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**Programa de Pós-Graduação em Biotecnologia**

Tese

# Calcogenobiotina e 7-cloroquinolina- 1,2,3-triazoil carboxamida com potencial antiproliferativo em linhagem de carcinoma de bexiga humano.

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Pelotas, 2019

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## Resumo

SONEGO, Mariana. **Calcogenobiotina e 7-cloroquinolina-1,2,3-triazoil carboxamidas com potencial antiproliferativo em linhagem de carcinoma de bexiga humano.** 2019. 76 p. Tese. Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Cerca de 90% dos carcinomas de bexiga são do tipo carcinoma urotelial, que se caracteriza por possuir altas taxas de recorrência e predisposição para progredir como tumor músculo invasivo, representando uma das neoplasias de maior custo para os sistemas de saúde. A quimioterapia intravesical é um padrão para o tratamento do câncer de bexiga não-músculo invasivo. Porém, a quimioterapia geralmente é agressiva e citotóxica, o que aumenta as taxas de morte causadas pelo câncer. Com isso, o objetivo desse trabalho foi avaliar duas classes distintas de compostos sintéticos, calcogenobiotinas e cloroquinolinas, mediante a linhagem tumoral de carcinoma de bexiga humano (5637), bem como suas capacidades citotóxicas em linhagem não tumoral de ovário de *hamster* (CHO-k1). Compostos heterocíclicos que apresentam propriedades farmacocinética e farmacodinâmica podem aumentar a afinidade do fármaco por uma proteína alvo, direcionando o tratamento. A avaliação biológica e atividade antioxidante (inibição da peroxidação lipídica, sequestrador de radical livre DPPH e tiol peroxidase) *in vitro* de novos derivados de calcogenobiotinas, bem como também foram investigadas as propriedades antitumorais de uma série de 7-cloroquinolina-1,2,3-triazoil-carboxamidas (QTCA), analisando suas atividades citotóxicas, mecanismos de parada do ciclo celular, indução de apoptose celular, *docking* molecular *in silico* e *western blotting*. Uma resposta proeminente foi obtida para os compostos selecionados, com derivados de biotina telúrio exibindo atividade antioxidante e antiproliferativa efetiva. O composto eficaz (5af) também não demonstrou toxicidade em estudos *in vitro* ou *in vivo*.
O ensaio de citotoxicidade identificou efeitos citotóxicos proeminentes, dependentes da dose e do tempo, após o tratamento com moléculas 1-(7-Cloroquinolin-4-ilo))-5-metil-N-fenil-1H-1,2,3-triazoil-4-carboxamida (QTCA-1) e 1-(7-Cloroquinolin-4-il)-N-(4-fluorofenil)-5-metil-1H-1,2,3-triazoile-4-carboxamida (QTCA-4), com efeito mínimo nas células normais. O ensaio de *live/dead* confirmou apoptose celular tardia significativa, enquanto a análise do ciclo celular demonstrou parada nas fases G0/G1 e o ensaio de citometria de fluxo com Anexina V-FITC/PI identificou morte celular apoptótica. Os resultados *in silico* indicam que estes compostos atuam através de diferentes mecanismos para induzir a parada do ciclo celular e a apoptose. O *western blotting* confirmou a ligação destas moléculas a proteínas pró e anti-apoptóticas. Em conclusão, os compostos heterocíclicos são candidatos promissores para induzir citotoxicidade e apoptose em células humanas de câncer de bexiga.



**Palavras-chave:** Câncer urotelial, Calcogênio, Biotina, Cloroquinolinas, Triazoil carboxamidas

## Abstract

SONEGO, Mariana. **Calcogenobiotin and 7-chloroquinoline-1,2,3-triazoyl carboxamides with antiproliferative potential in human bladder carcinoma line.** 2019. 76 p. Tese. Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Approximately 90% of bladder carcinomas are of the ureteral carcinoma type, which are characterized by high rates of recurrence and predisposition to progress as invasion tumor, representing one of the most costly neoplasms for health systems. Intravesical chemotherapy is a standard for the treatment of non-invasive bladder cancer. However, chemotherapy is usually aggressive and cytotoxic, which increases the death rates caused by cancer. Thus, the objective of the study was two distinct classes of drug synthesizers, calcogenobiotins and chloroquinolines, through a tumor of the human cell carcinoma (5637), as well as their cytotoxic capacity in non-tumor cells of hamster ovary (CHO-k1). Heterocyclic compounds which exhibit pharmacokinetic and pharmacodynamic properties may enhance drug affinity for a target protein by targeting the treatment. The biological and antioxidant evaluation (lipid peroxidation, free radical scavenger and thiol peroxidase) in vitro of new calcogenobiotin molecules have also been investigated as antitumor properties of a series of 7-chloroquinoline-1,2,3-triazoyl-carboxamides (QTCA), analyzing its cytotoxic activities, cell stop mechanisms, induction of cellular apoptosis, molecular docking in silico and western blotting. A prominent response was obtained for the sealing of the drug, with the advent of biotin exhibiting antioxidant and antiproliferative activity. The effective compound (5af) also did not demonstrate toxicity in vitro or in vivo studies. The cytotoxicity assay identified the dose-dependent and time-dependent cytotoxic effects after treatment with 1-(7-chloroquinolin-4-yl))- 5-methyl-N-phenyl-1H-1,2,3 4-carboxylic acid (QTCA-1) and 1-(7-chloroquinolin-4-yl)-N-(4-fluorophenyl)-5-methyl-1H-1,2,3-triazoyl-4-carboxamide (QTCA-4), which is minimal in normal cells. The live/dead assay confirmed significant late cell apoptosis, while a cell cycle analysis demonstrated correction at the G0/G1 phases and test flow cytometry with Annexin V-FITC/PI identified apoptotic cell death. The results in silico indicate these components through various tools to induce a cell cycle arrest and apoptosis. Western blotting confirmed the possibility of using pro and anti-apoptotic proteins. In conclusion, heterocyclic compounds are promising candidates for inducing cytotoxicity and apoptosis in human bladder cancer cells.



**Keywords:** Urothelial cancer, Calcogen, Biotin, Chloroquinolines, triazoyl carboxamides

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## Lista de Abreviaturas

AJCC – *American Joint Commission on Cancer* (Comissão Conjunta Americana de Câncer)

AUA – *American Urologic Association* (Associação Urológica Americana)

BCG – Bacilo *Calmette-Guérin*

CB – Câncer de Bexiga

CBMI-Carcinoma de bexiga músculo-invasivo

CBNMI – Carcinoma de bexiga não-músculo invasivo

C*is* – Carcinoma *in situ*

CU – Carcinoma Urotelial

EAU – *European Association of Urology* (Associação Europeia de Urologia)

ISUp – *International Society of Urology pathology* (Sociedade Internacional de patologia Urológica)

MMC – Mitomicina C

RTU – Ressecção Transuretral

TNM – Tumor-Nódulo-Metástase

UICC – *Union for International Cancer Control* (União para o Controle Internacional do Câncer)

WHO – *World Health Organization* (Organização Mundial da Saúde)

## Sumário

## 1 INTRODUÇÃO GERAL

O câncer é uma doença caracterizada pela formação e crescimento de populações celulares anormais que ameaçam a vida de um indivíduo ao interferir com as funções vitais do corpo. Embora se saiba que o câncer é causado por uma proliferação celular descontrolada, a principal causa biológica da doença ainda é pouco conhecida (López-lázaro, 2016)

O acúmulo de alterações de DNA em uma célula é imprescindível para que ocorra o câncer; visto que o DNA é o único componente celular que pode acumular e transmitir mudanças na informação genética, necessárias para a carcinogênese; no entanto, somente o acúmulo de tais alterações não é suficiente para a divisão celular descontrolada e a formação de câncer (López-lázaro, 2016).

Para o Brasil, estima-se no biênio 2018-2019 a ocorrência de 600 mil casos novos de câncer para cada ano. Sendo destes, 6.690 casos novos de câncer de bexiga em homens e 2.790 em mulheres (MINISTÉRIO DA SAÚDE & Instituto Nacional de Câncer José Alencar Gomes da Silva, 2017). A crescente na incidência do câncer de bexiga pode ser uma consequência dos efeitos do uso excessivo de tabaco, que é reconhecido como o principal fator de risco para o câncer (American Cancer Society, 2018).

O carcinoma urotelial é o câncer mais comum do trato urinário, podendo estar localizado no trato urinário inferior (bexiga e uretra) ou superior (ureter e cavidades pielocaliciais). Os carcinomas uroteliais representam 90-95% dos tumores de bexiga (Babjuk *et al.*, 2017). Sendo que destes, aproximadamente 75% são do tipo não-músculo invasivo, que inclui os limitados à mucosa ou submucosa, podendo ou não invadir a musculatura vesical, de acordo com a classificação Tumor Nódulo Metástase de 2009 (Cheung *et al.*, 2013; Babjuk *et al.*, 2017).

A heterogeneidade apresentada em coortes de pacientes com câncer de bexiga faz com que múltiplas modalidades de tratamento sejam ofertadas e apresentar uma conclusão sólida a respeito de sua eficácia é difícil de ser imposta. A ressecção transuretral (RTU) do tumor de bexiga com indução e manutenção por terapia adjuvante intravesical com Bacilo *Calmette-Guérin* (BCG) ou Mitomicina C (MMC) é a base da terapia para o CU. No entanto, a terapia com BCG demonstra algumas limitações, como intolerância por pacientes imunocomprometidos,

toxicidade local e sistêmica e elevadas taxas de progressão (Fuge *et al.*, 2015; Tomaszewski and Smaldone, 2010), enquanto que não há relatos na literatura clínica sobre a eficácia, segurança e tolerabilidade da administração de mitomicina C como adjuvante (Metcalfe *et al.*, 2017).

Grupos de pesquisa vêm trabalhando com o intuito de melhorar a qualidade de vida dos pacientes durante a administração dos quimioterápicos e a recorrência no período de cinco anos após o tratamento. Com isso, compostos heterocíclicos como a quinolina e a biotina, têm sido alternativas interessantes, em termos farmacológicos, pois apresentam importantes atividades biológicas, antitumorais e antioxidantes (Kumar, Bawa & Gupta, 2009). Enquanto a introdução de átomo de calcogênio na biotina pode agregar características biológicas adicionais, pois estes também são relatados na literatura como compostos biologicamente ativos e alvos importantes do câncer (Ba *et al*., 2010).

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 Câncer de Bexiga

O câncer de bexiga (CB) é o câncer geniturinário mais comum e o nono tipo mais incidente no mundo. A incidência é de 2-3 vezes mais comum no sexo masculino que no feminino (Ferlay *et al.*, 2013). É uma neoplasia altamente prevalente e está associado a morbidade, mortalidade e custos substanciais em todas as regiões do mundo, independentemente dos níveis de recursos (Sanli *et al.*, 2017). O carcinoma urotelial (CU) é o tipo histológico mais comum do CB presente em 90-95% dos casos (Babjuk *et al.*, 2017). A definição de CU é a invasão da membrana basal ou da lâmina própria. Esta definição imposta pela *World Health Organization (WHO)* substituiu o antigo termo carcinoma de células transicionais (Humphrey *et al.*, 2016).

Existem vários fatores de risco conhecidos para o CB, sendo os mais importantes o tabagismo, a infecção urinária crônica ou por esquistossomose, e a exposição ocupacional a determinados produtos químicos da indústria têxtil, petrolífera ou da imprensa, tais como, corantes à base de anilina e benzinas, aminas aromáticas e agentes alquilantes (Health, 2017).

Cerca de 9 em cada 10 pessoas com esse câncer têm mais de 55 anos sendo raro antes dos 40 anos. A idade média no momento do diagnóstico é de 73 anos. Os brancos são mais propensos a serem diagnosticados com câncer de bexiga do que os afro-americanos ou hispânicos americanos (Babjuk *et al.*, 2013; American Cancer Society, 2018). No entanto, muitos casos surgem sem estar associados a qualquer um destes fatores. Um fator de risco aumenta a probabilidade de vir a desenvolver câncer, mas não é condição determinante para a causa.

Para o diagnóstico patológico do tumor é realizado uma cistoscopia, que consiste na introdução de um tubo muito fino ligado a uma câmara com luz (cistoscópio), através da uretra (Health, 2017). É possível ligar instrumentos cirúrgicos na extremidade do tubo para obter o material para exame patológico (biópsia ou ressecção transuretral), com o objetivo de determinar o grau e o estágio tumor-nódulo-metástase do tumor de bexiga. É importante, na realização da biópsia, obter amostras do músculo detrusor para diferenciar a capacidade de invasão e

infiltração do tumor para o músculo, diferenciando músculo-invasivo de não-músculo invasivo (Babjuk *et al.*, 2017). Para melhor compreender essa capacidade de invadir outros tecidos da parede da bexiga é necessário que se conheça a arquitetura da parede da bexiga (Figura 1).

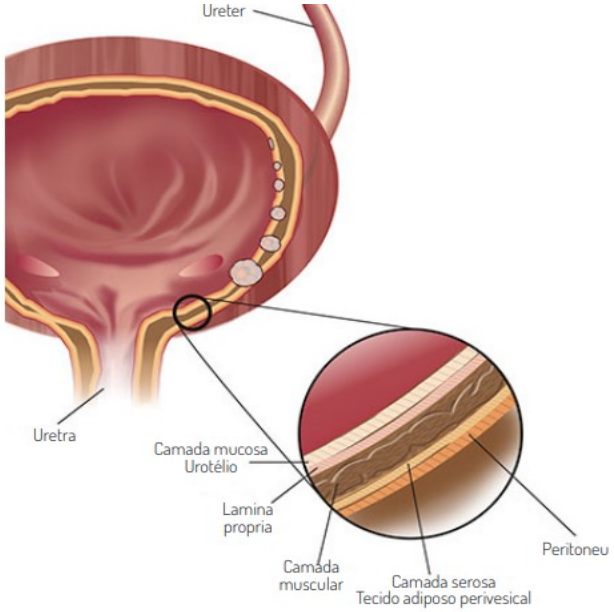


**Figura 1.** Camadas que constituem a parede da bexiga. Mucosa ou urotélio, é a camada mais interna da bexiga que fica em contato direto com a urina; lâmina própria, formada por tecido conjuntivo, vasos sanguíneos, nervos e glândulas; muscular, formada por três camadas de músculos liso; e a serosa, camada de revestimento externa formada por tecido adiposo e que separa a bexiga dos outros órgãos do abdômen. https://msd.pt/oncologia/

Em termos histológicos, a classificação consiste em: carcinoma de bexiga não-músculo invasivo (CBNMI) ou carcinoma de bexiga músculo-invasivo (CBMI), sendo aquele responsável por aproximadamente 75% dos diagnósticos (Figura 2) (Tan, 2018). Enquanto o carcinoma não-músculo invasivo (Tis, Ta e T1) permanece na camada mais interna da bexiga, o carcinoma invasivo cresce para as camadas mais profundas da bexiga afetando a lamina própria, podendo ou não penetrar na musculatura. Este tipo de câncer é potencialmente mais agressivo e propaga-se frequentemente para outros tecidos ou órgãos do corpo causando metástase (Magers *et al.*, 2019). No entanto, o CBNMI tem um risco de recorrência de 50-70% e risco de progressão de aproximadamente 20% em 5 anos, invadindo a musculatura própria e tornando-se carcinomas invasivos (T2-4); mesmo com o uso de tratamentos adjuvantes intravesicais (como BCG ou mitomicina C) recomendados por diretrizes clínicas (Tan, 2018).

Essa classificação é baseada no padrão Tumor-Nódulo-Metástase (TNM) para tumores malignos, permitindo estabelecer a dimensão da doença. Tal classificação foi desenvolvida em conjunto pela *American Joint Commission on Cancer* (AJCC) e pela *Union for International Cancer Control* (UICC) e atualizada em sua 8ª edição (Paner *et al.*, 2018; Magers *et al.*, 2019).

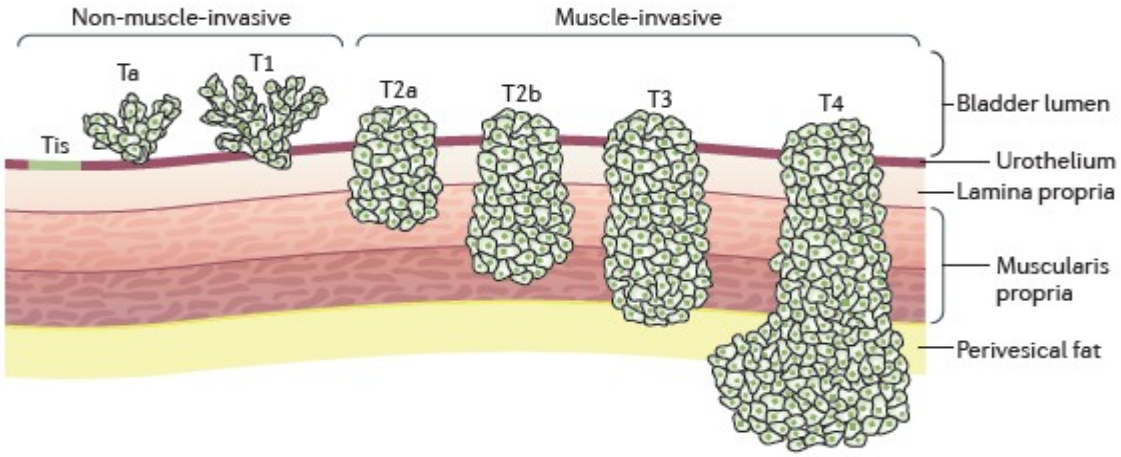


**Figura 2.** Tipos e estadiamento de tumores da bexiga urinária. O estadiamento do câncer de bexiga de acordo com o sistema Tumor, Nódulo, Metástases (TNM) é mostrado. Os tumores papilares que estão confinados à mucosa ou invadiram a lâmina própria (submucosa) são classificados como Ta e T1, respectivamente. O carcinoma *in situ* (CIS; Tis no sistema TNM) é um tumor plano, pouco diferenciado, confinado à mucosa. Os tumores do estágio 2 invadiram a camada muscular superficialmente (T2a) ou profundamente (T2b). Os tumores T3 têm invadido além da muscular própria a gordura perivesical. Os tumores T4 invadem órgãos próximos. Adaptado de DOI:10.1038/nrdp.2017.22

O grau histológico do tumor, assim como tamanho do tumor, a multifocalidade do tumor, o status de recidiva e a coexistência de carcinoma *in situ* são importantes influenciadores do fator prognóstico do tumor (Maclennan, Kirkali and Cheng, 2007).

Em 2004, a *World Health Organization* e a *International Society of Urological pathology* (ISUp) publicaram uma nova classificação histológica dos carcinomas uroteliais, fornecendo uma estratificação de pacientes diferente entre as categorias individuais em comparação com a classificação anterior de 1973 da WHO (Tabela 1) (Maclennan, Kirkali and Cheng, 2007). A classificação de 2004, é uma tentativa de padronizar a classificação do tumor urotelial, expandindo e definindo claramente as características morfológicas de células papilares não invasivas (Babjuk *et al.*, 2017).

Enquanto que as lesões planas são apresentadas como: hiperplasia, atipia reativa, atipia de significado desconhecido, displasia urotelial e C*IS* urotelial (Babjuk *et al.*, 2015).

**Tabela 1.** Grau tumoral em 1973 e em 2004 pela WHO

| | |
|---|---|
| **1973 WHO** | |
| Papiloma urotelial | |
| Grau 1: (G1) | Bem diferenciado |
| Grau 2: (G2) | Moderadamente diferenciado |
| Grau 3: (G3) | Pouco diferenciado |
| **2004 WHO** | |
| Lesões planas | |
| Hiperplasia (lesão plana sem atipia ou papiloma) | |
| Atipia reativa (lesão plana com atipia) | |
| Atipia de significado desconhecido | |
| Displasia urotelial | |
| Carcinoma urotelial *in situ* | |
| Lesões papilares | |
| Papiloma urotelial (com lesão completamente benigna) | |
| Neoplasia urotelial papilar de baixo potencial maligno | |
| Carcinoma urotelial papilar de baixo grau | |
| Carcinoma urotelial de alto grau | |

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A heterogeneidade molecular característica de todos os CU faz com que existam diferentes opções de tratamento; decidir a melhor modalidade dependerá de quão avançado é o câncer e da categoria de risco de disseminação em que ele está, somado a preferência do paciente e quais efeitos adversos estão dispostos e/ou capazes de tolerar, tornando a terapêutica do câncer de bexiga um grande desafio (Jose Sherly, 2018; Magers *et al*., 2019).

### 2.1.1 Terapias para carcinoma superficial de bexiga

A maioria dos tumores é diagnosticada em um estágio superficial e é removida cirurgicamente por ressecção transuretral, conforme as recomendações da "*European Association of Urology*" (EAU) (Babjuk *et al.*, 2017). Dependendo do estágio e grau dos tumores não-músculo invasivo, a terapia adjuvante é

recomendada como uma estratégia para reduzir a recorrência e diminuir o risco de progressão (Babjuk *et al.*, 2013).

As propriedades anatômicas da bexiga fazem dela um órgão excelente para esse tipo de terapia. A sua estrutura oca com uma via de acesso pronta através da uretra facilita a administração intravesical direta de agentes antitumorais que são relativamente contidos, minimizando assim, embora não prevenindo completamente, efeitos sistêmicos tóxicos (Siegel, Miller and Jemal, 2017).

Desde o trabalho de Morales em 1976, a instilação intravesical terapêutica do BCG vivo, que consiste em seis instilações intravesicais semanais logo após a ressecção transuretral, tem sido o padrão de tratamento para carcinoma urotelial de alto risco: carcinoma *in situ* e lesões de bexiga Ta/T1 de alto grau; com 50 a 70% de resposta clínica. (Morales, Eidinger and Bruce, 2002; Biot *et al.*, 2012).

No entanto, as reduções nos riscos de recorrência e progressão só foram observadas se a terapia de manutenção for utilizada com um tempo de manutenção variando entre 18 semanas e 3 anos (Meijden *et al.*, 2003), pois a repetição das instilações é necessária para uma infiltração robusta de células-T na bexiga, o que pode ser contornado pela exposição parenteral ao BCG antes da instilação, melhorando significativamente a sobrevida livre de recidiva (Biot *et al.*, 2012).

Como em qualquer tratamento, os pacientes podem não completar todo o regime de manutenção do BCG pela ocorrência de efeitos adversos, pois quanto mais BCG é administrado, mais provável que os efeitos adversos apareçam. Em outras palavras, quanto mais tempo as instilações são dadas, mais provável que o paciente desenvolva toxicidade grave (Fuge *et al.*, 2015; Grossman *et al.*, 2008). Até 65% dos pacientes tratados com BCG relatam efeitos tóxicos locais e 12% dos pacientes não completam a terapia de manutenção (Ojea and Martı, 2007).

As reações adversas são divididas em efeitos adversos locais (relacionados à bexiga) e efeitos adversos sistêmicos. A toxicidade local incluiu cistite bacteriana, cistite induzida por BCG, hematúria macroscópica. Outros efeitos adversos locais incluíram prostatite granulomatosa, epididimite, obstrução ureteral e bexiga contraída. Já os efeitos adversos sistêmicos foram classificados como febre (39 ºC), sintomas semelhantes aos da gripe, incluindo mal-estar geral e calafrios, infecção pulmonar induzida por BCG, toxicidade hepática e sepse por BCG. Erupção cutânea, artralgia e artrite foram classificadas como possíveis reações alérgicas (Meijden *et al.*, 2003). Embora raramente, a imunoterapia com BCG pode ser fatal

para paciente imunocomprometidos, possibilidade que deve ser considerada antes do início do tratamento (Fuge *et al.*, 2015).

Desde 2012, a disponibilidade do BCG tornou-se um problema mundial, e a produção da cepa deverá cessar em 2019, limitando ainda mais a disponibilidade (Bandari *et al.*, 2018). Pois ao contrário das vacinas, as instilações intravesicais são administradas em quantidades muito maiores, o que requer uma maior produção de BCG. Como o BCG é derivado do *Mycobacterium bovis*, os fabricantes devem considerar as diferenças biológicas inatas no substrato inicial. Os períodos de incubação são longos, o equipamento especializado é caro e o desenvolvimento bacteriano é propenso a contaminação. Esses fatores limitam a escalabilidade, como evidenciado pelas dificuldades experimentadas pela Merck® em responder a um aumento na demanda global quando os laboratórios Sanofi® foram fechados em 2012 (Magers *et al.,* 2019).

Para contornar os efeitos adversos, a fatalidade da terapia em pacientes imunocomprometidos, a falha na resposta ao uso de BCG e a falta do BCG no mercado, a instilação de agentes quimioterápicos tem sido uma estratégia (Grossman *et al.*, 2008). A mitomicina C é um agente quimioterápico bem conhecido e muito usado para terapia intravesical em câncer de bexiga não-músculo invasivo para reduzir o risco de recorrência, que é alto nesta doença; é provavelmente o agente mais comumente usado, devido a sua baixa taxa de efeitos adversos e sua eficácia (Ragonese *et al.*, 2016). Esse agente é derivado de culturas de *Streptomyces caespitosus* e atua como agente alquilante, inibindo a síntese do RNA, proteínas e DNA, principalmente.

Ambos a *American Urologic Association* (AUA) e a *European Association of Urology* (EAU) consideram a MMC um tratamento padrão para o tratamento pós-operatório de dose única imediata e para terapia adjuvante em CBNMI de baixo e médio risco. Mas apesar da popularidade deste agente no tratamento de CBNMIs, muitas questões sobre a abordagem ideal para a terapia MMC permanecem sem resposta e o esquema amplamente utilizado é empírico (Tan, 2019). Mesmo quando as abordagens ideais para administração de MMC são utilizadas, a grande maioria dos tumores volta. Esta aparente resistência ao tratamento pode ser superada pela terapia sequencial de MMC ou o uso deste agente em combinação com outros agentes que possuem diferentes mecanismos de ação, como o BCG (Deng *et al.*, 2017). Mesmo tentando contornar os efeitos adversos, a taxa de recorrência ainda é

alta e nem todos os pacientes obtêm bons resultados após a quimioterapia com MMC (Ragonese *et al.*, 2016).

A quimioterapia ainda é o tratamento de primeira linha do câncer, mesmo que as drogas utilizadas atualmente apresentem alta toxicidade e baixa seletividade. Esforços para potencializar a ação de drogas e melhorar os resultados nos pacientes, estão sendo realizados; incluindo técnicas de farmacocinética para maximizar a entrega de drogas e estratégias para melhorar a absorção e a ação do medicamento (Tabayoyong *et al.*, 2018; Tan, 2019).

## 2.2 Moléculas conjugadas para entrega seletiva da droga em células tumorais

Alcançar uma real seletividade de agentes quimioterapêuticos ainda é um desafio na pesquisa do câncer. Para contornar esse desafio, o desenvolvimento de sistemas de distribuição de fármacos específicos para tumores que reconhecem as diferenças intrínsecas entre as células normais e as células tumorais, é uma necessidade urgente na quimioterapia e tem recebido crescente atenção nos últimos anos (Marc, 2008).

Um direcionamento mais específico da droga pode ser alcançado utilizando-se de agentes de direcionamento específicos, como anticorpos, vitaminas, partículas magnéticas, hormônios ou peptídeos (Nan *et al.*, 2005; Lammers *et al.*, 2012; Bareford *et al.*, 2013; Gibiansky and Gibiansky, 2014). Nesse contexto, o direcionamento de drogas mediado por pequenas moléculas, como as vitaminas, têm se demonstrado promissor em transportar especificamente drogas anticâncer para os tumores (Lee *et al.*, 2002; Lu and Low, 2002; Leamon and Reddy, 2004).

Como se sabe, para sua sobrevivência, as células vivas precisam consumir vitaminas durante seu ciclo de vida e a intensa atividade metabólica das células cancerígenas é acompanhada por um grande uso de vitaminas essenciais para sustentar seu rápido crescimento (Russell-jones *et al.*, 2004; Russell-jones, Mctavish and Mcewan, 2011). Consequentemente, os receptores envolvidos na absorção das vitaminas são superexpressos na superfície de células cancerígenas e servem para o direcionamento de drogas ao tumor. A vitamina B12, o ácido fólico, a biotina e a riboflavina são vitaminas essenciais para a divisão de todas as células, mais

particularmente para o crescimento de células cancerígenas (Knox *et al.*, 2000; Kolhatkar, Lote and Khambhati, 2011; Kozyraki and Cases, 2013).

Estas vitaminas, particularmente a biotina, têm um imenso potencial para serem utilizadas como agentes de direcionamento para a entrega de moléculas citotóxicas a uma grande variedade de tumores. Existe também um enorme potencial para usar a biotina em combinação com o folato ou a vitamina B12 como alvo duplo para células tumorais (Russell-jones, Mctavish and Mcewan, 2011). Biotina é uma vitamina solúvel em água, de baixo custo e sua facilidade de modificação química tem o potencial de superar os agentes de direcionamento mais comuns, como anticorpos monoclonais e pequenos peptídeos (Grana *et al.*, 2002).

Os sistemas de liberação/entrega de drogas podem ser modificados por estímulos externos ou internos específicos para liberar seletivamente a droga em células tumorais sem afetar as células normais, minimizando os efeitos adversos indesejáveis (Maiti *et al.*, 2013). A base dessa seletividade é o uso do ambiente diferente das células tumorais em comparação com células normais, por exemplo, pH mais baixo (Muhammad *et al.*, 2011), hipóxia (Thambi *et al.*, 2014), enzimas superexpressas (Lee *et al.*, 2012) ou maiores níveis de tiois (Lee *et al.*, 2013). O ambiente de pH mais baixo particular dos tumores primários e metastáticos em relação aos tecidos normais e a corrente sanguínea é considerado uma vantagem adicional do sistema de entrega, pois o sistema de carreamento não liberaria o fármaco antes de atingir o tecido-alvo (Tannock and Hot, 1989; Rijcken *et al.*, 2007).

Esta estratégia de direcionamento mediada por vitaminas emergiu de forma valiosa. De fato, a ligação de fármacos citotóxicos a vitaminas selecionadas, formando conjugados de fármaco droga, resulta na entrega específica de grandes quantidades do fármaco as células-alvo. Entre as vitaminas estudadas e os trabalhos realizados até o momento, a biotina tem se mostrado o agente de direcionamento mais promissor (Tripodo *et al.*, 2014).

## 2.3 Calcogênios

Calcogênios são compostos não metálicos, pertencentes ao grupo 16 da tabela periódica, e dentro deste grupo está o telúrio, elemento raro não essencial que devido as semelhanças químicas com o selênio acaba por ser considerado um

elemento de grande interesse investigativo (Markert, 1992; Chasteen, 1993). Entre as características que ligam algumas estruturas contendo telúrio e selênio estão a capacidade de apresentarem-se como miméticos a selenoenzima glutationa peroxidase (Braga *et al.*, 2009), a capacidade antioxidante (Tabarelli *et al.*, 2017) e antitumoral (Sandoval and Leve, 2010; Sredni, 2012; Shaaban *et al.*, 2014).

Em 1987, o composto de telúrio codificado como AS101, apresentou pela primeira vez propriedades imunomoduladoras e, quando administrado em camundongos apresentou efeitos antitumorais (Kalechman and Albeck, 1992). Com o sucesso desse composto o interesse nos organocalcogênios aumentou continuamente entre os pesquisadores (Ba *et al.*, 2010).

Há alguns anos, o grupo de pesquisa LabSelen-NanoBio da Universidade Federal de Santa Maria (UFSM) vem se dedicando na química dos organocalcogênios, em especial ao átomo de telúrio (De Souza *et al.*, 2015; Da Rosa *et al*., 2016; Tabarelli *et al.*, 2017; Munchen *et al.*, 2018; Wagner *et al.*, 2018). Em 2016, Da Rosa e colaboradores demonstraram a síntese e atividade de derivados ACAT em linhagem 5637. Os resultados desse trabalho foram promissores para o composto 3j, o qual contém telúrio em sua estrutura, que demonstrou ser o mais ativo contra o carcinoma de bexiga humano, assim como potencial antioxidante. Adicionalmente, toxicidade *in vitro* e *in vivo* para esse composto foi avaliada e não demonstrou danos celulares ou sinais de toxicidade (Da Rosa *et al.,* 2016).

No ano seguinte, em 2017, foi demonstrado o efeito maior do composto contendo Te, como antioxidante e antitumoral, em linhagem A549 de carcinoma de pulmão e nenhum sinal de toxicidade *in vivo* foi observado (Tabarelli *et al.,* 2017). Mais recentemente, em 2018, foi demonstrado por Munchen e colaboradores o efeito antioxidante e antitumoral *in vitro*, de enxofre, selênio e telúrio, em células 5637, onde os compostos contendo telúrio foram mais uma vez melhores em relação aos outros calcogênios, assim como demonstraram toxicidade *in vitro* e *in vivo* em roedores, menor que os compostos contendo selênio (Munchen *et al.*, 2018). Com esses resultados é possível desmistificar a toxicidade do telúrio, assim como vê-lo como potencial antitumoral.

Tem sido sugerido que o potencial antitumoral juntamente com a toxicidade seletivas dos compostos de telúrio, é devido a alta capacidade desses compostos em transformar o ambiente oxidante redox presente nas células cancerígenas em

espécies reativas de oxigênio letais que podem levar à morte celular por apoptose. As células normais permanecem inalteradas devido a baixa carga de estresse oxidativo presente, desencadeando uma toxicidade de natureza seletiva desses compostos (Vij & Hardej, 2012).

## 2.4 Compostos heterocíclicos

Heterocíclicos são qualquer classe de compostos orgânicos cujas moléculas contenham um ou mais anéis com, pelo menos, um átomo (o heteroátomo) sendo um elemento diferente de carbono, mais comumente oxigênio, nitrogênio ou enxofre. Essas estruturas podem consistir em anéis aromáticos ou não aromáticos (Julio Alvárez-Builla and José Barluenga, 2011).

O papel dos compostos heterocíclicos tem se tornado cada vez mais importante na concepção de novas classes de entidades estruturais de importância medicinal (Eswaran, Adhikari and Shetty, 2009). Entre os compostos heterocíclicos farmacologicamente importantes, a quinolina e os seus derivados são bem conhecidos na química farmacêutica, devido ao seu largo espectro de atividades biológicas e à sua presença em compostos de ocorrência natural (Eswaran, Adhikari and Shetty, 2009).

Dentre as atividades biológicas de seus derivados destacam-se agentes anticonvulsivante (Bian *et al.*, 2013), anticâncer (Wallace *et al.*, 2003), antibacteriano (Naik *et al.*, 2009), atividade vaso relaxante (Grazia *et al.*, 2002), anti-inflamatória (Wilhelm *et al.*, 2014) e antioxidante (Dorey *et al.*, 2000), incluindo produtos farmacêuticos que estão, atualmente, disponíveis como fármacos. Os proeminentes são os antimaláricos (Quinina, Cloroquina), antivirais (Saquinavir), antibacterianos (Ciprofloxacino), antiparasitário (Oxamniquina), anestésico local (Dibucaína), antiasmático (Montelucaste), anticancerígeno (Camptotecina, Irinotecano, Topotecano), antipsicótico (Aripiprazol), antiglaucoma (Cartiolol) e cardiotônico (Vesnarinona) (Afzal *et al.*, 2015).

Com o advento do alcaloide Camptotecina em 1966, por Wall e Wani, seus análogos sintéticos como Topotecano, Irinotecano e Exatecano foram introduzidos (Wall *et al.*, 1966). Essas descobertas adicionaram uma nova dimensão no campo do desenvolvimento de drogas anticâncer contendo anéis quinolônicos. Como

mostra a figura 3, onde diversos agentes cancerígenos surgiram, três inibidores da proteína quinase (Bosutinib, Lenvatinib e Cabozantinib) e um inibidor da farnesiltransferase (Tipifarnib), dentre outros (Afzal *et al.*, 2015).

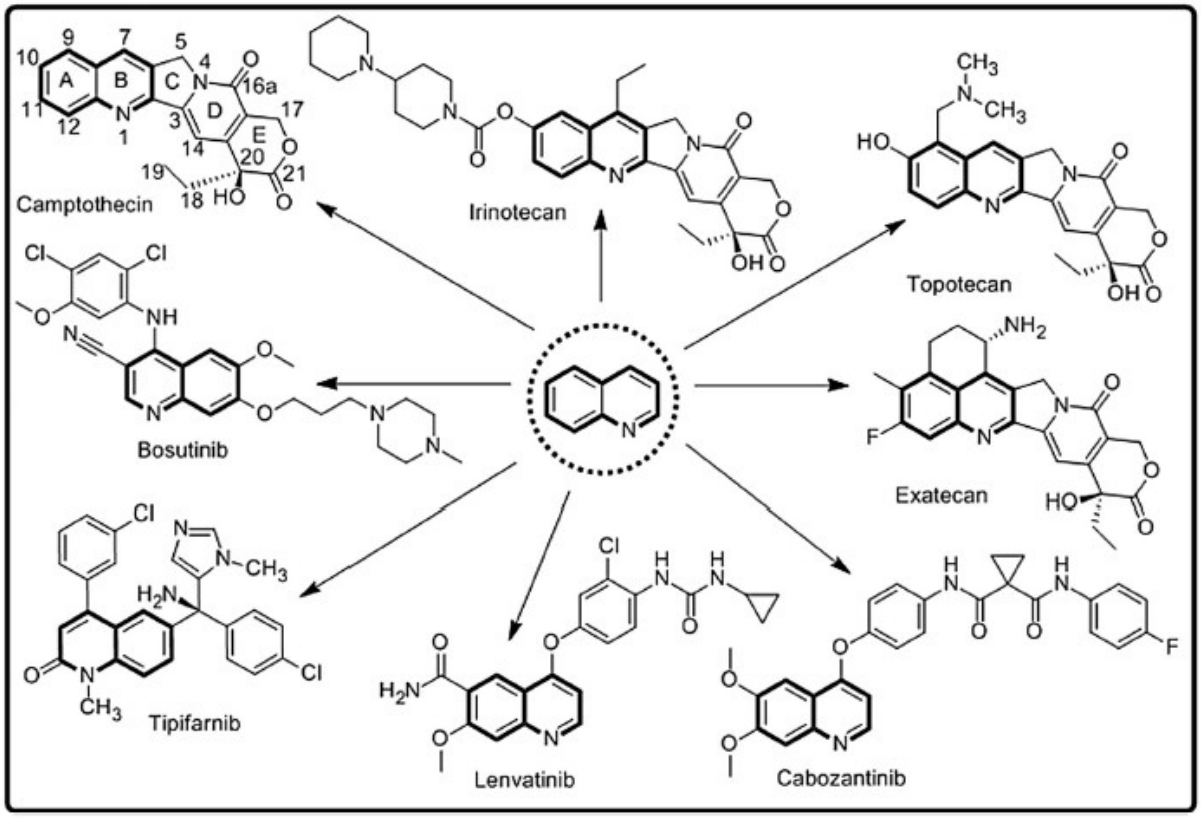


**Figura 3.** Drogas anticâncer contendo quinolina. DOI: 10.1016/j.ejmech.2014.07.044

Os derivados de quinolina atuam como inibidores do câncer através de diferentes mecanismos de ação, como inibidores de crescimento por parada do ciclo celular, apoptose, inibição da angiogênese, inibidores da topoisomerase, inibidores da proteína quinase, inibidores da telomerase, inibidor da farnesiltransferase, inibidores da anidrase carbônica, inibidor da Hsp90 e muitos mais, relatados em diferentes linhagens tumorais (Kumar, Bawa and Gupta, 2009; Bawa *et al.*, 2010).

A cloroquina demonstrou induzir a interrupção do ciclo celular e a apoptose em muitas células cancerígenas humanas. Além de seu próprio efeito anticancerígeno, evidências mostram que a cloroquina pode sensibilizar as células cancerígenas à radiação ou à quimioterapia. No entanto, o papel preciso da cloroquina desempenhado no tratamento anticâncer não é claro, estando sob investigação atraindo grande atenção (Zhang Yuan, Liao Zhi, Zhang Li-juan, 2015).

Cloroquina é um fármaco antimalárico e atualmente empregado na terapia das leishmanioses (Gemma *et al.*, 2006) que também tem sido historicamente utilizado com sucesso para o tratamento do lúpus eritematoso sistêmico e da artrite reumatoide, foi testado sobre linhagens tumorais de bexiga humana demonstrando inibição da autofagia e indução de apoptose de linhagens avançadas como 5637 e T24, dose e tempo dependentes e não demonstrou toxicidade em células epiteliais, sendo uma estratégia terapêutica eficaz (Lin *et al.*, 2017)

Dentre os sistemas heterocíclicos mais estudados encontram-se os triazóis, os quais têm despertado interesse pelo fato de possuírem um vasto campo de aplicações. Todos os triazóis são de origem sintética e não há indicações, até o momento, de que estes heterocíclicos possam ser encontrados na natureza (Melo *et al.*, 2006).

1,2,3-triazóis têm atraído contínuo interesse dos cientistas orgânicos e medicinais ao longo dos anos por causa de suas variadas atividades biológicas, como antibacteriana (Ulusoy *et al.*, 2003), antifúngica (Jiang *et al.*, 2014), anti-HIV (Danel *et al.*, 1996), anticonvulsivante (Wilhelm *et al.*, 2014), propriedades anti-inflamatórias (Wilhelm *et al.*, 2014), β-lactamase (Whiting *et al.*, 2006) e antioxidante (Saraiva *et al.*, 2016). É evidente que as propriedades favoráveis do anel 1,2,3-triazoil como caráter dipolar moderado, capacidade de ligação de hidrogênio, rigidez e estabilidade sob condições *in vivo* são responsáveis por suas atividades biológicas aumentadas (Thomas *et al.*, 2011). Assim como o fato desses compostos serem bioisósteros dos anéis heterocíclicos imidazólico, 1,2,4-triazólico e tetrazólico, encontrados em substâncias com atividades farmacológicas diversas, como antifúngica, antidepressiva, antiviral, antitumoral e anti-hipertensiva (Melo *et al.*, 2006).

Kumar, Bawa e Gupta demonstraram em 2009, as várias atividades biológicas de quinolina e seus derivados fundidos com outros heterocíclicos (Kumar, Bawa and Gupta, 2009). A síntese de moléculas híbridas contendo essas duas unidades heterocíclicas têm grande importância, pois esses compostos combinam as atividades biológicas bem conhecidas das unidades de quinolina com as atividades do 1,2,3-triazoil (Julio Alvárez-Builla and José Barluenga, 2011).

# 3 HIPÓSTESE E OBJETIVOS

## 3.1 Hipótese

A hipótese deste trabalho é a de que derivados calcogenobiotina contendo telúrio e derivados 7-cloroquinolina-1,2,3-triazoil carboxamidas (QTCA) demonstram atividade biológica *in vitro* em células de carcinoma de bexiga humano.

## 3.2 Objetivo geral

Este trabalho teve como objetivo geral avaliar a capacidade antiproliferativa *in vitro* de derivados calcogenobiotina contendo telúrio e derivados 7-cloroquinolina-1,2,3-triazoil carboxamidas, em linhagem 5637 de carcinoma de bexiga humano, bem como avaliar a citotoxicidade desses compostos em células não tumorais de ovário de *hamster* (CHO-k1).

## 3.3 Objetivos específicos

- Avaliar o potencial antitumoral *in vitro*, de derivados calcogenobiotina em linhagem de carcinoma de bexiga humano, 5637;
- Avaliar o potencial antitumoral *in vitro* de uma série de 7-cloroquinolina-1,2,3-triazoil carboxamida em linhagem de carcinoma de bexiga humano, 5637;
- Investigar a capacidade de indução de apoptose celular e parada de ciclo celular de ambas as classes de compostos;
- Avaliar a citotoxicidade *in vitro* dos derivados calcogenobiotina e da série de 7-cloroquinolina-1,2,3-triazoil carboxamida em célula não tumoral de ovário de *hamster* (CHO-k1);
- Avaliar a atividade antioxidante *in vitro,* através da inibição da peroxidação lipídica, capacidade de sequestrar o radical livre DPPH e capacidade semelhante a tiol peroxidase; e
- Simular através de ensaio de *docking* molecular, a possível ligação dos compostos com proteínas-alvo em células tumorais.

Os ensaios de toxicidade *in vitro e in vivo* e *docking* molecular foram realizados por grupos parceiros.

## 4 CAPÍTULOS

### 4.1 Manuscrito - Synthesis and biological evaluation of new antioxidant and antiproliferative chalcogenobiotin derivatives for bladder carcinoma treatment

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# Synthesis and biological evaluation of new antioxidant and antiproliferative chalcogenobiotin derivatives for bladder carcinoma treatment

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[#] These authors contributed equally to this work.
[†] Electronic Supplementary Information (ESI) available: $^{1}H$ and $^{13}C$ NRM spectra, mass spectra, and a full description of the synthesis of the compounds and biological activities are available free of charge.

**Abstract:** The synthesis, characterization, and *in vitro* biological evaluation of new antioxidant (inhibition of lipid peroxidation, scavenging of free radical DPPH, and thiol peroxidase-like activity) and antiproliferative chalcogenobiotin derivatives are tested against Bladder Carcinoma 5637 cells. A prominent response was obtained for the selected compounds, with tellurium biotin derivatives displaying effective antioxidant and antiproliferative activity. The effective compounds also demonstrated no toxicity in *in vitro* or *in vivo* studies.

## Introduction

Organochalcogenium compounds are an important class of organic compounds. The introduction of sulfur, selenium, or tellurium to organic substrates allows for modulation of the properties of these molecules, facilitating application in a variety of fields including organic synthesis, asymmetric catalysts for enantioselective reactions,[1] and key intermediates in organic transformations.[2] In material science, organochalcogenium compounds display liquid crystal properties,[3] and some selenium nanostructures show potential for electronic applications.[4] From a biological perspective, molecules containing chalcogenium display a range of antiviral,[5] antimicrobial,[6] anti-inflammatory,[7] antioxidant,[8] and antitumoral activities.[9] Recently, our research group published results demonstrating the antitumoral activity of chalcogenozidovudine derivatives.[10,11] These compounds presented prominent action against bladder carcinoma 5637 cells. Today, the search for drugs with selective action against a specific biological target is a prominent research field. This specificity may enhance therapeutic actions and, at the same time, reduce the number of side effects associated with treatments. In antitumoral targeting can be specified based on pH levels in antitumoral tissue, [12] or over expression of various receptors on cancerous cells. In this context, the use of biotin as a vector agent for cancer cells can improve targeted delivery due to increased dependence on vitamins and elevated levels of biotin receptors in tumor tissues compared to healthy cells. [13,14 15] Therefore, this study developed a small library of chalcogenobiotin derivatives from commercially available biotin using a two reaction approach. The antioxidant and antiproliferative activity of the resulting agents was explored using the bladder carcinoma 5637 cell line (Figure 1).

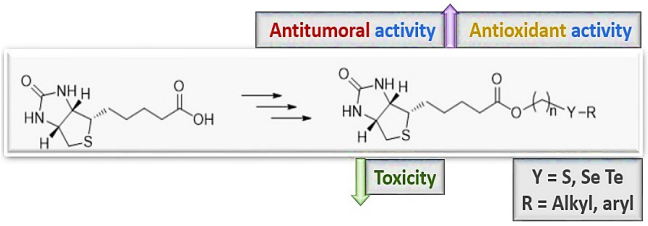
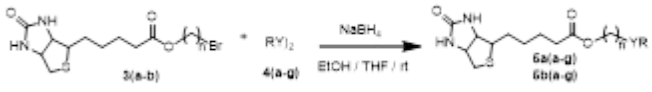



**Figure 1.** Synthesis and biological evaluation of chalcogenobiotin derivatives

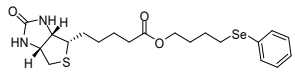
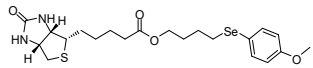
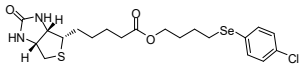
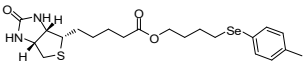
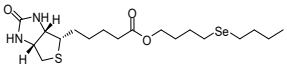
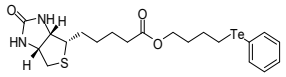
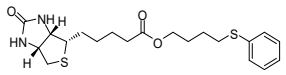
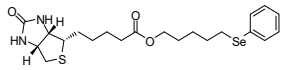
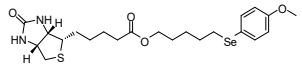
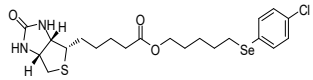
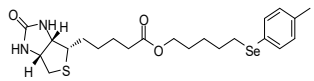
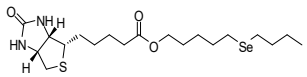
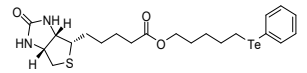
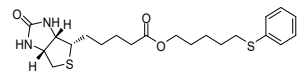
## Results and Discussion

Chalcogenobiotin derivatives **5a(a-g)** and **5b(a-g)** were synthesized from commercially available biotin utilizing two reactional steps for all derivatives, as depicted in Scheme 1 (and see SI file).




**Scheme 1.** Synthesis and biological evaluation of chalcogenobiotin derivatives **5a(a-g)** and **5b(a-g)**.

**Table 1.** General scheme for the synthesis of chalcogenobiotin derivatives **5a (a-g)** and **5b (a-g)**, for **Y** = **Se, Te or S**.

| Entry | Comp | Y | Product | Yield (%)[a] |
|---|---|---|---|---|
| 1 | 5aa | Se |  | 90 |
| 2 | 5ab | Se |  | 82 |
| 3 | 5ac | Se |  | 73 |
| 4 | 5ad | Se |  | 81 |
| 5 | 5ae | Se |  | 78 |
| 6 | 5af | Te |  | 66 |
| 7 | 5ag | S |  | 76 |
| 8 | 5ba | Se |  | 92 |
| 9 | 5bb | Se |  | 93 |
| 10 | 5bc | Se |  | 80 |
| 11 | 5bd | Se |  | 92 |
| 12 | 5be | Se |  | 78 |
| 13 | 5bf | Te |  | 68 |
| 14 | 5bg | S |  | 79 |

[a] Yields refer to the products after purification.

As observed in Figure 2, the respective chalcogenobiotin derivatives were obtained with yields ranging from 66% to 93%. In terms of electronic effects, the reactions were sensitive to the substituents attached to the aryl ring in chalcogenium moiety. For instance, higher yields were obtained by activating groups present in the original dichalcogenides, and a slight decrease in the efficiency was observed for the products containing deactivating groups (Figure 2, Entries 2, 3, 4 and 9, 10, 11, respectively). This may be related to the higher nucleophilicity of the chalcogenolates containing activating groups. Additionally, yields for selenium counterparts were higher than sulfur or tellurium derivatives. These results may be related to the lower nucleophilicity of sulfur compared to selenium nucleophiles and the lower stability of tellurolates, due to its higher tendency for oxidation. Furthermore, two alkyl derivatives were also obtained (Entries 5 and 12), showing the versatility of the methodology.

## In vitro cytotoxicity

It is known that cells need vitamins to survive. Thus, tumor cells with increased metabolic activity have higher requirements for essential vitamins.[16] Biotin is an exogenous vitamin necessary for cellular functions and growth, and its uptake by mammalian cells is mediated by biotin receptors. These receptors display increased expression in tumor cells in order to facilitate greater vitamin uptake.[17]

The cytotoxicity of the **Biotin**, **Heptyl(phenyl)tellane, 5aa**-**5ad**, **5af** and **5bf** derivatives (Figure 2 A-H) was tested within the range of 0.4 to 50 μM during 24 and 48 h of exposure. Dimethyl sulfoxide (DMSO 0.5%) was used as a vehicle for drug dilution and non-treated cells were used as negative controls. Results are shown in Table 2 and Figure 2. Biotin did not show cytotoxicity in all evaluated concentrations (Figure 2A).

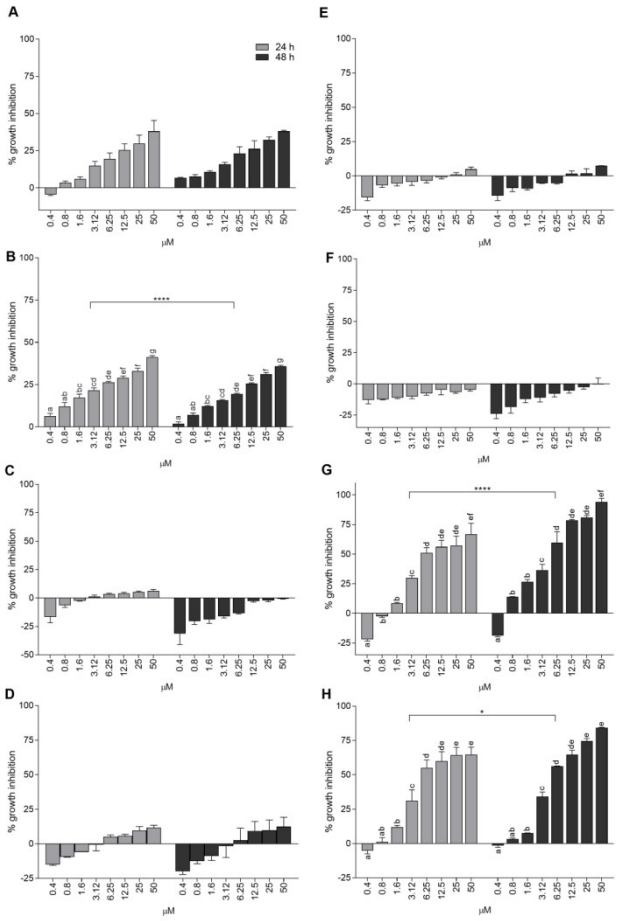



**Figure 2.** Antiproliferative effect of Chalcogenobiotin derivatives investigated by MTT assay. Growth inhibition of 5637 cancer cells following exposure to **biotin (A), heptyl(phenyl)tellane (B), 5aa (C), 5ab (D), 5ac (E), 5ad (F), 5af (G)** and **5bf (H)** for 24 and 48h. Data are expressed as means ± SEM from three independent experiments. The letters indicate the differences between concentrations within each time. * p=0.0222, **** p<0.0001.

Analyzing the obtained results, an effective influence of the chalcogenium atom on anti-proliferative activity was observed. Selenium derivatives **5aa**, **5ab**, **5ac,** and **5ad** caused no cytotoxicity against the evaluated tumor cells despite the variation of the substituent in the aromatic ring attached to the selenium atom (Figure 1, graphs **C-F**). Nevertheless, when selenium was modified for tellurium in compounds **5af** and **5bf**, variation in the antiproliferative activity of the chalcogenobiotin derivatives was observed, demonstrating a potent action against 5637 cells at lower concentrations (Figure 1, graphs **G**, H).

To study the influence of biotin on the antitumoral activity of the tellurobiotin derivatives, we prepared heptyl(phenyl)tellane, a compound containing tellurophenyl attached to a alkylic chain (Figure 3; for experimental procedure, see SI file).

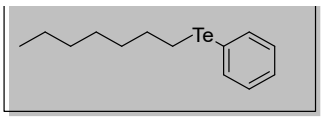



**Figure 3.** heptyl(phenyl)tellane

Like biotin, heptyl(phenyl)tellane displayed low cytotoxicity (Figure **2B**) which reduced over time. This result demonstrates the importance of biotin conjugated with the phenyltellurium group for improving antitumoral activity. In addition, the tellurium biotin derivatives **5af** and **5bf** significantly reduced 5637 cell line viability *in vitro* in a time-dose-dependent manner (Figures **2G** and **2H**), while biotin derivatives containing selenium (**5aa**-**5ad**) did not cause cytotoxicity.

The $IC_{50}$ for compounds **5af, 5bf,** and **heptyl(phenyl)tellane** was determined using a MTT reduction assay. [18] $IC_{50}$ values were calculated using the non-linear regression test in GraphPad Prism 7.04 using the growth inhibition rate (%) at 24 and 48 h. **5af** and **5bf** derivatives displayed cytotoxicity for the 5637 cell line in low concentrations (Table 2). These results demonstrate chalcogenobiotin derivatives are promising candidates for antitumor drugs for bladder cancer.

**Table 2.** Susceptibility of Bladder Carcinoma 5637 cells to chalcogenobiotin derivatives[a]

| Compound | $IC_{50}$ 24 h | $IC_{50}$ 48 h |
|---|---|---|
| **5af** | 5.8 ± 3.11 | 4.73 ± 2.13 |
| **5bf** | 7.63 ± 3.17 | 6.71 ± 1.87 |
| **heptyl(phenyl)tellane** | - | - |

[a] $IC_{50}$ values are given in μM. All data were obtained from three independent experiments and are presented as mean ± SEM inhibition. It was not possible to calculate the IC50 for heptyl(phenyl)tellane, as this compound failed to inhibit 50% of cell growth.

As described, the use of biotin represents an interesting strategy to increase targeted delivery of new drugs to tumor cells and reduce the cytotoxic effects of drug candidates on healthy cells. Thus, the **5af** and **5bf** derivatives as well as the biotin and heptyl(phenyl)tellane compounds were selected for testing of cytotoxicity in Chinese Hamster Ovary Cells (CHO-K1) (Figure 4 and Table 3). CHO-K1 cells were exposed to concentrations ranged from 0.4-50 μM for 24 and 48 h. While biotin did not display cytotoxicity against CHO-K1 cells (Figure 4**A**), heptyl(phenyl)tellane inhibited 50% of cell growth at a concentration of 12.5 μM for 24 h (Figure **4B** and Table 3).

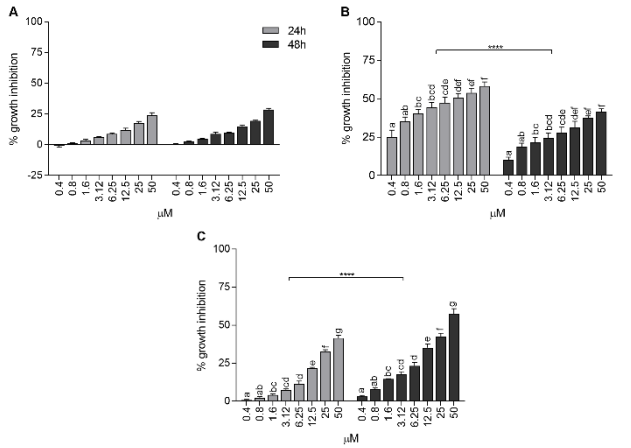



**Figure 4.** Antiproliferative effect of Chalcogenobiotin derivatives investigated by MTT assay. Growth inhibition of CHO-K1 cells following exposure to **biotin** (**A**), **heptyl(phenyl)tellane** (**B**) and **5af** (**C**) after 24 and 48 h. Data are expressed as means ± SEM from three independent experiments. The letters indicate the differences between concentrations within each time. **** p<0.0001.

The tellurobiotin derivative **5af** demonstrated good results in CHO-K1 cells regarding cytotoxicity in 5637 cells (Figure 3C). Both compounds required higher concentrations to inhibit cell growth in 50%, highlighting compound **5af** that in 24 h of treatment required approximately 70 μM to reach the $IC_{50}$ in CHO-K1 cells, as seen in Table 3, whereas in cells of the 5637 lineage required only 5.8 μM (Table 2, Figure 2).

**Heptyl(phenyl)tellane** displayed similar cytotoxicity against CHO-K1 cells and bladder carcinoma cells. Therefore, the incurporation of tellurophenyl moiety in a biotin nucleus allowed for tuning of the toxicity rates of this nucleus for healthy and cancerous cells, improving the targeting of these new drugs candidates.

**Table 3.** Susceptibility of Chinese Hamster Ovary Cells to chalcogenobiotin derivatives[a]

| Compound | $IC_{50}$ 24 h | $IC_{50}$ 48 h |
|---|---|---|
| 5af | 70.02 ± 9.47 | 34.19 ± 5.39 |
| heptyl(phenyl)tellane | - | - |

[a]$IC_{50}$ values are given in μM. All data were obtained from three independent experiments and are presented as mean ± SEM inhibition. Due to the noisy and incomplete data obtained for heptyl(phenyl)tellane, which reduces the ability of nonlinear regression methods to provide scientifically meaningful results, it was not possible to calculate the $IC_{50}$ of heptyl(phenyl)tellane.

## Cell cycle analysis

Tellurobiotin derivatives are organic molecules that reduce cell growth in a dose dependent manner by increasing the percentage of cells in the S (**5af**) and G2/M (**5bf**) phases of the cell cycle at concentrations of 12.5 μM (Figure 5). The results indicate that 5af and 5bf interfere with important processes such as DNA replication and possibly others related to cell division. In this sense, the accumulation of cells in the S and G2/M phases of the cell cycle, are in agreement with the reports in the literature, in which the organotrexate AS101 presented growth stop in G2/M and the results presented by Sailer et al. 2003, show that the diphenyl diteluret causes the cell cycle to stop at the S and G2/M phases.[19,20] Compounds containing tellurium have been recently used as an adjuvant in anticancer therapy and their use is further supported by this study.[21] The use of tellurobiotin as an adjuvant and the biotin molecule as a vector may further enhance tellurium action on its own.

In addition, the conventional biotin displays the same behavior as the control (Figure 6), increasing the relevance of the activity of the **5af** and **5bf** derivatives.

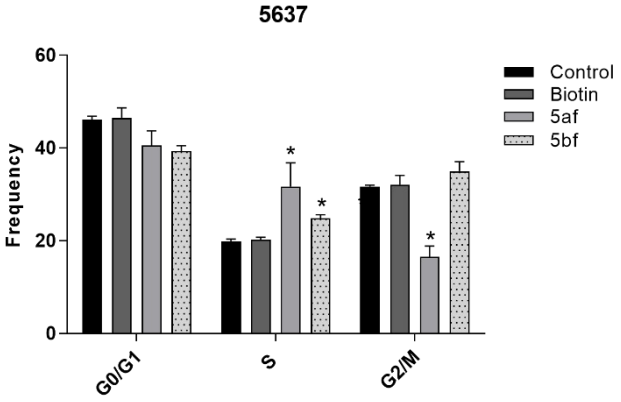



**Figure 5.** Frequency of 5637 cells at each stage of the cell cycle following treatment. Untreated cells and cells treated for 24 h with biotin, **5af,** and **5bf** (12.5 μM) were analyzed for DNA content by flow cytometry. Each column represents a media of three independent treatments. The standard deviation of the mean is represented by an error bar. * compared to control (p<0.05).

Conversely, tellurophenyl in the heptyl(phenyl)tellane did not display activity against bladder carcinoma cells. Together, these results demonstrate the antiproliferative activity observed is due to a combination of the tellurophenyl unity and biotin (Figure 6).

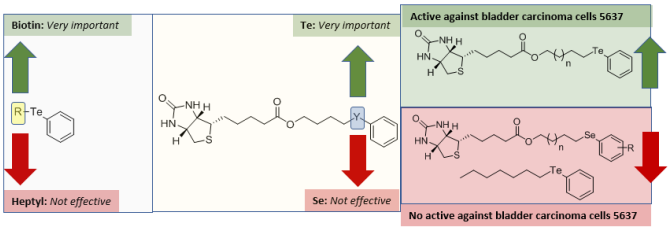



**Figure 6.** Structure-Activity Relationship (SAR) for antiproliferative activity in 5637 bladder carcinoma cells

## *In silico* docking study

The phosphatidylinositol 3-kinase (PI3K) family is a critical signal transduction pathway that regulates multiple cellular functions (cell growth, proliferation, differentiation, motility, and survival), and is the most frequently activated signaling pathway in human cancer, including bladder câncer.[22-24] Previous studies have indicated that PI3K inhibition represents a potential target for

new anticancer agentes.[22,25-27] Based on these observations, we used the molecular docking approach to predict the potential binding mode between the most active compounds in this study (**5af** and **5bf**) and the PI3Kα enzyme. The docking results indicated that both **5af** and **5bf** interact at the same site as the PI3K inhibitor PIK-93 (Figure 7). [22] According to the docking, the **5af** and **5bf** present practically the same binding position, interacting with the Tyr864 and Asp961 residues by H-bonds, and with Met800 and Met950 by hydrophobic interactions (Figures 7B, C). In addition, we verified that the tellurium atom interacts with the amino group from the backbone of the Val879 residue, similarly to PIK-93 (Figure 7A). The predicted binding energy ($\Delta G_{bind}$) indicated that both compounds bind spontaneously with the enzyme ($\Delta G_{bind}$: PIK-93 = -7.7 kcal/mol; **5af** = -7.0 kcal/mol; **5bf** = -7.3 kcal/mol). Together, these data support the hypothesis that **5af** and **5bf** are potential PI3K inhibitors.

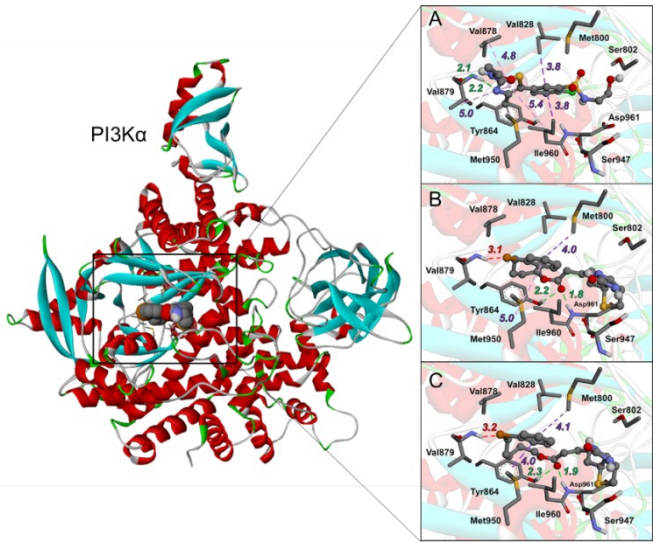



**Figure 7.** Docking simulations with the PI3Kα enzyme. (**A**) The PIK-93, (**B**) **5af,** and (**C**) **5bf** molecules interact with the PI3Kα ATP binding site. Purple and green dotted lines represent hydrophobic interactions and H-bonds, respectively. The intermolecular interaction distances are in Å. PIK-93 is a well know PI3K inhibitor and was used to validate the docking protocol and to compare the interactions with the active site. For comparison, the Val879, Tyr864, Met800, Met950, and Ile960 residues from the PI3K model are represented by Val882, Tyr867, Met804, Met953, and Ile963 in the PDB ID 2CHZ structure, respectively.

## Antioxidant and toxicity studies

A serious drawback to the development and application of drugs for some therapies - especially antitumoral compounds - is their toxicity, particularly those related to oxidative stress. On the other hand, compounds containing chalcogenium, especially selenium and tellurium, appear as efficient protective antioxidants. As such, the chalcogenobiotin derivatives **5af** and **5bf** that displayed antiproliferative activity against 5637 bladder cancer cells were selected for *in vitro* evaluation as antioxidant agents and for *in vitro* and *in vivo* assays to determine their toxicity.

## Thiobarbituric acid reactive substances (tbars)

Cellular membrane has mainly phospholipids in its composition. Lipids undergo peroxidation and form aldehydes, such as malondialdehyde. The products of lipid peroxidation react with thiobarbituric acid forming a pink-colored complex that can be quantified in TBARS assay. The antioxidant capacity of chalcogenobiotin derivative compounds was evaluated. All compounds (final concentration 200 μM) inhibited phosphatidylcholine (lipid source) peroxidation induced by Fe(II) (Figure 8). The compounds were more effective than one of the positive controls (200 μM diphenyl diselenide) and presented antioxidant activity statistically equal to α-tocopherol (positive control). Inhibitory concentration curves were then calculated. The calculated $IC_{50}$ for lipid peroxidation inhibition was about 5 μM (**5af**) and 1 μM (**5bf**) (Table 4). The $IC_{50}$ values were lower than those found for diphenyl diselenide (248 μM). Nevertheless, compounds **5af** and **5bf** presented $IC_{50}$ values statistically lower than those obtained with α-tocopherol (60 μM). These results indicated that these compounds are effective in preventing lipid peroxidation even at low concentrations.

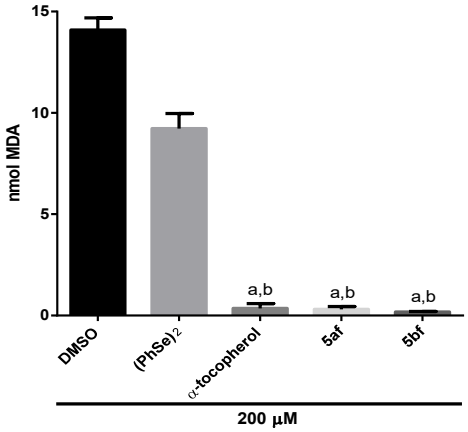



**Figure 8.** Effect of the chalcogenobiotin derivative compounds (200 μM) on lipid peroxidation induced by iron. Diphenyl diselenide ($(PhSe)_2$) and α-tocopherol (200 μM) were used as positive controls. Data were expressed as means ± S.E.M. from three independent experiments and differences are considered significant at $p < 0.05$. a ≠ DMSO; b ≠ $(PhSe)_2$ (post hoc test: Tukey $p < 0.05$).

## Scavenger activity of radical 2,2-diphenylpicrylhydrazyl (dpph)

One of the ways to test the antioxidant effect is by scavenging free radicals. Here, 2,2-diphenylpicrylhydrazyl (DPPH) was used as a free radical to test the ability of compounds **5af** and **5bf** to scavenge this radical. At 1 mM, compounds **5af** and **5bf** scavenged approximately 50% of the DPPH (Figure 9). The time required to scavenge 50% (t1/2) of the DPPH radical was calculated using a final concentration of 500 μM, with the compounds presenting a $t_{1/2}$ statistically equal to Butylated Hydroxy Toluene (positive control) at the same concentration (Table 4).

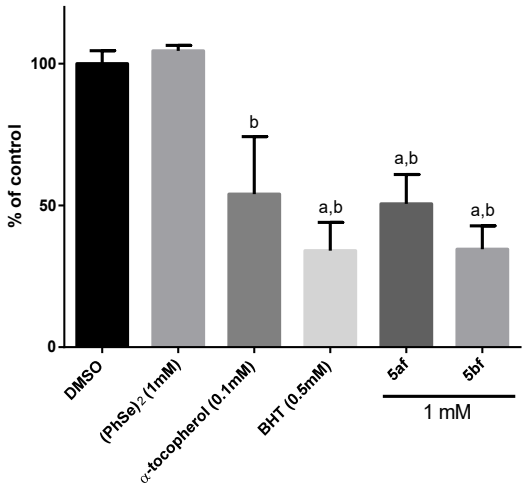



**Figure 9.** DPPH scavenging activity induced by chalcogenobiotin derivative compounds (1 mM). ($(PhSe)_2$), butylated hydroxytoluene (BHT, 0.5 mM), and α-tocopherol (0.1 mM) were used as positive controls. Data were expressed as means ± S.E.M. from three independent experiments and differences are considered significant at $p < 0.05$. a ≠ DMSO; b ≠ $(PhSe)_2$; (post hoc test: Tukey $p < 0.05$).

## Tiol peroxidase-like activity

This methodology demonstrates the efficacy of chalcogenobiotin derivatives in mimicking the antioxidant enzyme glutathione peroxidase (GPx), which transforms hydrogen peroxide into water. Compounds **5af** and **5bf** efficiently decomposed $H_2O_2$ at high concentrations (450 μM) similar to the rate of the positive control $(PhSe)_2$ (Figure 10). When a low concentration was tested (55 μM), compounds **5af** and **5bf** presented a $t_{1/2}$ statistically equal to the positive control Ebselen and lower than diphenyl disselenide at the same concentration (Table 4).

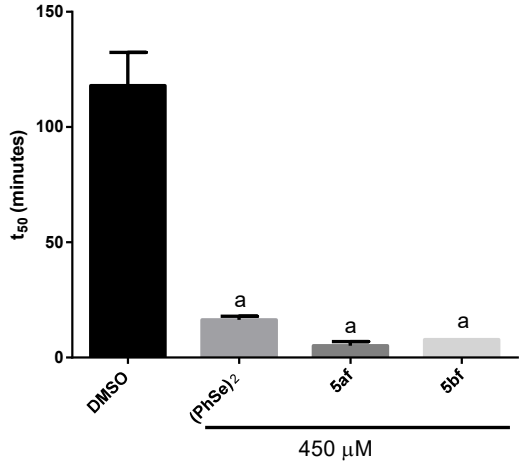



**Figure 10.** Thiol peroxidase-like activities with chalcogenobiotin derivatives (450 μM). Diphenyl diselenide ($(PhSe)_2$) was used as positive control. Data are expressed as means ± S.E.M. from three independent experiments and differences are considered significant at $p < 0.05$. a ≠ DMSO. (post hoc test: Tukey $p < 0.05$).

**Table 4** $IC_{50}$ values for inhibition of lipid peroxidation and $t_{1/2}$ to scavenge DPPH and GPx-like.

| Compound | TBARS IC50 (μM) | DPPH t1/2 (min) | GPx-like $t_{50}$ (min) |
|---|---|---|---|
| $(PhSe)_2$ | 248.30±9.28 [a] | > 180 | 120.5±27.62 [a] |
| Ebselen | n.d. | n.d. | 55.99±6.86 [b] |
| α-tocopherol | 60.33±15.94 [b] | n.d. | n.d. |
| BHT | n.d. | 20.67±7.22 [a] | n.d. |
| 5af | 5.00±1.00 [c] | 18.00±3.00 [a] | 15.78±0.93 [b] |
| 5bf | 1.33±0.33 [c] | 23.00±1.53 [a] | 38.86±0.66 [b] |

n.d.: not determined. Different letters mean significant difference among the compounds (post hoc test: Tukey $p<0.05$)

## Cell viability

Sample preparations and assays were performed in accordance with Bueno *et al*.[28] and Waczuk *et al*.[29] Incubation of human leucocytes with *t*-butyl hydroperoxide (positive control) caused a significant decrease in cell viability when compared to control (DMSO). On the other hand, incubation with compounds **5af** and **5bf** (100 μM) did not change cell viability, as observed in Figure 11.

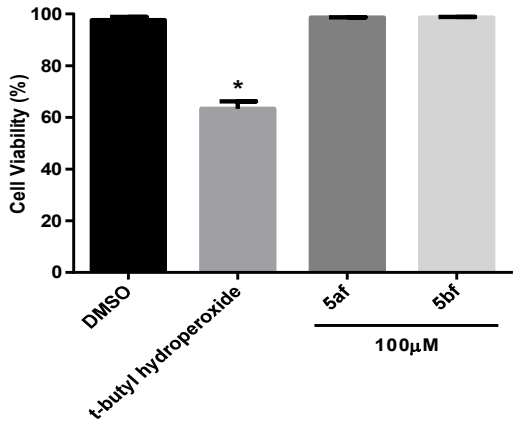



**Figure 11.** Effect of chalcogenobiotin derivative compounds on human leukocyte viability *in vitro*. t-butyl hidroperoxide (1 mM) was used as positive control. Data are expressed as means ± S.E.M. from three independent experiments and differences are considered significant at $p < 0.05$. * ≠ DMSO (post hoc test: Tukey $p < 0.05$).

## *In vivo* toxicity

Having compound 5af exhibiting more efficiently in the antitumor analyzes, in vivo toxicity studies using a more complex model, Swiss male mice were performed. Mice (n = 4 per group) were injected subcutaneously (s.c.) with compound **5af** (100 µmol/kg body weight) or DMSO (1 mL/kg body weight) and observed for 7 days (168 h). Compound **5af** did not induce overt signs of toxicity during the observation period (Figure 12).

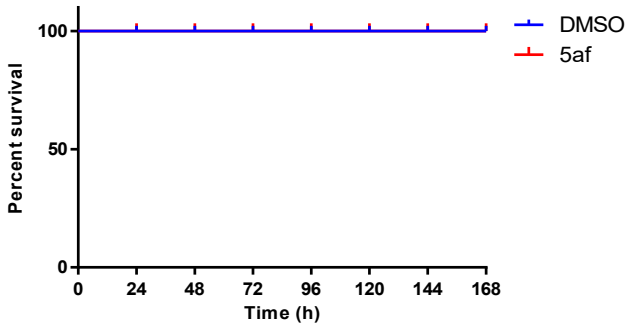



**Figure 12.** Survival rate of mice treated with **5af** compound (100 µmol/kg s.c.). Data were analyzed using Log-rank (Mantel-Cox) test.

Urea and creatinine (renal function markers) blood plasma levels were not affected 7 days (168 h) following treatment (Figure 12), indicating **5af** does not exhibit renal toxicity.

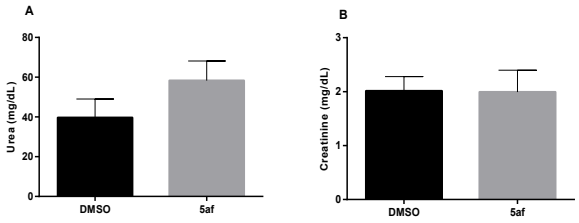



**Figure 11.** Effect of **5af** (100 µmol/kg, s.c.) on urea (**A**) and (**B**) creatinine plasma levels. Data were analyzed by unpaired t test. There was no significant difference between groups.

Aspartate aminotransferase (AST) (Figure 12A), alanine aminotransferase (ALT) (Figure 12B), and alkaline phosphatase activity (Figure 12C) are classical markers of liver function. Compound **5af** altered AST activity, but not ALT and alkaline phosphatase activity. Increased activity of one of the three hepatic markers suggests minimal hepatotoxicity of **5af**.

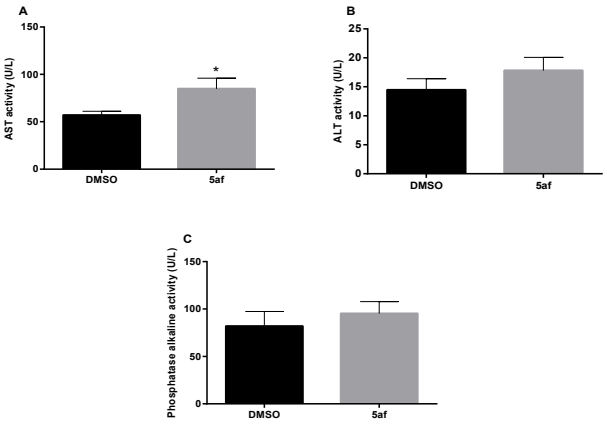



**Figure 12.** Effect of **5af** (100 µmol/kg, s.c.) on urea (**A**) and (**B**) creatinine plasma levels. Data were analyzed by unpaired t test. There was no significant difference between groups.

Additionally, evaluations for locomotor and exploratory behavior as well as for organ-to-body weight ratio (brain, liver, kidneys, and spleen) were also evaluated (see SI file).

## Conclusions

The synthesis of new chalcogenobiotin derivatives using simple two-step reactions and their application as antioxidant and antitumoral agents against bladder carcinoma 5637 cells was demonstrated. The selected compounds demonstrated both antioxidant and antitumoral activity. Additionally, *in vitro* and *in vivo* toxicity was evaluated and the effective compounds did not show signs of toxicity in the performed assays.

## Experimental Section

**1. Chemistry:** All reactions were run under an atmosphere of dry nitrogen or argon unless otherwise noted. DMSO, Thiobarbituric Acid (TBA), and Malondialdehyde was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers. The NMR spectra were recorded with a Bruker DPX-400 spectrometer with chemical shifts expressed in parts per million (in $CDCl_3$ and $Me_4Si$ as internal standard). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, brs = broad singlet, m = multiplet), coupling constants, and number of protons. All test compounds showed >95% purity as determined by LC-MS. LC-MS used the follows conditions: Shimadzu LC-20AD pump, Shimadzu SIL-20A autosampler, Shimadzu CTO-20A column oven (40 °C), Shimadzu SPD-M20A DAD Detector. Column: Phenomenex ref. 00F-4252-E0, Luna C18(2)5u, 5 µm, 100 Å, 4.6 mm × 150 mm. Gradient: 20−100% acetonitrile in water containing 0.01% of formic acid, Flow: 0.7 mL/min. MS: MicrOTOF QII Brucker, with ESI-source.

**2. Synthetic procedures:**

**2.1. Preparation of Bromobiotinyl derivatives 3**

***2.1.1*** *Preparation of 4-Bromobutyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (3a)*: In a two-necked rounded bottom flask under argon atmosphere, fitted with a

reflux condenser and magnetic stirring, biotin (4.08 mmol, 1.0 g), 1,4-dibromobutane (12.86 mmol, 1.52 ml), and DBU (12.40 mmol, 1, 85 mL) were dissolved in acetonitrile (100 mL). The reaction mixture was heated to reflux while stirring for 3 hours. The solvent was then evaporated under reduced pressure. The resulting white solid was purified by column chromatography on silica gel using ethyl acetate and methanol (90:10) as eluents to give the desired product 3a. The solvent was removed on a rotary evaporator and then the product was dried under vacuum pump, resulting in a 55% yield for compound 3a; Physical state: white solid; Yield: 55%; M.P.: 80,7 – 84,5 °C; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 6.04 (s, 1H), 5.69 (s, 1H), 4.52 – 4.49 (m, 1H), 4.33 – 4.29 (m, 1H), 4.10 (t, 2H, *J* = 6.4 Hz), 3.44 (t, 2H, *J* = 6.6 Hz), 3.18 – 3.14 (m, 1H), 2.91 (dd, 1H, $J^1$ = 12.8 , $J^2$ = 4.8 Hz), 2.74 (d, 1H, *J* = 12.8 Hz), 2.34 (t, 2H, *J* = 7.5 Hz), 1.96 – 1.91 (m, 2H), 1.81 – 1.66 (m, 6H) 1.49 – 1.43 (m, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.6, 163.8, 63.3, 61.9, 60.1, 55.4, 40.5, 33.9, 33.1, 29.3, 28.3, 28.2, 27.3, 24.7.

***2.1.2*** *Preparation of 5-Bromobutyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (3b)*: In a two-necked rounded bottom flask under argon atmosphere, fitted with a reflux condenser and magnetic stirring biotin (4.08 mmol, 1.0 g), 1,5-dibromopentane (12.86 mmol, 1.73 mL), and DBU (12.40 mmol, 1.85 mL) was dissolved in acetonitrile (100 mL). The reaction mixture was heated to reflux while stirring for 3 hours. After this period the solvent was evaporated under reduced pressure. The resulting white solid was purified by column chromatography on silica gel using ethyl acetate and methanol (90:10) as eluents to give the desired product 3a. The solvent was removed on a rotary evaporator and then the product was dried under vacuum pump, resulting in a 68% yield for compound 3b; Physical state: white solid; Yield: 68%; M.P.: 90.3 – 91.0 °C; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 6.06 (s, 1H), 5.72 (sl, 1H), 4.52 – 4.49 (m, 1H), 4.33 – 4.29 (m, 1H), 4.08 (t, 2H, *J* = 6.6 Hz), 3.44 – 3.40(m, 4H), 3.18 – 3.14 (m, 1H), 2.91 (dd, 1H, $J^1$ = 12.8 Hz, $J^2$ = 4.8 Hz), 2.74 (d, 1H, *J* = 12.8 Hz), 2.33 (t, 2H, *J* = 7.4 Hz), 1.68-1.47 (m, 10H), $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.6, 163.5, 64.0, 62.0, 60.2, 55.4, 40.5, 33.9, 33.4, 32.3, 28.3, 28,3; 27.8, 24.8, 24.64.

**2.2. Synthesis of Chalcogenobiotinyl derivatives**

***2.2.1*** *Preparation of Compounds* ***5a(a-g)*** *and* ***5b(a-g)*****:** In a two-necked rounded bottom flask under argon atmosphere and magnetic stirring, diorganoyl diselenide (0.5 mmol) solubilized in 9 mL of THF, $NaBH_4$ (2.5 mmol, 0,095 g), and 3 mL of ethanol were combined. The reaction was stirred until chalcogenolate formed (colorless solution). Subsequently, biotinyl bromoalkyl ester 3 (1 mmol) dissolved in 10 mL of THF was added and the resulting mixture was stirred for 6 hours. The reaction was then washed with an aqueous $NH_4Cl$ saturated solution (3 x 20 mL) and ethyl acetate. The organic phase was dried over $MgSO_4$ and removed by filtration and by heating under reduced pressure. The compounds **5a(a-g)** and **5b(a-g)** were purified by chromatographic column on silica gel using ethyl acetate and methanol as eluents.

*Synthesis of 4-(phenylseleno)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (5aa)***:** Physical state: pasty beige solid; Yield: 90%; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.49 – 7.47 (m, 2H), 7.26 – 7.24 (m, 3H), 5.78 (s, 1H), 5.39 (s, 1H), 4.50 – 4.47 (m, 1H), 4.30 – 4.28 (m, 1H), 4.10- 4.05 (m, 2H), 3.16 – 3.12 (m, 1H), 2.93 – 2.88 (m, 3H), 2.72 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.4 Hz. 2H), 1.76 – 1.61 (m, 8H), 1.47 – 1.39 (m, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.4, 163.9, 132.4, 130.0, 128.9, 126.7, 63.5, 61.9, 60.0, 55.3, 40.3, 33.8, 28.5, 28.2, 28.1, 27.1, 26.4, 24.6; HRMS (TOF MS ESI+) *m/z* calcd for $C_{20}H_{28}N_2O_3SSe$ [(M + H)$^+$]: 457.1064; found: 457.1100.

*Synthesis of 4-(4-methoxyphenylseleno)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ab**)*: Physical state: pasty yellow solid; Yield: 82%; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.44 (d, *J* = 8.8 Hz. 2H), 6.80 (d, *J* = 8.8 Hz. 2H), 6.27 (s, 1H), 6.02 (s, 1H), 4.48 – 4.45 (m, 1H), 4.29 – 4.25 (m, 1H), 4.04 (t, *J* = 6.0 Hz. 2H), 3.78 (s, 3H), 3.15 – 3.10 (m, 1H), 2.86 (d, *J* = 4.8 Hz. 1H), 2.81 (t, *J* = 6.8 Hz. 2H), 2.72 (d, *J* = 12.8 Hz. 1H), 2.29 (t, *J*=7.68 Hz, 2H*),* 1.72 – 1.61 (m, 8H), 1.46 – 1.40 (m, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.4, 163.9, 159.1, 135.4, 119.6, 114.6, 63.5, 61.8, 59.9, 55.3, 55.1, 40.3, 33.7, 28.4, 28.2, 28.1, 28.0, 26.4, 24.6; HRMS (TOF MS ESI+) *m/z* calcd for $C_{21}H_{30}N_2O_4SSe$ [(M + H)$^+$]: 487.1170; found: 487.1202.

*Synthesis of 4-(4-chlorophenylseleno)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ac**):* Physical state: pasty yellow solid; Yield: 73%; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.40 (d, *J* = 8.8 Hz. 2H), 7.22 (d, *J* = 8.8 Hz. 2H), 6.35 (s, 1H), 6.11 (s, 1H), 4.49 – 4.46 (m, 1H), 4.29 – 4.26 (m, 1H), 4.06 – 4.04 (m, 2H), 3.15 – 3.11 (m, 1H), 2.91 – 2.86 (m, 3H), 2.73 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.6 Hz. 2H), 1.75 – 1.63 (m, 8H), 1.46 – 1.40 (m, 2H). $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.5, 163.9, 133.8, 132.8, 129.0, 128.2, 63.4, 61.8, 60.0, 55.4, 40.4, 33.8, 28.5, 28.2, 28.1, 27.5, 26.3, 24.6; HRMS (TOF MS ESI+) *m/z* calcd for $C_{20}H_{27}ClN_2O_3SSe$ [(M + H)$^+$]: 491.0674; found: 491.0706.

*Synthesis of 4-(4-toluylseleno)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ad**)*: Physical state: pasty beige solid; Yield: 81%; $^{1}$H NMR ($CDCl_3$, 400 MHz) δ = 7.38 (d. *J* = 8 Hz. 2H), 7.07 (d, *J* = 8 Hz. 2H), 5.94 (s, 1H), 5.57 (s, 1H), 4.50 – 4.47 (m, 1H), 4.30 – 4.28 (m, 1H), 4.06 – 4.03 (m, 2H), 3.16 – 3.12 (m, 1H), 2.92 – 2.85 (m, 3H), 2.73 (d, *J* = 12.8 Hz. 1H), 2.31 – 2.28 (m, 5H), 1.74 – 1.65 (m, 8H), 1.46 – 1.41 (m, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.6, 163.7, 136.9, 133.2, 129.8, 126.1, 63.7, 61.9, 60.1, 55.4, 40.5, 33.9, 28.6, 28.3, 28.2, 27.6, 26.6, 24.7, 21.0; HRMS (TOF MS ESI+) *m/z* calcd for $C_{21}H_{30}N_2O_3SSe$ [(M + H)$^+$]: 471.1221; found: 471.1225.

*Synthesis of 4-(butylselanyll)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ae**)*: Physical state: pasty white solid; Yield: 78%; $^{1}$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 6.36 – 6.34 (m, 1H), 6.12 – 6.09 (m, 1H), 4.51 – 4.48 (m, 1H), 4.31 – 4.28 (m, 1H), 4.07 – 4.06 (m, 2H), 3.17 – 3.13 (m, 1H), 2.92 – 2.87 (m, 1H), 2.74 (d, *J* = 12.8 Hz. 1H), 2.58 – 2.54 (m, 4H), 2.32 (t, *J* = 7.4 Hz. 2H), 1.73 – 1.60 (m, 10H), 1.47 –

1.37 (m, 4H), 0.91 (t, *J* = 7.4 Hz. 3H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.5, 163.9, 63.6, 61.8, 59.9, 55.3, 40.3, 33.7, 32.5, 28.6, 28.2, 28.0, 26.8, 24.6, 23.5, 23.0, 22.8, 13.4; HRMS (TOF MS ESI+) *m/z* calcd for $C_{18}H_{32}N_2O_3SSe$ [(M + H)$^+$]: 437.1377; found: 437.1405.

*Synthesis of 4-(phenyltelluro)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5af**)*: Physical state: pasty yellow solid; Yield: 66%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.73 – 7.70 (m, 2H), 7.30 – 7.17 (m, 3H), 5.83 (sl. 1H), 5.47 (sl. 1H), 4.50 – 4.47 (m, 1H), 4.31 – 4.27 (m, 1H), 4.05 (t, *J* = 6.4 Hz. 2H), 3.17 – 3.12 (m, 1H), 2.92 – 2.88 (m, 3H), 2.73 (d, *J* = 12.8 Hz. 1H), 2.29 (t, *J* = 7.4 Hz. 2H), 1.87 – 1.62 (m, 8H), 1.47 – 1.38 (m, 2H). $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.6, 163.6, 138.4, 129.2, 127.6, 111.5, 63.5, 61.9, 60.1, 55.4, 40.5, 33.9, 30.8, 28.3, 28.2, 24.8, 7.7; HRMS (TOF MS ESI+) *m/z* calcd for $C_{20}H_{28}N_2O_3STe$ [(M + H)$^+$]: 507.0961; found 507.0995.

*Synthesis of 4-(phenylthio)butyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ag**)*: Physical state: pasty white solid; Yield: 76%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.32 – 7.25 (m, 4H), 7.18 – 7.14 (m, 1H), 6.27 (s, 1H), 5.99 (s, 1H), 4.47 – 4.44 (m, 1H), 4.28 – 4.25 (m, 1H), 4.06 (t, *J* = 6.2 Hz. 2H), 3.15 – 3.10 (m, 1H), 2.95 – 2.85 (m, 3H), 2.71 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.4 Hz. 2H), 1.74 – 1.62 (m, 8H), 1.45 – 1.40 (m, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.5, 163.9, 136.2, 129.0, 128.7, 125.8, 63.6, 61.8, 60.0, 55.4, 40.4, 33.8, 33.1, 28.2, 28.1, 27.5, 25.4, 24.6; HRMS (TOF MS ESI+) *m/z* calcd for $C_{20}H_{28}N_2O_3S_2$ [(M + H)$^+$]: 409.1620; found: 409.1638.

*Synthesis of 5-(phenylseleno)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5ba**)*: Physical state: pasty beige solid; Yield: 92%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm):7.47 – 7.45 (m, 2H), 7.28 – 7.20 (m, 3H), 6.25 (s, 1H), 6.03 (s, 1H), 4.46 – 4.43 (m, 1H), 4.27 – 4.26 (m, 1H), 4.08 – 4.01 (m, 2H), 3.14 – 3.10 (m, 1H), 2.91 – 2.84 (m, 3H), 2.71 (d, *J* = 12.8 Hz. 1H), 2.33 – 2.28 (m, 2H), 1.72 – 1.61 (m, 8H), 1.51 – 1.44 (m, 4H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.7, 164.2, 132.4, 130.5, 129.0, 126.7, 64.2, 62.1, 60.2, 55.5, 40.5, 33.9, 29.7, 28.4, 28.3, 28.1, 27.6, 26.1, 24.8; HRMS (TOF MS ESI+) *m/z* calcd for $C_{21}H_{30}N_2O_3SSe$ [(M + H)$^+$]: 471.1221; found: 471.1207.

*Synthesis of 5-(4-methoxyphenylseleno)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5bb**)*: Physical state: pasty beige solid; Yield: 93%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.44 (d, *J* = 8.6 Hz. 2H), 6.80 (d, *J* = 8.6 Hz. 2H), 6.17 (s, 1H), 5.90 (s, 1H), 4.48 – 4.45 (m, 1H), 4.29 – 4.26 (m, 1H), 4.03 (t, *J* = 6.6 Hz. 2H), 3.78 (s, 3H), 3.16 – 3.12 (m, 1H), 2.90 – 2.86 (m, 1H), 2.80 (t, *J* = 7.4 Hz. 2H), 2.72 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.4 Hz. 2H), 1.71 – 1.58 (m, 8H), 1.48 – 1.40 (m, 4H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.5, 163.9, 159.1, 135.3, 119.9, 114.6, 63.9, 61.9, 60.0, 55.3, 55.1, 40.3, 33.8, 29.6, 28.6, 28.2, 28.1, 27.9, 25.9, 24.6; HRMS (TOF MS ESI+) *m/z* calcd for $C_{22}H_{32}N_2O_4SSe$ [(M + H)$^+$]: 501.1326; found: 501.1340.

*Synthesis of 5-(4-chlorophenylseleno)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5bc**)*: Physical state: pasty white sold; Yield: 80%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.40 (d, *J* = 8.4 Hz. 2H), 7.22 (d, *J* = 8.4 Hz. 2H), 5.67 (s, 1H), 5.32 (s, 1H), 4.51 – 4.47 (m, 1H), 4.31 – 4.28 (m, 1H), 4.04 (t, *J* = 6.6 Hz. 2H), 3.17 – 3.12 (m, 1H), 2.92 – 2.87 (m, 3H), 2.73 (d, *J* = 12.8 Hz. 1H), 2.31 (t, *J* = 7.4 Hz. 2H), 1.73 – 1.60 (m, 8H), 1.49 – 1.42 (m, 4H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.7, 163.5, 133.9, 132.9, 129.2, 128.5, 64.2, 61.9, 60.1, 55.4, 40.5, 33.9, 29.6, 28.3, 28.2, 28.1, 28.0, 26.1, 24.8; HRMS (TOF MS ESI+) *m/z* calcd for $C_{21}H_{29}ClN_2O_3SSe$ [(M + H)$^+$]: 505.0831; found: 505.0861.

*Synthesis of 5-(4-toluylseleno)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5bd**)*: Physical state: pasty white solid; Yield: 92%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.38 (d, *J* = 8.2 Hz. 2H), 7.06 (d, *J* = 8.2 Hz. 2H), 4.52 – 4.49 (m, 1H), 4.33 – 4.30 (m, 1H), 4.04 (t, *J* = 6.6 Hz. 2H), 3.18 – 3.13 (m, 1H), 2.93 – 2.84 (m, 3H), 2.74 (d, *J* = 12.8 Hz.1H), 2.33 – 2.29 (m, 5H), 1.73 – 1.60 (m, 8H), 1.49 – 1.42 (m, 4H); $^{13}$C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 173.6, 163.4, 136.8, 133.1, 129.8, 126.4, 64.3, 62.1, 60.3, 55.3, 40.5, 33.9, 29.8, 28.4, 28.3, 28.1, 28.0, 26.1, 24.8, 21.0; HRMS (TOF MS ESI+) *m/z* calcd for $C_{22}H_{32}N_2O_3SSe$ [(M + Na)$^+$]: 507.1197; found: 507.1205.

*Synthesis of 5-(butylselanyll)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5be**)*: Physical state: pasty white solid; Yield: 78%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 6.22 (s, 1H), 5,90 (sl. 1H), 4.51 – 4.48 (m, 1H), 4.32 – 4.29 (m, 1H), 4.06 (t, *J* = 6.8 Hz. 2H), 3.18 – 3.13 (m, 1H), 2.90 (dd, $J^1$ = 12.6 Hz. $J^2$ = 5.0 Hz. 1H), 2.74 (d, *J* = 12.8 Hz. 1H), 2.55 (t, *J* = 7.4 Hz. 4H), 2.33 (t, *J* = 7.4 Hz. 2H), 1.72 – 1.60 (m, 10H), 1.48 – 1.37 (m, 6H), 0.91 (t, *J* = 7.4 Hz. 3H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.6, 163.9, 64.1, 61.9, 60.1, 55.4, 40.4, 33.8, 32.6, 30.1, 28.3, 28.1, 28.0, 26.2, 24.7, 23.6, 23.5, 22.9, 13.5; HRMS (TOF MS ESI+) *m/z* calcd for $C_{19}H_{34}N_2O_3SSe$ [(M + H)$^+$]: 451.1534; found: 451.1518.

*Synthesis of 5-(phenyltelluro)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5bf**)*: Physical state: pasty yellow solid; Yield: 68%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.70 – 7.68 (m, 2H), 7.30 – 7.16 (m, 3H), 6.28 (s, 1H), 6.04 (s, 1H), 4.47 – 4.43 (m, 1H), 4.28 – 4.25 (m, 1H), 4.02 (t, *J* = 6.6 Hz. 2H), 3.15 – 3.10 (m, 1H), 2.90 – 2.84 (m, 3H), 2.71 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.4 Hz. 2H), 1.75 – 1.57 (m, 8H), 1.46 – 1.39 (m, 4H); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ (ppm): 173.5, 163.9, 138.0, 128.9, 127.3, 111.5, 63.9, 61.8, 59.9, 55.3, 40.3, 33.8, 31.1, 28.2, 28.1, 28.0, 27.8, 24.6, 8.1; HRMS (TOF MS ESI+) *m/z* calcd for $C_{21}H_{30}N_2O_3STe$ [(M + Na)$^+$]: 543.0937; found: 543.0967.

*Synthesis of 5-(phenylthio)pentyl 5-{(1R,5S,6S)-3-oxo-7-thia-2.4-diazabicyclo[3.3.0]oct-6-yl}valerate (**5bg**)*: Physical state: pasty white solid; Yield: 79%; $^1$H-NMR (400 MHZ, $CDCl_3$) δ (ppm): 7.32 – 7.24 (m, 4H), 7.16 – 7.13 (m,1H), 6.20 (s, 1H), 5.95 (s, 1H), 4.46 – 4.43 (m, 1H), 4.27 – 4.24 (m, 1H), 4.04 (t, *J* = 6.4 Hz. 2H), 3.15 – 3.10 (m, 1H), 2.93 – 2.84 (m, 3H), 2.72 (d, *J* = 12.8 Hz. 1H), 2.30 (t, *J* = 7.4 Hz. 2H), 1.70 – 1.61 (m, 8H), 1.52 – 1.41 (m, 4H); $^{13}$C NMR ($CDCl_3$, 100 MHz) δ = 173.5, 163.9, 136.6, 128.9, 128.7, 125.6, 63.9, 61.9, 60.0, 55.3, 40.3, 33.8, 33.3, 28.6, 28.2, 28.1, 28.0, 24.9, 24.6; HRMS (TOF MS

ESI+) *m/z* calcd for $C_{21}H_{30}N_2O_3S_2$ [(M + H)$^+$]: 423.1776; found: 423.1783.

## 3. Antitumoral studies

### *3.1 Cell culture*

Human bladder carcinoma cells (line 5637) were obtained from the bank of the Rio de Janeiro cells (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil) and cultured in *DMEM* medium (Vitrocell Embriolife, Brasil) supplemented with 10% fetal bovine serum (Gibco, USA) in a humidified incubator at 37°C with 5% $CO_2$ and 95% air. All experiments were performed with cells in its logarithmic growth phase.

### *3.2 MTT Cell Proliferation and Cytotoxicity Assay*

The viability of 5637 cells was determined by measuring the reduction of MTT (3-[4,5-dimethyl-thiazol-2-il]-2,5-diphenyltetrazolium bromide) to formazan crystals[1]. Briefly, 5637 cells seeded at a density of $2x10^4$ cells per well in 96 well plates were cultured for 24 hours in ideal conditions. The medium was then aspirated and replaced by an equal volume (100 µL per well) DMEM/FBS containing different concentrations of the conditioning chalcogenobiotin derivatives (0.4 - 100 µM). In controls wells 100 µL DMEM/FBS were added, and vehicle controls were added with a corresponding DMSO concentration used in the dilution of the stock concentration of the test substance (DMSO concentration did not exceeded 0.5%). Following the above treatments, MTT at 5 mg/mL in phosphate-buffered saline (PBS) was added to each well, and the cells were incubated at 37 °C for an additional 3 hours. The supernatant was discarded, and 150 µl of dimethyl sulfoxide was added to each well for 10 min to dissolve the formazan crystals. The optical density levels of the cell cultures were measured spectrophotometrically using a dual beam microplate reader (Thermo-TP Plate Reader) at 492 nm. The inhibition (%) of cell proliferation was determined as follows: growth inhibition rate (%) = [1- ($Abs492_{treated\ cells}$/$Abs492_{control\ cells}$)] x 100.

### *3.3 Cell cycle assay with PI*

The evaluation of DNA content of cell strain 5637 after 24 h treatment with the compounds 5af and 5bf (concentration 12,5 µM) was based on the use of fluorescent DNA intercalator, propidium iodide (PI). The detection was performed in a Guava® Flow Cytometry easyCyte™ System plus flow cytometer. Cell cycle evaluations were performed according to the manufacturer's protocol using the Guava® Cell Cycle (Merck KGaA) reagent kit.

### *3.4 Data Analysis*

Data sets from MTT were analyzed using two-way ANOVA followed by Tukey's test for multiple comparisons. Cell cycle distribution was analyzed by two-way ANOVA followed by T-test for multiple comparisons. $p < 0.05$ was considered significant for all analyses. All data were expressed as mean ± SEM of triplicates.

## 4. Antioxidant studies

### *4.1 Thiobarbituric acid reactive substances (TBARS)*

Analysis was performed as previously described by Ohkawa et al (1979).[2] The incubation system to induce lipid peroxidation consisted of phosphatidylcholine (0.4 mg), iron sulfate (55 µM), Tris-HCl buffer pH 7.4 (1.85 mM), and biotin derivatives (final concentration 0-200 µM). The system was incubated for 30 minutes at 37 °C. Afterward, acetic acid buffer pH 3.4 and thiobarbituric acid (0.22%) were added and the samples were then incubated for one hour at 100 °C. To extract the thiobarbituric acid reactive substances (pink color) 400 µL of N-butanol was added. Then, the tubes were stirred for 30 s and centrifuged for 10 min at 6000 rpm. The supernatant was read at 532 nm. A MDA curve was prepared as a standard. Diphenyl diselenide (0-400 µM) and α-tocopherol (0-200 µM) were tested as positive control. The biotin derivative compounds were dissolved in DMSO.

### *4.2 DPPH radical scavenging assay*

Analysis was performed as previously described by Pereira et al (2014). [3] Biotin derivative compounds (1mM) were mixed with 0.3 mM DPPH in ethanol. A time curve was made for the 5af and 5bf compounds (final concentrations 500 µM). Absorbance was read at 518 nm every 30 min for 180 min. BHT (final concentration 500 µM) and diphenyl diselenide (final concentration 2 mM) were used as positive controls.

### *4.3 Thiol peroxidase-like activity*

Analysis was performed as previously described by Iwaoka and Tomoda (1994) [4] with few modifications. This method determines whether the derivatives of biotin can mimic the enzyme glutathione peroxidase activity (GPx). Thiophenol was used as an alternative to glutathione. The incubation system consisted of ethanol, thiophenol (2.5 mM), DMSO (blank) or compounds (final concentration of 0 - 450 µM), and hydrogen peroxide (2.3 mM). Ebselen and Diphenyl diselenide (55 µM) were used as a positive control. The reaction was monitored spectrofotometricaly at 305 nm for 20 minutes.

## 5. Cell viability

Analysis was performed as previously described by Bueno et al. (2013)[5]. Heparinized venous blood was obtained from healthy donors. The protocol was approved by the Ethical Committee of UFSM (n. 089.0.243.000-07). Isolated leukocytes ($2 \times 10^6$/mL) were incubated for 3 h with DMSO (0.5%), 1 mM t-butyl hidroperoxide (positive control), or indicated compounds (100 µM) in a Hank's buffer solution containing 10% human plasma. Cell viability were determined by Trypan blue exclusion.

## 6. *In vivo* study

All experiments were performed in accordance with Law 11.794, of October 8 2008, Decree 6899, of July 15, 2009, with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was approved by the Ethic

Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/31/2016 (Protocol number CEUA 4622031115).

### *6.1 Treatment*

The toxicity study was conducted using adults Swiss male mice (25 - 30g). Animals were randomized in two groups (n = 4 per group), and DMSO (1 mL/kg of body weight) or 5af (100 µmol/kg of body weight) were subcutaneously administered. Mice were monitored by a closed circuit TV for 168 h after the injection. Body weight, food, and water intake were evaluated daily. After seven days, mice were euthanized by decapitation and the blood and organs were collected.

The markers of kidney (urea and creatinine) and liver (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) function were analysed in serum using commercial kits (Labtest Diagnostica SA, Lagoa Santa, Minas Gerais, Brazil).

## 7. Data analysis

The *in vitro* analyses were repeated three times. Data were analyzed using one or two-way ANOVA, followed by Tukey test. The difference was considered to be significant when $p < 0.05$. Regarding the *in vivo* study, the data were analyzed using Log-rank (Mantel-Cox) test for analysis of survival and the unpaired t test for biochemical analysis. The difference was considered to be significant when $p < 0.05$. Data analysis was performed with the software GraphPad v. 6

## Associated content

*Electronic Supplementary Information (ESI) available: $^{1}H$ and $^{13}C$ NRM spectra, mass spectra, and a full description of the synthesis of the compounds and biological activities are available free of charge.

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**Author Contributions**

OEDR., AL, ACFS, LD contributed in the synthesis of chalcogenobiotin derivatives; MSS, CSO, FKS, TC contributed in the antitumoral evaluation; BCP, FDS, JBTR contributed in the antioxidant and toxicity evaluation; PAN contributed in the docking studies. AL and MSS contributed equally to this work.

All authors wrote and revised the manuscript.

## Notes

The authors declare no competing financial interest.

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## Abbreviations

DPPH, 2,2-Diphenyl-1-picrylhydrazyl; DBU, 1,8-Diazabicyclo(5.4.0)undec-7-ene; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); TBARS, Thiobarbituric Acid Reactive Substances; BHT, 2,6-bis(1,1-dimethylethyl)-4-methylphenol; AST, Aspartate aminotransferase; ALT, alanine aminotransferase.

**Keywords:** Antioxidants • Antiproliferative Activity • Biological Activity • Biotin • Chalcogeno-vitamins

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## 4.2 Manuscrito 2 - Synthesis and evaluation of 7-Chloroquinoline-1,2,3-triazoyl carboxamides as potent antiproliferative agents against human bladder carcinoma cells

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**Synthesis and evaluation of 7-Chloroquinoline-1,2,3-triazoyl carboxamides as potent antiproliferative agents against human bladder carcinoma cells**

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**Conflict of Interest Statement:** The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Abstract**

In the present study, the antitumoral properties of a series of 7-chloroquinoline-1,2,3-triazoyl-carboxamides (QTCA) were investigated by analyzing their cytotoxic activities against human bladder cells (5637; grade II carcinoma), in addition to the resulting cell viability, cell cycle arrest mechanisms, cell apoptosis induction, *in silico* molecular docking, and western blotting were performed. The cytotoxicity assay identified prominent dose- and time-dependent cytotoxic effects in 5637 cells after treatment with QTCA molecules, with minimal effect on normal cells. The live/dead assay confirmed the significant cell death, while cell cycle analysis demonstrated arrest in the G0/G1 phase and the Annexin V-FITC/PI flow cytometric assay identified apoptotic cell death caused by 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazoile-4-carboxamide (QTCA-1) and 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazoile-4-carboxamide (QTCA-4). The *in silico* results indicate that these compounds act through different mechanisms to induce cell cycle arrest and apoptosis. Western blotting confirmed the binding of these molecules to pro and anti-apoptotic proteins. In conclusion, QTCA's are promising candidates for inducing cytotoxicity, cell cycle arrest, and apoptosis in human bladder cancer cells.

Keywords

Anticancer; Bladder cancer cytotoxicity; Quinoline derivatives; Apoptosis; Cell cycle arrest

## 1. Introduction

Bladder cancer is the most common form of urinary cancer in developing countries, the second most common urinary cancer in developed countries, and one of the leading causes of cancer death worldwide (Siegel, Miller and Jemal, 2017). Urothelial carcinoma accounts for 90% of all histological subtypes, with non-invasive muscle bladder cancer (NMIBC) responsible for about 70% of all bladder cancers (Siegel, Miller and Jemal, 2017; American Cancer Society, 2018). Timely follow-up is extremely important due to the high rate of recurrence and progression, which makes bladder cancer the most expensive neoplasm to treat and highlights the need for development of new therapies for NMIBC (Yeung, Dinh and Lee, 2014; Eifler, Scarpato and Clark, 2015; Sanli *et al.*, 2017).

Currently, transurethral resection plus intravesical postoperative adjuvant instillation (chemotherapy and immunotherapy) is the first line treamtment for invasive non-muscular bladder cancer (NMIBC) (Kang, Li and Kang, 2019). In addition, researchers are investigating whether introduction of a number of new compounds into the bladder after surgery can help to decrease the risk of recurrence. The hope is to identify better and/or safer compounds than those used currently (Logan, Brown and Hayne, no date; Tomaszewski and Smaldone, 2010). New medicines are also needed to treat bladder cancer that does not respond or have intolerance to Bacillus Calmette-Guérin (BCG) therapy (Grossman *et al.*, 2008).

The goal of cancer therapies is to gain control or possibly terminate the uncontrolled growth of cancer cells. Targeting apoptosis is an effective approach for many cancer types, as apoptosis evasion is a nonspecific hallmark of cancer. Common measures for physical conditioning are antiapoptotic and inhibition of pro-apoptotic molecules (Pfeffer and Singh, 2018). Many research groups have specialized in development of pharmacological

compounds that are more potent and better targeted than current standard-of-care compounds, resulting in increased tumor cell killing and fewer adverse effects in patients.

Heterocycles play a very important role in drug development, since they represent pharmacophoric groups—the part of the substance responsible for its biological activity (Lednicer, 2007). Quinolines are naturally occurring *N-heterocyclic* compounds with increasing technological interest due to their pharmacological characteristics. However, few biological applications have been reported so far (Kumar, Bawa and Gupta, 2009). Nevertheless, synthetic derivatives of *N-heterocyclic* compounds have demonstrated a wide variety of biological activities, such as anticonvulsant and antinociceptive (Wilhelm *et al.*, 2014), antifungal (Manera *et al.*, 2007), antioxidant (Dorey *et al.*, 2000; Saraiva *et al.*, 2016), antibacterial (Naik *et al.*, 2009), antimycobacterial (Nayyar *et al.*, 2006), antimalarial (Bawa *et al.*, 2010), and anti-inflammatory (Clemence *et al.*, 1988). In addition, some synthetic derivatives also display significant antitumorigenic activities (Wallace *et al.*, 2003; Begnini *et al.*, 2017). These findings add a new dimension in the pharmaceutical development of anticancer drugs containing quinoline motifs, such as camptothecin and its two analogues irinotecan and topotecan (Ethyl, 1966; Afzal *et al.*, 2015). The derivatives demonstrate several activities, including growth inhibition by cell cycle arrest, apoptosis, and inhibition of angiogenesis (Kumar, Bawa and Gupta, 2009).

Among the heterocycles, 1,2,3-triazoiles act as a pharmacophoric group as well as a connector between two active substances, a strategy referred to as molecular hybridization (Melo *et al.*, 2006). Other groups have previously demonstrated the biological activity of quinolone an and triazoyl heterocycles in a single compound, with the derivatives demonstrating higher antibacterial and antifungal activity than the standard compounds (Thomas, Adhikari and Shetty, 2010).

These findings have added a new dimension to the field of anticancer drug development containing nitrogen-based heterocycles, which may contribute to the development of drugs with higher pharmacological potential. The present work aimed to evaluate the antitumoral activity of synthetized 7-chloroquinoline-1,2,3-triazoyl carboxamides (QTCA) in order to demonstrate its potential as a new therapy for bladder carcinomas.

## 2. Materials and methods

### 2.1 Synthesis of 7-chloroquinoline-1,2,3-triazoyl carboxamides

Synthesis of 7-chloroquinoline-1,2,3-triazoyl carboxamides is described in detail by Wilhelm, E. et al., 2014 [11]. Briefly, a solution of 4-azido-7-chloroquinoline 1 (0.3 mmol, 0.061 g) was diluted in DMSO (0.6 mL) (0.3 mmol) followed by the pyrrolidine catalyst (5 mol%). The crude product was purified by column chromatography on silica gel using a mixture of hexane-ethyl acetate (5:1) as eluent. For these studies, five 7-chloroquinoline-1,2,3-triazoyl carboxamides were screened: 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-1**); 1-(7-Chloroquinolin-4-yl)-*N*,5-diphenyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-2**); 1-(7-Chloroquinolin-4-yl)-*N*-phenyl-5-(trifluoromethyl)-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-3**); 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-4**); and 1-(7-Chloroquinolin-4-yl)-*N*-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-5**) (Figure 1).

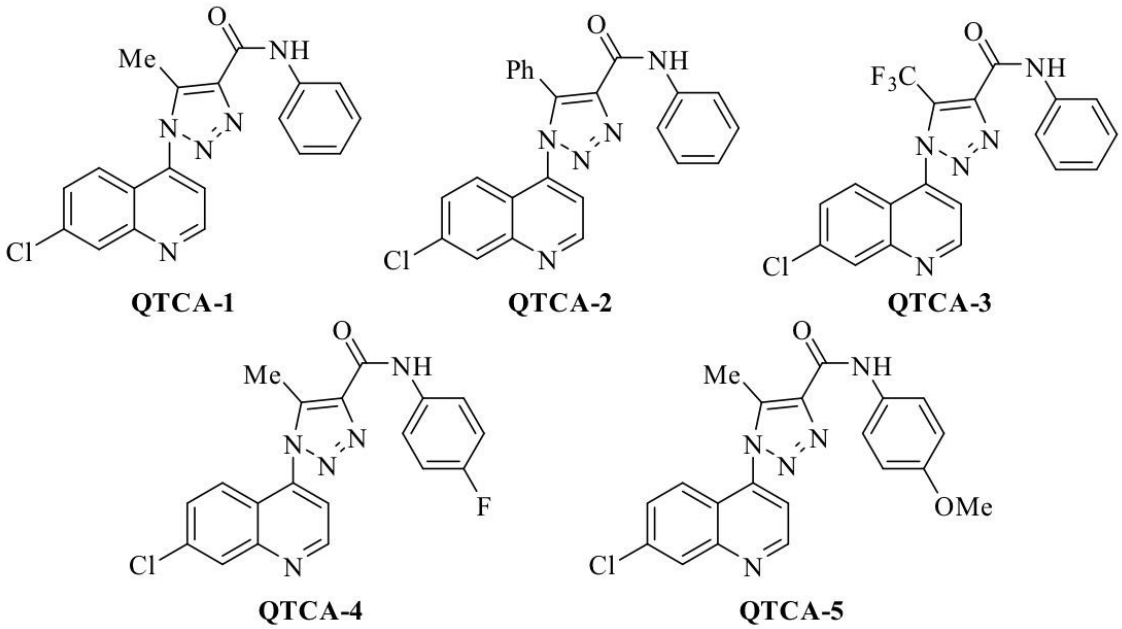


**Figure 1.** For these studies, five 7-chloroquinoline-1,2,3-triazoyl carboxamides were screened: 1-(7-Chloroquinolin-4-yl)-5-methyl-*N*-phenyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-1**); 1-(7-Chloroquinolin-4-yl)-*N*,5-diphenyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-2**); 1-(7-Chloroquinolin-4-yl)-*N*-phenyl-5-(trifluoromethyl)-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-3**); 1-(7-Chloroquinolin-4-yl)-*N*-(4-fluorophenyl)-5-methyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-4**) and 1-(7-Chloroquinolin-4-yl)-*N*-(4-methoxyphenyl)-5-methyl-1*H*-1,2,3-triazoile-4-carboxamide (**QTCA-5**).

**2.2 Cell culture and anticancer activity assay**

The human bladder carcinoma cell line 5637 and hamster ovary epithelium cell line (CHO-k1) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in liquid media (Dulbecco Minimal Essential Medium) supplemented with 10% fetal bovine serum (FBS) at 37 ºC with 5% $CO_2$. *In vitro* cytotoxicity of the compounds was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described (Mosmann, 1983). Cells were

incubated for 3 h at 37 °C. After this period, precipitated materials were dissolved in 100 µL DMSO and quantified by measuring absorbance at 492 nm in an automatic plate reader. Inhibition (%) of cell proliferation was determined as follows: inhibitory growth = (1-Abs492treated cells/Abs492control cells) x 100 (Ferrari, Fornasiero and Isetta, 1990). The $IC_{50}$ value corresponds to the concentration of test compound causing a decrease of 50% in cellular viability of drug-treated cells compared with untreated cells. This experiment was performed in triplicate.

**2.4. Viability assessment and LIVE/DEAD assay**

Live/dead solution (Invitrogen™, Carlsbad, USA) was prepared according to the supplier's protocol, where 2 µL of calcein AM and 0.5 µL of ethidium homodimer-1 were added to 1 mL of PBS. After the 48 hour treatment, media was removed from the wells and live/dead solution was added followed by 30 min incubation at room temperature in the dark. Then, the solution was removed and the wells were gently rinsed twice with PBS. Confocal microscopy was performed with a Leica TCS SP8 confocal laser scanning microscope at 200 x magnification. Live cells were analyzed by green fluorescent light emission (488 nm) resulting from calcein uptake. The permeable membrane of dead cells allows diffusion of ethidium bromide and binding to DNA, which was detected by the red fluorescent signal (546 nm). The recorded images were analyzed using Cell^F software (Cell^F, Olympus, Tokyo, Japan). The data was expressed as the mean ± SEM of percentage of dead cell, based 3 different fields of view.

**2.3 Cell cycle assay**

The number of cells at the different phases of the cell cycle (G0/G1, S, and G2/M) were analyzed by flow cytometry via propidium iodide (PI) staining. Cells ($1\times10^5$ cells per well) were cultured in a 12-well plate. After cellular synchronization in G1 phase, the treatments of 20 μM of **DCQ**, **QTCA-1,** or **QTCA-4** were administered for 48 h. After this period, 200 μL of cells were stained with 200 μL of Guava Cell Cycle Reagent according to the manufacturer's description. Data acquisition and analysis were performed on a Muse Cell Analyser (Millipore Corporation). The experiment was performed in triplicate.

**2.5 Measurement of apoptosis by Annexin V staining**

Apoptosis was determined by flow cytometry using the Annexin V- 7AAD apoptosis detection kit (Muse™ Annexin V & Dead Cell) following the manufacturer's instructions. The Annexin V/PI double staining assay recognizes the externalization of phosphatidylserine (PS) on the cell membrane, a hallmark of apoptotic cells. Briefly, $1 \times 10^5$ cells per well were seeded in 12-well plates and treated with 20 μM of **DCQ**, **QTCA-1,** or **QTCA-4** for 48 h. Treated and untreated cells (100 μL) were stained using 100 μL Muse Annexin V reagent. Cells were then incubated in the dark at room temperature for 20 min and analyzed by flow cytometry (Muse Cell Analyzer; Millipore Corporation). The results were reported as the percentage of cells in each apoptotic phase (early and late apoptosis and dead cells). The experiment was performed in triplicate.

**2.6 Molecular docking analyses**

In order to improve our understanding of the QTCA's pharmacological mechanisms of action, we performed molecular docking analyses to describe the binding mode of the tested

ligands (**DCQ**, **QTCA-1**, and **QTCA-4**) and their therapeutic targets (BAX, BCL-2 and MDM-2). First, 2D structures of each molecule were draw with Chemdraw, converted to 3D using the software Avogadro 0.9.4, followed by geometry optimization using the MMFF94 method (Hanwell *et al.*, 2012). Auto Dock Tools 1.5.4 was used to allow all the rotatable bonds of the ligands to rotate freely, while the protein receptors were considered rigid (Morris *et al.*, 2009). The available crystal structures of BAX (IF16), BCL-2 (4AQ3), and MDM2 (3JZK) were downloaded from Protein Data Bank.

The CHIMERA 1.5.3 software was used to remove molecules, ions, and water, as well as minimize the structure of proteins using the Gasteiger charges with 500 steps of minimization (Pettersen *et al.*, 2004). We conducted the molecular docking using the software autodock vina (version 1.1.1) with a grid box centered on the binding pocket of targets, allowing the program to search for additional places of probable interactions (Trott and Olson, 2009). The protein−ligand interactions were analyzed by Discovery studio visualizer 2016.

**2.7 Western blot analysis**

For the analysis of crude lysates, western blot analyses were performed. Briefly, cells ($1x10^6$ per well) were treated with **DCQ**, **QTCA-1,** or **QTCA-4** (20 μM) for 48 h. Cells were then washed twice with PBS and resuspended in 150 μL of lysis buffer plus PMSF (1mM). The lysate was centrifuged and the cell lysate proteins were separated by electrophoresis on 12% SDS polyacrylamide gel and then transferred to nitrocellulose membrane for 1h30. The membrane was blocked overnight in 5% nonfat dried milk solution and probed with the primary antibody against β-actin, p53, BAX, and BCL-2 (Sigma-Aldrich) for 1 h with gentle shaking at room temperature. Monoclonal antibodies against β-actin (1:1000), p53 (1:4000),

BAX (1:4000), and BCL-2 (1:2000) (Cell Signaling Biotech) were diluted in PBS-T. The blots were washed one time with PBS-T and incubated with anti-rabbit antibody for β-actin and anti-mouse antibody for p53, BAX, and BCL-2. All antibodies were conjugated with peroxidase (Sigma-Aldrich) and diluted in PBS-T for 1 h. After two washes of 30 min each with PBS-T, reactions were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Illinois, United States).

### 2.8 Statistical Analyses

Data analysis was performed on Graphpad software (Prism 7.4), where the MTT, cell cycle, and apoptosis assay data sets were analyzed using two-way ANOVA followed by the Tukey test for multiple comparisons. The $IC_{50}$ values for each compounds were calculated by a non-linear regression method using Graphpad software (Prism 7.4). For the live/dead assay, ANOVA was used followed by Bonferroni test for multiple comparisons. The mean of each treatment was compared with the mean of the control. $P < 0.05$ were considered significant for all analyses. Data for all figures are expressed as means ± SEM of at least three independent experiments.

## 3 Results and Discussion

### 3.1 QTCA-1 and QTCA-4 inhibit human bladder carcinoma cell proliferation

We previously described the organocatalytic synthesis and pharmacological properties of QTCA's (Wilhelm *et al.*, 2014), in addition to the antioxidant activity of their ester

analogues (Saraiva *et al.*, 2016). Since then, the anticancer activity of QTCA's was described for the first time in triple negative breast cancer cells (Begnini *et al.*, 2017).

As shown in Figure 2, cell viability decreased with increased concentrations of QTCA's as well as duration of treatment. The 50% growth-inhibitory concentrations ($IC_{50}$) for **QTCA-1** and **QTCA-4** after 48 h were 16.0 ± 3.03 μM and 17.2 ± 3.15 μM, respectively. DMSO vehicle alone demonstrated no growth inhibition (data not shown). These results suggested that the compounds **QTCA-1** and **QTCA-4** inhibits the growth of human bladder carcinoma cells *in vitro*. Our results are in accordance with what has been shown in the literature. A large number of quinoline derivatives have been marketed for their cytotoxic activity in various types of cancer, through different mechanisms of action (Afzal *et al.*, 2015; Jain *et al.*, 2016).

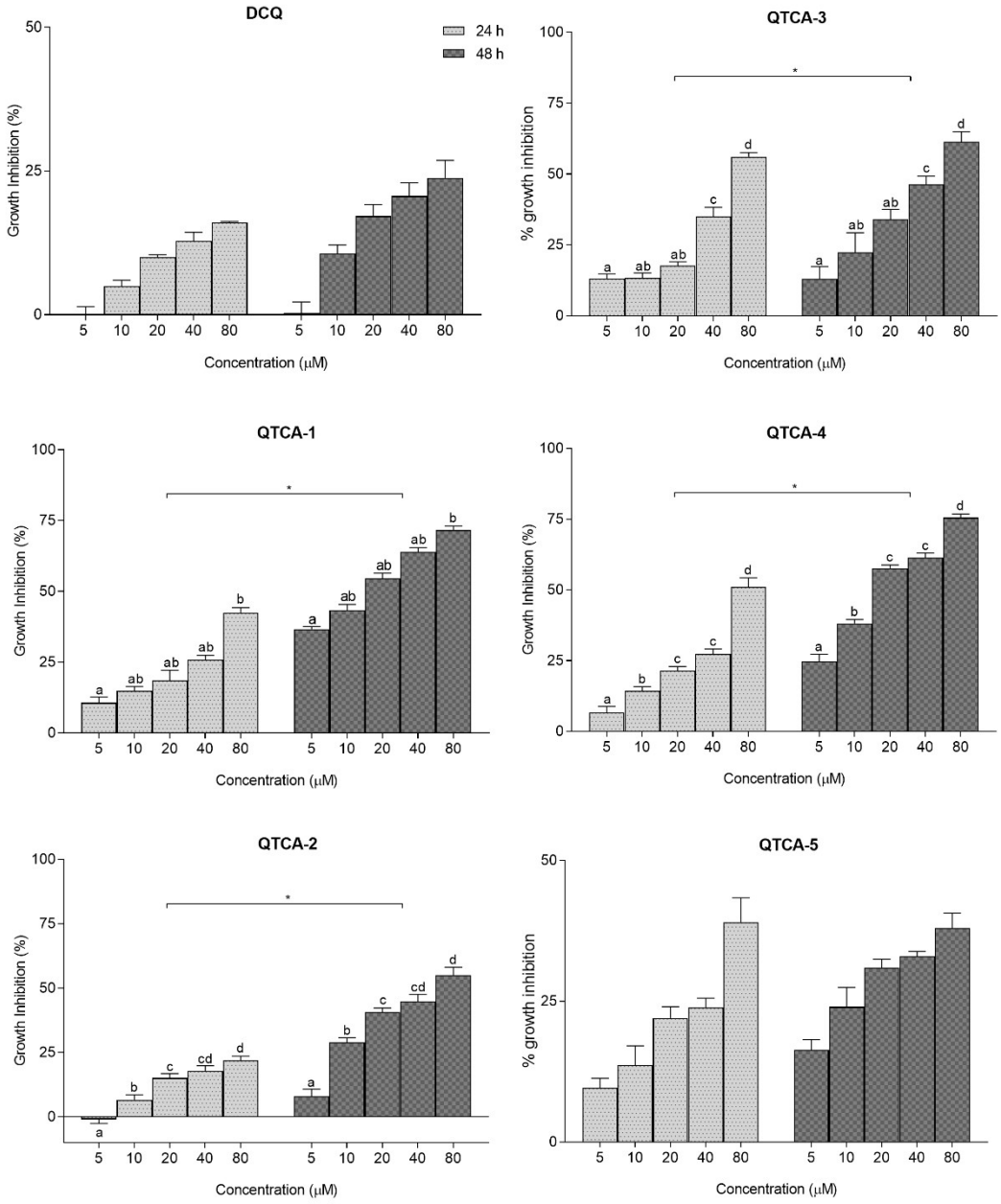


**Figure 2.** Inhibition cell proliferation of 5637 cells. The relative cell viability of 5637 cells was determined by MTT assay. 5637 cells were treated with 0 (control without treatment), 10, 20, 40, or 80 μM of 7-chloroquinoline-1,2,3-triazoyl carboxamides derivatives for 24 and 48 h. Mean ± SEM, n = 3. * represent the difference between times of exposure (24 and 48h), while the lower case letters represent the difference between concentrations within each time. Significance was considered at $P<0.05$.

Non-tumorigenic Chinese hamster ovary cells (CHO-k1) have been widely used in our group as a control for toxicity studies to demonstrate the selectivity of compounds towards tumor cells (Munchen *et al.*, 2018; Silvestre *et al.*, 2018; Wagner *et al.*, 2018). As demonstrated in Figure 3, **DCQ** was slightly more toxic to the CHO-k1 compared to the 5637 cells (40% compared to 23% inhibition, respectively). It was not possible to calculate the $IC_{50}$ for either cell line since the highest concentration of DCQ tested did not result in 50% growth inhibition. Therefore, DCQ was not considered cytotoxic against either line. As for the 5637 line, **QTCA-1** and **QTCA-4** demonstrated dose- and time-dependent toxicity for the CHO-k1 cells. In addition, the $IC_{50}$ values for **QTCA-1** and **QTCA-4** were similar to those calculated for the 5637 cells at 48h (22.41 ± 0.19 μM and 28.60 ± 0.30 μM, respectively). These results indicate that the tested derivatives do not demonstrate selective tumor cell killing.

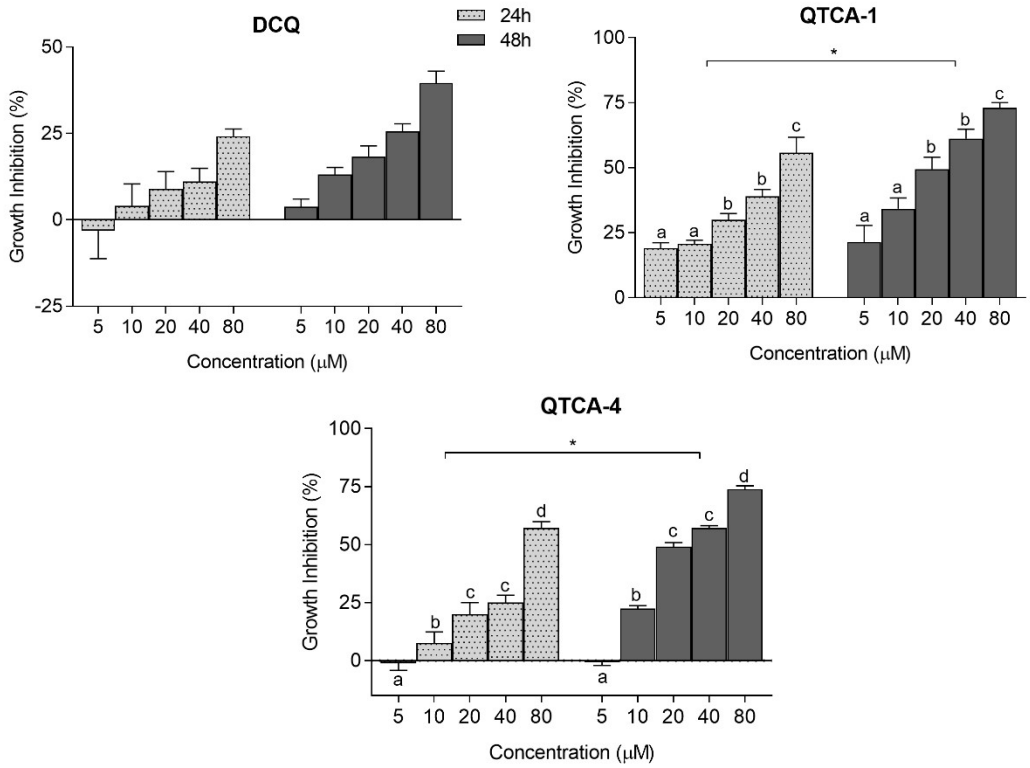


**Figure 3.** Effect of **DCQ**, **QTCA-1**, and **QTCA-4** on CHO-k1 cell viability after 24 and 48 hours of treatment. Mean ± SEM, n = 3. * represent the difference between times of exposure

(24 and 48h), while the lower case letters represent the difference between concentrations within each time. Significance was considered at $P<0.05$.

### 3.2 Derivatives 7-cloroquinolina-1,2,3-triazoyl carboxamidas reduce cell viability

Live/dead assays were performed to confirm the cytotoxic activity of QTCA's towards the bladder cancer cell line. The cells were treated with **DCQ**, **QTCA-1,** and **QTCA-4** at a dose based on the $IC_{50}$ values at 48h. Then, dual staining with fluorescence dyes (calcein-AM and ethidium homodimer) was performed to assess the percentage of dead cells present. A significant decrease in viable cells was observed following treatment with **QTCA-1** and **QTCA-4** for 48 h compared to the untreated control.

Figure 4 shows the average number of live and dead cells following exposure to each of the tested compounds. **DCQ** (B), **QTCA-1** (C), and **QTCA-4** (D) demonstrated a significant reduction in the number of live cells compared to control (A). In addition, the percentage of dead cells increased following treatment with **QTCA**-1 and **QTCA-4** (5.65% and 11.7%, respectively) compared to 0.15% in the control group.

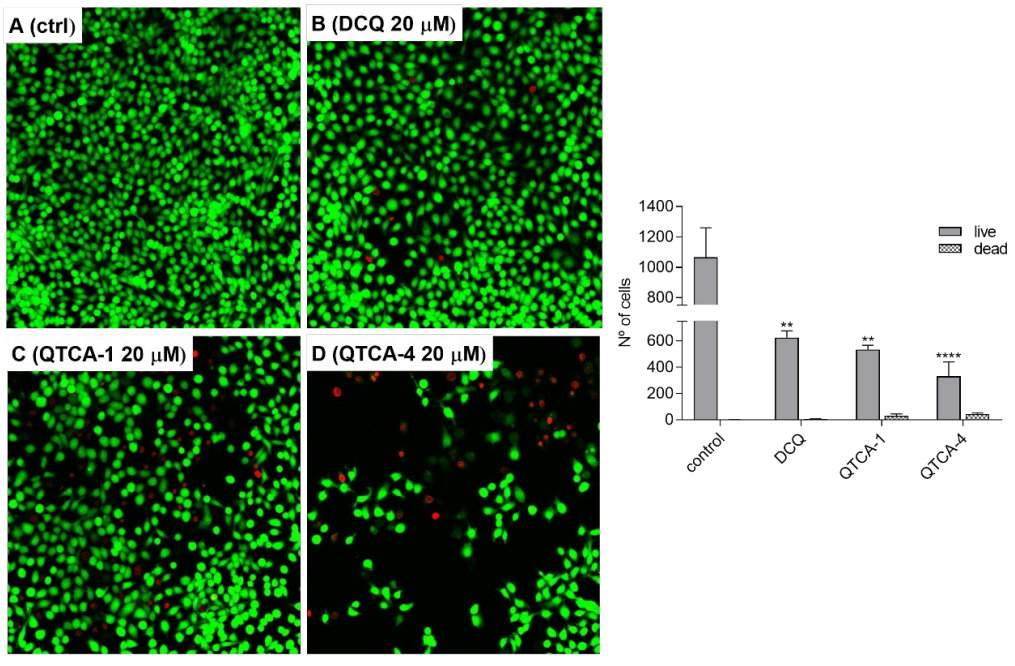

**Figure 4.** Live/dead test. (A-D) Live/dead fluorescence image of 5637 cells *in vitro*. Green fluorescent cells are alive and red fluorescent nuclei indicate dead cells. (E) Quantification of the number of dead cells after treatment for 48 hours with 20μM of each compound. Three distinct fields were evaluated. ** ($p = 0.0052$) **** ($p < 0.0001$)

### 3.3 QTCA-4 induces G0/G1 phase cell cycle arrest.

Cell cycle checkpoints ensure the preservation of genomic integrity by preventing subsequent cell divisions for cells with damaged DNA and cells with incomplete DNA (Ooi *et al.*, 2016). Many cytotoxic agents elicit their cytotoxic properties through alteration of cell cycle regulation (Sri Ramya *et al.*, 2018). Progression through G1 and G2 is delayed by blocking mechanisms if the DNA in the chromosomes is damaged by radiation or chemical compounds. In addition, most of the genetic changes that promote tumorigenesis involve a deregulation of cell cycle progression in G1 (Ooi *et al.*, 2016). To further study the mechanisms responsible for the cytotoxic effect of **DCQ**, **QTCA-1,** and **QTCA-4** in 5637 human bladder carcinoma cells, the proportion of cells in each cell cycle phase was assessed by flow cytometry. Cells were treated with 20 μM of each compound for 48 h.

As illustrated in Figure 5, the percentage of cells in the G0/G1 phase increased from 54.7% in the untreated control to 65.56% in QTCA-1 and 69.78% in QTCA-4 treated cells. These results indicate QTCA-1 and QTCA-4 treatment results in cell cycle arrest in the G0/G1 phase.

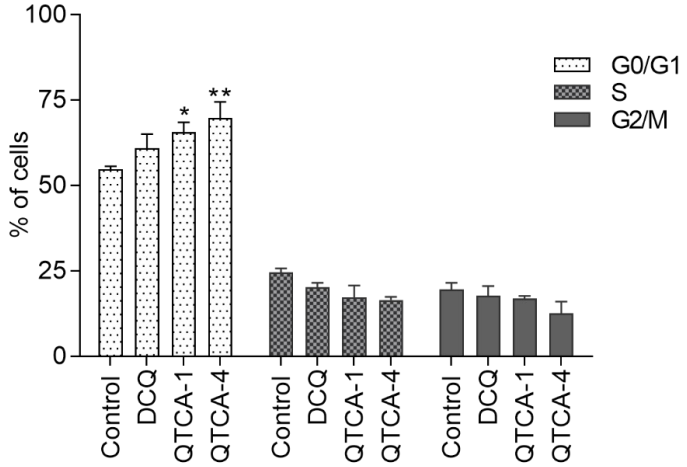


**Figure 5.** Representative results showing the distribution of 5637 cells in G0/G1, S, or G2 phase following treatment or with **DCQ**, **QTCA-1,** or **QTCA-4** (20 μM) for 48 h. Controls represent untreated cells. * (p=0.0021) ** (p=0.0002).

### 3.4 Apoptosis Assay

Cytotoxic and radiotherapy treatments generally depend on activation of apoptotic pathways. Therefore, compounds have been synthesized with the intention of selectively activating apoptosis to treat diseases where this cellular process is insufficient or inefficient, such as cancer (Nicholson, 2000). A common feature of apoptosis is the presence of phosphatidylserine phospholipid (PS) on the outer cell membrane, which is normally confined to the inner membrane in healthy cells. PS on the outer cell membrane can be detected via flow cytometry using a calcium-dependent phospolipid-binding protein called annexin V (Lauber *et al.*, 2004; Taylor, Cullen and Martin, 2008).

The results presented in Figure 6 indicate that there were no differences in the rate of early apoptosis or dead cells between the treatment groups and the control. However, the sample incubated with **QTCA-1** and **QTCA-4** presented significant increases in the percentage of cells in late apoptosis (47.11% and 43.96 % cells, respectively) compared to

samples treated with **DCQ** and untreated control, with no more than 12% of cells in late apoptosis in these groups. Furthermore, no difference in early apoptosis and late apoptosis was observed between **QTCA-1** and **QTCA-4**. Interestingly, the evaluated compounds demonstrated similar activity to the quinoline derivative, diethyl 4,6-bis-(3-dimethylaminopropylamino)-10-methylpyrido[3,2-g]quinoline-3,7-dicarboxylate in LNCaP, described by Li et al in 2006 (Li, Chen and Tzeng, 2006), where it was shown that inhibition of cell growth was due to cell cycle arrest in the G1 phase followed by apoptosis.

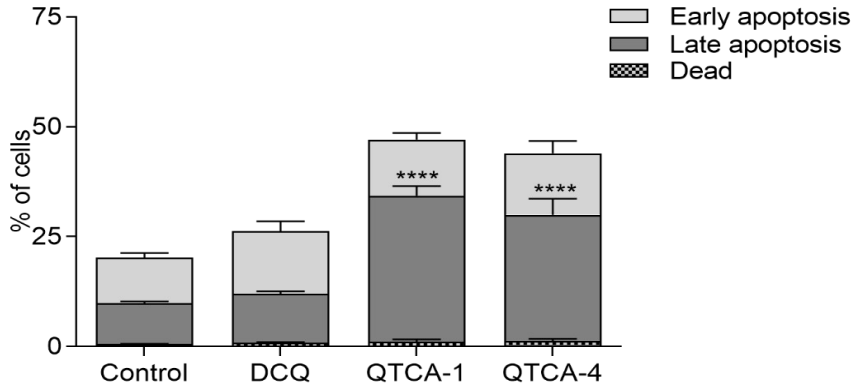


**Figure 6.** The double staining Annexin V/7-AAD assay was performed to determine the percentage of apoptotic cells following treatment with **DCQ**, **QTCA-1**, or **QTCA-4**. 5637 cells were exposed to 20 μM of each compound for 48 h**.** **** ($p<0.0001$) demonstrates the difference in the proportion of cells in late apoptosis following **QTCA-1** and **QTCA-4** treatment compared to untreated cells.

### 3.5 Binding affinity to BCL-2 and MDM2

Molecular docking analyses revealed high binding affinity between **QTCA-1** and the macromolecule MDM2 as well as between **QTCA-4** and Bcl-2 (Table 1), suggesting these

compounds act through different mechanisms of action to induce cytotoxicity. The interaction value for **QTCA-4** and Bcl-2 was significantly higher (-8.6 kcal/mol) than **QTCA**-1 (-7.6 kcal/mol) and **DCQ** (-7.1 kcal/mol). The best binding position for **QTCA-4** is in the BH3 (subfamily of pro-apoptotic proteins) domain, with mainly hydrophobic interactions with residues PHE63, LEU96, VAL92, and MET74, and an additional fluorine interaction with ASP70 (Figure 7 A and B). These residues are located in the Bcl-2 hydrophobic cleft, and the position of **QTCA-4** is very similar to BH3 mimetic inhibitors *ABT-263 and* 8-Chrysoeriol, suggesting Bcl-2 inhibition by QTCA-4 triggers cancer cell apoptosis (Ding *et al.*, 2014; Chandrashekhar *et al.*, 2017; Zhang *et al.*, 2018).

Table 1. Binding affinity assessed by molecular docking analyses based on thermodynamic parameters.

| | **Docking Score (Kcal/mol)** | | |
|---|---|---|---|
| **Molecules** | **BCL-2** | **BAX** | **MDM2** |
| **QTCA-1** | -7.6 | -6.9 | -8.1 |
| **QTCA-4** | -8.6 | -6.8 | -7.3 |
| **DCQ** | -7.1 | -5.3 | -5.5 |

In addition, the interaction value for **QTCA-1** and MDM2 was significantly higher (-8.1 kcal/mol) than **QTCA-4** (-7.3 kcal/mol) and **DCQ** (-5.5 kcal/mol). Ligand protein interactions indicate the most favorable binding position for **QTCA-1** is in the hydrophobic cavity of MDM2, consisting of a hydrogen bond with GLN24 and hydrophobic interactions with LYS51, LEU54, LEU57, and ILE99, which are also part of the p53 biding site (Figure 7 C and D). These interactions are similar to several Mdm2–p53 inhibitors, including nutlins (Shangary and Wang, 2009), imidazoles, and indoles (New *et al.*, 2015), indicating **QTCA-1** may occupy the p53-binding site and prevent formation of p53-MDM2 complexes. MDM2 is an oncogene and functions as an inhibitor of the N-terminal transactivation domain (TAD) of

p53, promoting its degradation through the ubiquitin–proteasome system. Thus, the inhibition of p53/MDM2 binding represents a viable strategy to reactivate p53 and enhance its tumor suppression properties (Estrada-ortiz, Neochoritis and Dçmling, 2016; Rawashdeh and Sorkhy, 2018; Song *et al.*, 2018).

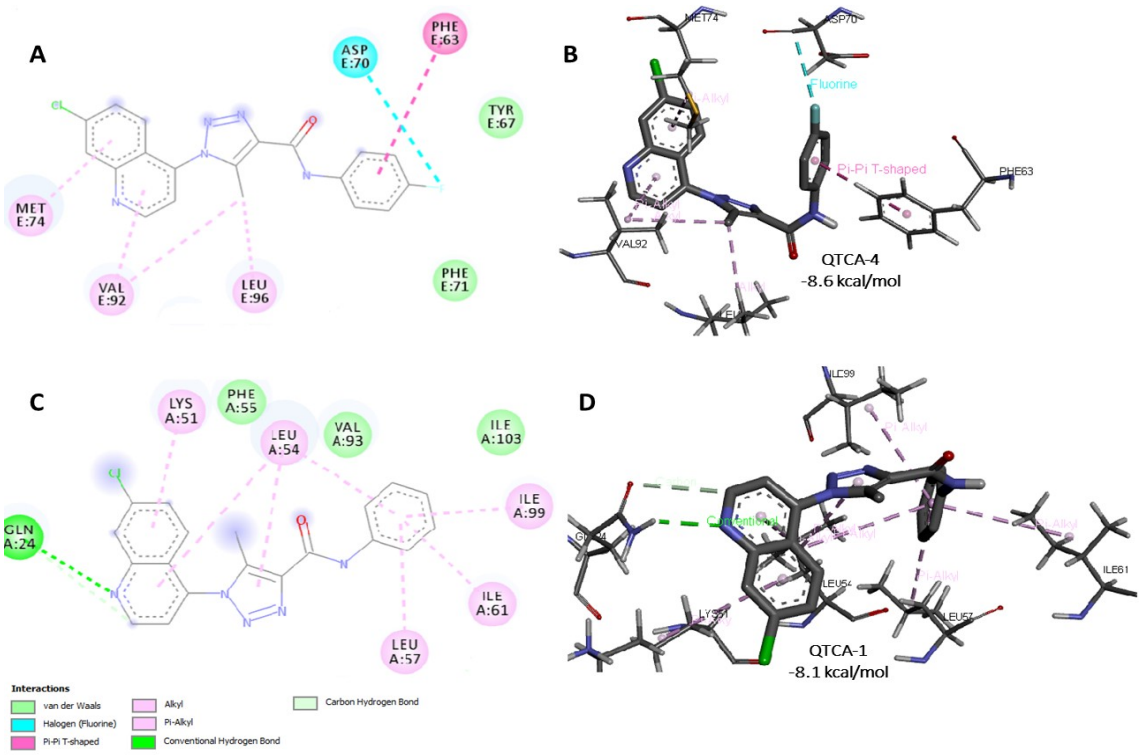


**Figure 7.** Predicted binding site and protein-ligand interactions of QTCA-4 and BCL-2 residues represented in (A) 2D, (B) 3D and QTCA-1 and MDM2 residues represented in (C) 2D, (D) 3D.

Although QTCA-1 and QTCA-4 display similar behaviors in *in vitro* assays, our *in silico* analyses suggest these compounds act through two different mechanisms of action. Although the molecules are similar, the addition of a fluorine atom in the **QTCA-4** structure may be the cause of this difference, although further work is required to confirm this hypothesis.

### 3.6 Detection of pro-apoptotic and anti-apoptotic proteins by western blot

The intrinsic pathway leading to apoptosis is regulated by the BCL-2 family of proteins. BCL-2 is an anti-apoptotic protein that can decrease mitochondrial permeability and inhibit apoptosis, whereas BAX, a pro-apoptotic protein, can accelerate apoptosis by enhancing mitochondrial permeability and cytochrome C release (Campbell and Tait, 2018; Pfeffer and Singh, 2018). BCL-2 protein expression was detected in control (untreated) human bladder carcinoma cells and those treated with DCQ, but not cells treated with QTCA-1 and QTCA-4 (Figure 8). This result further confirms QTCA-1 and QTCA-4 activate the intrinsic apoptosis pathway in 5637 cells through inhibition of BCL-2.

The tumor suppressor p53 accumulates in tumor cells and activates transcription of pro-apoptotic proteins of the BCL-2 family, including BAX, resulting in elimination of the tumor cell (Elkholi *et al.*, 2014). The QTCA-1 and QTCA-4 molecules promoted greater p53 protein expression compared to untreated cells (Figure 8). In the results obtained by molecular docking, QTCA-1 showed binding affinity for the MDM2 protein at the p53 binding site, suggesting that QTCA-1 interferes with formation of the p53-MDM2 complex and consequently reactivates p53 and its tumor suppressor properties.

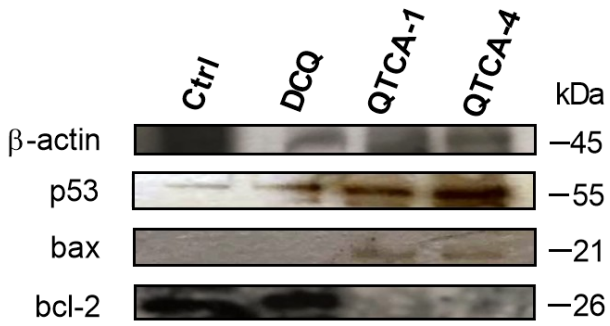


**Figure 8.** Western blot analysis of β-actin, p53, BAX, and BCL-2 proteins. Cell lysates from 5637 cells treated with or without (control) 7-chloquinoline-1,2,3-triazoyl-carboxamides (20 μM) for 48 h were used for western blotting.

In conclusion, the results confirm that QTCA-1 and QTCA-4 exhibited antiproliferative and apoptotic activity in human bladder carcinoma grade II (5637) cell line by decreasing expression of the anti-apoptotic protein BCL-2 and increasing expression of the pro-apoptotic protein BAX.

## 4 Acknowledgements

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## 5 CONCLUSÕES

Os novos calcogenobiotinas, 5af e 5bf demonstraram atividade antioxidante e antitumoral. Adicionalmente, a toxicidade *in vitro* e *in vivo* foi avaliada e o composto efetivo, 5af, não mostrou sinais de toxicidade nos ensaios realizados. Quanto aos derivados de quinolina, os resultados confirmam que QTCA-1 e QTCA-4 apresentam atividade antiproliferativa e apoptótica em células 5637.

## APOIO

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