FABRICIO SEIDY RIBEIRO INOUE

ATIVIDADE ANTITUMORAL *IN VITRO* DO 3,3',5,5'-TETRAMETOXIBIFENIL-4,4'-DIOL SOBRE AS LINHAGENS HUH7.5 E HEPG2/C3A DE CARCINOMA HEPATOCELULAR HUMANO

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BANCA EXAMINADORA

Prof. Dr. Wander Rogério Pavanelli
Universidade Estadual de Londrina - UEL

Dr.ª Bruna Taciane da Silva Bortoleti
Icahn School of Medicine at Mount Sinai

Prof.ª Dr.ª Milena Menegazzo Miranda-Sapla
Universidade do Vale do Itajaí - UNIVALI

Londrina, 11 de março de 2022.

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"O sentimento de admiração que a ciência pode nos dar é uma das maiores experiências das quais a psique humana é capaz".

Richard Dawkins

"Acima de tudo, não tema os momentos difíceis. O melhor vem deles."

Rita Levi-Montalcini

RESUMO

INOUE, Fabricio Seidy Ribeiro. **Atividade Antitumoral *in vitro* do 3,3',5,5'-Tetrametoxibifenil-4,4'-Diol Sobre as Linhagens Celulares HuH7.5 e HepG2/C3A de Carcinoma Hepatocelular Humano**. 2021. 76 p. Dissertação (Mestrado em Patologia Experimental) – Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, 2021.

Dentre os cânceres hepáticos primários, o carcinoma hepatocelular (CHC) é o mais incidente e agressivo, apresentando apenas 18% de sobrevida em 5 anos, baixa responsividade e recorrente resistência à quimioterapia devido a heterogeneidade molecular encontrada nos tumores. Neste contexto, a utilização de compostos fenólicos como alternativa terapêutica para o câncer tem sido o foco de muitos estudos devido às suas propriedades biológicas como antimutagenicidade, anti-inflamatória e antiproliferativa. O 3,3',5,5'-tetrametoxibifenil-4,4'-diol (TMBP) é um composto biossintético obtido através da oxidação do 2,6-dimetoxifenol por meio da biotransformação enzimática. Em estudos prévios utilizando o TMBP, foi demonstrado sua capacidade antiproliferativa sobre a linhagem A549 (adenocarcinoma pulmonar). O presente trabalho propôs explorar a atividade antitumoral do TMPB sobre as linhagens HuH7.5 e HepG2/C3A (CHC), assim como os mecanismos de morte celular envolvidos. Inicialmente, foi avaliada a viabilidade celular das linhagens tratadas com o TMBP (12,5–150 µM) nos tempos de 24 e 48h por ensaio de MTT. Posteriormente foram calculadas as concentrações inibitórias mínimas de 50% (IC_{50}), utilizadas para os experimentos seguintes. Para a confirmação do ensaio de viabilidade, foi realizado contagem através do método de exclusão azul de tripan. Para a análise dos efeitos celulares foram avaliadas as alterações morfológicas, ultraestruturais e capacidade de migração celular por microscopia eletrônica e ensaio de cicatrização de ferida. Como forma de avaliar as alterações metabólicas após tratamento com IC_{50} foram mensuradas espécies reativas de oxigênio (ERO) total for fluorescência, utilizando a sonda de diacetato de 2',7'-diclorohidrofluoresceína (H_2DCFDA), óxido nítrico (NO) pelo método de Griess, potencial de membrana mitocondrial, vacúolos autofágicos e gotículas lipídicas por métodos de fluorescência utilizando as marcações tetrametilrodamina (TMRE), monodansilcadaverina (MDC) e vermelho neutro (NR), respectivamente. A distribuição do ciclo celular também foi avaliada, juntamente com o ensaio de caracterização de morte celular por citometria de fluxo. O tratamento com TMBP reduziu a viabilidade celular em todas as concentrações testadas, chegando aos valores de IC_{50} de 68 e 50 µM em 24h para as linhagens HuH7.5 e HepG2/C3A, respectivamente, os quais foram utilizados para os seguintes experimentos. As células tratadas apresentaram migração comprometida, além de severas alterações morfológicas. O tratamento induziu aumento nos níveis de ERO e NO, despolarização da membrana mitocondrial e ativação de mecanismos de adaptação, como aumento da biosíntese de gotículas lipídicas e vacúolos autofágicos em ambas as linhagens, tais alterações foram validadas através das alterações ultraestuturais. Além disso o tratamento com TMBP promoveu similarmente nas duas linhagens a parada do ciclo celular na fase G2/M, e induziu a morte celular por apoptose. Desse modo, foi possível verificar a ação citotóxica e antiproliferativa direta do TMBP sobre as linhagens tumorais, indicando este composto como um candidato promissor ao desenvolvimento de uma droga antitumoral.

Palavras-chave: Carcinoma hepatocelular; TMBP; Compostos fenólicos; Apoptose.

ABSTRACT

INOUE, Fabricio Seidy Ribeiro. **In vitro Antitumoral Activity of 3,3',5,5'-Tetramethoxybiphenyl-4,4'-Diol on HuH7.5 and HepG2/C3A Cell Lineages of Human Hepatocellular Carcinoma**. 2021. 76 p. Dissertation (Masters in Experimental Pathology) – Biological Sciences Center, State University of Londrina, Londrina, 2021.

Among primary liver cancers, hepatocellular carcinoma (HCC) is the most incident and aggressive, presenting only 18% 5-year survival, low responsivity and recurrent resistance to chemotherapy considering its molecular heterogeneity found in different tumors. In this context, the use of phenolic compounds as an alternative to cancer therapy has been the focus of several studies due to their biological properties as antimutagenicity, anti-inflammatory and antiproliferative. 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol (TMBP) is a biosynthetic compound obtained by 2,6-dimethoxyphenol oxidation by enzymatic biotransformation. In previous studies with TMBP, was demonstrated its antiproliferative capacity against the A549 cell line (lung adenocarcinoma). This study has proposed exploring TMBP antitumoral activity on HuH7.5 and HepG2/C3A (HCC) cell lines, as well as involved cell death mechanisms. Initially was evaluated the cell viability of cells treated with TMBP (12.5–150 μM) for 24 and 48 hours by MTT assay. Subsequently, was calculated the minimum inhibitory concentration of 50% (IC_{50}), used for the next experiments. To confirm the cell viability assay, a cell counting by trypan blue exclusion assay was performed. For cellular effects analysis were evaluated morphological, ultrastructural and cell migration alterations by electron microscopy and wound healing assay. As a way to evaluate metabolic alterations after treatment with IC_{50}, was mensurated total reactive oxygen species (ROS), using a 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) probe, nitric oxide (NO) by Griess method, mitochondrial membrane potential, autophagic vacuoles and lipid droplets by tetramethylrhodamine (TMRE), monodansylcadaverine (MDC) and nile red (NR) staining, respectively. Cell cycle distribution was also evaluated, along with cell death assay by flow cytometry. Treatment with TMBP decreased cell viability in all concentrations tested, leading to IC_{50} values of 68 and 50 μM in 24 hours for HuH7.5 and HepG2/C3A cell lines, respectively, which were used for the following experiments. Treated cells presented severe morphological alterations and migration impairment. The treatment resulted in increased ROS and NO levels, mitochondrial membrane depolarization and activation of adaptation mechanisms, as increased autophagic vacuoles and lipid droplets biosynthesis in both lineages. Those alterations were validated through ultrastructural changes. Hence, TMBP treatment promoted similarly in both lines cell cycle arrest in the G2/M phase, as well as induced cell death by apoptosis. As demonstrated, it was possible to verify TMBP's cytotoxic and antiproliferative activity against these tumoral cell lines, indicating this compound as a promising candidate to the development of an antitumoral drug.

Key-words: Hepatocellular carcinoma; TMBP; Phenolic Compounds; Apoptosis.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

2,6-DMP	2,6-Dimetoxifenol
ATG	Gene Relacionado à Autofagia
ATM	Proteína Kinase Ataxia-Telangiectasia Mutada
ATR	Proteína Kinase Relacionada A ATM E Rad3
AXL	Receptor de Tirosino Kinase AXL
CAK	Kinase ativadora de CDK
CDK	Kinase Dependente de Ciclina
CF	Compostos Fenólicos
CHC	Carcinoma Hepatocelular
Chk1/2	Checkpoint Kinase 1 e 2
Ck1/2	Caseína Kinase 1 e 2
CKI	Inibidor de CDK
CTNNB1	Catenina Beta 1
ERO	Espécies Reativas de Oxigênio
FGF	Fator de Crescimento Fibroblástico
IARC	Agência Internacional de Pesquisa em Câncer
JNK	Kinase c-Jun N-terminal
MCDA	Morte Celular Dependente De Autofagia
MET	Receptor de Tirosino Kinase Codificado por Proto-oncogene MET
OMS	Organização Mundial da Saúde
PDGF	Fator de Crescimento Derivado de Plaquetas
Rb	Retinoblastoma
TERT	Transcriptase Reversa da Telomerase
TERTp	promotores de TERT

TMBP	3,3’,5,5’-tetrametoxibifenil-4,4’-diol
TNFR	Receptor do Fator de Necrose Tumoral
TP53	Proteína Tumoral 53
TRAILR1/2	Receptor de TRAIL 1 e 2
VEGF	Fator de Crescimento do Endotélio Vascular

SUMÁRIO

1. INTRODUÇÃO

1.1. ASPECTOS GERAIS DO CÂNCER

De acordo com a Organização Mundial da Saúde (OMS) (2020) , o câncer se tornou a principal causa de morte nos países desenvolvidos e a segunda em países subdesenvolvidos nas populações abaixo dos 70 anos. Como prediz o estudo realizado pela Agência Internacional de Pesquisa em Câncer (do inglês, *International Agency for Research on Cancer* - IARC) (2020), estima-se que para o ano de 2040 tenha cerca de 30 milhões de novos casos e 16 milhões de mortes relacionadas ao câncer. Este número tende a aumentar devido ao rápido crescimento e envelhecimento populacional, assim como a maior exposição a fatores de risco relacionados ao desenvolvimento socioeconômico e industrial em todo o mundo (SUNG et al., 2021).

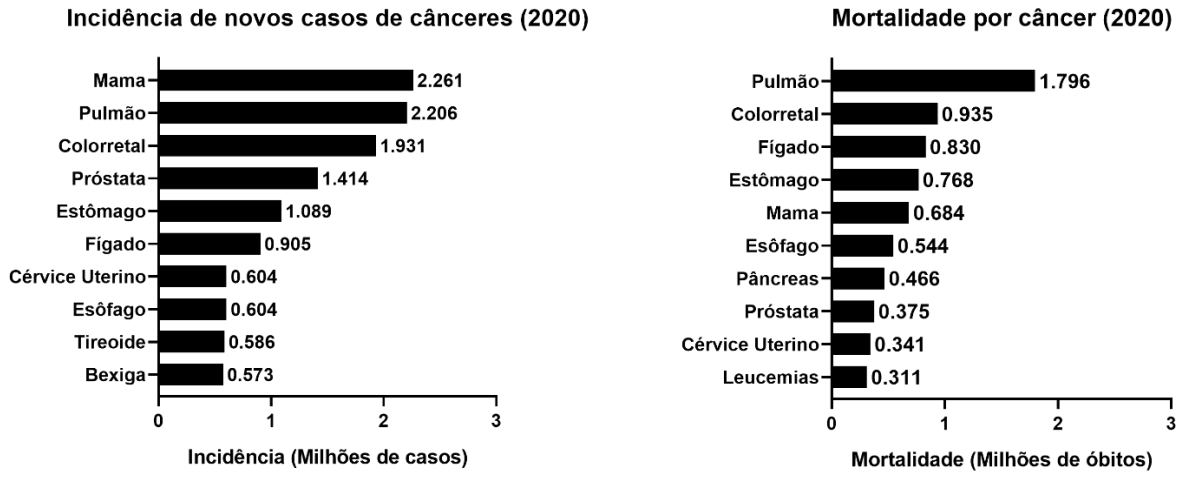
O desenvolvimento de um câncer é entendido como um processo de diversas etapas que envolve o acúmulo de mutações ou alterações epigenéticas em genes importantes para a fisiologia e homeostase celular, levando à sua transformação e evolução para um fenótipo maligno (TAKESHIMA; USHIJIMA, 2019). Este fenótipo compartilha características em comum entre as células de diversos tipos de cânceres, descritas por Hanahan & Weinberg (2000) como as “marcas registradas do câncer” (do inglês *hallmarks of cancer*). As seis características encontradas são a produção e sinalização autócrina e parácrina de fatores de crescimento, insensibilidade a sinais inibidores de crescimento, evasão dos mecanismos de morte celular, potencial proliferativo indefinido, angiogênese e capacidade de invasão tecidual e metástase.

Essas marcas registradas são causadas e intensificadas por meio da instabilidade genômica e inflamação, o que dá origem à cânceres com alta heterogeneidade. Em um posterior estudo realizado por Hanahan & Weinberg (2011), foram avaliadas outras duas características no desenvolvimento do câncer, a reprogramação do metabolismo energético e evasão dos mecanismos de destruição do sistema imune, as quais contribuem para a sobrevivência e propagação das células tumorais.

1.2. CÂNCERES HEPÁTICOS

Os cânceres hepáticos demonstram um grande problema de saúde pública mundialmente, sendo considerado o sexto câncer mais incidente e o terceiro com a maior taxa de mortalidade (**Fig. 1**), com um aumento progressivo em sua incidência ao longo dos anos. De acordo com a IARC (2021), em 2020 foram diagnosticados 905.677 casos de cânceres hepáticos. E estima-se um aumento progressivo de óbitos anualmente, de 896.569 mortes em 2020 para mais de 1,6 milhões até o ano de 2060 em todo o mundo (**Fig. 2**) (OMS, 2016).

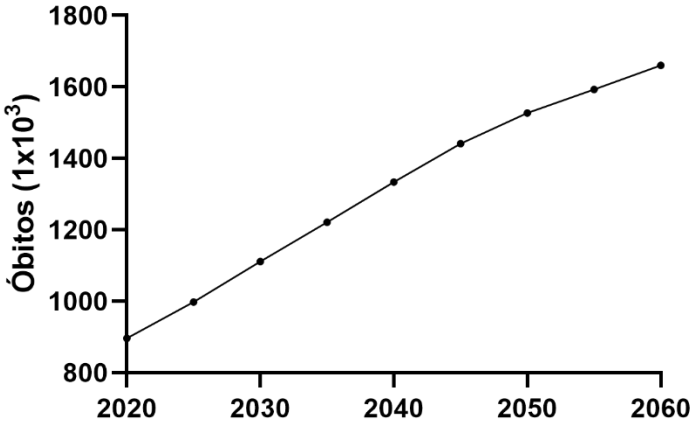
Figura 1 – Incidência e mortalidade por câncer (2020)



Fonte: Modificado de IARC, 2020.

No Brasil o carcinoma hepatocelular (CHC) é o décimo câncer de maior incidência e o sétimo com a maior taxa de mortalidade, apresentando 12.674 novos casos diagnosticados e em torno de 12.139 óbitos no ano de 2020 (IARC, 2021; INCA, 2021). Nas projeções realizadas por Santos et al. (2019) foi encontrada uma tendência no aumento da taxa de mortalidade em homens por este câncer até o ano de 2030 em todas as regiões brasileiras, com um aumento significativo para a região sul.

Figura 2 – Estimativa de óbitos por cânceres hepáticos 2020-2060



Fonte: Modificado de OMS, 2016.

Os cânceres hepáticos primários podem se originar a partir dos hepatócitos, epitélio ducto biliar ou tecido mesenquimal. Sendo os principais subtipos e de maior incidência o CHC seguido pelo colangiocarcinoma (KEW, 2017), compreendendo cerca de 80% e 15% de todas as neoplasias malignas no fígado, respectivamente (BANALES et al., 2020; INCA, 2018; JEMAL et al., 2011). Ambos os cânceres possuem características comuns entre si, como fatores de risco, desenvolvimento assintomático, alta heterogeneidade, além de serem altamente agressivos e pouco responsivos para os tratamentos disponíveis (BANALES et al., 2020; MARQUARDT; ANDERSEN; THORGEIRSSON, 2015).

1.3. Carcinoma Hepatocelular

O CHC é considerado o segundo câncer de maior letalidade, apresentando uma taxa de sobrevida de apenas 18% em 5 anos, estando atrás apenas dos tumores pancreáticos (JEMAL et al., 2017; YANG; ROBERTS, 2010). Isso se dá principalmente pelo envelhecimento populacional e progressão silenciosa da doença, que dificulta o diagnóstico precoce e tratamento adequado (JEMAL et al., 2011; YANG et al., 2019). Além disso, sua prevalência é cerca de 4 vezes maior em homens do que em mulheres, o que pode ser explicado devido à maior predisposição aos fatores de risco para a doença (BALOGH et al., 2016). O diagnóstico desta doença ocorre por volta dos 60 anos de idade, no entanto, em regiões de maior incidência, como no leste asiático e África subsaariana, a doença pode também afetar indivíduos com menos de 30 anos (KEW, 2017; YANG et al., 2019).

Dentre os fatores de risco relacionados ao CHC, está incluso o desenvolvimento prévio de doenças hepáticas crônicas (FORNER; REIG; BRUIX, 2018), sejam elas ocasionadas por infecções virais (hepatite B ou C) (EL-SERAG, 2012; ZAMOR; DELEMOS; RUSSO, 2017), exposição à aflatoxina B1 por meio da dieta (KIMANYA et al., 2021), etilismo (MORGAN; MANDAYAM; JAMAL, 2004; RAMADORI et al., 2017), tabagismo, infecção pelo vírus da imunodeficiência humana (VILLANUEVA, 2019) e doenças metabólicas e genéticas (WATSON; HYDON; LODGE, 2016; JAYACHANDRAN et al., 2020; KEW, 2014a).

1.3.1. Hepatocarcinogênese

O processo de hepatocarcinogênese envolve um ambiente inflamatório com constante dano hepático e regeneração com deposição de tecido fibroso, progredindo à cirrose e comprometimento funcional (FORNER; REIG; BRUIX, 2018). Isso possibilita o acúmulo de mutações somáticas e alterações epigenéticas em genes *driver*, os quais são de extrema relevância para a iniciação e progressão tumoral (MARQUARDT; ANDERSEN; THORGEIRSSON, 2015).

Os genes *driver* são denominados desta forma, uma vez que ao sofrerem alterações, estes genes se tornam responsáveis pelo desenvolvimento e progressão tumoral (WAKS et al., 2016). Genes *driver* são geralmente classificados de acordo com sua função, podendo ser oncogenes, onde tais mutações levam ao ganho de função, estimulando de forma exacerbada o crescimento e proliferação celular, ou genes supressores tumorais, que são geralmente afetados por perda de função, são impedidos de ativar os pontos de checagem do ciclo celular e promoverem o reparo do DNA (LEE; MULLER, 2010; LYU et al., 2020).

As principais mutações encontradas em lesões pré-neoplásicas hepáticas e no CHC estão relacionadas aos genes transcriptase reversa da telomerase (TERT), catenina beta 1 (CTNNB1) e proteína tumoral 53 (TP53) (SCHULZE; NAULT; VILLANUEVA, 2016; TORRECILLA et al., 2017).

Mutações nos promotores de TERT (TERTp) resultam em maior produção da enzima telomerase, levando ao aumento e manutenção dos telômeros, que por sua vez confere proliferação celular indefinida (NAULT et al., 2013; STEWART; WEINBERG, 2000). Já a β-catenina, principal proteína da via Wnt-β-catenina, frente a mutações no gene CTNNB1 induz maior ativação e acúmulo da proteína no citoplasma, a qual é translocada para o núcleo e promove a transcrição

de oncogenes envolvidos com sobrevivência e proliferação celular, como c-Myc e Ciclina D-1, indução de transição epitélio mesenquimal, pelo aumento de Claudina, SNAIL e Twist (CHEN et al., 2018; SHANG; HUA; HU, 2017) e mecanismos de evasão do sistema imune, que suprimem a resposta de células T CD8+ (LIANG et al., 2014).

A ativação da p53 ocorre por meio da detecção de estresse celular, seja ele induzido por dano em DNA, expressão de oncogenes, hipóxia, disfunção metabólica ou estresse replicativo (AUBREY; STRASSER; KELLY, 2016). Após ativação, a p53 participa de vários processos celulares a fim de voltar à homeostase celular por meio de mecanismos relacionados ao reparo de dano ao DNA, parada de ciclo, indução de apoptose e senescência celular, assim como em processos de adaptação metabólica e diferenciação celular (AUBREY; STRASSER; KELLY, 2016; HELTON; CHEN, 2007; LIEBERMAN et al., 2017; TOUFEKTCHAN; TOLEDO, 2018). Alterações epigenéticas ou mutações no gene TP53 podem levar a diminuição da proteína ou à produção de proteínas não funcionais (DHANASEKARAN; BANDOH; ROBERTS, 2016). No entanto, algumas mutações em TP53 geram uma proteína mutante com ganho de função, garantindo a progressão tumoral por meio do aumento proliferativo, sobrevivência celular, promoção de migração e invasão, levando a metástase e também ativando mecanismos de adaptação, tornando as células quimiorresistentes (D'ORAZI; CIRONE, 2019; ZHU et al., 2020).

1.4. Ciclo Celular

O ciclo celular compreende uma cascata de eventos altamente regulado, relacionados com o desenvolvimento embrionário, manutenção e regeneração tecidual, todavia, sua desregulação pode levar à proliferação celular anormal e desenvolvimento de tumores (BITEAU; HOCHMUTH; JASPER, 2011; KALDIS, 2016; RODRIGUES et al., 2019). O ciclo pode ser dividido em 4 fases (**Fig. 3**): fase G0 (quiescente), estágio onde as células são estimuladas por fatores de crescimento; fase G1 (do inglês, *gap 1*), crescimento celular; fase S (Síntese), duplicação do material genético; fase G2 (do inglês, *gap 2*), preparação para a divisão celular e fase M (Mitose), onde ocorre a divisão celular (VERMEULEN; VAN BOCKSTAELE; BERNEMAN, 2003; YANG; SHERIDAN, 2014).

A progressão através das fases do ciclo é controlada por um grupo de proteínas que compreendem as ciclinas, CDKs (do inglês, *cyclin-dependent kinases)* e as inibidoras de CDKs (CKIs) (**Fig. 3**) (MATSUDA et al., 2013; MURRAY, 2004),

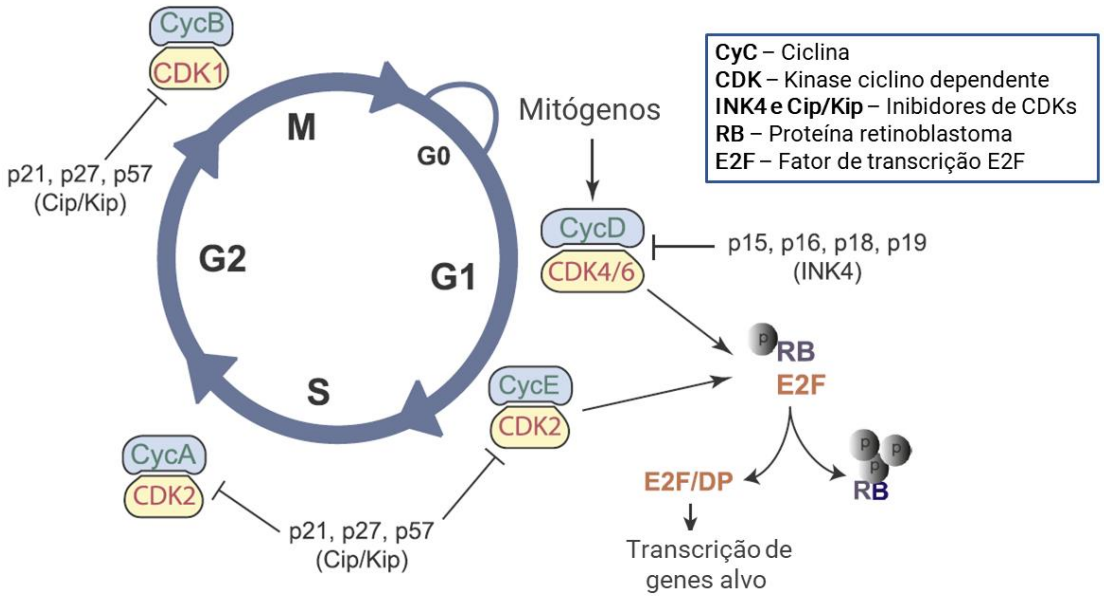
sendo estas reguladas por meio de sinais mitogênicos e inibidas através da ativação dos pontos de checagem, presentes no final de cada fase, detectando se as condições para proliferação celular são adequadas. No entanto, na presença de danos ao DNA, o ciclo pode ser interrompido até que o material genético seja reparado ou ocorre a indução de morte celular (KASTAN; BARTEK, 2004; OTTO; SICINSKI, 2017; VISCONTI; DELLA MONICA; GRIECO, 2016).

A entrada na fase G1 depende fisiologicamente de um estímulo mitogênico, ou seja, é dependente da sinalização de fatores de crescimento, que dessa forma levará a produção de ciclina D, que formam complexos com as CDKs (D-CDK4 e D-CDK6). Tais complexos são responsáveis pelo início da fosforilação da proteína Retinoblastoma (Rb), que permite os fatores de transcrição da família E2F a induzir a expressão de ciclina E, formando E-CDK2 (MALUMBRES; BARBACID, 2001; SANTO; SIU; RAJE, 2015). Este complexo induz a hiperfosforilação de Rb, interrompe sua interação com E2F e permite a progressão do ciclo para a fase S, que em conjunto com o complexo A-CDK2 induzem a produção de proteínas envolvidas com a síntese de DNA (BERTOLI; SKOTHEIM; DE BRUIN, 2013; SCHWARTZ; SHAH, 2005).

Durante a fase S, há a destruição das ciclinas E e inativação de E2F pelo complexo A-CDK2, a fim de cessar estímulos para síntese de DNA e progredir para a fase G2. Durante o fim da fase S e início de G2, há o aumento das ciclinas A e B, que complexam com a CDK1, formando complexos que regulam a passagem para a fase mitótica. A transição e início da mitose se dá pela fosforilação de proteínas mitóticas pelo complexo B-CDK1 (SCHWARTZ; SHAH, 2005; YANG; SHERIDAN, 2014).

Durante a progressão do ciclo celular as atividades dos complexos ciclina-CDK podem também ser reguladas através de fosforilação e desfosforilação por meio de kinases ativadoras de CDK (CAKs). Entretanto, frente à estímulos nocivos ou fisiológicos ocorre o acúmulo das CKIs, as quais inativam a atividade dos heterodímeros ciclina-CDK (SCHWARTZ; SHAH, 2005; YANG; SHERIDAN, 2014). As CKIs são divididas em duas classes principais, as proteínas da classe INK4 (p16, p15, p18 e p19) que inibem a ação das CDK4 e 6 por meio do bloqueio da ligação com a ciclina D, que resulta em parada na fase G1/S; e a classe KIP/CIP (p21, p27 e p57), que possui atividade mais ampla, induz parada durante todo o ciclo por meio da inibição das CDK1 e 2 (BESSON; DOWDY; ROBERTS, 2008; LEAL-ESTEBAN; FAJAS, 2020; SHERR; ROBERTS, 1999).

Figura 3 – Regulação do ciclo celular



Fonte: Adaptado de LEAL-ESTEBAN; FAJAS, 2020.

Outro componente crítico dos pontos de verificação de danos ao DNA é o fator de transcrição p53 (VERMEULEN; VAN BOCKSTAELE; BERNEMAN, 2003). Sua ativação ocorre através da detecção de danos pela proteína quinase ataxia telangiectasia mutada (ATM) e proteína quinase relacionada a ATM e Rad3 (ATR), das quais induzem a ativação de CHK1 e 2 (BARNUM; O'CONNELL, 2014; MALUMBRES; BARBACID, 2009; VISCONTI; DELLA MONICA; GRIECO, 2016). Além disso, p53 induz a produção de p21, e consequentemente inibe a progressão do ciclo (CHEN, 2016; GARDINO; YAFFE, 2011; TAMURA et al., 2012; TAYLOR; STARK, 2001). Quando o dano no DNA é irreparável, a p53 leva a produção de proteínas envolvidas na indução de morte celular por apoptose (OZAKI; NAKAGAWARA, 2011).

1.5. Morte Celular

O processo de morte celular é caracterizado por diversos mecanismos fisiológicos e patológicos que levam à destruição de células alvo que sofreram danos irreparáveis ou com potencial nocivo, como células imunes autorreativas, infectadas ou mesmo células tumorais, com a finalidade de manter a homeostase do organismo

(FUCHS; STELLER, 2011, 2015; GALLUZZI et al., 2016a, 2016b; JORGENSEN; RAYAMAJHI; MIAO, 2017).

Células em processo de morte apresentam diversas características morfológicas, sendo classificadas em três tipos distintos. O tipo I ou apoptose, apresenta diminuição do conteúdo citoplasmático, condensação (picnose) e fragmentação nuclear (cariorréxe), formação de bolhas na membrana (*blebbing*) e fragmentação em corpos apoptóticos, sem extravasamento do conteúdo intracelular, seguido de rápida fagocitose pelas células residentes (GALLUZZI et al., 2007; GALLUZZI; VITALE; ET AL., 2018; KROEMER et al., 2009).

O tipo II, também conhecido por morte celular autofágica, é caracterizada por grande vacuolização citoplasmática por meio da produção de fagolisossomos, dos quais são responsáveis pela degradação de componentes intracelulares. E o tipo III, denominado necrose, apresenta aumento do volume celular, inchaço das organelas e rompimento da parede celular, com extravasamento do conteúdo citoplasmático, culminando em sua destruição e ativação de células inflamatórias (GALLUZZI; VITALE; ET AL., 2018; JUNG; JEONG; YU, 2020; KATAYAMA et al., 2007; KROEMER et al., 2009).

Mais recentemente, o comitê em nomenclatura de morte celular estabeleceu diversas vias de morte molecularmente distintas dentre as denominadas mortes celulares reguladas, como apoptose, morte celular dependente de autofagia, necroptose, *anoikis*, entose, partanatos, piroptose e netose (GALLUZZI; VITALE; ET AL., 2018). Todavia, serão descritas abaixo somente as vias de morte pertinentes ao presente trabalho, limitadas a apoptose e ao envolvimento da autofagia no processo de morte celular.

1.5.1. Apoptose

A ativação do processo apoptótico pode ocorrer de duas formas, pela via intrínseca, também denominada mitocondrial, e a via extrínseca ou via dos receptores de morte. A via intrínseca pode ocorrer de maneira fisiológica ou devido a estresse celular causado por insultos ou agentes genotóxicos, como hipóxia e dano ao DNA. A via extrínseca, por sua vez, ocorre por meio de estímulos externos, como ativação dos receptores Fas, TNFR e TRAIL-R1/2, que induzem a transdução de sinais através de seus domínios de morte presentes no citoplasma (FUCHS; STELLER, 2011; NAGATA, 2018).

A via mitocondrial é regulada por proteínas da família Bcl-2, as quais são divididas em três classes: anti-apoptóticas (Bcl-2, Bcl-xL, Mcl-1, A1 e Bcl-B); pró-apoptóticas com único domínio BH3 (Noxa, Puma, Bim, Bid, Hrk, Bmf e Bad) e pró-apoptóticas formadoras de poro (Bax e Bak), também denominadas efetoras (CZABOTAR et al., 2014). Em homeostase, as proteínas anti-apoptóticas bloqueiam o desencadeamento da apoptose por meio da ligação e neutralização da atividade das proteínas pró-apoptóticas (SINGH; LETAI; SAROSIEK, 2019).

No entanto, frente à sinais de morte, as proteínas pró-apoptóticas que possuem apenas um domínio BH3 são expressas por meio de regulação transcricional ou pós-transcricional, que então inativam as proteínas anti-apoptóticas, inibindo a ligação entre estas (NAGATA, 2018). As proteínas pró-apoptóticas efetoras se oligomerizam de forma a produzir poros na membrana externa da mitocôndria, induzindo a permeabilização da membrana e permitindo o extravasamento do citocromo c para o citoplasma, cuja associação com Apaf-1 leva à formação do apoptossomo, ativação de caspases e por fim, morte celular (UREN; IYER; KLUCK, 2017; WESTPHAL; KLUCK; DEWSON, 2014).

A via extrínseca é iniciada através da ativação de um receptor de morte por seu respectivo ligante (por exemplo, Fas-FasL, TNFR-TNF-α e TRAILR-TRAIL) (THORBURN, 2004), no qual, posteriormente, promove o recrutamento de proteínas adaptadoras com domínio de morte (FADD e TRADD). Ambas levam ao recrutamento das caspases iniciadoras (8 e 10) e formam o complexo de sinalização indutor de morte (DISC), o que promove a ativação autocatalítica destas enzimas (JIN; EL-DEIRY, 2006).

Esta ativação dá início a fase executora, a qual pode ocorrer de duas formas no contexto da via extrínseca: via de células do tipo I (timócitos e linfócitos maduros), que consiste na clivagem direta da caspase 3 e 7 pela caspase 8, e por conseguinte leva a clivagem e ativação da caspase 6; e via de células do tipo II (hepatócitos e células tumorais), onde a ativação direta das caspases 3 e 7 são inibidas por XIAP, levando então a clivagem de Bid pela caspase 8 em sua forma truncada (tBid), responsável por interagir com as proteínas Bax e Bak, e desencadear a via intrínseca (BAIG et al., 2016; GALLUZZI; VITALE; ET AL., 2018; TAYLOR; CULLEN; MARTIN, 2008).

De maneira geral, todas as vias de morte culminam na ativação das caspases 3, 6 e 7, das quais são essenciais para a indução da apoptose (MCILWAIN;

BERGER; MAK, 2013) e externalização da fosfatidilserina (ELMORE, 2007; NAGATA, 2018).

1.5.2. Morte Celular por Autofagia

A autofagia é um processo catabólico celular onde componentes citoplasmáticos são destruídos e reciclados a fim de manter a homeostase sob condições de estresse, garantindo a sobrevivência celular (JUNG; JEONG; YU, 2020; MIZUSHIMA et al., 2008). Entretanto, existe um envolvimento entre o processo de autofagia e morte celular, podendo preceder e modular a ativação da apoptose, necroptose ou mesmo ocorrer de forma independente (DASARI et al., 2017).

A morte celular dependente de autofagia (MCDA) é mediada através de genes relacionados à autofagia (ATG), como o ATG5, BECLIN-1 e LC3 e posterior fosforilação da kinase c-Jun N-terminal (JNK), responsável por gerar sinais de morte (JUNG; JEONG; YU, 2020; SHIMIZU et al., 2014), levar a intensa produção de fagolisossomos e degradação de componentes citoplasmáticos, além de afetar a membrana nuclear (DASARI et al., 2017). No entanto, sem a ativação de JNK, a via é redirecionada à apoptose ou necroptose (CHOI et al., 2016; DASARI et al., 2017; GONZALEZ et al., 2012).

No contexto tumoral, a autofagia possui um duplo papel, tanto anti quanto pró-tumoral (LIN; BAEHRECKE, 2015; PANG; LIU, 2018). Durante o início da tumorigênese, a autofagia age como um supressor tumoral por meio da degradação de moléculas potencialmente oncogênicas e organelas danificadas, levando à diminuição da produção de espécies reativas de oxigênio (ERO) e por consequência, evitando danos no DNA e preservando a integridade genômica (GALLUZZI et al., 2015; GOLDSMITH; LEVINE; DEBNATH, 2014; PANDA et al., 2015; WHITE, 2012). Em estágios avançados, a autofagia passa a promover a sobrevivência das células tumorais pelo mesmo mecanismo, diminuindo o estresse oxidativo e fornecendo nutrientes suficientes para a sobrevivência e proliferação destas células (CHAVEZ-DOMINGUEZ et al., 2020; YUN; LEE, 2018).

Desta forma, diversos estudos focam em bloquear o mecanismo de autofagia, impedindo a reciclagem de proteínas e impossibilitando a adaptação das células tumorais a condições de estresse, como hipóxia e falta de nutrientes, favorecendo o uso de quimioterápicos e radioterapia (LEVY; THORBURN, 2020; LIN et al., 2017; PANG; LIU, 2018). Inúmeros compostos fenólicos, como o 3-decilcatecol,

kaempferol, luteolina, ácido salvianólico B e berberina, apresentaram aumento substancial de autofagia em linhagens de CHC por meio da inibição da via AKT/mTOR através da ativação de AMPK, que permite a expressão dos genes ATG e ativação da via JNK, revelando alternativas promissoras para o tratamento do CHC e outros cânceres (CAO et al., 2017; GO et al., 2017; GONG et al., 2016; HAN et al., 2017; YU et al., 2014).

1.6. TRATAMENTO

Dentre os vários tipos de tratamentos contra o CHC, somente os pacientes diagnosticados em estágios iniciais são submetidos à procedimentos curativos, como ablação, ressecção dos nódulos e transplante hepático (ALLEMANN et al., 2013; MAZZAFERRO et al., 1996), entretanto, a recorrência do CHC é de 70% no período de 5 anos (FORNER; REIG; BRUIX, 2018).

Para os pacientes em fase intermediária, onde procedimentos cirúrgicos não são recomendados, os pacientes com função hepática preservada podem se beneficiar da quimioembolização transarterial, que consiste na injeção de um quimioterápico como doxorrubicina, cisplatina e mitomicina seguido por embolização arterial a partir da infusão de particulas como microesferas de amido, miçangas de polivinil ou bobinas metálicas (BALOGH et al., 2016; CRISSIEN; FRENETTE, 2014; LEWANDOWSKI et al., 2011).

Entretanto, a maior parte dos pacientes acabam sendo diagnosticados em estágios avançados, apresentando pior prognóstico, alta recorrência e baixa resposta aos quimioterápicos e radioterapia (KEW, 2014b; TORRECILLA et al., 2017).

Os antineoplásicos utilizados como primeira linha de tratamento no CHC em estágio avançado são o sorafenibe e lenvatinibe, inibidores de tirosina kinases, que atuam bloqueando a sinalização do fator de crescimento derivado de plaquetas (PDGF), fator de crescimento do endotélio vascular (VEGF), fator de crescimento fibroblastico (FGF), proteína c-kit e RAF, tanto nas células tumorais quanto nas células endoteliais circundantes (COUTINHO, 2011; KUDO et al., 2018). Entretanto, ambos os tratamentos apresentam sobrevida livre de progressão de apenas 3 a 7 meses e média de sobrevida de 10 a 13 meses após o início do tratamento (LLOVET et al., 2021).

No estudo realizado por FINN et al. (2020), foi avaliado que a

combinação entre atezolizumabe (anticorpo monoclonal anti-PDL1) e bevacizumabe (anticorpo monoclonal anti-VEGF) aumentou a sobrevida dos pacientes comparado com os outros tratamentos disponíveis, entretanto, foi observado a diminuição da qualidade de vida dos pacientes após 11 meses de tratamento.

Como terapia de segunda linha, após falha com as opções terapêuticas anteriores, três quimioterápicos estão aprovados para tratamento, são eles: regorafenibe, cabozantinibe e ramucirumabe (LLOVET et al., 2021). O regorafenibe, assim como o cabozantinibe, são inibidores multi-kinase, que bloqueiam principalmente os receptores de VEGF e o segundo agindo também sobre o Receptor de Tirosino Kinase AXL (AXL) e o Receptor de Tirosino Kinase Codificado por Proto-oncogene MET (MET), cuja ativação está associada a proliferação celular e metástase, sendo um dos mecanismos de resistência ao sorafenibe. Ambos apresentaram respostas parecidas, tendo uma média de sobrevida de somente 10 meses (ABOU-ALFA et al., 2018; BRUIX et al., 2017; HARA et al., 2020).

O ramucirumabe é um anticorpo monoclonal que inibe seletivamente o receptor VEGFR-2, e é a atual escolha de tratamento para pacientes com níveis >400 ng/mL de alfa fetoproteina, e também apresenta poucos efeitos adversos graves, comparado as outras opções terapêuticas (GILABERT; RAOUL, 2018; LUCA; MARINO; MAIO, 2020). No entanto, a sobrevida dos pacientes é menor, apresentando uma média de apenas 8 meses após o tratamento (LLOVET et al., 2021)

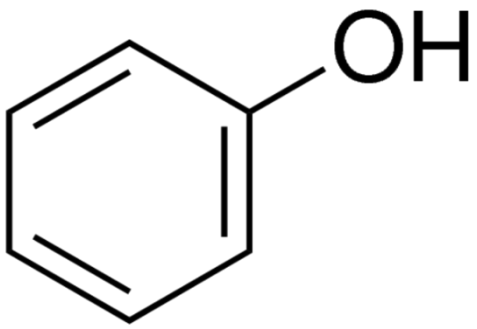
Neste contexto, tem sido cada vez mais estudada a utilização de compostos que possam atuar como agentes terapêuticos sobre o CHC, devido à alta letalidade e à todas as complicações encontradas no tratamento desta doença, como toxicidade e intensos efeitos colaterais, levando ao interrompimento do tratamento em mais de 10% dos casos. Por muitos anos, compostos fenólicos têm sido intensamente estudados por seus efeitos antitumorais, pró-apoptóticos, anti-angiogênicos e quimiopreventivos no câncer e em diversas doenças (BASLI; BELKACEM; AMRANI, 2017).

1.7. Compostos Fenólicos

Os compostos fenólicos (CF) compreendem um grande grupo com mais de oito mil moléculas, as quais possuem pelo menos um anel aromático com um ou mais radicais hidroxilas (-OH) (**Fig. 4**) e também são amplamente distribuídas em todo o mundo, podendo ser encontradas em todo o reino Plantae (BHUYAN; BASU,

2017). Este numeroso grupo de moléculas apresenta ainda uma grande variabilidade estrutural, sendo encontradas desde moléculas fenólicas simples, até mesmo complexos polímeros (BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

Figura 4 – Estrutura química de um composto fenólico



Fonte: Universidade da California Santa Cruz, 2014.

Os CF são também conhecidos como um grupo de fitoquímicos bioativos sintetizados a partir do metabolismo secundário de plantas, atuando como parte estrutural da parede celular, em mecanismos de defesa, sequestro de ERO e na sinalização de modulação gênica (GRIGORE, 2017; PETROPOULOS et al., 2018). Esta ampla família de moléculas pode ser classificada em diversos grupos, sendo os principais, os flavonóides, estilbenos, ácidos fenólicos e lignanas (FRAGA et al., 2019).

Estudos realizados em diversos modelos demonstram a relevância das pesquisas com os CF, uma vez que possuem propriedades preventivas e/ou terapêuticas para várias doenças, como cardiovasculares, neurodegenerativas e até mesmo o câncer, através de suas atividades antioxidante, anti-inflamatória e antimutagênica (CORY et al., 2018; HUANG; CAI; ZHANG, 2010). Também são capazes de promoverem parada de ciclo celular, diminuição de proliferação celular, bloqueio do processo de angiogênese, inibição de metástase, além de restituir a capacidade de diferenciação de células tumorais e ressensibilizar tumores quimiorresistentes (ANANTHARAJU et al., 2016; RAVISHANKAR et al., 2013).

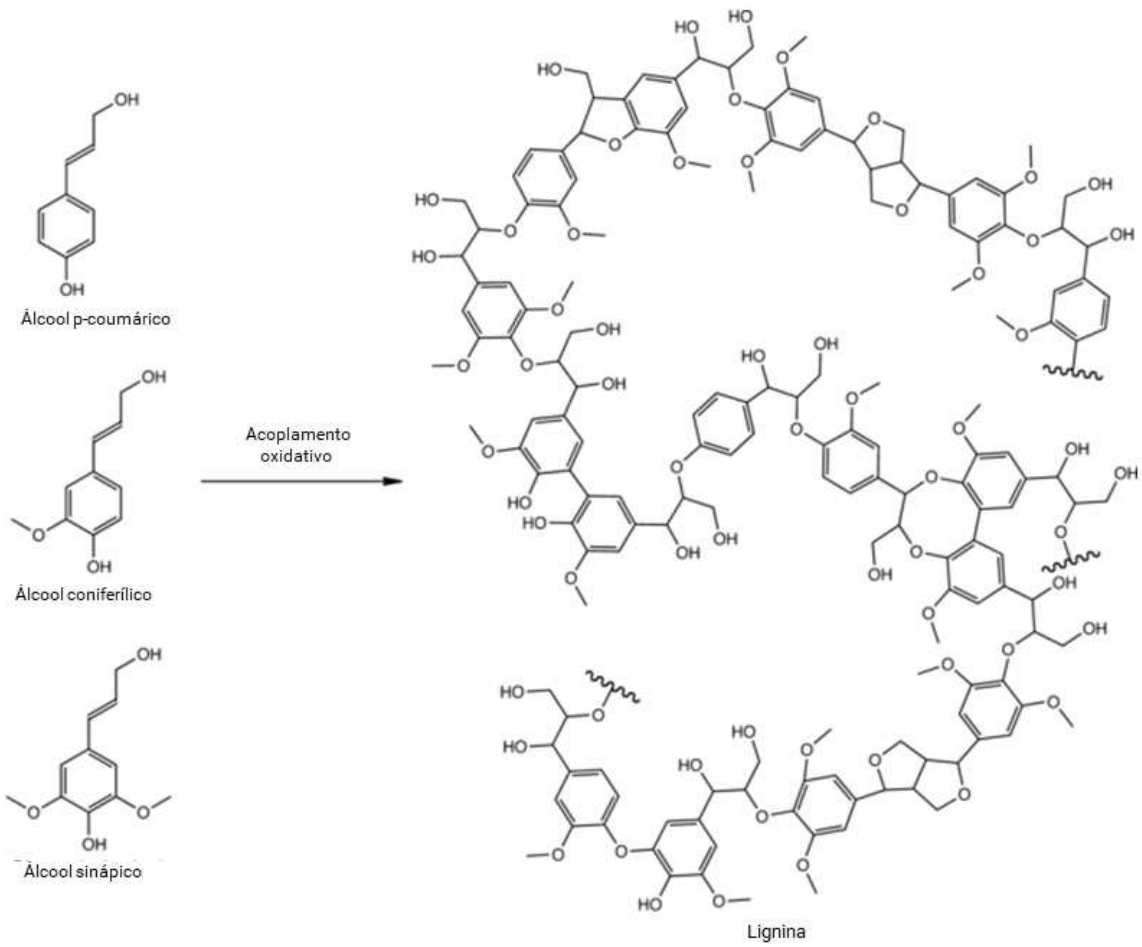
Alguns dos mecanismos importantes comumente desencadeados pelos CF sobre células tumorais, incluem a capacidade em induzir dano e despolarização da membrana mitocondrial, aumento da produção de ERO e lactato desidrogenase e consequente bloqueio do ciclo e morte por apoptose, sem induzir

efeitos deletérios sobre linhagens normais (ANANTHARAJU et al., 2016; MUTALIB et al., 2016; ZAMBONIN et al., 2012).

1.7.1. Lignina

A lignina (**Fig. 5**) consiste na maior classe de biopolímeros fenólicos, dos quais compreendem cerca de 35% da biomassa de todas as plantas vasculares (CHRISTOPHER; YAO; JI, 2014) e são sintetizados a partir da via dos 4-hidroxifenilpropanoides, que também resulta na produção de outros CF como os flavonoides, lignanos e ácidos hidroxicinâmicos (FRASER; CHAPPLE, 2011; RALPH et al., 2004). Estes polímeros possuem grande variação estrutural, sendo formados por três álcoois, sendo eles p-coumarico, coniferílico e sinápico (**Fig. 6**) em diferentes concentrações, dando origem as subunidades H (p-hidrofenil), G (guaiacil) e S (siringil) (CHRISTOPHER; YAO; JI, 2014; GLASSER, 2019).

Figura 5 – Estrutura da lignina e seus álcoois precursores



Fonte: Adaptado de WENG; CHAPPLE, 2010

Este polímero passou a ser produzido evolutivamente por plantas vasculares, como as pteridófitas, gimnospermas e angiospermas de forma a conferir rigidez estrutural, facilitar o transporte de água por toda a extensão da planta, assim como garantir a proteção contra a radiação UV e patógenos (WENG; CHAPPLE, 2010). Sua formação é também um importante processo para a manutenção do ecossistema, sendo o resultado do processo de fotossíntese, desta forma garantindo a absorção de dióxido de carbono e produção de água e oxigênio (GLASSER, 2019).

Sua disponibilidade no ambiente, assim como suas funções biológicas e estruturais vem atraindo a atenção de muitos grupos de pesquisa, tanto para aplicação biotecnológica como para o desenvolvimento de terapias alternativas para diversas doenças, dentre elas o câncer (RENAULT; WERCK-REICHHART; WENG, 2019; SUN et al., 2018).

A degradação da lignina ocorre por meio da atividade de duas grandes famílias enzimáticas, as lacases e as heme peroxidases, sendo a primeira a mais bem caracterizada e conhecida (ABDEL-HAMID et al., 2013). As lacases compõem uma classe de enzimas multicobre oxidases produzidas por vários gêneros de fungos, plantas, bactérias e insetos. Sua principal atividade envolve a síntese ou degradação de compostos orgânicos em CF de baixo peso molecular por meio da catalização oxidativa de seus substratos, reduzindo O_2 e produzindo H_2O como subproduto de sua atividade catalítica (CANNATELLI; RAGAUSKAS, 2017; SHARMA et al., 2018). As heme peroxidases por sua vez, são enzimas extracelulares de origem fúngica, que agem similarmente as lacases, degradando a lignina por meio da catalização oxidativa dos substratos através da redução em moléculas de H_2O_2 em H_2O (JANUSZ et al., 2017).

As ligninas com alta concentração da subunidade S são menos condensadas e mais facilmente digeridas pelas lacases, sendo o tipo mais utilizado para as aplicações industriais e biotecnológicas (RENAULT; WERCK-REICHHART; WENG, 2019). Por serem originadas majoritariamente pelo ácido sinápico, a degradação deste tipo de lignina dá origem a compostos como o siringaldeído (4-hidroxi-3,5-dimetoxibenzaldeído), ácido siringico (ácido 4-hidroxi-3,5-dimetoxibenzóico) e o siringol (2,6-dimetoxifenol (2,6-DMP)) (SRINIVASULU et al., 2018).

O 2,6-DMP é o produto mais conhecido da degradação da lignina, sendo amplamente utilizado para determinar a atividade das lacases (WAN; DU; MIYAKOSHI, 2008). Este composto também apresenta potencial antioxidante superior ao do ácido ascórbico e α-tocoferol (LAKANI; HEDJAZI; ABDULKHANI, 2019), além de atividade anti-inflamatória através do bloqueio da via dos eicosanoides, com redução de fosfolipase A2 e ciclooxigenase 2 (GOWDA et al., 2021). Seu efeito citotóxico sobre células tumorais também foi sugerido por Intisar e colaboradores (2013), contudo, se faz necessário posteriores investigações sobre o mecanismo de ação deste composto.

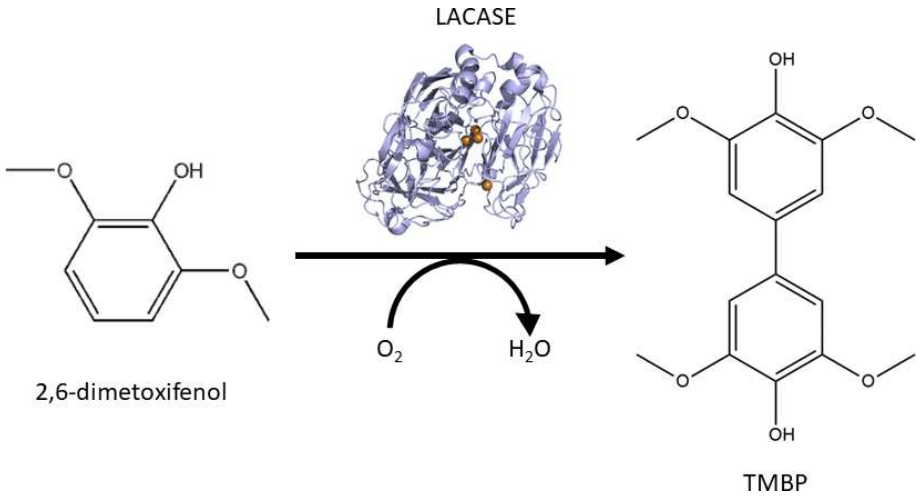
1.7.2. 3,3’,5,5’-Tetrametoxibifenil-4,4’-Diol

A síntese de compostos orgânicos por meio da biotransformação tem como objetivo de reduzir a toxicidade e melhorar a atividade biológica de moléculas, e possui como vantagem o menor custo, produção acelerada, em maiores quantidades e com maior especificidade de síntese da estrutura química desejada (SHAH et al., 2014; SMITHA; SINGH; SINGH, 2017). Por este motivo, a biotransformação tem ganhado reconhecimento nas indústrias químicas e farmacêuticas, dando origem a diversas moléculas biologicamente ativas com potencial terapêutico para diversas doenças, como o câncer e também por ser uma metodologia de química verde, gerando menos resíduos tóxicos e com menor consumo de energia (HEGAZY et al., 2015; ROH; KANG, 2014; SULTANA, 2018).

O 3,3’,5,5’-tetrametoxibifenil-4,4’-diol (TMBP) é um dímero biossintetizado a partir da oxidação de duas moléculas de 2,6-DMP catalisada por uma enzima lacase de origem fúngica (Fig. 6), como demonstrado por Adelakun et al. (2012) e Schirmann et al. (2018) utilizando *Trametes pubescens* e *Botryosphaeria rhodina*, respectivamente, para obtenção da enzima.

O desenvolvimento deste composto biossintético, como apresentado por Adelakun et al. (2012) teve como principal objetivo a busca por compostos com alta capacidade antioxidante para aplicação industrial. E seu desenvolvimento foi aplicado como um estabilizador de biodiesel por Schirmann et al. (2019), que se mostrou superior ao tert-butilhidroxitolueno, um antioxidante utilizado comercialmente, e garantiu estabilidade antioxidante ao biodiesel mesmo em altas temperaturas.

Figura 6 - Biossíntese do 3,3’,5,5’-tetrametoxibifenil-4,4’-diol (TMBP).



Fonte: adaptado de Schirmann et al. (2019).

Recentemente Concato e colaboradores (2020) demonstraram pela primeira vez o efeito do tratamento *in vitro* com TMBP sobre a linhagem tumoral humana A549 (adenocarcinoma pulmonar), o qual apresentou efeitos citotóxicos com redução da proliferação, indução de parada do ciclo celular na fase G2/M e apoptose tardia, sem causar citotoxicidade em células normais. Além disso, o estudo *in silico* revelou que esta molécula não viola nenhum dos parâmetros de Lipinski e Veber, possui alta absorção intestinal, propriedades anfipáticas, e potencial em se tornar uma droga oral para o tratamento de neoplasias.

Diante disso, como anteriormente apresentado, o atual tratamento para o CHC não é satisfatório, principalmente em seu estágio avançado. Deste modo, o TMBP pode exercer atividade antiproliferativa e citotóxica sobre as linhagens HuH7.5 e HepG2/C3A, demonstrando um potencial tratamento alternativo para este câncer, visto que o TMBP apresentou ótimo efeito citotóxico sobre a linhagem tumoral A549 (CONCATO et al., 2020). Subsequentemente, devido à carência de estudos sobre as atividades biológicas deste composto sobre células tumorais, este trabalho propõe avaliar os efeitos antitumorais induzidos pelo TMBP nestas células, possibilitando elucidar os mecanismos e vias de ação envolvidas.

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2. OBJETIVOS

2.1. OBJETIVO GERAL

- Avaliar a ação do TMBP *in vitro* sobre as linhagens tumorais humanas de CHC, HuH7.5 e HepG2/C3A.

2.2. OBJETIVOS ESPECÍFICOS

- Determinar *in vitro* a viabilidade celular das linhagens tumorais HuH7.5 e HepG2/C3A após o tratamento com TMBP;
- Observar as alterações morfológicas das células tumorais após o tratamento com TMBP;
- Averiguar se o tratamento com TMBP altera a capacidade migratória das linhagens HuH7.5 e HepG2/C3A;
- Verificar se o TMBP possui efeito sobre os níveis de ERO e NO após o tratamento;
- Avaliar se o tratamento altera o potencial de membrana mitocondrial nas células tratadas;
- Observar se o tratamento é capaz de induzir a formação de vacúolos autofágicos e síntese de corpos lipídicos;
- Analisar a distribuição do ciclo celular e indução de morte nas células após o tratamento com TMBP.

3. PRODUÇÃO CIENTÍFICA

ANEXO A

3,3',5,5'-tetramethoxybiphenyl-4,4'-diol exerts a cytotoxic effect on hepatocellular carcinoma cell lines by inducing morphological and ultrastructural alterations, cell cycle arrest in G2/M phase and death by apoptosis

Fabricio Seidy Ribeiro Inoue[a*], Virginia Marcia Concato[a], Bruna Taciane da Silva Bortoleti[a], Ellen Mayara Souza Cruz[a], Mariana Barbosa Detoni[a], Fernanda Tomiotto-Pellissier[a,b], Manoela Daiele Gonçalves[c], Juliana Maria Bitencourt de Morais-Valentim[a], Rayanne Regina Beltrame[d], Jéseka Gabriela Schirmann[e], Aneli M. Barbosa-Dekker[e,f], Robert F. H. Dekker[f], Ivete Conchon-Costa[a], Mário Sérgio Mantovani[g], Danielle Lazarin-Bidóia[a,d], Carolina Panis[h], Wander Rogério Pavanelli[a].

[a] Laboratory of Immunoparasitology of Neglected Diseases and Cancer, Department of General Pathology, Center of Biological Sciences, State University of Londrina, Londrina, PR, Brazil.
[b] Department of Medical Pathology, Federal University of Paraná, Curitiba, PR, Brazil.
[c] Laboratory of Biotransformation and Phytochemical, Department of Chemistry, Center of Exact Sciences, State University of Londrina, Londrina, PR, Brazil
[d] Laboratory of Technological Innovation in the Development of Drugs and Cosmetics, Department of Basic Health Sciences, State University of Maringá, Maringá, PR, Brazil.
[e] Laboratory of Research of Bioactive Molecules, Department of Chemistry, Center of Exact Sciences, State University of Londrina, Londrina, PR, Brazil.
[f] Beta-Glucan Produtos Farmoquímicos-EIRELI, Universidade Tecnológica Federal do Paraná, Londrina, PR, Brazil.
[g] Laboratory of Toxicological Genetics, Department of General Biology, Center of Biological Sciences, State University of Londrina, Londrina, PR, Brazil.
[h] Laboratory of Tumor Biology, Center of Health Sciences, State University of Western Parana, Francisco Beltrão, PR, Brazil.

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Affiliations:
[a] Laboratory of Immunoparasitology of Neglected Diseases and Cancer, Department of General Pathology, Center of Biological Sciences, State University of Londrina, Londrina, PR, Brazil.
[b] Department of Medical Pathology, Federal University of Paraná, Curitiba, PR, Brazil.
[c] Laboratory of Biotransformation and Phytochemical, Department of Chemistry, Center of Exact Sciences, State University of Londrina, Londrina, PR, Brazil
[d] Laboratory of Technological Innovation in the Development of Drugs and Cosmetics, Department of Basic Health Sciences, State University of Maringá, Maringá, PR, Brazil.
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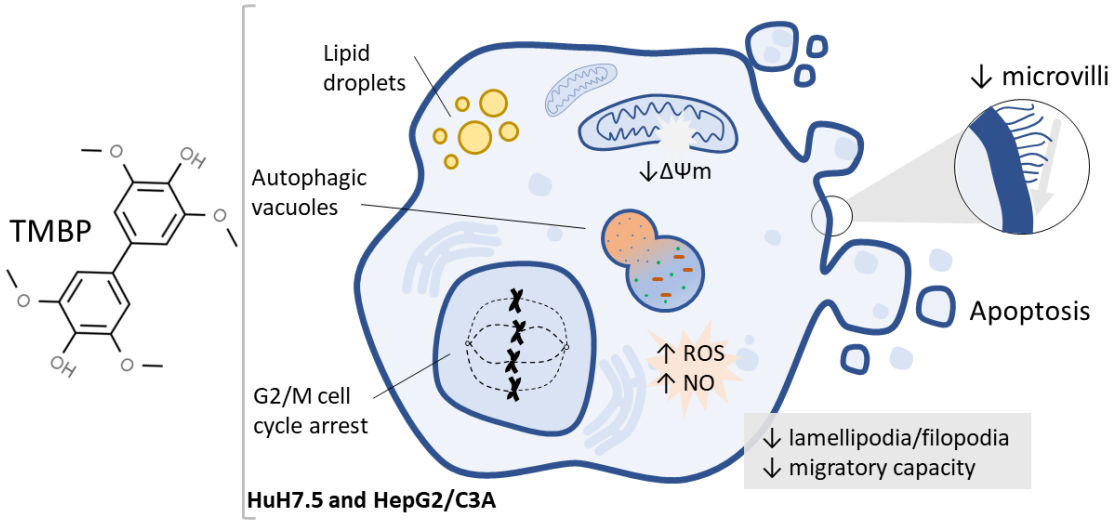
[f] Beta-Glucan Produtos Farmoquímicos-EIRELI, Universidade Tecnológica Federal do Paraná, Londrina, PR, Brazil.
[g] Laboratory of Toxicological Genetics, Department of General Biology, Center of Biological Sciences, State University of Londrina, Londrina, PR, Brazil.
[h] Laboratory of Tumor Biology, Center of Health Sciences, State University of Western Parana, Francisco Beltrão, PR, Brazil.
***Corresponding author:** Fabricio Seidy Ribeiro Inoue
Department of General Pathology, Center of Biological Sciences, State University of Londrina, Celso Garcia Cid Highway University Campus - Zip Code 86057-970, Post Box 10.011. Londrina, PR, Brazil.
Email: fabricioseidyinoue@gmail.com.

Graphical abstract



Highlights

- TMBP promotes severe morphological alterations and impaired migration in HCC cells.
- HCC-treated cells present increased ROS/NOx levels along with loss of ΔΨm, increased lipid droplets and autophagic vacuoles.
- TMBP induces cell cycle arrest at G2/M phase and apoptosis in HCC-treated cells.

Abstract

Hepatocellular carcinoma (HCC) is one of the leading incidences and causes of mortality worldwide. HCC is a very aggressive disease, frequently presenting tumor recurrence and chemoresistance. Therefore, the need for new treatment strategies with less toxicity requires attention. 3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol (TMBP) showed cytotoxicity against lung cancer cell lines without deleterious effects on normal cells. Thus, in this study, we proposed evaluating the antitumoral effect of TMBP on

two HCC cell lineages, HuH7.5 and HepG2/C3A. Cell lines were treated with TMBP (12.5–150 μM) for 24 and 48 h to measure metabolic cellular activity by MTT, and the determination of 50% inhibitory concentrations (IC_{50}). TMBP cytotoxicity was determined by trypan blue exclusion assay, and morphological and ultrastructural alterations were evaluated by electron microscopy. Cell migration was analyzed by wound healing assay, and the mechanisms involved in the induction of cell death by fluorometric assays using H_2DCFDA, tetramethylrhodamine ethyl ester (TMRE), Nile Red (NR) and monodansylcadaverine (MDC) staining. Cell cycle regulation and cell death were characterized by flow cytometry. TMBP reduced the viability of both cell lines, presenting IC_{50} values of 68 and 55 μM for HuH7.5 and 50 and 42 μM for HepG2/C3A in 24 and 48h, respectively. For both cells, the IC_{50} of 24h were chosen for the subsequent tests. TMBP treatment showed significant cytotoxicity and induced severe morphological alterations, such as membrane damage, the presence of apoptotic bodies, reduction of microvilli and reduction of lamellipodia and filopodia, resulting in impaired cell migration. TMBP also increased the generation of ROS, which resulted in mitochondrial depolarization, increased lipid droplets, and formation of autophagic vacuoles, as observed by transmission electron microscopy. These alterations lead to G2/M phase cell cycle arrest and death by apoptosis. Our data demonstrated that TMBP can be a useful antitumoral drug and highlights its mechanisms against HCC.

Keywords: Phenolic compounds, Antiproliferative, Reactive Oxygen Species, Mitochondrial Dysfunction, Cellular Migration.

1. INTRODUCTION

Liver cancer is the sixth most incident neoplasia worldwide and ranks

third in mortality rates [1]. According to the International Agency for Research on Cancer, 896,569 deaths by liver cancer occurred in 2020 [1], and the World Health Organization projects an annual increase of about 1.6 million deaths by 2060 [2]. These data reflect population aging, growth, and increasing risk factors related to socioeconomic development [3]. The most incident primary liver cancer is hepatocellular carcinoma (HCC), accounting for 80% of cases [4].

HCC is the most aggressive type of liver cancer, often diagnosed in advanced stages with a prognosis. Tumor recurrence and low responsivity to chemotherapy and radiotherapy are frequent, with the majority of patients dying in less than a year after diagnosis [5–8]. For advanced HCC stages, systemic therapy is based on tyrosine kinase inhibitors, such as sorafenib, lenvatinib and regorafenib, although the median survival is only 10 to 13 months [9–11]. In addition, all the available chemotherapeutic options present severe adverse events, leading to impaired quality of life, therapy discontinuation, as well as chemoresistance [11–13].

3,3’,5,5’-Tetramethoxybiphenyl-4,4’-diol (TMBP) is a biphenolic compound obtained by laccase catalysis of 2,6-dimethoxyphenol molecules, resulting in a dimeric structure [14,15]. Although TMBP has been synthesized for industrial purposes as a potent antioxidant [14,15], we demonstrated its selective *in vitro* antiproliferative activity against non-small cell lung carcinoma, with no detrimental effects on normal primary cell lines [16]. Moreover, we showed the favorable drug-likeness characteristics of TMBP *in silico*, and its potential to become a new oral antineoplastic drug by respecting all Lipinski and Veber parameters [16].

Given the lack of curative options in HCC treatment and the serious adverse effects associated with current available drugs, along with the induction of chemoresistance, there is an emergence for the development of new antineoplastic

drugs with greater selective activity against tumor cells and less cytotoxic effect on normal cells. Thus, the present study aimed to evaluate the antitumoral activity of TMBP on HCC cell lineages, HuH7.5, and HepG2/C3A. Our goals include exploring the possible mechanisms of action of TMBP on these cells and its potential to induce cell death.

2. METHODOLOGY

2.1. Cell Culture

Human hepatocellular carcinoma cell lineages, HuH7.5 and HepG2/C3A, were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, EUA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Santa Cruz Biotechnologies, Dallas, TX, USA) and incubated at 37°C in 5% CO_2.

2.2. Synthesis of TMBP

3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol (TMBP), $C_{16}H_{18}O_6$ (MW: 306) was synthesized from 2,6-dimethoxyphenol by laccase from the ascomyceteous fungus *Botryosphaeria rhodina* (MAMB-05) under the conditions previously described by Schirmann et al. [15]. The compound was obtained wad a yellow solid, identified by ^{1}H NMR (CDCl3, 400 MHz) δ (ppm): 3,98 (s, 12H), 6,73 (s, 4H), 5,56 (s, 2H), purity of 99% and melting point between 185-188°C. A stock solution of TMBP was prepared in 1% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). The DMSO concentration in all experiments did not exceed 0.06%.

2.3. Cell viability assay

HCC cell lines, HuH7.5 and HepG2/C3A (1×10^4) were seeded in 96-well plates and treated with TMBP at the following concentrations (12.5, 25, 50, 100, and 150 µM) for 24 and 48 h. Cells were then washed and incubated for 3 h in a solution of MTT (0.05 mg/mL). The MTT product formed (formazan crystals) was solubilized by adding 100 µL of DMSO, and the absorbance read at 540 nm in a GloMax® Discover Microplate Reader (Promega, GM3000). Experimental groups were defined as follows: control group (DMEM medium only), vehicle group (0.06% DMSO), TMBP groups and hydrogen peroxide (H_2O_2) group (0.4%) (Sigma-Aldrich) as the positive control. All experiments were performed in quadruplicate in three independent experiments.

Data obtained by the MTT assay were used to calculate the logarithmic regression for the 50% inhibitory concentration (IC_{50}). The following experiments were conducted using only the IC_{50} from the 24 h treatment period.

2.4. Cytotoxicity by trypan blue exclusion

HuH7.5 and HepG2/C3A cells (1×10^6) were seeded in 6-well plates and treated with TMBP IC_{50} (68 and 50 µM, respectively) for 24 h. For the evaluation of cytotoxicity, the cell suspension was diluted in 0.4% trypan blue solution in the ratio 1:1. The total number of viable cells was counted by light microscopy (Olympus BX41, Olympus Optical Co., LTD., Tokyo, Japan) in a Neubauer chamber.

2.5. Cell morphology evaluation by Scanning Electron Microscopy (SEM)

For morphological analysis, HuH7.5 and HepG2/C3A cells were seeded (5×10^5) in round coverslips inside a 24-well plate and treated with their respective IC_{50} of TMBP for 24h. The cells were then washed with PBS and fixed with

a solution of 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer for 24h. After fixation, samples were washed with 0.1 M sodium cacodylate buffer and dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100%). Subsequently, the cells were submitted to the critical point by substitution of ethanol to CO_2, and coated with gold nanoparticles. Microphotographs were obtained on a high-resolution double-beam electron microscope (FEI Scios).

2.6. Wound healing assay

HuH7.5 and HepG2/C3A cells lines were seeded (1 x 10^6) in 6-well plates and incubated until confluency. Subsequently, a wound was made in the cell monolayer by scratching a 200 µL pipette tip at the bottom of the wells. This was followed by IC_{50} TMBP treatment. After the treatment, photomicrographs (200x magnification) were taken using an EVOS microscope (Life Technologies) at different times (0, 12, and 24 h). Cellular migration was evaluated as a cell-free area and measured by ImageJ software. The percentual decrease in the area characterized the cell migration.

2.7. Reactive Oxygen Species (ROS) production

Cells were seeded (1 x 10^4) in black 96-well plates, treated with IC_{50} TMBP and incubated for 24 h. Cells were washed with PBS and a solution of 10 µM 2',7'-dichlorofluoresceindiacetate (H_2DCFDA) (Sigma-Aldrich) was added and left for 30 min in the dark at 37°C. H_2DCFDA, a cell-permeant probe, after it diffuses into the cell, is deacetylated by cellular esterases to form 2′,7′-dichlorodihydrofluorescein (H_2DCF). In the presence of ROS, predominantly H_2O_2, H_2DCF is rapidly oxidized to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent. The fluorimeter was set at

488 nm excitation and 530 nm emission on a GloMax® Discover Microplate Reader (Promega, GM3000). H_2O_2 was used as a positive control. Obtained data was normalized by cell counting.

2.8. Measurement of nitric oxide metabolites (NOx)

NOx levels were determined by the Griess method according to Gonçalves et al. [17]. Griess reagent (60 µL; 1% sulfanilic acid, 0,1% N-naphthylethylenediamine and 5% H_3PO_4) were added to 60 µL of cell culture supernatant following TMBP treatment and incubated for 10 min at room temperature. A calibration curve was constructed using serial dilutions of $NaNO_2$. Nitrite concentration was read at 550 nm on a microplate reader (Thermo Scientific, Multiskan GO).

2.9. Determination of mitochondrial membrane potential (∆Ψm)

∆Ψm was determined by tetramethylrhodamine ethyl ester (TMRE; Sigma-Aldrich) staining, a cell-permeable, cationic, red-orange fluorescent dye that readily accumulates in active mitochondria due to its relative negative charge. Inactive mitochondria have limited ΔΨm and do not sequester TMRE. HuH7.5 and HepG2/C3A cells were seeded (1×10^4) in black 96-well plates, treated with IC_{50} TMBP and incubated for 24 h. Thereafter, aliquots of 25 nM TMRE solution (2 µL/well) were added and incubated for 30 min at 37 °C. The wells were then washed with 200 µL PBS and immediately analyzed fluorometrically at 480 nm excitation and 580 nm emission in a GloMax® Discover Microplate Reader (Promega, GM3000). Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as the positive control as it is a known inducer of mitochondrial permeability and loss of ∆Ψm.

2.10. Lipid droplets (LD) detection

HCC cells were seeded (1 x 10^4) and treated as described above. Lipid droplets were evaluated by staining with Nile red (NR); a lipophilic stain used to localize and quantitate lipids, particularly neutral lipid droplets within cells. In the presence of lipids, the fluorescence colors range from golden yellow to deep red. Cells were stained with 10 µg/mL NR (Sigma-Aldrich) for 30 min and analyzed by fluorimetry at 530 and 635 nm for excitation and emission, respectively. PBS were used as the positive control.

2.11. Autophagic vacuoles formation

The quantification of autophagic vacuoles was based on the monodansylcadaverine (MDC) method. MDC is a fluorescent lysosomotropic compound, used for the identification of cadaverine protein, present in autophagic vesicles. Cells were seeded (1 x 10^4) in black 96-well plates and treated with IC_{50} TMBP. One hour before the end of the treatment period, the cells were washed twice with 200 µL PBS, followed by the addition of 100 µL MDC (50 µM) (Sigma-Aldrich) to each well and incubated for 1 h. Afterward, the cells were washed with PBS, and 100 µL PBS was added for the reading by fluorimetry at 380 nm excitation and 525 nm emission. PBS were used as a positive control.

2.12. Ultrastructure alterations evaluated by Transmission Electron Microscopy (TEM)

HuH7.5 and HepG2/C3A cells (1 x 10^6) were treated with 68 µM and 50 µM of TMBP, respectively, for 24 h at 37 °C, 5 % CO_2. Subsequently, the cells were

washed with PBS and fixed by immersion in glutaraldehyde 2.5% in sodium cacodylate buffer (0.1 M) for 24 h, post-fixed with 1 % OsO_4, 0.8 % potassium ferrocyanide and 10 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer for 1 h. Samples were dehydrated in increased concentrations of acetone (30, 50, 70, 90, 95, 100%) and embedded in EPON™ epoxy resin for 72h at 60°C. Finally, ultrathin sections (60–70 nm) were obtained, mounted on copper grids and contrasted with uranyl acetate and lead citrate. Sections were visualized on a JEOL JEM-1400 transmission electron microscope.

2.13. Cell cycle analysis

Cells were seeded at a density of 1 x 10^6 in 6-well plates and treated as described previously. Next the cell suspension was centrifuged at 1500 rpm for 5 min and the pellet resuspended in 300 µL PBS. Subsequently, a solution of 0,05% ribonuclease A (RNAse A; Sigma-Aldrich) was added and incubated for 30 min followed by the addition of a solution of 0,1% sodium citrate and 1% Triton-X100 and 50 µg/mL of propidium iodide (PI; Sigma-Aldrich) for 30 min. The fluorescence reading was estimated on a Muse® Cell Analyzer (Merck Millipore), with 5000 events. DNA content and cell population percentage were analyzed in different cell cycle phases (G1, S and G2/M) by fluorescence intensity.

2.14. Apoptosis assay

Cells were seeded (1 x 10^6) and treated with TMBP. After treatment, the cell suspensions were washed with PBS, and 50 µL of FITC Annexin V Apoptosis Detection Kit (BD Biosciences) was added in each sample for 20 min at room temperature to determine apoptosis. Samples were analyzed using a flow cytometer Muse® Cell Analyzer and data analyzed using the Muse Cell Analyzer™ software.

2.15. Statistical analysis

Statistical differences were obtained after one-way or two-way analysis of variance (ANOVA), followed by Tukey test for multiple comparisons and unpaired Student t-test for two groups using GraphPadPrism 8 (GraphPad Software, San Diego, California, USA). Data were expressed as mean ± standard error of the mean (SEM) of three independent experiments in triplicate or quadruplicate, and were considered significant differences when the *p*-value was $p < 0.05$. Values were characterized by: * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

3. RESULTS

3.1. TMBP reduces cellular metabolic activity of HCC cell lines

The effects of TMBP on HuH7.5 and HepG2/C3A metabolic activities were performed by the MTT assay. Both cell lines were treated with TMBP (12.5, 25, 50, 100, and 150 μM) for 24 and 48 h. Cell metabolic activity in HuH7.5 and HepG2/C3A, after 24 h of TMBP treatment, decreased to 87.09% (± 2.97), 81.53% (± 1.66), 75.16% (± 1.88), 34.97% (± 2.49) and 12.76% (± 0.73) ($p < 0.0001$), and 90.23% (± 0.90), 75.03% (± 1.13), 49.91% (±1.22), 30.49% (± 1.23) and 27.88% (± 0.66) ($p < 0.0001$), respectively (**Figure 1A, D**). After 48 h of treatment, the metabolic activity was reduced by more than 50% at the concentration of 50 μM in the HuH7.5 and HepG2/C3A cell lines. TMBP impaired cellular metabolic activity in a time and concentration dependent manner (**Table 1**).

The TMBP IC_{50} was determined for both cell lines at all of the analyzed times. The IC_{50} values were 68 and 50 μM in 24 h and 55 and 42 μM in 48h for HuH7.5 and HepG2/C3A, respectively (**Table 2**). The IC_{50} at 24 h for HuH7.5 (68 μM) and

HepG2/C3A (50 μM) were chosen for all of the experiments to follow. A significant reduction of viable cells to 52.50% (± 3.44) (p <0.0001) was observed for HuH7.5, and 36.67% (± 5.00) (p <0.0001) for the HepG2/C3A cells as evidenced by the trypan blue exclusion assays (**Figure 1B, E**). Also, representative images of the cell morphology during culture showed cellular alterations, with retracted and rounded morphologies, characteristic of *in vitro* apoptotic morphology in both cell lines after treatment with TMBP IC_{50} for 24h (**Figure 1C, F**).

3.2. TMBP induces morphological alterations and impairment of cell migration on HuH7.5 and HepG2/C3A cell lines.

SEM was used to assess alterations in cell morphology on TMBP treated cells. Comparative morphologies of the two HCC cell lines treated with TMBP are represented in Figure 2. The control groups of both cell lines showed firm adherence to the glass substrate, intact cellular membrane, presence of lamellipodia, and microvilli throughout the cell surface (**Figure 2A-C, G-I**).

The treatment with TMBP IC_{50} induced severe morphological alterations by reduction and rupture of lamellipodia and filopodia, irregular cell surface, a decrease of microvilli, formation of apoptotic bodies, as well as damage and rupture to the cell membranes, in HuH7.5 (**Figure 2D-F**) and HepG2/C3A (**Figure 2G-I**) cell lines.

The wound healing assay was performed to assess cell migration after TMBP treatment. The HuH7.5 control group presented a wound gap from 100% (± 3.43) to 25.62% (± 5.01) and 13.85% (± 2.58) for 12 h and 24 h, respectively. For the HepG2/C3A control group, the gap decreased from 100% (± 5.34) to 88.68% (± 6.02) and 65.38% (± 9.39) in the same times evaluated. The TMBP-treated groups showed

less migratory activity, presenting a gap of 70.16% (± 5.01) (p <0.0001) and 95.68% (± 7.06) (p <0.01) for HuH7.5 (**Figure 2M**) and HepG2/C3A cell lines (**Figure 2N**) at 24h, respectively.

3.3. TMBP promotes ultrastructural alterations and induces cellular metabolism stress by redox-based mechanisms.

To comprehend the mechanisms by which TMBP treatment exerts its cytotoxic effect on the HCC cells, we assessed the production of ROS, NOx, mitochondrial membrane potential, the formation of autophagic vacuoles and lipid droplets.

Measurement of total ROS generated through induction by TMBP was evaluated via the H_2DCFDA probe, and fluorescence readings were normalized by cell counting for each group. ROS increased from 0.080 (± 0.003) to 0.213 (± 0.025) arbitrary units of fluorescence (AUF) on HuH7.5 (p <0.001) and from 0.093 (± 0.003) to 0.221 (± 0.019) AUF on HepG2/C3A cells (p <0.0001) (**Figure 3A**) following TMBP treatment with the respective IC_{50} for each cell lineage when compared to controls. We observed an increase in NOx levels from 4.01 (± 0.09) to 4.67 (± 0.11) µM (p <0.001) after treatment on HuH7.5 and from 3.92 (± 0.13) to 4.95 (± 0.20) µM on HepG2/C3A cells (**Figure 3B**).

Subsequently, we assessed TMBP effects on the mitochondrial membrane potential by TMRE staining. A significant mitochondrial membrane depolarization was observed in both lineages, accompanied by a decrease in TMRE fluorescence from 144.30 (± 19.09) to 71.66 (± 9.42) AUF (p <0,001) on HuH7.5 and from 165.8 (± 23.23) to 73.65 (± 15.54) AUF (p <0,01) on HepG2/C3A cells (**Figure 3C**).

Considering the increased cellular stress and mitochondrial alterations induced by TMBP treatment, we evaluated lipid droplets by the NR staining procedure. An increase in fluorescence in the HuH7.5 and HepG2/C3A cells (**Figure 3D**) were detected in TMBP-treated groups from 0.21 (± 0.01) and 0.29 (± 0.01) to 0.33 (± 0.01) and 0.46 (± 0.03) AUF (p <0.0001), respectively.

The formation of autophagic vacuoles was evaluated by the MDC staining assay. TMBP-treated cells presented increases in the detection of autophagic vacuoles from 0.113 (± 0.001) to 0.226 (± 0.004) AUF (p <0.0001) for HuH7.5 cells and 0.097 (± 0.004) to 0.161 (± 0.010) AUF (p <0.0001) for the HepG2/C3A cells (**Figure 3E**).

TEM analysis was performed to observe ultrastructural alterations on the HuH7.5 and HepG2/C3A cells when treated with their respective TMBP IC_{50} concentrations. Both cell lines of the control group presented well-preserved structures, as mitochondria, nucleus, endoplasmic reticulum, Golgi complex, cytoplasmic membrane and the presence of microvilli (**Figure 3 F, G, L, M**).

HuH7.5 and HepG2/C3A cells treated with TMBP presented ultrastructural alterations characterized by increased cytoplasmic lipid droplets and autophagic vacuoles, mitochondrial damage, vacuolization and cytoplasm disarrangement, severe reduction of microvilli and cell membrane damage and rupture (**Figure 3 H-K, N-Q**).

3.4. Treatment with TMBP causes cell cycle arrest in the G2/M phase and apoptotic cell death in both HCC cell lines.

To consider the direct cytotoxic effect of TMBP on HCC cells, we evaluated the cell cycle distribution after IC_{50} treatment for 24h. TMBP treatment of

HuH7.5 resulted in the reduction of 23.71% (± 0.85) of cells in the G0/G1 phase (p <0.0001), a decrease of 2.85% (± 0.71) in the S phase, but was accompanied by an increase of 25.75% (± 1.23) of cells in the G2/M phase (p <0.001) (**Figure 4A**). HepG2/C3A cells presented a similar trend, with a decrease of 26.72% (± 1.77) of cells in the G0/G1 phase (p <0.001), reduction of 3.46% (± 0.47) in the S phase, and an increase in the G2/M phase of 23% (± 2.24) (p <0.001) (**Figure 4B**). These results are indicative of treatment-induced disturbances in the cell cycle. In addition, HepG2/C3A also showed an increase in the sub-G1 phase (data not shown), an effect suggestive of cell death.

Cell cycle disturbances can cause quiescence or induce the activation of cell death machinery. Therefore, the pattern of cell death was investigated by marking apoptotic and necrotic events. Our results revealed a decrease of 41.76% (± 4.90) (p <0.0001) of HuH7.5 live cells after treatment with TMBP, and a significant increase in the percentage of early and late stage apoptosis of 15.35% (± 1.96) (p <0.001), and 25.42% (± 2.58) (p <0.0001), respectively (**Figure 5A**). In the HepG2/C3A cell line, there was a decrease of live cells of 39.53% (± 3.15) (p <0.0001), with an increase of early and late stage apoptosis of 15.69% (± 1.16) (p <0.0001), and 23.05% (± 2.50) (p <0.0001), respectively (**Figure 5B**).

4. DISCUSSION

Systemic therapy for HCC is accompanied by several adverse effects, treatment discontinuation [12,18,19] and chemoresistance development, resulting in disease relapse and metastasis [20–22]. In this context, 3,3’,5,5’-tetramethoxybiphenyl-4,4’-diol (TMBP) exhibited potential to become a new antitumoral oral drug as demonstrated in our previous *in silico* study, in which the

molecule was shown to present favorable drug-likeness properties, and a potential to accumulate in mitochondria, inducing cell death by apoptosis [16]. Our findings included a redox-mediated stress condition, leading to morphological and ultrastructural alterations, impaired cell migration and death by apoptosis.

We first investigated the cytotoxic effects induced by TMBP on HCC cell lines. Hepatocytes are the primary site of xenobiotic metabolism, and even though HCC cells present dysregulated expression of genes involved in drug uptake, excretion and metabolism, resulting in chemoresistance [22], TMBP induced toxicity in both cell lines at lower concentrations when compared to our previous study on A549 cells of lung carcinoma [16]. Treatment decreased cell viability at all concentrations tested and induced severe morphological and ultrastructural alterations, confirming the cytotoxic effects of TMBP on HCC cell lineages.

Lamellipodia and filopodia are F-actin-rich protrusions, involved in cellular migration [23–25], and in the process of invasion and metastasis [26,27]. For this reason, we investigated the integrity of such structures, as well as cellular migratory capacity. TMBP-treated cells exhibited reduction and breakage of lamellipodia and filopodia, impairing migration, as observed by a larger wound area compared to the control group. These results indicate that TMBP can hinder the migratory activity and invasion of adjacent tissues by tumoral cells.

We further investigated the redox-based mechanisms of action involved in TMBP tumor elimination. The redox cellular status is frequently high due to ROS and reactive nitrogen species (RNS) generation in cancer cells due to high metabolic rate and gene mutations, generating genomic instability and tumor progression [28–32]. It can trigger programmed cell death through extensive oxidative damage to cellular components, including proteins, lipids, DNA and promoting

mitochondrial dysfunction [33–35], which can result in mitochondrial structure alterations, overproduction of ROS by mitochondrial Ca^{2+} overload and loss of ΔΨm [36]. This hypothesis supports our findings, which include a significant increase in ROS and NOx levels as well as a loss of ΔΨm.

Since the redox imbalance impacts cell homeostasis and structure, we investigated the effect of TMBP treatment on LDs and autophagic vacuoles. LDs are storage organelles composed of neutral lipids [37], produced due to energetic and redox imbalances as a way to maintain cell homeostasis [38]. LDs are involved in the maintenance of cell membranes, in the prevention of lipid peroxidation, and also as a delivery system of fatty acids to the mitochondria for energy production [39]. However, when faced with mitochondrial dysfunction and increased ROS, cells push a rapid accumulation of LDs in the cytosol, as demonstrated in HCC TMBP-treated cells, that presented a significant increase of NR fluorescence, as a result of fatty acid β-oxidation inhibition, culminating in the activation of apoptotic cell death [40].

Autophagy is a defense mechanism against oxidative stress in cancer, since tumoral cells frequently present increased ROS and RNS [41,42]. However, chemotherapeutic drugs can induce even more ROS and RNS production [43]. Overactivation of autophagy results in the accumulation of lysosomes in the cytoplasm, overconsumption of intracellular components [44] and promotes membrane damage as these organelles are highly sensitive to ROS, which promotes lysosomal membrane permeabilization and/or rupture, culminating in cell demise due to the release of hydrolytic enzymes into the cytoplasm [45,46]. This series of events corroborates with our observations using SEM, as diminished microvilli, cytoplasmic membrane damage and the formation of apoptotic bodies [47,48]. Accordingly, we have observed a significant increase in autophagic vacuoles in both HCC cell lines, which suggests a

process of autophagy-mediated cell death.

Cell cycle progression is tightly regulated by the checkpoints, which can detect cellular and DNA damage and lead to cell cycle arrest until the damage is resolved or may result in the activation of the mechanisms of cell death [49,50]. Our cell cycle distribution analysis presented an accumulation of cells in the G2/M phase, implying that TMBP can block cell proliferation by arresting the cycle before cells can enter mitosis due to promotion of extensive cellular damage [51,52].

TMBP can further induce apoptosis as indicated by an increase of DNA fragmentation observed as the sub-G1 population in the cell cycle distribution [53] and by the increased apoptotic population in treated cells. These events can happen through oxidative damage of mitochondria, resulting in the loss of ΔΨm, and subsequently, the release of cytochrome c in the cytosol [54].

In concluding, we observed that TMBP exerted a cytotoxic effect on HCC cells by promoting morphological alterations and migration impairment, increased ROS and NOx levels, loss of ΔΨm, increased LDs and autophagic vacuoles as well as cell cycle arrest and death by apoptosis.

5. CONCLUSION

Treatment with TMBP, exhibited reduction of cell viability by promoting cytotoxicity towards HCC cell lines, HuH7.5 and HepG2/C3A. These cytotoxic effects were mediated by morphological and ultrastructural alterations, impairment of migratory capacity, increased ROS and NOx levels, mitochondrial damage, cell cycle arrest in the G2/M phase and subsequently death by apoptosis.

These findings showed a promising application of TMBP and unraveled its intricate mechanisms of action as a potential candidate for development

of an antitumoral drug. This study determined the mechanism of action involved in *in vitro* TMBP treatment against HCC tumor cell lines and highlights an innovative therapeutic approach that can be explored in future investigations; however, more studies are needed to fully comprehend TMBP mechanisms and effects in different experimental models.

Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCF, 2', 7' – dichlorofluorescein; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethylsulfoxide; FBS, fetal bovine serum; H_2DCFDA, 2',7' –dichlorofluorescin diacetate; H_2O_2, hydrogen peroxide; HCC, hepatocellular carcinoma; LD, lipid droplets; MDC, monodansylcadaverine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOx, nitric oxide metabolites; NR, Nile red; PBS, phosphate buffered saline; PI, propidium iodide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TMBP, 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol; TMRE, tetramethylrhodamine ethyl ester.

CRediT authorship contribution statement:

Fabricio Seidy Ribeiro Inoue: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review and editing **Virginia Marcia Concato**: Methodology, Investigation, Formal analysis, Writing – review and editing. **Bruna Taciane da Silva Bortoleti**: Methodology, Investigation, Formal analysis, Writing – review and editing. **Ellen Mayara Souza Cruz**: Investigation. **Mariana Barbosa Detoni**: Investigation, Writing – review and editing. **Fernanda Tomiotto-Pellissier**: Investigation, Writing – review and editing. **Manoela Daiele Gonçalves**:

Investigation. **Juliana Maria Bitencourt de Morais-Valentim**: Investigation. Rayanne Regina Beltrame Machado: Investigation. **Rayanne Regina Beltrame Machado**: Methodology. **Jéseka Gabriela Schirmann**: Methodology. **Aneli M. Barbosa-Dekker**: Methodology, Writing – review and editing. **Robert F. H. Dekker**: Methodology, Writing – review and editing. **Ivete Conchon-Costa**: Validation. **Mário Sérgio Mantovani**: Validation. **Danielle Lazarin-Bidóia**: Investigation, Formal analysis, Validation. **Carolina Panis**: Investigation, Writing – review and editing. **Wander Rogério Pavanelli**: Conceptualization, Validation, Writing – review and editing, Supervision.

Conflict of interest

The authors have no conflict of interests to declare.

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TABLES

Table 1. Analysis of concentration and time response to TMBP on HuH7.5 and HepG2/C3A treated cells for 24 and 48 h.

		HuH7.5		HepG2/C3A	
		24 h	**48 h**	**24 h**	**48 h**
Control		A	A	A	A
Vehicle		A	A	A	A
TMBP	12.5 μM	B	B	B	B
	25 μM	B	B	C	C
	50 μM	B	c #	D	d #
	100 μM	C	d #	E	e #
	150 μM	D	e #	E	f #

TMBP: 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol; Different letters mean statistical difference compared to the previous row, while same letters mean no statistical significance; # represents the statistical difference between treatment periods with the same drug concentration.

Table 2. TMBP Inhibitory concentrations of 50% in HCC cell lines after 24 and 48 h treatment.

	IC_{50} (±SEM)	
Cell Line	**24 h**	**48 h**
HuH7.5	68 μM (± 0.01)	55 μM (± 0.01)
HepG2/C3A	50 μM (± 0.01)	42 μM (± 0.01)

IC_{50} – Inhibitory Concentration of 50%; SEM – Standard Error Mean.

FIGURES

Figure 1. TMBP treatment reduces viability in HuH7.5 and HepG2/C3A tumoral lineages. HCC cells were treated with different concentrations (12.5 – 150 μM) of TMBP for 24 and 48 h. Cell viability of (A) HuH7.5 and (D) HepG2/C3A cells were analyzed by the MTT assay. HuH7.5 (B) and HepG2/C3A (E) were treated with IC_{50} (68 and 50 μM) for 24h and counted by light microscopy by the trypan blue exclusion assay. Representative images of HuH7.5 (C) and HepG2/C3A (F) cell morphology by optical microscopy. Data are expressed as the mean ± SEM of three independent experiments performed in quadruplicate. Statistical difference from control, **** (p < 0.0001).

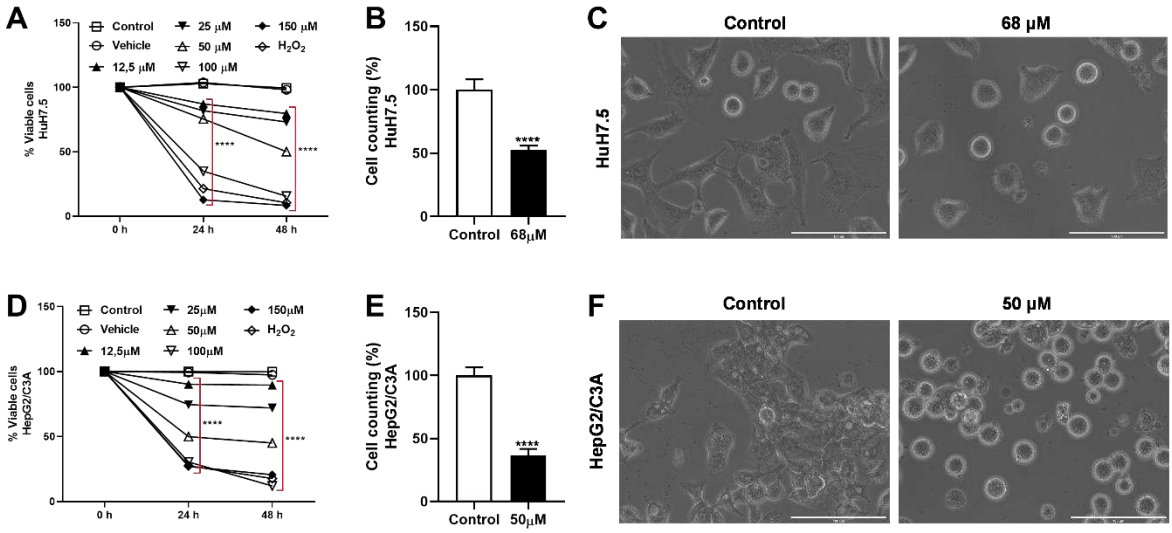

Figure 2. **TMBP induces alterations in cell morphology along with decreased migration capacity.** (A-C) Control HuH7.5 cell morphology; (D-F) HuH7.5 IC_{50} (68 µM) treated cells; (G-I) Control HepG2/C3A cell morphology; (J-L) HepG2/C3A IC_{50} (50 µM) treated cells; (M) HuH7.5 and (N) HepG2/C3A wound healing assay representative images at 0, 12, and 24 h. Migratory distance into the free area was quantified after 24 h. Values represent the mean ± SEM of three independent experiments performed in triplicate. Significant differences from control, ** (p <0.01), **** (p < 0.0001). Filopodia (F); Lamellipodia (L); Microvilli (Mi); Reduction of microvilli (Mi*); Apoptotic bodies (➤); Cell membrane damage (➔); Plasma membrane rupture (⏩); Lamellipodia breakage (*).

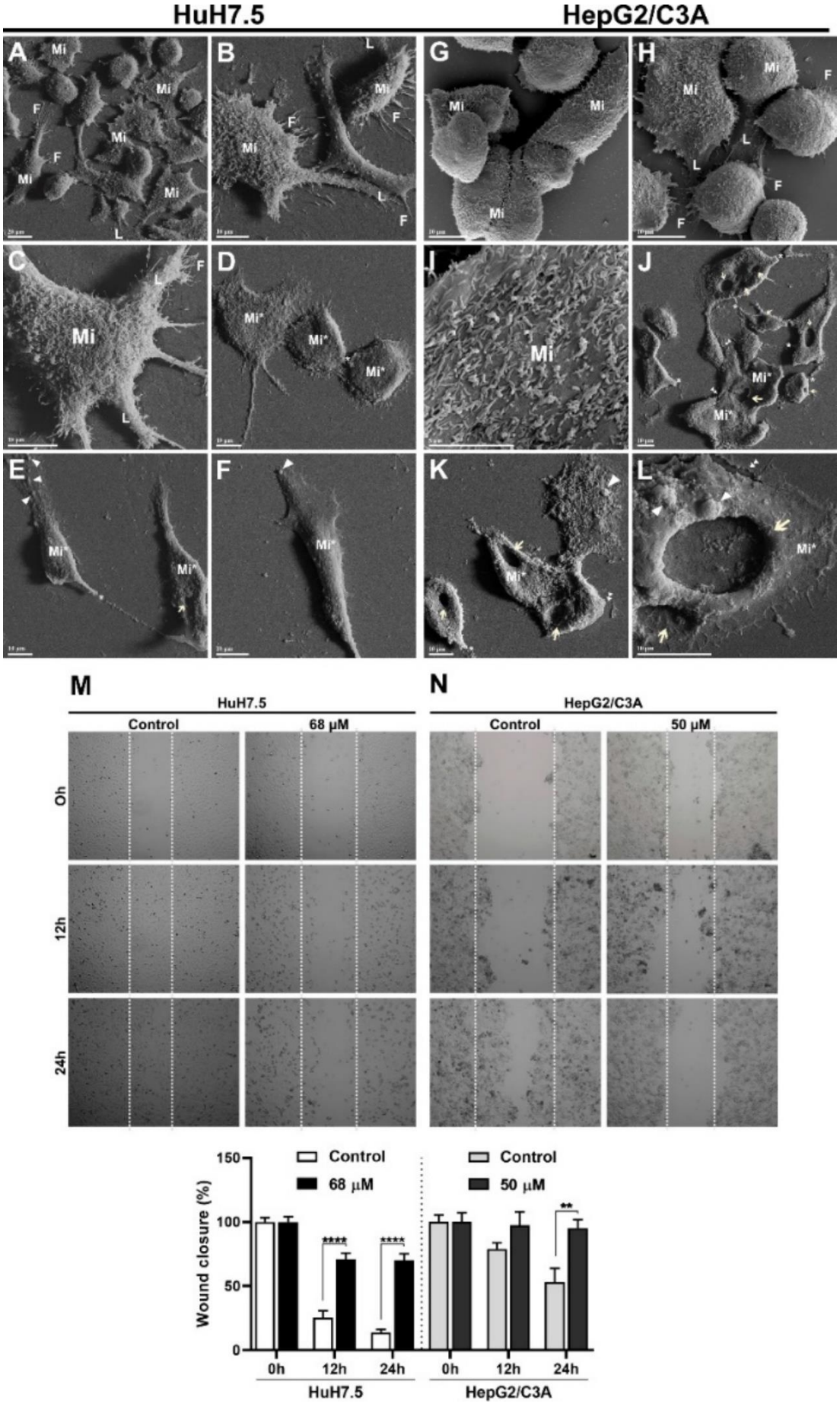
HuH7.5
HepG2/C3A
A
B
G
H
C
D
I
J
E
F
K
L
Mi
F
L
M
HuH7.5
Control
68 μM
N
HepG2/C3A
Control
50 μM
0h
12h
24h
Wound closure (%)
150
100
50
0
Control
68 μM
Control
50 μM
****
****
**
0h
12h
24h
0h
12h
24h
HuH7.5
HepG2/C3A

Figure 3. TMBP treatment induces increase in the levels of ROS and NOx, mitochondrial damage, formation of lipid droplets, autophagic vacuoles and ultrastructure alterations in HuH7.5 and HepG2/C3A cell lines. After 24 h treatment with TMBP (68 and 50 μM), arbitrary units of fluorescence (AUF) were normalized for the HuH7.5 and HepG2/C3A cell lines to determine the levels of total reactive oxygen species (A), lipid droplets (LD) (D) and autophagic vacuoles (AV) (E). Nitric oxide (B) levels were obtained by spectrophotometry and ΔΨm (C) by measuring AUF. Ultrastructure alterations were observed on HuH7.5 control group (F,G), HuH7.5 IC_{50} group (H-K), HepG2/C3A control (L,M) and HepG2/C3A IC_{50} group (N-Q). Golgi complex (Gc); *Microvilli* (Mi); Mitochondria (m); Nucleus (Nu); Endoplasmic Reticulum (er); Mitochondrial alterations (m*); Cytoplasm membrane damage and *microvilli* reduction (▶); Autophagic vacuoles (➔); Lipid droplets (*). Values were expressed as mean ± SEM. ** (p <0.01); *** (p <0.001); **** (p <0.0001) vs. control.

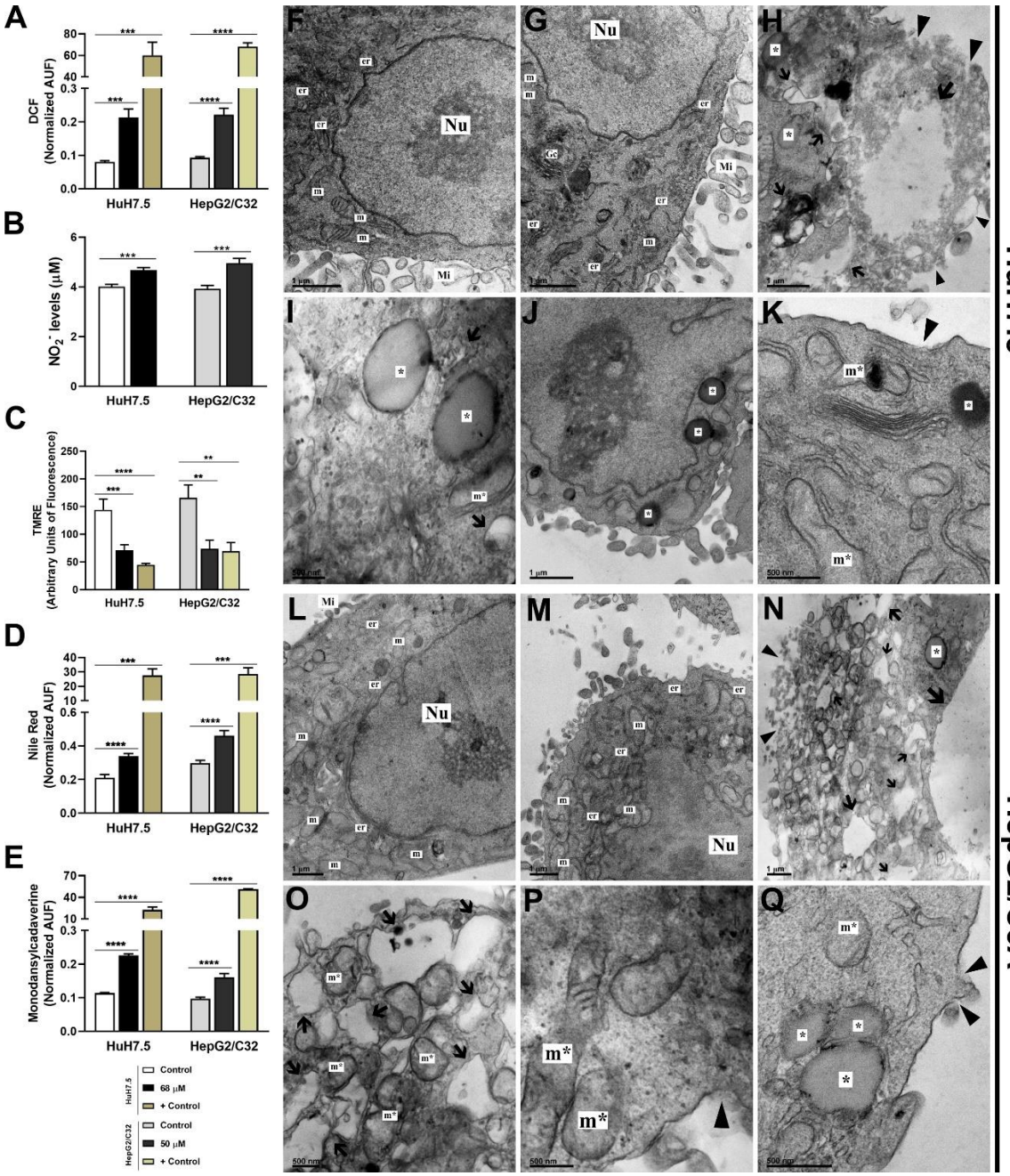
A
DCF (Normalized AUF)
HuH7.5
HepG2/C32
B
NO_2^- levels (μM)
C
TMRE (Arbitrary Units of Fluorescence)
D
Nile Red (Normalized AUF)
E
Monodansylcadaverine (Normalized AUF)
Control
68 μM
+ Control
50 μM
F
G
H
I
J
K
L
M
N
O
P
Q
Nu
Mi
er
m
m*
HuH7.5
HepG2/C3A

Figure 4. HCC cell lines treated with TMBP presented cell cycle arrest in the G2/M phase. HuH7.5 and HepG2/C3A cells were treated with the IC_{50} of TMBP (68 and 50 µM) for 24 h, and the cell cycle distribution by flow cytometry was plotted and quantitative analysis of cells at different stages of the cell cycle were evaluated (A) and (B), respectively. Peaks in green were considered as the Sub-G1 population. Values were expressed in % vs. control; and mean ± SEM. * (p <0.05); *** (p <0.001); **** (p <0.0001) vs. control.

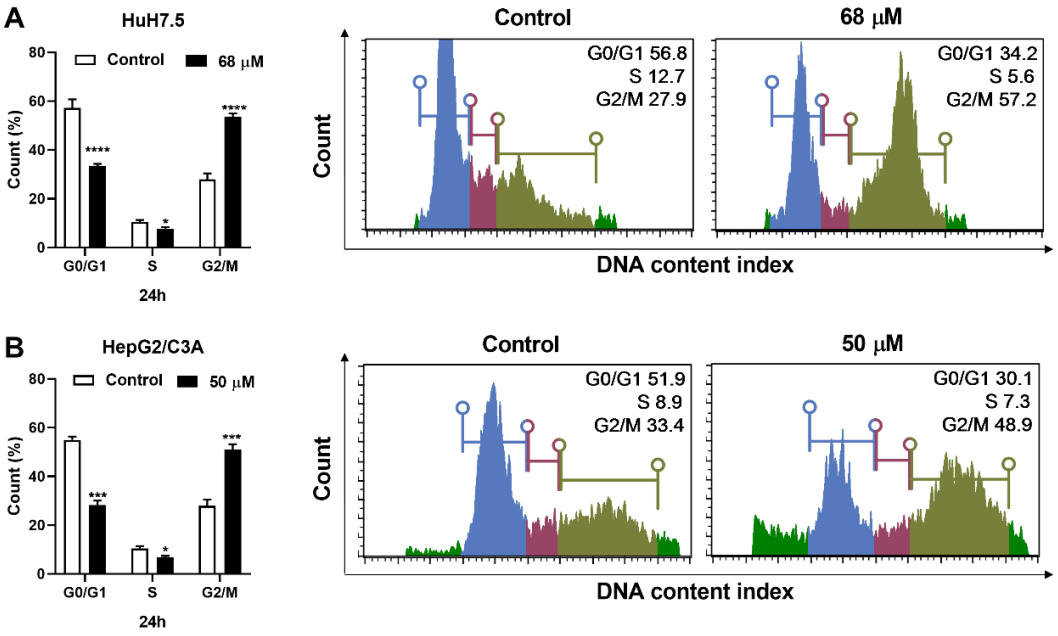

Figure 5. TMBP treatment induces apoptosis in HCC cell lines. HuH7.5 and HepG2/C3A cells were treated with the IC_{50} of TMBP (68 and 50 µM) for 24 h, and the cell death types were evaluated by flow cytometry, and were plotted and quantitatively analyzed (A) and (B), respectively. Values were expressed in % vs. control; and mean ± SEM. * (p <0.05); *** (p <0.001); **** (p <0.0001) vs. control.