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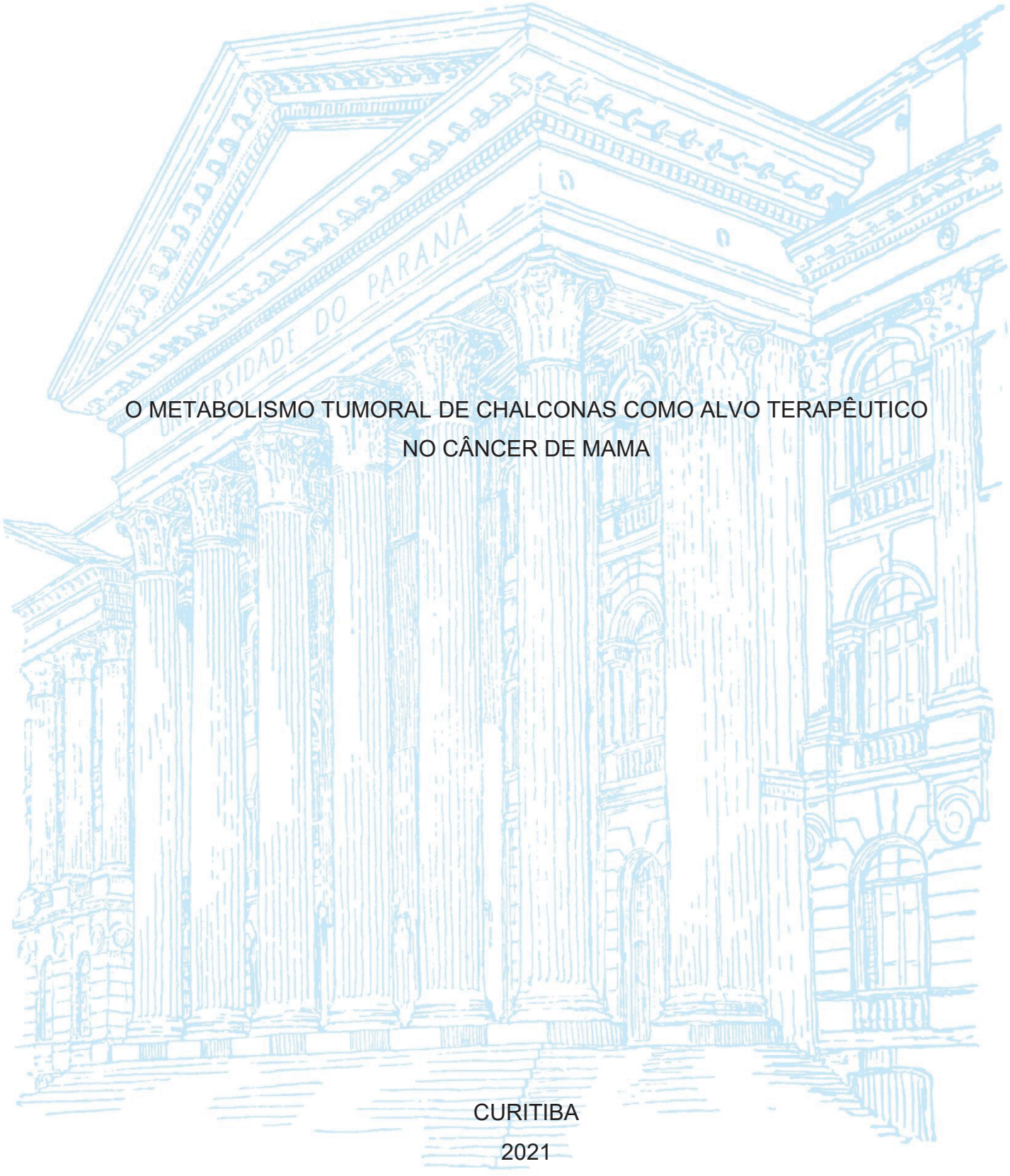
CLAUDIA MARTINS GALINDO



O METABOLISMO TUMORAL DE CHALCONAS COMO ALVO TERAPÊUTICO NO CÂNCER DE MAMA

CURITIBA

2021

CLAUDIA MARTINS GALINDO

O METABOLISMO TUMORAL DE CHALCONAS COMO ALVO TERAPÊUTICO NO CÂNCER DE MAMA

Tese apresentada ao curso de Pós-Graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Farmacologia.

Orientadora: Profª. Drª. Alexandra Acco

Coorientadora: Profª. Drª. Edneia A. S. R. Cavalieri

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **CLAUDIA MARTINS GALINDO** intitulada: **O metabolismo tumoral de chalconas como alvo terapêutico no câncer de mama**, sob orientação da Profa. Dra. ALEXANDRA ACCO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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

CURITIBA, 30 de Setembro de 2021.

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NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo – artigos publicados ou submetidos para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e dois artigos científicos, além das considerações finais.

Eu dedico esta tese aos animais de experimentação que tive a grande benção de cruzar pelo caminho e a todos aqueles que dia a dia ajudam no avanço da ciência.

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Ao meu pai José pelo apoio incondicional. Ao meu noivo Valdir por partilhar de cada plano, sucesso e decepção, com infinita confiança e amor.

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Dalai Lama

RESUMO

O câncer pode ser considerado um desafio inerente ao aumento da longevidade humana e estilo de vida atual. Observa-se, sobretudo, um crescimento notável nos casos de câncer de mama, considerado o tipo mais diagnosticado no mundo. Os impactos desta condição não se limitam à perda de qualidade de vida das mulheres vítimas da doença, principal público deste tipo de câncer, mas é também considerado um problema de saúde pública emergente. O remodelamento metabólico é uma das bases da transformação celular maligna e, por isso, um alvo interessante para a terapia anticâncer, utilizado em associação com outras modalidades terapêuticas para otimizar o tratamento e superar a resistência adquirida aos fármacos. Uma via clássica de plasticidade metabólica é do mTOR, um propulsor da biossíntese de macromoléculas que garante o crescimento e divisão celular. Esta via, dentre outras, foi investigada neste trabalho como possível alvo da chalcona sintética 4-nitrochalcona (4NC). A 4NC apresentou um efeito antiproliferativo em modelo *in vitro* de câncer de mama (linhagem MCF-7), associado à atenuação de Raptor e S6K1, efetores a jusante de mTOR, com consequente redução da síntese proteica; além de queda da razão lactato/piruvato, um indicador importante da glicólise aeróbica ou efeito Warburg. Adicionalmente, o potencial anticâncer da 4NC foi confirmado *in vivo* no carcinoma sólido de Ehrlich, com administração de 4NC por via oral, na dose de 25 mg kg^{-1} ao dia por 21 dias. Em ambos os modelos, houve também um incremento gênico e proteico dos níveis de LC3, um marcador autofágico. No entanto, demonstrou-se *in vitro* que os níveis de outros efetores autofágicos como p62 e Beclina-1 não se alteraram, sugerindo um efeito de bloqueio da autofagia mediado pela 4NC. Portanto, a 4NC apresenta efeito dual na modulação metabólica tumoral. Afora as vias de metabolismo clássicas, novos marcadores de interesse neste campo despontam, como é o caso da enzima 6-fosfofruto-2-quinase/frutose-2,6-bifosfatase 3 (PFKFB3). Trata-se de uma potente fomentadora do efeito de Warburg, com atividade enzimática colateral à glicólise. Tendo em vista a heterogeneidade do câncer de mama em termos de classificação morfológica, molecular e comportamento clínico, fez-se uma revisão de literatura para avaliar os benefícios da terapia anti-PFKFB3 no câncer de mama, com destaque para as chalconas 3PO, PFK15 e PFK158. Cada subtipo de câncer de mama apresentou uma resposta característica, vinculada principalmente às vias de sinalização PI3K/AKT/mTOR e MAPK e ao status hormonal. Independente do contexto de modulação metabólica, anti-mTOR ou anti-PFKFB3, as chalconas se mostram como possibilidades consistentes de terapia no câncer de mama, especialmente com base em estudos pré-clínicos.

Palavras-chave: Chalconas. mTOR. Autofagia. PFKFB3. Câncer de mama.

ABSTRACT

Cancer can be considered an inherent challenge to increasing human longevity and current lifestyle. In particular, there is a remarkable growth in cases of breast cancer, considered the most diagnosed cancer in the world. The impacts of this condition are not limited to the loss of quality of life of women victims of the disease, but it is seen as an emerging public health problem. Metabolic remodeling is one of the bases of malignant cell transformation and, therefore, an interesting target for anticancer therapy, used in association with other therapeutic modalities to optimize treatment and overcome acquired resistance to drugs. A classic pathway of metabolic plasticity is mTOR, a macromolecule biosynthesis booster that ensures cell growth and division. This pathway was investigated herein as a possible action mechanism of the synthetic chalcone 4-nitrochalcone (4NC). 4NC showed an antiproliferative effect in an *in vitro* model of breast cancer (MCF-7 cells lineage), associated with attenuation of Raptor and S6K1, downstream effectors of mTOR, with a consequent reduction in protein synthesis; in addition to a drop in the lactate/pyruvate ratio, an important indicator of aerobic glycolysis or Warburg's effect. Additionally, the anticancer potential of 4NC was confirmed *in vivo* using the solid Ehrlich carcinoma model, with oral administration of 4NC at a dose of 25 mg kg^{-1} per day. In both models, there was also an increase in gene and protein levels of LC3, an autophagic marker. However, it was demonstrated *in vitro* that the levels of other autophagic effectors such as p62 and Beclin-1 did not change, suggesting a 4NC-mediated autophagy blocking effect. Therefore, 4NC has a dual and synergistic effect on tumor metabolic modulation. Apart from the classical metabolism pathways, new markers of interest in this field are arising, such as the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3). It is a potent promoter of the Warburg effect, with enzymatic activity that is collateral to glycolysis. Considering the heterogeneity of morphological, molecular classification, and clinical behavior of breast tumor, a literature review was carried out to assess the benefits of anti-PFKFB3 therapy in breast cancer, with emphasis on the chalcones 3PO, PFK15, and PFK158. Each breast cancer subtype showed a characteristic response, mainly linked to the PI3K/AKT/mTOR and MAPK signaling pathways and hormonal status. Regardless of the context of metabolic modulation, anti-mTOR or anti-PFKFB3, chalcones have been shown to be consistent possibilities for breast cancer therapy at the preclinical level.

Keywords: Chalcones. mTOR. Autophagy. PFKFB3. Breast cancer.

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LISTA DE ABREVIATURAS OU SIGLAS

INTRODUÇÃO

AKT, proteína quinase B; ATGs, genes relacionados à autofagia; AMPK, proteína quinase ativada por monofosfato de adenosina; ATP, trifosfato de adenosina; CAT, ciclo do ácido tricarboxílico; CDK4/6, quinase dependente de ciclina 4/6; FO, fosforilação oxidativa; DNA, ácido desoxirribonucleico; DNC, doenças não comunicáveis; FADH, dinucleotídeo de flavina e adenina; JAK/STAT, janus quinase/transdutor de sinal e ativador de transcrição; HER2, receptor do fator de crescimento epidérmico humano 2; IHC, imunohistoquímica; LC3, proteínas associadas a microtúbulos 1A/1B de cadeia leve 3B; MDR, canais de resistência a múltiplas drogas; mTOR, alvo mecanístico da rapamicina; mTORC1, complexo 1 do alvo mecanístico da rapamicina; NAD, nicotinamida adenina dinucleotídeo; NADPH, nicotinamida adenina dinucleotídeo fosfato reduzido; NF-κB, fator nuclear kappa B; PD1; proteína 1 de morte celular; PDL1, ligante de morte celular programada 1; PI3K, fosfatidilinositol 3-quinase; Raptor, proteína regulatória associada a mTOR; RE, receptor de estrogênio; RNA, ácido ribonucleico; RP, receptor de progesterona; TN, triplo-negativo; ULK1; cinase 1 semelhante à unc-51.

ARTIGO CIENTÍFICO 1 – ‘4-NITROCHALCONE AS A POTENTIAL ANTIMETABOLIC DRUG IN NON-CLINICAL BREAST CANCER STUDIES’

4NC, 4-nitrochalcone; AA, ascorbic acid; AHA, azidohomoalanine; ALT, alanine transaminase; ANOVA, analysis of variance; AP, alkaline phosphatase; AST, aspartate aminotransferase; BAX, BCL2 associated X; Cat, catalase; cDNA, complementary DNA; CASP8, caspase 8; CDNB, 1-Chloro-2,4-dinitrobenzene; CEUA/BIO: ethics committee of animal experimentation/biological CM-H2DCFDA, chloromethyl-dichlorodihydrofluorescein diacetate; CQ, chloroquine; CV, central vein; DL_{50}, median lethal dose; DMEM, dulbecco’s modified eagle’s medium; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, nitrobenzoic acid; EC_{50}, half maximal effective concentration; EAC, Ehrlich ascites carcinoma; ELISA, enzyme linked immuno sorbent assay; ER, estrogen

receptor; ERα, estrogen receptor alpha; ETC, Ehrlich tumor cells; FBS, fetal bovine serum; FOX , ferrous oxidation - xylenol orange method; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GHS, globally harmonized classification system; GSH, reduced glutathione; GR, glutathione reductase; GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; HB4a, human breast cell line; HBSS, hank's balanced salt solution; H&E, hematoxylin and eosin; HER2; human epithelial receptor 2; *HPRT,* hypoxanthine phosphoribosyltransferase; KOT51, mouse mutant epidermal fibroblasts; LC3B, microtubule associated protein 1 light chain 3 beta; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MCF-7, human breast adenocarcinoma cell line; MDA-MD-231, human breast adenocarcinoma cell line; MRP1, multidrug resistance protein 1; mTOR, mammalian/mechanistic target of rapamycin; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX, methotrexate; NO_2, nitrogen dioxide; PCR, polymerase chain reaction; Phospho-S6K1, phosphorylated Ribosomal protein S6 kinase beta-1; PR, progesterone receptor; PT, Portal tract; PVDF, polyvinylidine difluoride; RAPA, rapamycin; Raptor, regulatory-associated protein of mTOR; RIPA, radioimmunoprecipitation assay buffer; RNA, ribonucleic acid; Rplp0, ribosomal protein lateral stalk subunit P0; ROS, reactive oxygen species; RPMI 1640, growth medium used in cell culture; S6K1, ribosomal protein S6 kinase beta-1; SEC, solid Ehrlich carcinoma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of mean; SI, selectivity index; SOD, superoxide dismutase; TNB, 5'-thio-2-nitrobenzoic acid; VEGF, vascular endothelial growth factor; Veh, vehicle; WST-1, water soluble tetrazolium salt 1; WT12, mouse wild type epidermal fibroblasts.

ARTIGO CIENTÍFICO 2 – ‘NUANCES OF PFKFB3 IN BREAST CANCER’

3PO, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; AKT, protein kinase B; APC/C-Cdh1, anaphase-promoting complex/cyclosome-cadherin 1; ATP, adenosine triphosphate; BRCA1, breast cancer gene 1; BRCT, BRCA1 C terminus; E2F1, E2F transcription factor 1; AMPK, adenosine monophosphate-activated protein kinase; EC, endothelial cell; E2, 17β-estradiol; EGR-1, early growth response 1; EGFR, epidermal growth factor receptor 1; ER, estrogen receptor; ERα, estrogen receptor

subtype α; ERE, estrogen response element; ERK, extracellular signal-regulated kinase; F6P, fructose-6-phosphate; F2,6BP, fructose-2,6-biphosphate; F2,6BPase, fructose-2,6-bisphosphatase; G6Pase, glucose-6-phosphatase; GLUT4, glucose transporter type 4; GPER, G protein-coupled estrogen receptor; HER2, human epidermal growth factor receptor type 2; HIF-1, hypoxia-inducible factor 1; HK, hexokinase; HRE, hormone-responsive element; HUVEC, human umbilical vein endothelial cell; IKKβ, inhibitor of nuclear factor κB kinase subunit β; iPFK-2, inducible PFK-2; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; miRNA, microRNA; MSK1, mitogen- and stress-activated protein kinase-1; mTOR, mammalian/mechanistic target of rapamycin; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation; P4, progesterone; p90RSK, 90 kDa ribosomal protein S6 kinase; PIM2, proviral insertion in murine lymphomas 2; PK, pyruvate kinase; PFK-1, 6-phosphofructo-1-kinase; PFK-2, phosphofructokinase 2; PFK15, 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one; PFK158, 1-(4-pyridinyl)-3-[7-(trifluoromethyl)-2E-quinolinyl]-2-propen-1-one; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PI3K, phosphatidylinositol 3-kinase; PKC, *protein kinase C*; PPP**,** pentose phosphate pathway; PR, progesterone receptor; PRB, PR isoform B; SHR, steroid hormone receptor; SQSTM1/p62, sequestosome 1; STAT, signal transducers and activators of transcription; SCF–β-TrCP, SKP1/CUL-1/F-box protein–β-transducin repeats-containing proteins; TIGAR, TP53 glycolysis and apoptosis regulator; TNBC, triple negative breast cancer; uPFK-2, ubiquitous PFK-2; VEGF, vascular endothelial growth *factor*; V_{max}, maximum velocity.

SUMÁRIO

1 INTRODUÇÃO

1.1 SITUAÇÃO ATUAL DE CÂNCER E DO CÂNCER DE MAMA

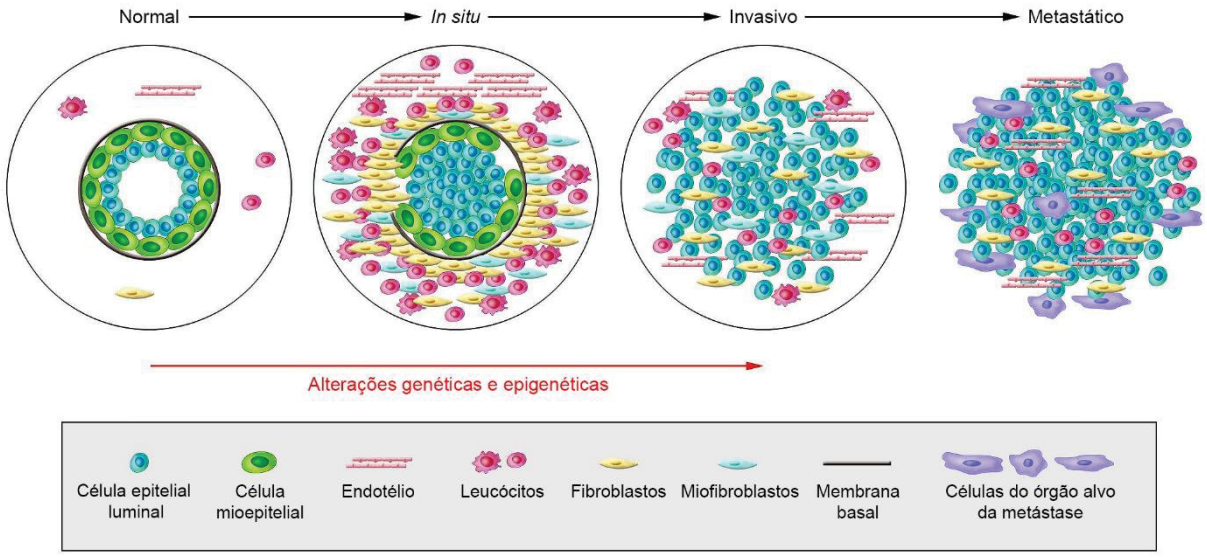
Ao contrário do ocorrido em grande parte da história humana, as doenças não comunicáveis (DNC) converteram-se na principal causa de morte no mundo (WHO, 2021a). Estas doenças incluem disfunções cardíacas, respiratórias, diabetes e câncer e são em geral de longa duração e natureza complexa, resultantes de múltiplos fatores intrínsecos e extrínsecos ao indivíduo (WHO, 2021a). O câncer é a segunda maior causa de morte de DNC, com mais de 9 milhões de mortes ao ano e a primeira causa de morte nos países desenvolvidos (WILD; WEIDERPASS; STERWART, 2020). Com o aumento da expectativa de vida e crescimento da população, as projeções indicam expressivos aumentos nas taxas de morte e incidência do câncer, com cerca de 100.000 casos de câncer em 2040 apenas nos Estados Unidos (RAHIB et al., 2021).

Câncer é um termo genérico para denominar centenas de enfermidades que cursam com alterações genéticas e epigenéticas progressivas e acumulativas, levando à perda de controle na maquinaria celular responsável pela proliferação celular e homeostase (HANAHAN; WEINBERG, 2000; TAKESHIMA; USHIJIMA, 2019). Os derivados celulares malignos apresentam seis atributos básicos: autossuficiência em sinais de crescimento, insensibilidade a sinais inibidores de crescimento, evasão de morte celular programada, potencial replicativo ilimitado, angiogênese sustentada, e invasão e metástase de tecidos (HANAHAN; WEINBERG, 2000). Dois atributos adicionais foram descritos posteriormente: a reprogramação metabólica e a evasão da resposta imune (HANAHAN; WEINBERG, 2011). O resultado final é um circuito celular de crescimento ilimitado, que dá origem a inúmeras gerações celulares com potencial de colonizar múltiplos sítios no corpo.

O câncer de mama é atualmente a principal causa de morte por câncer na população feminina e o tipo de câncer mais comum no mundo (BRAY et al., 2018; WHO, 2021b). No Brasil, o câncer de mama segue a mesma tendência, com uma mortalidade de 18.068 em 2019 e 66.280 novos casos estimados para 2020 (INCA, 2021). Além do histórico familiar da doença, o risco de câncer de mama é aumentado por fatores reprodutivos e de estilo de vida como menarca precoce, nuliparidade ou idade posterior na primeira gravidez, uso de hormônios exógenos, sobrepeso e inatividade física (WILD; WEIDERPASS; STERWART, 2020).

O câncer de mama é uma doença multiforme, compreendendo vários tipos histológicos e subtipos moleculares, que tem impacto direto na terapêutica e resposta clínica (POLYAK, 2007). A expansão clonal a partir da célula mioepitelial (basal) ou luminal transformada desencadeia o crescimento de uma massa mamária anormal, que varia desde um carcinoma de mama *in situ* a carcinomas invasivos e metastáticos (Figura 1).

FIGURA 1. PROGRESSÃO DO CÂNCER DE MAMA



FONTE: Modificado de POLYAK (2007).

A partir dos estudos de perfil de expressão gênica de PEROU et al., (2000) e SØRLIE et al. (2001) foi possível identificar subtipos de câncer de mama que preveem com maior acurácia o comportamento da neoplasia que os parâmetros clínicos e morfológicos até então utilizados, além de delinear potenciais alvos terapêuticos e indicadores de prognóstico. O câncer de mama passou a ser classificado de acordo com a expressão do receptor de estrogênio (RE), receptor de progesterona (RP) e receptor do fator de crescimento epidérmico humano 2 (HER2) em pelo menos 4 subtipos distintos: luminal A ($RE^+/RP^+/HER2^-$), luminal B ($RE^+/RP^{+/-}/HER2^{+/-}$), superexpressão de HER2 ($RE^-/RP^-/HER2^+$) e triplo-negativo (TN; $RE^-/RP^-/HER2^-$) (SØRLIE, 2004).

A assinatura molecular dos tumores é avaliada na rotina clínica por imunohistoquímica (IHC), juntamente com outros marcadores importantes como o Ki67 (proliferação celular), e são preditores independentes de morte por câncer de

mama (JOHANSSON et al., 2021). Os tumores do tipo luminal representam 60-70% dos casos de câncer de mama, seguidos dos com superexpressão de HER2 (15–20%) e TN (10–20%); o prognostico é bom a intermediário para os tipos luminais, enquanto que para os demais o prognostico é pouco favorável (NASCIMENTO; OTONI, 2020). A terapia do câncer de mama envolve uma estratégia multimodal com uma combinação de quimioterapia neoadjuvante, cirurgia, radioterapia, quimioterapia adjuvante e/ou endócrina e combinações de agentes quimioterápicos com terapia hormonal ou imunoterapia (FISUSI; AKALA, 2019).

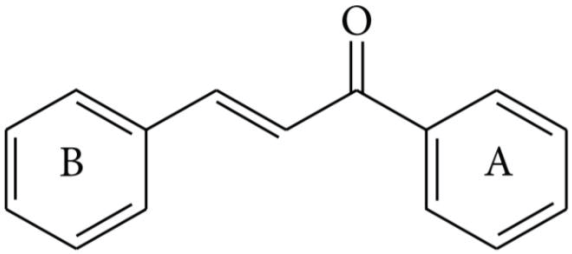
Os fármacos antitumorais visam erradicar o tumor ou, pelo menos, reduzir o tamanho tumoral. Tais agentes interferem em funções fundamentais para o crescimento e sobrevivência celular, como os agentes alquilantes que se ligam a várias macromoléculas celulares, especialmente o DNA, impedindo a divisão celular; os inibidores de microtúbulos, que perturbam a síntese e a degradação do citoesqueleto celular; e os antimetabólicos, que inibem etapas-chave na síntese de DNA e RNA (NYGREN, 2001). Apesar do alvo destes fármacos ser classicamente mais ativo em células tumorais, a falta de seletividade é um grande problema, assim como a resistência ao tratamento (CHABNER; ROBERTS, 2005). Assim, a necessidade de terapias anticâncer é emergente e o mercado para o desenvolvimento de novos fármacos contra o câncer de mama está em expansão, com destaque para os 5 principais alvos: terapia alvo para HER2, inibidores de quinase 4/6 dependente de ciclina (CDK4/6), agentes hormonais, inibidores da proteína 1 de morte celular (PD1)/ ligante de morte celular programada 1 PDL1, agentes citotóxicos e inibidores da fosfatidilinositol 3-quinase (PI3K)/proteína quinase B(AKT)/ alvo mecanístico da rapamicina (mTOR) (WILCOCK; WEBSTER, 2021).

1.2 CHALCONAS COMO ALTERNATIVAS TERAPÊUTICAS PARA O CÂNCER

O termo chalcona tem origem do grego “*chalcos*” (bronze), devido à tonalidade amarelada destes compostos. Trata-se de precursores na biossíntese de flavonoides e isoflavonoides, classificados como flavonoides menores (BOHM, 1975; NINOMIYA; KOKETSU, 2013). As chalconas (1,3-diaril-2-propen-1-ona) apresentam uma estrutura química de cadeia aberta, composta por dois anéis aromáticos (A e B) interligados por um sistema carbonil α,β-insaturado de três carbonos (NINOMIYA;

KOKETSU, 2013). As chalconas naturais estão presentes em chás, frutas, vegetais e alimentos à base de soja e são hidroxiladas em algum grau (BOHM, 1975; NOWAKOWSKA, 2007). A estrutura geral das chalconas pode ser observada na Figura 2.

FIGURA 2. ESTRUTURA QUÍMICA GERAL DAS CHALCONAS



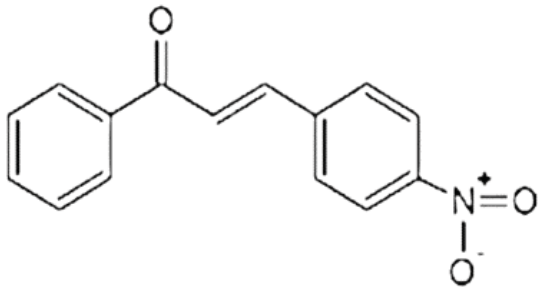
FONTE: DAS; MANNA (2016).

Chalconas naturais têm várias atividades biológicas *in vitro* e *in vivo* incluindo antifilarial, larvicida, antiprotozoária antimalárica, antibacteriana, antiviral, anti-HIV, antilipidêmica, antihiperglicêmica, antiplaquetária, anticonvulsivante, neuroprotetora, antiangiogênica, antioxidante, anticâncer e anti-inflamatória (BANOTH; THATIKONDA, 2020). Ademais, a estrutura das chalconas (C6-C3-C6) é uma matriz privilegiada e flexível para a síntese de compostos derivados, aumentando o arsenal de possibilidades de uso destes compostos (DÍAZ-TIELAS et al., 2016).

No campo das pesquisas anticâncer, as chalconas naturais e sintéticas mostraram ótimos efeitos devido ao seu potencial inibitório sobre: transportadores de resistência a múltiplas drogas (MRP1, MDR1 e BCRP), vias de hormônios sexuais, desacetilação de proteínas, degradação de p53, angiogênese, tubulina, topoisomerase, proteínas quinases e sobre as vias de sinalização Janus quinase (JAK)/ transdutor de sinal e ativador de transcrição (STAT), Wnt, fator nuclear kappa B (NF-κB) e mTOR (MAHAPATRA; BHARTI; ASATI, 2015). Estas ações estão de acordo com a abordagem das novas gerações de fármacos anticâncer, baseados na modulação de vias de crescimento, receptores e transdução de sinais, ou que regulam o ciclo celular ao invés de objetivar o dano citotóxico ou ao DNA de forma direta (DAS; MANNA, 2016). Recentemente, a aplicação das chalconas tem despontado nas pesquisas de câncer de mama (Ruparelia et al., 2018; Muchtaridi et al., 2019; Lim et al., 2020), como fármacos e pró-fármacos.

A 4-nitrochalcona (4NC) é uma chalcona sintética, com a adição do substituinte NO_2 na posição *para-* do anel B (Figura 3), relacionado ao aumento da potência e seletividade da chalcona (MAI et al., 2014). As propriedades anticâncer da 4NC foram demonstradas por vários autores *in vitro* (CARPIO ARÉVALO et al., 2019; CORDEIRO et al., 2021; DIMMOCK et al., 2002; DOS SANTOS et al., 2020; VIA et al., 2009), todavia no câncer de mama seu efeito biológico é ainda desconhecido.

FIGURA 3. ESTRUTURA QUÍMICA DA 4NC



FONTE: CARPIO ARÉVALO et al. (2019).

1.3 O METABOLISMO TUMORAL

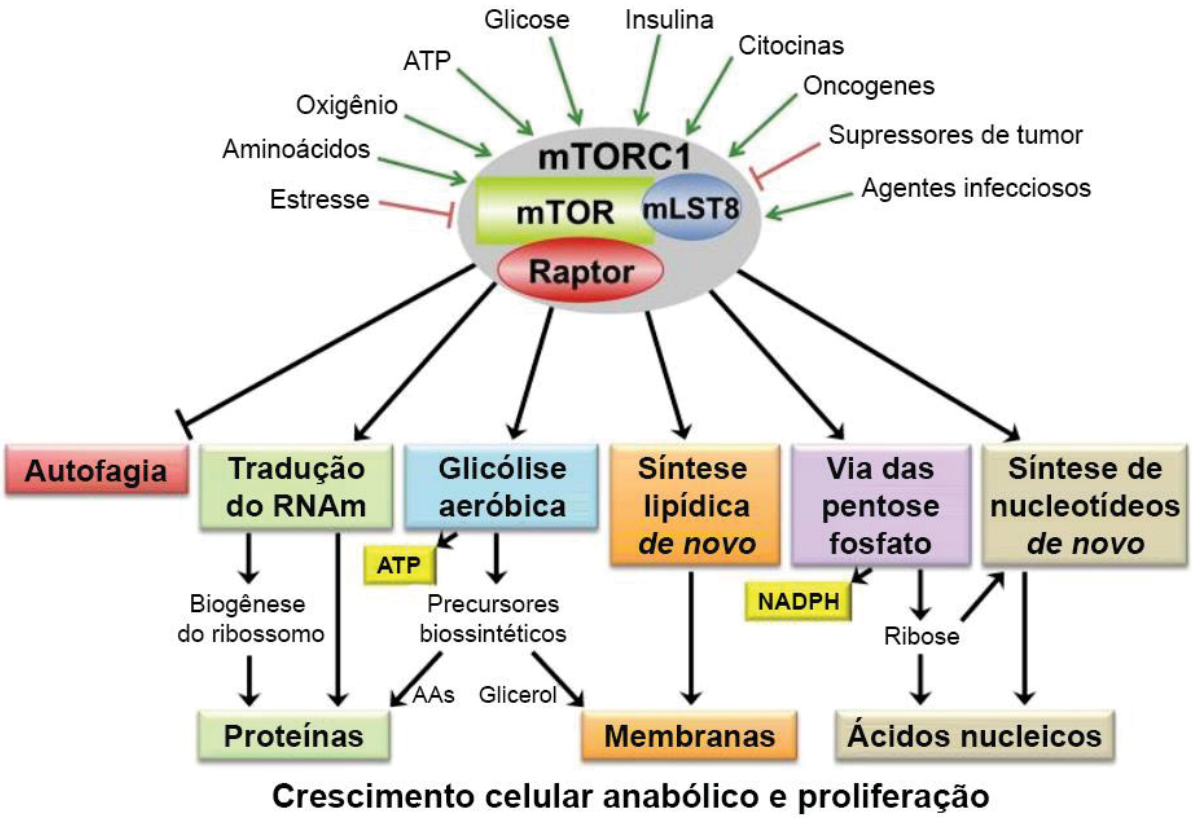
A reprogramação metabólica é uma dos oito atributos registrados do câncer (HANAHAN; WEINBERG, 2011), mencionados no item 1.1. Este termo é usado para descrever o incremento ou supressão de vias metabólicas em células tumorais decorrentes de mutações ou demais alterações relacionadas à transformação maligna (DE BERARDINIS; CHANDEL, 2016). A proliferação celular irrefreada é criticamente dependente da remodelação da capacidade de gerar energia, a fim de sustentar o crescimento e a divisão das células, que estão aumentados. De uma forma simplificada, o catabolismo completo da glicose é dependente de 3 vias celulares: a glicólise, o ciclo do ácido tricarboxílico (CAT) e a fosforilação oxidativa (FO) (PARKER, 2020). Inicialmente, a glicose é processada a piruvato pela glicólise e, na presença de oxigênio, é convertido a acetil-CoA, a qual segue para a mitocôndria. A acetil-CoA é, por sua vez, processada a oxaloacetato pelo CAT, reduzindo os aceitadores de elétrons nicotinamida adenina dinucleotídeo (NAD) e dinucleotídeo de flavina e adenina (FADH), os quais são direcionados para a FO para produzir 32 - 38 moléculas de trifosfato de adenosina (ATP)/ mol de glicose

(FADAKA et al., 2017). Em condições de hipóxia, a glicólise é a principal fonte energética da célula, a partir da conversão do piruvato a lactato, com o modesto rendimento de 4 moléculas de ATP/ mol de glicose (FADAKA et al., 2017). Embora contraintuitivo, a célula tumoral direciona boa parte de seu metabolismo para a glicólise, mesmo na presença de oxigênio, processo descrito por Otto Warburg e conhecido como efeito Warburg ou glicólise aeróbia (WARBURG, 1925). A subordinação das células tumorais à glicólise é um evento observado nos cânceres hematológicos e sólidos, mas é melhor compreendido nestes últimos, devido ao seu frequente aporte irregular de oxigênio (HANAHAN; WEINBERG, 2011); porém, a principal vantagem deste desvio metabólico é a notável habilidade da glicólise em produzir intermediários metabólicos, que são combustível para várias vias biossintéticas subsidiárias, como a via das pentoses fosfato e a síntese de lipídeos (DE BERARDINIS; CHANDEL, 2016).

O programa anabólico das células tumorais é impulsionado pela perda de supressores tumorais, como é o caso do p53 (LIU et al., 2019) e também pela ativação de oncogenes, como o MYC (DONG et al., 2020). Outrossim, vias de sinalização intracelular podem ser potentes indutores das reações anabólicas, em especial a via PI3K/AKT/mTOR, que com frequência apresenta-se alterada em vários canceres, incluindo o câncer de mama (HOXHAJ; MANNING, 2020; LI et al., 2021).

A ativação de PI3K/AKT na membrana plasmática estimula diretamente enzimas metabólicas e transportadores de nutrientes e ativa importantes efetores a jusante como a via mTOR. O complexo mTOR contém duas subunidades, o complexo mTOR 1 (mTORC1) e mTOR 2 (mTORC2). O mTORC1 é responsável por mediar a conexão de sinais de crescimento a montante, como a ligação de fatores de crescimento e a disponibilidade de nutrientes, com a maquinaria responsável pela biogênese e crescimento celular (LAPLANTE; SABATINI, 2012), como representado na figura 4, sendo um regulador chave de vias metabólicas.

FIGURA 4. REGULAÇÃO DO ANABOLISMO CELULAR VIA mTORC1

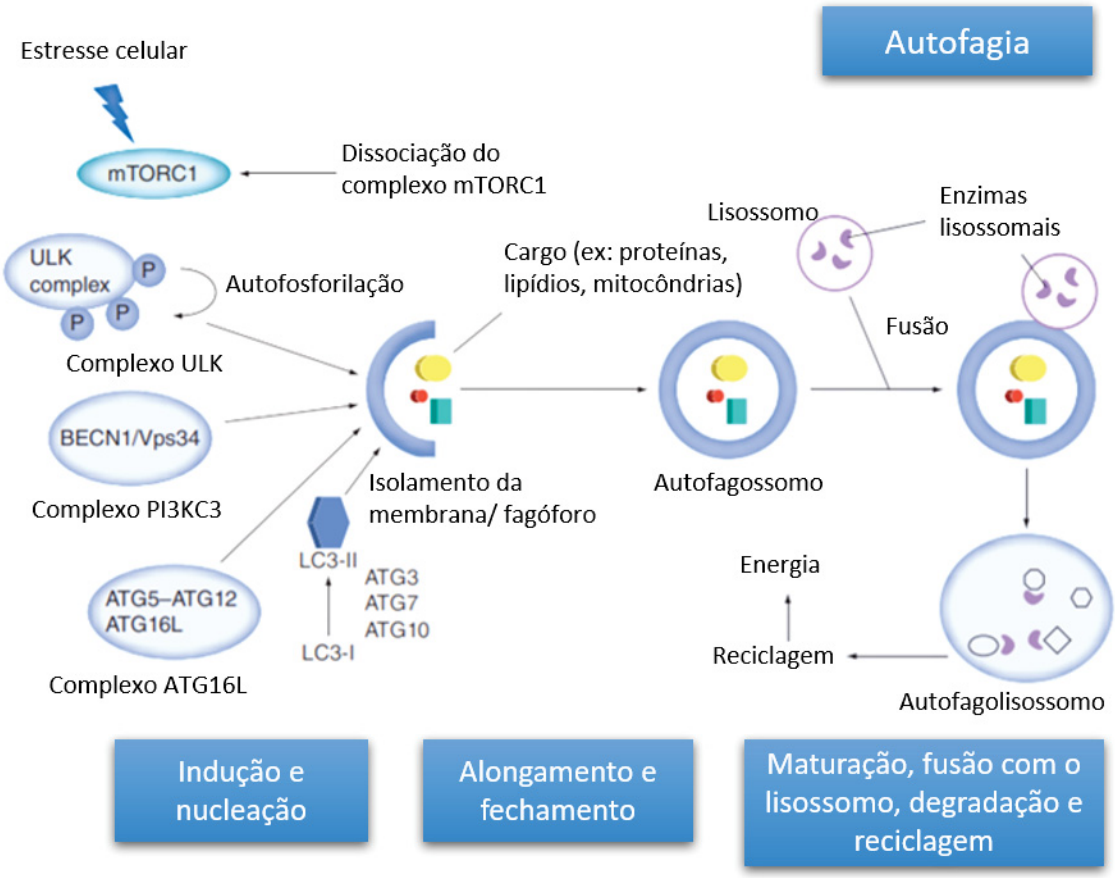


FONTE: Modificado de DIBBLE; MANNING (2013). Legenda: AAs, aminoácidos; ATP, trifosfato de adenosina; mLST8, alvo da subunidade LST8 do complexo de rapamicina; mTOR, alvo mecanístico da rapamicina; mTORC1, complexo 1 do alvo mecanístico da rapamicina; NADPH, nicotinamida adenina dinucleotídeo fosfato reduzido; Raptor, proteína regulatória associada a mTOR.

Ao contrário, em condições de limitação de nutrientes, a célula ativa um programa de catabolismo para garantir sua sobrevivência. Neste caso é um processo oposto à função de mTORC1 e diretamente regulado por ele: a autofagia, também referida como macroautofagia (DE BERARDINIS; CHANDEL, 2016).

A autofagia é uma resposta celular altamente conservada, baseada em um processo de degradação lisossomal bem regulado de proteínas mal dobradas, organelas danificadas e microorganismos invasores; entretanto, a autofagia pode ser ativada para garantir a sobrevivência celular em períodos de privação energética, pela reciclagem de conteúdo intracitoplasmático para fornecer nutrientes (Figura 5) ou mesmo atuar como uma ferramenta de morte celular, neste caso uma morte celular programada do tipo II (LEVINE; KLIONSKY, 2004).

FIGURA 5. VIA AUTOFÁGICA



FONTE: Modificado de GIL; PESZ; SĄSIADEK (2016). Legenda: ATG, gene relacionado à autofagia; PI3KC3, complexo da classe III PI3K; mTORC1, complexo 1 do alvo mecanístico da rapamicina; ULK, cinase semelhante à unc-51.

A autofagia consiste de uma maquinaria intrincada, com a participação de pelo menos 18 genes relacionados à autofagia (ATGs) (BEDNARCZYK et al., 2018). Na ocorrência de inibição de mTORC1 ou ativação da proteína quinase ativada por monofosfato de adenosina (AMPK), a cinase 1 semelhante à unc-51 (ULK1) torna-se ativada e inicia uma cascata de reações com a participação do complexo PI3K de classe III, com destaque para a proteína Beclina-1, havendo a formação de uma membrana lipídica dupla de isolamento, denominada fagóforo. Subsequentemente, há o recrutamento de várias proteínas responsáveis pelo alongamento e maturação do autofagossomo, como o complexo ATG5-ATG12/ATG16L e o sistema das proteínas associadas a microtúbulos 1A/1B de cadeia leve 3B (LC3)-fosfatidiletanolamina (LC3-II). O LC3-II é incorporado na membrana interna e externa do autofagossomo e com o auxílio do adaptador p62 faz a ligação com o conteúdo a ser degradado na superfície interna. O autofagossomo assim formado funde-se ao

lisossomo e ocorre a degradação dos substratos sequestrados (LEVINE; KROEMER, 2008). Deste modo, LC3-II e p62 podem ser utilizados para o monitoramento do fluxo autofágico (MIZUSHIMA; YOSHIMORI, 2007).

No câncer a autofagia apresenta um papel dualístico, a depender da fase: ela é considerada um supressor tumoral nas fases de iniciação e promoção da carcinogênese, pois protege as células do estresse e degrada os componentes danificados; mas quando superativada em um câncer já estabelecido, ela é uma ferramenta de plasticidade metabólica, promovendo a persistência tumoral (YUN; LEE, 2018). A autofagia é um alvo terapêutico promissor no tratamento do câncer em estudos pré-clínicos e clínicos, principalmente através do viés farmacológico inibitório (LEVY; TOWERS; THORBURN, 2017; LIM; MURTHY, 2020).

1.4 HIPÓTESE

A partir do imenso repertório de atividades biológicas das chalconas e a perspectiva de seu uso como droga anticâncer descritos na literatura, tem-se a hipótese de que a 4-nitrochalcona (4NC) apresenta atividade antitumoral no câncer de mama. Para testar esta hipótese foram utilizados modelos in *vitro* e *in vivo* de tumor mamário, a fim de determinar os mecanismos moleculares subjacentes a esta ação. O presente estudo pré-clínico pode nortear pesquisas futuras na área e estabelecer a 4NC como uma potencial alternativa na terapêutica do câncer de mama.

2 OBJETIVOS

2.1.1 Objetivo geral

Avaliar a atividade antitumoral da 4NC em modelo *in vitro* (linhagens de células mamárias) e em modelo singênico *in vivo* (carcinoma sólido de Ehrlich) e determinar os possíveis mecanismos de ação envolvidos, com ênfase em vias metabólicas tumorais.

2.1.2 Objetivos específicos

- Avaliar o efeito antiproliferativo da 4NC *in vitro* em células mamárias não-transformadas (HB4a) e transformadas (MCF-7 e MDA-MB-231);
- Investigar os possíveis mecanismos de ação antitumoral *in vitro* da 4NC na linhagem MCF-7 em relação a:
    - Marcadores de estresse oxidativo: GSH, ROS e citotoxicidade diferencial em relação à presença do transportador de efluxo MRP1;
    - Marcadores de morte celular: expressão gênica de *BAX*, *CASPASE 8*, *VEGF*, *LC3B* e *Raptor* e expressão proteica de LC3-II, p53, p62, Beclina-1 e S6K1 total e fosforilado;
    - Quantificação da síntese de proteínas nascentes em resposta ao tratamento com a 4NC;
    - Razão lactato/piruvato excretado no meio celular em resposta ao tratamento com a 4NC;
    - Expressão gênica da enzima *PFKFB3* em resposta ao tratamento com a 4NC;
- Avaliar a DL_{50} da 4NC frente ao teste de toxicidade aguda em camundongos;
- Avaliar o potencial antitumoral da 4NC no modelo tumoral de Ehrlich, a partir do acompanhamento do volume e peso tumoral;
- Investigar os efeitos gerais da 4NC no organismo animal através de análises hematológicas e bioquímicas;
- Investigar os possíveis mecanismos de ação antitumoral *in vivo* da 4NC em relação a:
    - Marcadores de estresse oxidativo tumoral e hepático: SOD, Cat, GST, GSH, LPO e ROS;
    - Marcadores morfológicos do tumor: análise histopatológica;
    - Marcadores de morte celular: expressão gênica de *Bax*, *Caspase 8*, *Vegf* e *Lc3b* e expressão proteica de LC3-II;
- Fazer um compilado da literatura sobre um marcador emergente de metabolismo tumoral (PFKFB3) no contexto do câncer de mama.

3 ARTIGO CIENTÍFICO 1 – '4-NITROCHALCONE AS A POTENTIAL ANTIMETABOLIC DRUG IN NON-CLINICAL BREAST CANCER STUDIES'

A ser submetido.

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3.1 ABSTRACT

Breast cancer is a high-magnitude public health problem, continually challenging physicians and scientists worldwide in the field of drug therapy. 4-nitrochalcone (4NC) is a phenolic compound that has promising antitumor activity *in vitro*, but its application in breast cancer treatment is still poorly explored. This study aimed to evaluate the action of 4NC in *in vitro* and *in vivo* breast cancer models. The cytotoxic potential of 4NC was tested towards MCF-7 and MDA-MD-231 breast cancer cells, with a lower impact in the non-tumor lineage HB4a. For *in vivo* studies, solid Ehrlich carcinoma (SEC) was used, a syngeneic mouse model with non-nuclear estrogen and progesterone positivity, characterized by immunohistochemistry. Daily oral administration of 4NC (25 mg kg^{-1}) for 21 days led to a consistent reduction in tumor growth compared to the vehicle group. No signs of toxicity evaluated by hematological, biochemical, histological, and oxidative stress parameters were observed in mice, and the DL_{50} was >2000 mg kg^{-1}. The molecular mechanisms underlying the action of 4NC are related to transcriptional and post-transcriptional attenuation of mTOR and autophagy pathway. The effectors Raptor and S6K1 showed decreased activation, with a consequent reduction in protein synthesis; concomitantly, there was an increase in LC3-II levels, but the autophagic response was not completed, with the maintenance of p62 levels. These results open new possibilities for the use of 4NC as a tumor cell metabolism modulating agent.

Keywords: 4-nitrochalcone; Breast cancer, Solid Ehrlich Carcinoma; MCF-7 cell; mTOR pathway; Autophagy.

3.2 INTRODUCTION

Cancer is one of the greatest challenges to human longevity (TØRRING, 2017). Despite advances in life expectancy in recent years, especially in developed countries, cancer mortality is expressive and has decreased very slowly with protective and curative measures (CAO et al., 2017). Breast cancer is the most diagnosed type of cancer in the world and the leading cause of cancer death in the female population (BRAY et al., 2018; WHO, 2021b). Intertumoral and intratumoral heterogeneity is an important feature of breast cancer, which reflects differences in

clinical manifestation and response to treatment (TURASHVILI; BROGI, 2017). Various pharmacological therapies including chemotherapy, targeted therapy, and endocrine therapy are available for the treatment of breast cancer at the systemic level, but the unfeasibility of single therapy and acquired drug resistance is constantly moving towards the need for new drugs and co-therapy protocols (DONG et al., 2021; HARBECK et al., 2019).

Chalcones are privileged compounds in medicinal chemistry. These molecules, natural or by synthetic origin, have an α,β-unsaturated ketone system, also known as chalconoid (ZHUANG et al., 2017). Chalcones exhibit anti-proliferative, chemopreventive, anti-inflammatory, anti-angiogenic, and antioxidant properties that configure precious characteristics in anti-cancer therapy (DAS; MANNA, 2016; GO; WU; LIU, 2005; OUYANG et al., 2021). Recently, the antitumor activities of chalcones and their derivatives have been highlighted through the modulation of the mTOR pathway (ABBAS et al., 2019; JIN et al., 2019; MATEEVA et al., 2017; WANI et al., 2016). There is crescent interest in this pathway, which is responsible for cancer cell growth by regulating anabolic metabolism, immune response, cytoskeleton remodeling, therapy resistance, and tumor recurrence (MAGAWAY; KIM; JACINTO, 2019; MOSSMANN; PARK; HALL, 2018). mTOR is a vital fueling pathway for the Warburg effect and its modulation opens possibilities in the vast field of tumor metabolic vulnerability (SUN et al., 2011). Moreover, mTOR interfaces with cell growth and autophagy, promoting cancer persistence even under nutrient deprivation (JUNG et al., 2010). Autophagy is also a powerful tool in favor of the metabolic reprogramming process, as opposed to the mTOR pathway, but it is complementary in terms of providing resilience and plasticity to tumor cells (CHAVEZ-DOMINGUEZ et al., 2020).

Since chalcones are a large group of chemical compounds, the mechanisms of action of many of them are not known, including 4NC. The present study investigated the effect and the mechanism of action of 4NC in breast cancer tumor using human breast cancer cells MCF-7, MDA-MB-231, and HB4a for the *in vitro* experiments, and Ehrlich tumor cells (ETC) for the *in vivo* investigations on mice.

3.3 MATERIAL AND METHODS

Chemicals and drugs. Bovine serum albumin (BSA), dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), glutathione reductase, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), 4-Nitrochalcone (4NC, molecular weight: 253.257 g/mol), K_2HPO^4, KH_2PO^4, RPMI1640, tris(hydroxymethyl)aminomethane, sodium nitrite, and xylenol orange were acquired from Sigma-Aldrich (Missouri, USA). Absolute ethanol, aluminum potassium, ascorbic acid, acetic acid, 1-Chloro-2,4-dinitrobenzene (CDNB), citric acid, dibasic potassium phosphate, dibasic sodium phosphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), eosin, ferrous ammonium sulfate, formaldehyde, hematoxylin, hydrogen peroxide, methanol, monobasic potassium phosphate, potassium chloride, pyrogallol, sodium acetate, sodium chloride, sodium phosphate, trichloroacetic acid, Triton X-100, Trypan blue, Tween 20, xylene were purchased from Vetec (Rio de Janeiro, Brazil). Alanine transaminase (ALT), alkaline phosphatase (AP), Aspartate (AST), and urea kits were acquired from Kovalent (São Paulo, Brazil). Chloromethyl-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), Hank's Balanced Salt Solution (HBSS), High Capacity cDNA Reverse Transcription Kit, Pierce BCA Protein Assay Kit, primers, SYBR Green PCR Master Mix and TriZol were purchased from ThermoFisher (Massachusetts, USA). The Bradford Protein Assay was obtained from Bio-Rad Laboratories (California, USA). ketamine was purchased from Vetnil Industry (São Paulo, Brazil), xylazine was purchased from Syntec (São Paulo, Brazil). Rapamycin was purchased from Selleck chemicals (Texas, USA). Chloroquine sulfate was purchased from Sigma (Sigma-Aldrich, Missouri, USA).

Cell culture. Immortalized human normal breast cell HB4a and human breast cancer cells MCF-7 and MDA-MB-231 were kindly provided by Ludwig Institute for cancer research (São Paulo, Brazil). Regarding breast cancer subtyping based on estrogen receptor (ER), progesterone receptor (PR), and human epithelial receptor 2 (HER2) status, MCF-7 is considered luminal A subtype (ER+/PR+/HER2−) and MDA-MB-231 a triple-negative subtype (ER−/PR−/HER2−) (DAI et al., 2017). The cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine and were kept in an incubator with proper humidity (85-95%), temperature (37°C) and gas tension (5% CO2).

Immortalized mouse epidermal fibroblasts WT12 and mouse mutant epidermal fibroblasts KOT51 (mdr1 a-/-/b-/-/mrp1-/-) transfected with human MRP1 cDNA were kindly provided by Academic Medical Center (Amsterdam, The Netherlands). Mouse fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine under the same conditions described above. Vehicle (Veh) and treated groups received 0.1% of DMSO. All experiments were performed with cells from passage 3 to 5.

Methyl Thiazolyl Tetrazolium (MTT) assay. Breast cells were plated in 96-well plates (5,000 cells/well) and 24 h after exposed to Veh (0.1% DMSO) and 4NC (5, 10, 15, 20, 25, 30 and 40 μM) for 24 h. Inside the intact cells, MTT was reduced to a purple product, formazan, that was diluted in DMSO and then quantified in a spectrophotometer at 570 nm (Synergy HT, Biotek, Vermont, USA). The cell viability was represented as a percentage (%) in comparison with vehicle. Based on the dose-response relationship, the half-maximal effective concentration (EC50) was calculated using GraphPad Prism (v. 5.0). The Selectivity index (SI) was calculated adapting the formula described by INDRAYANTO; PUTRA; SUHUD (2021) $SI = EC_{50}\ non\_transformed\ cell\ line / EC_{50}\ tumoral\ cell\ line$, which values ≥3 were considered as highly selective (WEERAPREEYAKUL et al., 2012).

Clonogenic assay. MDA-MB-231 and MCF-7 cells were plated in 6-well plates (50 - 200 cells/well) and 24 h after were exposed to Veh (0,1% DMSO) or 4NC (1 μM) for 24 h. The next day the medium was replaced with fresh medium and sequentially replaced every 2 days for 14 days, when the colonies were stained with Crystal violet and counted (FRANKEN et al., 2006).

Crystal violet staining. MCF-7 cells were plated in 24-well plates (50,000 cells/well) and 24 h after exposed to Veh (0.1% DMSO) or 4NC (5, 10, 15, 20, 25, 30 and 40 μM) for more 24 h. At the end of the treatment, the wells were washed with PBS to remove detached and dead cells and the viable cells were stained with crystal violet. The cells were lysed with SDS and the absorbance was quantified in a

spectrophotometer (Synergy HT, Biotek, Vermont, USA) (FEOKTISTOVA; GESERICK; LEVERKUS, 2016).

LDH assay. MCF-7 cells were plated in 96-well plates (5,000 cells/well) and 24 h after exposed to Veh (0.1% DMSO) or 4NC (5, 10, 15, 20, 25, 30 and 40 µM) for 4 h in HBSS medium. The supernatant was collected and mixed into a solution with Tris-HCL and NADH. Triton X-100 (0.1%) was used as a positive control. LDH activity was measured by adding sodium pyruvate, which leads to NADH oxidation and decreasing in absorbance, quantified in CLARIOstar® (BMG LabTech, Cary, USA) (KENDIG; TARLOFF, 2007).

Scavenging effect of 4NC in a cell-free system. The antioxidant property of 4NC on 2,2-diphenyl-1-picrylhydrazyl (DPPH) followed the method suggested by BLOIS (1958) and CHEN; WU; CHEN (2004). Serial dilutions of 4NC (1, 3, 10, 30, 100, and 300 µg mL^{-1}) in distilled water with 0.1% Tween 20 (Veh) were performed and added to react with DPPH in methanolic solution (10 µg mL^{-1}). Then, the absorbance of the samples was evaluated at 517 nm in the UV spectrophotometer (Synergy HT, Biotek, Vermont, USA) and compared with negative control (distilled water with 0.1% Tween 20) and positive control (solution of 50 µg mL^{-1} of ascorbic acid).

WST-1 assay. The non-transformed cells WT12 and KOT51 were plated in 96-well plates (5,000 cells/well) and 24 h after exposed to Veh (0.1% DMSO) or 4NC (5, 10, 15, 20, 25, 30 and 40 µM) for 24 h. Reactions were processed according to manufacturer's protocol (CELLPRO-Roche, Mannheim, Germany).The colored product was measured by spectrophotometer at 450 and 690 nm (Synergy HT, Biotek, Vermont, USA) and the viability was represented as a percentage (%) in comparison with vehicle.

GSH content *in vitro*. The levels of GSH were assessed in MCF-7, WT12, and KOT51 cells. 0.5 x 10^6 cells were seeded in 6 wells plate and kept to adhere for 24 h. The next day, cells were incubated with vehicle (0.1% DMSO) or 4NC (40 µM) for 4 h. Subsequently, the supernatant was discarded and cells were scrapped with a 10% perchloric acid solution and treated with a potassium phosphate solution (3 M)

for subsequent freezing at -20ºC. The content of GSH in the samples was measured by the reduction of DTNB in TNB (5'-thio-2-nitrobenzoic acid), followed by the addition of glutathione reductase (GR) in the system, according to Tietze enzyme recycling assay (TIETZE, 1969). The absorbance of TNB was measured at 412 nm on the CLARIOstar® Plus Multi-mode Microplate Reader (BMG LabTech, Cary, USA).

ROS measurement *in vitro*. MCF-7 cells were seeded in 96-wells plates and cultivated until reaching 70-80% confluence. Cells were washed with Hank's Balanced Salt Solution (HBSS) without phenol red and were resuspended with a fresh solution of HBSS containing 5 µM of chloromethyl-dichlorodihydrofluorescein diacetate (CM-H2DCFDA – ThermoFisher, Massachusetts, USA). The system was allowed to react for 30 minutes, then the reagent was removed and Veh (0.1% DMSO) or 4NC (5, 10, 15, 20, 25, 30 and 40 µM) were added to measure the fluorescence within 1 hour at 488/20, 520/20 nm on CLARIOstar® (BMG LabTech, Cary, USA). The oxidative activity was stimulated with a solution of 10 µM H_2O_2 (positive control) and the results blanked by unstained cells (autofluorescence).

Animals. The *in vivo* experiments were performed with female Swiss mice (*Mus musculus*), between 8 and 12 weeks old, with approval of the Committee on Ethics in the Use of Animals (CEUA/BIO – UFPR, nº 1063). Animals were housed in a vivarium with controlled conditions (20±2°C and 12:12h dark/light cycle) and randomly confined into plastic cages (n=7-9/group), with an enriched environment. Animals had free access to water and conventional laboratory feed (Nuvilab, Nuvital S.A., Colombo – PR, Brazil).

Acute oral toxicity test. The chemical properties of 4NC (https://pubchem.ncbi.nlm.nih.gov/compound/4-Nitrochalcone#section=Computed-Properties) were evaluated by Lipinski's rule of five (LIPINSKI et al., 2001). The 4NC was considered a potential oral drug and followed the acute oral toxicity testing (OECD, 2001). The initial dose started at 5 mg kg^{-1} and increased to 50, 300, and 2,000 mg kg^{-1} of 4NC. For this purpose, 12 female mice were used, being three animals for each dose. Prior to the experiment, the animals were fasted for 3-4 h and kept fasting for more than 2 hours after the administration of 4NC. The animals were

observed individually during the first 4 hours and afterward daily for 14 days. The observations included changes in behavior pattern and physical conditions. Whether necessary, animals in pain or sick could be euthanized. Individual animal data were provided and weights were determined every two days. On day 15, animals were anesthetized (ketamine hydrochloride 80 mg kg^{-1} and xylazine 10 mg kg^{-1}) and killed according to welfare rules. The animals were subjected to necropsy and samples of lung, liver, kidney, and brain were collected for routine histological processing and microscopic analysis.

4NC biological activity *in vivo* – Ehrlich carcinoma model in mice. ETC are a hyperdiploid breast cancer cell line characterized as ER+ (ÖZCAN ARICAN; ÖZALPAN, 2007; ZOHNY; KHALIL; EL-SHISHTAWY, 2019) and with non-progesterone responsive growth (PAVELIĆ et al., 1983). At first, aliquots of ETC were subcultivated into the peritoneal cavity (2 $x10^6$ cells/mouse) to form the Ehrlich Ascites Carcinoma (EAC) and weekly transplanted to new mice (n=2), a process knew as passages. Amongst each passage, the viability of the cells was assessed by the Trypan blue exclusion assay. When the viability of EAC was higher than 98%, usually after 3 or 4 passages, it was possible to induce SEC by inoculation of 2 $x10^6$ cells into the subcutaneous area of the right pelvic limb of the mouse. Cells were inoculated on day 0 and treatment started on day 1, lasting until day 21. A vehicle solution (distilled water and 0.1% Tween 20) was used to prepare all the treatments. Four experimental groups were selected: Naive group (no tumor) and the negative control group (Vehicle), both treated with vehicle solution (10 mL kg^{-1}); the 4NC group, treated with 25 mg kg^{-1} of 4NC; in all these groups the treatments were administered by daily oral gavage (p.o.); and the positive control group (MTX), treated by intraperitoneal injection (i.p.) with 2.5 mg kg^{-1} of the cytotoxic drug methotrexate (ABDEL-RAHMAN; KABEL, 2012) on days 1, 5, 9, 13, 17 and 21. The animals were monitored for body weight throughout the experiment and for tumor size from day 7, measured with a caliper, every 2 days. The estimation of tumor volume was performed with the formula: $V(cm^3) = L\,x\,W^2 x\,0.52$ (MISHRA et al., 2018), where *L* and *W* denote the largest and smallest diameter of tumor, respectively. Following the last day of treatment, the animals fasted for 12 – 16 h and were anesthetized with 80 mg kg^{-1} ketamine hydrochloride and 10 mg kg^{-1} xylazine i.p. Blood samples were collected from inferior cava vein just before the euthanasia

by diaphragm perforation, under anesthesia. Tumor and organs were harvested, weighted and prepared for subsequent analysis.

Histopathology and immunohistochemistry. Fragments of tumor and organs (liver, kidneys, and lung) collected from mice were processed according to histological routine techniques: fixation in 10% buffered formaldehyde, dehydration in ethanol, clearing in xylene, embedding in paraffin, sectioning, and staining with hematoxylin and eosin (H&E), followed by blind optical microscopy analysis. The histological architecture of each tissue was examined (normal/distorted) and other important parameters such as degeneration and cell death, inflammatory infiltration, vacuolation, and fibrosis were evaluated. The intensity of the histopathological findings were specified as negative (-), mild (+), moderate (++), or intense (+++), and the SEC lesions were classified from grade 0 to grade IV concerning the percentage of tissue affected: 0 (< 5%), I (5 - 25%), II (26 - 50%), III (51 - 75%) and IV (> 75%) (ALVES DE SOUZA et al., 2017). Additional tumor sections were selected for immunohistochemical detection of ER-alpha (ERα) and PR. The primary antibodies Dako ERα clone EP1 (Agilent Diagnostics, California, USA) and Dako PR clone PgR 636 (Agilents Diagnostics, Santa Clara, CA, USA) were used in the automated platform Dako Autostainer 48S (Agilent Diagnostics, California, USA).

Hematological and biochemical parameters. Hematocrit, hemoglobin concentration and leukocyte distribution analysis were performed by the automatic hematology analyzer BC2800-Vet (Mindray, Shenzhen, China). The plasma samples were tested by clinical chemistry analyzer BS-200 (Mindray, Shenzhen, China) for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), albumin, globulin, total protein, creatinine, and urea.

***In vivo* oxidative stress status**. Samples of liver and tumor were homogenized in potassium phosphate buffer (0,1M, pH 6.5) and the resulting homogenate was used to evaluate reduced glutathione (GSH) by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) technique (SEDLAK; LINDSAY, 1968). The supernatant (centrifuged at 21,000 × g for 20 min, 4ºC) was used to measure: catalase (Cat) activity, which is based on the consumption of exogenous hydrogen peroxide (H_2O_2)

as a substrate (AEBI, 1984); superoxide dismutase (SOD) activity, by pyrogallol (1,2,3-trihydroxybenzene) autoxidation method (GAO et al., 1998), glutathione-S-transferase (GST) activity by the conjugation of 1-cloro-2,4-nitrobenzeno (CDNB) with GSH, as described by HABIG; PABST; JAKOBY (1974) and lipid peroxidation (LPO) rate by FOX test (Ferrous Oxidation - Xylenol Orange Method), proposed by JIANG; WOOLLARD; WOLFF (1991). Protein levels were assessed by Bradford method (BRADFORD, 1976).

Gene expression in cells and solid tumor. Total RNA was isolated from breast cancer cells (6-wells plates with confluent MCF-7 cells treated for 24 h with 18 µM of 4NC) and fresh SEC fragments. In both cases, TRIzol reagent (Sigma-Aldrich, Missouri, USA) was used, as specified by the manufacturer's instructions. RNA integrity was assessed by electrophoresis in agarose gel and the RNA quantification was performed with Nanodrop-2000 spectrophotometer (ThermoFisher, Massachusetts, USA). cDNA synthesis was implemented from 2,000 ng RNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Massachusetts, USA) and the enzymatic amplification of DNA was performed using the StepOnePlus Real-Time PCR System with 1x SYBER Green PCR Master Mix Kit (ThermoFisher, Massachusetts, USA). The $2^{-\Delta\Delta CT}$ method was used to quantify the relative changes in gene expression (LIVAK; SCHMITTGEN, 2001), normalized with *HPRT* and *Rplp0* as endogenous reference genes. The targeted genes and the sequence of primers can be found in Supplementary Table S1.

Quantification of vascular endothelial growth factor (VEGF) secretion. MCF-7 cells were plated in 24-well plates (200,000 cells/well) and after attachment for 24 h, exposed to Veh (0.1% DMSO) or 4NC (18 µM) during 24 h. The supernatant was collected and stored at -80 ºC until further analysis with the Human VEGF DuoSet® ELISA kit (R&D Systems, Minnesota, USA). The procedures were done according to the manufacturer's specifications and the ELISA readings were performed on a spectrophotometer at 450/570 nm (Synergy HT, Biotek, Vermont, USA).

Immunoblotting of cells and solid tumor. Confluent breast cancer cells seeded in 6-wells plates were treated during 3 - 24 h with vehicle (0.1% DMSO), 4NC

(18 μM), and positive controls. The following positive controls were selected: chloroquine (100 μM) and rapamycin (50 nM) for autophagy and mTOR pathways, respectively. SEC fragments were kept at -80°C until the homogenization process. The proteins of all samples were extracted with RIPA buffer (NaCl 150 mM, nonidet P-40 1%, SDS 0.1%, and Tris-HCl pH 8.0), containing cOmplete™ Protease Inhibitor Cocktail (Hoffmann–La Roche, Basel, Switzerland). The protein content was measured by Pierce™ BCA Protein Assay Kit (ThermoFischer, Massachusetts, USA). Approximately 40 μg of protein of each sample was diluted in Laemmli sample buffer 4X (200 mM Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 0.02% bromophenol blue, and 5% β-mercaptoethanol) prior the loading in 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred by wet and semidry blotting to polyvinylidine difluoride (PVDF) membranes and the resultant blots were blocked overnight with 5% non-fat dry milk. The membranes were probed with primary antibodies for 2 h in the following dilutions: Anti-LC3B (L7543) - (1:500 – Sigma-Aldrich, Missouri, USA), Beclin-1 (1:1000 – Abcam), p62 (1:1000 - BD Biosciences, New Jersey, USA), S6K1 (1:1000 - Cell Signaling Technology, Massachusetts, USA), Phospho-S6K1 (Thr389) - (1:1000 - Cell Signaling Technology, Massachusetts, USA), GAPDH (1:1000 - Cell Signaling Technology, Massachusetts, USA), Anti-p53 (DO-1) – (1:1000 - Santa Cruz Biotechnologies, Texas, USA), Actin ACTN05 (C4) – (1:1000 – ThermoFischer, Massachusetts, USA) at room temperature and then probed with a peroxidase-conjugated secondary antibody (1:5000 – Bio-rad, California, USA) for 1 h. The chemiluminescent signal was detected in ImageQuant LAS 4000 (GE Healthcare, Chicago, IL) or X-ray film, using ECL reagent (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, and 0.2 mM p-coumarin) and H_2O_2. The resultant bands were analyzed using the Image Studio Lite Western Blot Analysis Software (LI-COR Biosciences, Nebraska, USA).

Click-chemistry reaction for newly synthesized protein. The MCF-7 cells were seeded in 12-wells plates until reaching 70-80% confluence and submitted to the protocol described by Meijer et al. (2021), with some minor adaptions. The treatment group was Co-incubated with AHA (1mM) and 4NC (18 μM) for 4 hours. Methionine + dye AF488 was used as a negative control. The fluorescence detection has done using the flow cytometer LSR Fortessa (BD Biosciences, New Jersey,

USA) and the analysis performed with the FlowJo 8.0 software (BD Biosciences, New Jersey, USA).

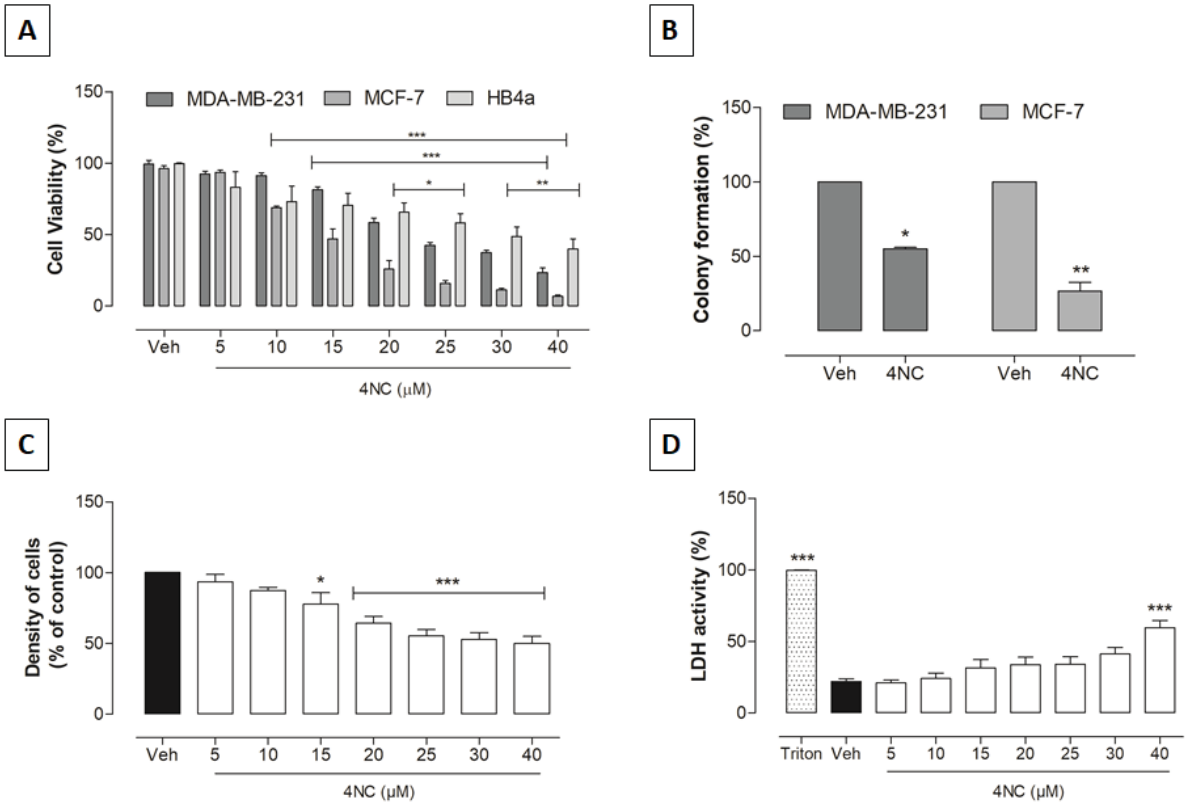
Determination of lactate-to-pyruvate ratio. To assess whether 4NC could alter the tumor metabolism, MCF-7 cells (5,000 cells/well) were seeded in 96-wells plate and 24 h after being exposed to Veh (0.1% DMSO) or 4NC (10, 20 and 40 μM) for 4 h. The medium was collected and processed according to GUTMANN; WAHLEFELD (1974) and CZOK; LAMPRECHT (1974) to measure lactate and pyruvate respectively. The resultant absorbance was measured at 340 nm in a spectrophotometer (Synergy HT, Biotek, Vermont, USA).

Statistics. The results are provided as mean ± standard error of mean (SEM). The statistical evaluation was performed using GraphPad Prism (v. 5.0), with $p < 0.05$. The comparisons between 2 groups were performed with Student's t test; for multiple comparisons one or two-way ANOVA with Newman Keuls test or Bonferroni post hoc were used, respectively.

3.4 RESULTS

4NC induces cytotoxicity in cells. The three breast cell lines exhibited some degree of sensitivity to 4NC. Based in MTT assay, for the MCF-7 cells, the effects of 4NC in viability could be observed from 10 μM, whereas for MDA-MB-231 cells the effects started from 15 μM. For the non-tumor cell line HB4a the cytotoxicity started only from 20 μM of 4NC (Fig. 1A). The EC_{50} was 14.97 μM, 21.71 μM, and 54.99 μM, respectively for the three cell lines. In parallel, the ability of cells to generate a progeny was reduced in MDA-MB-231 and MCF-7 cells treated with 4NC (Fig. 1B). A promising SI (3.68) was reached for MCF-7 cells; for MDA-MB-231 cells the SI was lower (2.53) than the proposed minimum threshold of 3. Therefore, the MCF-7 cell line was selected for further studies *in vitro*. Additional cell viability assays showed that in MCF-7 cells, treatment with 4NC led to a decrease in the number of adherent cells in treatments from 15 μM 4NC (Fig. 1C), measured by violet crystal; while the loss of membrane integrity, measured by LDH, was significantly increased only in cells treated with concentrations higher than 40 μM 4NC for 4 hours (Fig. 1D).

FIGURE 1. 4NC CYTOTOXICITY AGAINST HUMAN BREAST CELL LINES



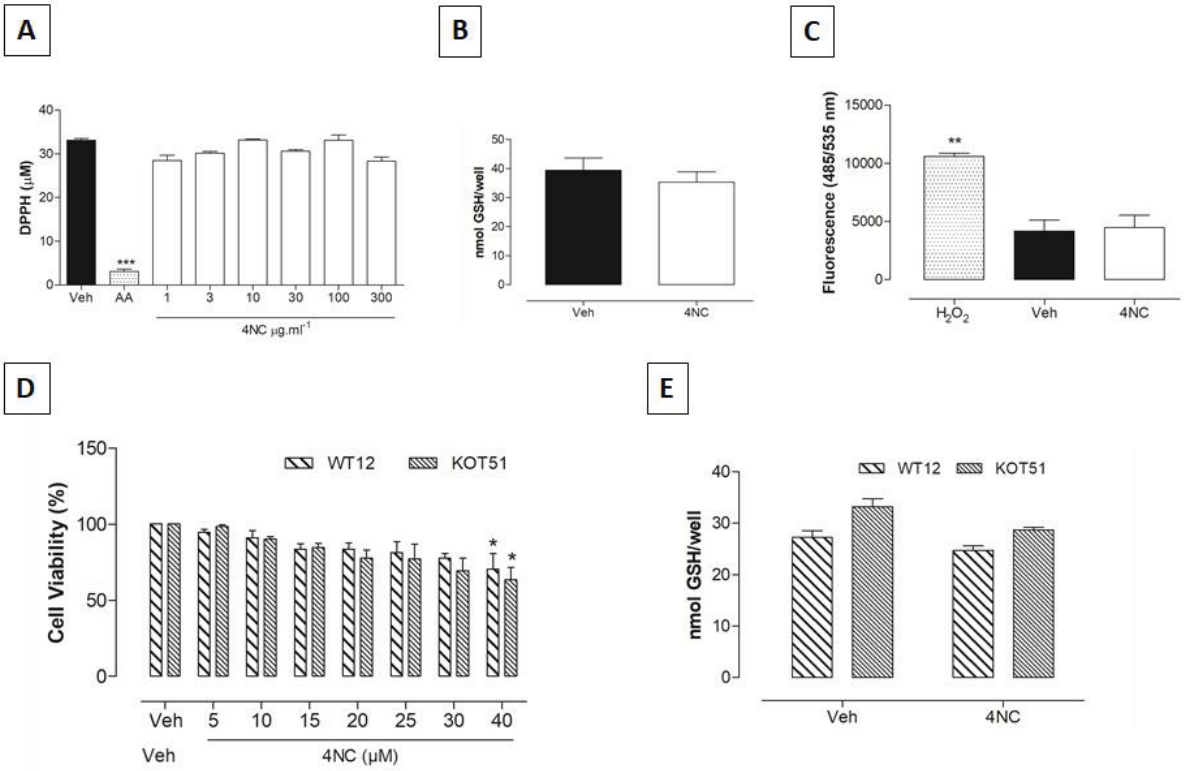
LEGEND: Cell viability was measured by MTT assay in MDA-MB-231, MCF-7, and HB4a cells (A) Clonogenic assay in MDA-MB-231 and MCF-7 cells (B), Crystal violet staining (C), and LDH assay (D), both last in MCF-7 cells. A range of 4NC from 0 (Veh) to 40 μM was used to treat cells during 24 h in A, C, and D; 1 μM of 4NC was used in B. The data are expressed as mean ± SEM (n=3-4). The statistical analyses were performed using one-way ANOVA followed by the post hoc Newman Keuls or Student t-test. * Significant difference compared with Veh group (one, two, and three symbols refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

4NC effects *in vitro* are not related to oxidative stress. 4NC showed negligible antioxidant properties against DPPH reagent, at least directly (Figure 2A). In a cell system, 4NC did not change the GSH and ROS levels in MCF-7 cells, even when exposed to a high concentration (40 μM 4NC), as showed in Figure 2 (B) e (C).

Differential cytotoxicity of 4NC related with efflux transporter. To assess the interaction of 4NC with efflux transporters and its participation as a substrate for these transporters, fibroblasts without efflux transporters (WT12) and fibroblasts expressing MRP1 (KOT51) were treated with 4NC and evaluated for cytotoxicity and GSH cell concentration, a known substrate for this transporter. At the highest concentration of 4NC, 30% and 37% viability was lost in WST12 and KOT51 cells,

respectively (Fig. 2D). GSH cell concentration did not change significantly after the 4NC treatment (Fig. 2E). The GSH decrease may be associated with the cell loss rather than with 4NC-associated GSH efflux.

FIGURE 2. ANTIOXIDANT AND PROOXIDANT PROPERTIES OF 4NC



LEGEND: Antioxidant attribute of 4NC through DPPH assay (A). GSH concentration (B) and ROS accumulation in MCF-7 cells under 4NC treatments (40 μM) (C). Fibroblast in the absence (WT12) or presence (KOT51) of MRP1 were treated for 24 h with 4NC (5 – 40 μM). (D) Viability analysis via WST-1 assay (E) GSH concentration inside the fibroblasts under the treatment with Veh and 4NC (40 μM). The data are expressed as mean ± SEM (n=3-4). The statistical analyses were performed using one-way ANOVA followed by the post hoc Newman Keuls or Student t-test. *Significant difference compared with Veh group (one, two, and three symbols refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). AA = 50 $\mu g.mL^{-1}$ ascorbic acid (positive control); H_2O_2 = 10 μM (positive control).

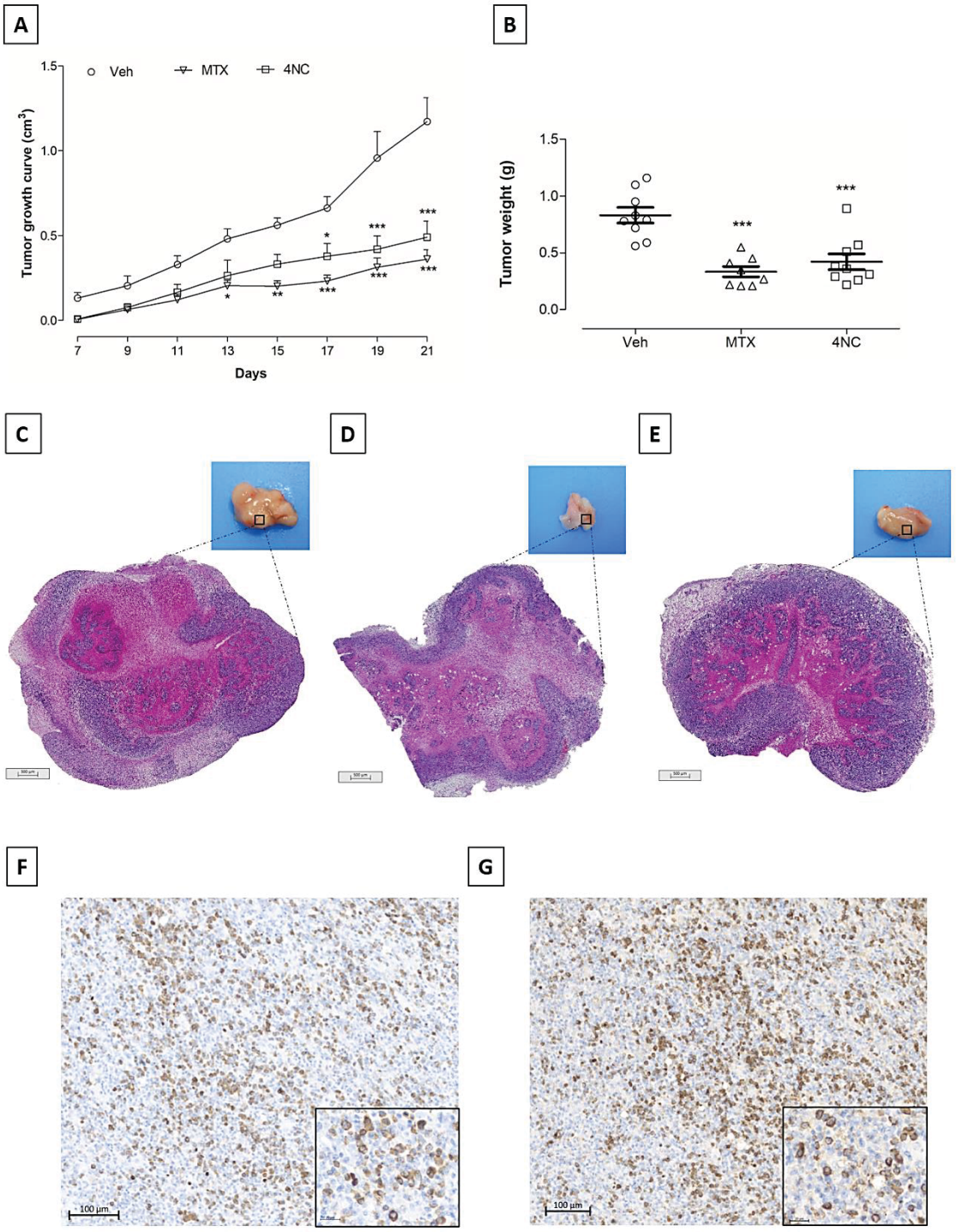
4NC is non-toxic via oral administration. No changes in behavior or physical condition were observed after the administration of 5, 50, and 300 mg kg^{-1} of 4NC. The group treated with 2,000 mg kg^{-1} had moderate yellowish diarrhea after 3 hours the onset of the test, however, this reaction was transitory and animals were alert, exhibiting normal appetite. Mortality was not present in any dosed animals, consequently, the GHS (Globally Harmonized Classification System) for 4NC was

Category 5 (> 2,000 – 5,000 mg kg^{-1} b.w.) and the median lethal dose (DL_{50}) higher than 2,000 mg kg^{-1}. Normal parameters were obtained on histological evaluation of all groups tested, as showed in the livers in Supplementary Fig. 1.

4NC impairs tumor growth. The treatment with 4NC reduced the development of SEC tumors. The dose of 25 mg kg^{-1} of 4NC significantly diminished the tumor volume by 44% from day 17, compared with vehicle group, and up to 58% on day 21. The MTX group, as expected, had a stronger effect on tumor growth, showing suppression of 58% from day 13 and 69% on day 21 (Fig. 3A). Similarly, both treatments were able to decrease tumor weight by 49% and 60% for 4NC and MTX, respectively, in comparison to vehicle (Fig. 3B).

SEC and organs microscopic evaluation. The SEC is characterized by polygonal cells highly pleomorphic and anaplastic, typical of undifferentiated carcinoma. In this experiment, necrosis was the predominant finding in all samples evaluated, but it was more prominent in the vehicle and MTX groups (grade IV), whilst for 4NC group the grade III was described (Fig. 3C, D and E). There were no differences between the pattern of inflammation: always peripheral, mild, and with polymorphonuclear cells. Histopathological analysis of liver, kidneys, and lung revealed normal cellularity and architecture in all groups (data not shown). The immunohistochemical examination confirmed the presence of hormonal receptors ERα and PR in all groups with SEC (Veh, MTX and 4NC treatments); however, surprisingly, the staining was restricted to the cytoplasmic membrane and cytoplasm (Fig. 3F and G), with no nuclear reactivity. Both ERα and PR stainings were coincident and predominantly with focal distribution.

FIGURE 3. MACROSCOPIC AND MICROSCOPIC CHARACTERISTICS OF TUMORS



LEGEND: Mice were treated for 21 days with vehicle (0.1% Tween, p.o.), MTX (2.5 mg kg^{-1}, i.p.), and 4NC (25 mg kg^{-1}, p.o.). 4NC reduces the tumor rise and weight as seen in (A) and (B). Representative histology from mice treated with vehicle (C), MTX (D), and 4NC (E) during 21 days. The amorphous-looking pink regions represent areas of necrotic tumor cells, more extensive in vehicle (C) and MTX (D) groups. Representative examples of ER-α (F) and PR (G) membrane staining in SEC (bottom right: details of staining cells). The data are expressed as mean ± SEM (n = 3-9/ group). The statistical analyses were performed using two-way ANOVA followed by Bonferroni´s post hoc test (A) and one-way ANOVA followed by Newman Keuls post hoc test (B). * Significant difference compared with Veh group (one, two, and three symbols refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

Biochemical parameters were altered in tumor-bearing groups. There were no differences in the hematological parameters, whereas biochemical parameters were strongly altered in tumor-bearing groups. AST, creatinine, and urea levels increased in tumor-bearing groups compared with a naive group. ALT levels were increased in the vehicle group versus naive group and decreased in the MTX group, compared with vehicle group. AP levels decreased in tumor-bearing groups compared with naive group. Total protein levels increased in MTX group compared with all other groups, the globulin followed the same pattern. However, 4NC did not induce alterations itself, since the alterations observed in this group (Table 1) are related to the tumor presence instead of the 4NC treatment.

TABLE 1. HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN EXPERIMENTAL GROUPS TREATED FOR 21 DAYS WITH VEHICLE (NAIVE –NO TUMOR-, AND VEHICLE –WITH TUMOR-, p.o.), MTX (2.5 mg kg^{-1}, i.p.), AND 4NC (25 mg kg^{-1}), p.o.)

PARAMETER	EXPERIMENTAL GROUPS			
	Naive	**Veh**	**MTX**	**4NC**
ALT (U L^{-1})	30.31±3.47	84.04±29.48**	42.95±12.31$^{\#\#}$	64.73±25.07
AST (U L^{-1})	75.72±4.51	370.01±67.23***	289.79±68.55***	287.02±72.49***
AP (U L^{-1})	75.35±8.29	51.81±7.25**	48.86±8.78***	57.3±11.12**
Glucose (mg dL^{-1})	91.47±34.13	114.33±42.55	142.89±51.69	109.61±38.62
Total protein (g dL^{-1}**)**	4.57±0.14	4.32±0.64	5.97±0.21$^{***,\#\#\#,bbb}$	4.01±0.30
Albumin (g dL^{-1})	1.66±0.12	1.99±0.34	2.58±1.13	1.73±0.25
Globulin (g dL^{-1})	2.91±0.12	2.37±0.77	3.97±0.29$^{***,\#\#\#,bbb}$	2.28±0.20
Creatinine (mg dL^{-1})	0.25±0.09	0.51±0.09***	0.49±0.05***	0.52±0.03***
Urea (mg dL^{-1})	40.62±4.93	54.31±8.19**	54.31±6.13**	54.8±3.52**
Hematocrit (%)	40.67±1.60	39.98±1.64	40.06±1.39	39.47±2.51
Hemoglobin (g dL^{-1})	12.58±0.42	12.14±0.38	12.15±0.43	12.07±0.56
White blood cells ($x10^3$ μL^{-1})	4.77±1.19	5.22±0.64	6.65±1.66	6.14±2.22
Lymphocytes ($x10^3$ μL^{-1})	3.27±0.99	3.83±0.69	4.47±1.61	3.94±1.3
Monocytes ($x10^3$ μL^{-1})	0.24±0.09	0.25±0.07	0.38±0.12	0.42±0.24
Granulocytes ($x10^3$ μL^{-1})	1.25±0.39	1.14±0.36	1.8±0.68	1.78±0.86

LEGEND: The data are expressed as mean ± SEM (n=7-9/group). The statistical analyses were performed using one-way ANOVA followed by Newman Keuls post hoc test. *, $^{\#}$, ^{b} Significant difference compared with naive, vehicle and 4NC groups, respectively (one, two, and three symbols

refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, and *AP* alkaline phosphatase

4NC does not alter oxidative stress in SEC. The analysis of oxidative stress parameters in tumor and hepatic tissues revealed no changes by the 4NC treatment. Slight changes were present only in the positive control group MTX: there was an increase in SOD activity in the liver (187%) and a decrease in the tumor tissue (-32.18%), as showed in Table 2.

TABLE 2. OXIDATIVE STRESS PARAMETERS IN MICE TISSUES IN EXPERIMENTAL GROUPS TREATED FOR 21 DAYS WITH VEHICLE (p.o.), MTX (2.5 mg kg^{-1}, i.p.), AND 4NC (25 mg kg^{-1}, p.o.)

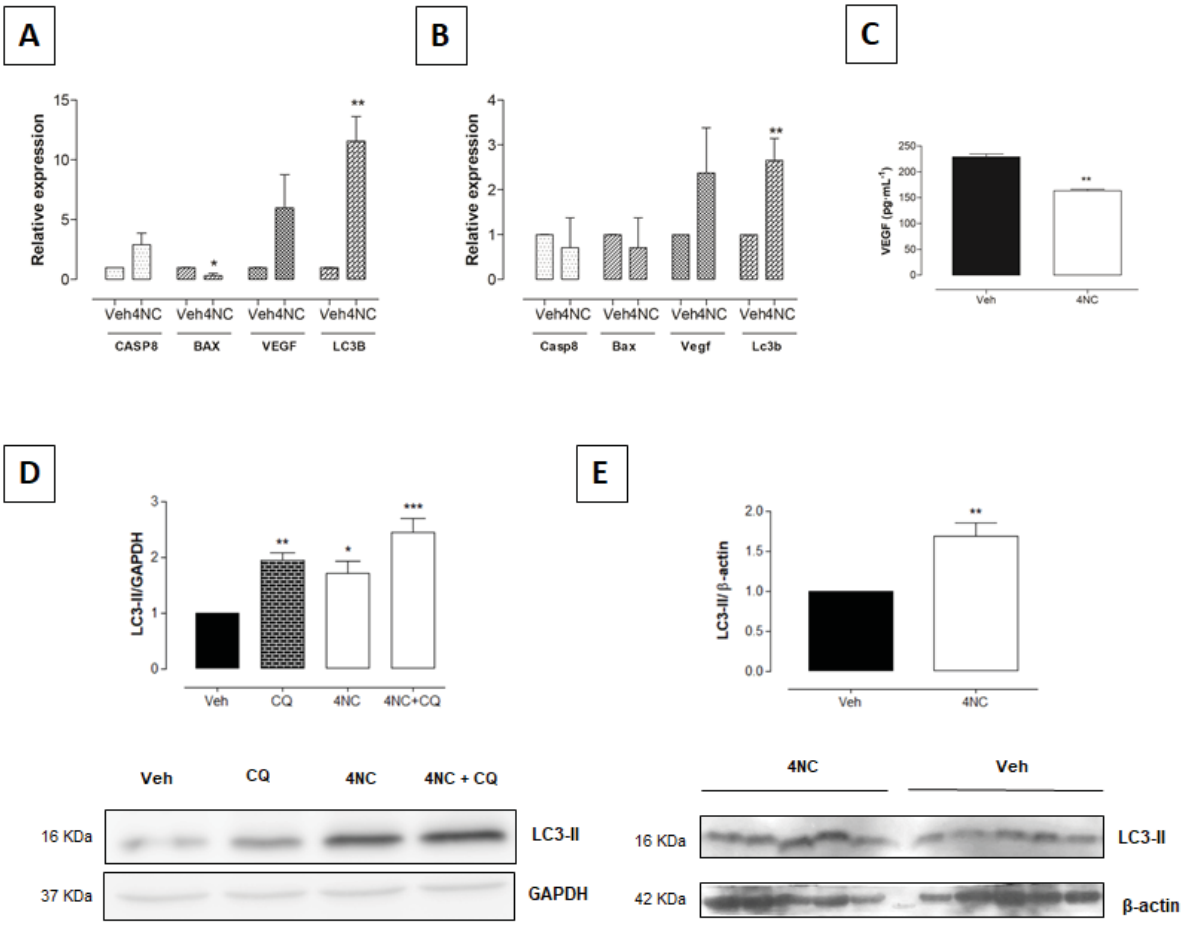
PARAMETER	EXPERIMENTAL GROUPS		
	Veh	**MTX**	**4NC**
GSH tumor (µg g tissue-^{1})	83.92±32.48	90.26±53.11	78.37±28.01
GSH liver (µg g tissue-^{1})	1027.51±123.06	888.90±163.87	874.78±190.58
GST tumor (mmol min mg protein^{-1})	4.012±0.96	3.64±0.81	2.66±0.73
GST liver (mmol min mg protein^{-1})	9.39±0.86	8.33±0.48	10.54±0.74
LPO tumor [hydroperoxides] (nmol mg protein^{-1})	9.07±1.3	10.05±3.44	8.04±2.46
LPO liver [hydroperoxides] (nmol mg protein^{-1})	34.77±3.59	28.94±3.63	28.48±2.00
SOD tumor (U mg protein^{-1})	229.26±42.04	155.50±33.25***	183.32±26.36
SOD liver (U mg protein^{-1})	242.72±11.51	454.08±84.50***	219.79±27.00
Cat tumor (nmol min mg protein^{-1})	10.39±2.24	10.78±3.41	11.06±1.01
Cat liver (nmol min mg protein^{-1})	464.48±130.34	493.15±83.18	437.81±101.77

LEGEND: GSH (reduced glutathione), GST (glutathione-S-transferase), SOD (superoxide dismutase), LPO (lipid peroxidation) and Cat (catalase). *** Significant difference compared with vehicle group ($p < 0.001$). The data are expressed as mean ± SEM (n= 6–11/group) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test.

The gene and protein profile after 4NC treatment *in vitro* and *in vivo*. The 4NC treatment leads to cell death by mechanisms that are not yet fully understood. To investigate the action of 4NC, genes related to apoptosis (Caspase 8 and Bax), necrosis (VEGF), and autophagy (LC3B) were evaluated. The response of *CASP8/Casp8* and *BAX/Bax* genes was variable (Fig. 3A, B) and does not indicate

activation of the apoptotic cell death pathway at the gene level; a fact that is confirmed by tumor histology, which showed that apoptosis is an incidental finding, associated only with transition areas between viable cells and necrotic areas (Fig. 2E). Furthermore, the protein levels of the tumor suppressor p53, an important regulator of apoptosis, did not change significantly *in vitro* (Supplementary Fig. S2A). The gene *VEGF*, related to hypoxia-induced death, showed a sharp increase, though not statistically significant (Fig. 4A, B). To check this data, a VEGF secretion measurement was performed in MCF-7 cells (Fig. 4C). A reduction of about 30% in the amount of VEGF secreted by cells was observed *in vitro*, which is in agreement with the *in vivo* histological analysis, in which cell necrosis was not an important finding in the tumors treated with 4NC (Fig. 3E). In MCF-7 cells, the treatment with 4NC caused more than 10-fold increase in the gene expression of *LC3B* (Fig. 4A), a gene related to the autophagy pathway. Similarly, an increase of 3-fold was also observed in the expression of *Lc3b* in SEC (Fig. 4B). Furthermore, at the protein level, there was a LC3B-II increase of 1.7-fold in comparison with Veh-treated group, both *in vivo* (Fig. 4E) and *in vitro* (Fig. 4D), confirming the major presence of autophagosomes. A preliminary experiment with short-term treatment (4 h) did not alter the LC3-II levels (Supplementary Fig. S2B).

FIGURE 4. 4NC-RELATED DEATH PATHWAYS IN BREAST CANCER

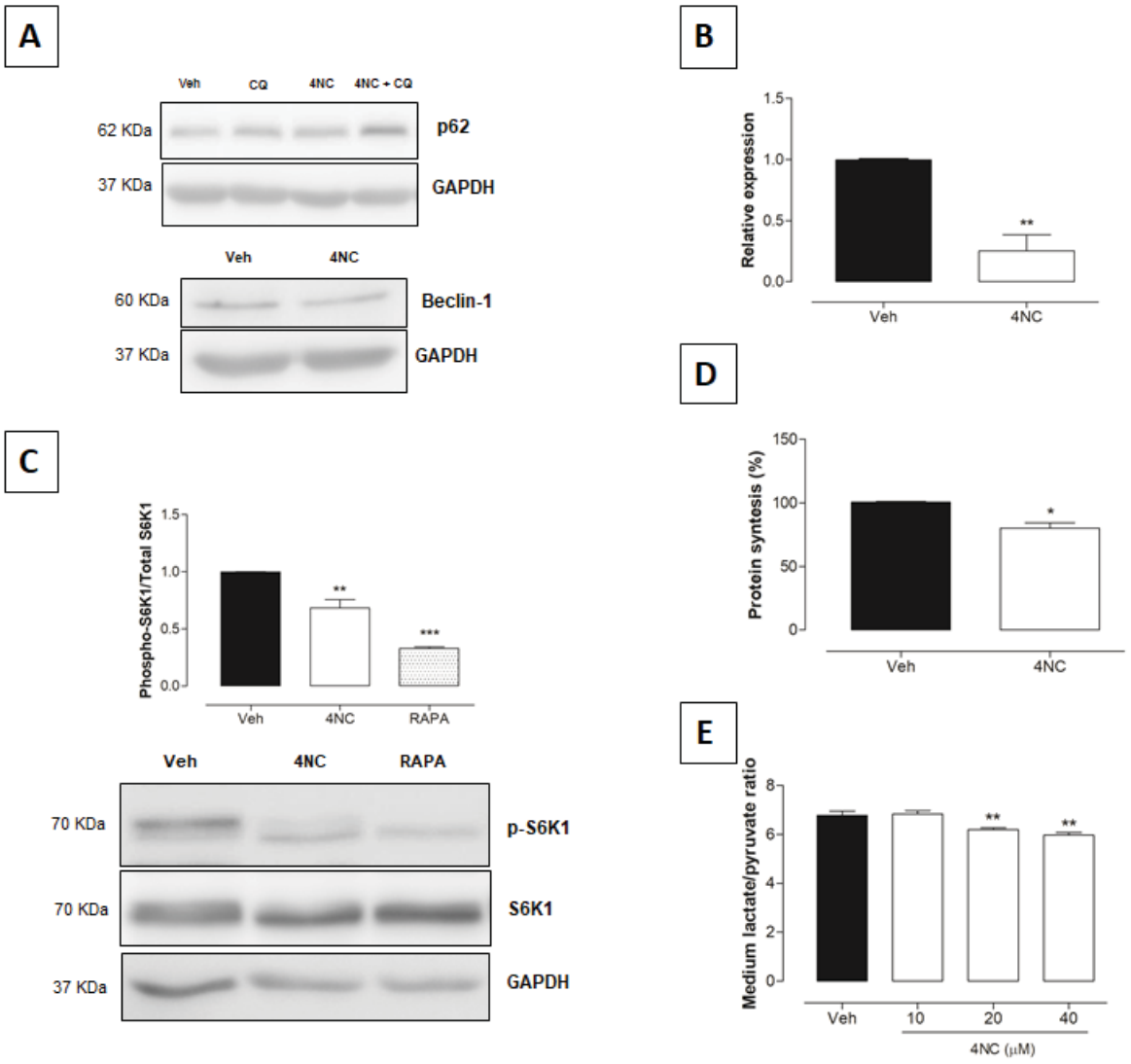


LEGEND: Gene expression in MCF-7 (A) and SEC (B). Quantitative real-time PCR was performed for the indicated genes using the endogenous reference gene *HPRT* and *Rplp0*. (C) Levels of VEGF in supernatants of MCF-7 cells treated with 4NC during 24 h. Representative image of LC3B-II expression *in vitro* (D). MCF-7 cells were stimulated for long-term (24 h) treatment with vehicle, chloroquine (CQ 100 μM), 4NC (18 μM), and chloroquine plus 4NC to determine LC3B-II levels. LC3-II expression *in vivo* (E). Mice were treated for 21 days with vehicle (p.o.) and 4NC (25 mg kg^{-1}, p.o.). The data are expressed as mean ± SEM (n=3-5). The statistical analyses were performed using one-way ANOVA followed by the post hoc Newman Keuls or Student t-test. * Significant difference compared with vehicle group (one, two, and three symbols refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

4NC act as a regulator of cancer cell metabolism. To confirm the activation of the autophagic pathway, additional autophagy-related markers were evaluated: p62 and Beclin-1. The 4NC treatment caused a slight accumulation of 14% on p62 in MCF-7 cells, higher than that found in the treatment with CQ, but lower than that observed with the association of 4NC+CQ (28%) (Fig. 5A). Beclin-1 levels remained stable (Fig. 5A). The autophagy upstream gene *RAPTOR* was downregulated by about 75%

(Fig. 5B), along with the protein levels of S6K1, a direct RAPTOR substrate. A reduction of about 30% in the phospho-S6K1/S6K1 ratio was observed (Fig. 5C). Considering the major goals of the mTOR pathway, which are the fine balance of metabolism and protein synthesis to promote cell growth, the effect of 4NC on these two aspects was finally evaluated. There was a reduction of approximately 20% in protein synthesis with only 4h of treatment with 4NC (Fig. 5D), and there was also a positive change in the metabolic profile concerning the lactate/pyruvate ratio (Fig. 5E). Considering that some chalcones regulate glycolysis by blocking the enzyme PFKFB3 (KOTOWSKI et al., 2021), the expression of this enzyme was tested *in vitro*. However, there was no change in its gene expression in MCF-7 cells (Supplementary Fig. S3).

FIGURE 5. 4NC MODULATION ON THE mTORC1 PATHWAY



LEGEND: Representative western blot of p62 and beclin-1, regarding autophagy pathway in MCF-7 cells stimulated for long-term (24 h) treatment with vehicle, chloroquine (100 μM), 4NC (18 μM), and chloroquine plus 4NC (A). MCF-7 cells were stimulated for long-term (24 h) treatment with Veh and 18 μM of 4NC to analyse the gene expression of *RAPTOR* (B). MCF-7 cells were stimulated for short-term (4 h) treatment with vehicle, rapamycin (50 nM), and 4NC (18 μM) to analyse phospho-S6K1/total S6K1 protein (C). (D) MCF-7 cells were labeled with AHA and treated with vehicle or 4NC (18 μM) treatment for 4 h to assess the nascent protein levels. (E) MCF-7 cells were treated with Veh or 4NC (18 μM) treatment for 4 h to determine the lactate/pyruvate ratio. The data are expressed as mean ± SEM (n=3-4). The statistical analyses were performed using one-way ANOVA followed by the post hoc Newman Keuls or Student t-test. * Significant difference compared with vehicle group (one, two, and three symbols refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

3.5 DISCUSSION

4NC exhibited potential antiproliferative and antimetabolic effects *in vitro* and *in vivo* in breast tumor models. The cell viability was reduced in a concentration-dependent way, with higher susceptibility of breast cancer cells, especially the luminal cell line MCF-7, than HB4a non-transformed cells. According to the viability assays performed, the impact of 4NC on cells seems to be associated with alterations in the intracellular machinery rather than a direct cytotoxic injury. Cell disruption, indirectly assessed by the LDH assay, was only significant from higher concentrations of 4NC (≥40 μM), while the ability to generate viable progeny cells was disturbed with 40-times lower concentration. 4NC has already been shown to reduce the viability in several *in vitro* models when used as a free drug and encapsulated in nanoparticles (CARPIO ARÉVALO et al., 2019; CORDEIRO et al., 2021; DIMMOCK et al., 2002; DOS SANTOS et al., 2020; VIA et al., 2009). The use of 4NC as an isolated agent or combined with other drugs and cancer therapies in some *in vitro* studies showed promising results in cancer therapy, corroborating our data.

The intrinsic reactivity of chalcones may have a dual nature: antioxidant and pro-oxidant (DÍAZ-TIELAS et al., 2016). Regarding the first characteristic, shared by several members of this group (CHEN et al., 2017; CHU; GUO, 2016; MONISHA et al., 2018), 4NC had poor activities in scavenging the free radical of the DPPH molecule. Despite conferring cytotoxic advantages to chalcones, the presence of the substituent NO_2 impairs the antioxidant abilities (LEE et al., 2020; SAITO et al.,

2021). Regarding the pro-oxidant feature, even with a high concentration of 4NC (40 μM) no change in the oxidative status was observed, both *in vitro* and *in vivo*. The interval of observation (1 – 4 h) for *in vitro* experiments does not exclude that this change occurs later, as observed by SUN et al (2010) with the metoxychalcone WJ9708011. An additional oxidative stress sensitization mechanism described for flavonoids and chalcones is the GSH depletion via MRP/ABCC transports, due to increase in the affinity of MRP1 to GSH and efflux of glutathione-adducts mediated by MRP1 (KACHADOURIAN; DAY, 2006; LESLIE; DEELEY; COLE, 2003; SABZEVARI et al., 2004). Treatment with 4NC in control and MRP1 transfected fibroblasts did not lead to selective cytotoxicity or a significant increase in GSH uptake by MRP1. Thus, the 4NC ability to deplete GSH seems to be irrelevant in the experimental models used in this study. At the same time, 4NC does not appear to be a substrate of MRP1 and, by inference, expression of MRP1 does not confer resistance to treatment with 4NC, by cotransport with GSH.

Treatment of SEC with 4NC prevented full tumor development, similarly to the standard cytotoxic drug used in this study (MTX), in comparison with the vehicle group. According to Eby, Tabatabai & Bursac (2010), the tumor growth rate increases with tumor size, becoming more prominent as time passes. Interestingly, treatment with MTX and 4NC delay this maximum tumor growth and tumor weight, leading to a longer period with a slower rate of growth. Our study demonstrated for the first time that there is cytoplasmic and membranous positivity for ERα and PR in SEC, however, such immunostaining pattern is considered as non-specific for the molecular classification of breast cancer subtypes (WELSH et al., 2012). Smart, Alejo & Frasor (2020) also detected ER immunostaining in the cytoplasm of a variety of murine breast cancer cell lines, with functional induction of resistance to oxidative damage, apoptosis, and cell death. It is suggested that ER cytoplasmic localization and estrogen independence may be signs of murine breast cancer progression (SMART; ALEJO; FRASOR, 2020; TORRES-ARZAYUS et al., 2010). Thus, the SEC could be used as model of an endocrine therapy-resistant human breast cancer, but further studies are needed to fully characterize these aspects.

Apoptosis is one of the most reported death mechanisms related to treatment with chalcones in breast cancer (BORTOLOTTO et al., 2016; DOS SANTOS et al., 2020; MOHAMED; IBRAHIM; EL-MANAWATY, 2021). CARPIO ARÉVALO et al. (2019) described morphological findings reminiscent of apoptosis in HeLa cervical

tumor cells treated with 4NC as well. A nitro-chalcone derivative synthesized by Khairul et al. (2021), in addition to morphological findings suggestive of apoptosis in MCF-7 cells, also caused potential autophagy and necrosis after 72 h of treatment. In our study, gene expression analysis did not show indications of apoptosis and necrosis, both *in vitro* and *in vivo*, as reinforced by *in vivo* histological findings. Unexpectedly there was an increase in the gene and protein expression of the autophagosome marker LC3-II for both cases. Increased LC3-II levels reflect an increase in the number of autophagosomes, which are induced by nutritional deprivation, but may also be increased due to the impaired degradation of these autophagosomes (YOSHII; MIZUSHIMA, 2017). The maintenance of levels of p62 assessed *in vitro* has discarded the occurrence of autophagy, which was confirmed by the normal levels of Beclin-1 (PANKIV et al., 2007). A similar finding was described by Singha et al. (2013), in which the abundance of p62 and LC3-II observed after treatment of triple-negative breast cancer cells with Manumycin A was interpreted as a sign of inhibition of autophagy.

The mTOR pathway is an important negative regulator of the initiation and maturation of autophagy (LAMPADA et al., 2017). Our study revealed that 4NC has an inhibitory effect on the mTORC1 pathway; however, the inhibition of the mTOR pathway was not enough for autophagy to occur in MCF-7 cells treated with 4NC. Furthermore, the rise in LC3-II levels is not exclusive of autophagy (KAR et al., 2009; LEE et al., 2015; SINGHA et al., 2013). 4NC, in addition to increasing the process from LC3-I to LC3-II, promoted the increase in *LC3B* expression, a finding that is not typical of autophagic activation in response to starvation and also occurs in non-apoptotic and non-autophagic cell death (KAR et al., 2009).

The relation between chalcones and the mTOR pathway has been previously reported. Synthetic chalcones have already been described as mTOR modulators (MATEEVA et al., 2017). Such chalcones reduced the viability of breast cancer cells due to the blockade of the mTOR pathway, with advantages over flavonoids, due to their more flexible chemical structure that favors their binding to mTOR. Moreover, the mTOR signaling is often over-activated in cancer, including breast cancer, since it offers growth advantages due to increased protein biosynthesis (HARE; HARVEY, 2017). Besides protein biosynthesis, the axis mTORC1-S6K1 is also responsible for regulating the biosynthesis of pyrimidines and fatty acids, with severe effects on the cell's ability to generate viable progeny (BEN-SAHRA et al., 2013; DÜVEL et al.,

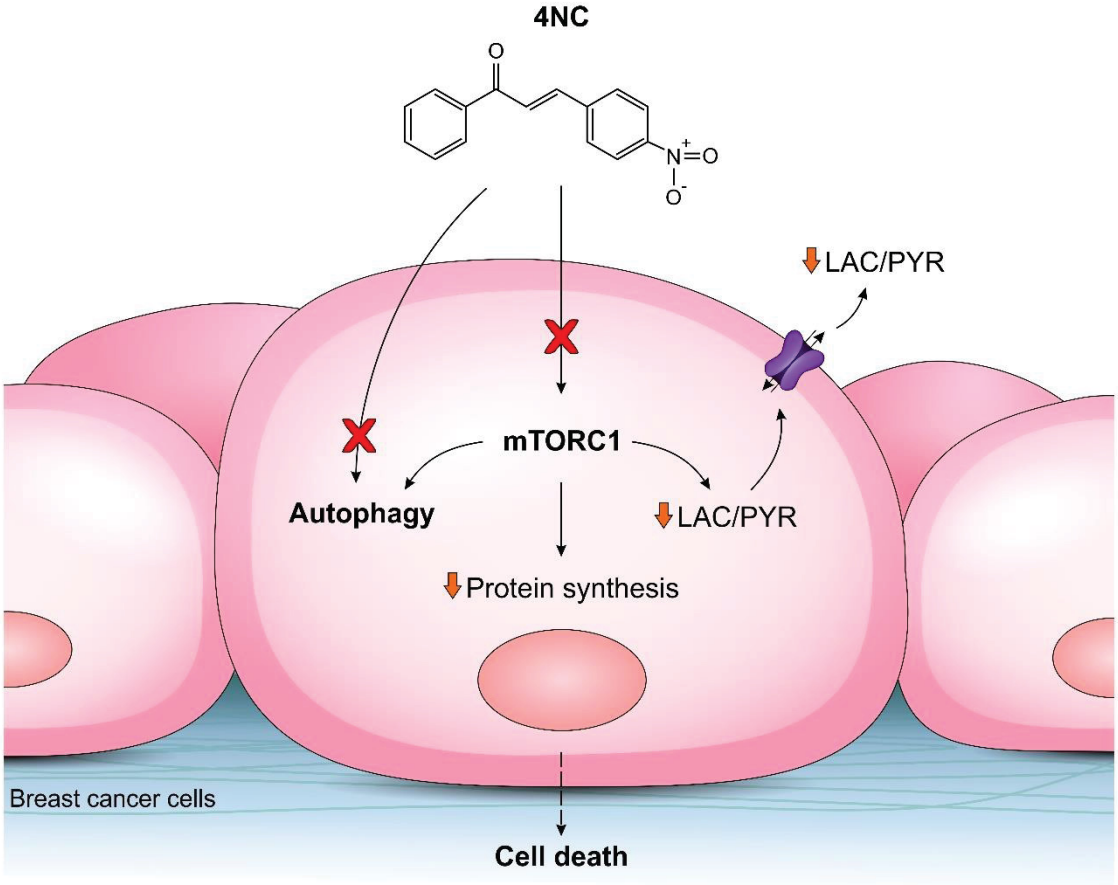
2010). Upregulation of mTOR signaling also leads to neoplastic cells acquiring resistance to chemotherapy and targeted drugs (GREMKE et al., 2020). Conversely, it makes cells metabolically more vulnerable as it reduces baseline levels of autophagy. The use of 4NC in this field appears to be very promising, as the mTORC1 blockade has been observed without the activation of the expected protective autophagic response.

The energy imbalance herein observed with 4NC treatment had effects on the lactate/pyruvate ratio, an important indicator of the Warburg effect on tumor cells (GO et al., 2021). SHI et al. (2018) observed a similar pattern of response when treating human ovarian cancer cells with the chalcone cardamonin, with suppression of mTORC1 and glycolysis. In human breast cancer, the lactate/pyruvate ratio is positively correlated with tumor volume (GALLAGHER et al., 2020). Thus for high lactate-consumers cells, as the luminal subtype, it represents an attractive therapeutic target (KENNEDY et al., 2013).

There is huge controversy about compounds with a nitroaromatic portion, generally related to high toxicity (KOVACIC; SOMANATHAN, 2014). The 4NC synthesized by Dimmock et al. (2002) caused neurotoxicity when administered intraperitoneally in mice, at the dose of 300 mg kg^{-1}. The commercial 4NC (Sigma-Aldrich, Missouri, USA) that we tested did not show any signs of change in behavior or tissue damage up to the oral dose of 2,000 mg kg^{-1}, just an isolated episode of yellowish diarrhea, probably related to high dosage and the occurrence of an osmotic/secretory diarrhea. The administration of 4NC for 21 days did not lead to alterations in hematological and biochemical profile, nor did it cause any tissue damage at the histological level. The greatest differences were observed in comparison with the naive group, but in this case, it is believed that the presence of the tumor itself was the main determinant of the alterations.

In conclusion, the results presented here indicate that 4NC has antiproliferative effects against human and murine mammary tumor cells, without inducing adverse effects and toxicity *in vivo*. The main cell mechanisms of 4NC are related to autophagy dysfunction and metabolic impairment (Figure 4). These findings certainly should be replicated in different cancer cell lines and tumor models to confirm the exposed results and determine further the therapeutic potential of 4NC to cancer patients.

FIGURE 1. 4NC TRIGGERS CELL DEATH IN BREAST CANCER CELLS THROUGH IMPAIRMENT OF mTOR PATHWAY AND AUTOPHAGY DYSFUNCTION



LEGEND: 4NC, 4-nitrochalcone; mTORC1, mechanistic target of rapamycin complex 1; LAC, lactate; LAC/PYR, lactate/pyruvate ratio; PYR, pyruvate.

3.6 ACKNOWLEDGEMENTS

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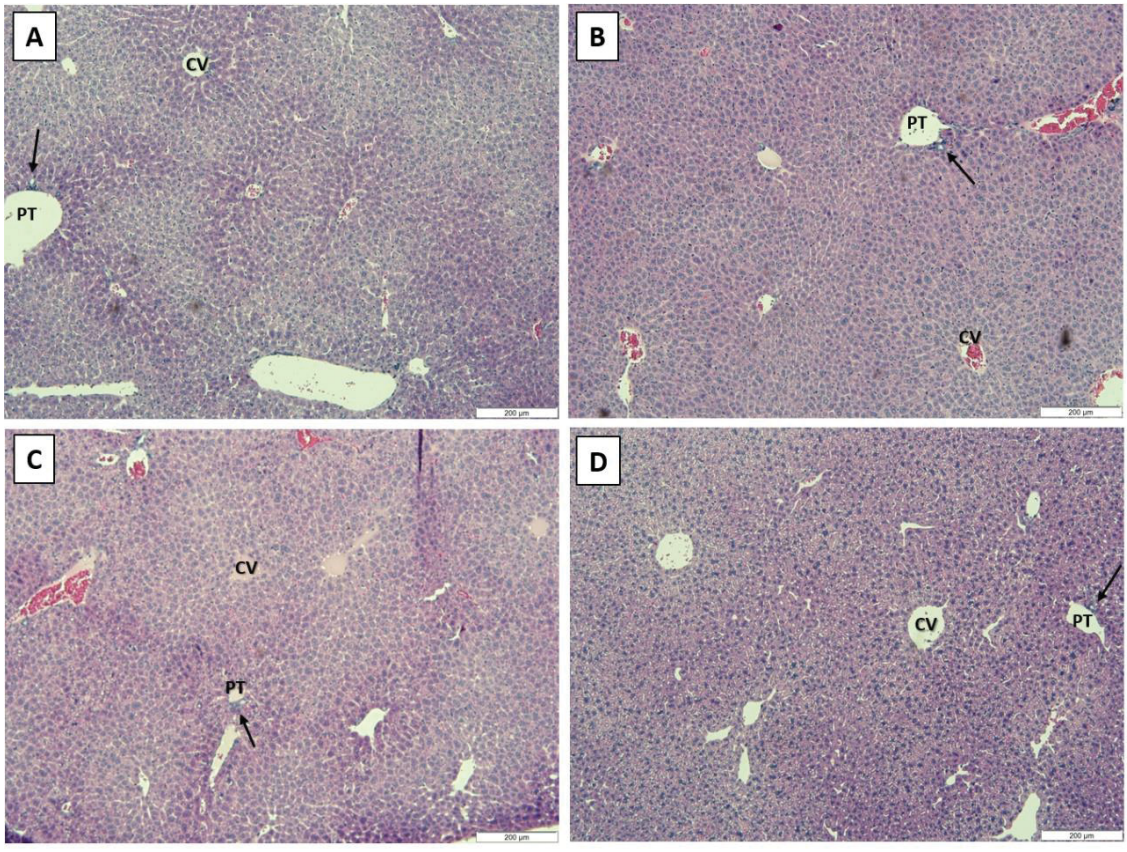
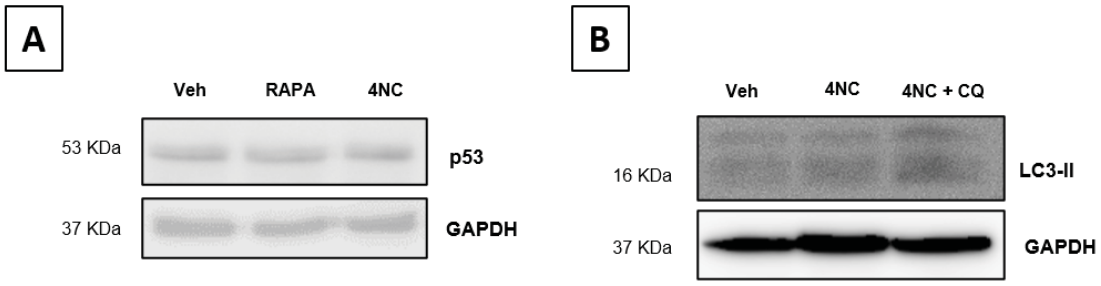
3.8 SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURE S1. 4NC DOES NOT INDUCE ACUTE CELL DAMAGE IN VIVO



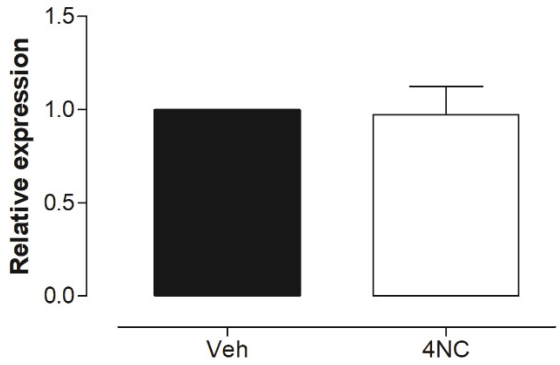
LEGEND: Mice were treated with the following doses of 4NC: 5 mg kg^{-1} (A), 50 mg kg^{-1} (B), 300 mg kg^{-1} (C) and 2000 mg kg^{-1} (D) for acute oral toxicity. There was no evidence of liver damage in all groups. PT (Portal tract); CV (central vein), arrow - cross-sections of the bile duct.

SUPPLEMENTARY FIGURE S2. 4NC DOES NOT PROMOTE ALTERATIONS IN p53 AND LC3-II ABUNDANCE IN MCF-7 CELLS



LEGEND: (A) MCF7 cells were treated with vehicle (Veh), rapamycin (RAPA 50 nM) and 4NC (18 μM) in (A) and with vehicle, 4NC (18 μM), and chloroquine plus 4NC (CQ 100 μM + 4NC 18 μM) in (B). A representative image of an independent experiment is show.

SUPPLEMENTARY FIGURE S3. *PFKFB3* EXPRESSION IN MCF-7 CELLS



LEGEND: MCF-7 cells were treated with vehicle (Veh) and 4NC (18 μM) for 24 h. The data are expressed as mean ± SEM (n=3).

SUPPLEMENTARY TABLE 1. SEQUENCE OF PRIMERS USED FOR QUANTITATIVE REAL TIME PCR

Gene	**Espécie**	**Universal and reverse primers** (5'→3')
HPRT	*Homo sapiens*	U: GAACGTCTTGCTCGAGATGTGA R: TCCAG CAGGTCAGCAAAGAAT
LC3B	*Homo sapiens*	U: ACCATGCCGTCGGAGAA R: ATCGTTCTATTATCACCGGGATT
BAX	*Homo sapiens*	U: TCCCCCCGAGAGGTCTTTT R: CGGCCCCAGTTGAAGTTG
CASP8	*Homo sapiens*	U: GGATGCCTTGATGTTATTCC R: AGTTCCCTTTCCATCTCCTC
VEGF	*Homo sapiens*	U: CCTATGTGCAGAGGAATTATG R: CCACTGTGTTGAGGGCAATG
RAPTOR	*Homo sapiens*	U: ACTGATGGAGTCCGAAATGC R: TCATCCGATCCTTCATCCTC
PFKFB3	*Homo sapiens*	U: GGAGGCTGTGAAGCAGTACA R: CAGCTAAGGCACATTGCTTC
Lc3b	*Mus musculus*	U: GTCCTGGACAAGACCAAGTTCC R: CCATTCACCAGGAGGAAGAAGG

Bax	*Mus musculus*	U: GCCTCCTCTCCTACTTC R: CCTCAGCCCATCTTCTT
Casp8	*Mus musculus*	U: CCAGGAAAAGATTTGTGTCTA R: GGCCTTCCTGAGTACTGTCAC
Vegf	*Mus musculus*	U: ACTGGACCCTGGCTTTACTGCT R: TGATCCGACTGATCTGCATGGTG
Rplp0	*Mus musculus*	U: CGACCTGGAAGTCCAACTAC R: ACTTGCTGCATCTGCTTG

4 ARTIGO CIENTÍFICO 2 – 'NUANCES OF PFKFB3 IN BREAST CANCER'

Artigo submetido no periódico *Cancer Chemotherapy and Pharmacology*.

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4.1 ABSTRACT

The enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) is a critical engine that supports glucose catabolism. PFKFB3 produces the signaling molecule fructose-2,6-biphosphate (F2,6BP), which activates the second gatekeeper in glycolysis, 6-phosphofructo-1-kinase (PFK-1), and favors the Warburg phenotype. Transcriptional and post-transcriptional processes regulate the abundance and phosphorylation of PFKFB3 in cells and its activation has been implicated in the progression of several types of cancer. PFKFB3 is important for sustaining glycolysis in the tumorigenesis scenario even under unpropitious conditions, thereby promoting metabolic reprogramming, cell proliferation, DNA repair, and drug resistance. Despite its heterogeneous phenotype, breast cancer presents unique characteristics that drive constitutive and inducible expression of PFKFB3 in this opportunistic glycolytic-shift. This enzyme is a point of convergence of multiple exogenous and endogenous growth-promoting and oncogenic signaling pathways, in special kinases cascades. The present review summarizes advances in *in vitro* and *in vivo* therapy studies that focus on PFKFB3 and the interplay between hormone receptor status and the underlying essential signal transduction system in breast cancer metabolic remodeling.

Keywords: PFKFB3; Breast cancer; Glycolysis; Kinase cascades; Metabolic therapy.

4.2 INTRODUCTION

Glycolysis is a remote and conserved metabolic pathway, serving two main purposes in the cell: the production of high-energy molecules (adenosine triphosphate [ATP]/nicotinamide adenine dinucleotide [NAD]) and intermediates for biosynthetic pathways [1, 2]. This first step in glucose catabolism consists of sequential enzymatic reactions, regulated at the level of three irreversible reactions coordinated by hexokinase (HK), phosphofructokinase 1 (PFK-1), and pyruvate kinase (PK) [2]**.** PFK-1, the second metabolic valve, is the central controller that directs glucose toward glycolysis or the pentose phosphate pathway (PPP), and it has a specific and unique regulator, fructose-2,6-bisphosphate (F2,6BP) [3, 4]**.** F2,6BP provides metabolic plasticity to cells by subverting the negative feedback of ATP on PFK-1, stimulating glycolytic flow and lactate production even when these levels are already high [2, 3, 5]. F2,6BP is the product of a collateral reaction in glycolysis, involving fructose-6-phosphate (F6P) and ATP, and it is mediated by the enzyme phosphofructokinase 2 (PFK-2) [3]. The reverse reaction is also possible but instead is mediated by fructose-2,6-bisphosphatase (F2,6BPase), generating fructose 6-phosphate and inorganic phosphate [3]. Actually, the kinase/phosphatase reactions are catalyzed by a sole enzyme with two functional domains**:** the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) [2].

4.3 PFKFB3 CONCEPTUALIZATIONS

Four isoforms of PFKFB have been described in mammals: PFKFB1 (liver isoenzyme), PFKFB2 (heart isoenzyme), PFKFB3 (brain/placenta isoenzyme), and PFKFB4 (testis isoenzyme) [6]. The PFKFB3 isoform is notable for its expressive

kinase/phosphatase (K:P) ratio, which favors metabolic flux in the glycolysis pathway and implies the reciprocal stimulation over HK expression whereas blocking glucose-6-phosphatase (G6Pase) [2, 3]. PFKFB3 is located in the cytoplasm where glycolysis occurs, but it is also found in the plasma membrane and mainly in the nucleus [7–10]. PFKFB3 trafficking between the cytoplasm and nucleus is controlled by acetylation processes [7]. At the molecular level, the human *PFKFB3* gene is expressed at low levels in all tissues, especially in highly proliferative tissues [11].

PFKFB3 is subjected to both short- and long-term regulation. Its long-term activation is based on the presence of several binding sites for transcription factors, such as hypoxia-inducible factor 1 (HIF-1), the estrogen receptor (ER), the progesterone receptor (PR), early growth response 1 (EGR-1), and nuclear factor-κB (NF-κB) in the promoter region of *PFKFB3* [12]. Its short-term activation occurs through phosphorylation and methylation. Some of its amino acid residues, such as Ser461 and Tyr194, are phosphorylation-dependent foci that respond to kinase proteins under propitious and unpropitious energy status, respectively [4, 13]. The phosphorylation of PFKFB3 decreases the Michaelis constant (K_m) and increases the reaction maximum velocity (*Vmax)* [14], which leads PFKFB3 to require a smaller concentration of F6P to generate F2,6BP and boosts glycolytic flux. Likewise, $R_{134/135}$ methylation facilitates F6P binding to PFKFB3 [13].

Despite its potent influence on glycolysis, PFKFB3 has a short half-life, and enzyme levels rise in a specific stage of the cell cycle: the mid-to-late G1 phase [15]. A very coordinated and sequential ubiquitin-proteasome system is activated. *The E3 ligase* anaphase-promoting complex/cyclosome (APC/C)-cadherin 1 (Cdh1) complex (APC/C-Cdh1) acts before the G1 peak, whereas SKP1/CUL-1/F-box protein (SCF)–β-transducin repeats-containing proteins (β-TrCP; SCF–β-TrCP) are activated

thereafter [15]. The phosphorylation by oncogene proviral insertion in murine lymphomas 2 (PIM2) reduced the levels of ubiquitination and proteasomal degradation of PFKFB3 as well [16]. PFKFB3 levels and activity increase in later phases of the cell cycle whether there is mitotic arrest [17]. Thus, PFKFB3 is an important intersection between cell-cycle machinery and metabolic supply. A summary of the main cell functions of PFKFB3 is shown in Figure 1.

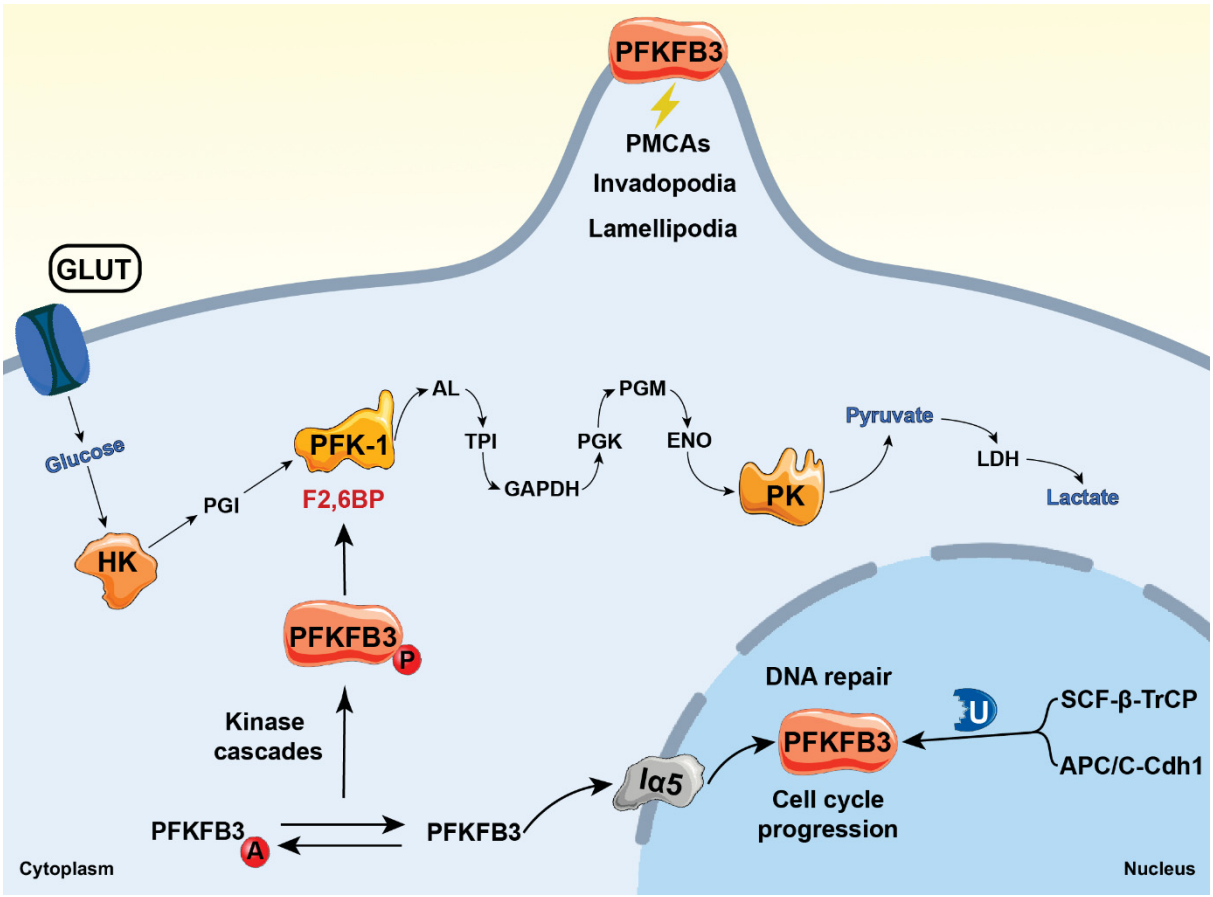

Fig. 1. PFKFB3 localization and function in cells. In the plasma membrane, PFKFB3 is responsible for glycolytic ATP supply to plasma membrane calcium ATPases (PMCAs), lamellipodia, and invadopodia. In the cytoplasm, PFKFB3 is phosphorylated by a series of kinase proteins and regulates the glycolytic enzyme PFK-1 through the production of F2,6BP. PFKFB3 contains a nuclear localization signal (NLS), allowing enzyme trafficking to the nucleus via importin complex α5 (Iα5). PFKFB3 acetylation prevents NLS recognition and causes PFKFB3 cytoplasmic retention. PFKFB3 has a non-canonical function in the nucleus, controlling cell-cycle progression and DNA repair in specific contexts. The ubiquitin ligase complexes APC/C-Cdh1 and SCF–β-TrCP target PFKFB3 for proteasomal destruction. PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3;

PMCAs, plasma membrane calcium ATPases; PFK-1, 6-phosphofructo-1-kinase; F2,6BP, fructose-2,6-biphosphate; NLS, nuclear localization signal; Iα5, importin complex α5; APC/C-Cdh1, anaphase-promoting complex/cyclosome-cadherin 1; SCF–β-TrCP, SKP1/CUL-1/F-box protein–β-transducin repeats-containing proteins. (Figure produced by Adobe Illustrator 2021 and Adobe Photoshop 2021 softwares).

4.4 MODULATION OF CARCINOGENESIS BY PFKFB3

A vigorous glycolytic rate is typical for cells that undergo rapid proliferation, such as cancer cells, becoming a sustained state and distinctive aspect of these cells [8]. This cancerous-metabolic shift was first described by Otto Warburg in rat carcinoma (Flexner-Jobling type) and sarcoma (Jensen type) [18] and is known as the Warburg effect. Tumor tissue utilizes glycolysis and diverts a large amount of the product, pyruvate, for fermentation instead of mitochondrial respiration, generating lactic acid [18]. This glycolytic fueling is described as a hallmark of cancer [19]. Some theories support the pro-tumor characteristics of glycolysis, such as its involvement in fast ATP synthesis, cell signaling, biosynthesis, and tumoral microenvironment maintenance [20].

PFKFB3 is abundant in several solid human tumors *in situ*, including carcinomas in colon, prostate, breast, ovary, liver, thyroid, tongue, nasopharynge, lung, head, and neck [4, 9, 21–25]. It has been described as an independent prognostic marker in lung cancer and hepatocellular carcinoma, although the phosphorylated form of PFKFB3 (p-PFKFB3) appears to be more accurate in gynecologic cancers [24, 26, 27]. PFKFB3 is essential to life and associated with circadian homeostasis, proliferative markers, and key cell cycle proteins [22, 24, 28]. However, its presence in a homozygote condition was associated with a higher chance of developing cancer in a model of intestinal neoplasia, linking PFKFB3 activity to cell transformation [4].

In addition to supporting the unrestrainable metabolism of neoplastic cells, PFKFB3 exerts an acidifying effect in the extracellular microenvironment, contributing to angiogenesis, immunosuppression, and metastasis [22, 23, 29]. To mitigate these pro-tumoral effects, several antiglycolytic (anti-PFKFB3) agents have been developed and tested [30]. The main effects of the pharmacological disruption of PFKFB3 include a reduction of F2,6BP production, impairments in glucose uptake, and lower rates of proliferation [31]. Additional outcomes have also been described, depending on cell type, antiglycolytic regimen, and synergistic effects with therapies (Table 1).

Tumor cells, especially under hypoxic conditions, undergo cell cycle arrest and cell death when PFKFB3 is blocked [25, 32]. The chalcone derivatives 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and 1-(4-pyridinyl)-3-[7-(trifluoromethyl)-2E-quinolinyl]-2-propen-1-one (PFK158) are among the most studied PFKFB3 antagonists, exerting either competitive or noncompetitive inhibitory effects on kinase activation [30, 32]. In general, they were well-tolerated drugs *in vitro* and *in vivo*, but with different pharmacokinetics properties, selectivity, and potency. The low solubility of 3PO limited its use, associated with the occurrence of nonspecific disturbances in normal cells, as cardiac cells, despite the fact that cardiac function was not affected. Conversely, the PFK15 inhibitor had limited effects in normal cells, whereas PFK158 inhibitor did not show adverse effects in animals or human trials [20, 32–34]. PFK158 was the first PFKFB3 antagonist that was tested in a Phase I clinical trial (NCT02044861) [35], which resulted in no toxicity up to a dose of 96 mg/m^2, but no further results were published.

Table 1 PFKFB3 as a target in anti-cancer therapy studies

Inhibitor	Type of study	Treatment combination	Concentration or dose range	Additional effect in cells or tumors	Reference
KAN0438757	*In vitro* (human cells: BJ TERT, BJ RAS; human cancer cells: MKN45, AGS, BCG823, MIA PaCa-2, PANC-1, NUGC-3, SW620, U2OS)	Ionizing radiation	10 μM	↓ Homologous recombination repair	[36]
Aspirin	*In vitro* (human cells: QSG-7701, LO2; human cancer cells: HCC-LM3, Huh7-R) *In vivo* (HCC-LM3 xenograft mouse model)	Sorafenib	*In vitro* (1-40 mM) *In vivo* (20, 50, and 100 mg/kg)	Inhibition of HIF-1α	[37]
3PO	*In vitro* (murine cancer cells: B16-F10, Panc02; human cells: HUVECs) *In vivo* (panc02 syngeneic mouse model, B16-F10 syngeneic mouse model)	—	*In vitro* (10-500 μM) *In vivo* (25-70 mg/kg)	Apoptosis 25 mg/kg – tumor vessel normalization; 70 mg/kg – tumor vessel disintegration and fragmentation	[38]
3PO	*In vitro* (human cells: HUVECs)	Sunitinib	5-15 μM	—	[39]
3PO	*In vivo* (patient-derived GBM orthotopic xenografts in mice)	Bevacizumab, Doxorubicin	25 mg/kg	Tumor vessel normalization; ↓ tumor hypoxia	[40]
3PO and PFK15	*In vitro* (human cancer cells: K562, K562IR, K562IDR, MEG-01) *In vivo* (CML syngeneic mouse model, K562, K562IR, and K562IDR xenograft mouse model)	Imatinib, Dasatinib	*In vitro* (0.0001-10 μM) *In vivo* (10 mg/kg)	Apoptosis; abolishment of tyrosine kinase inhibitor resistance	[41]
PFK15	*In vitro* (human cells: GES-1; human cancer cells: MKN45, AGS, BCG823) *In vivo* (MKN45 xenograft mouse model)	—	*In vitro* (2.5-10 μM) *In vivo* (25 mg/kg)	Apoptosis; cell cycle arrest in G0/G1 phase	[33]
PFK15	*In vitro* (human cancer cells: THP1, OCI-AML3)	Rapamycin	0.1-5 μM	—	[42]

PFK15	*In vitro* (human cancer cell: ACHN HeLa)	—	3-6 μM	Inhibition of basal autophagy; inhibition of AMPK pathway	[8]
PFK15	*In vitro* (human cells: 16HBE; human cancer cells: A549)	—	5-50 μM	Apoptosis; cell cycle arrest in G0/G1 phase	[7]
PFK15	*In vitro* (human cancer cells: HNSCC, Cal27, FaDu) *In vivo* (Cal27 xenograft mouse model)	—	*In vitro* (1.25-10 μM) *In vivo* (10-20 mg/kg)	Apoptosis; cell cycle arrest in G2/M phase	[24]
PFK15	*In vitro* (RPMI8226, ARP-1, OPM2)	Metformin	0.5-3 μM	Apoptosis; cell cycle arrest in S phase	[43]
PFK15	*In vitro* (human cancer cells: MIA PaCa-2, BxPC-3, PANC1, HPSCs)	—	0.3-10 μM	Apoptosis; inhibition of plasma membrane calcium ATPases	[10]
PFK15	*In vitro* (human cells: HEK293T; human cancer cells: NCI-H292, MDA-MB231, PC3; murine cells: MEF; murine cancer cells: ELT3) *In vivo* (MEF xenograft mouse model)	Rapamycin	*In vitro* (1-5 μM)	—	[30]
PFK15	*In vitro* (murine cancer cells: BNL 1ME A.7R.1) *In vivo* (BNL syngeneic mouse model)	—	*In vitro* (1-20 μM) *In vivo* (25 mg/kg)	Apoptosis; tumor vessel normalization; ↓ tumor hypoxia	[27]
PFK15 and PQP	*In vitro* (human cancer cells: 5637, HT1197, HT1376, RT4, SW780, T24, TCCSUP, UM-UC-3, Caco-2, HCT116, HT29)	Phenformin, NHI-2	PQP, 5 μM-20 mM; PKF15, 1-5 μM	—	[44]
PFK158	*In vitro* (human cancer cells: jurkat) *In vivo* (LLC syngeneic mouse model, CT-26 syngeneic mouse model, Bx-PC3 xenograft mouse model)	Paclitaxel, Gemcitabine, Irinotecan	*In vitro* (0.0001-100 μM) *In vivo* (5-30 mg/kg)	—	[34]
PFK158	*In vitro* (human cancer cells: HeyA8,	Cisplatin, Carboplatin, Paclitaxel	*In vitro* (5-15 μM) *In vivo* (25	Autophagy; lipophagy	[26]

	HeyA8MDR, OVCAR5, OV2008, C13, OV90) *In vivo* (HeyA8MDR xenograft mouse model)		mg/kg)		
PFK158	*In vitro* (human cancer cells: MCF7, MCF7:5C, LCC9)	4-Hydroxytamoxifen, Fulvestrant	0.5-25 μM	Apoptosis; ↑ reactive oxygen species	[45]
PFK158	*In vitro* (human cancer cells: EN1, HEC-1A, HEC-1B, ARK-2, SPAC1L) *In vivo* (HEC-1B, ARK-2 xenograft mouse model)	Carboplatin, Cisplatin	*In vitro* (0-20 μM) *In vivo* (35 mg/kg)	Apoptosis; autophagy; ↓ homologous recombination repair; inhibition of AKT and mTOR pathway	[46]
PFK158	Phase I clinical trial	—	24, 48, and 96 mg/m^2	—	[35]

LEGEND: KAN0438241, (4-{[(5'-fluoro-2'-hydroxybiphenyl-3-yl)sulfonyl]amino}-2-hydroxybenzoic acid; 3PO, (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one); PFK15, (1-[4-pyridinyl]-3-[2-quinolinyl]-2-propen-1-one); PFK158, (1-[4-pyridinyl]-3-[7-(trifluoromethyl)-2E-quinolinyl]-2-propen-1-one).

In preclinical studies, cell cycle arrest that is caused by PFKFB3 blockade has often been described, causing cytostatic or cytotoxic effects, depending on the treatment regimen. Cell death occurs mainly via apoptosis but also via autophagy. Despite these data, the clinical application and feasibility of anti-PFKFB3 therapy are still under consideration, particularly when considering the intrinsic complexity and variability of cancer. Although PFKFB3 is more expressed in neoplastic cells and therefore could be a potential therapeutic target, the matter of off-target issues that are observed in competitive kinase inhibition therapy is important. To date, studies indicate high specificity of binding to PFKFB3, especially with PKF158, in a group of more than 90 different kinase enzymes tested [30]. In this field, studies focusing on the activation of PFKFB3 bisphosphatase have been in evidence lately [47], because instead of blocking an essential enzyme for cell homeostasis, there is a normalization of the K:P ratio.

Considering the variability of tumor types, the following sections focus on the multi-level cancerous proprieties of PFKFB3 in breast cancer.

4.5 PFKFB3 IN BREAST CANCER

Breast cancer is the most prevalent and main cause of death in the female population [48]. Because of their highly heterogeneous properties, breast tumors are often classified based on their hormone receptor status (i.e., estrogen receptor [ER] and progesterone receptor [PR] expression) and the expression of human epidermal growth factor receptor type 2 (HER2). Four major immunophenotypes have been reported: luminal A ($ER^+/PR^+/HER2^-$), luminal B ($ER^+/PR^{+/-}/HER2^{+/-}$), HER2 enriched ($ER^-/PR^-/HER2^+$), and triple negative breast cancer (TNBC; $ER^-/PR^-/HER2^-$) [49]. Similar to other cancer types, breast cancer presents aberrant metabolism and its growth is supported by the Warburg effect.

Substantially high PFKFB3 expression is observed in human primary and metastatic breast cancer cells compared with healthy breast tissue, and higher levels of PFKFB3 were associated with poorer survival rates. Consequently, PFKFB3 expression increases proportionally to tumor staging, and it can be used as a prognostic marker for breast cancer [31, 50–52]. Additionally, PFKFB3 phosphorylation has been detected mostly in marginal and invading areas of breast carcinomas, suggesting that the modification of this enzyme might play a relevant role in metastasis [13, 21]. As expected, the differential distribution of PFKFB3 has been reported between breast cancer subtypes, and PFKFB3 expression is exacerbated in TNBC and HER2 enriched tumors [53]. PFKFB3 mRNA and protein expression is generally higher in more aggressive and metastatic cancers, representing an important malignancy factor when it is present [53]. In breast

cancer, it has been proposed that PFKFB3 beyond the regulation of aerobic glycolysis, cell proliferation, and migration, also functions as a mechanism of therapeutic escape, directly related to paclitaxel resistance [16]. Several factors contribute to the control of PFKFB3 activity in breast cancer. In the sections below, some of these interactions are summarized according to breast cancer subtype.

4.5.1 Luminal A/B subtypes

The imbalance in hormonal exposure is often linked to breast cancer risk, because ovarian steroid hormones are involved in controlling mammary gland expansion. Therefore, the expression of estrogen and progesterone receptors can create a selective advantage for growth of tumor cells. Estrogen and progesterone bind to steroid hormone receptors (SHRs), which act as transcription factors when binding to hormone-responsive elements (HREs) in the promoter region of regulated genes [54].

At the transcriptional level, PFKFB3 can be regulated by 17β-estradiol (E2), the main female sex hormone. The E2 transcriptional effect on MCF-7 (ER^+/PR^+) cells occurs within 1 h after the initiation of treatment and increases PFKFB3 mRNA and protein levels within 3-6 h [55]. This occurs through recruitment of the ERα subtype to estrogen response element (ERE) in the *PFKFB3* promoter [55]. Metabolic modifications that are activated by estrogen depend on ER membrane-initiated intracellular signaling that engages in crosstalk with kinase cascades, such as the mitogen-activated protein kinase (MAPK)/ERK and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways [56, 57]. Alternatively, ER-independent machinery can be engaged for estrogen to alter cell metabolism, including the G protein-coupled estrogen receptor (GPER). This machinery mediates

the interface between E2 and PFKFB3, decreasing the PFKFB3 turnover mainly via the ubiquitin-proteasome system [58].

Estrogen interferes with epigenetic RNA-mediated processes, specifically by modulating the microRNA (miRNA) miR-206, a tumor-suppressive factor. miR-206 binds to the 3'-untranslated region of PFKFB3 mRNA and prevents the activation of glycolysis, which was antagonized by E2 [59].

Progesterone (P4) has been reported to promote *PFKFB* gene expression [50]. This effect is achieved by long induction with P4 through the binding of PR isoform B (PRB) to progesterone response elements (PREs) in the *PFKFB3* promoter [5]. Genomic activation, however, depends on nongenomic activation of the p-PR_B/p-extracellular signal-regulated kinase (ERK)/p-mitogen- and stress-activated protein kinase-1 (MSK1) complex, which prepares chromatin for transcription [54]. The genomic and nongenomic modulation of steroid hormones are generally seen as independent pathways, but ERK phosphorylation leads to the robust phosphorylation and activation of 90 kDa ribosomal protein S6 kinase (p90RSK) and finally PFKFB3 [5, 54]. Thus, the upregulation of glycolytic fueling by PR signaling follows a bimodal and interconnected mechanism.

The synergistic control by ERs and PRs over PFKFB3 transcription and phosphorylation efficiently connects energy demand to PFKFB3 abundance in luminal cells. On the other hand, the presence of these receptors does not appear to be the main determinant of the glycolitic profile in luminal breast cancer, since it has an intermediate metabolic phenotype when considering glycolysis to oxidative phosphorylation (OXPHOS) [60].

4.5.2 HER2 enriched subtype

HER2 is an orphan ligand receptor that triggers the activation of cell proliferation survival, and cell cycle progression very efficiently. HER2-positive breast cancer cells exhibit a typical glycolytic phenotype, with high glucose uptake, a high rate of glucose-dependent proliferation, low mitochondrial activity, and high PFKFB3 expression compared with normal cells [53, 60–62]. The integration between upstream growth and proliferation pathways and PFKFB3 was demonstrated by the increase in PFKFB3 expression in non-tumoral cells after transfection with HER2 [31]. Treatment with the PFKFB3 antagonist 3PO led to a decrease in glycolysis rate and cell viability, and treatment with the HER2 antagonist lapatinib dose-dependently reduced the expression of PFKFB3 [31].

Massive glycolysis is a metabolic characteristic of $HER2^{+}$ tumors that are responsive to anti-HER2 treatment [61]. By comparison, genetically engineered mouse-derived breast tumors exhibited expected glycolytic rates that were associated with HER2 expression, whereas HER2-downregulated cells (i.e., in dormancy, regression, and regrowth states) switched glycolysis to OXPHOS [62]. Similar results were reported in several cell lines after the suppression of glycolysis, which led to the activation of autophagy, driving amino acids to feed mitochondrial OXPHOS [63]. In this case, an inverse relationship was found between autophagy and PFKFB3 [53, 64]. Breast cancer cells in the dormancy state elicited the $PFKFB3^{Low}Autophagy^{High}$ phenotype, whereas cells in the metastatic state exhibited a $PFKFB3^{High}Autophagy^{Low}$ phenotype, in which PFKFB3 expression was critical for sustaining stem cell-like characteristics [53]. The escape from dormancy is not driven by the *PFKFB3* gene alone—however its expression in HER2-enriched is related to lower relapse-free survival [53].

The presence of the HER2 receptor and the massive activation of glycolysis confer a favorable growth profile to these cells and is based on the activation of kinase cascades such as PI3K/AKT, MAPK and protein kinase C (PKC). In this case, the activation of PFKFB3 is a fast and synchronized post-transcriptional process.

4.5.3 TNBC subtype

TNBC cells exhibit the more aggressive and higher glycolytic phenotype [60, 65]. Glucose is the preferential substrate for TNBC cells, but these cells can survive, even without growing, in its absence [66]. To sustain this enriched glycolytic profile, the cells directly or indirectly upregulate PFKFB3. The latter occurs using the glycolytic intermediate F1,6BP, which phosphorylates epidermal growth factor receptors (EGRRs) to further enhance glycolysis signaling via c-Src-PFKFB3 [51, 65].

Among the factors that favor TNBC malignancy, breast cancer gene 1 (BRCA1) acts as an important maintainer of the metabolic resilience of tumors [67]. When a BRCA1-mutated TNBC cell line was transfected with a plasmid that encoded the wildtype BRCA1 gene, it downregulated five genes that are involved in glycolysis, including *PFKFB3* [68]. Additionally, BRCA1 mutation mediates the phosphorylation of AKT, which has been linked to an increase in PFKFB3 [25, 68, 69]. TNBC cell lines present high levels of PFKFB3 and are dependent on PFKFB3 to migrate, invade, and proliferate [51]. Distant metastasis, an important cause of death in TNBC patients, is associated with high PFKFB3 expression. Thus, the blockade of PFKFB3 decreases the levels and activity of signal transducers and activators of transcription 3 (STAT3), a pivotal protein in breast cancer metastasis [51, 70, 71].

Additionally, silencing of the *PFKFB3* gene in breast cancer cells drives the loss of angiogenic activity by suppressing *vascular endothelial growth factor* (VEGF),

the most abundant vascular growth factor that is expressed by breast cancer tumors [51, 72]. Besides this direct suppression of solid tumor growth, blocking PFKFB3 has a marked effect on tumor endothelial cells, leading since vascular barrier tightening to tumor vessel disintegration, depending on the 3PO concentration used [38]. Therefore, PFKFB3 may be used as a therapeutic target not only in TNBC but also in tumor endothelial cells to inhibits tumor growth and metastasis [38]. As seen for $HER2^{+}$ tumors, in TNBC tumors the main activators of PFKFB3 are the kinase enzymes.

The impact of PFKFB3 on different breast cancer subtypes is complex and certainly is not a main determinant of the glycolytic phenotype, which consists of a wide network of pro-survival pathways. However, PFKFB3 presents notable differences in distribution. In luminal tumors, hormonal effects of E2 and P4 on PFKFB3 are prominent. Independent of this control, however, the enzyme's actions are more intense in HER2 enriched and TNBC tumors. The presence of growth factor pathways appears to be paramount in this case. The non-receptor tyrosine kinase Src plays a key role in the direct activation of PFKFB3 and underlying kinase cascades. The levels of p-Src and phosphorylation of PFKFB3 at Tyr194 were higher in the MDA-MB-231 ($ER^{-}/PR^{-}/HER2^{-}$) TNBC cell line than in the MCF-7 luminal A (ER^{+}/PR^{+}) cell line. Additionally, levels of p-Src and phosphorylation of PFKFB3 were activated even under scarce glucose concentration, a condition in which PFKFB3 activation by AMPK would be expected [4]. By stimulating catabolic pathways, PFKFB3 makes available large amounts of metabolic intermediates for the synthesis of nucleic acids [4], thereby supporting the stronger cell proliferation of HER2 enriched and TNBC subtypes.

4.6 COMPONENTS OF PFKFB3 CELL SIGNALING CASCADES

A plethora of growth pathways and oncogenic factors can switch PFKFB3 to control glycolytic metabolism, even under conditions of low glucose availability [51]. The most important interconnection between PFKFB3 and these intrinsic and extrinsic effectors is outlined below and presented schematically in Figure 2.

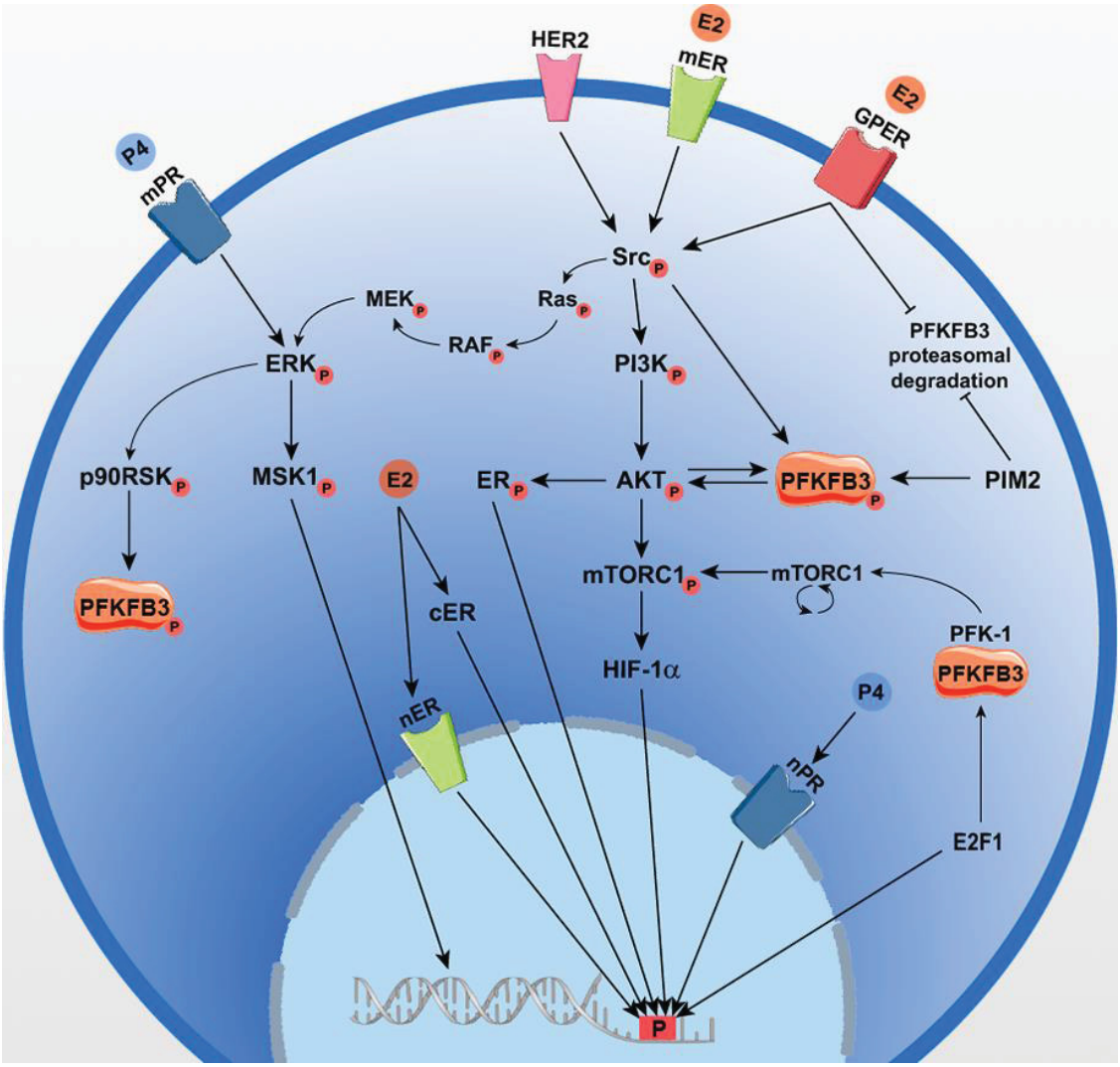

Fig. 2 PFKFB3 crosstalk with breast cancer-related receptors and cell signaling cascades. Estrogen receptors (GPER, membrane ER [mER]), progesterone receptors (membrane PR [mPR]), and HER2, once stimulated, activate downstream kinase cascades, with transcriptional and post-transcriptional effects on PFKFB3 activity. mPR activation leads to MSK1 phosphorylation, preparing chromatin for transcription, and P90RSK phosphorylation phosphorylates PFKFB3. Src is a convergence point for mER, GPER, and HER2 receptor activation, further activating the PI3K/AKT/mTOR and MAPK pathways. PFKFB3 is able to activate upstream kinases, such as mTORC1 and AKT. AKT directly phosphorylates PFKFB3 and ERs.

The stabilization of PFKFB3 occurs through the action of E2 and PIM2. Post-transcriptional regulation follows the binding of transcription factors in the PFKFB3 promoter region (P), which also involves nuclear ER (nER), cytoplasmic ER (cER), and nuclear PR (nPR) activation. The global effect is metabolic remodeling that favors glycolysis. AKT, protein kinase B; E2, 17β-estradiol; E2F1 E2F transcription factor 1; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GPER, G protein-coupled estrogen receptor; HER2, human epidermal growth factor receptor type 2; HIF-1, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; mTORC1, mammalian/mechanistic target of rapamycin complex 1; MSK1, mitogen- and stress-activated protein kinase-1; P4, progesterone; p90RSK**,** 90 kDa ribosomal protein S6 kinase; PIM2, proviral insertion in murine lymphomas 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor. (Figure produced by Adobe Illustrator 2021 and Adobe Photoshop 2021 softwares).

4.6.1 AKT/mTOR

The axis-PI3K/AKT/mammalian/mechanistic target of rapamycin (mTOR) is usually considered a linear signal transduction cascade that controls the destination of glucose in cells. However, it is not sequentially or equally activated in breast cancer tumors [73]. The enriched p-AKT signature is more common in the luminal A subtype and strongly related to ER activation even in the absence of E2 [74]. The p-mTOR signature is enriched in luminal B and $HER2^{+}$ subtypes and in endocrine-resistant breast cancer [73, 75].

AKT improves the translocation of glucose transporters to the plasma membrane at the same time that phosphorylates glycolytic enzymes, such as HK and PFKFB [76]. Specifically, PFKFB3 has a conserved site for phosphorylation by AKT at the Ser478 residue, beyond the usual Ser461 residue [77, 78]. Pharmacological interference in PFKFB3 activity restrains AKT signaling and insulin-stimulated glucose uptake by glucose *transporter* translocation to the plasma membrane [79]. PFKFB3 is believed to regulate the expression and phosphorylation of AKT [25]. Therefore, in addition to the cell's energy status, PFKFB3 increases rates of

glycolysis, with positive feedback control over both PFK-1 and the AKT pathway in a cyclic manner [79].

The mTOR pathway is known to induce glycolysis via downstream transcription factors, such as hypoxia-inducible factor 1α (HIF-1α) and Myc [80, 81]. HIF-1α, a subunit of HIF-1, binds to a specific HRE in the *PFKFB3* gene promoter region and enhances its transcription under hypoxic conditions [12]. Furthermore, it can be targeted by activating the mTORC1 pathway, especially in conditions of loss of function of the tuberous sclerosis complex gene (*TSC1*/*TSC2*), a negative regulator of this pathway [42, 82]. Similarly, the PI3K/AKT/mTOR negative regulator phosphatase and tensin homolog (*PTEN*) is suppressed in a wide variety of sporadic cancers and plays a protective role in inhibiting PFKFB3, augmenting its interaction with the E3 ligase APC/C-Cdh1 [77, 83]. At the same time, as a mTOR target, PFKFB3 associates with PFK-1 at the lysosomal surface to drive new mTORC1 activation via E2F transcription factor 1 (E2F1) stimulation [80]. The interplay between energy-sensing pathways is enhanced by PFKFB3, independent of AMP-activated protein kinase (AMPK) signaling.

4.6.2 AMPK

AMPK is a central energy gauge responsive to metabolic stress. A linear correlation was described between expression levels of AMPK and PFKFB3 in human breast tumors, although lower p-AMPK expression was observed in breast cancer compared with breast non-cancerous tissue [52, 84]. In this scenario, AMPK is often impaired because of PI3K/HER2 activation [85].

Genotoxic stress and mitotic arrest have a combined and sequential effect on AMPK and PFKFB3 phosphorylation, conferring resistance to treatment by protecting cancer cells from death [7, 17]. AMPK plays a fundamental role in adaptation of the

glycolytic response under conditions of physiological stress, in which the low-pH-adapted phenotype induces activation of the AMPK pathway and phosphorylation in PFKFB3 [86]. This process increases the glucose consumption and glycolysis rate but keeps ATP levels low compared with normal-pH cells. The benefit of this mechanism is to propagate the activation of AMPK and glycolysis shift [86].

Energy deprivation can induce activation of the AMPK pathway and autophagy to guarantee the cell survival, in a PFKFB3-dependent way. The PFKFB3 blocked by PFK15 mitigated the levels of basal autophagy and some autophagy-challenges mediated by AMPK [8]. This relationship is mainly due to the nuclear form of PFKFB3 and it may be associated with the positive effect of Fru-2,6-BP on the cell cycle machinery and the crucial role of PFKFB3 in DNA repair [36]. From this point of view, metabolic alterations are not the endpoint in cell survival but rather an active and adaptive process that rely in PFKFB3 activity.

4.6.3 MAPK

The MAPK pathway controls the transcription and activation of protein kinases relevant to the Warburg effect. Cellular processes of cell immortalization and transformation is closely coupled to the MAPK oncogenic downstream factor *RAS* and PFKFB3 [87].

In breast cancer, the expression of MAPK is related to ER^+ tumors and the apoptotic markers B-cell lymphoma 2 (BCL-2) and p53, suggesting a better outcome [57]. The MAPK association with $HER2^+$ tumors may occur, particularly in ER^- tumors, which present a poorer prognosis because of alterations of proliferative signals (i.e., p38 phosphorylation and p53 mutation) [57, 88].

Cancer cell lines that are exposed to stress stimuli initiate a cascade that involves the phosphorylation of p38 MAPK, MAPK-activated protein kinase 2 (MK2),

and PFKFB3, resulting in an increase in F2,6BP [14]. The intermediate phosphorylation of SRF mediated by MK2 lead to up-regulation in PFKFB3 gene expression after SRF binds in PFKFB3 promoter region [14]. The dual modulation of PFKFB3 through MAPK promotes a very rapid response, within 30 min in the case of transcriptional regulation, ensuring the adaptive potential of cancer cells to survive in an inhospitable microenvironment.

In parallel, p38 MAPK/MK2 signaling is the mechanism that underlies actions of the hedgehog (HH) signaling pathway on PFKFB3 phosphorylation [89]. The HH ligand sonic hedgehog (SHH) is a morphogen peptide that is secreted by epithelial cells and participates in the proliferation, differentiation, and cell polarity of normal mammary tissues [90]. Additionally to short-term activation of PFKFB3, aberrant activation of the HH pathway increases the transcription of glycolytic enzymes as HK and PK and presents a positive correlation between SHH and tumor size/stage in primary breast carcinomas [89].

4.6.4 NF-κB

The NF-κB pathway confers plasticity against physiological and environmental stressors. In breast cancer, NF-κB activation generally results in tumors with a higher grade (e.g., III), which translates into faster-growing cancers that are more likely to spread and be larger, ER^- and PR^-, and $HER2/neu^+$ and have a poorer prognosis [91].

The *PFKFB3* gene has a κB site in its promoter region and is positively regulated by NF-κB at the transcriptional level. PFKFB3 inhibition also suppress NF-κB signaling in hyperglycolytic tumor endothelial cells (ECs). This occurs because NF-κB is a lactate-responsive transcription factor [92]. A study of breast cancer cells

found that PFKFB3 interacted with a regulatory component of the NF-κB pathway, inhibitor of nuclear factor κB kinase subunit β (IKKβ) [93]. Glutamine deprivation guides IKKβ to phosphorylate PFKFB3 at Ser269, consequently decreasing its activity [93]. Low glutaminolysis requires a coordinated reduction of glycolysis to guarantee cell homeostasis.

Not only tumor cells benefit from PFKFB3 stimulation. Monocytes that are stimulated by tumor-derived soluble factors also exhibit PFKFB3 upregulation. This, in turn, stimulates NF-κB to induce programmed cell death ligand 1 (PD-L1) expression, resulting in impairments in T-cell activity and tumor escape [94]. Immunosuppressive cells, such as Th17 and myeloid-derived suppressor cells (MDSCs), also overexpress PFKFB3 [95]. From this point of view, anti-PFKFB3 therapy, in addition to antiproliferative properties, can be seen as an immunostimulatory therapy, overcoming the shift to a low immune response dictated by PFKFB3.

4.6.5 p53

p53 protein ensures the fidelity of cellular biological information over successive cell generations. The overall frequency of mutations of *p53* in breast cancer is lower than in other solid tumors and is heterogeneously distributed (e.g., less frequent in luminal A and B and present in the majority of $HER2^+$ and TNBC) [96, 97].

In cancer, the loss of p53 is an important source of genetic variation and contributes to the Warburg effect [98]. Interconnections between energy metabolism and tumor suppression arise from the fact that, as a tumor suppressor, p53 redirects glucose to PPP to maintain adequate nucleotide homeostasis to facilitate DNA repair

[99]. Thus, p53 activation causes the repressed expression of PFKFB3 through the binding of p53 in the *PFKFB3* promoter region [99].

p53 also orchestrates glycolysis by regulating posttranslational mechanisms. The p53-induced TP53 glycolysis and apoptosis regulator (TIGAR) has a catalytic domain that is similar to the bisphosphatase domain in PFKFB-family proteins, and it opposes the PFKFB3 production of F2,6BP [98]. TIGAR shunts glucose flux through the PPP and provides a reducing equivalent of NAPDH to neutralize reactive oxygen species, thereby avoiding apoptosis [1]. The long-noncoding RNA (lncRNA) actin γ 1 pseudogene (*AGPG*) is also a target of p53, the absence of which prevents APC/C-PFKFB3 ubiquitination, leading to PFKFB3 accumulation [100]. Therefore, metabolic reprogramming is under strict tumor-suppressive control, and PFKFB3 plays a vital role in sustaining survival and proliferative signals. Collectively, PFKFB3 is a robust biochemical interrupter in intricate cellular signaling machinery, directing metabolic reprogramming.

4.7 FINAL CONSIDERATIONS

PFKBF3 is a multifaceted enzyme that simultaneously modulates metabolism, cell proliferation, and DNA repair in tumor cells and the tumor environment. Its biochemical functions involve sustaining glucose fermentation, which benefits cell tumorigenesis through the Warburg effect. Additionally, PFKFB3 translocates to different compartments in the cell and can acquire non-canonical functions, such as controlling cell proliferation in the nucleus, invasiveness, and angiogenesis. Thus, PFKFB3 has recently been highlighted in preclinical cancer studies, including breast cancer, in which hormonal receptors and growth factor status are dominant drivers of malignancy. PFKFB3 is transcriptionally regulated by estrogen and progesterone.

The presence of ER, PR, or HER2 (or even their absence in the case of TNBC) leads to strong dependence on kinase cascades and their downstream effectors for PFKFB3 activation. PFKFB3 is a central integrating protein that interacts with important signaling pathways, such as AMPK, MAPK, AKT/mTOR, and NF-κB, and the mutant gene *p53* to orchestrate short- and long-term cancer-promoting responses (Fig. 3). PFKFB3 modulation appears to be a promising anti-cancer strategy that can break the feedback loop of the control of metabolism over cell survival and proliferation. Experimentally, PFKFB3 has been demonstrated to bypass the critical problem of intrinsic and acquired drug resistance when used in combination with standard chemotherapy. Special care must be taken with naturally glycolysis-addicted cells such as endothelial cells, stem cells and immune cells, that have aerobic glycolysis as a constitutive feature and that could suffer impairment of function with anti-PFKFB3 therapy. In the near future, an increasing number of *in vitro* and *in vivo* studies are expected to investigate features of PFKFB3 in cancer models and its translational application for the management of breast cancer.

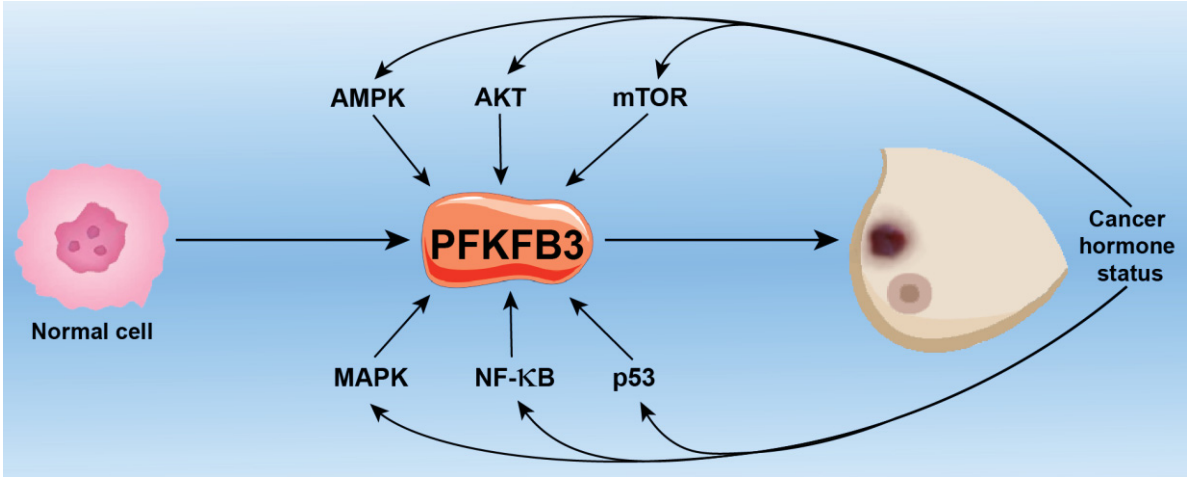

Fig. 3 The PFKFB3 enzyme is an oncogenic factor that modulates malignant transformation of breast cells and at the same time suffers positive feedback by the specific molecular characteristics of breast cancer**.** AKT, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; MAPK, mitogen-activated

protein kinase; mTOR, mammalian/mechanistic target of rapamycin; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation. (Figure produced by Adobe Illustrator 2021 and Adobe Photoshop 2021 softwares).

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5 CONSIDERAÇÕES FINAIS

Conforme o exposto neste trabalho, é possível propor a utilização de chalconas como terapêutica no contexto do câncer de mama em nível pré-clínico. A modulação metabólica parece ser um achado em comum das chalconas, embora com alvos farmacológicos diferentes. Determinou-se que a chalcona 4NC foi eficaz no tratamento do câncer de mama em linhagem de câncer de mama humana (MCF-7) e em um modelo tumoral murino singênico, sem apresentar toxicidade aguda e/ou durante o período experimental, neste último caso. O mecanismo de ação está associado à atenuação da via mTORC1-Raptor/S6K1, com impacto negativo sobre a síntese proteica e, ao mesmo tempo, indica que uma importante resposta fisiológica a esta privação biossintética - a autofagia - é concomitantemente bloqueada, limitando o crescimento e a proliferação das células tumorais. Os modelos experimentais utilizados, embora distintos em termos da classificação molecular clássica para o câncer de mama, sendo a linhagem MCF-7 hormonal dependente e o SEC hormonal independente, apresentaram resultados significativos de redução do crescimento tumoral, uma vez que a remodelação metabólica é uma qualidade universal do câncer. Ademais a descoberta de uma sinalização citoplasmática de estrógeno e progesterona observada no SEC é ainda um campo a ser estudado, para definir o potencial de transposição desta característica ao câncer de mama humano.

Por outro lado, na revisão de literatura abordando a enzima PFKFB3 em câncer de mama, a inibição enzimática foi o alvo das chalconas 3PO, PFK15 e PF158. A superexpressão da enzima PFKFB3 é uma base comum de vários cânceres para a manutenção da glicólise aeróbica e a resiliência ao dano celular e estresse. No câncer de mama, os tumores de fenótipo glicolítico como os que superexpressam HER2 e TN apresentam resultados superiores com a terapia anti-PFKFB3. Ainda que a enzima seja regulada positivamente por estrógeno e progesterona, a complexa sinalização de quinases PI3K/AKT/mTOR, AMPK e MAPK apresenta forte interação com a PFKFB3, em muitos casos com amplificação mútua sobre a sua ativação e um padrão de resposta rápida, mediada por processos de fosforilação. Entretanto, parece que a 4NC não interfere diretamente sobre PFKFB3 em células mamárias humanas. Isto confirma a variedade de mecanismos e efeitos biológicos dos diferentes compostos da ampla classe das chalconas.

A aplicação das chalconas no campo do metabolismo tumoral, pouco explorada até então, emerge como uma possibilidade interessante à terapia anticâncer. No entanto, estudos adicionais são necessários, envolvendo mais linhagens de câncer de mama e modelos animais para o câncer de mama, incluindo modelos xenográficos derivados de células tumorais de pacientes. A determinação do perfil de farmacocinética/farmacodinâmica das chalconas citadas, assim como de seu mecanismo de ação em detalhes, são desafios importantes à sua utilização futura em estudos clínicos.

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