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Avaliação de novas terapias *in vitro* contra o melanoma e câncer de bexiga

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Avaliação de novas terapias *in vitro* contra o melanoma e câncer de bexiga

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“Porque todas las cosas proceden de él, y existen por él y para él. ¡A él sea la Gloria por siempre!”

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Estrutura da tese

A presente tese, consiste na avaliação de novas terapias *in vitro* contra o melanoma e o câncer de bexiga e será está dividida na seguinte forma: Introdução geral, Objetivos (gerais e específicos), artigo 1 que descreve um mini-review de *Mycobacterium bovis* BCG na terapia de melanoma metastático, publicado na revista Applied Microbiology and Biotechnology. O manuscrito 1 mostra o efeito das cepas auxotróficas BCG Δ*leu*D e BCG recombinante Δ*leu*D/Ag85B em combinação com imiquimode, sobre a inibição do crescimento celular em linhagem celular de melanoma humano e sobre a resposta imune celular em macrófagos, a ser submetido a revista Applied Microbiology and Biotechnology. O manuscrito 2 reporta a avaliação da atividade antitumoral de compostos de azida de β-arilcalcogênio contendo telúrio em linhagem de carcinoma de bexiga e será submetido a revista indexada. O artigo e os manuscritos estão apresentados na formatação exigida por cada uma das revistas científicas.

Resumo

RUIZ, Martha. Avaliação de novas terapias *in vitro* contra o melanoma e câncer de bexiga. 2020. 141f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O câncer é considerado uma doença multifactorial causada por uma proliferação celular anormal e apresenta um impacto econômico no sistema de saúde a nível mundial. Os tipos de câncer abordados neste estudo foram melanoma e câncer de bexiga. No melanoma foram avaliadas as cepas auxotróficas de *Mycobacterium bovis* (BCG Δ*Leu*D e BCG recombinante Δ*Leu*D/Ag85B) e em combinação com o fármaco imiquimode sobre linhagem WM1366 de estágio IV para estudar o efeito na inibição celular e em linhagem de macrófago J774A.1 para determinar a resposta imune celular. Na linhagem WM1366 foram realizados os testes de MTT, Anexin e qRT-PCR, e em linhagem de macófago J774A.1 foi realizado qRT-PCR. No câncer de bexiga foram avaliados compostos organocalcogênios (azida de β-arilcalcogênio) contendo telúrio (**5c** e **5j**) sobre linhagem 5637 de grau II para avaliar a atividade citotóxica e antitumoral. Foram realizados o teste de citotoxicidade (MTT), live/dead, DAPI, qRT-PCR e docking molecular. Os resultados obtidos mostraram que para o melanoma (WM1366), o IC_{50} de inibição celular do imiquimode foi de 42.63 ± 4.28 μM em 48 horas. A combinação de Δ*leu*D/Ag85B+imiquimode em 40 μM mostrou 59,41% de inibição do crescimento, a combinação em 50 μM mostrou 61,30% e com 60 μM foi de um 80,08% de inibição do crescimento quando comparado ao imiquimode isolado (48,22%, 51,77%, e 57,97). A indução de apoptose mostrou as diferentes porcentagens de apoptose inicial e tardia na concentração de 60 μM nos tratamentos BCG Δ*leuD* + imiquimode (15,77%), BCG Δ*leuD*/Ag85B + imiquimode (13,96%), imiquimode (19,91%) quando comparado ao controle não tratado (3,78%) mostrando diferenças estatisticamente significantivas entre os tratamentos. Na expressão gênica em WM1366, BAX e Caspase 3 mostraram um incremento nos tratamentos BCG Δ*leu*D/Ag85B e Δ*leu*D/Ag85B + imiquimode; e em J774A.1 no tempo de 24 horas, a combinação BCG Δ*leu*D/Ag85B + imiquimode aumentou os níveis de mRNA de citocinas pró-inflamatórias *(IL-6*, *IL-12*, *TNF-α* e *IFN-γ*) quando comparadas ao imiquimode e as células não tratadas (P<0,05). Por outro lado, em câncer de bexiga (5637) os compostos **5c** e **5j** mostraram valores de IC_{50} de inibição celular de 1.57± 0.70 μM e 0.48± 0.13 μM (48h) respectivamente. Na análise da apoptose, observamos condensação da cromatina, mostrando 13% das células apoptóticas em **5c** e 20,3% em **5j**, em comparação às células não tratadas (2,34%). Na expressão gênica o composto **5c** aumentou a expressão relativa dos genes p53, Casp 3 e 9, SOD e CAT, e o composto **5j** aumentou os genes *p53*, *p21*, *Caspase-3* e *Caspase-9*, *Bax*, *SOD*, *CAT*, *GPx*, *GR* e *iNOS*. No docking molecular, **5c** apresentou afinidade de ligação à Survivin, Bcl-XL, RSK2, SGK1 e **5j** mostrou afinidade de ligação ao gene SGK1. Em conclusão, para o melanoma, a combinação do tratamento BCG *Δleu*D/Ag85B+imiquimode mostrou inibição do crescimento celular em WM1366, e aumento na resposta imune celular em J774A.1. Assim mesmo, para o câncer de bexiga os compostos **5c** e **5j** mostraram atividade citotóxica, e um aumento na expressão das proteínas pró-apoptóticas e enzimas antioxidantes, o que poderíamos sugerir de forma geral que os tratamentos avaliados neste estudo poderiam ter efeitos antitumorais promissores para estes tipos de cânceres.

Palavras chaves: atividade citotóxica, apoptose, compostos organocalcogênios, imiquimode, BCG.

Abstract

RUIZ, Martha. Evaluation of new *in vitro* therapies against melanoma and bladder cancer. 2020. 141f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Cancer is considered a multifactorial disease caused by abnormal cell proliferation and has an economic impact on the health system worldwide. The types of cancer studied in this study were melanoma and bladder cancer. For melanoma, auxotrophic strains of *Mycobacterium bovis* (BCG ΔLeuD and recombinant BCG Δ*Leu*D/Ag85B) were evaluated, and in combination with imiquimod on lineage WM1366 to study the effect on cell inhibition and on macrophage lineage J774A.1 to determine the cellular immune response. In WM1366, MTT, Annexin and qRT-PCR tests were performed, and in J774A.1 qRT-PCR was performed. For bladder cancer, organocalcogen compounds (β-arylcalcogen azide) containing tellurium (**5c** and **5j**) on line 5637 were evaluated to evaluate cytotoxic and antitumor activity. MTT, live/dead, DAPI, migration test, qRT-PCR, and molecular docking tests were performed. The results obtained showed that for melanoma (WM1366), the IC_{50} of cellular inhibition of imiquimod was 42.63 ± 4.28 µM in 48 hours. The combination of Δ*leu*D/Ag85B+imiquimod in 40 µM showed 59.41% growth inhibition, the combination in 50 µM showed 61.30% and with 60 µM it was 80.08% growth inhibition when compared to imiquimod isolated (48.22%, 51.77%, and 57.97). Apoptosis induction showed different percentages of early and late apoptosis at a concentration of 60 µM in BCG Δ*leu*D+imiquimod (15.77%), BCG Δ*leu*D/Ag85B+imiquimod (13.96%), imiquimod (19.91%) when compared to the untreated control (3.78%) showing statistically significant differences between treatments. In gene expression in WM1366, *Bax* and *Caspase- 3* showed an increase in BCG Δ*leu*D/Ag85B and Δ*leu*D/Ag85B+imiquimod treatments, and in J774A.1 in 24 hours, the BCG Δ*leu*D/Ag85B + imiquimod combination increased the mRNA levels of proinflammatory cytokines (*IL-6*, *IL-12*, *TNF-α*, and *IFN-γ*) when compared to imiquimod and untreated cells ($P < 0.05$). On the other hand, in bladder cancer (5637) compounds **5c** and **5j** showed IC_{50} cell inhibition values of 1.57 ± 0.70 µM and 0.48 ± 0.13 µM (48h) respectively. In the analysis of apoptosis, we observed condensation of chromatin, showing 13% of apoptotic cells in **5c** and 20.3% in **5j**, compared to untreated cells (2.34%). In gene expression, compound **5c** increased the relative expression of genes *p53*, c*aspase-3* and *caspase-9*, *SOD* and *CAT*, and compound **5j** increased genes p*53*, *p21*, *caspase-3* and caspase-*9*, *Bax*, *SOD*, *CAT*, *GPx*, *GR* and *iNOS*. In molecular docking, **5c** showed binding affinity to Survivin, Bcl-XL, RSK2, SGK1, and **5j** showed binding affinity only to the SGK1 gene. In conclusion, for melanoma, the combination of BCG Δ*leu*D / Ag85B + imiquimod treatment showed inhibition of cell growth in WM1366, and increased cellular immune response in J774A.1. Likewise, for bladder cancer compounds **5c** and **5j** showed cytotoxic activity, and an increase in the expression of pro-apoptotic proteins and antioxidant enzymes, which could suggest in general that the treatments evaluated in this study could have promising antitumor effects for these types of cancers.

Keywords: cytotoxic activity, apoptosis, organocalcogen compounds, imiquimod, BCG.

Lista de Figuras

Lista de Tabelas

Lista de Abreviaturas

ABCD – Assimetria, Bordas, Cores, Diâmetro, Extensão

ANVISA – Agencia Nacional de Vigilância Sanitária

BCG – Bacilo Calmete-Guérin

BRAF – Protooncogén B-Raf

CBMI – Câncer de bexiga músculo invasivo

CBNMI – Câncer de bexiga não músculo invasivo

CCT – Carcinoma de células de transição

EGFR – *Epidermal growth factor receptor* (Receptor do fator de crescimento epidérmico)

FDA – *Food and Drug Administration*

GSTM1 – Glutathione S-transferase Mu 1 (Glutationa S-transferase Mu 1)

IFN-ɣ – Interferon gama

INCA – Instituto Nacional do Câncer

IL-6 – Interleucina 6

IL-8 – Interleucina 8

IL-10 – Interleucina 10

IL-12 – Interleucina 12

NAT2 – *Enzyme N-acetyltransferase 2* (enzima N-acetiltransferase 2)

NRAS – ProtoOncogene, NRAS- GTPase

PIK3CA–phosphatidylinositol-4,5-bisphosphate 3-kinase (fosfatidilinositol-4,5-bifosfato 3-cinase)

PTEN – *Phosphatase and tensin homolog* (Fosfatase homóloga a tensina)

RB1 – Gene codificante do retinoblastoma

rBCG – BCG recombinante

RTU – Ressecção transuretral

DPDT – Ditelureto de difenila

UFC – Unidade Formadora de Colônia

TNF-α – Fator de necrose tumoral

TP53 – Gen da proteína supressora tumoral 53

SUMÁRIO

1 INTRODUÇÃO GERAL

O câncer é considerado uma doença multifactorial causada por uma proliferação celular anormal. Atualmente, o câncer está entre as principais causas de morbidade e mortalidade, apresentando um impacto econômico no sistema de saúde a nível mundial (AMERICAN CANCER SOCIETY, 2020).

Existem diversos tipos de cânceres como o câncer de bexiga, câncer de mama, melanoma, câncer de pulmão, entre outros (INCA, 2020), que são originados por mutações em genes envolvidos na programação e manutenção celular como os proto-oncogenes, genes supresores de tumores e genes de reparação (OLBRYT et al., 2020).

O melanoma representa 4% dos cânceres de pele, sendo uns dos tipos de cânceres de pele mais agressivos no mundo todo, em 2020, estima-se que haverá 100.350 novos casos de melanoma nos Estados Unidos (AMERICAN CANCER SOCIETY 2020; SIEGEL; MILLER; JEMAL, 2020). Por outro lado, o câncer de bexiga é o segundo câncer do trato genitourinário mais comum e a estimativa para este ano 2020 nos Estados Unidos são de 81.400 novos casos (SIEGEL; MILLER; JEMAL, 2020), sendo o câncer de bexiga não músculo invasivo (CBNMI) responsável por aproximadamente 70% de todos os casos (FERREIRA et al., 2019).

Existem diferentes tratamentos como a cirurgia, quimioterapia, radioterapia e imunoterapia (LUTHER et al., 2019). No entanto, algumas terapias podem apresentar efeitos adversos como pele seca, diarreia, vômito, problemas cardiovasculares, nefrotoxicidade, entre outros, ou podem ter baixas taxas de resposta contra o câncer (BROUSSARD et al., 2018; CORRÊA et al., 2019).

Uma das áreas de maior interesse na terapêutica contra o melanoma e câncer de bexiga é a imunoterapia que consiste na ativação do sistema imune (YU et al., 2019). O uso de bactérias como o BCG (Bacilo Calmette-Guérin) induz às próprias células do sistema imunológico dos pacientes para que possam agir contra as células cancerosas, através da expressão dos níveis de citocinas (YANG et al., 2017).

Atualmente existe a busca por novos compostos sintéticos e o uso de combinações de cepas recombinantes (rBCG) com outros agentes terapêuticos poderiam apresentar propriedades farmacológicas com potencial antitumoral e imunoduladores para o tratamento do câncer (BENITEZ et al., 2019; GAZZÉ, 2018; LUTHER et al., 2019).

Uns dos compostos que tem apresentado capacidade antioxidante e antitumoral são os compostos organocalcogênios que contem selênio e telúrio. Diversos estudos têm elucidado quais são os mecanismos de ação desses compostos e têm reportado que os compostos que contém telúrio estão envolvidos na apoptose celular (WU et al., 2019) e podem atuar como antioxidantes (HASSAN et al., 2011).

Estudos realizados pelo grupo de pesquisa em oncologia (Laboratório de Biotecnologia do Câncer) da Universidade Federal de Pelotas têm avaliado compostos *in vitro* em linhagens tumorais de diferentes tipos de cânceres onde foram avaliadas nanocápsulas contendo tretinoína em adenocarcinoma de pulmão (SCHULTZE et al., 2014), derivados de pirazolinas bexiga (TESSMANN et al., 2017) e 7-chloroquinolina (SONEGO et al., 2019) em linhagem 5637 de câncer de bexiga. Por outro lado, nanocápsulas contendo lapatinib foram testadas em linhagem T24 também em câncer de bexiga (BUSS et al., 2019), BCG recombinante em linhagem 5637 (BEGNINI et al., 2013) e porfirinas contendo platino na linhagem WM1366 de estágio IV de melanoma (COUTO et al., 2020), mostrando-se todos estes estudos anteriormente citados como potenciais antitumorais promissores para o câncer.

Neste contexto, o desenvolvimento e busca de novas estratégias terapêuticas, que visam o aumento na eficácia dos tratamentos contra o câncer de bexiga e melanoma são de grande revelância científica.

2 REVISÃO BIBLIOGRÁFICA

2.1 CÃNCER

O câncer é um conjunto de mais de 100 doenças de origem multifatorial, causado por uma proliferação celular anormal. Esta doença é originada pela transformação de células normais devido a mutações em genes associados ao controle, supervivência, reparação celular, levando a metástase através da disseminação das células neoplásicas a diferentes órgãos (BRÜCHER; JAMALL, 2014) (Figura 1).

O câncer é um dos principais problemas de saúde pública mundial, presentando um grande impacto econômico, e é a segunda causa de morte no mundo, depois das doenças cardiovasculares (AMERICAN CANCER SOCIETY, 2020).

O número de novos casos esperados nos Estados Unidos para o ano 2020 será de 1.806.590 e 606.520 mortes (SIEGEL; MILLER; JEMAL, 2020). No Brasil o número de casos novos será de 625 mil para o triênio 2020-2022 (INCA, 2020).

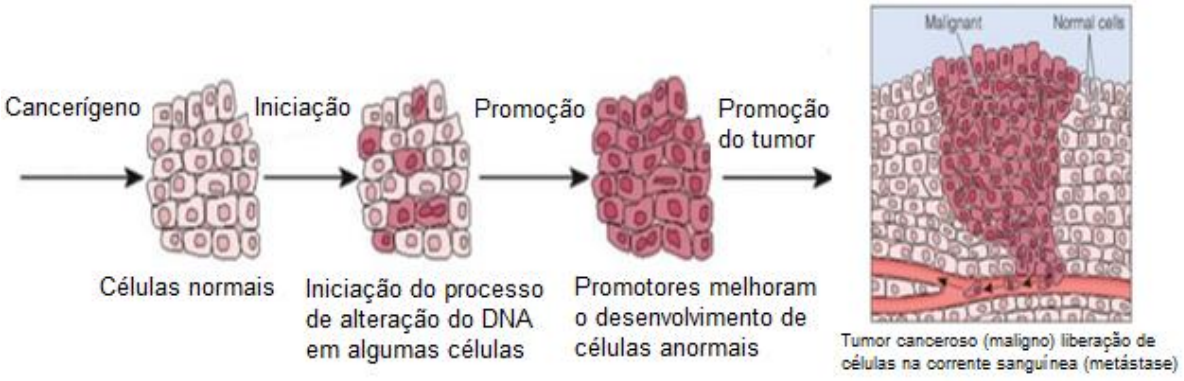


Figura 1. Etapas de formação de um câncer. Adaptado de (THOMSON, 2007). A figura representa as diferentes etapas de formação de um câncer como a iniciação, promoção, evolução de células anormais na formação do tumor, desencadeado pela exposição de um cancerígeno.

Diversos fatores de risco têm sido associados com o surgimento do câncer, como o consumo do cigarro e álcool, ao consumo de alimentos ultraprocessados, à exposição aos raios UV, exposição a químicos, exposição a fatores biológicos como no caso de infecções por vírus oncogênicos, ao envelhecimento, e devido a fatores genéticos (FIOLET et al., 2018; MADIA; WORTH; WHELAN, 2019).

Existem diferentes tratamentos como a cirurgia, quimioterapia, radioterapia, e imunoterapia que ajudam a controlar a progressão do câncer. No entanto, segundo a Organização Mundial da Saúde (OMS) e pela União Internacional para o Controle do Câncer (UICC), o câncer poderia ser prevenido mediante estratégias de prevenção, e detecção precoce da doença.

2. 2 MELANOMA

O melanoma é considerado um tipo de câncer, originado pela transformação maligna dos melanócitos, células encarregadas de produzir melanina que é o pigmento que determina a cor da pele (INFANTE CARBONELL et al., 2019).

Este tipo de neoplasia é heterogênea representando 4% dos cânceres de pele, mas é considerado uns dos cânceres mais invasivos e agressivos no mundo pela capacidade de gerar metástase, sendo responsável pela maioria das mortes relacionadas a esta doença e nos últimos anos tem se visto um aumento na sua incidência (CAMACHO et al., 2017). Em 2020, estima-se que haverá nos Estados Unidos 100.350 novos casos de melanoma e são esperadas 6.850 mortes no mesmo ano (AMERICAN CANCER SOCIETY 2020; SIEGEL; MILLER; JEMAL, 2020). No Brasil, o número de casos novos estimados para melanoma neste ano será de 4.200 em homens e de 4.250 em mulheres (INCA, 2020).

O câncer da pele começa na epiderme, que é composta por diferentes tipos de células como as escamosas, basais e os melanócitos. O melanoma comumente localiza-se na pele (95%) e pode-se manifestar raramente na boca, intestinos, esôfago ou olhos (AZOURY, 2014; CAMACHO et al., 2017).

Os diferentes tipos de melanomas incluem o melanoma extensivo superficial, considerado a forma mais comum no mundo todo, pode ocorrer em qualquer idade, em qualquer parte do corpo, principalmente nas costas ou nas extremidades inferiores (INFANTE CARBONELL et al., 2019). Este tipo corresponde cerca de um 70% dos casos e acomete geralmente a população de cor branca. O melanoma nodular é o segundo tipo mais comum e consiste em um tumor agressivo, atingindo outros locais do corpo, como o tronco e as extremidades, representando cerca de 15 a 30% dos casos. O melanoma lentigo maligno “carcinoma *in situ*” acomete áreas que estão mais expostas ao sol, como o rosto, pescoço, é mais comum em idosos, representando de 4 a 10% dos casos (BANDARCHI et al., 2010). Por fim, o melanoma lentiginoso acral é o mais raro, é encontrado geralmente nas palmas das mãos, solas dos pés e unhas, encontrado comumente em negros, asiáticos e hispânicos correspondendo 2 a 10% dos casos (MARKINSON et al., 2019) (Figura 2).

Figura 2. Tipos de melanoma. Adaptado de (NATIONAL CANCER INSTITUTE, 2016). As figuras representam os diferentes tipos de melanoma mais representativos, ordenados de acordo a sua incidência: melanoma extensivo superficial, melanoma nodular, melanoma lentigo maligno e melanoma lentiginoso acral.

2.2.1 FATORES DE RISCO

Existem diversos fatores de risco que estão associados ao desenvolvimento desta doença: como a exposição aos raios ultravioleta UVA e UVB sem proteção população branca, pessoas com um número elevado de nevos, exposição a câmaras de bronzeamento artificial, a idade, histórico familiar de melanoma e em pessoas imunocompremetidas) (FECHETE et al., 2019).

Por outro lado, outros fatores de risco são as mutações como no caso dos genes *CDKN2, CDK4* e mutações adquiridas nos genes *RB1, PTEN/MMAC1* (GARMAN et al., 2017). Os genes *NRAS* e *BRAF* são os genes principalmente mutados, encontram-se em 35% e 50% dos casos respectivamente, sendo a mutação V600K (substituição do aminoácido lisina pelo aminoácido valina) a de maior prevalência (CHENG et al., 2018), e o melanoma pode se originar em qualquer grupo étnico (ROVERE et al., 2016).

2.2.2 SINTOMAS E DIAGNÓSTICO

O melanoma apresenta diferentes sintomas como mudança em uma mancha ou pinta já existente, que apresentem modificações do tamanho, cor, forma, pigmentação irregular, presença de coceira, dor, inchaço, sangramento e não

cicatrização da área afetada (SEUMA, 2004). Comumente o melanoma pode se desenvolver de novo em um 75% dos casos ou pode crescer sobre um nevo preexistente (CAMACHO et al., 2017).

Para diagnosticar o melanoma através da avaliação clínica, precisa-se de uma dermatoscopia (através do uso de um aparelho: o dermatoscópio), das avançadas técnicas de imagem digital computarizada ou da microscopia láser confocal que auxilia na identificação e avaliação de lesões suspeitas, das características morfológicas e as mudanças nas sardas, manchas e nevos (MICHIELIN et al., 2019).

Existe uma regra nomeada ABCDE para estabelecer as diferentes características, como A: presença de simetria ou assimetria, B: presença de bordas regulares ou irregulares, C: cor, D: diâmetro (maior que 5mm) e E: evolução, aumentando assim a sensibilidade e especificidade do diagnóstico entre 57% a 90% (MICHIELIN *et al.*, 2019). Outros tipos de exames realizados são a ressonância magnética e a tomografia por emissão de pósitrons (PET) (AZOURY, 2014).

A confirmação do diagnóstico é realizada por um exame anátomopatológico do tecido suspeito (biópsia), seguido de um exame histopatológico e imunoistoquímico (por meio dos testes de detecção das proteínas S-100, HMB-45 e Melan A) (DA COSTA et al., 2019).

Para estabelecer o prognóstico do melanoma, o estadiamento do tumor ou o tipo histológico do melanoma, deve-se cumprir com diversos critérios e parâmetros como: a avaliação da medição de Breslow, detecção do nível de Clark: de I a V, o índice mitótico e o infiltrado inflamatório linfocitário peritumoral e intratumoral (DA COSTA et al., 2019).

Os sistemas de classsificação Breslow determinam a espessura do tumor, os níveis de Clark avaliam a invasão nas camadas da pele catalogando assim os tumores em Nível I até o nível V e o estadiamento TNM classifica o melanoma segundo as suas características locais, regionais e à distância como os estágios I, II III e IV (LATTANZI et al., 2019; MICHIELIN et al., 2019) (Figura 3).

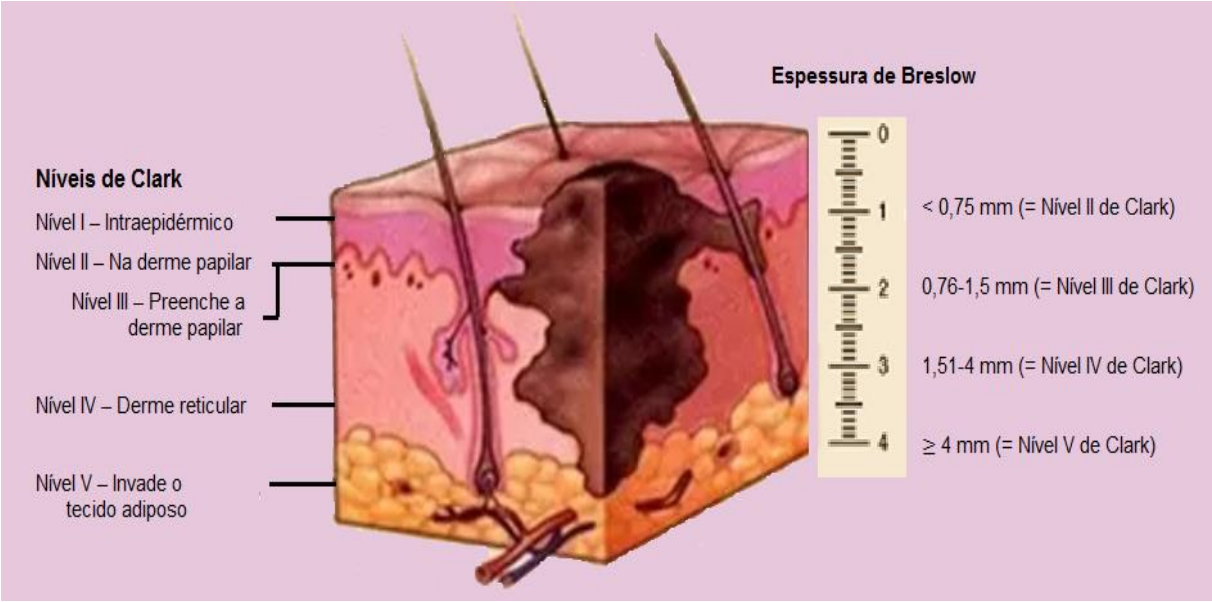


Figura 3. Classificação Breslow e níveis de Clark no estadiamento do melanoma. Adaptado de (LANE; DALTON; SANGUEZA, 2007). A figura presenta os diferentes níveis de clark que avaliam a invasão da infiltração nas diferentes camadas da pele, também mostra a classificação de breslow que determina a espessura do tumor.

O estadiamento TNM é usado para determinar a extensão do tumor inicial na pele, refere-se a T (tumor), N (nódulos ou gânglios linfáticos) e M (metástase) e os números representam o grau de malignidade, como no caso do número 0 (sem tumor ou gânglios afetados ou sem metástase) até IV com invasão a outros órgãos. A tabela 1 representa a classificação e estadiamento do melanoma de acordo ao TNM (GERSHENWALD; SCOLYER, 2018).

Tabela 1. Estadiamento do melanoma (TNM)

Estágio	T	N	M	Descrição
O	Tis	N0	M0	As células de melanoma são encontradas apenas entre a camada externa (epiderme). Esta lesão é considerada pré-cancerosa
IA	T1a	N0	M0	O melanoma não tem mais que 1 mm de espessura, sem ulceração e taxa mitótica menor que 1/mm2
IB	T1b	N0	M0	O melanoma não ultrapassa 1 mm, com ulceração
	T2a	N0	M0	O melanoma é mais grosso que 1 mm, mas não mais de 2 mm, sem ulceração

IIA	T2b	N0	M0	O melanoma é mais grosso que 1 mm, com ulceração
	T3a	N0	M0	O melanoma é mais grosso que 2 mm, mas não mais de 4 mm, sem ulceração
IIB	T3b	N0	M0	O melanoma é mais grosso que 2 mm, não maior de 4 mm, com ulceração
	T4a	N0	M0	O melanoma é mais grosso que 4 mm, sem ulceração
IIC	T4b	N0	M0	O melanoma é mais grosso que 4 mm, com ulceração
IIIA	T1-T4a	N1a	M0	Metástase microscópica encontrada em um linfonodo
	T1-T4a	N2a	M0	Metástase microscópica encontrada em dois a três linfonodos
IIIB	T1-T4b	N1a	M0	Metástase microscópica encontrada em um linfonodo
	T1-T4b	N2a	M0	Metástase microscópica encontrada em dois a três linfonodos
	T1-T4a	N1b	M0	Metástase macroscópica encontrada em um linfonodo
IIIC	T1-T4b	N1b	M0	Metástase macroscópica encontrada em um linfonodo
	T1-T4b	N2b	M0	Metástase macroscópica encontrada em dois a três linfonodos
	T1-T4b	N2c	M0	Melanomas em trânsito sem metástase para os linfonodos
	T (qualquer)	N3	M0	O melanoma é encontrado em quatro ou mais linfonodos, ou em dois ou mais linfonodos que parecem estar unidos
IV	T (qualquer)	N (qualquer)	M1	Presença de metástase

2.2.3 TRATAMENTO

No tratamento, a cirurgia é o primeiro procedimento realizado nos casos dos melanomas superficiais, e dependendo do estágio do tumor é realizada a quimioterapia, radioterapia ou imunoterapia, sendo este último usado no caso da resistência aos terapêuticos convencionais (MICHIELIN et al., 2019). O tratamento para o melanoma pode ser combinado, deve ser personalizado para cada paciente devido à heterogeneidade dos tumores a seu tamanho, localização, estadiamento, e à presença ou ausência de mutações em genes afetados como no caso dos genes *BRAF* e *NRAS* (HERNÁNDEZ; NIEWEG, 2014).

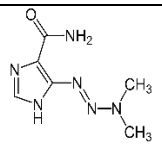
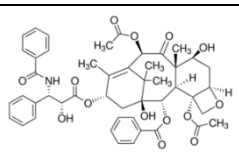
Em relação à cirurgia, este é o primeiro procedimento realizado restrito à pele, que pode ser feita através da cirurgia de ressecção de metástase, linfadenectomia ou a cirurgia plástica reparadora (CAMACHO et al., 2017).

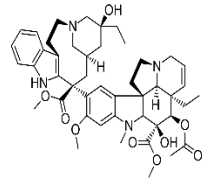
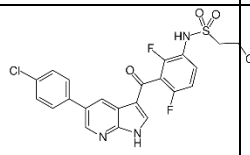
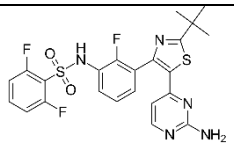
A radioterapia é usada para controlar o crescimento celular e as recidivas do tumor, usa raios de alta energia como os raios X e é um tratamento que se pode complementar ao tratamento adjuvante como à cirurgia de linfanectomia e/ou a doença metastática (MICHIELIN et al., 2019). A radioterapia tem sido usada para tratar o melanoma ocular, o lentigo maligno (através da radioterapia externa ou braquiterapia) e é usada também na terapia paliativa. Por outro lado, este tipo de tratamento e dependendo do tumor precisa de altas dose de radiação, podem também atingir as células sadias que estão na periferia, apresentando assim os difererentes efeitos adversos nos pacientes que podem ser limitados na área que recebe os raios X, produzindo problemas cutâneos, fatiga, queda do cabelo, diarreia e problemas para respirar, geralmente durante a segunda ou terceira semana após início deste tratamento (AMERICAN CANCER SOCIETY 2019).

A quimioterapia consiste no uso de quimioterápicos (medicamentos) de forma individual ou a combinação de vários deles que podem ser administrados de forma oral, intravenosa, subcutânea, intramuscular, tópica, tanto de forma local, ou de forma sistêmica, como no caso da doença metastática (DUFFY et al., 2014). A quimioterapia pode ser usada como tratamento adjuvante e neoadjuvante. Sua duração depende do tipo do tumor, geralmente é administrada por ciclos e pode ser combinada com a radioterapia.

Os diferentes fámacos usados para o tratamento do melanoma estão representados na tabela 2.

Tabela 2. Quimioterápicos usados no tratamento contra o melanoma.

Fármaco	Estrutura molecular	Mecanismo de ação	Efeitos adversos	Referencias
Dacarbazina (DTIC)		Agente alquilante no DNA	Perda de apetite, diarreia, queda de cabelo temporária, erupção cutânea leve, leucopenia.	(CORRÊA et al., 2019)
Paclitaxel		Agente antimicrotubular	Anemia, diarreia, queda de cabelo, mielossupressão, dor muscular e nas articulações.	(BHATIA et al., 2012)

Vinblastina		Agente antimicrotubular	Vômito, náusea, falta de apetite, neuropatia periférica, constipação.	(SCHINZARI et al., 2017)
Vemurafenibe		Inibidor da enzima serina/treonina quinase B-Raf	Erupção cutânea em 52%, artralgia em 58% dos indivíduos, e fotossensibilidade.	(HOPKINS et al., 2019)
Dabrafenibe		Inibidor da enzima quinase B-Raf	Dor de cabeça, náuseas, diarreia, febre, queda de cabelo.	(DA COSTA et al., 2019)

Um recente estudo, realizado por nosso grupo de pesquisa em oncologia celular e molecular (grupo GPO- Laboratório de Biotecnologia do câncer), mostrou que compostos com porfirinas de platina (II) usados como fotossensibilizadores são promissores para o tratamento do melanoma, mostrando inibição celular e atividade apoptótica na linhagem WM1366 (COUTO et al., 2020).

Atualmente, uns dos tratamentos de maior interesse é a imunoterapia, que consiste em auxilar o sistema imunológico dos pacientes a reconhecer o tumor e a combatê-lo de forma eficaz (YU et al., 2019). Os efeitos adversos são menos frequentes, quando comparados à quimioterapia levando a alterações gastrointestinais, endócrinas e na pele, e em casos mais graves poderiam mostrar colite e hipotireoidismo (COVENTRY, 2019).

Por outro lado, existem tratamentos adjuvantes com a expressão de diferentes interleucinas, de interferons (IFNs) (MICHIELIN et al., 2019), o uso de bactérias atenuadas e seus diferentes sistemas de expressão como no caso de *Mycobacterium bovis*- Bacilo Calmette-Guérin ou BCG que poderiam melhorar o desempenho na terapia antitumoral (BEGNINI et al., 2013).

Em relação à terapia alvo, atualmente existem inibidores de tirosina-quinasa comercialmente chamados vemurafenib, dabrafenib que atuam contra a mutação *BRAF* e alguns inibidores de MEK, como o cobimetinib e o trametinib, poderiam favorecer o tratamento neste tipo de mutação ou a combinação dos fármacos (DA COSTA et al., 2019; DROPPELMANN M. et al., 2016).

Outra terapia alvo usada no melanoma é o uso de anticorpos monoclonais que atuam como inibidores dos pontos de controle nomeados como pembrolizumab ou nivolumab (anti-PD-1), atezolizumab (anti-PD-L1), ipilimumab (anti-CTLA-4), ou suas combinações, usados geralmente nos casos de melanoma metastásico no estágio III e IV (DROPPELMANN M. et al., 2016).

2.2.3.1 IMIQUIMODE

O imiquimode (1-(2-metilpropil)-1H-imidazol[4,5-c]quinolina-amina)(Figura 4), é uma amida imidazoquinolina heterocíclica, agonista dos TLRs (*Toll like receptors*) 7 e 8, expresso em monócitos, macrófagos, células dendríticas e células malignas (DAJON et al., 2019).

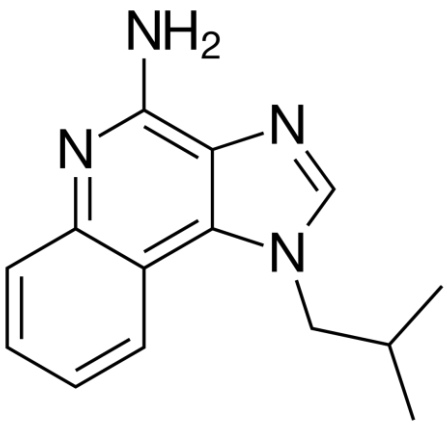


Figura 4. Representação da estrutura molecular do imiquimode.

O mecanismo de ação do imiquimode tem sido associado à ativação do MYD88 e posteriormente com à indução do fator de transcrição nuclear kappa B (NF-κB) que está associado à resposta inflamatória e à geração de citocinas, mostrando uma atividade imunomoduladora local, induzindo a liberação de citocinas pró-inflamatórias, incluindo a interleucina 6 (IL-6), interleucina 8 (IL-8), e fator de necrose tumoral alfa (TNF-α) (NARAYAN et al., 2012; TIO et al., 2019) (Figura 5).

Imiquimode é usado como creme dermatológico tópico a 5%, conhecido comercialmente como Aldara que apresenta atividade antitumoral, antiviral e os

efeitos adversos sistêmicos ao imiquimode administrado localmente são raros, levando a eritema e prurido (TIO et al., 2019; WESTER; EYLER; SWAN, 2017).

Aldara foi aprovado pela *Food and Drug Administration* (FDA) dos EUA e pela Administração de Produtos Terapéuticos (TGA) para seu uso em queratose actínica, verrugas genitais externas, e carcinoma basocelular superficial (AL-MAYAHY et al., 2019), e o imiquimode também pode ser usado em combinação com a interleucina 2 (IL-2) para tratamento do melanoma maligno, sendo tolerada pelos pacientes e mostrando taxas de resposta em lesões cutâneas refratárias (GREEN et al., 2007).

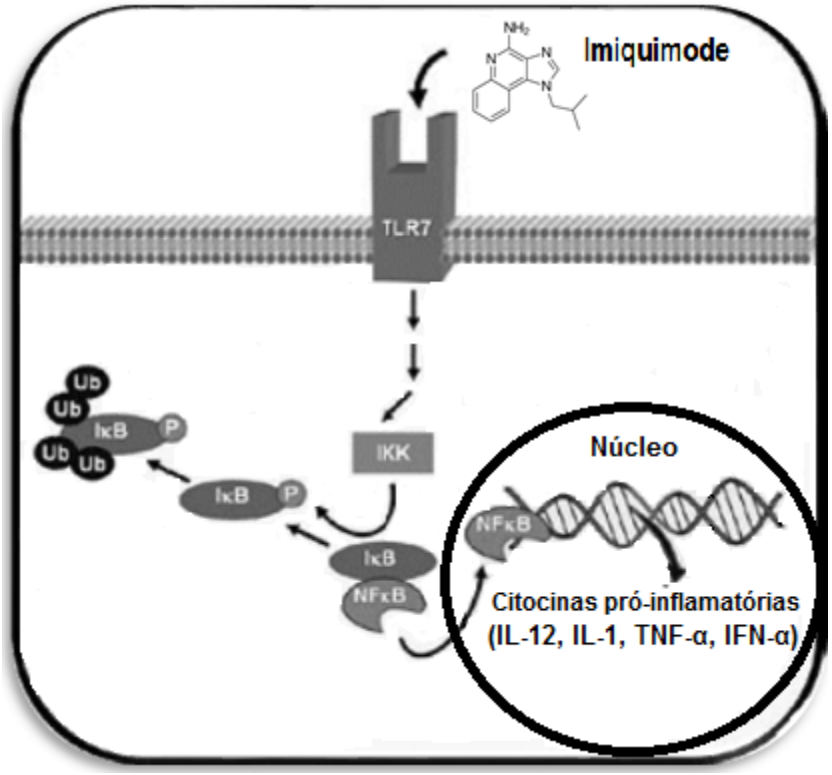


Figura 5. Mecanismo de ação do Imiquimode. Adaptado de (PERSAUD; LEBWOHL, 2002). IKK = inhibidor IkB cinase; IkB = inhibidor de NFkB; NFkB = fator nuclear kB. A figura mostra a ligação do imiquimode ao receptor tipo toll-like receptor (TLR7) que desencadeia uma cascata enzimática levando à fosforilação do inhibidor de NFkB e sua posterior ubiquitinação. Seguidamente, o fator de transcrição nuclear kB livre entra no núcleo e permite a expressão de genes que codificam citocinas pró-inflamatórias como: interleucina 12 (IL-12), interleucina 1 (IL-1), fator de necrose tumoral alfa (TNF-α) e interferon alfa (IFN-α).

Um estudo reportou que uma paciente com melanoma maligno que apresentava um grande tamanho na lesão e que devido a sua localização não foi possível usar a cirurgia nem radioterapia, foi usado o imiquimode a 5% mostrando benefícios para o tratamento não cirúrgico (VERGA; CHOHAN; VERDOLINI, 2019).

A apoptose induzida por imiquimode está associada à ativação das vias quinase1/c-Jun-N-terminalcinase/p38 desencadeando a indução de estresse endoplasmático e aumento da liberação intracelular de Ca2+, posteriormente à degradação da calpaína e finalmente à clivagem da caspase-4 (NYBERG; ESPINOSA, 2016). Também, o imiquimode pode desencadear desregulação mitocondrial pela perda do potencial da membrana mitocondrial que leva à liberação do citocromo C, clivagem da caspase-9, caspase-3 e ativação da polimerase poli (ADP-ribose) (PARP) (EL-KHATTOUTI et al., 2016).

Um estudo *in vitro* em células de um tumor da próstata, foi demonstrado que o imiquimode induz apoptose direta por uma via dependente das mitocôndrias, induzindo a produção de IL-6 (HAN et al., 2013). Por outro lado, um estudo realizado em células de adenocarcinoma endometrial, o imiquimode conseguiu diminuir a viabilidade celular provavelmente devido à redução dos níveis de *Bcl-2* e de *Bcl-XL*, mostrando também em estudos *in vivo* que imiquimode não mostrou toxicidade (ALMOMEN et al., 2016).

2.2.3.2 BCG (Bacilo Calmette-Guérin)

O BCG é uma vacina preparada a partir de uma cepa atenuada do *Mycobacterium bovis*(MARUF et al., 2016; MORALES; EIDEINGER; BRUCE, 2017)(MARUF et al., 2016; MORALES; EIDEINGER; BRUCE, 2017) que perdeu a virulência através das passagens nos cultivos artificiais, mantendo o poder antigênico (MORALES; EIDEINGER; BRUCE, 2017).

O BCG é usado no tratamento imunoterapêutico contra o câncer, sendo usado como uns dos tratamentos de primeira linha contra o câncer superficial não-músculo invasivo de bexiga) (BEGNINI et al., 2015). Por outro lado, faz parte da estratégia envolvida no tratamento do melanoma (MORTON et al., 1974), pois o BCG pode ser administrado pela via intratumoral e pode atuar como um adjuvante eficaz, cujo mecanismos de ação tem sido associado ao reconhecido pelos macrófagos, através dos TLR 2 e 4, levando à ativação de linfócitos T, produzindo Th1, e posteriormente à

expressão de diferentes citocinas como IL-2, IL-6, IL-8, TNF-α e IFN-γ (YANG et al., 2017) (Figura 6).

BCG tem sido envolvido na ativação da morte celular através da produção da apoptose em células uroteliais (YU et al., 2015) e na indução da autofagia (KHAN et al., 2019). Por outro lado, também está implicado na maturação das células dendríticas humanas, mostrando uma expressão aumentada de HLA-DR, CD86, e na produção de IL-6 (MIN et al., 2010).

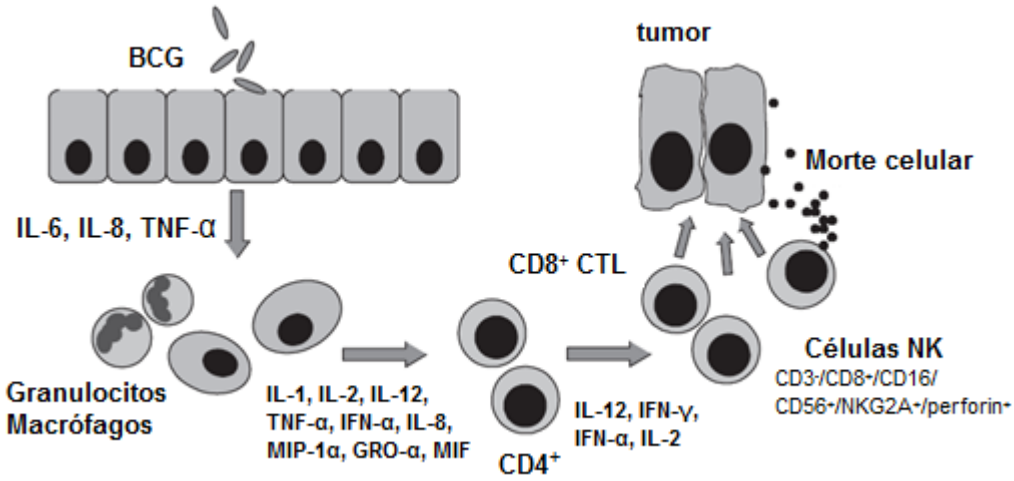


Figura 6. Imunoterapia por BCG. Adaptado de (BRANDAU; SUTTMANN, 2007). A figura representa a internalização do Bacilo Calmette-Guérin (BCG) em células tumorais, sua posterior liberação de citocinas pró-inflamatórias: interleucina 6 (IL-6), interleucina 8 (IL-8) e fator de necrose tumoral alfa (TNF-α). Seguidamente são atraídos ao local granulocitos e macrófagos que posteriormente ampliam a cascata inflamatória atraindo os linfócitos $CD4^+$, $CD8^+$ e as células NK que finalizam com a morte das células tumorais.

O BCG usado para o tratamento intralesional da metástase de melanoma cutâneo inoperável, em combinação com outros tratamentos, como no caso dos inibidores do ponto de controle estão associados à regressão da doença metastática (FARIES et al., 2017).

Um estudo reportou que a combinação de imiquimode a 5%, mais injeção intralesional de BCG (cepa Tice) não foi suficiente para causar regressão da doença (KIBBI et al., 2015). Neste contexto, a busca por novas combinações nos tratamentos, o desenvolvimento de novas construções de cepas de BCG recombinantes (rBCG)

que consigam expressar antígenos imunogênicos próprios ou antígenos heterólogos (BASTOS et al., 2009; RIZZI et al., 2017) que consigam melhorar o desempenho da terapia antitumoral são pouco explorados e são de grande relevância para o tratamento do câncer em forma geral (FARIES et al., 2017), e principalmente para o melanoma metastásico (BENITEZ et al., 2019).

O antígeno Ag85B é uma proteína imunogênica expressa da própria micobacteria, é reconhecida pela molécula HLA-A*0201 das células T $CD8^+$ humanas, ativa a resposta Th1, Th17 e induz a produção de IL-2, sendo assim um epítopo imunogênico importante alvo para o desenvolvimento de vacinas (BACK et al., 2019).

O grupo de pesquisa em oncologia (GPO) tem avaliado em parceria com outros grupos de pesquisa da UFPel (Universidade Federal de Pelotas), cepas recombinantes de BCG, como no caso da cepa auxotrófica usada neste estudo BCGΔ*leu*D/Ag85B (BORSUK et al., 2007; RIZZI et al., 2012) (Figura 7), avaliada como um possível tratamento contra o câncer superficial de bexiga *in vitro*, mostrando atividade antitumoral (BEGNINI et al., 2015).

A Universidade Federal de Pelotas (UFPel) tem depositado pedidos de patentes com diferentes construções de BCG expressando diferentes proteínas imunogênicas como a utilização da cepa auxotrófica (Δ*leu*D) - *Mycobacterium Bovis* recombinante superexpresando a proteína Ag85B como agente terapêutico e/ou imunoterapeutico para o câncer de bexiga mostrando diminuição na viabilidade celular e expressão génica de genes pró-apoptóticos (BR 10 2012021809 7 A2). Outra construção realizada foi a expressão da proteína CP40 de *Corynebacterium pseudotuberculosis* usado para imunoterapia no câncer de bexiga (BR 102015031737-9 A2 e BR 102016024822-1 A2). Por outro lado, a construção expressando a proteína fosfolipase D como agente terapêutico também usado no câncer de bexiga (BR 102016024833-7 A2), e a expressão da proteína Ag85B e a proteína p53 humana, para melhora no tratamento de câncer uroterial (BR 102013016531-0 A2 e BR 102015031739-5 A2).

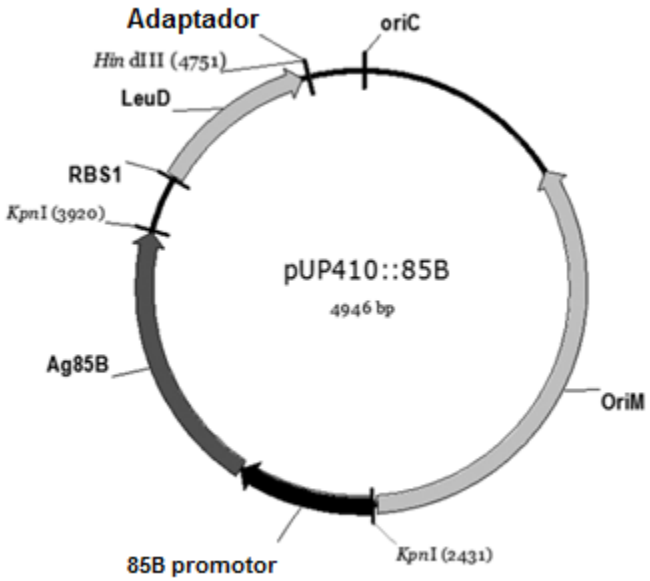


Figura 7. Ilustração do plásmido utilizado na construção. Adaptado de (BORSUK et al., 2007; RIZZI et al., 2012). A construção do plásmido pUP410::85B representa as origens de replicação de *E.coli* (oriC), de *Mycobacterrim* (oriM), a sequencia de reconhecimento de ribossomo RBS1, o promotor 85B, o gene que codifica para a proteína imunogênica Ag85B e o gene da leucina D (*Leu*D).

2. 3 CÂNCER DE BEXIGA

O câncer de bexiga origina-se por uma alteração das células de transição no tecido interno da bexiga chamado urotélio com capacidade de invasão a outros órgãos e é o segundo tumor urológico de maior ocorrência. A estimativa de novos casos nos Estados Unidos para o ano 2020 são de 81.400, com cerca de 17.980 óbitos por este tipo de câncer (AMERICAN CANCER SOCIETY 2020). Este tipo de tumor é considerado o quarto tipo de tumor mais comum em homens e o décimo terceiro mais comum em mulheres de forma global (SIEGEL; MILLER; JEMAL, 2020).

No Brasil, estimam-se 7.590 casos em homens e de 3.050 em mulheres para o ano do triênio 2020-2022 (INCA, 2020).

Os tumores são classificados de acordo aos tipos de alteração celular, de forma que o carcinoma de células de transição (CCT) origina-se por uma alteração nas células do tecido interno da bexiga (mucosa e submucosa), sendo o carcinoma superficial representando 90% dos casos e pode se encontrar como carcinoma urotelial papilar de baixo e alto grau, seguidamente, o carcinoma de células

escamosas é produzido pela alteração nas células planas e delgadas produto da inflamação ou irritação representando cerca de 8%, e o adenocarcinoma originado pela alteração das células glandulares de secreção com cerca de 1-2% (AMERICAN CANCER SOCIETY 2020).

Aproximadamente 75% dos pacientes apresentam câncer de bexiga não músculo invasivo (CBNMI) e 25% dos casos apresentam câncer de bexiga músculo invasivo (CBMI) (BABJUK et al., 2015).

Neste tipo de câncer, inclui o carcinoma *in situ* (TIS), o tumor confinado ao epitélio (Ta), e os tumores infiltrantes que invadem a lâmina própria (T1) onde podem se disseminar invadindo a parede muscular e posteriormente espalhando-se até os órgãos próximos ou gânglios linfáticos, transformando-se em um câncer invasivo (T3 e T4) respectivamente (AZEVEDO et al., 2017; BABJUK et al., 2015) (Figura 8).

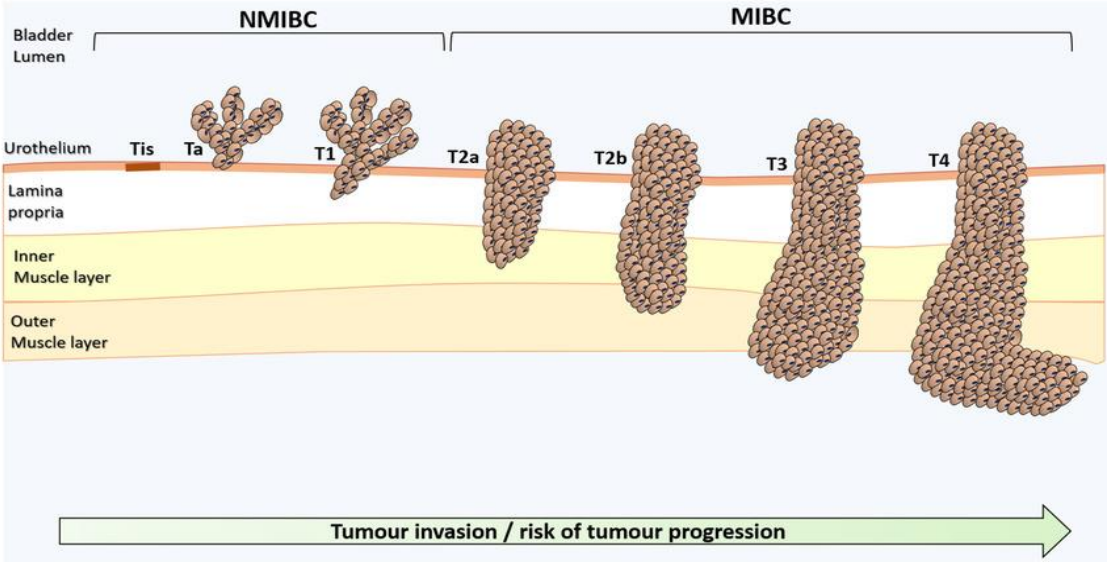


Figura 8. Classificação dos tumores no câncer de bexiga. Adaptado de (AZEVEDO et al., 2017). A imagens mostra a classificação do carcinoma uroterial dividido em: NMIBC (*Non-Muscle Invasive Bladder Cancer*) composto pelos tumores Tis, Ta e T1 e o MIBC (*Muscle Invasive Bladder Cancer*) composto pelos tumores T2-T4.

2.3.1 FATORES DE RISCO

Existem diversas causas que desencadeiam o câncer de bexiga, dentre os quais se destacam os fatores ambientais como o tabagismo, associado com este tipo de câncer em 50%-70% dos casos (WITJES et al., 2018). Por outro lado, a idade

avançada, devido a que tem se reportado em grande parte em pessoas maiores de 50 anos, e as infecções urinárias sobretudo devido à infecção por *Schistosoma hematobium* tem se visto associadas também na aparição deste tipo de câncer (WITJES et al., 2018).

Diversos fatores como a exposição ocupacional a aminas aromáticas, exposição a agentes químicos derivados do petróleo como as aminas aromáticas (benzeno), exposição a metais pesados, à radiação ionizante, ao aumento do consumo de produtos farmacêuticos como a ciclofosmida, ao desequilibrio entre as espécies reativas e as defesas antioxidantes do organismo causados pelo estresse oxidativo e pesticidas poderiam estar envolvidos (AMERICAN CANCER SOCIETY, 2020).

Entre outros fatores encontram-se pacientes com histórico familiar da doença, observa-se uma prevalência com maior frequência em homens do que em mulheres e polimorfismos em genes evolvidos na reparação do DNA (SIEGEL; MILLER; JEMAL, 2020).

Por outro lado, tem sido identificado regiões cromossômicas que estão associadas ao risco de desenvolver este tipo de câncer, podendo citar como exemplos as deleções no cromossomo 9 e ativação de oncogenes, como *c-erb-B2, HER-2/neu, H-ras*, *PIK3CA, EGFR, E2F3* e *CCND1* (KNOWLES; HURST, 2015).

Outros dos genes envolvidos na aparição deste tipo de tumor são as mutações nos genes supressores de tumores e em genes envolvidos no metabolismo celular como *TP53, PTEN*, *APC*, *STAG2, CDKN2A, RB1, NAT2, GSTM1* (BURGER et al., 2013), e uns dos genes de grande importância é a mutação no gene FGFR que está associado cerca de um 20% a 60% com carcinomas uroteliais, sobretudo o gene *FGFR3* presentado em um 15% dos casos de carcinoma urotelial metastático (FOTH et al., 2018).

No câncer de bexiga também tem se visto a associação do gene *SGK* que codifica a quinase induzida por glicocorticoides, com a proliferação, migração e invasão das células uroteliais através da via de sinalização da β-catenina/c-Myc (CHEN et al., 2018). *SGK1* tem se visto altamente expressa esta proteína em vários tumores como no caso do câncer coloretal (LIANG et al., 2017). Além disso, esta proteína está envolvida na adesividade e na transição epitélio-mesenquimal e posteriormente com sua invasividade (SULZMAIER; RAMOS, 2013).

Outro dos genes envolvidos no carcinoma urotelial é a expressão do gene *Bcl-XL* cuja função é controlar a morte celular através da inibição da apoptose que serve

como um marcador no prognóstico (YOSHIMINE et al., 2013). Finalmente, a proteína survivina é uma das proteínas que pertence à família de genes inibidores da apoptose (IAP) e sua expressão tem se visto durante o desenvolvimento embrionário e fetal, e além disso, está sobre-expressa em cânceres humanos como no câncer de bexiga, associado à progressão e mortalidade neste tipo de câncer (SHARIAT et al., 2007). Estudos realizados em pacientes e estudos com linhagem celular em câncer de bexiga determinaram as diferentes frequências genotípicas, mostrando que a expressão desta proteína esteve associada com o grau, estádio e a agressividade do tumor (KIU et al., 2014).

2.3.2 SINTOMAS E DIAGNÓSTICO

Os sintomas mais representativos no câncer de bexiga são devidos principalmente a alterações na micção, onde pode se apresentar hematúria (presença de sangue na urina) ocorrendo cerca de um 40% a 60% dos casos, necessidade frequente de urinar mais com problemas na hora de eliminar a urina e também pode estar acompanhado da irritação, dor e sensação de queimação na hora da micção (AMERICAN CANCER SOCIETY, 2020). Em relação ao diagnóstico, existem diversos estúdios para detectar o câncer de bexiga, onde incialmente deve ser realizado a palpação abdominal e toque retal, seguidamente a citologia urinaria que é de grande importância, onde são examinadas as células para a detecção de mudanças nas características morfológicas (WITJES et al., 2018).

Dentro dos métodos de imagens estão a ultrassonografia de vias urinárias como o ultrassom abdominal e pélvico onde permite visualizar presença de massas, a ressonância e tomografia computarizada são usadas para a detecção da presença dos tumores papilares e seguimento da progressão, e outras das alterativas é a urografia intravenosa (VAZ; ZAPAROLLI, 2020; WITJES et al., 2018). Um dos exames comumente utilizados, sendo considerado o método padrão no diagnóstico para este tipo de câncer é a citoscópia da bexiga (ou uretrocistoscopia) que auxilia na localização do tumor, determina o seu tamanho, e detecta o estadiamento através de uma colheita de tecido através de uma biópsia para indicar se o tumor é superficial ou invasivo (SANLI; DOBRUCH; KNOWLES, 2017).

Por outro lado, existem outros exames como os testes de biomarcadores urinários para detectar antígenos oncofetais como no caso do antígeno

carcinoembrionário (CEA) e alfafetoproteínas (AFP), comumente encontrados em células neoplásicas. Dentre desses marcadores estão o testes: BTA Stat (ensaio imunográfico), NMP22 BladderChek® (Nuclear matrix proteins, através da técnica de imunoensaio), Urovision e imumunocyt (testes de imunoflorescencia), usados para identificar e prever recidivas (FU et al., 2016; SCHULSTER, 2018).

O câncer de bexiga é classificado de acordo ao sistema de classificação de tumores da União Internacional Contra o Câncer (UICC) onde são classificados em três categorias: (T) correspondente às características do tumor primário, (N) envolvimento regional de linfonodos e (M) representa a presença ou ausência de metástases à distância (M) (UICC, 2017) (Tabela 3).

Tabela 3. Estadiamento do câncer de bexiga (TNM). Fonte: União Internacional do Câncer (UICC), atualizada em 2009 (7ª versão) (WITJES et al., 2018).

Estágio	Descrição
T - Tumor primário	
Ta	Tumor papilar limitado à mucosa
Tis	Carcinoma *in situ* (CIS); tumor plano
T1	Tumor invade submucosa (córion ou lâmina própria)
T2	Tumor invade músculo detrusor
T2a	Muscular superficial (metade interna)
T2b	Muscular profunda (metade externa)
T3	Tumor invade gordura perivesical
T3a	Microscopicamente
T3b	Macroscopicamente
T4	Tumor invade órgãos adjacentes ou parede pélvica
T4a	Tumor invade próstata, útero, vagina
T4b	Tumor invade parede pélvica ou abdominal
N – Linfonodos regionais	
N0	Sem metástases
N1	Metástases em linfonodo único < 2cm
N2	Metástases em múltiplos linfonodos na pelve
N3	Metástases em linfonodo da ilíaca comum
M- Metástase à distância	
M0	Sem metástases
M1	Metástase à distância

2.3.3 TRATAMENTO

Existem diferentes tratamentos para o câncer de bexiga e sua aplicação depende do estadiamento do tumor, sua localização e invasão (BABJUK et al., 2018).

Uns dos tratamentos comumente usados neste tipo de câncer é a ressecção transuretral (RTU) endoscópica é realizada através de uma citoscopia que consiste na utilização de uma sonda introduzida através da uretra para retirada tecidos do tumor (biópsia) através da raspagem (SCHULSTER, 2018).

A imunoterapia com BCG (Bacilo Calmette-Guérin) é uns dos tratamentos de primeira linha na terapia intravesical contra o câncer superficial de bexiga não-músculo invasivo, é administrado como terapia intravesical (onde o BCG é injetado diretamente na bexiga através de uma sonda) (BABJUK et al., 2018).

O BCG é uma cepa viva atenuada de origem bovina de *Mycobacterium bovis* (MORALES; EIDEINGER; BRUCE, 2017) é um adjuvante e é eficaz na estimulação e ativação do sistema imune do próprio paciente no combate às células tumorais e na redução da sua progressão (DONIN et al., 2017). A dose administrada de BCG é de uma dose semanalmente de 40-120 mg durante seis semanas, apresenta pouco efeito colateral sobre as células sadias do trato urinário (SYLVESTER, 2011). No entanto, alguns pacientes não respondem ao tratamento ou podem apresentar efeitos secundários como toxicidade local como cistite, febre e hematúria (GROSSMAN et al., 2008).

Atualmente o pembrolizumabe (KEYNOTE 052) é admintrado para o câncer de bexiga não musculo invasivo não responsivo ao tratamento com BCG com carcinoma *in situ* (CIS), é administrado a cada 3 semanas de forma endovenosa apresenta uma atividade antitumoral eficaz, mostrando uma tolerância aceitável (SCHULSTER, 2018).

Na área da quimioterapia os fármacos podem ser administrados antes da cirurgia (terapia neoadjuvante) ou depois da cirurgia (terapia adjuvante) podem ser administrados de forma local como é o uso da mitomicina, administrada intravesicalmente; ou de forma sistêmica como no caso da administração de gencitabina, cisplatina, vimblastina e metotrexato em casos do câncer de bexiga músculo invasivo ou carcinoma metastático (BABJUK et al., 2018; HUO et al., 2019)..

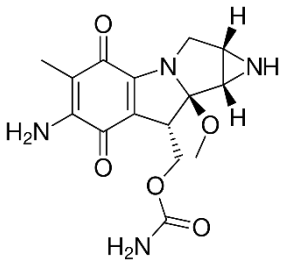
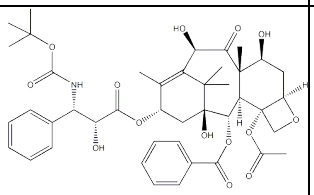
Geralmente a maioria dos fármacos atuam principalmente no RNA e no DNA, como o caso da gencitabina, que pertence a categoria de fármacos chamados antimetabolitos (2´-deoxy-2´,2´- citidina difluoro), é um análogo de nucleosído que atua no bloqueio da replicação do DNA, desencadeando a morte celular por apoptose (SKINNER et al., 2013), e este fármaco é usado para o tratamento de distintos tipos

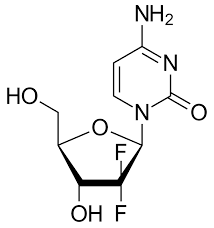
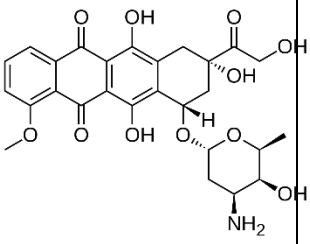
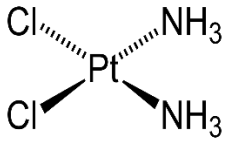
de cânceres como o câncer de bexiga, câncer de pulmão e câncer de mama metastático (WANG et al*.,* 2019).

Outros fármacos representados na tabela 4 são maiormente usados no tratamento contra o câncer de bexiga, e a terapia combinada de fármacos distintos pode minimizar a resistência destes pelas células tumorais (BABJUK et al., 2018). Neste tipo de câncer uns dos fármacos de interesse no tratamento nas células de transição de bexiga é a cisplatina (cis-diaminodicloroplatino), é um antineplásico e atua como agente alquilante devido a que este fármaco se liga ao DNA, interfere também na parade de ciclo celular na fase G2, causando apoptosis celular das células cancerígenas. Assim mesmo, induz dano mitocondrial pela diminuição da atividade *ATPase* alterando os mecanismos de transporte celular (GALLUZZI et al., 2012).

A cisplatina é usado para o tratamento de câncer de bexiga, testicular, ovário, pulmonar, mama, entre outros (GALLUZZI et al., 2012), induz efeitos adversos em curto prazo, produz efeitos secundários nos pacientes e pode-se combinar com o fármaco gemcitabina, induzindo o arresto do ciclo celular em fase G1, desencadeando a apoptose e evitando a progressão da doença (DA SILVA et al., 2010).

Tabela 4. Quimioterápicos usados no tratamento contra o câncer de bexiga.

Fármaco	**Estrutura molecular**	**Mecanismo de ação**	**Efeitos adversos**	**Referencias**
Mitomicina C		Antimetabólito que atua em nível celular bloqueando a replicação de DNA e RNA e inibindo a síntese protéica	Febre, náusea, diarreia, falta de apetite, hematúria, cistite, diminuição das plaquetas no sangue	(SCHMIDT et al., 2020)
Docetaxel		Agente antimicrotubular	Constipação, vômito, cansaço, queda de cabelo, dor nos músculos, articulações ou ossos.	(DANIELS et al., 2020)

Gencitabina		Antimetabolitos	Cistite, hematúria, náusea, vômito, erupção cutânea, mielossupressão.	(SKINNER et al., 2013; WANG et al., 2019)
Doxorrubicina		Liga-se ao DNA da célula impedindo a síntese de DNA, RNA e proteínas	Hipotensão, vômito, taquicardia, alopecia, miocardites.	(CRUZ; DUARTE-RODRIGUES; CAMPELO, 2016)
Cisplatina		Agente alquilante no DNA	Diarreia, vômito, hipomagnesemia, nefrotoxicidade e acidentes cardiovasculares.	(DA SILVA et al., 2010)

Recentemente, o fármaco erdafitinibe foi aprovado pela Agencia Nacional de Vigilância Sanitária (ANVISA) para o câncer de bexiga, é administrado por via oral, mostrando eficácia em um 20% dos pacientes que apresentam a mutação do receptor do fator de crescimento do fibroblasto (FGFR). É usado para o tratamento do câncer urotelial localmente avançado ou metastático, sendo a primeira terapia alvo anti-FGFR. Porém ainda existem efeitos colaterais como a toxicidade de pele e a ocular (MARANDINO et al., 2019).

Um dos problemas da maioria dos fármacos antitumorais na quimioterapia são sua baixa permanência plasmática, a destruição das células que estão em divisão tanto células cancerosas como as sadias atuando de forma não-específica. Por tanto, o desenvolvimento de sistemas de liberação controlada, o aumento na eficiência da quimioterapia são alvo de estudos, sobretudo visando a diminuição dos efeitos adversos e o impedimento da evolução da doença é de extrema importância (KAMAT et al., 2016).

A radioterapia é usada no controle da doença localmente avançada, pode ser usada em combinação com a quimioterapia no caso que a cirurgia não seja realizada (POMPEO et al., 2008). Este tratamento pode ser realizado de forma externa, onde os raios X incidem na bexiga somente, ou pode ser realizado de forma interna onde é colocado um dispositivo na bexiga, liberando uma substância radioativa. Uma das

desvantagens deste tratamento são os efeitos adversos nos pacientes como queimaduras na pele, vômitos, náuseas, diarreia, entre outros (POMPEO et al., 2008).

Por outro lado, dependendo da agressividade do tumor, pode ser realizada uma cirurgia chamada cistectomia parcial, onde é removida uma parte da bexiga ou a cistectomia radial, onde é removida a bexiga toda e se é o caso a remoção também dos orgãos próximos como próstata, vesículas seminais, útero e ovários (SCHULSTER, 2018).

2.4 COMPOSTOS ORGANOCALCOGÊNIOS

Os compostos organocalcogênios são compostos que apresentam na sua estrutura molecular elementos como Selênio (Se), Telúrio (Te) e enxofre (S), localizados no grupo 16 da tabela periódica. Atualmente estes compostos são de grande interesse na comunidade científica devido a que são usados como agentes intermediários na síntese química devido a sua estabilidade e também devido a suas propriedades biológicas (DE SOUZA et al., 2015).

De forma geral os compostos organocalcogênios têm mostrado propriedades antivirais, antioxidantes, antidepressiva e atividade antitumoral (TIEKINK, 2012). Em relação às propriedades imunoduladoras um estudo reportou a síntese e avaliação dos compostos diselenobisbenzamidas (DISeBAs), mostrando uma resposta eficaz na inibição da proteína nucleocapsídica 7 (NCp7) retroviral (SANCINETO et al., 2015).

O composto organocalcogênio nomeado ebselen (organoselênio) apresenta propriedades anti-inflamatórias, antioxidantes (MIORELLI et al., 2008), e alguns compostos sintético de organosselenio podem ser usados como neuroprotetores, como antioxidantes e inibidores de enzimas (ROSA et al., 2007).

Os compostos organocalcogênios com presença de selênio apresentam capacidade antioxidante, devido a que é capaz de mimetizar à atividade da enzima glutationa peroxidase (GPx) incorporado na enzima a selenocisteína que ajuda a proteger as células dos danos oxidativos, que é o mecanismo pelo qual a maioria dos autores atribuem sua atividade antioxidante (BANDEIRA et al., 2019). Assim mesmo, foi reportado que o composto organotelúrio 2,2-dimetil-4- (feniltelanilmetil) -1,3-dioxolano foi eficaz na inibição da peroxidação lipídica apresentando uma atividade antioxidante, relacionado à presença do átomo de telúrio na sua estrutura (NOBRE et al., 2014).

Por outro lado, o composto contendo selênio 3-((4-clorofenil) selanil)-1-metil-1H-indole mostrou que este ajuda na reversão do comportamento depressivo induzido associado à inflamação, estresse oxidativo *in vivo* (CASARIL et al., 2019), e por outro lado, este composto reduziu a indução de tumores de mama, a neuroinflamação e melhorou as alterações comportamentais nos camundongos (CASARIL et al., 2020).

BAMPI et al., (2020) reportaram que o composto organoselenio 1-metil-3-(fenilselanil) -1H-indole (MFSeI) foi capaz de reverter o estresse oxidativo, neuroinflamação, depressão e hiperglicemia em camundongos.

DA ROSA et al., (2016) sintetizaram e avaliaram compostos derivados de arilchalcogeno-3-amino-timidina (ACAT) em linhagem de carcinoma de bexiga (5637), mostrando uma atividade antitumoral atuando de maneira dependente da dose-tempo e obtendo valor de IC_{50} de 7.94 ± 1.92 µM em 48 horas. Além disso teve uma formação de TBARS baixo mostrando uma capacidade antioxidante.

Estudo realizado por TABARELLI et al., (2017) sintetizaram os compostos organocalcogênios (azida de β-arilcalcogênio) contendo telúrio e selênio e foram testados em linhagem de carcinoma de pulmão (A549) demonstrando atividade antioxidante, mais houve um incremento nos compostos que continham telúrio. Além disso, esses compostos foram testados em modelo animal (Swiss), e não mostram efeitos hepatotóxicos nem toxicidade renal evidentes nesses órgãos avaliados respectivamente.

O efeito tóxico dos compostos organocalcogênios ainda não estão completamente compreendidos, mas se sugerem que os grupos tióis endógenos poderiam estar envolvidos (HASSAN et al., 2011). Por outro lado, foram desenvolvidos compostos organochalcogênios com atividade de modular o balanço redox intracelular por catálise, sendo o estado redox a chave determinante para resposta ao estresse e função celular (BOOTY et al, 2019). Além disso, também, alguns relatos sugerem que os compostos organochalcogênios como o Ebselen (Ebs) e diselenido de difenila (PhSe)2 induzem uma depolarização e disfunção mitocondrial por oxidação de grupos tiol mitocondriais (PUNTEL et al., 2010).

Estudos tem pesquisado e elucidado os diferentes mecanismos de ação dos compostos organocalcogenios e também tem contribuído com a síntese de novos compostos, técnica de liberação (KRUG et al., 2020), e a introdução de diferentes substituintes como o oxigênio cloreto, bromo, fluoreto que fazem parte da síntese de

compostos, aumenta a lipofilicidade das moléculas, a penetração da membrana lipídica, e também poderiam conferir efeitos citotóxicos (TABARELLI et al., 2017).

2.4.1 COMPOSTOS ORGANOCALCOGÊNIOS DE TELÚRIO

O telúrio é considerado um metaloide tóxico, mas a sua toxicidade depende da estrutura química em que o telúrio esteja e tem se visto promissores devido a suas propriedades biológicas (SREDNI, 2012).

A propriedade antioxidante dos compostos organotelúricos poderia ser explicada devido a alterações na oxidação do estado divalente de Te (II) para o estado tetravalente de Te (IV) (HASSAN et al., 2011).

PASCHOALIN et al., (2019) avaliaram o efeito antitumoral de organoteluranos quirais (1-[Butyl(dichloro)-λ4 -tellanyl]-2-[1S-methoxyethyl]benzene (organotelluranes RF-13R e RF-13S) sobre um modelo murinho de melanoma (C57BL/6) e reportaram que esses compostos mostraram baixa toxicidade, foram inibidores de catepsina (proteases lisossômicas de cisteína que aumentam a expressão nas células tumorais) e poderiam ser agentes potenciais antitumorais devido a que interferiu na migração, proliferação na linhagem de melanoma B16F10-Nex2, evitando a angiogênese. Por outro lado, o composto organotelúrio (IV) RT-04 foi avaliado na linhagem celular de leucemia promielocítica humana HL60, reportando que esse composto conseguiu induzir apoptose nas células por modulação negativa da expressão de Bcl-2 (ABONDANZA et al., 2008).

Em células de câncer de colon humano (HT-29) os compostos difenil-ditellurida mostraram um aumento na atividade das caspases 3, 7 e 9 (VIJ; HARDEJ, 2012). No entanto, estudos envolvendo compostos organocalcogênios contendo telúrio para o tratamento do câncer de bexiga são escassos e suas propriedades farmacológicas ainda não foram exploradas para esse câncer.

Alguns estudos sugeriram o papel potencial dos organochalcogênicos e indicam a inibição da atividade do complexo I das mitocôndrias, possivelmente à atividade de oxidação do tiol, e também a alteração do metabolismo celular e a regulação da morte celular apoptótica (PUNTEL et al., 2013).

Estudos realizados com ditelureto de difenila (DPDT) demonstraram que este composto foi capaz de inibir a enzima humana topoisomerase I de DNA e sugerem

que a redução da viabilidade celular obtida neste estudo foi pela parada do ciclo celular (JORGE et al., 2014).

Por outro lado, o DPDT na faixa de concentração de 0,01 a 0,1 μM apresenta propriedades antimutagênicas e antigenotóxicas mostrando redução da frequência de micronúcleos em células V79 (fibroblastos pulmonares de hamster chinês) (TRINDADE et al., 2015). Recentemente, TRINDADE et al., (2019) reportaram que o DPDT mostrou atividade anticâncerigena induzida pelo aumento da geração de ERO, e também apresentou propriedades antioxidantes e pró-oxidantes.

Em relação a atividade imunoduladora e anticancerigena, os compostos AS101 [ammonium trichloro(dioxoethylene-o,o')tellurate] e o composto SAS [octa-O-bis-(R,R)-tartarate ditellurane] permitem a inativação de proteases de cisteína, como a catepsina B, que está envolvida em progresso tumoral, metástase e inflamação e também na inibição da proteína survivina (família das proteínas inibidoras da apoptose) e na interferência na produção de IL-10 tumoral (SREDNI, 2012).

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar novas terapias *in vitro* contra o melanoma mediante resposta aos tratamentos com cepas auxotróficas de BCG em combinação com o agente imunodulador imiquimode, assim mesmo como o efeito de compostos de azida de β-arilcalcogênio contendo telúrio sobre a indução de apoptose e resposta a genes associados ao estresse oxidativo em câncer de bexiga.

3.2 Objetivos Específicos

- Avaliar o efeito das cepas auxotróficas BCG Δ*leu*D, BCG recombinante Δ*leu*D/Ag85B e sua combinação com imiquimode sobre a inibição no crescimento celular e apoptose utilizando o ensaio de MTT e de anexin (citometria de fluxo) em linhagem de melanoma (WM1366);

- Determinar a expressão gênica dos genes *BAX*, *BCL-2* e *CASP3* em linhagem WM1366, e dos genes envolvidos na resposta imune como *IL-6*, *IL-12*, *TNF-α* e *IFN-γ* em linhagem de macrófago J774A.1, em resposta aos tratamentos das cepas BCG Δ*leu*D e BCG Δ*leu*D/Ag85B em combinação com imiquimode;

- Avaliar o potencial citotóxico dos compostos de azida de β-arilcalcogênio ((S)-(2-azido-1-phenyl-3-telurophenyl)-propane (**5c**) e (S)-(2-azido-1-phenyl-3-teluro-p-methoxy-phenyl)- propane) (**5j**) sobre linhagem transicional de câncer de bexiga (5637) por meio do ensaio colorimétrico de redução do MTT;

- Estudar o efeito dos compostos **5c** e **5j** sobre a viabilidade celular e a capacidade de indução de apoptose por meio dos ensaios de live/dead e DAPI;

- Determinar o efeito dos compostos de telúrio nos níveis de expressão dos genes anti-apoptóticos, pro-apoptóticos e de estresse oxidativo (*BCL-2*, *BAX*,

TP53, *p21*, *CASP3, CASP9*, *SOD*, *CAT*, *GPX*, *GR* e *iNOS*) sobre linhagem 5637 utilizando qRT-PCR;

- Avaliar a interação dos compostos **5c** e **5j** com proteinas alvos por meio de docking molecular.

4. CAPÍTULOS

4.1 Artigo 1- *Mycobacterium bovis* BCG in metastatic melanoma therapy

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Mycobacterium bovis BCG in metastatic melanoma therapy

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Abstract

Melanoma is the most aggressive form of skin cancer, with a high mortality rate and with 96,480 new cases expected in 2019 in the USS. BRAFV600E, the most common driver mutation, is found in around 50% of melanomas, contributing to tumor growth, angiogenesis, and metastatic progression. Dacarbazine (DTIC), an alkylate agent, was the first chemotherapeutic agent approved by the US Food and Drug Administration (FDA) used as a standard treatment. Since then, immunotherapies have been approved for metastatic melanoma (MM) including ipilimumab and pembrolizumab checkpoint inhibitors that help decrease the risk of progression. Moreover, *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) serves as an adjuvant therapy that induces the recruitment of natural killer NK, CD4+, and CD8+ T cells and contributes to antitumor immunity. BCG can be administered in combination with chemotherapeutic and immunotherapeutic agents and can be genetically manipulated to produce recombinant BCG (rBCG) strains that express heterologous proteins or overexpress immunogenic proteins, increasing the immune response and improving patient survival. In this review, we highlight several studies utilizing rBCG immunotherapy for MM in combination with other therapeutic agents.

Keywords Bacillus Calmette–Guérin, Recombinant BCG, Immunotherapy, Antitumor activity, Skin cancer

Introduction

Melanoma is the most aggressive form of skin cancer, representing 4% of all dermatological cancers. A total of 96,480 new cases of melanoma will be diagnosed, and 7230 deaths are expected in 2019 in the USA (Siegel et al. 2019). Its etiology is multifactorial, being associated with both environmental and genetic factors. BRAFV600E is the most common mutation, being observed in around 50% of melanomas and contributing to tumor growth, angiogenesis, and metastatic progression (Garnett and Marais 2004; Haass et al. 2004). Current treatments for advanced melanoma include chemotherapeutic and immunotherapeutic strategies, such as dacarbazine (DTIC), vemurafenib, interferon, interleukin 2 (IL-2), imiquimod, and checkpoint inhibitors. In addition, a number of recent studies are identifying new therapeutic strategies and targets for reducing melanoma's metastatic potential (Orgaz and Sanz-Moreno 2013; Mattia et al. 2018). One of these treatments is Mycobacterium bovis Bacillus Calmette– Guerin (BCG). BCG is an attenuated strain that has been used as an immunotherapeutic agent for melanoma and superficial urothelial carcinoma (Begnini et al. 2015; Maruf et al. 2016). Nowadays, wild-type BCG and recombinant strains are used in combination with chemotherapeutic and immunotherapeutic agents to enhance the immune response and tumor regression (Stewart and Levine 2011) (Fig. 1).

An improved understanding of the biological, genetic, molecular, and immunologic factors contributing to the progression of metastatic melanoma (Brandner and Haass 2013; Griewank et al. 2014; Shtivelman et al. 2014) may help identify novel therapeutic strategies (Ribas et al. 2011; Sullivan and Flaherty 2014). In addition, advanced proteomic technologies may be useful for detection and analysis of proteins involved in melanoma progression (Findeisen et al. 2009; Bougnoux and Solassol 2013). In this review, we highlight several studies utilizing BCG immunotherapy in combination with other therapeutic agents for the treatment of metastatic melanoma.

Melanoma

Melanoma is a neoplastic disorder caused by the malignant transformation of normal melanocytes and represents the most aggressive type of skin cancer (Bandarchi et al. 2010). Most cases originate in the skin, followed by the eyes and mucous membranes. Melanoma is

associated with environmental factors and patient demographics, such as lighter skin tone, sun sensitivity, presence of atypical nevi, multiple freckles, people with weakened immune systems, and family history of melanoma (Gallagher et al. 2005; Bishop et al. 2007). Its increasing incidence is due to different ethnicity, geographical location, and excessive exposure to ultraviolet radiation combined with its high metastatic potential which has resulted in a significant increase in mortality (Gallagher and Lee 2006; Ali et al. 2013; Arnold et al. 2018).

Melanoma is subdivided into four different types: superficial spreading melanoma (70% of melanomas), nodular melanoma (15–30%), acral lentiginous (5–10%), and lentigo malignant (5%) (Goldstein 2001; Bastian 2014).

Deregulation of the MAPK, PI(3)K-AKT, P16INK4A/ Rb), and Wnt/b-catenin signaling pathways is observed in metastatic melanoma and has been implicated in its etiopathogenesis and invasive behavior (Orgaz and Sanz- Moreno 2013; Alegre et al. 2014; Gurzu et al. 2018). Somatic mutations in the BRAF gene are observed in 40– 60% of melanoma cases (Curtin et al. 2005; Abildgaard and Guldberg 2015). The most commonly observed mutation results in the substitution of lysine for valine (V600K mutation) (Long et al. 2011; Lovly et al. 2012) and is responsible for the activation of the MAPK pathway, which regulates normal cell growth and survival (Shinozaki et al. 2007; Flaherty and McArthur 2010; Roskoski 2010). Other genes commonly mutated in melanomas include NRAS (15–30%) (Gorski et al. 2005), CDK4, CDKN2A (observed in 20 to 40% of families with melanoma susceptibility) (Puig et al. 2005; Potrony et al. 2015), p16, PTEN, AKT1, MAP2K1, MAP2K2, MAP3K5 and MAP3K9. In addition, a number of DNA methylation changes have been reported (Hodis et al. 2012; Nikolaev et al. 2012; Stark et al. 2012). These mutations and epigenetic alterations trigger and promote the secretion of growth factors that contribute to cell proliferation, angiogenesis, alterations in the extracellular matrix, cytoskeletal organization, and metastasis (Haass et al. 2004; Orgaz and Sanz-Moreno 2013) to various organs such as the lungs, liver, brain, and bones (Leong 2003).

Melanoma diagnoses are made by dermatoscopy examination and advanced digital computer imaging techniques following the morphologic features summarized by the asymmetry, border, color, diameter, and elevation (ABCDE), which are confirmed by biopsy and histopathological examination. This process also allows for melanoma staging and prognostic, as well as TNM (tumor, node metastasis) classification for detection of distant metastases (Abbasi et al. 2004; Balch et al. 2009; Ciudad-Blanco et al. 2014; Lattanzi et al. 2019).

Therapies for metastatic melanoma treatment

Treatment for metastatic melanoma (MM) is mostly based on systemic therapy, although radiotherapy and surgical treatments are also used. However, current treatments have not resulted in significant improvements in patient survival due to adverse effects (Smith et al. 2007). Surgery is mostly used for resection of distant metastases (Luther et al. 2019). Radiation therapy can be used as adjuvant therapy for systemic therapy; however, some patients with symptomatic metastases can benefit from radiotherapy (Bhatia et al. 2009).

The first systemic therapy used for MM treatment is the cytotoxic chemotherapeutic agent Dacarbazine (DTIC). DTIC is an alkylate agent and is the first chemotherapeutic agent approved for MM by the US Food and Drug Administration (FDA) in 1975. Another treatment that had been approved for MM t reatment in the USA is inter leukin-2, an immunostimulatory cytokine involved in T cell proliferation. Interleukin-2 trials have demonstrated a 15 to 20% response rate forMMpatients (Schwartzentruber et al. 2011); however, this drug failed to demonstrate survive prolongation (Garbe et al. 2011). Other cytokines being used to treat MM include interferon-alpha (Luther et al. 2019), the first cytokine to demonstrate activity inMM, with tumor response ranging from 10 to 20% (Schadendorf et al. 2009).

Since 2011, ten new agents have been approved by the FDA, including targeted therapies, immunotherapies, cancer vaccines, and other small molecules that can act as monotherapies or in combination (Simeone and Ascierto 2017; Luther et al. 2019). Ipilimumab is an IgG1 monoclonal antibody targeting cytotoxic T lymphocyte antigen 4 (CTLA-4). Treatment with ipilimumab was the first treatment to demonstrate a survival advantage for patients with MM (Wolchok et al. 2010). Other checkpoint inhibitors approved for MM include the programmed death 1 (PD-1) inhibitors nivolumab and pembrolizumab (Albertini 2018). These PD- 1 inhibitors were first approved for patients with melanoma refractory to vemurafenib and/or ipilimumab. Atezolizumab, avelumab, and durvalumab are PD-L1 inhibitors that have also been approved for use in MM patients with advanced melanoma (Arulananda et al. 2018; Callahan et al. 2018; Hogan et al. 2018).

Talimogene laherparepvec (TVEC) is the only oncolytic virus approved for melanoma treatment (Luther et al. 2019). Another immunotherapeutic agent is allovectin-7 (velimogene aliplasmid) which is well tolerated, reduction in tumor size, and seems to be safe for the treatment of stage III or IV melanoma (Gonzalez et al. 2006; Bedikian et al. 2010; Sloot et al. 2016).

Drugs targeting altered mitogen-activated protein kinase (MAPK) pathway signaling—commonly disrupted in MM due to BRAF V600E mutations—have been approved form MM treatment, including dabrafenib and vemurafenib (Heakal et al. 2011; Hauschild et al. 2012). Other approaches to treatMMinclude combination therapies. Combination therapies can be a solution for the treatment ofMMthat is resistant to individual therapies (Srivastava and McDermott 2014; Gazzé 2018). However, one potential problem regarding the use of combination therapies is increased toxicity. The combination of vemurafenib and cobimetinib, dabrafenib and trametinib, or encorafenib and binimetinib have become standard treatments for MM patients carrying the BRAFV600E mutation (Ribas et al. 2016). Immunotherapies can also be combined. The most well-established and studied combination is ipilimumab and nivolumab (Hodi et al. 2016).

Despite recent advances, these therapies still have limitations including low response rates, adverse side effects, and resistance, especially for anti-PD-1/PD-L1 checkpoint inhibitors (Broussard et al. 2018). For these reasons, novel treatment strategies for MM are currently being developed (Olszanski 2014; Arulananda et al. 2018; Sullivan et al. 2018) to improve targeted therapy through identification of new targets for MM and combine current and future drugs (Mitchell 2003; Finn et al. 2012; Broussard et al. 2018).

Bacillus Calmette–Guérin

BCG is an attenuated Mycobacterium bovis strain developed by Albert Calmette and Camille Guérin that is widely used as a vaccine to protect against tuberculosis (TB) (Hart and Sutherland 1977; Dietrich et al. 2003) and has also been used as the first-line immunotherapy for intravesical treatment of superficial urothelial carcinoma (Ahn et al. 2014; Begnini et al. 2015;Maruf et al. 2016), resulting in reduced recurrence and progression (Kresowik and Griffith 2009; Askeland et al. 2012;Morales et al. 2015; Donin et al. 2017). However, BCG administration can cause adverse side effects including hematuria, irritation, local inflammation, and cystitis (Koya et al. 2006). The exact mechanism of action is unknown but it involves BCG binding to the fibronectin of the bladder wall followed by its internalization via macropinocytosis and presentation of BCG antigens to Tcells (Lattime 1992; Zhao et al. 2000; Redelman-Sidi et al. 2013), causing tumor cell killing through signaling via the Toll-like receptors (TLR) 2, 3, 4, and 9, resulting in cytokine secretion and induction of the local inflammatory response (Miyazaki et al. 2006; Suttmann et al. 2006; Naoe et al. 2007).

There are several BCG modifications being used for immunotherapy and vaccination (Yuan et al. 2010; Zheng et al. 2015). BCG is sub-classified in different strains (Leung et al.

2008; Hayashi et al. 2009; Liu et al. 2009), and BCG exhibits anti-proliferative activities and results in the production of cytokines including IL-6 and IL-8 (Secanella-Fandos et al. 2013). Also, BCG has been genetically manipulated to secrete recombinant proteins (Borsuk et al. 2007; Begnini et al. 2013; Oliveira et al. 2017), foreign antigens from parasites, bacteria, and viruses (Bastos et al. 2009), and pro-inflammatory cytokines including IL-2, IL-8, and IL-18 that can enhance humoral and cellular immune responses (Biet et al. 2002; Luo et al. 2003; Luo et al. 2004).

rBCG::Ag85B-IFN-γ has been used in C57BL/6 mice to enhance immune responses and induced tumor necrosis factor TNF-α and IFN-γ expression (Liu et al. 2017). In addition, recombinant BCG::Rv2645 has been shown to improve dendritic cell (DC) antigen presentation and enhance Th1/Th17 immune responses against tuberculosis (Luo et al. 2018). The pantothenate auxotroph strain of *Mycobacterium bovis* BCG (BCGDpanCD) expressing HIV-1 Gag, Gp120, and RT induces a production of IL-2 and TNF-α by T cells (Chapman et al. 2013). Another recombinant BCG strain (ΔureC::hly) expressing a heterologous protein of Listeria monocytogenes has been shown to trigger production of caspases, IL-18, and IL-1β (Saiga et al. 2015). A similar study with ΔureC hly+ BCG was reported by Desel et al. (2011) and some vaccines with this construction are being tested for clinical efficacy to demonstrate their immunogenicity and safety (Nieuwenhuizen et al. 2017).

As an alternative to BCG, Mycobacterium vaccae (M. vaccae) has been shown to be safe, well tolerated, and results in long-term survival in patients with MM (Cananzi et al. 2013). In addition, the use of Mycobacterium indicus pranii (Mw) for the treatment of melanoma is being studied due to its ability to inhibit the matrix metalloproteinase (MMP-9), inhibiting melanoma tumor cell growth, and reducing invasiveness and metastatic potential (Halder et al. 2017).

BCG combination in melanoma treatment

BCG is used as an immunotherapeutic agent for the treatment of cutaneous MM. It is commonly administered alone or in combination with an autologous vaccine or drug by intralesional injection (Sloot et al. 2016) for different stages of melanoma, resulting in regression of local and regional tumors and improved patient survival (Lotem et al. 2002; Triozzi et al. 2011). There are many different BCG-based combination therapies currently in use for the treatment of melanoma (Table 1). One of the most common combinations is BCG with DTIC which results in a 36.5% increase in the 10-year survival rate for stage II melanoma patients (Cascinelli et al. 1989). Additional combinations such as BHD (hydroxyurea with DTIC), BHD plus BCG,

and DTIC plus BCG have been tested in patients with disseminated MM, resulting in overall response rates of 31%, 27%, and 18%, respectively (Costanzi et al. 1982). Even, the use of BCG and retinoids such as vitamin A palmitate and tretinoin have been shown to display antitumor activity for MM (Meyskens Jr. 1982).

In a study for patients with stage IV melanoma, the use of the allogeneic cancer vaccine canvaxin™in combination with BCG following complete resection resulted in antitumor immune responses and a 5-year overall survival rate of 39% compared with that of 20% for unvaccinated patients (Hsueh et al. 2002). This combination also increased the number of cytokine-producing CD4+ Tcells, which correlated with overall survival (Hsueh et al. 2004). Faries et al. (2017) performed the same study on 246 stage IV melanoma patients treated with canvaxin™and BCG following complete surgical resection and compared the results with 250 patients treated with BCG/placebo administered by intradermal injection. These results showed no improvement in patient outcomes and a 5- year survival rate of 34.9 months for patients treated with canvaxin™ and BCG compared with that of 39.1 months for patients treated with BCG/placebo. A similar study using BCG in combination with DTIC reported that this combination caused mild toxicity and was well tolerated, but did not improve patient outcomes compared with BCG alone (Agarwala et al. 2004).

Polyvalent vaccines have been shown to increase IgM responses and enhance the humoral immune response in patients with stage II melanoma, resulting in improved survival (DiFronzo et al. 2002). CancerVax has also been shown to induce an increase in TA90-IC antibodies in patients with advanced melanoma resulting in prolonged survival. TA90- IC is an antigen expressed on melanoma cells that serve as a prognostic marker, with higher TA90-IC levels present in patients with stage IV melanoma (Tsioulias et al. 2001). Another study reported immunization of MM patients with either smallpox (vaccinia) or BCG, showing that the vaccination reduced the risk of death and prolonged patient survival (Kölmel et al. 2005).

Additionally, the administration of BEC2, an anti-idiotypic mouse monoclonal antibody that mimics GD3 ganglioside mixed with BCG (BEC2-KLH/BCG) for patients with stage III and IV melanoma resulted in a 78% survival rate over 28 months (Yao et al. 1999). Furthermore, the GD3 ganglioside can be used as a target for melanoma immunotherapy, showing that 10 patients (42%) developed a detectable anti-GD3 antibody response, but did not correlate with survival outcomes (Chapman et al. 2004). It has been reported that BCG is a therapeutic option for stage III in-transit melanoma and can induce chemokine expression and regression of skin lesions (Yang et al. 2017). Another study developed a therapeutic vaccine named CSF-470 for cutaneous melanoma using a mixture of cell lines, BCG, and granulocyte-

macrophage colony- stimulating factor (GM-CSF) as adjuvants in phase II–III trials, which resulted in the recruitment of Ag-presenting cells and antitumor immune responses (Aris et al. 2015). Other studies have demonstrated the use of a M-VAX vaccine composed of autologous melanoma cells and BCG that induces interferon-gamma-mediated Tcell triggering inflammatory responses (Carretero et al. 2008).

The use of autologous melanoma vaccines and BCG plus recombinant human GM-CSF (rhGM-CSF) for treatment of stage IV melanoma has been shown to result in a complete response in 10% of patients with a median survival of 232 days (Leong et al. 1999). One such vaccine, VACCINEL, in combination with BCG and rhGM-CSF was administrated to patients with stage IIB/IV melanoma, resulting in well-tolerated, mildly toxic effects and increased antitumor immune responses (Barrio et al. 2006). Similar studies using the CSF-470 vaccine in combination with BCG and rhGM-CSF were well tolerated (Mordoh et al. 2017) and induced the release of pro-inflammatory cytokines such as IL-1β and TNF-α, stimulating long-term cellular and humoral immune responses (Pampena et al. 2018). On the other hand, Lotem et al. (2016) observed that the use of an autologous melanoma vaccine conjugated to dinitrophenyl and mixed with BCG in patients with stage III melanoma, followed by administration of ipilimumab, increased the response rate to 46% compared with 19% of patients treated with ipilimumab alone.

The continued generation of recombinant fusion proteins for use in melanoma immunotherapy is important as MUC1 andmaltose-binding protein (MBP) in combination with BCG induces a specific cellular immunity, synergistic activities, NK cell activity, and increased expression of IFN-γ and IgG2c (Fang et al. 2014).

BCG in melanoma immunotherapy

Recently, immunotherapeutic strategies have been used in cancer therapy to activate the immune system and destroy cancer cells. While the antitumor mechanism of BCG has not been clearly elucidated, BCG has been used as a nonspecific immune stimulant to induce long-lasting immune responses (Wada et al. 1996). Indeed, different tumor immunization strategies andmolecularly targeted therapies against the MAPK pathway are being studied to improve the response rate and survival for advanced melanoma patients (Davar et al. 2012). However, in order to translate these novel therapies to clinical practice, a better understanding of melanoma pathogenesis, its interaction between cancer and immune cells, and the genetic alterations resulting in targetable melanoma-associated antigens (i.e., neoantigens), such as MART-1

(Melan-A), gp100, and tyrosinase (Schumacher and Schreiber 2015; Cervinkova et al. 2017), is required. By utilizing vaccination strategies to increase the number of detectable neoantigens, we may be able to improve recognition of tumor cells by CD8+ T cells, circumventing the immune escape mechanism utilized by the tumor cells (Ragupathi et al. 2000; Terando et al. 2007), increasing tumor cell killing and reducing metastases (Lau et al. 2001; Hepner et al. 2017).

Vaccine development with a BCG adjuvant can induce immune responses contributing to antitumor immunity through the recruitment the NK, CD4+, and CD8+ T cells (Murphy et al. 1993; Kim and Cantor 2014; Kaufmann et al. 2016), resulting in the release of cytokines into the tumor microenvironment (Lardone et al. 2017). BCG-stimulated DCs secretes higher levels of TNF-α, IFN-γ, and IL-12p40 (Lalor et al. 2010; Kumar and Bhaskar 2019).

BCG causes the nonspecific identification of pathogenassociated molecular patterns (PAMPs) by TLR2, TLR4, TLR8, and TLR9, triggering an inflammatory cascade that results in the activation of macrophages (Mantovani and Sica 2010; Iqbal and Hussain 2014; Velmurugan et al. 2016), dendritic cells (Tsuji et al. 2000), and NK cells (Brandau et al. 2001; Kleinnijenhuis et al. 2014). BCG can also increase TRAIL expression and activation of TLR4 and TLR2 in neutrophils, triggering cancer cell apoptosis and improving melanoma patient responses (Ludwig et al. 2004).

It has been shown that the use of BCG as an immunotherapy agent for melanoma can result in tumor regression (Morton et al. 1976) and that topical or intratumoral injection of BCG induces immune cell infiltration and enhances the long-lasting expression of chemokines and cytokines, including CXCL9, CXCL10, CXCL11, IL-15, TNF-α, and IFN-γ (Yang et al. 2017). Due to the high levels of tumor heterogeneity observed clinically and relatively low immunogenicity of melanoma-associated antigens, immunotherapeutic strategies for melanoma utilizing rBCG strains capable of secreting functional cytokines that can stimulate antitumor immune responses show great promise (Sanlorenzo et al. 2014).

The development of recombinant BCG (rBCG) strains has resulted in new vaccines, capable of delivering different antigens that trigger induction of CD8 T cell immune responses with long-lasting memory compared with conventional systems (da Costa et al. 2014; Liang et al. 2015; Oliveira et al. 2017). Also, these rBCG strains have been genetically modified to display different phenotypes, for example through the utilization of Hsp60, Hsp7, pAN, and 18-kDa promoters (Newton-Foot and Gey Van Pittius 2013; Oliveira et al. 2017) that increase cytokine expression and enhance immune responses (Himmelrich et al. 2000; Slobbe et al. 1999). The use of different rBCG strains secreting a variety of cytokines such as IL-4, IL-6, IL-

2, IFN-γ, and GM-CSF can modify and potentially improve the antitumor response (Murray et al. 1996; Zhou et al. 2015).

In addition, this method also allows for the development of auxotrophic strains, including strain, where genes involved in the synthesis of metabolites are deleted, strains without antibiotic resistance markers, and strains displaying stable expression both in vitro or in vivo (Borsuk et al. 2007; Seixas et al. 2010; Rizzi et al. 2017).

Several studies have reported the administration of rBCG strains for melanoma treatment (Table 2). One of the most common rBCG strains used for anticancer therapy is rBCG strains expressing interleukin-2 (rBCG-IL-2) and GM-CSF (rBCG-GM-CSF). These combinations increase the production of INF-γ, cytokine important for inhibiting tumor cell growth (Fujimoto et al. 1996).

One of the most important strains used is a Pasteur strain, which contains the heat shock protein 60 (hsp60) promoter controlling the expression of IL-2. When administered by intratumoral injection, this rBCG strain displayed immunomodulatory properties and resulted in a 45% reduction in tumor size in a murine B16 melanoma model (C57BL/6 mice) (Duda et al. 1995). In addition, rBCG can express recombinant human interferon-alpha 2B (rhIFN-α) under control of the hsp60 promoter, resulting in increased IFN-γ production and immunostimulatory properties (Luo et al. 2001).

Conclusion

This review summarizes the used of wild-type and various rBCG strains as therapeutic agents for melanoma to induce immunomodulatory activities and enhance antitumor immune responses, as well as its combination with chemotherapy and immunotherapy agents. Few rBCG constructs have been explored and little is known about the immunological basis and mechanisms of action leading to BCG-induced melanoma cell killing. Further investigation of these mechanisms of action will allow for the development of improved treatment strategies to improve the survival of MM patients. Funding information This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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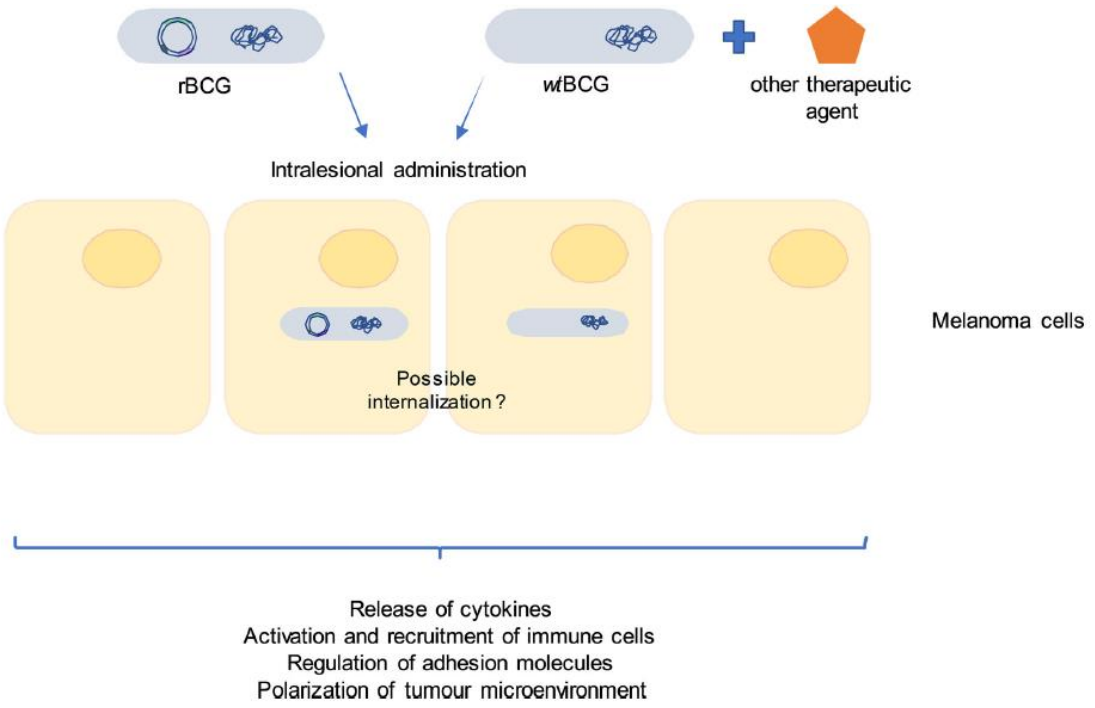

Fig. 1 Administration of wild-type or recombinant BCG strains alone or in combination with a chemotherapeutic or immunotherapeutic agent enhances immune responses and tumor regression in melanoma patients.

Table 1 Combination BCG treatments for melanoma

BCG Strain	BCG dose	Population	Drug/Vaccine	BCG Administration	Main results	Reference
Connaught	BCG plus 100,000 units of vitamin	49 patients with stage I and stage II	BCG BCG + high-dose vitamin A	BCG by scarification	-The difference in relapse-free survival in the two groups was not statistically significant ($p = 0.27$) -Vitamin A was well tolerated	Meyskens (1982)
Connaught	6×10^8 (range 4-8 x 10^8)	386 patients with disseminated malignant melanoma	*BHD (betahistine dihydrochloride) BHD BHD+BCG DTIC+BCG	BCG by scarification	-In patients older tan 60 years of age, the response rate appeared higher in the BCG groups, but this was not statistically significant - BHD plus BCG and DTIC plus BCG showed an overall response rate of 27% and 18% respectively	Costanzi et al. (1982)
Pasteur	6×10^8 CFU	196 patients	*DTIC: Dacarbazina DTIC DTIC+BCG DTIC+*Corynebacterium parvum*	BCG by scarification	-The duration of response was longer with DTIC+BCG treatment but the difference was not statistically significant -In nonresponders, survival was less than 7 months	Veronesi et al. (1984)
Pasteur	75 mg	668 patients with stage II	DTIC BCG BCG+DTIC	BCG by scarification	-The patients that were initially non-reactive to BCG developed skin reactivity after 6.7 + 9 BCG vaccinations. -Survival rate of patients submitted to BCG treatment was 34.3%, and patients who received BCG and DTIC was 36.5% overall and 30.1% event-free survival	Cascinelli et al. (1989)

Table 1 *(continued)*

BCG Strain	BCG dose	Population	Drug/Vaccine	BCG Administration	Main results	Reference
Connaught	3.4 x 10^8 CFU	18 patients with stage III, or stage IV	Mitumomab (*BEC-2*) KLH(keyhole limpet hemocyanin) BEC2 conjugated to KLH and mixed with BCG (BEC2-KLH/BCG)	Intradermal injection	-Four patients developed anti-GD3 IgM antibodies as a result of immunization with BEC2-KLH/BCG -Thirteen of the patients were free of melanoma, resulting in a 78% survival rate	Yao et al. (1999)
Tice	10^7 CFU	20 patients with stage IV	Autologous melanoma vaccine and BCG + rhGM-CSF	Intradermal injection	-Twelve patient (60%) had progression of disease during treatment -Two patients (10%) showed partial response	Leong et al. (1999)
Tice	CancerVax was administered with BCG at 8 x 10^6 CFU	219 patients with stage II, III, or IV	CancerVax+BCG	Intradermal injection	-CancerVax inhibited the metastatic process by activating cytotoxic T cells -There was an increased IgM response -TA90-IC levels were significantly higher for patients with stage IV melanoma than patients with stage II	Tsioulias et al. (2001)
Tice	2.7 to 10.8 x 10^6 CFU .	150 patients with stage IV	Canvaxin vaccine + BCG	Intradermal injection	-The incidence of recurrence was 69% in vaccinated patients and 88% in nonvaccinated patients	Hsueh et al. (2002)

Table 1 *(continued)*

BCG Strain	BCG dose	Population	Drug/Vaccine	BCG Administration	Main results	Reference
Tice	8 x 10^6 CFU	83 patients with stage II	Canvaxin, polyvalent vaccine (PV)+BCG	Intradermal injection	-PV induced cellular and humoral immune responses, there was an increase of Anti-TA90 immunoglobulin (IgM) associated with decreased, recurrence and improved survival	DiFronzo et al. (2002)
BCG was purchased from Pasteur Merieux Connaught (Toronto, Canada)	2 x 10^7 CFU	24 patients with stage III or IV	GD3 ganglioside+ BCG	Intradermal injection	-42% of patients developed anti-GD3 antibodies, but this response did not correlate with survival outcomes. -There was no significant difference in survival between responders and no-responders	Chapman et al. (2004)
BCG (Instituto Malbran (Buenos Aires, Argentina)	2 x 10^6 CFU	20 patients with stages IIB and IV	*VACCIMEL and BCG as adjuvant GM-CSF VACCIMEL+GM-CSF rhGM-CSF (Recombinant human granulocyte-monocyte-colony-stimulating factor)	Intradermal injection	-The systemic toxicity of VACCIMEL, BCG, and rhGM-CSF was mild -Regression of metastatic lesions was not observed	Barrio et al. (2006)
IL-BCG/ imiquimod treatment	3 × 10^6 CFU and 1.5 × 10^6 CFU two weeks later.	9 patients with stage III	Intralesional Bacillus Calmette-Guerin (ILBCG) and topical 5% imiquimod cream	intradermal injection	-The combination therapy was well tolerated -78% patients did not develop recurrent intransit disease	Kidner et al. (2012)

Table 1 *(continued)*

BCG Strain	BCG dose	Population	Drug/Vaccine	BCG Administration	Main results	Reference
BCG (Shanghai Institute of Biological Products Co., Ltd., Shanghai, China)	BCG (150 mg/kg)	C57BL/6 female mice (mouse melanoma model)	*Human mucin 1 (MUC1) *Maltose-binding protein (MBP) MBP MUC1 BCG (MUC1/BCG) MUC1-MBP (MUC1-MBP/BCG)	Subcutaneous injection	-MBP and BCG alone were found to induce NK cell activity - MUC1-MBP/BCG synergistically induced NK cell activity -Immunization with MUC1/BCG and MUC1-MBP induced lower levels of IgG1 and significantly higher levels of IgG2c compared with MUC1	Fang et al. (2014)
Tice	3 millions CFU and 1.5 million CFU 2 weeks later	3 patients with in-transit melanoma (ITM)	Intralesional Bacillus Calmette-Gue´rin (ILBCG) and/or topical imiquimod.	Intralesional Injection	-The ILBCG monotherapy was not sufficient to cause disease regression, but 3 years and 11 months later, 1 patient remained disease free	Kibbi et al. (2015)
BCG unknown	10^6 CFU	45-year-old Caucasian woman with stage III	Mixture of cell lines using BCG and GM-CSF as adjuvants	Intradermal injection	-MART-1 Ag was found throughout the vaccination site -Recruitment of Ag-presenting cells and immune response toward the tumor	Aris et al. (2015)

Table 1 *(continued)*

BCG Strain	BCG dose	Population	Drug/Vaccine	BCG Administration	Main results	Reference
BCG (Statens Serum Institut, Denmark)	10^7 CFU	126 patients with stage III	Autologous melanoma cells conjugated to dinitrophenyl and mixed with BCG	Intradermal injection	-Reduced toxicity of the vaccination and protective immunity was observed -Patients with strong delayed-type hypersensitivity (DTH) response showed an overall survival of 75% compared with 44% in patients without a strong response	Lotem et al. (2016)
Tice	BCG doses were 3 x 10^6 CFU on day 0 and 1.5 x 10^6 CFU on day 14 .	246 patients with stage IV	BCG plus CanVaxin vaccine Canvaxin: three irradiated whole cells melanoma lines (M10-VACC, M24-VACC, and M101-VACC).	Intradermal injection	-In the BCG/Cv group, survival was longer in responders than in nonresponders -There was no improvement in outcomes following adjuvant treatment with vaccine over BCG/placebo	Faries et al. (2017)
Pasteur	1 x 10^6 CFU	108 patients with stage II and III	CSF-470 vaccine plus BCG and rhGM-CSF	Intradermal injection	-CSF-470 vaccine plus BCG plus rhGM-CSF administered as adjuvant therapy was well tolerated	Mordoh et al. (2017)
BCG unknown	160,000 CFU	12 patients with stage IIB, IIC, and III	CSF-470 vaccine plus BCG and (rhGM-CSF)	Intradermal injection	-There was the release of pro-inflammatory cytokines such as IL-1b and TNF-a in some patients -The vaccination stimulated a long term cellular and humoral immune response	Pampena et al. (2018)

Table 2 Recombinant BCG developed for melanoma treatment

BCG Strain	Antigen/gene organism	promoter	Secretion signal	Model	rBCG dose	Administration	Main results	Reference
Pasteur	IL-2 (mouse)	hsp60	α-Antigen	C57BL/6 mice	rBCG 3A: 10^8 CFU BCG: 10^6	Intratumoral or subcutaneous injection	-There was a 45% reduction in tumor size in the rBCG 3A group and a 44% reduction in the WT BCG group, suggesting rBCG 3A promotes an effective response against melanoma in the murine B16 model	Duda et al. (1995)
Pasteur	IL-2, GM-CSF (mouse)	hsp60	alpha-antigen signal	C57BL/6 female mice	1.3 x 10^7 CFU	Intratumoral or subcutaneous injection	-BCG and this combination treatment stimulated the production of INF-γ by splenocytes and inhibited tumor cell growth	Fujimoto et al. (1996)
Prague	The recombinant BCG ΔureC::hly (rBCG) vaccine	hsp60	Dsred-monomer fluorescent protein	C57BL/6 female mice	1 × 10^6 CFU	Subcutaneous injection	-Increased activation of caspases 3 and 7 was observed -rBCG displayed early increased expression of Il-1β, Il-18, and Tmem173	Saiga et al. (2015)

4.2 Manuscrito 1 - Recombinant BCG strain combined with imiquimod promotes cell growth inhibition in melanoma cell line

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Recombinant BCG strain combined with imiquimod promotes cell growth inhibition in melanoma cell line

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Abstract

Melanoma is the most aggressive form of skin cancer, has increased in recent decades, being one of the leading causes of death in the world. We evaluated the cell growth inhibition and apoptosis induction by auxotrophic BCG Δ*leu*D, and recombinant BCG Δ*leu*D/Ag85B strains in combination with imiquimod drug in human melanoma cell line WM1366. The cytotoxic activity was determined by MTT assay, and apoptosis induction was evaluated through flow cytometry, and qRT-PCR. Also, the cytokine profile induced by BCG strains with imiquimod in macrophage mouse line JJ74A.1 was measured qRT-PCR. The results of the cytotoxic effects with imiquimod showed IC_{50} value of 42.63±4.28 µM (48h) in WM1366 cell line. The combination of Δ*leu*D/Ag85B + imiquimod in 40µM showed 59.41% of growth inhibition, the combination in 50 µM showed 61.30%, and with 60 µM was reported 80.08% of growth inhibition when compared to imiquimod drug alone (48.22%, 51.77%, and 57.97%, respectively). Apoptosis induction showed that early/late percentages of apoptosis at concentration 60 µM for combination treatments were: BCG Δ*leu*D + imiquimod (15.77%), BCG Δ*leu*D/Ag85B + imiquimod (13.96%), imiquimod (19.91%) when compared to the

untreated control (3.78%) showing statistically significant differences among treatments. The *Bax* showed relative expression in BCG Δ*leu*D/Ag85B treatment following an increase in BCG Δ*leu*D/Ag85B + imiquimod treatment, and *Caspase-3* had an expression increased in treatments with BCG Δ*leu*D, BCG Δ*leu*D/Ag85B, and BCG Δ*leu*D/Ag85B + imiquimod. The gene expression profile of cytokines measured in JJ74A.1 showed that the combination BCG Δ*leuD*/Ag85B+ imiquimod increased mRNA levels of pro-inflammatory cytokines (*IL-6*, *IL-12*, *TNF-α,* and *IFN-γ*) when compared to imiquimod, and untreated cells ($P < 0.05$). In conclusion, the combination of BCG Δ*leu*D/Ag85B + imiquimod treatment showed inhibition of cell growth, exhibited apoptotic activity in WM1366 cell line, and increased the pro-inflammatory response in J774A.1, being a promising treatment for melanoma.

Keywords

Citotoxic activity, toll like receptor, immunotherapy, apoptosis, antigen 85B, WM1366

1. Introduction

Cutaneous melanoma (CM) is originated from the malignant transformation of melanocytes, cells that produce melanin. Malignant melanoma (MM) is the most aggressive form of skin cancer due to several mutations that occur in cells affecting genes responsible for cell proliferation (Olbryt et al. 2020). This neoplasia accounts for 4% of all dermatological cancers, with an estimated 100.350 new cases in the US for 2020. MM has been the most increasing cause of death in the world over the past decades (Siegel et al. 2020).

The therapies for melanoma comprise surgery, chemotherapy, radiation therapy, and immunotherapy (Lattanzi et al. 2019; Luther et al. 2019). Immunotherapy can activate and potentiate the immune responses against cancer (Yu et al. 2019; Boudewijns et al., 2020) due to high mutation present in melanoma, and the neoantigens could be detectable by the immune system and target them for destruction (Braunlein and Krackhardt 2017). One of the main strategies involved in melanoma treatment is the use of *Mycobacterium bovis* bacille Calmette–Guérin (BCG) (Morton et al. 1974), and BCG-based therapy is the first-line treatment in intravesical therapy against non-muscle invasive superficial bladder cancer (Begnini et al. 2015; Maruf et al. 2016).

BCG is used for intralesional therapy in malignant melanoma, can be used in combination with other treatment (Faries et al. 2017) as autologous vaccine (Sloot et al. 2016), being a potent effective adjuvant for multivalent recombinant vaccines, recognized by macrophages that trigger the

activation of T cells, and produce Th1 and expression of different cytokines, including IL-2, IL-6, TNF-α, and IFN-γ (Zhou et al. 2015; Yang et al. 2017).

The use of recombinant BCG (rBCG) expressing foreign antigens or overexpressing antigens can provide good protection, induce humoral and cellular immune responses, improve the performance of BCG-antitumor therapy, inhibit tumor growth and progression of cancer (Luo et al. 2004; Faries et al 2017).

Imiquimod (Aldara) is a heterocyclic imidazoquinoline amide used as a topical cream, presents antitumor, and antiviral activity (Wester et al. 2017). Imiquimod acting as toll-like receptors TLR7 and TLR8 agonist expressed in monocytes, macrophages, dendritic cells, and malignant cells, modulating the immune responses (Narayan et al. 2012; Dajon et al. 2019) by inducing the release of pro-inflammatory cytokines including interferon alfa (IFN-α), interleukin 12 (IL-12), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor alfa (TNF-α) (Turza et al. 2010; Narayan et al. 2012). Aldara was approved by the US Food and Drug Administration (FDA) for external genital warts, actinic keratosis, and superficial basal cell carcinoma (Al-mayahy et al. 2019).

In this study, it was employed the auxotrophic strain rBCG Δ*leu*D/Ag85B. It was constructed with a selectable marker and to express the Ag85B immunogenic protein, encoded by *fbpB* gene from *M. tuberculosis* (Borsuk et al. 2007; Rizzi et al. 2012). Despite the advances, several therapies have low response rates against cancer (Broussard et al. 2018), and currently, new strategies and combinations of treatments are being found to reduce the advanced melanoma (Gazzé 2018; Luther et al. 2019). Few rBCG constructs have been investigate, and the combination of rBCG strains with other therapeutic agents is a great interest to treat metastatic melanoma (Benitez et al. 2019). In this context, the aim of this study was to determine the effects of combination of the recombinant strain rBCG Δ*leu*D/Ag85B with imiquimod through *in vitro* evaluation of the cytotoxic activity in human melanoma cancer cells (WM1366), and the pro-inflammatory response in cell line J774A.1.

2. Material and Methods

2.1. Cell line and cell culture

Mouse monocyte/macrophages cell line (JJ74A.1) was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil), and the human melanoma cell line (WM1366) was kindly provided by University of São Paulo (USP) Ribeirão Preto, SP-Brazil. The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin, purchased

respectively from Vitrocell Embriolife (Campinas, Brazil) and Gibco (Grand Island, NY, USA). The cells were grown at 37° C in an atmosphere of 95% humidity and 5% CO_2. The experiments were performed using cells in the exponential growth phase.

2.2. Bacterial strains and cell culture

The auxotrophic *Mycobacterium bovis* BCG Δ*leu*D, and BCG Δ*leu*D/Ag85B strains were grown as previously described (Begnini et al. 2013). Shortly, BCG strains were grown on Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco Laboratories, Detroit, MI, USA), 0.2% glycerol, and 0.05% Tween 80 (Sigma), or in 7H10 agar (Difco) supplemented with OADC. BCG Δ*leu*D strain was grown in media supplemented with 100 mg/ml L-leucine (Sigma-Aldrich, St. Louis, Missouri, USA), and bacterial growth (optical density at 600 nm) was measured. *M. bovis* BCG Δ*leu*D was developed by Borsuk et al. (2007), and the BCG Δ*leu*D/Ag85B (rBCG) (pUP410::*fbp*B) was constructed by Rizzi et al. (2012), and the Ag85B expression was realized through western blotting by Begnini et al. 2013.

2.3. Determination of cytotoxicity

2.3.1. *WM1366 culture*

Human melanoma cells (WM1366) were seeded at a density of 2×10^4 cells per well in 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere for 24h. After, cells were incubated with imiquimod drug obtained from InvivoGen (San Diego, CA, USA), diluted with pirogenic water at concentrations of 5, 10, 20, 40, 50, 60, 80 and 100 μM per well for 48h. The water was used as vehicle control, and untreated cells were used as a negative control. The culture medium was removed, and the cell viability was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide] to formazan salt (Mosmann 1983). Cells were washed and 5 mg of MTT solution was added to each well for 3h at 37 °C. The absorbance of each well was read on a microplate reader (MR-96A-Microplate Reader) at a wavelength of 492 nm. This assay showed the half-maximal inhibitory concentration (IC_{50}). The inhibition (%) of cell proliferation was determined by inhibitory growth = (1-Abs492 treated cells/Abs492 control cells) x 100% (Zheng et al. 2011). The results were validated by the mean of three independent experiments in triplicates for each experiment.

2.3.2. *WM1366 and BCG strains co-culture*

Human melanoma cells were grown at a density of 2×10^4 cells per well in 96-well plates and cells were incubated with different combinations of treatments: BCG Δ*leu*D, BCG Δ*leu*D/Ag85B, BCG Δ*leu*D + imiquimod, BCG Δ*leu*D/Ag85B + imiquimod, and imiquimod. Imiquimod was used at concentrations of 40, 50 e 60 μM (concentrations close to the IC_{50} values) respectively. BCG Δ*leu*D and BCG Δ*leu*D/Ag85B were used at concentration of 10^5 CFU (colony-forming unit) for all assays. Each experiment was performed in triplicate. The cytotoxic assay was measured by the colorimetric MTT assay previously described.

2.4. Apoptosis assay

WM1366 cells were seeded at a density of 1×10^5 cells per well in 12-well plates and incubated for 24h. After 24h, cells were treated with different treatments: BCG Δ*leu*D and BCG Δ*leu*D/Ag85B at concentration of 10^5 CFU, BCG Δ*leu*D + imiquimod, BCG Δ*leu*D/Ag85B + imiquimod, and imiquimod at concentration of 60 μM (because in this concentration there was a significant difference between treatments) for 48h. All assays were performed in three independent experiments. Apoptosis was determined by flow cytometry, the procedure was conducted according to the methodology provided in Annexin V-7AAD apoptosis detection kit (Guava Technologies, Millipore Corporation). Briefly, cells were incubated at room temperature for 20 min in the dark and the 7-AAD-positive and annexin V-negative indicates nuclear nebris, 7-AAD-positive and annexin V-positive indicates late apoptotic cells, 7-ADD negative and annexin V-negative indicates live health cells, and 7-AAD-negative and annexin V-positive indicates early apoptotic cells.

2.5. RNA isolation and quantitative Real-Time PCR (qRT-PCR)

The analyses of gene expression associated with apoptotic mechanisms in WM1366, and pro-inflammatory response in J774A.1 were investigated by qRT-PCR. Cells were seeded at a density of 2×10^5 cells per well in 6-well plates and grown at 37 °C, 5% of CO_2 for 24h. Then, cells were incubated with different combinations: BCG Δ*leu*D, BCG Δ*leu*D/Ag85B at concentration of 10^5 CFU, BCG Δ*leu*D + imiquimod, BCG Δ*leu*D/Ag85B + imiquimod and imiquimod at concentration of 60 μM for 24h. Afterward, the cells were washed with phosphate-buffered saline (PBS; Gibco). Total RNA was extracted with TRizol reagent (InvitrogenTM, Carlsbad, CA, United States) (Campos et al. 2010). The RNA concentration and quality were evaluated using the Nanovue 4282 spectrophotometer (GE Healthcare). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied BiosystemsTM, United Kingdom) according to the

manufacturer's instructions. Real-time PCR reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK). Genes and primer sequences used for PCR analysis are listed in Table 1. The housekeeping gene GAPDH was used as an endogenous control. The relative expression data were calculated according to the $2^{-\Delta\Delta Ct}$ (Delta-Delta Comparative Threshold) method and were presented as fold changes (Rao et al. 2013). Three independent experiments in triplicates were performed.

Table 1. Sequences of primers used for qRT-PCR in this study (Begnini et al. 2013)

Gene	**Sequence 5'-3'**
IL-6 For	AGTTGCCTTCTTGGGACTGA
IL-6 Rev	ACAGTGCATCATCGCTGTTC
IL-12 For	CATGGTGGATGCCGTTCA
IL-12 Rev	ACCTCCACCTGCCGAGAAT
TNF-α For	CATCTTCTCAAAATTCGAGTGACAA
TNF-α Rev	TGGGAGTAGACAAGGTACAACCC
IFN-γ For	GCGTCATTGAATCACACCTG
IFN-γ Rev	TGAGCTCATTGAATGCTTGG
Bax For	ATGCGTCCACCAAGAAGC
Bax Rev	ACGGCGGCAATCATCCTC
Bcl-2 For	GTGTGGAGAGCGTCAACC
Bcl-2 Rev	CTTCAGAGACAGCCAGGAG
Caspase-3 For	CAGTGGAGGCCGACTTCTTG
Caspase-3 Rev	TGGCACAAAGCGACTGGAT
GAPDH For	GGATTTGGTCGTATTGGG
GAPDH Rev	TCGCTCCTGGAAGATGG

2.6. Statistical analysis

Data set were analyzed by one or two-way analysis of variance (ANOVA) followed by Tukey´s post-test for multiple comparisons. The results were shown as mean ± SEM (standard error of the mean), and are representative of at least three independent experiments. The differences were considered significant when $P < 0.05$ in all analyses. Statistical analysis was performed using Graph Pad Prism 7.0 software (San Diego, CA, USA).

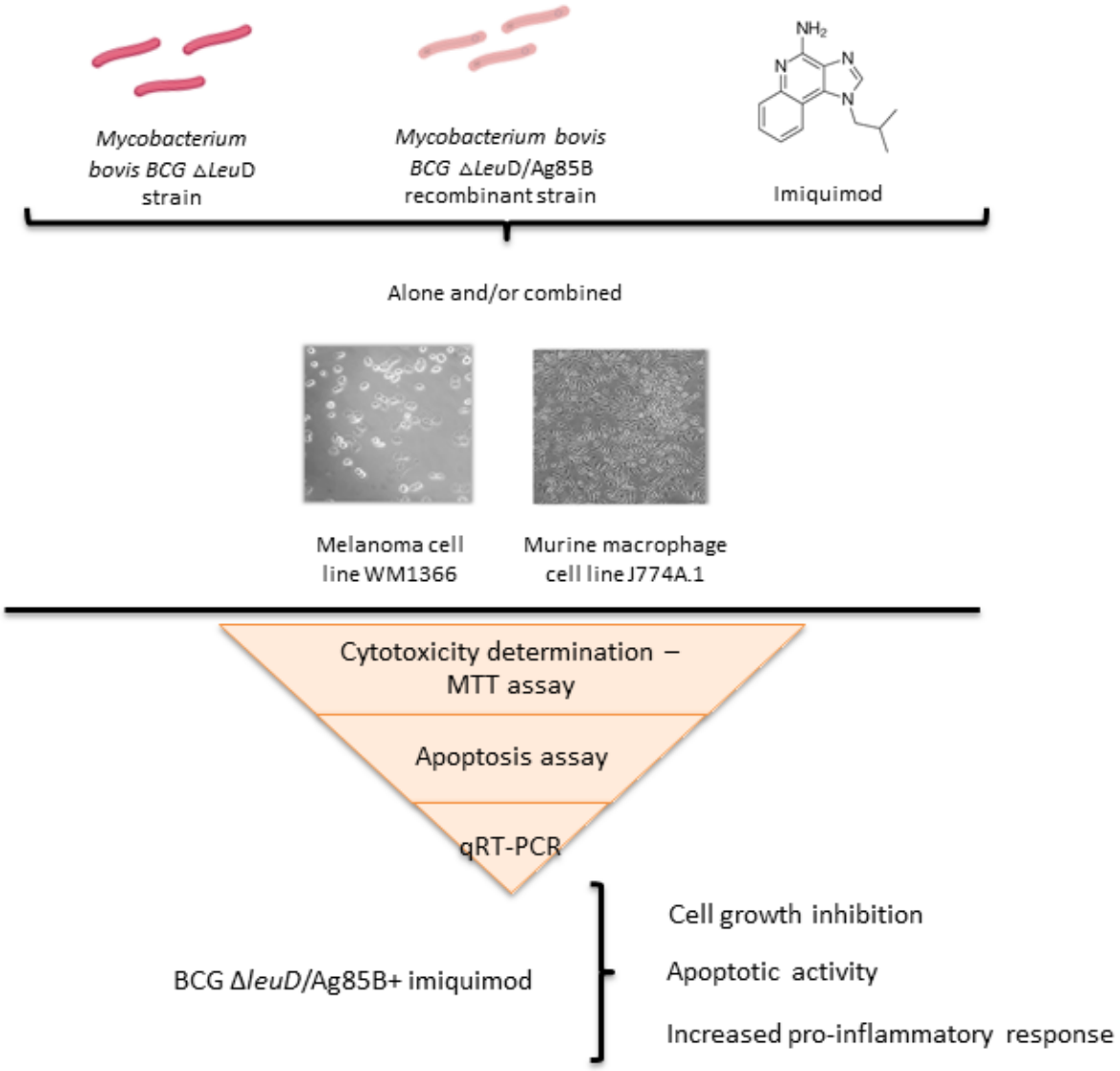


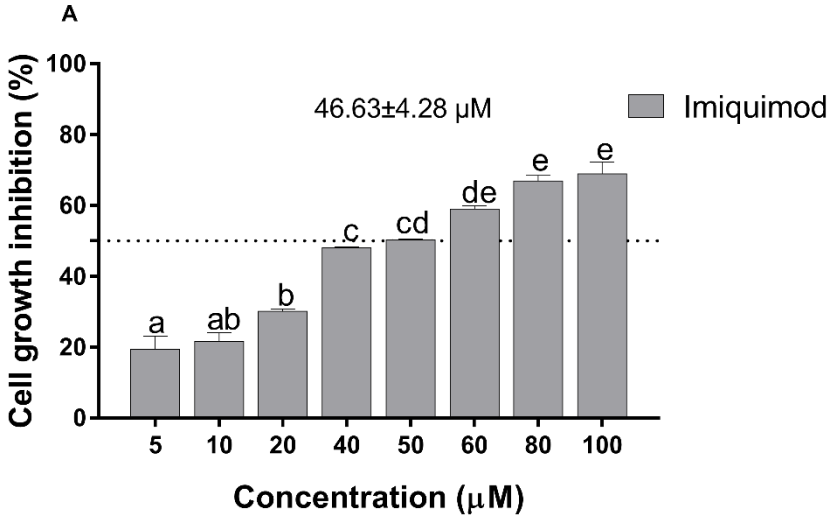
Fig. 1 Scheme of protocol of combinations of treatments in WM1366 and J774A.1 cell lines.

3. Results

3.1. Auxotrophic BCG strains in combination with imiquimod inhibited cell proliferation

Growth inhibition of melanoma cells (WM1366) treated with imiquimod and/or BCG was determinated by MTT assay. To determine which concentrations of imiquimod to use in combination with BCG we tested concentration ranging from 5 to 100 μM at 48 hours (Fig. 2A). Three imiquimod concentration were chosen (40 μM, 50 μM and 60 μM) because they were close to inhibiting 50% of cell growth (46.63 ± 4.28 μM). When combining BCG strains with imiquimod at 40 μM growth

inhibition of Δ*leu*D + imiquimod was 36.31% and for BCG Δ*leu*D/Ag85B + imiquimod was 59.41% for BCG Δ*leu*D was 20.71%, for BCG Δ*leu*D/Ag85B was 33.54% and imiquimod alone was 48.22% (Fig. 2B). At imiquimod concentration of 50 μM the growth inhibition for Δ*leu*D + imiquimod was 52.66%, BCG Δ*leu*D/Ag85B + imiquimod was 61.30%, for BCG Δ*leu*D was 26.42%, BCG Δ*leu*D/Ag85B was 31.92% and imiquimod 57.77% (Fig. 2C). Finally, at concentration of 60 μM growth inhibition for the combination of Δ*leu*D + imiquimod was 71.59%, BCG Δ*leu*D/Ag85B+imiquimod was 80.08%, BCG Δ*leu*D obtained 26.42% of growth inhibition, BCG Δ*leu*D/Ag85B 31.92% and imiquimod 57.97% (Fig. 2D), showing significant difference in this concentration. To determine whether imiquimod interferes with BCG growth viability, BCG Δ*leu*D and BCG Δ*leu*D/Ag85B strains were cultured in middlebrook 7H9 broth in the presence or absence of imiquimod (IC_{50}). Cell growth was assessed by measuring the concentration of BCG, absorbance at 600 nm over a period of 5 days (every 24 h). In addition, 100 μL of the strains (10^6/mL) were plated on 7H10 agar with or without imiquimod (IC_{50}), and the effect of imiquimod on BCG growth was determined by observing colonies on plates after incubation for 21 days at 37 °C. Interestingly, BCG strains remained alive after treatment with imiquimod (data not shown).



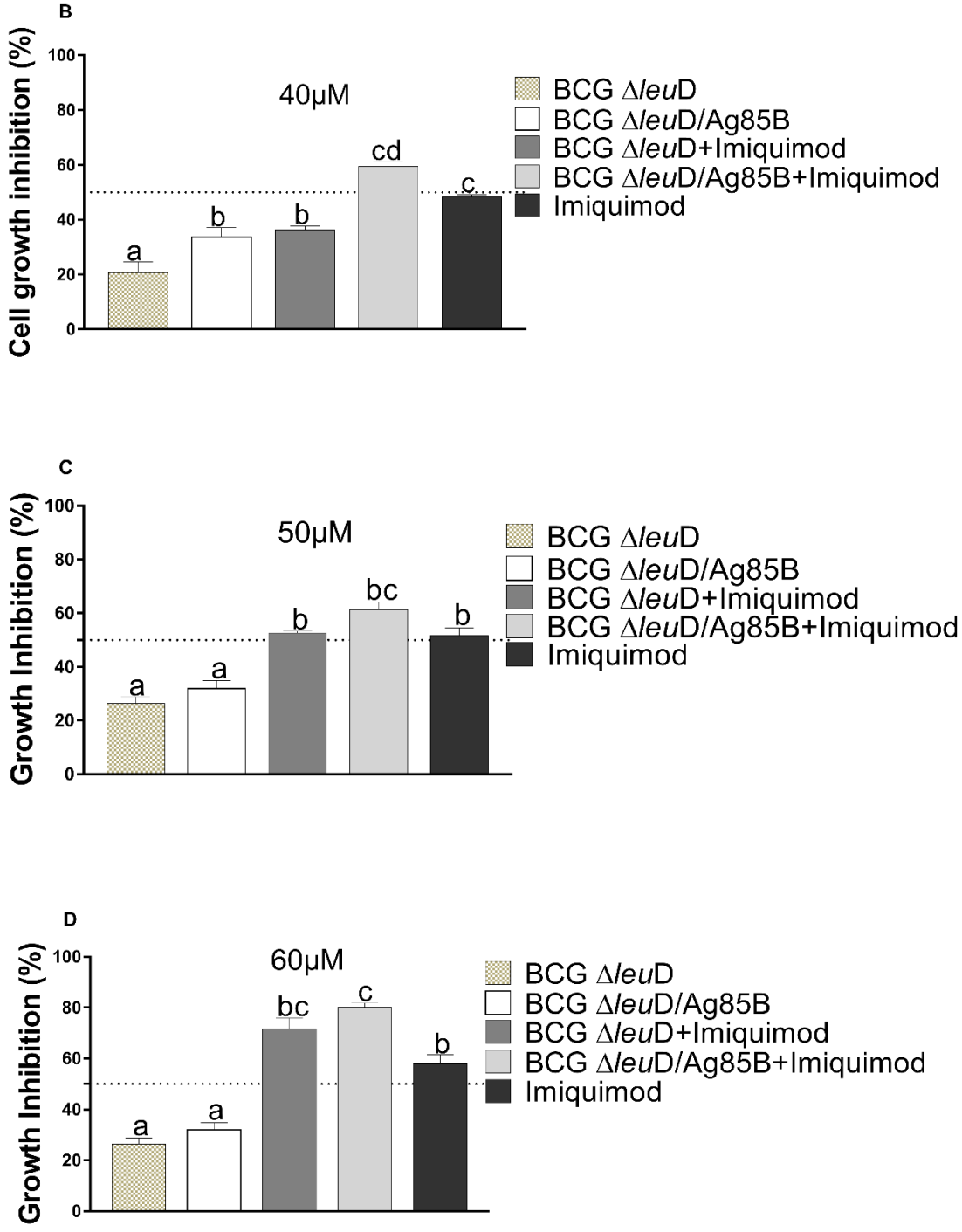


Fig. 2 Cell growth inhibition by MTT assay in WM1366 cell line. Cells were incubated with imiquimod at concentrations of 5-100 μM for 48h (A). The combination of BCG strains and imiquimod in concentrations of 40 μM, 50 μM and 60 μM were evaluated respectively (B-D). Data were represented as mean ± SEM of three independent experiments. One-way and Two-way ANOVA with Tukey´s post-test was used to analyze statistical significance. Different letters indicate significant differences between the means and differences were considered significant at $P < 0.05$.

3.2. Apoptosis determination

To determine which mechanism was involved in cell death, apoptosis assay was performed. Results obtained with BCG and imiquimod treatment showed that this combination was able to induce apoptotic cells. The percentage of early/late apoptosis at 60 μM concentration for combined treatments was: BCG Δ*leu*D (3.40%), BCG Δ*leu*D/Ag85B (3.77%), BCG Δ*leu*D + imiquimod (15.77%), BCG Δ*leu*D/Ag85B + imiquimod (13.96%), imiquimod (19.91%) when compared to the untreated control (3.78%). Also showed differences between live cells with survival percentages in BCG Δ*leu*D + imiquimod (77.45%), BCG Δ*leu*D/Ag85B + imiquimod (82.72%), imiquimod (74.21%) when compared to the untreated control (94.61%). (Fig 3).

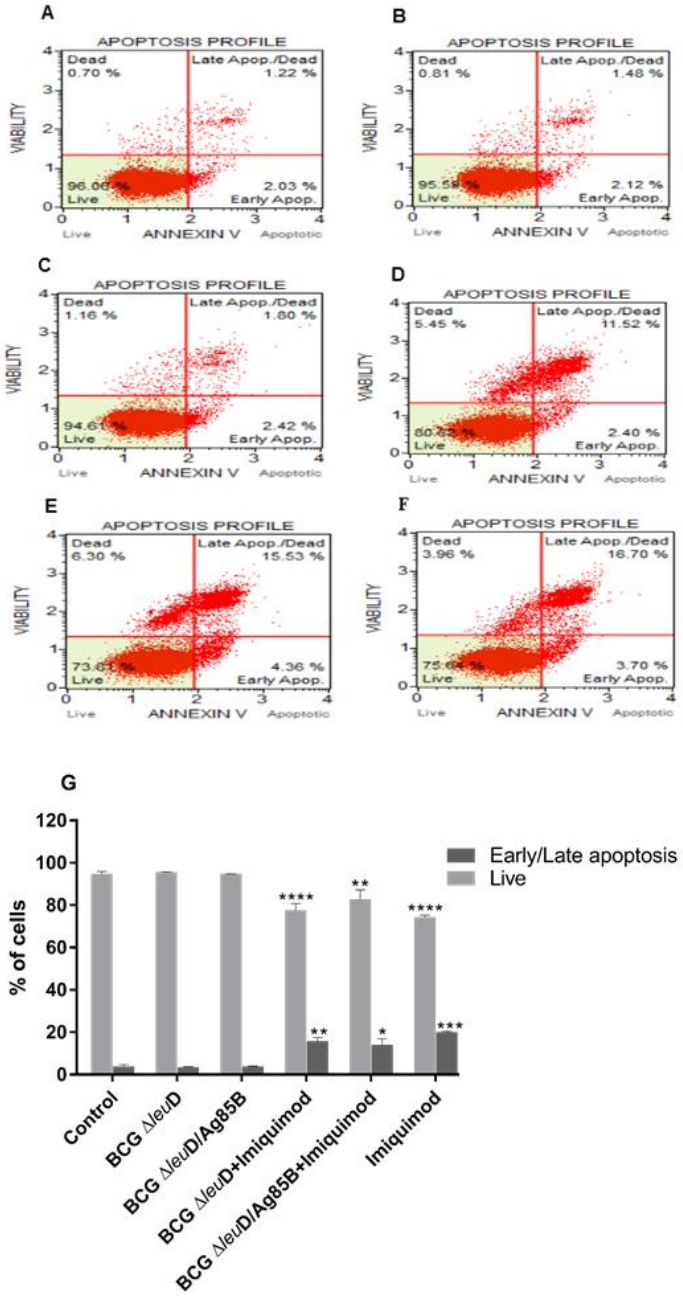
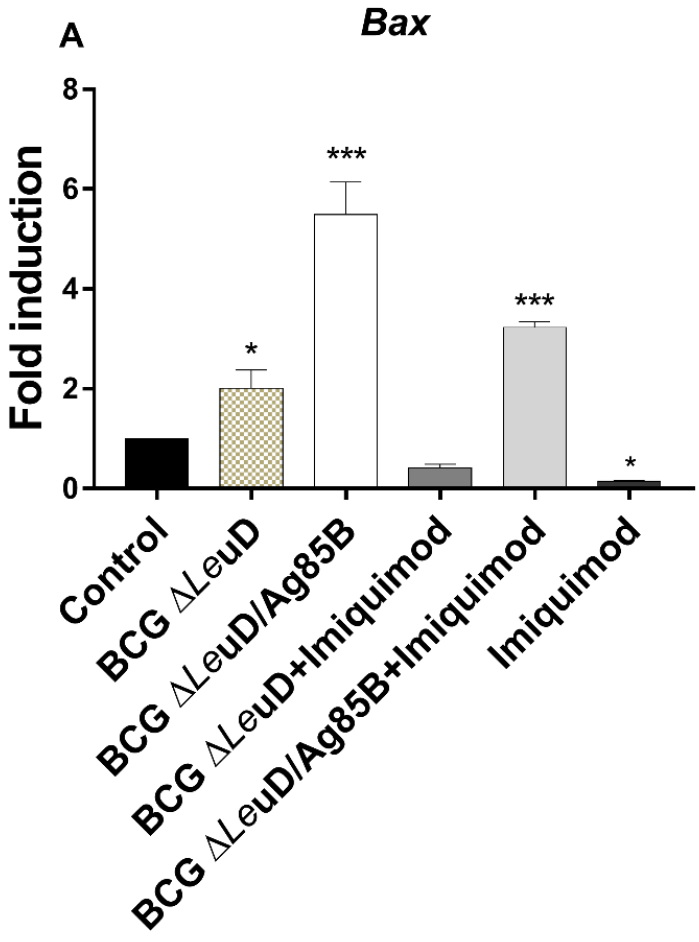
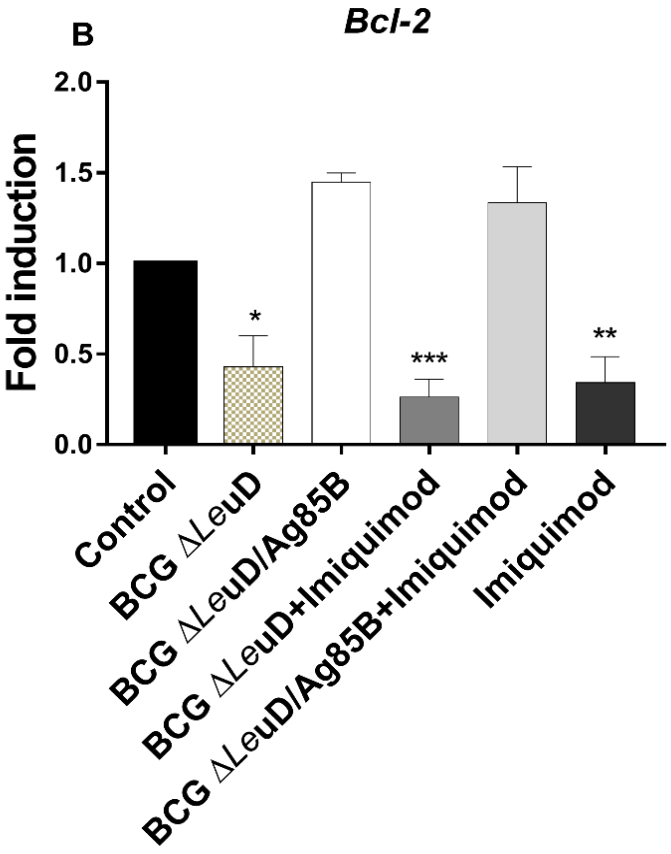


Fig. 3 Apoptosis was determined by flow cytometry in WM1366 after 48 h. The combination of BCG strains and imiquimod at concentration of 60 uM was evaluated respectively. The upper panel represents the percents obtained by flow cytometry, showing the untreated control (A), BCG Δ*leu*D (B), BCG Δ*leu*D/Ag85B (C), BCG Δ*leu*D + imiquimod (D), BCG Δ*leu*D/Ag85B + imiquimod (E), and imiquimod (F). The bottom panel represents the percentage of live or early/late apoptosis after each treatment (G). Data were represented as mean ± SEM of three independent experiments. Two-way ANOVA with Tukey´s post-test was used to analyze statistical significance. (*) represents a significant difference between the different treatments in relation to the control group. **P*< 0.05, ** *P*< 0.01, *** *P*< 0.001, and **** *P*< 0.0001. Significance was considered at *P*< 0.05.

3.3. Gene expression profile in WM1366 cells treated with rBCG strains, and imiquimod

Genes related to apoptotic mechanisms such as *Bax*, *Bcl-2*, and *Caspase-3* were evaluated by real-time PCR. *Bax* gene had its expression most increased in BCG Δ*leu*D/Ag85B treatment following an increase in BCG Δ*leu*D/Ag85B + imiquimod treatment (Figure 4A). *Bcl-2* gene had differences just in the BCG Δ*leu*D, BCG Δ*leu*D + imiquimod, and imiquimod where the fold induction was lower than in control (Figure 4B). *Caspase-3* had its gene expression increased in treatments with BCG Δ*leu*D, BCG Δ*leu*D/Ag85B and BCG Δ*leu*D/Ag85B + imiquimod (Figure 4C).

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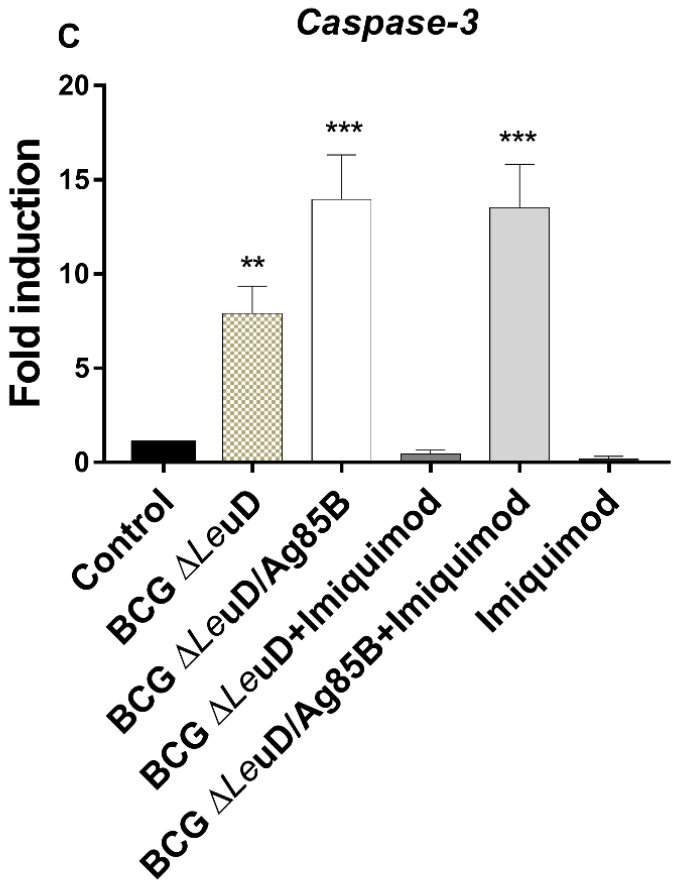
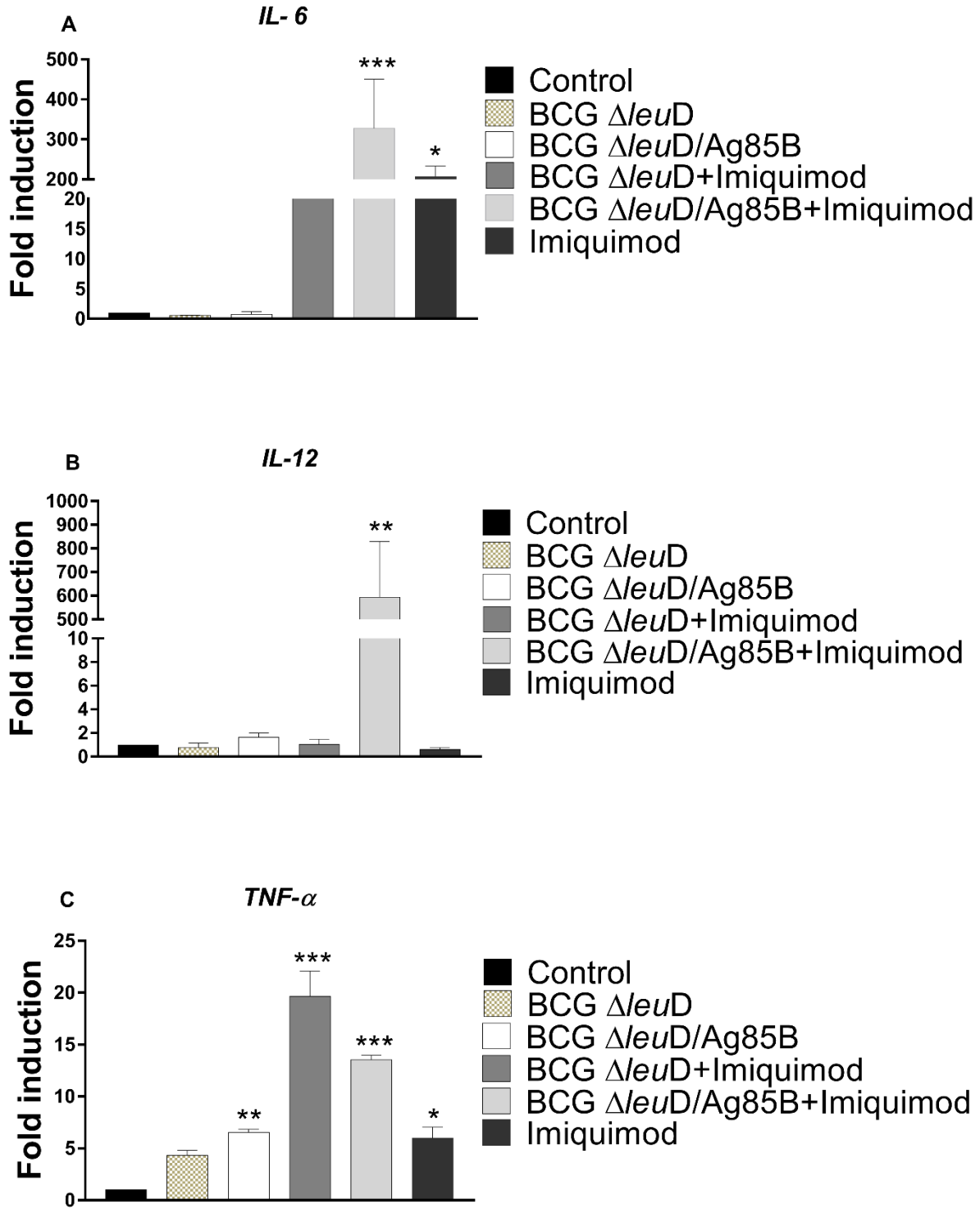


Fig 4. Gene expression of apoptotic genes in WM1366 cells treated with BCG Δ*leu*D, BCG Δ*leu*D/Ag85B and imiquimod alone or combined. (A): *Bax* gene; (B): *Bcl-2* gene; (C): *Caspase-3* gene. The results were presented as mean ± SEM. Two-way ANOVA with Tukey´s post-test was used to analyze statistical significance. (*) represents a significant difference between the different treatments in relation to the control group. **P*< 0.05, ***P*< 0.01, and ****P*< 0.001. Significance was considered at *P*< 0.05.

3.4. Gene expression profile in J774A.1 cells treated with rBCG strains, and imiquimod

Cellular immune response to different treatments with BCG strains and imiquimod was evaluated by measuring transcription levels of genes associated with the immune response as cytokines by qRT-PCR. The result for the expression profile of genes associated with pro-inflammatory cytokines such *as IL-6, IL-12, TNF-α,* and *IFN-γ* had a higher fold induction when compared to untreated cells. The combined treatment of BCG Δ*leu*D/Ag85B + imiquimod induced an increase in the mRNA levels of *IL-6, IL-12,* and *IFN-γ* (*P* < 0.05) in cells exposed to 60 µM

compared with other groups evaluated (Figure 5). On the other hand, *TNF-α* showed an increase in the fold induction in BCG Δ*leu*D + imiquimod, in BCG Δ*leu*D/Ag85B + imiquimod, imiquimod, and BCG Δ*leu*D/Ag85B. Moreover, imiquimod showed an increase in expression level in IL-6, and *TNF-α* only (Fig. 5A and C).



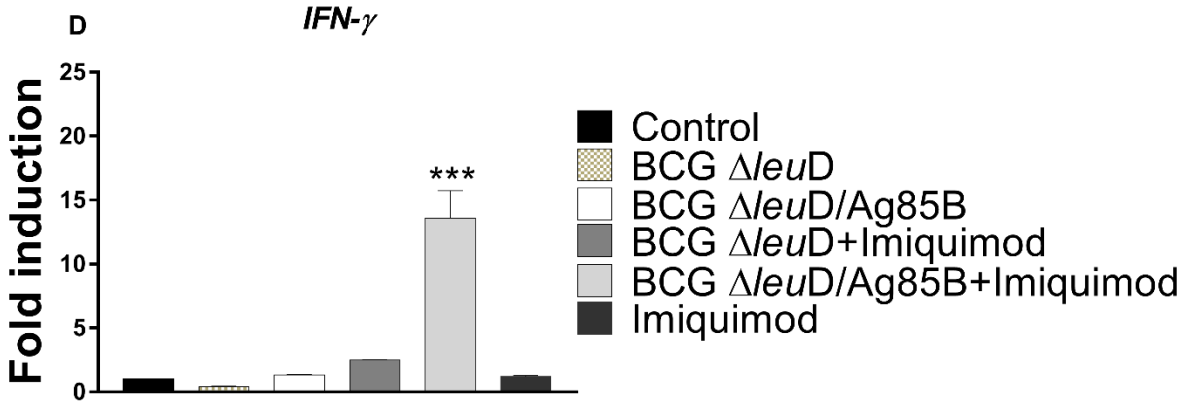


Fig. 5. The cytokine profile induced by BCG and imiquimod in J774A.1 was determined by qRT-PCR in 24h. (A): IL-6 production; (B): IL-12 production; (C): TNF-α production; (D): IFN-γ production. The results were presented as mean ± SEM. Two-way ANOVA with Tukey´s post-test was used to analyze statistical significance. (*) represents a significant difference between the different treatments in relation to the control group. **P*< 0.05, ***P*< 0.01, and ****P*< 0.001. Significance was considered at P< 0.05.

4. Discussion

Melanoma presents high rates of progression, metastasis due to its late diagnosis, thus, the discovery of new therapeutic alternatives that can inhibit the growth of tumor cells is of great importance (Couto et al. 2019; Boudewijns et al. 2020; Olbryt et al. 2020). Patients under immunotherapy has responded to treatment; however, some patients do not respond (Borcoman et al. 2019). An alternative is the use of BCG, which is associated with the activation of the immune system in the treatment of cancer (Schwarzer et al. 2010; Begnini et al. 2015). In general, different BCG strains have been constructed to increase the immune response, using several promotes, overexpressing own proteins, expressing heterologous antigens, by employing different strategies including auxotrophic complementation system (Borsuk et al. 2007; Yuan et al. 2015; Oliveira et al. 2017; Rizzi et al. 2017). BCG is used as an immunologic adjuvant due to the expression of various ligands that can bind to TLR2 and TLR4 in monocytes and stimulates cytokines IL-1β, and TNF-α (Tsuji et al. 2000). The antigen 85 (Ag85) is recognized by the HLA-A* 0201 of human CD8+T cells that induce the production of *TNF-α*, and *IFN-γ* (Geluk et al. 2000).

Currently, few studies have associated BCG in combinations with other chemotherapeutic or adjuvant immunotherapeutic agents in the treatment of melanoma (Benitez et al. 2019). The melanoma cell line WM1366 ($NRAS^{Q61L}$) used in this study represents 15% to 20% of the mutations in melanoma patients and our results showed the inhibition of cell growth by imiquimod with IC_{50} value of 46.63±4.28 μM. On the other hand, the combination of BCG Δ*leu*D/Ag85B + imiquimod (40 μM) showed 59.41% of inhibition cell growth compared to imiquimod alone which obtained 48.22% respectively, showing that the synergism between these two treatments could be interfering with the growth of tumor cells. A study by our research group evaluated the BCG Δ*leu*D/Ag85B strain in human bladder cancer cell line (5637) and had an enhanced cytotoxicity in this cell line *in vitro*, showing an expression of *AIF* and *EndoG* genes (Begnini et al. 2013).

In our analysis of apoptosis, the combination of the strain Δ*leu*D/Ag85B administered with imiquimod showed 13.96% of early and late apoptosis rate and imiquimod showed 19.91% of apoptotic cells. Imiquimod has shown efficacy against lentigo maligna (Tio 2019), and systemic side effects to locally administered imiquimod are rare, leading to erythema, and pruritus (Love et al. 2009).

Almomen et al. (2016) showed that imiquimod decreased the viability of endometrial adenocarcinoma cells, and probably induces apoptosis through the reduction of *Bcl-2* protein levels. Also, *Bax* expression was not affected, and were found an increase of gene expression levels of caspases 3 and 7. Likewise, in a prostate tumor line, it was demonstrated that imiquimod induces direct apoptosis through a mitochondrial-dependent pathway and induced the production of IL-6 (Han et al. 2013), data that are corroborated by our study.

The mechanism of action of the imiquimod is not yet completely clear, has been associated with the activation of MYD88, and induction of nuclear transcription factor-kappaB (NF-κB) which is associated with an inflammatory response, and the generation of cytokines. Studies suggest that imiquimod-induced apoptosis is associated with the activation of apoptosis signaling regulating kinase1/c-Jun-N-terminal kinase/p38 pathways, triggering the induction of endoplasmic stress, and increase in intracellular Ca^{2+} release, degradation of calpain, and cleavage of caspase-4 (Nyberg and Espinosa 2016). Imiquimod can trigger mitochondrial dysregulation by the loss of mitochondrial membrane potential which leads to the release of cytochrome C, cleavage of caspase-9, caspase-3, and activation of poly (ADP-ribose) polymerase (PARP) (El-Khattouti et al. 2016), and another study reported that imiquimod caused apoptosis by activating AMPK and was independent of TLR7/8 expression (Wang et al. 2015).

In the gene expression profile of apoptotic genes in WM1366, we observed an increase in mRNA levels of *Caspase-3,* and *Bax* in cells treated with BCG Δ*leu*D/Ag85B and BCG

Δ*leu*D/Ag85B + imiquimod. This result mainly the combination, corroborate with apoptosis detection analysis. On the other hand, the response observed in the J774A.1 cell line showed that there was an increase in the pro-inflammatory cytokines *IL-6, IL-12, IFN-γ,* and *TNF-α* compared to control. The combination of BCG Δ*leu*D/Ag85B + imiquimod showed an increase in the levels of *IL-6, IL-12,* and *IFN-γ,* compared to imiquimod which showed an increase in *IL-6* and *TNF-α.* However, the combination of BCG Δ*leu*D + imiquimod presented an increase in TNF-α. Similar data have been reported by Pampena et al. (2017) reporting that in cutaneous melanoma, the combination of BCG, and CSF-470 vaccine cells, increased the inflammatory cytokines *IFN-γ,* and *TNF-α.*

A study *in vivo* reported that imiquimod induced several cytokines like *IL-1, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α* (Sauder 2000), and that J774 cells respond to infection with *Mycobacterium* (Andreu et al. 2017). On the other hand, the combination of topical imiquimod and intralesional IL-2 in patients with metastatic malignant melanoma showed an increase in the activation of CD4+, CD8+ T cells, and an increase in the production of interferon-gamma (IFN-γ) and *IL-5* in the treatment (Green et al. 2008). Further, other combinations with imiquimod have been studied for regression of cutaneous metastases (Lee et al. 2007; Teulings et al. 2018).

Several clinical trial studies as Kibbi et al. (2015), reported three cases of metastatic stage III melanoma that were treated with the combination of imiquimod cream 5% plus intralesional BCG injection, reporting that the patient who had the combined therapy presented hypersensitivity to the treatment but there was a regression of the tumor compared to BCG alone that was not sufficient to cause disease regression.

In conclusion, the combination of BCG Δ*leu*D/Ag85B + imiquimod treatment evaluated in our study showed inhibition of cell growth, exhibited apoptotic activity in WM1366 cell line, and increased the pro inflammatory cytokine profile in J774A.1, being a promising treatment for melanoma.

Author contributions: Material preparation, data collection, and analysis were performed by Martha Lucia Ruiz Benitez, Fernanda Severo Sabedra Sousa, Camila Bender, João Rodrigues, Natália Vieira Segatto, Thaís Larré Oliveira. Study conception and design were supervised by Sibele Borsuk, Odir Antônio Dellagostin, Tiago Collares, and Fabiana Kömmling Seixas. The first draft of the manuscript was written by Martha Lucia Ruiz Benitez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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4.3 Manuscrito 2- Evaluation of the antitumor activity of chiral β-Aryl-chalcogenium azide compounds containing tellurium in bladder cancer cell

Evaluation of the antitumor activity of chiral β-Aryl-chalcogenium azide compounds containing tellurium in bladder cancer cell

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Abstract

Bladder cancer is the second urothelial cancer and non-muscle invasive bladder represents approximately 75% of all cases of this type of cancer. Currently, are finding new compounds with antitumoral and antioxidant activities, and organotellurium compounds are getting especially due to biological properties. In the present study, we evaluated the cytotoxic effect *in vitro* of chiral β-Aryl-chalcogenium azide compounds against the human bladder cancer cell line 5637. The inhibition of cell growth was evaluated by MTT assay, the induction of apoptosis, the viability cells were measured by DAPI staining and live/dead assay respectively. Also, the gene expression of pro-apoptotic, anti-apoptotic and oxidative stress were investigated by qRT-PCR, and *in silico* molecular docking was analyzed. Our results showed that **5c** and **5j** compounds presented concentration-dependent cytotoxicity, decreasing the cell viability in 48 hours, obtained IC_{50} values of 1.57 ± 0.70 µM and 0.48 ± 0.13 µM respectively. In apoptosis analysis, we observed chromatin condensation, showing 13% of apoptotic cells in **5c** and 20.3% in **5j**, compared to the untreated cells (2.34%). The compound **5c** increased the relative expression of *p53*, *Caspase-3*, *Caspase-9*, *SOD* and *CAT* genes. Meanwhile, the compound **5j** increased the *p53, p21*, *Caspase-3*, *Caspase-9*, *Bax*, *SOD*, *CAT*, *GPx*, *GR* and *iNOS* genes. Finally, molecular docking showed that **5c** had a binding affinity to Survivin, Bcl-XL, RSK2, SGK1, and **5j** showed binding affinity to gene SGK1 only. These findings suggest that β-Aryl-chalcogenium azide compounds could have a potential antitumoral effect against this type of cancer.

Key words: Organocalcogen compounds, antitumor activity, growth inhibition, tellurium.

Abbreviations

ATCC American Type Culture Collection

FSB Fetal Bovine Serum

IC_{50} The half maximal inhibitory concentration

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolidium bromide)

DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)

ROS reactive oxygen species

Bcl-XL B-cell lymphoma-extra large

ERK Extracellular signal-regulated kinases

MDM2 murine double minute 2

RSK2 The ribosomal S6 kinase 2

SGK1 Inactive serum and glucocorticoid-regulated kinase 1

1. Introduction

Cancer is a multifactorial disease, caused by abnormal cell proliferation due to mutatios in tumor suppressor genes, and protoncogenes. Currently, cancer is among the leading causes of morbidity, and mortality worldwide, and the global incidence of cancer could increase by 50%, and reach 15 million new cases by 2020 [1].

In this sense, the bladder cancer is the sixth most prevalent cancer in the United States, and the second urothelial cancer with 81.400 new cases and 17.980 deaths estimated for 2020 [2,3]. Approximately, 75% of all cases present non-muscle invasive bladder cancer (NMIBC), and this type of tumor has high recurrence rates and predisposition between 10-15% to progress to muscle-invasive tumor bladder cancer (MIBC) [4]. Furthermore, occupational exposure to aromatic amines, heavy metals, ionizing radiation, pharmaceuticals, urothelium irritation, an imbalance between reactive species and the body's antioxidant defenses caused by oxidative stress are the principal factors associated for this type of cancer [5].

The main treatment choices are surgery, intravesical therapy, chemotherapy, radiotherapy, and immunotherapy. However, some therapeutic options are often followed by strong adverse effects [6], and the best treatment option is chosen according to the specific tumor type of the individual concerned and according to the tumor staging. Despite the therapeutic advances, are required new alternatives, like chemotherapeutic agents more selective. It is necessary to find new compounds with pharmacological properties, with as high antioxidant and antitumoral activities that can help to decrease the adverse effects in the patients, and their risk of recurrence for this type of cancer. Antioxidant as well as antitumoral drugs can be obtained through chemical synthesis, and organochalcogenium compounds has increased since the discovery of their important biological activities of these molecules, being described as promising [7]. Organocalcogen compounds have mainly in their molecular structure elements such as Selenium (Se), Tellurium (Te) and sulfur (S) are in group 16 of the periodic table. Among these compounds, organotellurium have demonstrated great interest in the scientific community, and they are used in organic synthesis as intermediate agents.

Reports demonstrated that organochalcogen compounds are interesting due their antiviral activity [8], antioxidant [9], and antitumoral [7, 10], and these compounds that contain the tellurium atom in their structure have shown several interesting properties, such as immunomodulatory activity [11], antioxidant [12], and anticancer [13, 14].

The antioxidant and antitumoral activity of organochalcogens highlighted that these molecules showed inhibition of cell growth in bladder cancer (5637) [7], and in lung carcinoma (A549) [15]. Several studies have been investigated the possible mechanisms of action of these compounds, which

may contribute to elucidate its several biological properties related to molecules containing chalcogenium nucleus, and the introduction of methoxy groups or differents substituents that could confer cytotoxic effects [15-17]. Studies have indicated that the electronegativity of certain elements such as oxygen, and halogens such as chloride, bromine, fluoride that form part of the synthesis of compounds, increasing the lipophilicity of the molecules, their penetration of the lipid membrane, improving the effectiveness of various treatments [18].

The pharmacological properties intrinsic reported in the organotellurium compounds can be attributed to their ability to eliminate reactive oxygen species (ROS) and nitrogen reactive species (NRS) [11]. It also has been shown that tellurium compounds may have biological activities and may act as immunomodulators *in vitro,* and *in vivo* [19].

Organotellurium compounds can also mimetic the activity of the enzyme glutathione peroxidase (GPx), which are excellent catalysts for antioxidant reactions, and protect the body against potentially harmful effects of ROS through, protect cells from oxidative damage, which is the mechanism to which most authors attribute their antioxidant activity [20]. Also, this antioxidant property could be explained due to changes in the oxidation from the divalent Te (II) to the tetravalent Te (IV) state [21]. Currently, studies involving organochalcogen compounds containing tellurium in their structure to the treatment in bladder cancer are poor, and their pharmacological properties have not yet been explored for this cancer.

In this context, amino acid derivatives containing chalcogenium as chiral β-Aryl-chalcogenium azide are organochalcogen compounds that have presented a promising antitumor activity for lung cancer [15]. Therefore, the aim of this study was to evaluate the antitumor activity through cell growth inhibition, and apoptosis induction, induced by chiral β-Aryl-chalcogenium azide compounds containing tellurium in bladder cancer as well as, elucidate what is its mechanism of action of these compounds.

2. Materials and methods

2.1. Synthesis of chiral β-aryl-chalcogenium azides

The β-aryl-chalcogenium azides compounds **5c** (S)-(2-azido-1-phenyl-3-telurophenyl)-propane) and **5j** (S)-(2-azido-1-phenyl-3-teluro-p-methoxy-phenyl)- propane) containing tellurium (Fig. 1) were synthesized in the Department of Chemistry, University of Santa Maria (UFSM) and the synthesis procedure was performed according to methodology previously reported by Tabarelli et al., 2017 [15]. In this previous work, *in vitro* assays were performed to evaluate cytotoxic and antioxidant activity in lung carcinoma cells (A549 strain), as well as *in vivo* evaluation of liver and

kidney toxicity of swiss mice. According to the results obtained in this previous study, two compounds were selected (Fig. 1), which presented promising results. In this sense, both chosen compounds present in their chemical structure the element tellurium. For the experiments, the compounds were diluted in dimethylsulfoxide (DMSO) with concentration less than 0.5% will not be toxic to cells.

5c **5j**

Fig. 1 Representation of the molecular structure of β-aryl-chalcogenium azides containing tellurium (**5c)** (S)-(2-azido-1-phenyl-3-telurophenyl)-propane) and (**5j)** (S)-(2-azido-1-phenyl-3-teluro-p-methoxy-phenyl)- propane).

2.2. Cell culture

Human bladder cancer cells (5637; grade II carcinoma) were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). The cells were grown in RPMI (Dulbecco's modified Eagle's medium) (Vitrocell Embriolife, Campinas, Brazil), supplemented with 10% FBS Gibco (New York, USA), 1% L-glutamine, and 1% penicillin/streptomycin. Cultures were maintained in a controlled atmosphere with 37 °C, 5% CO_2 and 95% humidified air.

2.3. Cell viability measurement

The cytotoxicity of the **5c** and **5j** compounds was determined by measuring the reduction of soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolidium bromide). Cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well. After 24h, cells were treated with **5c** and **5j** in different concentrations (0.1, 1, 2.5, 6.25, 12.5, 25, 50 and 100 µM) for 48 hours. We chose this treatment time for the compounds according to the results found by Tabarelli et al. (2017) [15]. Subsequently, the time of treatment exposure, the medium was replaced with 5 mg/mL MTT for 3h at 37 °C and absorbance of formazan reduction product was measured by spectrophotometer at 492 nm using a microplate reader (MR-96A-Microplate Reader). Cell inhibition was determinate by the formula:

$$\%\ inhibition = \left(\frac{1-Abs492_{treated\ cells}}{Abs492_{control\ cells}}\right) \times 100 \quad [22]$$

The results obtained were used to determine the concentration necessary to reach 50% of the maximum inhibitory effect of the compound (IC_{50}). All assays were performed in three independent experiments, each repeated in triplicate.

2.4. LIVE/DEAD assay

5637 cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well. After 24h, cells were treated with **5c** and **5j** compounds in concentrations of 1.6 µM and 0.5 µM respectively for 48h. The live/dead cell viability assay (Invitrogen™, Carlsbad, CA, USA) was realized following the manufacturer´s instruction. Briefly, the media was removed, and the cells were labeled with calcein-AM, and ethidium homodimer (EthD-1) by 30 min at room temperature in dark. After incubation the solution was removed, and the cells were washed twice with phosphate-buffered saline (PBS) 1X. The assay was analyzed by confocal laser scanning microscopy (Leica TCS SP8) at 200 x magnification. Calcein uptake was measured in live cells by converted to green fluorescence via intracellular esterases analyzed by fluorescent light emission (488 nm). The diffusion of ethidium homodimer through the permeable membrane and binds to DNA was measured in dead cells, detected by the red fluorescent signal (546 nm). The recorded images were analyzed using Cell^F (Cell^F, New York, USA). The total number of live or dead cells obtained, and the results were expressed as the mean ± SD, based on three different experiments.

2.5. DAPI staining

Bladder human cells (5637) were seeded at a density of 2 x 10^4 cells per well in 96-well plates. After 24h, cells were treated with **5c (**1.6 µM**)** and **5j (**0.5 µM**)** for 48h. After this period, cells were fixed in acetone-methanol (solution 1:1) for 5 min, the solution was removed, and cells were washed with 1X PBS. DAPI staining solution (4′,6-diamidino-2-phenylindole, dihydrochloride) (Invitrogen™, Carlsbad, CA, USA) was following according to the manufacturer´s protocol. Cells were incubated with DAPI for 30 min at room temperature in the dark. Then, the DAPI was removed, and the cells were again washed twice with 1X PBS. The assay was analyzed by confocal laser scanning microscopy (Leica TCS SP8) at 200 x magnification. The apoptotic analysis was evaluated by the increase of fluorescent intensity when attached to DNA, through the excitation/emission peak at 480/510 nm. The recorded images were analyzed using Cell^F software (Cell^F, New York, USA).

2.6. Quantitative Real-Time PCR (qRT-PCR)

The gene expression profiles of apoptotic and oxidative stress related genes were evaluated by qRT-PCR. Cells were seeded in 12-well flat bottom plates at a density of 2×10^5 per well and grown at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air for 24 h. Then, the cells were treated with **5c** and **5j** compounds at a concentration of 1,6 μM and 0,5 μM, respectively, the IC_{50} values and incubated for 48 h. After this period, the total mRNA was extracted from the cells using TRIzol reagente (Invitrogen™, Carlsbad, USA) and quantified by Nanovue Plus Spectrophotometer™ (GE®). The cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) according to the manufacturer's protocol. Realtime PCR reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, UK) and the primer sequences used are indicated in Table 1. Gene expression were normalized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene. The negative control were cells maintained just with RPMI 1640 medium without compound treatment.

Table 1 – Primer sequences used [23]

Gene	**Sequence**
GAPDH	F - GGAGCGAGATCCCTCCAA
	R - GGCTGTTGTCATACTTCT
Bax	F – GCTTCAGGGTTTCATCCA
	R- ACGGCGGCAATCATCCTC
Bcl-2	F- GGTGGGGTCATGTGTGTGG
	R- CGGTTCAGGTACTCAGTCATCC
Caspase-3	F- CAGTGGAGGCCGACTTCTTG
	R- TGGCACAAAGCGACTGGAT
Caspase-9	F- GTCTCAATGCCACAGTCCAG
	R- GCATTAGCGACCCTAAAGCAC
p53	F- CACGCCCACGGATCTGAA
	R- AGCGAGCACTGCCCAACA
p21	F- TGTCCGTCAGAACCCATGC
	R- AAAGTCGAAGTTCCATCGCTC

SOD	F - GGAAGCCATCAAACGTGACT
	R - CTGATTTGGACAAGCAGCAA
CAT	F - TTTCCCAGGAAGATCCTGAC
	R - ACCTTGGTGAGATCGAATGG
GPx	F - TCCCGTGCAACCAGTTTG
	R - TTCACCTCGCACTTCTCGAA
GR	F – CCCGATGTATCACGCAGTTA
	R – TTCACTGCAACAGCAAAACC
iNOS	F- ACAAGCCTACCCCTCCAGAT
	R - TCCCGTCAGTTGGTAGGT

2.7. Molecular docking

To investigate possible locations where molecules **5c** and **5j** have greater interaction by the target proteins of interest and trying to explain its possible mechanism of action by inhibiting the different pathways of metabolism, signal transduction, inhibition, and cell death, molecular docking studies were performed. Crystallographic structures of proteins of interest were retrieved from Protein Data Bank. The target proteins evaluated were, Survivin, Bcl-XL, RSK2, SGK1, and. The proteins were prepared by the graphical interface AUTODOCKTOOLS 1.5.4 where water-repellent molecules were removed, all hydrogens were added, the Gasteiger charges were calculated, and non-polar hydrogens were linked to the carbon atoms. **5c** and **5j** compounds were drawn by ChemDraw 2018, to convert the molecules to 3D, the QuickPrep tool of the MOE software was used [24], in which corrected the bonds of the atoms of the molecule and added hydrogen, their charges were assimilated into each atom and the structure's energy was minimized (MMFF94x, gradient: 0.01) [25]. We used Autodock Tools 1.5.4 to all rotatable binder connections can rotate freely, while our receivers have been considered rigid [26]. All calculations for protein-ligand coupling were analyzed by the Lamarckian genetic algorithm method (LGA) [27]. We used the AUTODOCK 4 redocking technique to compare the results of docking of compounds to the crystallographic binder of the tested proteins, indicating how better the ligand was that which obtained its more negative binding free energy than the control, but that does not necessarily mean that the ligand does not interact with that protein. Visualization of protein-binder interactions, amino acid residue analysis was analyzed by Discovery Studio Visualizer 2019, and Pymol software was used to generate the surface image of proteins.

2.8. Statistical analysis

Data set were analyzed by one or two-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. The results were shown as mean, and standard deviation (SD) at least three independent experiments. The differences were considered statistically significant differences when $P < 0.05$ in all analyses. Statistical analysis was performed using GraphPad Prism 7.0, and Statistitix version 10.0 (Analytical Software, Tallahassee, FL).

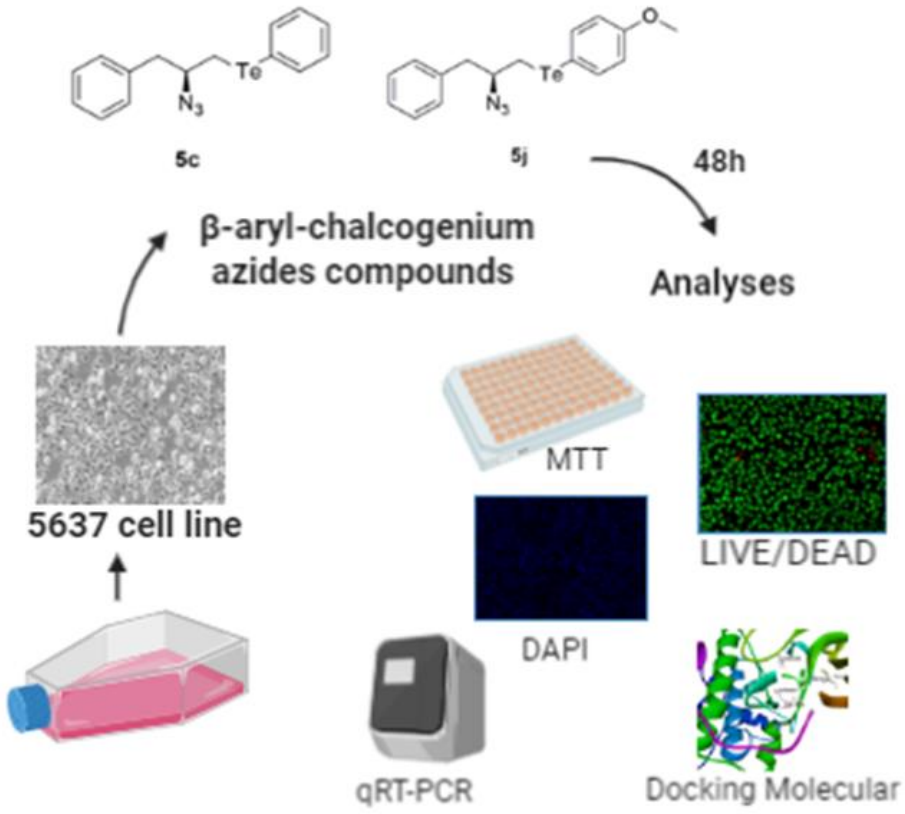


Fig 2. Scheme of protocol of **5c** and **5j** compounds in 5637 cell line.

3. Results

3.1. β-aryl-chalcogenium azides compounds reduce cell viability of bladder cancer cells

The results of MTT assay showed incubation with both **5c** and **5j** at 48h could reduce cell viability of the human bladder cancer cell line (5637) *in vitro* in a dose-dependent manner, as it shows the Fig. 3.

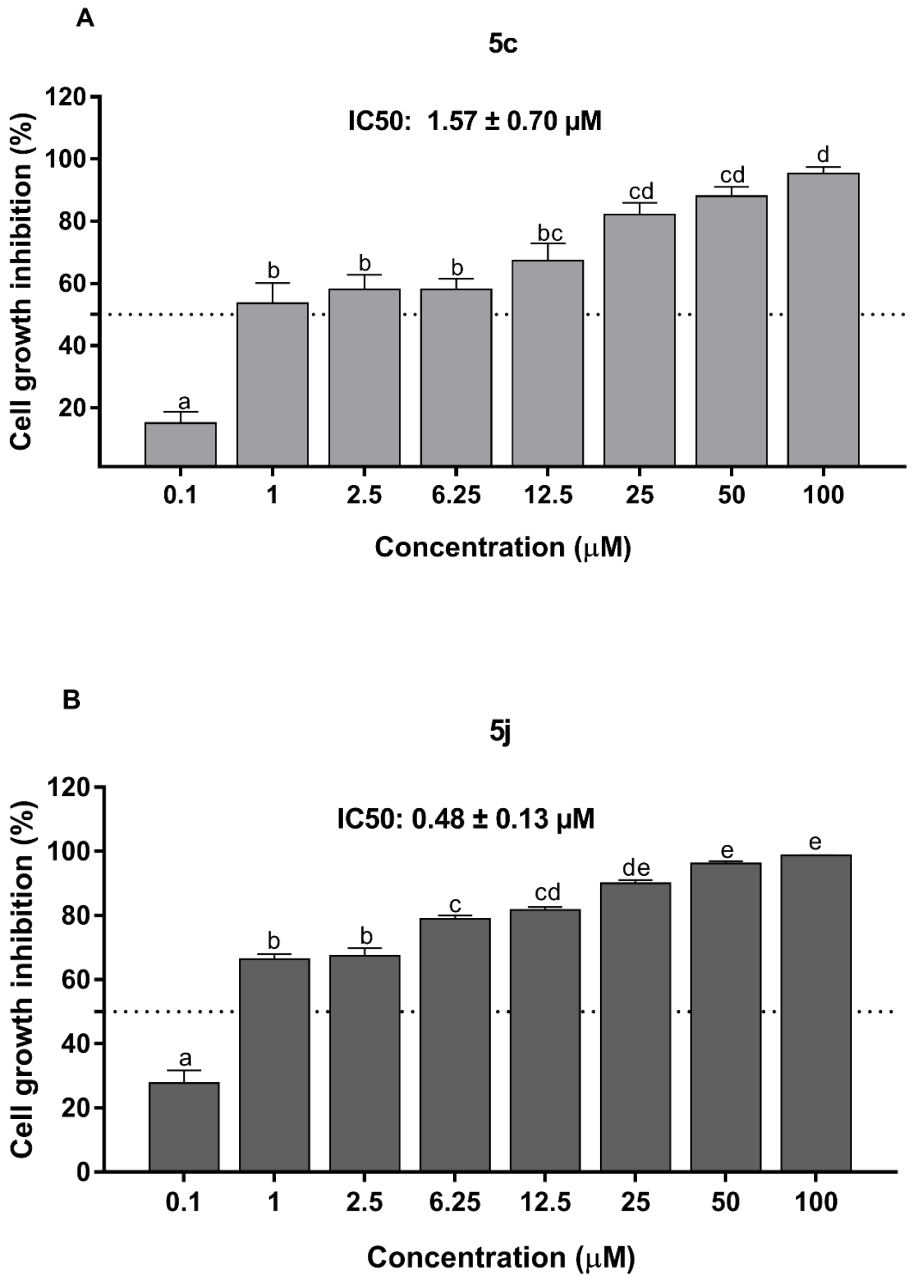


Fig. 3 Cell growth inhibition by MTT in 5637 bladder cells. Cells were treated with **5c** [A] and **5j** [B] compounds diluted in DMSO at concentrations of 0.1-100 µM for 48h. Data were obtained from three independent experiments. Different letters represent significant differences between the means of concentrations, and differences were considered significant at $P < 0.05$.

The organochalcogen compounds inhibited 50% of cancer cell viability. IC_{50} values after 48h of exposure to treatments with **5c** and **5j** were 1.57 ± 0.70 µM and 0.48 ± 0.13 µM, respectively

(Table 2). Evidencing that the compound **5j** has a higher potency since it was able to inhibit 50% of the cellular viability with a lower concentration.

Table 2. The IC_{50} values (μM) of β-aryl-chalcogenium azides compounds against human bladder cancer cells line 5637 in 48h of treatment.

Compounds	IC_{50} μM
5c	1.57 ± 0.70
5j	0.48 ± 0.13

IC_{50} Concentration that reduced cell growth by 50% compared to controls.

The IC_{50} values were calculated by a non-linear regression method

Data were expressed as means ± SEM

3.2. LIVE/DEAD assay

The analysis of cell viability measuring by live/dead assay in 5637 cells in 48 h showed that there was significant difference between **5c** and **5j** (0.5 μM) compounds in live cells (P = 0.0102 and P = 0.0018 respectively) compared to the untreated control, through the average of total number of cells estimated (Fig. 4). It was found that **5c** and **5j** compounds presented a little more cell death (represented in red fluorescent cells) compared to the untreated control, but here there was no difference between treatments.

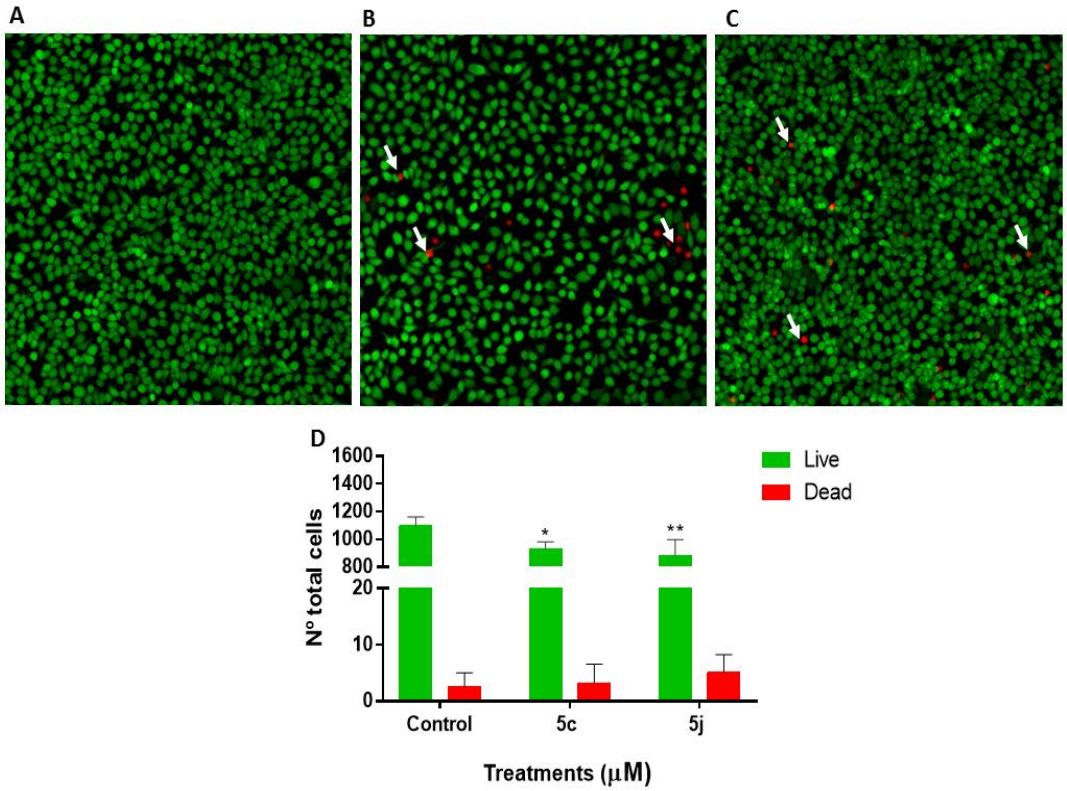


Fig. 4 Analysis of viability cells was estimated by LIVE/DEAD assay. The panel [A] indicates untreated cells/control, [B] indicate cells treated with **5c** (1.6 µM) and [C] cells treated with **5j** (0.5 µM) compounds for 48h. Three distinct fields were evaluated. The red fluorescent cells indicate dead cells and the green fluorescent cells indicate live cells. The panel [D] represents the number of total cells evaluated. Data are presented as mean and standard deviation (SD) from three independent experiments. $^{*}P$ <0.05, ** P <0.01 relative to the control group.

3.3. Determination of Apoptosis

The organochalcogen compounds tested in 48h increased the percentage of apoptotic cells in 5637 represented in blue fluorescent cells, showing chromatin condensation observed, when compared to control cells Fig. 5 (panel A, B, C). The results obtained in **5c** treatment at the concentration of 1.6 µM and **5j** treatment at the concentration of 0.5 µM showed percentages of 13% and 20.3% of apoptotic cells compared to the untreated cells (only culture medium) (2.34%) (Fig. 5, Panel D).

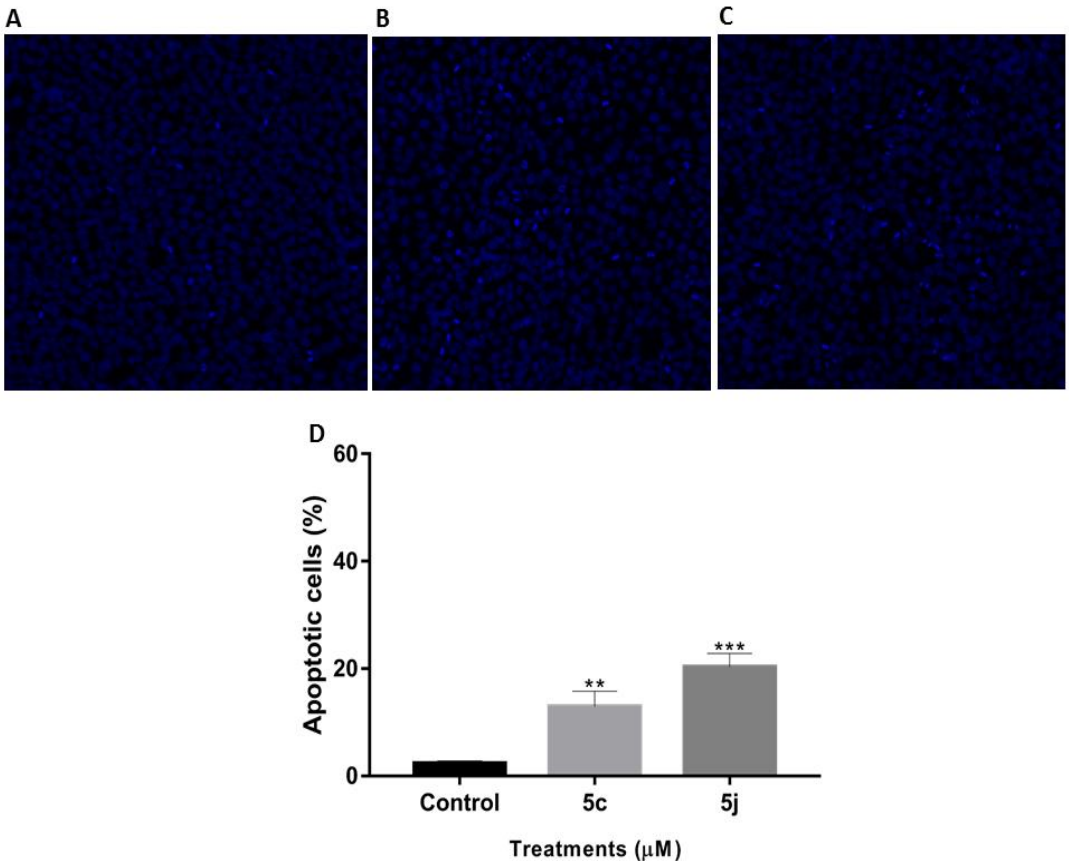
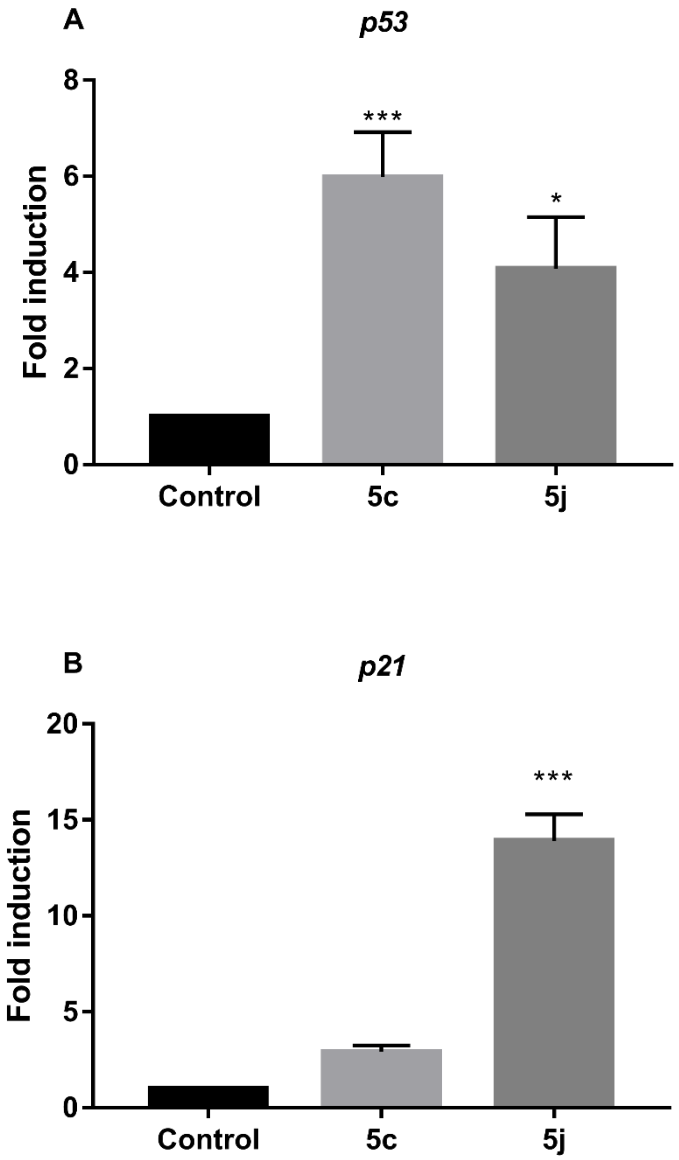


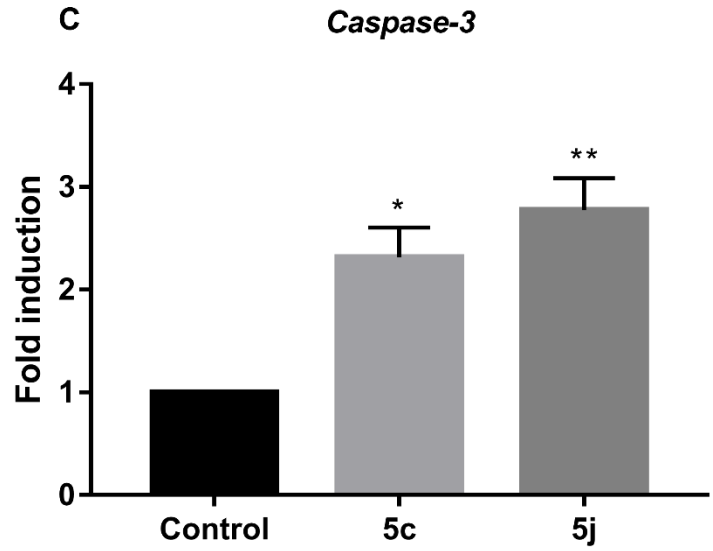
Fig. 5 The Analysis of apoptotic cells was evaluated by DAPI. [A] indicate untreated cells/control, [B] indicate cells treated with **5c** (1.6 μM) and [C] cells treated with **5j** (0.5 μM) compounds for 48h. Three distinct fields were evaluated. The blue fluorescent cells indicate apoptotic cells and the blue cells indicate live cells. The panel [D] represents the total number of apoptotic cells. Data are presented as mean and standard deviation (SD) from three independent experiments. ** P <0.01, *** P <0.001, relative to the control group.

3.4. qRT-PCR analysis

The results expressed in Figure 6A show that compound **5c** increased pro-apoptotic genes *p53* levels (P <0.001) compared to the control. In addition to these findings, compound **5j** also increased the levels of this protein P < 0.05 when compared to the control. The one-way analyses should the compound **5j** (Fig. 6B) increased the *p21* (P <0.001) when compared with the control. However, compound **5c** had no effect on altering the relative expression of the *p21* gene, compared with the control. Regarding caspase level, composition **5j** increased the relative expression of both *Caspase-3* and *Caspase-9* when compared to the control. While treatment of 5637 cells with compound **5c** did not cause significant differences between groups (Fig. 6C and 6D).

In the Fig. 6E, we observed the compound **5j** increased the fold induction of *Bax*, when compared with the control ($P < 0.01$) and the compound **5c** did not present statistical difference between the control group. However, in the Fig. 6F, there was no significant difference of relative expression of the *Bcl-2* gene between the groups treated with the compound **5c** and **5j** with the control. Considering the relationship between the *Bax* and *Bcl-2* genes, we can observe that compound **5j** has a greater interaction with this relationship than compound **5c**, corroborating the results presented separately.




C
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Control
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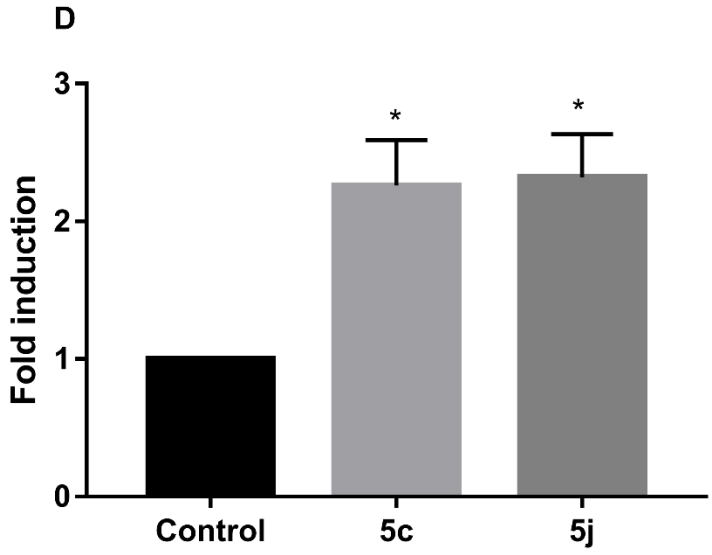
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Control
5c
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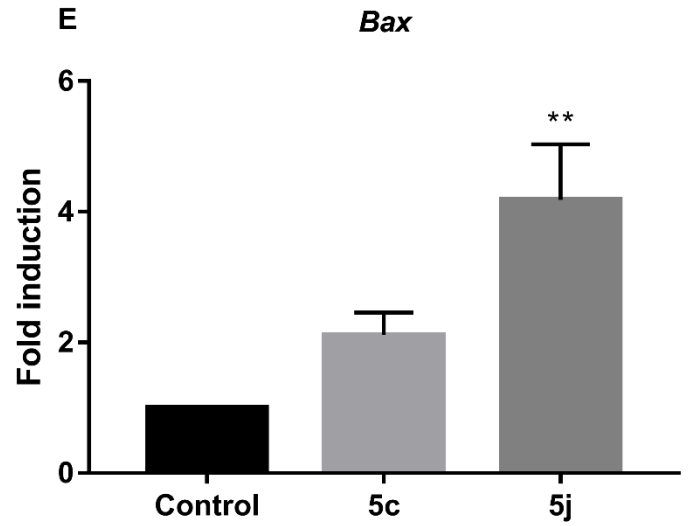
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Fold induction
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Control
5c
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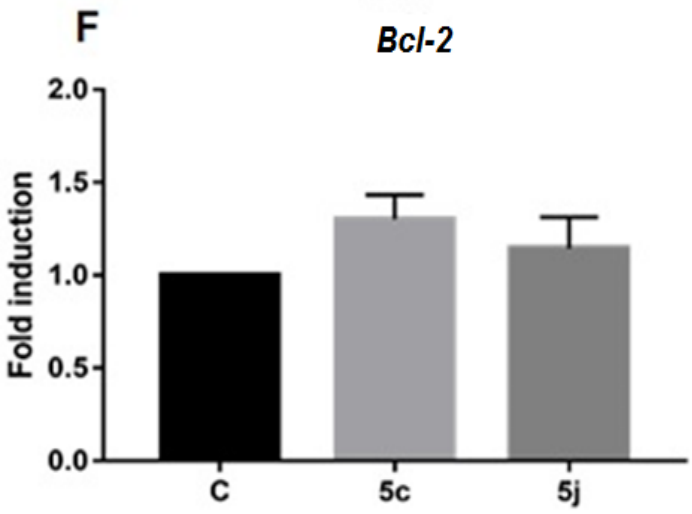
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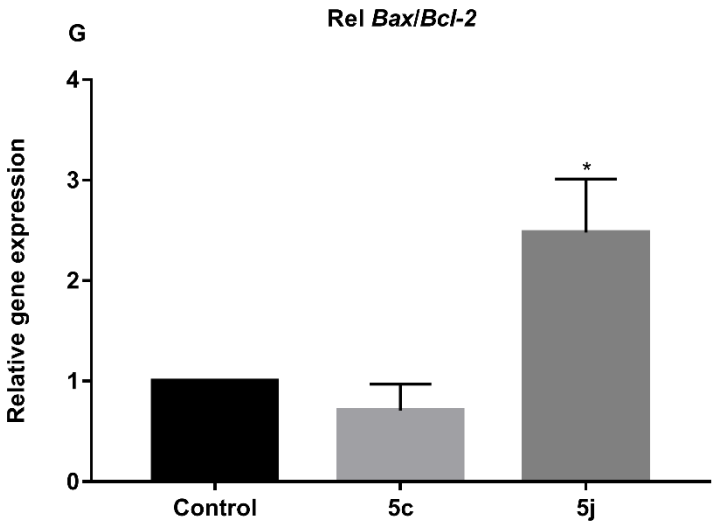
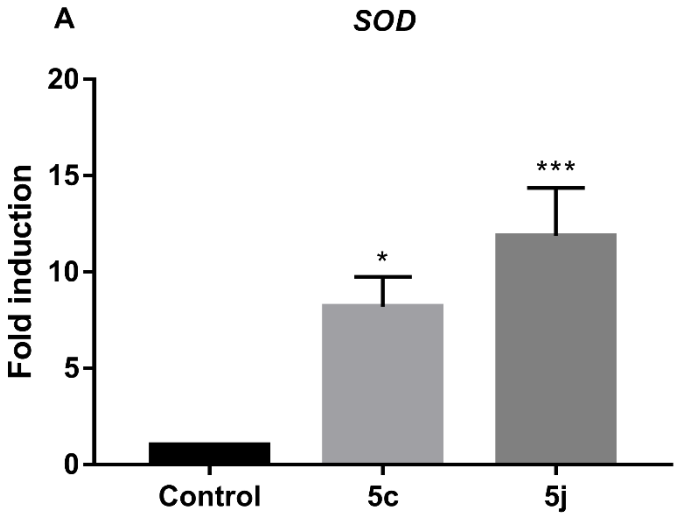


Fig. 6 Effect of the **5c** and **5j** treatment on the apoptotic gene expression of *p53* [A] *CDKN1A* (p21) [B], *Caspase-3* [C], *Caspase-9* [D], *Bcl-2* [E], *Bax* [F] and the relationship between *Bax* and *Bcl-2* [G] in 5637 cells. Analysis were performed by RT-PCR using total RNA extracted from 5637 cells pre-treated for 48 hours with **5c** and **5j** or RPMI (as control). Statistical analysis was performed by Two-way ANOVA followed by the Tukey's test when appropriate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared with the control group. *Abbreviations:* C: control.

In order to investigate the influence of the compounds **5c** and **5j** on the gene expression profile of genes related with oxidative stress, the following enzymes were evaluated: superoxide dismutase (*SOD*), catalase (*CAT*), glutathione peroxidase (*GPx*), glutathione reductase (*GR*) and nitric oxide synthase (*iNOS*). The *SOD* and *CAT* levels increased (P <0.01) in cells treated with both compounds compared with the control (Fig. 7). The *GPx*, *GR* and *iNOS* levels significantly increased in cells exposed to **5j** treatment, however there was no difference in the group treated with **5c** compound compared with the control and the same gene expression profile was observed in the iNOS levels (Fig. 7E).


A
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Control
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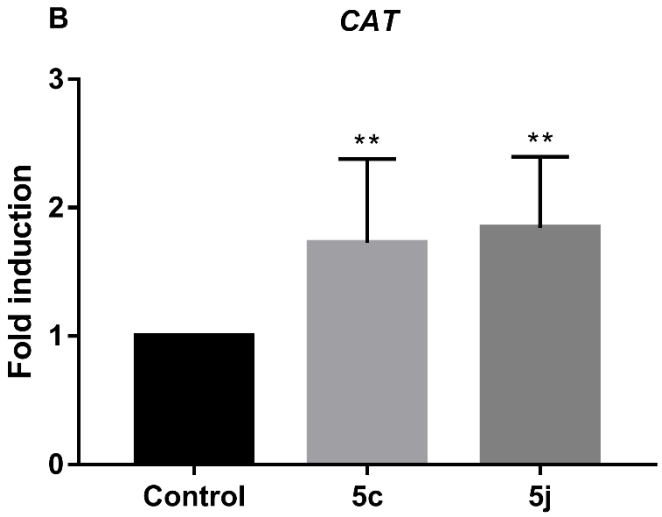
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Control
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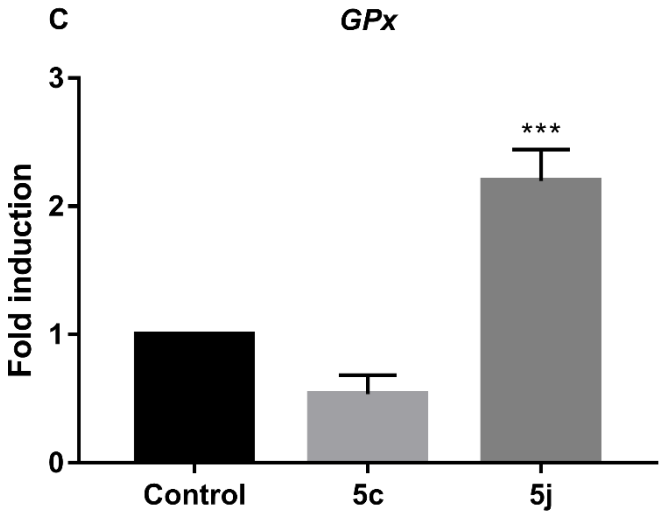
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Control
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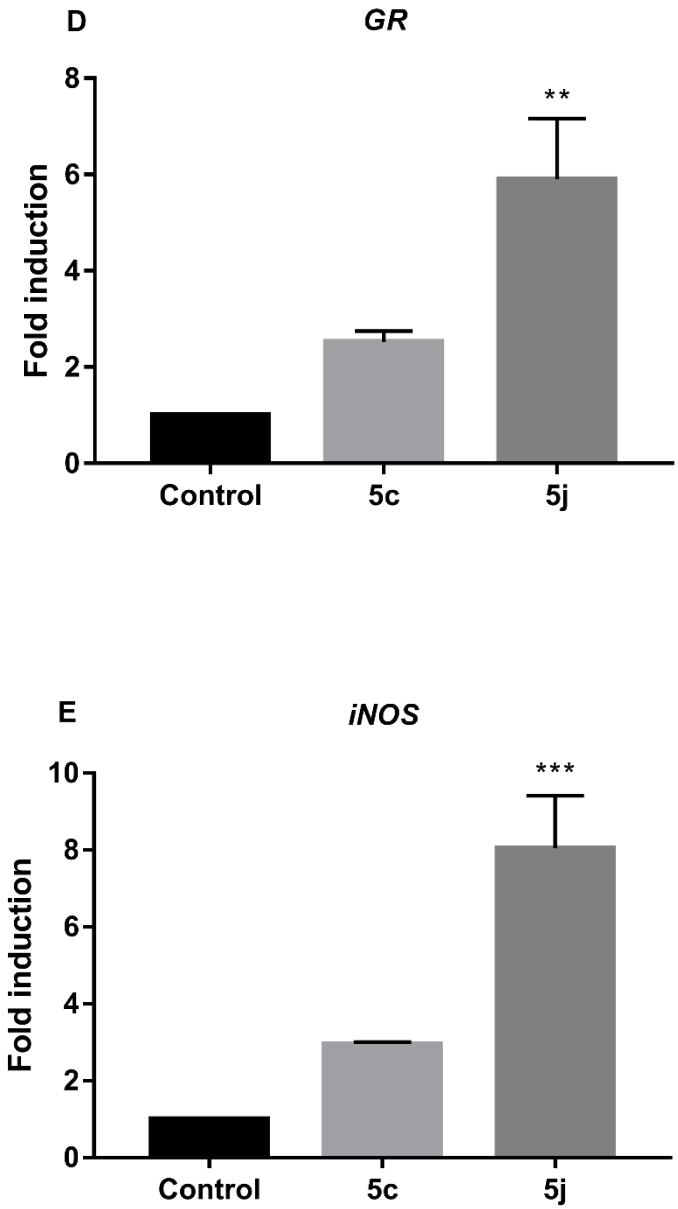


Fig. 7 Effect of the **5c** and **5j** treatment on the oxidative stress gene expression of *SOD* [A] *CAT* [B], *GPx* [C], *GR* [D] and *iNOS* [E] in 5637 cells. Analysis were performed by RT-PCR using total RNA extracted from 5637 cells pre-treated for 48 hours with **5c** and **5j** or RPMI (as control). Statistical analysis was performed by Two-way ANOVA followed by the Tukey's test when appropriate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared with the control group. *Abbreviations:* C: control.

3.5. Molecular Docking analysis

The docking results in the estimated free energy of binding (kcal/mol) are shown in Table 3

Table 3. Estimate free energy of binding affinity (Kcal/mol) of **5c** and **5j** compounds relative to the target proteins co-crystallized

Protein/PDB ID	Molecule	Free energy (Kcal/mol)	Common Amino Acids Interaction
Survivin (3UIH)	Co-crystallized	-6.68	LEU64,TRP67,ASP71,GLU76
	5c	-6.58	LEU64,TRP67,ASP71,GLU76
	5j	-5.45	LEU64,TRP67,GLU76
Bcl-XL (6RNU)	Co-crystallized	-5.17	ALA104,LEU108,LEU130,ARG132,ARG139
	5c	-5.45	LEU130,ARG132,ARG139
	5j	-4.07	ALA104,LEU108,LEU130,ARG132,ARG139
RSK2 (4D9T)	Co-crystallized	-6.94	CYS436,ALA449,LYS451,VAL491, THR493, MET496, LEU546
	5c	-6.98	CYS436,ALA449,LYS451,VAL491,THR493
	5j	-4.97	CYS436,ALA449,LYS451,MET496,LEU546
SGK1 (2R5T)	Co-crystallized	-5.34	VAL112,GLU226,THR239
	5c	-6.32	VAL112,GLU226
	5j	-5.61	VAL112

Molecular docking analyses predicted the interaction of compounds **5c** and **5j** with target proteins co-crystallized tested, showing for **5c** different docking scores: Survivin (-6,58 Kcal/mol), Bcl-XL (-5,45 Kcal/mol), RSK2 (-6,98 Kcal/mol). Moreover, **5c** and **5j** showed the best binding affinity for SGK1, showing a docking score of -6,32 Kcal/mol, and -5,61 Kcal/mol respectively when compared to the co-crystallized protein. The linkages found were Pi-Alkil, Pi-Anion, Pi-Pi T-shaped, Pi-Sigma, and conventional hydrogen bond. The interactions between survivin and both compounds presented binding sites with 4 amino acid residues. Bcl-XL with **5c** showed 7 amino acid residues, with **5j** showed 8 amino acid residues. The interaction between SGK1 presented 6 amino acid residues with **5c**, and 3 amino acid residues with **5j**. Finally, RSK2 showed for **5c** and **5j** compounds interaction with 7, and 6 amino acid residues respectively (Fig. 8-12, Table 3).

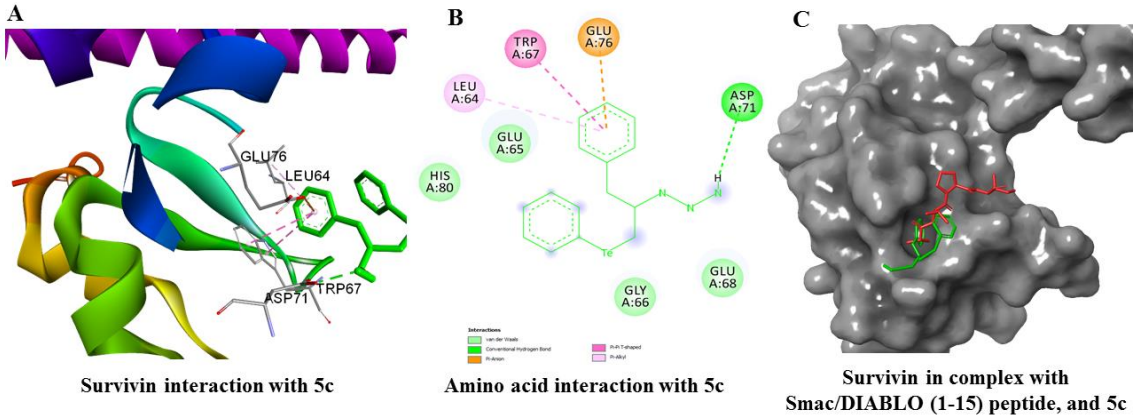


Fig. 8 Docking analysis demonstrated a binding affinity of **5c** compound to Survivin [A], [B], and [C]. Green indicates Smac/DIABLO peptide, and the red color indicates the **5c** compound

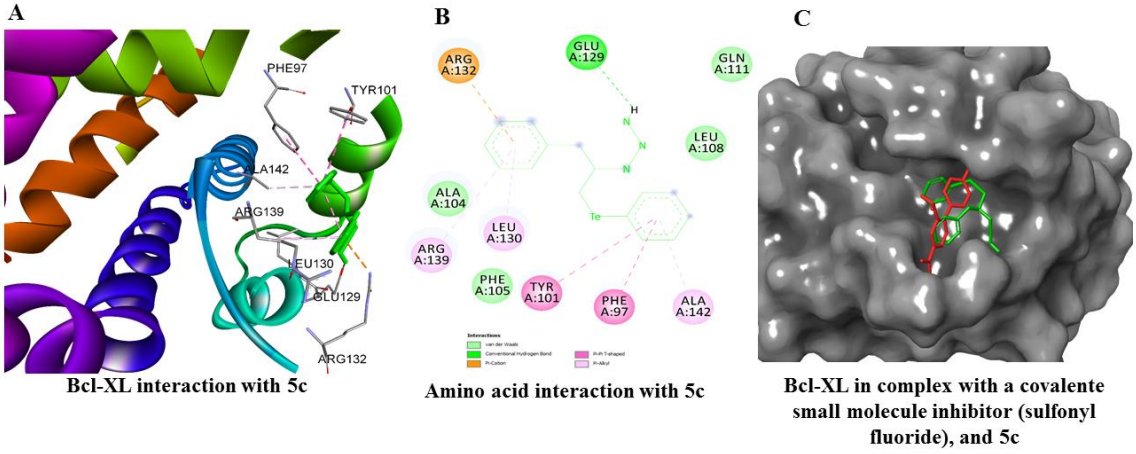
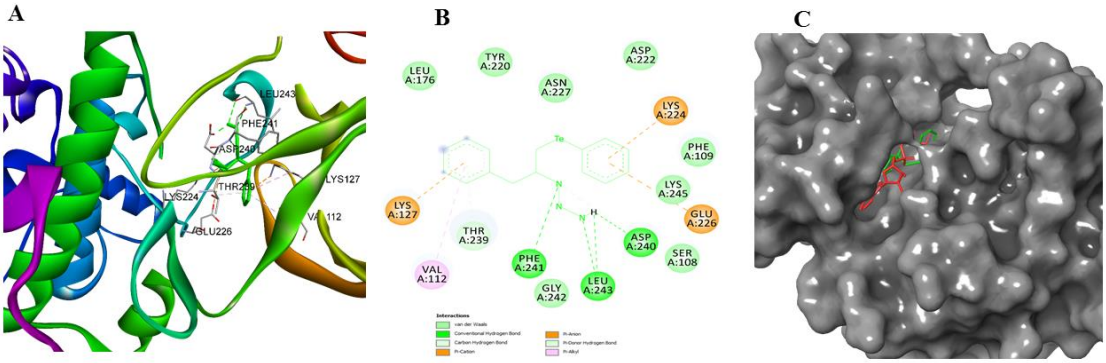
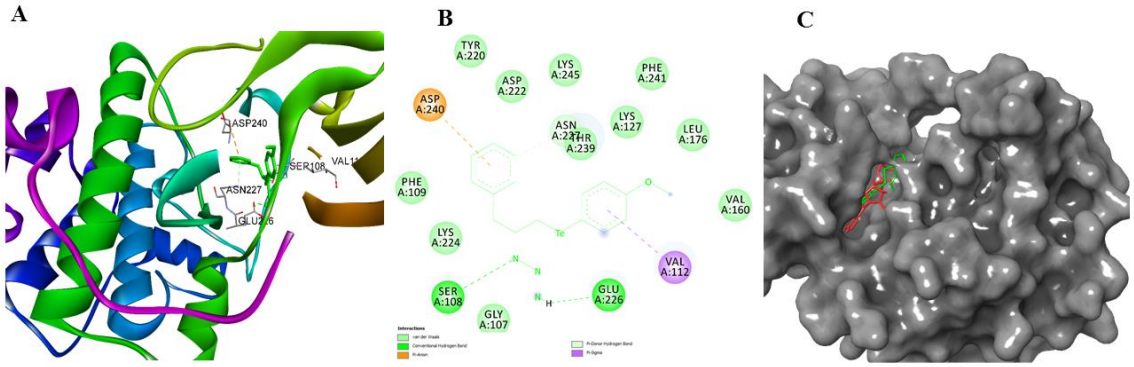


Fig. 9 Docking analysis demonstrated a binding affinity of **5c** compound to Bcl-XL [A], [B], and [C]. The color green indicates the small molecule inhibitor (sulfonyl fluoride), and the red color indicates the **5c** compound.



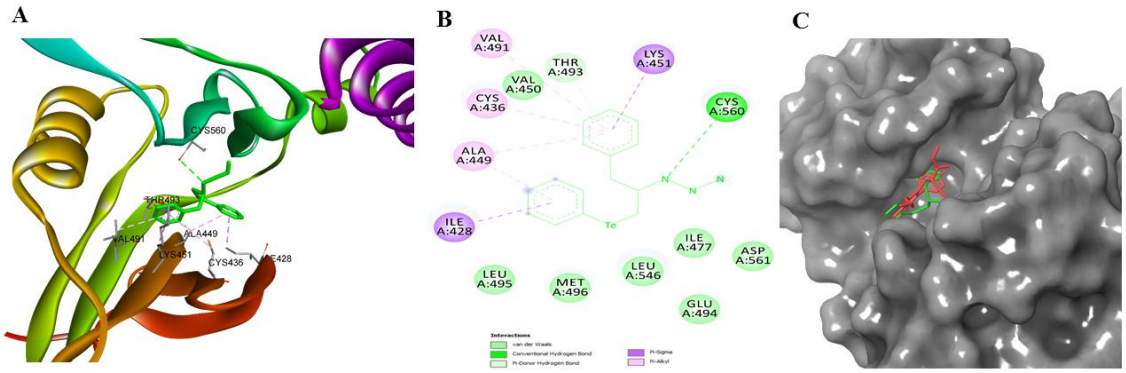
SGK1 interaction with 5c | **Amino acid interaction with 5c** | **SGK1 in complex with AMP-PNP, adenosine 5´(beta gamma-imido) triphosfate, and 5c**

Fig. 10 Docking analysis demonstrated a binding affinity of **5c** compound to SGK1 [A], [B], and [C]. The color green indicates AMP-PNP, adenosine 5´(beta gamma-imido) triphosphate, and the red color indicates the **5c** compound.



SGK1 interaction with 5j | **Amino acid interaction with 5j** | **SGK1 in complex with AMP-PNP, adenosine 5´(beta gamma-imido) triphosfate, and 5j**

Fig. 11 Docking analysis demonstrated a binding affinity of **5j** compound to SGK1 [A], [B], and [C]. The color green indicates AMP-PNP, adenosine 5´(beta gamma-imido) triphosphate, and the red color indicates the **5j** compound.



RSK2 C-terminal Kinase Domain interaction with 5c

Amino acid interaction with 5c

RSK2 C-terminal Kinase Domain in complex with inhibitor (E)-methyl 3-(4-amino-7-(3-hydroxypropyl)-5-p-tolyl-7H-pyrrolo[2,3-d]pyrimidin-6-yl)-2-cyanoacrylate, and 5c

Fig. 12 Docking analysis demonstrated a binding affinity of **5c** compound to RSK2 [A], [B], and [C]. The color green indicate inhibitor (E)-methyl 3-(4-amino-7-(3-hydroxypropyl)-5-p-tolyl-7H-pyrrolo[2,3-d]pyrimidin-6-yl)-2-cyanoacrylate, and the red color indicate the **5c** compound.

As it shows Table 3, was obtained the common amino acids interaction by estimate free energy of binding affinity. The docking analysis revealed that **5c** had a more binding affinity than **5j** on the docking energy estimated. However, **5j** showed common amino acids interaction when compared to the co-crystallized proteins, showing for Survivin 3 amino acid residues (LEU64, GLU76, and TRP67), for Bcl-XL 5 amino acid residues (ALA104, LEU108, LEU130, ARG132, and ARG139), for SGK1 1 amino acid residue (VAL112), and for RSK2 showed 5 amino acid residues (CYS436, ALA449, LYS451, MET496, and LEU546).

4. Discussion

In this study, we evaluated the cytotoxic activity of chiral β-Aryl-chalcogenium azide compounds containing tellurium, and we found that both **5c** and **5j** were capable of decrease the cell viability of 5637 cell line *in vitro* using MTT assay. Furthermore, **5j** presented greater cytotoxicity, showing lower IC_{50} value when compared with **5c**. Our results are in agreement with data from other studies [8, 28, 29]. In addition, the viability assay showed that **5c** and **5j** reduced cell number, compared to the untreated cells, and the determination of apoptosis for both compounds demonstrated morphological changes in chromatin. Similar studies were reported by Abondanza et al. (2008), where they demonstrated that organotellurium compounds are capable of inducing apoptosis in human promyelocytic leukemia cells HL-60 [30].

The compounds of this study were chosen because of the promising results found in the study in lung carcinoma by Tabarelli et al. (2017) reported that azide b-arylcalcogen compounds (**5c**, **5i, 5j**, and **5p**) containing the tellurium atom in their structure showed a greater antioxidant effect compared to compounds containing other chalcogen atoms and reported an increase in G2/M phase cell distribution compared to the control group. On the other hand, the compounds **5c** and **5j** did not induce overt signs of toxicity *Swiss* male mice [15].

Organochalcogen compounds have received special attention, mainly due to the interesting biological properties as the antitumor activities of the molecules that contain selenium and tellurium atoms in their structure [7, 29, 31]. In previous studies, the antioxidant and antitumoral activity of organochalcogens highlighted that these molecules showed prominent activity for both biological approaches in bladder cancer (5637) [7], and in lung carcinoma (A549) [15]. Nogueira et al., 2004 indicated that organic compounds with tellurium atoms have been associated with antioxidant effects in various oxidative stress models [11]. Furthermore, these compounds presented potential applications in medicine for acute myeloid leukemia, external genital warts treatments, and were promising for autoimmune diseases [32, 33]. Currently, studies involving organochalcogen compounds containing tellurium in their structure in bladder cancer are scarce, requiring greater attention to these compounds due to their pharmacological properties not yet investigated and the great representativeness of this cancer in the world population.

According to our results, we can see that compound **5j** had a better effect when compared to compound **5c**. We belived that this is due the presence of oxygen atom (electronegative element), and the *para* position (p-OMe-C_6H_4) in **5j**, showed a slight increase in apoptosis when compared to **5c**, and an previous study showed that the introduction of methoxy groups may enhance the anticancer activity due to these groups can promote the cellular uptake of platinum complexes in HepG-2 cells [17].

Also, studies have indicated that the electronegativity of certain elements such as oxygen, and halogens such as chloride, bromine, fluoride that form part of the synthesis of compounds, can form chemical bonds by attracting an electron by *van der Waals* forces, increasing the lipophilicity of the molecules, their penetration of the lipid membrane, improving the effectiveness of various treatments [34, 35]. In addition, the effect of compound **5j** appears to be involved with activation of the apoptosis pathway by increasing expression of *p53*, *p21*, *Bax*, and *caspase-3* and *caspase-9*. In addition, this anti-tumor effect involves pro and anti antioxidants genes. In contrast, the compound **5c** did not have the oxygen in the *para* position (p-OMe-C_6H_4), thereby, just increased the levels of pro-apoptotic protein p53 and activated downstream proteins such as caspases 3 and 9. Importantly, **5c** does not affect *p21*, *Bax*, *Bcl-2* expression in 5637 cells in the concentration tested.

In this study our data reveal that cell death was through apoptosis. However, when there is deregulation in this process, diseases such as autoimmune, degenerative diseases and even cancer can occur. There are components that are closely linked to the mechanism of death by apoptosis, such as *BCL-2* and the caspase family [36].

There are two pathways through which caspases can be activated, the intrinsic pathway, also called mitochondrial pathway, or extrinsic apoptosis. Both pathways lead to a common pathway of apoptosis [37]. The extrinsic pathway is initiated when TNF and FAS ligands bind to death receptors such as TNF type 1 (TNFR1) and a related protein called Fas (CD95). This binding triggers a series of effects including activation of caspase 8, which is an initiating caspase, which initiates apoptosis [37, 38]. Meanwhile, the intrinsic pathway is initiated within the cell. It may be activated by factors such as increased oxidative stress, irreparable genetic damage, hypoxia and high concentrations of cytosolic calcium. Irrespective of the stimuli, this pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c in the cytoplasm. This pathway is regulated by BCL2 family proteins, which may be anti-apoptotic, such as BCL2 or pro-apoptotic such as BAX protein. When there is intrinsic pathway activation, mitochondrial permeability is increased and pro-apoptotic molecules such as *Bax* are released. The release of pro-apoptotic proteins leads to the release of cytochrome C which activates caspase 3 and caspase 9 [39, 40]. In view of the above, we believe that compound **5j** is acting by the intrinsic pathway of apoptosis to induce death of 5637 cell lines. According to the results shown, compound **5j** increased expression of the protein Bax, a protein pro-apoptotic while listening for an increase in the relative expression of caspases 3 and 9. The activation of this pathway occurs by the increase of reactive oxygen species (ROS), such as nitric oxide, which leads to an imbalance with oxidizing defenses, thus generating oxidative stress. ROS serve as a signaling messenger to mediate various biological responses, excessive intracellular ROS, which can induce depolarization of the mitochondrial membrane potential, are also considered an apoptotic death signal that ultimately activates the intrinsic apoptotic pathway [41].

Besides that, the p53 is a well-known tumor suppressor able to drive cell cycle arrest, apoptosis, or senescence when DNA damaging occurred or the loss of cell integrity. indeed, during cell cycle, p53 associates with MDM2 in the nucleus and subsequently undergoes nuclear exclusion, allowing its ubiquitination and subsequent degradation [42]. Also, is involved in the apoptotic activity, triggering the activation of bax, caspases 3, 9, and subsequently cell death. A study realized in a human cancer cell line (ht-29) indicated that diphenyl ditelluride compounds showed an increase in the activity of caspases 3, 7, and 9 [43]. Our results corroborate these studies, which indicate that the action pathway of the compounds is through apoptosis. Compound 5c increases the relative

expression of *p53*, *caspase-3*, and *caspase-9*. The same is observed with compound 5j, which in addition to increasing expression of these genes, is also involved with increasing expression of p21, which is evidenced that p21 is a major inhibitor of p53-dependent apoptosis. It is not entirely clear how a cell chooses between apoptosis and p21-dependent cell cycle arrest following DNA damage and p53 stabilization, but generally high levels of *p21* expression mediate cell-cycle arrest and protect against p53-dependent apoptosis [44]. In our study we did not find significant results for cycle arrest, but we can see that p21 is closely intertwined with inhibitor of p53-dependent apoptosis.

In this study were evaluated gene expression levels of antioxidant enzymes SOD, CAT, GPx, GR besides iNOS enzyme. The qRT-PCR results showed that the **5j** compound obtained a better activity because it increased all gene levels when compared with control. In the other hand, **5c** compound only increased the antioxidant enzymes superoxide dismutase and catalase levels. This data indicate that even cancer cells has high ROS levels, that cells treated with **5c** and **5j** compound could induce expression of antioxidant genes enzymes and this expression is interesting to synthesis of antioxidant enzymes that would control and neutralize the ROS levels in the cellular environmental to keep the antioxidant homeostasis.

SOD is the enzyme responsible to break down the superoxide anion radical, a potential genotoxic radical. Previous studies demonstrated that low SOD levels could contribute to cellular malignant while higher levels could suppress this malignancy [45] corroborating with our results in this study. In the same way, hydrogen peroxide is a strong mutagenic reactive specie [46] that affects directly cell proliferation [47]. CAT is the enzyme that convert it and the capacity to modulate the expression of this enzyme is important to keep this antioxidant balance, the same as GPx enzyme that convert H_2O_2 too.

Cancer cells show higher levels of reactive species than to normal cells and a kind of difficulty to antioxidant enzymes to control them. Therefore, the capacity to modulate this process becomes an interesting anticancer approach [48]. The interest in potentialize the antioxidant defenses to prevent diseases is widespread, but in view of the alarming data of cancer cases and death in the world and the treatment´s difficulties, antioxidant supplementation during chemotherapy has the potencial to reduce the drug´s toxicity used in clinic [49].

Nitric oxide is dual in the body, mostly in cancer. Studies have suggest that increased NO levels synthetized by iNOS enzyme in the tumor environment is beneficial to stimulate phagocytic cells combat the tumor cells, because NO is a cytotoxic mediator [50]. Furthermore, nitric oxide in high concentration is capable to induce apoptosis modulating p53 and caspase expression [51]. In this sense, the results observed in our study corroborate with what is suggest in previous studies, that

when there is an increase in NO, there is an activation of p53, which induces an activation of the caspase pathway leading to apoptosis.

In order to understand a possible selectivity route of action of compounds **5c** and **5j** we performed molecular docking in silico analyzes. We evaluated the main proteins that could be involved.

Our results of molecular docking showed the binding affinity with different proteins system and in most tested targets **5c** demonstrated more free energy of binding (kcal/mol) interaction with Survivin, Bcl-XL, RSK2, and SGK1 proteins. Survivin is known to be an apoptosis inhibitor and has been shown to be overexpressed in tumors such as lung, breast, colon, pancreas, liver, uterus, ovaries, among others [52, 53]. Thus, our compounds (especially **5c**), by binding to it, could be disabling this inhibition of apoptosis. In the same vein, Bcl-XL also acts to inhibit apoptosis, where it contributes to tumor development, progression and drug resistance [54]. Leech *et al* in his study of lung cancer, he showed that regulation of BCL-XL protein had positive results and may be indicated as a candidate for cancer therapy [54]. Studies reported that cancer cell lines resistance to treatments have overexpression of Bcl-XL protein [55], and **5c** showed interaction with this protein. A serine/threonine kinase RSK2 also showed a binding affinity with **5c**, and studies indicated that this kinase regulates cell proliferation, responding to the environmental stresses, growth factors, induce cell cycle progression, and is involved in human skin cancer [56, 57].

About RSK2, it is a downstream target of ERK1/2 in the MAPK/ERK pathway and its inhibition suppresses tumorigenesis and metastasis of neoplasms [58]. Thus, it is important to try to regulate it. Our silicon study has shown that compound **5c** requires less energy to bind to RSK2. This data indicates that this compound may be a good candidate for inhibition of this kinase in autophagy, inhibiting its proliferation, activating and cellular invasion [59].

The protein with the highest binding affinity molecular coupling analysis was SGK1 (mostly for compound **5c**), a member of the serine/threonine kinases protein family, activated by PDK-1 phosphorylation, associated with resistance to chemotherapy and radiotherapy [60]. This protein plays an important role in the response to cell stress, being directed downstream of phosphatidylinositol 3 (PI-3) kinase, PI3K and AKT activation, involved in cell processes such as survival, cell growth, migration and apoptosis [61]. Although compound **5j** was not strongly bound, it showed a common amino acid interaction when compared to co-crystallized proteins. Demonstrating that the compound binds like these control regions.

Thus, our *in silico* results compared to real-time PCR when evaluating apoptosis death show that **5c** and **5j** may be promising because they increased the expression of apoptosis-related genes compared to control. Some reports indicate that organochalchogens could cause oxidative damage,

triggering a mitochondrial dysfunction by thiol groups oxidation related to cell death [62, 63], being the thiol redox status the key determinant to stress response, and cellular function [64, 65]. The antioxidant property of organotellurium compounds could be explained due to changes in the oxidation from the divalent Te (II) to the tetravalent Te (IV) state [21].

Recently, Trindade et al. (2019) reported that Diphenyl ditelluride (DPDT) showed anticancer activity induced by increasing ROS generation, also presented antioxidant, and prooxidant properties [66]. Also, tellurium compounds are involved in ROS formation, cell cycle arrest, and induction of programmed cell death [67]. Conversely, studies mentioned that the use of antioxidants could accelerate the tumor phenotype [68, 69]. Some studies have suggested the potential role of organochalcogens on molecular targets, and have reported inhibition of the complex I activity of the mitochondria, possibly to thiol oxidation activity, altering cellular metabolism, and regulation of apoptotic cell death [70, 71].

In this paper, we investigate possible mechanisms of action involved in the antitumor activity of β-Aryl-chalcogenium azide compounds containing chiral tellurium (**5c** and **5j**) in bladder cancer cell 5637. Our results suggest that compounds **5c** and **5j** are promising for treatment of bladder cancer. In this context, we observed that compound **5j** was more effective than compound **5c** in that it had a lower concentration *in vitro* effect. Still, its activity seems to be involved in the induction of apoptosis by increasing NO, leading to cell death by this pathway. Of course, the best performing *in silico* compound **5c** is also a very promising molecule because according to the results shown it also appears to have its anticancer effect by inducing apoptosis and increasing the expression of antioxidant enzymes. Additional assays will be performed on different cell lines in order to further broaden the application spectrum of these compounds and further elucidate the effects of these compounds and their cell death pathways.

Therefore, according to our results we can see that compounds **5c** and **5j** are promising for the treatment of bladder cancer. Compound **5j** has been shown to be more effective than compound **5c** because it has a lower concentration *in vitro* effect and it appears that its activity is involved in the induction of apoptosis through NO increase, this leads to cell death by this pathway. Of course, the **5c** compound which has shown better results *in silico* is also a very promising molecule, because according to the results shown it also seems to have its anti-cancer effect by inducing apoptosis and increasing the expression of antioxidant enzymes. However, further studies are needed to better elucidate the effects of these compounds and their cell death pathways.

Author contributions: Material preparation, data collection, and analysis were performed by Martha Lucia Ruiz Benitez, Fernanda Severo Sabedra Sousa, Izadora Peter Furtado, João Carlos Rodrigues

Junior, Victoria Mascarenhas Borba, Greice Tabarelli, Gabriela Klein Couto, Júlia Damé Fonseca Paschoal, Bruna Silveira Pacheco. Study conception and design were supervised by Fabiana Kömmling Seixas, Oscar E. D. Rodrigues, Tiago Collares. The first draft of the manuscript was written by Martha Lucia Ruiz Benitez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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5. CONCLUSÃO GERAL

A combinação da cepa BCG Δ*Leu*D/A85B + imiquimode avaliada em melanoma, mostraram uma porcentagem de inibição máxima de 80,08%, quando comparado com o imiquimode (57,97%), mostrando também uma atividade apoptótica na linhagem WM1366. Por outro lado, esta combinação evidenciou um aumento da resposta imune celular na linhagem de macrófago J774A.1, sugerindo que possivelmente esta estratégia empregada poderia ser promissora no tratamento para este tipo de câncer.

Os compostos de azida de β-arilcalcogênios avaliados em câncer de bexiga (5637), demonstraram inibição do crescimento celular, mostrando valores de inibição (IC_{50}) de 1,57 ± 0.70 μM **(5c)** e 0,48 ± 0.13 μM **(5j)** em 48h, evidenciando também um aumento na expressão das proteínas pro-apoptóticas e enzimas antioxidantes.

6. PERSPECTIVAS

De acordo aos resultados promissores obtidos neste projeto podemos considerar que:

- Em relação ao melanoma, é preciso realizar teste como o co-cultivo e ensaios *in vivo* para verificar a aplicação terapêutica da combinação da cepa BCG recombinante (Δ*Leu*D/Ag85B) com o imiquimode, testar esta combinação em células não tumorais e avaliar esta combinação em outras linhagens que representem diferentes estágios do melanoma.

- No câncer de bexiga, sugere-se testar estes compostos em outras linhagens celulares de carcinoma de bexiga, avaliar a geração de ROS, de óxido nítrico e geração de TBARS para complementar com nossos resultados obtidos da expressão génica de genes associados ao estresse oxidativo.

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