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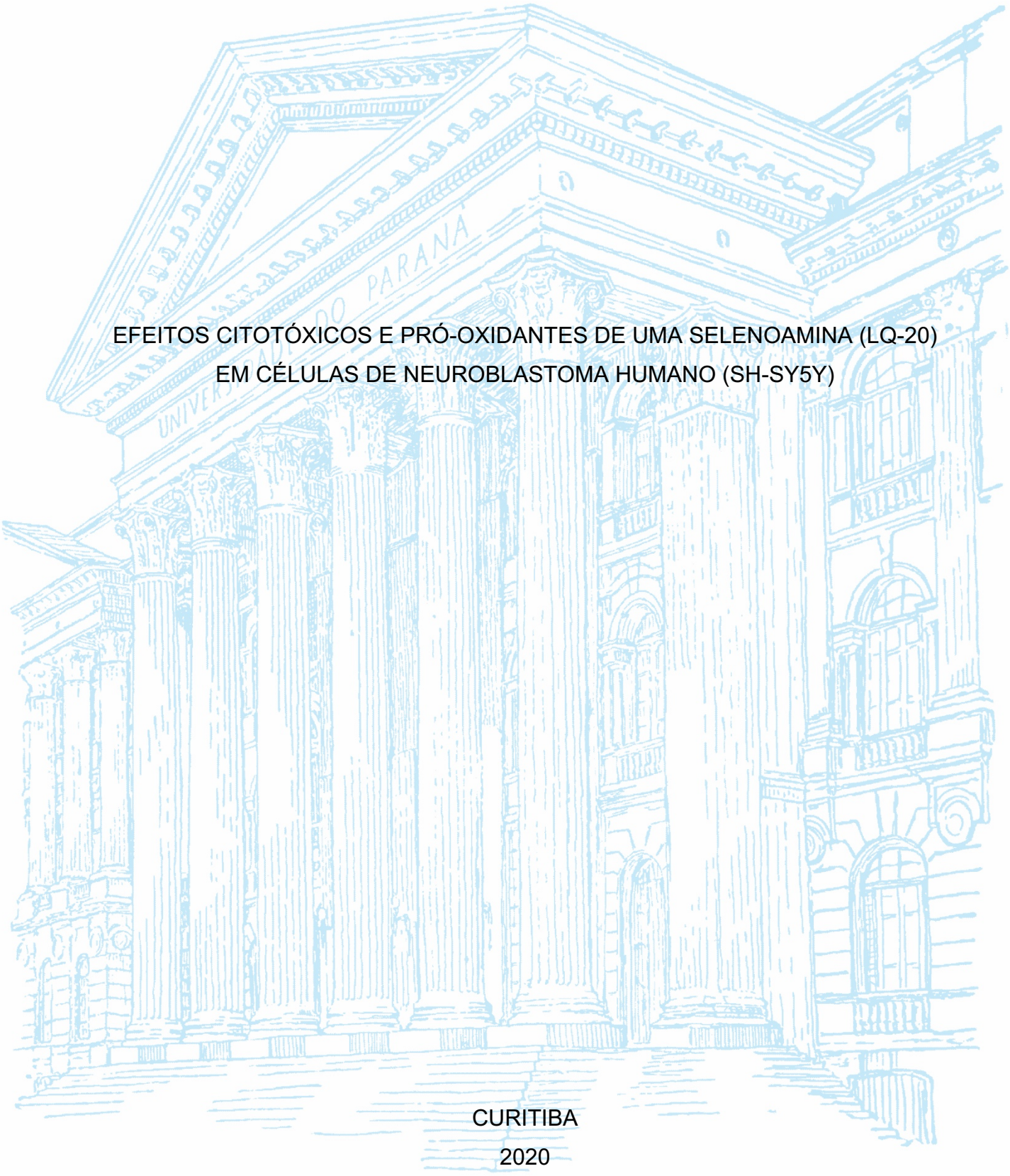

EFEITOS CITOTÓXICOS E PRÓ-OXIDANTES DE UMA SELENOAMINA (LQ-20) EM CÉLULAS DE NEUROBLASTOMA HUMANO (SH-SY5Y)

CURITIBA

2020

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EFEITOS CITOTÓXICOS E PRÓ-OXIDANTES DE UMA SELENOAMINA (LQ-20) EM CÉLULAS DE NEUROBLASTOMA HUMANO (SH-SY5Y)

Dissertação apresentada ao curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Ciências - Bioquímica.

Orientador(a): Prof(a). Dr(a). Maria Eliane Merlin Rocha

CURITIBA

2020

Universidade Federal do Paraná
Sistema de Bibliotecas
(Giana Mara Seniski Silva – CRB/9 1406)

Santos, André Vinicius Rederd de Oliveira dos
Efeitos citotóxicos e pró-oxidantes de uma selenoamina (LQ-20) em células de neuroblastoma humano (SH-SY5Y). / André Vinicius Rederd de Oliveira dos Santos. – Curitiba, 2020.
66 p.: il.

Orientadora: Maria Eliane Merlin Rocha

Dissertação (mestrado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências – Bioquímica.

1. Compostos de selênio 2. Estresse oxidativo 3. Neuroblastoma I. Título II. Rocha, Maria Eliane Merlin, 1965- III. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências – Bioquímica.

CDD (20. ed.) 618.929948

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIENCIAS BIOLOGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS (BIOQUÍMICA) - 40001016003P2

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **ANDRÉ VINICIUS REDERD DE OLIVEIRA DOS SANTOS** intitulada: **Efeitos citotóxicos e pró-oxidantes de uma selenoamina (LQ20) em células de neuroblastoma humano (SH-SY5Y)**, sob orientação da Profa. Dra. MARIA ELIANE MERLIM ROCHA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 25 de Março de 2020.

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MARIA ELIANE MERLIM ROCHA	31/03/2020 23:09:31.0	191.177.53.60	kzqx3ubfWf7rQH8D8Dkcb9P/Kh2V37eoVF5Xi8RkG/7km8hPQY...
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AGRADECIMENTOS

Agradeço a Deus, por sua infinita graça, cuidado e amor;

Agradeço à professora Dra. Maria Eliane, pela orientação, por ter me aceitado como aluno, por todos os aprendizados e conselhos;

À minha família, que sempre me apoiou com muito amor e carinho, e me incentivou a realizar esse sonho;

Aos meus colegas e amigos do laboratório de Oxidações Biológicas e Cultivo Celular pela boa convivência e amizade, em especial gostaria de agradecer ao Eduardo, Juliana, Lisley, Marília, Elaine, Stephane e Juan que de certa forma contribuíram para o desenvolvimento do meu trabalho;

Às técnicas Jainy e Beti pela dedicação e toda ajuda;

Aos amigos Philippe, Anna Luiza, Gustavo e Gabi que durante o mestrado tornaram engraçados os momentos difíceis;

Agradeço à Fernanda por todo carinho e suporte durante esses dois anos;

Agradeço ao professor Dr. Glaucio Valdameri e aos alunos Isadora, Manoella, Giovana, Diogo, Ingrind do Departamento de Análises Clínicas da UFPR pela colaboração nos experimentos de ciclo celular.

Agradeço ao professor Dr. Alfredo M. de Oliveira do Departamento de Química da UFPR pelo fornecimento do composto LQ-20;

Ao professor Dr. Silvio Marques Zanata do Departamento de Patologia Básica da UFPR pelo fornecimento das células SH-SY5Y e à professora Dra. Michele Dietrich Moura Costa do Departamento de Biologia Molecular, Estrutural e Genética da UEPG por toda ajuda com o protocolo de diferenciação celular;

Agradeço ao programa de Pós-Graduação em Ciências-Bioquímica e as agências financiadoras Capes e CNPQ pelo suporte financeiro.

RESUMO

O neuroblastoma é um tumor sólido encontrado comumente na infância e possui altos índices de morte. As principais formas de combater a doença são através de: cirurgia, radiação, terapia com retinoides, transplante de células-tronco, imunoterapia e quimioterapia. A alta heterogeneidade do tumor faz com que a doença desenvolva resistência a múltiplas drogas quimioterápicas reduzindo a eficácia do tratamento. Compostos pertencentes a classe de organosselênios tem sido estudado pela capacidade de afetar a homeostase redox de células tumorais por meio da oxidação de grupamentos tióis de enzimas antioxidantes com geração de espécies reativas de oxigênio (EROs) e consequentemente a indução da morte celular. Nesse trabalho foram avaliados pela primeira vez, os efeitos da selenoamina LQ-20 sobre a viabilidade e proliferação celular, fases do ciclo celular, geração de EROs, níveis de glutationa reduzida (GSH) em células SH-SY5Y não diferenciadas, bem como a toxicidade do composto em células SH-SY5Y diferenciadas com ácido retinoico (RA). Células SH-SY5Y incubadas com LQ-20 (IC_{50} 63 μM) sofreram redução da viabilidade e proliferação celular de forma dependente de concentração e tempo de incubação, acompanhado de aumento de DNA fragmentado. A quantificação de EROs por DCFH-DA revelou um aumento na geração de EROs para as concentrações de 50 e 100 μM de LQ-20, provavelmente desencadeado pela inibição observada nos níveis intracelulares de GSH. Além disso, células SH-SY5Y diferenciadas se mostraram mais resistentes quando incubadas com LQ-20 quando comparadas com as células não diferenciadas incubadas com LQ-20. Os resultados sugerem que o tratamento com LQ-20 pode induzir efeitos citotóxicos em células SH-SY5Y, com um mecanismo ainda não totalmente esclarecido, porém envolvendo, pelo menos em parte, depleção de GSH e aumento dos níveis de EROs e além disso, há uma diferença importante nos efeitos quando comparado com o modelo celular diferenciado com RA (*neuron-like*) versus o não diferenciado que precisariam ser melhor explorados. Estes resultados mostram que a LQ-20 é um composto com efeitos promissores para serem melhor estudados posteriormente visando a aplicação deste composto no tratamento do neuroblastoma.

Palavras-chave: Compostos com selênio. Selenoamina. Estresse oxidativo. Neuroblastoma. SH-SY5Y.

ABSTRACT

High-risk neuroblastoma is an aggressive extracranial solid tumor frequently diagnosed during infancy. Besides modern strategies of treatments, low survival rates and side effects still remain a challenge for patients, and there is an urgent need for effective treatments. Among new and synthesized compounds, selenium compounds are reported to have potential antitumor activity, and is a promisor chemotherapeutic agent. The present study, for the first time, investigated the cytotoxicity and antiproliferative activity of selenoamine LQ-20 using human neuroblastoma SH-SY5Y cells, and the relative toxicity in differentiated SH-SY5Y cells with retinoic acid (RA). SH-SY5Y cells were treated with different doses of LQ-20 (20-200 μM). Cell viability was detected using the MTT assay. Cell proliferation was detected using crystal violet staining and tryplan blue exclusion method, followed by analysis in cell cycle through flow cytometry. We also evaluated reactive oxygen species (ROS) and alterations on intracellular reduced glutathione (GSH) levels of SH-SY5Y cells treated with LQ-20. LQ-20 (IC_{50} 63 μM) reduced the SH-SY5Y cells viability and proliferation in a time- and dose-dependent manner. LQ-20 at 50 and 100 μM induced cell death and promoted increase in fragmented DNA after 72 h of incubation. In addition, LQ-20 was responsible for ROS overproduction and GSH depletion in SH-SY5Y cells treated for 72 h, which correlated with a property of selenium compounds in promoting cell oxidative stress. LQ-20 (50 and 100 μM) and cisplatin (4 μM) incubation promoted reduction of viability in differentiated SH-SY5Y cells with RA and undifferentiated SH-SY5Y cells. However, when LQ-20 was used, the reduction in viability was greater in undifferentiated cells than in those differentiated with RA (neuron-like cells). These results suggest a difference in the effects promoted by LQ-20 in neuron-like cells when compared to the tumor cell. Thus, it is also possible to conclude that the selenoamine LQ-20 is capable of inducing SH-SY5Y cells death possibly through a mechanism that, at least in part, involves depletion of GSH and increase in ROS levels and may even have differences in tumor SH-SY5Y cells when compared to cells differentiated with RA.

Keywords: Selenium compounds. Selenoamine. Oxidative stress. Neuroblastoma. SH-SY5Y.

SUMÁRIO

1 INTRODUÇÃO

O neuroblastoma, uma neoplasia do sistema nervoso simpático, representa o segundo tumor sólido mais comum na infância. O tumor pode estar localizado ao longo dos gânglios da cadeia simpática, mas é encontrado principalmente na região medular da glândula adrenal. Este tipo de tumor é considerado heterogêneo e o prognóstico pode variar em relação aos estágios da doença, o que dificulta formas de tratamento e desenvolvimento de terapias (PARK; EGGERT; CARON, 2010; LUCENA et al., 2018; VRENKEN et al., 2020).

A incidência global da doença foi estimada em 10,5 casos/milhão de crianças/ano. Na América do Norte foram diagnosticados em crianças de até 15 anos, mais de 650 casos por ano, sendo a taxa de sobrevivência de aproximadamente 50%, ainda que o paciente seja submetido a intensos tratamentos (STILLER; PARKIN, 1992; SAIT; MODAK, 2017; MOONEY et al., 2019). Devido a frequência da ocorrência de invasão e metástase em neuroblastoma de alto risco e a resistência a múltiplas drogas quimioterápicas, existe a urgência de novos agentes terapêuticos para melhorar a eficácia e reduzir a toxicidade dos tratamentos já existentes (FARAJ et al., 2017; CURTIN et al., 2018).

No Brasil entre as neoplasias malignas mais comuns em crianças e adolescentes estão as leucemias com uma frequência de 26%, seguida dos linfomas e tumores do sistema nervoso central (13-14%) e com 8-10% de frequência o neuroblastoma (INCA, 2017, 2018).

Os tratamentos modernos para neuroblastoma envolvem esquemas estratégicos de terapias combinadas, como cirurgias e sessões intensas de quimioterapias (REYNOLDS et al., 2003). As quimioterapias combinam agentes como a cisplatina, carboplatina, etoposido e compostos contendo selênio (GANDIN et al., 2018; JOHNSEN et al., 2018; PINTO et al., 2015). Outras formas de tratamento também são utilizadas como: cirurgias, radiação, terapia retinoide, transplante de células-tronco e imunoterapia (BHATNAGAR; SARIN, 2012; VANICHAPOL et al., 2018). Derivados de ácido retinoico são utilizados em associação com imunoterapias em uma das fases finais de tratamento combinado, conhecido como terapia multimodal (REYNOLDS et al., 2003; MATTHAY et al., 2016).

O selênio e seus derivados têm sido considerados promissores para pesquisas que visam desenvolver agentes quimioterápicos, pois além de ser um elemento importante para o funcionamento do organismo, está relacionado a atividades redox e antioxidantes (FERNANDES; GANDIN, 2015; GANDIN et al., 2018). Compostos contendo organosselênio são reconhecidos por seus efeitos antiproliferativos e pela sua capacidade de indução de morte celular por apoptose, podendo ser indicados como potentes agentes citotóxicos para diferentes modelos de células tumorais (CHENG et al., 2012; ZHAO et al., 2012; WEEKLEY et al., 2012).

A tolerância, segurança e perfis farmacocinéticos dos compostos: selenito de sódio, Se-metilselenocisteína e seleno-L-metionina foram testados em pacientes com leucemia linfática crônica e em pacientes com tumores sólidos metastáticos. Nesse teste clínico, os compostos com selênio foram bem tolerados e a dose (400 µg) determinada não foi tóxica para os pacientes (EVANS et al., 2019). Testes clínicos dos compostos com selênio em combinação com quimioterápicos comerciais, como a cisplatina por exemplo, apresentaram capacidade de modular a toxicidade do tratamento em relação a eficácia e amenização de efeitos colaterais (ASFOUR et al., 2006; ASFOUR et al., 2007; SIEJA et al., 2004).

Linhagens celulares imortalizadas são comumente usadas em pesquisas laboratoriais e possuem capacidade de se dividir indefinidamente, podendo oferecer maior reprodutibilidade em relação a culturas primárias quando aplicadas na pesquisa para desenvolvimento de agentes quimioterápicos (MASTERS, 2000). A linhagem celular de neuroblastoma humano SH-SY5Y tem sido utilizada em estudos visando o desenvolvimento de potenciais drogas terapêuticas antitumorais e também como modelo para estudo de doenças neurodegenerativas (LOPES et al., 2010; YAN et al., 2015; AGUIAR, 2016). Existem poucos relatos na literatura sobre a ação de compostos contendo selênio sobre células de neuroblastoma humano (POSSER et al., 2011; HUSSAIN et al., 2015).

A selenoamina N-(1-(4-(butilselanil)fenil)etil)-2-metilpropan-1-amina (LQ-20), utilizada neste trabalho, foi sintetizada no Departamento de Química da UFPR e ainda não foram investigados seus efeitos sobre células SH-SY5Y.

Desta forma, o presente trabalho tem como objetivo principal estudar a ação citotóxica e pró-oxidante da selenoamina sintética LQ-20 sobre o modelo celular de neuroblastoma humano SH-SY5Y.

1.1 JUSTIFICATIVA

Devido à necessidade no desenvolvimento de novas terapias para tratamento do tumor neuroblastoma e ainda os promissores efeitos citotóxicos que já foram observados com outros compostos com selênio sobre algumas linhagens de células tumorais, no presente trabalho foram propostos os seguintes objetivos:

1.2 OBJETIVOS

1.2.1 Objetivo geral

O objetivo geral deste trabalho é avaliar o potencial efeito citotóxico e pró-oxidante da selenoamina LQ-20 em células de neuroblastoma SH-SY5Y.

1.2.2 Objetivos específicos

- Avaliar citotoxicidade do composto LQ-20;
- Analisar o efeito sobre as fases do ciclo celular;
- Determinar os níveis de espécies reativas de oxigênio (EROs);
- Determinar os níveis de glutationa reduzida (GSH);
- Avaliar de forma preliminar o possível envolvimento da autofagia no mecanismo de ação da LQ-20;
- Avaliar os efeitos do composto LQ-20 sobre a viabilidade de células SH-SY5Y diferenciadas com ácido retinoico (*neuron-like*) com o objetivo de compará-los com o modelo não diferenciado.

2 REVISÃO DE LITERATURA

2.1 ASPECTOS GERAIS DE NEUROBLASTOMA

Durante o desenvolvimento embrionário, células da crista neural migram e diferenciam-se em diferentes linhagens celulares, incluindo melanócitos, neurônios sensoriais, entéricos e simpáticos (NAKAGAWARA et al., 2018). O processo de diferenciação e especificação das células derivadas da crista neural é regulada por uma complexa rede reguladora de genes. A desregulação desse processo normal de desenvolvimento com o envolvimento de oncogenes como MYCN ou ALK pode levar ao desenvolvimento de diferentes tipos de neuroblastoma (LOUIS; SHOHET, 2015; WHITTLE et al., 2017; TSUBOTA; KADOMATSU, 2018).

O termo neuroblastoma é comumente usado para descrever um grupo de tumores neuroblásticos que inclui: neuroblastomas (tipo mais comum), ganglioneuroblastomas e ganglioneuromas (SHOHET; FOSTER, 2017). Os tumores se diferenciam de acordo com os níveis de maturação celular. Ganglioneuroma é composto por células que alcançaram diferenciação celular, já neuroblastoma compõem um conjunto de células primárias imaturas. Um ganglioneuroblastoma possui ambas as características celulares dos outros dois tumores (TOLBERT; MATTHAY, 2018).

Tumores neuroblásticos podem ser manifestados por todo o sistema nervoso simpático, mas são geralmente localizados no abdômen em cerca de 70% dos casos, sendo 40% na medula adrenal e 25% em gânglios simpáticos (AHMED, et al.; 2017). A principal característica do neuroblastoma é a sua heterogeneidade, com uma probabilidade de cura muito variável, dependendo da extensão do tumor e idade do paciente (MARIS et al., 2007; AMANO et al., 2018).

Neuroblastoma é uma doença que afeta 1 a cada 8.000 recém-nascidos e representa cerca de 6-10% dos tumores infanto-juvenis, aparecendo comumente em crianças com até 15 anos de idade (JOHNSEN et al, 2019; BECKER; WILTING, 2018). No Brasil, segundo o estudo realizado pelo Instituto de Oncologia Pediátrico, crianças com idade entre 1-4 anos, representam o maior grupo dos 258 pacientes diagnosticados (49%) com neuroblastoma, no período de 1991 a 2012 (LUCENA, 2018). Em 2014 foram relatadas 2.724 mortes por câncer infanto-juvenil, sendo os

grupos de câncer infantil liderados por leucemia, tumores do sistema nervoso central, linfomas e neuroblastoma (INCA, 2017, 2018).

O tumor, uma vez diagnosticado, é identificado e classificado conforme o estágio de agressividade e a idade do paciente, para assim submeter a criança ao tratamento adequado, podendo ser realizado por meio de: cirurgias, radiação, terapia retinoide, transplante de células-tronco, imunoterapia e quimioterapia (BHATNAGAR; SARIN, 2012; VANICHAPOL et al., 2018).

Neuroblastoma classificado como de alto risco, continua sendo uma doença desafiadora para tratamentos antitumorais, recorrendo em especial aos tratamentos quimioterápicos adjuvantes (AMOROSO et al., 2017). Pacientes diagnosticados com esse tipo de tumor apresentam uma taxa de sobrevivência de 50%, mesmo sendo submetidos a tratamentos intensos como a terapia multimodal (MOONEY et al., 2019). As terapias multimodais, constituem um conjunto de tratamento intensivos para se tentar reduzir a carga tumoral e aumentar a sobrevida do paciente (MULLASSERY; LOSTY, 2015). Tratamentos para pacientes de alto risco incluem regimes de 5 ou 6 sessões de quimioterapias e cirurgias, seguido por terapias de consolidação com altas doses de quimioterápicos, irradiação e transplante autólogo de células troncos (PINTO et al., 2015).

Pacientes que alcançam a remissão da doença, após as outras duas fases, são submetidos a terapias de manutenção ou pós consolidação. Esse tipo de terapia visa reduzir ao máximo as chances de remissão do tumor. Testes clínicos utilizando derivados de ácido retinoico, foram inseridos nos regimes das terapias após consolidação, e resultados mostraram-se eficazes para a sobrevivência dos pacientes em relação ao grupo de não tratados (MATTHAY et al., 1999; MATTHAY et al., 2009; PEARSON et al., 2008; PINTO et al., 2015; PEINEMANN et al., 2017; CHALAPEK et al., 2018).

Os maiores desafios dos tratamentos para neuroblastoma estão em combater a metástase do tumor e a resistência a múltiplas drogas quimioterápicas (CURTIN et al., 2018). Sendo assim, tratamentos com novas drogas se fazem necessário para aumentar a eficácia e reduzir a toxicidade das terapias existentes (FARAJ et al., 2017).

Cisplatina, topotecano, irinotecano e etoposido são algumas das drogas quimioterápicas comumente usadas para tratamento de neuroblastoma (VENEZIANI et al., 2018). O dehidroeffusol é um composto fenantrenóide que demonstra

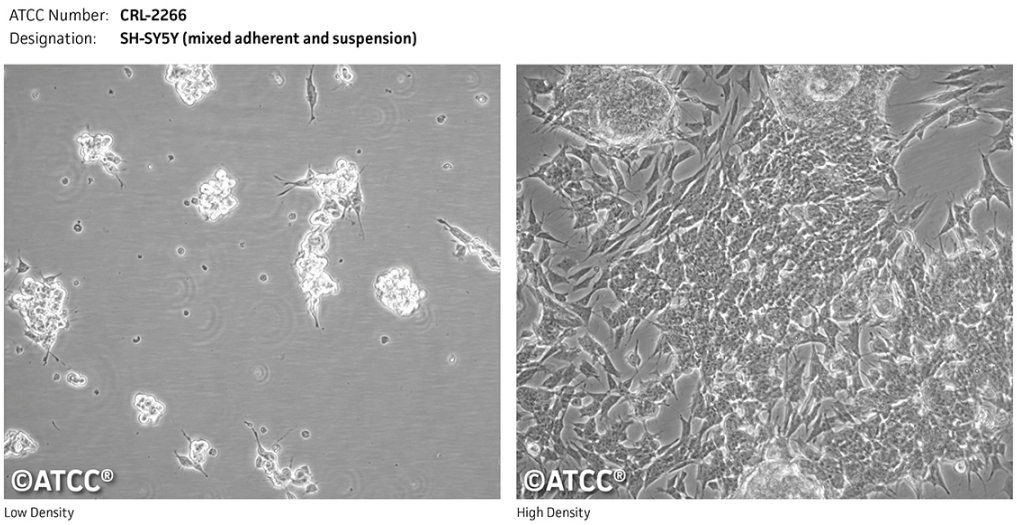
atividade citotóxica em cinco tipos de células de câncer humano, incluindo as linhagens de neuroblastoma SH-SY5Y e HeLa (MA et al., 2016). Schulte et al. (2018) verificaram a regressão espontânea de neuroblastoma em meio a tratamentos quimioterápicos e em relação a alterações genéticas como a amplificação do gene MYCN e membros da família RAS/MAPK, evidenciando a heterogeneidade e complexidade do tumor.

2.2 LINHAGEM CELULAR DE NEUROBLASTOMA HUMANO SH-SY5Y E O USO DO ÁCIDO RETINÓICO

Amplamente utilizada em pesquisas com células neuronais, a linhagem humana SH-SY5Y foi derivada por meio de subclonagens da linhagem celular SK-N-SH originalmente extraída de uma biópsia de medula óssea de neuroblastoma metastático, subclonada 3 vezes; primeiro em SH-SY, então em SH-SY5 e finalmente em SH-SY5Y, pela Dra. June L. Biedler (BIEDLER et al., 1978).

As células SH-SY5Y (FIGURA 1) possuem características importantes quando consideradas sua utilização em estudos *in vitro*: 1) Ambos os cultivos, aderente e em suspensão são possíveis. 2) possui fenótipo *neuroblast-like*, com características catecolaminérgicas 3) podem ser diferenciadas em um fenótipo mais maduro (*neuron-like*), caracterizado pela presença de marcadores neuronais (ROSS et al., 1983; CONROY; BERG, 1995; GILANY et al., 2008; AMINI; WHITE, 2013). O fato de que células SH-SY5Y consistem em uma população homogênea com características *neuroblast-like*, proporciona a pesquisadores, diferenciar estas células em células que possuem um fenótipo mais maduro (*neuron-like*) por meio da adição de agentes externos, capazes de induzir esse processamento.

FIGURA 1 – MICROGRAFIA DE CÉLULAS SH-SY5Y EM BAIXA DENSIDADE (*low density*) E ALTA DENSIDADE CELULAR (*high density*)



FONTE: American Type Culture Collection (ATCC)[1].

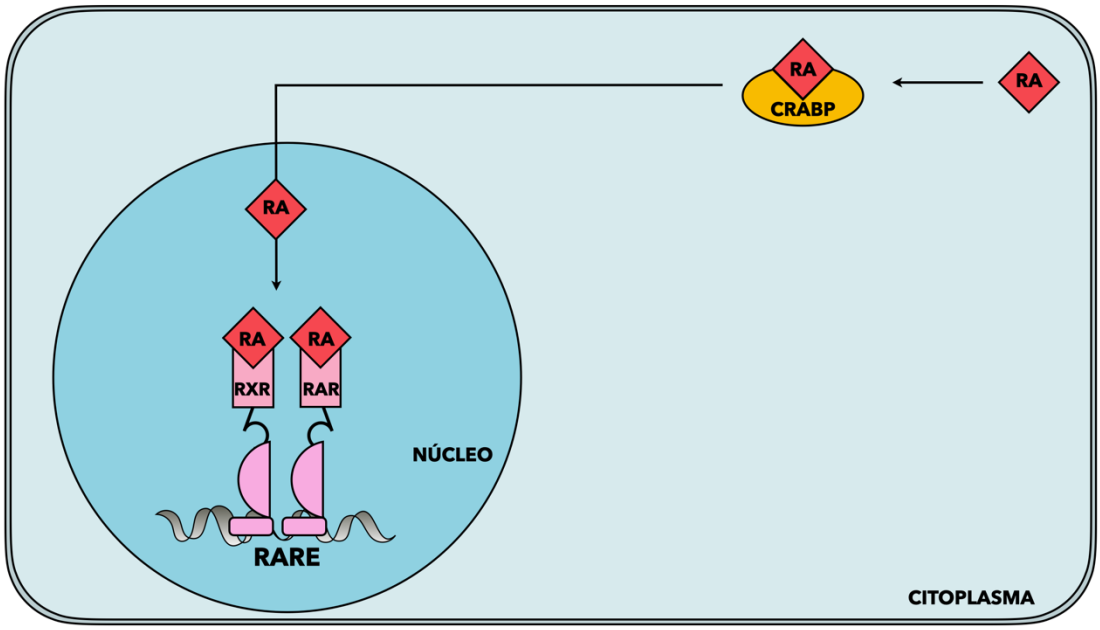
Pode-se citar como vantagens para o uso dessas células: a capacidade de expansão em larga escala (antes da diferenciação) acompanhados de fácil manipulação e baixo custo em relação a culturas primária de neurônios (AMINI; WHITE, 2013). Na forma não diferenciada, as células de neuroblastoma humano SH-SY5Y são caracterizadas morfologicamente por corpos celulares arredondados e pouco desenvolvimento de neuritos, tendem a crescer em *cluster* e formar aglomerados (BIEDLER et al., 1978; PAHLMAN et al., 1984). Células SH-SY5Y não diferenciadas mantêm sua capacidade de proliferar continuamente e expressam marcadores de neurônios não desenvolvidos (PAHLMAN et al., 1984).

O processo de diferenciação das células SH-SY5Y através de intermediários adicionados ao meio de cultura resulta em células morfologicamente mais maduras e similares a neurônios primários, com neuritos mais desenvolvidos e longos. Apresentam taxa de proliferação reduzida, com parada de ciclo celular em G0, bem com aumento na atividade da enzima enolase específica para neurônios (NSE) (PAHLMAN et al., 1984; ROSS et al., 1996; MELINO et al., 1997; ENCINAS et al., 2000). Na literatura são descritos vários métodos para indução da diferenciação em células SH-SY5Y, sendo o ácido retinoico (RA) o mais bem caracterizado e implementado agente indutor (YANG et al., 2016; XICOY et al., 2017).

[1] Disponível em: <https://www.atcc.org/products/all/CRL-2266.aspx#specifications> Acesso em abril de 2020.

RA, é a forma ácida da vitamina A, sendo essencial para desenvolvimento normal, manutenção de crescimento e diferenciação de células epiteliais, fibroblásticas e mielomonocítica de embriões (organogênese) (BASTIEN; ROCHETTE-EGLY, 2004; VITOBELLO et al., 2011). Conforme ilustrado na FIGURA 2, os efeitos biológicos de RA, são mediados por receptores de ácido retinoico (RAR) e de retinoides (RXR) (QUIAO et al., 2012). O RA é transportado para o núcleo por meio da proteína de ligação celular ao ácido retinoico (CRABP) e se liga ao heterodímero de receptores nucleares RXR/RAR, que por sua vez, está conectado a uma específica sequência de DNA conhecida como elemento de reposta ao ácido retinoico (RARE), funcionando como um regulador da expressão gênica (VILHAIS-NETO; POURQUIÉ, 2008).

FIGURA 2 – ESQUEMA REPRESENTATIVO DO MECANISMO DE AÇÃO DO ÁCIDO RETINOICO (RA)



FONTE: Adaptado de Maden (2007).

Portanto, para o desenvolvimento do sistema nervoso, o papel principal de RA é descrito como padronização e diferenciação do tubo neural (MADEN, 2007). RA é relatado na literatura como inibidor do crescimento de células neuroblastoma, induzindo a diferenciação celular, o que torna um agente adequado para a eliminação da doença residual (SIDELL, 1982; LOUIS; SHOHET, 2015; WAETZIG et al, 2018).

Entretanto, estudos que promovem indução da diferenciação celular exigem marcadores confiáveis para se distinguir entre um fenótipo diferenciado de um não diferenciado e assim não fazer correlações erradas em relação ao modelo celular (AMINI; WHITE, 2013). Células SH-SY5Y não diferenciadas são caracterizadas por marcadores que indicam proliferação, como antígeno nuclear celular em proliferação (PCNA) e marcadores neuronais imaturos, como Nestina. Após a diferenciação, células SH-SY5Y expressam níveis elevados de marcadores neuronais maduros como βIII-tubulina, proteína associada ao microtúbulo 2 (MAP2), sinaptofisina, proteína nuclear de neurônio (NeuN) e NSE (CHEUNG et al., 2009; LOPES et al., 2010; KOVALEVICH; LANGFORD, 2013; JAUHARI et al., 2017).

O desenvolvimento de neuritos e o processo de crescimento celular inibido são fortes indícios de que a diferenciação está ocorrendo em culturas de neuroblastoma, porém não são suficientes e únicos para atestar o estado diferenciado dessas células (RUUSALA et al., 1985). Além do ácido retinoico, novas classes de compostos têm sido estudadas para o tratamento do neuroblastoma, entre eles os compostos com selênio.

2.3 COMPOSTOS COM SELÊNIO

Compostos orgânicos com selênio fazem parte de um grupo de moléculas quimicamente diversas. A química dos compostos com selênio na literatura, foi negligenciada por mais de um século, porém nos últimos anos muitos estudos visaram projetar e desenvolver uma série de moléculas orgânicas contendo selênio, com o objetivo de identificar potenciais compostos quimioterápicos que pudessem ser direcionados a um tipo específico de câncer e superar resultados negativos de ensaios anteriores (COMASSETO, 2010; BARTOLINI et al., 2017; GANDIN et al., 2018). Os principais fatores que tem contribuído para o desenvolvimento desse campo são: 1) o selênio pode ser introduzido em substratos orgânicos como eletrófilo ou nucleófilo (WAITKINS; CLARK, 1945); 2) selênio quando ligado a grupos metálicos possui função catalítica potencializada. Alguns compostos com selênio podem ser precursores para síntese de nanopartículas (YAO et al., 2004; KEDARNATH; JAIN, 2013); 3) a descoberta de Ebselen como um promissor oxidante, fez com que se propusesse a síntese de novos compostos com selênio (SIES; MASUMOTO, 1997; WIRTH, 2015; REICH; HONDAL, 2016).

Dentro das classes de compostos orgânicos com selênio, podemos destacar o Ebselen como sendo a principal molécula estudada, sendo referência para estudos dos demais compostos com selênio (BARTOLINI et al., 2017). O composto Ebselen foi sintetizado pela primeira vez em 1924, por Lesse e Weiss, e considerado irrelevante até os anos 60. Só em 1984, quando descobriram a capacidade de mimetizar a atividade enzimática da glutationa peroxidase (GPx) que o composto se tornou relevante e estudos posteriores visaram estudar as propriedades bioquímicas e farmacológicas do composto (MULLER et al., 1984; AZAD; TOMAR, 2014). Recentemente o composto Ebselen tem sido aplicado em testes clínicos, porém os resultados da sua ação terapêutica não foram conclusivos para corroborar a eficácia do composto (BARTOLINI et al., 2017). Os testes *in vitro* e *in vivo* tem sido promissor, quando aplicados a modelos de célula tumorais e em camundongos (GANDIN et al., 2018).

Em geral os compostos com selênio são classificados em selenidos/diselenidos, derivados de ácidos selenoaminos, ácido selênico ou metabólitos do selênio inorgânico (BARTOLINI et al., 2017; GANDIN et al., 2018).

2.3.1 Metabolismo, farmacologia e toxicidade dos compostos com selênio

Estudos contínuos acerca da absorção, metabolismo e distribuição do selênio são necessários para relacionar com o melhoramento da saúde de pacientes e evitar efeitos adversos dos compostos (KURŠVIETIENĖ et al., 2020). Os efeitos tóxicos do selênio são estritamente dependentes da concentração e da natureza da espécie do organosselênio (SANTI et al., 2018). Os efeitos antitumorais de selênio são causados por diferentes mecanismos que incluem: indução de parada de ciclo celular e apoptose, inibição da capacidade de invasão e migração celular, bloqueio de angiogênese, modulação da proliferação celular (DRUTEL et al., 2013; WHANGER, 2004; CHEN et al., 2013; KURŠVIETIENĖ et al., 2020).

Embora o mecanismo de metabolização varie de um composto a outro, estudos demonstram que as funções pró-oxidantes de muitas estruturas de compostos com selênio são a chave para o efeito antitumoral (MISRA et al., 2015).

A atividade pró-oxidante pode ser um potente inibidor para o crescimento celular e até mesmo em algum tipo específico de tumor (MISRA et al., 2015). Reações de oxidorredução são essenciais para a manutenção da homeostase

fisiológica em organismos e quando ocorre um desequilíbrio entre a geração de espécies reativas e os níveis das defesas antioxidantes pode-se verificar o estresse redox (HELMUT et al., 2017). Esse efeito redox dos compostos com selênio os torna exímios candidatos a quimioterápicos devido a algumas particularidades das células tumorais que podem apresentar níveis elevados de espécies reativas de oxigênio (EROs) e sistema antioxidante superexpresso (DEBERARDINIS; CHANDEL, 2016; GALADARI et al., 2017). Por esses efeitos, estudos envolvendo compostos com selênio exploram a geração de EROs, oxidação de grupamento tióis em proteínas e ligação ao DNA a níveis tóxicos capazes de induzir morte em células tumorais (FERNANDES; GANDIN, 2015; GANDIN et al., 2018). Esses mecanismos podem induzir a morte da célula pelo processo de apoptose, como no estudo observado por Maraldi et al. (2011). Os compostos: selenito de sódio (0,1 μM), selenato (0,5 μM) e seleno-metionina (0,5 μM) foram capazes de induzir morte celular por apoptose em células de neuroblastoma SKNBE mediada por EROs, após 24 horas de incubação.

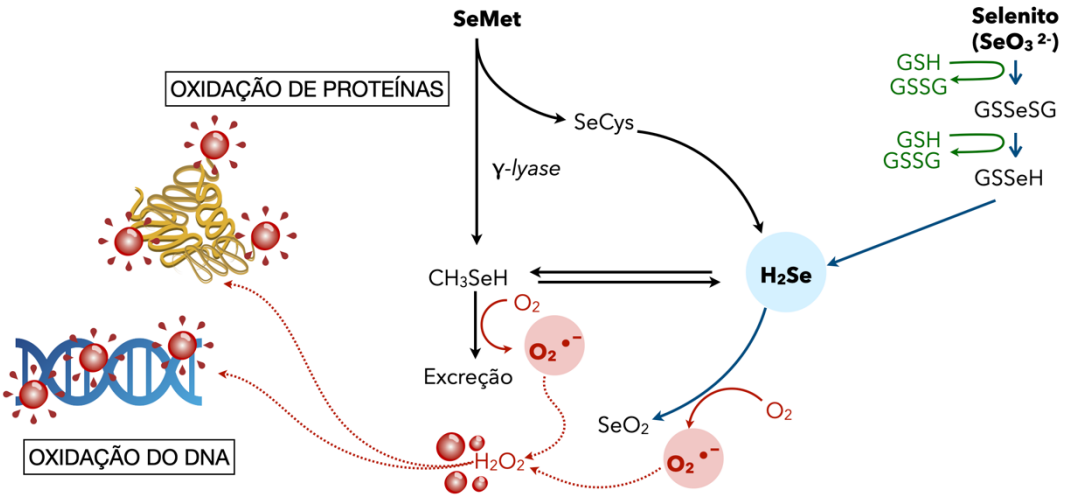
Estresse oxidativo resulta em danos mediados por radicais livres de forma direta ou indireta ao DNA e também tem sido relacionado com a inibição da maquinaria de reparo do DNA (WINTERBOURN, 2008; SANMARTÍN et al., 2012). Devido ao elevado nível basal de EROs, as células tumorais tornam-se mais suscetíveis a estresse oxidativo do que células normais, pois os sistemas antioxidantes endógenos podem ser sobrecarregados. Esta vulnerabilidade biológica que as células cancerosas possuem, as torna alvo para potenciais agentes terapêuticos que possuem a capacidade de elevar os níveis de EROs e por meio disso promover a morte celular (SANMARTÍN et al., 2012).

O estado redox das células é coordenado por sistemas dependentes de grupamentos tióis e quando ocorre a interrupção desses circuitos pode resultar no estresse oxidativo (JONES, 2008). Sendo a glutationa um desses reguladores para os sistemas glutationa reduzida (GSH)/glutationa oxidada (GSSH), glutaredoxina (Grx) e tioredoxina (TRx)/tioredoxina redutase (TRr) (TAKAHASHI et al., 2005; RAY et al., 2012). Stańczyk et al. (2010), em seu trabalho com o composto selenol (Se IV) (20 mg/kg) evidenciaram alteração nas concentrações de GSH relacionadas com grupos tióis quando aplicado em camundongos. No estudo feito por Chen et al. (2007) utilizando três compostos com selênio (selenito de sódio, selenocistamina e ácido disselenodipropiônico), observaram a oxidação da glutationa com geração de superóxido, sugerindo então que essas espécies catalíticas que produzem

superóxido eram os ânions $GSSe^-$ ou RSe^-. A formação desses ânions de selênio se ocorre a partir da reação do selenito com glutationa ou outros grupamentos tióis.

Na FIGURA 3 podemos observar esquematicamente a gerações de EROs através das reações metabólicas do composto inorgânico selenito e do composto orgânico seleno-L-metionina em humanos. Na reação de selenito com grupamentos tióis de glutationa reduzida inicialmente é gerado uma selenodiglutationa (GSSeSG) e após reduções subsequentes, forma-se o selenido (H_2Se). Esse útlimo reage com oxigênio gerando dióxido de selênio (SeO_2) e ânion superóxido. Em paralelo, a seleno-L-metionina (SeMet) é convertida enzimaticamente a monometilselenol (CH_3SeH) ou em selenocisteína (SeCys) através da via de trans-selenação, gerando o selenido como intermediário comum para reações metabólicas dos compostos com selênio (GANTHER et al., 1968; CHAUDIERE et al., 1992; SEKO et al., 1997; MISRA et al., 2015; KURŠVIETIENĖ et al., 2020).

FIGURA 3 – ESQUEMA GERAL DA METABOLIZAÇÃO DE COMPOSTOS ORGÂNICOS E INORGÂNICOS DE SELÊNIO



FONTE: Adaptado de Kuršvietienė et al. (2020).

Por outro lado, quando se trata de morte celular, designar o tipo de morte muitas vezes requer um grande processo de avaliação de marcadores e sinais celulares. Recentes relatos na literatura, indicam que a morte celular causada por compostos com selênio pode ocorrer por outras vias além da apoptose, como autofagia ou necrose e até combinação entre as vias, pois muitas delas podem se sobrepor (SINGLETARY; MILNER et al., 2008; KURŠVIETIENĖ et al., 2020). A autofagia e apoptose podem ser ativadas por sinais comuns entre si e alguns

compostos com selênio como a Se-DL-cistína (100 μM), nesse contexto foi capaz de induzir mais de um tipo de morte celular ao mesmo tempo em células HeLa, após 6 a 12 horas de tratamento. Esses dados colaboram com a pesquisa onde se busca respostas dos compostos em mais de um alvo molecular, a fim de prevenir resistência a medicamentos (WALLENBERG et al., 2014).

Embora estudos sugiram propriedades antitumorais dos compostos com selênio, é necessário mais dados para se tentar elucidar o papel dos compostos quando aplicados a terapias e até mesmo em combinações com outras drogas com a finalidade de modular a eficácia e toxicidade das terapias (KURŠVIETIENĖ et al., 2020).

2.3.2 Compostos com selênio aplicados em testes clínicos

Diferentes classes de compostos com selênio têm sido sintetizadas e exploradas como agente antiproliferativo e esse campo está emergindo cada vez mais, com vários compostos demonstrando atividade citotóxica em células tumorais (GANDIN et al., 2018).

Atualmente alguns compostos com selênio têm sido avaliados em ensaios pré-clinicos em pacientes diagnosticados com câncer. O Ethaselen, um inibidor de tioredoxina redutase, se mostrou capaz de inibir crescimentos de células tumorais e induzir apoptose em pacientes chineses acometidos de câncer no pulmão. Os resultados mostraram que a dose diária de 120 mg de ethaselen pode ser bem tolerada e segura (ClinicalTrials.gov identifier: NCT02166242) (WANG et al., 2012).

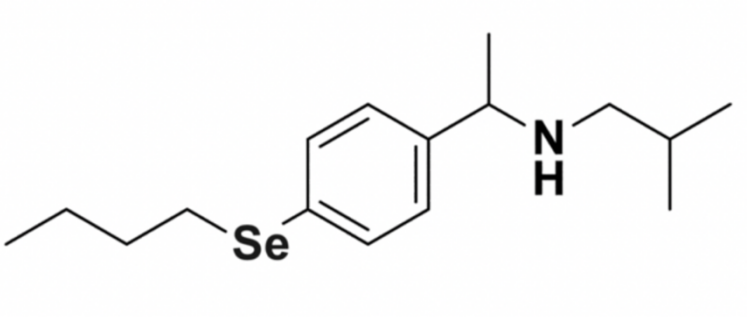
No estudo reportado por Sieja et al. (2004), suplementação com selênio (200 μg/dia) por 3 meses, reduziu significativamente os efeitos colaterais provocado pelo tratamento com cisplatina combinada com ciclofosfamida em pacientes com tumor ovariano, demonstrando a capacidade do selênio e seus compostos na aplicação terapêutica combinada.

2.3.3 LQ-20

Tendo em vista o interesse em aprimorar a seletividade, eficácia e especificidade, amenizando assim a toxicidade, novos compostos com selênio têm sido sintetizados.

Puccinelli e colaboradores sintetizaram novos compostos organosselenetos nitrogenados e verificaram de forma preliminar seus efeitos citotóxicos sobre a viabilidade de células tumorais e normal imortalizada. Avaliaram respostas distintas em células tumorais: HT-29 (câncer de cólon intestinal), PC-3 (câncer de próstata), HeLa (câncer colo útero) e em células não tumorais: HACAT (queratinócitos de pele humana) para um mesmo composto. Entre os compostos estudados o LQ-20 (FIGURA 4) foi o mais promissor por possuir maior efeito citotóxico em células tumorais (CC_{50} = 550,0 $\mu mol.L^{-1}$ para HeLa; 37,5 $\mu mol.L^{-1}$ para HT-29 e 78,3 $\mu mol.L^{-1}$ para PC-3) e menor em células não tumorais (CC_{50}= 195,5 $\mu mol.L^{-1}$). Contudo, seus mecanismos de ação ainda não são conhecidos, assim como suas propriedades redox. Por este motivo, neste trabalho avaliamos as propriedades pró-oxidantes e efeitos citotóxicos utilizando como modelo experimental as células SH-SY5Y.

FIGURA 4 – ESTRUTURA DA SELENOAMINA LQ-20 (N-(1-(4-(butilselanil)fenil)etil)-2-metilpropan-1-amina)



LQ-20

FONTE: Thiago Puccinelli (2018).

3 MANUSCRITO

Cytotoxic and prooxidant effects of a selenoamine (LQ-20) in human neuroblastoma cells (SH-SY5Y)

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Abstract

High-risk neuroblastoma is an aggressive extracranial solid tumor frequently diagnosed during infancy. Besides modern strategies of treatments, low survival rates and side effects still remain a challenge for patients, and there is an urgent need for effective treatments. Among new and synthesized compounds, selenium compounds are reported to have potential antitumor activity, and is a promisor chemotherapeutic agent. The present study, for the first time, investigated the cytotoxicity and antiproliferative activity of selenoamine LQ-20 using human neuroblastoma SH-SY5Y cells, and the relative toxicity in differentiated SH-SY5Y cells with retinoic acid (RA). SH-SY5Y cells were treated with different doses of LQ-20 (20-200 μM). Cell viability was detected using the MTT assay. Cell proliferation

was detected using crystal violet staining and tryplan blue exclusion method, followed by analysis in cell cycle through flow cytometry. We also evaluated reactive oxygen species (ROS) and alterations on intracellular reduced glutathione (GSH) levels of SH-SY5Y cells treated with LQ-20. LQ-20 (IC_{50} 63 μM) reduced the SH-SY5Y cells viability and proliferation in a time- and dose-dependent manner. LQ-20 at 50 and 100 μM induced cell death and promoted increase in fragmented DNA after 72 h of incubation. In addition, LQ-20 was responsible for ROS overproduction and GSH depletion in SH-SY5Y cells treated for 72 h, which correlated with a property of selenium compounds in promoting cell oxidative stress. LQ-20 (50 and 100 μM) and cisplatin (4 μM) incubation promoted reduction of viability in differentiated SH-SY5Y cells with RA and undifferentiated SH-SY5Y cells. However, when LQ-20 was used, the reduction in viability was greater in undifferentiated cells than in those differentiated with RA (neuron-like cells). These results suggest a difference in the effects promoted by LQ-20 in neuron-like cells when compared to the tumor cell. Thus, it is also possible to conclude that the selenoamine LQ-20 is capable of inducing SH-SY5Y cells death possibly through a mechanism that, at least in part, involves depletion of GSH and increase in ROS levels and may even have differences in tumor SH-SY5Y cells when compared to cells differentiated with RA.

Keywords Selenium compounds, Selenoamine, Oxidative stress, Chemotherapeutics, Neuroblastoma, SH-SY5Y

Introduction

Neuroblastoma is an extracranial solid tumor, diagnosed in the first year of life. The tumor arises from the developing sympathetic nervous system (from neural crest cell differentiation), frequently found in adrenal glands or sympathetic ganglia [1-3]. Worldwide, it is the most common sympathetic nervous system tumor and the incidence in children at age 0 to 4 years, accounts for approximately 15% of cancer deaths [4-6]. The clinical and biological presentations are extremely complex and heterogeneous, ranging from spontaneous regression to metastatic disease, and are associated with unfavorable outcomes in pediatric patients, which creates challenges for development of treatment and therapies [1, 7, 8].

Moderns treatments for neuroblastoma are defined according to risk groups, categorized into low, intermediate or high-risk group. Treatments for high-risk neuroblastoma involve strategic schematics of combined therapy, such as surgeries, radiotherapy, immunotherapy and intense sessions of chemotherapy. Chemotherapy combines agents such as cisplatin, carboplatin, topotecan, doxorubicin [9-11]. A lot of progress has been made in the treatment for high-risk neuroblastoma; however, the outcome for patients still remains poor, with long-term survival about 50% [10-11]. The main challenge in high-risk neuroblastoma treatment is to overcome tumor metastasis and resistance to chemotherapeutic drugs, thus there is an imminent need for new treatments and drugs [12].

Current studies with various structures of synthetic selenium compounds have been shown to promote anti-proliferative effects and cell death, clearly demonstrating a potential anticancer agent [13-16]. When selenium is applied in therapies, the progression of malignant cells are to be more sensitive to selenium cytotoxicity than normal cells [17]. Organic compounds with selenium produce a series of biological changes upon tumoral cells by activating or altering compartmentalization of molecular oxygen, interaction with protein thiols and selenoproteins, interference with redox homeostasis, and signaling to induce alterations in energy metabolism and cell cycle [18].

SH-SY5Y cell lines have certain morphological and biochemical properties in vivo that can be differentiated by specific externals agents, and all-trans retinoic acid (RA) is the most commonly used for this purpose, since it is a factor present in early neuronal development [19]. Differentiated SH-SY5Y cells have been used in studies aimed at the development of potential antitumor therapeutic drugs and also as a model for the study of cytotoxic effects on tumor cells and neurodegenerative diseases [20-21]. In this study we tested whether the selenoamine LQ-20 compound (Fig. 1) possessed a toxic effect on human neuroblastoma cell line (SH-SY5Y) and whether these effects are accompanied by generation of reactive species and alterations on cell viability, proliferation, and death pathways. In addition, SH-SY5Y cells were differentiated to make them suitable for investigating cytotoxic effects in cell lines with *neuronal-like* features. The findings might be important for understanding the detailed mechanism involved in the cytotoxic effect of LQ-20.

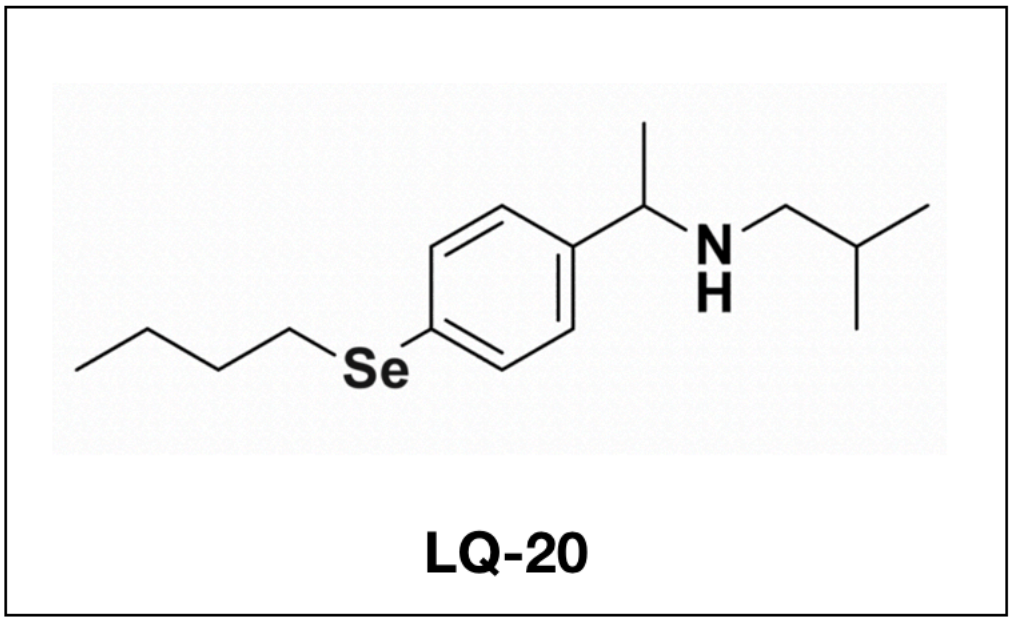

Fig. 1 Chemical structure of selenoamine LQ-20 [N-(1-(4-(butylselanil)phenyl)ethyl)-2-methylpropan-1-amine)].

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium-low glucose (DMEM), MEM non-essential amino acid solution (100×), thiazolyl blue tetrazolium bromide (MTT), trypan blue, propidium iodide (PI), ribonuclease A (RNase A), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), DL-buthionine-(S,R)-sulfoximine (BSO), N-acetyl-L-cysteine (NAC), tert-butyl hydroperoxide solution (TBH), monochlorobimane (mBCl), acridine orange hydrochloride hydrate (AO), cis-diammineplatinum(II) dichloride (cisplatin), triton X-100, hydrogen peroxide solution and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Êxodo Científica (Sumaré, SP, Brazil). All-trans retinoic acid (RA) was purchased from BioGems International (Westlake Village, ON, Canada). Fetal bovine serum (FBS) was obtained from Gibco (Invitrogen, NY, USA) and Nutrient Mixture Ham's F12 (F12) from Cultilab (Campinas, SP, Brazil). All other reagents were commercial products of the highest available purity grade.

LQ-20 solutions

The selenium compound LQ-20 was synthesized and kindly provided by Dr. Alfredo M. de Oliveira (Chemistry Department, Federal University of Paraná, Brazil) [22]. The compound LQ-20 was dissolved in DMSO to make a stock solution of 200 mM, and then further diluted in medium with equal amounts of DMEM and F12, to obtain different working concentrations (10-100 μM). In all of the experiments, the control condition established was a cell culture medium with 0.1% DMSO. The final concentration of DMSO in cell culture medium never exceeded 0.1% and had no effect on cell growth. The stock solution was stored at -20 ^{o}C.

Cell culture

Human neuroblastoma SH-SY5Y cells from ATCC (Manassas, VA, USA) were maintained in medium mixture DMEM/F 12 (1:1) supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acid solution (100×), 100 μg/mL streptomycin and 100 U/mL penicillin, in a 5% CO_2 humidified incubator at 37 ^{o}C. Cell medium was replaced every 48 h, and the cells were sub-cultured once confluence was reached [23].

Cell viability assay

Initially, the effect of LQ-20 on metabolic activity of cells was analyzed using the MTT assay [24] and after determining the IC_{50}. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well and were incubated at 37 °C for 24 h. After overnight incubation, medium was replaced and 200 μL of fresh medium containing a different concentration of LQ-20 was added (20, 50, 60, 100 and 200 μM). Cells were exposed for 24, 48, and 72 h. After treatment, each well was added with 20 μL of MTT (5 mg/ml) for 3 hours. Medium was removed, and the formazan crystals were dissolved with 200 μL of DMSO. The results were expressed as percentage of viable cells (MTT absorbance) in comparison to untreated cells (100%).

Cell proliferation assays

The Crystal violet and trypan blue exclusion assays were used to evaluate the effect of LQ-20 on cell proliferation [25, 26]. For crystal violet assay, adherent cells after incubation, were fixed with methanol, then cells were stained with crystal violet solution (0.2%). After washing, crystal violet was extracted with sodium citrate solution (0.05 M) in 50% ethanol. The absorbance of both experiments was measure at 550 nm using a microplate spectrophotometer (Epoch, BioTek). The results were expressed as percentage of cells (crystal violet absorbance) in comparison to untreated cells (100%).

In addition, for trypan blue exclusion assay, SH-SY5Y cells in a 24-well plate (5.85×10^4 cells/per well) were incubated with 50 μM of LQ-20. After 24, 48, and 72 h, cells were stained with 0.4% trypan blue solution. Cells which do not take up trypan blue were considered viable and were counted using an automated cell counter (Countess FL II, Thermo Fisher Scientific).

Cell cycle analysis

Cells were seeded in a 24-well plate at a density of 5.85×10^4 cells/per well. After 72 h of treatment with LQ-20 (50 μM), adherent cells were centrifuged; then immediately permeabilized with triton X-100 (0.01%) and stained with 50 mg/ml PI solution containing 100 μg/mL RNase A for 15 min, prior to analysis with a flow cytometry instrument (FACScalibur, BD Biosciences). PI staining is able to bind to DNA content of compromised cell membranes [27]. Results were express as percentage of cells in G0/G1, S, G2/M phases, and sub-G1.

Morphologic evaluation and staining with propidium iodide and acridine orange

The cells were plated at a density of 1×10^4 cells/well in 96-well plate and incubated with LQ-20 (50 and 100 μM) for 72 h. Images of morphological characteristics of the SH-SY5Y cells were obtained using an inverted fluorescence phase contrast (Axiovert 40 CFL Trinocular, Zeiss), and after treatment, PI (200

µg/mL) and AO (5 µg/mL) were added to cells for 15 min, and medium was removed before imaging [28].

Measurement of intracellular ROS generation

The intracellular ROS levels were measure using the fluorescent probe DCFH-DA [29]. SH-SY5Y cells were treated with LQ-20 (50 and 100 µM) for 72 h onto 96-well plates (1×10^4 cells/well). After the treatment, cells were washed with phosphate buffer solution ×1 (PBS) and then incubated for 30 min with DCFH-DA (10 µM). Following incubation with DCFH-DA, each well was washed with PBS again, and after that, the fluoresce was evaluated immediately using a microplate reader (Infinite M200, Tecan) at wavelengths of 485 nm for excitation and 520 nm for emission. Images of SH-SY5Y cells exposed to LQ-20 were obtained using an inverted fluorescence phase contrast microscope (Axiovert 40 CFL Trinocular, Zeiss). A positive control with TBH (250 µM) treatment and H_2O_2 (400 µM), for 4 h, was used in cells.

Determination of GSH levels

In order to investigate the effects on glutathione (GSH) levels, cells (1×10^4 cells/well) were assessed with a mBCl fluorescent probe [30]. After a 72 h exposure to LQ-20 (50 and 100 µM), the medium supernatant was discarded, then each well was washed with PBS. Cells were stained for 25 min with mBCl (10 µM) in PBS. After incubation with mBCl wells were washed twice and fluorescence was recorded before with PBS as a blank and after adding the probe into a microplate reader (Infinite M200, Tecan) at the excitation/emission wavelengths (385/485 nm). Images were captured with a fluorescence microscope (Axiovert 40 CFL Trinocular, Zeiss). Treatments with H_2O_2 (400 µM), BSO (50 µM), and NAC (5 mM) for 72 h were used as control groups [31, 32].

RA-induced differentiation

SH-SY5Y cells were seeded at an initial density of 2.5×10^4 cells/well in 24-well plate in medium DMEM/F12 supplemented with 10% FBS, 1% MEM non-

essential amino acid solution (100×), and antibiotics. After 24 h of cellular adhesion, the medium was replaced and reduced to DMEM/F12 with 1% FBS, 1% amino acids, and antibiotics, then cells were incubated for further 96 h with RA (2μM). Medium was replaced every 48 h. At day 4, the medium was substituted by determined treatments, and cells were used for experiments [33, 34]. Differentiated and treated cells were stained with hematoxylin and eosin. Images were captured with a fluorescence microscope (Axiovert 40 CFL Trinocular, Zeiss). Cisplatin (4 μM) treatment was used as control of cell death.

Statistical analysis

Data obtained were expressed as mean ± standard error of the mean. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by Tukey's multiple group comparison test. $P<0.05$ was considered significant.

Results

Cytotoxicity effect of LQ-20 compound on cell viability and proliferation of neuroblastoma cells

Initially, we evaluated the cytotoxic effect of LQ-20 (20, 50, 60, 100, and 200 μM) on SH-SY5Y cells treated for 24, 48, and 72 h by MTT assay. As shown in Fig. 2, LQ-20 significantly decreased cells viability, in a time and concentration-dependent manner (48 and 72h of incubation). After a 24 h incubation with LQ-20 60, 100, and 200 μM the viability of SH-SY5Y cells was reduced around of 20%. However, for a 48 h incubation, cell viability decreased about 24, 27, 30.6, and 55% in 50, 60, 100, and 200 μM concentrations, respectively. Indeed, at the later time-treatment (72 h) the cell viability reduction was of 30, 46, 66, and 75% to concentrations of 50, 60, 100, and 200 μM of LQ-20. The IC_{50} value (drug concentration required to inhibit 50% of cell growth) obtained was 63 μM for 72 h of treatment.

In the subsequent experiments using crystal violet staining we observed that LQ-20 50 and 100 μM caused a reduction in cell proliferation of about 30 and 70%,

respectively, after 72 h of incubation when compared to the control (100%) (Fig. 3A). These results agree with those obtained using the MTT assay using the same concentrations of LQ-20. In addition to the effect of LQ-20 on SH-SY5Y cell proliferation, the trypan blue exclusion assay was used. Results showed that, 50 μM of LQ-20 did not significantly reduce cell proliferation for 24 and 48 h, but significantly reduced cell proliferation to approximately 21% after 72 h of incubation with 50 μM of LQ-20 (Fig. 3B).

Moreover, morphological changes in the cells were observed through an inverted fluorescence phase contrast microscope, after 72 h following exposure to LQ-20 (50 and 100 μM), using propidium iodide (PI). The red fluorescent DNA-intercalating dye PI allow the microscopic to distinguish morphologically between living and dead cells. PI is membrane-impermeable and only stains the red nucleus of nonintegrable cells. In Figure 3C, an increase in cell death is observed when the cells were incubated with LQ-20 (50 and 100 μM).

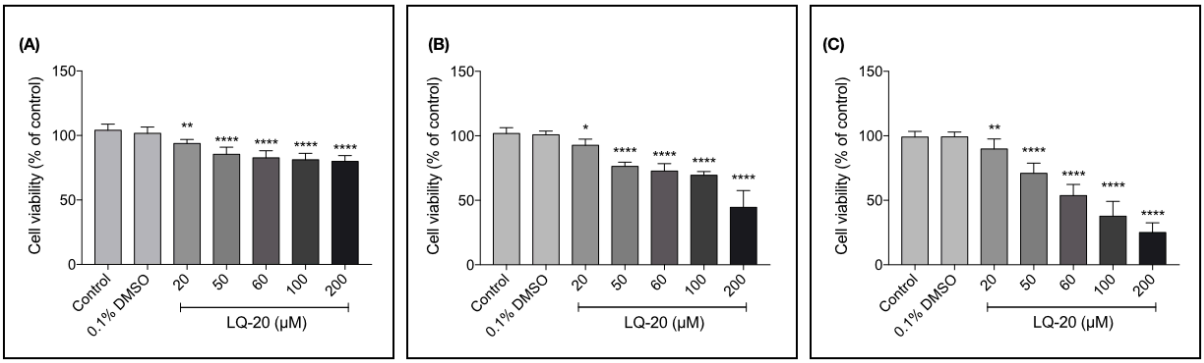

Fig. 2 Cytotoxic effects of LQ-20 on human neuroblastoma SH-SY5Y cells for **(A)** 24 **(B)** 48 **(C)** 72 hours incubation. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with (*) $p<0.05$; (**) $p<0.005$; and (****) $p<0.0001$.

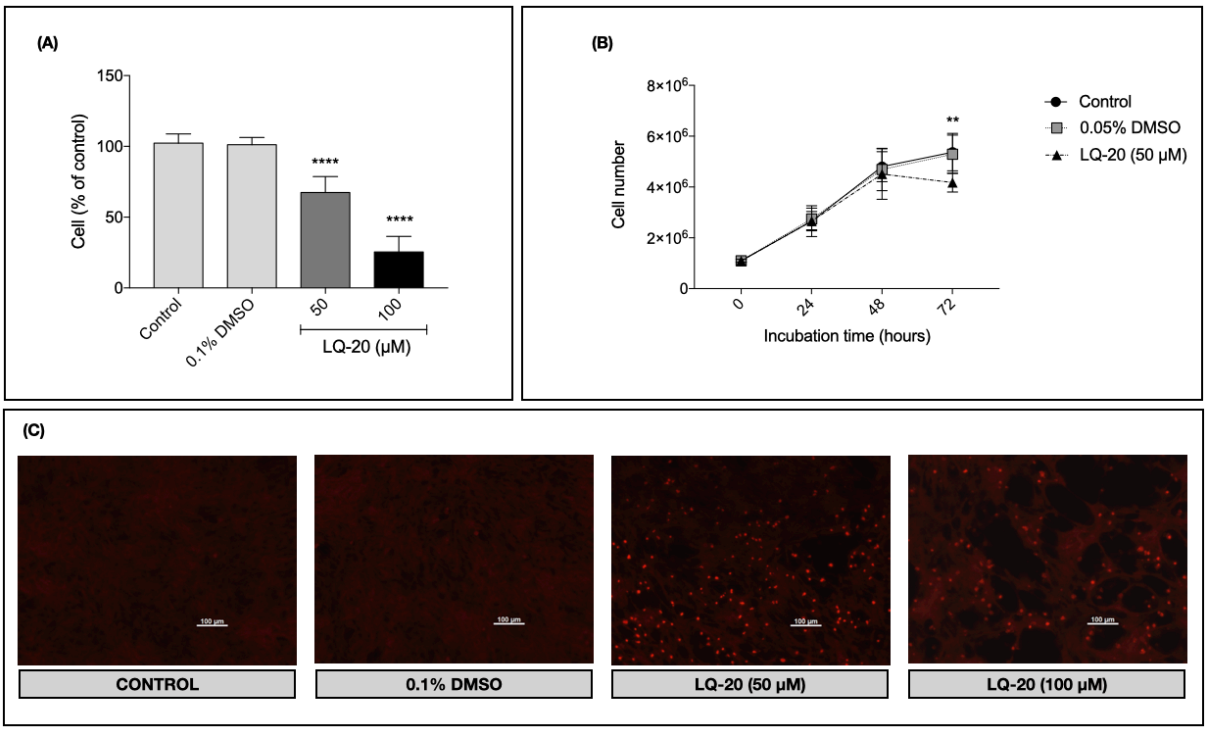

Fig. 3 LQ-20 induced cytotoxic effects on SH-SY5Y cell proliferation and number of viable cells. **(A)** LQ-20 significantly decreased the cell proliferation with 50 and 100 µM concentrations after 72 hours of incubation compared to control cells by crystal violet staining. **(B)** Cell proliferation curves of SH-SY5Y determined by trypan blue assay. Number of SH-SY5Y cells after exposure to 50 µM LQ-20, for 24, 48 and 72 hours. **(C)** Propidium iodide (PI, red) staining SH-SY5Y cells obtained under fluorescence microscope. Untreated control cells and DMSO control cells. Treatment with 50 µM LQ-20 for 72 h. and Treatment with 100 µM LQ-20 for 72 h. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with (**) $p<0.005$, (****) $p<0.0001$. Magnification ×10

LQ-20 effect on SH-SY5Y cells cycle progression

Flow cytometry analysis confirmed that LQ-20 produced SH-SY5Y cell death, shown by accumulation of sub-G1 cell debris (Fig. 4A). Approximately 40.5% of cells treated with 50 µM were observed in sub-G1 phase after 72 h of incubation (Fig. 4B), demonstrating an increase in DNA fragmentation. This compound also caused a significant decrease in the number of cells in the G0/G1, S, and G2/M phase about 22, 3.77, and 13% respectively (Fig. 4C). Therefore, 50 and 100 µM were selected to investigate its prooxidant effects.

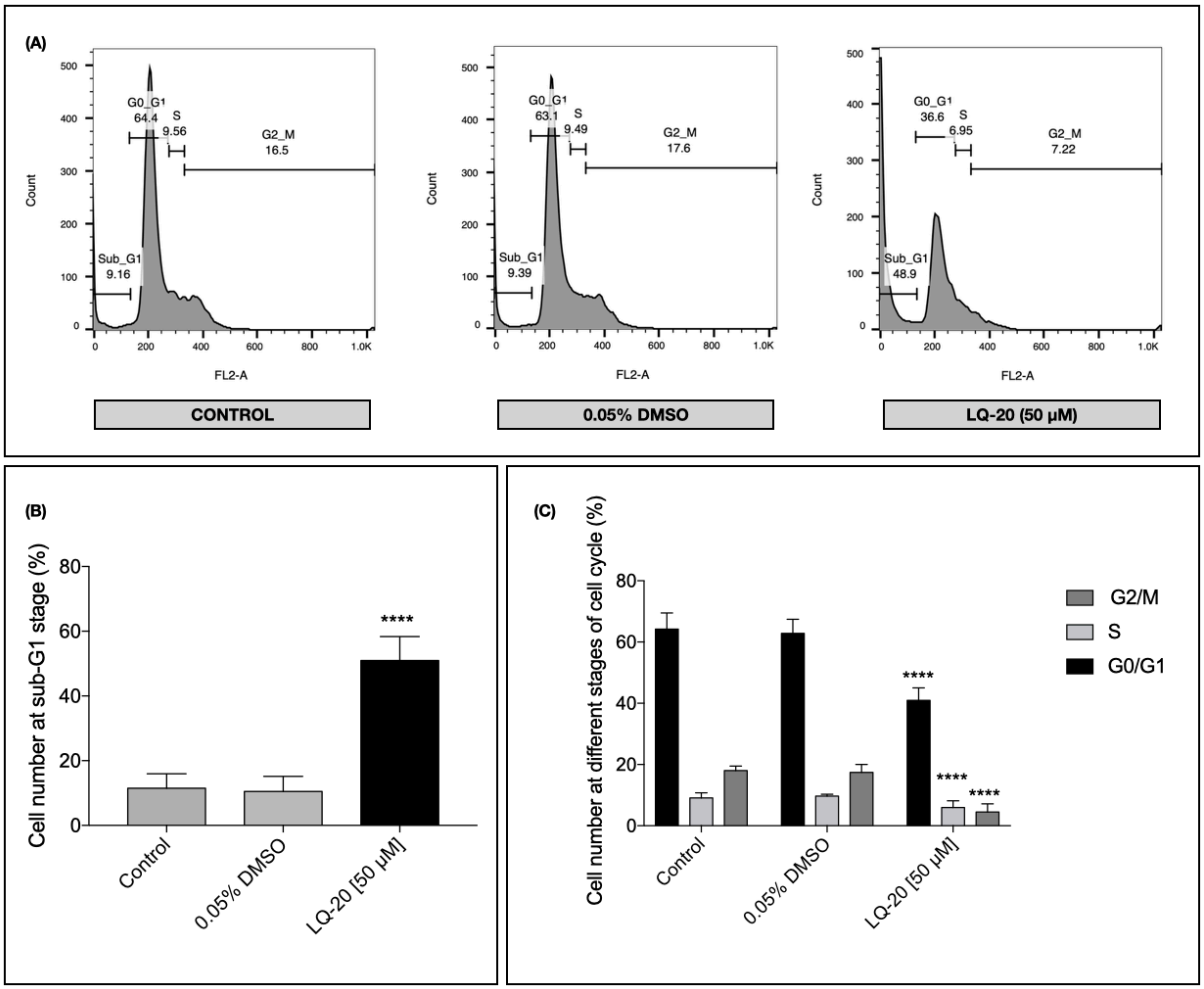

Fig. 4 Effects of LQ-20 on cell cycle in SH-SY5Y cells. The cells treated with 50 µM concentration of LQ-20 for 72 h, collected and stained with PI. **(A)** Representative histograms of DNA content of cells. **(B)** Quantification of the percentage of cells in sub-G1 stage. **(C)** Quantification of the percentage of cells in each cycle stage. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with (****) $p<0.0001$.

ROS generation in the SH-SY5Y cells induced by LQ-20 treatment

Oxidative stress is known to cause death cell after treatment of tumor cells with some selenium compounds. Therefore, to verify this hypothesis we used a DCFH-DA fluorescence probe to analyze intracellular ROS levels. Images of control cultures of SH-SY5Y cells and treated cells are shown in Fig. 5A. An approximate significant 2-fold increase in intracellular ROS levels was observed in SH-SY5Y cells treated with 50 and 100 µM for 72 h when compared to the control group (control and

DMSO) (Fig. 5B). Additionally, we used TBH (250 µM) and H_2O_2 (400 µM) treatments as positive controls of ROS generation.

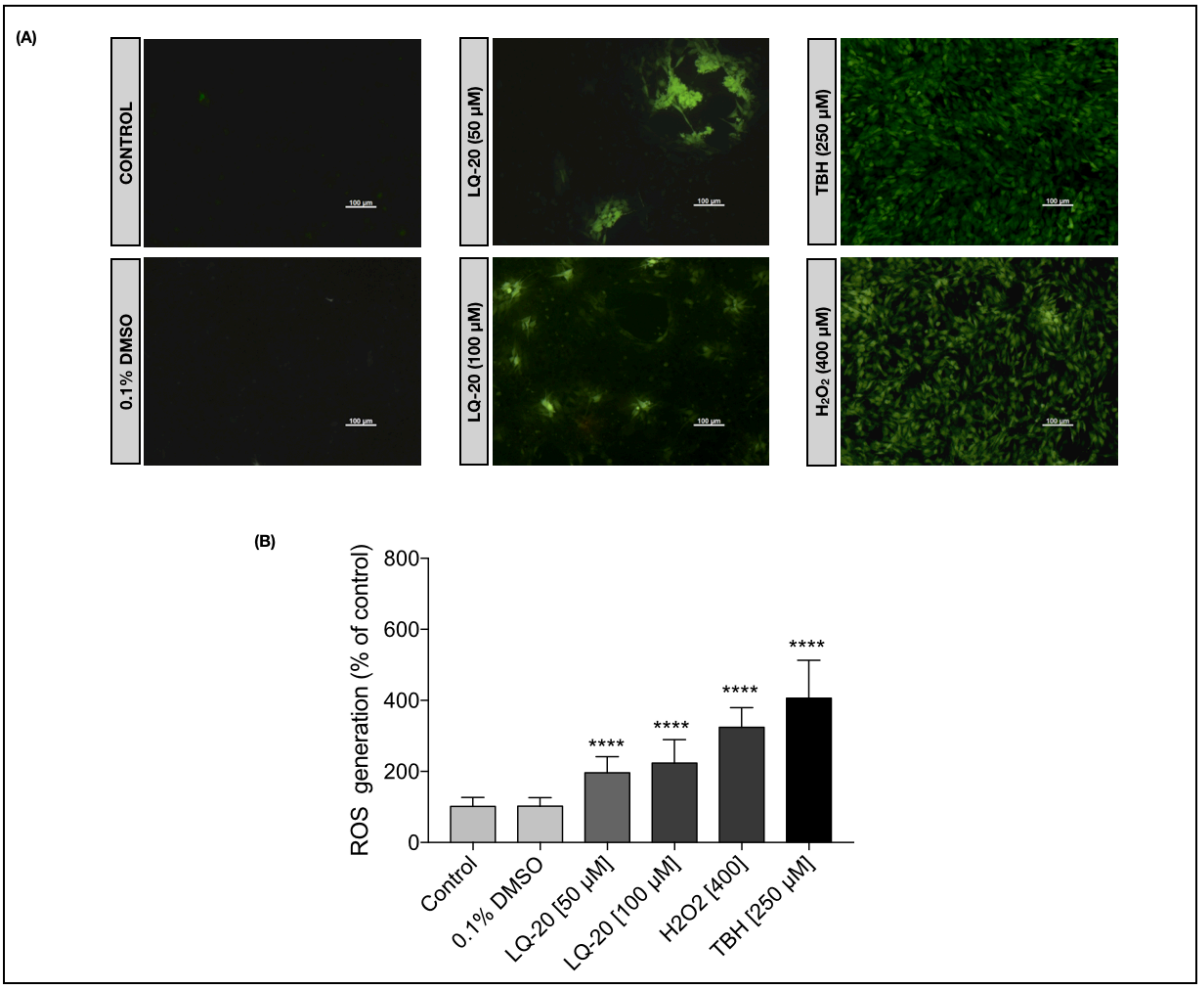

Fig. 5 Effects of LQ-20 on intracellular ROS levels of SH-SY5Y cells. **(A)** Fluorescence micrographs of SH-SY5Y cells: Untreated control cells, DMSO control cells, cells exposed to LQ-20 (50 and 100 µM) showing intracellular green DCF fluorescence as a result of ROS production. TBH (250 µM) and H_2O_2 (400 µM) were used as a positive control of ROS. **(B)** Levels of ROS in SH-SY5Y cells treated with LQ-20 (50 and 100 µM) for 72 hours. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with asterisks (****) $p<0.0001$. Magnification ×10

Depletion of cellular GSH levels

In order to check if the LQ-20 could deplete GSH, the mBCl fluorescent probe was used. As shown in Fig. 6A, incubation of SH-SY5Y cells with LQ-20 (50 and 100 μM) for 72 h resulted in a significant decrease of GSH levels, in a concentration-dependent manner. Incubation of SH-SY5Y cells with 50 μM of LQ-20 led to about 13% of reduction of cellular GSH levels, and when incubated with LQ-20 100 μM, it is 58% lower. Treatment of SH-SY5Y cells with H_2O_2 (400 μM) and BSO (50 μM), an inhibitor of γ-glutamylcysteine ligase (γGCL) in cellular GSH biosynthesis [35] were used as a positive depletion control. Another used control was NAC (5 mM) for its ability to be a cysteine precursor which is converted intracellularly into GSH and there was an increase of 78% in observed GSH levels [36].

Incubation of SH-SY5Y cells with LQ-20 100 μM for 72 h promoted reduction in cellular GSH content similar to that observed with BSO (50 μM) (40%) and with H_2O_2 (400 μM) treatment for 4 h (65% decrease) when compared with control and DMSO control, showing an important depletion of GSH levels promoted by LQ-20. In Fig. 6B, the blue fluorescence of mBCl was weaker than controls when LQ-20 was added.

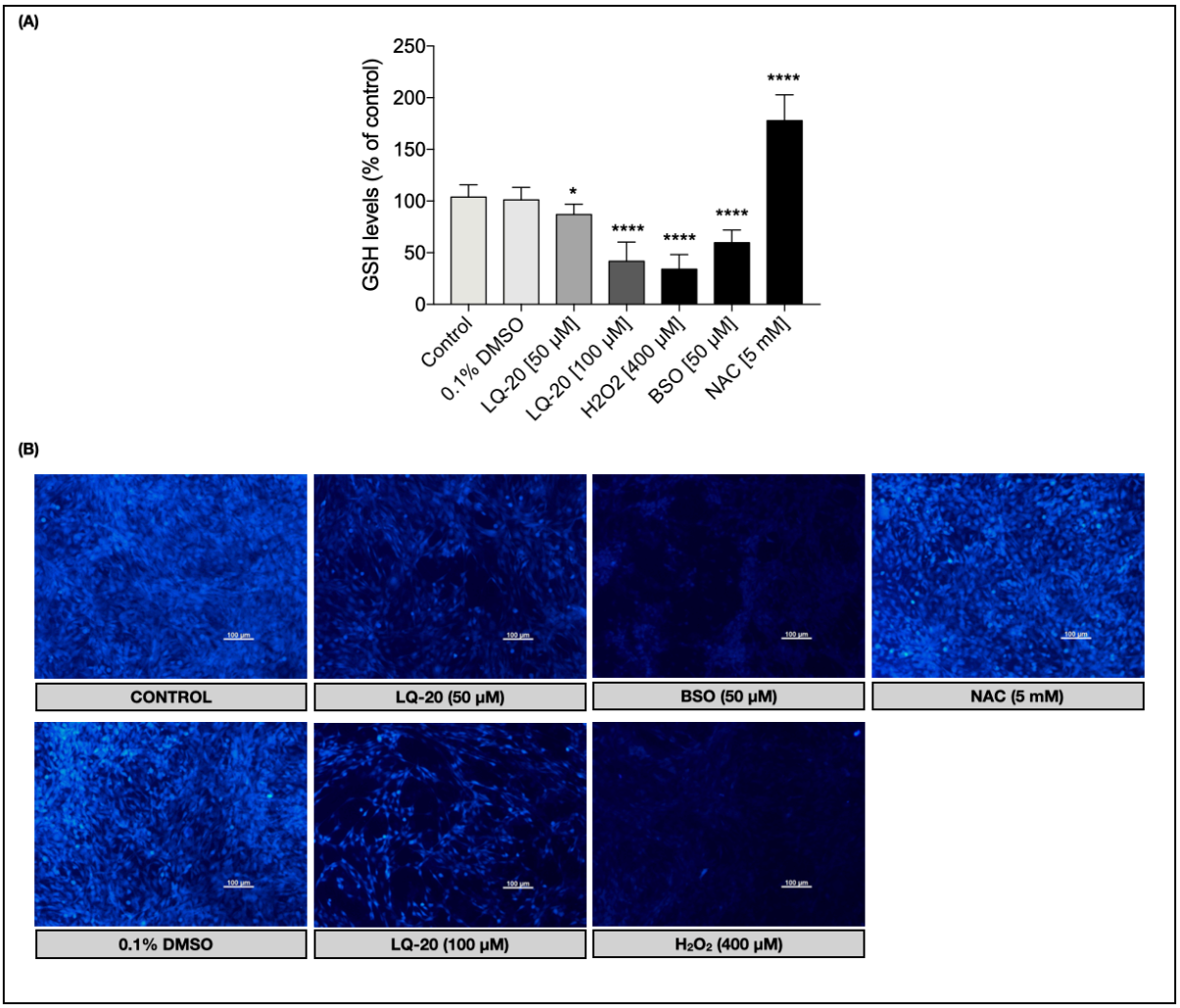

Fig. 6 Effects of LQ-20 on GSH content in SH-SY5Y cells. **(A)** SH-SY5Y cells were treated with LQ-20 (50 and100 µM) for 72 h, and then the effects of LQ-20 on GSH content were monitored. Treatment with BSO (50 µM) and H_2O_2 (400 µM) as a positive control group of GSH depletion, and with NAC (5mM) as a GSH precursor. Photomicrographs of SH-SY5Y cells stained with mBCl fluorescent probe. **(B)** Untreated control cells; DMSO control cells; Treatment with 50 µM LQ-20 for 72 h; Treatment with 100 µM LQ-20 for 72 h with BSO (50 µM), H_2O_2 (400 µM) as a positive control group of GSH depletion, and with NAC (5mM) as a GSH precursor. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with (****) $p<0.0001$. Magnification ×10

Cell death promoted by LQ-20 using Acridine orange assay

During the autophagy process, organelles and other cytoplasmic constituents can be sequestered to form the autophagosome that will fuse with lysosomes for their degradation to occur. The acridine orange (AO) dye is used because it is a membrane-permeable dye that diffuses through the membranes of living cells, and it

is a weak base that accumulates inside acidic vesicles within cells. The cytoplasm and nucleolus fluoresce in green and the acidic compartments fluoresce in orange.

The fluorescent dye acridine orange was used to observe the LQ-20 effects upon death pathways of SH-SY5Y cells. Control SH-SY5Y neuroblastoma cells showed integrity in green cell membranes and nuclei when stained with AO, indicating that the cells are viable (Fig. 7A). This can be observed in Figure 7A, where cell bodies are stained orange (yellow arrows) in treated cells with 50 μM of LQ-20, and clearly shown in treated cells with 100 μM concentration, representing hallmarks of cell death suggestive of autophagy. When chloroquine (25 μM) was added together with LQ-20, the number of cells marked with AO was greater than that observed only with the addition of chloroquine, indicating that the effects of inducing cell death by LQ-20 remained. However, further analysis would be needed to confirm this hypothesis.

Figure 7B shows the effects of LQ-20 (50 and 100 μM) on SH-SY5Y cell morphology, indicating a drastic decrease in cell number with loss of cell contact, even cytoplasm content. As shown in Fig. 2C, the nucleus of cells treated with LQ-20 (50 and 100 μM) are stained red when compared with the control group (control and DMSO) indicating that permeabilization and cell death occurred.

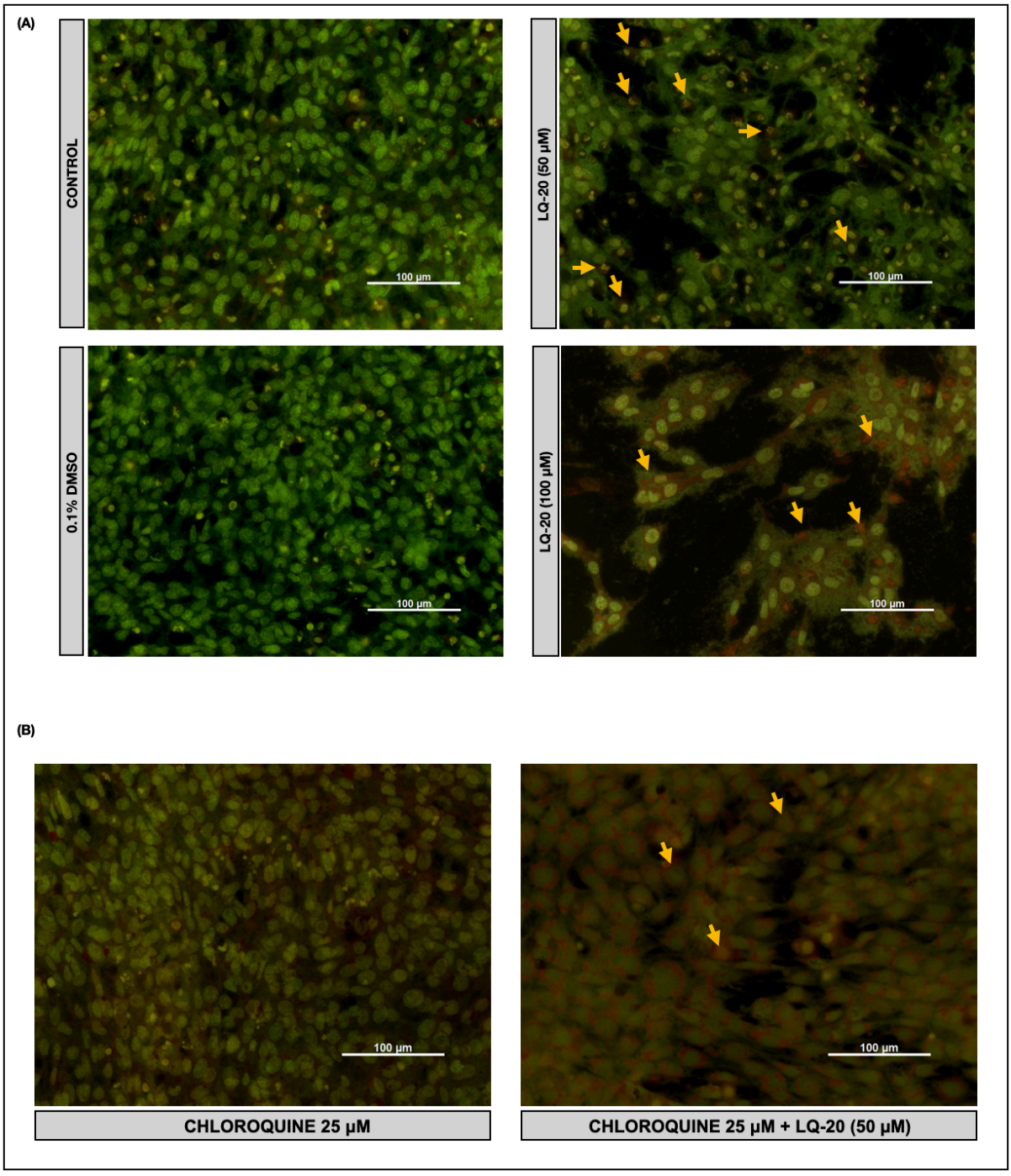

Fig. 7 Fluorescent micrographs of acridine orange (AO, green) staining SH-SY5Y cells obtained under fluorescence microscope. **(A)** Untreated control cells, DMSO control cells (green stained). Treatment with 50 µM LQ-20 for 72 h. Treatment with 100 µM LQ-20 for 72 h. **(B)** Cells incubated with chloroquine 25 µM only and treatment with 50 µM LQ-20 + chloroquine 25 µM, for 72 h. Yellow arrows indicating cell bodies. Magnification ×20

Cell differentiation by retinoic acid and treatment with LQ-20

In order to explore the possible different effects of LQ-20 in SH-SY5Y cells and differentiated SH-SY5Y cells (neuron-like), retinoic acid (RA) treatment was used. Initially the effects of different concentrations of RA were verified (data not shown) and the concentration of RA (2 μM) was chosen. Cells differentiated with RA (2 μM) for 96 h were then subjected to sequential incubation with LQ-20 (50 μM and 100 μM) for 72 h. Cisplatin (4 μM) was used to compare the effects because it is one of the primary drugs used for neuroblastoma treatment [37]. LQ-20 (50 and 100 μM) treatment leads to a reduction in cellular viability of about 12% and 17% respectively, with no statistical difference between concentrations (Fig. 8A). Cisplatin induced a decrease in SH-SY5Y cell viability 72 h post treatment, about 14.5%. As shown in Fig. 8C, SH-SY5Y cells exposed to RA (2μM) appear to undergo morphological changes when compared with undifferentiated cells, which is demonstrated by alterations in cell proliferation and increased neuronal characteristics (neuron-like); however, more analysis is necessary to confirm the differentiation, since differentiated SH-SY5Y cells have been demonstrated to express a variety of markers of mature neurons such as neuronal nuclei protein (NeuN), synaptophysin (SYN), neuron specific enolase (NSE), tyrosine hydroxylase (TH), β-III tubulin, and microtubule associated protein (MAP) [34].

In order to compare, the effects of RA (2 μM), LQ-20 (50 and 100 μM) and cisplatin (4 μM) for 72 h in DMEM/F12 (1% FBS) medium without differentiation, cell viability was evaluated using MTT assay (Fig. 8B). Cisplatin (4 μM) induced a decrease in SH-SY5Y cell viability of about 38%, LQ-20 (100 μM) treatment induced approximately 48%, but interestingly LQ-20 50 μM treatment had the same effect in viability as RA (2 μM) treatment, with about a 20% reduction in cell viability.

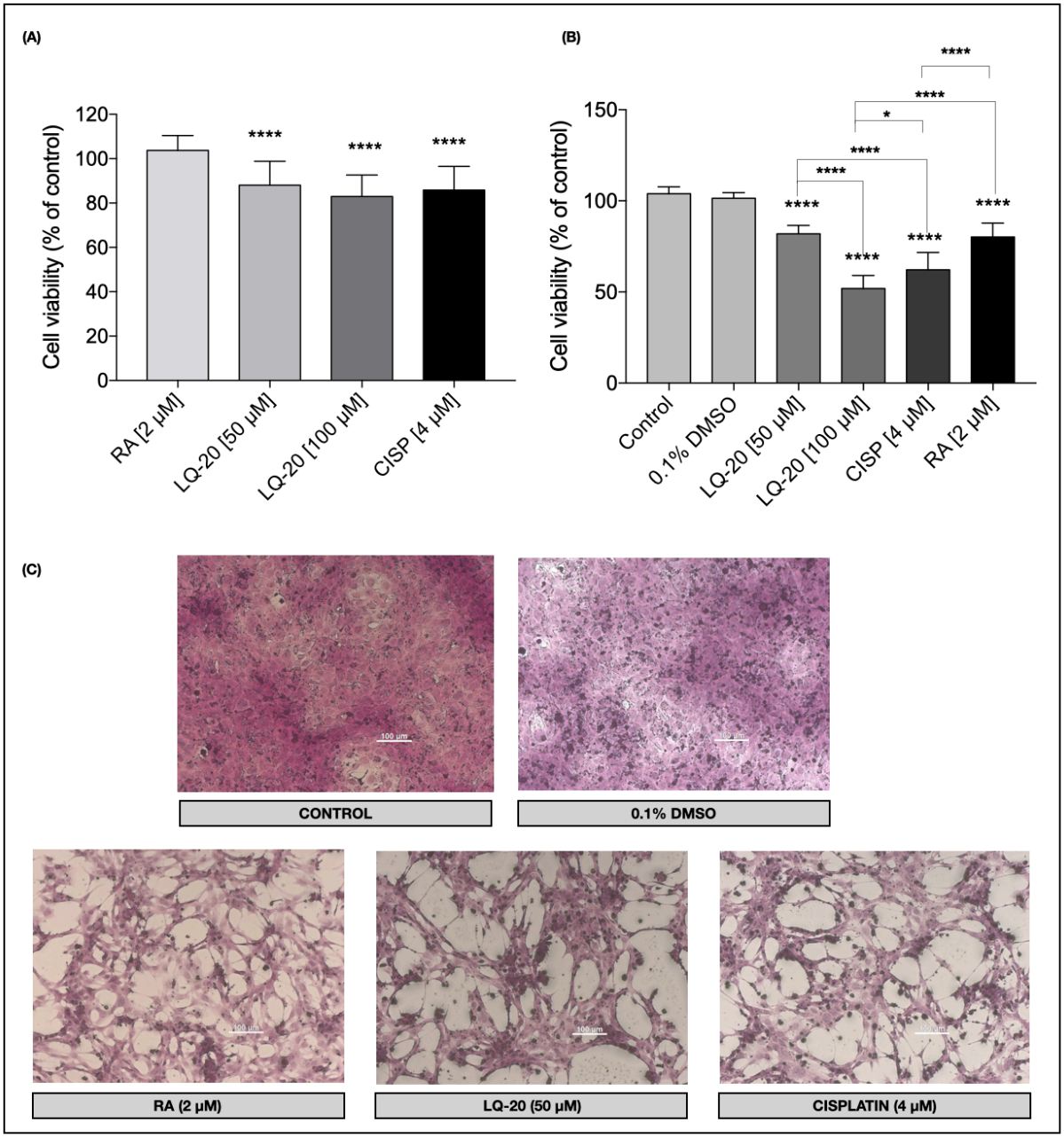

Fig. 8 Cell viability evaluation of RA-differentiated SH-SY5Y cells. SH-SY5Y cells were exposed to RA (2 µM) for 4 days, after that, treated with LQ-20 (50 and 100 µM), cisplatin 4 µM (CISP). **(A)** Viability of SH-SY5Y cells upon differentiation treatment followed by LQ-20 and cisplatin treatments using MTT. **(B)** Effects of LQ-20 (50 and 100 µM) on viability of undifferentiated SH-SY5Y cells for 72 hours incubation in medium with fetal bovine serum reduced to 1%; 2 µM RA (retinoic acid) and 4 µM CISP (cisplatin) as comparative group of drugs. **(C)** Differentiation followed by LQ-20 and cisplatin treatments measured in images of H&E-stained taken in day 7; In addition, control of undifferentiated SH-SY5Y cells and DMSO 0.1% control cells. The data represent the mean of 3 independent experiments. Statistically significant differences between control DMSO (0.1%) and treated cells are represented with (*) $p<0.05$ and (****) $p<0.0001$. Magnification ×10

Discussion

Neuroblastoma is an aggressive extracranial and solid tumor in children. Considering the low survival rates associated with the disease, the majority of studies focus on high-risk patients [38-40]. In order to reduce toxicity and enhance the outcome in high-risk neuroblastoma patients, there is a search for alternative compounds to develop novel therapies [4, 41, 42].

Studies in the literature report that selenium compounds seem to be promising candidates as chemotherapeutic agents, due to the potential to cause several toxic effects in tumor cells and in vivo models [43-46]. Several mechanisms have been suggested to explain the cytotoxicity and anti-cancer activity of selenium compounds, such as pro-oxidant properties, mostly related to ROS generation, oxidation of protein thiols, and direct or indirect DNA binding [47, 48]. These mechanisms can be crucial in understanding the process involved in anti-cancer effects and are relevant to the cancer field.

In the current study, it was shown that LQ-20 (Fig. 1), a new synthesized selenium compound is capable of promoting cytotoxic effects in SH-SY5Y cells (Fig. 2). It can be observed that LQ-20 reduced viability in 48 and 72 h of treatment and is dependent on the time and concentration used (Fig. 2B, 2C) with IC_{50} 63 μM. Similar results were obtained by crystal violet staining and trypan blue assay to confirm the reduction observed in MTT assay in 72 h of treatment (Fig. 3A, B). It can be observed an antiproliferative effect of LQ-20 50 μM on SH-SY5Y cells after 72 h of incubation. These results agree with those observed in the literature, in which studies showed that compounds with selenium have potent anti-tumor activity that correlates with this reduction in viability and proliferation effects [45-48].

It was also evaluated if LQ-20 could interfere in the cell cycle of SH-SY5Y, and there was an increase in percentage of cells in sub-G1 phase and reduction in the number of cells in the other phases of the cell cycle, after treatment with LQ-20 50 μM (Fig. 4). The aforementioned data may provide indications of an increase in cells containing fragmented DNA, and that LQ-20 did not promote cell cycle arrest but cell death. As reported by Kim and cols., synthetic 5-phenylselenyl-methyl-2’-deoxyuridine (PhSe-T) and 5-methylselenyl-methyl-2'-deoxyuridine (MeSe-T), at 150 μM and 10 μM respectively, induced apoptosis in human leukemia cell line (HL-60) after 40 and 32 h of treatment, and were linked to ROS generation and DNA

damage/caspase activation [49]. Thus, it possible that the DNA fragmentations observed in this study could be related to an ROS increase observed in Fig. 5.

Apoptotic induction of tumor cells is a possible mechanism to promote the anti-cancer activity by selenium compounds, but this property is mainly dependent on the structure of the compound [45]. Besides the apoptotic pathway, there is evidence that cell death caused by selenium compounds can also proceed by autophagy, necrosis, mitotic catastrophe, or overlaps among them [50, 51]. Therefore, to verify if this reduction could be related with cell death, microscopies of the SH-SY5Y cells in PI stained were performed. In Fig. 3C, it can be observed the nucleus of SH-SY5Y cells treated with LQ-20 stained red with higher intensity than control cells (control and DMSO), indicating signs of cell death. This staining method is based upon the alteration of morphological characteristics of dead cells, such as membrane integrity loss, which can distinguish them from living cells, but the method is not sufficient in understanding the mechanism that could lead to cell death [52]. For this reason, further analysis would be necessary to better understand the mechanism of cell death promoted by LQ-20.

Some compounds with selenium are also able to induce autophagy along with the process of apoptosis. Next, it was explored the ability of LQ-20 to induce autophagy in SH-SY5Y cells. As shown in Fig. 7A, micrographs of cells stained with AO indicate the presence of intracellular acidification vesicles, which in literature is one of the hallmarks of autophagic response [52, 53]. When chloroquine (25 μM) was added to SH-SY5Y cells for 72 h as an autophagy inhibitor, it can be observed an enhanced LQ-20 sensitivity in SH-SY5Y cells as shown in Fig. 7B. Also, it can be observed an increase of orange bodies (yellow arrows) in the presence of LQ-20 treatment, and these suggest that there is no resistance after autophagy inhibition when cells are treated with LQ-20 50 μM [55]. These results agree with those observed in the literature in which some compounds with selenium are capable of inducing more than one type of programmed cell death which can contribute to its therapeutic efficacy [54, 56]. Wallenberg and cols. observed effect of selenite (5 μM), selenodiglutathione (5 μM) or seleno-DL-cystine (100 μM) for 12 h in HeLa cells. [55]. The results obtained in this study are also in line with those of Karelia and cols. that tested synthesized selenium compounds: SelSA-1 (bis(5-phenylcarbamoylpentyl) diselenide), and SelSA-2 (5-phenylcarbamoylpentyl selenocyanide) as a histone deacetylase inhibitor and observed an increased autophagic process in lung cancer

cells (A549 cells), after 3 and 24 h of incubation, respectively, and at 5 μM of concentration [57]. However, further analysis would be necessary to confirm the importance and possibility of autophagy occurring in the cell death induction process promoted by LQ-20.

Many authors suggested that selenium compounds may increase ROS levels which can cause cellular damage [51–52, 58, 59]. Thus, it was used a fluorescent probe to investigate the effect of LQ-20 upon SH-SY5Y cells. These results showed approximately 3-fold increase in ROS levels, which is data suggestive of cell death with intracellular ROS generation after 72 h treatment with LQ-20 (Fig. 5). Additionally, selenium compounds can alter intracellular redox balance through oxidation of intracellular thiols, proteins and low molecular-weight compounds can suffer oxidation [47, 59-62]. In addition, during the metabolism of compounds with selenium, as in the conversion of selenide to selenite or even from methylselenol, ROS can be generated [17].

Many types of tumors have high GSH content which contributes to resistance in chemotherapy treatment [54, 63]. In line with this finding, in this study can be observed that treatment for 72 h with LQ-20 is able to reduce the content of intracellular GSH (Fig. 6), and this GSH depletion may contribute to the overproduction of ROS levels, leading to cell death [64]. These results are in line with those observed with Ebselen (10 μM), one of the most studied compounds that contains selenium, when incubated for 24 h, leads to the depletion of GSH levels in SH-SY5Y cells and an increased sensitivity to peroxide treatment [64]. Therefore, future experiments will need to elucidate the pro-oxidant mechanism of the compound LQ-20.

Considering the results obtained in this study, it was found a significant correlation between the pro-oxidant potential of LQ-20 and cytotoxicity in differentiated SH-SY5Y cells with retinoic acid. The human neuroblastoma SH-SY5Y cell line can be differentiated into neuron-like characteristics by combining FBS reduction to 1% and with RA treatment for 1 to 21 days, leading to the acquisition of morphological and biochemical characteristics of an in vitro cellular model [65]. During the differentiation process, RA treatment upregulates expression of neuronal and dopaminergic markers in SH-SY5Y cells, and it is important for the characterization of this cell model [20]. High-risk neuroblastoma patients are commonly first treated with a combination of chemotherapics such as

cyclophosphamide, doxorubicin, etoposide, and platinum compounds, this therapy is called induction chemotherapy [8]. Considering this combination of strategies, it was previously tested a concentration of RA in SH-SY5Y cell viability and it was possible differentiate neuroblastoma human SH-SY5Y cells with 2 μM RA for 4 days of pre-treatment. After the pre-treatment it was measured the differences between cytotoxic effects and analyzed their responsiveness to LQ-20 and cisplatin. The differentiated SH-SY5Y cells treated with 50, 100 μM of LQ-20 responded equally to 4 μM cisplatin when compared with RA control. In Fig. 8 it can be observed the effect of cytotoxicity compounds in SH-SY5Y cell viability cultured with the same medium and incubation time but without pre-treatment with RA. It was possible to observe that differentiated cells were less affected by LQ-20 than the undifferentiated SH-SY5Y cells. These data suggested a model with protective effect of RA pre-treatment. Cheung et al., verified that SH-SY5Y cells differentiated with 10 μM RA for 7 days prior to treatment, demonstrated a modulated response to neurotoxins, altering survival signaling mechanisms [66]. It can be found in literature that selenite is selectively toxic to tumor cells capable to induce cell death in certain concentrations and less toxic when compared to its effects on non-tumor cells [54, 67, 68].

The procedure of differentiation leads to a homogeneous neuronal population with expression of neuronal characteristic (neurites growth) and a decrease of proliferation [20] as captured in micrographs in Fig. 8C. Techniques and markers used to determine the dopaminergic phenotype of SH-SY5Y cells are required to get the differentiation experimentally validated [65]. An interesting study was reported by Schneider et al., when SH-SY5Y cells differentiated with RA exhibited alterations in cells bioenergetic, resulting in increased levels of cytochrome c oxidase and MnSOD, and an increase in mitochondrial membrane potential by approximately 60%, at day 5 after RA (10 μM) treatment was observed [69].

This is the first time that are exploring the effects of selenoamines in SH-SY5Y cell systems, such as differentiated SH-SY5Y cells with RA.

Conclusion

Thus, we can conclude that selenoamine LQ-20 is capable of inducing SH-SY5Y cell death possibly by a mechanism that, at least in part, involves depletion of GSH and increases ROS levels and may even have differences for SH-SY5Y cells when compared to cells differentiated with RA.

This is the first study demonstrating that LQ-20 treatment can induce cytotoxic effects upon SH-SY5Y cells, providing important new insights into further investigations to clarify the potential use of LQ-20 as a chemotherapeutic agent.

Acknowledgement

The authors woud like to thank the financial support from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The financial support organizations had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest.

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4 CONSIDERAÇÕES FINAIS

Sendo assim, é possível concluir que a selenoamina LQ-20 é capaz de induzir a morte das células SH-SY5Y por um mecanismo, que, ao menos, pode ter relação com a depleção de GSH, e aumento dos níveis de EROs, além das possíveis diferenças com células SH-SY5Y diferenciadas com RA. Este é o primeiro trabalho com o composto LQ-20 e a ação de induzir efeitos citotóxicos sobre células SH-SY5Y, fornecendo novas informações para investigações futuros, com o intuito de esclarecer o potencial uso de LQ-20 como um agente quimioterápico.

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