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Diego de Sousa Gomes da Anunciação

Avaliação da citotoxicidade do BRACO-19 em células de glioma humano da linhagem U251.

BRASÍLIA, 2019

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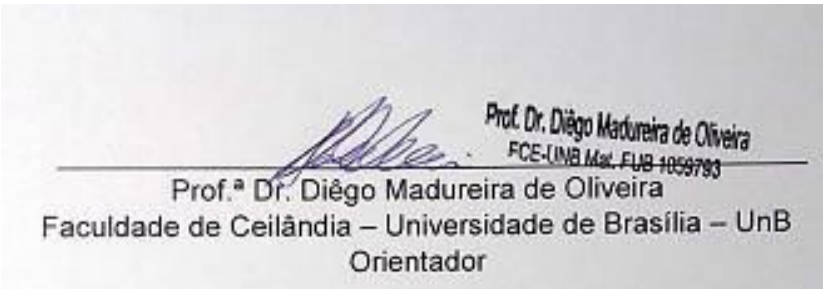

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Sumário

Resumo

Este estudo teve como objetivo investigar a provável atividade antitumoral de Braco-19 em células de glioblastoma U251. Foram analisados aspectos da biologia celular, tempo de duplicação, cálculo de $IC_{50\%}$, tratamentos de longo prazo, análise morfológica e citometria de fluxo. Com o tempo de duplicação de aproximadamente 32 horas, os testes de $IC_{50\%}$ podem ser realizados. Em apenas 24 horas de tratamento e com concentrações variando de 0,05μM a 50μM, uma resposta estatisticamente significativa pode ser observada com um $IC_{50\%}$ calculado em 4,214 μM. Em 72 horas, a $IC_{50\%}$ era de 0,9788μM. Os tratamentos a longo prazo mostraram uma resposta profunda da linha celular com Braco-19. A concentração escolhida de 1μM foi baseada no resultado obtido em $IC_{50\%}$ de 72 horas e achados na literatura. A análise morfológica mostrou uma maior variação no comprimento celular no grupo tratado do que no grupo controle. Diferenças estatisticamente significativas foram encontradas no cálculo do comprimento e da relação do eixo perpendicular, mostrando que as células são menos alongadas com o tratamento a longo prazo. A sensibilidade da célula ao composto ocorreu não apenas em testes de curto prazo, mas também em testes de longo prazo. A citometria mostra que o tratamento, por mais curto que seja, pode levar a linha celular a um estado quiescente. Os resultados obtidos foram promissores para a nova classe de medicamentos que podem surgir, tendo o G-quadruplex como alvo terapêutico. *In vitro*, os resultados foram positivos.

Palavras chave: Braco-19; U251; Tratamento à longo prazo; Glioma.

Abstract

This study aimed to investigate the probable antitumor activity of Braco-19 in U251 glioblastoma cells. Aspects of cell biology were analyzed, such as doubling time characterization, calculation of $IC_{50\%}$, long term treatments, morphological analysis and flow cytometry. With the doubling time of approximately 32 hours, the $IC_{50\%}$ tests can be conducted. In just 24 hours of treatment and with concentrations ranging from 0.05 μM to 50 μM, a statistically significant response can be observed with an $IC_{50\%}$ calculated at 4.214μM. At 72 hours, the calculated $IC_{50\%}$ was 0.9788μM. Long-term treatments showed a profound response of the Braco-19 in U251 cell line. The chosen concentration of 1μM was based on the result obtained at $IC_{50\%}$ of 72 hours and findings in the literature. The morphological analysis showed a greater variation in cell length in the treated group than in the control group. Statistically significant differences were found when calculating the length and perpendicular axis ratio, showing that the cells are less elongated with long-term treatment. The sensitivity of the cell to the compound occurred not only in short term tests, but also in long term tests. Cytometry shows that treatment, however short, can bring the cell line to a quiescent state. The results obtained were promising to the new class of drugs that may arise, having the G-quadruplex as a therapeutic target. *In vitro* the results were positive.

Keywords: Braco-19; U251 Cell Line; Long term treatment; glioma.

Lista de Figuras

Lista de abreviaturas

1p/19q (Co-deleção do braço curto do cromossomo 1 e do braço longo do cromossomo 19)

ALT (Alongamento Alternativo de Telômero)

ATRX (Alpha-thalassemia/mental retardation syndrome X-linked)

C-Myc (Gene regular de proliferação)

CP-31398, APR-246, PK083, PK11007, NSC319726 (Compostos ativadores de P53)

DMEM (Dulbecco's Modified Eagle Medium)

DNA (Ácido desoxirribonucleico)

FSC (Forward Scater)

IC50% (Metade da concentração inibitória máxima)

IDH 1 e 2 (Isocitrato desidrogenase)

MS (Ministério da Saúde Brasileiro)

MTT (Brometo de 3-(4,5-dimetil-2-tiazolil)-2, 5-difenil-2H-tetrazólio)

NAD (Dinucleótido de nicotinamida e adenina)

NADH (Dinucleótido de nicotinamida e adenina reduzido)

OMS (Organização Mundial da Saúde)

P53 (Proteína Reguladora de Ciclo)

R^2 (Coeficiente de correlação)

SDS (Dodecil sulfato de sódio)

SNC/ CNS (Sistema Nervoso Central / Central Nervous System)

SSC (Side Scater)

TERC (Componente RNA da Telomerase)

TERT (Telomerase Reverse Transcriptase)

TMZ (Temozolomida)

TP53 (Gene que codifica e proteína P53)

U251 (Linhagem tumoral de Osteossarcoma)

Capítulo I

Introdução, Revisão de Literatura, Justificativa e Objetivos

1. Introdução

Existem mais de cem tipos de tumores histopatologicamente diferentes, de origem primária, que acometem o sistema nervoso central (SNC). Embora sejam raros os casos de tumores no SNC, com a incidência de 2% do total de cânceres primários, eles representam uma total de 7% das mortes causadas por câncer de origem primária (DAVIS, 2018).

Entre os tumores malignos primários que afetam o SNC os gliomas são os mais frequentes em adultos, possuindo como características clínicas resistência a vários quimioterápicos tradicionais sendo, portanto, de difícil tratamento (ALENTORN et al., 2015).

Boa parte da dificuldade e falta de resposta a tratamentos convencionais se deve ao fato do glioma apresentar heterogeneidade celular, impossibilitando um tipo de tratamento universal, bem como os sintomas clínicos que são inespecíficos, tais como cefaléia e convulsões. Devido às características citadas, essa morbidade é de difícil diagnóstico em estágios iniciais do desenvolvimento, sendo um desafiador campo de estudo, mas que propicia o surgimento de diversas pesquisas na área com a finalidade de melhorar o prognóstico e sobrevida dos pacientes acometidos (JOVČEVSKA et al., 2013).

Entender os mecanismos que levam à patogênese do câncer é de suma importância para a definição de um alvo farmacológico. Sabe-se que as células cancerosas têm em sua essência a característica de proliferar indeterminadamente, sem um limite no número de ciclos celulares a que se submetem, sendo esse fenômeno conhecido como imortalização celular (MENDER et al., 2016).

A imortalização celular é um fenômeno imprescindível para a tumorigênese. Cerca de 85 a 90% dos tumores possuem em comum a expressão de uma enzima chamada telomerase (MENDER et al., 2016). Essa enzima é responsável por manter e alongar uma região repetitiva do DNA chamada de telômero, que possui o papel de proteger partes codificantes do material genético (MACIEJOWSKI; DE LANGE, 2017). Tal proteção é necessária pois a enzima responsável pela duplicação do material genético, DNA polimerase, possui uma limitação, sendo incapaz de replicar a extremidade final do DNA. Esse fenômeno é conhecido como “problema do fim da replicação” (MARTÍNEZ; BLASCO, 2015).

Devido a esse problema, a célula está limitada quanto à sua divisão, pois a cada nova replicação o telômero se encurta, podendo levar à crise telomérica e a perda de genes importantes. Para contornar esse problema e manter níveis mitóticos elevados a grande maioria dos tumores reconstroem os telômeros por desreprimir o gene da telomerase, como supracitado (MACIEJOWSKI; DE LANGE, 2017).

Os telômeros e suas estruturas secundárias tem sido um importante alvo farmacológico, para a pesquisa de variados quimioterápicos, nesse cenário surgiu o composto sintético Braco-19, com uma promissora ação antitumoral *in vitro*, com a estabilização da estrutura secundária do G-quadruplex, formado pela interação entre guaninas, sendo a região telomérica rica nessas bases nitrogenadas.

Tendo em vista a exposição de argumentos supracitada, o presente trabalho visa avaliar uma possível ação citotóxica de um composto experimental, tendo como alvo farmacológico a ação da telomerase em células de glioblastoma humano (U251) que a expressam, usando para tal um modelo *in vitro* de estudo.

2. Revisão Bibliográfica

2.1. Tumores do sistema nervoso central, uma visão geral

Tumores do sistema nervoso central (SNC) são raros. Embora raros são causa significativa de mortalidade em crianças e adultos jovens, sendo representantes respectivamente de 30 e 20% de todas as mortes relacionadas ao câncer (MCNEILL, 2016)

Existem mais de cinquenta tipos de entidades patológicas diferentes, quando se trata de tumores cerebrais, formando um grupo complexo e diverso devido a grandes variáveis como a localização, morfologia, comportamento clínico e aspectos da biologia molecular (PIÑEROS et al., 2016). No Brasil, a mortalidade causada por esse tipo de neoplasia é crescente, estima-se que no biênio de 2018 a 2019 haverá cerca de 11.320 novos casos de câncer no SNC, sendo o risco estimado de 5,6 novos casos a cada 100 mil homens e 5,17 a cada 100 mil mulheres (PARREIRA et al., 2018).

Devido a importante função que o sistema nervoso central desempenha no ser humano, qualquer alteração de sua fisiologia, mesmo que mínima, pode

acarretar em problemas graves e até irreversíveis. A depender do sítio anatômico da neoplasia, haverá diferenciadas sintomatologias apresentadas pelo paciente acometido, influenciando vias de diagnóstico que, a depender do quão inespecífico forem os sintomas, podem prolongar o correto reconhecimento da neoplasia. Hodiernamente há um foco em acelerar o processo de diagnóstico, sendo extremamente importante para melhorar a sobrevida do paciente (WALKER et al., 2017).

Existem diversas formas de classificar as neoplasias que acometem o SNC, seja pela origem histológica, localização, padrões de diferenciação característicos e ou aspectos anaplásicos. Na classificação histológica, os tumores mais comuns sãos os gliomas e os meningiomas. Sendo os gliomas representantes de aproximadamente 30% de todos os tumores cerebrais primários e 80% de todos os malignos, são responsáveis pela maioria das mortes de neoplasias de origem primária (WELLER et al., 2015).

Classificações adicionais podem ocorrer levando em conta a localização e padrões de diferenciação, como por exemplo o astrocitoma pilocítico e o mixopapilar, ou mesmo aspectos anaplásicos, como a atividade mitótica, proliferação microvascular e necrose (WELLER et al., 2015).

Com a finalidade de diminuir a quantidade de classificações e poder padronizar melhor os tipos de tumores cerebrais e suas características clínicas, a Organização Mundial da Saúde (OMS) estabeleceu um sistema de graus, variando do I ao IV (em algarismos romanos), dessa forma facilitando a classificação, o diagnóstico e possíveis abordagens de tratamento. A classificação tem como base aspectos morfológicos visto ao microscópio e aspectos moleculares (LOUIS et al., 2016). Quanto maior o número, maior o risco e consequentemente menor é a sobrevida do paciente (RASMUSSEN et al., 2017).

O grau I, possui como características, um crescimento lento e não infiltrante, sendo geralmente tratado com ressecção cirúrgica. O grau II também é de crescimento lento, mas já pode possuir características infiltrativas, podendo recidivar após ressecção, a longo prazo pode adquirir características de crescimento rápido. Já o grau III possui características morfológicas mais anormais, quando visto ao microscópio, são infiltrativos e frequentemente necessitam de tratamentos

adjuvantes à cirurgia, como quimioterapia e radioterapia. Por fim, os de grau IV, são de rápido crescimento e necessitam de tratamentos mais agressivos (LOUIS et al., 2016).

2.2 Gliomas

Os principais tipos de tumores que afetam o SNC são os meningiomas, meduloblastomas, gangliogliomas, Schwannomas, craniofaringiomas e gliomas. Existem também tumores que se desenvolvem perto do tecido cerebral ou mesmo no tecido cerebral, sendo eles os cordomas, linfomas não Hodgkin e tumores da glândula pituitária (AMERICAN CANCER SOCIETY, 2016).

Embora as neoplasias sejam bastante variadas e com muitos tipos de classificações, o presente trabalho focou-se em neoplasias que acometem as células da glia (gliomas), mais especificamente os glioblastomas. Dentre variadas opções de estudo o glioblastoma foi escolhido devido a alta agressividade desse tumor, além de baixa sobrevida dos pacientes acometidos. Além da agressividade, possui alta prevalência, sendo 54% de todos os gliomas e 16% de todos os tumores cerebrais de origem primária (TAMIMI; JUWEID, 2017).

O glioblastoma está contido dentro do subgrupo dos tumores astrocíticos, que fazem parte de um grupo maior, os gliomas. Além do glioblastoma, fazem parte do subgrupo dos tumores astrocíticos os astrocitomas e os astrocitomas anaplásicos. Glioma é um termo geral para tumores primários que acometem o SNC, sendo derivados de células da neuroglia ou células progenitoras (HANIF et al., 2017).

As neoplasias primárias possuem a sua origem em células que compõem o órgão, ou seja, possuem características próximas ao tecido original. Já os tumores de origem metastática são formados por células que podem migrar para outros tecidos saindo de sua origem e formando as metástases ou tumores secundários. O tipo de origem impacta na malignidade e no tipo de tratamento (HANIF et al., 2017).

Em análise histopatológica, os glioblastomas são difusos e heterogêneos, podendo ser classificados de acordo com a OMS em grau IV de malignidade, representando o mais alto grau. É extremamente raro gliomas formarem metástase, especialmente os glioblastomas, também devido aos pacientes possuírem uma

sobrevida curta, cerca de 1 a 2 anos depois de um diagnóstico confirmatório, não havendo muito tempo de acompanhamento clínico (PERRY; WESSELING, 2016).

Avanços na biologia molecular puderam identificar os vários subtipos de gliomas e consequentemente sub classificá-los. Os perfis genéticos e epigenéticos distintos revelaram vários possíveis biomarcadores, que servirão para ajudar no diagnóstico preditivo e prognóstico, com a finalidade de melhorar a resposta à terapia e direcionar o desenvolvimento de novos antitumorais (MASUI; MISCHEL; REIFENBERGER, 2016).

2.3 Tipos atuais de tratamento para glioblastoma

Pela natureza difusa do tumor, o glioblastoma é de demasiada complexidade para uma ressecção cirúrgica. Outras características como a rápida taxa proliferativa, que leva ao surgimento de novas mutações, dificultam a ação de quimioterápicos, tornando as células resistentes ao longo do tratamento (LÓPEZ; GARCÍA, 2016).

O local do glioblastoma também é um fator de dificuldade em um tratamento, pois por se localizar no parênquima cerebral, vários possíveis quimioterápicos não conseguem adentrar e exercer a sua ação, devido em boa parte a eficiente barreira hemato-encefálica. Essa barreira natural protege o encéfalo de possíveis agentes danosos que possam estar presentes no sangue, impedindo diversas moléculas de adentrar no tecido. Essa característica dificulta em boa parte a ação de quimioterápicos (VAN TELLINGEN et al., 2015).

O uso de radioterapia para tratamento de glioblastoma mostrou-se eficaz como adjuvante à terapia cirúrgica, mas o ganho médio de sobrevida do paciente continua baixo. Além do ganho ser pequeno, estudos demonstram que o uso de radioterapia por períodos prolongados, no tecido cerebral, aceleram processos fisiopatológicos crônicos, levando a declínios cognitivos permanentes. Logo após a incidência da radiação no tecido cerebral, ocorre uma inflamação com a capacidade de alterar o microambiente de sinalização celular em células progenitoras e hipocampais, as principais células relacionadas com a memória e cognição (MAKALE et al., 2017).

De acordo com o mais recente protocolo clínico e diretrizes terapêuticas em oncologia do Ministério da Saúde Brasileiro (MS), existem alguns tipos de esquemas a serem seguidos de acordo com o resultado de exames, estado clínico geral do paciente e o diagnóstico médico.

As recomendações mais usuais do MS são as técnicas de ressecção cirúrgica e como adjuvante o uso da radioterapia. Em alguns casos pode ser usado em conjunto as três técnicas supracitadas (ressecção cirúrgica, radioterapia e quimioterapia). Em casos em que uma cirurgia não é possível, é recomendado o uso da radioterapia junto com a quimioterapia. Além dos esquemas citados, existem dois de uso paliativo, sendo eles a radioterapia paliativa e a quimioterapia paliativa (BRASIL. Ministério da Saúde, 2014).

O principal quimioterápico recomendado pelo ministério da saúde para o tratamento de câncer cerebral é temozolomida (TMZ). O medicamento supracitado possui como mecanismo de ação a alquilação e metilação do ácido nucleicos (DNA). Sendo a temozolomida o tratamento de primeira linha para glioblastomas, é um pró fármaco de característica lipofílica, o que melhora de forma considerável a passagem pela barreira hematoencefálica e consequentemente sua biodisponibilidade no tecido (BARCISZEWSKA et al., 2015).

A temozolomida combinada com a radioterapia demonstrou uma sensível melhora na sobrevida de pacientes, principalmente idosos (PERRY et al., 2017). Mas, embora seja eficaz, a quimioterapia com TMZ associada a outras abordagens de tratamento, como a ressecção e a radioterapia, não são capazes de levar a cura clínica da comorbidade, apenas elevando a sobrevida e em raríssimos casos uma remissão do tumor. Cerca de 75% dos pacientes que participam de ensaios clínicos morrem em cerca de 2 anos de diagnóstico (BLAKELEY et al., 2019).

Estudos recentes já demonstram uma certa resistência de glioblastomas com o tratamento usual, cerca de 50% dos pacientes em tratamento com temozolomida não respondem adequadamente. Boa parte dessa resistência está sendo associada a uma deficitária via de reparo de DNA em células de glioblastoma, bem como a superexpressão de O-6 metilguanina metiltransferase, responsável por remover metilação e alquilação no ácido nucleico (LEE, 2016).

Devido ao limitado tratamento de glioblastoma, com apenas um único mecanismo de dano às células cancerosas e, a uma baixa sobrevida dos pacientes acometidos, se faz necessário investimentos e constante busca de novos quimioterápicos para melhorar essa conjuntura atual. Para o desenvolvimento de novos antitumorais é necessário definir alvos moleculares para a ação de possíveis quimioterápicos, sendo de grande importância os investimentos em pesquisas para tal.

2.4 Marcadores e alvos moleculares em gliomas

A quimioterapia é um dos importantes tratamentos para o câncer, por isso é constante o investimento em pesquisas e desenvolvimento de novos antitumorais. Infelizmente, levando em consideração a natureza da neoplasia e sua semelhança a nível genético com células saudáveis é muito corriqueiro efeitos adversos, pois todos os antitumorais afetam em algum nível células normais do corpo (PEARCE et al., 2017).

Os efeitos adversos podem conduzir a uma piora geral do quadro do indivíduo submetido ao tratamento, afetando sua qualidade de vida e estados físicos e mentais. A toxicidade de muitos antitumorais podem levar a complicações clínicas graves, como falência renal e efeitos em células que possuem um alto metabolismo, como células do sistema imunológico, da pele e do folículo capilar. A queda de cabelo pode ser devastador para o emocional do paciente (BARNETT, 2016).

Devido aos aspectos supracitados, há grandes investimentos em formas de aumentar a eficácia e diminuir os efeitos adversos da quimioterapia. Embora tenham muitas semelhanças com células somáticas, as neoplasias possuem diversas mutações em seu código genético, alterando seu fenótipo e portanto a expressão de genes e produção de metabólitos em excesso ou de forma alterada. Visando essas diferenças, a ciência tem buscado alterações específicas e importantes para a tumorigênese (GRAHAM; SOTTORIVA, 2017).

Recentes estudos mostraram que alterações genéticas específicas estão relacionadas com os gliomas, podendo inclusive ser objeto de estudo para definir alvos farmacológicos para possíveis terapias oncológicas. Alterações genéticas no IDH1/2, ATRX, TERT, TP53 e a codeleção 1p/19q, possuem o potencial de

reclassificar os gliomas em correlações mais lógicas para melhorar a escolha de tratamento, do que apenas levar em consideração características histológicas (FOOTE; PAPADOPOULOS; DIAZ JR, 2015). Os genes supracitados possuem bastante potencial para terapias e diagnósticos oncológicos, sendo subsequentemente descritos em separado cada um.

O gene que codifica a enzima IDH está sendo classificado como um oncogene de relevância no surgimento de gliomas. O nome completo da enzima é Isocitrato Desidrogenase e participa do metabolismo celular no ciclo de Krebs, fazendo a descarboxilação oxidativa do ácido isocítrico, tendo como seus produtos o alfa-cetoglutarato e dióxido de carbono, possuindo como finalidade de reação a formação de NAD reduzido (Dinucleotídeo de Nicotinamida e Adenina), o NADH (ZENG; CUI; GAO, 2015).

Alterações na enzima do IDH, relacionadas a 80% dos gliomas de baixo e alto grau, conduz a formação em excesso de um intermediário, o 2-hidroxiglutarato. Esse intermediário se liga a sítios catalíticos de enzimas chaves, importantes para o enovelamento do DNA e regiões promotoras de genes, causando hipermetilação. Mutações do IDH provocam hipermetilação de promotores, silenciando genes importantes como os de supressão de tumor. Além do silenciamento de genes, alterações da enzima fazem com que se acumulem espécies reativas de oxigênio, levando a mais mutações, promovendo a carcinogênese (ZENG; CUI; GAO, 2015).

Outro gene de importância é o que codifica a proteína ATRX sendo associado a vários tumores da glia e incluído no diagnóstico para averiguar variantes de gliomas pela OMS. A proteína ATRX tem um papel na remodelação da cromatina, mantendo a estabilidade genômica através da deposição conjunta com a histona variante H3.3 nos telômeros e regiões repetitivas do DNA (HAASE et al., 2018).

A perda da expressão do gene do ATRX deixa a região telomérica não silenciada, podendo sofrer influência de outros fatores. Alterações na expressão dessa proteína têm sido correlacionadas ao fenômeno ALT (Alongamento alternativo de telômeros), promovendo mais replicações celulares pois mantém os telômeros e contribui para a não erosão de genes codificantes durante a duplicação celular. A perda de ATRX também pode levar a um estresse replicativo e danos ao DNA, promovendo novas mutações (HAASE et al., 2018).

Outro gene de importância é o que codifica a enzima telomerase e, está presente em cerca de 80 a 90% dos glioblastomas, também é amplamente difundido em vários cânceres. A enzima telomerase tem um papel muito importante na manutenção dos telômeros, que são regiões repetitivas do DNA (TTAGGG, sendo T timina, A adenosina e G guanina). Essas regiões possuem um importante papel de recobrir o final dos cromossomos, impedindo a erosão de genes codificantes, devido ao problema do fim da replicação (HUANG et al., 2015).

A subunidade catalítica da enzima telomerase é conhecida pela sua abreviação TERT (do inglês: transcriptase reversa da telomerase) que juntamente com o componente RNA da telomerase (TERC) compreendem a unidade mais importante do complexo da telomerase. A expressão dessa enzima está relacionada com células indiferenciadas e com alto poder mitótico. Na carcinogênese, essa enzima tem um papel de alongar os telômeros e permitir que a célula possa replicar sem fim e também contribui para a desdiferenciação celular (HUANG et al., 2015).

Um dos principais genes supressores de tumor é o TP53, sendo um dos mais frequentemente mutados em vários cânceres. Inicialmente tinha se classificado o TP53 como um oncogene, mas através de pesquisas se chegou à conclusão que a forma selvagem do gene é um supressor de tumor, participando da regulação da transcrição celular. O gene de transcrição da proteína é ativado quando há dano no DNA, promovendo a parada do ciclo celular e apoptose. Mutações no gene TP53 conduz a inativação da proteína P53, sendo um dos mecanismos de evasão da morte celular por uma célula cancerosa, conduzindo a uma rápida progressão tumoral (KASTENHUBER; LOWE, 2017).

A P53 como alvo terapêutico é um tanto quanto complicada, pois iria requerer a ativação do gene funcional, podendo a terapêutica de ativação se focar em mediadores que intensifiquem a expressão de P53 naturalmente. Vários compostos estão em desenvolvimento para ativar o tipo selvagem da p53, sendo eles CP-31398, APR-246, PK083, PK11007, NSC319726 e ácido estítico. O objetivo geral é estabilizar a conformação nativa do domínio do núcleo p53, a fim de restaurar a ligação e transativação do DNA específico da sequência de genes alvo p53 e, finalmente, para induzir a morte celular e eliminar o tumor (BYKOV et al., 2018).

Uma das primeiras alterações em gliomas descritas, foi a co-deleção do braço curto do cromossomo 1 e do braço longo do cromossomo 19, devido a erros de translocação. Essa mutação é mais associada aos oligodendrogliomas e pode representar um bom prognóstico de tratamento, pois é mais responsiva a agentes alquilantes de DNA. Nem todos os gliomas apresentam essa co-deleção, sendo alguns gliomas possuindo apenas uma das deleções, ou mesmo nenhuma delas, sendo esses os de pior prognóstico (ECKEL-PASSOW et al., 2015).

A temozolomida, um dos poucos tratamentos para gliomas é bastante eficaz quando se há a dupla deleção dos braços dos cromossomos supracitados (SPEIRS et al., 2015). Essa identificação pode conduzir a um diagnóstico e uma estimativa de sobrevida melhor. Outras inferências que se pode fazer é que a identificação da co-deleção demonstra diferenças quanto a origem da patologia e se pode fazer associações com variantes germinativas, visto que é mais importante para o surgimento de oligodendrogliomas do que outros tumores da glia (ECKEL-PASSOW et al., 2015).

Todos os marcadores supracitados podem ser usados como ferramentas de diagnóstico e classificação de tumores que afetam o sistema nervoso central. Algumas alterações sejam nas proteínas ou nos genes que as codificam são passíveis de algum tipo de intervenção farmacológica. Há na clínica apenas a temozolomida como quimioterápico eficaz para o tratamento de gliomas.

Mesmo alguns gliomas possuindo uma boa resposta ao quimioterápico, os glioblastomas não possuem uma boa resposta sendo ainda de sobrevida baixa. Por isso se faz necessário a busca por novos compostos que possam ser mais eficazes na cura clínica da patologia. Uma forma inicial de triagem de fármacos oncológicos se faz através de um ramo da biologia conhecido como oncologia experimental, que pode desenvolver modelos experimentais para determinação do mecanismo de ação de moléculas com potencial atividade anticâncer, assim como o estudo dos mecanismos responsáveis pela gênese das neoplasias.

2.5 Oncologia experimental

A oncologia é o ramo da ciência médica que se dedica ao estudo e tratamento de neoplasias, o que inclui sua etiologia e desenvolvimento. Como uma forma de

melhor entender o câncer e poder traçar estratégias terapêuticas, vários modelos experimentais foram desenvolvidos. Ao longo das décadas formas mais robustas de experimentação foram sendo desenvolvidas, novas formas de imageamento e avanços no campo da computação que ajudaram a predizer riscos de câncer (QIU et al., 2016).

Modelos de estudos *in vitro* e *in vivo* para gliomas humanos, tem um potencial enorme para a compreensão da biologia do glioma, bem como também estratégias para o tratamento desses tumores. Os modelos de estudos em gliomas necessitam atender a demandas específicas e diferentes do que outros modelos necessitam, boa parte devido a várias aberrações genéticas e também ao microambiente cerebral (LENTING et al., 2017).

O microambiente de desenvolvimento dos gliomas é um grande obstáculo para as quimioterapias vigentes, pois esse tecido apresenta uma barreira de proteção (barreira hemato-encefálica) que pode ser intransponível para variados fármacos, o que limita a ação dos mesmos. Novos sistemas de entrega de fármacos estão sendo desenvolvidos, com o auxílio da nanotecnologia, para melhorar a biodisponibilidade de quimioterápicos no tecido cerebral (GANIPINENI; DANHIER; PRÉAT, 2018).

O modelo de estudo usado neste artigo foi a cultura celular convencional, sendo um dos modelos em que mais se assemelham às características genéticas dos gliomas na clínica, pois se usam células que vieram de humanos, sendo por isso o modelo de pesquisa amplamente usado. As células padronizadas mantém as aberrações genéticas dos tumores originais e, permitem o estudo detalhado de vias de sinalização oncogênicas de maneira controlada e reprodutível. Além disso, tais linhagens celulares também permitem testes rápidos e reprodutíveis de fármacos dirigidos *in vitro*, como pré-teste para testes adicionais em modelos *in vivo* e pré-clínicos apropriados (LENTING et al., 2017).

2.6 Promissor composto Braco-19

O composto antitumoral escolhido como objeto de estudo deste presente trabalho foi o composto sintético Braco-19, que é um composto derivado da acridina que demonstrou uma boa ação antitumoral *in vitro* (HARRISON et al., 2003). Produz

ação de diminuir o crescimento de colônias celulares tumorais, fusão cromossômica e atividade anticancerígena em xenoenxertos. Mas boa parte da caracterização do composto se limitou a parâmetros farmacológicos, como captação celular e permeabilidade em membranas, ainda não muito estudado *in vivo* (PAQUIM; PONTINHA; BRETT, 2015).

O Braco-19 tem uma ação diferenciada dos compostos mais comuns usados na clínica (como exemplo a Temozolomida que é um agente alquilante). Boa parte dos tumores, cerca de 85-90% possuem em comum a desrepressão do gene da telomerase (PAQUIM; PONTINHA; BRETT, 2015). Vários compostos possuem ação direta, inibindo a enzima da telomerase. O composto usado neste estudo tem uma ação indireta na via da telomerase, estabilizando estruturas secundárias do DNA (BURGER et al., 2005).

A estrutura secundária de DNA na qual o composto tem a sua ação é o G-quadruplex. O telômero é uma região rica em uma base nitrogenada, a guanina (sequência de bases nitrogenadas do telômero TTAGGG). Por possuírem hidrogênios disponíveis para a ligação esses interagem com o átomo de nitrogênio do anel de outra guanina, formando um pareamento de Hoogsteen (descreve pareamentos incomuns de bases nitrogenadas) que se apresenta em estruturas tridimensionais pela interação de quatro guaninas, formando assim o G-quadruplex (NEIDLE, 2017).

O G-quadruplex é uma estrutura natural formada pela interação das guaninas, o composto Braco-19 tem a ação de estabilizar essa estrutura tridimensional. Para a ação da enzima telomerase o telômero necessita estar na forma de fita, ao estabilizar o G-quadruplex o DNA telomérico passa a ter barreiras físicas para a ação da enzima. Dessa forma o composto consegue frear um mecanismo de alongamento de telômeros, impedindo a progressão tumoral (RHODES; LIPPS, 2015).

Para avaliar a ação promissora desse composto foi escolhida uma linhagem celular que expressa a enzima telomerase e pertencente ao tipo mais mortal de tumor da glia (o glioblastoma), a linhagem U251. Essa linhagem é amplamente usada em estudos sobre glioblastomas, mas a ação do composto Braco-19 nessa linhagem foi pouco caracterizada, necessitando de mais estudos como este para

definir a ação e quem sabe um promissor antitumoral para a clínica, podendo elevar a baixa sobrevida dos pacientes acometidos.

2.7 Linhagem U251

A linhagem U251 foi estabelecida primeiramente no laboratório de Wallenberg, na cidade de Uppsala na Suécia, a aproximadamente 40 anos (TORSVIK et al., 2014). A linhagem celular foi descrita em 1973, sendo extraída de um astrocitoma maligno proveniente de um paciente do sexo masculino (WESTERMARK; PONTEN; HUGOSSON, 1973). A célula tem um comportamento e perfil similares ao glioblastoma humano multiforme *in vivo,* tornando sua cultura viável e de grande relevância de estudo para a patologia (TORSVIK et al., 2014).

Essa linhagem foi escolhida como objeto de estudo pois mantém um perfil similar ao glioblastoma humano multiforme, bem como expressa a enzima telomerase, que pode ser detectada em grandes quantidades na linhagem. Sendo a inibição da atividade da telomerase um importante fator para aumentar a susceptibilidade da linhagem a tratamentos mais convencionais ou mesmo diretamente causando morte celular por apoptose (KONDO et al., 1998).

3. Justificativa

O composto sintético Braco-19 tem uma promissora ação indutora de senescência e apoptose demonstrada em outros modelos *in vitro,* mas ainda não teve seu efeito estudado em células de glioma, um tipo de câncer que carece de novas alternativas terapêuticas. A linhagem U251 foi escolhida para estudo da ação do composto, pois apresenta características comuns a cerca de 90% dos tumores, que é a expressão da enzima telomerase, bem como a de ser uma célula de glioma humano. Dentre os tumores primários que acometem o sistema nervoso central, o glioma é o mais comum. A complexidade, a refratariedade ao tratamento, o difícil diagnóstico de glioma e a carência de estudos na área, fazem dessa neoplasia um interessante alvo de pesquisa.

4. Objetivos

4.1 Objetivo geral:

Avaliar o potencial citotóxico do composto sintético Braco-19 em um modelo *in vitro* de glioma humano.

4.2 Objetivos específicos:

a) Determinar a $IC_{50\%}$ do composto Braco-19 em células de glioma U251.

b) Avaliar o efeito do composto no tempo de duplicação da linhagem.

c) Determinar o potencial do composto no ciclo celular.

d) Determinar os efeitos do tratamento na morfologia celular.

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Capítulo II

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Original Article

Evaluation of BRACO-19 cytotoxicity in human glioma U251 cell line.

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Background: This study aimed to investigate the potential antitumor activity of Braco-19 in U251 glioblastoma cells.

Methods: U251 cells were cultured in specific plates for this purpose. Aspects of cell biology were analyzed, such as doubling time characterization, calculation of IC50% at 24 and 72 hours, long term treatments, morphological analysis and flow cytometry.

Results: With the doubling time of approximately 32 hours, the $IC_{50\%}$ tests can be conducted. In just 24 hours of treatment and with concentrations ranging from 0.05 µM to 50 µM, a statistically significant response can be observed with an $IC_{50\%}$ calculated at 4.214µM. At 72 hours, theoretically after a doubling pass, the calculated $IC_{50\%}$ was 0.9788µM, showing a dose dependent and time dependent response. Long-term treatments showed a profound response of the Braco-19 cell line, requiring a larger amount of cells to be plated to obtain a considerable sample for morphology analysis. The chosen concentration of 1µM was based on the result obtained at $IC_{50\%}$ of 72 hours and findings in the literature, which suggest this appropriate concentration to evidence the action of the compound. The morphological analysis showed a greater variation in cell length in the treated group than in the control group. Statistically significant differences were found when calculating the length and perpendicular axis ratio, showing that the cells are less elongated with long-term treatment. The cytometry shows an cytostatic effect on cell cycle.

Conclusions: The action of Braco-19 on telomerase expressing glioblastoma cells has shown promise. The sensitivity of the cell to the compound occurred not only in short term tests, but also in long term tests. In the long term, the compound was equally effective, probably by more direct action on the telomere, preventing its stretching and leading to senescence after a few doubling passages. The morphological analysis showed that although the average length between the treated and the controls is not very different, the ratio between the length and the perpendicular axis (width) is significantly different between the groups, leading to the conclusion that the treatment leaves the cells less elongated and consequently wider. Cytometry shows that treatment, however short, can bring the cell line to a quiescent state. The results obtained were promising to the new class of drugs that may arise, having the G-quadruplex as a therapeutic target. *In vitro* the results were positive.

Keywords: Braco-19; U251 Cell Line; Long term treatment; Glioma.

Introduction

There are more than one hundred types of histopathologically different tumors of primary origin that affect the central nervous system (CNS). Although CNS tumors are rare, with an incidence of 2% of all primary cancers, they account for a total of 7% of deaths from primary cancer (1). Among primary malignant tumors that affect the CNS, gliomas are the most frequent in adults, with resistance to several traditional chemotherapy as clinical characteristics and, therefore, difficult to treat (2).

Much of the difficulty and lack of response to conventional treatments is due to the fact that glioma has cellular heterogeneity, precluding a universal type of treatment, as well as nonspecific clinical symptoms such as headache and seizures. Due to the mentioned characteristics, this morbidity is difficult to diagnose in the early stages of development, being a challenging field of study, but which allows the emergence of several researches in the area aiming to improve the prognosis and survival of affected patients (3).

Understanding the mechanisms that lead to the pathogenesis of cancer is extremely important, for defining pharmacological targets. Cancer cells are known to have in essence the characteristic of proliferating indefinitely, without a limit on the number of cell cycles, which is known as cell immortalization (4).

Cell immortalization is an indispensable phenomenon for tumorigenesis. About 85 to 90% of tumors have in common the expression of an enzyme called telomerase (4). This enzyme is responsible for maintaining and lengthening a repetitive region of DNA called telomere, which has as its function the protection of coding parts of genetic material (5). Such protection is necessary because the enzyme responsible for the duplication of genetic material, DNA polymerase, has a limitation, being unable to replicate the final end of the DNA. This phenomenon is known as the "end of replication problem" (6).

Telomerase has been an important pharmacological target for the research of various chemotherapeutic agents, largely due to its high selectivity and prevalence in tumors. But the Braco-19 compound, used in this work, has a differentiated mechanism of action because it indirectly intervenes in the telomerase pathway, stabilizing secondary DNA structures (7).

The secondary DNA structure in which the compound has its action is the G-quadruplex. The telomere is a region rich in guanine. Having hydrogens available for bonding, the guanines interact with the nitrogen and oxygen atom of another guanine ring, forming a Hoogsteen pairing (which describes unusual nitrogenous base pairings) that presents in three-dimensional structures by interaction of four guanines, thereby forming a G-quadruplex (8).

The Braco-19 compound has the action of stabilizing this three-dimensional structure. For the action of the telomerase enzyme the telomere needs to be in the unfolded form. By stabilizing the G-quadruplex the telomeric DNA has physical barriers to the enzyme's action. Stabilizing this structure, the compound can stop the telomere elongation mechanism, preventing tumor progression (9).

To evaluate the promising action of this compound was chosen the U251 cell line which expresses the telomerase enzyme and belongs to the deadliest type of glial tumor (the glioblastoma). This cell line is widely used in studies on glioblastomas, but the action of the Braco-19 compound on these cells has been poorly characterized, requiring further studies, such as this one, to define the action and perhaps a promising antitumor chemotherapy for the clinic, which may elevate the poor survival of affected patients.

Methods

Cell Culture

Glioblastoma U251 cells were plated into 10 cm diameter polystyrene culture plates (Kasvi) for amplification. After amplification the culture plate was trypsinized (0.5%) and centrifuged at 1500 RPM (500G) for 3 minutes to separate the culture medium and trypsin from the cells, being resuspended in fresh medium for different tests to be described. Cells were cultured with DMEM (Dulbecco's Modified Eagle's Medium - Sigma), supplemented with 10% (v/v) sterile fetal bovine serum (Cultilab), 100 IU/ml penicillin, 100 µG/ml streptomycin and 44mM sodium bicarbonate ($NaHCO_3$) in a humid atmosphere consisting of 95% air and 5% CO_2 at a temperature of 37°C.

Doubling Time

The doubling time test was performed using a 96 well plate where 1,000 cells were plated in each well using the classic MTT reduction test to assess cell quantity. This test is based on the ability of viable cells to reduce the yellow colored compound called 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT (Sigma Aldrich)) to formazan, which has purplish color. After 4 hours of plating, the culture medium of the first column was changed using a culture medium plus MTT at a concentration of 1mg/ml. After 2 hours the MTT medium was exchanged for a cell lysis solution (40% dimethylformamide, 40% water and 20% by weight powdered sodium dodecyl sulfate (SDS)) (10).

After 12 hours of lysis buffer action, the first 8 well column was transferred to another 96 well plate, where absorbance was measured at 490 nm wavelength, by an ELISA reader (Tp-Reader type B model).

The experiment was repeated on each column every 24 hours following the MTT reaction of the previous column. The experiment lasted approximately 5 days, where the confluence was already close to 100 percent in the wells.

Viability test for $IC_{50\%}$ Determination

For viability test the cells were counted using a Neubauer chamber and trypan blue as a dye. Cells were seeded in 96 well plates having a number of 10,000 cells per well and incubated for 24 hours. After 24 hours of incubation the cells were treated with DMEM medium in the first column, DMEM plus drug diluent vehicle (water) in the second column and Braco-19 compound diluted at different concentrations in subsequent columns for 24 and 72 hour periods.

Cell viability was measured by MTT assay in a 96 well plate. The medium with the drug and diluent being replaced with the MTT reagent (Sigma Aldrich) at the same concentration and time of action as the previous experiment (10). After the elapsed time the medium was removed and the cells lysed with 100 µL per well of SDS. The absorbance at 490 nm of wavelength was subsequently measured on a Thermo Plate reader (Tp-Reader type B model).

Long Term Treatment with predefined fields

The long-term treatment was initially done in specific 10 cm diameter polystyrene (Kasvi) plates, 50,000 cells were plated per plate and divided into two groups for comparison. The first only with DMEM medium and diluent vehicle, water (Milli-Q water) and the second with Braco-19 compound in toxic concentration (1 µM).

The culture medium of the two plates was changed every four days in the same atmosphere as the cell culture described later.

Before the cells were plated, 6 fields were pre-defined on the outer bottom of the culture plates, numbered 1 through 6, to observe the growth of the culture. The diameter of the field of view was defined in a way that comprised the full focus of the 4x microscope lens without the field edge appearing in the image.

Morphological analysis

All photos used in the experiments were taken using an inverted phase contrast Nikon Eclipse microscope (TS 100). For morphological analysis, the image editing software ImageJ (version 1.8.0_112 for Windows) was used. The micrographs chosen for quantification were at the treatment time of approximately 288 hours, and 3 random observation fields were chosen in each plate (control and treated), randomly counting 20 cells per field.

The length was determined by counting the micrograph pixels of the cells by the program and compared to a known scaled image (image from a Neubauer), thus having the cell dimensions in µm. For the evaluation to be true, all images, including the scale, were taken under the same conditions, with the same resolution, magnification and capture software.

The cell length was measured by drawing a line that passed the longest distance between the cell poles, the width was measured by passing a line on the longest distance between two points of the width and forming an angle of 90° with the length line.

Flow Cytometry

The U251 cell line was seeded in a 12 well plate ($5x10^4$ cells per well) and incubated in the same cell culture environment as described above. After 24 hours the treatments of interest were initiated, and 3 control wells (Milli-Q™ water) and 3 treated wells were selected, with Braco-19 at a concentration of 5µM (using the $IC_{50\%}$ of the treatment for 24 hours).

After 24 hours of treatment, the supernatant was removed and reserved in corresponding microtubes for each well. Being replaced by 400 µL trypsin (0,5%) in each well and incubated for 5 minutes at 37 °C. With the cells completely detached from the plate, the previously reserved medium was added to counteract the action of trypsin.

The entire contents of the wells were resuspended and transferred to corresponding 1.5 mL tubes. The tubes were centrifuged at 2000 rpm for 5 minutes at 4 °C. After centrifugation, the supernatant was discarded in sufficient quantity to leave only 100 µL in each tube. The pellet formed was resuspended and 500 µL of 1x PBS was added and the tube was vortexed.

Again the tubes were centrifuged at 2000 rpm for 5 minutes at 4°C, the supernatant was discarded until 100 µL and 1 mL of 70% ice cold ethanol (Sigma) was added, after homogenization, the tubes were stored in the freezer (-20°C) for 24 hours for further analysis.

After 24 hours, the tubes were centrifuged, discarded the supernatant, added PBS and vortexed (as described above) two more times. After the washing procedure, 100 µL of RNAse (50 µg/mL) was added to the 100 µL of the cell suspension and all tubes were vortexed.

The tubes were placed in the incubator at 37 °C for 30 minutes. After this time, 100 µL of 20 µg/mL propidium iodide (PI) solution was added to each tube, vortexed and stored at room temperature protected from light for 30 minutes. After the previous procedure the samples were centrifuged at 2000 rpm for 5 minutes, discarded the supernatant and resuspended in 1x PBS with final volume of 300 µL of sample. The tubes were then placed on ice and analyzed by the BD FACSCalibur™ cytometer.

Statistical Analysis

GraphPad Prism (GraphPad Int., USA) version 5.00 for Windows was used for all graphs. Being used appropriate statistical test for each experiment performed. For each data, the measure of central tendency was quantified, and parametric or nonparametric tests were chosen depending on the data distribution. The complete description of the statistical tests used for each experiment are specified in the results of this work. The main tests used were: exponential growth equation, one-way ANOVA, Kruskal Wallis with Dunn's posttest, Student's t-test, Mann Whitney, nonlinear regression in logarithmic function. The p values were considered significant when less than 0.05.

Results

Doubling time

Initially, the doubling time of U251 was evaluated, which is important to characterize normal cell growth, a parameter for

established times when conducting the $IC_{50\%}$ tests and on the following of the long term tests.

The U251 cell line had a doubling time calculated with a time interval between 30.17 to 34.29 hours. An average of 32.1 hours for a duplication pass **(Figure 1)**.

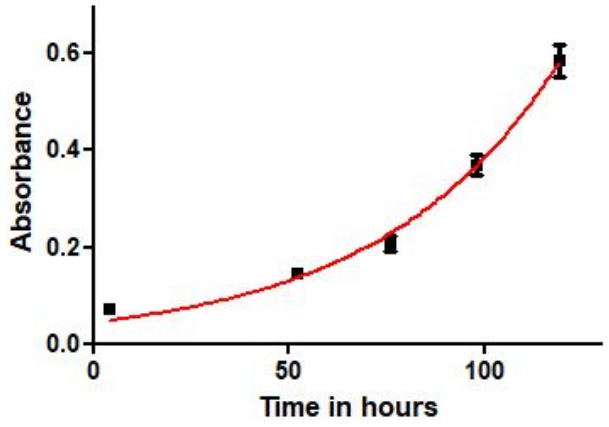

Figure 1) Doubling time, lasting approximately 119 hours. R^2 value of 0.98. Data expressed as mean with error bars. Exponential growth equation: A = 0.04449 * exp (T * 0.02159), where A is the absorbance and T the time in hours.

$IC_{50\%}$ Determination

After doubling time results, $IC_{50\%}$ tests were conducted (concentration at which 50% of the culture cells are unviable), it is important parameter in deciding which concentration to use in long term tests. In the short term the U251 cell line was sensitive to Braco-19. Even in short periods of time, such as 24 hours, the cell line showed a statistically significant response to 10 µM of the drug **(Figure 2A)**.

In 72 hours the response was even higher, in theory the cell had already gone through the first duplication. Braco-19 interference was perceived from 1 µM, but statistically significant at 1.5 µM concentration **(Figure 2B)**.

Subsequently, the data obtained in the viability graphs **(Figures 2A and 2B)** were subjected to nonlinear regression between concentration and response, generating a logarithmic graph with the drug concentration in log in relation to cell viability in percentage to established the $IC_{50\%}$ **(Figure 3).**

Viability was observed to decrease as concentration increased, an expected result. Thus obtaining the $IC_{50\%}$ values, which at 24 hours was a concentration between 3.52 to 4.92 µM and at 72 hours the $IC_{50\%}$ range was between 0.92 and 1.02 µM **(Figure 3)**. 24 hour average was 4.214µM and 72 hours was 0.9788µM.

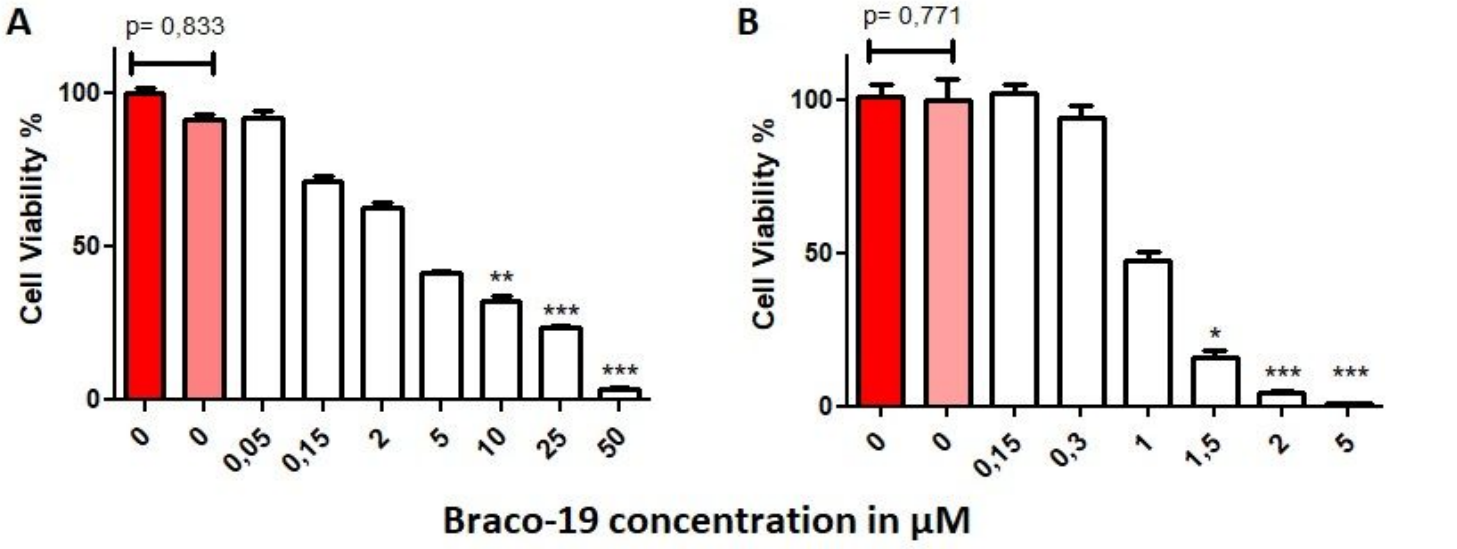

Figure 2) Viability test at 24h (A) and 72h (B) treatment at different concentrations in the U251 glioblastoma cell line. The data are in percentage in relation to the control (DMEM), and the bars show the medians of the groups with the standard deviation. The first column (red) is the DMEM control only and the second (pink) is the DMEM control plus 0.02% of water as diluent. For comparison between the second column and the others, the Kruskal Wallis test was used, a nonparametric test with Dunn's posttest. At 10µM, 25µM and 50µM concentrations showed a significant P value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within 24 hours, while within 72 hours from the concentration of 1.5µM all after were significant. A Student's t-test was performed to compare the controls (red and pink) at 24h and, Mann Whitney between the 72h controls, P values expressed in the figure. Only a few concentrations were chosen to better show the results graphically, statistical analyzes were performed with all data, statistical tests of each analysis were chosen using as symmetry criteria of the data, using nonparametric tests for asymmetric data.

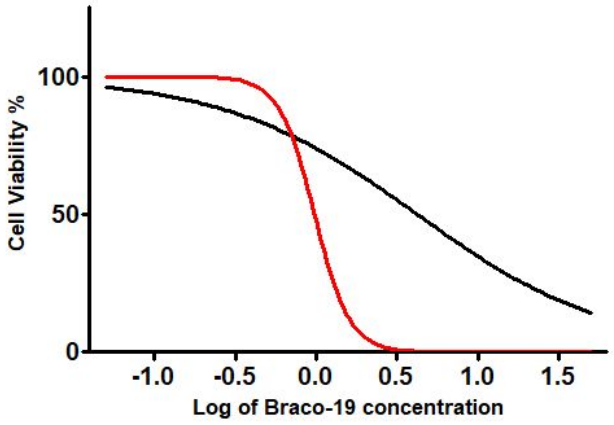

Figure 3) Nonlinear regression between concentration and response. Graph in logarithmic function, log concentration in relation to cell viability. In red $IC_{50\%}$ in 72 hours and in black $IC_{50\%}$ in 24 hours. The R^2 value was 0.98 for the 72 hour $IC_{50\%}$ and 0.91 for the 24 hour IC_{50}. The $IC_{50\%}$ in 24h ranging from 3.62 to 4.90 µM and in 72h from 0.94 to 1.02µM. Being the black curve (24h) governed by the equation: V = 100/(1 + 10 ^ ((Log 0.6247 - C) * (-0.7245))). And the red curve (72h): V = 100 / (1 + 10% ((Log -0.009320 - C) * (-3.986))). V being the cell viability (%) and C the concentration in µM.

Long Term Treatment

After the short term sensitivity tests, the long term tests were started. The cell line was divided into two groups, one only with DMEM medium plus diluent (0.02% water), called group W (Water) and the second with Braco-19 at a concentration of 1µM, called group B.

Such concentration was chosen because it is standard in several other articles and has proven efficacy in this concentration in telomerase expressing cells (7), the $IC_{50\%}$ value at 72 hours was close to that used in the long term.

As with short-term treatment, the cell was sensitive to long-term treatment, at 144 hours a large difference between groups was already noticeable **(Figure 4)**.

The whole experiment lasted about 408 hours, with approximately 13 doubling passes. The sensitivity of the U251 cell line to Braco-19 was reaffirmed. In conducting the experiment, there was a smaller number of cells in group B compared to group W, the morphology was similar between groups, but had some size variations in group B.

Micrographs were chosen that were representative of the experiment **(Figure 4)**. The criteria for choosing the fields shown in figure 4 were image quality and representativeness of plate confluence.

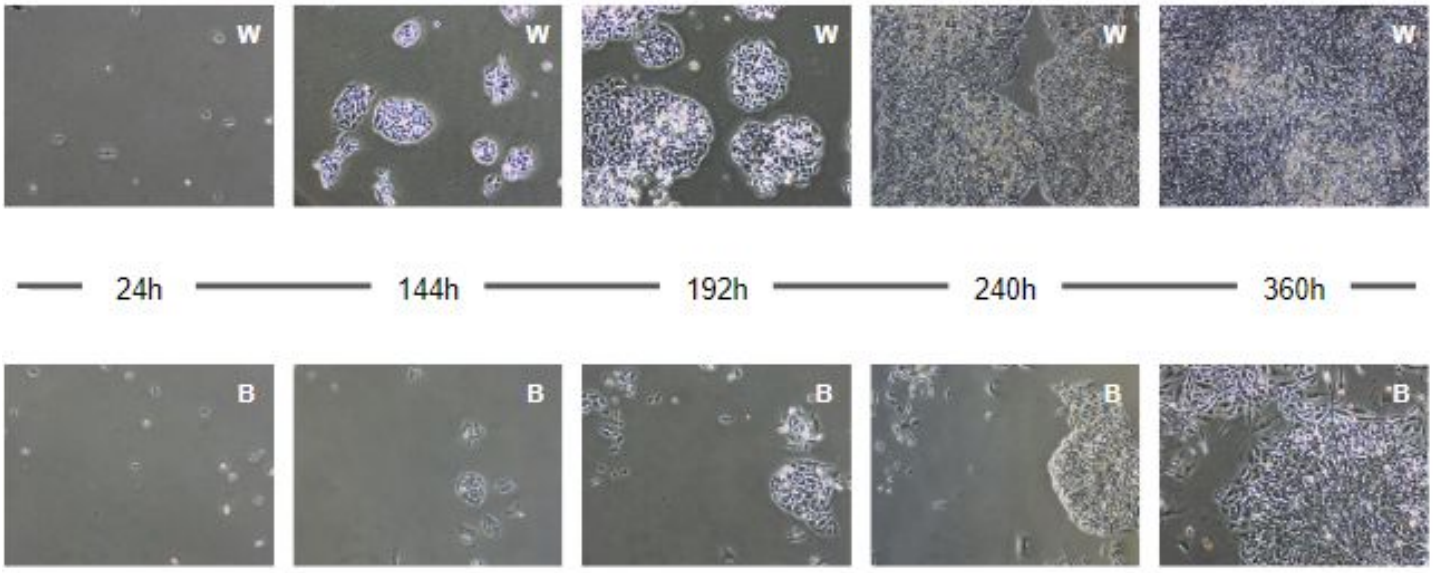

Figure 4) Long-term treatment, 50,000 cells were dropped into each plate (counted using a Neubauer chamber), with predefined fields to maintain a more faithful comparison between groups at times 24, 144, 192, 240. and 360 hours. Micrographs obtained with phase contrast microscope at 40x magnification. B represents the group with the drug Braco-19 at a concentration of 1µM and W represents the medium with the diluent (water) at a concentration of 0.02%. The whole experiment lasted 408 hours, approximately 13 doubling pass.

Morphological analysis

Some morphological changes were observed in long-term treatment, especially in the predefined fields of observation.

To evaluate such morphological changes, micrographs were chosen from six observation fields, three from group B and three from group W.

In each field, 20 cells were randomly chosen, with a total of 60 on group B and 60 cells on the group W (water) was counted. The chosen time was approximately 188 hours of treatment, because at that time the cells had visible limits for quantitation and confluence was not high, avoiding morphological change by contact inhibition.

In analysis, the data showed that there is no statistically significant difference in cell length **(Figure 5)**, although in the treated group (group B), the variation in cell length was much larger than in the control group (group W).

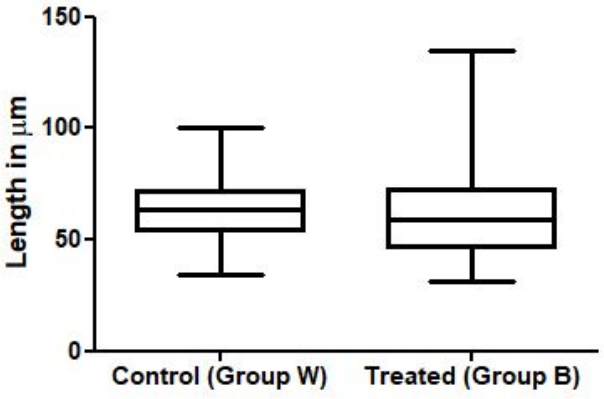

Figure 5) Graph showing cell length and its variation between treated and control groups. The median in the control was 63.30µm and in the treated was 59.09µm. Minimum values of 34.22µm (control) and 30.98µm (treated) and maximum values of 100.3µm (control) and 134.7µm (treated). Mann Whitney test did not show statistically significant differences between groups, having a P value of 0.2963.

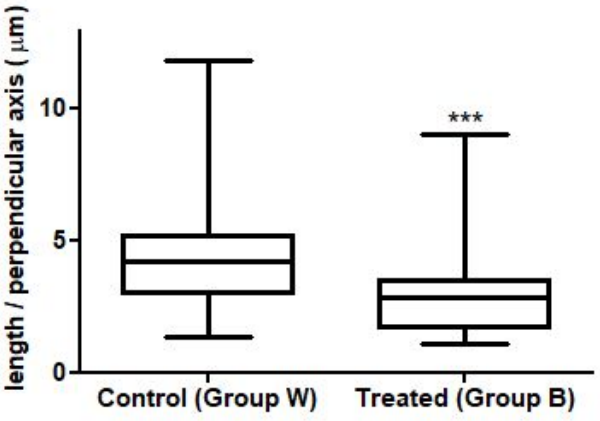

Figure 6) Graph showing the polar morphology index of the cells. Statistically significant difference, P <0.0001, obtained by the Mann Whitney test.

Although there was no statistical difference in length, statistically significant differences were found in the polar morphology index, which is the ratio between the length and the perpendicular axis (width) of the cells **(Figure 6)**.

This shows that the treatment makes the cells less elongated, having a larger perpendicular axis than the control group, although the average length does not change much.

Flow Cytometry

Cytometry was conducted to define some mechanism of cell cycle interference induced by the action of Braco-19 compound on U251 human glioblastoma cells, using propidium iodide compound as DNA intercalating agent.

Figures 7a and b set the parameters for the analyzes contained in **figures 8a and b**. **Figure 7a** shows the events measured by the equipment in a dot plot graph, with red representing the population about 90% of the events measured.

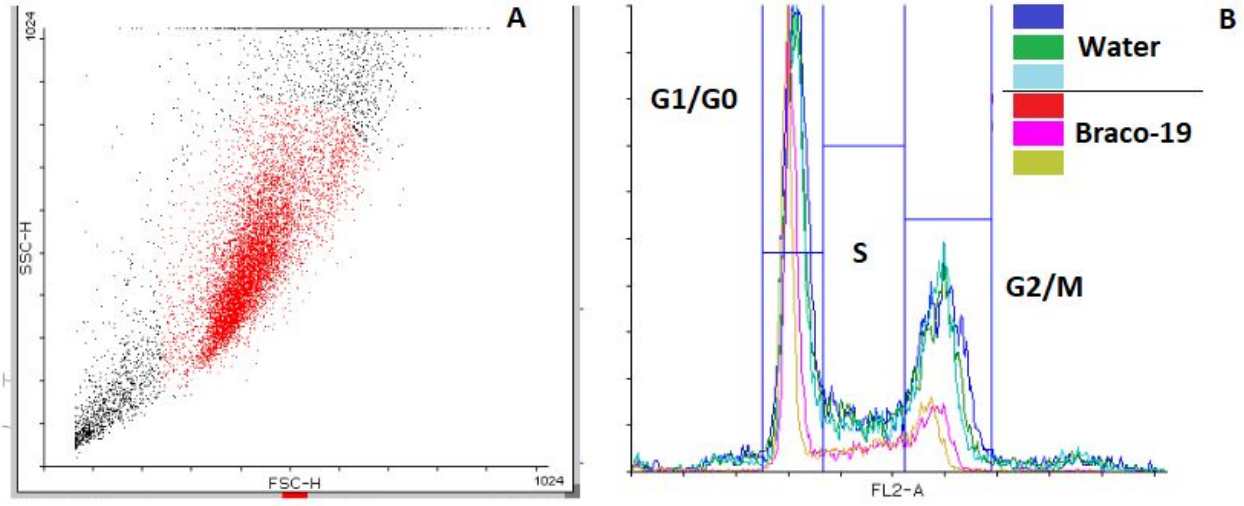

Figure 7) Figure 7a: Graph showing the dispersion of events measured by the cytometer, on x axis FSC (Forward Scater) values and SSC (Side Scater) values on y axis, representing cell size and complexity respectively. In red the data chosen to represent the population and construction of the subsequent graphs, being over 90% of the total of events. Figure 7b: Fluorescence histogram (Propidium Iodide) by number of events with the 3 controls (Water) and 3 treated (Braco-19). The quadrant division histogram show which group is in G1/G0 or S or G2/M. There is no statistically significant difference in fsc and ssc values between the three controls and between the three treated

In **figure 7b** are the histograms demonstrating the reproducibility of the experiment, where the peaks are close between the controls and between the treated ones. In **figure 7b** are defined in quadrants what was considered as G0/G1 (first peak), G2/M (second peak) and S (between the 2 peaks).

In **Figure 8a** is the histogram of only one control and one treated data, showing a smaller number of cells in G2/M phase. In the data analysis it can be observed a statistically significant difference between the control (with 0.02% water) and treated (with Braco-19 at 5µM). In just 24 hours of treatment the group containing the compound showed significantly more cells at phase G0 or G1 **(Figure 8b)**.

A lower number of cells in G2 or M was also observed in the

treated group (B) compared to the control (W) **(Figure 8b)**. Even short-term treatment with Braco-19 has shown a cytostatic effect on cell cycle arrest, probably by interference with other regions of guanine-rich DNA, where G-quadruplex formation occurs and Braco-19 can stabilize, somehow hindering the progression of the tumor cell leaving it in a quiescent state, which may lead to apoptosis in the future.

This result confirms that observed in long-term treatments, where there was clearly a visible difference in colony growth between groups W and B. At 144 hours of treatment, the difference in cell number between groups was noticeable **(Figure 4)**. Several morphological changes were observed during treatment.

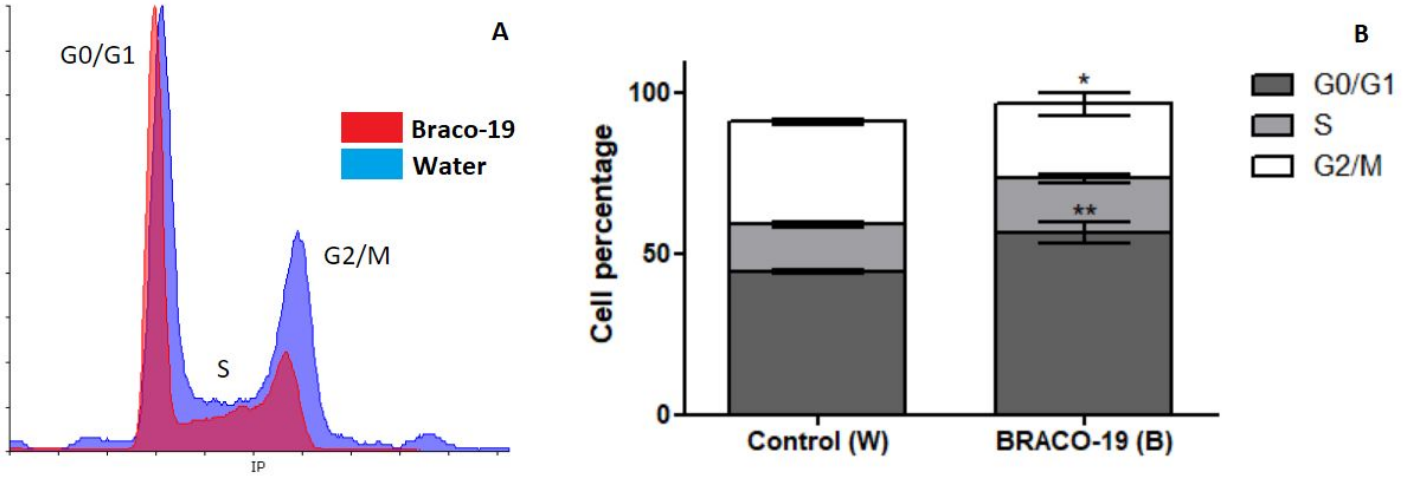

Figure 8) Figure 8a: Demonstrates the histogram of one of the controls and one of the treated, and on the x axis is the relative amount of fluorescence of the propidium iodide compound (an indirect way of quantifying the amount of DNA) and on the y axis the amount of events. The first peak represents the amount of initial fluorescence that demonstrates the cells in the G1/G0 phase, twice its fluorescence represents the second peak, demonstrates the cells in the G2/M phase, and the intermediate data between the peaks represents the cells in the S phase. Figure 8b: Represents all experiments, with 100% representing all events and each peak with a relative percentage. Unpaired t-test was used to compare the means between control and treated. In phases G1/G0 and G2/M they presented a significant P value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) within 24 hours of treatment.

Discussion

Synthetic antitumor compound Braco-19, which is an acridine-derived compound, has demonstrated a good antitumor action *in vitro* in several studies (11, 12, 13). In this study, the antitumor action of Braco-19 on human glioblastoma cells was reaffirmed. There was both short and long term cytotoxic action. Several aspects and results of the experiments performed are consistent with the results found in the literature, corroborating the data obtained.

The doubling time of approximately 32 hours is close to that found in the literature, the times found are between 28 and 38 hours with DMEM medium as a source of nutrients, as well as supplemented with 10% fetal bovine serum in a humid atmosphere at 37°C and 5% CO_2 (14). With the result within the expected standard, the $IC_{50\%}$ tests can be conducted.

The chosen times (24 and 72 hours) were thought to obtain a possible response of the U251 cell line to Braco-19 in a short treatment period, evidencing a possible response before the first doubling and after a doubling pass.

In just 24 hours of treatment and with concentrations ranging from 0.05 µM to 50 µM, a statistically significant response can be observed with an $IC_{50\%}$ calculated at 4.214µM. At 72 hours, theoretically after a doubling pass, the calculated $IC_{50\%}$ was 0.9788µM, showing a dose-dependent and time dependent-response. Literature data suggest an approximate $IC_{50\%}$ of 1.55µM in 72 hours of exposure, the result showed a more sensitive cell line to treatment (15).

Long-term treatments showed a profound response of the Braco-19 cell line, requiring a larger amount of cells to be plated to obtain a considerable sample for morphology analysis. The chosen concentration of 1µM was based on the result obtained at $IC_{50\%}$ of 72 hours and findings in the literature, which suggest this appropriate concentration to evidence the action of the compound (16).

The morphological analysis showed a greater variation in cell length in the treated group than in the control group. Statistically significant differences were found when calculating the length and perpendicular axis ratio, showing that the cells are less elongated with long-term treatment.

Some studies suggest that Braco-19 may not only act by stabilizing the G-quadruplex and thus inhibiting the maintenance of the rangefinder and leading to senescence with telomeric erosion, but also, interacting with the telomer and other cellular mechanisms.

In addition to inhibiting the action of telomerase, articles point out that Braco-19 can undo the T-loop (structure that stabilizes the end of the chromosome), favoring chromosomal fusion, activating DNA damage pathways and translocation of nucleus telomerase to cytoplasm (15).

Data observed on cytometry suggest that Braco-19 has an action beyond telomeres, interfering with the cell cycle. It was observed that although it did not have a significant action on the DNA synthesis phase (Phase S), in only 24 hours there was an increase in G1/G0 phase quiescent cells and a decrease in G2/M phase doubling cells. Studies show that several regions of DNA also rich in guanine, besides telomere, or even RNA that have nearby guanines can spontaneously form G-quadruplex (17).

G-quadruplex stabilization in certain regions of genomic DNA can act as a gene expression regulator, decreasing or increasing transcription (18). It has been reported that there is in the c-myc gene promoter a guanine-rich region, which when used a stabilizer can decrease its expression (19). C-myc is an important oncogene and its expression is related to a wide variety of other genes that are involved in apoptosis, proliferation and cell differentiation (20). Maybe the interference of Braco-19 stabilizing the G-quadruplex may have downregulated the gene and thus promoted a decrease in duplication observed on cytometry, more test will be conducted to evaluate this potential mechanism.

As mentioned, the G-quadruplex complex can occur in RNA as well, and in interaction with this molecule can disrupt translation, protein binding and also in splicing (21; 22; 23).

In literature, it was correlated, in addition to an interference on telomere elongation by G-quadruplex stabilizers, an action of downregulation of the protein responsible for its elongation, telomerase. It has been reported that stabilizers may induce alternative splicing in enzyme messenger RNA, as RNA has guanine-rich regions, thereby decreasing its action in elongating telomeres.

Conclusion

In summary, the action of Braco-19 on glioblastoma cells has shown promise. The sensitivity of the cell to the compound occurred not only in short term tests, but also in long term tests.

In the long term treatment, the compound was equally effective, the G-quadruplex stabilization is probably cause DNA damage, especially in tumor cells, where elongated telomeres are theoretically more sensitive to G-quadruplex stabilizers. The morphological analysis showed that the ratio between the length and the perpendicular axis (width) is significantly different between the groups, leading to the conclusion that the treatment leaves the cells. less elongated and consequently wider. The cytometry showed a cytostatic effect of Braco-19 compound.

The results obtained were promising to the new class of drugs that may arise, having the G-quadruplex as a therapeutic target, *in vitro* the results were positive. Direct inhibitors of telomerase require long treatment periods to shorten the telomere and inhibit its growth and consequently tumor progression (24, 25, 26). Braco-19 has solid evidence of *in vitro* antitumor action, *in vivo* efficacy in early stages of treatment in nude mice (7), evidence has been found that the drug can sensitize cells resistant to chemotherapeutic treatment by telomerase inhibition (27). Further testing should be conducted to improve data for in vivo treatments using Braco-19, as well as to evaluate possible adverse reactions that the antitumor may cause, as its action takes place in various ways in the human genome. The toxicity of this compound should be evaluated and taken as a precautionary measure for future treatments using the G-quadruplex.

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Anexos

Anexo 1 - Perspectivas futuras

Perspectivas Futuras

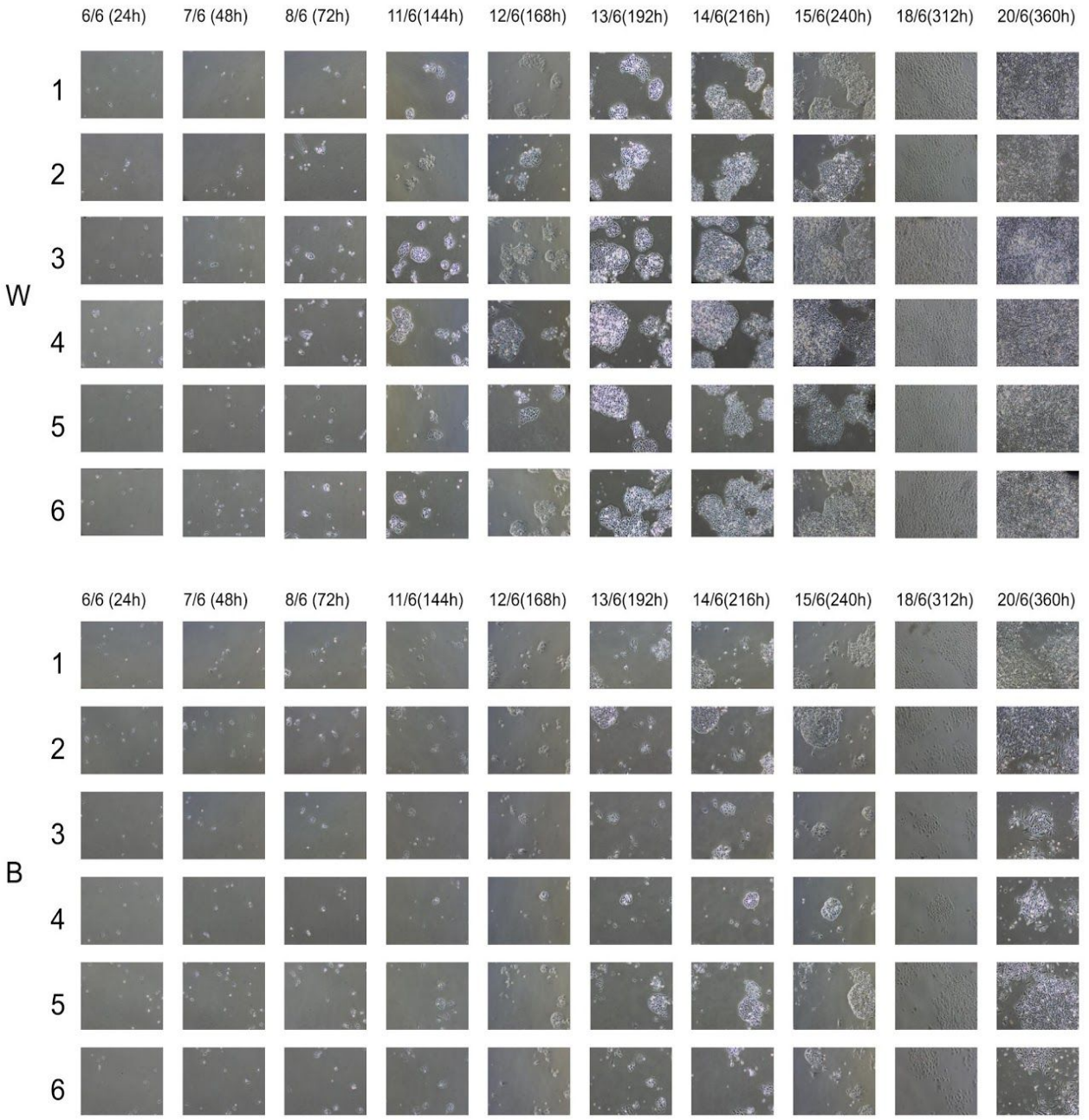
Para melhor elucidar alguns mecanismos que a ação do composto Braco-19 pode estar causando em células de glioblastoma humano U251 e, também, melhor correlacionar os achados *in vitro* com possíveis extrapolações para a clínica, novos testes e experimentos deverão ser conduzidos.

Para avaliar uma interferência direta no tamanho dos telômeros que a estabilização do G-quadruplex possa causar, estão sendo conduzidos testes para o tamanho de telômero, com uma cultura que já foi previamente tratada por cerca de 286 horas ou aproximadamente 9 passos de duplicação.

Avaliação na expressão de genes também está sendo conduzida, o Braco-19 possui como efeito secundário uma possível interferência na enzima telomerase, responsável por alongar os telômeros, para avaliar e quantificar uma possível diferença na expressão da enzima telomerase no controle e no tratado, testes usando a RT-qPCR estão sendo feitos, avaliando a expressão do RNA mensageiro do gene hTERT, responsável pela enzima telomerase.

O uso de Braco-19 como sensibilizante em tratamentos já usados na clínica pode se mostrar eficaz e reduzir a citotoxicidade do composto. Testes de $IC_{50\%}$ estão sendo conduzidos comparando o Braco-19 com o fármaco Docetaxel.

Anexo 2 - Fotos do tratamento com os campos pré definidos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Anexo 3 - Formato da revista Translational Cancer Research (TCR)

Translational Cancer Research

Print ISSN 2218-676X
Online ISSN 2219-6803

INSTRUCTION FOR AUTHORS

Thank you for your interest in *Translational Cancer Research* (TCR). Please consult the following instructions to help you prepare your manuscript, and feel free to contact us with any questions. To ensure fast peer review and publication, manuscripts that do not adhere to the following instructions will be returned to the corresponding author for technical revision before undergoing peer review. We are looking forward to your submission.

TABLE OF CONTENT

1. ABOUT THE JOURNAL

Translational Cancer Research (TCR, Transl Cancer Res; ISSN: 2218-676X; www.thetcr.org) is an Open Access, Peer Review journal launched in June 2012, indexed by Science Citation Index Expanded (SCIE) in October 2015. The indexation covers from the very first issue of the journal Volume 1 (1), to be online at the Web of Science™ core collection. It publishes the results of novel research investigations which bridge the laboratory and clinical settings including risk assessment, cellular and molecular characterization, prevention, detection, diagnosis and treatment of human cancers with the overall goal of improving the clinical care of cancer patients. TCR publishes laboratory studies of novel therapeutic interventions as well as clinical trials which evaluate new treatment paradigms for cancer. The focus of TCR is original, peer-reviewed, science-based research that successfully advances clinical medicine toward the goal of improving cancer patients' lives. The editors and an international advisory group of scientists and clinician-scientists as well as other experts will hold TCR articles to the high-quality standards. We accept Original Articles as well as Review Articles, Editorials and Brief Articles.

Editor-in-Chief: Eric Y Chuang, Sc.D
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Tung-Sung Tseng, DrPH, MS, CHES/MCHES
Frequency: Monthly
Print ISSN 2218-676X
Journal Abbreviation: Transl Cancer Res
Publisher: AME Publishing Company

2. REVIEW PROCESS

Manuscripts are assigned sequentially to Science Editors. The Science Editor solicits reviewers (typically, two external reviews

are sought). The reviewers' evaluations and Science Editor's comments are compiled by the Editors-in-Chief for disposition and transmittal to the authors. A decision is made usually within six weeks of the receipt of the manuscript.

The Editors-in-Chief will advise whether a manuscript is accepted, should be revised or is rejected. Minor revisions are expected to be returned within two weeks of decision; major revisions within three weeks. Manuscripts not revised within these time periods are subject to withdrawal from consideration for publication unless the authors can provide extenuating circumstances.

A number of manuscripts will have to be rejected on the grounds of priority and available space. A manuscript may be returned to the authors without outside review if the Editors-in-Chief and Science Editor find it inappropriate for publication in the Journal. Similarly, the Editors may expedite the review process for manuscripts felt to be of high priority in order to reach a rapid decision. Such 'fast-track decisions' will normally occur within one week of receipt of the manuscript.

Authors may recommend preferred reviewers by providing the Editors-in-Chief with the names, addresses and email addresses of up to three suitably qualified individuals of international standing but the Editors-in-Chief will not be bound by any such nomination. Likewise, authors may advise of any individual who for any reason, such as potential conflict of interest, might be inappropriate to act as a referee, again without binding the Editors-in-Chief.

The Editors-in-Chief's decision is final. If, however, authors dispute a decision and can document good reasons why a manuscript should be reconsidered, a rebuttal process exists. In the first place, authors should write to the Editors-in-Chief.

All journals Manuscripts should be written in a clear, concise, direct style so that they are intelligible to the professional reader who is not a specialist in the particular field. Where contributions are judged as acceptable for publication, the Editor and the Publisher reserve the right to modify manuscripts to eliminate ambiguity and repetition and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

3. MANUSCRIPT CATEGORY

(1) ORIGINAL ARTICLE

Word limit: 5,000 words maximum including abstract but excluding references, tables and figures.
Abstract: Structured. 450 words maximum.
References: No maximum.
Figures/tables: No maximum, but 8 figures should be sufficient.
Description: Full-length reports of current research in either basic or clinical science. The abstract should contain the following subheadings: **Background**, **Methods**, **Results and Conclusions**. Original articles should entail a section describing the contribution of each author to the manuscript. See section "Authors' Contribution" for details. Meta-analysis will be categorized into this type.

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Word limit: 6,000 words maximum including abstract but excluding references, tables and figures.
Abstract: Unstructured. 450 words maximum.
References: No maximum.
Figures/tables: Minimum 1 image or figure.
Description: Reviews are comprehensive analyses of specific topics. They are submitted upon invitation by the Editors. Proposals for reviews may be submitted; however, in this case authors should only send an outline of the proposed paper for initial consideration. Both solicited and unsolicited review articles will undergo peer review prior to acceptance. Review articles should entail a section describing the contribution of each author to the manuscript. See section " Authors' contribution' for details.

(3) EDITORIAL

Word Limit: 2,500 words maximum excluding references, tables and figures.
Abstract: Not required.
References: 25 maximum.
Figures/tables: 2 maximum in total.
Description: Editorial is written by recognized leader(s) in the field. It is generally solicited by the (Deputy) Editor(s)-in-Chief.

(4) EDITORIAL COMMENTARY

Word Limit: 2,500 words maximum excluding references, tables and figures.
Abstract: not required for this manuscript type.
References: 25 maximum.
Figures/Tables: 2 maximum.
Description: The Editors will invite an expert in the field to discuss a paper or report or event within the past few months or so, or in the near future and provide a commentary on the importance of each accepted paper to outline its strengths and weaknesses. It should set the problems addressed by the paper/report/event in the wider context of the field.

(5) CLINICAL GUIDELINE

Word limit: 6,000 words maximum including abstract but excluding references, tables and figures.
Abstract: Unstructured. 450 words maximum.
References: No maximum.
Figures/tables: Minimum 1 image or figure.
Description: Guidelines need to be the product of a large group of individuals who are recognized authorities in their field. Guidelines will be written by a working party to include a steering committee (usually at least 4 members) and other authors representing a wide range of those with special relevant expertise as well as those whose everyday practice will be

influenced by the guidelines.

(6) TECHNICAL NOTE

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References: 35 maximum.
Figures/tables: 10 maximum in total.
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(7) CASE REPORT

Word limit: 2,500 words maximum excluding references, tables and figures.
Abstract: Unstructured. 250 words maximum.
References: 20 maximum.
Figures/tables: 8 maximum in total.
Description: New observations of diseases, clinical findings or novel/unique treatment outcomes relevant to practitioners in oncology. The text should be arranged as follows: Introduction, Case Report, Discussion.

The author should prepare the manuscript according to the CARE guideline and indicate at the end of the Introduction section that the case report is presented in accordance with the CARE Guideline. The wording of the indication could be "We present the following case in accordance with the CARE Guideline." The CARE checklist (https://data.care-statement.org/wp-content/uploads/2019/10/CARE-checklist-English-2013.pdf) should be provided as an additional file. Submissions received without these elements will be returned to the authors as incomplete. The checklist will not be used as a tool for judging the suitability of manuscripts for publication, but it is intended as an aid to authors to entirely and transparently let reviewers and readers know what authors did and found.

The authors should also provide a statement at the end of the main text to confirm that the patient has given their consent for the Case reports to be published. The editorial office may request copies of the informed consent documentation at any time. We recommend the following wording is used for the consent section: "Written informed consent was obtained from the patient for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal."

If the patient has died, then consent for publication must be sought from the next of kin of the patient. If the patient is a minor, or unable to provide consent, then consent must be sought from the parents or legal guardians of the patient. In these cases, the statement in the 'Consent' section of the manuscript should be amended accordingly.

Only cases of exceptional interest and novelty are considered. For manuscripts that do not qualify, Editors may ask authors to shorten manuscripts and rewrite as other article types.

For the example of the published case report, please refer to: http://jtd.amegroups.com/article/view/17372/html.

(8) CORRESPONDENCE

Word limit: 1,000 words maximum excluding references, tables and figures.
Abstract: Not required.
References: 10 maximum.
Figures/tables: 1 maximum in total.
Description: Correspondence on content published in TCR or on other topics of interest to our readers is welcomed. The journal might invite replies from the authors of the original publication, or pass on letters to these authors. Correspondence is also referred to as Letter to the Editor.

4. STRUCTURE OF THE MANUSCRIPT

The length of manuscripts must adhere to the specifications under the section Manuscript Categories.

Manuscripts should be presented in the following order: (i) title page, (ii) abstract and key words, (iii) text, (iv) acknowledgments, (v) footnote, (vi) references, (vii) supplementary material, (viii) figure legends, (ix) tables (each table complete with title and footnotes) and (x) figures. Footnotes to the text are not allowed and any such material should be incorporated into the text as parenthetical matter.

TITLE PAGE

The title page should contain (i) the title of the paper. Concise titles are easier to read than long, convoluted ones. Titles that are too short may, however, lack important information, such as study design (which is particularly important in identifying randomized controlled trials). Authors should include all information in the title that will make electronic retrieval of the article both sensitive and specific. (ii) the full names of the authors and (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers, of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote. The title should be short, informative and contain the major key words so that readers and in particular online users will discover the article easily in online search. Do not use abbreviations in the title. A running title of no more than 60 characters including spaces.

ABSTRACT AND KEYWORDS

The length of abstracts must adhere to the word count specifications under the section Manuscript Categories. The

abstract should state the main problem, methods, results, and conclusions. Do not use reference, table or figure in the abstract. It must be factual and comprehensive. The use of abbreviations and acronyms should be limited and general statements (e.g. "the significance of the results is discussed") should be avoided. The abstract of an original article, systematic review and meta-analysis should be structured into four paragraphs with headings of Background, Methods, Results and Conclusions. The abstracts for all other manuscript types should be unstructured.

Three to five key words should be supplied below the abstract, in alphabetical order, and should be taken from those recommended by the US National Library of Medicine's Medical Subject Headings (MeSH) browser list at:

http://www.nlm.nih.gov/mesh/meshhome.html

TEXT

Authors must use the following subheadings to divide the sections of their Original Article manuscript: Introduction, Methods, Results, Discussion, Conclusions, Acknowledgment, Footnote, References, and when relevant, Supplementary Material. Authors should follow the same structures in Systematic Review and Meta-analysis. However, review, perspective, viewpoint and commentary articles do not have those clear sections, they can be written in several sections with their own headings according to the topic.

AUTHOR CONTRIBUTIONS

This section is only required for original article, review article, systematic review and meta-analysis article. It describes the contribution each author made to the manuscript. Authorship credit should be based on 1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3. Please note that acquisition of funding, collection of data, language editing or general supervision of the research group alone does not constitute authorship.

The Author contributions section should be completed as follow:

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(II) Administrative support:
(III) Provision of study materials or patients:
(IV) Collection and assembly of data:
(V) Data analysis and interpretation:
(VI) Manuscript writing: All authors
(VII) Final approval of manuscript: All authors

Note: 1. VI and VII of all authors are obligatory while the rest information are case based; 2. Contributions section is not required when there is only one author.

ACKNOWLEDGMENT

Textual material that names the parties which the author wishes to thank or recognize for their assistance in, for example, producing the work, funding the work, inspiring the work, or assisting in the research on which the work is based.

All contributors who do not meet the criteria for authorship should be listed in an acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing or language editing assistance, or a department chairperson who provided only general support. Financial and material support should also be acknowledged. When there is no one to be acknowledged, authors should also indicate 'Acknowledgements' section as 'None'.

TCR policy requires that all authors of all manuscripts sign a statement revealing: 1) Any financial interest in or arrangement with a company whose product was used in a study or is referred to in an article, 2) Any financial interest in or arrangement with a competing company, 3) Any other financial connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications or opinions stated including pertinent commercial, governmental, private or other sources of funding for the individual author(s) or for the affiliated department(s) or organization(s), personal relationships, or direct academic competition. Statements related to study design, such as providers of the drugs used in the study should be indicated in the Methods section of the article, and other financial interests which are not directly related to carrying out the study should be stated in the Acknowledgements.

FOOTNOTE

a. Conflicts of Interest: See section "Conflict of interest" for details.
b. Financial Disclose: Some variables, such as "measures of income inequality and degree of financial openness, are not included in our study because of the limited availability of good-quality data across countries over the sample period". When there is no financial disclose, authors should also indicate "Financial Disclose" section as "None".
c. Ethical statement: the authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Please note that the above statement must be included in the footnote of the article as part of the Ethical Statement.

REFERENCE

The Vancouver system of referencing should be used (examples are given below). In the text, references should be identified using numbers in round brackets in which they appear consecutively [e.g., "cancer-related mortality (19)"; "denocarcinoma (29,30)"; "malignancies (14-18)"]. If cited in tables or figure legends, number according to the first identification of the table or figure in the text. In the reference list, cite the names of all authors when there are three or fewer;

when four or more, list the first three followed by et al. Do not use ibid. or op cit. Reference to unpublished data and personal communications should not appear in the list but should be cited in the text only (e.g. Smith A, 2000, unpublished data). All citations mentioned in the text, tables or figures must be listed in the reference list. Names of journals should be abbreviated in the style used in Pubmed. Authors are responsible for the accuracy of the references.

• Journal article

1. Gibas Z, Prout DF Jr, Pontes JR. Chromosome changes in germ cell tumours of the testis. Cancer Genet Cytogenet 1986; 19: 254-52.

•Online article not yet published in an issue

An online article that has not yet been published in an issue (therefore has no volume, issue or page numbers) can be cited by its Digital Object Identifier (DOI). The DOI will remain valid and allow an article to be tracked even after its allocation to an issue.

1. Furuya R, Takahashi R, Furuya S, et al. Is urethritis accompanied by seminal vesiculitis? Int J Urol. DOI: 10.1111/j.1442-2042.2009.02314.x

• Book

2. Ernstoff M. Urologic Cancer. Blackwell Science, Boston, 1997.

• Chapter in a Book

3. Gilchrist RK. Further commentary: Continent stroma. In: King LR, Stone AR, Webster GD (eds). Bladder Reconstruction and Continent Urinary Diversion. Year Book Medical, Chicago, 1987; 204-5.

TABLE

Tables should be self-contained and complement, but not duplicate, information contained in the text. Number tables consecutively in the text in Arabic numerals. Type tables on a separate page with the legend above. Legends should be concise but comprehensive – the table, legend and footnotes must be understandable without reference to the text. Vertical lines should not be used to separate columns. Column headings should be brief, with units of measurement in parentheses; all abbreviations must be defined in footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values. Statistical measures such as SD or SEM should be identified in the headings. If tables have been reproduced from another source, a letter from the copyright holder (usually the Publisher), stating authorization to reproduce the material, must be attached to the covering letter.

FIGURE

All illustrations (line drawings and photographs) are classified as figures. Figures should be cited in consecutive order in the text. Magnifications should be indicated using a scale bar on the illustration. If figures have been reproduced from another source, a letter from the copyright holder (usually the Publisher), stating authorization to reproduce the material, must be attached to the covering letter.

Size: Figures should be sized to fit within the column (82 mm), intermediate (118 mm) or the full text width (173 mm).

Resolution: Figures must be supplied as high resolution saved as .eps or .tif. Halftone figures 300 dpi (dots per inch), Color figures 300 dpi saved as CMYK, figures containing text 400 dpi, Line figures 1,000 dpi.

Color figures: Files should be set up as CMYK (cyan, magenta, yellow, black) and not as RGB (red, green, blue) so that colors as they appear on screen will be a closer representation of how they will print in the Journal.

Line figures: Must be sharp, black and white graphs or diagrams, drawn professionally or with a computer graphics package.

Text sizing in figures: Lettering must be included and should be sized to be no larger than the journal text or 8 point (Should be readable after reduction – avoid large type or thick lines). Line width between 0.5 and 1 point.

Figure legends: Type figure legends on a separate page. Legends should be concise but comprehensive – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

VIDEO

TCR will accept digital files in mp4, flash video (flv.), MPEG(MPEG video file), DVD video format, mov., avi., and mwv. formats or video on CD/DVD. Contributors are asked to be succinct, and the Editor-in-chief reserves the rights to require shorter video duration if necessary. Video files can be submitted with a manuscript online: http://tcr.amegroups.com/pages/view/submit-multimedia-files.

Duration: Video files should be limited to 20 minutes.

Quality: Please set the video aspect ratio as 4:3 or 16:9 (widescreen). The original video should be of high quality. The resolution is no less than 1280*720, the frame rate no less than 24 frames per second and the bit rate no lower than 5Mbps.

Text in video: All the text notes, explanations or descriptions, etc. in the video must be in English. And the logo or watermark of hospital should not be stick on the screen. Plus, the information of patients should be erased from the video.

Video legends: Legends for the video files should be provided. The video files should be numbered consecutively in the order of reference in the text.

EQUATION

Equations should be numbered sequentially with Arabic numerals; these should be ranged right in parentheses. All

variables should appear in italics. Use the simplest possible form for all mathematical symbols.

APPENDIX

The Supplementary Appendix should be paginated, with a table of contents, followed by the list of investigators (if there is one), text (such as methods), figures, tables, and then references. The supplementary appendix should not be included in the article's reference list.

The Appendix must be submitted in a Word file. The Appendix will not be edited for style. It will be presented online as additional information provided by the authors.

The published article will contain a statement that supplementary material exists online and will provide the reader with a URL and link. To reference the supplementary appendix in the text of the article, refer to it as in the following example:

"Many more regressions were run than can be included in the article. The interested reader can find them in a supplementary appendix online."

5. ETHICAL CONSIDERATION

Authors must state that the protocol for the research project has been approved by a suitably constituted Ethics Committee of the institution within which the work was undertaken and that it conforms to the provisions of in accordance with the Helsinki Declaration as revised in 2013, available at: http://www.wma.net/en/30publications/10policies/b3/%20index.html. The journal retains the right to reject any manuscript on the basis of unethical conduct of either human or animal studies. All investigations on human subjects must include a statement that the subject gave informed consent. Patient anonymity should be preserved. Photographs need to be cropped sufficiently to prevent human subjects being recognized (or an eye bar should be used).

◆ **For studies in the following categories:**

Randomized controlled trials or other intervention research: This category includes any study that carries out medical intervention(s) on patients or healthy individuals.

Case-control study: A case-control study is designed to retrospectively analyze the exposure to the risk factor of interest in subjects with known outcomes (with or without disease; dead or alive; or, with or without other pre-determined endpoints).

Prospective cohort study: In a prospective cohort study, patients with known exposure to a risk factor are followed and then the outcomes (with or without disease; or, dead or alive) were identified.

Cross-sectional studies: Cross-sectional studies are performed to investigate the occurrence of a specific disease or the status quo of a clinical condition.

Basic or translational medical research using human specimens:

- Authors must state whether their studies had been approved by an institutional review board (IRB) (if yes, please provide the number of approval document). For a multi-center study, IRB approval must be obtained from each center.
- The authors must state whether all the subjects had signed the informed consent forms. For subjects under 18 years of age or those with limited capacity for civil conduct, the authors must state whether their caregivers had signed the informed consent forms.
- Also, the authors should state whether the study outcomes will affect the future management of the patients.

◆ **For other categories:**

Retrospective and ambispective cohort studies: In these studies, the patients' exposure to risk factor(s) were retrospectively identified, followed by the retrospective follow-up of the patients to determine the relationship between the future or current endpoints (with or without disease; or, dead or alive) and the exposure.

- For studies in this category, authors must state whether their study had been approved by an institutional review board (IRB) (if yes, please provide the number of approval document). For a multi-center study, IRB approval must be obtained from each center.
- Also, the authors should state whether the study outcomes will affect the future management of the patients.
- The authors must state whether all the subjects had signed the informed consent forms before enrollment. For subjects under 18 years of age or those with limited capacity for civil conduct, the authors must state whether their caregivers had signed the informed consent forms. For deceased patients or those who had lost capacity for civil conduct, the informed consent forms could be signed by their family members or caregivers. For studies on patient data retrieved from hospital medical record system or social insurance systems, an informed consent form is not required; however, the authors still need to declare whether the patient's personal data have been secured.

Systematic review and meta-analysis, review, hypothesis, and editorial

- No statement on medical ethics is required.

Case report and visualized surgery:

- No statement on medical ethics is required. However, in cases of involving new and controversial treatments, approval from IRC might be required.
- Informed consent must be obtained from the subjects or their caregivers.

Diagnostic accuracy tests: These studies are performed to evaluate the efficiency of a specific index test in disease diagnosis.

- For studies in this category, authors must state whether their study had been approved by an institutional review board (IRB) (if yes, please provide the number of approval

document). For a multi-center study, IRB approval must be obtained from each center.
- Also, the authors should state whether the study outcomes will affect the future management of the patients.
- If the study has a prospective design: the authors must state whether all the subjects had signed the informed consent forms before enrollment. For subjects under 18 years of age or those with limited capacity for civil conduct, the authors must state whether their caregivers had signed the informed consent forms. However, for retrospective studies based on a hospital medical record system, no informed consent is required.

Nested case-control study: In a nested case-control study, the patients were followed up after the biological samples are obtained from the subjects, and then a subset of patients are chosen for the analysis.

If the study has a prospective design:
- Authors must state whether their study had been approved by an institutional review board (IRB) (if yes, please provide the number of approval document). For a multi-center study, IRB approval must be obtained from each center.
- Also, the authors should state whether the study outcomes will affect the future management of the patients.
- The authors must state whether all the subjects have signed the informed consent forms before they enter the study, no matter whether they enter the final analysis. For subjects under 18 years of age or those with limited capacity for civil conduct, the authors must state whether their caregivers had signed the informed consent forms.

If the study is based on a previously available specimen bank, the authors must:
- State whether the specimen bank had been approved by the IRB upon its establishment;
- State whether all the subjects had signed the informed consent forms during the establishment of the bank (attached with the numbers of approval documents).

Post hoc analysis: In a post hoc analysis, the authors re-examines the currently available data from different perspectives.
- The authors need to state whether the previous studies had been approved by the local medical ethics committee(s)
- Also, it is important to state whether all the subjects had signed the informed consent forms in the previous studies.

For more information on statement of ethics, please feel free to consult our editorial staff.

6. INFORMED CONSENT

Identifying information, including names, initials, or hospital numbers, should not be published in written descriptions, photographs, or pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent is required for **Case report, original/research articles and visualized surgery**. The statement should be included in the footnote.

It may be possible to publish without explicit consent if the report is important to public health (or is in some other way important); consent would be unusually burdensome to obtain; and a reasonable individual would be unlikely to object to publication (all three conditions must be met).

7. POLICIES ON CONFLICT OF INTEREST

Our journal complies with the International Committee of Medical Journal Editors' uniform requirements on Conflict of Interest statement.

Conflict of Interest exists when an author (or the author's institution), reviewer, or editor has financial or personal relationships with other persons or organizations that inappropriately influence (bias) his or her actions. The existence of such relationships does not necessarily represent true conflict of interest. The potential for conflict of interest can exist whether or not an individual believes that the relationship affects their judgment. Financial relationships (such as employment, consultancies, stock ownership, honoraria, paid expert testimony, patents) are the most easily identifiable conflicts of interest and the most likely to undermine the credibility of the journal, the authors, and of science itself (http://www.icmje.org/index.html). Conflict of interest would be included in the FOOTNOTE section.

(1). PARTICIPANT

All participants in the peer-review and publication process—not only authors but also peer reviewers, editors, and editorial board members of journals—must consider their conflicts of interest when fulfilling their roles in the process of article review and publication and must disclose all relationships that could be viewed as potential conflicts of interest.

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When authors submit a manuscript of any type or format they are responsible for disclosing all financial and personal relationships that might bias or be seen to bias their work.

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Reviewers should be asked at the time they are asked to critique a manuscript if they have conflicts of interest that could complicate their review. Reviewers must disclose to editors any conflicts of interest that could bias their opinions of the manuscript, and should recuse themselves from reviewing specific manuscripts if the potential for bias exists. Reviewers must not use knowledge of the work they're reviewing before its publication to further their own interests.

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Editors who make final decisions about manuscripts should recuse themselves from editorial decisions if they have conflicts of interest or relationships that pose potential conflicts related to articles under consideration. Other editorial staff members who participate in editorial decisions must provide editors with a current description of their financial interests or other conflicts (as they might relate to editorial judgments) and recuse themselves from any decisions in which a conflict of interest exists. Editorial staff must not use information gained through working with manuscripts for private gain. Editors should publish regular disclosure statements about potential conflicts of interests related to the commitments of journal staff. Guest editors should follow these same procedures.

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