial wall, where it may be shielded from various antioxidants present in plasma/serum (Schwartz et al., 1991; Witztum and Steinberg, 1991). For this reason, we performed assays using serum in conditions similar to the assays conducted using isolated LDL. Notably, the Cu^{2+}-induced CD formation was reduced and the lag phase was significantly increased in a concentration-dependent manner in the presence of IBTC. When we tested TBARS formation in plasma, we found the same result as that obtained using the isolated LDL. Moreover, the IBTC protection from serum oxidation was greater than the protection from LDL oxidation. This result could be due in part to the presence of other antioxidants in plasma/serum (de Lima Portella et al., 2008).

Besides, aortic slice viability assay using MTT indicates that IBTC has no cytotoxic effect in aortic slices at the concentrations used in our experiments, even using a concentration 100 times higher than the effective concentrations used in our previous tests.

When IBTC was tested with tissue homogenates, we observed that the compound reduced lipid peroxidation, as confirmed by reduced levels of TBARS. Different IBTC concentrations were required for an effective decrease in TBARS production among the tissues tested. This finding that may be related to different capacities of these tissues to respond against an oxidizing agent (Puntel et al., 2009).

Taken together, results obtained in the present study strongly indicate that IBTC may be acting as an antioxidant molecule and, more importantly, as an antiatherogenic agent as evidenced by results of LDL oxidation assays. We hypothesize that the antioxidant activity displayed by IBTC relies on its interaction with ROS. Scheme 1 demonstrates our suggested mechanism of action for IBTC. IBTC can react with these ROSs generating its oxidized form (ox-IBTC) a sulfoxide molecule formed in an environment rich in oxygen molecules such as peroxides, hydroperoxides, or another oxygen free radical species (Fraaije et al., 2004; Vannelli et al., 2002).

Conclusion

In conclusion, our results show that IBTC is capable of protecting LDL and serum from oxidation caused by cooper and AAPH in vitro. In addition, IBTC prevents lipid hydroperoxide formation and protects Trp from oxidation. These findings clearly indicate a high potential for IBTC to act as an antiatherogenic molecule. Thus, this study shows that thiosemicarbazones may exert atheroprotective activities; however, further studies are needed to identify the exact molecular mechanisms for this activity. Furthermore, IBTC shows an antioxidant activity in homogenates of different tissues obtained from rats. These results are in agreement with the data obtained with LDL and serum. Therefore, we suggest that IBTC may be a useful compound to serve as an antioxidant in other experimental paradigms that involve oxidative damage in brain, liver, and kidney.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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4. CONCLUSÕES

4.1 Conclusão geral:

Ainda há um conhecimento limitado sobre o processo de oxidação da LDL na aterogênese e o uso de antioxidantes para a sua prevenção. Por isso há um grande interesse na procura por novas moléculas que sejam efetivas em prevenir a oxidação da LDL. Neste contexto, os resultados obtidos neste trabalho, apesar de serem bastante preliminares, permitem concluir que a IBTC têm grande potencial antioxidante, o qual foi representado pela sua eficácia em prevenir as modificações oxidativas na LDL, em exibir propriedade scavenger de radicais livres e em reduzir a peroxidação lipídica.

Considerando que oxidação da LDL um evento crucial para o desenvolvimento da aterosclerose, sugerimos que a IBTC pode ser considerada um agente anti-aterogênico promissor para posteriores pesquisas *in vivo*. No entanto, mais estudos precisam ser feitos com esse composto, tanto em modelos *in vitro* quanto *in vivo*, para comprovar a sua eficácia e sua baixa toxicidade.

4.2 Conclusões específicas:

De acordo com os resultados apresentados nesta dissertação podemos concluir que a IBTC:

- ✓ Reduz a formação de dienos conjugados (DC) e aumenta o tempo de fase lag de maneira concentração-dependente durante a oxidação da LDL induzida tanto por $CuSO_4$ como por AAPH;
- ✓ Reduz a formação de DC no soro humano induzida por $CuSO_4$;
- ✓ Previne a oxidação da parte protéica da LDL;
- ✓ Tem atividade antioxidante contra a formação de DC similar a alguns antioxidantes clássicos;
- ✓ Previne a formação de TBARS induzida por nitroprussiato de sódio em diferentes tecidos de ratos e por $CuSO_4$ em LDL e plasma humano;
- ✓ Possui atividade scavenger de radicais livres;

- ✓ Não apresenta toxicidade em fatias da artéria aorta;
- ✓ Não é quelante do metal Cu^{+2}.

5. PERSPECTIVAS

Tendo em vista os resultados obtidos neste trabalho, as perspectivas para trabalhos posteriores são em modelos de aterosclerose:

- Ampliar os estudos sobre a toxicidade da IBTC in vivo;
- Investigar o efeito da IBTC sobre a atividade e expressão de diferentes enzimas do sistema antioxidante in vivo;
- Verificar a atividade da IBTC em prevenir a formação de células espumosas in vitro;
- Investigar o efeito da IBTC sobre enzimas e marcadores inflamatórios in vivo;
- Determinar a farmacocinética da IBTC in vivo.

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