UNIVERSIDADE FEDERAL DE PELOTAS
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Tese

Avaliação *in vitro* de novas moléculas (metabólitos secundários e lectinas) com potencial na terapêutica do câncer

Fernanda Nedel

Pelotas, 2012

FERNANDA NEDEL

AVALIAÇÃO IN VITRO DE NOVAS MOLÉCULAS (METABÓLITOS SECUNDÁRIOS E LECTINAS) COM POTENCIAL NA TERAPÊUTICA DO CÂNCER

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Orientador: Fabiana Kömmling Seixas

Co-Orientador (o): Tiago Veiras Collares

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RESUMO

NEDEL, Fernanda. **Seleção de novas moléculas e modalidades de tratamento no combate ao câncer.** 2012. 89 f. Tese (doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O câncer é uma das principais causas de morte no mundo, onde os índices devem aumentar 50% até 2020. Embora a ressecção cirúrgica e terapias adicionais (como a quimioterapias e radioterapias) sejam capazes de curar tumores primários bem delimitados, o mesmo não se aplica a metástase devido ao seu envolvimento sistêmico e a resistência a terapias convencionais. Portanto, atualmente o desfio clínico é desenvolver novas drogas e modalidades de tratamentos que irão impactar significativamente as taxas de cura do câncer. Neste sentido, o presente trabalho objetivou avaliar o efeito antineoplásico e investigar a rota de apoptose induzido pelo disseleneto de diarila e seus derivados substituídos - $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ e $(4\text{-}MeOC_6H_4Se)_2$ - em células de adenocarcinoma de colorretal humano (HT-29). Verificamos que os compostos $(3\text{-}CF_3C_6H_4Se)_2$ e $(4\text{-}MeOC_6H_4Se)_2$ induziram um efeito citotoxidade por meio de apoptose, onde os genes pró-apoptoticos (Bax, caspase-9, caspase-8, fator indutor de apoptose (AIF) e endonuclease G (EndoG)) foram altamente expressos e os genes anti-apoptótico (Bcl-2 e survivin) mostraram uma redução na sua expressão. Em um segundo momento avaliamos o potencial antineoplásico das lectinas *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) e *Canavalia ensiformis* (ConA) em células HT-29, as quais se mostraram efetivas em reduzir a viabilidade celular. Uma vez confirmado o efeito antineoplásico, as lectinas forma marcadas com FITC e a sua interação com as células tumorais foi investigado. As lectinas FITC-ConA e FITC-ConBol demonstraram potencial de se ligar as células HT-29 ao contrário da FITC-ConBr. A fim de investigar uma nova modalidade de tratamento foi avaliada a interação entre as respectivas lectinas com as células HT-29 quando associadas à nanotubos de carbonos funcionalizados de paredes múltiplas (f-MWCNTs). Quando os f-MWCNTs foram incorporados as lectinas FITC-ConA e FITC-ConBol houve um aumentaram na intensidade de fluorescência.

Palavras-chaves: adenocarcinoma de colorretal humano; disseleneto de diarila e seus derivados substituídos; lectinas; nanotubos de carbono.

ABSTRACT

NEDEL, Fernanda. **Selection of new molecules and treatment modalities to fight cancer.** 2012. 89 p. Tese (doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Cancer is a leading cause of death and its rates are expected to increase 50% by 2020. Although surgical resection and additional therapies (such as chemotherapy and radiotherapy) are able to cure well-confined, primary tumors, the same does not apply during metastasis due to the systemic involvement and its resistance to conventional therapies. Therefore, the current clinical challenge is to develop new drugs and treatment modalities that will significantly impact the cure rates. In this sense, the present study aimed to evaluate the anticancer effect and study the underlying cell death mechanisms of diaryl diselenides and its substituted structures - $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ e $(4\text{-}MeOC_6H_4Se)_2$ - on the human colon adenocarcinoma cell line (HT-29). We verified that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ induced cytotoxicity through apoptosis mechanisms in HT-29 cells, where pro-apoptotic genes were up-regulated (Bax, caspase-9, caspase-8, apoptosis-inducing factor (AIF) and endonuclease G (EndoG), and anti-apoptotic genes were down-regulated (Bcl-2 and survivin). In a second moment we evaluated the anticancer potential of *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) and *Canavalia ensiformis* (ConA) lectins in HT-29 cells, which showed an effective capacity to reduce cell viability. Once the anticancer effect was confirmed, lectins were labeled with FITC and its interaction with the tumor cells was investigated. The FITC-ConA and FITC-ConBol demonstrated the potential to bind to HT-29 cells unlike FITC-ConBr. In order to investigate a new treatment modality, the interaction between the respective lectins with HT-29 was evaluated when associated with functionalized multi-walled carbon nanotubes (f-MWCNTs). When f-MWNT was incorporated to FITC-ConBol and FITC-ConA lectins there was an increase in fluorescence intensity.

Keywords: human colon adenocarcinoma; substituted diaryl diselenides; lectins; carbon nanotubes.

Sumário

1. INTRODUÇÃO GERAL

Atualmente o câncer é uma das principais causas de morte no mundo, onde esse número deve aumentar significativamente, em parte, devido ao envelhecimento da população global (VISVADER,J.E., 2011). Segundo a Organização Mundial de Saúde (OMS), a incidência de câncer, pode aumentar em 50% nos próximos 20 anos, passando de 10 milhões de pessoas acometidas em 2000, para 15 milhões em 2020. Com base no Relatório Mundial sobre o Câncer, recentemente divulgado, a OMS solicitou que governos, autoridades e público em geral empreendam ações urgentes para evitar a ampliação do número de vítimas dessa enfermidade.

Os tratamentos mais comuns contra o câncer são restritos e envolvem a quimioterapia, radioterapia e intervenções cirúrgicas (MISRA,R. et al., 2010). Estes tratamentos são capazes de curar tumores primários bem delimitados, no entanto, o mesmo não se aplica a metástase devido ao seu envolvimento sistêmico e resistência a terapias convencionais (CHAFFER,C.L.;WEINBERG,R.A., 2011;VALASTYAN,S.;WEINBERG,R.A., 2011). Portanto, atualmente o desafio clínico é desenvolver novos medicamentos e modalidades de tratamento que irão impactar significativamente as taxas de cura, eliminando à resistência as drogas pelas células tumorais, promovendo o mínimo de toxicidade (MIURA,K. et al., 2011;SANTANDREU,F.M. et al., 2011).

O câncer se desenvolve em função de mutações em genes envolvidos no controle da proliferação e apoptose celular (morte celular programada), permitindo que as células obtenham a habilidade de invadir tecidos promovendo a formação de metástases (BARBELLIDO,S.A. et al., 2008). Considerando que um dos processos mais importantes na regulação do equilíbrio entre o crescimento e morte células é a apoptose, esta tem atraído uma crescente atenção no desenvolvimento de terapias para o tratamento do câncer (LIU,J.J. et al., 2011). De fato as terapias que envolvem a eliminação de células tumorais por meio da citotoxidade, como por exemplo, a quimioterapia, imunoterapia e radiação γ, são predominantemente mediadas pela indução de morte celular programada (FULDA,S.;DEBATIN,K.M., 2004).

Uma molécula crucial para a apoptose são as caspases, pois são tanto iniciadoras como executoras do processo. Existem três vias através das quais podem ser ativadas as caspases. As duas vias de inicialização mais comumente

descritas são a via extrínseca (receptor da morte) e a via intrínseca (mitocondrial). A via extrínseca é desencadeada pelo receptor da morte Fas, o qual é dependente da combinação com o Fas ligante (Fas-L). Quando o estímulo para a morte celular ocorre, Faz-L pode se ligar diretamente com o receptor Faz, formando um complexo, o qual promove o recrutamento dos adaptadores de proteínas intracelulares que ligam e agregam moléculas de procaspase-8, as quais clivam e ativam uma a outra. Esse complexo, por sua vez, irá ativar a procaspase-3 para induzirem o processo de apoptose (LIU,J.J. et al., 2011;WONG,R.S., 2011). A via intrínseca é uma outra estratégia que leva a apoptose, onde a mitocôndria desempenha um papel central. Quando a célula é exposta a um estímulo extracelular (por exemplo, UV, Raio-X e toxina) ou sinais intracelular (danos ao DNA, instabilidade nuclear) a membrana mitocondrial externa torna-se permeável, liberando citocromos-c. A liberação do citocromo-c promove o recrutamento de uma proteína adaptadora Apaf-1 e a procaspase-9 formando o apoptossomo e desencadeando a morte celular. Esta via é rigorosamente regulada por um grupo de proteínas pertencentes à família Bcl-2. Existem dois grupos principais de proteínas Bcl-2 uma pró-apoptotica como exemplo o Bax, e outra anti-apoptótica como o Bcl-2. Enquanto as proteínas anti-apoptóticas regulam a apoptose através do bloqueio da liberação do citocromo-c pela mitocondria, as proteínas pró-apoptóticas agem estimulando a sua liberação (LIU,J.J. et al., 2011;WONG,R.S., 2011).

Assim, durante algumas vias da apoptose a membrana externa da mitocôndria pode ser despolarizada, tornando-se parcialmente permeável a proteínas. O resultado deste processo pode acarretar na liberação de proteínas como a endonuclease G (EndoG) e fator indutor de apoptose (apoptosis-inducing factor - AIF). Acredita-se que ambas as proteínas uma vez liberadas da membrana mitocondrial translouquem para o núcleo da célula, onde elas participam da degradação da cromatina, atuando, portanto, em uma via independente de caspases (VARECHA,M. et al., 2012). Portanto, a indução da apoptose tem sido reconhecida como uma estratégia ideal para quimioterápicos, onde compostos com a habilidade de induzir apoptose em tumores possuem o potencial de serem utilizados na terapia do câncer (YAN,Q. et al., 2009).

O selênio é um elemento traço essencial, o qual sob a forma de selenocisteína compõe proteínas e enzimas antioxidantes, as quais são exigidas para as diversas funções biológicas (ZENG,H.;COMBS,G.F., 2008). Os compostos

de selênio demonstraram a capacidade de prevenir o câncer em diversos modelos animais e aumentar a eficácia quimiopreventiva em humanos com câncer de pulmão, colorretal, cabeça e pescoço e próstata, embora a mesma taxa de sucesso não tenha sido observado com o câncer de mama e de pele (ROSA,R.M. et al., 2007;SUZUKI,M. et al., 2010). O selênio parece ter uma ação antimutagênico durante os estágios iniciais do câncer, prevenindo a ativação de oncogenes e a transformação de células normais em um fenótipo maligno (ROSA,R.M. et al., 2007). Além da atividade quimiopreventiva, evidências recentes tem apontado para o potencial quimioterapêutico destes compostos (SUZUKI,M. et al., 2010). Os compostos derivados de selênio podem ser inorgânicos, tais como o selenito e o selenato, ou compostos orgânicos, como o selenometionina e disselentos de diarila (NOGUEIRA,C.W.;ROCHA,J.B., 2011). O selenito de sódio pode induzir a apoptose em linhagens de células tumorais. No entanto, a apoptose ocorre como resultado da toxicidade do selênio, causando quebras no DNA e danos cromossomais nas células de linhagens tumorais e linfócitos humanos, respectivamente (SUZUKI,M. et al., 2010). Por outro lado, os compostos orgânicos de selênio têm menos efeitos colaterais e ausência de efeitos genotóxico quando comparados com os selênios inorgânicos (RIKIISHI,H., 2007;SUZUKI,M. et al., 2010). O disseleneto de diarila, um composto orgânico de selênio, é estável e vem sendo utilizado na síntese de uma variedade de compostos organoselênio farmacologicamente ativos. Estudos experimentais tem demonstrado o potencial de proteção do disseleneto de diarila, com propriedade antioxidantes, anti-hiperglicêmicas e anti-inflamatórias (ROSA,R.M. et al., 2007;SAVEGNAGO,L. et al., 2008;NOGUEIRA,C.W.;ROCHA,J.B., 2011). Recentemente este composto tem demonstrado a capacidade de induzir efeitos citotóxicos na linhagem de neuroblastoma humano (SH-SY5Y), o qual é possivelmente mediado pela via de ERK1/2 (POSSER,T. et al., 2011). No entanto, a introdução de grupos funcionais (por exemplo, cloro, flúor e metoxila) no anel aromático do disseleneto de diarila pode alterar as propriedades da molécula proporcionando uma alternativa aos atuais agentes terapêuticos (MACHADO M.A., et al., 2009;SAVEGNAGO,L. et al., 2009). De fato a introdução do grupamento cloro no grupo aril do disseleneto de diarila confere um menor efeito citotóxico sobre as células V79 (células de fibroblastos de pulmão de hamster chinês) em comparação com o dissleneto de diarila (MACHADO M.A., et al., 2009).

No entanto, embora muitas pesquisas tem sido realizadas com a finalidade de identificar novas drogas para o tratamento do câncer, alguns importantes inconvenientes tem limitado o seu sucesso. Dentre estes estão: a falta de seletividade adequada contra as células tumorais e consequente toxicidade para os tecidos saudáveis; biodisponibilidade limitada para o tecido tumoral, exigindo altas dosagens de medicamentos; incapacidade de algumas drogas de atravessarem as barreiras celulares; e especialmente, o aparecimento frequente de resistência a múltiplas drogas (JI,S.R. et al., 2010;SHAPIRA,A. et al., 2011), fenômeno que contribui para a falha do tratamento em 90% dos pacientes com metástase (GAO,Z. et al., 2012). A fim de superar estas questões, não somente o desenvolvimento de novos medicamentos deve ser considerado, mas também os sistemas pelo qual as drogas podem ser entregues (*drug delivering systems*). A partir desta perspectiva, uma maneira plausível de entrega eficiente de drogas para o tecido tumoral seria associar drogas antineoplásica com nanopartículas (HU,C.M.;ZHANG,L., 2009;SHAPIRA,A. et al., 2011).

De fato a nonotecnologia - área que envolve o desenho e construção de estruturas com um espectro de tamanho de 1-1.000 nm - fornece uma alternativa inovadora e promissora a quimioterapias convencionais (PETROS,R.A.;DESIMONE,J.M., 2010). Esta estratégia emergente tem demonstrado uma melhor eficácia terapêutica com efeitos colaterais reduzidos, em comparação com modalidades terapêuticas clássicas, envolvendo drogas não alvo específicas (HU,C.M.;ZHANG,L., 2009;SHAPIRA,A. et al., 2011). Devido a sua capacidade de encapsular, aderir e/ou conjugar drogas ou produtos biológicos terapêuticos a sua estrutura, as nanopartículas tem sido vislumbradas como passiveis de sobrepujarem a resistência das células tumorais a múltiplas drogas. Simultaneamente os nanocarreadores multifuncionais podem aumentar a penetrabilidade por barreiras fisiológicas e proteger os fármacos ou produtos biológicos terapêuticos do processo de degradação. Ainda, os nanocarreadores podem auxiliar na solubilização de drogas hidrofóbicas, diminuir a depuração de fármacos, regular a liberação de drogas assim como garantir a entrega no alvo específico de interesse (GAO,Z. et al., 2012;HU,C.J. et al., 2012).

Entre as diversas classes de nanomateriais os nanotubos de carbono (CNT) tem atraído atenção especial devido as suas propriedades espectroscópicas, térmicas e elétricas (JI,S.R. et al., 2010;SHAPIRA,A. et al., 2011) Vários estudos tem

demonstrado que CNT funcionalizados são capazes de internalizar em uma variedade de tipos celulares, através do cruzamento da membrana celular por endocitose ou por meio de outros mecanismos (JI,S.R. et al., 2010). Este processo se torna possível uma vez que as dimensões destas estruturas tubulares variam tipicamente de 0,4-2 nm de diâmetro nos nanotubos de carbonos de parede única (SWCNTs), 1-3 nm para os nanotubos de carbono de parede dupla (DWCNTs) e 2-100 nm de nanotubos de carbono de paredes múltiplas (MWCNTs). A despeito das suas nanodimensões, os CNT tem uma elevada área superficial que permite o carregamento de componentes ativos em densidades elevadas no seu interior, e/ou através de ligações funcionais do composto as paredes externas dos CTNs. No entanto, os CNTs possuem uma superfície muito hidrofóbica e, portanto, não são facilmente suspensos em solventes fisiológicos, o que pode ser uma desvantagem importante na aplicação clínica (RYBAK-SMITH,M.J.;SIM,R.B, 2011). Desta forma as paredes laterais dos CNTs têm sido alteradas de forma não covalente e covalente. Através da introdução de grupos funcionais polares as forças de van der Waals entre os CNTs individualizados ou agregados são eliminados, modificando a sua interação com os fluídos biológicos e permitindo a introdução de moléculas alvos, tais como fragmentos de anticopos (CHENG,W. et al., 2008;ZHANG,X. et al., 2010;VEDALA,H. et al., 2011;XUE,Y. et al., 2011).

Os CNTs têm sido utilizados como sistemas de entrega para uma variedade de componentes, incluindo tipicamente fármacos anticancerígenos e antifúngicos; bimoléculas, tais como proteínas, peptídeos, DNA e siRNA; ligando alvos tais como vitaminas, peptídeos e anticorpos que tem possibilitado o fornecimento de drogas a um tecido ou subpopulação de células específicas. Alguns estudos têm associado lectinas, CNTs, e células tumorais. No entanto o foco principal tem sido no desenvolvimento de tecnologias de monitoramento que sejam práticas e de alta sensibilidade e rendimento, para a análise do status de glicosilação das células tumorais e para fornecer uma ferramenta de diagnóstico que possa guiar o tratamento do câncer (CHENG,W. et al., 2008;ZHANG,X. et al., 2010;VEDALA,H. et al., 2011;XUE,Y. et al., 2011).

As lectinas são proteínas ou glicoproteínas de origem não imune que contêm pelo menos um domínio não catalítico, o que lhes permite seletivamente reconhecer e se ligar reversívelmente a açucares livres ou glicanos específicos presentes em glicoproteínas e glicolipídeos, sem alterar a estrutura do carboidrato (KOMATH,S.S.

et al., 2006;MONIRA,P. et al., 2009;FU,L.L. et al., 2011;RUSSI,M.A. et al., 2012). Esta especificidade vem sendo profundamente correlacionada com o câncer, uma vez que a transformação neoplásica das células é geralmente associada com alterações nos glicoconjugados da superfície celular, tais como alterações na ramificação de carboidratos complexos e ocasionalmente o aparecimento de estruturas incomuns (MODY,R. et al., 1995;KAUR,M. et al., 2006;SUJATHAN,K. et al., 2009). De fato, as lectinas de plantas foram utilizadas como ferramentas simples de reconhecimento tumoral para diferenciar tumores malignos de tumores benignos e avaliar o grau de glicosilação associada à metástase (MODY,R. et al., 1995;KAUR,M. et al., 2006;SUJATHAN,K. et al., 2009). Recentemente, as lectinas têm sido utilizadas em análises de *microarrays* para melhorar o reconhecimento de tumores malignos (GUPTA,G. et al., 2010;FU,L.L. et al., 2011). Além disso, as lectinas de plantas possuem uma atividade antitumoral, induzido apoptose predominantemente através de vias dependentes de caspases (LIU,B. et al., 2009).

Assim este trabalho foi delineado visando avaliar o potencial antineoplásicos de moléculas distintas: do disseleneto de diarila e seus derivados substituídos e de três tipos de lectinas - *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) e *Canavalia ensiformis* (ConA) -. Mediante os resultados de citotoxidade para o disseleneto de diarila e seus derivados substituídos objetivamos avaliar a possível rota de apoptose pelo qual as moléculas poderiam estar promovendo a morte celular. Para as três lectinas uma vez comprovada a sua citotoxidade avaliamos se estas eram capazes de interagir com as células tumorais. E considerando os avanços na área de nanotecnologia no câncer investigamos se as lectinas associadas à nanotubos de carbono de paredes múltiplas funcionalizados (f-MWNT) poderia aumentar a interação célula-lectina.

Os dados gerados nesta tese estão na forma de artigos científicos, visando, assim, proporcionar uma divulgação objetiva e rápida dos resultados obtidos. Neste contexto, o artigo 1 teve por objetivo investigar os efeitos e o mecanismo de ação do disseleneto de diarila e seus derivados substituídos sobre as células de adenocarcinoma de colorretal (HT-29). Este trabalho está formatado de acordo com as normas do periódico **Life Sciences**.

O artigo 2 descreve a capacidade antineoplásica das lectinas *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) e *Canavalia ensiformis* (ConA) e a

sua associação a f-MWNT. Este trabalho está formatado segundo as normas do periódico **Carbon**.

2. ARTIGO 1

Substituted diaryl diselenides: Cytotoxic and apoptotic effect in human colon adenocarcinoma cells

(Artigo formatado segundo as normas do periódico *Life Sciences*)

Substituted diaryl diselenides: cytotoxic and apoptotic effect in human colon adenocarcinoma cells

Fernanda Nedel[a], Vinicius F. Campos[a], Diego Alves[b], Alan J. A. McBride[a], Odir A. Dellagostin[a], Tiago Collares[a], Lucielli Savegnago[a,*], Fabiana K. Seixas[a,*]

[a]Grupo de Oncologia Celular e Molecular, Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico/Biotecnologia, Universidade Federal de Pelotas, Capão do Leão, RS 96010-900, Brazil.

[b]Laboratório de Síntese Orgânica Limpa – LASOL, Universidade Federal de Pelotas, Capão do Leão, RS 96010-900, Brazil.

*Corresponding authors at: Universidade Federal de Pelotas, Programa de Pós-Graduação em Biotecnologia, Campus Universitário s/n, Capão do Leão, RS, 96010-900, Brazil.

Phone: +55 53 32757350

E-mail address: luciellisavegnago@yahoo.com.br (L. Savegnago); seixas.fk@gmail.com (F. Seixas)

ABSTRACT

Aims: To investigate the effects and study the underlying cell death mechanisms of diaryl diselenides, including: diphenyl diselenide $(C_6H_5Se)_2$; 4-chlorodiphenyl diselenide $(4\text{-}ClC_6H_4Se)_2$; 3-(trifluoromethyl)-diphenyl diselenide $(3\text{-}CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide $(4\text{-}MeOC_6H_4Se)_2$, on the human colon adenocarcinoma cell line HT-29.

Main methods: The viability of HT-29 cells after exposure to the diaryl diselenides and its substituted structures was based on the MTT assay. To verify if cell death was mediated throughout apoptosis mechanisms, flow cytometry and real-time PCR (qPCR) analyses were conducted.

Key findings: The MTT assay and flow cytometry analyses showed that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ induced cytotoxicity through apoptosis mechanisms in HT-29 cells. qPCR revealed there was an up-regulation of pro-apoptotic (Bax, casapase-9, caspase-8, apoptosis-inducing factor (AIF) and Endonuclease G (EndoG) and cell-cycle arrest genes (p53 and p21) and down-regulation of anti-apoptotic (Bcl-2 and survivin) and Myc genes.

Significance: These results demonstrate that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ have the potential to induce apoptosis in HT-29 cells through the activation of caspase-dependent and independent pathways and through cell-cycle arrest.

Keywords: Human colon adenocarcinoma; Apoptosis; Substituted diaryl diselenides; Selenium; Cancer

Introduction

Colorectal cancer is one of the leading causes of cancer mortality (Limami et al. 2011), corresponding to 9.4% of all cases of cancer worldwide (Cantero-Muñoz et al. 2011). Fifty percent of all recently diagnosed patients ultimately develop metastatic disease. Regardless of the advances in developing new chemotherapy agents, no drug has been able to treat colorectal cancer metastasis with a non-relapsing cure rate. Currently the clinical challenge is to develop new drugs that will have a significant impact on cure rates, by reversing drug resistance, and with minimal toxicity (Miura et al. 2011).

Selenium is an essential trace element, (Zeng and Combs 2008) that has the ability to prevent cancer in several animal models and to enhance chemopreventive efficacy in human lung, colorectal, head and neck and prostate cancer (Suzuki et al. 2010). The chemopreventive role of selenium is well supported by epidemiological, preclinical, and clinical evidence (Clark et al. 1998). Furthermore, emerging evidence has indicated the potential of selenium compounds in cancer chemotherapy (Suzuki et al. 2010).

Diphenyl diselenide $(C_6H_5Se)_2$, an organic selenium compound, has raised great interest due to its antioxidant, antidepressant-like, neuroprotective and antinociceptive properties (Nogueira and Rocha 2011; Savegnago et al. 2008a; Savegnago et al. 2008b; Savegnago et al. 2007). Recently, Posser et al. (2011) showed, for the first time, that $(C_6H_5Se)_2$ was cytotoxic to human cancer cells (SH-SY5Y) in vitro, possibly mediated by the ERK1/2 pathway (Posser et al. 2011). However, to date no study has evaluated the cytotoxic effect of $(C_6H_5Se)_2$ in other human cancer cell types.

In addition, studies have demonstrated that the introduction of a substitute (e.g., chloro, fluor or methoxyl) in the aromatic ring of $(C_6H_5Se)_2$ can alter its molecules properties (Machado et al. 2009; Savegnago et al. 2009; Wilhelm et al. 2009). The introduction of chloro into the aryl group of diaryl diselenide conferred a weak cytotoxic effect on V79 cells (Chinese hamster

lung fibroblast cells) compared to $(C_6H_5Se)_2$ (Machado et al. 2009; Savegnago et al. 2009; Wilhelm et al. 2009). Although this substitute could alter the biological effects of $(C_6H_5Se)_2$, their potential as cytotoxic agents for cancer chemotherapy has not yet been explored.

Therefore, our objective was to investigate the effect and the underlying cell death mechanisms of $(C_6H_5Se)_2$ and its substituted structures, 4-chlorodiphenyl diselenide (4-$ClC_6H_4Se)_2$, 3-(trifluoromethyl)-diphenyl diselenide (3-$CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide (4-$MeOC_6H_4Se)_2$ on the human colon adenocarcinoma cell line (HT-29). In addition, we also verified whether the introduction of an electron donating (-methoxyl) or an electron withdrawing group (-chloro and -trifluoromethyl) into the aryl group of diaryl diselenide altered its biological effect. To the best of our knowledge this is the first study that demonstrates the effect of $(C_6H_5Se)_2$ and its substituted structures on HT-29 cells.

Materials and Methods

Chemicals

$(C_6H_5Se)_2$, (4-$ClC_6H_4Se)_2$, (3-$CF_3C_6H_4Se)_2$ and (4-$MeOC_6H_4Se)_2$ (Fig. 1) were prepared according to methods in the literature. Analysis of ^{1}H and ^{13}C NMR spectra showed that the analytical and spectroscopic data was in full agreement with its assigned structure. The chemical purity of these compounds was determined by gas chromatography/mass spectrometry.

Cell Culture

The HT-29 cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS), purchased from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA),

respectively. Cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth.

Determination of cytotoxicity

The viability of the HT-29 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water insoluble formazan (Ali et al. 2010; Henn et al. 2011). Briefly, cells were seeded at a density of 2 x 10^4 cell per well in a volume of 100 µL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h before being used in the MTT assay. Cells were incubated with different concentration of $(C_6H_5Se)_2$, $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ or $(4\text{-}MeOC_6H_4Se)_2$ (5 – 80 µM) for 24, 48 and 72 h. These compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the DMEM supplemented with 10% FBS to the desired concentrations. The final DMSO concentration in the culture medium never exceeded 0.8% and a control group exposed to an equivalent concentration of DMSO was evaluated. After incubation the media was removed and 180 µL of DMEM and 20 µL MTT (5 mg MTT/mL solution) was added to each well. The plates were incubated for an additional 3 h and the medium was discarded. 200 µL of DMSO was added to each well, and the formazan was solubilised on a shaker for 5 min at 100 × g. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 492 nm. The percentage inhibition of cell growth was determined as follows: inhibitory rate = $(1\text{- } Abs_{4\text{treated cells}}/Abs_{492\text{control cells}}) \times 100$ (Zheng et al. 2011). All observations were validated by at least three independent experiments and for each experiment the analyses were performed in triplicate.

Apoptotic assay

The Guava Nexin assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, 2.0×10^4 to 1.0×10^5 of the treated HT-29 cells (100 μL) were added to 100 μL of Guava Nexin reagent. Cells were incubated in the dark at room temperature for 20 min and samples (2000 cells per well) were acquired on the flow cytometry Guava EasyCyte System. In this assay, an annexin V-negative and 7-AAD-positive result indicated nuclear debris, an annexin V-positive and 7-AAD-positive result indicated late apoptotic cells, while an annexin V-negative and 7-AAD-negative result indicated live healthy cells and annexin V-positive and 7-AAD-negative result indicated the presence of early apoptotic cells.

Gene expression evaluation by real-time PCR

The HT-29 cells were seeded in 6-well flat bottom plate at a density of 2 x 10^5 per well and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Cells were then exposed to 20, 40 and 80 μM of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ or $(4\text{-}MeOC_6H_4Se)_2$ for 48 h. After this period the cells were washed with 1x phosphate-buffered saline (PBS; Gibco) and the RNA was extracted from the cells. Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) were carried out as previously described (Campos et al. 2010). Briefly, RNA samples were isolated using TRIzol Reagent (Invitrogen) and samples were DNase-treated with a DNA-free kit (Ambion, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) according to the manufacturer's protocol. The qPCR reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK) using the primers described in Table 1.

Data analysis

Data sets from the MTT assay and qPCR were analysed using a two-way ANOVA followed by a Tukey test for multiple comparisons. Two factors were considered: the compound used (four levels) and the concentration of the compound (three levels). Significance was considered at $p < 0.05$ in all analyses. The data are expressed as the means ± SEM.

Results

Determination of cytotoxicity

Both the $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$ compounds had a significant cytotoxic effect on the HT-29 cells at 80 μM and this effect improved significantly with exposure time (Fig. 2). Both the $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds achieved significant cytotoxicity at a concentration of 20 μM. After 48 h exposure to 20 μM $(3\text{-}CF_3C_6H_4Se)_2$, cytotoxicity was 24% ($p < 0.05$) and this increased significantly to 96% at 80 μM (Fig. 2). The cytotoxicity of the $(4\text{-}MeOC_6H_4Se)_2$ compound at 20 μM, after 24 h exposure, was 44% and further increases in the concentration of the compound resulted in significant reduction in the viability of the HT-29 cells (62 and 75% cytotoxicity, Fig. 2). The exposure time had no significant effect on the cytotoxicity of the $(3\text{-}CF_3C_6H_4Se)_2$ compound. Only the $(4\text{-}MeOC_6H_4Se)_2$ compound showed a significant improvement with exposure time, for example, at 20 μM and after 24 and 48 h exposure, cytotoxicity increased from 44 to 65%, respectively, although there was no further improvement at 72 h (Fig. 2). The presence of 0.8% DMSO in the culture medium had no effect on cell viability, as compared to the control cells without DMSO.

Apoptosis analysis

The Annexin-PE staining assay was performed to further characterize the observation that the $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds could induce apoptosis in HT-29 cells after exposure for 48h. Annexin V binds to those cells that express phosphatidylserine on the outer

layer of the cell membrane, a characteristic feature of cells entering apoptosis. The results indicated that $(C_6H_5Se)_2$ induced apoptosis at a concentration of 80 µM (22.5%, Fig. 3B). The lower concentrations (20 and 40 µM) of $(C_6H_5Se)_2$ where not effective in causing cell death through apoptosis, inducing similar levels of apoptosis (5.2 and 6.1%, respectively) seen in the control groups (3.0 and 6.1%, respectively). The $(3\text{-}CF_3C_6H_4Se)_2$ compound induced a higher percentage of apoptosis at the 40 and 80 µM concentrations (22.3 and 84.7%, respectively) compared to the controls and the $(C_6H_5Se)_2$ compound. At the 20 µM concentration the percentage of apoptotic cells was 7.8%, similar to that observed in the control groups. The $(4\text{-}MeOC_6H_4Se)_2$ compound was able to induce significant apoptosis in the HT-29 cells at 20 µM (38.6%), this increased to 58.9% upon exposure to a concentration of 40 µM, although a further increase in concentration to 80 µM did not increase apoptosis (54.7%). Apoptosis induction from exposure of the HT-29 cells to 0.8% DMSO had no effect.

Gene expression

In order to evaluate the likely apoptosis pathways activated by $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ in HT-29 cells (48 h exposure), anti-apoptotic and pro-apoptotic gene expression were investigated. Bax mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (20, 40 and 80 µM) when compared to the control groups (Fig. 4A). However, $(C_6H_5Se)_2$ had no effect on Bax mRNA levels when compared to the control groups ($p>0.05$). Bcl-2 mRNA levels decreased significantly ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM) when compared to control groups. HT-29 cells exposed to $(3\text{-}CF_3C_6H_4Se)_2$ (40 µM), $(4\text{-}MeOC_6H_4Se)_2$ (20 µM) and $(C_6H_5Se)_2$ (40 and 80 µM) decreased Bcl-2 mRNA levels when compared to control groups ($p<0.05$) (Fig. 4B). Caspase 9 was up-regulated ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM), $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM)

(Fig 4C). Exposure to $(3\text{-}CF_3C_6H_4Se)_2$ (20 and 40 µM), $(4\text{-}MeOC_6H_4Se)_2$ (20 µM) and $(C_6H_5Se)_2$ (20, 40 and 80 µM) had no effect on caspase 9 gene expression ($p>0.05$). However, caspase 8 mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM) when compared to the control groups. $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ (20 µM) did not affect caspase 8 gene expression ($p>0.05$) (Fig. 4D). Survivin expression was significantly down-regulated ($p<0.05$) in HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)_2$ (40 and 80 µM), $(4\text{-}MeOC_6H_4Se)_2$ (20, 40 and 80 µM) and $(C_6H_5Se)_2$ (80 µM) when compared to the control group (Fig. 4E). The $(3\text{-}CF_3C_6H_4Se)_2$ (20 µM) and $(C_6H_5Se)_2$ (20 and 40 µM) compounds had no effect on survivin expression ($p>0.05$).

The mRNA levels for AIF and EndoG were also evaluated. AIF expression was significantly up-regulated ($p<0.05$) upon exposure to $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (20, 40 and 80 µM) when compared to the control group (Fig. 4F). However, $(C_6H_5Se)_2$ and $(3\text{-}CF_3C_6H_4Se)_2$ (20 and 40 µM) had no effect on AIF mRNA levels when compared to control groups ($p>0.05$). EndoG mRNA expression was up-regulated ($p<0.05$) when the HT-29 cells were treated with $(C_6H_5Se)_2$ (20, 40 and 80 µM), $(3\text{-}CF_3C_6H_4Se)_2$ (20, 40 and 80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (20, 40 and 80 µM) compared to the control group (Fig. 4G). HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM) had altered levels of cell cycle-related gene expression, p53 expression was significantly up-regulated ($p<0.05$), in comparison to the control groups. $(C_6H_5Se)_2$, at all concentrations tested, had no effect on p53 mRNA levels (Fig. 5A). p21 gene expression showed the same expression pattern as p53, where $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM) caused significant up-regulation ($p<0.05$) and $(C_6H_5Se)_2$ had no effect (Fig. 5B). MYC gene expression was significantly reduced ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM). $(C_6H_5Se)_2$ had no effect on MYC gene expression (Fig. 5C).

Gene expression upon exposure to 0.8% DMSO was similar to the control group in all experiments.

Discussion

Previous studies have confirmed that organoselenium compounds, such as $(C_6H_5Se)_2$ and its substituted structures, exhibit a remarkable spectrum of pharmacological properties (Machado et al. 2009; Savegnago et al. 2009; Wilhelm et al. 2009). Indeed, $(C_6H_5Se)_2$ has exhibited antioxidant, antidepressant-like, neuroprotective and antinociceptive properties and recently it was demonstrated that $(C_6H_5Se)_2$ had a cytotoxic effect, mediated by the ERK1/2 pathway, on SH-SY5Y cancer cells (Posser et al. 2011). Posser et al. (2011) reported that 30 µM $(C_6H_5Se)_2$ significantly decreased cell viability in 50% of cells and, at a concentration of 10 µM, induced changes in cell morphology (Posser et al. 2011). To the best of our knowledge no study has evaluated the effect of $(C_6H_5Se)_2$ and the substituted diaryl diselenides $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ as cytotoxic and apoptotic agents against cancer cells in vitro or in vivo.

In the present study, $(C_6H_5Se)_2$ and one of its substituted structures, $(4\text{-}ClC_6H_4Se)_2$, only presented significant cytotoxic effects against the HT-209 cells at a concentration of 80 µM. A similar study that used a neuroblastoma cell line reported cytotoxic effects at lower concentrations (10-30 µM $(C_6H_5Se)_2$). However, this discrepancy may be related to differences between the SH-SY5Y and HT-29 tumour cell lines, as they exhibit different gene profiles when exposed to potent toxic substances (Thirunavukkarasusx et al. 2011). These results suggest that $(C_6H_5Se)_2$ has a selective action and therefore offers an opportunity to investigate its use as a therapeutic agent. This selectivity has been observed with other selenium compounds, where cancer cells, including lung (A549) and head and neck (HSC-3), were substantially more sensitive to selenite and prone to induction of apoptosis than the

breast cancer cell line MCF-7 (Suzuki et al. 2010). The $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds induced cytotoxicity and alterations in cell morphology in HT-29 cells in a dose-dependent manner: 20 µM (24.4 vs. 65.2%), 40 µM (81.8 vs. 81.7%) and 80 µM (91.2 vs. 96.1%), respectively. A recent study evaluated the ability of different selenium compounds (selenate, selenite, MeSeA, MeSeCys and SeMet) to induce cell death in HT-29 cells (Lunøe et al. 2011). The most effective compound was selenite, an inorganic selenium, the percentage of cell death was 21 (10 µM) and 39% (100 µM), followed by two organic selenium compounds, MeSeA (methylseleninic acid) 2 (10 µM) and 14% (100 µM), and MeSeCys (Se-methylselenocysteine) 3% (100 µM). This suggests that the $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds evaluated in the current study are potentially cytotoxic against human colon adenocarcinoma cells, albeit in vitro. The substitution of a hydrogen atom on the aryl group of diaryl diselenide by an electron withdrawing group (-trifluoromethyl) or an electron donating group (-methoxyl) altered the cytotoxicity when compared to diphenyl diselenide. However, these effects were independent of the nature of the aromatic ring in the diaryl diselenide. Both molecules demonstrated greater cytotoxicity compared to $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$. It has been reported that selenium can inhibit cell proliferation, inducing injury via generation of reactive oxygen species (ROS) (Rudolf et al. 2008). ROS levels can activate the JNK pathway and caspases-3 and 9 via cytochrome c, with down-regulation of Bcl-2 and up-regulation of Bax (Chen et al. 2011). Also, it has been demonstrated that $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$ present higher thiol peroxidase activity and an improved antioxidant potential than $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ in vivo (Meotti et al. 2004). Since, selenium-induced apoptosis in cancer cells can be suppressed by antioxidants (Wu et al. 2010), it is possible that the higher antioxidant potential of $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$ could trigger a less effective cytotoxic effect on HT-29 cells than $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$.

Since apoptosis is thought to be the mediator of selenium anticancer activity, we verified, by an Annexin-PE staining assay, that the cytotoxicity effect caused by the $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds was mediated by apoptosis. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. One pathway by which caspases can be activated involves the extrinsic death receptor pathway, where death ligands bind to death receptors, activating caspase 8 and subsequently initiating apoptosis by cleaving other downstream or executioner caspases (Wong 2011). When $(C_6H_5Se)_2$ and its substituted structures were tested for their ability to stimulate expression of caspase-8, $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM) was the only compound that induced high levels of caspase-8 mRNA. Since the upstream caspase for the extrinsic death receptor pathway is caspase-8, this suggests that $(4\text{-}MeOC_6H_4Se)_2$ could be activating a death receptor and therefore contributing to apoptosis in the HT-29 cells. In addition, $(4\text{-}MeOC_6H_4Se)_2$ could present a different biological effect from the other substituted structures due to its electron donating group (-methoxyl).

A second pathway involved in caspase activation is the mitochondrial release of cytochrome c (Wong 2011). Cytoplasmatic release of cytochrome c activates capase-3 via formation of a complex (apoptosome) which is made of cytochrome c, APAF-1 and caspase-9 (Jackson and Combs 2008). Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) are closely involved in this process, an increase in Bcl-2 expression prevents cytochrome c release from the mitochondria, inhibiting the activation of caspase-9 and caspase-3, and preventing apoptosis (Santandreu et al. 2011). In the present study, Bcl-2 expression was down-regulated by $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM) and $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM), whereas Bax expression was up-regulated. These findings suggest that Bax and Bcl-2 were involved in mediating the apoptotic effects associated with the cytotoxicity of $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ in HT-29 cells. In addition, caspase-9 mRNA levels were significantly increased by treatment

with (3-$CF_3C_6H_4Se)_2$ (80 µM) and (4-$MeOC_6H_4Se)_2$ (40 and 80 µM) showing that caspase-9 was involved in mediating the apoptotic effects associated with these compounds. Apoptosis induced by selenium has been reported to involve activation of the caspases. It was shown that MeSeA induced apoptosis in human prostate cancer (Yamaguchi et al. 2005) and leukaemia cells (Kim et al. 2001) by the activation of multiple caspases (caspase-3, -7, -8 and -9), mitochondrial release of cytochrome c and DNA fragmentation. Other organic and inorganic selenium compounds have been shown to induce caspase-mediated apoptosis, including MeSeCys, selenite (Suzuki et al., 2010), sodium selenite (Chen et al. 2011), selenium dioxide (SeO_2) (Rikiishi 2007).

Additional apoptotic factors that can be released from the mitochondrial intermembrane space into the cytosol are AIF and EndoG, which translocate to the nucleus, triggering chromatin condensation and DNA degradation in a caspase-independent manner (Vařecha et al. 2012; Wong 2011). In the current study AIF gene expression was up-regulated by (3-$CF_3C_6H_4Se)_2$ (80 µM) and (4-$MeOC_6H_4Se)_2$ (20, 40 and 80 µM) and EndoG was up-regulated by exposure to the two substituted diaryl diselenides as well as to $(C_6H_5Se)_2$. These results suggest that diaryl diselenide and its substituted structures could induce apoptosis not only through the activation of multiple caspases but also through a caspase-independent pathway.

Survivin has been implicated in the inhibition of apoptosis, cell proliferation, angiogenesis, and cellular stress response. In HT-29 cells, (3-$CF_3C_6H_4Se)_2$ (40 and 80 µM), (4-$MeOC_6H_4Se)_2$ (20, 40 and 80 µM) and $(C_6H_5Se)_2$ (80 µM) down-regulated the gene expression of survivin. Survivin expression was down-regulated in cell lines derived from prostate cancer cells, such as LNCaP, C4-2 (Chun et al. 2007), DU145 and PC-3 (Hu et al. 2008) treated with selenium. However, when the same selenium compound was tested with a metastatic cell line derived from PC-3 (PC-3M) and two other prostate cancer cell lines (C4-2B and 22Rv1), it had no effect on survivin expression, indicating that the apoptosis induced

by selenium was not mediated by decreasing survivin expression (Liu et al. 2010). These results indicated that selenium could trigger different responses depending on the type of cell. Furthermore, p53 and p21 mRNA expression levels were increased while MYC gene expression was down-regulated upon exposure to $(3\text{-}CF_3C_6H_4Se)_2$ (80 µM), $(4\text{-}MeOC_6H_4Se)_2$ (40 and 80 µM). The expression of p53, p21 and MYC induced by $(C_6H_5Se)_2$ did not differ from that of the control groups. Investigators have shown that cells deficient in p21 escaped G2/M phase cell cycle arrest when exposed to DNA damaging agents (Rosa et al. 2007b), and that p53 arrested the cell cycle by lowering Cyclin B1 levels (Rosa et al. 2007a). In addition, reduction of MYC expression was associated with cell cycle arrest in SH-SY5Y cells (Posser et al. 2011). Our results suggest that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ influenced the expression of p53, p21 and MYC and that they could be effective as anti-proliferative agents by inducing G2/M cell cycle arrest. Selenite was shown to elevate the levels of phosphorylated p53 protein at Ser-15 and concomitantly increase the expression of p21. In addition, the pro-apoptotic Bax levels were elevated and when a p53-specific inhibitor was used Bax expression was reduced by 50%, suggesting that selenium compounds could mediate tumour cell death by the p53 pathway. However, other mechanisms may also contribute to the expression of Bax. In addition, it was observed that cytochrome c, capspases-9 and -8 did not participate in the execution of apoptosis in selenite-exposed cells (Rudolf et al. 2008). In the present study, the $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds appeared to mediate apoptosis in a caspase-dependent manner, since the expression of caspase-9 was significantly higher in treated HT-29 cells. However, p53 phosphorylation could also contribute to elevated Bax expression leading to apoptosis.

It is important to clarify that the benefit of selenium compounds is related to its bioavailability in the intestine and its ability to enter the bloodstream where it can be distributed to the various organs and tissues. Of note, the bioavailability of selenium is closely related to its

chemical form (Thiry et al. 2012). In this study the most cytotoxic compound, (4-$MeOC_6H_4Se)_2$, exhibited a significant inhibitory effect (> 40%) on HT-29 cells at a concentration of 20 µM that increased to >75% at a concentration of 80 µM following exposure for 24 h. Furthermore, these concentrations are similar to those used in other studies that reported induction of apoptosis in cancer cells with similar doses (10-100 µM) of selenium compounds (Lunøe et al. 2011; Posser et al. 2011). Further work will need to be carried out to verify the cytotoxic effects of the compounds in animal models and to confirm their bioavailability at these concentrations.

Conclusion

In summary, for the first time the cytotoxic potential of $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ was demonstrated in human colon adenocarcinoma cells and the cytotoxic effect was likely mediated through the induction of apoptosis. In addition, several molecular targets of these compounds were investigated and the evidence suggests that apoptosis was stimulated by a caspase-dependant pathway as well as by a caspase-independent pathway and that cell-cycle arrest was mediated by the p53, p21 and MYC genes.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

Ali D, Ray RS, Hans RK. UVA-induced cyototoxicity and DNA damaging potential of benz (e) acephenanthrylene. Toxicol Lett. 2010;199:193-200.

Campos VF, Collares T, Deschamps JC, Seixas FK, Dellagostin OA, Lanes CF et al. Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the Brazilian flounder Paralichthys orbignyanus. J Biosci. 2010;35:405-13.

Cantero-Muñoz P, Urién MA, Ruano-Ravina A. Efficacy and safety of intraoperative radiotherapy in colorectal cancer: a systematic review. Cancer Lett. 2011;306:121-33.

Chen B, Wang X, Zhao W, Wu J. Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549. J Exp Clin Cancer Res. 2010;29:99.

Chen XJ, Duan FD, Zhang HH, Xiong Y, Wang J. Sodium Selenite-Induced Apoptosis Mediated by ROS Attack in Human Osteosarcoma U2OS Cells. Biol Trace Elem Res. 2011.

Chun JY, Hu Y, Pinder E, Wu J, Li F, Gao AC. Selenium inhibition of survivin expression by preventing Sp1 binding to its promoter. Mol Cancer Ther. 2007;6:2572-80.

Clark LC, Dalkin B, Krongrad A, Combs GF, Turnbull BW, Slate EH et al. Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. Br J Urol. 1998;81:730-4.

Gochhait S, Dar S, Pal R, Gupta P, Bamezai RN. Expression of DNA damage response genes indicate progressive breast tumors. Cancer Lett. 2009;273:305-11.

Henn S, Nedel F, de Carvalho RV, Lund RG, Cenci MS, Pereira-Cenci T et al. Characterization of an antimicrobial dental resin adhesive containing zinc methacrylate. J Mater Sci Mater Med. 2011;22:1797-802.

Hu H, Li GX, Wang L, Watts J, Combs GF, Lü J. Methylseleninic acid enhances taxane drug efficacy against human prostate cancer and down-regulates antiapoptotic proteins Bcl-XL and survivin. Clin Cancer Res. 2008;14:1150-8.

Hu L, Sun Y, Hu J. Catalpol inhibits apoptosis in hydrogen peroxide-induced endothelium by activating the PI3K/Akt signaling pathway and modulating expression of Bcl-2 and Bax. Eur J Pharmacol. 2010;628:155-63.

Huang TC, Huang HC, Chang CC, Chang HY, Ou CH, Hsu CH et al. An apoptosis-related gene network induced by novel compound-cRGD in human breast cancer cells. FEBS Lett. 2007;581:3517-22.

Jackson MI, Combs GF. Selenium and anticarcinogenesis: underlying mechanisms. Curr Opin Clin Nutr Metab Care. 2008;11:718-26.

Kim T, Jung U, Cho DY, Chung AS. Se-methylselenocysteine induces apoptosis through caspase activation in HL-60 cells. Carcinogenesis. 2001;22:559-65.

Limami Y, Pinon A, Leger DY, Mousseau Y, Cook-Moreau J, Beneytout JL et al. HT-29 colorectal cancer cells undergoing apoptosis overexpress COX-2 to delay ursolic acid-induced cell death. Biochimie. 2011;93:749-57.

Lin JJ, Hsu HY, Yang JS, Lu KW, Wu RS, Wu KC et al. Molecular evidence of anti-leukemia activity of gypenosides on human myeloid leukemia HL-60 cells in vitro and in vivo using a HL-60 cells murine xenograft model. Phytomedicine. 2011;18:1075-85.

Lin SS, Huang HP, Yang JS, Wu JY, Hsia TC, Hsai TC et al. DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. Cancer Lett. 2008;272:77-90.

Liu X, Gao R, Dong Y, Gao L, Zhao Y, Zhao L et al. Survivin gene silencing sensitizes prostate cancer cells to selenium growth inhibition. BMC Cancer. 2010;10:418.

Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL et al. Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res. 2010;54:967-76.

Lunøe K, Gabel-Jensen C, Stürup S, Andresen L, Skov S, Gammelgaard B. Investigation of the selenium metabolism in cancer cell lines. Metallomics. 2011;3:162-8.

Machado MaS, Villela IV, Moura DJ, Rosa RM, Salvador M, Lopes NP et al. 3'3-ditrifluoromethyldiphenyl diselenide: a new organoselenium compound with interesting antigenotoxic and antimutagenic activities. Mutat Res. 2009;673:133-40.

Meotti FC, Stangherlin EC, Zeni G, Nogueira CW, Rocha JB. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ Res. 2004;94:276-82.

Miura K, Fujibuchi W, Ishida K, Naitoh T, Ogawa H, Ando T et al. Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today. 2011;41:175-82.

Nogueira CW, Rocha JB. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch Toxicol. 2011;85:1313-59.

Posser T, de Paula MT, Franco JL, Leal RB, da Rocha JB. Diphenyl diselenide induces apoptotic cell death and modulates ERK1/2 phosphorylation in human neuroblastoma SH-SY5Y cells. Arch Toxicol. 2011;85:645-51.

Rikiishi H. Apoptotic cellular events for selenium compounds involved in cancer prevention. J Bioenerg Biomembr. 2007;39:91-8.

Rosa RM, do Nascimento Picada J, Saffi J, Henriques JA. Cytotoxic, genotoxic, and mutagenic effects of diphenyl diselenide in Chinese hamster lung fibroblasts. Mutat Res. 2007a;628:87-98.

Rosa RM, Hoch NC, Furtado GV, Saffi J, Henriques JA. DNA damage in tissues and organs of mice treated with diphenyl diselenide. Mutat Res. 2007b;633:35-45.

Rudolf E, Rudolf K, Cervinka M. Selenium activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells. Cell Biol Toxicol. 2008;24:123-41.

Santandreu FM, Valle A, Oliver J, Roca P. Resveratrol potentiates the cytotoxic oxidative stress induced by chemotherapy in human colon cancer cells. Cell Physiol Biochem. 2011;28:219-28.

Savegnago L, Jesse CR, Nogueira CW. Structural modifications into diphenyl diselenide molecule do not cause toxicity in mice. Environ Toxicol Pharmacol. 2009;27:271-6.

Savegnago L, Jesse CR, Pinto LG, Rocha JB, Barancelli DA, Nogueira CW et al. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. Pharmacol Biochem Behav. 2008a;88:418-26.

Savegnago L, Jesse CR, Santos AR, Rocha JB, Nogueira CW. Mechanisms involved in the antinociceptive effect caused by diphenyl diselenide in the formalin test. J Pharm Pharmacol. 2008b;60:1679-86.

Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JB, Nogueira CW et al. Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur J Pharmacol. 2007;555:129-38.

Suzuki M, Endo M, Shinohara F, Echigo S, Rikiishi H. Differential apoptotic response of human cancer cells to organoselenium compounds. Cancer Chemother Pharmacol. 2010;66:475-84.

Thirunavukkarasusx N, Ghosal KJ, Kukreja R, Zhou Y, Dombkowski A, Cai S et al. Microarray analysis of differentially regulated genes in human neuronal and epithelial cell lines upon exposure to type A botulinum neurotoxin. Biochem Biophys Res Commun. 2011;405:684-90.

Thiry C, Ruttens A, De Temmerman L, Schneider YJ, Pussemier L. Current knowledge in species-related bioavailability of selenium in food. Food Chem. 2012;130:767-84.

Vařecha M, Potěšilová M, Matula P, Kozubek M. Endonuclease G interacts with histone H2B and DNA topoisomerase II alpha during apoptosis. Mol Cell Biochem. 2012;363:301-7.

Wang WL, McHenry P, Jeffrey R, Schweitzer D, Helquist P, Tenniswood M. Effects of Iejimalide B, a marine macrolide, on growth and apoptosis in prostate cancer cell lines. J Cell Biochem. 2008;105:998-1007.

Wilhelm EA, Jesse CR, Nogueira CW, Savegnago L. Introduction of trifluoromethyl group into diphenyl diselenide molecule alters its toxicity and protective effect against damage induced by 2-nitropropane in rats. Exp Toxicol Pathol. 2009;61:197-203.

Wong RS. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res. 2011;30:87.

Wu M, Kang MM, Schoene NW, Cheng WH. Selenium compounds activate early barriers of tumorigenesis. J Biol Chem. 2010;285:12055-62.

Yamaguchi K, Uzzo RG, Pimkina J, Makhov P, Golovine K, Crispen P et al. Methylseleninic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis. Oncogene. 2005;24:5868-77.

Zeng H, Combs GF. Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. J Nutr Biochem. 2008;19:1-7.

Zheng D, Wang Y, Zhang D, Liu Z, Duan C, Jia L et al. In vitro antitumor activity of silybin nanosuspension in PC-3 cells. Cancer Lett. 2011;307:158-64.

Figure legends

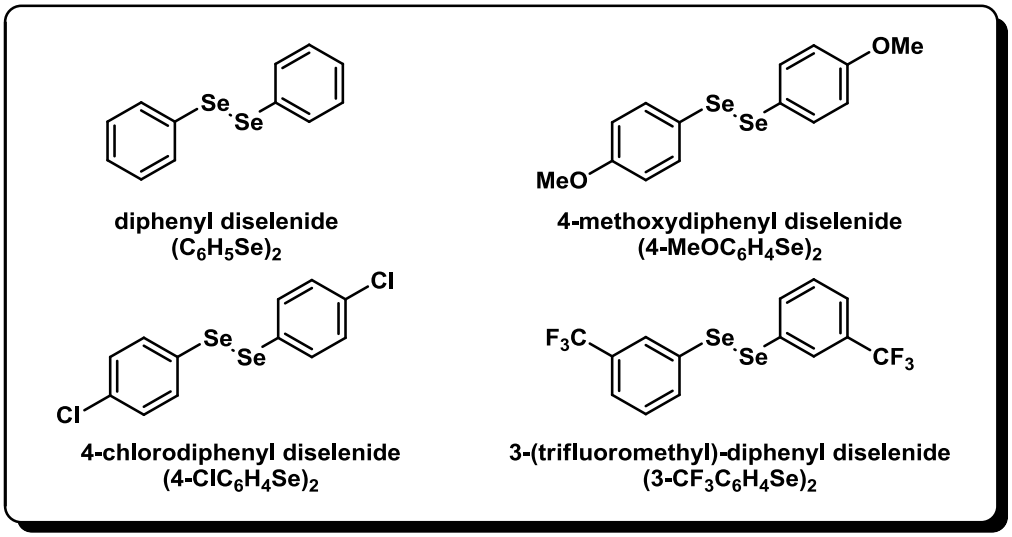

Fig. 1. Chemical structure of diaryl diselenides

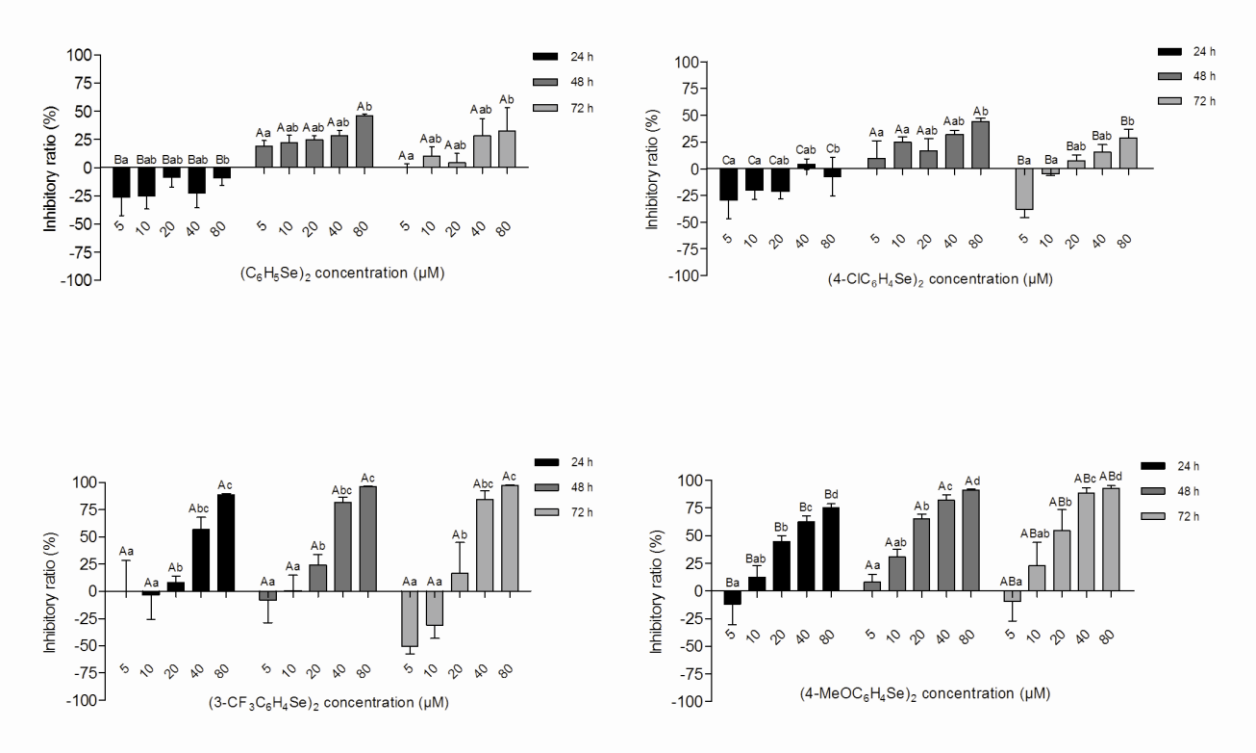

Fig. 2. Effect of the different concentration of substituted diaryl diselenides, $(C_6H_5Se)_2$ (4-$ClC_6H_4Se)_2$, (3-$CF_3C_6H_4Se)_2$ and (4-$MeOC_6H_4Se)_2$ following exposure for 24, 48 and 72 h on the inhibition of HT-29 cells. Data are expressed as the means ± SEM. Uppercase letters indicate significant differences between treatment times and lowercase letters indicate significant differences in the concentrations used. A p-value < 0.05 was considered significant (Tukey test).

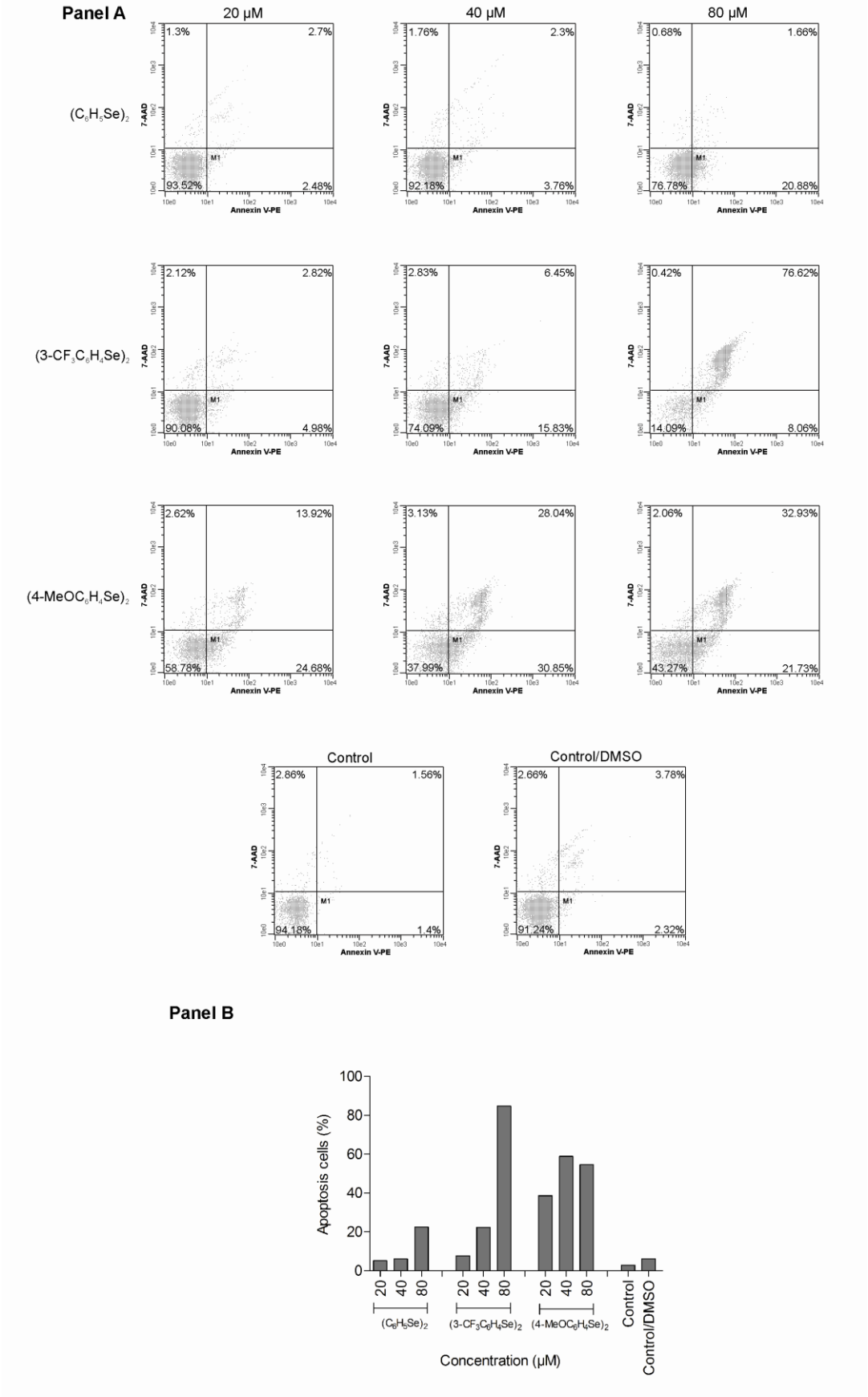
Panel A
20 μM
40 μM
80 μM
(C6H5Se)2
(3-CF3C6H4Se)2
(4-MeOC6H4Se)2
Control
Control/DMSO
7-AAD
Annexin V-PE
M1
1.3%
2.7%
93.52%
2.48%
1.76%
2.3%
92.18%
3.76%
0.68%
1.66%
76.78%
20.88%
2.12%
2.82%
90.08%
4.98%
2.83%
6.45%
74.09%
15.83%
0.42%
76.62%
14.09%
8.06%
2.62%
13.92%
58.78%
24.68%
3.13%
28.04%
37.99%
30.85%
2.06%
32.93%
43.27%
21.73%
2.86%
1.56%
94.18%
1.4%
2.66%
3.78%
91.24%
2.32%
Panel B
Apoptosis cells (%)
100
80
60
40
20
0
20
40
80
20
40
80
20
40
80
Control
Control/DMSO
(C6H5Se)2
(3-CF3C6H4Se)2
(4-MeOC6H4Se)2
Concentration (μM)

Fig. 3. Annexin V-PE analysis of HT-29 cells treated with 20, 40 and 80 μM of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, and control groups after exposure for 48 h. Panel A. Flow cytometry graphs. Panel B. Percentage of apoptotic cells.

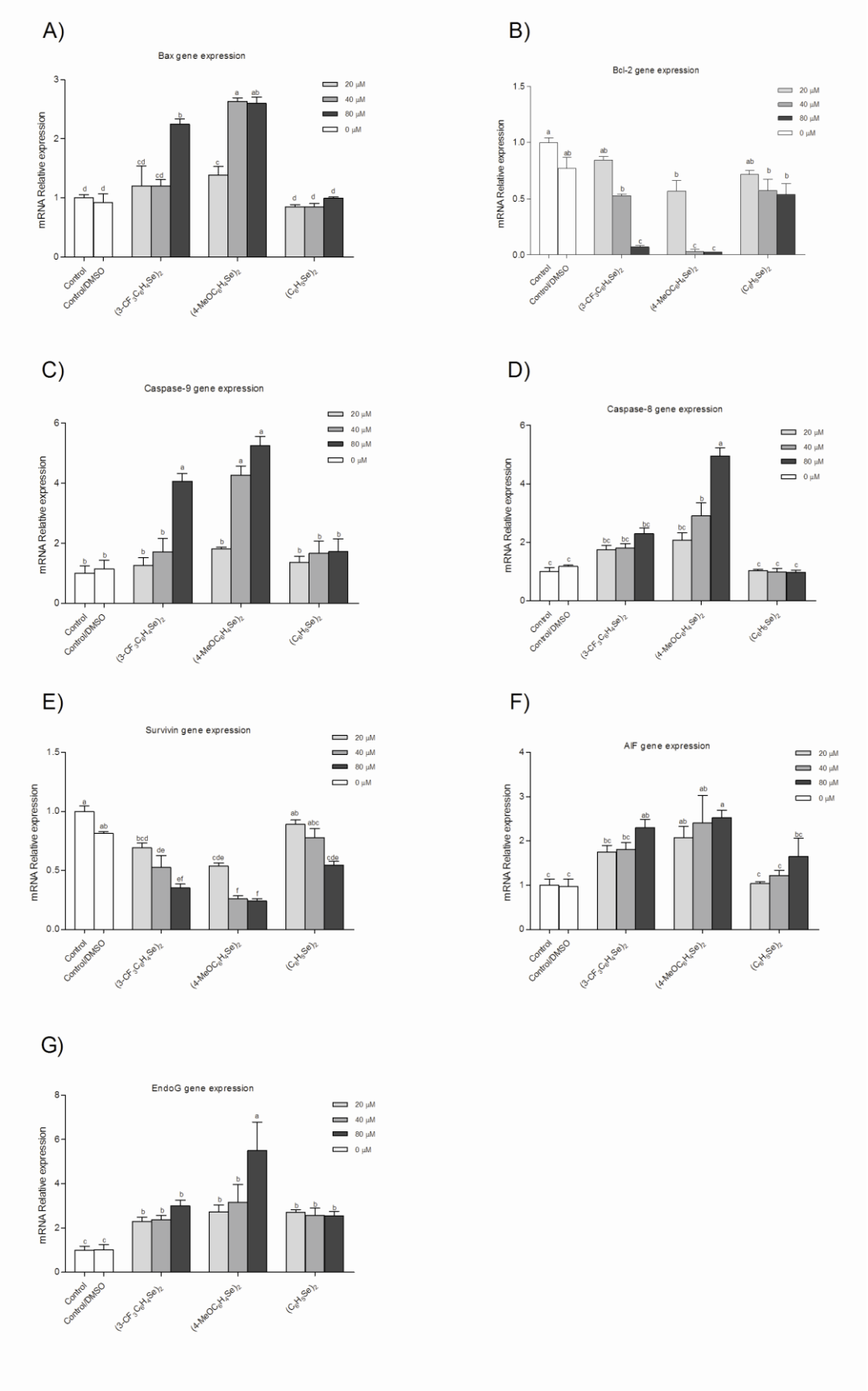
A)
Bax gene expression
mRNA Relative expression
20 µM
40 µM
80 µM
0 µM
Control
Control/DMSO
(3-CF3C6H4Se)2
(4-MeOC6H4Se)2
(C6H5Se)2
B)
Bcl-2 gene expression
C)
Caspase-9 gene expression
D)
Caspase-8 gene expression
E)
Survivin gene expression
F)
AIF gene expression
G)
EndoG gene expression

Fig. 4. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in apoptotic-related gene expression. A - Bax, B - Bcl-2, C – Caspase 9, D – Caspase 8, E - Survivin, F - AIF and G - EndoG. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

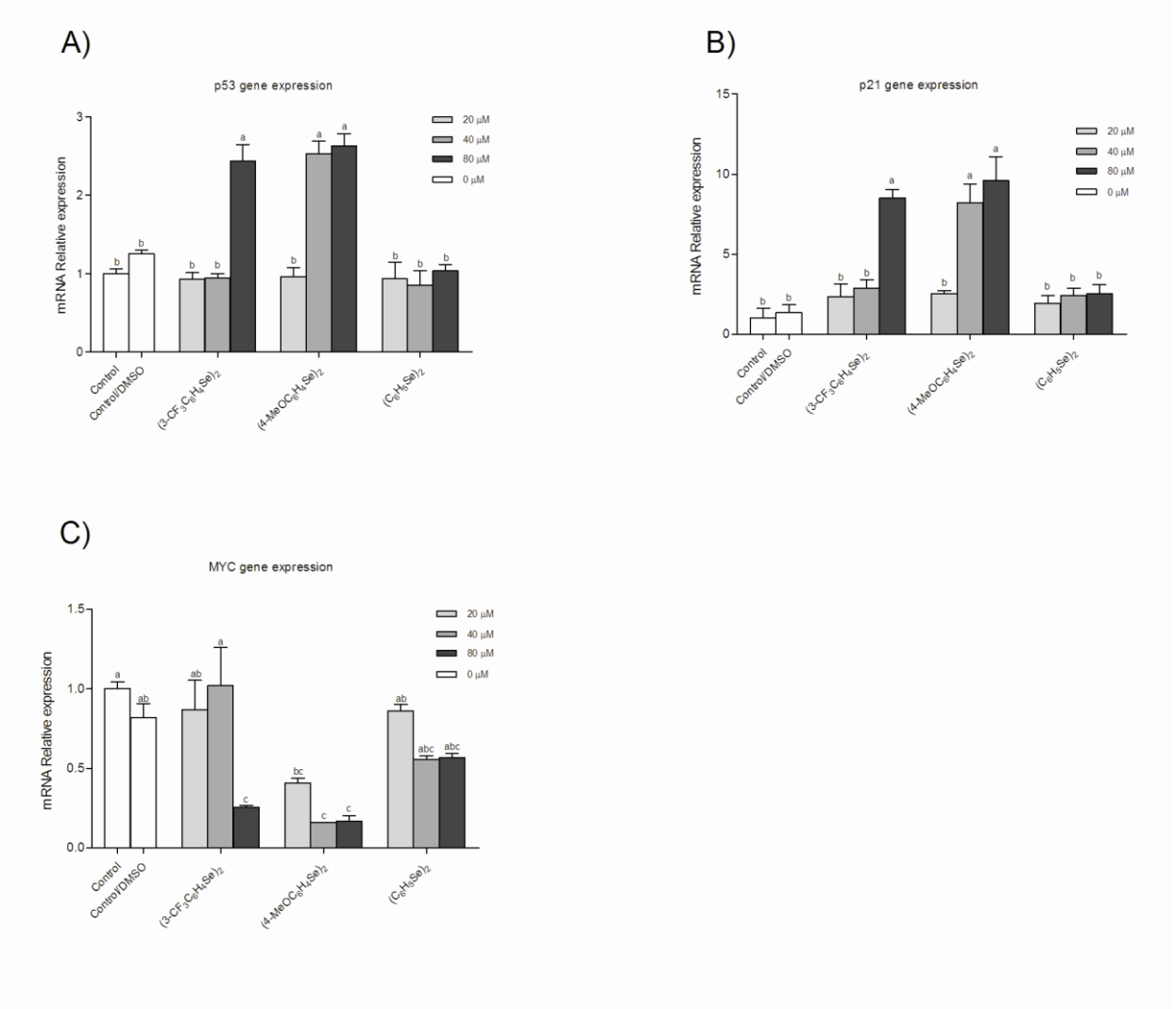

Fig. 5. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in cell-cycle arrest-related gene expression. A – p53, B – p21 and C - Myc. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

Table 1. Primers sequences used in this study.

Primers	**Sequence 5'→ 3'**	**Reference**
p53 For	AGCGAGCACTGCCCAACA	(Gochhait et al. 2009)
p53 Rev	CACGCCCACGGATCTGAA	
Bcl-2 For	GTGTGGAGAGCGTCAACC	(Chen et al. 2010)
Bcl-2 Rev	CTTCAGAGACAGCCAGGAG	
Bax For	ATGCGTCCACCAAGAAGC	(Chen et al. 2010)
Bax Rev	ACGGCGGCAATCATCCTC	
Casp9 For	CCAGAGATTCGCAAACCAGAGG	(Huang et al. 2007)
Casp9 Rev	GAGCACCGACATCACCAAATCC	
Survivin For	CTGTGGGCCCCTTAGCAAT	(Wang et al. 2008)
Survivin Rev	TAAGCCCGGGAATCAAAACA	
p21 For	CCTAATCCGCCCACAGGAA	(Wang et al. 2008)
p21 Rev	ACCTCCGGGAGAGAGGAAAA	
MYC For	TCAGCAACAACCGAAAATGC	(Wang et al. 2008)
MYC Rev	TTCCGTAGCTGTTCAAGTTTGTG	
GAPDH For	GGATTTGGTCGTATTGGG	(Hu et al. 2010)
GAPDH Rev	TCGCTCCTGGAAGATGG	
Casp8 For	GGATGGCCACTGTGAATAACTG	(Lin et al. 2011)
Casp8 Rev	TCGAGGACATCGCTCTCTCA	
AIF For	GGGAGGACTACGGCAAAGGT	(Lu et al. 2010)
AIF Rev	CTTCCTTGCTATTGGCATTCG	
EndG For	GTACCAGGTCATCGGCAAGAA	(Lin et al. 2008)
EndG Rev	CGTAGGTGCGGAGCTCAATT	

3. ARTIGO 2

CNTLectins: new insights in cancer therapy.

(Artigo formatado segundo as normas do periódico *Carbon*)

CNTLectins: new insights in cancer therapy

Fernanda Nedel[1]; Flavio Fernando Demarco[2]; Sandra Beatriz Chaves Tarquinio[3]; Oscar Endrigo Dorneles Rodrigues[4]; Luciano da Silva Pinto[1]; Odir Antônio Dellagostin[1]; Francisco V. S. Arruda[5]; Edson H. Teixeira[5]; Kyria S. Nascimento[5]; Tiago Collares[1]; Benildo Sousa Cavada[5*]; Fabiana Kömmling Seixas[1*]

[1] *Molecular and Cellular Oncology Group, Biotechnology Unit, Technology Development Center, Federal University of Pelotas, RS 96010-900, Brazil.*

[2] *Department of Operative Dentistry, Federal University of Pelotas, RS, Brazil.*

[3] *Department of Semiology and Clinics, Federal University of Pelotas, Pelotas, RS 96015-560, Brazil*

[4] *Department of Chemistry,Federal University of Santa Maria 97105-900, Santa Maria, RS 96015-560, Brazil*

[5] *Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE 60440-970, Brazil*

*Corresponding authors.

Tel/Fax: +55 85 33669818/ 85 33669818. E-mail address: bscavada@gmail.com (Cavada, BS).

Tel/Fax: +55 53 32757587. E-mail address: seixas.fk@gmail.com (Seixas, FK)

Abstract

Considering the independent potential of plant lectins and carbon nanotubes (CNTs) in cancer therapy, we proposed a combination of the two for a cancer therapeutic approach: CNTLectin. Additionally, we investigated the antineoplastic effects of three plant lectins isolated from the seeds of *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) and *Canavalia ensiformis* (ConA) in human colon adenocarcinoma (HT-29) cells, as well as the lectin-cell interaction. The viability of the HT-29 cells towards the three lectins was determined using the MTT assay. To verify the lectin-cell interaction, the lectins were labeled with FITC and associated with f-MWCNTs. The results indicated that all lectins were cytotoxic to HT-29 cells. The FITC-ConBol conjugates demonstrated an intense fluorescence signal associated with the extracellular membrane. Controversially, the FITC-ConBr conjugate did not bind to the HT-29 cells. The interaction of ConBol and ConA with cells improved when the fluorescent lectins were associated with the f-MWCNTs. These results demonstrate that ConA, ConBr and ConBol are potential anti-neoplastic agents for HT-29 cells. Additionally, our study demonstrates, for the first time, that ConBol and ConA enhance the lectin-cell interaction when associated with f-MWCNTs.

1. Introduction

Cancer is a leading cause of death and is a major health problem in both industrialized and developing nations [1, 2]; its rates are expected to increase 50% by 2020 [3, 4]. Although surgical resection and additional therapies are able to cure well-confined, primary tumors, the same does not apply during metastasis due to the systemic involvement and the resistance of metastatic tumors to conventional therapies [5, 6]. Therefore, the current clinical challenge is to develop new drugs and treatment modalities that will significantly impact the cure rates by reversing drug resistance with minimal toxicity [7, 8]

Neoplastic cell transformations are usually associated with alterations in cell surface glycoconjugates, such as increases in sialylation, alterations in the branching of complex carbohydrates and occasionally the emergence of unusual structures. Therefore, researchers have focused on using such variations in future therapies and as diagnostic or prognostic targets [9-11]. Lectins are proteins or glycoproteins of non-immune origin that contain at least one non-catalytic domain, which enables them to selectively recognize and reversibly bind specific free sugars or glycans present on glycoproteins and glycolipids without altering the structure of the carbohydrate [12-15]. Previously, plant lectins have been used as simple tumor recognition tools to differentiate malignant tumors from benign tumors and to evaluate the degree of glycosylation associated with metastasis [9, 15]. Recently, plant lectins have been used in microarray analyses to enhance the recognition of malignant tumors [15, 16]. Additionally, plant lectins possess antitumor activities, inducing apoptosis, predominantly through caspase-dependent pathways [17]. This programmed cell death is a highly regulated mechanism that allows a cell to self-degrade [18]; the failure of this process is responsible for tumor promotion and progression, as well as treatment resistance [7]. Thus, apoptosis signaling systems serve as promising targets for the development of novel anticancer agents [18].

Although extensive and profound research has been conducted to identify new drugs for cancer treatment, some major drawbacks have limited the success of chemotherapy. Some treatments lack sufficient selectivity towards cancer cells and therefore are toxic to healthy tissues. Some drugs have limited bioaccessibility to the tumor tissues, requiring high drug doses, while other drugs are unable to cross cellular barriers. Another factor limiting successful cancer treatment is the frequent emergence of drug resistance [19, 20]. To overcome these issues, not only the development of novel pharmaceuticals but also the system of drug delivery require consideration. From this perspective, a plausible way to efficiently deliver cancer drugs is to associate anticancer drugs with nanoparticles. The emerging use of nanoparticle-based strategies has demonstrated enhanced therapeutic efficacy with reduced side effects compared with the classic, non-targeted therapeutic drug combination modalities currently used [20, 21].

Diverse classes of nano-material carbone nanotubes (CNTs) have attracted particular attention due to their unique properties, such as their spectroscopic, thermal and electrical properties [19, 22]. Several studies have demonstrated that the functionalized CNTs internalize a wide variety of cell types by crossing cell membranes through endocytosis or using other mechanisms [19]. The dimensions of these tubular structures typically range from 0.4 to 2 nm in diameter for single-walled CNTs (SWCNTs), 1 to 3 nm for double-walled CNTs (DWCNTs) and 2 to 100 nm for multi-walled CNTs (MWCNTs) [22]. Aside from their nano-dimension, CNTs have a large surface area that enables to load their interior with a high density of active components, or/and use their exterior walls to make functional attachments with essential compounds. CNTs have been used as delivery systems for a variety of components: typical anticancer and antifungal drugs; biomacromolecules such as proteins, peptides, DNA, siRNA and antisense oligomers; and targeting ligands such as vitamins, peptides and antibodies have all been used in complex CNT-based nanodrugs to enable

delivery into a specific tissue or cell subpopulation. In addition, some studies have associated lectins, especially ConA, with CNTs and cancer cells. However, the focus of CNT-cancer research is to develop sensitive, practical and high-throughput monitoring technologies that analyze the glycosylation status of cancer cells and provide the diagnostic tools that guide cancer treatment [23-26].

Thus, considering the independent potential of plant lectins and CNTs, we initially aimed to investigate the antineoplastic effects of three plant lectins in human colon adenocarcinoma (HT-29) cells; the plant lectins were isolated from seeds of *Canavalia brasiliensis* (ConBr), *Canavalia boliviana* (ConBol) and *Canavalia ensiformis* (ConA). In addition, we investigated the interaction between these lectins and HT-29 cells when associated with functionalized multi-walled carbone nanotubes (f-MWCNTs). To the best of our knowledge, this is the first study that demonstrates the antineoplastic potential of ConBol and ConBr and their association with CNTs. Finally, we use the association between lectins and CNTs in a cancer therapeutic approach, which can serve as a new drug and treatment modality for cancer therapy: CNTLectins.

2. Experiments

2.1 Lectins

The lectins used in this study were purified from leguminous plant seeds growing in Ceara state, Brazil. The steps for purification have been described previously [27, 28]. Briefly, the defatted (with *n*-hexane) seed flour from each species (*C. brasiliensis*, *C. boliviana* and *C. ensiformis*) was extracted with 0.15 M NaCl (1:10, m/v) for 3 h under continuous stirring at room temperature and then centrifuged at 16,000 g for 20 min at 4 °C. The clear supernatant was then applied to a Sephadex G-50 column (40 × 2.5 cm) that was equilibrated and eluted with 0.15 M NaCl containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$. After elution of the unbound

protein, the lectin was desorbed from the column using a 0.1 M solution of the specific inhibitor sugar, d-glucose, added to the equilibrium solution or using a 0.05 M glycine–HCl buffer pH 2.6 containing 0.15 M NaCl. The fractions containing the lectin were pooled, dialyzed against 1 M acetic acid for 1 h, dialyzed exhaustively against distilled water, lyophilized and used for the preparation of the solution to be assayed. Prior to its use in the bioassays, the purity of each lectin was evaluated using denaturing electrophoresis (SDS–PAGE).

2.2 FITC-Lectins

FITC-labeled lectins were prepared in the inhibition buffer (0.1 mol L^{-1} D-mannose in 0.1 mol L^{-1} carbonate-bicarbonate buffer, pH 9.0), the conjugation buffer (0.1 mol L^{-1} carbonate-bicarbonate buffer, pH 9.0) and the washing buffer (phosphate-buffered saline (PBS): 0.01 mol L^{-1} sodium phosphate buffer, 0.027 mol L^{-1} KCl and 0.15 mol L^{-1} NaCl, pH 7.4). Initially, the lectins were dissolved in the inhibition buffer and incubated at 37 °C for 1 h. Then, 250 µL of fluorescein isothiocyanate (FITC) (500 µg/mL in conjugation buffer) was added dropwise. The solution was incubated for 2 h at room temperature with gentle stirring. Subsequently, unconjugated FITC was separated from FITC-lectin by size exclusion chromatography using a Sephadex G-25 column previously equilibrated and eluted with washing buffer. The absorbance for all of the fractions was determined at 280 nm (protein) and 495 nm (FITC) to verify the chromatographic efficiency. The FITC-labeled lectins were dialyzed against 1 mol L^{-1} acetic acid for 1 h to remove the inhibitor carbohydrate and then dialyzed against distilled water and lyophilized.

2.3 MWCNT-oxidation

For the MWCNT functionalization, we employed the methodology described by [29]. Briefly, the MWCNTs (Sigma-Aldrich, St. Louis, MO, USA) were refluxed in a 9.0 mol/L HNO_3 solution (200 mL) for 24 h at 150 °C. Then, the system was cooled to room temperature, filtered through a 0.2 µm PTFE membrane and washed with deionized water until a neutral pH was obtained. The HNO_3-treated MWCNTs were dried using a vacuum for 24 h and washed as described in the related protocol.

2.4 Cell Culture

The human colon adenocarcinoma cell line (HT-29) was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), which were purchased from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA), respectively. The cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells that were in the logarithmic growth phase.

2.5 Determining the cytotoxicity of Lectins

The viability of HT-29 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water-insoluble formazan [30-32]. Briefly, cells were seeded at a density of 2×10^4 cells per well in a volume of 100 µL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h before being used in the cell viability assay. The HT-29 cells were then incubated with different concentrations of ConA, ConBol and ConBr (5 – 100 $\mu g.mL^{-1}$) for 24, 48 and 72 h. The lectins were dissolved in DMEM/10% FBS to the desired concentrations. The f-MWCNTs were dissolved in PBS.

After the incubation periods, the medium was removed, and 180 µL of medium and 20 µL of MTT (5 mg MTT^{mL-1} solution) were added to each well. The plates were incubated for an additional 3 h, and the medium was discarded. DMSO (200 µL) was added to each well, and the formazan was solubilized on a shaker for 5 min at 100 *g*. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a test wavelength of 492 nm. The cell inhibitory growth rate was determined as follows: inhibitory rate = (1- Abs_{492} treated cells/Abs_{492} control cells) x 100% [33]. All observations were validated by at least two independent experiments and in triplicate for each experiment.

2.6 Lectin-cell association

The HT-29 cells were cultured in 96-well culture plates at a density of 2 x 10^4 cells per well and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Ten microliters ($mg.mL^{-1}$) of FITC-ConA, FITC-ConBr and FITC-ConBol was dissolved in DMEM/10% FBS, added to the cells containing DMEM/10% FBS (200 µL) and incubated for 1, 3 and 6 h. At the end of the incubation period, the cells were washed twice with PBS and then viewed and photographed using an inverted fluorescence Olympus IX71 microscope (Olympus Optical Co., Ltd. Tokyo, Japan).

2.7 CNTLectin

The HT-29 cells were cultured in 96 well culture plates at a density of 2 x 10^4 cells per well and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Functionalized-MWCNTs were added to PBS (50 $\mu g.mL^{-1}$) and subsequently added to the existent 100 µL of DMEM/10% SFB in each well to obtain a final concentration of 5 $\mu g.mL^{-1}$. The FITC-ConA, FITC-ConBr or FITC-ConBol was dissolved in DMEM/10% FBS (1 $mg.mL^{-1}$), added to f-MWCNT/DMEM/10% FBS in a final concentration of 100 $\mu g.mL^{-1}$ and incubated for 1, 3

and 6 h. At the end of the incubation period, the cells were washed twice with PBS and then were viewed and photographed with an inverted fluorescence Olympus IX71 microscope (Olympus Optical Co., Ltd. Tokyo, Japan).

2.8 Data analysis

The data sets from the MTT analysis were analyzed using a two-way ANOVA followed by a Tukey test for multiple comparisons. Two factors were considered: amount of used compound (four levels) and compound concentration (three levels). In all analyses, $p < 0.05$ was considered statistically significant. The data are expressed as the mean ± SEM.

3. Results

3.1 Determination of cytotoxicity

All of the lectins tested demonstrated a significant *in vitro* cytotoxic activity against the HT-29 cells; this cytotoxic effect increased in a time-dependent manner. The most effective period was 72 h for ConA and 48 h for ConBr and ConBol ($p<0.05$) (Fig. 1).

Table 1 shows a summary of the cytotoxic effects that each lectin had on the HT-29 cells, including the concentration, time and inhibition rate. Lower concentrations (5 - 25 μg mL^{-1}) of all lectins were less effective inhibitors of HT-29 cell proliferation, as the cell inhibitory rates were lower than 50% (Fig. 1). The f-MWCNTs did not present a significant cytotoxic effect against Chinese hamster ovary cells (CHO-K1) (data not shown).

Table 1. The inhibitory ratio (%) effect of lectins on HT-29 cells after 48 and 72 h of treatment.

Lectin	% Inhibitory ratio (48 h)		% Inhibitory ratio (72 h)	
	50 µg.mL^{-1}	100 µg.mL^{-1}	50 µg.mL^{-1}	100 µg.mL^{-1}
ConBr	53.1	81	66.8	94.9
ConBol	47.1	75.7	68.2	97.1
ConA	57.3	74	85.6	97.2

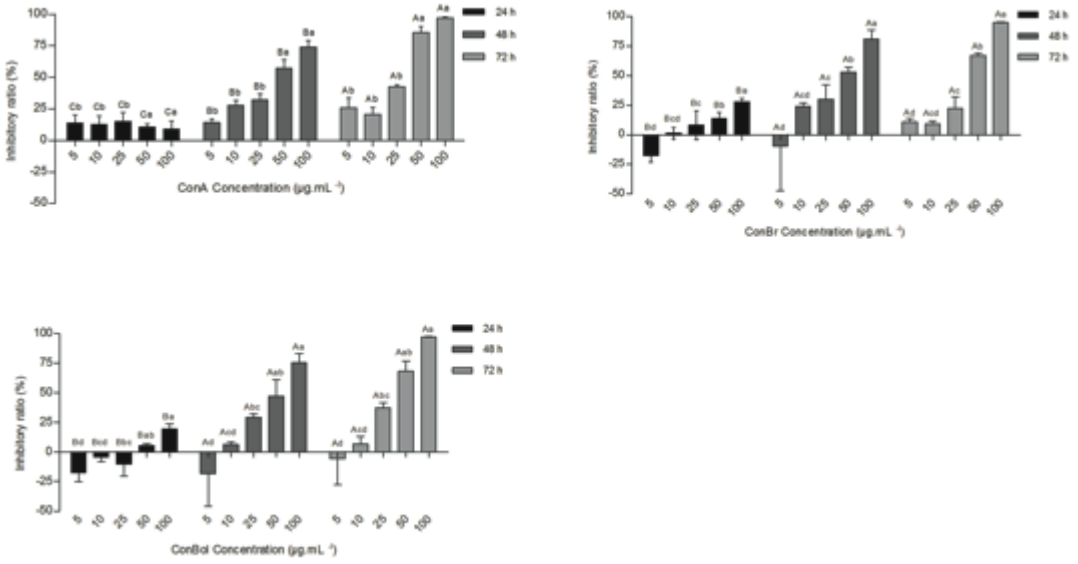

Figure 1. The effect of different concentrations of ConA, ConBol and ConBr on the inhibition of HT-29 cells after 24, 48 and 72 h. The data are expressed as the mean ± SEM. Uppercase letters indicate differences among treatment times, and lowercase letters indicate differences among concentrations. A $p < 0.05$ was considered statistically significant (Tukey test).

3.2 Lectin-cell interaction

ConA, ConBr and ConBol showed effective cytotoxicity against the HT-29 cells; therefore, the interaction between these cells and lectins was investigated using FITC-lectins. The results demonstrated that ConA and ConBol were able to bind the HT-29 cells within 6 h. The most intense interaction was observed with ConBol (Fig. 4), followed by a slight fluorescence with ConA after 3-6 h (Fig. 2). After 1 h and 3 h of exposure, ConBol exhibited fluorescence, mostly in the extracellular membrane, conferring a honeycombed shape to the cell cluster; however, a slight fluorescence was observed in the intracellular domains as well (Fig. 4 – A5 and B5). Between 3 and 6 h, ConBol was seen in the extracellular domain (Fig. 4 – B1-2 and C1-2). On the contrary, ConBr did not bind to the HT-29 cells during the 6 h period (Fig. 3)

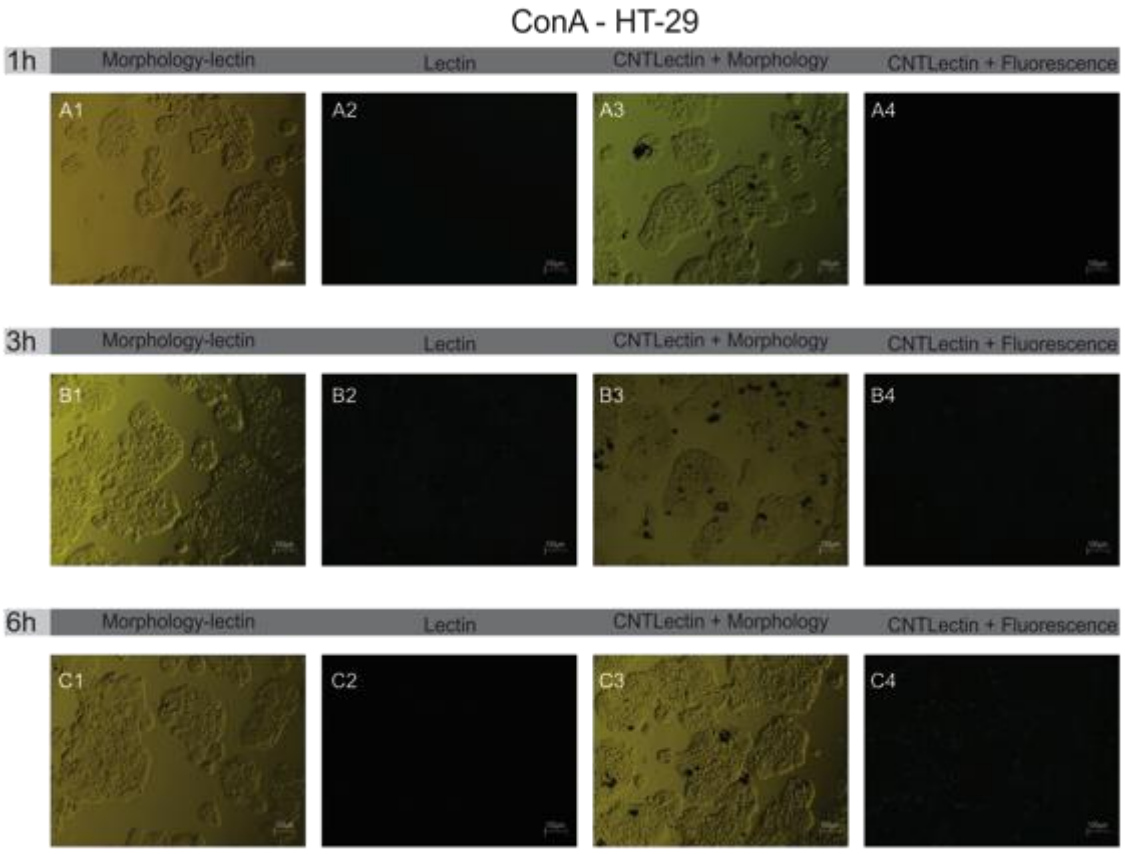

Figure 2. Fluorescent microscopy of the ConA/cell-lectin interaction. Fig. 2 represents the interaction between the HT-29 cells, the fluorescent ConA lectin, and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4).

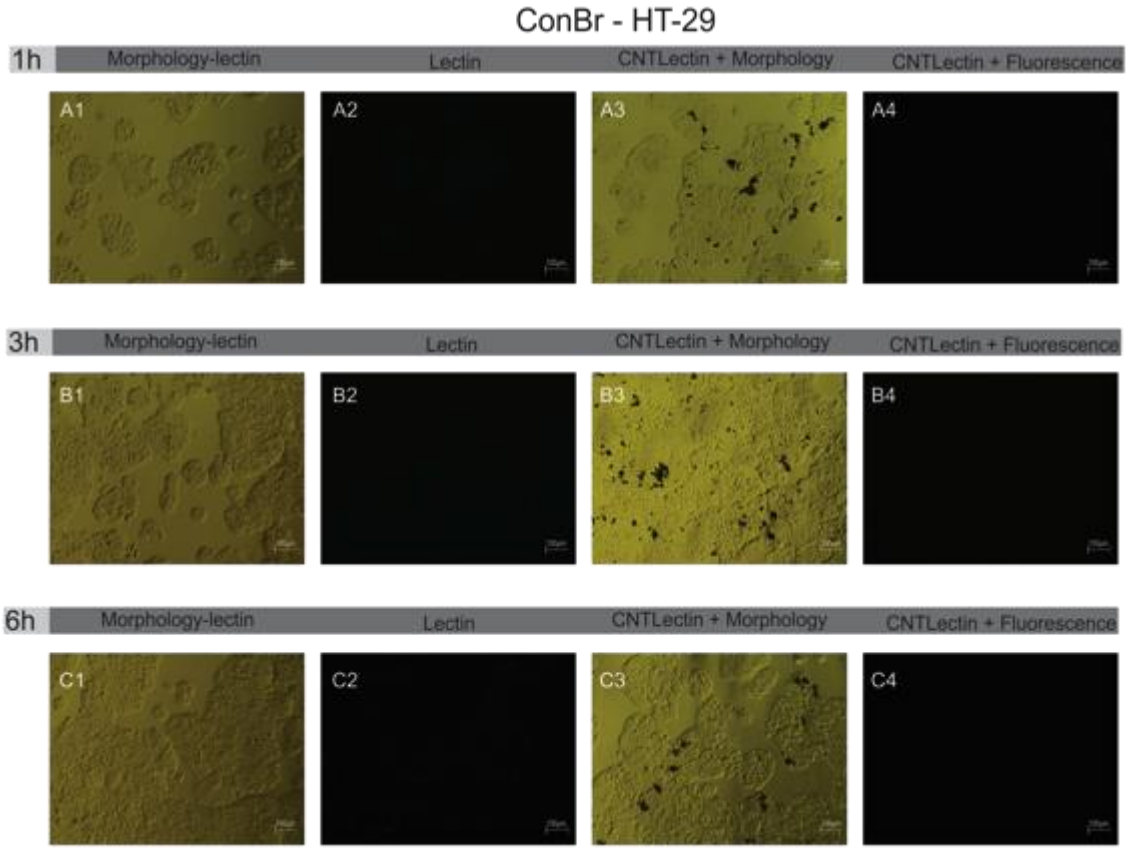

Figure 3. Fluorescent microscopy of the ConBr/cell-lectin interaction. Fig. 3 represents the interaction between the HT-29 cells, the fluorescent ConBr lectin and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4).

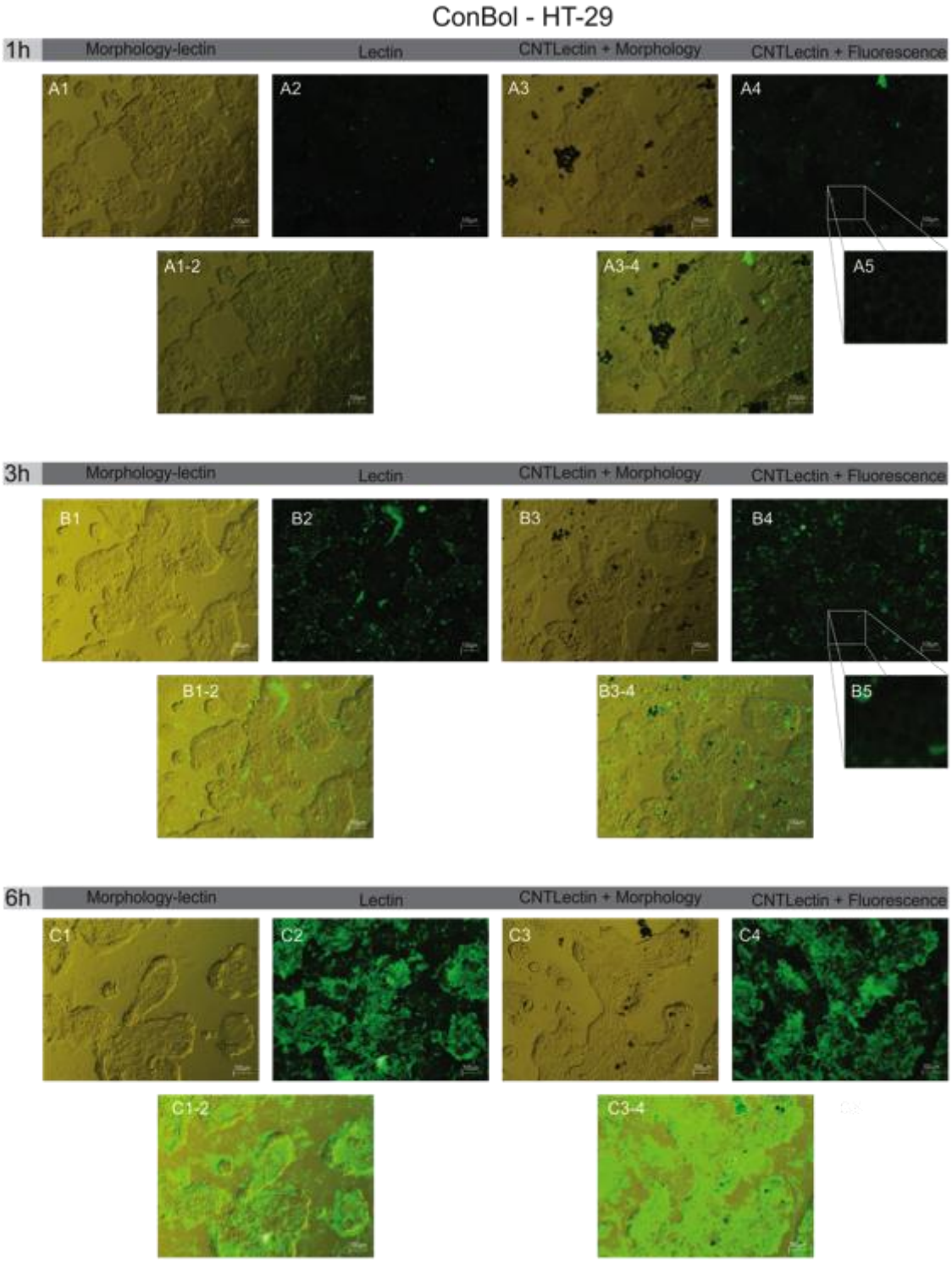

Figure 4. Fluorescent microscopy of the ConBol/cell-lectin interaction. Fig. 4 represents the interaction between the HT-29 cells, the fluorescent ConBol lectin and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4). Fig 4.A5 and B5 show a close-up of the ConBol interaction with the HT-29 cells,

demonstrating the honeycomb shape of cell clusters, as well as the higher level of fluorescence in the extracellular membrane.

3.3 CNTLectin

Since the f-MWCNTs were not cytotoxic, and ConA, ConBr and ConBol inhibited the rate of HT-29 cell growth, we combined these components aiming to increase the lectin-tumor cell interaction. In the f-MWCNT-ConA group, fluorescence was increased in the HT-29 cells after 6 h of exposure (Fig. 2), and these f-MWCNT-ConA showed an affinity for the extracellular membrane (Fig. 2). The f-MWCNT-ConBol group exhibited increased fluorescence in the first hour of cell contact (Fig. 4). After 3 h, a higher amount of fluorescence of ConBol was observed in the extracellular domain; however, when the f-MWCNTs were associated with ConBol, the fluorescence was greater in the cell clusters (Fig. 4 – B1-2/B3). Meanwhile, ConBr showed no fluorescence when associated with the carbon nanotubes (Fig. 3).

4. Discussion

Plant lectins are a group of proteins/glycoproteins with important biological activities and have been used as probes and diagnostic and activator tools. Notably, their anticancer properties have been demonstrated *in vitro, in vivo* and in case studies, suggesting their role as therapeutic agents [34, 35]. Concanavalin A (Con A), the first reported legume lectin, was isolated from the jack bean (*Canavalia ensiformis*) and is a Ca^{2+} /Mn^{2+} - dependent, mannose/glucose-binding lectin [36]. ConA has been gaining attention due to its antitumor and antiproliferative activity; ability to trigger apoptosis, autophagy and anti-angiogenesis; and immunomodulatory effects [36]. Studies have demonstrated that ConA can trigger the apoptotic events mediated by the mitochondrial pathway in diverse cell types including

human melanoma (A375) [17] and human hepatocellular liver carcinoma (HepG2) [37]. ConBr, which is a lectin isolated from the seeds of *Canavalia brasiliensis* [38], has been shown to stimulate cultivated human lymphocytes and interferon-γ production [39], cause mast cells to release histamine *in vitro*[40] and increase the production of cytokines (IL-2, IL-6 and IFN-γ) and decrease the production of IL-10 in cultivated splenocytes [41]. ConBr stimulates the production of high levels of IFN-γ and TNFα in human peripheral blood mononuclear cells [38], and it stimulates the production of nitric oxide by murine macrophages *in vitro* and *in vivo* [42]. Finally, it releases and induces apoptosis in lymphocytes *in vivo* [43]. Meanwhile, ConBol is a lectin purified from *Canavalia boliviana* seeds, and its biological effects have not been well characterized, in contrast to ConBr and ConA [28]. ConBol exhibits antinociceptive effects of both central and peripheral origins, involving the opioid system [44], and demonstrates an inhibitory activity on the growth of *S. mutans* and biofilms [45].

Although several studies have focused on the anticancer potential of ConA, the same aspects have not been evaluated for ConBr and ConBol. In the current study, ConBr and ConBol had similar cytotoxic effects on HT-29 cells. ConA has been suggested as a potential antineoplastic agent in pre-clinical and clinical trials for cancer therapy; therefore, the similar cytotoxic results obtained with ConBr and ConBol could indicate a new potential use for both lectins.

To verify the interaction between the HT-29 cells and either ConBr or ConBol, lectins were labeled with FITC. The data showed that both ConBol and ConA bind to the HT-29 cells in a similar way. However, with ConBol, a higher fluorescence can be observed in the extracellular membrane that confers a honeycombed shape to the cell cluster. On the other hand, ConA has a high level of fluorescence that is concentrated in the intracellular space. In agreement with our observation, it has been shown that ConA binds to the cisternal space of

the nuclear envelop, the rough endoplasmic reticulum (RER) and the cisternae along the proximal face of the Golgi stack [46, 47], and it has been used to identify the ER in biological assays [48]. Our findings suggest that ConBol binds the nuclear envelope, the RER and the Golgi complex, which is similar to ConA. However, ConBol also binds to the extracellular membrane with high affinity, indicating that different receptors may be involved in the interactions between both lectins and HT-29 cells. Despite the high level of similarity shared between the Diocleinae lectins, ConBr did not bind to the HT-29 cells. These results suggest that ConBr induces its cytotoxic effects through another mechanism, as in hemagglutination, and not by directly binding HT-29 cells. Liu et al. [17] demonstrated a link between ConA's hemagglutinating activity, mannose-binding activity and antiproliferative activity. Interestingly, ConBr shares a 99% amino acid sequence identity with ConA [49] and ConBol [28]. Despite this similarity, they possess different biological activities [38].

Once we verified the cytotoxic potential of ConBol and ConBr in HT-29 cells and the lectin-cell interaction, we investigated whether the incorporation of a functionalized multi-walled carbone nanotube (f-MWCNT) could increase the binding of lectin to HT-29 cells. Thus, it was demonstrated that the incorporation of f-MWCNTs increased the interaction between HT-29 cells and either ConA (6 h) or ConBol (1 – 6 h). Proteins can adsorb spontaneously onto the sidewalls of CNTs, forming protein-CNT conjugates [50, 51]. The formation of these conjugate seems to be very specific and depends on the protein structure [52]. Salvador-Morales et al. [51] showed that out of 35 proteins in the human complement activation system, only fibrinogen and apolipoprotein bind in great quantity to CNTs. Recently, Ge et al. [52] investigated the interaction of SWCNTs with human serum proteins and observed that Tyr and Phe amino acids were present in the adsorption region and directly contacted the surface of SWCNTs, thereby playing a critical role in determining the adsorption capacity of these proteins. Additionally, as previously described, ConA, ConBr and ConBol display a

high degree of similarity in their primary structures and in the amino acid residues that determine their carbohydrate binding site (Tyr12, Asn14, Leu99, Tyr100, Asp208 and Arg228), their metal binding site (Glu8, Asp10, Tyr12, Asn14, Asp19, His24, Val32, Ser34, Asp208 and Arg228) and their hydrophobic cavity (Tyr54, Leu81, Leu85, Val89, Val91, Phe111, Ser113, Val179, Ile181, Phe191, Phe212 and Ile214), which are conserved in their primary structure [38, 53]. Because the hydrophobic cavity of these lectins contain Tyr54, Phe191 and Phe212, which are the preferential sites for SWCNT-binding, it is possible that ConA, ConBr and ConBol interact with the f-MWCNTs through Tyr and Phe residues, leaving the carbohydrate-binding sites of ConBol and ConA free to bind HT-29 cells. In fact, it was recently suggested that ConA can adsorb to SWCNTs in a nonspecific manner [23].

In addition, when ConBol was associated with the f-MWCNTs (3 h), the fluorescence in the extracellular domain was significantly decreased and the fluorescence in the cell clusters increased (Fig. 4 – B1-2/B3). This result indicates that f-MWCNTs provide HT-29 cells with more lectins to bind and internalize, resulting in a faster incorporation of lectin in the tumor cell. Likewise, single MWCNTs can enter the cell through direct penetration, while MWCNT bundles undergo endocytosis [54]. In addition, MWCNT-conjugated proteins form endosomes after endocytosis and undergo retrograde-transport to the ER, where they are translocated to the cytosol [55]. Therefore, the endocytosis promoted by MWCNTs may induce faster incorporation of lectin in the HT-29 cells. In fact, Weng et al. [56] demonstrated that when recombinant ricin A chain protein (RTA), a lectin from the castor bean plant *Ricinus communis*, was incubated with L-929 cells, the endocytosis was a slow process, and even after 15 h, there was no significant cell death. However, when RTAs were conjugated with the MWCNTs for a period of 20 h, cell death was induced in approximately 40% of the cells. This study also demonstrated that in the HeLa cell lines, some MWCNT-RTAs are translocated to the cytoplasm, while others are localized near the endoplasmic reticulum,

ribosomes and the Golgi apparatus, where the localization of these conjugates was correlated to an increase in cell death. Finally, when tumor cell lines (MCF-7 e HeLa) were treated with MWCNT-RTAs, cell death was significantly increased.

ConBr showed no fluorescence when associated with the f-MWCNTs. This result demonstrates that, although CNTs can enhance lectin binding to HT-29 cells when present, their activity is linked to the existing lectin-binding properties.

5. Conclusion

In summary, we demonstrated that both ConBr and ConBol have the potential to act as antineoplastic agents in HT-29 cells. However, ConBol and ConBr seem to differ in their respective mechanisms for cytotoxic induction. ConBol exhibited a large number of cell-lectin interactions, indicating a possible mechanism for cell death via a cell receptor. However, ConBr did not bind to the HT-29 cells. Additionally, our study demonstrated that the association of ConBol or ConA with f-MWCNTs increases the cell-lectin interactions, thereby increasing the amount of lectin available for tumor cells and potentially contributing to their cytotoxicity. This interaction enables a new possible cancer treatment modality: CNTLectin.

5. References

[1] Tang FY, Cho HJ, Pai MH, Chen YH. Concomitant supplementation of lycopene and eicosapentaenoic acid inhibits the proliferation of human colon cancer cells. J Nutr Biochem. 2009;20(6):426-34.

[2] Stein K, Borowicki A, Scharlau D, Schettler A, Scheu K, Obst U, et al. Effects of synbiotic fermentation products on primary chemoprevention in human colon cells. J Nutr Biochem. 2011.

[3] Clark LC, Dalkin B, Krongrad A, Combs GF, Turnbull BW, Slate EH, et al. Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. Br J Urol. 1998;81(5):730-4.

[4] Xu W, Du M, Zhao Y, Wang Q, Sun W, Chen B. γ-Tocotrienol inhibits cell viability through suppression of β-catenin/Tcf signaling in human colon carcinoma HT-29 cells. J Nutr Biochem. 2011.

[5] Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science. 2011;331(6024):1559-64.

[6] Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275-92.

[7] Miura K, Fujibuchi W, Ishida K, Naitoh T, Ogawa H, Ando T, et al. Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today. 2011;41(2):175-82.

[8] Santandreu FM, Valle A, Oliver J, Roca P. Resveratrol potentiates the cytotoxic oxidative stress induced by chemotherapy in human colon cancer cells. Cell Physiol Biochem. 2011;28(2):219-28.

[9] Mody R, Joshi S, Chaney W. Use of lectins as diagnostic and therapeutic tools for cancer. J Pharmacol Toxicol Methods. 1995;33(1):1-10.

[10] Sujathan K, Jayasree K, Remani P. Significance of a galactose specific plant lectin for the differential diagnosis of adenocarcinoma cells in effusion. J Cytol. 2009;26(4):134-9.

[11] Kaur M, Singh K, Rup PJ, Saxena AK, Khan RH, Ashraf MT, et al. A tuber lectin from Arisaema helleborifolium Schott with anti-insect activity against melon fruit fly, Bactrocera cucurbitae (Coquillett) and anti-cancer effect on human cancer cell lines. Arch Biochem Biophys. 2006;445(1):156-65.

[12] Komath SS, Kavitha M, Swamy MJ. Beyond carbohydrate binding: new directions in plant lectin research. Org Biomol Chem. 2006;4(6):973-88.

[13] Russi MA, Vandresen-Filho S, Rieger DK, Costa AP, Lopes MW, Cunha RM, et al. ConBr, a Lectin from Canavalia brasiliensis Seeds, Protects Against Quinolinic Acid-Induced Seizures in Mice. Neurochem Res. 2012;37(2):288-97.

[14] Monira P, Koyama Y, Fukutomi R, Yasui K, Isemura M, Yokogoshi H. Effects of Japanese mistletoe lectin on cytokine gene expression in human colonic carcinoma cells and in the mouse intestine. Biomed Res. 2009;30(5):303-9.

[15] Fu LL, Zhou CC, Yao S, Yu JY, Liu B, Bao JK. Plant lectins: targeting programmed cell death pathways as antitumor agents. Int J Biochem Cell Biol. 2011;43(10):1442-9.

[16] Gupta G, Surolia A, Sampathkumar SG. Lectin microarrays for glycomic analysis. OMICS. 2010;14(4):419-36.

[17] Liu B, Li CY, Bian HJ, Min MW, Chen LF, Bao JK. Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells. Arch Biochem Biophys. 2009;482(1-2):1-6.

[18] Vangestel C, Van de Wiele C, Mees G, Peeters M. Forcing cancer cells to commit suicide. Cancer Biother Radiopharm. 2009;24(4):395-407.

[19] Ji SR, Liu C, Zhang B, Yang F, Xu J, Long J, et al. Carbon nanotubes in cancer diagnosis and therapy. Biochim Biophys Acta. 2010;1806(1):29-35.

[20] Shapira A, Livney YD, Broxterman HJ, Assaraf YG. Nanomedicine for targeted cancer therapy: towards the overcoming of drug resistance. Drug Resist Updat. 2011;14(3):150-63.

[21] Hu CM, Zhang L. Therapeutic nanoparticles to combat cancer drug resistance. Curr Drug Metab. 2009;10(8):836-41.

[22] Rybak-Smith MJ, Sim RB. Complement activation by carbon nanotubes. Adv Drug Deliv Rev. 2011;63(12):1031-41.

[23] Vedala H, Chen Y, Cecioni S, Imberty A, Vidal S, Star A. Nanoelectronic detection of lectin-carbohydrate interactions using carbon nanotubes. Nano Lett. 2011;11(1):170-5.

[24] Cheng W, Ding L, Lei J, Ding S, Ju H. Effective cell capture with tetrapeptide-functionalized carbon nanotubes and dual signal amplification for cytosensing and evaluation of cell surface carbohydrate. Anal Chem. 2008;80(10):3867-72.

[25] Zhang X, Teng Y, Fu Y, Xu L, Zhang S, He B, et al. Lectin-based biosensor strategy for electrochemical assay of glycan expression on living cancer cells. Anal Chem. 2010;82(22):9455-60.

[26] Xue Y, Bao L, Xiao X, Ding L, Lei J, Ju H. Noncovalent functionalization of carbon nanotubes with lectin for label-free dynamic monitoring of cell-surface glycan expression. Anal Biochem. 2011;410(1):92-7.

[27] Sumner JB, Howell SF. Identification of Hemagglutinin of Jack Bean with Concanavalin A. J Bacteriol. 1936;32(2):227-37.

[28] Moura TR, Bezerra GA, Bezerra MJ, Teixera CS, Bezerra EH, Benevides RG, et al. Crystallization and preliminary X-ray diffraction analysis of the lectin from Canavalia boliviana Piper seeds. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2009;65(Pt 3):213-5.

[29] Stéfani D, Paula AJ, Vaz BG, Silva RA, Andrade NF, Justo GZ, et al. Structural and proactive safety aspects of oxidation debris from multiwalled carbon nanotubes. J Hazard Mater. 2011;189(1-2):391-6.

[30] Ali D, Ray RS, Hans RK. UVA-induced cyototoxicity and DNA damaging potential of benz (e) acephenanthrylene. Toxicol Lett. 2010;199(2):193-200.

[31] Henn S, Nedel F, de Carvalho RV, Lund RG, Cenci MS, Pereira-Cenci T, et al. Characterization of an antimicrobial dental resin adhesive containing zinc methacrylate. J Mater Sci Mater Med. 2011;22(8):1797-802.

[32] Nedel F, Campos VF, Alves D, McBride AJ, Dellagostin OA, Collares T, et al. Substituted diaryl diselenides: Cytotoxic and apoptotic effect in human colon adenocarcinoma cells. Life Sci. 2012.

[33] Zheng D, Wang Y, Zhang D, Liu Z, Duan C, Jia L, et al. In vitro antitumor activity of silybin nanosuspension in PC-3 cells. Cancer Lett. 2011;307(2):158-64.

[34] Yan Q, Li Y, Jiang Z, Sun Y, Zhu L, Ding Z. Antiproliferation and apoptosis of human tumor cell lines by a lectin (AMML) of Astragalus mongholicus. Phytomedicine. 2009;16(6-7):586-93.

[35] dos Santos AF, Cavada BS, da Rocha BA, do Nascimento KS, Sant'Ana AE. Toxicity of some glucose/mannose-binding lectins to Biomphalaria glabrata and Artemia salina. Bioresour Technol. 2010;101(2):794-8.

[36] Li WW, Yu JY, Xu HL, Bao JK. Concanavalin A: a potential anti-neoplastic agent targeting apoptosis, autophagy and anti-angiogenesis for cancer therapeutics. Biochem Biophys Res Commun. 2011;414(2):282-6.

[37] Liu Z, Li X, Ding X, Yang Y. In silico and experimental studies of concanavalin A: insights into its antiproliferative activity and apoptotic mechanism. Appl Biochem Biotechnol. 2010;162(1):134-45.

[38] Cavada BS, Barbosa T, Arruda S, Grangeiro TB, Barral-Netto M. Revisiting proteus: do minor changes in lectin structure matter in biological activity? Lessons from and potential biotechnological uses of the Diocleinae subtribe lectins. Curr Protein Pept Sci. 2001;2(2):123-35.

[39] Barral-Netto M, Santos SB, Barral A, Moreira LI, Santos CF, Moreira RA, et al. Human lymphocyte stimulation by legume lectins from the Diocleae tribe. Immunol Invest. 1992;21(4):297-303.

[40] Lopes FC, Cavada BS, Pinto VP, Sampaio AH, Gomes JC. Differential effect of plant lectins on mast cells of different origins. Braz J Med Biol Res. 2005;38(6):935-41.

[41] de Oliveira Silva F, das Neves Santos P, de Melo CM, Teixeira EH, de Sousa Cavada B, Arruda FV, et al. Immunostimulatory activity of ConBr: a focus on splenocyte proliferation and proliferative cytokine secretion. Cell Tissue Res. 2011;346(2):237-44.

[42] Andrade JL, Arruda S, Barbosa T, Paim L, Ramos MV, Cavada BS, et al. Lectin-induced nitric oxide production. Cell Immunol. 1999;194(1):98-102.

[43] Barbosa T, Arruda S, Cavada B, Grangeiro TB, de Freitas LA, Barral-Netto M. In vivo lymphocyte activation and apoptosis by lectins of the Diocleinae subtribe. Mem Inst Oswaldo Cruz. 2001;96(5):673-8.

[44] Figueiredo JG, da Silveira Bitencourt F, Beserra IG, Teixeira CS, Luz PB, Bezerra EH, et al. Antinociceptive activity and toxicology of the lectin from Canavalia boliviana seeds in mice. Naunyn Schmiedebergs Arch Pharmacol. 2009;380(5):407-14.

[45] Cavalcante TT, Anderson Matias da Rocha B, Alves Carneiro V, Vassiliepe Sousa Arruda F, Fernandes do Nascimento AS, Cardoso Sá N, et al. Effect of lectins from Diocleinae subtribe against oral Streptococci. Molecules. 2011;16(5):3530-43.

[46] Tartakoff AM, Vassalli P. Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. J Cell Biol. 1983;97(4):1243-8.

[47] Guasch RM, Guerri C, O'Connor JE. Study of surface carbohydrates on isolated Golgi subfractions by fluorescent-lectin binding and flow cytometry. Cytometry. 1995;19(2):112-8.

[48] Ofner LD, Hooper NM. The C-terminal domain, but not the interchain disulphide, is required for the activity and intracellular trafficking of aminopeptidase A. Biochem J. 2002;362(Pt 1):191-7.

[49] Sanz-Aparicio J, Hermoso J, Grangeiro TB, Calvete JJ, Cavada BS. The crystal structure of Canavalia brasiliensis lectin suggests a correlation between its quaternary conformation and its distinct biological properties from Concanavalin A. FEBS Lett. 1997;405(1):114-8.

[50] Foldvari M, Bagonluri M. Carbon nanotubes as functional excipients for nanomedicines: II. Drug delivery and biocompatibility issues. Nanomedicine. 2008;4(3):183-200.

[51] Salvador-Morales C, Flahaut E, Sim E, Sloan J, Green ML, Sim RB. Complement activation and protein adsorption by carbon nanotubes. Mol Immunol. 2006;43(3):193-201.

[52] Ge C, Du J, Zhao L, Wang L, Liu Y, Li D, et al. Binding of blood proteins to carbon nanotubes reduces cytotoxicity. Proc Natl Acad Sci U S A. 2011;108(41):16968-73.

[53] Bezerra EH, Rocha BA, Nagano CS, Bezerra GeA, Moura TR, Bezerra MJ, et al. Structural analysis of ConBr reveals molecular correlation between the carbohydrate recognition domain and endothelial NO synthase activation. Biochem Biophys Res Commun. 2011;408(4):566-70.

[54] Mu Q, Broughton DL, Yan B. Endosomal leakage and nuclear translocation of multiwalled carbon nanotubes: developing a model for cell uptake. Nano Lett. 2009;9(12):4370-5.

[55] Wang M, Yu S, Wang C, Kong J. Tracking the endocytic pathway of recombinant protein toxin delivered by multiwalled carbon nanotubes. ACS Nano. 2010;4(11):6483-90.

[56] Weng X, Wang M, Ge J, Yu S, Liu B, Zhong J, et al. Carbon nanotubes as a protein toxin transporter for selective HER2-positive breast cancer cell destruction. Mol Biosyst. 2009;5(10):1224-31.

Figure captions

Figure 1. The effect of different concentrations of ConA, ConBol and ConBr on the inhibition of HT-29 cells after 24, 48 and 72 h. The data are expressed as the mean ± SEM. Uppercase letters indicate differences among treatment times, and lowercase letters indicate differences among concentrations. A $p < 0.05$ was considered statistically significant (Tukey test).

Figure 2. Fluorescent microscopy of the ConA/cell-lectin interaction. Fig. 2 represents the interaction between the HT-29 cells, the fluorescent ConA lectin, and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4).

Figure 3. Fluorescent microscopy of the ConBr/cell-lectin interaction. Fig. 3 represents the interaction between the HT-29 cells, the fluorescent ConBr lectin and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4).

Figure 4. Fluorescent microscopy of the ConBol/cell-lectin interaction. Fig. 4 represents the interaction between the HT-29 cells, the fluorescent ConBol lectin and the f-MWCNT-ConA after 1 (A1 and A2/ A3 and A4), 3 (B1 and B2/ B3 and B4) and 6 h (C1 and C2/ C3 and C4). Fig 4.A5 and B5 show a close-up of the ConBol interaction with the HT-29 cells, demonstrating the honeycomb shape of cell clusters, as well as the higher level of fluorescence in the extracellular membrane.

Table caption

Table 1. The inhibitory ratio (%) effect of lectins on the HT-29 cells after 48 and 72 h of treatment

4. Conclusões

A) Os derivados substituídos de disseleneto de diarila $(3\text{-}CF_3C_6H_4Se)_2$ e $(4\text{-}MeOC_6H_4Se)_2$, mostraram ser capazes de induzir a citotoxidade em células HT-29.

B) Os compostos $(3\text{-}CF_3C_6H_4Se)_2$ e $(4\text{-}MeOC_6H_4Se)_2$ mostraram promover esta citotoxidade por meio da apoptose, por ativação de vias independentes e dependentes de caspapses.

C) As lectinas ConA, ConBr e ConBol demonstraram o potencial de induzir um efeito antineoplásico sobre as células HT-29.

D) As lecitnas ConA e ConBol mostraram-se capazes de se ligar as células HT-29, onde a ConBol mostrou uma maior intensidade de florescência na membrana plasmática da célula quando comparada com a ConA.

E) Quando as três lectinas foram associadas aos f-MWCNTs, a ConA e ConBol demonstraram um aumento na intensidade da fluorescência.

5. Referências

BARBELLIDO SA, TRAPERO JC, SÁNCHEZ JC, GARCÍA MAP, CASTANO NE, and MARTÍNEZ AB. Gene therapy in the management of oral cancer: review of the literature. *Medicina Oral Patollogía Oral y Cirugía Bucal*, 13: 15-21, 2008.

CHAFFER CL, and WEINBERG RA. A perspective on cancer cell metastasis. *Science*, 331: 1559-1564, 2011.

CHENG W, DING L, LEI J, DING S and JU H. Effective cell capture with tetrapeptide-functionalized carbon nanotubes and dual signal amplification for cytosensing and evaluation of cell surface carbohydrate. *Analytical Chemistry*, 80: 3867-72, 2008.

FU LL, ZHOU CC, YAO S, YU JY, LIU B and BAO JK. Plant lectins: targeting programmed cell death pathways as antitumor agents. *The International Journal Biochemistry & Cell Biolology*, 43: 1442-9, 2011.

FULDA S, and DEBATIN KM. Apoptosis signaling in tumor therapy. *Annals of the New York Academy of Sciences*, 1028: 150-156, 2004.

GAO Z, ZHANG L, and SUN Y. Nanotechnology applied to overcome tumor drug resistance. *Journal of Controlled Release*, 162: 45-55, 2012.

GUPTA G, SUROLIA A and SAMPATHKUMAR SG. Lectin microarrays for glycomic analysis. *OMICS*, 14: 419-36, 2010.

HU CM, and ZHANG L. Therapeutic nanoparticles to combat cancer drug resistance. *Current Drug Metabolism*, 10: 836-841, 2009.

HU CM, and ZHANG L. Nanoparticle-based combination therapy toward overcoming drug resistance in cancer. *Biochemical Pharmacology*, 83: 1104-1111, 2012.

JI SR, LIU C, ZHANG B, YANG F, XU J, LONG J, JIN C, FU DL, NI QX, and YU XJ. Carbon nanotubes in cancer diagnosis and therapy. *Biochimica et Biophysica Acta*, 1806: 29-35, 2010.

KAUR M, SINGH K, RUP PJ, SAXENA AK, KHAN RH, ASHRAF MT, KAMBOJ SS and SINGH J. A tuber lectin from Arisaema helleborifolium Schott with anti-insect activity against melon fruit fly, Bactrocera cucurbitae (Coquillett) and anti-cancer effect on human cancer cell lines. *Archives of Biochemistry Biophysics*, 445: 156-65, 2006.

KOMATH SS, KAVITHA M and SWAMY MJ. Beyond carbohydrate binding: new directions in plant lectin research. *Organic & Biomolecular Chemistry*, 4: 973-88, 2006.

LIU B, LI CY, BIAN HJ, MIN MW, CHEN LF and BAO JK. Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells. *Archives of Biochemistry Biophysics*, 482: 1-6, 2009.

LIU JJ, LIN M, YU JY, LIU B, and BAO JK. Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Letters*, 300: 105-114, 2011.

MACHADO MS, VILLELA IV, MOURA DJ, ROSA RM, SALVADOR M, LOPES NP, BRAGA AL, ROESLER R, SAFFI J and HENRIQUES JA. 3'3-ditrifluoromethyldiphenyl diselenide: a new organoselenium compound with interesting antigenotoxic and antimutagenic activities. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 673: 133-140, 2009.

MISRA R, ACHARYA S, and SAHOO SK. Cancer nanotechnology: application of nanotechnology in cancer therapy. *Drug Discovery Today*, 15: 842-850, 2010.

MIURA K, FUJIBUCHI W, ISHIDA K, NAITOH T, OGAWA H, ANDO T, YAZAKI N, WATANABE K, HANEDA S, SHIBATA C, and SASAKI I. Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. *Surgery Today*, 41: 175-182, 2011.

MODY R, JOSHI S and CHANEY W. Use of lectins as diagnostic and therapeutic tools for cancer. *Journal of Pharmacological and Toxicological Methods*, 33: 1-10, 1995.

MONIRA P, KOYAMA Y, FUKUTOMI R, YASUI K, ISEMURA M and YOKOGOSHI H. Effects of Japanese mistletoe lectin on cytokine gene expression in human colonic carcinoma cells and in the mouse intestine. *Biomedical Research*, 30: 303-9, 2009.

NOGUEIRA CW and ROCHA JB. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. *Archives Toxicology*, 85: 1313-1359, 2011.

Oragnização Munidal da Saúde. Disponível em: <http://www.who.int/research/en/> Acesso em 20 de agosto de 2012.

PETROS RA, and DESIMONE JM. Strategies in the design of nanoparticles for therapeutic applications. *Nature Reviews Drug Discovery*, 9: 615-627, 2010.

POSSER T, PAULA MT, FRANCO JL, LEAL RB and ROCHA JB. Diphenyl diselenide induces apoptotic cell death and modulates ERK1/2 phosphorylation in human neuroblastoma SH-SY5Y cells. *Archives of Toxicology*, 85: 645-651, 2011.

RIKIISHIi H. Apoptotic cellular events for selenium compounds involved in cancer prevention. *Journal of Bioenergetics and Biomembranes*, 39: 91-98, 2007.

ROSA RM, PICADA JN, SAFFI J and HENRIQUES JA. Cytotoxic, genotoxic, and mutagenic effects of diphenyl diselenide in Chinese hamster lung fibroblasts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 628: 87-98, 2007.

RUSSI MA, VANDRESEN-FILHO S, RIEGER DK, COSTA AP, LOPES MW, CUNHA RM, TEIXEIRA EH, NASCIMENTO KS, CAVADA BS, TASCA CI and LEAL RB. ConBr, a Lectin from Canavalia brasiliensis Seeds, Protects Against Quinolinic Acid-Induced Seizures in Mice. *Neurochemical Research*, 37: 288-97, 2012.

RYBAK-SMITH MJ and SIM RB. Complement activation by carbon nanotubes. *Advanced Drug Delivery Reviews*, 63: 1031-41, 2011.

SANTANDREU FM, VALLE A, OLIVER J, and ROCA P. Resveratrol potentiates the cytotoxic oxidative stress induced by chemotherapy in human colon cancer cells. *Cellular Physiology and Biochemistry*, 28: 219-228, 2011.

SAVEGNAGO L, JESSE CR, PINTO LG, ROCHA JB, BARANCELLI DA, NOGUEIRA CW and ZENI G. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. *Pharmacology Biochemistry and Behavior*, 88: 418-426, 2008.

SAVEGNAGO L, JESSE CR and NOGUEIRA CW. Structural modifications into diphenyl diselenide molecule do not cause toxicity in mice. *Environmental Toxicology and Pharmacology*, 27: 271-276, 2009.

SHAPIRA A, LIVNEY YD, BROXTERMAN HJ, and ASSARAF YG. Nanomedicine for targeted cancer therapy: towards the overcoming of drug resistance. *Drug Resistance Updates*, 14: 150-163, 2011.

SUJATHAN K, JAYASREE K and REMANI P. Significance of a galactose specific plant lectin for the differential diagnosis of adenocarcinoma cells in effusion. *Journal of Cytology*, 26:134, 2009.

SUZUKI M, ENDO M, SHINOHARA F, ECHIGO S and RIKIISHI H. Differential apoptotic response of human cancer cells to organoselenium compounds. *Cancer Chemotherapy and Pharmacology*, 66: 475-484, 2010.

VALASTYAN S, and WEINBERG RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*, 147: 275-292, 2011.

VARECHA M, POTESILOVÁ M, MATULA P, and KOZUBEK M. Endonuclease G interacts with histone H2B and DNA topoisomerase II alpha during apoptosis. *Molecular and Cellular Biochemistry*, 363: 301-307, 2012.

VEDALA H, CHEN Y, CECIONI S, IMBERTY A, VIDAL S, STAR A. Nanoelectronic detection of lectin-carbohydrate interactions using carbon nanotubes. *Nano Letters*, 11: 170-5, 2011.

VISVADER JE. Cells of origin in cancer. *Nature*, 469: 314-322, 2011.

WONG RS. Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, 30: 87, 2011.

XUE Y, BAO L, XIAO X, DING L, LEI J, JU H. Noncovalent functionalization of carbon nanotubes with lectin for label-free dynamic monitoring of cell-surface glycan expression. *Analytical Biochemistry*, 410:92-7, 2011.

YAN Q, LI Y, JIANG Z, SUN Y, ZHU L, and DING Z. Antiproliferation and apoptosis of human tumor cell lines by a lectin (AMML) of Astragalus mongholicus. *Phytomedicine*, 16: 586-593, 2009.

ZENG H and COMBS GF. Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. *The Journal of Nutritional Biochemistry*, 19: 1-7, 2008.

ZHANG X, TENG Y, FU Y, XU L, ZHANG S, HE B, WANG C and ZHANG WI. Lectin-based biosensor strategy for electrochemical assay of glycan expression on living cancer cells. *Analytical Chemistry*, 82: 9455-60, 2010.

6. Anexos

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Substituted diaryl diselenides: Cytotoxic and apoptotic effect in human colon adenocarcinoma cells

Fernanda Nedel [a], Vinicius F. Campos [a], Diego Alves [b], Alan J.A. McBride [a], Odir A. Dellagostin [a], Tiago Collares [a], Lucielli Savegnago [a,*], Fabiana K. Seixas [a,*]

[a] Grupo de Oncologia Celular e Molecular, Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico/Biotecnologia, Universidade Federal de Pelotas, Capão do Leão, RS 96010-900, Brazil

[b] Laboratório de Síntese Orgânica Limpa—LASOL, Universidade Federal de Pelotas, Capão do Leão, RS 96010-900, Brazil

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ABSTRACT

Aims: To investigate the effects and study the underlying cell death mechanisms of diaryl diselenides, including: diphenyl diselenide $(C_6H_5Se)_2$; 4-chlorodiphenyl diselenide $(4\text{-}ClC_6H_4Se)_2$; 3-(trifluoromethyl)-diphenyl diselenide $(3\text{-}CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide $(4\text{-}MeOC_6H_4Se)_2$, on the human colon adenocarcinoma cell line HT-29.

Main methods: The viability of HT-29 cells after exposure to the diaryl diselenides and its substituted structures was based on the MTT assay. To verify if cell death was mediated throughout apoptosis mechanisms, flow cytometry and real-time PCR (qPCR) analyses were conducted.

Key findings: The MTT assay and flow cytometry analyses showed that $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ induced cytotoxicity through apoptosis mechanisms in HT-29 cells. qPCR revealed there was an up-regulation of pro-apoptotic (Bax, casapase-9, caspase-8, apoptosis-inducing factor (AIF) and Endonuclease G (EndoG)) and cell-cycle arrest genes (p53 and p21) and down-regulation of anti-apoptotic (Bcl-2 and survivin) and Myc genes.

Significance: These results demonstrate that $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)_2$ have the potential to induce apoptosis in HT-29 cells through the activation of caspase-dependent and independent pathways and through cell-cycle arrest.

Introduction

Colorectal cancer is one of the leading causes of cancer mortality (Limami et al., 2011), corresponding to 9.4% of all cases of cancer worldwide (Cantero-Muñoz et al., 2011). Fifty percent of all recently diagnosed patients ultimately develop metastatic disease. Regardless of the advances in developing new chemotherapy agents, no drug has been able to treat colorectal cancer metastasis with a non-relapsing cure rate. Currently the clinical challenge is to develop new drugs that will have a significant impact on cure rates, by reversing drug resistance, and with minimal toxicity (Miura et al., 2011).

Selenium is an essential trace element (Zeng and Combs, 2008) that has the ability to prevent cancer in several animal models and to enhance chemopreventive efficacy in human lung, colorectal, head and neck and prostate cancer (Suzuki et al., 2010). The chemopreventive role of selenium is well supported by epidemiological, preclinical, and clinical evidence (Clark et al., 1998). Furthermore, emerging evidence has indicated the potential of selenium compounds in cancer chemotherapy (Suzuki et al., 2010).

Diphenyl diselenide $(C_6H_5Se)_2$, an organic selenium compound, has raised great interest due to its antioxidant, antidepressant-like, neuroprotective and antinociceptive properties (Nogueira and Rocha, 2011; Savegnago et al., 2007, 2008a, 2008b). Recently, Posser et al. (2011) showed, for the first time, that $(C_6H_5Se)_2$ was cytotoxic to human cancer cells (SH-SY5Y) in vitro, possibly mediated by the ERK1/2 pathway (Posser et al., 2011). However, to date no study has evaluated the cytotoxic effect of $(C_6H_5Se)_2$ in other human cancer cell types.

In addition, studies have demonstrated that the introduction of a substitute (e.g., chloro, fluor or methoxyl) in the aromatic ring of (C_6H_5Se) can alter its molecular properties (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). The introduction of chloro into the aryl group of diaryl diselenide conferred a weak cytotoxic effect on V79 cells (Chinese hamster lung fibroblast cells) compared to (C_6H_5Se) (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). Although this substitute could alter the biological effects of (C_6H_5Se), their potential as cytotoxic agents for cancer chemotherapy has not yet been explored.

Therefore, our objective was to investigate the effect and the underlying cell death mechanisms of $(C_6H_5Se)_2$ and its substituted structures,

* Corresponding authors at: Universidade Federal de Pelotas, Programa de Pós-Graduação em Biotecnologia, Campus Universitário s/n, Capão do Leão, RS, 96010-900, Brazil. Tel.: +55 53 32757350.
E-mail addresses: luciellisavegnago@yahoo.com.br (L. Savegnago), seixas.fk@gmail.com (F.K. Seixas).

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4-chlorodiphenyl diselenide (4-$ClC_6H_4Se)_2$, 3-(trifluoromethyl)-diphenyl diselenide (3-$CF_3C_6H_4Se)_2$ and 4-methoxydiphenyl diselenide (4-$MeOC_6H_4Se)_2$ on the human colon adenocarcinoma cell line (HT-29). In addition, we also verified whether the introduction of an electron donating (-methoxyl) or an electron withdrawing group (-chloro and -trifluoromethyl) into the aryl group of diaryl diselenide altered its biological effect. To the best of our knowledge this is the first study that demonstrates the effect of $(C_6H_5Se)_2$ and its substituted structures on HT-29 cells.

Materials and methods

Chemicals

$(C_6H_5Se)_2$, (4-$ClC_6H_4Se)_2$, (3-$CF_3C_6H_4Se)_2$ and (4-$MeOC_6H_4Se)_2$ (Fig. 1) were prepared according to methods in the literature. Analysis of ^1H and ^{13}C NMR spectra showed that the analytical and spectroscopic data was in full agreement with its assigned structure. The chemical purity of these compounds was determined by gas chromatography/mass spectrometry.

Cell culture

The HT-29 cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS), purchased from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA), respectively. Cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth.

Determination of cytotoxicity

The viability of the HT-29 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water insoluble formazan (Ali et al., 2010; Henn et al., 2011). Briefly, cells were seeded at a density of 2×10^4 cells per well in a volume of 100 μL in 96-well plates and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h before being used in the MTT assay. Cells were incubated with different concentrations of $(C_6H_5Se)_2$, (4-$ClC_6H_4Se)_2$, (3-$CF_3C_6H_4Se)_2$ or (4-$MeOC_6H_4Se)_2$ (5–80 μM) for 24, 48 and 72 h. These compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the DMEM supplemented with 10% FBS to the desired concentrations. The final DMSO concentration in the culture medium never exceeded 0.8% and a control group exposed to an equivalent concentration of DMSO was evaluated. After incubation the media were removed and 180 μL of DMEM and 20 μL MTT (5 mg MTT/mL solution) were added to each well. The plates were incubated for an additional 3 h and the medium was discarded. 200 μL of DMSO was added to each well, and the formazan was solubilized on a shaker for 5 min at 100×g. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 492 nm. The percentage inhibition of cell growth was determined as follows: inhibitory rate $=(1-Abs_{4treated\ cells}/Abs_{492control\ cells})\times100$ (Zheng et al., 2011). All observations were validated by at least three independent experiments and for each experiment the analyses were performed in triplicate.

Apoptotic assay

The Guava Nexin assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, 2.0×10^4 to 1.0×10^5 of the treated HT-29 cells (100 μL) were added to 100 μL of Guava Nexin reagent. Cells were incubated in the dark at room temperature for 20 min and samples (2000 cells per well) were acquired on the flow cytometry Guava EasyCyte System. In this assay, an annexin V-negative and 7-AAD-positive result indicated nuclear debris, an annexin V-positive and 7-AAD-positive result indicated late apoptotic cells, while an annexin V-negative and 7-AAD-negative result indicated live healthy cells and annexin V-positive and 7-AAD-negative result indicated the presence of early apoptotic cells.

Gene expression evaluation by real-time PCR

The HT-29 cells were seeded in a 6-well flat bottom plate at a density of 2×10^5 per well and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. Cells were then exposed to 20, 40 and 80 μM of $(C_6H_5Se)_2$, (3-$CF_3C_6H_4Se)_2$ or (4-$MeOC_6H_4Se)_2$ for 48 h. After this period the cells were washed with phosphate-buffered saline (PBS; Gibco) and the RNA was extracted from the cells. Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) were carried out as previously described (Campos et al., 2010). Briefly, RNA samples were isolated using TRIzol Reagent (Invitrogen) and samples were DNase-treated with a DNA-free kit (Ambion, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK)

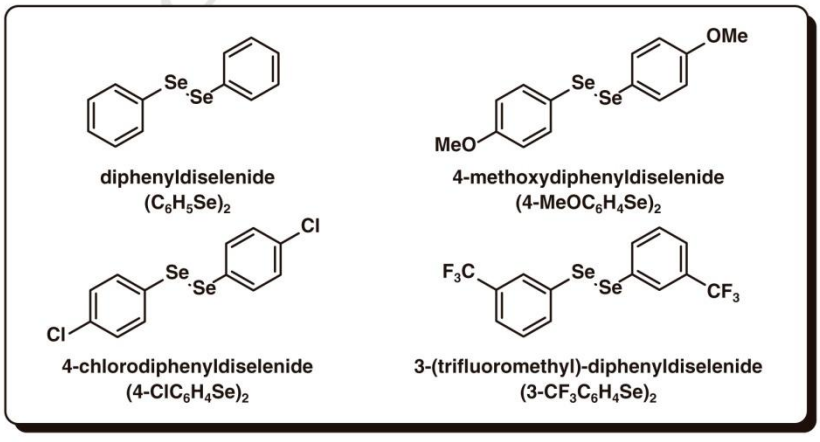

Fig. 1. Chemical structure of diaryl diselenides.

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according to the manufacturer's protocol. The qPCR reactions were run on a Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK) using the primers described in Table 1.

Data analysis

Data sets from the MTT assay and qPCR were analysed using a two-way ANOVA followed by a Tukey test for multiple comparisons. Two factors were considered: the compound used (four levels) and the concentration of the compound (three levels). Significance was considered at $p<0.05$ in all analyses. The data are expressed as the means ± SEM.

Results

Determination of cytotoxicity

Both the $(C_6H_5Se)_2$ and $(4\text{-}ClC_6H_4Se)_2$ compounds had a significant cytotoxic effect on the HT-29 cells at 80 μM and this effect improved significantly with exposure time (Fig. 2). Both the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds achieved significant cytotoxicity at a concentration of 20 μM. After 48 h exposure to 20 μM $(3\text{-}CF_3C_6H_4Se)$, cytotoxicity was 24% ($p<0.05$) and this increased significantly to 96% at 80 μM (Fig. 2). The cytotoxicity of the $(4\text{-}MeOC_6H_4Se)$ compound at 20 μM, after 24 h exposure, was 44% and further increases in the concentration of the compound resulted in a significant reduction in the viability of the HT-29 cells (62 and 75% cytotoxicity, Fig. 2). The exposure time had no significant effect on the cytotoxicity of the $(3\text{-}CF_3C_6H_4Se)$ compound. Only the $(4\text{-}MeOC_6H_4Se)$ compound showed a significant improvement with exposure time, for example, at 20 μM and after 24 and 48 h exposure, cytotoxicity increased from 44 to 65%, respectively, although there was no further improvement at 72 h (Fig. 2). The presence of 0.8% DMSO in the culture medium had no effect on cell viability, as compared to the control cells without DMSO.

Apoptosis analysis

The annexin-PE staining assay was performed to further characterize the observation that the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)_2$ compounds could induce apoptosis in HT-29 cells after exposure for 48 h. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. The results indicated that (C_6H_5Se) induced apoptosis at a concentration of 80 μM (22.5%, Fig. 3B). The lower concentrations (20 and 40 μM) of (C_6H_5Se) were not effective in causing cell death through apoptosis, inducing similar levels of apoptosis (5.2 and 6.1%, respectively) seen in the control groups (3.0 and 6.1%, respectively). The $(3\text{-}CF_3C_6H_4Se)$ compound induced a higher percentage of apoptosis at the 40 and 80 μM concentrations (22.3 and 84.7%, respectively) compared to the controls and the (C_6H_5Se) compound. At the 20 μM concentration the percentage of apoptotic cells was 7.8%, similar to that observed in the control groups. The $(4\text{-}MeOC_6H_4Se)$ compound was able to induce significant apoptosis in the HT-29 cells at 20 μM (38.6%), this increased to 58.9% upon exposure to a concentration of 40 μM, although a further increase in concentration to 80 μM did not increase apoptosis (54.7%). Apoptosis induction from exposure of the HT-29 cells to 0.8% DMSO had no effect.

Gene expression

In order to evaluate the likely apoptosis pathways activated by $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in HT-29 cells (48 h exposure), anti-apoptotic and pro-apoptotic gene expressions were investigated. Bax mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) when compared to the control groups (Fig. 4A). However, (C_6H_5Se) had no effect on Bax mRNA levels when compared to the control groups ($p>0.05$). Bcl-2 mRNA levels decreased significantly ($p<0.05$) in cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) when compared to control groups. HT-29 cells exposed to $(3\text{-}CF_3C_6H_4Se)$ (40 μM), $(4\text{-}MeOC_6H_4Se)$ (20 μM) and (C_6H_5Se) (40 and 80 μM) decreased Bcl-2 mRNA levels when compared to control groups ($p<0.05$) (Fig. 4B). Caspase 9 was up-regulated ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM), $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) (Fig. 4C). Exposure to $(3\text{-}CF_3C_6H_4Se)$ (20 and 40 μM), $(4\text{-}MeOC_6H_4Se)$ (20 μM) and (C_6H_5Se) (20, 40 and 80 μM) had no effect on caspase 9 gene expression ($p>0.05$). However, caspase 8 mRNA levels were significantly higher ($p<0.05$) in cells exposed to $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) when compared to the control groups. (C_6H_5Se), $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ (20 μM) did not affect caspase 8 gene expression ($p>0.05$) (Fig. 4D). Survivin expression was significantly down-regulated ($p<0.05$) in HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)$ (40 and 80 μM), $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and (C_6H_5Se) (80 μM) when compared to the control group (Fig. 4E). The $(3\text{-}CF_3C_6H_4Se)$ (20 μM) and (C_6H_5Se) (20 and 40 μM) compounds had no effect on survivin expression ($p>0.05$).

The mRNA levels for AIF and EndoG were also evaluated. AIF expression was significantly up-regulated ($p<0.05$) upon exposure to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) when compared to the control group (Fig. 4F). However, (C_6H_5Se) and $3\text{-}CF_3C_6H_4Se)$ (20 and 40 μM) had no effect on AIF mRNA levels when compared to control groups ($p>0.05$). EndoG mRNA expression was up-regulated ($p<0.05$) when the HT-29 cells were treated with $(C_6H_5Se)_2$ (20, 40 and 80 μM), $(3\text{-}CF_3C_6H_4Se)$ (20, 40 and 80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) compared to the control group (Fig. 4G). HT-29 cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) had altered levels of cell cycle-related gene expression, p53 expression was significantly up-regulated ($p<0.05$), in comparison to the control groups. (C_6H_5Se), at all concentrations tested, had no effect on p53 mRNA levels (Fig. 5A). p21gene expression showed the same expression pattern as p53, where $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) caused significant up-regulation ($p<0.05$) and (C_6H_5Se) had no effect (Fig. 5B). MYC gene expression was significantly reduced ($p<0.05$) in cells treated with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM). (C_6H_5Se) had no effect on MYC gene expression (Fig. 5C). Gene

Table 1
Primer sequences used in this study.

Primers	Sequence 5′→3′	Reference
p53 for	AGCGAGCACTGCCCAACA	Gochhait et al. (2009)
p53 rev	CACGCCCACGGATCTGAA	
Bcl-2 for	GTGTGGAGAGCGTCAACC	Chen et al. (2010)
Bcl-2 rev	CTTCAGAGACAGCCAGGAG	
Bax for	ATGCGTCCACCAAGAAGC	Chen et al. (2010)
Bax rev	ACGGCGGCAATCATCCTC	
Casp9 for	CCAGAGATTCGCAAACCAGAGG	Huang et al. (2007)
Casp9 rev	GAGCACCGACATCACCAAATCC	
Survivin for	CTGTGGGCCCCTTAGCAAT	Wang et al. (2008)
Survivin rev	TAAGCCCGGGAATCAAAACA	
p21 for	CCTAATCCGCCCACAGGAA	Wang et al. (2008)
p21 rev	ACCTCCGGGAGAGAGGAAAA	
MYC for	TCAGCAACAACCGAAAATGC	Wang et al. (2008)
MYC rev	TTCCGTAGCTGTTCAAGTTTGTG	
GAPDH for	GGATTTGGTCGTATTGGG	Hu et al. (2010)
GAPDH rev	TCGCTCCTGGAAGATGG	
Casp8 for	GGATGGCCACTGTGAATAACTG	Lin et al. (2011)
Casp8 rev	TCGAGGACATCGCTCTCTCA	
AIF for	GGGAGGACTACGGCAAAGGT	Lu et al. (2010)
AIF rev	CTTCCTTGCTATTGGCATTCG	
EndG for	GTACCAGGTCATCGGCAAGAA	Lin et al. (2008)
EndG rev	CGTAGGTGCGGAGCTCAATT	

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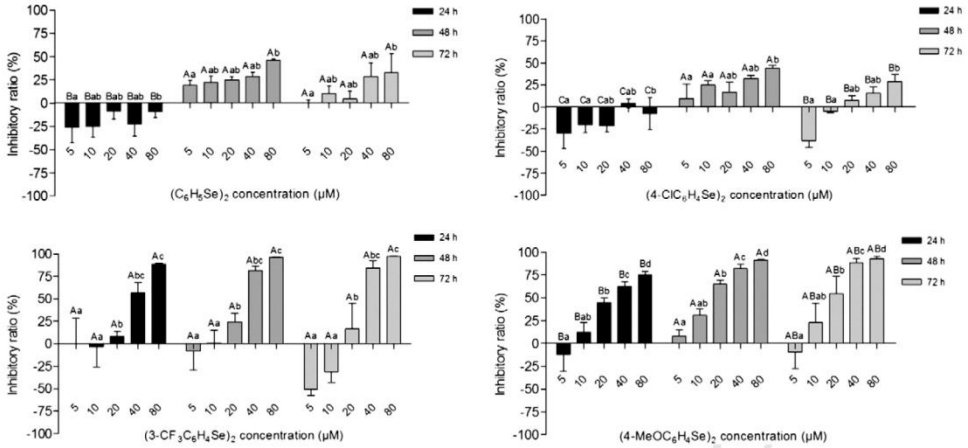

Fig. 2. Effect of the different concentration of substituted diaryl diselenides, $(C_6H_5Se)_2$ $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$ following exposure for 24, 48 and 72 h on the inhibition of HT-29 cells. Data are expressed as the means ± SEM. Uppercase letters indicate significant differences between treatment times and lowercase letters indicate significant differences in the concentrations used. A p-value<0.05 was considered significant (Tukey test).

expression upon exposure to 0.8% DMSO was similar to the control group in all experiments.

Discussion

Previous studies have confirmed that organoselenium compounds, such as (C_6H_5Se) and its substituted structures, exhibit a remarkable spectrum of pharmacological properties (Machado et al., 2009; Savegnago et al., 2009; Wilhelm et al., 2009). Indeed, (C_6H_5Se) has exhibited antioxidant, antidepressant-like, neuroprotective and antinociceptive properties and recently it was demonstrated that (C_6H_5Se) had a cytotoxic effect, mediated by the ERK1/2 pathway, on SH-SY5Y cancer cells (Posser et al., 2011). Posser et al. (2011) reported that 30 μM (C_6H_5Se) significantly decreased cell viability in 50% of cells and, at a concentration of 10 μM, induced changes in cell morphology (Posser et al., 2011). To the best of our knowledge no study has evaluated the effect of $(C_6H_5Se)_2$ and the substituted diaryl diselenides $(4\text{-}ClC_6H_4Se)_2$, $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ as cytotoxic and apoptotic agents against cancer cells in vitro or in vivo.

In the present study, (C_6H_5Se) and one of its substituted structures, $(4\text{-}ClC_6H_4Se)_2$, only presented significant cytotoxic effects against the HT-209 cells at a concentration of 80 μM. A similar study that used a neuroblastoma cell line reported cytotoxic effects at lower concentrations (10–30 μM (C_6H_5Se)). However, this discrepancy may be related to differences between the SH-SY5Y and HT-29 tumor cell lines, as they exhibit different gene profiles when exposed to potent toxic substances (Thirunavukkarasusx et al., 2011). These results suggest that (C_6H_5Se) has a selective action and therefore offers an opportunity to investigate its use as a therapeutic agent. This selectivity has been observed with other selenium compounds, where cancer cells, including lung (A549) and head and neck (HSC-3), were substantially more sensitive to selenite and prone to induction of apoptosis than the breast cancer cell line MCF-7 (Suzuki et al., 2010). The $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds induced cytotoxicity and alterations in cell morphology in HT-29 cells in a dose-dependent manner: 20 μM (24.4 vs. 65.2%), 40 μM (81.8 vs. 81.7%) and 80 μM (91.2 vs. 96.1%), respectively. A recent study evaluated the ability of different selenium compounds (selenate, selenite, MeSeA, MeSeCys and SeMet) to induce cell death in HT-29 cells (Lunøe et al., 2011). The most effective compound was selenite, an inorganic selenium, the percentage of cell death was 21 (10 μM) and 39% (100 μM), followed by two organic selenium compounds, MeSeA (methylseleninic acid) 2 (10 μM) and 14% (100 μM), and MeSeCys (Se-methylselenocysteine) 3% (100 μM). This suggests that the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds evaluated in the current study are potentially cytotoxic against human colon adenocarcinoma cells, albeit in vitro. The substitution of a hydrogen atom on the aryl group of diaryl diselenide by an electron withdrawing group (-trifluoromethyl) or an electron donating group (-methoxyl) altered the cytotoxicity when compared to diphenyl diselenide. However, these effects were independent of the nature of the aromatic ring in the diaryl diselenide. Both molecules demonstrated greater cytotoxicity compared to (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$. It has been reported that selenium can inhibit cell proliferation, inducing injury via generation of reactive oxygen species (ROS) (Rudolf et al., 2008). ROS levels can activate the JNK pathway and caspases-3 and 9 via cytochrome c, with down-regulation of Bcl-2 and up-regulation of Bax (Chen et al., 2011). Also, it has been demonstrated that (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$ present higher thiol peroxidase activity and an improved antioxidant potential than $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in vivo (Meotti et al., 2004). Since, selenium-induced apoptosis in cancer cells can be suppressed by antioxidants (Wu et al., 2010), it is possible that the higher antioxidant potential of (C_6H_5Se) and $(4\text{-}ClC_6H_4Se)_2$ could trigger a less effective cytotoxic effect on HT-29 cells than $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$.

Since apoptosis is thought to be the mediator of selenium anticancer activity, we verified, by an Annexin-PE staining assay, that the cytotoxicity effect caused by the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds was mediated by apoptosis. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners. One pathway by which caspases can be activated involves the extrinsic death receptor pathway, where death ligands bind to death receptors, activating caspase 8 and subsequently initiating apoptosis by cleaving other downstream or executioner caspases (Wong, 2011). When $(C_6H_5Se)_2$ and its substituted structures were tested for their ability to stimulate expression of caspase-8, $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM) was the only compound that induced high levels of caspase-8 mRNA. Since the upstream caspase for the extrinsic death receptor pathway is caspase-8, this suggests that $(4\text{-}MeOC_6H_4Se)$ could be activating a death receptor and therefore contributing to apoptosis in the HT-29 cells. In addition, $(4\text{-}MeOC_6H_4Se)$ could present a different biological effect from the other substituted structures due to its electron donating group (-methoxyl).

A second pathway involved in caspase activation is the mitochondrial release of cytochrome c (Wong, 2011). The cytoplasmatic release of cytochrome c activates capase-3 via the formation of a complex (apoptosome)

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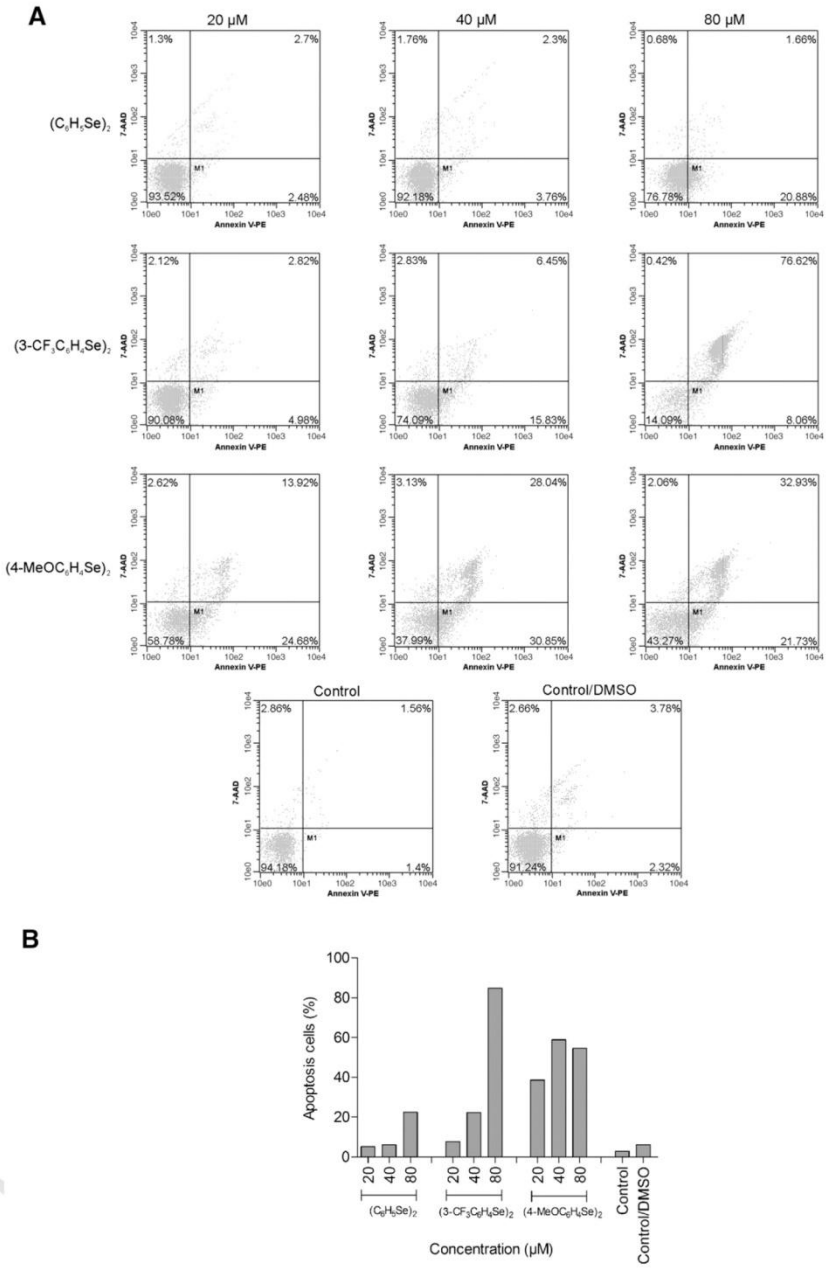

Fig. 3. Annexin V-PE analysis of HT-29 cells treated with 20, 40 and 80 μM of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, and control groups after exposure for 48 h. Panel A. Flow cytometry graphs. Panel B. Percentage of apoptotic cells.

which is made of cytochrome c, APAF-1 and caspase-9 (Jackson and Combs, 2008). Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) are closely involved in this process, an increase in Bcl-2 expression prevents cytochrome c release from the mitochondria, inhibiting the activation of caspase-9 and caspase-3, and preventing apoptosis (Santandreu et al., 2011). In the present study, Bcl-2 expression was down-regulated by $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM), whereas Bax expression was up-regulated. These findings suggest that Bax and Bcl-2 were involved in mediating the apoptotic effects associated with the cytotoxicity of $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ in HT-29 cells. In addition, caspase-9 mRNA levels were significantly increased by treatment with $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM)

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showing that caspase-9 was involved in mediating the apoptotic effects associated with these compounds. Apoptosis induced by selenium has been reported to involve the activation of caspases. It was shown that MeSeA induced apoptosis in human prostate cancer (Yamaguchi et al., 2005) and leukemia cells (Kim et al., 2001) by the activation of multiple caspases (caspases-3, -7, -8 and -9), mitochondrial release of cytochrome

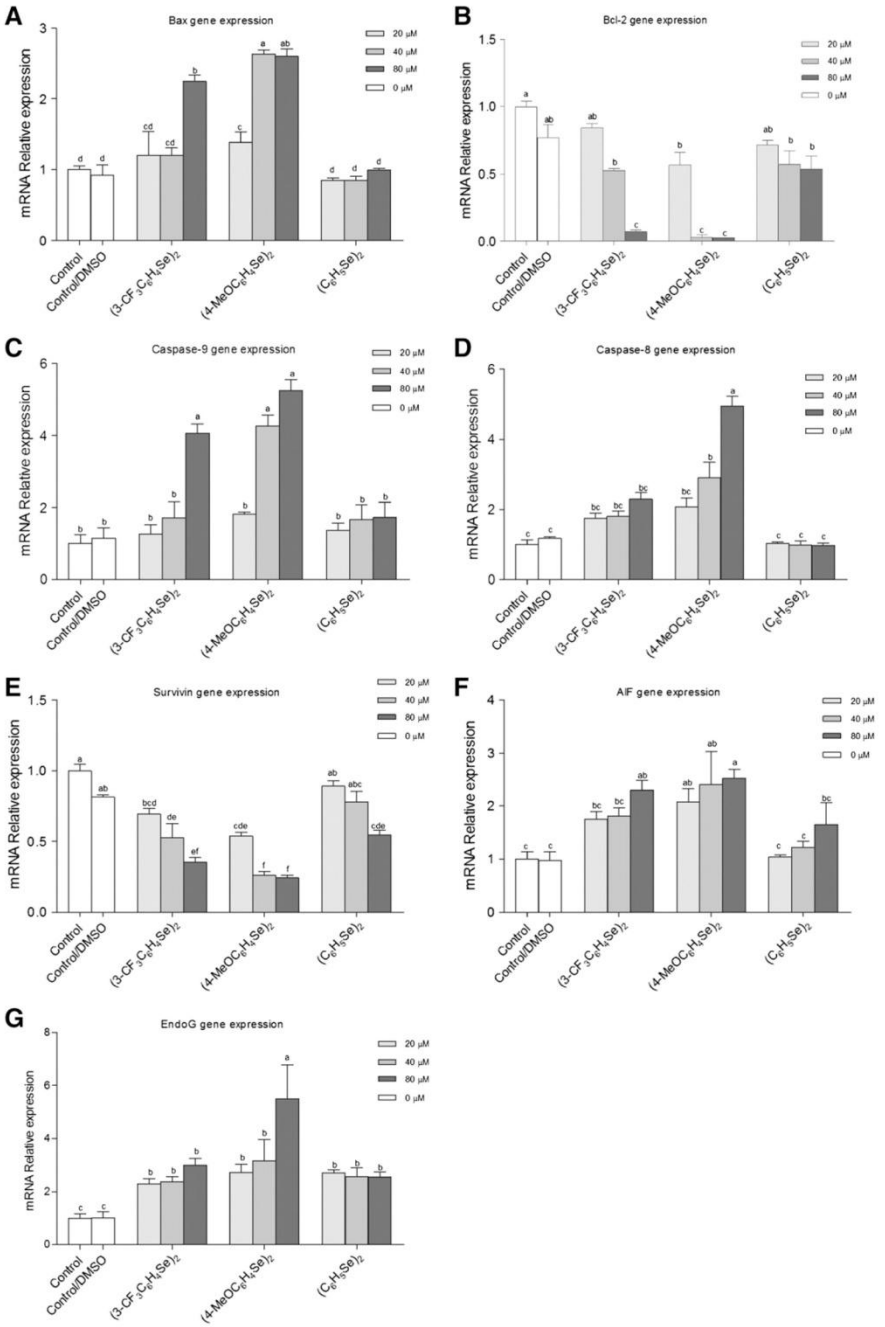

Fig. 4. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in apoptotic-related gene expression. A—Bax, B—Bcl-2, C—caspase 9, D—caspase 8, E—survivin, F—AIF and G—EndoG. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

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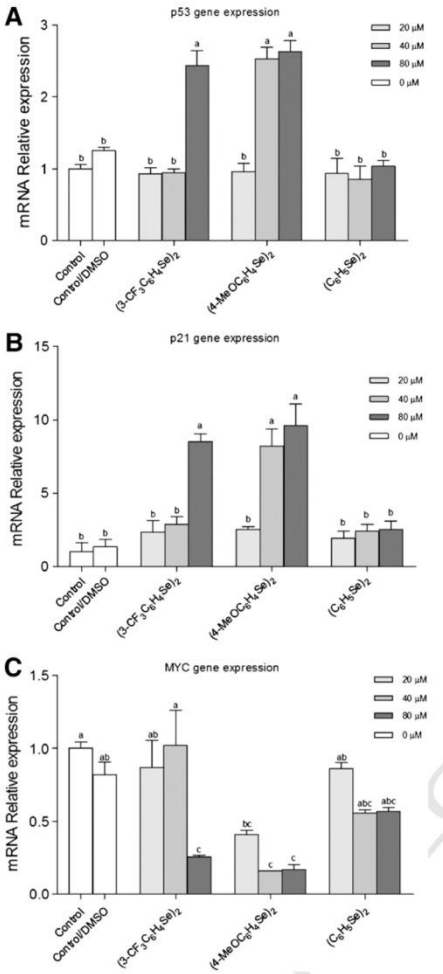

Fig. 5. Effect of $(C_6H_5Se)_2$, $(3\text{-}CF_3C_6H_4Se)_2$ and $(4\text{-}MeOC_6H_4Se)_2$, in cell-cycle arrest-related gene expression. A—p53, B—p21 and C—Myc. The data shown are expressed as the means ± SEM of a representative experiment performed in triplicate (n = 3). Letters above the bars indicate significant differences. A p-value < 0.05 was considered significant (Tukey test).

c and DNA fragmentation. Other organic and inorganic selenium compounds have been shown to induce caspase-mediated apoptosis, including MeSeCys, selenite (Suzuki et al., 2010), sodium selenite (Chen et al., 2011), and selenium dioxide (SeO_2) (Rikiishi, 2007).

Additional apoptotic factors that can be released from the mitochondrial intermembrane space into the cytosol are AIF and EndoG, which translocate to the nucleus, triggering chromatin condensation and DNA degradation in a caspase-independent manner (Vařecha et al., 2012; Wong, 2011). In the current study AIF gene expression was up-regulated by $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and EndoG was up-regulated by exposure to the two substituted diaryl diselenides as well as to $(C_6H_5Se)_2$. These results suggest time that diaryl diselenide and its substituted structures could induce apoptosis not only through the activation of multiple caspases but also through a caspase-independent pathway.

Survivin has been implicated in the inhibition of apoptosis, cell proliferation, angiogenesis, and cellular stress response. In HT-29 cells, $(3\text{-}CF_3C_6H_4Se)$ (40 and 80 μM), $(4\text{-}MeOC_6H_4Se)$ (20, 40 and 80 μM) and (C_6H_5Se) (80 μM) down-regulated the gene expression of survivin. Survivin expression was down-regulated in cell lines derived from prostate cancer cells, such as LNCaP, C4-2 (Chun et al., 2007), DU145 and PC-3 (Hu et al., 2008) treated with selenium. However, when the same selenium compound was tested with a metastatic cell line derived from PC-3 (PC-3M) and two other prostate cancer cell lines (C4-2B and 22Rv1), it had no effect on survivin expression, indicating that the apoptosis induced by selenium was not mediated by decreasing survivin expression (Liu et al., 2010). These results indicated that selenium could trigger different responses depending on the type of cell. Furthermore, p53 and p21 mRNA expression levels were increased while MYC gene expression was down-regulated upon exposure to $(3\text{-}CF_3C_6H_4Se)$ (80 μM) and $(4\text{-}MeOC_6H_4Se)$ (40 and 80 μM). The expression of p53, p21 and MYC induced by (C_6H_5Se) did not differ from that of the control groups. Investigators have shown that cells deficient in p21 escaped G2/M phase cell cycle arrest when exposed to DNA damaging agents (Rosa et al., 2007b), and that p53 arrested the cell cycle by lowering cyclin B1 levels (Rosa et al., 2007a). In addition, reduction of MYC expression was associated with cell cycle arrest in SH-SY5Y cells (Posser et al., 2011). Our results suggest that $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ influenced the expression of p53, p21 and MYC and that they could be effective as antiproliferative agents by inducing G2/M cell cycle arrest. Selenite was shown to elevate the levels of phosphorylated p53 protein at Ser-15 and concomitantly increase the expression of p21. In addition, the pro-apoptotic Bax levels were elevated and when a p53-specific inhibitor was used Bax expression was reduced by 50%, suggesting that selenium compounds could mediate tumor cell death by the p53 pathway. However, other mechanisms may also contribute to the expression of Bax. In addition, it was observed that cytochrome c, capspases-9 and -8 did not participate in the execution of apoptosis in selenite-exposed cells (Rudolf et al., 2008). In the present study, the $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ compounds appeared to mediate apoptosis in a caspase-dependent manner, since the expression of caspase-9 was significantly higher in treated HT-29 cells. However, p53 phosphorylation could also contribute to elevated Bax expression leading to apoptosis.

Of note, the role of apoptosis in the current study was determined using real-time PCR and this is a potential limitation as it is known that mRNA does not necessarily reflect protein concentration, this will be part of future work on these compounds. Furthermore, it is important to clarify that the benefit of selenium compounds is related to its bioavailability in the intestine and its ability to enter the bloodstream where it can be distributed to various organs and tissues. Of note, the bioavailability of selenium is closely related to its chemical form (Thiry et al., 2012). In this study the most cytotoxic compound, $(4\text{-}MeOC_6H_4Se)$, exhibited a significant inhibitory effect (> 40%) on HT-29 cells at a concentration of 20 μM that increased to >75% at a concentration of 80 μM following exposure for 24 h. Furthermore, these concentrations are similar to those used in other studies that reported induction of apoptosis in cancer cells with similar doses (10–100 μM) of selenium compounds (Lunøe et al., 2011; Posser et al., 2011). Further work will need to be carried out to verify the cytotoxic effects of the compounds in animal models and to confirm their bioavailability at these concentrations.

Conclusion

In summary, for the first time the cytotoxic potential of $(3\text{-}CF_3C_6H_4Se)$ and $(4\text{-}MeOC_6H_4Se)$ was demonstrated in human colon adenocarcinoma cells and the cytotoxic effect was likely mediated through the induction of apoptosis. In addition, several molecular targets of these compounds were investigated and the evidence suggests that apoptosis was stimulated by a caspase-dependant pathway as well as by a caspase-independent pathway and that cell-cycle arrest was mediated by the p53, p21 and MYC genes. However, mRNA levels do not necessarily reflect protein concentration and further work will be required to confirm these findings.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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References

Ali D, Ray RS, Hans RK. UVA-induced cyototoxicity and DNA damaging potential of benz(e)acephenanthrylene. Toxicol Lett 2010;199:193–200.
Campos VF, Collares T, Deschamps JC, Seixas FK, Dellagostin OA, Lanes CF, et al. Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the Brazilian flounder *Paralichthys orbignyanus*. J Biosci 2010;35:405–13.
Cantero-Muñoz P, Urién MA, Ruano-Ravina A. Efficacy and safety of intraoperative radiotherapy in colorectal cancer: a systematic review. Cancer Lett 2011;306:121–33.
Chen B, Wang X, Zhao W, Wu J. Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549. J Exp Clin Cancer Res 2010;29:99.
Chen XJ, Duan FD, Zhang HH, Xiong Y, Wang J. Sodium selenite-induced apoptosis mediated by ROS attack in human osteosarcoma U2OS cells. Biol Trace Elem Res 2011.
Chun JY, Hu Y, Pinder E, Wu J, Li F, Gao AC. Selenium inhibition of survivin expression by preventing Sp1 binding to its promoter. Mol Cancer Ther 2007;6:2572–80.
Clark LC, Dalkin B, Krongrad A, Combs GF, Turnbull BW, Slate EH, et al. Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. Br J Urol 1998;81:730–4.
Gochhait S, Dar S, Pal R, Gupta P, Bamezai RN. Expression of DNA damage response genes indicate progressive breast tumors. Cancer Lett 2009;273:305–11.
Henn S, Nedel F, de Carvalho RV, Lund RG, Cenci MS, Pereira-Cenci T, et al. Characterization of an antimicrobial dental resin adhesive containing zinc methacrylate. J Mater Sci Mater Med 2011;22:1797–802.
Hu H, Li GX, Wang L, Watts J, Combs GF, Lü J. Methylseleninic acid enhances taxane drug efficacy against human prostate cancer and down-regulates antiapoptotic proteins Bcl-XL and survivin. Clin Cancer Res 2008;14:1150–8.
Hu L, Sun Y, Hu J. Catalpol inhibits apoptosis in hydrogen peroxide-induced endothelium by activating the PI3K/Akt signaling pathway and modulating expression of Bcl-2 and Bax. Eur J Pharmacol 2010;628:155–63.
Huang TC, Huang HC, Chang CC, Chang HY, Ou CH, Hsu CH, et al. An apoptosis-related gene network induced by novel compound-cRGD in human breast cancer cells. FEBS Lett 2007;581:3517–22.
Jackson MI, Combs GF. Selenium and anticarcinogenesis: underlying mechanisms. Curr Opin Clin Nutr Metab Care 2008;11:718–26.
Kim T, Jung U, Cho DY, Chung AS. Se-methylselenocysteine induces apoptosis through caspase activation in HL-60 cells. Carcinogenesis 2001;22:559–65.
Limami Y, Pinon A, Leger DY, Mousseau Y, Cook-Moreau J, Beneytout JL, et al. HT-29 colorectal cancer cells undergoing apoptosis overexpress COX-2 to delay ursolic acid-induced cell death. Biochimie 2011;93:749–57.
Lin SS, Huang HP, Yang JS, Wu JY, Hsia TC, Hsai TC, et al. DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. Cancer Lett 2008;272:77–90.
Lin JJ, Hsu HY, Yang JS, Lu KW, Wu RS, Wu KC, et al. Molecular evidence of anti-leukemia activity of gypenosides on human myeloid leukemia HL-60 cells in vitro and in vivo using a HL-60 cells murine xenograft model. Phytomedicine 2011;18:1075–85.
Liu X, Gao R, Dong Y, Gao L, Zhao Y, Zhao L, et al. Survivin gene silencing sensitizes prostate cancer cells to selenium growth inhibition. BMC Cancer 2010;10:418.
Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL, et al. Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res 2010;54:967–76.
Lunøe K, Gabel-Jensen C, Stürup S, Andresen L, Skov S, Gammelgaard B. Investigation of the selenium metabolism in cancer cell lines. Metallomics 2011;3:162–8.
Machado MaS, Villela IV, Moura DJ, Rosa RM, Salvador M, Lopes NP, et al. 3′ 3-ditrifluoromethyldiphenyl diselenide: a new organoselenium compound with interesting antigenotoxic and antimutagenic activities. Mutat Res 2009;673:133–40.
Meotti FC, Stangherlin EC, Zeni G, Nogueira CW, Rocha JB. Protective role of aryl and alkyl diselenides on lipid peroxidation. Environ Res 2004;94:276–82.
Miura K, Fujibuchi W, Ishida K, Naitoh T, Ogawa H, Ando T, et al. Inhibitor of apoptosis protein family as diagnostic markers and therapeutic targets of colorectal cancer. Surg Today 2011;41:175–82.
Nogueira CW, Rocha JB. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. Arch Toxicol 2011;85:1313–59.
Posser T, de Paula MT, Franco JL, Leal RB, da Rocha JB. Diphenyl diselenide induces apoptotic cell death and modulates ERK1/2 phosphorylation in human neuroblastoma SH-SY5Y cells. Arch Toxicol 2011;85:645–51.
Rikiishi H. Apoptotic cellular events for selenium compounds involved in cancer prevention. J Bioenerg Biomembr 2007;39:91–8.
Rosa RM, Hoch NC, Furtado GV, Saffi J, Henriques JA. DNA damage in tissues and organs of mice treated with diphenyl diselenide. Mutat Res 2007a;633:35–45.
Rosa RM, do Nascimento Picada J, Saffi J, Henriques JA. Cytotoxic, genotoxic, and mutagenic effects of diphenyl diselenide in Chinese hamster lung fibroblasts. Mutat Res 2007b;628:87–98.
Rudolf E, Rudolf K, Cervinka M. Selenium activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells. Cell Biol Toxicol 2008;24:123–41.
Santandreu FM, Valle A, Oliver J, Roca P. Resveratrol potentiates the cytotoxic oxidative stress induced by chemotherapy in human colon cancer cells. Cell Physiol Biochem 2011;28:219–28.
Savegnago L, Pinto LG, Jesse CR, Alves D, Rocha JB, Nogueira CW, et al. Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. Eur J Pharmacol 2007;555:129–38.
Savegnago L, Jesse CR, Pinto LG, Rocha JB, Barancelli DA, Nogueira CW, et al. Diphenyl diselenide exerts antidepressant-like and anxiolytic-like effects in mice: involvement of L-arginine-nitric oxide-soluble guanylate cyclase pathway in its antidepressant-like action. Pharmacol Biochem Behav 2008a;88:418–26.
Savegnago L, Jesse CR, Santos AR, Rocha JB, Nogueira CW. Mechanisms involved in the antinociceptive effect caused by diphenyl diselenide in the formalin test. J Pharm Pharmacol 2008b;60:1679–86.
Savegnago L, Jesse CR, Nogueira CW. Structural modifications into diphenyl diselenide molecule do not cause toxicity in mice. Environ Toxicol Pharmacol 2009;27:271–6.
Suzuki M, Endo M, Shinohara F, Echigo S, Rikiishi H. Differential apoptotic response of human cancer cells to organoselenium compounds. Cancer Chemother Pharmacol 2010;66:475–84.
Thirunavukkarasusx N, Ghosal KJ, Kukreja R, Zhou Y, Dombkowski A, Cai S, et al. Microarray analysis of differentially regulated genes in human neuronal and epithelial cell lines upon exposure to type A botulinum neurotoxin. Biochem Biophys Res Commun 2011;405:684–90.
Thiry C, Ruttens A, De Temmerman L, Schneider YJ, Pussemier L. Current knowledge in species-related bioavailability of selenium in food. Food Chem 2012;130:767–84.
Vařecha M, Potěšilová M, Matula P, Kozubek M. Endonuclease G interacts with histone H2B and DNA topoisomerase II alpha during apoptosis. Mol Cell Biochem 2012;363:301–7.
Wang WL, McHenry P, Jeffrey R, Schweitzer D, Helquist P, Tenniswood M. Effects of iejimalide B, a marine macrolide, on growth and apoptosis in prostate cancer cell lines. J Cell Biochem 2008;105:998-1007.
Wilhelm EA, Jesse CR, Nogueira CW, Savegnago L. Introduction of trifluoromethyl group into diphenyl diselenide molecule alters its toxicity and protective effect against damage induced by 2-nitropropane in rats. Exp Toxicol Pathol 2009;61:197–203.
Wong RS. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 2011;30:87.
Wu M, Kang MM, Schoene NW, Cheng WH. Selenium compounds activate early barriers of tumorigenesis. J Biol Chem 2010;285:12055–62.
Yamaguchi K, Uzzo RG, Pimkina J, Makhov P, Golovine K, Crispen P, et al. Methylseleninic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis. Oncogene 2005;24:5868–77.
Zeng H, Combs GF. Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. J Nutr Biochem 2008;19:1–7.
Zheng D, Wang Y, Zhang D, Liu Z, Duan C, Jia L, et al. In vitro antitumor activity of silybin nanosuspension in PC-3 cells. Cancer Lett 2011;307:158–64.

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