



UNIVERSIDADE  
ESTADUAL DE LONDRINA

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FABRICIO SEIDY RIBEIRO INOUE

**3,3',5,5'-TETRAMETOXIBIFENIL-4,4'-DIOL INDUZ  
APOPTOSE E POTENCIALIZA A CITOTOXICIDADE DE  
DOXORRUBICINA VIA ESTRESSE OXIDATIVO,  
DISFUNÇÃO MITOCONDRIAL E AUTOFAGIA EM  
LINHAGENS DE CÂNCER DE MAMA TRIPLO NEGATIVO.**

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutor.

Orientador: Prof. Dr. Wander Rogério Pavanelli  
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Londrina  
2026

Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

- I583      Inoue, Fabricio Seidy Ribeiro.  
3,3',5,5'-Tetrametoxibifenil-4,4'-diol induz apoptose e potencializa a citotoxicidade de doxorubicina via estresse oxidativo, disfunção mitocondrial e autofagia em linhagens de câncer de mama triplo negativo / Fabricio Seidy Ribeiro Inoue. - Londrina, 2026.  
129 f. : il.
- Orientador: Wander Rogério Pavanelli.  
Coorientador: Carolina Panis.  
Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2026.  
Inclui bibliografia.
1. Câncer de mama triplo negativo - Tese. 2. Apoptose - Tese. 3. Estresse oxidativo - Tese. 4. Sinergismo - Tese. I. Pavanelli, Wander Rogério. II. Panis, Carolina. III. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. IV. Título.

CDU 616

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Londrina, 06 de Fevereiro de 2026.

Dedico este trabalho às pessoas que fizeram parte da minha trajetória científica e pessoal. Àquelas que, de diferentes formas, estiveram presentes, ofereceram apoio, incentivo e confiança, mesmo nos momentos mais difíceis. Cada gesto, palavra e aprendizado compartilhado contribuíram para que este caminho fosse possível e para que este trabalho se tornasse realidade.

## **AGRADECIMENTOS**

Agradeço, primeiramente, a Deus, que tem me abençoado e guiado diariamente, concedendo-me a força, a coragem e a perseverança necessárias para chegar até aqui. A Ele, toda a admiração e glória. À Santa Filomena, por ouvir minhas preces e interceder por mim diante das dificuldades que, em muitos momentos, pareceram impossíveis. Obrigado por transformar o impossível em possível.

Ao meu orientador, Prof. Dr. Wander Pavanelli, por ter estendido as mãos, aberto as portas de seu laboratório e me acolhido como parte de sua equipe. Sou profundamente grato por todo o apoio, pelos ensinamentos, pelas conversas e pela confiança depositada em mim. Agradeço, ainda, por ter me integrado à família que é o Laboratório de Imunoparasitologia de Doenças Negligenciadas e Câncer (LIDNC), do qual tenho imenso orgulho de fazer parte.

À minha coorientadora, Prof.<sup>a</sup> Dr.<sup>a</sup> Carolina Panis, que me inspira diariamente por seu vasto conhecimento em câncer de mama e por seu trabalho na região sul do país. Agradeço pelas conversas, orientações e por mediar a colaboração com a Dr.<sup>a</sup> Renata Binato, do Instituto Nacional do Câncer (INCA), a quem também agradeço pelo suporte técnico e científico na realização das análises transcriptômicas.

Agradeço às professoras do LIDNC, Prof.<sup>a</sup> Dr.<sup>a</sup> Maria Isabel Busch Pereira e Prof.<sup>a</sup> Dr.<sup>a</sup> Maiara Providello, pela amizade, pelas discussões científicas enriquecedoras e pelos conselhos ao longo dessa caminhada. Agradeço, de forma especial, à Prof.<sup>a</sup> Dr.<sup>a</sup> Danielle Bidóia, por todo o apoio, sobretudo por sua disposição em ir para a bancada e conduzir experimentos comigo até altas horas da noite. Eu não teria percorrido nem metade desse caminho sem você. Minha eterna gratidão.

Minha profunda gratidão ao Prof. Dr. Mario Sergio Mantovani e à sua equipe, por terem aberto as portas de seu laboratório e permitido a condução de diversos experimentos essenciais para a realização deste trabalho. Essa colaboração foi imprescindível para sua concretização.

Minha gratidão à Prof.<sup>a</sup> Dr.<sup>a</sup> Aneli Dekker, Prof. Dr. Robert Dekker e seus colaboradores do Laboratório de Moléculas Bioativas, pelo desenvolvimento e fornecimento do TMBP para a realização desse trabalho.

Meus agradecimentos à CAPES, ao CNPq e à Universidade Estadual de Londrina pelo financiamento e apoio institucional que tornaram este trabalho possível.

Agradeço novamente ao Prof. Dr. Mario Sergio Mantovani e à Prof.<sup>a</sup> Dr.<sup>a</sup> Flávia Guarnier por aceitarem contribuir com este trabalho em meu exame de qualificação. Estendo meus agradecimentos aos Professores Dr.<sup>a</sup> Flávia Guarnier, Dr. Fábio Seiva, Dr.<sup>a</sup> Danielle Bidóia e Dr.<sup>a</sup> Maria Isabel Busch Pereira por aceitarem compor esta banca de defesa e contribuírem com o resultado final deste trabalho.

Agradeço, de forma especial, à coordenadora do Programa de Pós-Graduação em Patologia Experimental (PPGPE), Prof.<sup>a</sup> Dr.<sup>a</sup> Flávia Guarnier, por todo o apoio desde meu ingresso no mestrado. Sou grato pela orientação durante o estágio em docência em Patologia Geral, experiência ímpar para minha formação acadêmica, bem como pelas discussões científicas que iluminaram minha trajetória em diferentes etapas. Agradeço por sua dedicação e desejo que continue sendo fonte de inspiração para muitos outros alunos.

A todo o time do LIDNC, minha sincera gratidão pela amizade, apoio e companheirismo. Agradeço, em especial, a Ana Carolina Rodrigues, Angélica Nunes, Giovana Sita e Heloisa Gomes pela parceria e amizade ao longo do doutorado. Às minhas amigas Ellen Cruz e Mariana Detoni, agradeço pela amizade construída ao longo desses seis anos. Obrigado por todo o apoio, conselhos, sessões de choro no laboratório e experimentos que se estenderam até tarde da noite. Vocês tornaram essa jornada mais leve.

Ao meu amigo João Manoel, jamais esquecerei sua chegada a Londrina e como nosso vínculo evoluiu de colegas de departamento para grandes amigos e parceiros de pesquisa. Agradeço por todos os experimentos que nos levaram a sair do laboratório de madrugada. Hoje não os vejo mais como dias exaustivos de trabalho, mas como tempo de qualidade e fortalecimento de nossa amizade. Saudades, meu amigo.

Agradeço aos meus alunos de iniciação científica e TCC, Miriã de Paula e Vinicius Sampaio, dos quais tenho imenso orgulho. Foi uma jornada de aprendizado mútuo, marcada por confiança e crescimento. Vinicius, continue seguindo seus sonhos, um futuro brilhante o aguarda. Miriã, que se tornou uma grande amiga, hoje também minha nutricionista, e em breve mestranda em Patologia Experimental, desejo que continue sendo essa pessoa sonhadora e encantadora. Que nossa amizade perdure por toda a vida; quero estar por perto para ver você brilhar.

Um agradecimento especial às amigas que fiz no primeiro ano no PPGPE, Liara Cavalcante, Raquel Nakama e Maria Isabel Bruno, por tudo o que

enfrentamos juntos, pelas lágrimas, pelo ombro amigo, pelos conselhos, pelos momentos de acolhimento e por terem me ajudado a enfrentar crises de ansiedade. Obrigado por tudo.

Minha eterna gratidão aos meus pais, meu alicerce e minha casa. Não existem palavras que alcancem a dimensão do amor, do cuidado e da entrega que sempre me ofereceram. Vocês foram força quando eu caí, luz quando tive medo e abrigo quando tudo parecia estar além das minhas forças. Obrigado por acreditarem quando eu duvidei, por me sustentarem quando eu pensei em desistir e por sonharem comigo antes mesmo que eu soubesse sonhar. Cada passo que dou carrega a marca do amor de vocês, cada conquista tem o reflexo da dedicação que sempre tiveram por mim. Amo vocês além do que cabe em palavras, além do tempo, além da distância. Amo vocês daqui até a lua e de volta.

Ao meu namorado, Renan Pimentel, com quem escolhi compartilhar a vida, deixo meu mais sincero agradecimento. Obrigado por ser presença constante, por seu carinho, lealdade e cuidado, e por essa personalidade encantadora e sorriso bobo, capaz de aliviar até os dias mais difíceis. Obrigado por seu apoio incondicional, por me dar forças quando pensei em desistir e trazer calma quando tudo parecia perdido. Obrigado por caminhar ao meu lado, por me fazer companhia no laboratório quando todos já foram embora, por acreditar e me apoiar diariamente na busca pelos meus sonhos. Sua chegada tornou meus dias mais leves, meus passos mais firmes e essa jornada mais possível. Que possamos seguir dividindo a vida, o amor e os sonhos, por muito tempo.

Aos meus melhores amigos, Déborah Souza, Fernanda Rebello e Wellington Silva, minha profunda gratidão por mais de uma década de amizade construída com presença, lealdade e afeto. Obrigado por estarem sempre ali, prontos para ouvir, aconselhar e acolher, especialmente nos momentos mais difíceis. Obrigado pela companhia, pelas conversas que confortam, pelos risos que aliviam e pelos instantes de leveza que tornaram essa caminhada mais tranquila. Vocês são minha família de coração, escolhida com amor. Obrigado por serem exatamente quem são.

Por fim, a todos que, de forma direta ou indireta, contribuíram para a realização deste trabalho e não foram aqui citados, meus sinceros agradecimentos.

“Entre dúvidas, noites longas e fé, aprendi que fazer ciência também é um ato de resistência.”

## RESUMO

INOUE, Fabricio Seidy Ribeiro. **3,3',5,5'-TETRAMETOXIBIFENIL-4,4'-DIOL INDUZ APOPTOSE E POTENCIALIZA A CITOTOXICIDADE DE DOXORRUBICINA VIA ESTRESSE OXIDATIVO, DISFUNÇÃO MITOCONDRIAL E AUTOFAGIA EM LINHAGENS DE CÂNCER DE MAMA TRIPLO NEGATIVO**. 2026. 129 p. Tese em Patologia Experimental – Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, 2026.

O câncer de mama triplo negativo (CMTN) é o subtipo mais agressivo e metastático, caracterizado por alta instabilidade genômica, mutações em *TP53*, dependência de CDK1 para progressão do ciclo celular e estresse oxidativo elevado. O 3,3',5,5'-tetrametoxibifenil-4,4'-diol (TMBP) demonstrou, por meio de análises de docking molecular, interação com CDK1 no sítio inibitório Tyr15, redução da expressão de *CDK1* e seletividade para células tumorais *TP53* mutantes. Neste trabalho, investigamos o perfil transcriptômico e os processos biológicos modulados pelo TMBP, bem como seus efeitos antitumorais isolado e em combinação com doxorubicina (DOX) em linhagens de CMTN. A atividade metabólica celular (resazurina) foi utilizada para determinar os valores da concentração inibitória de 50% (IC<sub>50</sub>) nas linhagens HB4a (não tumoral), MDA-MB-231 e MDA-MB-468. Na etapa transcriptômica, as células MDA-MB-231 tratadas com TMBP foram analisadas por microarranjo (Affymetrix Human GeneChip 1.0 ST), identificando genes diferencialmente expressos (DEGs) e vias biológicas enriquecidas. Esses achados foram validados por ensaios de apoptose, atividade de caspase-3/7 e ensaio de migração. Em seguida, as três linhagens foram tratadas com combinações TMBP+DOX e avaliadas por modelagem de sinergismo (ZIP), ensaio do cometa, ciclo celular, apoptose, ERO,  $\Delta\Psi_m$ , gotículas lipídicas e organelas vesiculares ácidas, atividade de caspase-3/7, quantificação de vacúolos autofágicos e análises bioquímicas para marcadores de estresse oxidativo. O tratamento com TMBP em células MDA-MB-231 resultou em 108 DEGs associados à apoptose, via TGF- $\beta$ /SMAD e reconhecimento/reparo de DNA, além de aumento de apoptose, ativação de caspase-3/7 e redução da migração celular. O tratamento combinado (TMBP+DOX) apresentou efeitos citotóxicos aditivos a sinérgicos seletivamente nas células de CMTN, preservando a viabilidade das células não tumorais. Mecanicamente, a associação potencializou a parada do ciclo celular em G1, potencializou a morte celular por apoptose, promoveu maior acúmulo de ERO e disfunção mitocondrial, evidenciada pela perda do  $\Delta\Psi_m$  e danos ultraestruturais. O tratamento induziu autofagia e elucidou seu papel citotóxico no processo de morte celular. A depleção de defesas antioxidantes causadas pelo tratamento, incluindo SOD, GPx e GSH, revelou colapso redox, particularmente em células MDA-MB-231, destacando a suscetibilidade específica desse subtipo ao estresse oxidativo. Em conjunto, os dois estudos demonstram que o TMBP apresenta potente atividade antitumoral, isolado ou associado à DOX, reforçando o TMBP como um candidato terapêutico promissor para terapias direcionadas ao CMTN e outros tumores que compartilham de vulnerabilidades similares.

**Palavras-chave:** Câncer de mama triplo negativo; TMBP; Apoptose; Estresse oxidativo; Sinergismo.

## ABSTRACT

INOUE, Fabricio Seidy Ribeiro. **3,3',5,5'-TETRAMETHOXYBIPHENYL-4,4'-DIOL INDUCES APOPTOSIS AND POTENTIATES THE CYTOTOXICITY OF DOXORUBICIN VIA OXIDATIVE STRESS, MITOCHONDRIAL DYSFUNCTION, AND AUTOPHAGY IN TRIPLE-NEGATIVE BREAST CANCER CELL LINES**. 2026. 129 p. Thesis in Experimental Pathology – Center of Biological Sciences, State University of Londrina, Londrina, 2026.

Triple-negative breast cancer (TNBC) is the most aggressive and metastatic subtype, characterized by high genomic instability, *TP53* mutations, elevated basal oxidative stress, and strong dependence on CDK1 for cell cycle progression. The compound 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol (TMBP) has been shown, through molecular docking analyses, to interact with CDK1 at the inhibitory Tyr15 site, reduce *CDK1* expression, and exhibit selectivity toward *TP53*-mutant tumor cells. In this study, we investigated the transcriptomic profile and biological processes modulated by TMBP, as well as its anticancer effects alone and in combination with doxorubicin (DOX) in TNBC cell lines. Cell metabolic activity was used to determine the inhibitory concentration of 50% (IC<sub>50</sub>) values for HB4a (non-tumoral), MDA-MB-231, and MDA-MB-468. For transcriptomic analysis, TMBP-treated MDA-MB-231 cells were profiled using the Affymetrix Human GeneChip 1.0 ST array to identify differentially expressed genes (DEGs) and enriched biological pathways. These findings were functionally validated by apoptosis, caspase-3/7 activity, and migration assays. Subsequently, the three cell lines were treated with TMBP+DOX combinations and evaluated through ZIP synergy modeling, cell cycle, apoptosis, ROS,  $\Delta\Psi_m$ , lipid droplets, and acidic vesicular organelles, comet assay, autophagic vacuole quantification, and biochemical measurements of oxidative stress markers. TMBP-treated MDA-MB-231 cells resulted in 108 DEGs associated with apoptosis, TGF- $\beta$ /SMAD signaling, and DNA recognition/repair, along with increased apoptosis, enhanced caspase-3/7 activity, and reduced cell migration. Combined treatment (TMBP+DOX) produced additive to synergistic cytotoxic effects selectively in TNBC cells while preserving the viability of non-tumorigenic cells. Mechanistically, the combination enhanced G1 cell cycle arrest, potentiated apoptotic cell death, and promoted increased ROS accumulation and mitochondrial dysfunction, as evidenced by  $\Delta\Psi_m$  loss and ultrastructural damage. The treatment also induced autophagy and elucidated its cytotoxic role in the cell death process. Depletion of antioxidant defenses induced by treatment, including SOD, GPx, and GSH, revealed redox collapse, particularly in MDA-MB-231 cells, highlighting the specific susceptibility of this subtype to oxidative stress. Collectively, these findings demonstrate that TMBP exhibits potent antitumor activity, either alone or in combination with DOX, reinforcing TMBP as a promising therapeutic candidate for TNBC and other tumors that share similar biological vulnerabilities.

**Key-words:** Triple-negative breast cancer; TMBP; Apoptosis; Oxidative stress; Drug synergy.

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## LISTA DE ABREVIATURAS E SIGLAS

|         |                                                           |
|---------|-----------------------------------------------------------|
| 2,6-DMP | 2,6-Dimetoxifenol                                         |
| AKT     | AKT Serina/Treonina Quinase 1                             |
| ATG     | Gene Relacionado à Autofagia                              |
| BL1/2   | CMTN Basal- <i>like</i> 1/2                               |
| BRCA1/2 | Gene do Câncer de Mama 1/2                                |
| CAK     | Quinase ativadora de CDK                                  |
| CDH1    | Gene da Proteína E-caderina                               |
| CDK     | Quinase Dependente de Ciclina                             |
| CKI     | Inibidor de CDK                                           |
| CMTN    | Câncer de Mama Triplo Negativo                            |
| DEGs    | Genes Diferencialmente Expressos                          |
| DOX     | Doxorrubicina                                             |
| E2F3    | Fator de Transcrição E2F 3                                |
| EGFR    | Receptor do Fator de Crescimento Epidérmico               |
| ERO     | Espécies Reativas de Oxigênio                             |
| FGF     | Fator de Crescimento Fibroblástico                        |
| HER2    | Receptor tipo 2 do Fator de Crescimento Epidérmico Humano |
| ICESP   | Instituto de Câncer do Estado de São Paulo                |
| IARC    | Agência Internacional de Pesquisa em Câncer               |
| JAK     | Janus quinase                                             |
| JNK     | Kinase c-Jun N-terminal                                   |
| Ki67    | Marcador da proteína Ki-67                                |
| LAR     | CMTN Receptor Luminal Androgênico                         |
| MCDA    | Morte Celular Dependente De Autofagia                     |

|              |                                                                             |
|--------------|-----------------------------------------------------------------------------|
| MCMA         | Morte Celular Mediada por Autofagia                                         |
| MES          | CMTN Mesenquimal                                                            |
| MET          | Receptor do fator de crescimento de hepatócitos                             |
| MMPs         | Metaloproteinases de matriz                                                 |
| mTOR         | Alvo de Rapamicina em Mamíferos                                             |
| MYC          | Proto-oncogene MYC                                                          |
| NF-kB        | Fator Nuclear Kappa B                                                       |
| PI3K         | Fosfatidilinositol 3-Quinase                                                |
| PIK3CA       | Gene Fosfatidilinositol-4,5-bisfosfato 3-quinase Subunidade Catalítica Alfa |
| PTEN         | Gene Homólogo de Fosfatase e Tensina                                        |
| Rb           | Retinoblastoma                                                              |
| RE           | Receptor de Estrogênio                                                      |
| RP           | Receptor de Progesterona                                                    |
| SKI          | Proto oncogene Sloan-Kettering Institute                                    |
| SKIL         | Proto oncogene tipo Sloan-Kettering Institute                               |
| STAT         | Transdutor de Sinal e Ativador de Transcrição                               |
| STK11        | Gene da Serina/Treonina Quinase 11                                          |
| TEM          | Transição Epitélio-Mesenquimal                                              |
| TGF- $\beta$ | Fator de crescimento transformador beta                                     |
| TMBP         | 3,3',5,5'-tetrametoxibifenil-4,4'-diol                                      |
| TNFR         | Receptor do Fator de Necrose Tumoral                                        |
| TNF          | Fator de Necrose Tumoral                                                    |
| TP53         | Gene da Proteína Tumoral 53                                                 |
| TVAE         | Taxa de Variação Anual Estimada                                             |

|      |                                               |
|------|-----------------------------------------------|
| UICC | União Internacional para o Controle do Câncer |
| VEGF | Fator de Crescimento do Endotélio Vascular    |

## SUMÁRIO

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## **APRESENTAÇÃO DA TESE**

Esta tese foi elaborada em conformidade com o Regimento do Programa de Pós-Graduação em Patologia Experimental (Resolução CEPE nº 029/2018), o qual estabelece que dissertações e teses sejam redigidas no formato de artigos científicos. Nesse modelo, o trabalho é organizado a partir de manuscritos estruturados de forma independente, elaborados de acordo com os padrões científicos e metodológicos exigidos por periódicos da área.

Embora apresentados como artigos científicos, os manuscritos que compõem esta tese estão interligados por uma hipótese central e por objetivos complementares, constituindo um conjunto coeso e integrado. As seções de Introdução e Conclusão Geral têm como finalidade contextualizar o tema investigado e integrar os principais resultados obtidos, contribuindo para uma compreensão global do estudo desenvolvido.

Durante o processo de elaboração desta tese, foram utilizadas ferramentas de inteligência artificial como apoio à escrita científica, incluindo revisão linguística, aprimoramento da clareza e fluidez textual, organização de ideias e auxílio na estruturação do texto.

Ressalta-se que o uso dessas ferramentas teve caráter exclusivamente auxiliar, não substituindo a concepção experimental, a condução dos experimentos, a análise e interpretação dos dados, nem a tomada de decisões científicas, as quais são de inteira responsabilidade do autor. Todos os resultados, análises, discussões e conclusões apresentados neste trabalho foram desenvolvidos de forma independente e refletem o conhecimento científico do autor.

## 1. INTRODUÇÃO

O câncer é considerado a principal causa de morte nos países desenvolvidos e a segunda maior em países subdesenvolvidos (*World Health Organization (WHO)*, 2020). A tendência é que esses números continuem aumentando, conforme o estudo realizado pela Agência Internacional de Pesquisa em Câncer (IARC), que estima 35 milhões de novos casos e 18 milhões de mortes para o ano de 2050 (Bray *et al.*, 2024).

O aumento na incidência de câncer está associado ao rápido crescimento e envelhecimento populacional, bem como à maior exposição a fatores de risco ao longo da vida, que estão relacionados ao desenvolvimento socioeconômico global (Sung *et al.*, 2021). Outra grande preocupação é o recente aumento na incidência de cânceres de desenvolvimento precoce, como o câncer de mama em mulheres entre 18 e 49 anos, que apresenta maior agressividade, especialmente em mulheres de descendência africana (Chelmow *et al.*, 2020). Esse cenário ocorre devido às mudanças no estilo de vida atual, com aumento do consumo de álcool, cigarro, obesidade, sedentarismo, privação de sono e dietas ricas em gordura, açúcar e alimentos ultraprocessados (Ugai *et al.*, 2022).

Estima-se que houve cerca de 10 milhões de mortes por câncer em 2020, sendo 70% dos óbitos em países de baixa e média renda, como o Brasil e outros países da América Latina. Isso se deve não apenas ao baixo investimento em saúde nesses países, mas também ao elevado custo dos medicamentos quimioterápicos, tornando as drogas para terapia-alvo menos acessíveis para grande parte da população (Barrios *et al.*, 2023; Jalali *et al.*, 2022). Em resposta a esse cenário, esforços têm sido feitos a fim de desenvolver novas terapias de menor custo e com menos efeitos adversos a partir de moléculas de origem natural ou sintéticas análogas (Sharifi-Rad *et al.*, 2019).

### 1.1. CÂNCER DE MAMA

#### 1.1.1. Epidemiologia

O câncer de mama representa uma grande preocupação mundial, sendo o câncer de maior incidência e mortalidade em mulheres no Brasil e no mundo, com aproximadamente 95 mil casos e mais de 22 mil óbitos em território nacional (*International Agency For Research On Cancer*, 2024) e 2,3 milhões de casos e 670

1 mil mortes mundialmente somente em 2022 (Bray *et al.*, 2024).

2 No Brasil, a taxa de mortalidade por câncer de mama vem  
3 aumentando em todas as faixas etárias nas últimas décadas (Chávez-Penha;  
4 Bustamante-Teixeira; Nogueira, 2023). Embora mulheres de 40 a 74 anos apresentem  
5 maior mortalidade (30,7 por 100.000) em comparação às de 20 a 39 anos (3,1 por  
6 100.000), dados do IARC indicam um aumento expressivo entre mulheres jovens. A  
7 TVAE foi de +0,7% ao ano para mulheres de 40 a 74 anos no período de 1980 a 2021  
8 e de +1,2% para mulheres de 20 a 39 anos, correspondendo a aumentos acumulados  
9 de 32% e 62%, respectivamente (*International Agency For Research On Cancer*,  
10 2024).

11 Um estudo realizado no Instituto de Câncer do Estado de São Paulo  
12 (ICESP) reforça essa tendência, demonstrando um aumento proporcional tanto na  
13 incidência quanto na mortalidade por câncer de mama em mulheres jovens. Nesse  
14 estudo, foi observado um aumento de casos em mulheres abaixo dos 40 anos de 9,9%  
15 (2009-2014) para 12,9% (2015-2020), acompanhado pelo aumento proporcional da  
16 mortalidade de 9,6% para 12,4% no mesmo período (Bonadio; Moreira; Testa, 2022).

17 Esse aumento da mortalidade em mulheres jovens está associado  
18 principalmente ao diagnóstico tardio e ao comportamento tumoral agressivo,  
19 frequentemente relacionado ao subtipo triplo negativo (Chávez-Penha; Bustamante-  
20 Teixeira; Nogueira, 2023; Erić, 2018; Silva *et al.*, 2021), cujas abordagens terapêuticas  
21 permanecem limitadas e frequentemente baseadas em quimioterapia sistêmica,  
22 caracterizada por baixa seletividade tumoral e alta toxicidade (Obidiro; Battogtokh;  
23 Akala, 2023), reforçando a necessidade do desenvolvimento de novas alternativas  
24 terapêuticas mecanisticamente orientadas.

### 25 1.1.2. Classificação do câncer de mama

26 A classificação do câncer de mama baseia-se principalmente na  
27 caracterização molecular e imunofenotípica do tumor, sendo fundamental para a  
28 determinação do prognóstico e abordagem terapêutica. Nesse contexto, a  
29 classificação é realizada através da expressão dos receptores hormonais de  
30 estrogênio (RE), progesterona (RP) e do receptor tipo 2 do fator de crescimento  
31 epidérmico humano (HER2) (Weiss *et al.*, 2017). Além dos receptores hormonais, a  
32 classificação também inclui o marcador Ki67 como definido pelo consenso de St.  
33 Gallen de 2013, subdividindo o câncer de mama em luminal A, luminal B, HER2

positivo (HER2+) e triplo negativo (**Tabela 1**) (Goldhirsch *et al.*, 2013).

**Tabela 1** – Classificação molecular do câncer de mama

|             | Luminal A | Luminal B     | HER2+  | Triplo negativo |
|-------------|-----------|---------------|--------|-----------------|
| Frequência  | 30-40%    | 20-30%        | 12-20% | 15-20%          |
| RE          | +         | +             | +/-    | -               |
| RP          | +         | +/-           | +/-    | -               |
| HER2        | -         | +/-           | +      | -               |
| Ki67        | <14%      | >14%          | >14%   | >14%            |
| Prognóstico | Bom       | Intermediário | Ruim   | Ruim            |

**Fonte:** Adaptado de (Orrantia-Borunda *et al.*, 2022).

A maior detecção da proteína Ki67 está diretamente relacionada com um alto índice de proliferação celular, auxiliando na identificação de tumores de progressão rápida e pior prognóstico. Em alguns casos, mutações no gene *TP53* também são avaliadas, e a perda ou diminuição de função da p53 resulta em maior agressividade tumoral (Eroles *et al.*, 2012).

Os tumores luminais A e B possuem um perfil de expressão semelhante às células epiteliais do lúmen ductal mamário, com alterações em genes relacionados à ativação do receptor de estrógeno. O subtipo luminal B também apresenta superexpressão de genes relacionados a proliferação celular e expressão variável de genes relacionados ao HER2 (Perou *et al.*, 2000). Já o subtipo HER2+ é caracterizado pela superexpressão de genes associados a sinalização de HER2 e genes presentes no amplicon do HER2 localizado no cromossomo 17q12 (Perou *et al.*, 2000).

Os cânceres de mama triplo negativo (CMTN) apresentam expressão de genes de forma semelhante a células mamárias basais e células mioepiteliais, como as citoqueratinas basais (Perou *et al.*, 2000). Este subtipo também apresenta superexpressão de genes associados a proliferação sem expressão de genes relacionados aos RE, RP e HER2 (Perou *et al.*, 2000).

### 1.1.3. Fatores de risco para o CMTN

O câncer de mama está associado a diversos fatores de risco, classificados em não modificáveis e modificáveis (Łukasiewicz *et al.*, 2021). Embora o CMTN compartilhe alguns desses fatores, esse subtipo apresenta características epidemiológicas e biológicas peculiares que o diferem dos demais subtipos.

O CMTN acomete preferencialmente mulheres mais jovens, com maior incidência antes dos 40 anos (McGuire *et al.*, 2015), e apresenta maior prevalência em mulheres negras, as quais, apesar de menor incidência global, apresentam maior mortalidade associada a esse subtipo (Yedjou *et al.*, 2019).

Entre os fatores não modificáveis, destaca-se a forte influência genética e histórico familiar, especialmente associada a mutações no gene *BRCA1* (do inglês, *Breast Cancer Gene 1*), que estão presentes em 70-80% dos casos hereditários de CMTN (Choi *et al.*, 2023; Kumar *et al.*, 2024). Além disso, mutações em TP53, associadas a maior instabilidade genômica e agressividade tumoral, também são observadas com maior frequência nesse subtipo (Łukasiewicz *et al.*, 2021).

Entre os fatores modificáveis, o sedentarismo e a obesidade, principalmente em mulheres pré-menopáusicas, estão associados a um risco aumentado de CMTN. Diferentemente do observado nos subtipos luminais, onde a obesidade exerce maior impacto na pós-menopausa, no CMTN esse fator está fortemente relacionado a alterações metabólicas sistêmicas, como resistência à insulina e inflamação crônica de baixo grau (Berger; Iyengar, 2021).

## 1.2. CÂNCER DE MAMA TRIPLO NEGATIVO

O CMTN compreende 15 a 20% dos tumores mamários e é caracterizado pela ausência dos receptores RE, RP e HER2 e alta marcação para Ki67 (Fragomeni; Sciallis; Jeruss, 2018). Este subtipo está associado a pior prognóstico, maior agressividade tumoral, altas taxas de recidiva e metástases (Kumar; Aggarwal, 2016).

Do ponto de vista molecular, o CMTN constitui um grupo altamente heterogêneo de tumores, tendo sido classificado inicialmente em seis subtipos por Lehmann *et al* (2016) e posteriormente refinado para quatro subgrupos moleculares: Basal-like 1 (BL1), Basal-like 2 (BL2), Mesenquimal (MES) e Receptor Luminal Androgênico (LAR) (Lehmann *et al.*, 2021).

O subtipo MES, devido ao seu envolvimento no remodelamento da matriz extracelular, motilidade celular, transição epitélio-mesenquimal (TEM) e aquisição de fenótipo semelhante a células-tronco, confere aos tumores mesenquimais uma alta capacidade de invasão, migração e metástase, sendo associado a prognósticos desfavoráveis (Lehmann *et al.*, 2021). Linhagens celulares

representativas desse subtipo, como a linhagem MDA-MB-231, amplamente utilizada em estudos in vitro, apresenta resistência à quimioterapia convencional, sendo de grande relevância para o estudo e desenvolvimento de terapêuticas alternativas direcionadas (Li *et al.*, 2021).

Em contraste, o subtipo BL1, representado por linhagens como a MDA-MB-468 (Li *et al.*, 2021), apresenta alta expressão de genes associados à proliferação e aos mecanismos de reparo de danos ao DNA, sendo mais sensível a quimioterápicos genotóxicos. O subtipo BL2 é enriquecido em vias de sinalização mediadas por receptores de fatores de crescimento, como EGFR e MET, apresentando menor resposta às quimioterapias convencionais. Por fim, o subtipo LAR distingue-se pela expressão elevada do receptor de andrógeno e genes luminais, apresentando comportamento menos proliferativo e sendo potencialmente vulnerável a terapias antiandrogênicas (Lehmann *et al.*, 2021).

#### 1.2.1. Alterações somáticas no CMTN

O CMTN possui alta instabilidade genômica, sendo o subtipo com maior carga mutacional e frequência de alterações no número de cópias, como amplificações e deleções, que contribuem para a heterogeneidade tumoral, agressividade e resistência terapêutica (Asleh; Riaz; Nielsen, 2022)

Dentre as principais mutações, a mais prevalente ocorre no gene *TP53*, alterado em cerca de 70% dos casos, resultando em instabilidade genômica, desregulação dos mecanismos de reparo do DNA e resistência à apoptose. Outras mutações recorrentes incluem *PIK3CA* e *BRCA1* (aproximadamente 15% dos casos). As mutações em *PIK3CA* levam a ativação constitutiva da via PI3K/AKT, estimulando a proliferação celular e evasão às vias de morte celular. Enquanto que mutações em *BRCA1* compromete a via de reparo por recombinação homóloga, contribuindo para a alta instabilidade genômica desses cânceres (Ibragimova; Tsyganov; Litviakov, 2021; Shah *et al.*, 2012; The Cancer Genome Atlas Network, 2012).

Os CMTN também apresentam vasta alteração de números de cópias, geralmente com amplificações e deleções em regiões envolvendo genes oncogênicos e supressores tumorais (Kawazu *et al.*, 2017). O gene *MYC* é o gene de maior amplificação nos CMTN (80% dos casos), seguido pelos genes *E2F3*, *IRS2*, *CCNE1*, *EGFR*, *NFIB* e *CCND1* em mais de 40% dos casos. Entre as principais deleções estão *CHD1* (71%), *PTEN* (58%), *RB1* (54%) e *CDKN2A* (43%) (Jiang *et al.*,

2019).

A amplificação de *MYC* resulta em maior expressão de genes relacionados a biogênese ribossomal, metabolismo e síntese proteica, favorecendo a proliferação celular (Miller *et al.*, 2012). Enquanto que a amplificação de *EGFR* e *IRS2* promovem proliferação, motilidade, invasão e resistência terapêutica através da ativação sustentada da via PI3K/Akt, que é exacerbada frente a perda de *PTEN*, interrompendo a regulação negativa da via (Hassan; Aubel, 2025; Lee *et al.*, 2020; Uribe; Marrocco; Yarden, 2021). Já o aumento de *NFIB* favorece a plasticidade e fenótipo metastático dessas células (Denny *et al.*, 2016).

As amplificações de *CCNE1* (ciclina E1), *CCND1* (ciclina D1) e *E2F3* aumentam a atividade das CDK4/6, promovendo fosforilação e inativação de Rb, que resulta na liberação de E2F e avanço para a fase S (Singh *et al.*, 2025). As deleções de *RB1* e *CDKN2A* (p16/p14) inativam os principais *checkpoints* do ciclo, através da eliminação do bloqueio transcricional sobre E2F e ativação constitutiva das CDK4/6 (Rubin; Sage; Skotheim, 2020). A amplificação de *E2F3* por sua vez, impacta todo o ciclo celular, persistindo desde a fase S até G2, resultando no aumento de ciclina B1 via FOXM1 e hiperativação de CDK1 (Singh *et al.*, 2025). Além disso, a deleção de *CHD1* compromete o reparo de DNA por recombinação homóloga e a organização da cromatina, aumentando a instabilidade genômica que agrava a desregulação do ciclo e facilita o acúmulo de alterações somáticas (Kari *et al.*, 2016).

Essas alterações genéticas causam inúmeras alterações no perfil de expressão gênica no CMTN, resultando em centenas de genes diferencialmente expressos (DEGs) no transcriptoma dessas células. Dentre os DEGs, através de análises de enriquecimento e interação proteína-proteína, oito genes com expressões aumentadas (*CCNB1*, *CDK1*, *TOP2A*, *MKI67*, *TTK*, *CCNA2*, *BUB1* e *PLK1*) foram associados ao pior prognóstico e menor sobrevida das pacientes com CMTN (Wei *et al.*, 2021).

Os DEGs no CMTN são enriquecidos para processos metabólicos, divisão celular, mitocôndria e cadeia respiratória. Entre estes genes, a *CDK1* apresenta a maior expressão e demonstra a dependência desses cânceres na atividade da CDK1 para sustentação da proliferação celular. A expressão de *CDK1* é controlada pelo microRNA miR-143-3p, que se liga em sua porção 3'UTR, impedindo a poliadenilação e tradução do mRNA. Entretanto, o miR-143-3p encontra-se depletado nos tumores, explicando a superexpressão de *CDK1* (Pan; Cong; Ni, 2022).

Esse aumento e ativação de *CDK1* foi observado principalmente nos subtipos BL1 (MDA-MB-468) e MES (MDA-MB-231), sendo este um alvo terapêutico de extrema relevância para o tratamento destes cânceres (Lehmann *et al.*, 2021).

#### 1.2.2. Reprogramação de vias de sinalização no CMTN

A biologia complexa dos CMTN é caracterizada por múltiplas vias de sinalização aberrantes que promovem proliferação celular, resistência à morte, evasão imunológica, inflamação crônica e metástase. Dentre essas vias, destacam-se PI3K/Akt, NF- $\kappa$ B e TGF- $\beta$ , que frequentemente interagem entre si, regulando genes críticos para sobrevivência celular, TEM, evasão imunológica e progressão do ciclo celular, incluindo a ativação sustentada de *CDK1* (Guo *et al.*, 2024; Oliveira *et al.*, 2025).

##### 1.2.2.1. Eixo PI3K/Akt-NF- $\kappa$ B

A via PI3K/Akt (**Fig. 1**) é uma das vias mais importantes na regulação da proliferação, sobrevivência celular e resposta ao estresse, estando frequentemente ativada no CMTN. Sua ativação no câncer pode ocorrer por múltiplos mecanismos, que incluem mutações em *PIK3CA*, deleções ou perda funcional de PTEN e amplificação de receptores de fatores de crescimento (EGFR, FGFR, MET etc) que tenham PI3K como alvo *downstream* (Hassan; Aubel, 2025).

A ativação constitutiva da via PI3K/AKT promove a fosforilação e inativação de proteínas pró-apoptóticas, como Bad, além de favorecer o aumento de Bcl-2, a estabilização de MYC e o aumento da expressão de ciclinas, como a ciclina D1 (Liang; Slingerland, 2003). A ativação da via também leva à ativação de *CDK1* através da fosforilação de CDC25B e inibição de seus repressores (Wee1 e Myt1), facilitando a ativação da proteína e progressão do ciclo celular (Baldin *et al.*, 2003; Kalous; Aleshkina; Anger, 2023).

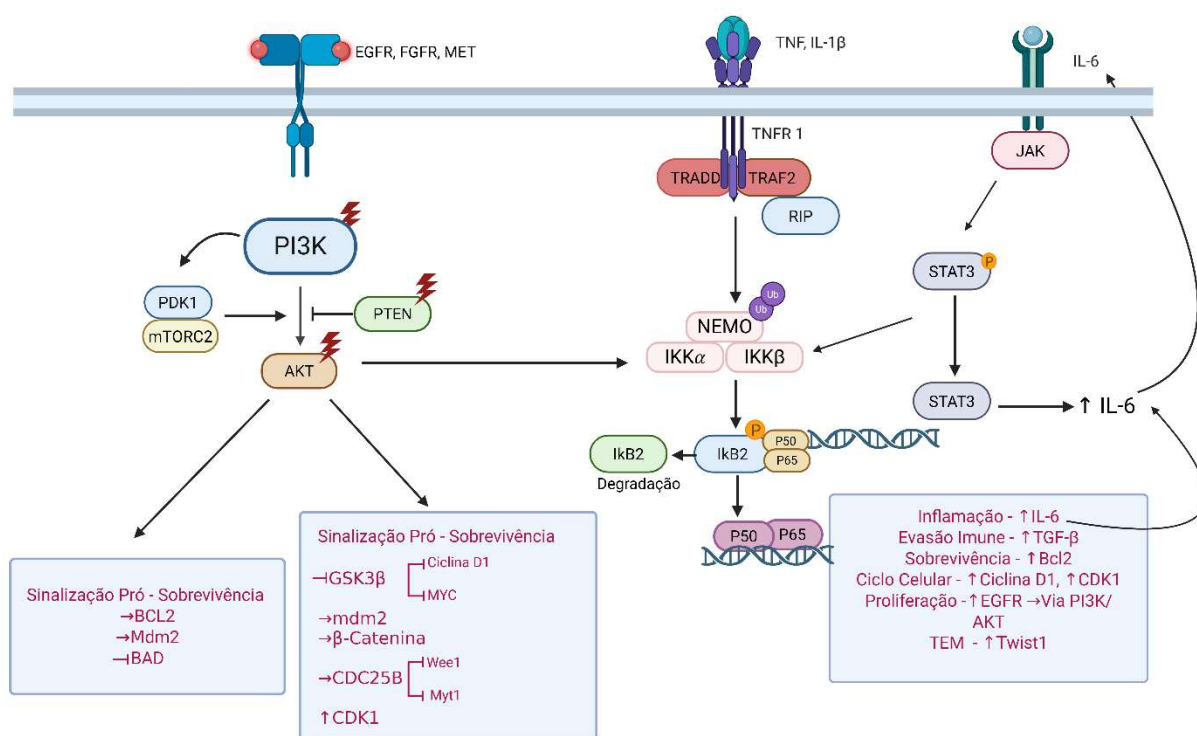
A via NF- $\kappa$ B (**Fig. 1**) é outra via relevante que está comumente desregulada no CMTN, atuando na sobrevivência celular, ativação de genes antiapoptóticos, TEM e regulação da resposta inflamatória no microambiente tumoral (Chakraborty *et al.*, 2024; Tang *et al.*, 2020).

A ativação deste fator de transcrição é mediada por citocinas inflamatórias como TNF e IL-1 $\beta$ , TLRs e receptores tirosina-quinase (Guo *et al.*, 2024). A via clássica, que ocorre através da fosforilação de I $\kappa$ B e a translocação do dímero p65/p50 para o núcleo, promove a transcrição de genes envolvidos nos processos de

evasão imune (via TGF- $\beta$ ) (Du *et al.*, 2021), sobrevivência celular (família Bcl-2) (Pavitra *et al.*, 2023), TEM/metástase (Twist1) (Li *et al.*, 2012) e reguladores do ciclo celular (Ciclina D1 e CDK1) (Voce *et al.*, 2021; Zhang *et al.*, 2017).

Além da via clássica, o NF- $\kappa$ B pode interagir com a via PI3K/AKT, estabelecendo um *feedback* positivo, frequentemente amplificado pela ativação concomitante de STAT3. A ativação da via EGFR/PI3K/AKT ativa NF- $\kappa$ B através da ativação de IKK, que por sua vez induz aumento da expressão de EGFR (Li *et al.*, 2016). A via JAK/STAT3 é ativada a partir da sinalização por IL-6, que uma vez ativada, ativa NF- $\kappa$ B e induz o aumento de IL-6 de forma sinérgica com NF- $\kappa$ B, uma vez que ambos os fatores de transcrição tem como alvo este mesmo gene (Hendrayani *et al.*, 2016; Hirano, 2021).

**Figura 1 - Eixo PI3K/AKT-NF- $\kappa$ B no CMTN.**



A via PI3K/AKT é ativada a partir de receptores tirosina-quinase (EGFR, FGFR e MET), promovendo a ativação de PI3K e AKT, sendo negativamente regulada por PTEN. Alterações somáticas frequentes em componentes dessa via são indicadas por ⚡, e resultam em ativação constitutiva da sinalização. A ativação de AKT promove sinalizações de sobrevivência e proliferação celular. A via canônica de NF- $\kappa$ B é ativada por citocinas inflamatórias (TNF e IL-1 $\beta$ ), levando à ativação do complexo IKK, fosforilação e degradação de I $\kappa$ B e liberação do dímero p50/p65. A ativação nuclear de NF- $\kappa$ B induz a transcrição de genes associados à inflamação, evasão imune, sobrevivência, regulação do ciclo celular, proliferação e TEM. A via não canônica de NF- $\kappa$ B é representada pela ativação do complexo IKK por AKT e STAT3. A via JAK/STAT3 é ativada por IL-6, levando a fosforilação de STAT3, que transloca-se ao núcleo, induzindo transcrição de genes pró-tumorais, incluindo IL-6 e estabelecendo um *feedback* positivo, além de interagir com o complexo IKK, amplificando a ativação da via NF- $\kappa$ B. **Fonte:** o autor

#### 1.2.2.2. Via TGF- $\beta$ e transição epitélio mesenquimal

No CMTN, a via TGF- $\beta$  possui um papel dicotômico na carcinogênese, atuando tanto como supressor tumoral em estágios iniciais e como promotor do tumor nas fases avançadas através da indução de TEM, angiogênese, invasão, metástase e manutenção do fenótipo semelhante a células-tronco tumorais (Baba *et al.*, 2022; Chandra Jena *et al.*, 2021).

Essa dualidade do TGF- $\beta$  ocorre devido às diferenças entre a sinalização canônica (SMAD dependente) e não canônica (SMAD independente) (**Fig. 2**). A ativação canônica desta via se dá através da ativação dos receptores T $\beta$ RI e II, que leva a fosforilação de SMAD2/3 e a formação de um complexo com SMAD4. A translocação do complexo para o núcleo induz a transcrição de genes envolvidos em parada do ciclo, apoptose e manutenção da polarização e integridade do epitélio. Já a via não canônica interage diretamente com vias como PI3K/Akt, MAPKs e NF- $\kappa$ B, contribuindo para a TEM, metástase e quimiorresistência (Baba *et al.*, 2022; Deng *et al.*, 2024; Zhang *et al.*, 2021).

Nos cânceres, a via canônica do TGF- $\beta$  é suprimida devido a mutações, deleções ou inibição de mediadores da via (Zhang *et al.*, 2021). Apesar de mutações em SMAD4 serem pouco frequentes no CMTN (3%) (Roy-Chowdhuri *et al.*, 2015), mutações em *TP53* e *PTEN* e hiperativação da via PI3K/Akt são comuns (The Cancer Genome Atlas Network, 2012). Essas alterações induzem uma reprogramação da via do TGF- $\beta$  ao longo da progressão tumoral, onde a via canônica interrompe a sinalização citostática e apoptótica e passa a sinalizar para TEM, aumentando a expressão de marcadores mesenquimais (Giarratana *et al.*, 2024; Sato *et al.*, 2014). Contudo, a via não canônica continua hiperativada no CMTN, cooperando com a via canônica na manutenção do fenótipo mesenquimal e nos processos de invasão, migração e metástase (Giarratana *et al.*, 2024).

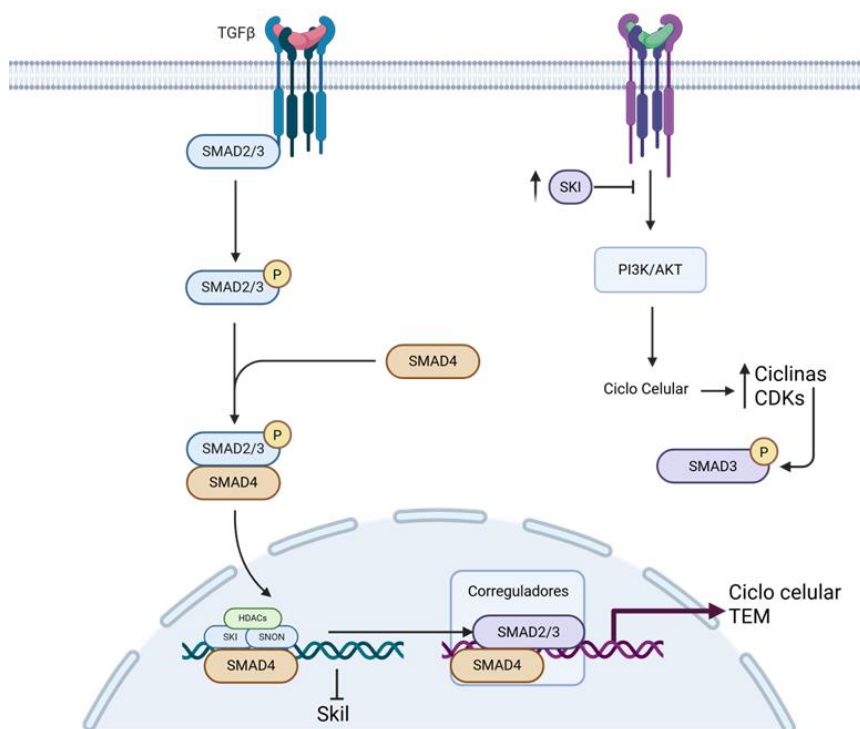
A ativação dos fatores de transcrição *SNAIL*, *SLUG*, *ZEB* e *TWIST* levam a repressão de marcadores epiteliais como a E-caderina e promovem marcadores mesenquimais como N-caderina e Vimentina. Em conjunto a essa reprogramação molecular, o TGF- $\beta$  induz o aumento de metaloproteinases de matriz (MMPs), principalmente MMP2 e MMP9, que degradam os componentes da matriz extracelular, promovendo a invasão e disseminação das células cancerosas (Luo *et al.*, 2025).

Os genes *SKI* e *SKIL* (SnoN) são repressores nucleares que inibem a

1 sinalização canônica do TGF- $\beta$  através da inibição da ligação do complexo  
2 SMAD2/3/4 ao DNA e através do recrutamento de histonas desacetilases. Essa  
3 repressão ocorre de forma a controlar e prevenir a sinalização excessiva pelo TGF- $\beta$   
4 (Tecalco-Cruz *et al.*, 2018). O gene *SKIL*, em condições basais, é inibido pelo  
5 complexo SnoN/Ski/Smad4, entretanto, após ativação dos receptores T $\beta$ RI/II pelo  
6 TGF- $\beta$  ocorre a degradação de Ski e SnoN, possibilitando a transcrição gênica pelo  
7 complexo de SMADs. De forma concomitante, os níveis de SnoN são aumentados  
8 como *feedback* negativo. Desta forma, essas proteínas realizam o fino controle da via  
9 durante o ciclo celular, apoptose e diferenciação celular (Tecalco-Cruz *et al.*, 2012).

10               No CMTN, a função de Ski e SnoN se encontram desreguladas e  
11 estão associadas ao papel dicotômico do TGF- $\beta$  no câncer. A proteína Ski apresenta  
12 atividade antitumoral e quando aumentada é capaz de inibir as sinalizações pró-  
13 oncogênicas ao impedir a ativação das vias não canônicas, limitando a TEM, invasão  
14 e metástase (Rashidian *et al.*, 2015). Já a superexpressão e estabilização de SnoN  
15 comumente apresenta atividade pró-tumoral no CMTN por permitir que as células  
16 cancerosas evadam os efeitos citostáticos da sinalização canônica enquanto promove  
17 motilidade e sobrevivência celular através das vias não canônicas (Zhang *et al.*, 2020;  
18 Zhu *et al.*, 2016). Essas vias convergem para a promoção do ciclo celular através do  
19 aumento e ativação de ciclinas e CDKs (Lee; Choi; Joo, 2013). As CDKs por sua vez  
20 podem fosforilar SMAD3, gerando um *feedback* positivo que enfraquece a via  
21 canônica supressora tumoral e reforça o processo de TEM e metástase (Ooshima;  
22 Park; Kim, 2019; Tarasewicz *et al.*, 2014). Por conseguinte, a desregulação de Ski e  
23 SnoN não só suprime o papel supressor do TGF- $\beta$ , levando a TEM e metástase, como  
24 também induz uma proliferação celular descontrolada mediada por CDKs.

## 1 Figura 2 – Vias canônica e não canônica do TGF- $\beta$ na progressão tumoral.



Esquema das vias de sinalização canônica (1) e não canônica (2) do TGF- $\beta$  e seu papel na progressão tumoral. Na via canônica, a ligação do TGF- $\beta$  aos seus receptores promove a fosforilação de SMAD2 e SMAD3, levando à degradação dos reguladores negativos SKI e SnoN e à liberação de SMAD4. A formação do complexo SMAD resulta na transcrição de genes associados à transição epitélio-mesênquima (TEM) e à regulação do ciclo celular. A via não canônica envolve a ativação de vias pró-sobrevivência, como PI3K/AKT, que reforçam a sinalização do TGF- $\beta$  por meio da fosforilação de SMAD3 e do aumento da expressão de ciclinas e CDKs, estabelecendo circuitos de retroalimentação positiva. No CMTN, a desregulação de SKI e SnoN favorece a ativação sustentada dessas vias, contribuindo para agressividade tumoral, invasão e progressão da doença. **Fonte:** o autor.

### 1.3. CICLO CELULAR E CDK1 NO CMTN

O ciclo celular é um processo rigorosamente regulado por diferentes proteínas regulatórias que permitem a progressão do ciclo ou induzem a sua parada (**Fig. 3**). Este processo é de suma importância desde o desenvolvimento embrionário até a manutenção da integridade tecidual (Aly, 2023).

A progressão do ciclo ocorre em resposta a estímulos mitogênicos externos por meio de fatores de crescimento e sinalizações proliferativas. Desta forma, a célula sai de sua fase quiescente (G0) e progride pelas fases da intérfase, G1 (do inglês, *Gap 1*), S (síntese de DNA) e G2 (do inglês, *Gap 2*) e culmina na fase M (mitose), resultando em duas células filhas geneticamente idênticas (Alberts *et al.*, 2017).

Após o estímulo mitogênico, a dinâmica celular é alterada e ocorre a

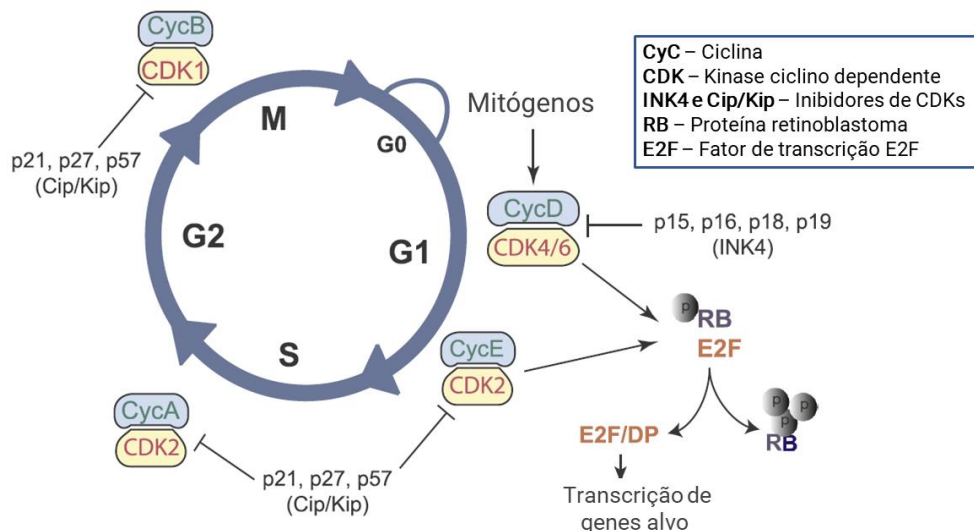
1 ativação de diversas vias de sinalização, como MAPK, PI3K/Akt/mTOR, NOTCH, SHH  
2 e Wnt, que regulam de forma fina a produção das proteínas regulatórias de forma a  
3 garantir a progressão do ciclo celular. Dentre as proteínas reguladas por essas vias,  
4 as de maior relevância são as ciclinas, quinases dependentes de ciclinas (CDKs),  
5 inibidores de CDKs (CKIs) e os supressores tumorais p53 e pRb, que atuam como  
6 promotores do ciclo ou como agentes de parada frente a estresse celular ou danos ao  
7 DNA (Pasha; Munuwar; Qureshi, 2021).

8               Nesse contexto, a CDK1 exerce um papel de regulador central do ciclo  
9 celular. Embora conhecida classicamente por seu papel na transição da fase G2 para  
10 a fase M, a CDK1 é indispensável para a viabilidade celular. Como demonstrado no  
11 estudo de Santamaría *et al.* (2007), ao contrário das CDKs 2, 3, 4 e 6, cuja deleção  
12 não compromete a organogênese em modelos murinos, a perda de CDK1 é letal. Isso  
13 ocorre devido a sobreposição de funções da CDK1 sobre outras CDKs, sendo capaz  
14 de interagir com todas as ciclinas, compensando a ausência de outras CDKs em todas  
15 as fases do ciclo celular.

16               Ao longo da fase G2, a fim de evitar a entrada prematura na fase  
17 mitótica e permitir o reparo de possíveis danos ao DNA, o complexo ciclina B/CDK1 é  
18 mantido inativo pela fosforilação inibitória nos resíduos T14 e Y15 da CDK1 mediada  
19 por Myt1 e Wee1, respectivamente. A ativação desse complexo ocorre pela ação da  
20 fosfatase CDC25C, que remove os grupos fosfato. Além disso, a fosforilação em T161  
21 pelo complexo quinase ativadora de CDK (CAK, formado por CDK7/ciclina H/MAT1)  
22 é indispensável para a total ativação enzimática da CDK1 (Massacci; Perfetto; Sacco,  
23 2023).

24

1 **Figura 3 – Regulação do ciclo celular.**



Representação esquemática das fases do ciclo celular e dos complexos ciclina-CDK que controlam sua progressão. A entrada em G1 é mediada pelo complexo Ciclina D-CDK4/6, responsável pela fosforilação inicial da proteína do retinoblastoma (Rb), processo que pode ser inibido por proteínas da família INK4. A transição G1/S ocorre por meio do complexo Ciclina E-CDK2, que promove a hiperfosforilação de Rb e a liberação dos fatores de transcrição E2F. Durante a fase S, o complexo Ciclina A-CDK2 inicia e sustenta a replicação do DNA, enquanto, após sua conclusão, prepara a célula para a mitose. A transição G2/M e a entrada em mitose são controladas pelo complexo Ciclina B-CDK1. A atividade dos complexos contendo CDK2 e CDK1 é negativamente regulada por inibidores da família Cip/Kip, assegurando o controle adequado da progressão do ciclo celular. **Fonte:** Adaptado de (Leal-Esteban; Fajas, 2020).

Mutações e deleções em genes supressores tumorais (*TP53* e *RB1*) ou ativação aberrante de oncogenes (*MYC* e *CCND1*) gera uma desregulação desses mecanismos (Ibragimova; Tsyganov; Litviakov, 2021; Jiang *et al.*, 2019; Thu, K. L. *et al.*, 2018). Isso resulta na ativação desregulada e sustentada de CDK1, que leva a uma hiperativação mitótica, instabilidade cromossômica e evasão dos pontos de checagem, comprometendo a resposta frente ao estresse genômico, promovendo proliferação celular descontrolada e resistência a mecanismos pró-apoptóticos (Mir; Haq, 2023). Neste contexto, o aumento de atividade de CDK1 exacerba a evasão aos pontos de checagem e acúmulo de danos ao DNA devido a sua capacidade de inibir a atividade da p73, um supressor tumoral da família da p53 (Wang; Bode; Zhang, 2023).

Devido à alta dependência funcional da CDK1 para proliferação e sobrevivência celular no CMTN, sua inibição resulta em apoptose e comprometimento dos mecanismos de reparo de DNA, o que aumenta a sensibilidade a agentes que promovem estresse replicativo e dano genômico, configurando um alvo terapêutico

promissor (Liao *et al.*, 2017).

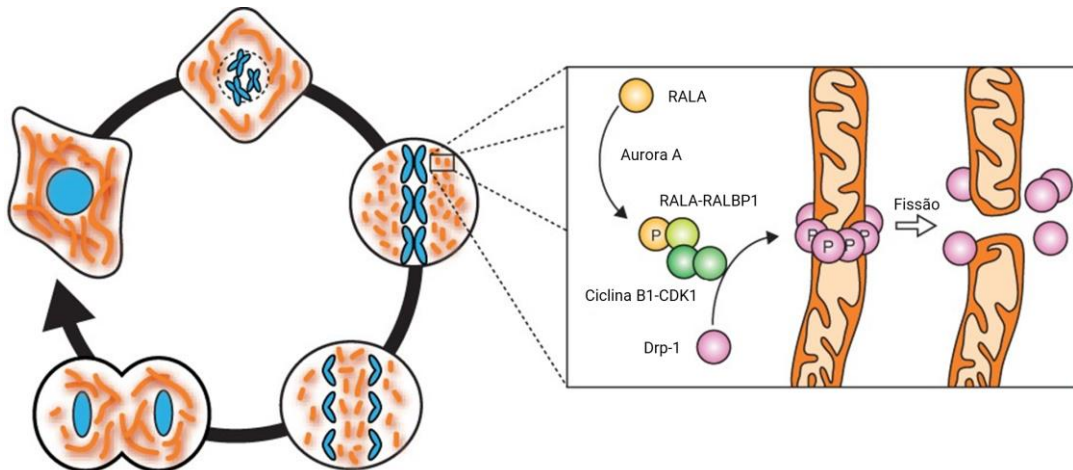
#### 1.4. CDK1 E SEU PAPEL NA DINÂMICA MITOCONDRIAL

Além de sua função clássica na transição G2/M do ciclo celular, a CDK1 desempenha um papel central na regulação da dinâmica mitocondrial, do metabolismo energético e do controle redox celular (Massacci; Perfetto; Sacco, 2023). Essas funções são particularmente relevantes em células tumorais, que apresentam elevada demanda bioenergética, aumento basal de espécies reativas de oxigênio (ERO) e reprogramação metabólica para sustentar proliferação, sobrevivência e invasividade (Gregg *et al.*, 2019; Xie *et al.*, 2019).

A dinâmica mitocondrial é um processo altamente regulado e contínuo de fissão e fusão mitocondrial, que controla o formato, tamanho, número e distribuição celular. Esses eventos opostos ocorrem de forma coordenada de acordo com a demanda celular. A fissão mitocondrial, mediada por Drp-1, é responsável pela segregação mitocondrial na mitose e pela eliminação seletiva de mitocôndrias danificadas. Enquanto a fusão, mediada pelas proteínas mitofusinas 1 (Mfn1), 2 (Mfn2) e OPA1, tem como função a otimização da eficiência bioenergética celular (Tábara; Segawa; Prudent, 2025).

Durante a progressão do ciclo celular, o complexo ciclina B1/CDK1 ativa Drp-1 (**Fig. 4**) e promove fissão a fim de promover a correta segregação mitocondrial durante a mitose (Xie *et al.*, 2019). No contexto tumoral, a hiperativação de CDK1 induz intensa fissão mitocondrial e reprogramação metabólica, estando fortemente associado à alta proliferação e invasividade (Chen; Chan, 2017). Isso ocorre devido ao aumento de atividade de Drp-1 e redução nos níveis de Mfn2. Entretanto, a inibição de Drp-1 (**Fig. 5**) ou aumento de Mfn1/2 é capaz de impedir a proliferação celular ao inibir a fragmentação e segregação mitocondrial (Malhotra *et al.*, 2016; Rehman *et al.*, 2012), bem como reduzir a capacidade de migração e invasão celular ao diminuir a formação de lamelipódios e impedir o acúmulo de mitocôndrias nessas estruturas, necessário para a polimerização de F-actina (Zhao *et al.*, 2013).

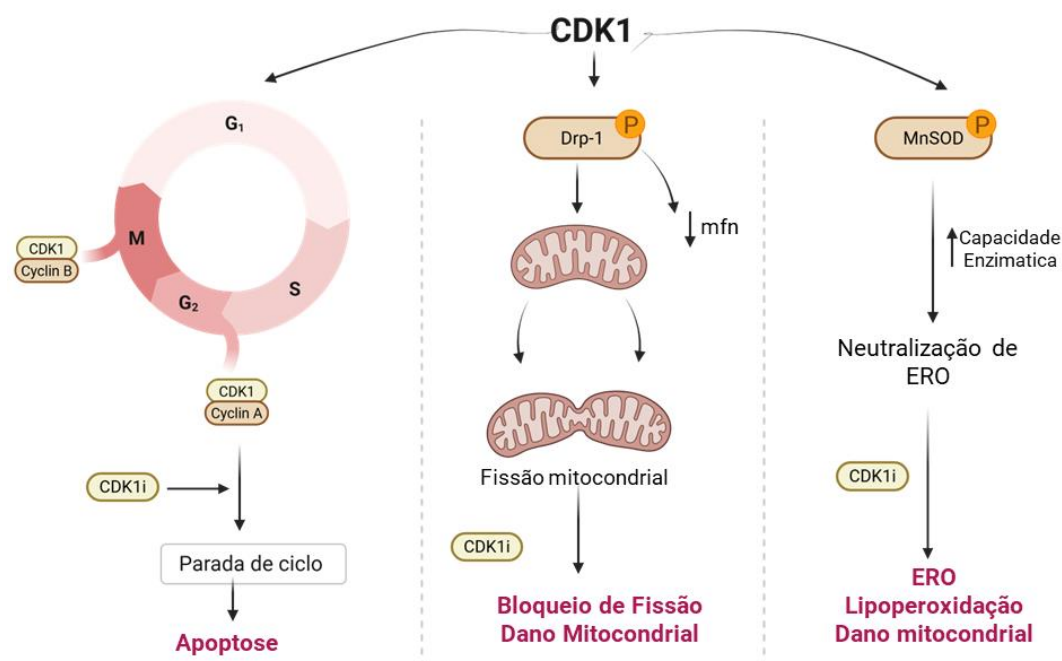
1 **Figura 4 – Fissão mitocondrial mediada por Ciclina B1-CDK1 durante a mitose.**



2  
3 Durante a mitose, Aurora A promove a relocação de RALA para a superfície mitocondrial, onde se  
4 complexa com RALBP1, atuando como uma estrutura de ancoragem que aproxima o complexo Ciclina  
5 B1–CDK1 de Drp-1, facilitando a fosforilação a ativação de Drp-1. Essa modificação é essencial para  
6 o recrutamento de Drp-1 à membrana mitocondrial externa, promovendo a fissão mitocondrial e  
7 assegurando a correta segregação das mitocôndrias entre as células-filhas. **Fonte:** adaptado de  
8 (Yamano; Youle, 2011)  
9

10 A CDK1 também modula diretamente a resposta antioxidante  
11 mitocondrial (**Fig. 5**) por meio da fosforilação de manganês superóxido dismutase  
12 (MnSOD), aumentando sua capacidade enzimática de converter o ânion superóxido  
13 ( $O_2^{\bullet-}$ ) em oxigênio ( $O_2$ ) e peróxido de hidrogênio ( $H_2O_2$ ), reduzindo o acúmulo de ERO  
14 e protegendo a célula contra danos oxidativos (Candas *et al.*, 2013; Kalen *et al.*, 2017).  
15  
16

1 **Figura 5 – Papéis da CDK1 e consequências de sua inibição em células tumorais.**



2  
3 No ciclo celular, a inibição de CDK1 resulta em parada sustentada do ciclo, culminando na ativação de  
4 apoptose. No contexto mitocondrial, o complexo ciclina B–CDK1 promove a fosforilação de Drp-1,  
5 induzindo fissão mitocondrial, e regula a defesa antioxidante por meio da fosforilação de MnSOD. A  
6 inibição de CDK1 bloqueia a fissão mitocondrial, favorece o acúmulo de mitocôndrias disfuncionais,  
7 compromete a neutralização de ERO e resulta em aumento do estresse oxidativo, lipoperoxidação e  
8 dano mitocondrial. **Fonte:** o autor.

## 9 1.5. MORTE CELULAR

11 A morte celular é um processo fisiológico essencial desde a fase de  
12 desenvolvimento embrionário até o indivíduo adulto, com a finalidade de eliminar  
13 células danificadas ou com potencial nocivo, como células infectadas e tumorais, de  
14 forma a manter a homeostase do organismo (Lee *et al.*, 2023; Newton *et al.*, 2024).

15 Do ponto de vista funcional, os mecanismos de morte celular podem  
16 ser classificados em dois grupos, a forma não imunogênica e a imunogênica, que  
17 induz a ativação de respostas pró-inflamatórias devido a liberação de padrões  
18 moleculares associado a dano (DAMPs) presentes no citoplasma (Newton *et al.*,  
19 2024).

20 Os diferentes tipos de morte celular são caracterizados por vias  
21 moleculares distintas, que podem interagir entre si ou serem ativadas  
22 simultaneamente em diferentes contextos (Lee *et al.*, 2023; Wang; Li; Wu, 2025). Com  
23 os avanços das últimas décadas, vários tipos de morte celular foram descritos além

da apoptose, autofagia e necrose, como a necroptose, piroptose, ferroptose, metuose e mitoptose (Hajibabaie; Abedpoor; Mohamadynejad, 2023).

Uma das características dos cânceres é a capacidade de resistir às sinalizações pró-apoptóticas, impactando negativamente as terapias que possuem como mecanismo de ação a ativação deste tipo de morte (Hanahan; Weinberg, 2011). No CMTN a desregulação em vias de sinalização como PI3K/Akt e NF- $\kappa$ B contribuem para que essas células resistam aos estímulos pró-apoptóticos, tornando essas vias pontos críticos a serem utilizados como alvos terapêuticos (Liu *et al.*, 2025).

Nesse contexto, estratégias que promovam estresse celular exacerbado, como o aumento de ERO, disfunção mitocondrial e comprometimento do reparo de DNA, têm se mostrado particularmente eficazes ao ultrapassar a capacidade adaptativa das células tumorais (An *et al.*, 2024; Labrie *et al.*, 2022; Zhao *et al.*, 2023). Além disso, a indução simultânea de múltiplos tipos de morte celular também se destaca como forma de contornar os mecanismos de resistência das células tumorais (Cao *et al.*, 2024; Li *et al.*, 2025; Lin *et al.*, 2016; Park *et al.*, 2023; Yu *et al.*, 2025).

#### 1.5.1. Apoptose mitocondrial e sua relevância no CMTN

A apoptose, ou morte celular programada, ocorre principalmente por duas vias, a via intrínseca (mitocondrial) e a extrínseca (mediada por receptores de morte). A via intrínseca é ativada por sinais internos, como estresse oxidativo, danos ao DNA ou hipóxia (Cavalcante *et al.*, 2019; Lossi, 2022).

A via mitocondrial é regulada por proteínas da família Bcl-2, composta por proteínas antiapoptóticas (Bcl-2, Bcl-xL, Mcl-1, A1 e Bcl-B), pró-apoptóticas de domínio BH3 único (Noxa, Puma, Bim, Bid, Hrk, Bmf e Bad) e pró-apoptóticas efetoras formadoras de poro (Bax e Bak). Em condições de homeostase, as proteínas anti-apoptóticas neutralizam as pró-apoptóticas, impedindo a permeabilização da membrana mitocondrial e a liberação do citocromo c (Carneiro; El-Deiry, 2020; Jan; Chaudhry, 2019; Lossi, 2022). Quando há sinalização de morte, as proteínas de domínio BH3 único passam por alterações pós-transcricionais que liberam Bax e Bak, permitindo sua oligomerização e formação de poros na membrana mitocondrial, resultando na liberação do citocromo c, formação do apoptossomo (com Apaf-1) e ativação das caspases 3 e 7, levando a externalização da fosfatidilserina e fragmentação celular (McComb *et al.*, 2019).

A via extrínseca é iniciada através da ativação de receptores de morte por seu respectivo ligante (Fas-FasL, TNFR-TNF- $\alpha$  e TRAILR-TRAIL), recrutando proteínas adaptadoras com domínio de morte (FADD e TRADD) (Ashkenazi, 2015; Lossi, 2022; Tummers; Green, 2017). Ambas levam ao recrutamento das caspases iniciadoras (8 e 10) e formam o complexo de sinalização indutor de morte (DISC). Em células tumorais, a caspase 8 cliva Bid em tBid, que interage com Bax e Bak, conectando as vias extrínseca e intrínseca, amplificando a sinalização pró-apoptótica (Carneiro; El-Deiry, 2020; Jan; Chaudhry, 2019; McComb *et al.*, 2019).

No CMTN, entretanto, essas sinalizações são limitadas pela superexpressão de proteínas antiapoptóticas e pela ativação sustentada de vias de sobrevivência, reforçando a necessidade de estratégias que promovam dano mitocondrial e estresse oxidativo intenso para efetiva indução de apoptose (Labrie *et al.*, 2022; Liu *et al.*, 2025).

#### 1.5.2. Autofagia, estresse oxidativo e morte celular dependente de ERO

A autofagia é um processo catabólico onde componentes citoplasmáticos são degradados e reciclados por meio do sistema lisossomal a fim de manter a homeostase celular. Esse mecanismo é ativado em condições de estresse, promovendo a sobrevivência celular ao manter o equilíbrio bioenergético e a integridade estrutural celular (Huang *et al.*, 2025; Jung; Jeong; Yu, 2020).

No contexto tumoral, a autofagia possui um papel dual. Nas fases iniciais da tumorigênese, a autofagia age como um supressor tumoral por meio da degradação de moléculas oncogênicas e organelas disfuncionais, levando à redução da geração de ERO e por consequência, prevenindo danos ao DNA e instabilidade genômica. Em tumores estabelecidos, a autofagia passa a promover a sobrevivência celular pelo mesmo mecanismo, diminuindo o estresse oxidativo e fornecendo nutrientes suficientes para a sobrevivência e proliferação destas células (Huang *et al.*, 2025; Jung; Jeong; Yu, 2020).

De forma conceitual, a autofagia pode atuar como um mecanismo citoprotetor ou citotóxico, dependendo do contexto metabólico, redox e da intensidade de estresse celular. Quando ativada de maneira exacerbada ou prolongada, a autofagia pode resultar em morte celular dependente de autofagia (MCDA) ou atuar como facilitadora de outras vias de morte, configurando a morte celular mediada por autofagia (MCMA). A MCDA é mediada pelas proteínas Genes Relacionados à

Autofagia (ATG), como o ATG5, BECLIN-1 e LC3 e posterior fosforilação da quinase c-Jun N-terminal (JNK), responsável pela intensa produção de autofagolisossomos e degradação de componentes citoplasmáticos, além de afetar a integridade da membrana nuclear (Bialik; Dasari; Kimchi, 2018; Denton; Kumar, 2019). A MCMA por sua vez, ocorre de forma similar, entretanto, dependendo do contexto é capaz de interagir com outras vias de morte, como apoptose, piroptose, necroptose e ferroptose (Liu *et al.*, 2025; Wang *et al.*, 2025).

As ERO são subprodutos do metabolismo oxidativo e abrangem um grupo de moléculas derivadas do oxigênio molecular. Podem ser divididas em espécies não radicais (como o  $H_2O_2$ ) e radicais livres (como o  $O_2^{\bullet-}$  e  $\bullet OH$ ) (Sies; Jones, 2020). Seus níveis são regulados por sistemas antioxidantes enzimáticos como superóxido dismutase (SOD), catalase (CAT), glutational peroxidase (GPx), glutational redutase (GR), peroxirredoxinas (Prx) e tioredoxina (Trx), e não enzimáticos como glutational (GSH), vitaminas e seus derivados (A, C e E) e outros metabólitos endógenos (Moloney; Cotter, 2018).

O acúmulo exacerbado de ERO desempenha um papel decisivo na transição entre sobrevivência e morte. As ERO são produzidas principalmente pela cadeia transportadora de elétrons mitocondrial e, quando excedem a capacidade antioxidante celular, estabelece-se o estresse oxidativo, desencadeando danos moleculares e estruturais a proteínas, lipídeos e DNA (Moloney; Cotter, 2018).

Esse acúmulo leva à disfunção mitocondrial por oxidação de componentes da cadeia transportadora de elétrons e abertura do poro de transição de permeabilidade mitocondrial (mPTP), culminando em perda do potencial de membrana mitocondrial ( $\Delta\Psi_m$ ), inibição da fosforilação oxidativa e diminuição da produção de ATP. Desta forma, a permeabilização da membrana mitocondrial favorece a liberação de fatores pró-apoptóticos, como citocromo c, que promove a formação do apoptossomo e ativação de caspases efetoras, ativando a via intrínseca da apoptose. Níveis elevados de ERO também pode resultar na oxidação de proteínas antiapoptóticas como Bcl-2 e Bcl-XL, bem como ativação de proteínas pró-apoptóticas como Bax e Bak, intensificando a permeabilização mitocondrial e o processo apoptótico (Moloney; Cotter, 2018).

Além disso, as ERO modulam diretamente a autofagia por meio da inibição de mTOR e ativação de AMPK, levando à ativação de ULK1 e início da formação do autofagossomo. A oxidação direta de proteínas como ATG4 também

regula a conjugação de LC3 e a dinâmica da membrana durante a biogênese do autofagossomo (Hasan *et al.*, 2022). Em células de CMTN, a ativação sustentada de AMPK e a inibição de mTOR estão associadas à supressão de vias proliferativas, como PI3K/Akt, IL-6/JAK/STAT3 e c-Myc, podendo levar a um fluxo autofágico exacerbado, degradação de proteínas antiapoptóticas e permeabilização lisossomal, culminando em apoptose mediada por autofagia (Cao *et al.*, 2019; Seo *et al.*, 2018; Yuan *et al.*, 2023).

Dessa forma, a convergência entre disfunção mitocondrial, estresse oxidativo e ativação exacerbada da autofagia gera um cenário de vulnerabilidade para células de CMTN, limitando sua capacidade de adaptação metabólica e favorecendo a ativação coordenada de mecanismos de morte celular (Munkácsy; Santarpia; Györffy, 2023; Pei *et al.*, 2025).

#### 1.6. ESTRATÉGIAS TERAPÊUTICAS NO CMTN

Comparado aos outros subtipos, o CMTN apresenta o pior prognóstico, com mais da metade das pacientes tendo recidiva nos primeiros 5 anos após o diagnóstico e sobrevida média de apenas 10 meses com as terapias atualmente disponíveis (Li *et al.*, 2022). A ausência de alvos moleculares clássicos, como os receptores hormonais e HER2, limita as opções terapêuticas disponíveis. Assim, para o CMTN a quimioterapia permanece como a principal estratégia, utilizando esquemas à base de antraciclinas e taxanos (Obidiro; Battogtokh; Akala, 2023).

A doxorubicina (DOX), uma antraciclina amplamente utilizada, atua intercalando-se ao DNA e inibindo a topoisomerase II, promovendo quebras duplas na fita de DNA e causando a parada do ciclo celular. Ao mesmo tempo, a DOX se acumula nas mitocôndrias e promove intensa geração de ERO, despolarizando a membrana mitocondrial e liberando o citocromo c, ativando a apoptose. A DOX também potencializa sua atividade citotóxica ao induzir a apoptose por meio dos receptores de morte (Kciuk *et al.*, 2023).

Além da apoptose, a DOX induz outros tipos de morte, como ferroptose, piroptose e morte mediada por autofagia. A indução de morte celular imunogênica também modula o microambiente tumoral através da exposição de calreticulina e liberação de ATP, levando a ativação de células dendríticas e resposta imune antitumoral (Kciuk *et al.*, 2023).

Os taxanos, como o paclitaxel e o docetaxel, atuam principalmente na estabilização dos microtúbulos, o que leva à parada do ciclo celular em mitose e ativa a apoptose. Contudo, os taxanos também induzem a formação de múltiplos micronúcleos em células tumorais deficientes em laminina A e C, como os cânceres de mama e ovário. Esses micronúcleos facilmente se rompem, desencadeando uma resposta pró-inflamatória, sendo um mecanismo de morte interessante em células tumorais resistentes à apoptose (Xu *et al.*, 2024).

Como forma de ampliar as opções terapêuticas para estes cânceres, diversas terapias foram aprovadas, como a quimioterapia combinada aos inibidores de checkpoint imunológico, o pembrolizumabe (anti-PD-1) e atezolizumabe (anti-PD-L1), entretanto, sendo benéfico para um pequeno grupo de tumores triplo negativos que expressam PD-L1 (Corti *et al.*, 2025). Para as pacientes com mutações em BRCA1/2 são indicados os inibidores de PARP, como olaparibe e talazoparibe, que atuam no bloqueio da via de reparo de quebras simples no DNA, o que leva ao colapso genômico em células que já possuem deficiência na recombinação homóloga (Barchiesi *et al.*, 2021).

Apesar dos avanços, a resistência terapêutica permanece como uma barreira no tratamento do CMTN. A ativação compensatória de vias pró-sobrevivência, como PI3K/Akt/mTOR, NF- $\kappa$ B, TGF- $\beta$  e MAPK/ERK, contribui para a resistência à apoptose, TEM, metástase, evasão imune e falha terapêutica (Guo *et al.*, 2024; Oliveira *et al.*, 2025; Soundararajan *et al.*, 2019).

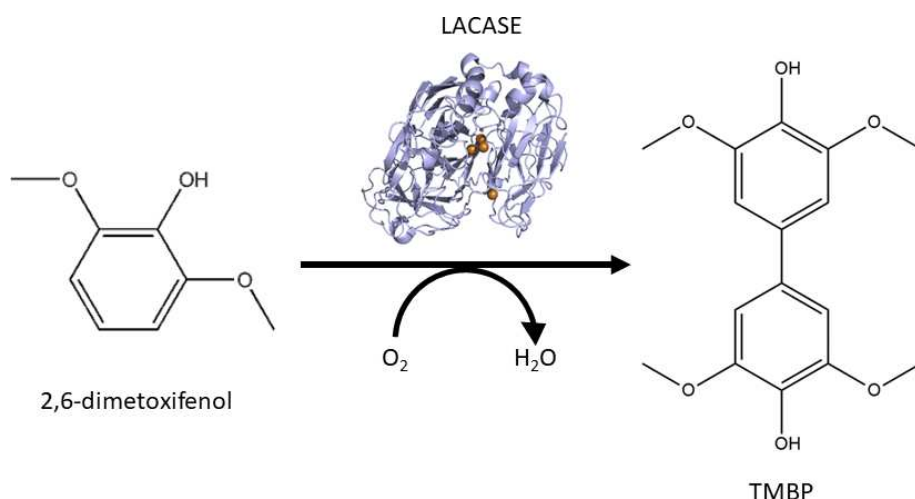
Nesse contexto, a inibição farmacológica de CDK1 tem emergido como uma estratégia terapêutica promissora para o CMTN, especialmente em tumores com mutação em *TP53*, que apresentam dependência de CDK1 para sobreviver ao estresse genotóxico (Jabbour-Leung *et al.*, 2016) e em tumores altamente proliferativos com hiperativação de MYC (Horiuchi *et al.*, 2012). Todavia, estudos clínicos utilizando inibidores de CDK1 e multi-CDKs como tratamento isolado demonstraram toxicidade não seletiva, limitando a dose e eficácia do tratamento. Portanto, para contornar as limitações dos inibidores de CDKs, a síntese de moléculas sintéticas com maior seletividade ou combinações com outros agentes anticâncer (Thu, K. *et al.*, 2018; Wang; Bode; Zhang, 2023) podem ser empregados, como demonstrado na associação de dinaciclibe com pembrolizumabe em pacientes em estágio avançado de CMTN (Chien *et al.*, 2020).

### 1 1.7. 3,3',5,5'-TETRAMETOXIBIFENIL-4,4'-DIOL (TMBP)

2 O 3,3',5,5'-tetrametoxibifenil-4,4'-diol (TMBP) é um dímero bifenólico  
3 biossintetizado a partir da oxidação de moléculas de 2,6-dimetoxifenol (2,6-DMP)  
4 catalisada por uma enzima lacase de origem fúngica (*Botryosphaeria rhodina*) (**Fig. 6**)  
5 (Schirmann *et al.*, 2018). Inicialmente, o desenvolvimento deste composto teve como  
6 principal objetivo a obtenção de moléculas com alta capacidade antioxidante para  
7 aplicações industriais (Adelakun *et al.*, 2012).

8 Posteriormente, seu desenvolvimento foi aplicado como um  
9 estabilizador de biodiesel, que se mostrou superior ao tert-butilhidroxitolueno, um  
10 antioxidante utilizado comercialmente, e garantiu estabilidade antioxidante ao  
11 biodiesel mesmo em altas temperaturas (Schirmann *et al.*, 2019).

12 **Figura 6 - Biossíntese do TMBP.**



14 Representação esquemática da biossíntese do TMBP a partir da oxidação de moléculas de 2,6-DMP,  
15 catalisada por lacase de *Botryosphaeria rhodina*. **Fonte:** adaptado de Schirmann *et al.* (2019).

17 Os efeitos biológicos do TMBP foram demonstrados pela primeira vez  
18 sobre a linhagem celular A549 (adenocarcinoma pulmonar), apresentando parada do  
19 ciclo celular em G2/M e apoptose tardia, sem causar citotoxicidade em macrófagos  
20 peritoneais de camundongos Balb/c. Paralelamente, análises *in silico* demonstraram  
21 que a molécula não viola nenhuma das diretrizes de Lipinski e Veber, apresenta alta  
22 absorção intestinal, propriedades anfipáticas e acúmulo em mitocôndrias, sugerindo  
23 um perfil farmacocinético favorável e potencial para desenvolvimento como fármaco  
24 oral antineoplásico (Concato *et al.*, 2020).

25 Além de seus efeitos antiproliferativos, o TMBP também demonstrou  
26 impacto sobre o fenótipo invasivo em células NCI-H460 (carcinoma pulmonar de

1 células não pequenas). O tratamento reduziu significativamente a migração celular e  
2 os marcadores de TEM, como N-caderina e  $\beta$ -catenina. Esses efeitos foram  
3 acompanhados pela redução da fosforilação de PI3K, AKT e NF- $\kappa$ B, resultando em  
4 morte por apoptose (Concato-Lopes *et al.*, 2024).

5 Evidências mais recentes indicam um possível mecanismo molecular  
6 específico associado à ação do TMBP. Inoue *et al.* (2025) demonstraram, por meio de  
7 análises *in silico* de docking molecular, que o TMBP apresenta afinidade de ligação  
8 ao resíduo Tyr15 da CDK1, um sítio crítico para a regulação da atividade dessa  
9 quinase. Corroborando esse achado, foi observado que o TMBP exerce maior  
10 atividade citotóxica e redução da expressão de *CDK1* em células com mutação em  
11 TP53 (HuH7.5; carcinoma hepatocelular) quando comparadas a células com TP53  
12 selvagem (HepG2/C3A), sugerindo uma maior dependência funcional de CDK1 em  
13 tumores TP53 mutantes.

14 É importante destacar que, até o momento, a interação entre TMBP e  
15 CDK1 foi avaliada exclusivamente por abordagens *in silico* e por expressão de mRNA,  
16 não havendo ainda validação experimental direta nos níveis proteicos ou no estado  
17 de fosforilação da CDK1. Ainda assim, esses achados fornecem um forte racional  
18 mecanístico para investigações funcionais adicionais.

19 Diante disso, a elevada agressividade e heterogeneidade molecular  
20 do CMTN, associadas à alta frequência de mutações em *TP53*, dependência  
21 exacerbada de CDK1 e limitação de terapias direcionadas eficazes, torna-se  
22 necessário investigar estratégias terapêuticas eficazes contra vulnerabilidades  
23 celulares específicas desse subtipo tumoral. Estudos recentes indicam que a  
24 desregulação da dinâmica mitocondrial, estresse oxidativo basal aumentado e a  
25 dependência de mecanismos adaptativos de sobrevivência constituem alterações  
26 críticas para a manutenção da viabilidade e progressão tumoral no CMTN. Nesse  
27 contexto, o TMBP se destaca como um candidato promissor, uma vez que apresenta  
28 atividade antitumoral seletiva, potencial interação funcional com CDK1 e capacidade  
29 de modular vias associadas à proliferação, ao reparo de DNA e à morte celular. Assim,  
30 este trabalho parte da premissa de que o TMBP é capaz de comprometer a  
31 sobrevivência de células de CMTN por meio da interferência em eixos centrais de  
32 controle do ciclo celular, metabolismo mitocondrial e resposta ao estresse oxidativo,  
33 além de potencializar a eficácia de quimioterápicos genotóxicos, como a DOX.

## 2. OBJETIVOS

### 2.1. OBJETIVO GERAL

- Elucidar os mecanismos moleculares e celulares envolvidos na atividade antitumoral do TMBP, administrado de forma isolada ou em associação com DOX, em modelos *in vitro* de CMTN, utilizando as linhagens tumorais humanas MDA-MB-231 e MDA-MB-468, bem como a linhagem mamária não tumoral HB4a, com ênfase em análises de bioinformática, regulação do ciclo celular, função mitocondrial, perfil redox e indução de morte celular.

### 2.2. OBJETIVOS ESPECÍFICOS

#### Etapa 1 – Caracterização dos efeitos do TMBP

- Analisar a expressão diferencial de transcritos do genoma global após o tratamento com TMBP na linhagem MDA-MB-231;
- Identificar por meio de ferramentas de bioinformática as principais vias de sinalização e processos celulares modulados pelo TMBP;
- Validar os processos celulares identificados através de ensaios funcionais;

#### Etapa 2 – Avaliação da associação entre TMBP e DOX

- Avaliar o efeito do TMBP, da DOX e de suas associações sobre a atividade metabólica celular das linhagens MDA-MB-231, MDA-MB-468 e HB4a, determinando os valores de IC<sub>50</sub>, a interação farmacológica pelo índice de combinação (ZIP) e índice de seletividade;
- Investigar o efeito dos tratamentos isolados e combinados na morfologia e ultraestrutura celular;
- Analisar o efeito dos tratamentos na distribuição do ciclo e indução de morte celular;
- Avaliar o impacto dos tratamentos sobre a função mitocondrial, estresse celular, perfil redox e o envolvimento da autofagia;

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### 3. PRODUÇÃO CIENTÍFICA

#### ANEXO A

Transcriptomic profiling of 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol-treated MDA-MB-231 cells reveals increased apoptosis, disrupted DNA damage response, and suppression of TGF- $\beta$  signaling

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Jornal: a ser submetido em Cancer Letters (Fator de Impacto 2024: 10.1)

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## Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by high genomic instability, *TP53* mutations, and dependence on Cyclin-Dependent Kinase 1 (CDK1)-driven cell cycle progression. The 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol (TMBP) was previously shown by molecular docking analysis to interact with CDK1 at Tyr15 inactivation site, along with *CDK1* mRNA downregulation and apoptosis more efficiently on *TP53*-mutant cells, without presenting cytotoxicity in non-tumoral cells. Here, we investigated the transcriptional and functional effects of TMBP on MDA-MB-231 cell line. Cells were treated with TMBP (5 – 100  $\mu$ M) for 24 h, and cellular metabolic activity (resazurin) data was used to determine the 50% inhibitory concentration ( $IC_{50}$ ) value. Transcriptome profiling was conducted to evaluate the effects of TMBP on genome-wide gene expression. Annexin V/propidium iodide staining, caspase 3/7 activity, and wound healing assays were conducted to corroborate the enriched biological processes. TMBP reduced cell viability in a concentration-dependent manner, yielding an  $IC_{50}$  value of 29.29  $\mu$ M. Transcriptomic profiling identified 108 differentially expressed genes. Gene set enrichment analysis revealed modulation of apoptosis, TGF- $\beta$ /SMAD signaling, and DNA damage recognition and repair pathways. Functional assays demonstrated a marked increase in late apoptosis, elevated caspase 3/7 activity, and impaired migratory capacity. These results support TMBP as a potential therapeutic candidate for TNBC, and other tumors characterized by *TP53* mutations, CDK1 dependency and compromised DNA damage checkpoints.

**Keywords:** CDK1; Triple-negative breast cancer; SMADs; Metastasis.

## 1. INTRODUCTION

Triple-negative breast cancer (TNBC) is the most aggressive and metastatic of all subtypes of breast cancer. The absence of hormone receptors and HER2 amplification leaves TNBC with no actionable therapeutic targets, making cytotoxic chemotherapy the standard treatment (1,2). Although recent advances such as immune checkpoint and PARP inhibitors have presented benefits in selected patient subsets, treatment responses remain suboptimal and long relapse-free survival is uncommon (3,4). Lehmann (5) classified TNBC in four subtypes, Basal-like (BL) 1, BL2, Mesenchymal (MES), and Luminal androgen receptor (LAR). Among them, MES TNBC represents the most challenging subtype, underscoring the urgency for therapeutic strategies targeting epithelial-to-mesenchymal transition (EMT), specifically PI3K/AKT, Wnt/ $\beta$ -catenin, and TGF- $\beta$  signaling pathways (6).

TNBC tumors frequently harbor *TP53* mutations, rendering them reliant on compensatory mechanisms such as CDK1-driven cell cycle progression, DNA repair, and robust redox control (5,7–9). Inhibiting these adaptive programs can shift the balance toward apoptosis, particularly in cells already burdened by oxidative and genotoxic stress (8,10,11). Previous studies have shown that 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol (TMBP) disrupts cancer cell survival by inducing oxidative stress, promoting mitochondrial dysfunction, and downregulation of *CDK1* (12,13), an essential kinase for G2/M regulation and homologous recombination repair (14). These features position TMBP as a potentially effective agent against TNBC, where CDK1 dependency and DNA repair defects constitute major therapeutic vulnerabilities.

In this study, we investigated the effects of TMBP on MDA-MB-231 TNBC cells through genome-wide transcriptomic profiling, and functional approaches.

Through differentially expressed genes (DEGs) and gene set enrichment analyses (GSEA) we aimed to identify biological programs altered by TMBP exposure. By integrating these results with known TNBC vulnerabilities, we provide mechanistic evidence supporting TMBP as a promising therapeutic candidate for the highly aggressive TNBC.

## 2. METHODOLOGY

### 2.1. Cell Culture

The MDA-MB-231 cell line was provided by Dr. Juliana Mara Serpeloni from the State University of Londrina. The cell line was cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Santa Cruz Biotechnologies, Dallas, TX, USA), and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were confirmed to be mycoplasma-negative using reverse transcription–quantitative real-time PCR (RT–qPCR).

### 2.2. Laccase-catalyzed synthesis of TMBP

3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol (TMBP), C<sub>16</sub>H<sub>18</sub>O<sub>6</sub> (MW: 306) was synthesized from 2,6-dimethoxyphenol by laccase from the ascomyceteous fungus *Botryosphaeria rhodina* (MAMB-05) under the conditions previously described by Schirmann et al. (15). A stock solution of TMBP was prepared in 1% (w/v) dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). The DMSO concentration in all experiments did not exceed 0.03%, except for the cellular metabolic activity assay (0.1%).

### 2.3. Cellular metabolic activity assay

MDA-MB-231 cells ( $5 \times 10^3$ ) were seeded in 96-well plates, and treated with TMBP (5 - 100  $\mu$ M) for 24 h. Cells were then incubated for 2 h in a solution of 7-Hydroxy-3H-phenoxazin-3-one 10-oxide (Resazurin; 60  $\mu$ M). The fluorescence intensity was quantified in a Glomax® Discover Microplate Reader (Promega, GM3000) at Ex: 520 nm and Em: 580 nm. Following the resazurin assay, the dose-response curve was generated from normalized metabolic activity values, and the IC<sub>50</sub> was estimated using a four-parameter non-linear regression model.

### 2.4. Transcriptomic analysis

The MDA-MB-231 cells ( $5 \times 10^5$ ) were seeded in 6-well plates and treated for 24 h. Total RNA was obtained from triplicate pools using a RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. For the chip array assay, the RNA was processed, hybridized to a GeneChip Human Exon 1.0 ST Array (Affymetrix, CA, USA), washed, stained and scanned as previously described (16). Partek® Discovery Suite™. Version 6.3 (Partek, Inc, 2008) was used for data analysis.

### 2.5. Gene Set Enrichment and Network Analysis

Gene Set Enrichment Analysis (GSEA) was performed to identify pathways associated with transcriptional alterations in the dataset. Genes were ranked based on  $-\log_{10}(\text{p-value})$  obtained from differential expression analyses. Enrichment analyses were conducted using Reactome and Hallmark gene sets from the Molecular Signatures Database (MSigDB), enabling both mechanistic and consolidated pathway-level interpretation. GSEA was implemented in the R statistical environment, and

pathway significance was assessed using normalized enrichment scores (NES) and false discovery rate (FDR)–adjusted p-values. Gene sets meeting the predefined significance threshold were considered significantly enriched. For visualization of functional relationships, genes contributing to significantly enriched pathways were exported and analyzed using Cytoscape software. Network representations were used to qualitatively explore pathway interconnections and highlight central genes involved in enriched biological processes.

## **2.6. Apoptosis assay**

The MDA-MB-231 cells ( $5 \times 10^5$ ) were seeded in 6-well plates and treated for 24 h. Subsequently, the cell suspension was centrifuged at 2000 rpm for 5 minutes, and the cell pellet resuspended in 100  $\mu$ L of annexin V and propidium iodide solution (Millipore, Billerica, MA, USA) for 20 min at room temperature (RT). After staining procedures, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at RT, washed three times with PBS and resuspended in 300  $\mu$ L of PBS. The analyses were performed using the FACSCalibur (BD Biosciences, New Jersey, USA) flow cytometer at 10,000 events.

## **2.7. Caspase 3/7 activity assay**

Caspase-3/7 enzyme activity was performed using the EnzChek™ Caspase-3/7 Assay Kit (Invitrogen™) as described previously (12). Briefly, after seeding ( $1 \times 10^6$  cells) and treatment steps, the cell pellet was incubated at -80 °C for lysis. Subsequently, a lysis buffer (1% Triton X-100 in PBS) was added and incubated in ice for 30 min. This was followed by the addition of kit reagents and incubated for 30 min. The fluorescence intensity readings (GloMax, Promega; Ex: 342 nm, Em: 441 nm)

were normalized and expressed in arbitrary units of fluorescence.

## **2.8. Wound healing assay**

MDA-MB-231 cells were seeded ( $1 \times 10^6$ ) in 6-well plates and incubated until confluency. Subsequently, a wound was made in the cell monolayer by scratching a 200  $\mu$ L pipette tip at the bottom of the wells, followed by TMBP treatment with culture media at 2% FBS to decrease cell proliferation. After the treatment, photomicrographs (200x magnification) were taken using an EVOS microscope (Life Technologies) at 0 and 24 h. Cell migration was evaluated as a cell-free area was measured by ImageJ software. The percentile decreases in the area characterized the cell migration.

## **2.9. Statistical analysis**

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

# **3. RESULTS**

## **3.1. Cell metabolic activity in MDA-MB-231 cells following TMBP treatment.**

The metabolic activity of MDA-MB-231 was assessed by the resazurin reduction assay after exposure to increasing concentrations of TMBP (5 - 100  $\mu$ M) for

24 h. TMBP reduced cellular metabolic activity in all concentrations tested (**Figure 1A**), presenting a reduction to 90.68%, 85.12%, 70.83%, 5.10%, 1.48%, and 0.67% at 5, 10, 25, 50, 75, and 100  $\mu$ M, respectively ( $p < 0.0001$  for all concentrations vs. control). Non-linear regression analysis yielded an  $IC_{50}$  value of 29.29  $\mu$ M, and therefore 30  $\mu$ M was determined for all the subsequent assays (**Figure 1B**).

### 3.2. Differentially expressed genes (DEGs) and Gene set enrichment analysis (GSEA)

Transcriptomic profiling of TMBP-treated MDA-MB-231 cells identified 108 DEGs (Supplementary table 1), of which 21 were upregulated and 87 were downregulated (**Figure 2A**).

To characterize the functional programs modulated by TMBP in the MDA-MB-231 cell line, we performed GSEA using the Hallmark and Reactome databases. The Hallmark enrichment showed significant modulation of apoptosis ( $p = 0.0301$ ; represented by *ETF1*, *ANXA1*, and *MMP2*), and TGF- $\beta$  signaling ( $p = 0.0215$ ; through the altered expression of *SKI* and *SKIL* as the top regulated processes (**Figure 2B,C**).

Reactome revealed a wider overview of the biological alterations induced by TMBP, highlighting a strong enrichment of pathways related to DNA damage recognition and repair, including terms as global-genome nucleotide excision repair (GG-NER;  $p = 0.0004$ ), DNA Damage Recognition in GG-NER ( $p > 0.0001$ ), Nucleotide Excision Repair ( $p = 0.0011$ ), and DNA Repair ( $p = 0.0026$ ), represented by *BAP1*, *H2AC20*, *COPS3*, *INO80C*, *RBX1*, and *RAD23A* transcripts (**Figure 2B,C**). Pathways regulating TGF- $\beta$ /SMAD signaling were also significantly enriched, such as downregulation of SMAD2/3–SMAD4 transcriptional activity ( $p = 0.0061$ ),

transcriptional activity of the SMAD2/3/4 heterotrimer ( $p = 0.0159$ ), and signaling by the TGF- $\beta$  receptor complex ( $p = 0.0395$ ), represented by the *SKI*, and *SKIL* genes (Figure 2B,C).

Other enriched pathways included components of Notch1-related processes (*TLE4*, *RBX1*), Wnt/ $\beta$ -catenin–associated pathways (*H2AC20*, *TLE4*, *SRY*, *GNG2*, *RBX1*), deubiquitination and UCH proteinases (*BAP1*, *H2AC20*, *INO80C*, *RAD23A*), and additional regulatory categories including extra-nuclear estrogen signaling, creatine metabolism, BH4 synthesis, and modulation of BACH1 activity.

### 3.3. Validation of transcriptomic signatures through functional assays

Annexin V/PI staining revealed a marked reduction in viable MDA-MB-231 cells following TMBP treatment, decreasing from 99.38% to 20.01% ( $p < 0.0001$ ). Early apoptosis and necrosis populations did not differ between groups. However, late apoptosis increased considerably, increasing from 0.38% to 73.85% in treated cells ( $p < 0.0001$ ; Figure 3A). Consistent with this increase in apoptotic cells, caspase-3/7 activity was also significantly elevated after TMBP exposure, increasing from 28.34 AU to 47.07 AU ( $p < 0.001$ ; Figure 3B).

Cell migration was assessed using a wound healing assay. The migratory capacity was assessed at two time-points, at zero h (100% gap) and after 24 h. Control cells reduced the wound area to 17.35%, whereas TMBP-treated cells exhibited impaired migratory capacity, closing the gap to only 54.04% ( $p < 0.0001$ ; Figure 3C).

## 4. DISCUSSION

TNBC is the most aggressive and lethal breast cancer subtype,

accounting for the highest number of breast cancer related deaths despite representing only 20% of cases (17). Its poor prognosis is due to the rapid tumor progression, early metastatic dissemination, and high rates of recurrence (6). Unlike hormone receptor positive or HER2-amplified tumors, TNBC lacks targeted therapies, leaving cytotoxic chemotherapy as the standard treatment (2,18). Although recent advances such as immune checkpoint and PARP inhibitors have provided incremental benefit in selected patients, therapeutic responses remain highly variable, and durable remissions are rare (3,4). The absence of targeted therapies, combined with intrinsic genomic instability, high intra-tumoral heterogeneity, and therapy-induced resistance mechanisms, underscores the urgent need for novel strategies capable of exploiting TNBC-specific vulnerabilities (19,20).

In this study, we investigated the transcriptional and functional responses of MDA-MB-231 cells to TMBP, a compound previously shown to downregulate *CDK1* expression, induce oxidative stress, and promote mitochondrial damage in cancer cells (12,13). Our findings indicate that TMBP triggers a multifaceted cellular response characterized by apoptosis activation, disruption of DNA damage repair programs, modulation of TGF- $\beta$  pathway, and reduced migratory capacity. Together, these results help expand the understanding of the mechanics through which TMBP exerts its anticancer effects.

The transcriptomic profiling revealed an enrichment of apoptosis-related processes, which was supported by the marked increase in late apoptotic cells and elevated caspase-3/7 activity. The downregulation of *ETF1*, *ANXA1*, and *MMP2*, is consistent with the shift toward a pro-death phenotype. *ETF1*, also known as eukaryotic release factor 1 (eRF1), is a key translation termination factor that ensures proper stop-codon recognition and prevents the production of aberrant proteins

through its role in termination and nonsense-mediated mRNA decay (21). Its downregulation results in ribosome stalling and the accumulation of misfolded or truncated proteins, triggering ribosome-associated quality control and the integrated stress response (22–24). Persistent activation of these stress pathways increases proteotoxic and endoplasmic reticulum stress, culminating in caspase 3 activation and apoptosis (23,25). *ANXA1* and *MMP2* are both implicated in cell growth, invasion, and metastasis in TNBC (26,27). *ANXA1* inhibition has shown to inhibit MMP2/9 activities, while arresting the cell cycle, and promoting apoptosis through the inhibition of PI3K/Akt pathway (26,28).

Reactome analysis exhibit strong enrichment of pathways involved in DNA damage recognition and nucleotide excision repair, including GG-NER and global DNA repair programs. The upregulation of chromatin-associated H2A variant, *H2AC20*, and increased *BAP1* expression are consistent with activation of DNA damage recognition machinery (29,30). In contrast, the downregulation of *INO80C* (31), *RAD23A* (32), *RBX1* (33), and *COPS3* (34) indicates suppression of essential DNA repair and chromatin remodeling functions. Together, these alterations suggest that TMBP disrupts genome maintenance, which aligns with the known role of CDK1 in homologous recombination-dependent repair of DNA double-strand breaks (35), therefore, its downregulation likely promotes the accumulation of lethal DNA lesions that trigger apoptosis. Given that *TP53*-mutant TNBC cells are highly dependent on CDK1 for cell cycle progression, and control of oxidative and genotoxic stress (14,36), the dysfunction of DNA repair signatures observed here reflect this vulnerability.

Our findings also highlight significant modulation of TGF- $\beta$ /SMAD signaling, primarily through the differential expression of *SKI* and *SKIL*, two important repressors of SMAD-mediated transcription (37). Increased *SKI* expression combined

with decreased *SKIL* suggests inhibition of canonical TGF- $\beta$  signaling. In TNBC, TGF- $\beta$  promotes EMT, migration and metastasis, while also promoting cell proliferation (38). Repression of TGF- $\beta$  signaling, as well as decreased *MMP2* by TMBP may thus contribute both to impaired motility and to enhanced apoptotic susceptibility. This is supported by our wound healing assay, in which TMBP reduced migratory capacity by more than 35% compared to control cells.

In summary, these transcriptomic signatures integrate with previous evidence that TMBP induces oxidative stress and mitochondrial damage (12,13). Mitochondrial dysfunction is known to activate intrinsic apoptosis pathways, and limit the energy supply required for migration and metastasis (39). Oxidative stress also amplifies DNA damage by overwhelming redox homeostasis (40), reinforcing the impaired repair capacity observed in the enriched DNA repair pathways.

## CONCLUSION

These results indicate that TMBP exerts coordinated anticancer effects by simultaneously targeting key vulnerabilities of TNBC cells, as CDK1 dependency, genomic instability, redox imbalance, and invasive activity. The integration of transcriptomic signatures with functional approaches strengthens the mechanistic understanding of the compound and suggests that TMBP disrupts multiple essential processes in parallel, by inducing DNA damage, attenuating motility, and ultimately driving apoptotic cell death. These findings support further investigation of TMBP as a potential therapeutic agent for TNBC, particularly in tumors characterized by CDK1 dependency and deficient DNA damage checkpoints.

**Abbreviations:** BL, Basal-like; CDK, cyclin-dependent kinase; DEGs, differentially

expressed genes; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethylsulfoxide; EMT, epithelial-to-mesenchymal transition; eRF1, eukaryotic release factor 1; FBS, fetal bovine serum; GG-NER, global-genome nucleotide excision repair; GSEA, gene set enrichment analyses; LAR, luminal androgen receptor; MES, mesenchymal; SEM, standard error of the mean; TMBP, 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol; TNBC, Triple-Negative Breast Cancer.

#### **CRedit authorship contribution statement:**

**Fabricio Seidy Ribeiro Inoue:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review and editing. **Ellen Mayara Souza Cruz:** Investigation, Writing – review and editing. **Danielle Lazarin-Bidóia:** Investigation, Formal analysis, Validation. **Mariana Barbosa Detoni:** Investigation, Writing – review and editing. **Renata Binato:** Methodology. **Paulo Rohan:** Methodology. **Jéseka Gabriela Schirmann:** Resources, Methodology. **Aneli M. Barbosa-Dekker:** Resources, Writing – review and editing. **Robert F. H. Dekker:** Resources, Writing – review and editing. **Mário Sérgio Mantovani:** Resources, Methodology. **Carolina Panis:** Conceptualization, Resources, Formal analysis, Writing – review and editing. **Wander Rogério Pavanelli:** Resources, Writing – review and editing, Supervision.

#### **Conflict of interest**

The authors have no conflict of interests to declare.

#### **Funding**

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível

Superior (CAPES, Brazil), Conselho Nacional de Pesquisa (CNPq, Brazil), Financiadora de Estudos e Projetos (FINEP, Brazil), and Universidade Estadual de Londrina (UEL, Brazil).

## Acknowledgements

During the preparation of this manuscript, the authors used generative artificial intelligence tools to assist with language refinement and improvement of clarity. The authors take full responsibility for the scientific content, data interpretation, and conclusions presented in this manuscript.

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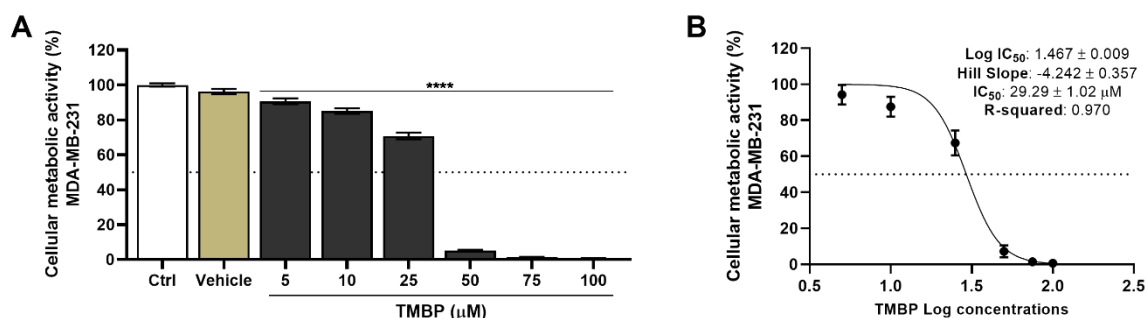
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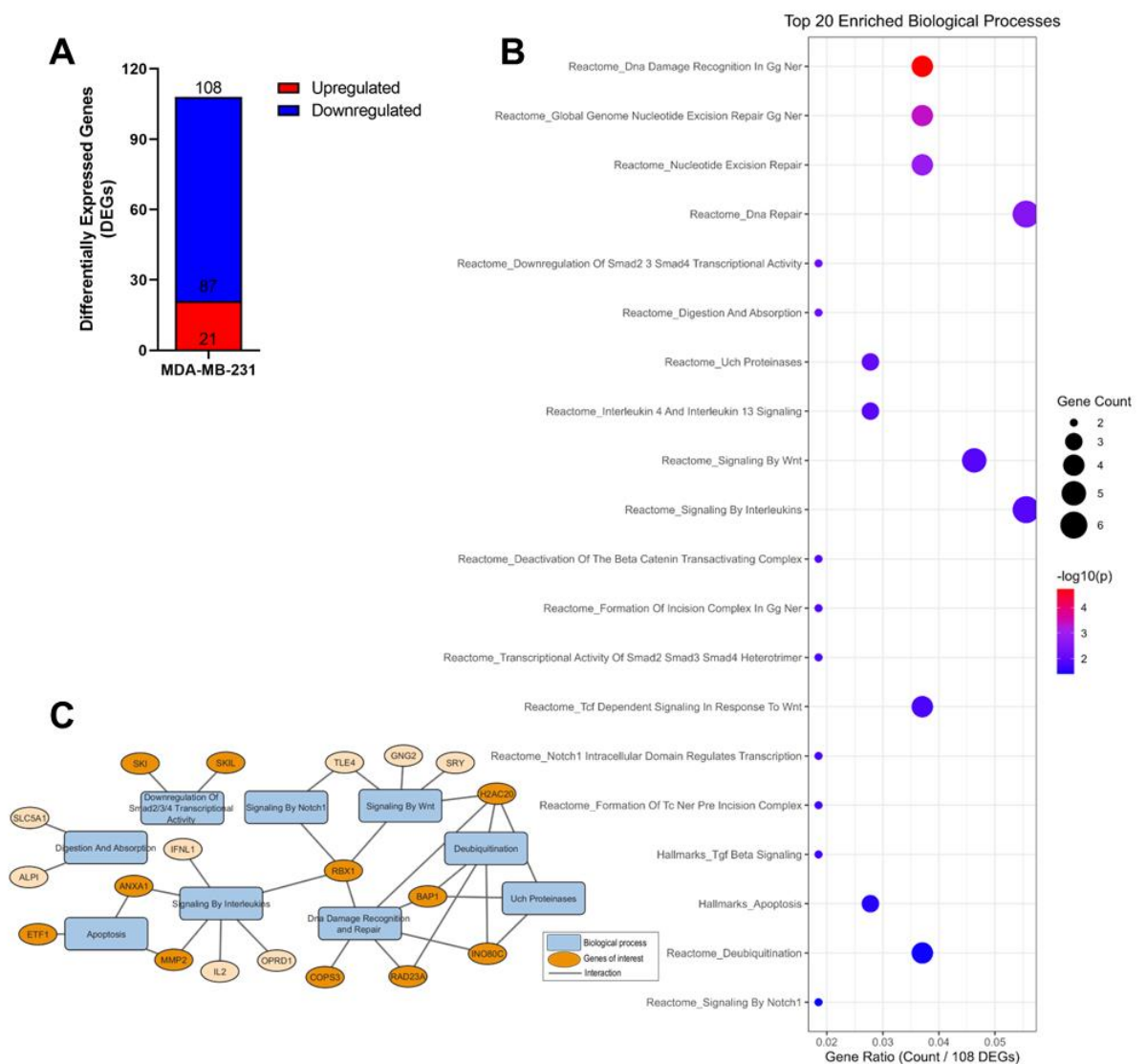
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## FIGURES

**Figure 1. TMBP reduces metabolic activity in MDA-MB-231 cells.** (A) Cellular metabolic activity was assessed by resazurin reduction following 24 h exposure to increasing concentrations of TMBP (5–100  $\mu\text{M}$ ). Fluorescence values were normalized to untreated control cells. (B) Dose–response curve and non-linear regression analysis used to determine the half-maximal inhibitory concentration ( $\text{IC}_{50}$ ). Data are presented as mean  $\pm$  SEM from independent experiments. Statistical significance was determined relative to control; \*\*\*\* $p < 0.0001$ .



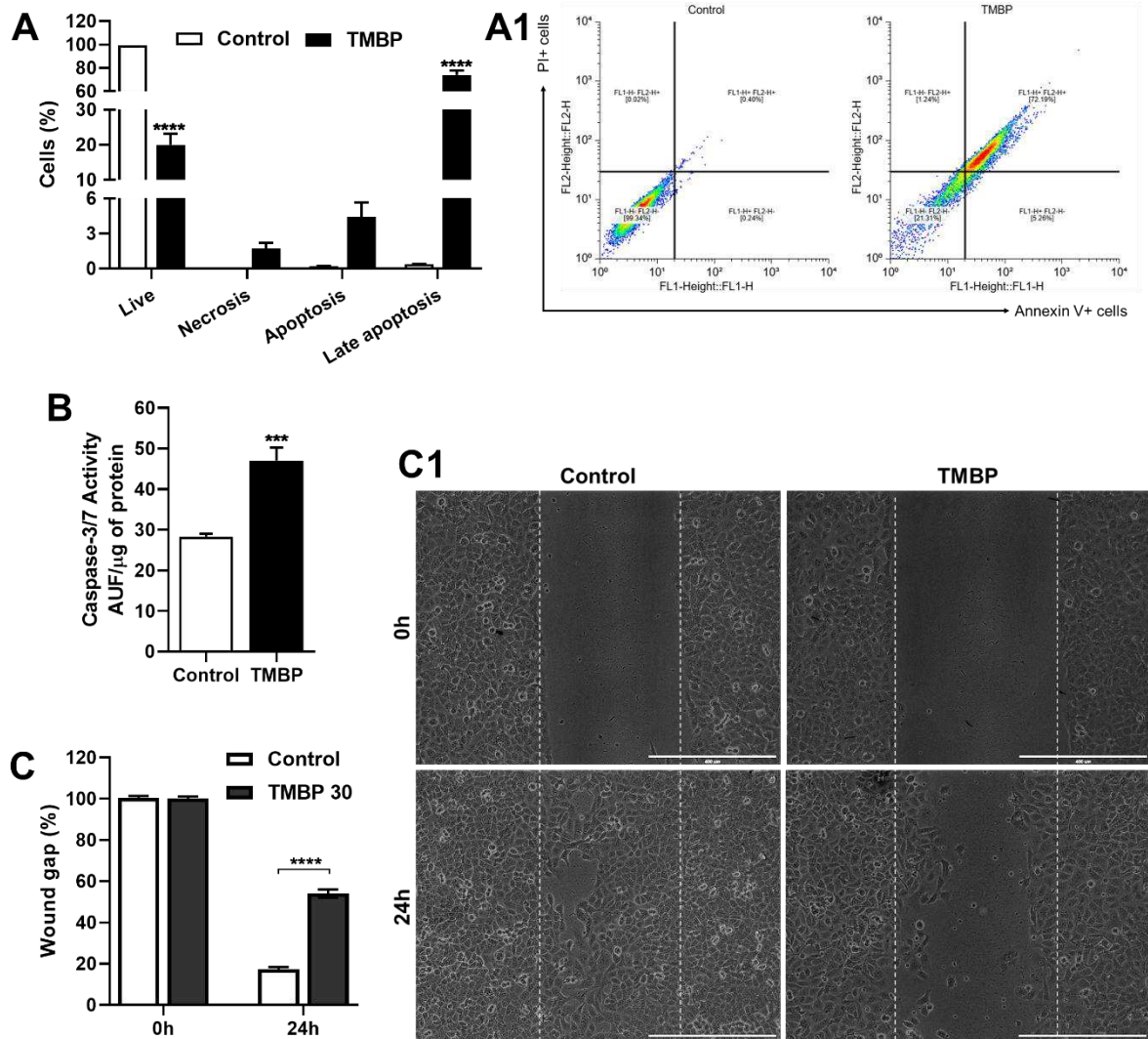
1 **Figure 2. Transcriptomic profiling reveals TMBP-associated modulation of pathways related to**  
2 **DNA damage response, apoptosis, and TGF-β signaling.** (A) DEGs identified following TMBP  
3 treatment, determined by comparative transcriptomic analysis between treated and control MDA-MB-  
4 231 cells. (B) Gene Set Enrichment Analysis (GSEA) highlighting biological processes significantly  
5 enriched upon TMBP exposure. (C) Interaction network of genes involved in the major cellular processes  
6 identified by GSEA.



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**Figure 3. TMBP induces apoptotic cell death and impairs migratory capacity in MDA-MB-231 cells.** (A) Quantification of apoptotic and necrotic cell populations by Annexin V/PI staining following TMBP treatment. (A1) Representative flow cytometry scatter plots illustrating Annexin V/PI staining profiles. (B) Caspase-3/7 activity as a measure of executioner caspase activation. (C) Quantitative analysis of cell migration assessed by wound-healing assay. (C1) Representative light microscopy micrographs of wound closure at 0 and 24 h. Data are presented as mean  $\pm$  SEM from independent experiments. Statistical significance was determined relative to control; \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001.



## 1 Supplementary materials

### 2 **Supplementary table 1.** List of DEGs of TMBP-treated MDA-MB-231 cells.

| Gene         | Gene full name                                                | Log2FC |
|--------------|---------------------------------------------------------------|--------|
| GCOM1        | GCOM1, MYZAP-POLR2M combined locus                            | 3,67   |
| UQCC6        | ubiquinol-cytochrome c reductase complex assembly factor 6    | 3,37   |
| GGPS1        | geranylgeranyl diphosphate synthase 1                         | 3,15   |
| PMS2P4       | PMS1 homolog 2, mismatch repair system component pseudogene 4 | 2,38   |
| NDUFAF3      | NADH:ubiquinone oxidoreductase complex assembly factor 3      | 2,30   |
| SKI          | SKI proto-oncogene                                            | 2,30   |
| GOLGA1       | golgin A1                                                     | 2,25   |
| SARS1        | seryl-tRNA synthetase 1                                       | 2,20   |
| YPEL2        | yippee like 2                                                 | 2,20   |
| OPRD1        | opioid receptor delta 1                                       | 2,19   |
| FADS6        | fatty acid desaturase 6                                       | 2,19   |
| OR10C1       | olfactory receptor family 10 subfamily C member 1             | 2,19   |
| H2AC20       | H2A clustered histone 20                                      | 2,17   |
| IL2          | interleukin 2                                                 | 2,10   |
| RPS10P7      | ribosomal protein S10 pseudogene 7                            | 2,08   |
| LOC100506253 | uncharacterized LOC100506253                                  | 2,08   |
| LINC00052    | long intergenic non-protein coding RNA 52                     | 2,05   |
| CCNF         | cyclin F                                                      | 2,05   |
| UPK3A        | uroplakin 3A                                                  | 2,03   |
| BAP1         | BRCA1 associated deubiquitinase 1                             | 2,01   |
| DMXL2        | Dmx like 2                                                    | 2,01   |
| PSMG3        | proteasome assembly chaperone 3                               | -2,00  |
| CT75         | cancer/testis associated transcript 75                        | -2,01  |
| SUCLA2       | succinate-CoA ligase ADP-forming subunit beta                 | -2,01  |
| MS4A6E       | membrane spanning 4-domains A6E                               | -2,01  |
| FSTL5        | folliculin like 5                                             | -2,02  |
| BFAR         | bifunctional apoptosis regulator                              | -2,02  |
| ANO3         | anoctamin 3                                                   | -2,03  |
| ALPI         | alkaline phosphatase, intestinal                              | -2,04  |
| WDR53        | WD repeat domain 53                                           | -2,04  |
| OR10G4       | olfactory receptor family 10 subfamily G member 4             | -2,05  |
| LINC01003    | long intergenic non-protein coding RNA 1003                   | -2,06  |
| ANXA1        | annexin A1                                                    | -2,06  |
| TCAP         | titin-cap                                                     | -2,06  |
| CAMKMT       | calmodulin-lysine N-methyltransferase                         | -2,08  |
| SRY          | sex determining region Y                                      | -2,08  |
| SLC25A26     | solute carrier family 25 member 26                            | -2,08  |
| BLK          | BLK proto-oncogene, Src family tyrosine kinase                | -2,08  |
| LOC401191    | uncharacterized LOC401191                                     | -2,10  |
| RNF113B      | ring finger protein 113B                                      | -2,11  |
| LINC00592    | long intergenic non-protein coding RNA 592                    | -2,11  |
| GALNT2       | polypeptide N-acetylgalactosaminyltransferase 2               | -2,12  |
| HMGN4        | high mobility group nucleosomal binding domain 4              | -2,13  |

|           |                                                                                 |       |
|-----------|---------------------------------------------------------------------------------|-------|
| GNG2      | G protein subunit gamma 2                                                       | -2,13 |
| DCST2     | DC-STAMP domain containing 2                                                    | -2,14 |
| NPS       | neuropeptide S                                                                  | -2,15 |
| ZNF23     | zinc finger protein 23                                                          | -2,15 |
| KRT26     | keratin 26                                                                      | -2,16 |
| OR4K14    | olfactory receptor family 4 subfamily K member 14                               | -2,16 |
| CKMT2     | creatine kinase, mitochondrial 2                                                | -2,16 |
| TLE4      | TLE family member 4, transcriptional corepressor                                | -2,18 |
| ZNRF2P1   | zinc and ring finger 2 pseudogene 1                                             | -2,22 |
| IFT70A    | intraflagellar transport 70A                                                    | -2,22 |
| COPS3     | COP9 signalosome subunit 3                                                      | -2,24 |
| RAB8B     | RAB8B, member RAS oncogene family                                               | -2,24 |
| CITED4    | Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 4 | -2,25 |
| NPVF      | neuropeptide VF precursor                                                       | -2,25 |
| FAM240A   | family with sequence similarity 240 member A                                    | -2,26 |
| PCBP1     | poly(rC) binding protein 1                                                      | -2,26 |
| GPR137C   | G protein-coupled receptor 137C                                                 | -2,28 |
| PKHD1L1   | PKHD1 like 1                                                                    | -2,30 |
| IFNL1     | interferon lambda 1                                                             | -2,32 |
| FSCN3     | fascin actin-bundling protein 3                                                 | -2,33 |
| LHFPL6    | LHFPL tetraspan subfamily member 6                                              | -2,34 |
| TNFAIP8L3 | TNF alpha induced protein 8 like 3                                              | -2,34 |
| TBCC      | tubulin folding cofactor C                                                      | -2,36 |
| ANKRD65   | ankyrin repeat domain 65                                                        | -2,36 |
| LINC00488 | long intergenic non-protein coding RNA 488                                      | -2,36 |
| RAD23A    | RAD23 homolog A, nucleotide excision repair protein                             | -2,37 |
| FAM98A    | family with sequence similarity 98 member A                                     | -2,40 |
| POM121L12 | POM121 transmembrane nucleoporin like 12                                        | -2,40 |
| ARMS2     | age-related maculopathy susceptibility 2                                        | -2,44 |
| KLHDC9    | kelch domain containing 9                                                       | -2,47 |
| STOML1    | stomatin like 1                                                                 | -2,48 |
| RBX1      | ring-box 1                                                                      | -2,48 |
| OR6C68    | olfactory receptor family 6 subfamily C member 68                               | -2,49 |
| SKIL      | SKI like proto-oncogene                                                         | -2,49 |
| H1-9P     | H1.9 linker histone, pseudogene                                                 | -2,50 |
| INO80C    | INO80 complex subunit C                                                         | -2,51 |
| GCHFR     | GTP cyclohydrolase I feedback regulator                                         | -2,56 |
| FBP2      | fructose-bisphosphatase 2                                                       | -2,56 |
| PLA2G3    | phospholipase A2 group III                                                      | -2,59 |
| PDPR2P    | pyruvate dehydrogenase phosphatase regulatory subunit 2, pseudogene             | -2,59 |
| MMP2      | matrix metalloproteinase 2                                                      | -2,60 |
| DRAM2     | DNA damage regulated autophagy modulator 2                                      | -2,62 |
| BUB1      | BUB1 mitotic checkpoint serine/threonine kinase                                 | -2,64 |
| FDPSP2    | farnesyl diphosphate synthase pseudogene 2                                      | -2,66 |
| BUD31     | BUD31 homolog                                                                   | -2,67 |
| HPDL      | 4-hydroxyphenylpyruvate dioxygenase like                                        | -2,76 |

|           |                                                     |       |
|-----------|-----------------------------------------------------|-------|
| GPRC5D    | G protein-coupled receptor class C group 5 member D | -2,78 |
| AGGF1     | angiogenic factor with G-patch and FHA domains 1    | -2,78 |
| NFKBID    | NFKB inhibitor delta                                | -2,81 |
| KRT4      | keratin 4                                           | -2,90 |
| SLC5A1    | solute carrier family 5 member 1                    | -3,03 |
| MS4A10    | membrane spanning 4-domains A10                     | -3,08 |
| ZCCHC4    | zinc finger CCHC-type containing 4                  | -3,09 |
| PPM1L     | protein phosphatase, Mg2+/Mn2+ dependent 1L         | -3,17 |
| TIFAB     | TIFA inhibitor                                      | -3,46 |
| MXRA8     | matrix remodeling associated 8                      | -3,49 |
| C1orf50   | chromosome 1 open reading frame 50                  | -3,58 |
| SLC16A13  | solute carrier family 16 member 13                  | -3,64 |
| ANGEL1    | angel homolog 1                                     | -3,76 |
| MANCR     | mitotically associated long non coding RNA          | -3,86 |
| ANKRD29   | ankyrin repeat domain 29                            | -3,91 |
| SEPTIN12  | septin 12                                           | -3,92 |
| KDM4A-AS1 | KDM4A antisense RNA 1                               | -4,26 |
| CCNYL4    | cyclin Y like 4 (pseudogene)                        | -5,63 |
| ETF1      | eukaryotic translation termination factor 1         | -8,43 |

1  
2

**ANEXO B**

3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol potentiates Doxorubicin efficacy via mitochondrial dysfunction, ROS overload and autophagy-mediated apoptosis in triple-negative breast cancer cell lines

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Jornal: a ser submetido em Free Radical Biology & Medicine (Fator de Impacto 2024: 8.2)

3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol potentiates Doxorubicin efficacy via mitochondrial dysfunction, ROS overload and autophagy-mediated apoptosis in triple-negative breast cancer cell lines

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## Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype characterized by *TP53* mutations, dependence on CDK1-driven cell cycle progression, and elevated oxidative stress, rendering these tumors vulnerable to therapies that disrupt redox and proliferative homeostasis. The 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol (TMBP), has previously demonstrated anticancer activities in several tumor cell lines. Thus, we investigated whether TMBP enhances the therapeutic efficacy of doxorubicin (DOX) in TNBC cell lines. Non-tumoral HB4a, and TNBC MDA-MB-231, and MDA-MB-468 cells were evaluated using metabolic activity assays, ZIP-based synergism modeling, along with mechanistic analyses including scanning and transmission electron microscopy, flow cytometry (cell cycle, apoptosis, ROS,  $\Delta\Psi_m$ , lipid droplets, acidic vesicles), caspase-3/7 activity, comet assay, autophagic vacuole quantification, and biochemical assessment of oxidative stress markers. TMBP+DOX exhibited additive to synergistic interactions in TNBC cells, while sparing non-tumoral HB4a cells. Mechanistically, TMBP potentiated DOX-induced G1 arrest, leading to catastrophic ROS overload and mitochondrial structural disruption. Autophagy inhibition partially rescued cell viability, indicating a cytotoxic rather than cytoprotective role. Consistently, depletion of antioxidant defenses (SOD, GPx, and GSH) further indicated redox collapse, especially in MDA-MB-231 cells, highlighting subtype-specific vulnerability to redox imbalance. In conclusion, TMBP synergizes with DOX by exacerbating oxidative stress and engaging ROS-dependent, autophagy-mediated apoptotic pathways. This mechanistically informed combination strategy selectively targets TNBC cells while preserving non-tumoral cell viability, supporting its potential as a redox-based therapeutic approach for aggressive TNBC.

**Keywords:** Programmed cell death; Chemoresistance; Oxidative damage

## 1. INTRODUCTION

Triple-negative breast cancer (TNBC) accounts for approximately 20% of breast cancers and represents the most aggressive and therapeutically challenging subtype, characterized by the absence of estrogen receptor, progesterone receptor, and HER2 amplification, limiting targeted therapeutic strategies (1,2). Despite initial responsiveness to chemotherapy, most patients present recurrence and metastasis. Current regimens are largely based on anthracyclines, taxanes, and platinum agents, which are associated with significant toxicity and chemoresistance (3), reinforcing the urgent need for rationally designed combination strategies that exploit TNBC-specific vulnerabilities.

One hallmark of TNBC is the high frequency of *TP53* loss-of-function mutations, that leads to impaired G1/S checkpoint, rendering tumor cells heavily dependent on cyclin-dependent kinase 1 (CDK1)-driven cell cycle progression (4,5). Its pharmacological inhibition has the potential to selectively impair TNBC cells and enhance the efficacy of DNA-damaging standard therapies such as doxorubicin (DOX), while sparing non-transformed tissues that retain functional *TP53* (6,7). TNBC cells also exhibit elevated baseline reactive oxygen species (ROS) yet relying on amplified antioxidant pathways to survive (8). By targeting mitochondrial function and inducing ROS generation overload might be an interesting target for tumors with this phenotype.

The 3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol (TMBP), a synthetic biphenyl molecule, was previously shown by molecular docking analysis to interact with CDK1 at T15 inactivation site, along with *CDK1* mRNA downregulation, cell cycle arrest at G2/M, increased ROS, mitochondrial damage, and apoptosis more efficiently on *TP53*-mutant cells (9), with no toxicity on BALB/c mouse peritoneal macrophages (10). Integrating these vulnerabilities, CDK1 reliance due to *TP53* mutation and

heightened oxidative stress, offers a strong mechanistic rationale for combination therapy.

In this study, we investigated whether TMBP enhances DOX activity in TNBC by inducing mitochondrial damage and overloading oxidative stress. By comparing responses in TNBC and non-tumoral cells, we also assess the translational relevance and therapeutic window of this combination. Collectively, our findings aim to advance TMBP+DOX as a mechanistically therapeutic strategy for TNBC, laying the groundwork for future preclinical validation and biomarker-driven patient selection.

## **2. METHODOLOGY**

### **2.1. Cell Culture**

The non-tumoral HB4a cell line was kindly provided by Dr. Maria Lucia Hirata Katayama from the University of São Paulo. The MDA-MB-231 cell line was provided by Dr. Juliana Mara Serpeloni from the State University of Londrina. The MDA-MB-468 cell line was provided by Dr. Jaqueline Carvalho de Oliveira from the Federal University of Parana. All human mammary cell lineages were confirmed to be mycoplasma-negative using reverse transcription–quantitative real-time PCR (RT–qPCR).

The HB4a cell line was cultured in RPMI-1640 medium (Gibco, Invitrogen, New York, USA), supplemented with hydrocortisone and insulin at a concentration of 5 µg/mL. The MDA-MB-231 and MDA-MB-468 were cultured in DMEM (Lifetechnologies, Carlsbad, CA, USA), and DMEM/F12 medium (Gibco), respectively. All culture media were supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Santa Cruz Biotechnologies, Dallas, TX, USA), and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere.

Cells were seeded at different concentrations due to proliferation rate and/or cell size, so confluency does not reach >90%. HB4a and MDA-MB-468 cells were seeded at the following concentrations for all experiments, unless otherwise specified:  $1 \times 10^4$  (96-well plate),  $1 \times 10^5$  (24-well plate), and  $1 \times 10^6$  (6-well plate); while for MDA-MB-231:  $5 \times 10^3$  (96-well plate),  $5 \times 10^4$  (24-well plate), and  $5 \times 10^5$  (6-well plate). For all experiments, the cells were incubated for 24 h for stabilization.

## 2.2. Laccase-catalyzed synthesis of TMBP

3,3',5,5'-Tetramethoxybiphenyl-4,4'-diol (TMBP),  $C_{16}H_{18}O_6$  (MW: 306) was synthesized from 2,6-dimethoxyphenol by laccase from the ascomyceteous fungus *Botryosphaeria rhodina* (MAMB-05) under the conditions previously described by Schirmann et al. (11). A stock solution of TMBP was prepared in 1% (w/v) dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). The DMSO concentration in all experiments did not exceed 0.03%, except for the cellular metabolic activity assay (0.1%).

## 2.3. Cellular metabolic activity assay

The cell lines, HB4a, MDA-MB-231, and MDA-MB-231 were seeded in 96-well plates, and treated with TMBP (6 - 100  $\mu$ M) or DOX (0.01 - 5  $\mu$ M) for 24 h. Cells were then incubated for 2 h in a solution of 7-hydroxy-3H-phenoxazin-3-one 10-oxide (resazurin; 60  $\mu$ M). The fluorescence intensity was quantified in a Glomax® Discover Microplate Reader (Promega, GM3000) at Ex: 520 nm and Em: 580 nm. The data were used to calculate the 50% inhibitory concentration ( $IC_{50}$ ), and determine the range of concentrations (TMBP 6-50  $\mu$ M; DOX 0.01-0.5  $\mu$ M) for the drug associations. Subsequently, the selectivity index, and the combination index (CI) was evaluated

1 using the Zero Interaction Potency (ZIP) model through the SynergyFinderPlus  
2 platform.

3 To elucidate the involvement of ROS or autophagy in the treatments'  
4 cytotoxic effects, cells were pretreated with N-acetyl cysteine (NAC; 5 mM; Sigma-  
5 aldrich) or hydroxychloroquine (HCQ; 10  $\mu$ M; PHLO) for 2 h or wortmannin (WMN; 100  
6 nM; Invitrogen) for 1 h prior to adding the treatments. After treatment, the experiment  
7 was conducted as described previously.

#### 9 2.4. Scanning and Transmission Electron Microscopy

10 For morphological analyzes, MDA-MB-231, and MDA-MB-468 cells  
11 were seeded in round coverslips inside a 24-well plate and treated for 24 h. The cells  
12 were then washed with PBS and fixed with a solution of 2.5% glutaraldehyde and 0.1  
13 M sodium cacodylate buffer (pH 7.2) for 24 h. The cells were then dehydrated in  
14 increasing concentrations of ethanol (30, 50, 70, 90, 95, and 100% v/v). Subsequently,  
15 the cells were submitted to the critical point by substituting ethanol for CO<sub>2</sub>, and coated  
16 with gold nanoparticles (Leica Geosystems, St. Gallen, Switzerland).  
17 Microphotographs were obtained on a FEI Quanta 250 scanning electron microscope.

18 For ultrastructural analyzes, the cells (MDA-MB-468:  $1.5 \times 10^6$ ; MDA-  
19 MB-231:  $1 \times 10^6$ ) were seeded in T25 flasks, treated and fixed as described previously.  
20 The cells were then post-fixed with 1 % OsO<sub>4</sub>, 0.8 % potassium ferrocyanide and  
21 10 mM CaCl<sub>2</sub> in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h. Samples were  
22 dehydrated in increased concentrations of acetone (30, 50, 70, 90, 95, 100% v/v) and  
23 embedded in EPON™ epoxy resin for 72 h at 60 °C. Finally, ultrathin sections (60–  
24 70 nm slices) were obtained, mounted on copper grids and contrasted with 5% uranyl  
25 acetate and 2% lead citrate. Sections were visualized on a JEOL JEM-1400

transmission electron microscope.

## 2.5. Flow Cytometry

The cells were seeded in 6-well plates and treated for 24 hours. Subsequently, the cell suspension was centrifuged at 2000 rpm for 5 minutes, and the cell pellet resuspended in 300  $\mu$ L of PBS. After the following staining procedures, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at 4° C, washed three times with PBS and resuspended in 300  $\mu$ L of PBS. Specifically for cell cycle analysis, cells were fixed with 70% ethanol at 4° C overnight before washing and staining steps. The analyses were performed using the FACSCalibur (BD Biosciences, New Jersey, USA) flow cytometer at 10,000 events.

### 2.5.1. Cell Cycle Analysis

After fixed, a solution of 0.05% ribonuclease A (RNAse A; Sigma-Aldrich) was added and incubated for 30 min, followed by the addition of a solution of 0.1% sodium citrate and 1% Triton-X 100 along with 50  $\mu$ g/mL of propidium iodide (PI) (Sigma, St. Louis, MO, USA) for 30 min. The percentage of cells in different phases of the cell cycle (G0/G1, S, and G2/M) were estimated based on fluorescence intensity.

### 2.5.2. Apoptosis Assay

The assay was performed by adding 50  $\mu$ L of annexin V and propidium iodide solution (Millipore, Billerica, MA, USA) for 20 minutes at room temperature before fixing cells with 4% PFA as described.

### 2.5.3. Reactive Oxygen Species (ROS) generation

A solution of 2',7'-dichlorofluoresceindiacetate (H<sub>2</sub>DCFDA; 10  $\mu$ M; Sigma-Aldrich) was added in the supernatant for 30 min in the dark at 37°C, and then fixed with 4% PFA. H<sub>2</sub>O<sub>2</sub> was used as a positive control.

#### 2.5.4. Determination of mitochondrial membrane potential ( $\Delta\Psi_m$ )

$\Delta\Psi_m$  was determined by tetramethylrhodamine ethyl ester (TMRE; 250 nM; Sigma-Aldrich) staining. Cells were incubated for 45 min at 37 °C with TMRE solution in PBS. The wells were then washed with PBS and fixed with 4% PFA. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as the positive control.

#### 2.5.5. Lipid droplets (LD) detection

Lipid droplets were evaluated by staining with Nile red (NR; 1  $\mu$ M; Sigma-Aldrich) for 15 min, then washed with PBS, and fixed with 4% PFA. H<sub>2</sub>O<sub>2</sub> were used as the positive control.

#### 2.5.6. Detection of acidic vesicular organelles (AVOs)

The AVOs were detected by Acridine Orange (AO; 1  $\mu$ g/mL w/v; Sigma-Aldrich) staining. The cells were incubated for 15 min at room temperature, washed with PBS, and fixed with 4% PFA before analysis.

### 2.6. Comet assay

The assay was performed as previously described (12). Cells were seeded in 6-well plates and treated for 3 h. After treatment, cells were suspended in PBS and mixed with low-melting point agarose (0.8%). The cell solution was then added to slides coated with agarose (1.5%). After solidification, they were subjected to

chemical lysis for 24 h at 4 °C, followed by alkaline treatment for 20 min. Subsequently, the slides were electrophoresed in alkaline buffer (pH > 13) at 1 V/cm and 300 mA for 20 min, followed by neutralization (pH 7.5), fixation with absolute ethanol, and storage at 4 °C. The slides were stained with ethidium bromide (2 µg/mL), and 100 nucleoids per treatment were evaluated using photomicrographs at 200× magnification (EVOS® FL Auto Cell Imaging System (Thermo Fisher). Tail DNA damage was visually classified according to the damage index (classes 0 to 4).

## 2.7. Caspase 3/7 activity assay

Caspase-3/7 enzyme activity was performed using the EnzChek™ Caspase-3/7 Assay Kit (Invitrogen™). The cells were seeded in 6-well plates and treated for 24 h, washed with PBS, and incubated at -80 °C for lysis. Lysis buffer (1% Triton X-100 in PBS) was subsequently added and incubated in ice for 30 min. This was followed by the addition of kit reagents (i.e., reaction buffer, dithiothreitol, and 5.0 mM Z-DEVD-AMC substrate) and incubated for 30 min. The increase in fluorescence intensity readings (GloMax, Promega; Ex: 342 nm, Em: 441 nm) are indicative of cleavage of the Z-DEVD-AMC substrate. Data were normalized and expressed in arbitrary units of fluorescence.

## 2.8. Autophagic vacuoles formation

Cells were seeded in 6-well plates, treated for 24 hours, and stained with monodansylcadaverine (MDC; 50 µM; Sigma-Aldrich) for 30 min at 37° C. Thereafter, the cells were washed with PBS, and the concentration normalized to 1x10<sup>5</sup> cells per sample. Subsequently, 100 uL of each sample were added to a black 96-well plate in three technical replicates, and read by fluorimetry at 380 nm excitation and 525 nm emission. H<sub>2</sub>O<sub>2</sub> were used as positive control.

## 2.9. Redox profile

For the analysis of oxidative stress markers, treated cells in 6-well plates were collected, centrifuged at 2000 rpm, the supernatant was discarded, and the pellet was frozen for subsequent quantifications. The oxidative stress assessment included the determination of total protein concentration using the Bradford method (Quick Start™ Bradford Protein Assay, Bio-Rad). Lipid peroxidation was quantified using a reaction system composed of 1.15% KCl, 1 M FeCl<sub>3</sub>, 0.25 M ascorbic acid, 2.8% TCA, and 1% TBA. Additionally, the activities of the main antioxidant enzymes were determined: superoxide dismutase (SOD; EC 1.15.1.1) (13), catalase (CAT; EC 1.11.1.6) (14), glutathione peroxidase (GPx; EC 1.11.1.9) (15), and glutathione-S-transferase (GST; EC 2.5.1.18) (16). All enzymatic assays were performed at 25 °C and analyzed using a microplate reader (μQuant-MQX 200), with Kcjunior 675 software (Bio-Tek Instruments, Winooski, VT, USA) for kinetic or spectrophotometric measurements.

## 2.10. Public Transcriptomic Data Processing and Heatmap Visualization

Publicly available gene expression datasets (GSE26599, GSE133049, and GSE82298) were retrieved from the Gene Expression Omnibus (GEO) database. All datasets were generated using the Affymetrix Human Genome U133 Plus 2.0 Array (GPL570) platform, ensuring technical consistency across studies and enabling direct comparison without cross-platform normalization. Only untreated control samples were included in the analysis, allowing comparison of basal transcriptional profiles across cell lines.

Expression data were imported into the R statistical environment using Bioconductor packages, including GEOquery for data acquisition. Probe identifiers were annotated and mapped to official gene symbols using the hgu133plus2.db annotation package. Probes lacking valid gene symbol annotation were excluded. When multiple probes mapped to the same gene, the probe exhibiting the highest mean expression across samples was selected to represent the corresponding gene. When required, expression data were log2-transformed and normalized using standard workflows implemented in the limma package.

For pathway-focused visualization, genes associated with the PI3K/AKT signaling and apoptosis pathways were curated based on established pathway databases and published literature, prioritizing core regulators and effectors with well-documented roles in cancer biology. To enable comparison of relative gene expression patterns across samples and cell lines, expression values were z-score normalized on a per-gene basis. Heatmaps were generated using the pheatmap package, allowing visualization of expression patterns and pathway-associated transcriptional profiles.

#### 2.10.1. Pathway Activity Analysis by ssGSEA and PLAGE

Pathway-level activity was assessed at the individual-sample level using single-sample Gene Set Enrichment Analysis (ssGSEA) and Pathway Level Analysis of Gene Expression (PLAGE), both implemented in the GSVA package. Hallmark gene sets from the Molecular Signatures Database (MSigDB) were used for the PI3K/AKT/mTOR signaling and apoptosis pathways.

ssGSEA enrichment scores were calculated independently for each sample based on normalized expression data, providing quantitative estimates of

pathway engagement under basal conditions. In parallel, PLAGE scores were computed using the same Hallmark gene sets following z-score normalization of gene expression values.

## 2.11. Statistical analysis

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

## 3. RESULTS

### 3.1. TMBP and DOX exhibit additive to synergistic cytotoxic effects in TNBC cells while preserving non-tumoral cell viability

Resazurin assays performed after 24 h demonstrated a concentration-dependent reduction in metabolic activity following TMBP (6–100  $\mu$ M) and DOX (0.01–5  $\mu$ M) treatment. TMBP reduced metabolic activity from 12.5  $\mu$ M onward in all cell lines, with the most pronounced cytotoxic effects observed at 50 and 100  $\mu$ M ( $p < 0.0001$ ). In non-tumoral HB4a cells, viability decreased to 66.64% and 36.12% (**Figure 1A**), whereas MDA-MB-231 viability dropped to 20.47% and 0.68% (**Figure 1B**), and MDA-MB-468 to 34.19% and 5.37% (**Figure 1C**), respectively. DOX reduced viability in all cell lines at all concentrations ( $p < 0.0001$ ; **Figure 1A-C**), with MDA-MB-468 being the most sensitive.

The IC<sub>50</sub> analysis further confirmed the selective cytotoxic profile of TMBP toward TNBC cells (HB4a: 71.75 µM; MDA-MB-231: 35.84 µM; MDA-MB-468: 29.45 µM), whereas DOX IC<sub>50</sub> values, 0.731 µM, 0.600 µM, and 0.275 µM (**Table 1**), indicated limited selectivity, mainly toward MDA-MB-468 cells.

Based on the IC<sub>50</sub> values and differential sensitivity observed across cell lines, combination treatments were designed to evaluate potential pharmacological interactions between TMBP and DOX (**Figure 1D-F**). All statistical analyses comparing monotherapies vs. combinations are presented in **Supplementary Table 1**. Among the tested combinations, 25 µM TMBP + 0.1 µM DOX demonstrated the most effective cytotoxic response in both TNBC cell lines, inhibiting MDA-MB-231 and MDA-MB-468 by 51.68% and 57.81%, while sparing non-tumoral HB4a cells (22.61%;  $p < 0.05$  vs TMBP;  $p < 0.0001$  vs DOX; **Figure 1G**).

Consistently, selectivity index values were 2.29 and 2.56 for MDA-MB-231 and MDA-MB-468, respectively (**Table 2**). ZIP-based interaction analysis (**Figure 1H**) revealed additive to synergistic interactions between TMBP and DOX, with synergy scores of 21.440 in MDA-MB-231 cells and 3.190 in MDA-MB-468 for the 25 µM + 0.1 µM combination.

### 3.2. TMBP, DOX, and TMBP+DOX induce pronounced morphological alterations of TNBC cell lines

Scanning electron microscopy was conducted to evaluate surface morphological changes in the TNBC cell lines MDA-MB-231 and MDA-MB-468 following treatment with TMBP, DOX, or their combination (TMBP+DOX) for 24 h (**Figure 2**).

Control MDA-MB-231 (Figure 2A1–2) and MDA-MB-468 cells (Figure 2E1–2) exhibited firm substrate adhesion and preserved cellular morphology, characterized by intact cell membrane and abundant membrane protrusions, including lamellipodia, filopodia, and microvilli, consistent with a healthy and metabolically active phenotype.

In contrast, all treatment conditions induced marked morphological alterations in both TNBC cell lines. Treated cells displayed cell rounding, loss of substrate adhesion, substantial reduction in surface microvilli, and retraction or fragmentation of lamellipodia and filopodia (**Figure 2**). Notably, MDA-MB-231 cells treated with TMBP alone (**Figure 2B2**) or in combination with DOX (**Figure 2D1–2**) exhibited severe plasma membrane damage, including membrane rupture and surface irregularities. Similar structural alterations were observed in MDA-MB-468 cells following TMBP (**Figure 2F2**) and TMBP+DOX (**Figure 2H1–2**) treatments, indicating extensive membrane compromise.

### 3.3. TMBP and DOX differentially modulate cell cycle arrest, with combined treatment promoting G1 arrest selectively in TNBC cells

Cell cycle analysis revealed distinct responses to TMBP, DOX, and their combination across cell lines. TMBP treatment increased the G2/M population in all cells, with an increase to 31.48% in HB4a ( $p < 0.01$ ; **Figure 3A**), 34.39% in MDA-MB-231 ( $p < 0.05$ ; **Figure 3B**), and 48.34% in MDA-MB-468 ( $p < 0.001$ ; **Figure 3C**). In contrast, DOX induced G1 arrest, with populations reaching 54.94% in HB4a ( $p < 0.05$ ), 50.36% in MDA-MB-231 ( $p < 0.01$ ), and 55.55% in MDA-MB-468 ( $p < 0.001$ ).

The combination treatment shifted TNBC cells toward a predominant G1 arrest. MDA-MB-231 and MDA-MB-468 exhibited significant increases in the G1

phase (58.22%,  $p < 0.0001$ ; and 55.23%,  $p < 0.001$ , respectively), consistent with DOX-driven checkpoint activation. In HB4a cells, however, the combination did not alter the cell cycle profile relative to control (histograms are available at **supplementary figure 1**).

### 3.4. **TMBP+DOX selectively potentiates apoptotic cell death in TNBC cells while preserving viability in non-tumoral cells**

Annexin V/PI staining (representative scatter plots are available at **supplementary figure 2**) and caspase 3/7 activity assays were conducted to characterize treatment-induced cell death. In HB4a cells, both TMBP and DOX increased early apoptosis (11.77% and 17.70%;  $p < 0.01$  and  $p < 0.0001$ ), while the combination preserved viability (94.67%; **Figure 4A**) and maintained total apoptosis at near-control levels (4.60%; **Figure 4B**). DOX alone significantly elevated total apoptosis (19.76%;  $p < 0.01$ ). Caspase 3/7 activity increased following DOX (1.760-fold;  $p < 0.0001$ ) and TMBP+DOX (1.912-fold;  $p < 0.0001$ ; **Figure 4C**), indicating that apoptotic signaling remains limited in non-tumorigenic cells despite treatment.

In MDA-MB-231 cells, TMBP and DOX significantly increased early apoptosis (16.40% and 11.34%;  $p < 0.0001$ ), while the combination strongly promoted late apoptosis (35.96%;  $p < 0.0001$ ) and reduced viability to 60.46% (**Figure 4D**). Total apoptosis followed this pattern, with TMBP (18.84%;  $p < 0.0001$ ) and DOX (13.85%;  $p < 0.001$ ) both inducing apoptosis, but the combination reaching the highest level (39.52%;  $p < 0.0001$  vs. all groups; **Figure 4E**). Caspase 3/7 activity was strongly induced by TMBP (2.416-fold;  $p < 0.0001$ ) and further elevated by the combination (2.790-fold;  $p < 0.0001$  vs. DOX; **Figure 4F**), confirming potentiation of apoptotic signaling.

Similarly, in MDA-MB-468 cells, TMBP (10.23%) and DOX (4.77%) induced early apoptosis, while the combination greatly increased both early (29.27%) and late apoptosis (30.77;  $p < 0.0001$ ), reducing viability to 39.38% (**Figure 4G**). Total apoptosis was highest with the combination (60.04%;  $p < 0.0001$  vs. all groups), exceeding the effects of TMBP (13.72%) and DOX (10.49%) alone (**Figure 4H**). Caspase 3/7 activity followed this pattern, showing strong induction by TMBP (9.214-fold;  $p < 0.0001$ ) and an even greater increase under the combination (12.410-fold;  $p < 0.0001$  vs. TMBP and DOX; **Figure 4I**).

#### 3.4.1. Basal transcriptional profiling indicates physiological PI3K/AKT and apoptosis signaling in non-tumoral mammary cells

To investigate whether insulin supplementation could account for the preserved viability and limited apoptotic response observed in HB4a cells, we performed transcriptomic analyses using publicly available datasets. Z-score-normalized heatmaps revealed that genes associated with PI3K/AKT signaling and apoptosis were globally downregulated in non-tumoral mammary cells compared to TNBC cell lines (**Supplementary Figure 3A–B**). Consistently, ssGSEA analysis demonstrated higher pathway enrichment scores in MDA-MB-231 cells, intermediate scores in HB4a cells, and lower scores in MDA-MB-468 cells (**Supplementary Figure 3C–D**), reflecting distinct modes of pathway regulation. PLAGE analysis further confirmed reduced coordinated transcriptional activation of both PI3K/AKT and apoptosis gene sets in HB4a cells relative to TNBC cells (**Supplementary Figure 3E–F**). Together, these data indicate that insulin supplementation maintains physiological signaling in non-tumoral cells rather than inducing aberrant pro-survival transcriptional programs.

### 3.5. **TMBP attenuates DOX-induced genotoxic stress in non-tumoral HB4a cells**

DNA integrity was assessed using the comet assay, scoring damage from 0 (no damage) to 4 (maximal fragmentation) to calculate the DNA Damage Index (**Figure 5A**). In HB4a cells, DOX significantly increased DNA damage (20.33 vs. control 2.33;  $p < 0.0001$ ), while the combination treatment (TMBP+DOX) significantly reduced this genotoxic effect (10.00;  $p < 0.0001$  vs DOX), indicating a protective response in non-tumorigenic cells. TMBP alone did not induce significant DNA damage (4.00; **Figure 5B**).

In contrast, TMBP increased DNA damage in both MDA-MB-231 (12.33 vs. control 1.66;  $p < 0.01$ ; **Figure 5C**), and MDA-MB-468 cells (13.00 vs. control 3.00;  $p < 0.05$ ; **Figure 5D**). DOX induced genotoxicity only in MDA-MB-468 (20.67;  $p < 0.001$ ). The TMBP+DOX combination also increased DNA damage on both TNBC lines when compared to control, but DNA damage index did not differ from either single-drug treatment.

### 3.6. **TMBP+DOX induces mitochondrial dysfunction and lipid droplet accumulation in TNBC cells**

$\Delta\Psi_m$  and intracellular lipid accumulation were evaluated to characterize metabolic stress induced by TMBP, DOX, and their combination in TNBC cells.

In MDA-MB-231 cells, TMBP significantly reduced  $\Delta\Psi_m$  (773.7 AU vs. control 1455 AU;  $p < 0.0001$ ), with a similar depolarization after DOX treatment (875.9 AU;  $p < 0.0001$ ). The combination treatment led to the strongest mitochondrial

depolarization (640.7 AU;  $p < 0.0001$ ; **Figure 6A**). A similar pattern was observed in MDA-MB-468 cells, where both TMBP (731.3 AU) and DOX (854.7 AU) significantly decreased  $\Delta\Psi_m$  compared to control (1678 AU;  $p < 0.0001$  for both), with the lowest  $\Delta\Psi_m$  detected following TMBP+DOX treatment (681.2 AU;  $p < 0.0001$ ; **Figure 6B**).

Transmission electron microscopy (TEM) corroborated these findings, revealing intact mitochondrial ultrastructure only in control cells, characterized by preserved cristae organization (**Figure 6A1, B1**). In contrast, treated cells exhibited swollen mitochondria with disrupted or lost cristae following TMBP, DOX, and particularly TMBP+DOX exposure (**Figure 6A2-4, B2-4**).

LD accumulation, assessed by NR staining, was also markedly increased under treatment conditions. In MDA-MB-231 (**Figure 6C**), TMBP elevated NR fluorescence from 6.56 AU to 17.69 AU ( $p < 0.0001$ ), and DOX produced a similar increase (17.55 AU;  $p < 0.0001$ ). The combination further amplified this effect (30.13 AU;  $p < 0.0001$  vs. all groups). In MDA-MB-468 (**Figure 6D**), LD accumulation increased from 10.07 AU (control) to 19.82 AU after TMBP ( $p < 0.05$ ), to 39.93 AU after DOX ( $p < 0.0001$ ), and reached the highest levels following TMBP+DOX treatment (98.72 AU;  $p < 0.0001$  vs. all groups).

Consistent with NR staining, TEM revealed increased cytoplasmic LDs in treated cells, with accumulation after single treatments (**Figure 6C2-3, D2-3**), and extensive LDs deposition after TMBP+DOX exposure (**Figure 6C4, D4**).

### 3.7. TMBP+DOX presents autophagy-dependent cytotoxicity in TNBC cells

Autophagic activation was assessed using AO and MDC staining. In MDA-MB-231 cells, TMBP significantly increased the percentage of AO red fluorescent positive cells (23.02% vs. control 0.55%;  $p < 0.01$ ), whereas DOX induced only a

modest non-significant increase. The combination treatment induced the highest autophagic response (42.09%;  $p < 0.0001$ ; **Figure 7A**). Similarly, MDC staining increased with TMBP (129.90 AU vs. control 64.06 AU;  $p < 0.0001$ ) and with TMBP+DOX (111.10 AU;  $p < 0.01$ ; **Figure 7A'**), while DOX alone did not significantly differ from control. In MDA-MB-468 cells, TMBP increased AO red fluorescence positivity (14.86% vs. 0.38%;  $p < 0.01$ ), which was further enhanced by TMBP+DOX treatment (18.43%;  $p < 0.05$ ; **Figure 7B**). MDC staining also increased after TMBP (404.60 AU vs. 232.10 AU;  $p < 0.0001$ ) and TMBP+DOX (455.90 AU;  $p < 0.0001$ ; **Figure 7B'**), while DOX again showed no significant effect.

Ultrastructural analysis by TEM confirmed autophagic activation, as autophagic vacuoles were absent in control cells (**Figure 7A1, B1**), but readily detected following TMBP (**Figure 7A2, B2**), and TMBP+DOX (**Figure 7A4, B4**) treatments in both cell lines. DOX treatment induced limited autophagic vacuoles formation (**Figure 7A3, B3**).

To determine whether autophagy contributed to survival or cytotoxicity, cells were pretreated with WMN (early-stage inhibitor) or HCQ (late-stage inhibitor) prior to resazurin assays.

In MDA-MB-231, autophagy inhibition significantly rescued metabolic activity across all treatment groups. WMN increased viability in TMBP (22,204 AU vs. 31,399 AU;  $p < 0.0001$ ), DOX (23,394 AU vs. 28,524 AU;  $p < 0.0001$ ), and TMBP+DOX (14,762 AU vs. 19,511 AU;  $p < 0.0001$ ) groups (**Figure 7C**). HCQ produced a similar protection, increasing viability in TMBP (18,227 vs 20,717;  $p < 0.01$ ), DOX (24,471 vs 22,324;  $p < 0.05$ ), and TMBP+DOX treated cells (12,173 vs 14,796;  $p < 0.001$ ; **Figure 7D**).

In MDA-MB-468, WMN (30,070 vs. 36,388;  $p < 0.0001$ ; **Figure 7E**) and HCQ (29,115 vs. 39,902;  $p < 0.0001$ ; **Figure 7F**) significantly increased viability only in TMBP-treated cells, with no significant effect in DOX or TMBP+DOX groups.

### 3.8. TMBP-induced cytotoxicity is ROS-dependent in TNBC cell lines

ROS levels were quantified using H<sub>2</sub>DCFDA to determine whether oxidative stress contributes to the cytotoxic effects of TMBP, DOX, and TMBP+DOX in TNBC cell lines. In MDA-MB-231 cells, TMBP and DOX led to a significant increase in ROS (27.92 AU and 29.15 AU, respectively vs control 7.64 AU;  $p < 0.0001$ ). The TMBP+DOX combination further enhanced ROS generation (34.15 AU;  $p < 0.0001$  vs. control;  $p < 0.05$  vs. TMBP; **Figure 8A**). MDA-MB-468 cells exhibited an even stronger response. TMBP and DOX elevated ROS from 9.68 AU to 55.91 AU and 60.15 AU, respectively ( $p < 0.0001$ ), whereas the combination induced a prominent increase (190.1 AU;  $p < 0.0001$  vs. all groups; **Figure 8B**). Pre-treatment with the antioxidant NAC effectively suppressed ROS generation in all conditions (**Figure 8A,B**).

To determine whether ROS generation was functionally linked to cytotoxicity, cell viability was evaluated following NAC pre-treatment. In MDA-MB-231 cells, NAC significantly rescued TMBP-induced loss of viability (from 8,972 to 15,660 AU;  $p < 0.0001$ ) and also partially restored viability in the TMBP+DOX group (5,561 to 10,771 AU;  $p < 0.0001$ ; **Figure 8C**). Similarly, in MDA-MB-468 cells, NAC significantly reversed viability loss induced by TMBP (17,882 to 26,634 AU;  $p < 0.0001$ ) and DOX (16,206 to 19,014 AU;  $p < 0.001$ ), with the strongest rescue observed in the TMBP+DOX group (4,884 to 14,568;  $p < 0.0001$ ; **Figure 8D**).

### 3.9. Redox profile reveals distinct oxidative responses underlying ROS-dependent cytotoxicity

To further characterize oxidative stress induced by the treatments, we quantified antioxidant enzymes, GSH and lipid peroxidation levels in both TNBC cell lines.

In MDA-MB-231, SOD activity remained unchanged with TMBP and DOX, but was significantly reduced in the TMBP+DOX group (240.3 U/mg;  $p < 0.001$  vs. control 270.8 U/mg; **Figure 9A**). Catalase activity increased from 12.05  $\mu\text{M}$  in control to 18.83  $\mu\text{M}$  with TMBP ( $p < 0.05$ ), 19.44  $\mu\text{M}$  with DOX ( $p < 0.01$ ), and 18.83  $\mu\text{M}$  in TMBP+DOX ( $p < 0.05$ ; **Figure 9C**). GSH levels (**Figure 9E**) and GPx activity (**Figure 9G**) were significantly reduced only in TMBP+DOX group, from 77.07 to 71.69  $\mu\text{g/mL}$  ( $p < 0.01$ ) and 15.06 to 12.25  $\mu\text{M}$  ( $p < 0.01$ ), respectively. GST activity (control: 31.87  $\mu\text{M}$ ) increased significantly only in DOX group (46.01  $\mu\text{M}$ ;  $p < 0.05$ ; **Figure 9I**). TBARS levels increased significantly after TMBP (3.08 O.D. vs. control 2.08 O.D.;  $p < 0.05$ ) and DOX (3.02 O.D.;  $p < 0.05$ ) and further rose after combination treatment (4.28 O.D.;  $p < 0.0001$  vs. control;  $p < 0.05$  vs. TMBP;  $p < 0.01$  vs. DOX; **Figure 9K**).

In MDA-MB-468, SOD activity increased from 209.9 to 260.5 U/mg with TMBP ( $p < 0.01$ ), 263.6 U/mg with DOX ( $p < 0.01$ ), and 271.3 U/mg with TMBP+DOX ( $p < 0.01$ ; **Figure 9B**). Catalase increased only in TMBP+DOX (62.83  $\mu\text{M}$  vs. control 49.80  $\mu\text{M}$ ;  $p < 0.05$ ; **Figure 9D**). GSH levels rose significantly with TMBP (71.55  $\mu\text{g/mL}$ ;  $p < 0.001$ ), DOX (68.84  $\mu\text{g/mL}$ ;  $p < 0.05$ ), and T+D (71.00  $\mu\text{g/mL}$ ;  $p < 0.001$ ) compared to control (63.97  $\mu\text{g/mL}$ ; **Figure 9F**). GPx activity increased only in DOX treatment (16.83  $\mu\text{M}$  vs. control 12.29  $\mu\text{M}$ ;  $p < 0.01$ ; **Figure 9H**). GST activity was significantly reduced by TMBP (30.06  $\mu\text{M}$ ;  $p < 0.001$ ), DOX (21.01  $\mu\text{M}$ ;  $p < 0.0001$ ), and TMBP+DOX (22.14  $\mu\text{M}$ ;  $p < 0.0001$ ), with the combination also significantly lower

than TMBP ( $p < 0.001$ ; **Figure 9J**). TBARS levels increased sharply with TMBP (1.90 O.D. vs. control 0.32 O.D.;  $p < 0.0001$ ) and DOX (2.64 OD;  $p < 0.0001$ ) compared to control (0.32 O.D.), but were lower in TMBP+DOX (1.83 O.D.;  $p < 0.0001$  vs. control;  $p < 0.01$  vs. DOX; **Figure 9L**).

#### 4. DISCUSSION

TNBC remains one of the most therapeutically challenging breast cancer subtypes due to its aggressive biology, high proliferative capacity, metabolic plasticity, and lack of targeted therapies (1,3). In this context, the CDKs, but specially CDK1, has emerged as an essential survival kinase, and its inhibition is increasingly recognized as a promising therapeutic approach (17,18). A major reason for the CDK1 dependency in TNBC, specially on Basal-like 1 (BL1) and Mesenchymal (MES) subtypes (4,5) is the high prevalence of *TP53* mutations and *MYC* overexpression (19–22). Without p53-mediated G1/S checkpoint control, MYC promotes cell cycle progression by upregulating cyclins and suppressing CDK inhibitors, then TNBC cells have to rely on CDK1-driven G2/M progression to maintain the high proliferation rate and to tolerate oxidative and genotoxic stress (6,23,24). Thus, CDK1 inhibition or downregulation removes a critical compensatory checkpoint, leading to mitotic vulnerability and sensitization to stress-inducing therapies (6,7).

Our findings show that TMBP, a synthetic biphenyl compound previously characterized by our group in other tumoral cell lines (9,10,25), induces stronger cytotoxicity in *TP53*-mutated TNBC cells compared to wild-type A549 (325  $\mu$ M) and NCI-H460 (152  $\mu$ M), lung cancer cells, IC<sub>50</sub> values at 24 h treatment. We have previously shown that wild-type HepG2/C3a can activate compensatory mechanisms resulting in increased *CDK1* mRNA expression as a way to overcome TMBP

mechanism of action, while mutant HuH7.5 shows intense *CDK1* downregulation with more prominent G2 arrest (9).

Moreover, its combination with DOX not only enhanced cytotoxic efficacy but also increased selectivity toward TNBC cells, demonstrating additive to synergistic effects on MDA-MB-468 and MDA-MB-231, respectively. While demonstrating antagonistic effect on HB4a cells. Similarly to what was observed in mammary cells treated with roscovitine, a multi-CDK inhibitor, and doxorubicin, depicting synergy on TNBC cells and antagonistic response on *TP53* wild-type cells (6).

Consistent with our previous findings, TMBP alone induced G2 arrest, whereas DOX induced G1 arrest, and the TMBP+DOX combination further enhanced G1 arrest in TNBC cells. DOX-induced cell cycle arrest normally depends on the p53/p21 axis (26), however, in *TP53*-mutant cells, DOX can induce p21 via p73, a p53-family member with overlapping transcriptional functions, but to a lesser extent (27–29). Moreover, the loss of p53/p21 axis disrupts the G1/S checkpoint, blocking their ability to restrain CDK2–cyclin E and CDK4/6–cyclin D activities (30,31). Under these conditions, *TP53*-mutant cancers become highly dependent on CDK1 to sustain proliferation. CDK1 assumes a broader role in this context, supporting cell cycle progression by compensating for other CDKs with dysregulated activities and maintaining sufficient CDK signaling to drive G1/S and G2/M transitions (32,33).

In this context, TMBP likely disrupts the CDK signaling that these cells rely on, lowering their threshold for checkpoint activation. As a consequence, the attenuated p73-mediated p21 induction triggered by DOX becomes sufficient to impose a G1 arrest when CDK1 is inhibited or downregulated. This explains why the TMBP+DOX combination shifted the arrest toward G1 despite TMBP alone inducing

G2 arrest. Importantly, in HB4a cells, which retain an intact p53/p21 axis and do not exhibit CDK1 dependency, the combination treatment maintained a cell cycle distribution similar to the control group, indicating that the cooperative G1 arrest is specific to the checkpoint-deficient, CDK1-dependent background of TNBC cells.

Both TNBC cell lines also displayed robust apoptotic signaling under TMBP+DOX, characterized by a pronounced increase in late apoptotic populations and marked activation of caspase-3/7. The combination intensified apoptosis beyond either monotherapy, consistent with a model in which cell cycle disruption, potentially involving CDK1 inhibition, may compromise DNA damage tolerance, allowing DOX-induced genotoxicity to trigger irreversible apoptotic commitment (6). Importantly, this amplification of apoptosis was not observed in non-tumoral HB4a cells, further reinforcing treatment selective cytotoxicity of the combined treatment toward TNBC cells.

A potential concern when using insulin-supplemented media is the artificial activation of pro-survival signaling pathways, particularly PI3K/AKT, which could mask cytotoxic effects in non-tumoral cells. However, transcriptomic analyses using publicly available datasets demonstrated that non-tumoral mammary epithelial cells exhibit globally reduced transcriptional engagement of PI3K/AKT and apoptosis-related gene networks compared to TNBC cell lines. Z-score-normalized expression profiles, ssGSEA enrichment scores, and PLAGE coordination analyses consistently indicated that HB4a cells operate within a physiological signaling range, whereas TNBC cells, particularly MDA-MB-231, display heightened pathway engagement. These findings are consistent with previous multi-omic studies showing that non-tumoral mammary epithelial cells, such as MCF10A, despite requiring hEGF and insulin supplementation for proliferation, exhibit significantly lower PI3K/AKT pathway

enrichment compared to TNBC cell lines, MDA-MB-231 and HCC1937 (34). Collectively, these observations suggest that insulin supplementation maintains basal metabolic homeostasis in non-tumoral cells rather than inducing aberrant pro-survival transcriptional programs. Consequently, the preserved viability of HB4a cells following TMBP+DOX treatment likely reflects intrinsic signaling balance rather than pharmacological protection, supporting the selective vulnerability of TNBC cells to combined treatment.

A defining finding of this study is that TMBP-induced cytotoxicity occurs in a ROS-dependent manner, consistent with Concato-Lopes et al. (25), who reported oxidative stress and ROS accumulation after TMBP treatment in NCI-H460 cells. We show that NAC fully suppresses TMBP-driven ROS overproduction and substantially rescues cell viability, effects that are especially pronounced for the TMBP+DOX combination. Doxorubicin itself is a well-documented generator of ROS via mitochondrial disruption and redox cycling, and ROS contributes to its cytotoxic signaling (35,36). Taken together, the pharmacologic rescue experiments corroborate with prior reports on TMBP's pro-oxidant mechanism and indicate that ROS overload underlies the enhanced cytotoxicity observed when TMBP is combined with DOX.

The mitochondrial dysfunction was accompanied by distinct redox adaptations in the two TNBC cell lines, along with a prominent accumulation of LDs. CDK1 plays a direct role in mitochondrial homeostasis by phosphorylating Drp-1 to promote mitochondrial fission (37) and by phosphorylating MnSOD to enhance its stability and antioxidant activity (38). Studies have shown that blocking Drp-1-mediated fission results in enlarged, swollen mitochondria with disrupted cristae (39,40), while loss of MnSOD activity increases mitochondrial ROS, overproduction of 4-hydroxynonenal (4-HNE), reduced oxidative phosphorylation (OXPHOS), severe

1 cristae disruption and vacuolization (41,42). These data are consistent with our  
2 ultrastructural findings of swollen mitochondria, cristae loss, decreased membrane  
3 potential, and accumulation of LDs.

4               The LD accumulation is a direct adaptive response to mitochondrial  
5 dysfunction and oxidative stress, serving both as a storage site for damaged lipids and  
6 as a protective mechanism against lipid peroxidation through membrane maintenance  
7 (43,44). However, excessive LD buildup due to oxidative damage has been associated  
8 with impaired  $\beta$ -oxidation (45), and increased susceptibility to oxidative cell death (46).

9               Our data showing decreased SOD, GPx and GSH in MDA-MB-231  
10 after combination treatment versus compensatory upregulation of SOD, catalase and  
11 GSH in MDA-MB-468 is due to the redox heterogeneity in TNBC. Optical redox imaging  
12 studies show that MDA-MB-231 cells displays a more oxidized basal redox state,  
13 presenting greater reliance on OXPHOS and thus increased demand on antioxidant  
14 systems, whereas MDA-MB-468 cells display a more reduced profile with increased  
15 reduced nicotinamide adenine dinucleotide (NADH) and GSH, which is consistent with  
16 a more glycolytic metabolic phenotype and a greater capacity for ROS scavenging  
17 (8,47,48). Regardless of these baseline differences, strong and persistent ROS  
18 overload exceeds the capacity of antioxidant systems, leading to oxidative damage  
19 accumulation and cell death (49).

20               Our results also demonstrate that autophagy also occurs in a context-  
21 dependent manner, contributing to cell death in MDA-MB-231 cells but remaining  
22 predominantly cytoprotective in MDA-MB-468 under DOX-containing conditions.  
23 Pharmacologic inhibition of autophagy rescued viability in MDA-MB-231, revealing a  
24 pro-death autophagic mechanism, similar to what was observed in carnosol-induced  
25 excessive mitochondrial damage, oxidative stress, and autophagy-mediated apoptosis

in mesenchymal-like MDA-MB-231 cells (50,51). In contrast, MDA-MB-468 cells, that presents a more glycolytic and antioxidant phenotype, maintained the classical cytoprotective role of autophagy upon DOX or combination treatment, likely using autophagic flux to buffer damaged organelles and recycle substrates to sustain metabolic homeostasis. Only TMBP alone was sufficient to push autophagy toward a pro-death outcome in MDA-MB-468, suggesting that the extent and nature of oxidative stress and mitochondrial damage dictates whether the pathway promotes survival or accelerates cell death.

Taken together, this study provides a unified mechanistic framework in which TMBP interaction and downregulation of CDK1 destabilizes mitochondrial homeostasis, impairs antioxidant defense, and drives ROS accumulation, ultimately triggering mitochondrial dysfunction, autophagy dysregulation, lipid peroxidation, and apoptosis. DOX synergizes with this vulnerability by enhancing oxidative and genotoxic stress while promoting G1 arrest. The selective lethality toward TNBC cells is closely tied to their *TP53*-mutated, CDK1-dependent phenotype, highlighting a clear therapeutic rationale for this combination.

## 5. CONCLUSION

In this study, we demonstrate that TMBP exerts a selective cytotoxicity against TNBC cells by exploiting their dependence on CDK1, a vulnerability driven, in part, by *TP53* loss-of-function mutation. TMBP induces G2 arrest, disrupts mitochondrial dynamics likely through impaired Drp1 and MnSOD activation, and triggers mitochondrial depolarization, lipid accumulation, and excessive ROS production. The TMBP+DOX combination amplifies oxidative stress, enhances G1 arrest, and promotes apoptosis in a synergistic and TNBC-selective manner. Redox

profiling reveals distinct adaptive responses between TNBC cell lines, yet both converge on ROS-driven cytotoxicity. Importantly, this work identifies pro-death autophagy as a novel mechanism contributing to TMBP's anticancer activity. Together, these findings provide a comprehensive mechanistic rationale for combining TMBP with DOX to exploit mitochondrial and redox vulnerabilities inherent to TNBC, positioning this strategy as a promising therapeutic avenue for further preclinical development.

**Abbreviations:**  $\Delta\Psi_m$ , mitochondrial membrane potential; 4-HNE, 4-hydroxynonenal; AO, acridine orange; AVOs, acidic vesicular organelles; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CDK, cyclin-dependent kinase; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethylsulfoxide; Drp-1, dynamin related protein 1; F12, Ham's F-12 nutrient mixture; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSH, glutathione; H<sub>2</sub>DCFDA, 2',7' -dichlorofluorescein diacetate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HCQ, hydroxychloroquine; LD, lipid droplets; MDC, monodansylcadaverine; MnSOD, manganese superoxide dismutase; NAC, N-acetyl cysteine; NR, Nile red; OXPHOS, oxidative phosphorylation; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute; SEM, standard error of the mean; SOD, superoxide dismutase; TEM, transmission electron microscopy; TMBP, 3,3',5,5'-tetramethoxybiphenyl-4,4'-diol; TMRE, tetramethylrhodamine ethyl ester; TNBC, triple-negative breast cancer; WMN, wortmannin; ZIP, zero interaction potency

**CRedit authorship contribution statement:**

**Fabricio Seidy Ribeiro Inoue:** Conceptualization, Methodology, Investigation, Formal

analysis, Writing – original draft, Writing – review and editing. **Danielle Lazarin-Bidóia**: Investigation, Formal analysis, Validation. **João Manoel de Sousa**: Investigation. **Ellen Mayara Souza Cruz**: Investigation. **Mariana Barbosa Detoni**: Investigation, Writing – review and editing. **Miriã França de Paula**: Investigation. **Vinícius Ferreira Sampaio**: Investigation. **Rodolfo Bento Balbinot**: Investigation. **Luan Vitor Alves de Lima**: Investigation. **Milena Cremer de Souza**: Investigation. **Fabio Rodrigues Ferreira Seiva**: Resources, Methodology. **Jéseka Gabriela Schirmann**: Resources, Methodology. **Aneli M. Barbosa-Dekker**: Resources, Writing – review and editing. **Robert F. H. Dekker**: Resources, Writing – review and editing. **Mário Sérgio Mantovani**: Resources, Methodology. **Celso Vataru Nakamura**: Resources, Methodology. **Carolina Panis**: Resources, Investigation, Writing – review and editing, Supervision. **Wander Rogério Pavanelli**: Conceptualization, Resources, Writing – review and editing, Supervision.

## **Conflict of interest**

The authors have no conflict of interests to declare.

## **Funding**

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Conselho Nacional de Pesquisa (CNPq, Brazil), Financiadora de Estudos e Projetos (FINEP, Brazil), and Universidade Estadual de Londrina (UEL, Brazil).

## **Acknowledgements**

## **Declaration of Generative AI and AI-assisted technologies in the writing process.**

During the preparation of this manuscript, the authors used generative artificial intelligence tools (ChatGPT) to assist with language refinement and improvement of clarity. The authors take full responsibility for the scientific content, data interpretation, and conclusions presented in this manuscript.

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1 **TABLES**2 **Table 1.** TMBP and DOX Inhibitory concentrations of 50% in mammary cell lines after 24 h treatment.

|                   | <b>TMBP</b>                   | <b>DOX</b>                    |
|-------------------|-------------------------------|-------------------------------|
| <b>Cell Lines</b> | <b>IC<sub>50</sub> (±SEM)</b> | <b>IC<sub>50</sub> (±SEM)</b> |
| <b>HB4a</b>       | 71.75 µM (± 1.06)             | 0.731 µM (± 0.001)            |
| <b>MDA-MB-231</b> | 38.84 µM (± 1.11)             | 0.600 µM (± 0.001)            |
| <b>MDA-MB-468</b> | 29.45 µM (± 1.08)             | 0.275 µM (± 0.001)            |

IC<sub>50</sub> – Inhibitory Concentration of 50%; SEM – Standard Error Mean.

3

4 **Table 2.** Selectivity index of TMBP+DOX treatment in TNBC cells.

| <b>Comparison (T 25 + D 0.1)</b> | <b>Selectivity Index (SI)</b> |
|----------------------------------|-------------------------------|
| <b>MDA-MB-231 / HB4a</b>         | 2.29                          |
| <b>MDA-MB-468 / HB4a</b>         | 2.56                          |

5

6

1 FIGURES

2 **Figure 1. TMBP and DOX reduce cell viability in mammary cell lines and exhibit selective additive**  
3 **to synergistic interactions in TNBC cells.** (A–C) Resazurin assays after 24 h of treatment with TMBP  
4 (6–100  $\mu$ M) or DOX (0.01–5  $\mu$ M) show a concentration-dependent reduction in metabolic activity in  
5 HB4a (A), MDA-MB-231 (B), and MDA-MB-468 (C) cells. (D–F) Inhibition heatmaps of TMBP+DOX  
6 combinations in HB4a (D), MDA-MB-231 (E), and MDA-MB-468 (F) cells show selective toxicity to TNBC  
7 cells. (G) Comparative statistical analysis of 25  $\mu$ M TMBP + 0.1  $\mu$ M DOX combination. (H) ZIP analysis  
8 indicates additive to synergistic interactions in both TNBC cell lines. Data are expressed as the mean  $\pm$   
9 SEM. Statistical difference: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ).

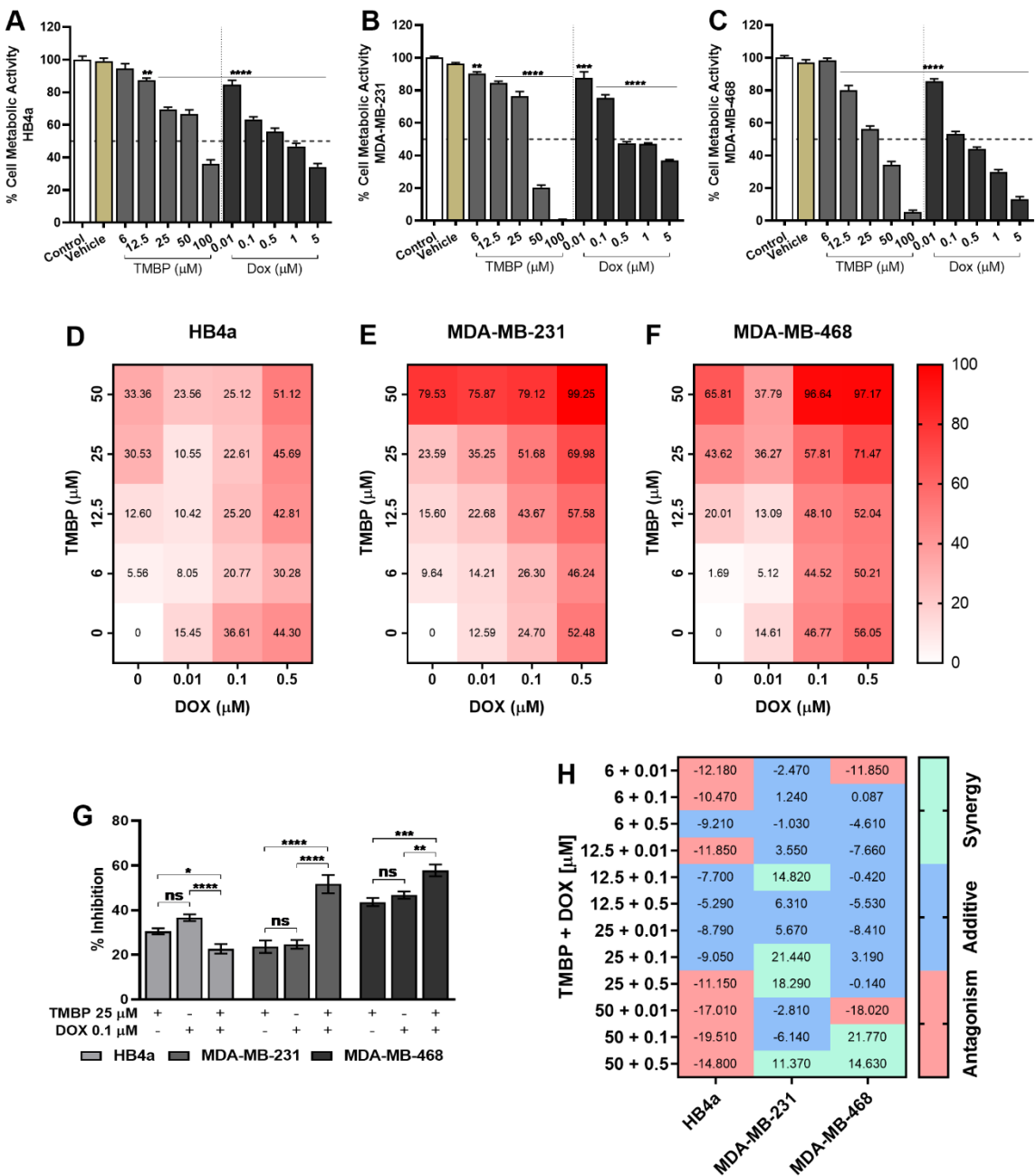


**Figure 2. Morphological alterations in TNBC cells following treatment with TMBP, DOX, and TMBP+DOX assessed by scanning electron microscopy.** (A–D) MDA-MB-231 cells: control (A1–2), TMBP (B1–2), DOX (C1–2), and TMBP+DOX (D1–2). (E–H) MDA-MB-468 cells: control (E1–2), TMBP (F1–2), DOX (G1–2), and TMBP+DOX (H1–2). Control cells display preserved membrane integrity with abundant filopodia (F), lamellipodia (L), and microvilli (Mi). Treated cells show reduction of microvilli (►), retraction or fragmentation of membrane protrusions, and cell membrane damage (→). Scale bars: 10  $\mu$ m.

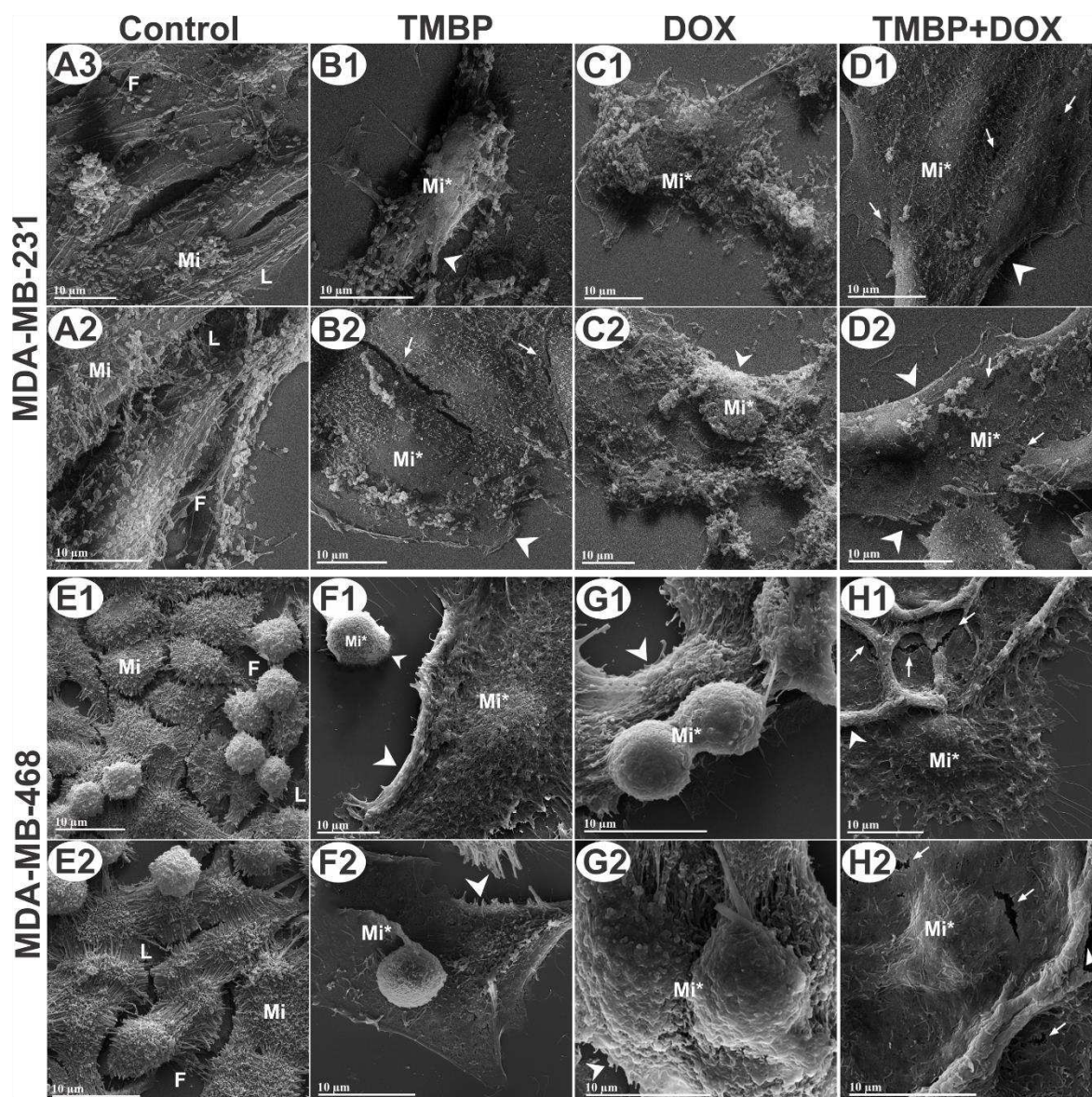


**Figure 3. TMBP and DOX combination shifts TNBC cells toward G1 arrest.**  
 (A–C) Cell cycle distributions of HB4a (A), MDA-MB-231 (B), and MDA-MB-468 (C) cells following treatment with TMBP, DOX, or TMBP+DOX. Data are expressed as the mean  $\pm$  SEM. Statistical difference: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ).

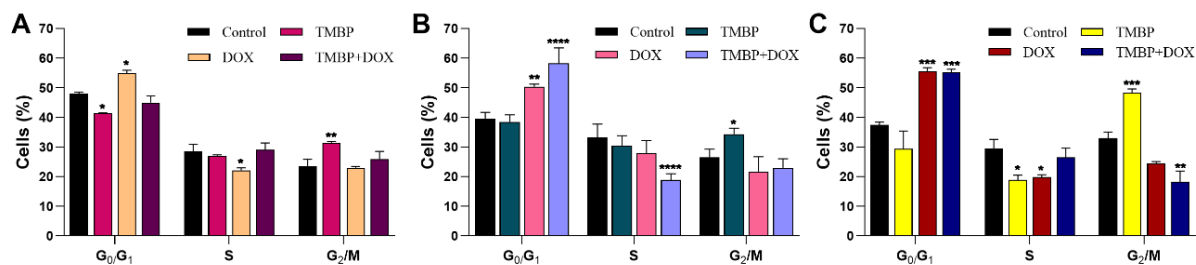


**Figure 4. TMBP+DOX selectively induces apoptosis in TNBC cells while preserving viability in non-tumoral HB4a cells.** After treatments with TMBP, DOX or TMBP+DOX, (A, D, G) Annexin V/PI assay was conducted to assess apoptotic/necrotic events, (B, E, H) Total apoptotic population (%), and (C, F, I) Caspase 3/7 activity in HB4a, MDA-MB-231, and MDA-MB-468, respectively. Data are expressed as the mean  $\pm$  SEM. Statistical difference: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ).

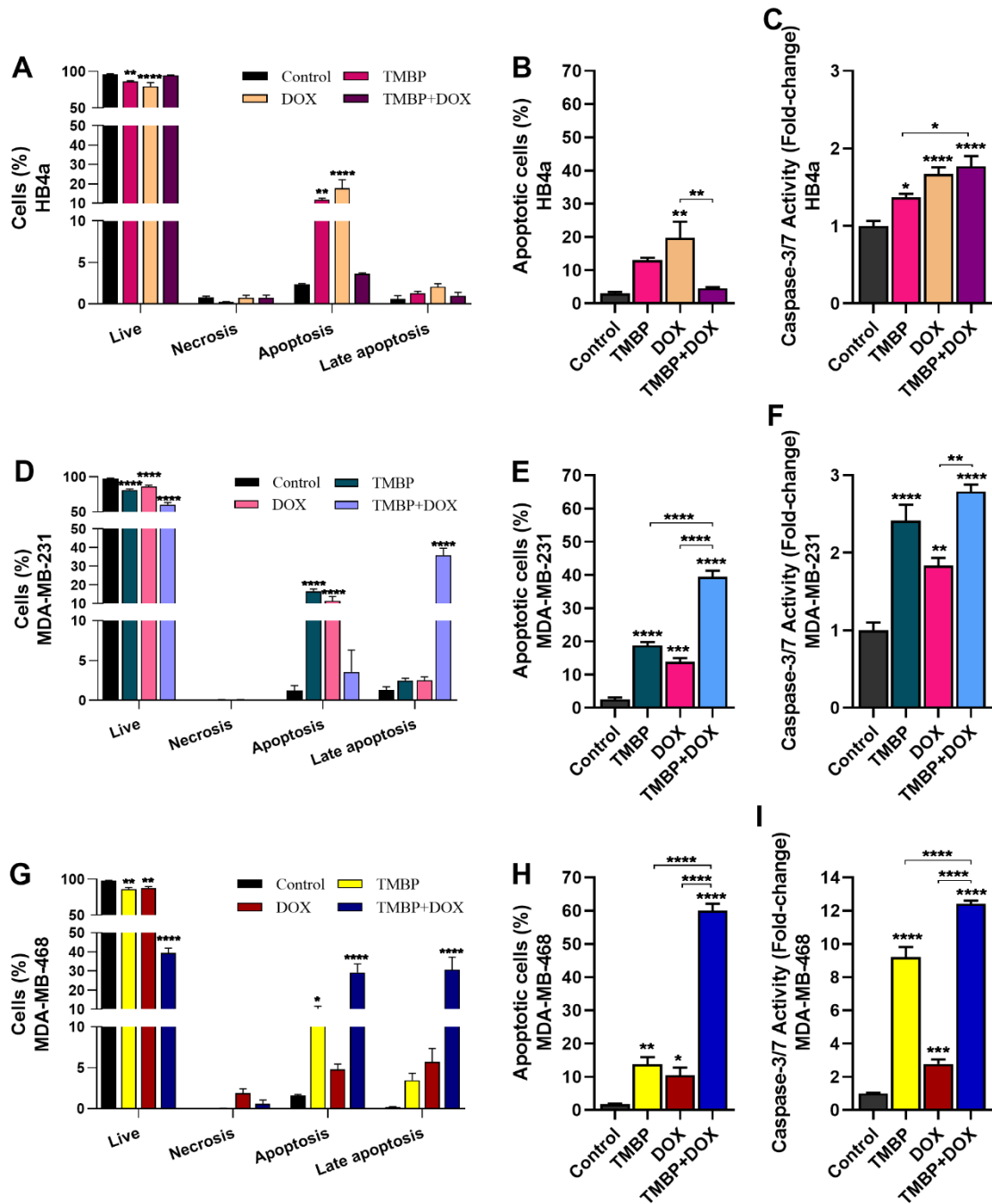


**Figure 5. TMBP reduces DOX-induced DNA damage in non-tumoral HB4a cells following short-term exposure.** (A) Representative images of comet assays after TMBP, DOX, and TMBP+DOX 3 h treatments and nucleoids representing DNA damage classes (0-4) used to calculate the DNA Damage Index. (B-D) Quantification of DNA Damage Index in HB4a (B), MDA-MB-231 (C), and MDA-MB-468 (D) cells. Data are expressed as the mean  $\pm$  SEM. Statistical difference: \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ).

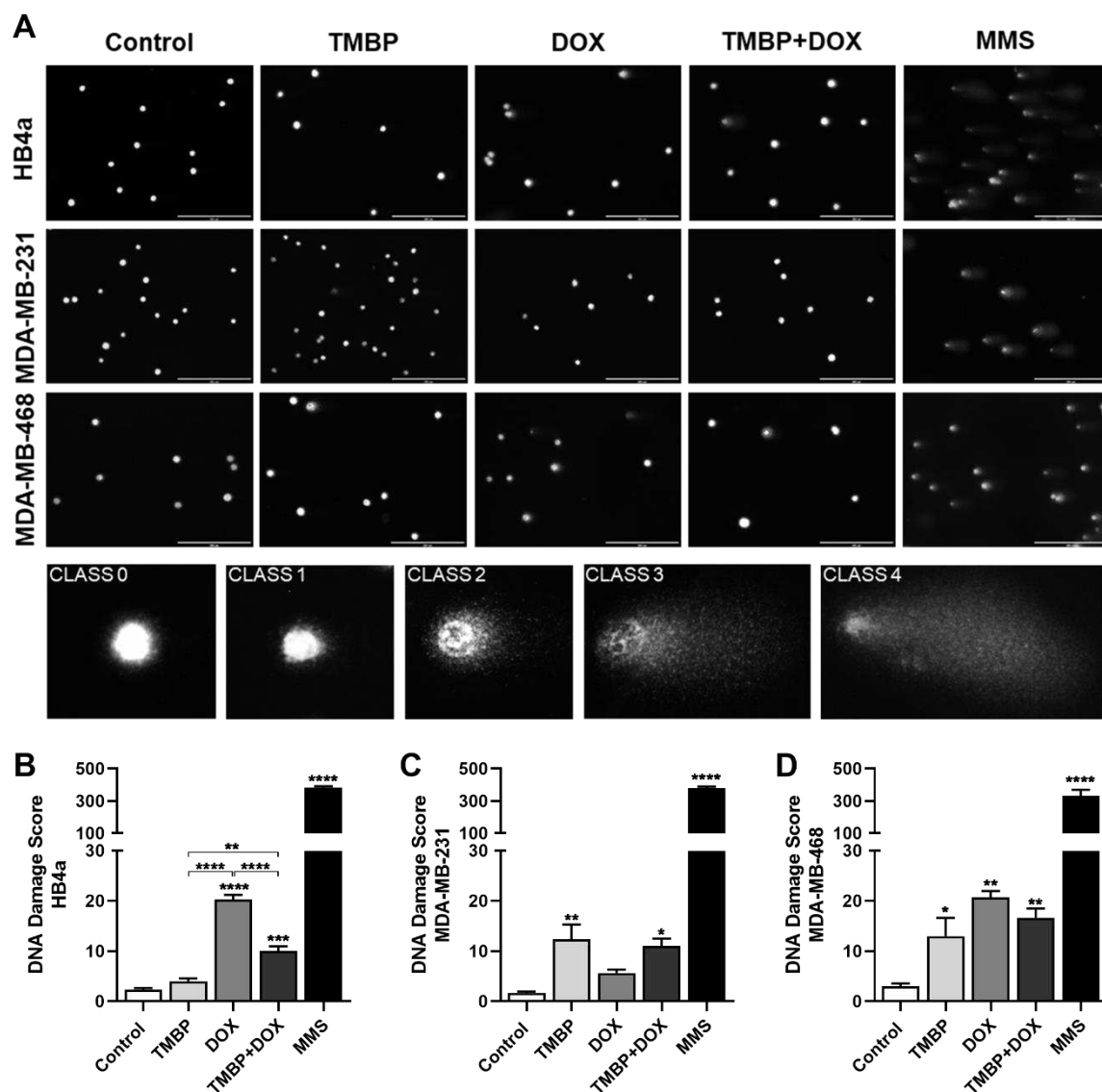


**Figure 6. TMBP+DOX induces mitochondrial dysfunction and lipid accumulation in TNBC cells.**

(A-B)  $\Delta\Psi_m$  was assessed by TMRE staining in MDA-MB-231 (A) and MDA-MB-468 (B) cells following treatments with TMBP, DOX, or TMBP+DOX. (A1-4, B1-4) Representative TEM images illustrate mitochondrial ultrastructural alterations induced by the treatments. (C, D) Lipid droplet accumulation was quantified by NR staining in MDA-MB-231 (C) and MDA-MB-468 (D) cells, with (C1-C4, D1-D4) corresponding TEM representative images highlighting lipid droplets cytoplasmic accumulation. Ultrastructural features include nucleus (n), mitochondria (m), endoplasmic reticulum (er), mitochondrial alterations ( $\rightarrow$ ), endoplasmic reticulum alterations ( $\blacktriangleright$ ), and lipid droplets (\*). Data are expressed as the mean  $\pm$  SEM. Statistical difference: \* ( $p < 0.05$ ), \*\*\*\* ( $p < 0.0001$ ).

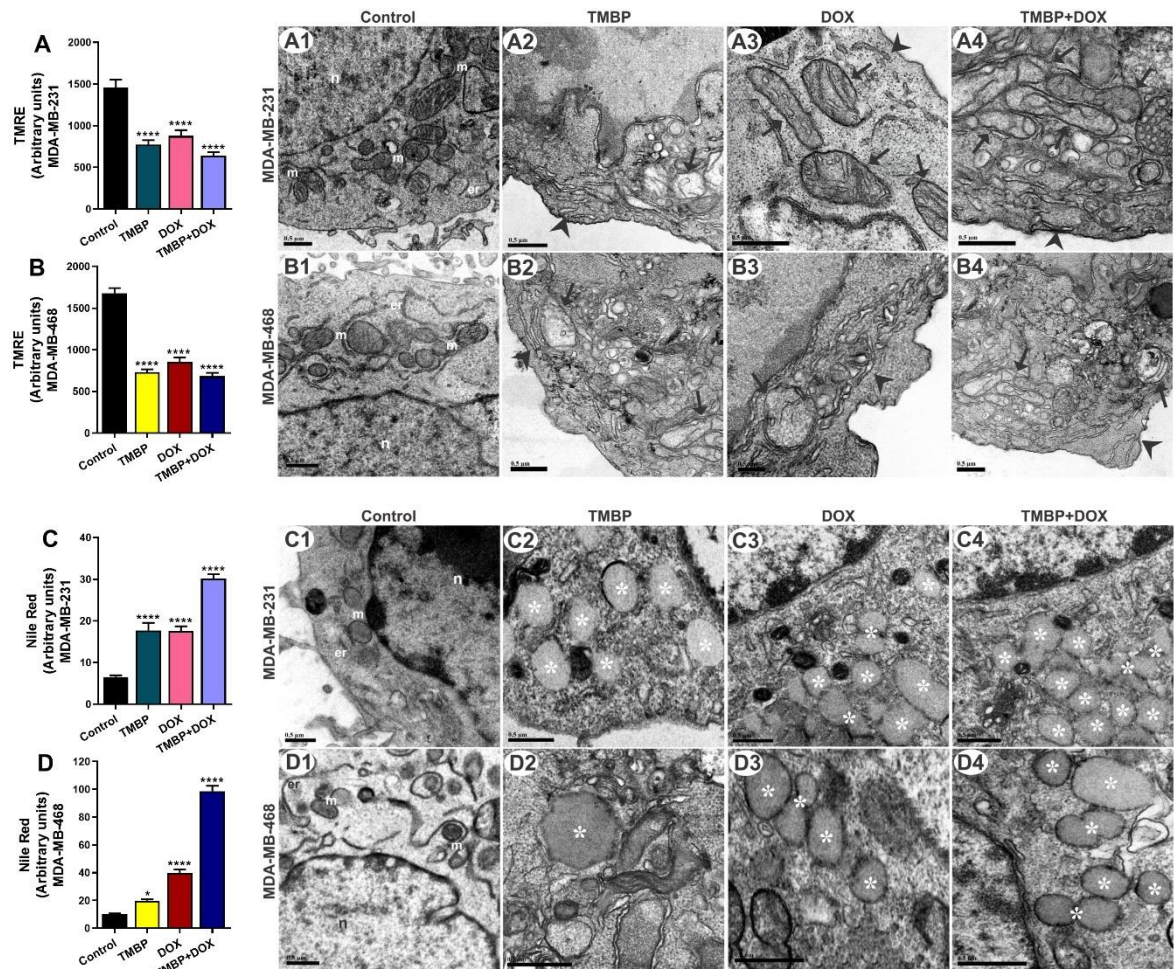
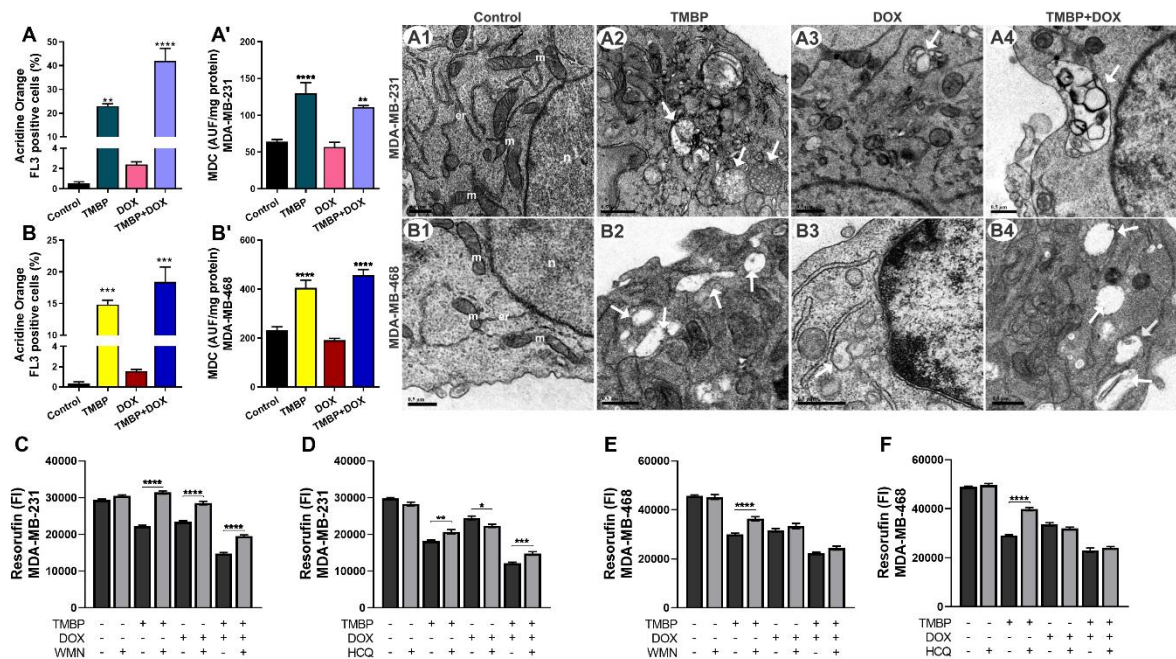
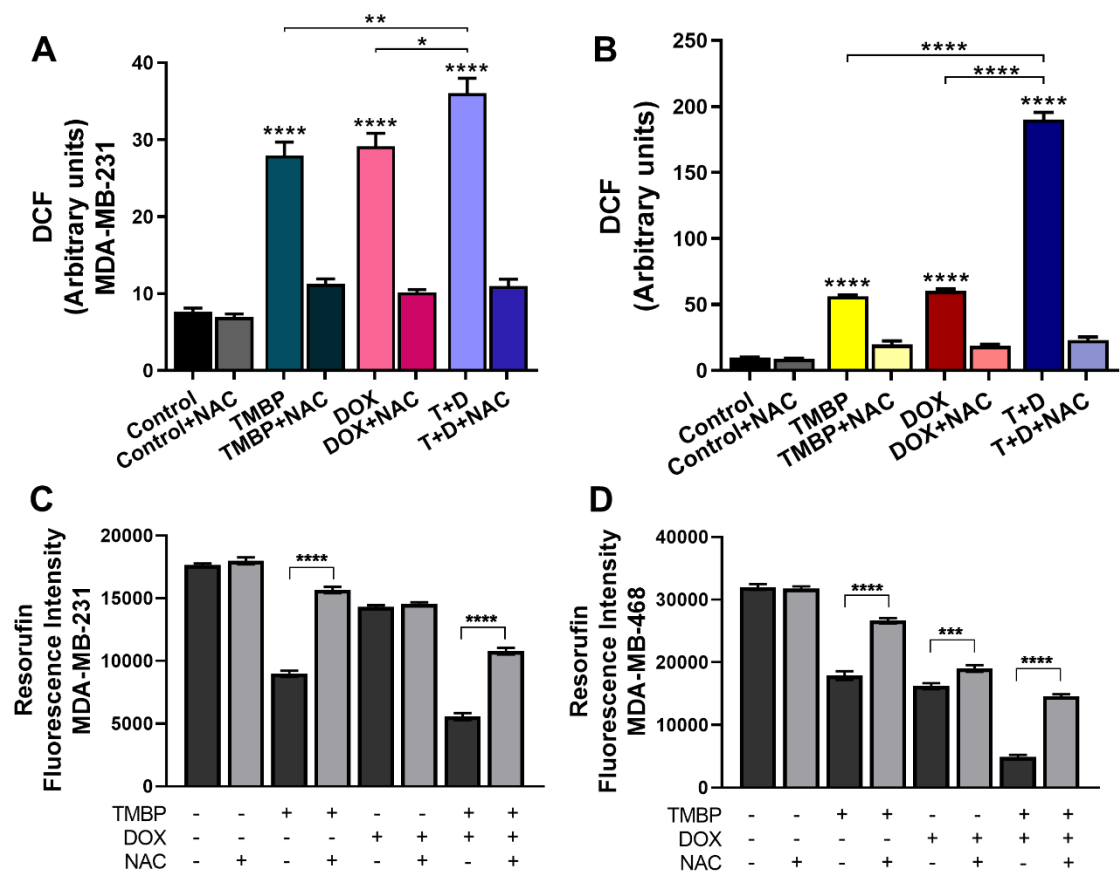


**Figure 7. TMBP+DOX exerts autophagy-dependent cytotoxicity in TNBC cells.** (A-B) AVOs were detected by AO staining in MDA-MB-231 (A) and MDA-MB-468 (B) cells following treatment with TMBP, DOX, or the combination. (A', B') Autophagic vacuoles were further quantified using monodansylcadaverine (MDC) staining. (A1–A4, B1–B4) Representative TEM images depict autophagic vacuoles and associated ultrastructural features. (C, E) Pharmacological inhibition of autophagy was performed using wortmannin (WMN), an early-stage autophagy inhibitor, and (D, F) hydroxychloroquine (HCQ), a late-stage autophagy inhibitor, in MDA-MB-231 and MDA-MB-468 cells, respectively. Cellular ultrastructures are indicated as nucleus (n), mitochondria (m), endoplasmic reticulum (er), and autophagic vacuoles (→). Data are expressed as the mean  $\pm$  SEM. Statistical differences: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .



1 **Figure 8. TMBP-induced cytotoxicity is ROS-dependent in TNBC cells.** (A–B) Total ROS levels  
2 were measured by H<sub>2</sub>DCFDA staining, and (C, D) cell viability was assessed by resazurin assay in  
3 MDA-MB-231 (A, C) and MDA-MB-468 (B, D) cells following treatment with TMBP, DOX, or  
4 TMBP+DOX, in the presence or absence of NAC. Data are expressed as the mean ± SEM. Statistical  
5 differences: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

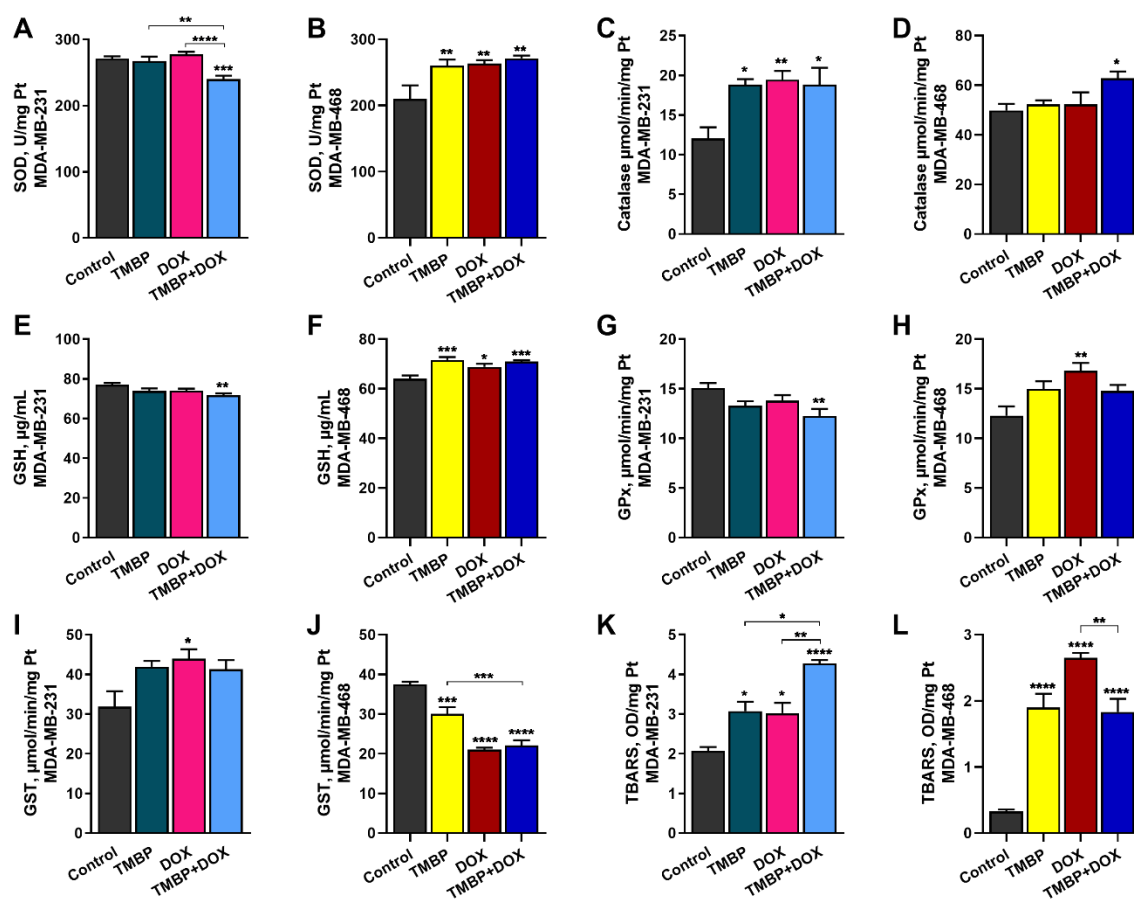


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**Figure 9. Mesenchymal oxidative MDA-MB-231 and Basal-like 1 glycolytic MDA-MB-468 exhibit distinct redox profiles after treatments.** To evaluate treatment-induced redox remodeling, antioxidant enzymes, (A, B) SOD, (C, D) Catalase, (G, H) GPx, (I, J) GST; (E, F) GSH and (K, L) lipid peroxidation (TBARS) were assessed in MDA-MB-231 and MDA-MB-468 cells following exposure to TMBP, DOX, or TMBP+DOX. Data are expressed as the mean  $\pm$  SEM. Statistical differences: \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001; \*\*\*\* $p$  < 0.0001.

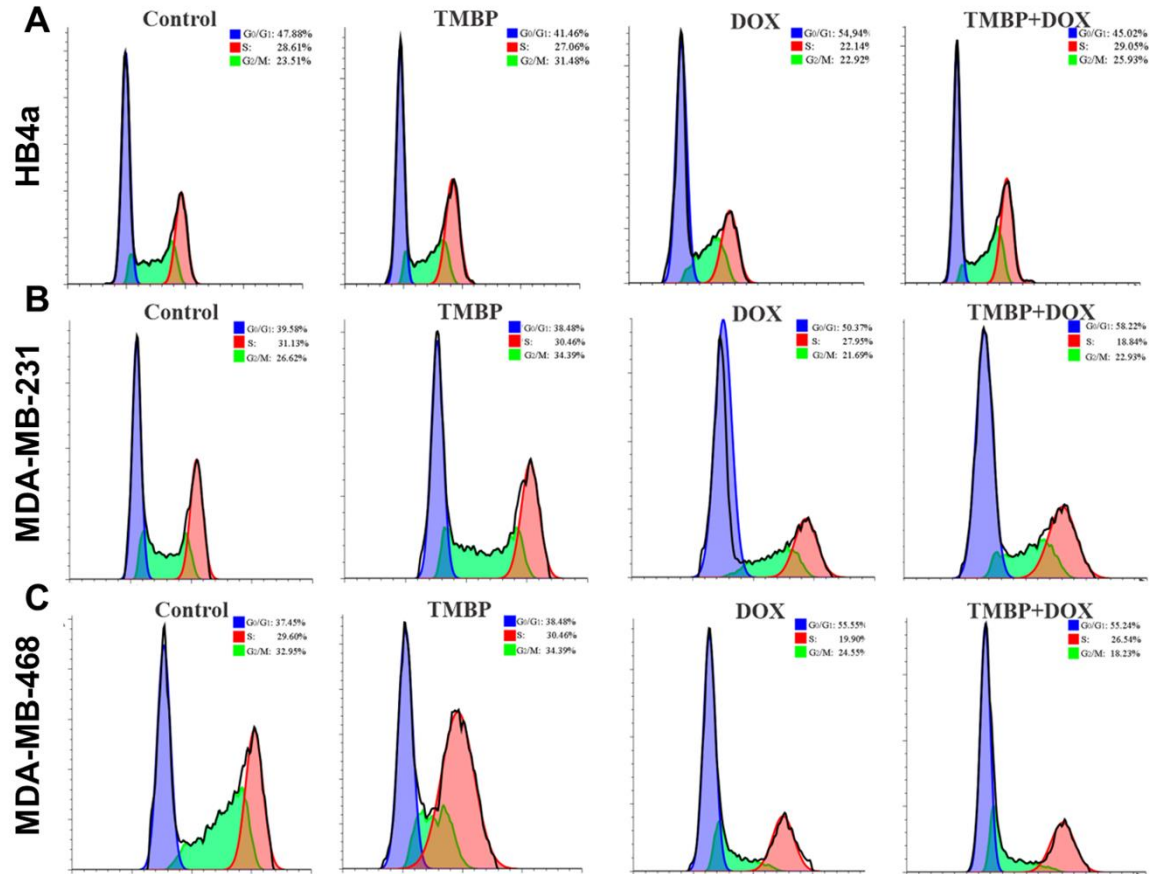


## SUPPLEMENTARY MATERIALS

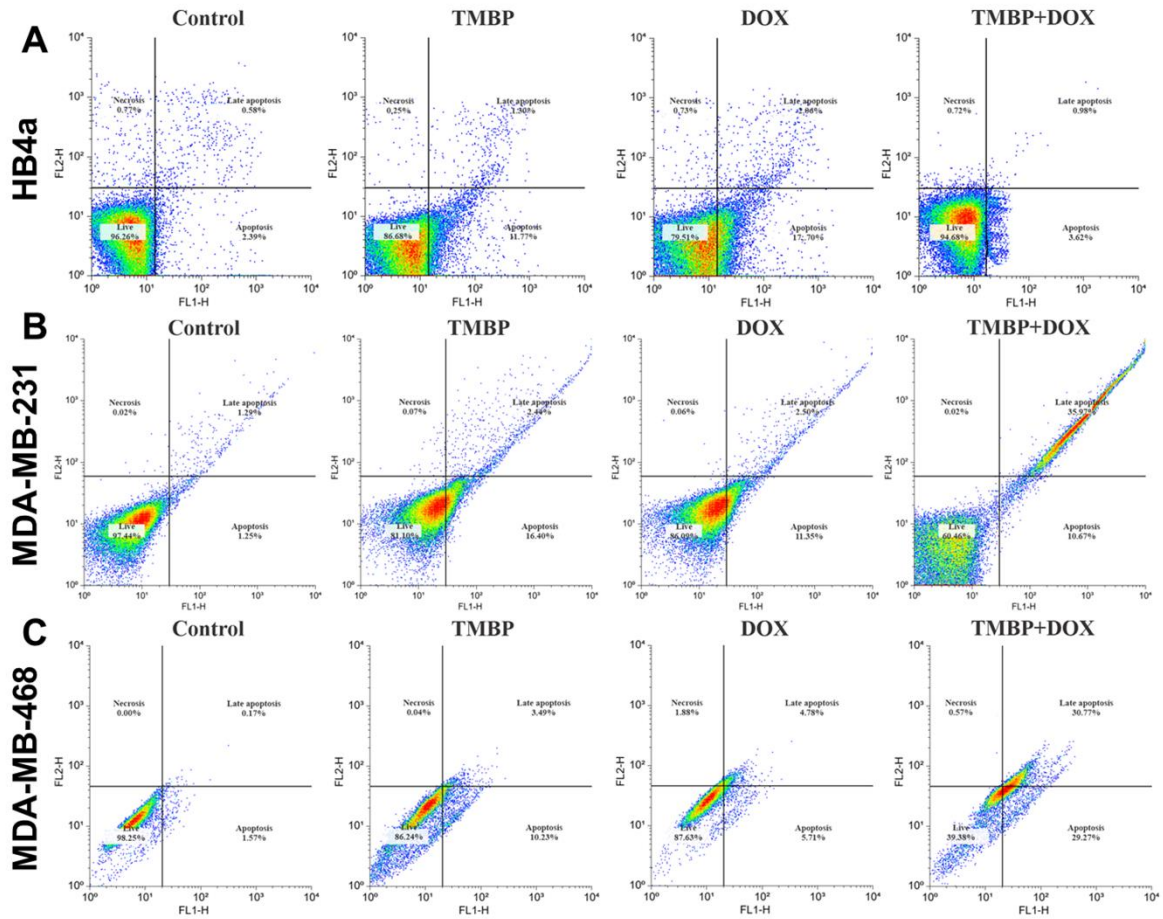
**Supplementary Table 1.** Percent inhibition for each drug condition across MDA-MB-231, MDA-MB-468, and HB4a cell lines. Values represent mean  $\pm$  SEM. TMBP (6-50) vs combinations \* ( $p < 0.05$ ), \*\*\* ( $p < 0.001$ ), \*\*\*\* ( $p < 0.0001$ ); DOX (0.01-0.5) vs combinations # ( $p < 0.05$ ), ## ( $p < 0.01$ ), ### ( $p < 0.001$ ), #### ( $p < 0.0001$ ).

| TMBP ( $\mu$ M) | DOX ( $\mu$ M) | MDA-MB-231 (%)            | MDA-MB-468 (%)            | HB4a (%)                 |
|-----------------|----------------|---------------------------|---------------------------|--------------------------|
| 6               | 0              | 9.64 $\pm$ 0.96           | 1.69 $\pm$ 1.31           | 5.56 $\pm$ 2.99          |
| 12.5            | 0              | 15.60 $\pm$ 1.13          | 20.01 $\pm$ 2.92          | 12.60 $\pm$ 1.30         |
| 25              | 0              | 23.59 $\pm$ 2.79          | 43.62 $\pm$ 1.77          | 30.53 $\pm$ 1.35         |
| 50              | 0              | 79.53 $\pm$ 1.42          | 65.81 $\pm$ 2.08          | 33.36 $\pm$ 2.52         |
| 0               | 0.01           | 12.59 $\pm$ 3.96          | 14.61 $\pm$ 1.60          | 15.45 $\pm$ 2.75         |
| 0               | 0.1            | 24.70 $\pm$ 1.96          | 46.77 $\pm$ 1.64          | 36.61 $\pm$ 1.49         |
| 0               | 0.5            | 52.48 $\pm$ 0.99          | 56.05 $\pm$ 1.25          | 44.30 $\pm$ 2.26         |
| 6               | 0.01           | 14.21 $\pm$ 1.93          | 5.12 $\pm$ 1.31####       | 8.05 $\pm$ 1.99          |
| 6               | 0.1            | 26.30 $\pm$ 3.04****      | 44.52 $\pm$ 1.80****      | 20.77 $\pm$ 2.49***,#### |
| 6               | 0.5            | 46.24 $\pm$ 4.97****      | 50.21 $\pm$ 2.17****,#    | 30.28 $\pm$ 0.98****,### |
| 12.5            | 0.01           | 22.68 $\pm$ 3.18#         | 13.09 $\pm$ 1.13*         | 10.42 $\pm$ 3.60         |
| 12.5            | 0.1            | 43.67 $\pm$ 4.24****,#### | 48.10 $\pm$ 1.25****      | 25.20 $\pm$ 2.17****,### |
| 12.5            | 0.5            | 57.58 $\pm$ 3.29****      | 52.04 $\pm$ 1.78****      | 42.81 $\pm$ 2.09****     |
| 25              | 0.01           | 35.25 $\pm$ 3.44*,###     | 36.27 $\pm$ 1.76*,####    | 10.55 $\pm$ 2.18****     |
| 25              | 0.1            | 51.68 $\pm$ 4.11****,#### | 57.81 $\pm$ 2.67***,##    | 22.61 $\pm$ 2.12*,####   |
| 25              | 0.5            | 69.98 $\pm$ 3.74****,###  | 71.47 $\pm$ 1.92****,#### | 45.69 $\pm$ 1.74****     |
| 50              | 0.01           | 75.87 $\pm$ 1.54####      | 37.79 $\pm$ 3.15****,#### | 23.56 $\pm$ 3.17         |
| 50              | 0.1            | 79.12 $\pm$ 1.66####      | 96.64 $\pm$ 0.40****,#### | 25.12 $\pm$ 2.17*,###    |
| 50              | 0.5            | 99.25 $\pm$ 0.15****,#### | 97.17 $\pm$ 0.40****,#### | 51.12 $\pm$ 1.89 ****    |

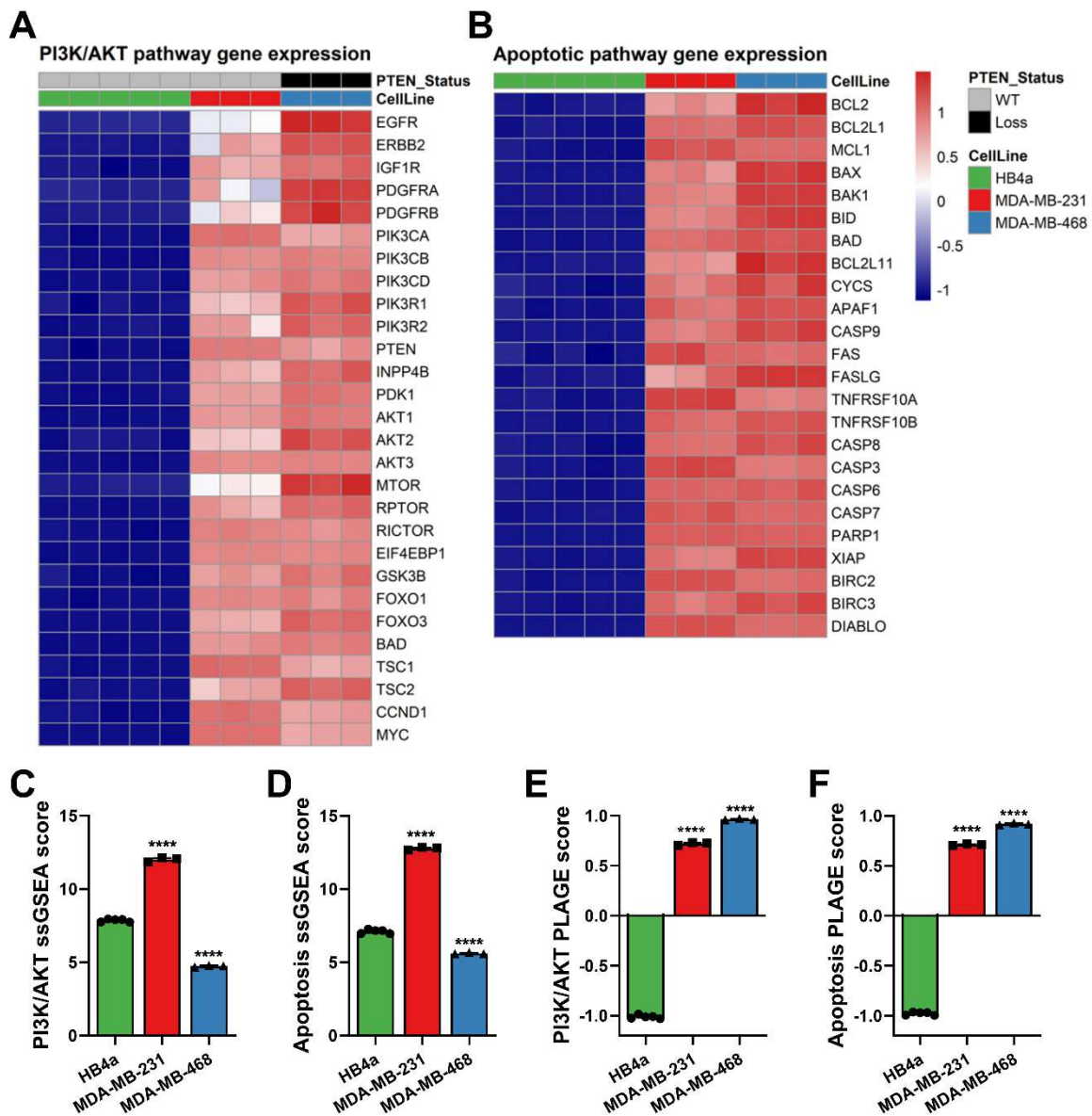
**Supplementary Figure 1.** Representative cell cycle distributions histograms of (A) HB4a, (B) MDA-MB-231, and (C) MDA-MB-468 cells following treatment with TMBP, DOX, or TMBP+DOX, as assessed by flow cytometry. Histograms correspond to the quantitative data shown in Figure 3.



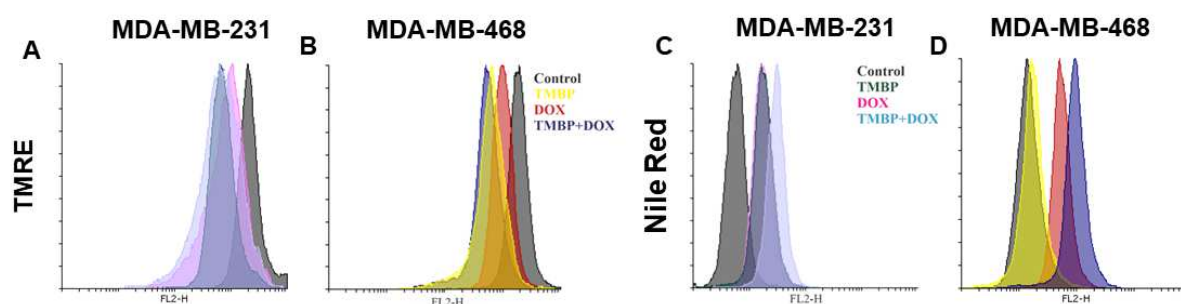
1 **Supplementary Figure 2.** Representative flow cytometry scatter plots of Annexin V/PI staining assay  
 2 of (A) HB4a, (B) MDA-MB-231, and (C) MDA-MB-468 cells after treatment with TMBP, DOX, or  
 3 TMBP+DOX, illustrating the distribution of viable, early apoptotic, late apoptotic, and necrotic cell  
 4 populations. Quantitative analyses are presented in Figure 4.



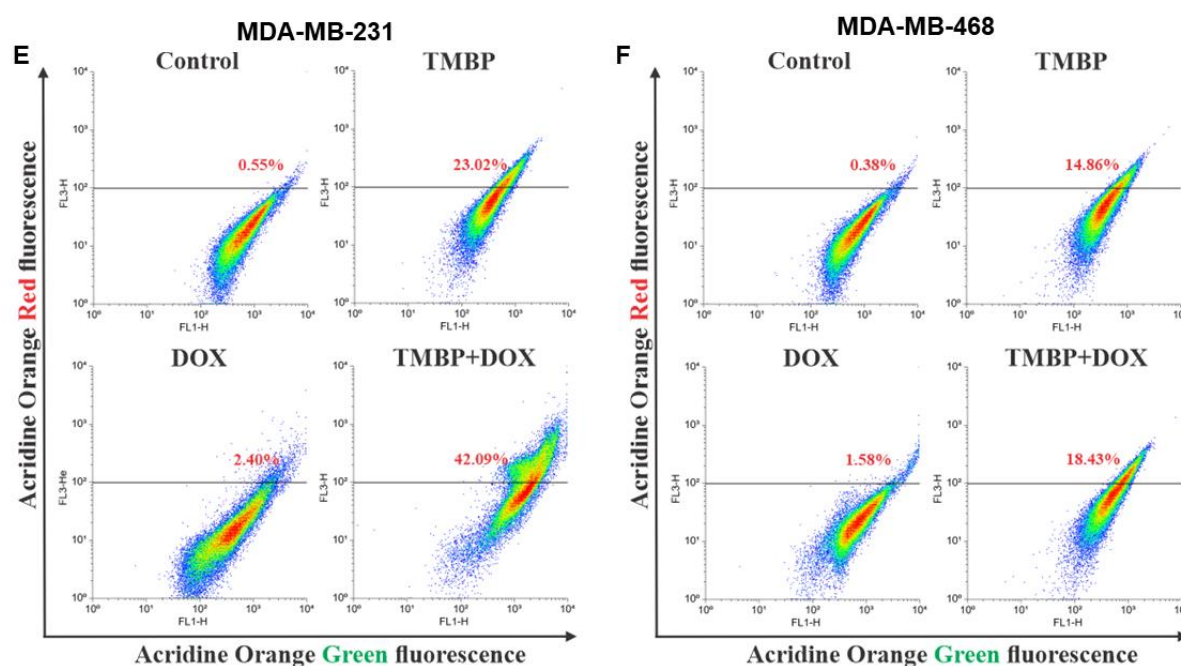
1 **Supplementary Figure 3.** Transcriptomic analysis of PI3K/AKT signaling and apoptosis pathway  
2 engagement in non-tumoral and TNBC cell lines using publicly available datasets.  
3 (A–B) Z-score–normalized gene expression heatmaps of genes associated with the (A) PI3K/AKT  
4 pathway and (B) apoptosis, highlighting differential transcriptional profiles between HB4a and TNBC  
5 cells. (C–D) Single-sample gene set enrichment analysis (ssGSEA) scores for (C) PI3K/AKT signaling  
6 and (D) apoptosis, representing ranked pathway activity across cell lines.  
7 (E–F) PLAGE analysis of (E) PI3K/AKT and (F) apoptosis gene sets, comparing coordinated  
8 transcriptional activation among HB4a, MDA-MB-231, and MDA-MB-468 cells.



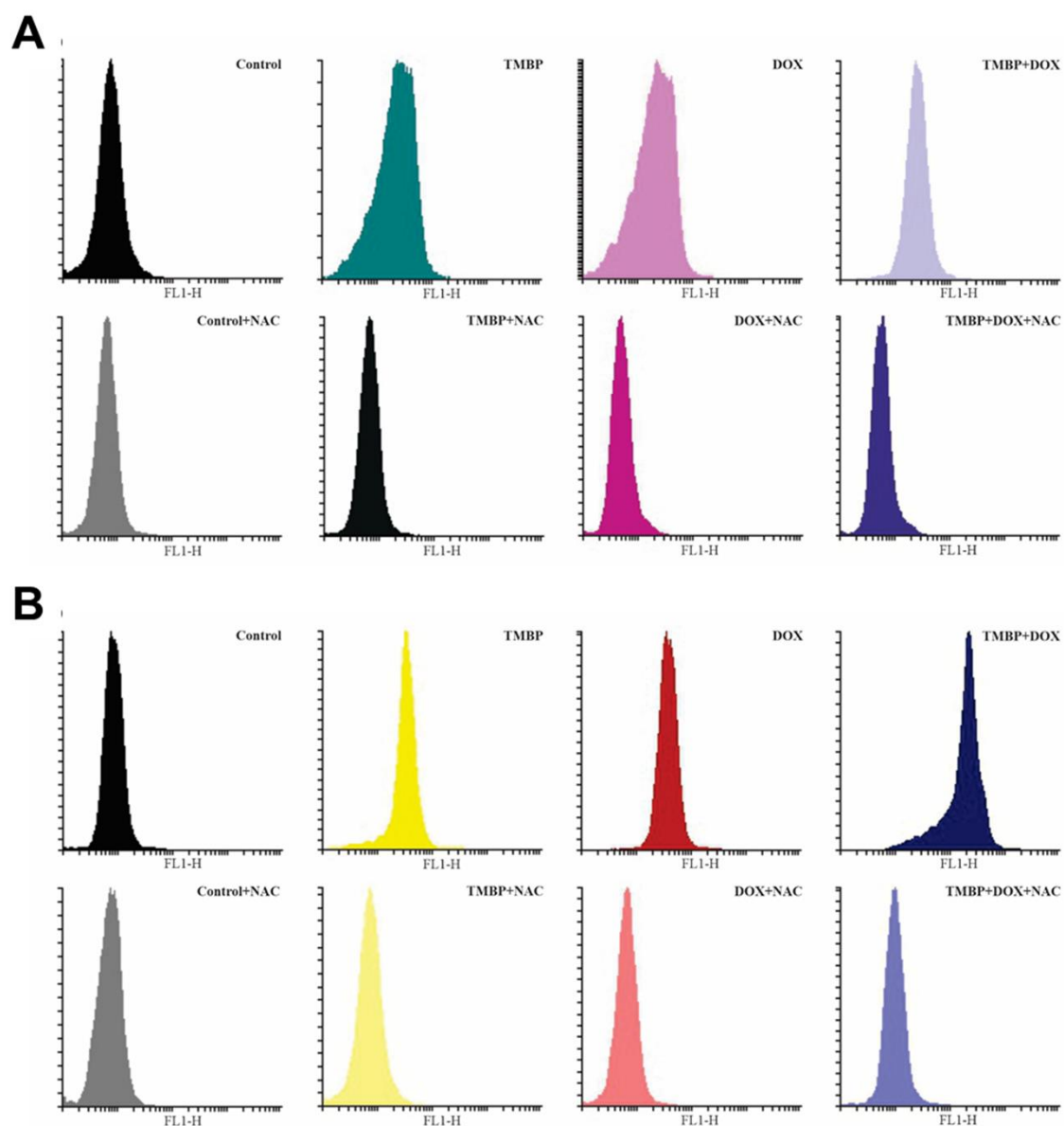
**Supplementary Figure 4.** Representative flow cytometry histograms of (A–B)  $\Delta\Psi_m$  assessed by TMRE staining and (C–D) intracellular lipid accumulation assessed by Nile Red staining in (A, C) MDA-MB-231 and (B, D) MDA-MB-468 cells following treatment with TMBP, DOX, or TMBP+DOX. Quantitative analyses are shown in Figure 6.



**Supplementary Figure 5.** Representative flow cytometry scatter plots of acridine orange red fluorescence (FL3) used to assess acidic vesicular organelles (AVOs) in (A) MDA-MB-231 and (B) MDA-MB-468 cells after treatment with TMBP, DOX, or TMBP+DOX. Corresponding quantitative data are presented in Figure 7.



1 **Supplementary Figure 6.** Representative flow cytometry histograms of ROS levels measured by  
 2  $H_2DCFDA$  staining in (A) MDA-MB-231 and (B) MDA-MB-468 cells treated with TMBP, DOX, or  
 3 TMBP+DOX in the presence or absence of the antioxidant N-acetyl-L-cysteine (NAC). Quantitative  
 4 analyses are shown in Figure 8.



## CONCLUSÃO GERAL

O TMBP, previamente caracterizado como um composto com atividade antitumoral seletiva, demonstrou capacidade de atuar sobre vulnerabilidades centrais do CMTN. De forma isolada, o tratamento com TMBP em células MDA-MB-231 evidenciou a modulação de vias associadas à apoptose, à sinalização TGF- $\beta$ /SMAD e à resposta a danos ao DNA, levando a ativação de caspases 3/7, aumento de apoptose tardia e redução da capacidade de migração dessas células. Posteriormente, a associação entre TMBP e DOX resultou em interações aditivas a sinérgicas em células de CMTN com seletividade superior a 2, induziu maior parada do ciclo em G1 e aumento de apoptose tardia em células de CMTN, sem comprometer a regulação do ciclo e viabilidade da linhagem não tumoral HB4a. Observou-se ainda que essa combinação induziu maior aumento de ERO e desorganização do controle redox, levando à disfunção e ao dano mitocondrial, ao acúmulo de gotículas lipídicas citoplasmáticas e à ativação de processos autofágicos.

A neutralização de ERO e a inibição farmacológica de autofagia por WMN e HCQ restauraram parcialmente a viabilidade celular nas células de CMTN, corroborando o papel desses mecanismos na morte celular induzida pelo TMBP. Esses resultados sustentam a hipótese de que o TMBP compromete a sobrevivência tumoral por interferir em eixos críticos do controle do ciclo celular, do metabolismo mitocondrial e da resposta ao estresse oxidativo, efeitos que se mostram mais pronunciados quando o composto é utilizado em associação com a DOX.

Em conjunto, os resultados destacam o TMBP como um agente terapêutico promissor, com potencial para integrar estratégias direcionadas ou combinatórias voltadas ao tratamento do CMTN e de outros tumores que compartilham vulnerabilidades biológicas semelhantes.