UNIVERSIDADE FEDERAL DO CEARÁ

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

NÚCLEO DE PESQUISA E DESENVOLVIMENTO DE MEDICAMENTOS

EMERSON LUCENA DA SILVA

AVALIAÇÃO DE NOVOS ALVOS DO FÁRMACO MEBENDAZOL NO TRATAMENTO DO ADENOCARCINOMA GÁSTRICO

FORTALEZA

2024

EMERSON LUCENA DA SILVA

AVALIAÇÃO DE NOVOS ALVOS DO FÁRMACO MEBENDAZOL NO TRATAMENTO DO ADENOCARCINOMA GÁSTRICO

Tese apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutor em Farmacologia. Área de concentração: Farmacologia Geral.

Orientadora: Professora Dr.ª Raquel Carvalho Montenegro.
Coorientador: Professor Dr. Pedro Filho Noronha de Souza.

FORTALEZA
2024

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

S579a Silva, Emerson Lucena da.
Avaliação de novos alvos do fármaco Mebendazol no tratamento do adenocarcinoma gástrico / Emerson Lucena da Silva. – 2024.
162 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Faculdade de Medicina, Programa de Pós-Graduação em Farmacologia, Fortaleza, 2024.
Orientação: Profa. Dra. Raquel Carvalho Montenegro.
Coorientação: Prof. Dr. Pedro Filho Noronha de Souza.

1. Alvo farmacológico. 2. Câncer gástrico. 3. Reposicionamento de fármacos. 4. Reprogramação metabólica. 5. Transcriptoma. I. Título.

CDD 615.1

EMERSON LUCENA DA SILVA

AVALIAÇÃO DE NOVOS ALVOS DO FÁRMACO MEBENDAZOL NO TRATAMENTO DO ADENOCARCINOMA GÁSTRICO

Tese apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Ceará como parte dos requisitos para obtenção do título de Doutor em Farmacologia. Área de concentração: Farmacologia Geral.

Aprovada em:___/___/___

BANCA EXAMINADORA

_____

Profª. Drª. Raquel Carvalho Montenegro (Orientadora)
Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Paulo Michel Pinheiro Ferreira
Universidade Federal do Piauí (UFPI)

_____

Prof. Dr. Daniel Pereira Bezerra
Fundação Oswaldo Cruz (Fiocruz) - Bahia

_____

Profª. Drª. Leticia Veras Costa Lotufo
Universidade de São Paulo (USP)

_____

Profª. Drª. Gerusa Neyla Andrade Senhorinho
Laurentian University of Sudbury

Dedico este trabalho in memoriam a Alesson Lucena, meu irmão e amigo, que sempre acreditou nos meus sonhos.

AGRADECIMENTOS

Muitos passos foram necessários para a chegada até esse momento e em todos eles, tive a ajuda e a companhia de pessoas que tornaram essa jornada mais leve. Tantas pessoas estiveram comigo nesses quatro anos, muitas experiências, risadas, choros, e que de forma direta ou indireta, me ajudaram a chegar até o fim, a todos eu agradeço por terem estado ao meu lado.

A minha mãe Dona Maria, ao meu pai Seu Antoniel e a minha tia Lucélia eu agradeço por serem meu porto seguro sempre que precisei, por me guiarem desde pequeno e me fazerem ver mais longe. Agradeço por sempre me apoaiarem a voar mais alto, mesmo quando isso significava estar longe, amo vocês.

Ao meu companheiro Leonardo, por todas as vezes que me fez sentir seguro com um abraço, que me escutou atentamente e me aconselhou. Obrigado por dividir comigo o peso do dia a dia, por me suportar em momentos de estresse e me alegrar em dias tristes, que ainda possamos conquistar muitas coisas juntos.

Agradeço ao meu irmão de coração Felipe, que foi muito mais doque um colega com quem dividi apartamento e parceiro de laboratório. Um exemplo de profissional e um professor/pesquisador sem igual, muito obrigado por toda a ajuda e parceria nesse processo.

A minha orientadora Profa. Dra. Raquel Montenegro e ao meu co-orientador Prof. Dr. Pedro Souza, obrigado por guiarem esse trabalho e me ajudarem a ser um pesquisador e um profissional melhor, agradeço por todas as dicas e puxões de orelha, foi engrandecedor poder contar com vocês nesse processo.

Aos meus amigos do Laboratório de Farmacogenética e do NPDM, a minha segunda casa, em especial a Luína, Laís, Thâmara, Laio, Rhaynan, Lívia, Caio, Carol, Emanuel, Adrhyan, Aline, Dyane, Arlene, Pedrinho, Ana Beatriz, Bea, Flávia, Daiane e Elmer, obrigado por toda a ajuda nos experimentos, nas análises e pelos momentos de descontração e alegria.

Aos meus amigos de longe ou de perto, que sempre estiveram comigo quando eu precisei, Neil, Iago, Wallace, Danilo, Priscila, Vanessa, Sabrina, Letícia, Ingryd, Carla, Laís, Lorena e Carol, obrigado pelo ombro amigo.

Agradeço aos meus novos amigos, Prof. John Ashley Scott, Prof. Corey Laamanen, Profa. Gerusa Senhorinho, Débora, Elsa (Mixiang Du), Carly, Sean, William, Rahul, Ibrahim,

Rowan, Laurel, Mike e Poppy que fizeram da minha estadia no Canadá a mais maravilhosa possível, obrigado por toda hospitalidade e amizade.

Aos órgãos de fomento, especialmente ao CNPq, assim como ao Núcleo de Pesquisa de Desenvolvimento de Medicamentos (NPDM) e a unidade multiusuário, obrigado por terem tornado a minha pesquisa possível.

"You is Kind,

You is Smart,

You is Important"

(Tate Taylor; **Histórias Cruzadas**, 2011)

RESUMO

O câncer gástrico (CG) está entre as neoplasias mais incidentes do mundo, sendo considerado o quinto tipo de câncer mais frequente e quarto mais letal no mundo. Apesar dos benefícios terapêuticos observados na adição da quimioterapia/quimiorradioterapia a cirurgia, as taxas de recorrência local e do aparecimento de tumores em sítios distantes continuam elevados. Dessa forma, o estudo de novas terapias deve ser incentivado com o intuito de oferecer novos tratamentos a indivíduos não responsivos aos esquemas atuais. Estudos vem avaliando o reposicionamento do anti-helmíntico Mebendazol (MBZ) na clínica oncológica, demonstrando seu alto potencial antitumoral em diferentes tipos de câncer, incluindo o CG. Apesar de vários alvos já terem sido relacionados ao mecanismo de ação desse fármaco, seu potencial sobre a reprogramação do metabolismo tumoral e sob e regulação dos transcritos na linhagem tumoral metastática gástrica AGP-01 ainda não foi elucidado. Dessa forma esse trabalho tem como objetivo avaliar novos alvos farmacológicos do MBZ na linhagem metastática gástrica AGP-01, por meio das análises do perfil metabólico e molecular *in vitro* e *in silico*, assim como, do transcriptoma dessa célula após o tratamento com o fármaco. Resultados mostraram que o MBZ tem atividade antitumoral e seletiva a linhagem AGP-01, tendo a capacidade de modular o metabolismo tumoral pela inibição da expressão de enzimas da via glicolítica (*SLC2A1*, *HK1*, *GAPDH* e *LDHA*) e da síntese de nucleotídeos (*PRPS1*, *TYMS*, *MTHFD1*, *DHODH* e *HPRT1*) em tempo não citotóxico. Os transcritos que codificam para essas enzimas, quando hiperexpressos tem relação com um pior prognósticos em pacientes com CG. Os dados também demonstraram que, após a inibição da expressão desses transcritos, o MBZ leva a alterações morfológicas, aumento na fragmentação da membrana celular e diminuição do potencial de membrana mitocondrial, em conjunto com a ativação de Caspase 3/7, parada no ciclo celular em G0/G1 e diminuição na formação de colônias, mostrando uma relação entre a atividade antitumoral do MBZ e sua capacidade de modulação do metabolismo tumoral. A análise do transcriptoma da célula AGP-01 após o tratamento com o MBZ demonstrou que esse leva ao aumento na expressão dos genes *CCL2*, *IL1A* e *CDKN1A*, enquanto os genes *H3C7*, *H3C11* e *H1-5* tiveram sua expressão reduzida de forma significativa. Essas alterações foram validadas por PCR em tempo real, a análise de expressão em bancos de dados demonstrou que a baixa expressão de *ILI1A* e a alta expressão *de H3C11* e *H1-5* está associado a uma menor expectativa de vida dos pacientes com GC. Dessa forma, os dados encontrados até o momento demonstraram que o fármaco MBZ altera o metabolismo

tumoral da linhagem AGP-01, tendo relação com sua atividade antitumoral e antiproliferativa e que o fármaco modula a expressão gênica de proteínas histonas e citocinas inflamatórias, indicando um possível efeito epigenético e imunológico em células tumorais. Os achados demonstram novos alvos do fármaco MBZ ainda não descritos na literatura, podendo levar ao melhor esclarecimento do seu mecanismo de ação e beneficiar clinicamente pacientes com CG que não respondem as terapias atuais.

Palavras-chave: Alvo farmacológico; Câncer gástrico; Reposicionamento de fármacos; Reprogramação metabólica; Transcriptoma.

ASSESSMENT OF THE MEBENDAZOLE DRUG'S NEW TARGETS IN THE TREATMENT OF GASTRIC ADENOCARCINOMA

ABSTRACT

Gastric cancer (GC) is among the most common cancers in the world, being considered the fifth most common and fourth most lethal type of cancer in the world. Despite the therapeutic benefits observed in the addition of chemotherapy/chemoradiotherapy to surgery, the rates of local recurrence and the appearance of tumors in distant sites remain high. Therefore, the study of new therapies should be encouraged to offer new treatments to individuals who are not responsive to current regimens. Studies have evaluated the repositioning of the anthelmintic Mebendazole (MBZ) in the oncology clinic, demonstrating its high antitumor potential in different types of cancer, including GC. Although several targets have already been related to its mechanism of action, its potential in the reprogramming of tumor metabolism and the regulation of transcripts in the AGP-01 gastric metastatic cell line has not yet been elucidated. These findings may provide new dimensions to the pharmacological potential of MBZ, as well as clarify its mechanism of action *in vitro*. Therefore, this work aims to evaluate new pharmacological targets of MBZ in the AGP-01 gastric cancer cell line, through the analysis of metabolic pathways and the transcriptome of this cell after MBZ treatment. Initial results showed that MBZ has antitumor and selective activity against the AGP-01 cell line, modulating the tumor metabolism by inhibiting the expression of enzymes from glycolytic pathway (*SLC2A1*, *HK1*, *GAPDH*, and *LDHA*) and nucleotide synthesis (*PRPS1*, *HPRT1*, *TYMS*, and *DHODH*) in a non-cytotoxic time. These enzymes, when overexpressed, are related to reducing the overall survival rates of patients with GC. Data also demonstrated that, after transcription inhibition of these enzymes, MBZ leads to morphological changes, increased cell membrane fragmentation, and decreased mitochondrial membrane potential, in conjunction with Caspase 3/7 activation and cell cycle arrest. in G0/G1, showing a relationship between the antitumor activity of MBZ and its ability to modulate tumor metabolism. Analysis of the AGP-01 cell line's transcriptome after treatment with MBZ demonstrated an increase in the expression of the *CCL2*, *IL1A*, and *CDKN1A* genes, while the *H3C7*, *H3C11*, and *H1-5* transcripts had their expression significantly reduced. These modifications in the mRNA expression were validated by Real-Time PCR, and expression analysis in online databases demonstrated that low expression of *ILI1A* and high expression of *H3C11* and

H1-5 are associated with a reduction in overall survival rates in patients with GC. Thus, the data found so far have demonstrated that the drug MBZ alters the tumor metabolism of the AGP-01 cell line, being related to its antitumor activity, and that MBZ modulates the gene expression of histone proteins and inflammatory cytokines, indicating a possible epigenetic and immunological effect in tumor cells. The findings demonstrate new targets of MBZ that have not yet been described in the literature, leading to a better clarification of its mechanism of action and to benefit clinically patients with GC that do not respond to current therapies.

Keywords: Drug Repurposing; Gastric cancer; Metabolic reprogramming; Pharmacological target; Transcriptome.

LISTA DE ILUSTRAÇÕES

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE TABELAS

CAPÍTULO II

CAPÍTULO IV

LISTA DE ABREVIATURAS E SIGLAS

3-BP	3-Bromopyruvate
5-FU	5-Flourouracil
ACRG	Grupo Asiático de Pesquisa do Câncer
Adj.P.Val	Valor de p ajustado
AJCC	Comitê Misto americano de câncer
ANOVA	Análise de Variância
ATP	Adenosina Trifosfato
BNBZ	Benitrobenrazide
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCL2	Ligante de quimiocina 2
CDKN1A	Inibidor de Quinase Dependente de Ciclina 1A
CG	Câncer Gástrico
CI_{50}	Concentração e Inibição de 50%
CIN	Instabilidade Cromossômica
CIMP	*CpG island methylator phenotype*
CN	Controle Negativo
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CTP	Citidina-5-trifosfato
DEGs	Genes Diferencialmente Expressos
DHODH	Di-hidro-orotato desidrogenase
DMSO	Dimetilsulfóxido
DNMTS	*DNA methyltransferases*
EBV	Vírus Epstein-Barr
ECF	Epirrubicina, Cisplatina e 5-FU
EGA	*European Genome-phenome Archive*
ELFE	Epirrubicina, Leucovorin, 5-Flourouracil e Etoposídeo
EMR	Ressecções endoscópicas de mucosa
ENTREZID	Número de identificação do gene no NCBI
ES	Pontuação de enriquecimento da via
ESD	Ressecções endoscópicas de submucosa
EUS	Ultrassonografia Endoscópica

FC	*Fold Change*
FDA	*US Food and Drug Administration*
FDR	Taxa de descoberta falsa
FOLFOX	Ácido fólico, 5-Fluorouracil e Oxaliplatina
FOLFIRI	Ácido fólico, 5-Flourouracil e Irinotecano
fRMA	*Robust Multiarray Averaging*
FUNCAP	Fundação Cearense de Amparo à Pesquisa
FWER	*Family-wise error rate*
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GEO	*Gene Expression Omnibus*
GLUT	*Glucose transporters*
GMP	Guanosina monofosfato
GSEA	Análise de enriquecimento de conjunto de genes
GTEx	*Genotype-Tissue Expression*
HK	Hexoquinase
H1-5	H1 histona ligante 5
H3C11	H3 histona agrupada 11
H3C7	H3 histona agrupada 7
HDACS	*Histone deacetylases*
HPRT1	Hipoxantina-guanina fosforribosiltransferase
HR	*Hazard Ratio*
IDH	Índice de Desenvolvimento Humano
ISGs	Interferon-stimulated genes
IL1A	Interleucina 1 alfa
IMP	Inosina monofosfato
IHQ	Imuno-histoquímica
INCA	Instituto Nacional do Câncer
LDH	*Lactate dehydrogenase*
LG	*Low Glucose Medium*
MBZ	Mebendazol
mM	milimolar
MSI	Instabilidade de Microssatélites
MSS	Microssatélites Estável

MTHFD1	Enzima Trifuncional Metilenotetrahidrofolato desidrogenase, ciclohidrolase e formiltetrahidrofolato sintetase 1
MTX	Metrotexato
NGS	Sequenciamento de nova geração
NCI	Instituto Nacional do Câncer Americano
ND	Não Determinado
NES	Pontuação de enriquecimento normalizada
NOM p-val	*p* valor nominal
NPDM	Núcleo de Pesquisa e Desenvolvimento de Medicamentos
OMS	Organização Mundial da Saúde
OXPHOS	Fosforilação oxidativa
PCI	Índice de Carcinomatose Peritoneal
PRPP	Fosforribosil pirofosfato
PDB	*Protein Data Bank*
PI	Iodeto de Propídio
PRPS1	Fosforibosil-pirofosfato sintetase
RMSD	Raiz do desvio quadrático médio das posições atômicas
RH 123	Rodamina 123
ROT	Rotenona
SD	Desvio Padrão
SMILE	*Simplified Molecular Input Line Entry Specification*
STAD	Adenocarcinoma de Estômago
TC	Tomografia computadorizada
TCA	Ciclo dos ácidos tricarboxílicos
TCGA	*The Cancer Genome Atlas*
THF	Tetra-hidrofolato
TSG	Genes supressores tumorais
TYMS	Timidilato sintase
UICC	União Internacional para o Controle do Câncer
UFC	Universidade Federal do Ceará
UMP	Monofosfato de uridina
VEGF2	Fator de crescimento vascular 2
XELOX	Oxaliplatina e Capecitabina

SUMÁRIO

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

EMERSON LUCENA DA SILVA

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Câncer

O câncer é um termo utilizado para caracterizar um conjunto de mais de 200 doenças que possuem em comum algumas da características como a capacidade de crescimento desordenado e o aumento no potencial migratório e invasivo, podendo situar outros sítios do corpo que não o seu sítio de crescimento primário, destacando seu potencial maligno (Sameri *et al.*, 2021). O câncer pode ser classificado de acordo com o tecido de onde se origina, visto que quando essa doença de desenvolve de célula do tecido epitelial (pele ou mucosa) é denominada como carcinoma, quando se desenvolve de tecido conjuntivo (osso, músculo ou cartilagem) é denominado sarcoma. Ainda pode ser derivado de tecidos precursores de células hematopoiéticas e de células do sistema imune, conhecidas como leucemias e linfomas/mielomas, respectivamente (Carbone, 2020; Gupta; Dwivedi, 2017; Hassanpour; Dehghani, 2017).

No desenvolvimento da neoplasia, ocorrem modificações que levam a progressão celular, as interações do tumor com o ambiente tumoral também são alteradas, isso resulta de disfunções em genes vitais que teriam como função manter a homeostasia tecidual. As mutações ocorrem em genes conhecidos como proto-oncogenes e em genes supressores tumorais, isso acarreta na proliferação celular descontrolada e permite a aquisição das características das células cancerosas observadas na maioria das neoplasias (Kontomanolis *et al.*, 2020).

Proto-oncogenes, são genes que modulam características como proliferação celular, sobrevivência e a capacidade de invasão e motilidade das células normais, por isso a expressão desses genes é cuidadosamente regulada para evitar a proliferação celular desordenada (Stasevich *et al.*, 2021). Quando mutados, esses genes são denominados como oncogenes, causando hiperproliferação celular, aumento da capacidade de sobrevivência e disseminação devido ao descontrole na maquinaria de divisão. Esses genes são fenotipicamente dominantes, sendo necessária apenas uma mutação em uma de suas cópias para alterar o fenótipo. Esses podem ter sua expressão intensificada através de modificação na sequência do gene, de uma amplificação desse gene no genoma da célula ou pela translocação do mesmo para outra região, aonde sob a influência de um novo promotor pode ter aumento na produção de transcritos (Kontomanolis *et al.*, 2020; Wang *et al.*, 2018).

Por sua vez, os genes supressores tumorais (*Tumor Supressor genes*, TSGs), tem como função inibir/diminuir a proliferação e a sobrevivência celular por estarem relacionados com a repressão de processos como ciclo celular e controle de vias de morte, tentando assim, manter a homeostase do organismo e a sua integridade celular. Em 1997, Kinzler e Vogelstein dividiram os TSGs dentro de duas categorias: os genes guardiões (*gatekeepers*) que controlam o progresso celular, através de ciclos de crescimento e divisão e os genes cuidadores (*caretakers*), que possuem a função de manter a integridade do genoma celular (Kinzler; Vogelstein, 1997). Quando esses genes são inativados tendem a aumentar a vantagem de crescimento seletiva das células mutadas sobre as células normais (Wang *et al.*, 2018).

Os TSGs são fenotipicamente recessivos, ou seja, é necessário o silenciamento de ambas as cópias do gene para levar ao desenvolvimento da doença. A inativação de apenas uma cópia do gene é geralmente seguida pela perda da cópia restante, levando assim, ao surgimento do fenótipo tumoral (Morris; Chan, 2015). Podem sofrer inativação por diversos mecanismos, como deleções locais ou em larga escala e pela perda de heterozigosidade, modificações epigenéticas, introdução de códons da parada em regiões aleatórias da sequência ou pela modificação no quadro de leitura (*frameshifts*), além disso, essa inativação pode ocorrer por mutações em íntrons que inativam sítios de *splice* de pré-mRNA, levando a formação de proteínas não funcionais (Kim, Soo Yeon *et al.*, 2019; Vogelstein *et al.*, 2013).

Dessa forma, com o intuito de melhor entender a natureza do câncer e assim traçar novas terapias contra a doença, em 2000, Hanahan e Weinberg descreveram seis marcas características, do inglês *hallmarks*, do desenvolvimento do câncer, dentre elas estão: evasão da via apoptótica, alto suficiência em sinais de crescimento celular, insensibilidade a sinais que impedem o crescimento, invasão tecidual e metástase, potencial replicativo ilimitado e a capacidade das células cancerosas em criar novos vasos para nutrir o tumor (Hanahan; Weinberg, 2000).

Cerca de 11 anos depois, os mesmos autores adicionaram quatro novas características dentro de duas classes, uma delas ficou conhecida como a das características facilitadoras do desenvolvimento do câncer o que inclui a instabilidade genômica e mutação e a inflamação promovida pelo tumor como *hallmarks*, e dentre as características emergentes foram descritas a desregulação do metabolismo celular e o mecanismo de escape do sistema imune, todas estando relacionadas ao processo de tumorigenêse (Hanahan; Weinberg, 2011).

Mais recentemente, Douglas Hanahan publicou uma nova versão do estudo, baseado nas pesquisas e no conhecimento adquiro sobre o câncer nos últimos anos, outras características surgiram como essenciais para o desenvolvimento e progressão da célula tumoral. Como novas características emergentes temos o desbloqueio da plasticidade fenotípica e a senescência celular, já entre as novas características possibilitadoras do desenvolvimento do tumor a reprogramação epigenética não mutacional e o microbioma tumoral (micróbios polimórficos) entram como agentes essenciais para o processo de carcinogênese (Hanahan, 2022). Na figura 1 abaixo são ilustradas todas as características associadas que os tumores têm em comum e que estão relacionadas ao desenvolvimento de diferentes tipos de câncer.

Figura 1. Características fenotípicas associadas ao desenvolvimento do câncer. Na figura são descritas todas as características relacionadas ao desenvolvimento do tumor elucidadas até agora, em preto temos as características originais, publicadas em 2000. Em cinza as características elucidadas em 2011, e em vermelho as mais recentes.

Fonte: Adaptada de Hanahan (2022) (Criada com (BioRender.com).

Devido a sua importância para o desenvolvimento e progressão tumoral, as mesmas tem sido amplamente estudadas como alvos farmacológicos para a terapia do câncer (Hanahan; Weinberg, 2000).

1.1.1 Metabolismo tumoral

A desregulação do metabolismo tumoral foi descrita pela primeiramente pelo médico fisiologista Otto Warburg como uma marca relacionada ao desenvolvimento do câncer (Warburg, 1956; Warburg; Dickens, 1931; Warburg; Wind; Negelein, 1927). Essa reprogramação tem como função principal permitir o crescimento e divisão contínua das células tumorais, pela modificação do fluxo de vias bioquímicas celulares e ao aumento na produção de intermediários biossintéticas e assim, de macromoléculas essenciais para o desenvolvimento celular (Yang *et al.*, 2024).

A reprogramação tumoral está intimamente ligada a ação de oncogenes e a diminuição na expressão dos TSGs, essa ação em conjunta leva a modificação no atividade de diferentes vias biossintéticas, como as relacionadas a produção de ácidos graxos, aminoácidos, nucleotídeos e energia na forma de adenosina trifosfato (ATP) (Kocianova; Piatrikova; Golias, 2022; Yang *et al.*, 2024). Devido a dependência tumoral pelos intermediários biossintéticos gerados por essas vias para o seu crescimento e propagação, muitas destas têm sido estudadas como fonte de novos alvos terapêuticos, com destaque para as vias glicolítica e a de síntese de nucleotídeos (Wu, Huai-liang *et al.*, 2022; Zhang, Guilian *et al.*, 2022).

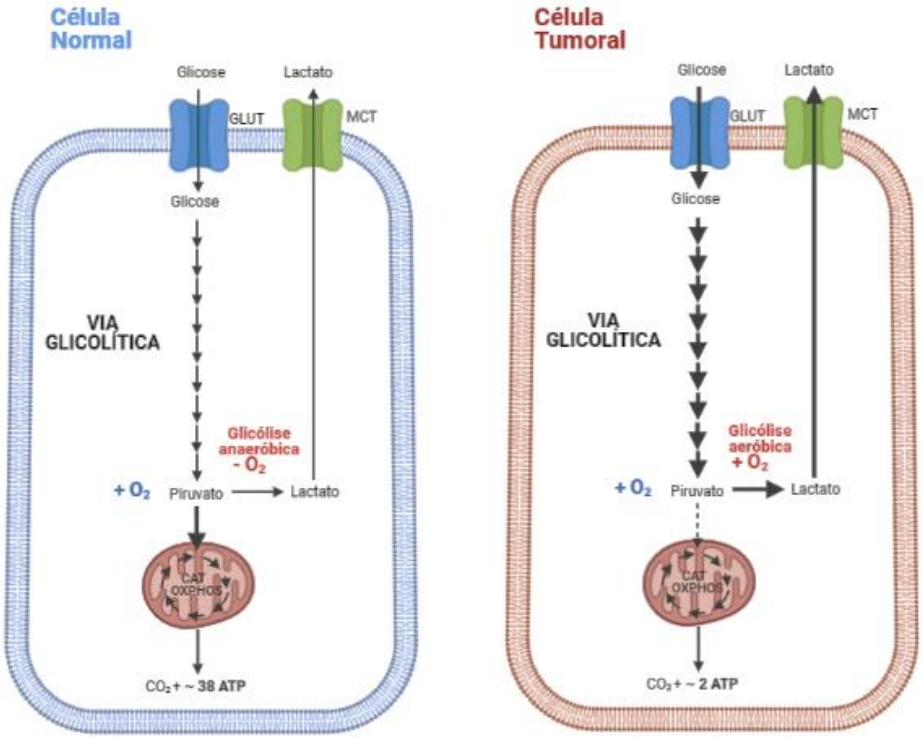
1.1.1.2 Via glicolítica

A via glicolítica, ou glicólise, consiste em uma série de dez reações bioquímicas no citosol na qual ocorre a oxidação de glicose, levando a produção de duas moléculas de piruvato, quatro moléculas de ATP e duas moléculas equivalentes de dinucleótido de nicotinamida e adenina ($NADH^{+}$) por molécula de glicose, esses por sua vez podem seguir para outras reações que ocorrem na mitocôndria ou serem reduzidos a lactato dependendo da quantidade de oxigênio disponível (Chandel, 2021a).

Como visto, o pesquisador Otto Warburg foi o pioneiro no estudo das alterações no metabolismo de células tumorais quando comparadas as células normais. Em relação a via glicolítica, esse verificou que em condições aeróbicas (presença de oxigênio) as células normais metabolizam glicose, primeiramente em piruvato, pela via glicolítica e então em dióxido de carbono (CO_2) na mitocôndria, através de um conjunto de reações conhecidos como ciclo de Krebs/ciclo dos ácidos tricarboxílicos (TCA), e sem seguida pela fosforilação oxidativa (OXPHOS). Já em condições anaeróbicas (ausência de oxigênio), a glicólise é priorizada, e a concentração de piruvato enviado à

mitocôndria diminui. Entretanto, nas células tumorais, mesmo na presença de oxigênio, ocorre uma modificação metabólica e a produção de energia ocorre majoritariamente pela via glicolítica, com a fermentação de glicose e produção de lactato, esse fenômeno ficou conhecido como glicólise aeróbica ou efeito Warburg (Fig. 2) (Warburg, 1956; Warburg; Dickens, 1931).

Figura 2. Comparação esquemática do metabolismo normal com o tumoral. A modificação na via glicolítica na célula tumoral ficou conhecida como glicólise aeróbica ou efeito Warburg. GLUT: Transportador de glicose; MCT: Transportadores de monocarboxilatos.

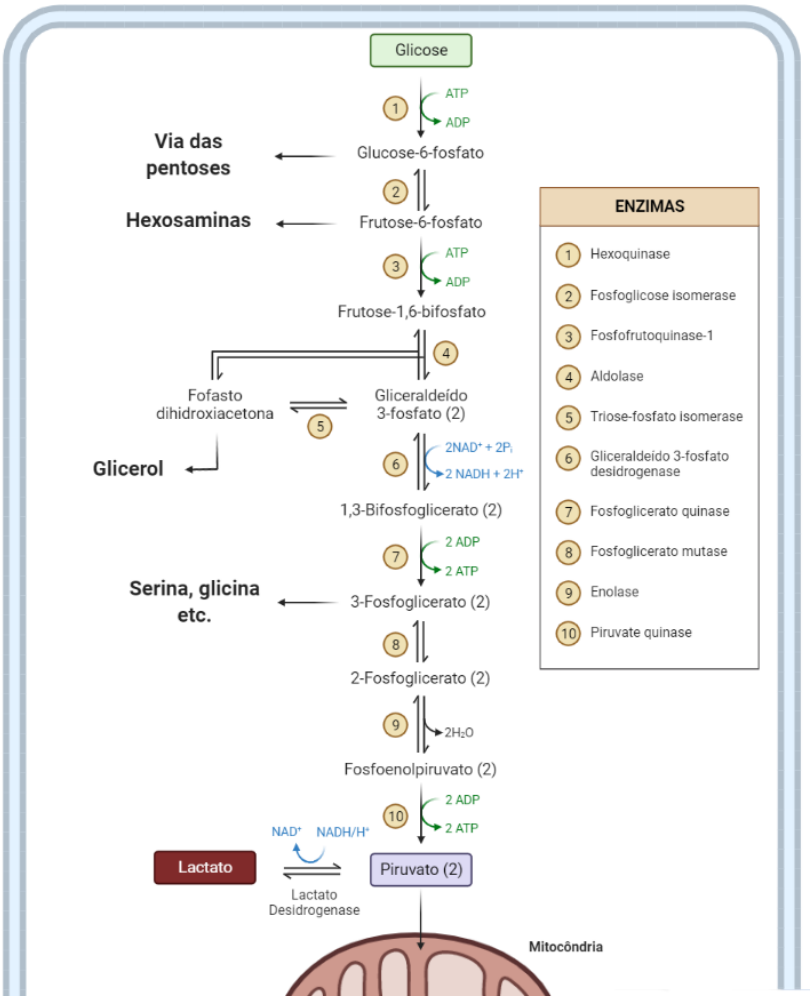


Fonte: Adaptada de da Silva e colaboradores (2022).

Como visto na figura 2, na célula normal, para cada molécula de glicose podem ser formadas até 38 moléculas de ATP através da OXPHOS, enquanto na célula tumoral com a fermentação são formados apenas 2, uma eficiência cerca de 18 vezes menor. Essa troca parece ser desvantajosa energeticamente para o tumor, entretanto, na célula tumoral ocorre uma maior expressão de transportares de glicose (GLUT), assim como de outras enzimas da via, levando dessa forma a uma maior produção de intermediários e de ATP, essenciais para a progressão do tumor (Angulo-Elizari *et al.*, 2023)

A geração de intermediários biossintéticos pela via glicolítica é imprescindível para a manutenção da replicação tumoral, vias de síntese de macromoléculas utilizam dos intermediários da via glicolítica para produção de DNA, RNA, proteínas e lipídios, permitindo a manutenção de processos replicativo (Figura 3) (Schiliro; Firestein, 2021).

Figura 3. Via glicolítica e a produção de intermediários para outras vias de biossíntese de macromoléculas. São enumeradas em ordem as dez enzimas e as suas respectivas reações bioquímicas na via glicolítica, levando a produção de piruvato a partir da glicose e de outros intermediários.



Fonte: Adaptada de da Silva e colaboradores (2022).

Devido a importância do metabolismo da glicose e a via glicolítica na geração de energia para o desenvolvimento do câncer, cada vez mais estudos tem buscado investigar a inibição de enzimas e transportadores dessa via como promissores alvos farmacológicos na oncologia (Xintaropoulou *et al.*, 2018; Zhang, Guilian *et al.*, 2022).

Inibidores glicolíticos como STF-31, 3-bromopiruvato (3-BP), Benitrobenrazida (BNBZ), DC-5163 e ácido oxâmico têm sido amplamente estudados como ferramentas farmacológicas na terapêutica do câncer, visando transportadores de

glicose (GLUT) (Chan *et al.*, 2011; Martin; Kornmann; Fuhrmann, 2003), hexoquinases (HK) (Cardaci; Desideri; Ciriolo, 2012; Zheng *et al.*, 2021), gliceraldeído-3-fosfato desidrogenase (GAPDH) (Li et al., 2020; Pacchiana et al., 2022), lactato desidrogenases (LDH) (Kim, Eun-Yeong et al., 2019; Qiao et al., 2021; Shibata et al., 2021; Zhang, Wenjing et al., 2022) entre outros, esses são estudados em diferentes modelos de câncer e estão em diferentes fases de estudo, tanto pré-clínico quanto clínico (da Silva; Montenegro; Moreira-Nunes, 2022).

1.1.1.3 Via da síntese de nucleotídeos

Como visto, a hiperproliferação celular depende do aumento do número de ciclos replicativos, incluindo a síntese de novas macromoléculas celulares como DNA, RNA que sustentam a replicação descontrolada (Hanahan; Weinberg, 2011). Genes relacionados a vias de síntese de nucleotídeo estão hiper expressos em diversos tipos de câncer, e tem demonstrado interesse como novos alvos farmacológicos na terapia do câncer (Akhavan *et al.*, 2021; Wu *et al.*, 2021).

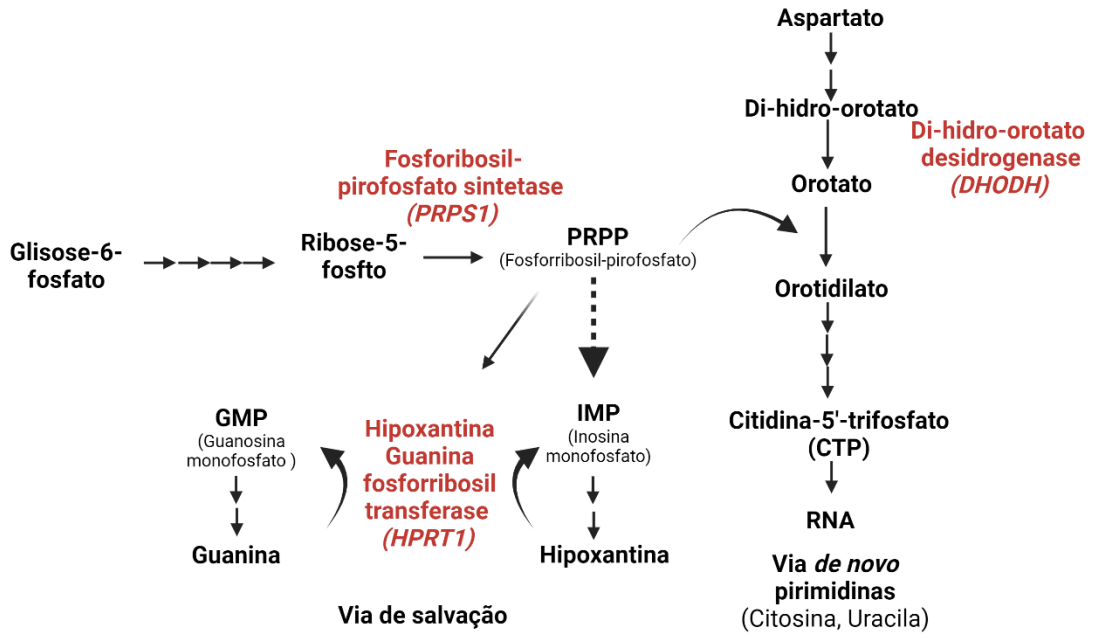
O intermediário biosintético da via glicolítica glicose-6-fostato pode ter outros destinos catabólicos, um deles é a produção de DNA, RNA pela via das pentoses (Figura 3). São conhecidos dois tipos de vias responsáveis pela síntese de nucleotídeos: a vias de novo e as vias de salvação. Essas vias não estão correlacionadas e são independentes uma da outra principalmente no que se refere a seus mecanismos e em sua regulação. Enquanto a via *de novo* sintetiza nucleotídeos tendo como precursores metabolitos como aminoácidos e derivados de glicose, a via de salvação recupera intermediários (nucleosídeos) da degradação de ácidos nucléicos, usando-os como precursores para síntese de nucleotídeos (Walter; Herr, 2022). Devido ao alto potencial proliferativo das células tumorais, o metabolismo de nucleotídeos pela via *de novo* é hiper regulado, mas diferente das células não malignas, o tumor pode trocar para a via de salvação para manter a eficiência na replicação em caso de necessidade bioenergética (Vander Heiden; DeBerardinis, 2017; Walter; Herr, 2022; Wu, Huai-liang *et al.*, 2022).

A glicose-6-fostato é oxidada a ribose-5-fosfato, molécula essencial para a produção do aminoácido histidina, assim como de nucleotídeos e ácidos nucleicos. A ribose-5-fostato é então convertida em fosforribosil-pirofosfato (PRPP) por meio da enzima Fosforibosil-pirofosfato sintetase (*PRPS1*), esse serve de intermediária para diversas reações biossintéticas na célula (Chandel, 2021b).

Como visto na figura 4, o PRPP pode servir de substrato para via de salvação ou para a via *de novo* da síntese de nucleotídeos. Esse pode ser catabolizado em um conjunto reações até inosina monofosfato (IMP) (Pedley; Benkovic, 2017). Na síntese de salvação, a enzima Hipoxantina-guanina Fosforribosiltransferase, codificada pelo gene *HPRT1*, realiza a síntese de IMP e de guanosina monofosfato (GMP) a partir de bases hipoxantina e guanina livres, respectivamente, utilizando o PRPP como substrato para transferência do grupamento fosforibosil, assim permitindo a sua utilização em novas reações de síntese de nucleotídeos (Torres; Puig, 2007; Walter; Herr, 2022).

Por sua vez, a enzima Di-hidro-orotato desidrogenase (*DHODH)* têm um papel chave na via *de novo* da síntese de pirimidinas, sendo utilizada na produção monofosfato de uridina (UMP) e Citidina-5-trifosfato (CTP) para a síntese de uracila e citosina a partir de aspartato. A enzima DHODH é a única enzima mitocondrial da via de síntese de pirimidinas, estando localizada na superfície externa da membrana mitocondrial interna e tem sua atividade relacionada com a cadeia transportadora de elétrons . O PRPP também tem papel importante nessa via, fornece a cadeia lateral de ribose-5-fosfato formando orotilidato, levando posteriormente a formação de nucleotídeos para a síntese de RNA (Figura 4) (Mullen; Singh, 2023; Zhou *et al.*, 2021).

Figura 4. Vias de salvação *de novo* na síntese de nucleotídeos. Em vermelho são representadas enzimas importantes em vias de síntese de DNA e RNA, cada seta representa uma reação bioquímica da via, enquanto a seta tracejada representa um conjunto com 10 reações sequenciais para a síntese de IMP a partir de PRPP.

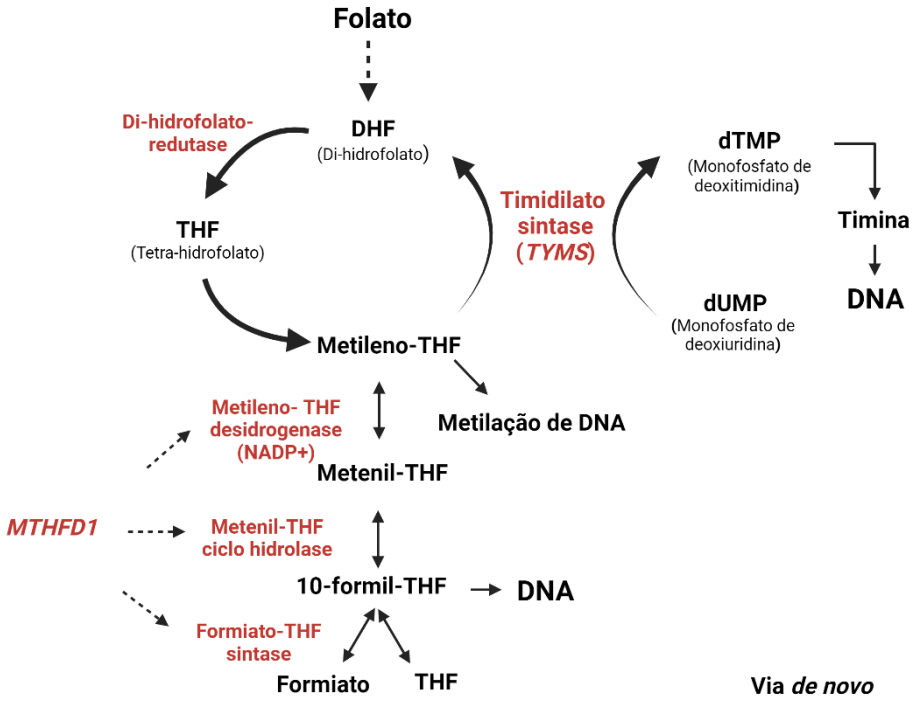


Fonte: Própria autoria, 2024 (Criada com BioRender.com).

O metabolismo de um carbono (1C) compreende uma série de vias metabólicas interligadas que incluem os ciclos de metionina e folato, esse desenvolve um papel crucial em vias de biossíntese de aminoácidos e nucleotídeos. O folato (vitamina B9), ou seu composto de origem sintética ácido fólico, tem um influência na produção de células vermelhas, na metilação do DNA e na proliferação celular por meio da via *de novo* de síntese de nucleotídeos (He *et al.*, 2018; Robinson; Eich; Varambally, 2020).

No ciclo do folato ocorre uma série de reações resultando em diferentes espécies de tetra-hidrofolato (THF), esse é a forma ativada do folato que tem a capacidade de transferência de grupamentos 1C (Figura 5). Nesse ciclo, a molécula metileno-THF é utilizada para síntese do nucleotídeo timina por meio de uma reação de oxidação da enzima Timidilato sintase (codificada por *TYMS*), ou ainda esse pode ser reduzido para formação de metionina para reações de metilação do DNA (Morscher *et al.*, 2018). Como visto na figura 5, o THF também pode ser convertido novamente em metileno-THF por meio da ação da enzima trifuncional Metilenotetrahidrofolato desidrogenase, ciclohidrolase e formiltetrahidrofolato sintetase 1 (*MTHFD1*), realimentando o ciclo metabólico do folato. O intermediário 10-formil-THF ainda age como cofator para outo ciclo de síntese *de novo* de purinas, levando a formação de IMP (Lee *et al.*, 2017; Robinson; Eich; Varambally, 2020).

Figura 5. Via do folato e intermediários importantes na síntese de nucleotídeos. O THF é um intermediário crucial para diversas reações bioquímicas, em vermelho são representadas enzimas importantes da via de síntese de nucleotídeos, cada seta representa uma reação bioquímica da via.



Fonte: Própria autoria, 2024 (Criada com BioRender.com).

Como visto, a geração de THF e sua metabolização pode diferentes vias pode dar origem a aminoácidos importantes para a metilação do DNA, assim como, funciona como intermediário na síntese de nucleotídeos por vias *de novo*, sendo uma essencial para o crescimento celular. Essa via tem sido muito estudada do ponto de visto clínico, tendo fármacos já aprovados que tem como alvo as enzimas Di-hidrofolato redutase e Timidilato sintase (Mullen; Singh, 2023)

O estudo do relacionamento entre o metabolismo de nucleotídeos e a sua relação com o desenvolvimento e a progressão tumoral vem ganhando cada vez mais importância na pesquisa clínica (Aird; Zhang, 2015; Halbrook; Wahl; Lyssiotis, 2019). Quimioterápicos citotóxicos da classe dos antimetabólicos como 5-flourouracil (5-FU), Metrotexato, Capacitabina e Citarabina são exemplos de sucesso clínico que tem alvo no metabolismo de nucleotídeos, levando a inibição direta ou indireta de vias de síntese de purinas e pirimidinas e assim impedindo a formação de RNA e DNA e a progressão tumoral (da Silva; Montenegro; Moreira-Nunes, 2022; Luengo; Gui; Vander Heiden, 2017), nesse sentido a busca e o estudo de novas moléculas com alvo no metabolismo celular, e especificamente, no metabolismo de nucleotídeos traz grandes expectativas para descoberta de novos alvos moleculares na terapia do câncer (Wu, Huai-liang *et al.*, 2022).

Devido a importância dessas vias para o crescimento do tumor algumas enzimas dessas vias têm sido escolhidas como alvos farmacológicos de drogas com potencial antitumoral, como destacado anteriormente. Genes que codificam para enzimas das vias *de novo* de nucleotídeos como a *PRPS1*, *DHODH* , *TYMS* e *MTHFD1*, assim como a enzima da via de salvação *HPRT1* tem sido descrito como hiper expressos no câncer, estando associado ao aumento do perfil proliferativo, migratório, e quimioresistente em diferentes tipos de neoplasias (Bárcenas-López *et al.*, 2021; Lee *et al.*, 2017; Liu *et al.*, 2019; Olsen *et al.*, 2022; Wu, Tong *et al.*, 2022; Xiong *et al.*, 2022; Yuan; Xiao; Lu, 2023), podendo ser bons alvos farmacológicos para terapia do câncer (Feng *et al.*, 2020; Mullen; Singh, 2023; Tan *et al.*, 2022).

1.2 Câncer Gástrico

1.2.1 Epidemiologia

O câncer gástrico (CG) está entre as neoplasias mais incidentes do mundo, sendo considerado o quinto tipo de câncer mais frequente, excluindo o câncer de pele

não melanoma, acometendo mais de 1 milhão de pessoas (5,6% dos casos). Se torna um grave problema de saúde mundial, visto que, apesar de ocupar a quinta posição na incidência, é considerado o quarto mais letal com cerca de 769 mil mortes notificadas em 2020 (7,7% de todas as mortes causadas por neoplasias) (Sung *et al.*, 2021). O CG é duas vezes mais frequente em homens (incidência de 15,8% em homens para 7% em mulheres), sendo o quarto câncer mais diagnosticado e incidente em homens (7,1% e 9,1%, respectivamente), na população feminina é e sétimo mais diagnosticado (4%) e o quinto mais letal (6%) (Morgan *et al.*, 2022; Sung *et al.*, 2021).

Em relação a localidade, as incidências mais altas de CG são observadas em países da Ásia oriental tanto em homens como em mulheres (32,5% e 13,2%, respectivamente), homens que residem no Japão (88,1%), Mongólia (47,2%) e Coréia (39,7%) apresentam a maior incidência no mundo. As incidências mais baixas são notificadas na África, com índices menores que 5%. As taxas de mortalidade são mais elevadas na Ásia Oriental tanto em homens como em mulheres (21,1% e 8,8%, respectivamente). Uma menor parcela de mortes é observada em países com Indice de Desenvolvimento Humano (IDH) muito altos, quando comparados com países com médio e baixo IDH. As projeções afirmam que a incidência anual do CG tendem a aumentar para cerca de 1.8 milhões de novos casos e 1.3 milhões de mortes até 2040 (Morgan *et al.*, 2022).

No Brasil, de acordo com o Instituto Nacional do Câncer (INCA), as estimativas para o triênio 2023-2025 são de aproximadamente 21 mil novos casos (9,94% dos casos) de CG, sendo a quinta neoplasia mais frequente no país. Dentre esses novos casos, 13.340 serão diagnosticados em homens (12,6%) e 8.140 em mulheres (7,4%), fazendo dessa neoplasia a quarta e a sexta mais frequente em cada sexo, respectivamente. Dados reportam que, diferente de outras regiões do país, o CG tem apresentado aumento no número de casos e óbitos em regiões com menor IDH, como norte e nordeste, nessas regiões essa neoplasia é segunda (12,5%) e terceira (12,2%) mais frequente em homens, com um aumento em 3,1% na mortalidade na população em ambas as regiões (Braga *et al.*, 2022; INCA, 2023). No estado de Ceará são esperados 1.460 novos casos para o ano de 2023, sendo o terceiro tipo de câncer mais diagnosticado na população do estado, por gênero é o segundo mais frequente em homens (19,78%) e o sexto (11,63%) em mulheres (INCA, 2023).

Apesar da diminuição na incidência e mortalidade nas últimas décadas, dados vem demonstrado uma projeção de aumento de casos de CG entre a população de jovens adultos (< 50 anos) tanto em países de baixo como alto IDH (Arnold *et al.*, 2020a). O desenvolvimento do CG está associado a vários fatores em conjunto, sendo determinados pela combinação de fatores genéticos e ambientais. Cerca de metade dos casos de CG estão relacionados ao acúmulo de mutações causadas por fatores ambientais, como fumo, consume de bebidas alcoólicas, consumo de comidas contendo conservantes e uma dieta rica em sal e carboidratos, redução na inserção de frutas e vegetais na alimentação e o sedentarismo (Kobayashi, 2018; Tomasetti; Li; Vogelstein, 2017; Trinh *et al.*, 2023). Infecções crônicas pela bactéria *Helicobacter pilory* (*H. pilory*) e pelo vírus *Epstein-Barr* (EBV) também tem forte contribuição para o desenvolvimento do CG, já os fatores hereditários correspondem em menos de 3% dos casos apresentados (Arnold *et al.*, 2020b; Rustgi; Ching; Kastrinos, 2021).

1.2.2 Classificação histológica e molecular

Aproximadamente 90% dos CG são do tipo adenocarcinoma, podendo se desenvolver em diferentes regiões do órgão, como cardia ou na região não cardia **(**corpo**,** fundo **e** piloro). Dependendo da localidade, o tumor pode estar associado a diferentes fatores, tumores que acometem a cardia estão mais ligados ao histórico familiar, estilo de vida, a doença do refluxo esofágico e infecções pela bactéria *H. pylori* (Abdi *et al.*, 2019). Apesar de aumento dos adenocarcinomas nas regiões da cardia, os da região da não cardia representam aproximadamente 80% dos casos de CG (Colquhoun *et al.*, 2015). Existem diversas classificações histopatológicas do CG, as mais utilizadas são a de Lauren que foi instaurada em 1965, e a da Organização Mundial da Saúde (OMS) que é a mais utilizada em países ocidentais (Lordick *et al.*, 2022).

1.2.2.2 Classificação de Lauren

De acordo com a classificação de Lauren os adenocarcinomas que acometem as regiões do corpo, fundo e piloro (não cardia) são subdivididos em outras duas variantes principais, essas são diferentes histológicas e possuem características distintas, sendo conhecidas como tipo intestinal e do tipo difuso (Lauren, 1965). O tipo intestinal é caracterizado pela presença de células coesas dispostas em formações glandulares, estando associado a metaplasia intestinal, inflamação crônica, infecção por *H. pilory*, com aumento na capacidade invasiva e sendo mais incidente em pacientes de mais idade

e do sexo masculino (Ma *et al.*, 2016; Sitarz *et al.*, 2018). O tipo intestinal é geralmente precedido por uma fase pré-cancerosa, tendo início com a transição da mucosa normal para uma gastrite atrófica, em seguida a essa alteração, ocorre o desenvolvimento de uma metaplasia intestinal, que evolui para uma displasia e para formação do adenocarcinoma (Lauren, 1965).

No tipo difuso, entretanto, as células possuem pouca adesão, sendo são pouco diferenciadas, infiltram-se no estroma como células únicas ou pequenos subgrupos e comumente formam células em anel de sinete. Esse é mais associado a fatores genéticos, sendo ainda prevalente em pacientes mais jovens do sexo feminino e descreve um pior prognostico (Sarriugarte Lasarte *et al.*, 2021). Esse tipo de adenocarcinoma também é originado de uma lesão na mucosa normal (gastrite), ainda assim, possui menor relação com fatores ambientais do que o tipo intestinal, embora a infecção por *H. pylori* também esteja envolvida na carcinogênese do tipo difuso. Entretanto, diferentemente do tipo intestinal, no tipo difuso ocorre o desenvolvimento do adenocarcinoma como resultado da inflamação crônica, sem ser necessário os passos intermediários característicos do tipo intestinal. O tipo difuso, pode acometer toda a superfície do órgão, tem incidência em homens e mulheres na mesma proporção e está associado com um pior prognóstico e taxa de sobrevida (Castaño-Rodríguez; Kaakoush; Mitchell, 2014; Ma *et al.*, 2016; Sitarz *et al.*, 2018).

1.2.2.3 Classificação da Organização Mundial da Saúde (OMS)

Desde 2010, O GC também pode ser classificado de acordo com a OMS, sendo divididos em tubulares, papilares, mucinosos, pouco coesos (incluindo carcinoma de células em anel de sinete (*Signet ring cell*, SRC)) e carcinomas mistos (Bosman *et al.*, 2010). Quando comparamos com a classificação de Lauren, os carcinomas tubulares, papilares e mucinosos se enquadram no tipo intestinal previamente descrito, já o carcinoma de células em SRC outros carcinomas poucos coesos correspondem ao tipo difuso (Hu *et al.*, 2012; Van Cutsem *et al.*, 2016).

O adenocarcinoma tubular é o tipo histológico mais comum entre esses. Tende a formar massas polipóides ou com perfil ulcerado em várias formas e demonstra histologicamente túbulos irregularmente distendidos, fundidos ou ramificados de vários tamanhos, muitas vezes com muco intraluminal, restos nucleares e inflamatórios (Hu *et al.*, 2012). O adenocarcinoma papilar é outra variante histológica, acometendo de 1,5%

– 2% de todos os casos, sendo frequentemente observada em estágios iniciais do desenvolvimento do CG. Tende a afetar pessoas com maior faixa etária (> 60 anos), ocorre na região do estômago proximal e está frequentemente associada a metástases hepáticas e a uma maior taxa de envolvimento de linfonodos, levando a com baixo prognóstico. Histologicamente, é caracterizada por projeções epiteliais sustentadas por um núcleo fibrovascular central (Hu *et al.*, 2012; Shiratori *et al.*, 2020).

O adenocarcinoma mucinoso é responsável por 10% do carcinoma gástrico. Histologicamente é caracterizado pela alta produção de mucinas extracelulares (proteínas glicosiladas que ajudam na constituição do muco) que constituem pelo menos 50% do volume do tumor. Nesse subtipo histológico, as células tumorais podem formar arquitetura glandular e aglomerados celulares irregulares (Cai *et al.*, 2018; Hu *et al.*, 2012).

Os carcinomas SRC e outros carcinomas poucos coesos são compostos geralmente por uma mistura de células em SRC e células em anel não-sinete. Se assemelham estruturalmente a histiócitos, linfócitos e células plasmáticas com alta produção de mucina, que geralmente permanece dentro da célula (Zhao *et al.*, 2023). Pode apresentar invasão linfovascular, linfonodal e metástase duodenal. Devido ao grande potencial invasivo e metastático, atenção especial precisa ser dada a essas subtipo no momento de ressecção cirúrgica. Colorações especiais, como a imuno-histoquímica (IHQ) da citoqueratina ou marcação de reticulina, podem ajudar a detectar células do anel de sinete morfologicamente ocultas (Hughes; Greywoode; Chetty, 2011).

1.2.2.4 Outras classificações histológicas

Existem ainda outras duas classificações histológicas descritas, a do sistema Carneiro divide os adenocarcinomas gástricos em 4 classes, sendo tumores glandulares, de células isoladas, sólidos e mistos, de acordo com a expressão de marcadores de IHQ (MUC6, MUC5AC e marcadores gástricos peptídicos TFF1 ou marcadores intestinais MUC2, CDX2, CD10 e pepsinogênio-1, entre outros) (Carneiro, 1997).

A classificação de Goseki também divide em quatro subtipos os tumores gástricos, esses são classificados ou acordo com o nível de produção de mucina (alta ou baixa) ou pelo nível de diferenciação tubular (baixa diferenciação ou alta diferenciação), dessa forma são classificados em tumores com: 1) alta diferenciação tubular e pouca

produção de mucina intracelular; 2) alta diferenciação tubular e alta produção de mucina intracelular; 3) baixa diferenciação tubular e pouca produção de mucina intracelular e 4) com baixa diferenciação tubular e alta produção de mucina intracelular (Cisło *et al.*, 2018; Seeneevassen *et al.*, 2021).

1.2.2.5 Classificação Molecular

Os tumores gástricos não variam só morfologicamente, mas também apresentam perfis moleculares distintos. Baseado nos avanços da biologia molecular e das técnicas de sequenciamento e nova geração (NGS), assim como nos estudos epigenéticos, novas classificações baseadas no perfil de mutação do CG foram desenvolvidas (Chia; Tan, 2016; Wang; Liu; Hu, 2019).

A rede de pesquisa do programa de estudos genômicos denominada Atlas do Genoma do Câncer (*The Cancer Genome Atlas*, TCGA) reportou em 2014 uma das caracterizações moleculares do CG mais bem caracterizadas e utilizadas, nesse estudo foram utilizadas seis diferentes plataformas moleculares de análise em 295 tumores primários gástricos. Os dados propuseram uma classificação dividida em quatro subgrupos de CG (Figura 6), sendo eles: Tumores positivos para EBV (8,8 % das amostras), tumores com instabilidade de microssatélites (MSI) (21,7% das amostras), tumores genomicamente estáveis (GS) (19,7%) e tumores com instabilidade cromossômica (CIN) (49,8%) (TCGA Research Network, 2014).

Os tumores EBV positivos foram caraterizados por serem mais frequente na região do fundo ou corpo gástrico, sendo mais frequente em homens. Como o nome já cita, esses tumores apresentam grande grau de infecção por EBV, alta taxa de metilação no DNA, e mutações importantes nos genes *PIK3CA*, *BCOR*, hiperexpressão de *CD274* e *PDCD1LG2* (PD-L1/2, respectivamente), amplificação de *JAK2* e *HER 2* (TCGA Research Network, 2014; Wang; Liu; Hu, 2019).

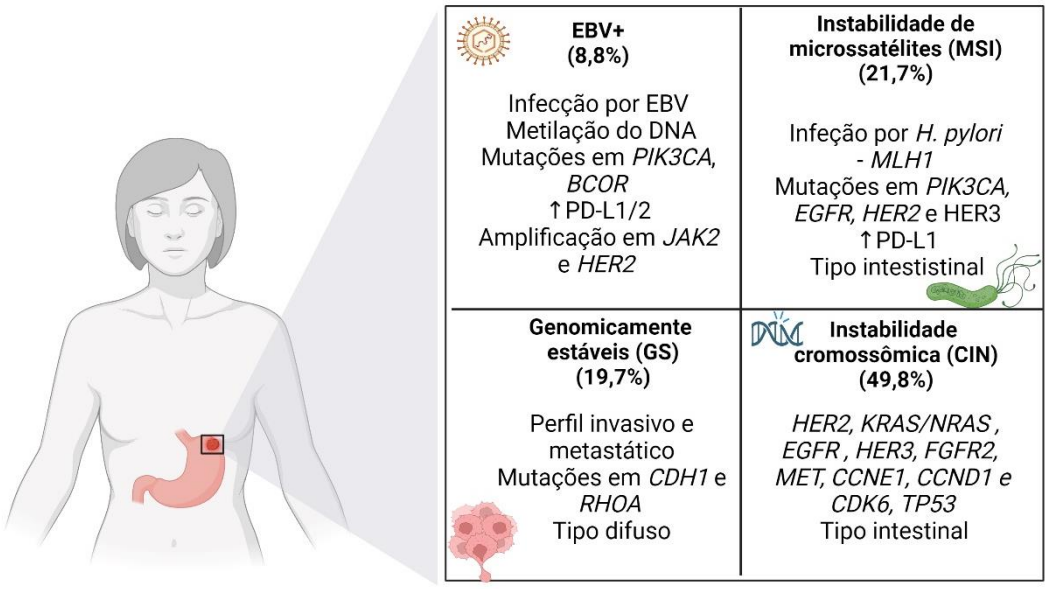
Os tumores reconhecidos com MSI foram mais frequentes em populações com maior faixa etária (>70 anos) e do sexo feminino, estando mais localizada no antro gástrico e estando associado a infecção por *H. pylori*. Nesses casos, a modificação epigenética é importante, uma vez que é comum a metilação na região promotora do gene *MLH1* acarretando no seu silenciamento, esse gene está relacionado ao reparo de incompatibilidade de DNA, resultando no aumento no nível de mutações no material genético e no fenótipo mutacional MSI (Jung *et al.*, 2001). Mutações em *IK3CA*, *EGFR*, *HER2 e HER3* são observadas nesse subtipo, com alta taxa de expressão da proteína

PD-L1. Tanto os tumores EBV positivos como os com fenótipo MSI mostraram possuir o fenótipo mutilador das ilhas CpG (*CpG island methylator phenotype*, CIMP), também estando mais associados a histologia do tipo intestinal (Wang; Liu; Hu, 2019).

Os tumores genomicamente estáveis possuem baixa número de alterações no número de copias, sendo diagnosticados em pacientes mais jovens (<60 anos). Estão mais relacionados a histologia do tipo difuso e tem alta expressão em proteínas relacionadas a adesão celular e angiogênese, com mutações em *CDH1* e *RHOA*, favorecendo o perfil invasivo e metastático (Seeneevassen *et al.*, 2021; Wang; Liu; Hu, 2019).

Por outro lado, os tumores com CIN foram mais diagnosticados na junção gastroesofágica/cardia, com perfil histológico intestinal. Nesse subtipo amplificações no gene *HER2* (25%), *KRAS/NRAS* (18%), *EGFR* (10%), *HER3*(8%), *FGFR2* (8%) e *MET* (8%) são as mais observadas. Além disso, tumores CIN possuem alta frequência de mutação no gene *TP53* (73% dos casos), assim como, amplificações em genes relacionados ao ciclo celular como *CCNE1*, *CCND1* e *CDK6* (Wang; Liu; Hu, 2019).

Figura 6. Classificação molecular do câncer gástrico baseado no TCGA.



Fonte: Própria autoria, 2024 (Criada com BioRender.com).

Baseado na expressão de mRNA, na análise do número de cópias somáticas e no sequenciamento genético, o grupo Asiático de Pesquisa do Câncer (*Asian Cancer Research Group*, ACRG) também propôs uma classificação molecular baseada na taxa de expressão de 300 tumores, classificando-os em quatro grupos distintos, sendo eles

pacientes com alta taxa de MSI (22,7%), pacientes com taxa de microssatélites estável (MSS) atrelado a presença de marcadores de transição epitélio mesenquimal (MSS/EMT, 15,3%), pacientes MSS sem mutação em *TP53 (*MSS/*TP53+*, 26,3%) e pacientes MSS com perca de *TP53 (*MSS/*TP53-*, 35,7%) (Cristescu *et al.*, 2015).

Os pacientes com alta taxa MSI estão mais relacionados a região do antro gástrico, com histologia do tipo intestinal. Apresentam mutações em *ARID1A*, *KRAS* e *ALK*, tem o melhor prognóstico dentro os subtipos. Pacientes MSS/EMT são geralmente mais novos com diagnóstico já em estágio avançado da doença, com alto nível de infiltração peritoneal, nesse, são encontradas células com a morfologia de SRC e com peca da expressão do gene *CDH1*. Pacientes MSS/*TP53+* possuem diversas mutações catalogadas, como nos genes *APC, ARID1A, KRAS, PIK3CA*m e *SMAD4*, tem o segundo melhor prognóstico entre os subtipos. Pacientes MSS/*TP53-* possuem alto nível de mutações no *TP53* (60%) com baixa frequência em outras mutações. Possem amplificações recorrentes nos genes *HER2*, *EGFR*, *CCNE1*, *CCND1*, *MDM2*, *ROBO2*, *GATA6* e *c-Myc* (Cristescu *et al.*, 2015; Wang; Liu; Hu, 2019).

Embora a subclassificação pelo perfil molecular pareça deixar ainda mais complexo o processo de classificação de tumores gástricos, essa ajuda a identificar subtipos de GC baseados nas semelhanças moleculares e características genéticas, dando informações essenciais para a seleção de terapias mais direcionadas para esses pacientes (Bijlsma *et al.*, 2017; Deng *et al.*, 2012).

1.2.3 Diagnóstico e estadiamento

O GC é geralmente assintomático em estádios iniciais, em etapas avançadas da progressão da doença os pacientes podem apresentar sintomas como dificuldade de deglutição (disfagia), fraqueza (astenia), indigestão, vômitos, perda de peso, saciedade precoce e/ou anemia por deficiência de ferro. Entretanto, na maioria dos casos, os sintomas são inespecíficos e não levantam grande preocupação do paciente no momento, o que leva geralmente a diagnósticos tardios da doença com a piora na sintomatologia, diminuindo as chances de um tratamento curativo e levando a redução na probabilidade de cura quando comparado ao diagnóstico em estágios iniciais (Allum *et al.*, 2018).

A endoscopia é o método padrão outro de diagnóstico do CG, múltiplas biópsias devem ser realizadas com o objetivo de fornecer material com tamanho

adequado para a interpretação histológica e/ou molecular (Tominaga *et al.*, 2016). A Ultrassonografia Endoscópica (EUS) ou ecoendoscopia é um método combinado de encospia digestiva e ultrassom, ajudando na obtenção de imagens e na aquisição de biópsias, ajudando na identificação de regiões infiltradas pelo tumoral na parede gástrica (Hamada *et al.*, 2021). As ressecções endoscópicas de mucosa (EMR) e de submucosa (ESD) além de poderem ser utilizadas como a finalidade diagnóstica, também podem ter intuito curativo em caso de lesões menores que 20 mm, ou maiores, respectivamente. Além disso, ambos os procedimentos podem ajudar na aquisição de informações sobre o estadiamento tumoral (Ono *et al.*, 2021).

Para tumores es estádios avançados a tomografia computadorizada (TC) padrão de tórax/abdômen/pélve costuma ser suficiente para exames de imagem, mas a Tomografia Computadorizada com Emissão de Pósitrons com fluodesoxiglicose (FDG-PET)/TC pode ser considerada para indicações clínicas específicas, como avaliação adicional de lesões indeterminadas (Gertsen *et al.*, 2021; Joshi; Badgwell, 2021).

A laparoscopia e a realização da citologia do lavado peritoneal são recomendadas para todos os casos de CG em estágios IB-III, que são considerados ressecáveis, para diminuir a possibilidade de doença metastática peritoneal oculta (Ramos *et al.*, 2023). Em caso positivo, as metástases peritoniais devem ser documentados de acordo com o Índice de Carcinomatose Peritoneal (PCI) que varia de 0 a 39, conforme a gravidade da doença (Ye *et al.*, 2022).

O estadiamento do CG é realizado de acordo com a Comitê Misto americano de câncer (AJCC) e a União Internacional para o Controle do Câncer (UICC), pelo sistema TNM. Esses dados levam em conta as características do tumor primário (T), as características dos linfonodos das cadeias de drenagem linfática do órgão em que o tumor se localiza (N), e a presença ou ausência de metástases a distância (M). Essas características possuem graduações em escalas crescentes, variando de T0 a T4, de N0 a N3 e de M0 a M1, respectivamente (Amin *et al.*, 2017).

1.2.4 Tratamento

Para doenças em estágio inicial IA (T1N0M0), bem diferenciadas e não ulceradas a ressecção cirúrgica por endoscopia pode ser potencialmente curativa (Pimentel-Nunes *et al.*, 2015; Tan, 2019). A realização da cirurgia depende da localização do tumor, do índice TNM e do subtipo histológico, tumores T1 que não tem o critério para a ressecção cirúrgica por endoscopia necessitam, mesmo que menos

radicais, de cirurgia. Para tumores mais avançados a cirurgia de gastrectomia, retirada parcial (subtotal) ou total (radical) do estômago, é a indicada. Quando é observado que a lesão se encontra com margens proximais de ressecção satisfatória, a gastrectomia subtotal é a ideal para manter a qualidade de vida do paciente, já em estágios mais avançados (IB-III) a gastrectomia radical é recomendada (Maspero *et al.*, 2022).

Estudos vem tentando mostrar o benefício da ressecção endoscópica de acesso minimamente invasivo (laparoscopia) na diminuição do morbidade pós-operatória e no tempo de recuperação quando comparada em cirurgias mais invasivas em lesões mais avançadas (Quan *et al.*, 2016). Estudos no leste asiático e em países ocidentais também demonstraram os benefícios da laparoscopia em tumores em estádios iniciais e mais avançados, tornando o método laparoscópio uma boa alternativa para realização de gastrectomia total ou parcial com linfadenectomia (retirada dos linfonodos) em pacientes com CG (Smyth *et al.*, 2020).

Embora a completa retirada do tumor e dissecação linfonodal sejam considerados parâmetros de cura para o CG, uma alta taxa de recidivas locais em tem sido observada. Os sítios de recidivas mais frequentes são as regiões próximas ao tumor (31% - 68% dos casos), peritônio (41,5% - 45,7%) e fígado (20% - 32%). Um benefício na sobrevida dos pacientes tem sido observado na adição da quimioterapia ou quimiorradioterapia a cirurgia (Cascinu *et al.*, 2022; Kang *et al.*, 2015).

Diferentes regimes de quimioterápicos têm sido estudados juntamente a cirurgia para a avaliar a resposta do paciente, a sua sobrevida e recorrência da doença. A quimioterapia perioperatória, tratamento antes e após a cirurgia, foi utilizada no estudo clínico de fase III (MAGIC) na qual foram administrados três ciclos de uma combinação de Epirrubicina, Cisplatina e 5-FU (ECF) antes da cirurgia, e três ciclos de Epirrubicina e Cisplatina no dia 1 após o procedimento, com continua administração de 5-FU por 21 dias. Os resultados mostraram que a utilização de ECF de forma perioperatória melhoraram a sobrevida dos pacientes diagnosticados com CG metastático em estágio avançado, tendo uma taxa de sobrevida em 5 anos de 36%, enquanto que para os pacientes que realizaram a cirurgia de forma isolada, esse taxa foi de 23% (Cunningham David *et al.*, 2006).

Resultados similares foram obtidos através da utilização de Cisplatina e 5-FU em conjunto com o procedimento cirúrgico, foi observada um taxa de 38% de sobrevida na utilização da quimioterapia perioperatória com a cirurgia, enquanto para cirurgia de forma isolada a taxa foi de 24%. A sobrevida livre de doença, ou seja, o período em que

um paciente permanece livre de sinais e sintomas da doença após o tratamento, também foi superior, sendo de 34% para a quimioterapia perioperatória e de 19% para pacientes apenas tratados com cirurgia (Ychou *et al.*, 2011).

No estudo clínico de fase III utilizando a terapia adjuvante, regime quimioterápico após o procedimento cirúrgico, com os quimioterápicos Epirrubicina, Leucovorin (Ácido Fólico), 5-FU e Etoposídeo em combinação (ELFE) para pacientes com CG, foi verificado que após 5 anos de acompanhamento os pacientes não demonstram aumento na sobrevida significativo quando comparada a utilização apenas da cirurgia (De Vita *et al.*, 2007). Por outro lado, para pacientes asiáticos portadores de CG em estágio avançado a mono-quimioterapia utilizando fluoropirimidinas (pró-drogas do 5-FU) levou a um aumento significativo na taxa de sobrevida, em comparação com aqueles que fizeram uso apenas da cirurgia de remoção, entretanto os ensaio precisam ser repetidos em uma população mais heterógena para demonstrar sua eficácia clínica (Cascinu *et al.*, 2022; Sakuramoto *et al.*, 2007).

Esquemas como a combinação dupla com derivados de platina e fluoropirimidina(s) [Ácido fólico, 5-fluorouracil e Oxaliplatina (FOLFOX) ou Oxaliplatina e Capecitabina (XELOX)] são recomendados como tratamento de primeira escolha em protocolos internacionais para CG metastático. Uma alternativa terapêutica é a combinação de Ácido fólico, 5-Flourouracil e Irinotecano (FOLFIRI), que também vem demonstrando bons resultados em caso de falha terapêutica inicial com melhor tolerabilidade (Cascinu *et al.*, 2022; Guimbaud *et al.*, 2014).

A utilização de quimiorradioterapia adjuvante demonstrou bons resultados no tratamento do CG, em estudo de fase clínica II combinando radioterapia com a utilização do regime de 5-FU e ácido fólico por um mês em adição a cirurgia de remoção. Após o acompanhamento por 5 anos, o grupo de pacientes que recebeu a quimiorradiação obteve um aumento na taxa de sobrevida e na sobrevivência livre de progressão quando comparo aos pacientes que apenas receberam a cirurgia, além disso, após 10 anos de acompanhamento o benefício terapêutico se manteve (Macdonald *et al.*, 2001, 2009).

Recentemente, com a introdução da terapia alvo direcionada houve um aumento no número de estudos como objetivo de identificar e validador novos biomarcadores e alvos moleculares que visam fornecer novas possibilidades terapêuticas aos pacientes com CG (Sato *et al.*, 2023).

Para pacientes com HER2 positivo (score IHQ 3+ ou 2+ mais confirmação pela técnica de hibridização *in situ* (FISH)) as diretrizes apontam para adição do anticorpo monoclonal anti-HER2 Transtuzumabe à quimioterapia. Entretanto, poucos pacientes se beneficiam dessa terapia, visto que apenas aos 10-15% desses apresentam essa condição (Bang *et al.*, 2010; Makiyama *et al.*, 2020).

Outros agentes alvo direcionados como os inibidores de PD-L1, PD-1, VEGFR também podem ser combinados a quimioterapia citotóxica para pacientes que apresentam esse perfil genético, podendo acarretar em melhora significativa em uma parcela pequena dos pacientes com CG (Fuchs *et al.*, 2014; Janjigian *et al.*, 2021; Shitara *et al.*, 2020; Smyth *et al.*, 2020). Outros estudos também mostram que pacientes com o fenótipo de alta instabilidade de microssatélites (MSI-H) podem se beneficiar de terapias com inibidores de PD-1, como Pembrolizumabe em combinação com a quimioterapia (Chao *et al.*, 2021).

Apesar dos benefícios terapêuticos observados na adição da quimioterapia/quimiorradioterapia, e das terapias alvo a cirurgia, as taxas de recorrência local e do aparecimento de tumores em sítios distantes continuam elevados (Cascinu *et al.*, 2022; Mokadem *et al.*, 2019). Dessa forma, o estudo de novas terapias assim como melhor compreender essa neoplasia deve ser incentivado com o intuito de oferecer novos tratamentos a indivíduos não responsivos as terapias atuais.

1.3 Reposicionamento de Fármacos

O reposicionamento de fármacos é uma ferramenta que visa a descoberta de novos usos para medicamentos já aprovados por agencias fiscalizadoras na prática clínica. Esse processo leva a vantagens substanciais em comparação com os métodos de estudos tradicionais de substâncias ainda não conhecidas, uma vez que a descoberta e desenvolvimento *de novo* pode levar de 10 a 17 anos, com elevado custo (1 a 2 bilhões de dólares) e baixa taxa de sucesso visto que menos de 1% das drogas estudadas chegam realmente a prática clínica (Sun *et al.*, 2022; Xia *et al.*, 2024).

O processo de estudo e investigação de novas drogas passa por várias fases, primeiramente ocorrem estudos pré-clínicos envolvendo testes de eficácia, toxicidade, avaliação dos perfis farmacocinético e farmacodinâmico em modelos de estudo *in vitro* e *in vivo*. Em seguida, ao atestar sua eficácia terapêutica, as drogas passam para as fases de estudo clínicos de fase I, II e III em humanos para determinar sua segurança e efetividade (American Cancer Society, 2020; Jaki *et al.*, 2023; Yang *et al.*, 2019).

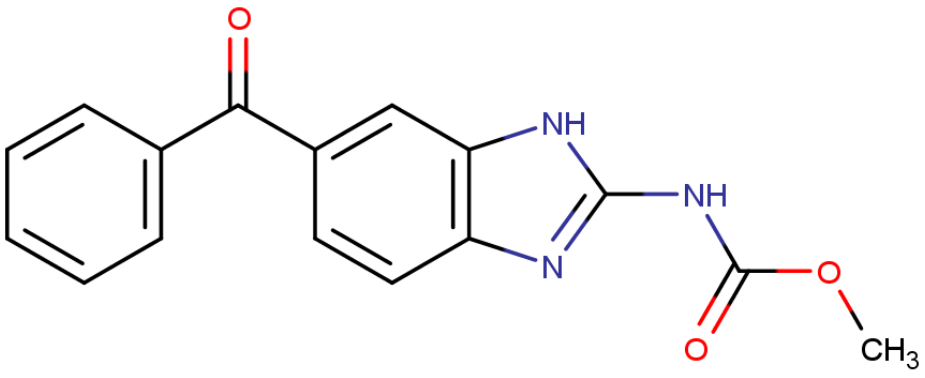
Recentemente o interesse no reposicionamento de drogas tem aumentado, principalmente na oncologia. Essa alternativa pode levar a uma diminuição no tempo e nos custos de estudo e desenvolvimento de uma substância já aprovada, uma vez que essa já possui seus perfis de segurança, dosagem e toxicidade elucidados e documentados (Xia *et al.*, 2024). Nas últimas décadas, o número de novas substâncias aprovadas pela agência americana de regulamentação FDA (*US Food and Drug Administration*) têm caído significativamente, aumentando assim o interesse por terapias reposicionadas na oncologia (Kirtonia *et al.*, 2021).

São exemplos de drogas reposicionadas para o manejo oncológico os antibióticos citotóxicos da classe das antraciclinas como Doxorrubicina, Daunorrubicin e Epirubicina que integram o tratamento quimioterápico de diferentes tipos de câncer (Mattioli *et al.*, 2023). Muitos outros medicamentos, entre eles os utilizados para tratativa de doenças como diabetes (Meformina), inflamações (Aspirina, Diclofenaco), infecção fúngica (Itraconazol) e parasitárias (Albendazol), tem sido estudos de forma isolada ou em combinação para avaliar seu potencial antitumoral, apresentando resultados significativos (Kirtonia *et al.*, 2021). Nesse último, os fármacos da classe dos benzimidazóis tem atraído interesse clínico, com diversos estudos mostrando seu potencial antitumoral (Petersen; Baird, 2021; Song *et al.*, 2022; Wang *et al.*, 2020)

1.3.1 Mebendazol

O fármaco Mebendazol (MBZ, N-[6-(benzoil)-1H-benzimidazol-2-il] carbamato de metilo), de formula molecular $C_{16}H_{13}N_3O_3$ (Figura 7) pertence a classe dos benzimidazóis, sendo um droga aprovada pela FDA para o tratamento de infecções parasitárias e tem seu feito sob a inibição da polimerização da tubulina e sob a captação de glicose no parasita (Keystone; Murdoch, 1979; Laclette; Guerra; Zetina, 1980).

Figura 7. Estrutura química do fármaco Mebendazol.



Fonte: PUBCHEM, 2023.

O MBZ é uma medicamente altamente lipofílico, sendo que menos de 10% é absorvido pelo trato gastrointestinal, esta pequena porção sofre metabolismo rapidamente por enzimas hepáticas. Fármacos como Carbamazepina ou Fenitoína, e indutores das enzimas metabolizadoras CYP450 podem diminuir sua concentração plasmática, possui metabolismo principalmente hepático, levando a produção do metabólico 2-amino-5-benzoil benzonidazol, sendo também metabolizado em compostos inativos de hidroxi e hidroxiamina, esses não possuem ação anti-helmíntica. É majoritariamente excretado por meio das vezes de forma inalterada u como metabólito secundário, cerca de 2% são excretado pela urina (Allan; Watson, 1983; MacDonald *et al.*, 2004).

Os efeitos adversos mais comuns associados ao uso do MBZ a perda de apetite, dor abdominal, diarreia, flatulência, náusea, vomito, dor de cabeça e o aumento nos níveis de enzimas hepáticas. Apesar da toxidade do fármaco estar majoritariamente, mas não apenas, atrelada a irritação gastrointestinal, efeitos como neutropenia (redução da contagem de neutrófilos no sangue) e/ou trombocitopenia (redução na contagem de plaquetas no sangue) também foram observados em pacientes que utilizaram doses elevadas ou que realizaram o tratamento por um tempo mais longo doque o recomendado (Thakur; Patel, 2024; Wilson; Rausch, 1982). Entretanto, efeitos tóxicos do fármaco não são usualmente observados (Dayan, 2003).

O primeiro trabalha relatando a atividade antitumoral do MBZ foi publicado em 2002, por Mukhopadhyay e colaboradores. Nesse estudo, o MBZ foi analisado em modelos de câncer de pulmão, tendo elevado o grau de células em apoptose, causou parada do ciclo celular em G2/M em modelo *in vitro*, assim como, diminuiu o tamanho do tumor e o número de metástases pulmonares em modelos xenográficos.

Após isso, outros estudos tentando esclarecer a atividade antitumoral do MBZ foram realizados, dessa forma foi observado que esse composto levou a parada do ciclo mitótico, a morte celular por apoptose relacionada a despolimerização da tubulina em linhagens de câncer pulmonar (Sasaki *et al.*, 2002). Estudos *in vitro* e *in silico* demonstraram que o MBZ se liga ao receptor de fator de crescimento vascular 2 (VEGF2), diminuindo a angiogênese (Dakshanamurthy *et al.*, 2012). Estudos *in vitro* realizados pelo nosso grupo, mostraram o efeito citotóxico do MBZ em linhagens de CG, além de esclarecer seu potencial genotóxico, antimetastático e na regulação de fatores de transcrição *c-Myc* e de genes que são codificam para proteínas transportadoras de drogas (Pinto *et al.*, 2015, 2017, 2019).

O fármaco MBZ se encontra em estudos de fase clínica I e II, utilizado de maneira isolada ou em combinação com outros quimioterápicos para o tratamento de neoplasias como glioblastoma, meduloblastoma e câncer de colón (Guerini *et al.*, 2019). Isso reafirma a importância do reposicionamento de drogas para o estudo de novos quimioterápicos, sendo alternativas baratas e eficazes para a terapia de outras doenças, como o câncer (Sleire *et al.*, 2017).

Como visto, o fármaco MBZ demonstrou ter diversos alvos no tratamento do câncer, entretanto seu papel no metabolismo celular tumoral e na regulação do transcriptoma permanece inexplorado. Esses achados podem dar novas dimensões do potencial farmacológico do MBZ, assim como melhore esclarecer seu mecanismo de ação *in vitro*. Dessa forma nesse trabalho pretendemos avaliar novos alvos farmacológicos do MBZ na linhagem metastática gástrica AGP-01, por meio da análise de vias metabólicas e do transcriptoma dessa linhagem após o tratamento com MBZ.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar potenciais novos alvos do fármaco Mebendazol para o tratamento do adenocarcinoma gástrico

2.2 Objetivos Específicos

- Avaliar a citotoxicidade e seletividade do MBZ em diferentes linhagens gástricas;
- Avaliar o efeito do MBZ sob o consumo de glicose e atividade da enzima LDH na linhagem metastática AGP-01;
- Avaliar o efeito do MBZ sob o ciclo celular, morte celular e integridade mitocondrial na linhagem metastática AGP-01;
- Comparar a expressão dos genes de vias de síntese de nucleotídeos entre as linhagens MNP-01 e AGP-01;
- Analisar o efeito do tratamento com o MBZ na expressão dos genes de vias metabólicas na linhagem tumoral AGP-01;
- Avaliar por meio de bancos de dados online a expressão dos genes de vias metabólicas em amostras clínicas de adenocarcinoma gástrico;
- Avaliar por meio de bancos de dados online a relação da expressão dos genes de vias metabólicas com a sobrevida de pacientes portadores de adenocarcinoma gástrico;
- Avaliar a interação do fármaco MBZ com as proteínas das vias do metabolismo tumoral;
- Avaliar o efeito do MBZ na regulação dos transcritos (transcriptoma) e na linhagem metastática AGP-01.

REFERÊNCIAS

ABDI, Esmat *et al.* Risk factors predisposing to cardia gastric adenocarcinoma: Insights and new perspectives. **Cancer Medicine**, v. 8, n. 13, p. 6114–6126, 2019.

AIRD, Katherine M.; ZHANG, Rugang. Nucleotide metabolism, oncogene-induced senescence and cancer. **Cancer Letters**, v. 356, n. 2, Part A, Cancer Metabolism, p. 204–210, 2015.

AKHAVAN, Homa *et al.* Revealing novel biomarkers involved in development and progression of gastric cancer by comprehensive bioinformatics analysis. **Informatics in Medicine Unlocked**, v. 25, p. 100630, 2021.

ALLAN, R. J.; WATSON, T. R. The metabolic and pharmacokinetic disposition of mebendazole in the rat. **European Journal of Drug Metabolism and Pharmacokinetics**, v. 8, n. 4, p. 373–381, 1983.

ALLUM, William *et al.* ECCO essential requirements for quality cancer care: Oesophageal and gastric cancer. **Critical Reviews in Oncology/Hematology**, v. 122, p. 179–193, 2018.

AMERICAN CANCER SOCIETY. **Types and Phases of Clinical Trials**. 2020. Disponível em: https://www.cancer.org/cancer/managing-cancer/making-treatment-decisions/clinical-trials/what-you-need-to-know/phases-of-clinical-trials.html. Acesso em: 24 maio 2024.

AMIN, Mahul B. *et al.* The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. **CA: A Cancer Journal for Clinicians**, v. 67, n. 2, p. 93–99, 2017.

ANGULO-ELIZARI, Eduardo *et al.* Unmasking the Warburg Effect: Unleashing the Power of Enzyme Inhibitors for Cancer Therapy. **Drugs and Drug Candidates**, v. 2, n. 3, p. 728–769, 2023.

ARNOLD, Melina *et al.* Global burden of oesophageal and gastric cancer by histology and subsite in 2018. **Gut**, v. 69, n. 9, p. 1564–1571, 2020a.

ARNOLD, Melina *et al.* Is gastric cancer becoming a rare disease? A global assessment of predicted incidence trends to 2035. **Gut**, v. 69, n. 5, p. 823–829, 2020b.

BANG, Yung-Jue *et al.* Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. **Lancet (London, England)**, v. 376, n. 9742, p. 687–697, 2010.

BÁRCENAS-LÓPEZ, Diego Alberto *et al.* Promising genes and variants to reduce chemotherapy adverse effects in acute lymphoblastic leukemia. **Translational Oncology**, v. 14, n. 1, p. 100978, 2021.

BIJLSMA, Maarten F. *et al.* Molecular subtypes in cancers of the gastrointestinal tract. **Nature Reviews Gastroenterology & Hepatology**, v. 14, n. 6, p. 333–342, 2017.

BIORENDER.COM. **BioRender**. Disponível em: https://app.biorender.com/. Acesso em: 9 dez. 2023.

BOSMAN, F. T. *et al.* WHO classification of tumours of the digestive system. **WHO classification of tumours of the digestive system.**, n. Ed. 4, 2010. Disponível em: https://www.cabdirect.org/cabdirect/abstract/20113051318. Acesso em: 13 dez. 2023.

BRAGA, Lucia Libanez Bessa Campelo *et al.* Temporal trends and spatial clusters of gastric cancer mortality in Brazil. **Revista Panamericana de Salud Pública**, v. 46, p. e101, 2022.

CAI, Lei *et al.* Prognostic significance of mucinous component in gastric adenocarcinoma after radical D2 gastrectomy. **OncoTargets and Therapy**, v. 11, p. 967–973, 2018.

CARBONE, Antonino. Cancer Classification at the Crossroads. **Cancers**, v. 12, n. 4, p. 980, 2020.

CARDACI, Simone; DESIDERI, Enrico; CIRIOLO, Maria Rosa. Targeting aerobic glycolysis: 3-bromopyruvate as a promising anticancer drug. **Journal of Bioenergetics and Biomembranes**, v. 44, n. 1, p. 17–29, 2012.

CARNEIRO, Fátima. Classification of gastric carcinomas. **Current Diagnostic Pathology**, v. 4, n. 1, p. 51–59, 1997.

CASCINU, Stefano *et al.* The evolving strategies for the management of patients with metastatic gastric cancer: A narrative review and expert opinion. **Frontiers in Medicine**, v. 9, 2022. Disponível em: https://www.frontiersin.org/articles/10.3389/fmed.2022.1002435. Acesso em: 14 dez. 2023.

CASTAÑO-RODRÍGUEZ, Natalia; KAAKOUSH, Nadeem O.; MITCHELL, Hazel M. Pattern-Recognition Receptors and Gastric Cancer. **Frontiers in Immunology**, v. 5, 2014. Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4105827/. Acesso em: 21 nov. 2019.

CHAN, Denise A. *et al.* Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. **Science Translational Medicine**, v. 3, n. 94, p. 94ra70, 2011.

CHANDEL, Navdeep S. Glycolysis. **Cold Spring Harbor Perspectives in Biology**, v. 13, n. 5, p. a040535, 2021a.

CHANDEL, Navdeep S. Nucleotide Metabolism. **Cold Spring Harbor Perspectives in Biology**, v. 13, n. 7, p. a040592, 2021b.

CHAO, Joseph *et al.* Assessment of Pembrolizumab Therapy for the Treatment of Microsatellite Instability-High Gastric or Gastroesophageal Junction Cancer Among Patients in the KEYNOTE-059, KEYNOTE-061, and KEYNOTE-062 Clinical Trials. **JAMA oncology**, v. 7, n. 6, p. 895–902, 2021.

CHIA, N.-Y.; TAN, P. Molecular classification of gastric cancer. **Annals of Oncology: Official Journal of the European Society for Medical Oncology**, v. 27, n. 5, p. 763–769, 2016.

CISŁO, Magdalena *et al.* Distinct molecular subtypes of gastric cancer: from Laurén to molecular pathology. **Oncotarget**, v. 9, n. 27, p. 19427–19442, 2018.

COLQUHOUN, A. *et al.* Global patterns of cardia and non-cardia gastric cancer incidence in 2012. **Gut**, v. 64, n. 12, p. 1881–1888, 2015.

CRISTESCU, Razvan *et al.* Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. **Nature Medicine**, v. 21, n. 5, p. 449–456, 2015.

CUNNINGHAM DAVID *et al.* Perioperative Chemotherapy versus Surgery Alone for Resectable Gastroesophageal Cancer. **New England Journal of Medicine**, v. 355, n. 1, p. 11–20, 2006.

DA SILVA, Emerson Lucena; MONTENEGRO, Raquel Carvalho; MOREIRA-NUNES, Caroline Aquino. Tumor Metabolism: Challenges and Future Perspectives. *In*: REZAEI, Nima (org.). **Handbook of Cancer and Immunology**. Cham: Springer International Publishing, 2022. p. 1–27. Disponível em: https://doi.org/10.1007/978-3-030-80962-1_103-1. Acesso em: 15 abr. 2023.

DAKSHANAMURTHY, Sivanesan *et al.* Predicting new indications for approved drugs using a proteochemometric method. **Journal of Medicinal Chemistry**, v. 55, n. 15, p. 6832–6848, 2012.

DAYAN, A. D. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. **Acta Tropica**, v. 86, n. 2–3, p. 141–159, 2003.

DE VITA, F. *et al.* Adjuvant chemotherapy with epirubicin, leucovorin, 5-fluorouracil and etoposide regimen in resected gastric cancer patients: a randomized phase III trial by the Gruppo Oncologico Italia Meridionale (GOIM 9602 Study). **Annals of Oncology: Official Journal of the European Society for Medical Oncology**, v. 18, n. 8, p. 1354–1358, 2007.

DENG, Niantao *et al.* A comprehensive survey of genomic alterations in gastric cancer reveals systematic patterns of molecular exclusivity and co-occurrence among distinct therapeutic targets. **Gut**, v. 61, n. 5, p. 673–684, 2012.

FENG, Xing *et al.* UHMK1 promotes gastric cancer progression through reprogramming nucleotide metabolism. **The EMBO Journal**, v. 39, n. 5, p. e102541, 2020.

FUCHS, Charles S. *et al.* Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. **The Lancet**, v. 383, n. 9911, p. 31–39, 2014.

GERTSEN, Emma C. *et al.* 18F-Fludeoxyglucose-Positron Emission Tomography/Computed Tomography and Laparoscopy for Staging of Locally

Advanced Gastric Cancer: A Multicenter Prospective Dutch Cohort Study (PLASTIC). **JAMA surgery**, v. 156, n. 12, p. e215340, 2021.

GUERINI, Andrea Emanuele *et al.* Mebendazole as a Candidate for Drug Repurposing in Oncology: An Extensive Review of Current Literature. **Cancers**, v. 11, n. 9, p. 1284, 2019.

GUIMBAUD, Rosine *et al.* Prospective, randomized, multicenter, phase III study of fluorouracil, leucovorin, and irinotecan versus epirubicin, cisplatin, and capecitabine in advanced gastric adenocarcinoma: a French intergroup (Fédération Francophone de Cancérologie Digestive, Fédération Nationale des Centres de Lutte Contre le Cancer, and Groupe Coopérateur Multidisciplinaire en Oncologie) study. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, v. 32, n. 31, p. 3520–3526, 2014.

GUPTA, Anshu; DWIVEDI, Tanima. A Simplified Overview of World Health Organization Classification Update of Central Nervous System Tumors 2016. **Journal of Neurosciences in Rural Practice**, v. 8, n. 4, p. 629–641, 2017.

HALBROOK, Christopher J.; WAHL, Daniel R.; LYSSIOTIS, Costas A. Running the Light: Nucleotide Metabolism Drives Bypass of Senescence in Cancer. **Trends in Biochemical Sciences**, v. 44, n. 12, p. 991–993, 2019.

HAMADA, Kazu *et al.* Examination of Endoscopic Ultrasonographic Diagnosis for the Depth of Early Gastric Cancer. **Journal of Clinical Medicine Research**, v. 13, n. 4, p. 222–229, 2021.

HANAHAN, Douglas. Hallmarks of Cancer: New Dimensions. **Cancer Discovery**, v. 12, n. 1, p. 31–46, 2022.

HANAHAN, Douglas; WEINBERG, Robert A. Hallmarks of Cancer: The Next Generation. **Cell**, v. 144, n. 5, p. 646–674, 2011.

HANAHAN, D.; WEINBERG, R. A. The hallmarks of cancer. **Cell**, v. 100, n. 1, p. 57–70, 2000.

HASSANPOUR, Seyed Hossein; DEHGHANI, Mohammadamin. Review of cancer from perspective of molecular. **Journal of Cancer Research and Practice**, v. 4, n. 4, p. 127–129, 2017.

HE, Donglin *et al.* Methylenetetrahydrofolate Dehydrogenase 1 (MTHFD1) is Underexpressed in Clear Cell Renal Cell Carcinoma Tissue and Transfection and Overexpression in Caki-1 Cells Inhibits Cell Proliferation and Increases Apoptosis. **Medical Science Monitor : International Medical Journal of Experimental and Clinical Research**, v. 24, p. 8391–8400, 2018.

HU, Bing *et al.* Gastric cancer: Classification, histology and application of molecular pathology. **Journal of Gastrointestinal Oncology**, v. 3, n. 3, p. 251–261, 2012.

HUGHES, Caroline; GREYWOODE, Godman; CHETTY, Runjan. Gastric pseudo-signet ring cells: a potential diagnostic pitfall. **Virchows Archiv**, v. 459, n. 3, p. 347–349, 2011.

INCA. **Estimativa 2023: incidência de câncer no Brasil**. 2023. Disponível em: https://www.inca.gov.br/publicacoes/livros/estimativa-2023-incidencia-de-cancer-no-brasil. Acesso em: 5 set. 2023.

JAKI, Thomas *et al.* Early phase clinical trials in oncology: realising the potential of seamless designs. **European journal of cancer (Oxford, England : 1990)**, v. 189, p. 112916, 2023.

JANJIGIAN, Yelena Y. *et al.* First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. **The Lancet**, v. 398, n. 10294, p. 27–40, 2021.

JOSHI, Smita S.; BADGWELL, Brian D. Current treatment and recent progress in gastric cancer. **CA: A Cancer Journal for Clinicians**, v. 71, n. 3, p. 264–279, 2021.

JUNG, H. Y. *et al.* Methylation of the hMLH1 promoter in multiple gastric carcinomas with microsatellite instability. **Pathology International**, v. 51, n. 6, p. 445–451, 2001.

KANG, Wei-Ming *et al.* Factors associated with early recurrence after curative surgery for gastric cancer. **World Journal of Gastroenterology: WJG**, v. 21, n. 19, p. 5934–5940, 2015.

KEYSTONE, J. S.; MURDOCH, J. K. Mebendazole. **Annals of Internal Medicine**, v. 91, n. 4, p. 582–586, 1979.

KIM, Eun-Yeong *et al.* A Novel Lactate Dehydrogenase Inhibitor, 1-(Phenylseleno)-4-(Trifluoromethyl) Benzene, Suppresses Tumor Growth through Apoptotic Cell Death. **Scientific Reports**, v. 9, n. 1, p. 3969, 2019.

KIM, Soo Yeon *et al.* Aberrantly hypermethylated tumor suppressor genes were identified in oral squamous cell carcinoma (OSCC). **Clinical Epigenetics**, v. 11, n. 1, p. 116, 2019.

KINZLER, K. W.; VOGELSTEIN, B. Cancer-susceptibility genes. Gatekeepers and caretakers. **Nature**, v. 386, n. 6627, p. 761, 763, 1997.

KIRTONIA, Anuradha *et al.* Repurposing of drugs: An attractive pharmacological strategy for cancer therapeutics. **Seminars in Cancer Biology**, v. 68, p. 258–278, 2021.

KOBAYASHI, Jun. Effect of diet and gut environment on the gastrointestinal formation of N-nitroso compounds: A review. **Nitric Oxide: Biology and Chemistry**, v. 73, p. 66–73, 2018.

KOCIANOVA, Eva; PIATRIKOVA, Viktoria; GOLIAS, Tereza. Revisiting the Warburg Effect with Focus on Lactate. **Cancers**, v. 14, n. 24, 2022. Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9776395/. Acesso em: 19 jun. 2024.

KONTOMANOLIS, Emmanuel N. *et al.* Role of Oncogenes and Tumor-suppressor Genes in Carcinogenesis: A Review. **Anticancer Research**, v. 40, n. 11, p. 6009–6015, 2020.

LACLETTE, J. P.; GUERRA, G.; ZETINA, C. Inhibition of tubulin polymerization by mebendazole. **Biochemical and Biophysical Research Communications**, v. 92, n. 2, p. 417–423, 1980.

LAUREN, P. THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. **Acta Pathologica Et Microbiologica Scandinavica**, v. 64, p. 31–49, 1965.

LEE, Derek *et al.* Folate cycle enzyme MTHFD1L confers metabolic advantages in hepatocellular carcinoma. **The Journal of Clinical Investigation**, v. 127, n. 5, p. 1856–1872, 2017.

LI, Ting *et al.* Discovery of novel glyceraldehyde-3-phosphate dehydrogenase inhibitor via docking-based virtual screening. **Bioorganic Chemistry**, v. 96, p. 103620, 2020.

LIU, Tingting *et al.* MYC predetermines the sensitivity of gastrointestinal cancer to antifolate drugs through regulating TYMS transcription. **eBioMedicine**, v. 48, p. 289–300, 2019.

LORDICK, F. *et al.* Gastric cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up☆. **Annals of Oncology**, v. 33, n. 10, p. 1005–1020, 2022.

LUENGO, Alba; GUI, Dan Y.; VANDER HEIDEN, Matthew G. Targeting Metabolism for Cancer Therapy. **Cell chemical biology**, v. 24, n. 9, p. 1161–1180, 2017.

MA, Junli *et al.* Lauren classification and individualized chemotherapy in gastric cancer (Review). **Oncology Letters**, v. 11, n. 5, p. 2959–2964, 2016.

MACDONALD, Louisa M. *et al.* Characterisation of benzimidazole binding with recombinant tubulin from Giardia duodenalis, Encephalitozoon intestinalis, and Cryptosporidium parvum. **Molecular and Biochemical Parasitology**, v. 138, n. 1, p. 89–96, 2004.

MACDONALD, J. S. *et al.* Chemoradiation of resected gastric cancer: A 10-year follow-up of the phase III trial INT0116 (SWOG 9008). **Journal of Clinical Oncology**, v. 27, n. 15_suppl, p. 4515–4515, 2009.

MACDONALD, J. S. *et al.* Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. **The New England Journal of Medicine**, v. 345, n. 10, p. 725–730, 2001.

MAKIYAMA, Akitaka *et al.* Randomized, Phase II Study of Trastuzumab Beyond Progression in Patients With HER2-Positive Advanced Gastric or Gastroesophageal Junction Cancer: WJOG7112G (T-ACT Study). **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, v. 38, n. 17, p. 1919–1927, 2020.

MARTIN, Hans-Jörg; KORNMANN, Frank; FUHRMANN, Günter Fred. The inhibitory effects of flavonoids and antiestrogens on the Glut1 glucose transporter in human erythrocytes. **Chemico-Biological Interactions**, v. 146, n. 3, p. 225–235, 2003.

MASPERO, Marianna *et al.* Impact of Surgical Margins on Overall Survival after Gastrectomy for Gastric Cancer: A Validation of Japanese Gastric Cancer Association Guidelines on a Western Series. **Annals of Surgical Oncology**, v. 29, n. 5, p. 3096–3108, 2022.

MATTIOLI, Roberto *et al.* Doxorubicin and other anthracyclines in cancers: Activity, chemoresistance and its overcoming. **Molecular aspects of medicine**, v. 93, p. 101205, 2023.

MOKADEM, I. *et al.* Recurrence after preoperative chemotherapy and surgery for gastric adenocarcinoma: a multicenter study. **Gastric Cancer**, v. 22, n. 6, p. 1263–1273, 2019.

MORGAN, Eileen *et al.* The current and future incidence and mortality of gastric cancer in 185 countries, 2020–40: A population-based modelling study. **eClinicalMedicine**, v. 47, 2022. Disponível em: https://www.thelancet.com/journals/eclinm/article/PIIS2589-5370(22)00134-1/fulltext. Acesso em: 18 out. 2022.

MORRIS, Luc G. T.; CHAN, Timothy A. Therapeutic targeting of tumor suppressor genes. **Cancer**, v. 121, n. 9, p. 1357–1368, 2015.

MORSCHER, Raphael J. *et al.* Mitochondrial translation requires folate-dependent tRNA methylation. **Nature**, v. 554, n. 7690, p. 128–132, 2018.

MUKHOPADHYAY, Tapas *et al.* Mebendazole Elicits a Potent Antitumor Effect on Human Cancer Cell Lines Both in Vitro and in Vivo. **Clinical Cancer Research**, v. 8, n. 9, p. 2963–2969, 2002.

MULLEN, Nicholas J.; SINGH, Pankaj K. Nucleotide metabolism: a pan-cancer metabolic dependency. **Nature Reviews Cancer**, v. 23, n. 5, p. 275–294, 2023.

OLSEN, Thale Kristin *et al.* DHODH is an independent prognostic marker and potent therapeutic target in neuroblastoma. **JCI Insight**, v. 7, n. 17, p. e153836, 2022.

ONO, Hiroyuki *et al.* Guidelines for endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer (second edition). **Digestive Endoscopy: Official Journal of the Japan Gastroenterological Endoscopy Society**, v. 33, n. 1, p. 4–20, 2021.

PACCHIANA, Raffaella *et al.* 3-Bromo-Isoxazoline Derivatives Inhibit GAPDH Enzyme in PDAC Cells Triggering Autophagy and Apoptotic Cell Death. **Cancers**, v. 14, n. 13, p. 3153, 2022.

PEDLEY, Anthony M.; BENKOVIC, Stephen J. A New View into the Regulation of Purine Metabolism – The Purinosome. **Trends in biochemical sciences**, v. 42, n. 2, p. 141–154, 2017.

PETERSEN, Jakeb S. S. M.; BAIRD, Sarah K. Treatment of breast and colon cancer cell lines with anti-helmintic benzimidazoles mebendazole or albendazole results in selective apoptotic cell death. **Journal of Cancer Research and Clinical Oncology**, v. 147, n. 10, p. 2945–2953, 2021.

PIMENTEL-NUNES, Pedro *et al.* Endoscopic submucosal dissection: European Society of Gastrointestinal Endoscopy (ESGE) Guideline. **Endoscopy**, v. 47, n. 9, p. 829–854, 2015.

PINTO, Laine Celestino *et al.* Mebendazole, an antiparasitic drug, inhibits drug transporters expression in preclinical model of gastric peritoneal carcinomatosis. **Toxicology in vitro: an international journal published in association with BIBRA**, v. 43, p. 87–91, 2017.

PINTO, Laine Celestino *et al.* Mebendazole induces apoptosis via C-MYC inactivation in malignant ascites cell line (AGP01). **Toxicology in Vitro**, v. 60, p. 305–312, 2019.

PINTO, Laine Celestino *et al.* The anthelmintic drug mebendazole inhibits growth, migration and invasion in gastric cancer cell model. **Toxicology in vitro: an international journal published in association with BIBRA**, v. 29, n. 8, p. 2038–2044, 2015.

QIAO, Tianyun *et al.* Inhibition of LDH-A by Oxamate Enhances the Efficacy of Anti-PD-1 Treatment in an NSCLC Humanized Mouse Model. **Frontiers in Oncology**, v. 11, 2021. Disponível em: https://www.frontiersin.org/articles/10.3389/fonc.2021.632364. Acesso em: 6 nov. 2022.

QUAN, Yingjun *et al.* Comparison of laparoscopic versus open gastrectomy for advanced gastric cancer: an updated meta-analysis. **Gastric Cancer: Official Journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association**, v. 19, n. 3, p. 939–950, 2016.

RAMOS, Marcus Fernando Kodama Pertille *et al.* QUIMIOTERAPIA INTRAPERITONEAL PARA O CÂNCER GÁSTRICO COM CARCINOMATOSE PERITONEAL: PROTOCOLO DE ESTUDO DE UM ENSAIO DE FASE II. **ABCD. Arquivos Brasileiros de Cirurgia Digestiva (São Paulo)**, v. 36, p. e1744, 2023.

ROBINSON, Alyncia D.; EICH, Marie-Lisa; VARAMBALLY, Sooryanarayana. Dysregulation of de novo nucleotide biosynthetic pathway enzymes in cancer and targeting opportunities. **Cancer Letters**, v. 470, p. 134–140, 2020.

RUSTGI, Sheila D.; CHING, Charlotte K.; KASTRINOS, Fay. Inherited Predisposition to Gastric Cancer. **Gastrointestinal Endoscopy Clinics**, v. 31, n. 3, p. 467–487, 2021.

SAKURAMOTO, Shinichi *et al.* Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. **The New England Journal of Medicine**, v. 357, n. 18, p. 1810–1820, 2007.

SAMERI, Saba *et al.* Targeting the hallmarks of cancer: the effects of silibinin on proliferation, cell death, angiogenesis, and migration in colorectal cancer. **BMC Complementary Medicine and Therapies**, v. 21, n. 1, p. 160, 2021.

SARRIUGARTE LASARTE, Aingeru *et al.* From Lauren's diffuse gastric cancer to WHO's poorly cohesive carcinoma. Clinicopathological and prognostic characteristics. **Revista Espanola De Enfermedades Digestivas**, v. 113, n. 5, p. 324–331, 2021.

SASAKI, Ji-ichiro *et al.* The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells. **Molecular Cancer Therapeutics**, v. 1, n. 13, p. 1201–1209, 2002.

SATO, Yasushi *et al.* Novel Biomarkers of Gastric Cancer: Current Research and Future Perspectives. **Journal of Clinical Medicine**, v. 12, n. 14, p. 4646, 2023.

SCHILIRO, Chelsea; FIRESTEIN, Bonnie L. Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation. **Cells**, v. 10, n. 5, p. 1056, 2021.

SEENEEVASSEN, Lornella *et al.* Gastric Cancer: Advances in Carcinogenesis Research and New Therapeutic Strategies. **International Journal of Molecular Sciences**, v. 22, n. 7, p. 3418, 2021.

SHIBATA, Sachio *et al.* Identification of the first highly selective inhibitor of human lactate dehydrogenase B. **Scientific Reports**, v. 11, n. 1, p. 21353, 2021.

SHIRATORI, Yasutoshi *et al.* Rapidly Growing Papillary Well-Differentiated Adenocarcinoma in a Patient With a Helicobacter pylori-Naive Gastric Mucosa. **ACG Case Reports Journal**, v. 7, n. 8, p. e00444, 2020.

SHITARA, Kohei *et al.* Efficacy and Safety of Pembrolizumab or Pembrolizumab Plus Chemotherapy vs Chemotherapy Alone for Patients With First-line, Advanced Gastric Cancer: The KEYNOTE-062 Phase 3 Randomized Clinical Trial. **JAMA oncology**, v. 6, n. 10, p. 1571–1580, 2020.

SITARZ, Robert *et al.* Gastric cancer: epidemiology, prevention, classification, and treatment. **Cancer Management and Research**, v. 10, p. 239–248, 2018.

SLEIRE, Linda *et al.* Drug repurposing in cancer. **Pharmacological Research**, v. 124, p. 74–91, 2017.

SMYTH, Elizabeth C. *et al.* Gastric cancer. **Lancet (London, England)**, v. 396, n. 10251, p. 635–648, 2020.

SONG, Bomi *et al.* Repurposing of Benzimidazole Anthelmintic Drugs as Cancer Therapeutics. **Cancers**, v. 14, n. 19, p. 4601, 2022.

STASEVICH, Ekaterina Mikhailovna *et al.* The Role of Non-Coding RNAs in the Regulation of the Proto-Oncogene MYC in Different Types of Cancer. **Biomedicines**, v. 9, n. 8, p. 921, 2021.

SUN, Duxin *et al.* Why 90% of clinical drug development fails and how to improve it?. **Acta Pharmaceutica Sinica. B**, v. 12, n. 7, p. 3049–3062, 2022.

SUNG, Hyuna *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. **CA: a cancer journal for clinicians**, 2021.

TAN, Jia-nan *et al.* Long noncoding RNA OVAAL enhances nucleotide synthesis through pyruvate carboxylase to promote 5-fluorouracil resistance in gastric cancer. **Cancer Science**, v. 113, n. 9, p. 3055–3070, 2022.

TAN, Zhaoyang. Recent Advances in the Surgical Treatment of Advanced Gastric Cancer: A Review. **Medical Science Monitor : International Medical Journal of Experimental and Clinical Research**, v. 25, p. 3537–3541, 2019.

TCGA RESEARCH NETWORK. Comprehensive molecular characterization of gastric adenocarcinoma. **Nature**, v. 513, n. 7517, p. 202–209, 2014.

THAKUR, Rahul K.; PATEL, Shivani P. Mebendazole. *In*: STATPEARLS. Treasure Island (FL): StatPearls Publishing, 2024. Disponível em: http://www.ncbi.nlm.nih.gov/books/NBK557705/. Acesso em: 20 ago. 2024.

TOMASETTI, Cristian; LI, Lu; VOGELSTEIN, Bert. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. **Science (New York, N.Y.)**, v. 355, n. 6331, p. 1330–1334, 2017.

TOMINAGA, Naoyuki *et al.* Five biopsy specimens from the proximal part of the tumor reliably determine HER2 protein expression status in gastric cancer. **Gastric Cancer: Official Journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association**, v. 19, n. 2, p. 553–560, 2016.

TORRES, Rosa J.; PUIG, Juan G. Hypoxanthine-guanine phosophoribosyltransferase (HPRT) deficiency: Lesch-Nyhan syndrome. **Orphanet Journal of Rare Diseases**, v. 2, n. 1, p. 48, 2007.

TRINH, Thao Thi Kim *et al.* Cluster of lifestyle risk factors for stomach cancer and screening behaviors among Korean adults. **Scientific Reports**, v. 13, n. 1, p. 17503, 2023.

VAN CUTSEM, Eric *et al.* Gastric cancer. **Lancet (London, England)**, v. 388, n. 10060, p. 2654–2664, 2016.

VANDER HEIDEN, Matthew G.; DEBERARDINIS, Ralph J. Understanding the Intersections between Metabolism and Cancer Biology. **Cell**, v. 168, n. 4, p. 657–669, 2017.

VOGELSTEIN, Bert *et al.* Cancer genome landscapes. **Science (New York, N.Y.)**, v. 339, n. 6127, p. 1546–1558, 2013.

WALTER, Melanie; HERR, Patrick. Re-Discovery of Pyrimidine Salvage as Target in Cancer Therapy. **Cells**, v. 11, n. 4, p. 739, 2022.

WANG, Liang-Jun *et al.* Albendazole-Induced SIRT3 Upregulation Protects Human Leukemia K562 Cells from the Cytotoxicity of MCL1 Suppression. **International Journal of Molecular Sciences**, v. 21, n. 11, 2020. Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7312678/. Acesso em: 13 jan. 2021.

WANG, Li-Hui *et al.* Loss of Tumor Suppressor Gene Function in Human Cancer: An Overview. **Cellular Physiology and Biochemistry**, v. 51, n. 6, p. 2647–2693, 2018.

WANG, Qianqian; LIU, Ganglei; HU, Chunhong. Molecular Classification of Gastric Adenocarcinoma. **Gastroenterology Research**, v. 12, n. 6, p. 275–282, 2019.

WARBURG, O. On the origin of cancer cells. **Science (New York, N.Y.)**, v. 123, n. 3191, p. 309–314, 1956.

WARBURG, Otto; DICKENS, F. The metabolism of tumours. Investigations from the Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Edited by Otto Warburg, Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Translated from the German edition, with accounts of additional recent researches, by Frank Dickens, M.A., Ph.D., whole-time worker for the Medical Research Council, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Demy 8vo. Pp. 327 + xxix. Illustrated. 1930. London: Constable & Co. Ltd. 40s. net. **BJS (British Journal of Surgery)**, v. 19, n. 73, p. 168–168, 1931.

WARBURG, Otto; WIND, Franz; NEGELEIN, Erwin. THE METABOLISM OF TUMORS IN THE BODY. **The Journal of General Physiology**, v. 8, n. 6, p. 519–530, 1927.

WILSON, J. F.; RAUSCH, R. L. Mebendazole and alveolar hydatid disease. **Annals of Tropical Medicine and Parasitology**, v. 76, n. 2, p. 165–173, 1982.

WU, Tong *et al.* HPRT1 Promotes Chemoresistance in Oral Squamous Cell Carcinoma via Activating MMP1/PI3K/Akt Signaling Pathway. **Cancers**, v. 14, n. 4, p. 855, 2022.

WU, Zhengxin *et al.* Identification of crucial genes of pyrimidine metabolism as biomarkers for gastric cancer prognosis. **Cancer Cell International**, v. 21, p. 668, 2021.

WU, Huai-liang *et al.* Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy. **Journal of Hematology & Oncology**, v. 15, n. 1, p. 45, 2022.

XIA, Ying *et al.* Drug repurposing for cancer therapy. **Signal Transduction and Targeted Therapy**, v. 9, n. 1, p. 1–33, 2024.

XINTAROPOULOU, Chrysi *et al.* Expression of glycolytic enzymes in ovarian cancers and evaluation of the glycolytic pathway as a strategy for ovarian cancer treatment. **BMC cancer**, v. 18, n. 1, p. 636, 2018.

XIONG, Guohang *et al.* NRF2-directed PRPS1 upregulation to promote the progression and metastasis of melanoma. **Frontiers in Immunology**, v. 13, p. 989263, 2022.

YANG, Jianqiang *et al.* Cancer metabolism and carcinogenesis. **Experimental Hematology & Oncology**, v. 13, n. 1, p. 10, 2024.

YANG, Wei *et al.* Preclinical pharmacodynamic evaluation of a new Src/FOSL1 inhibitor, LY-1816, in pancreatic ductal adenocarcinoma. **Cancer Science**, v. 110, n. 4, p. 1408–1419, 2019.

YCHOU, Marc *et al.* Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase

III trial. **Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology**, v. 29, n. 13, p. 1715–1721, 2011.

YE, Zeyao *et al.* Prediction of Peritoneal Cancer Index and Prognosis in Peritoneal Metastasis of Gastric Cancer Using NLR-PLR-DDI Score: A Retrospective Study. **Cancer Management and Research**, v. 14, p. 177–187, 2022.

YUAN, Li; XIAO, Zhiqiang; LU, Ruohuang. Hypoxanthine guanine phosphoribosyltransferase 1, a target of miR-125b-5p, promotes cell proliferation and invasion in head and neck squamous cell carcinoma. **Heliyon**, v. 9, n. 9, p. e20174, 2023.

ZHANG, Wenjing *et al.* Inhibition of LDHA suppresses cell proliferation and increases mitochondrial apoptosis via the JNK signaling pathway in cervical cancer cells. **Oncology Reports**, v. 47, n. 4, p. 1–11, 2022.

ZHANG, Guilian *et al.* Targeting tumor glycolysis metabolism in oral squamous cell carcinoma cells by brusatol. **Pharmacological Research - Modern Chinese Medicine**, v. 5, p. 100172, 2022.

ZHAO, Weizhu *et al.* Single-cell analysis of gastric signet ring cell carcinoma reveals cytological and immune microenvironment features. **Nature Communications**, v. 14, n. 1, p. 2985, 2023.

ZHENG, Mengzhu *et al.* Novel selective hexokinase 2 inhibitor Benitrobenrazide blocks cancer cells growth by targeting glycolysis. **Pharmacological Research**, v. 164, p. 105367, 2021.

ZHOU, Yue *et al.* DHODH and cancer: promising prospects to be explored. **Cancer & Metabolism**, v. 9, n. 1, p. 22, 2021.

CAPÍTULO II

MEBENDAZOLE TARGETS ESSENTIAL PROTEINS IN GLUCOSE METABOLISM LEADING GASTRIC CANCER CELLS TO DEATH

EMERSON LUCENA DA SILVA

Toxicology and Applied Pharmacology

Volume 475, 15 September 2023, 116630

MEBENDAZOLE TARGETS ESSENTIAL PROTEINS IN GLUCOSE METABOLISM LEADING GASTRIC CANCER CELLS TO DEATH

Emerson Lucena da Silva[a], Felipe Pantoja Mesquita[a], Dyane Rocha Aragão[a], Adrhyann Jullyanne de Sousa Portilho[a], Aline Diogo Marinho[a], Lais Lacerda Brasil de Oliveira[a], Luina Benevides Lima[a], Maria Elisabete Amaral de Moraes[a]; Pedro Filho Noronha Souza[a,b]; Raquel Carvalho Montenegro[a]*

[a]*Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Cel. Nunes de Melo, 1000 – Rodolfo Teófilo. Fortaleza, Brazil.*
[b]*Department of Biochemistry and Molecular Biology, Federal University of Ceará. Mister Hull Avenue-Pici. Fortaleza, Brazil*

*Address correspondence to this author: Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Street Cel. Nunes de Melo, 1000 – Rodolfo Teófilo, Fortaleza, Ceará, Brazil.
E-mail: **rmontenegro@ufc.br**

Artigo publicado na revista internacional *Toxicology and Applied Pharmacology*
(Qualis CAPES Ciências Biológicas II: A2)
DOI: 10.1016/j.taap.2023.116630

ABSTRACT

Gastric cancer (GC) is among the most-diagnosed and deadly malignancies worldwide. Deregulation in cellular bioenergetics is a hallmark of cancer. Based on the importance of metabolic reprogramming for the development and cancer progression, inhibitors of cell metabolism have been studied as potential candidates for chemotherapy in oncology. Mebendazole (MBZ), an antihelminthic approved by FDA, has shown antitumoral activity against cancer cell lines. However, its potential in the modulation of tumoral metabolism remains unclear. Results evidenced that the antitumoral and cytotoxic mechanism of MBZ in GC cells is related to the modulation of the mRNA expression of glycolic targets *SLC2A1*, *HK1*, *GAPDH*, and *LDHA*. Moreover, *in silico* analysis has shown that these genes are overexpressed in GC samples, and this increase in expression is related to decreased overall survival rates. Molecular docking revealed that MBZ modifies the protein structure of these targets, which may lead to changes in their protein function. *In vitro* studies also showed that MBZ induces alterations in glucose uptake, LDH's enzymatic activity, and ATP production. Furthermore, MBZ induced morphologic and intracellular alterations typical of the apoptotic cell death pathway. Thus, this data indicated that the cytotoxic mechanism of MBZ is related to an initial modulation of the tumoral metabolism in the GC cell line. Altogether, our results provide more evidence about the antitumoral mechanism of action of MBZ towards GC cells and reveal metabolic reprogramming as a potential area in the discovery of new pharmacological targets for GC chemotherapy.

Keywords: Antitumoral; Drug repurposing; Glycolytic pathway; Metabolic reprogramming; Molecular docking; Pharmacologic targets.

1 INTRODUCTION

Deregulation in cellular bioenergetics was first explained by Otto Warburg being a hallmark of cancer development (Hanahan and Weinberg, 2011; Warburg, 1956; Warburg et al., 1927; Warburg and Dickens, 1931). Glucose metabolism and the glycolytic pathway play a central role in the energy generation for cancer development and supply biochemical precursors supporting the synthesis of DNA, RNA, protein, and lipids for cellular replication (Schiliro and Firestein, 2021). Glycolytic inhibitors like STF-31, 3-bromopyruvate (3-BP), Benitrobenrazide (BNBZ), DC-5163, and Oxamic acid have been widely studied as pharmacological tools in cancer therapeutics, targeting glucose transporters (GLUT) (Chan et al., 2011; Martin et al., 2003), hexokinases (HK) (Cardaci et al., 2012; Zheng et al., 2021), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Li et al., 2020; Pacchiana et al., 2022), lactate dehydrogenases (LDH) (Kim et al., 2019; Qiao et al., 2021; Shibata et al., 2021; Zhang et al., 2022).

Drug development has relied on standardized nonclinical and clinical steps that can take up to 15 years and billions of dollars. Repurposing or repositioning emerges as a strategy to identify new applications for approved drugs outside the scope of their original clinical indication with a promise of reducing the drug development process's risk, cost, and timeline (World Health Organization, 2021). Many drugs are already commonly used off-label; however, there are few in standard clinical practice outside cancer treatment being moved into oncology (Pantziarka et al., 2018; World Health Organization, 2021).

Mebendazole (MBZ), a benzimidazole derivative, is widely known for its antihelminthic potential with action in inhibiting tubulin polymerization and glucose uptake in helminths (Keystone and Murdoch, 1979; Laclette et al., 1980). MBZ is a safe cheap, and widely used drug in developing and low incoming countries (Guerini et al., 2019; Karra et al., 2016; Thakur and Patel, 2022). Recently, our group has investigated the potential of MBZ and identified its mechanism of action linked to the disruption of microtubes, the mitigation of metastatic behavior, induction of DNA damage, modulation in gene expression, and apoptosis (Pinto et al., 2019, 2017, 2015). Moreover, several studies have reported the antitumoral potential of MBZ in different types of cancer, such as lung (Sasaki et al., 2002), colon (Nygren et al., 2013; Williamson et al., 2016), brain (Bai et al., 2015; Bodhinayake et al., 2015; De Witt et al., 2017; Gallia et al., 2021; Patil et al., 2022; Skibinski et al., 2018), skin (Doudican et al., 2013), hepatocarcinoma (Q. Li et al., 2022), head and neck (Kralova et al., 2018; Zhang et al., 2017), thyroid (Williamson et al.,

2020), pancreas (Williamson et al., 2021), and leukemia (Daniel et al., 2022; Freisleben et al., 2019; Li et al., 2019), making it a potential candidate to a repurposing drug into oncology practice.

Lately, cancer care expenses have increased due to rising numbers of patients diagnosed, drug failure, and new treatments involving costly anticancer drugs such as target therapies. Despite the high expenditure of money and new technologies, many types of cancer still lack efficient treatment options and are still associated with unfavorable outcomes across different countries (World Health Organization, 2021). Gastric cancer (GC) is one of these tumors associated with poor prognosis where early disease stage rarely causes symptoms. GC contributes to global cancer incidence and mortality, with more than 1 million new cases estimated annually, the fifth most diagnosed malignancy globally. Due to its often advanced stage at diagnosis, mortality from gastric cancer is high, making it the fourth most common cause of cancer-related deaths worldwide (Machlowska et al., 2020; Sung et al., 2021a). Despite combined treatment, GC still has a poor prognosis, with 5-year overall survival in less than 30% of cases (Joshi and Badgwell, 2021; Song et al., 2017). In addition, metastatic GC has long been considered less responsive to surgical treatment and more resistant to chemotherapy (Joshi and Badgwell, 2021; Y. Li et al., 2022).

Based on the importance of metabolic reprogramming for the development and progression of cancer and the vast arsenal of existing approved drugs with established safety profiles, inhibitors of cell metabolism may be an attractive strategy to offer a more effective treatment option to patients with gastric cancer (Falzone et al., 2018). To the best of our knowledge, the metabolic reprogramming profile of this drug is not yet known. Therefore, this study aimed to provide new insights into the action of MBZ on the tumor metabolism of GC cell lines and sought to find new metabolic targets of clinical interest in the therapy of GC.

2 METHODS

2.1 Chemicals

Mebendazole (MBZ) (Medley®, 500 mg) and Rotenone (ROT) (Merck®) were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM and stored at -20 °C until use. ROT is a mitochondrial complex I inhibitor, leading to metabolic alterations and modulation in cellular bioenergetics (Karlsson et al., 2016). Therefore, this compound

was chosen as the positive control for the metabolic modulation of cancer cells during experiments.

2.2 Cell culture and conditions

Gastric adenocarcinoma tumor cell lines AGP-01, ACP-03, and ACP-02 were established from malignant ascitic fluid (AGP-01) of metastatic primary intestinal-type (ACP-03), and primary diffuse-type (ACP-02) tumor (Leal et al., 2009). Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco ®) with Low Glucose (LG, 5.5 mM) based on previous studies to evaluate metabolic parameters of GC cell lines (da Silva et al., 2022). Media were supplemented with 10% (v/v) fetal bovine serum (Gibco®), 1% (v/v) penicillin (100 U mL^{-1}), and streptomycin (100 mg mL^{-1}) (Invitrogen®), and cell lines were maintained in a 5% CO_2 air-humidified atmosphere at 37 °C.

2.3 Cytotoxicity by Alamar Blue Assay

Alamar Blue assay (Rampersad, 2012) was employed to define the half-maximal inhibitory concentration (IC_{50}) of MBZ to measure the potency of MBZ in inhibiting cell proliferation. AGP-01, ACP-02, and ACP-03 cells were seeded in a 96-well plate with 3 x 10^3 cells per well in DMEM Low Glucose (LG) conditions. After attachment, cells were treated in a concentration-response curve (20 µM – 0.3125 µM) of MBZ for 72 hours. Then, the Alamar Blue solution (0.2 mg mL^{-1}) (Merck®) was added to each well for 3 hours, and the fluorescence intensity (Ex/Em: 530/590 nm) was measured in a microplate reader (Beckman Coulter Microplate Reader DTX 880) and IC_{50} calculated.

2.4 Cell viability by Trypan Blue

The Trypan Blue exclusion assay was performed to determine the non-cytotoxic concentration of MBZ for metabolic studies (Strober, 2015). The AGP-01 cell line was plated ($7x10^4$ cells/well) in a 24-well plate. After 24 hours, cells were treated with MBZ (0.1 µM) and ROT (1 µM) for 24 hours, then cells were treated with 0.025% (m/v) trypsin to release cells of the bottom of wells and collected, and all content was centrifugated to obtain the cell pellet. In sequence, cells were stained with Trypan Blue solution (0.4%), and the cell viability was estimated in the Neubauer chamber by counting the viable and non-viable cells.

2.5 Cell cycle analysis

Possible alterations in cell cycle progression induced by MBZ in a non-cytotoxic concentration were evaluated. AGP-01 cells were seeded in ($7x10^4$ cells/well) a 24-well plate and, after attachment, treated with MBZ (0.1 µM) or ROT (1 µM) for 24 hours. Then cells and the supernatant were centrifugated, and the pellet was fixed in 80% ethanol solution at 4 ºC overnight. After, the cells were centrifugated again, and the pellet was resuspended in a solution with propidium iodide (50 µg mL^{-1}) for 30 minutes at 37 ºC. Cells were resuspended in PBS 1X, and DNA content was evaluated using flow cytometry (BD FACSVerse™). Ten thousand events were analyzed using FlowJo® software (Mesquita et al., 2018).

2.6 Glucose uptake

To determine the glucose consumption rate alterations, AGP-01 cells were seeded in a 24-well plate ($7x10^4$ cells/well). After cell attachment, the AGP-01 cell line was treated with MBZ (0.1 µM) or ROT (1 µM) for 24 hours. Next, the supernatant was collected, centrifuged, and glucose concentration (mg/dL) was determined by enzymatic-colorimetric assay (Labtest®, Brazil) following the manufacturer's manual. The absorbance was estimated at 450 nm using Beckman Coulter Microplate Reader DTX 880 (da Silva et al., 2022), and glucose uptake was determined.

2.7 Lactate dehydrogenase (LDH) activity

To evaluate alterations in the lactate dehydrogenase (LDH) enzymatic activity, AGP-01cells were plated at $7x10^4$ cells/well in a 24-well plate. After 24 hours, cells were treated with MBZ (0.1 µM) or ROT (1 µM) 24 hours, and all cellular content was collected and centrifuged. Pellet was resuspended in 100 µL of Triton X-100 (1x) for 30 min at 37 °C to disrupt the cellular membrane and enzyme release. Further, the LDH activity (U/L) was estimated using a commercial biochemical test (Labtest®, Brazil), following the manufacturer's manual. The enzymatic activity was determined by measuring the absorbance at 340 using Beckman Coulter Microplate Reader DTX 880 (da Silva et al., 2022).

2.8 Quantification of intracelular ATP levels

We sought to investigate if MBZ modulates ATP levels in gastric cell lines, so the AGP-01 cell line was seeded ($7x10^4$ cells/well) in a 24-well-plate and, after cell attachment, was treated with MBZ (0.1 µM) or ROT (1 µM) for 24 hours. After exposure, cells were trypsinized, and the cellular pellet was resuspended in 200 µL of lysis buffer (2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100, 25 mM Tris-HCl, pH 7.8) for 15 min (Xie et al., 2019). After centrifugation, the supernatant was collected and the ATP concentration was determined using the ATP Determination Kit (Invitrogen™) according to the manufacturer's protocol. The luminescence intensity (Maximum emission of 560 nm) was measured in the Cytation 3 Cell Imaging Reader (BioTek®), and ATP concentration was determined.

2.9 Morphological analysis

Initial modulation of metabolism can lead to modification in cellular phenotype and morphology (Sobiepanek et al., 2021). As such, the AGP-01 cell line was seeded at 4 x 10^4 cells/well in a 24-well plate and treated with MBZ (0.1 µM) or ROT (1 µM) for 24, 48, and 72 hours. After exposure, the supernatant was removed, wells were washed twice with PBS 1X, and cells were stained with a Fast Panoptic kit (LaborClin®, Brazil) following the manufacturer's manual. Then, cell morphology was analyzed, classified and photographed by optical microscopy at 10X magnification (Nikon® Eclipse Ni/DSRi2) (da Silva et al., 2022).

2.10 Caspase 3/7 activity

To visualize activation of the apoptotic inducers, caspase 3 and 7, the CellEvent® Caspase-3/7 kit (Thermo Fisher Scientific) was used (Mesquita et al., 2020). The AGP-01 cell line was seeded at a concentration of $4x10^4$ cells/well in a 24-well plate, after cell attachment, cells were treated for different times (24, 48, and 72 hours) with MBZ (0.1 µM) or ROT (1 µM). Next, the supernatant was removed and stored, cells were detached and all cell content was centrifuged. In sequence, the cellular pellet was resuspended in the staining solution (5 µM of CellEvent® Caspase-3/7 reagent detection into PSB with 5% serum) for 30 min at 37 °C following the manufacturer's protocol. Finally, activation of Caspase 3/7 was analyzed by flow cytometry (BD FACSVerseTM), the sum of 10,00

events was analyzed. The evaluation of Caspase 3 and 7 activations proceeded in FlowJo® software (da Silva et al., 2022).

2.11 Analysis of cellular and mitochondrial membrane by flow cytometry

In sequence, to analyze modifications in cellular membrane integrity and the mitochondrial polarization induced by MBZ treatment over time, it was used dual staining with propidium iodide (PI) and rhodamine 123 (Rh123), respectively (da Silva et al., 2022; Zou et al., 2010). Briefly, AGP-01 cells were plated in a 24-well plate ($4x10^4$ cells/well) and treated with MBZ (0.1 µM) or ROT (1 µM) for 24, 48, and 72 hours. Then, cells were detached, centrifuged, and the pellet was resuspended in 1X PBS solution with PI/Rh123 (5 µg mL^{-1} each) for 25 min at 37 °C. To further analysis, cells were centrifuged, and the pellet was resuspended in PBS 1x and analyzed by flow cytometry (BD FACSVerse™). A sum of 10,000 events was evaluated and cell populations were determined by FlowJo® software (Lugli et al., 2010).

2.12 Total RNA extraction and transcript analysis by qRT-PCR

In previous studies, the metabolic targets *SLC2A1* (GLUT1), *HK1*, *GAPDH*, and *LDHA* showed significant modification in gene expression correlated with enhancement of the proliferative profile in the AGP-01 cell line (da Silva et al., 2022). So, we sought to investigate the influence of MBZ in their modulation. First, the *in vitro* mRNA expression of metabolic genes was analyzed in cancer cells after treatment with MBZ. AGP-01 cells were seeded (7×10^4 cells per well) for 24 hours and then treated with MBZ (0.1 µM) or ROT (1 µM) for 24 hours. Afterward, the total RNA was extracted using TRIzol® Reagent (Life Technologies®, USA). The RNA concentration and quality were determined using NanoDrop (Thermo Scientific). Further, reverse transcription was performed using a High-Capacity cDNA kit, according to the manufacturer's protocol (Life Technologies, USA).

Quantitative real-time PCR (qRT-PCR) was executed by the Fast SyberGreen kit (Applied Biosystems, USA). The Relative expression levels of *SLC2A1* (NM_006516.2), *HK1* (NM_001322364.1), *GAPDH* (NM_002046.6), and *LDHA* (NM_005566.3) were normalized and determined using *RPLP0* (NM_001002.4) gene as an endogenous control. Primer efficiency > 95% was determined for all genes described.

All requirements proposed in Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed (Bustin et al.,

2009). The expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), considering non-treated cells (Negative Control) as a calibrator of the experiments.

2.13 Gene expression analysis of metabolic targets through online databases

The expression of metabolic genes evaluated previously in the AGP-01 cell line was correlated with disease outcome by an *in silico* global gene expression analysis in databases with free access to transcriptome data from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and Genotype-Tissue Expression (GTEx). Expression patterns in tumor samples and the correlation of gene expression with the survival rate of *SLC2A1*, *HK1*, *GAPDH*, and *LDHA* genes were assessed by GEPIA (http://gepia.cancer-pku.cn/) and Kaplan Meier plotter (https://kmplot.com/analysis/) software, respectively (Lánczky and Győrffy, 2021; Tang et al., 2017).

2.14 Proteins and mebendazole molecules preparation for docking analysis

Molecular docking was performed to observe glycolic constituents' protein interaction with MBZ. The PDB (Protein Data Bank – https://www.rcsb.org/) was used to download the "pdb." files of three-dimensional structures (3D) for GLUT1 (PDB ID: 6THA), HK1 (PDB ID: 1CZA), GAPDH (PDB ID: 6YND), and LDHA (PDB ID: 5W8J) proteins. As a criterion for choosing the best structures, it was looked at those with an X-ray resolution lower than 2 Angstroms (Å). The two-dimensional (2D) structure of mebendazole was designed using MarvinSketch® software to obtain the SMILE code (Simplified Molecular Input Line Entry Specification). Then, the structure was converted into a three-dimensional (3D) skeleton using the Avogadro® program and geometrically optimized.

The proteins and MBZ structures were processed using PyMol software to remove ligands and water molecules. Then the protonation state of the amino acid residues of all molecules tested in simulations was adjusted at pH 7.4 using the PDB2PQR-PropKa server (https://server.poissonboltzmann.org/).

2.15 Molecular docking analysis

The molecular docking was performed in the DockThor server (https://dockthor.lncc.br/v2/), which uses flexible ligands and rigid receptors as a

methodology, using a genetic algorithm and the MMFF94S molecular force field to predict the score of each pose (de Magalhães et al., 2014; Halgren, 1996). Supplementary Table 1 summarizes the coordinates and dimensions of the grid box, automatically defined by the server itself.

Four dockings were performed for each enzyme. 1) only MBZ was used as a ligand. 2) the enzymes and their native ligands. 3) the native ligands and the drug were used concomitantly. 4) the protein already anchored with the MBZ drug was used, and the native ligands were added. The poses of MBZ were analyzed e compared with native ligands of each enzyme to evaluate the correct pose of interaction.

2.16 Statistical analyses

All assays were performed in three independent experiments, in triplicates, and the results are expressed as mean ± standard deviation (SD). Normality distribution analysis was performed by the Kolmogorov- Smirnov test. The negative control (NC) group was compared with mebendazole and the positive control rotenone by Analysis of Variance (ANOVA) followed by Bonferroni's post-test. Significant differences were considered with a confidence interval of 95% ($p<0.05$). GraphPad Prism 5.01 software was used for data analysis and graph design.

3 RESULTS

3.1 MBZ presents antitumoral effect in Gastric Cancer cell lines in low glucose medium

Firstly, we sought to assess the cytotoxic concentration of MBZ against gastric cancer cell lines after 72 hours of treatment. The cytotoxic potential of MBZ was determined in three gastric adenocarcinoma cell lines (AGP-01, ACP-02, and ACP-03) cultivated in the Low Glucose (5.5 mM) medium (Table 1). The present study showed that the growth of all cell lines was affected by MBZ after 72 hours of treatment. AGP-01, ACP-03, and ACP-02 cell lines presented IC_{50} values of 0.11, 0.21, and 0.24, respectively. The AGP-01 cell line showed the best results among tumoral cells with an IC_{50} of 0.11 µM, being chosen to continue the evaluation of the effect of MBZ on tumor metabolism modulation.

Table 1. *In vitro* cytotoxic activity of MBZ. Cells were cultured in Low Glucose (LG) media and treated with a concentration-response curve of MBZ for 72 hours, then Alamar Blue was added, and the half-maximal inhibitory concentration (IC_{50}) was estimated. The IC_{50} values (μM) and confidence interval of 95% were obtained from three independent experiments.

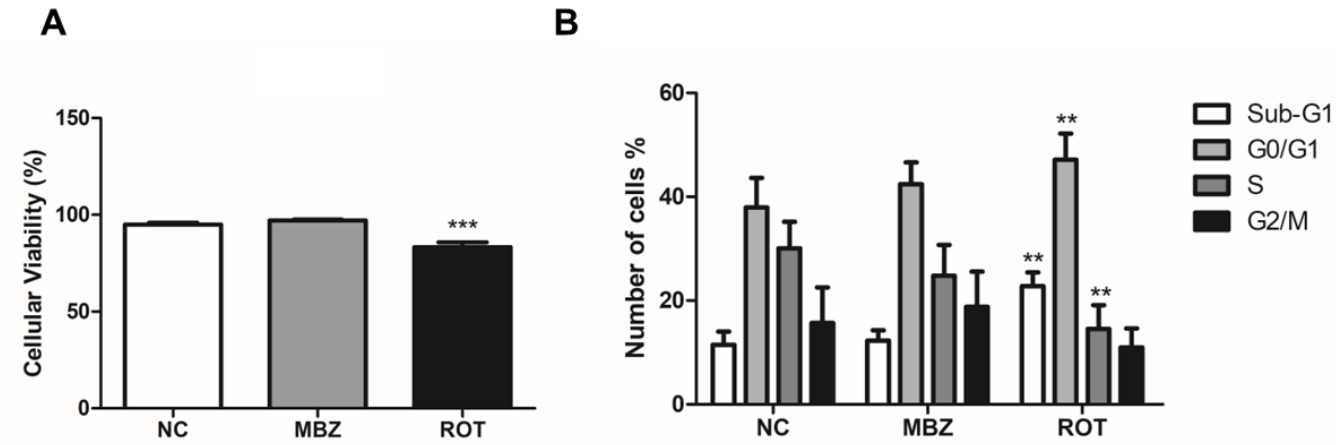
Cell line	Origin	MBZ IC_{50} (μM)
AGP-01	Malignant ascitic fluid of the metastatic primary intestinal type	0.11 (0.067 – 0.18)
ACP-03	Primary intestinal type	0.21 (0.11 – 0.39)
ACP-02	Primary diffuse type	0.24 (0.13 – 0.44)

3.2 Low MBZ concentration shows no cytotoxic nor cell cycle disruption after 24 hours of exposure in AGP-01 cell line

To better comprehend the potential of MBZ in metabolic modulation, without interference from cell death, and to ensure that the cells were initially viable. Non-cytotoxic nor cytostatic concentration and time were evaluated, thus, cells were treated at 0.1 μM of MBZ within 24 hours of exposure. Results showed that there was no modification in the cellular viability and the cell cycle pattern after 24 hours of cell exposure to MBZ (Fig. 1A). However, rotenone 1 μM (ROT), a metabolic inhibitor of the electron transport chain, significantly reduces cell viability (p<0.0001), enhanced cell fragmentation (cells in Sub-G1) and lead to G0/G1 cell cycle arrest (p<0.001) (Fig. 1B).

Since MBZ (0.1 μM) was not cytotoxic nor changed the cell cycle after 24 h of exposure, this concentration and treatment time were defined for subsequent metabolic assays once metabolism and its modulation by MBZ could only be observed in viable cells.

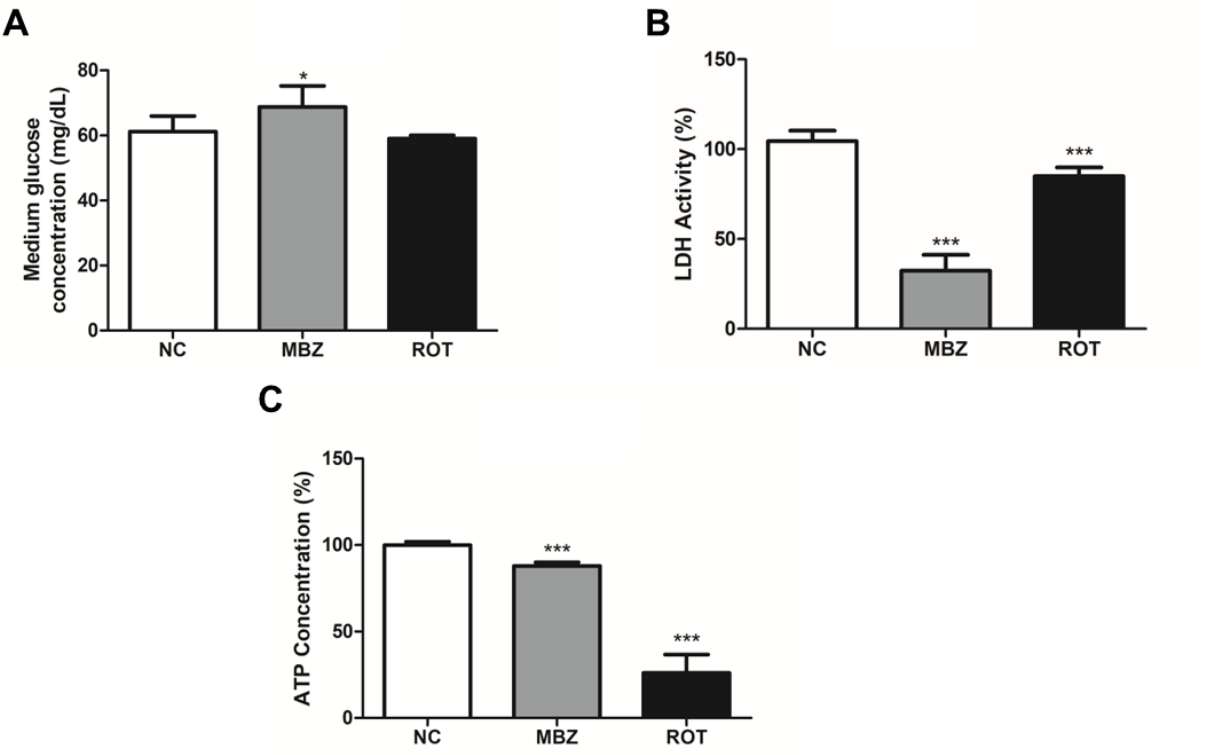
Figure 1. MBZ did not affect cellular viability or cell cycle distribution within 24 hours of exposure. Assays were performed after 24 hours of cell exposure to IC_{50} (0.1 μM) of MBZ and ROT (1 μM). **(A)** Average of cell viability in different treatments by Trypan blue exclusion. Non-treated cells (NC) were set as 100% **(B).** The average percentage of the number of cells (%) in the Sub-G1, G0/G1, S, and G2/M phases was calculated using FlowJo® software. Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: **p<0.001, ***p<0.0001. MBZ: Mebendazole. NC: Negative control. ROT: Rotenone.



3.3 MBZ alters glucose uptake, LDH activity, and ATP production in gastric cancer cell line AGP-01

Then, we sought to visualize if MBZ modulates the metabolic profile of the AGP-01 cell line by assessing the glucose uptake, LDH activity, and ATP production after 24 hours of treatment. MBZ inhibits significantly glucose uptake ($p<0.05$), observed by the presence of glucose content in the extracellular compartment when compared to the negative control (Fig. 2A). Results also showed that MBZ reduced the intrinsic activity of LDH enzyme and hence the intracellular ATP concentration ($p<0.0001$) (Fig. 2B and C) in non-cytotoxic concentration (0.1 µM). Furthermore, the inhibitor of the electron transport chain ROT significantly reduces LDH activity and ATP production after the same exposure time (Fig. 2C). Thus, the results demonstrate that MBZ, in a short treatment time, induces metabolic modulation in the AGP-01 gastric cancer cell line.

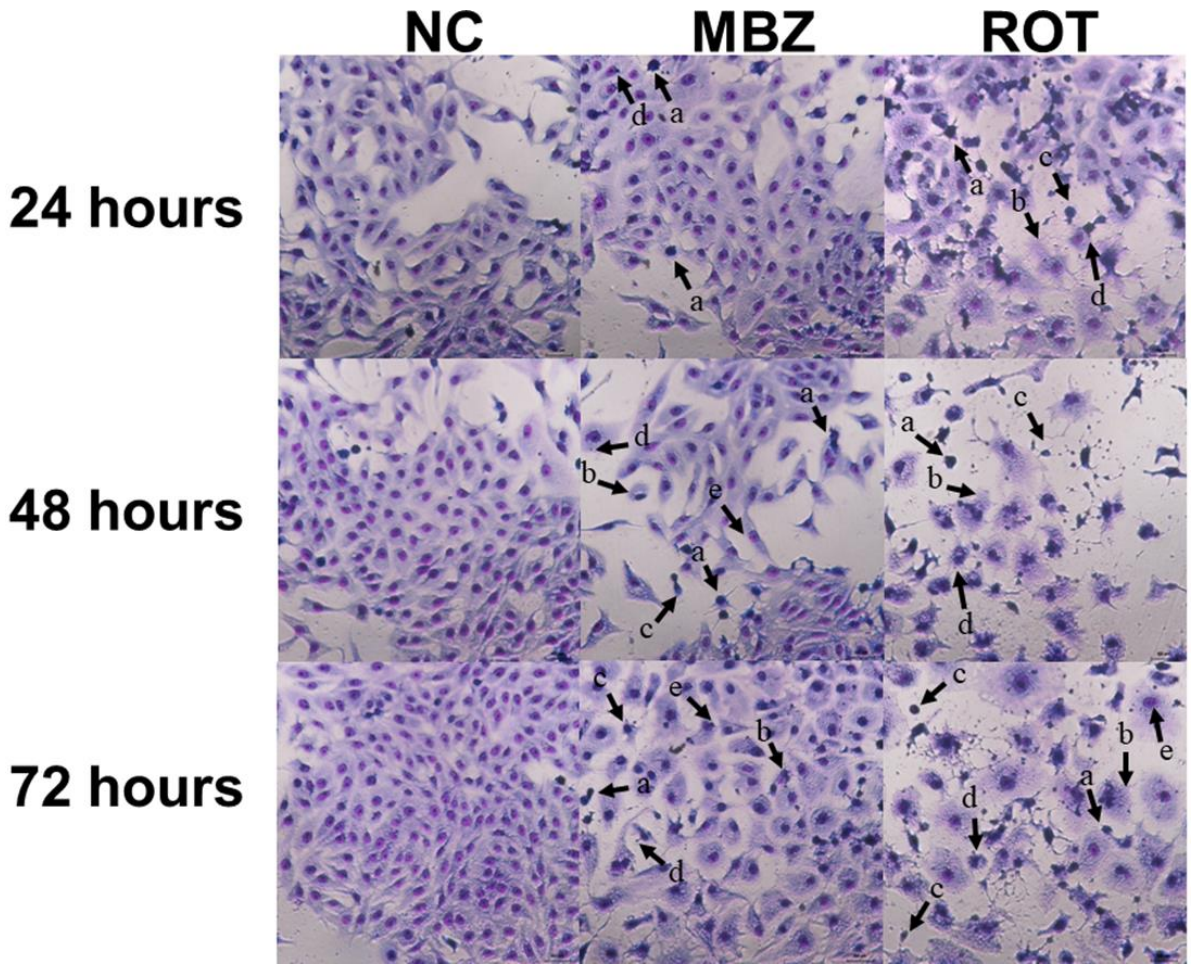
Figure 2. The cytotoxic mechanism of MBZ is related to the metabolic modulation of the AGP-01 cell line. Assays were performed after 24 hours of cell exposure to IC_{50} (0.1 µM) of MBZ. **(A)** Glucose uptake was estimated by the concentration of glucose (mg/dL) that remained in the culture media. **(B)** Intrinsic LDH activity. **(C)** ATP production. Non-treated cells (NC) were set as 100%. Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: *p<0.05, ***p<0.0001. MBZ: Mebendazole. NC: Negative control. ROT: Rotenone.



3.4 MBZ induces morphologic alterations in the AGP-01 cell line

The analysis of modifications in cell morphology caused by MBZ was performed by cell staining to differentiate the nucleus from the cytoplasm. As shown in Figure 3, cells treated with MBZ (0.1 µM) or ROT (1 µM) showed morphological modification as acidification (Fig. 3 “a” and black arrow), cell lysis (Fig. 3 “b” and black arrow), reduction in cytoplasmatic volume (Fig. 3 “c” and black arrow), blebs formation (Fig. 3 “d” and black arrow), and nuclear condensation (Fig. 3 “d” and black arrow) from 24 hours of exposure. In cells treated with MBZ, time-dependent damage was noticed, with the enhancement of morphologic alterations throughout treatment, indicating the triggering of the apoptotic process in the AGP-01 cell line.

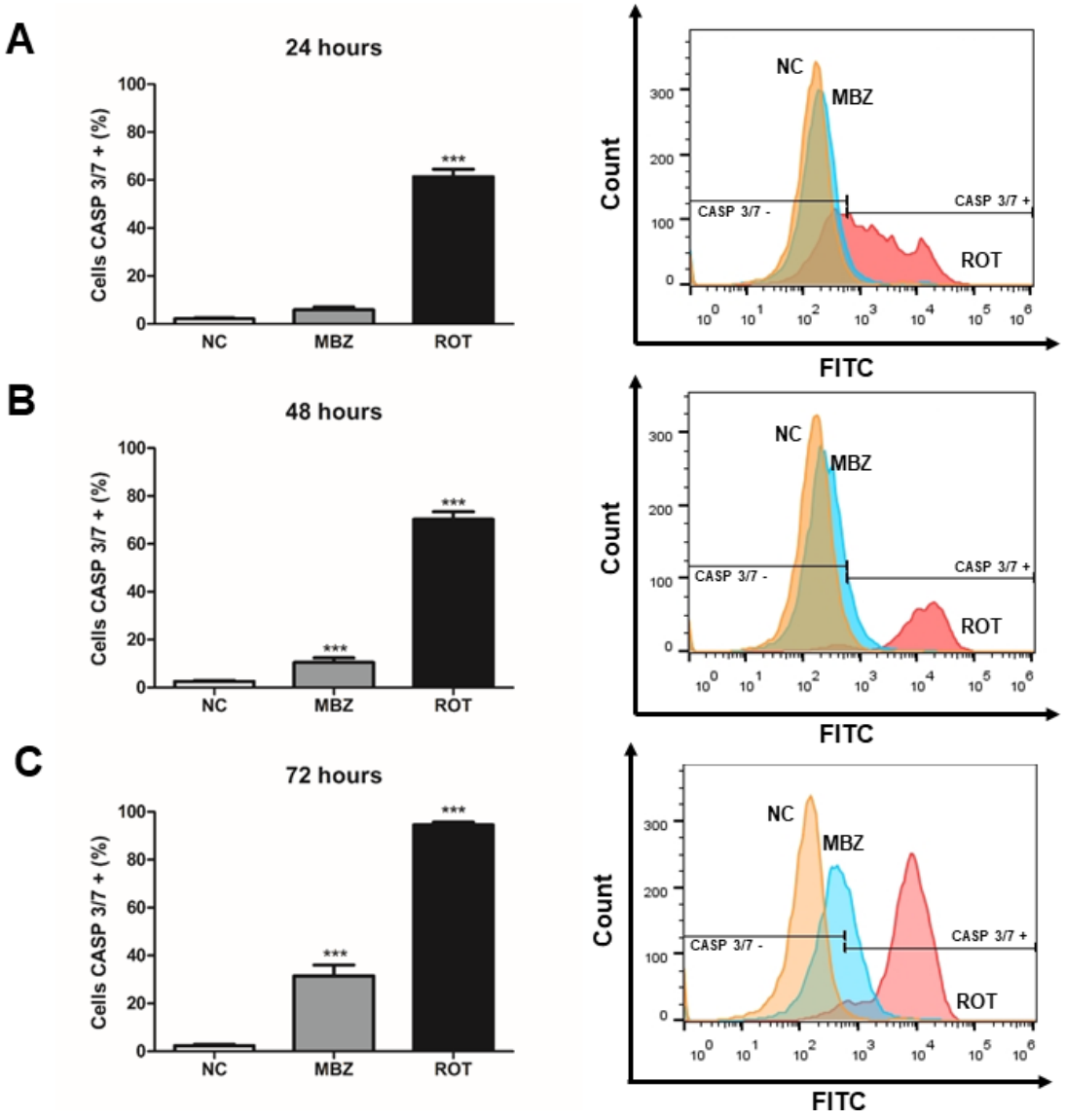
Figure 3. The treatment with MBZ causes cellular morphologic alteration typical of the apoptotic process over time. The AGP-01 cell line was treated with MBZ for 24, 48, and 72 hours and stained with the Panoptic kit. Morphological alterations (black arrows) were observed, such as (a) acidification, (b) cell lysis, (c) reduction in cytoplasmatic volume, (d) blebs formation, and (e) nuclear condensation. The figures represent three independent triplicated experiments (10× magnification, scale = 100 µm).



3.5 MBZ triggers Caspase-3/7 activation in the AGP-01 cell line

To further comprehend the cell death mechanism displayed by MBZ, the activation of the apoptotic inducers, caspase 3 and 7, was analyzed. Results showed that MBZ did not enhance caspase 3 and 7 activations in the first 24 hours of treatment (Fig. 4). Nevertheless, after 48 and 72 hours of treatment was observed a significant enhancement of apoptotic inducers, in around 10% and 30 %, respectively (p<0.0001). Results show that MBZ takes more than 24 hours to initiate the cell death signaling cascade in the AGP-01 cell line.

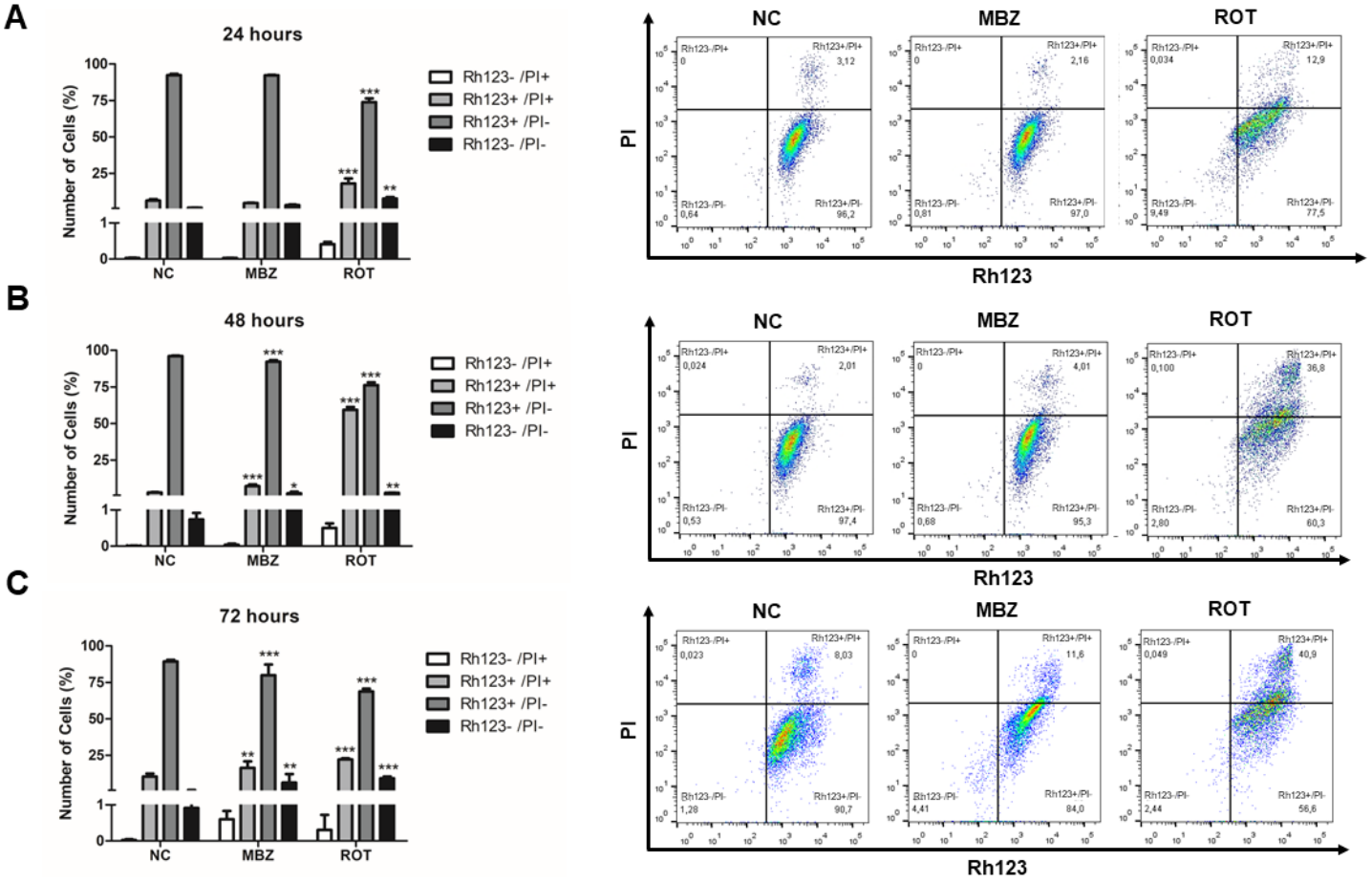
Figure 4. MBZ enhances caspase 3 and 7 activations in the AGP-01 cell line. Gastric cancer cell line AGP-01 was treated with MBZ for **(A)** 24, **(B)** 48, and **(C)** 72 hours. CellEvent® Caspase 3/7 kit was assessed to analyze caspase 3 and 7 activations by flow cytometry. Data are presented as the mean ± SD of three independent experiments of the average percentage of Caspase 3/7-FITC positive cells (CASP 3/7+) was analyzed by FlowJo software and is presented in bars on the left side. The representative histogram of Caspase-3/7-FITC staining (10,000 events) is shown on the right side. Cells without previous treatment (NC) were compared with cells treated with MBZ and ROT with ANOVA followed by Bonferroni's posttest. Significant differences: ***p<0.0001. Data are presented as the mean ± SD of three independent experiments. FITC: Fluorescein-5-isothiocyanate. MBZ: Mebendazole. NC: Negative control. ROT: Rotenone.



3.6 MBZ increases cellular membrane fragmentation and mitochondrial membrane depolarization in the AGP-01 cell line

The alterations in the cell membrane integrity and in the mitochondrial membrane potential induced by MBZ were analyzed by double staining with PI and Rh123. Mitochondrial dysfunction may predict metabolic stress due to its relation with cellular energy production. Additionally, analysis of the cellular membrane integrity could reveal intrinsic cellular mechanisms (Zou et al., 2010). Results evidenced that in the first 24 hours of treatment, MBZ did not alter the cellular membrane integrity as well as the mitochondrial membrane polarization (Fig. 5A). However, after 48 hours and 72 of treatment (Fig. 5B and C), MBZ caused enhancement in cellular membrane disruption (Rh123+ /PI+), and mitochondrial membrane depolarization (Rh123- /PI-) with a significant reduction in cells with viable profile (Rh123+ /PI-). Data evidenced that the initial metabolic stress caused by MBZ results in mitochondrial damage and cellular membrane integrity disruption related to its intracellular cytotoxic mechanism.

Figure 5. MBZ leads to modifications in cellular membrane integrity and causes mitochondrial membrane depolarization in the AGP-01 cell line. The AGP-01 cell line was treated with MBZ for (A) 24, (B) 48, and (C) 72 hours, stained with PI and Rh123, and analyzed by flow cytometry. The histograms on the right size show a representative cell distribution in two-dimension scatter plots of double staining (10,000 events). The graphs on the left side represent the average of cells analyzed. Cell populations were analyzed by FlowJo software. Data are presented as the mean ± SD of three independent experiments. Cells without previous treatment (NC) were compared to cells treated with MBZ and with the positive control ROT by ANOVA followed by Bonferroni's posttest. Significant differences: *p<0.05, **p<0.001, ***p<0.0001. MBZ: Mebendazole. NC: Negative control. PI: Propidium Iodite. Rho123: Rhodamine 123. ROT: Rotenone.



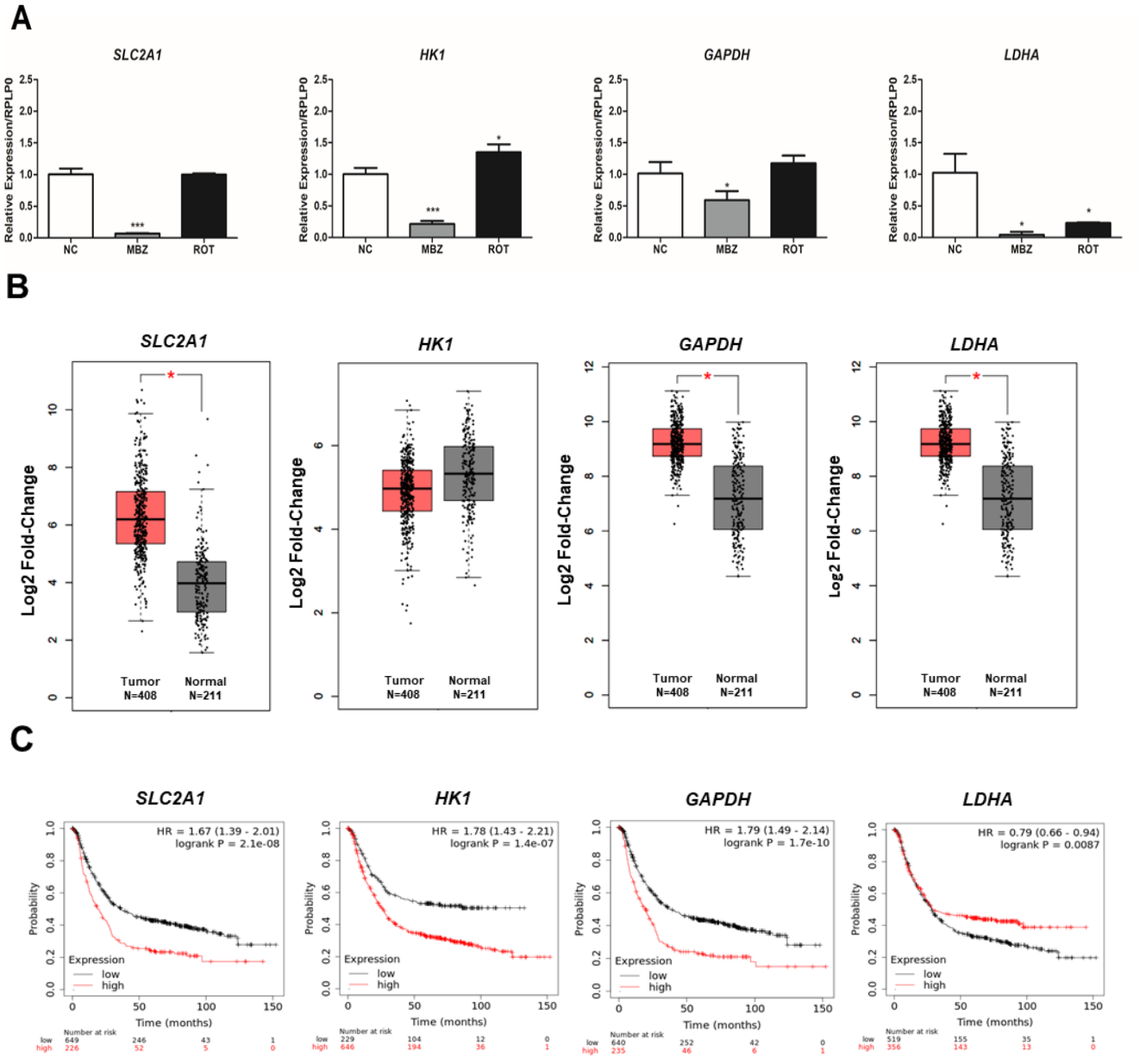
3.7 *In vitro* and *in silico* analyses reveals that MBZ reduces the mRNA expression of metabolic-related genes predictors of poor prognosis in gastric cancer

Based on metabolic reprogramming, the glycolytic pathway has a central role in the energy generation for cancer development (Schiliro and Firestein, 2021). Glycolytic proteins as glucose transporter (GLUT1), hexokinase 1 (HK1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Lactate Dehydrogenase A (LDHA) have been studied as targets for antitumoral drugs, once they are frequently overexpressed in cancer cells (da Silva et al., 2022; Xintaropoulou et al., 2015).

Thus, the gene expression of the glycolytic pathway targets, *SLC2A1*, *HK1*, *GAPDH*, and *LDHA*, were analyzed by RT-qPCR in AGP-01 cells after 24 hours of MBZ exposure. As shown in Figure 6A, MBZ decreases the expression of *SLC2A1*, *HK1*, *GAPDH*, and *LDHA* ($p<0.05$) after 24 hours. The most affected targets were *SLC2A1* and *LDHA*, with approximately a 90% reduction in transcripts. The less affected transcript was *GAPDH*, showing a reduction of only 50% in mRNA expression. Thus, results attested to the initial modulation of the transcript levels of the glycolytic targets by MBZ.

In sequence, we perform the *in silico* global expression analyses of the metabolic genes affected by MBZ treatment. The GEPIA database evaluated the gene expression of 408 CG samples and 211 samples from adjacent normal tissues. The analyses indicated that genes from the glycolytic pathway (Fig. 6B), such as *SLC2A1*, *GAPDH*, and *LDHA*, showed hyperexpression in CG samples compared to normal tissue. *HK1* did not show a statistical difference in CG samples compared to normal samples ($p<0.05$). The online analysis also showed that the highly expressed *SLC2A1*, *HK1*, and *GAPDH* led to a reduction in the overall survival (OS) rate in patients with CG ($p<0.05$). Otherwise, low expression of *LDHA* seems to be related to the reduction in the OS in GC's patients. Altogether, the data reaffirm the importance of metabolic reprogramming to cancer development, being potential biomarkers of poor prognosis in GC, mainly genes of the glycolytic pathways, which may become critical metabolic targets in GC therapy. Furthermore, *in vitro* results showed the potential of MBZ in reducing the level of these genes in the GC AGP-01 cell line, being potentially pharmacological targets of MBZ in the clinic.

Figure 6. MBZ modulates gene expression of metabolic-related genes predictors of poor prognosis in gastric cancer. (A) After 24 hours of cell exposure to MBZ, total mRNA was extracted, and analyzed the transcripts levels for SLC2A1, HK1, GAPDH, and LDHA. Gene expression was normalized by endogenous gene RPLP0, and the NC group was used as a calibrator of the experiment. (B) Global expression of metabolism-related genes SLC2A1, HK1, GAPDH, and LDHA were analyzed by online gene expression databases. The GEPIA dataset shows the hyperexpression of genes in CG samples (red boxplot). (C) Kaplan–Meier Plotter analysis evidenced reduced OS rate in patients with high gene expression of transcripts related to metabolism pathways. Data are presented as the mean ± SD of three independent experiments, and statistical analysis was performed with ANOVA followed by Bonferroni's posttest. Significant differences: *p<0.05, ***p<0.0001. MBZ: Mebendazole. NC: Negative control. HR: Hazard Ratio. ROT: Rotenone.



3.8 *In silico* analyses shows the interaction of MBZ with metabolic proteins

As observed in the previous result, MBZ alters the levels of mRNAs related to the glycolytic pathway in the AGP-01 cell line. Thus, molecular docking was performed towards

GLUT1, HK1, GAPDH, and LDHA, which are the product of genes evaluated above, to verify the interaction and pharmacological potential of MBZ as a metabolic modulator (Figures 7-10).

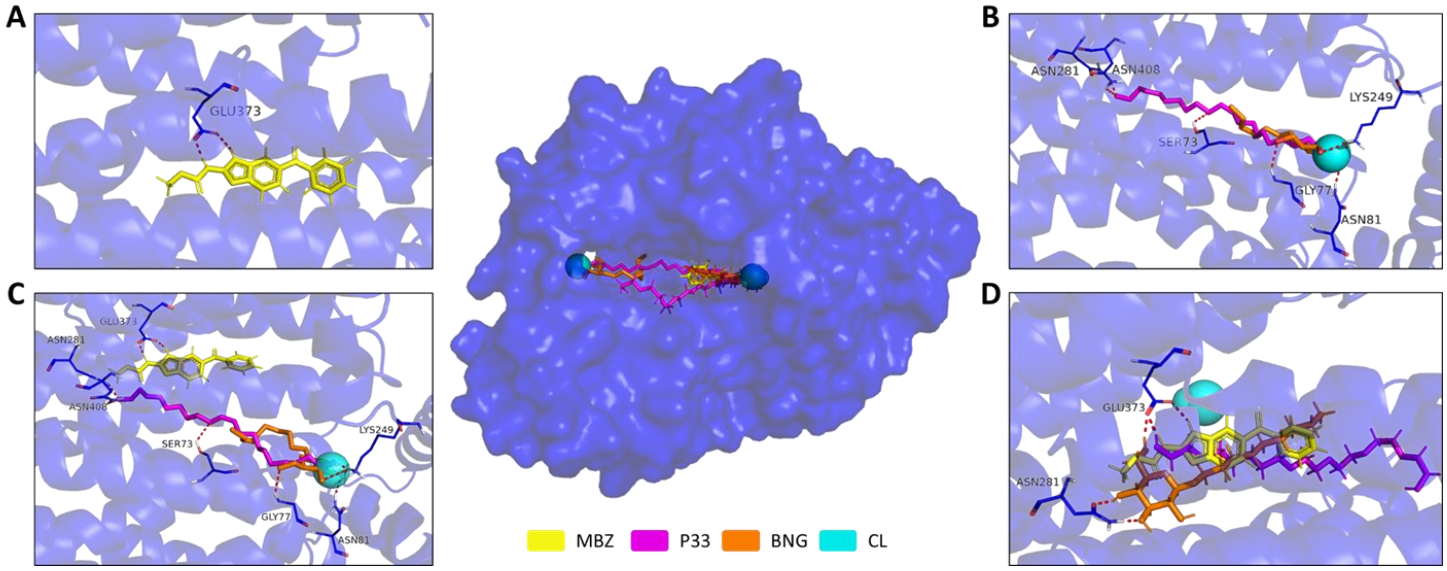
3.8.1 GLUT1

MBZ was evaluated by molecular docking against GLUT1 protein alone and in the presence of its ligands (Figure 7). Alone, MBZ interacts with GLUT by an ionic interaction with the amino acid residue Glu^{373} (Figure 7A). It is possible to see only one interaction with the amino acid Glu^{373} with an energy of -8.56 kCal Mol^{-1} (Supplementary Table 2). The root means square deviation (RMSD) of GLUT1 after interaction with MBZ indicated a value of 1.242 Å, suggesting an alteration in the structure of GLUT1 caused by MBZ.

In Figure 7B, the native ligands bound to GLUT1 near the active site supported by interactions Ser^{73}, Gly^{77}, Asn^{81}, Lys^{249}, Asn^{281} and Asn^{408} and different energies for each ligand (Supplementary Table 2). Interestingly, MBZ in the presence of GLUT1 native ligands (Figure 7C) revealed that the ligands remained in the same place they were in the previous dockings, including performing polar interactions with the same residues. However, it is possible to see the P33 and BNG underwent a slight change in their conformation. Additionally, the interaction of MBZ with GLUT1 also changes.

Alone, MBZ interacts only with Glu^{373}. In the presence of ligand, MBZ acquires a new interaction with Asn^{281} residues (Figure 7C). Curiously, the same residue Asn^{281} interacts with both MBZ and P33 (Figure 7C), which we can infer as an effect caused by adding MBZ. The energy of interaction of MBZ with GLUT1 in the presence of ligands slightly changes from -8.560 to -8.601 kCal mol^{-1} (Supplementary Table 2). Another docking assay was performed to evaluate if MBZ could change the interaction of GLUT1 with its ligand. So, GLUT1, in the presence of MBZ, was redocked against its ligand (Figure 7D). It noticed changes in the position of ligands interaction. All ligands were attracted to the binding site where the MBZ was, including changing the interactions with the amino acids. In this case, there are only connections with residues Asn^{281} and Glu^{373} (Figure 7D). As revealed in supplementary table 2, all interaction energies of ligands changed.

Figure 7. Molecular docking analysis of the GLUT1 protein and MBZ. (A) Docking the GLUT1 with the MBZ; (B) Docking of GLUT1 and native ligands; (C) Docking the GLUT1 bound to MBZ and then with native ligands simultaneously; (D) docking of GLUT1 bound with the native and thus with MBZ.



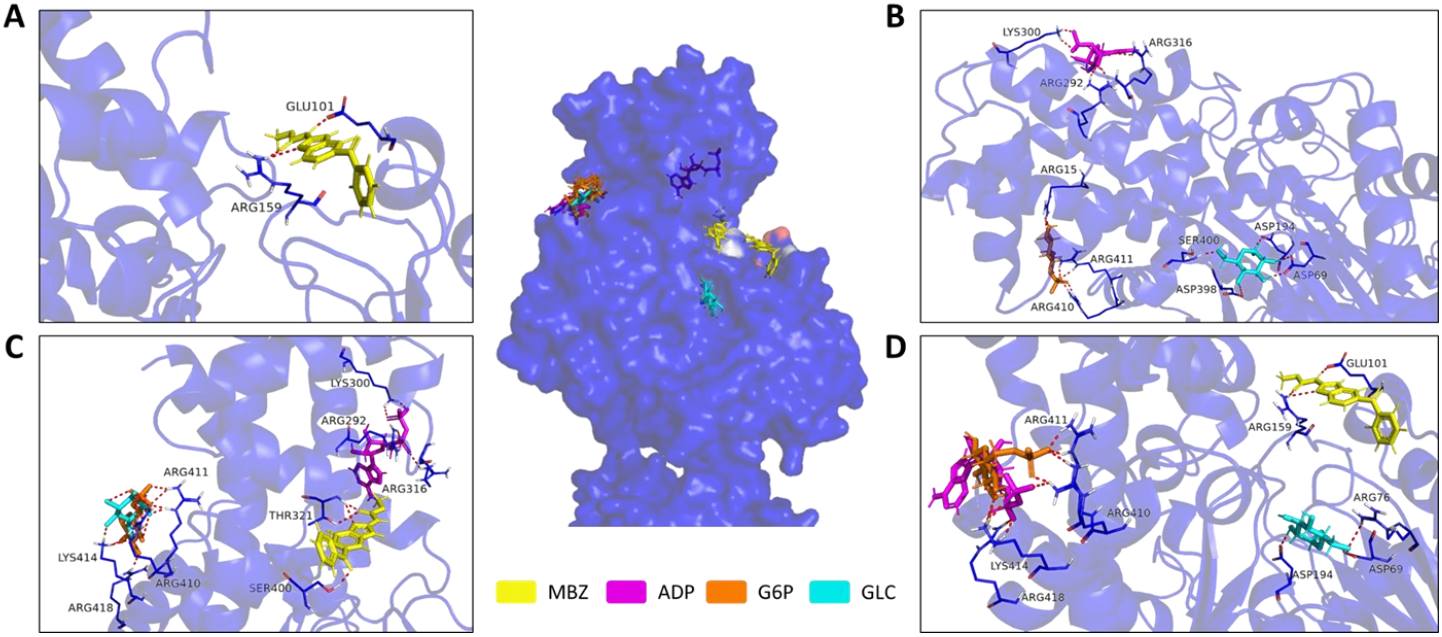
3.8.2 HK1

Regarding the interaction with HK1 protein alone, MBZ was able to interact with two amino acid residues, Glu^{101} and Arg^{159} (Figure 8A), with an energy of interaction of 7.547 kcal mol^{-1} (Supplementary Table 3) and an RMSD value of 1.248 Å also indicating a change in the HK1 structure. Different than GLUT1, in the HK1, the natural ligand interacts at different sites in the proteins as revealed by Figure 8B with interaction Arg^{15}, Asp^{69}, Asp^{194}, Arg^{292}, Lys^{300}, Arg^{316}, Asp^{398}, Ser^{400}, Arg^{410} and Arg^{411} amino acid residues.

In Figure 8C, in the presence of all native ligands of HK1, MBZ interacted in a different region. Now, MBZ is closer to the ADP ligand and occupying the position previously occupied by the GLC (Figure 8C), which moved to the site where the G6P is anchored (Figure 8C). These conformational changes led MBZ to interact with the amino acids Thr^{321} and Ser^{400} and native ligands bound to residues Arg^{292}, Lys^{300}, Arg^{316}, Thr^{321}, Ser^{400}, Arg^{410}, Arg^{411}, Lys^{414}, and Arg^{418} (Figure 8C).

The docking of HK1 with its ligands in the presence of MBZ also caused alterations. In the presence of MBZ, HK1 interact with ligand in different amino acid residues Asp^{69}, Arg^{76}, Arg^{194}, Arg^{410}, Arg^{411}, Lys^{414}, and Arg^{418} (Figure 8D). In the presence of MBZ, G6P and ADP interact in the same site, contrasting with the normal interaction (Figure 8D). The energies of interaction also changed and are summarized in Supplementary Table 3.

Figure 8. Molecular docking analysis of the HK1 protein and MBZ. (A) Docking the HK1 with the MBZ; (B) Docking of HK1 and native ligands; (C) Docking the HK1 bound to MBZ and then with native ligands simultaneously; (D) docking of HK1 bound with the native and thus with MBZ.

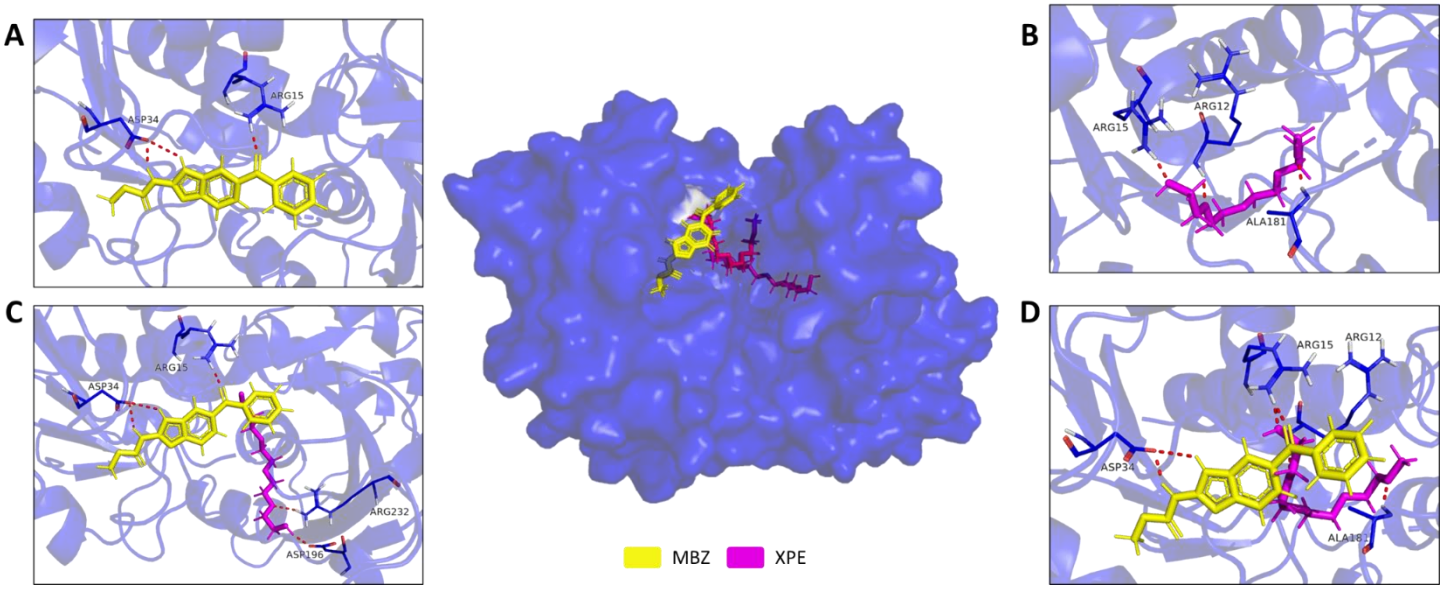


3.8.3 GAPDH

MBZ also interacted with the GAPDH protein (Figure 9A) by performing ionic bonds with Asp^{34} and Arg^{12} with an energy of interaction of -7.195 kcal mol^{-1} (Supplementary Table 4) and RMSD value of 1.462 Å. In the docking of the XPE native ligand, it was seen that it makes connections with Arg^{12}, Arg^{15}, and Ala^{181} (Figure 9B).

The docking of MBZ with GAPDH in the presence of XPE revealed that MBZ remains in the same position as alone but induces changes in the interaction of XPE with GAPDH (Figure 9C). In the presence of XPE, is interacting with Asp^{196} and Arg^{232} from GAPDH rather than Arg^{12}, Arg^{15}, and Ala^{181} (supplementary figure 3C), suggesting that MBZ interferes in the interaction of GAPDH with its natural ligand. The energies of these interactions are summarized in supplementary table 4. In contrast, when XPE is docked with GAPDH in the presence of MBZ, the interactions are the same as XPE alone (Figure 9D); however, with a slight difference in the interaction energy (Supplementary table 4).

Figure 9. Molecular docking analysis of the GAPDH protein and MBZ. (A) Docking the GAPDH with the MBZ; (B) Docking of GAPDH and native ligands; (C) Docking the GAPDH bound to MBZ and then with native ligands simultaneously; (D) docking of GAPDH bound with the native and thus with MBZ.



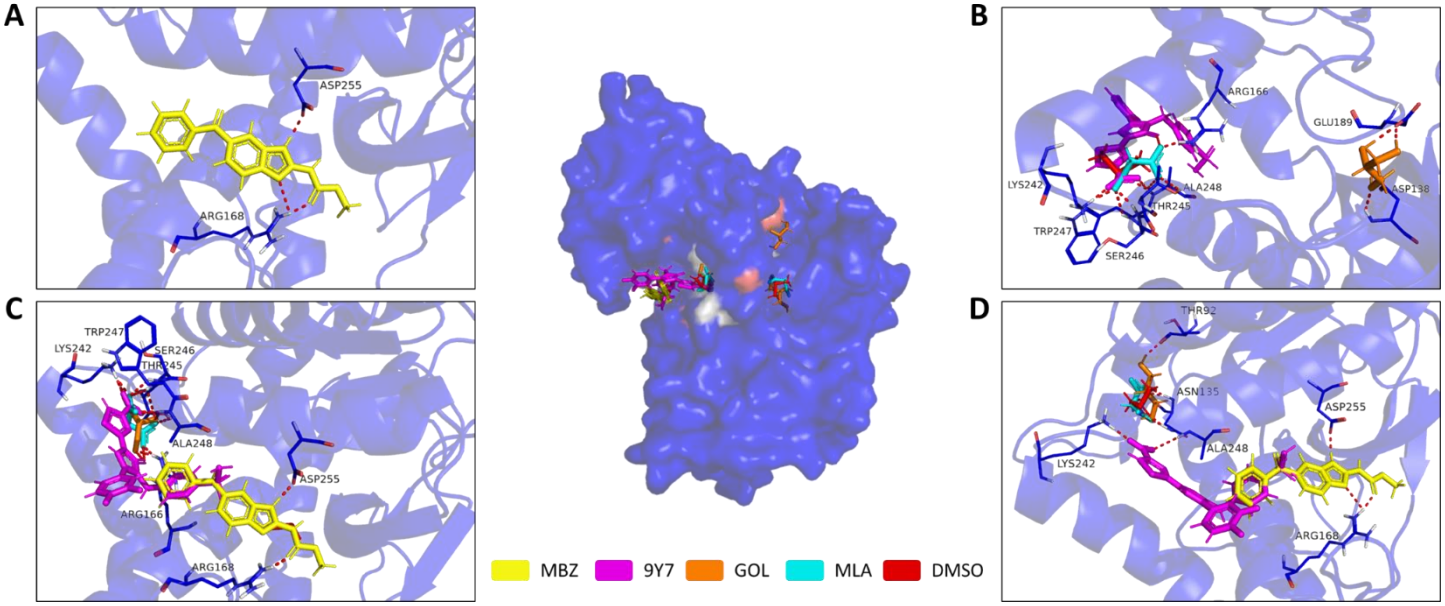
3.8.4 LDHA

The complex of LDHA and MBZ alone is supported by the ionic interaction of MBZ with Asp^{255} and Arg^{168} (Figure 10A) with an interaction energy of -7.550 kcal mol^{-1} (Supplementary Table 5) and RMSD value of 1.385 Å. In Figure 10B, native substrates bound to residues Asp^{138}, Arg^{166}, Glu^{189}, Lys^{242}, Thr^{245}, Ser^{246}, Trp^{247}, and Ala^{248} (Figure 10A) and the energy of interaction of each ligand is summarized in Supplementary Table 5.

The docking analysis of MBZ with LDHA in the presence of its natural ligand did not change the amino acid residues of interaction with MBZ and remained the same as MBZ alone, Asp^{255} and Arg^{168} (Figure 10A and C). However, the interaction of MBZ with LDHA interferes with the precise attachment of the GOL (Figure 10C). Without MBZ, GOL interacts with Glu189 and Asp138 amino acid residues (Figure 10B). However, in the presence of MBZ, GOL interacts with Ala248 and Thr245 (Figure 10C). The interaction of MBZ with GAPDH alters the interaction with ligands already bound to the enzyme, which alters the energy of interaction of GOL with LDHA (Supplementary Table 5).

The docking analysis of LDHA ligands in the presence of MBZ anchored to the enzyme led to modifications in the ligand's interaction with LDHA (Figure 10D). In this case, the native ligands interacted only with residues Thr^{92}, Asn^{135}, Arg^{168}, Lys^{242}, Ala^{248} and Asp^{255} (Figure 10D). In all cases, the presence of MBZ alters the energy of interaction of LDHA with substrates (Supplementary Table 5).

Figure 8. Molecular docking analysis of the LDHA protein and MBZ. (A) Docking the LDHA with the MBZ; (B) Docking of LDHA and native ligands; (C) Docking the LDHA bound to MBZ and then with native ligands simultaneously; (D) docking of LDHA bound with the native and thus with MBZ.



4 DISCUSSION

Although the development of new techniques and surgical equipment, along with new therapies, the increase in 5 years survival rates and decreased the number of deaths of patients diagnosed with GC, the number of deaths caused by this neoplasia worldwide remains high (Arnold et al., 2020; Miao et al., 2022; Sung et al., 2021b). Metabolic reprogramming is an emergent hallmark of cancer progression and antitumoral drugs with activity in malignant metabolic modulation have become potential compounds in cancer therapy (Hanahan and Weinberg, 2011; Luengo et al., 2017; Stine et al., 2022). In this sense, studying drugs and their role in malignant metabolism modulation presents a novel approach to discovering new targets in GC treatment. Our results showed that MBZ, a potential drug undergoing a pharmacological repositioning process, presented highly antitumoral activity in GC cell lines (Table 1) driven by multiple mechanisms that alter cancer cells' energetic metabolism, depleting the glucose employment in ATP production.

The antimetabolic potential of MBZ was first described in 1987. In the study, the antihelmintic activity was linked to the inhibition of enzymes related to carbohydrate metabolism leading to parasite death (Ahmad and Nizami, 1987). Although there are studies describing the antitumor mechanism of MBZ in different types of cancer (Bai et al., 2015; Bodhinayake et al., 2015; Daniel et al., 2022; De Witt et al., 2017; Doudican et al., 2013; Freisleben et al., 2019; Gallia et al., 2021; Kralova et al., 2018; Nygren et al., 2013; Patil et al.,

2022; Pinto et al., 2015; Sasaki et al., 2002; Skibinski et al., 2018; Williamson et al., 2021, 2020), this is the first one to demonstrate its primary activity on the metabolic inhibition of GC cell lines, which may lead to the discovery of new relevant targets in cancer chemotherapy (Bin et al., 2022).

Several studies do not consider the glucose content in the cultured medium. However, cells maintained in high glucose conditions (25 mM of glucose) would present experimental bias due to the high amount of nutrients, which is not even similar to that presented in normal glycemic conditions (4–6 mM) nor in tumor (ranging from 0.4 mM in the tumor for 1.3 mM in the surrounding tissue) (da Silva et al., 2022; Han et al., 2011; Hirayama et al., 2009; Razzak et al., 2018; Zhuang et al., 2014). Interestingly, cytotoxicity results of MBZ in GC cell lines were better in cells maintained in low glucose conditions when compared to the same cell line maintained in high glucose (Pinto et al., 2015). Results showed around 5-6 fold reduction in IC_{50} of metastatic (AGP-01 cells) and primary tumor (ACP-03 cells), respectively, evidencing that low glucose (5.5 mM) environment is better for understanding the GC metabolism.

Non-cytotoxic exposure times and concentrations are ideal for helping the understanding of modifications in intracellular pathways without the final process of cellular death. This condition is essential to shedding more light on the mechanism by which MBZ drives cancer cells to death by modulation of metabolism (Fujita et al., 2020). Thus, the non-cytotoxic or cytostatic time of 24 hours was chosen (Fig. 1). Other studies evidenced that MBZ can lead to G0/G1 or G2/M cell cycle arrest depending on the concentration and time used for cell treatment (Mukhopadhyay et al., 2002; Pinto et al., 2019; Williamson et al., 2020). This set of experiments was essential to show that the effects of MBZ go beyond the inhibition of energetic cellular metabolism. Somehow, in GC cells and other types of cancer, MBZ could interfere with nuclear function, impacting cell division cycles and thus jeopardizing cancer cell development (Pinto et al., 2019).

MBZ led to a reduction in glucose uptake, LDH activity, and ATP production in the AGP-01 cell line (Fig. 2). The reduced expression of metabolic enzymes and transporters is related to a decrease in metabolic flow and, consequently, in the final energy production, studies have shown this mechanism as commonly used by potential antitumoral drugs with the target in metabolism deregulation of tumoral cell lines (Brockmueller et al., 2021; Han et al., 2015; He et al., 2019; Xu et al., 2020). Remarkably, these results are exciting and in agreement with in silico analysis. The reduction in glucose uptake agrees with the reduction of *SLC2A1* mRNA expression and the interaction of MBZ with GLUT1 itself (Fig. 6 and 7). Additionally, the reduction of LHD activity is a result of either the reduction with the accumulation of LDH

or direct effect of MBZ in the LDH activity (Fig. 2, 6, and 10). In both cases, the *in silico* prediction of dockings interaction was corroborated by in vitro experiments.

The modulation of metabolism by MBZ enhanced the apoptotic mechanism of cell death of the AGP-01 cell line throughout treatment time, as shown in Figures 3, 4, and 5. Improvement of morphologic alterations typical of apoptosis, enhancement in caspase 3 and 7 activity, as well as the propidium iodide incorporation (PI+) indicates activation of the intracellular mechanisms of cell death (da Silva et al., 2022; Vasconcelos et al., 2018). Results also showed a reduction in Rhodamine 123 staining (Rh123-) (Fig. 5), indicating mitochondrial membrane depolarization after treatment with MBZ. Mitochondria is crucial for energy formation as ATP during the process known as oxidative phosphorylation (Moindjie et al., 2021). Insults in the mitochondrial architecture and polarization are strongly related to ATP production and cell death mechanisms (Vakifahmetoglu-Norberg et al., 2017). Thus, results show that MBZ also disrupts the energy-producing machinery of the cancer cell line AGP-01, triggering the apoptotic process.

Glucose metabolism is essential for cell growth and development because it provides the primary source of energy and carbon skeleton for cellular biosynthesis (Stine et al., 2022). Based on that, glucose metabolism became a recent area to seek for new metabolic targets in oncology (Stine et al., 2022). The solute carriers of the glucose transporter (GLUT) family mediate the first step of the glucose metabolism pathway allowing glucose transport into the cell. Studies have identified hyperexpression of GLUT1, encoded by the *SLC2A1* gene, in metabolic reprogramming and malignant phenotype development in GC (Min et al., 2021; Yan et al., 2015).

Once glucose is in the cytosol, a sequence of biochemical reactions (glycolytic pathway) is responsible for glucose conversion into pyruvate. Therefore, some enzymes such as hexokinase (HK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) exert pivotal roles in glucose transformation and alterations in their gene expression and activity are associated with GC malignant phenotype acquisition (da Silva et al., 2022; Nowak et al., 2018).

Our analysis showed that MBZ reduced the mRNA expression of *SLC2A1*, *HK1*, and *GAPDH* in the metastatic AGP-01 cell line (Fig. 6). Glycolysis is an ancient energetic pathway employed by cells to produce ATP. In biochemical terms, glycolysis is employed by both anaerobic and aerobic organisms, being more profitable in aerobic ones (Zhang et al., 2021). Moreover, in cancer cells, glycolysis serves much more than just oxidate glycolysis (Hu et al., 2014). In cancer cells, as revealed by Otto Warburg, anaerobic glycolysis is predominant, even being less productive for ATP levels. The Warburg effect provides a fruitful environment for

cancer cells to grow, proliferation, aggressiveness, and drug resistance. The Warburg effect leads to a high accumulation of lactate outside the cell, acidifying the medium and favoring cancer cell growth (de la Cruz-López et al., 2019; Hu et al., 2014; San-Millán and Brooks, 2017).

Based on that, it is clear that by reducing the expression of genes related to glucose uptake (*SLC2A1*), glycolysis pathway (*HK1* and *GAPDH*), and lactate production (*LDHA*) drastic changes in cellular metabolism occur, occasionally triggering cell death mechanisms. *SLC2A1* encodes for GLUT1 transporter and is the highest expressed isoform in cancer types, increasing the intracellular glucose levels and keeping the glycolysis pathway active (Guo et al., 2011; Lai et al., 2012). Also, to keep the energy cycle moving, enzymes must be produced. *HK1* and *GAPDH* are important glycolytic genes responsible for critical steps of the glycolysis pathway. HK is responsible for the first step in the glycolytic pathway by phosphorylating glucose to produce glucose-6-phosphate consuming one mol of ATP (Guo et al., 2023). GAPDH catalyzes the sixth step of the glycolytic pathway by converting glyceraldehyde-3-phosphate into 2-phosphoglycerate, producing one mol of NADH, the first compensatory event in the pathway (Zhu et al., 2021).

Lactate dehydrogenase (LDH) facilitates the glycolytic process by converting pyruvate to lactate. Lactate production in glycolysis contributes largely to malignant progressions, like replenishing NAD+ for glycolysis, lowering pH for invasion, and eliciting immune system escape. Aberrant expression and activation of LDHA are strictly related to different cancers (Feng et al., 2018; Sheng et al., 2012). Moreover, high serum LDH levels are usually associated with a poor prognosis in many cancer types. Even the most effective drugs, which have entirely improved patient outcomes in clinics over the past decade, provide minimal benefit to those with high serum LDH levels (Claps et al., 2022; Hatami et al., 2023).

It is well-known that glycolytic genes are essential to promote carcinogenesis, protecting cancer cells from apoptosis events and malignant proliferation (Claps et al., 2022; Zhang et al., 2021). Additionally, the non-canonical activity of these glycolytic-related genes has been reported to favor cancer cells (Hu et al., 2014). The higher expression of these genes is also associated with low survival in patients (Fig. 6C). Therefore, seeking a drug that interacts with tumor metabolism is relevant. Here, it is shown that one of the mechanisms employed by MBZ toward GC is by reducing the expression of these genes (Fig. 6A), suggesting the interference of vital pathways to cancer establishment.

Even though the reduction of these genes by MBZ is high, it did not reach zero (Fig. 6A). For example, *GAPDH* gene expression remains with values around 50%. Nevertheless, the lower expression of these genes indicates the presence of proteins in cells. Based on that, a question emerged: Can MBZ interact with the product of those genes and inhibit their activity? Docking analysis suggested that MBZ interacts with those proteins and induces conformational changes in their 3D structures (Figures 7-10 and supplementary tables 2-5). By inducing changes in the 3D structures of those proteins, MBZ also interferes with their interaction with respective substrates, indicating interference in activity.

A study performed by Chan and co-workers (2011) (Chan et al., 2011) evaluated the interaction of STF-31 with GLUT1 as a renal cell carcinoma target, the interactions of STF-31 with Arg^{126} and Trp^{412} residues from GLUT1. MBZ interacted with a different residue (Fig. 7). Another study with HK1 by Kulkarni and co-workers (2022) (Kulkarni et al., 2022) used the same structure we used (Fig. 8). The authors evaluated the interaction of Glycyrrhizin with HK1 with residues Asp^{79}, His^{99}, Lys^{146}, Lys^{147}, Leu^{148}, Pro^{149}, Ala^{458}, Tyr^{461}, Lys^{481}, Lys^{880}, and Lys^{885}. MBZ interacted with different residues than those reported.

Regarding GAPDH, in another study by Noh and co-workers (2021) (Noh et al., 2021), it was shown that a folic acid derivate interacts with GAPDH by the following residues Asn^{8}, Gly^{9}, Gly^{11}, Arg^{12}, Gly^{14}, Asn^{33}, Asp^{34}, Arg^{78}, Ala^{96}, and Thr^{97}. One residue common to MBZ is the Asp^{34} (Fig. 9A). The interaction of MBZ with GAPDH is close to the folic acid derivate, suggesting this spot could be important to the function of GAPDH.

For LDHA, Sun and co-workers (2016) (Sun et al., 2016) tested compounds to inhibit pancreatic cancer. Through *in silico* analysis, it was verified that the compounds interacted with Asp^{52}, Gly^{97}, $Arg^{99,}$ and Arg^{112}. In another study, carried out by Pandey and co-workers (2019) (Pandey et al., 2019), involving the molecular docking analysis of the anti-inflammatory drug diclofenac for possible uses in cancer therapy, interactions were found with residues Arg^{105}, Asn^{137}, Pro^{138}, Val^{139}, Asp^{140}, Ile^{141}, Glu^{191}, His^{192}, Gly^{193}, Asp^{194}, Ser^{195}, Tyr^{238}, and Leu^{322}. In all these cases, the interaction residues were completely different than the one presented by MBZ.

In contrast, Amr and co-workers (2020) (Amr et al., 2020) analyzed other compounds with anticancer potential and found interactions with the amino acids Asn^{137}, Arg^{168}, His^{192}, Tyr^{238} and Thr^{247}. Among the residues found, only Arg^{168} showed a link in common with MBZ during the interaction with LDHA. All the results suggested that MBZ present unique interactions with all these proteins mentioned above, indicating a new interaction model with

them and thus reinforcing the potential of MBZ to be employed in developing a new drug against GC.

Here, in addition to the interaction of the MBZ with enzymes, we also showed MBZ interferes in the interaction of the enzymes with respective substrates, which potentialize the effect of MBZ on these enzymes. Additionally, it was shown that MBZ has a unique interaction with those enzymes compared to other molecules in the literature. Our results revealed that MBZ affects the metabolisms of GC cells in two ways. First, shutting down the expression of genes by an unknown mechanism. Second, the molecular docking analysis revealed that even the proteins produced could be inhibited by MBZ present in the cytoplasm of GC cells.

In context, MBZ inhibits at different points the energetic metabolism. By inhibiting the expression of the gene and the activity of GLUT1, MBZ interferes with glucose uptake in the GC cell line. Targeting both *HK1* and *GAPDH* genes and activities, MBZ blocks the glycolysis pathway. Lastly, affecting the pattern of *LDHA* gene expression and LDH enzyme activity, MBZ did not allow the conversion of pyruvate in lactate and NAD^+ regeneration. The pharmacologic or genetic inhibition of *SLC2A1*, *HK1*, and *GAPDH* genes has been shown to reduce cell growth, drug resistance, migration, invasion, and trigger cell death *in vitro* and *in vivo* cancer models (Ding et al., 2017; Fu et al., 2022; Pacchiana et al., 2022; Shima et al., 2022; Zhou et al., 2018).

Targeting metabolic constituents is relevant to GC therapy. Our searches in gene expression databases of patients with GC demonstrated that *SLC2A1* and *GAPDH* are hyperexpressed in GC samples and that the enhancement in mRNA levels of *SLC2A1*, *HK1*, and *GAPDH* are correlated with a reduction in the overall survival rate of these patients (Fig. 6). The dysregulation in the expression of these proteins are also shown in others studies that correlate this alteration with improvement in proliferative and metastatic profile, as well as with the poor prognosis in GC patients (Gao et al., 2015; Wang et al., 2017; Yan et al., 2015; Yang, 2020).

Changed energy metabolism has emerged as one of the critical cancer biochemical fingerprints. Our results suggest that MBZ can interfere with the glycolysis pathway, inhibiting critical targets related to metabolism evolved in cancer progression and drug failure in gastric cancer. Moreover, low glucose uptake predisposes cells to higher cytotoxicity and, thus, apoptosis in gastric cancer cells. Low-calorie uptake during chemotherapy cycles might sensitize cells to chemotherapy by interfering with cell metabolism. These findings may change understanding how metabolism can interfere with drug efficacy and chemotherapy regimens.

5 CONCLUSION

Therefore, it was shown that MBZ induced changes in the energetic metabolism related to glucose starting on uptake through the oxidation pathway. By altering the gene expression and activity of respective proteins, all associated with a poor outcome in the clinic, MBZ has driven AGP01 cancer cells to death by intracellular apoptosis-related mechanisms. This study shed new light on the cytotoxic mechanism of MBZ in GC cell line AGP-01, providing more information that can help this drug become a potential chemotherapy in the clinical therapy of GC.

Declaration of Competing Interests

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful to the Brazilian funding agencies: National Council for Scientific and Technological Development (CNPq) (Productive scholarship – CNPq-PQ: 308586/2022-0, and CNPq graduate scholarship: 140436/2020-0), Coordination for the Improvement of Higher Level Personnel (CAPES), Federal University of Ceará (UFC), and the Cearense Foundation of Scientific and Technological Support (FUNCAP) for financial assistance. Also, we thank the University Federal of Ceará and the multiuser unit of the Drug Research and Development Center (NPDM) for fellowships and structural support.

REFERENCES

Ahmad, M., Nizami, W.A., 1987. In vitro effects of mebendazole on the carbohydrate metabolism of Avitellina lahorea (Cestoda). J. Helminthol. 61, 247–252. https://doi.org/10.1017/S0022149X00010105

Amr, A.E.-G.E., Mageid, R.E.A., El-Naggar, M., M. Naglah, A., S. Nossier, E., Elsayed, E.A., 2020. Chiral Pyridine-3,5-bis- (L-phenylalaninyl-L-leucinyl) Schiff Base Peptides as Potential Anticancer Agents: Design, Synthesis, and Molecular Docking Studies Targeting Lactate Dehydrogenase-A. Molecules 25, 1096. https://doi.org/10.3390/molecules25051096

Arnold, M., Abnet, C.C., Neale, R.E., Vignat, J., Giovannucci, E.L., McGlynn, K.A., Bray, F., 2020. Global Burden of 5 Major Types Of Gastrointestinal Cancer. Gastroenterology 159, 335-349.e15. https://doi.org/10.1053/j.gastro.2020.02.068

Bai, R.-Y., Staedtke, V., Wanjiku, T., Rudek, M.A., Joshi, A., Gallia, G.L., Riggins, G.J., 2015. Brain Penetration and Efficacy of Different Mebendazole Polymorphs in a Mouse Brain Tumor Model. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 21, 3462–3470. https://doi.org/10.1158/1078-0432.CCR-14-2681

Bin, Y.-L., Hu, H.-S., Tian, F., Wen, Z.-H., Yang, M.-F., Wu, B.-H., Wang, L.-S., Yao, J., Li, D.-F., 2022. Metabolic Reprogramming in Gastric Cancer: Trojan Horse Effect. Front. Oncol. 11.

Bodhinayake, I., Symons, M., Boockvar, J.A., 2015. Repurposing mebendazole for the treatment of medulloblastoma. Neurosurgery 76, N15-16. https://doi.org/10.1227/01.neu.0000460594.93803.cb

Brockmueller, A., Sameri, S., Liskova, A., Zhai, K., Varghese, E., Samuel, S.M., Büsselberg, D., Kubatka, P., Shakibaei, M., 2021. Resveratrol's Anti-Cancer Effects through the Modulation of Tumor Glucose Metabolism. Cancers 13, 188. https://doi.org/10.3390/cancers13020188

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Cardaci, S., Desideri, E., Ciriolo, M.R., 2012. Targeting aerobic glycolysis: 3-bromopyruvate as a promising anticancer drug. J. Bioenerg. Biomembr. 44, 17–29. https://doi.org/10.1007/s10863-012-9422-7

Chan, D.A., Sutphin, P.D., Nguyen, P., Turcotte, S., Lai, E.W., Banh, A., Reynolds, G.E., Chi, J.-T., Wu, J., Solow-Cordero, D.E., Bonnet, M., Flanagan, J.U., Bouley, D.M., Graves, E.E., Denny, W.A., Hay, M.P., Giaccia, A.J., 2011. Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. Sci. Transl. Med. 3, 94ra70. https://doi.org/10.1126/scitranslmed.3002394

Claps, G., Faouzi, S., Quidville, V., Chehade, F., Shen, S., Vagner, S., Robert, C., 2022. The multiple roles of LDH in cancer. Nat. Rev. Clin. Oncol. 19, 749–762. https://doi.org/10.1038/s41571-022-00686-2

da Silva, E.L., Mesquita, F.P., Portilho, A.J. de S., Bezerra, E.C.A., Daniel, J.P., Aranha, E.S.P., Farran, S., de Vasconcellos, M.C., de Moraes, M.E.A., Moreira-Nunes, C.A., Montenegro, R.C., 2022. Differences in glucose concentration shows new perspectives in gastric cancer metabolism. Toxicol. In Vitro 82, 105357. https://doi.org/10.1016/j.tiv.2022.105357

Daniel, J.P., Mesquita, F.P., Da Silva, E.L., de Souza, P.F.N., Lima, L.B., de Oliveira, L.L.B., de Moraes, M.E.A., Moreira-Nunes, C. de F.A., Burbano, R.M.R., Zanatta, G., Montenegro, R.C., 2022. Anticancer potential of mebendazole against chronic myeloid leukemia: in silico and in vitro studies revealed new insights about the mechanism of action. Front. Pharmacol. 13.

de la Cruz-López, K.G., Castro-Muñoz, L.J., Reyes-Hernández, D.O., García-Carrancá, A., Manzo-Merino, J., 2019. Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches. Front. Oncol. 9.

de Magalhães, C.S., Almeida, D.M., Barbosa, H.J.C., Dardenne, L.E., 2014. A dynamic niching genetic algorithm strategy for docking highly flexible ligands. Inf. Sci. 289, 206–224. https://doi.org/10.1016/j.ins.2014.08.002

De Witt, M., Gamble, A., Hanson, D., Markowitz, D., Powell, C., Al Dimassi, S., Atlas, M., Boockvar, J., Ruggieri, R., Symons, M., 2017. Repurposing Mebendazole as a Replacement for Vincristine for the Treatment of Brain Tumors. Mol. Med. Camb. Mass 23, 50–56. https://doi.org/10.2119/molmed.2017.00011

Ding, X., Liu, J., Liu, T., Ma, Z., Wen, D., Zhu, J., 2017. miR-148b inhibits glycolysis in gastric cancer through targeting SLC2A1. Cancer Med. 6, 1301–1310. https://doi.org/10.1002/cam4.1008

Doudican, N.A., Byron, S.A., Pollock, P.M., Orlow, S.J., 2013. XIAP downregulation accompanies mebendazole growth inhibition in melanoma xenografts. Anticancer. Drugs 24, 181–188. https://doi.org/10.1097/CAD.0b013e32835a43f1

Falzone, L., Salomone, S., Libra, M., 2018. Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium. Front. Pharmacol. 9, 1300. https://doi.org/10.3389/fphar.2018.01300

Feng, Y., Xiong, Y., Qiao, T., Li, X., Jia, L., Han, Y., 2018. Lactate dehydrogenase A: A key player in carcinogenesis and potential target in cancer therapy. Cancer Med. 7, 6124–6136. https://doi.org/10.1002/cam4.1820

Freisleben, F., Stamm, H., Muschhammer, J., Thaden, V., Modemann, F., Krispien, A., Brauneck, F., Wellbrock, J., Fiedler, W., 2019. Mebendazole Mediates Its Anti-Leukemic Effects By Proteasomal Degradation of GLI Transcription Factors Via Inhibition of HSP70/90-Chaperone Activity in Acute Myeloid Leukemia in a Preclinical and Clinical Setting. Blood 134, 5050. https://doi.org/10.1182/blood-2019-129973

Fu, M., Liu, Y., Cheng, H., Xu, K., Wang, G., 2022. Coptis chinensis and dried ginger herb combination inhibits gastric tumor growth by interfering with glucose metabolism via LDHA and SLC2A1. J. Ethnopharmacol. 284, 114771. https://doi.org/10.1016/j.jep.2021.114771

Fujita, M., Imadome, K., Somasundaram, V., Kawanishi, M., Karasawa, K., Wink, D.A., 2020. Metabolic characterization of aggressive breast cancer cells exhibiting invasive phenotype:

impact of non-cytotoxic doses of 2-DG on diminishing invasiveness. BMC Cancer 20, 929. https://doi.org/10.1186/s12885-020-07414-y

Gallia, G.L., Holdhoff, M., Brem, H., Joshi, A.D., Hann, C.L., Bai, R.-Y., Staedtke, V., Blakeley, J.O., Sengupta, S., Jarrell, T.C., Wollett, J., Szajna, K., Helie, N., Mattox, A.K., Ye, X., Rudek, M.A., Riggins, G.J., 2021. Mebendazole and temozolomide in patients with newly diagnosed high-grade gliomas: results of a phase 1 clinical trial. Neuro-Oncol. Adv. 3, vdaa154. https://doi.org/10.1093/noajnl/vdaa154

Gao, Y., Xu, D., Yu, G., Liang, J., 2015. Overexpression of metabolic markers HK1 and PKM2 contributes to lymphatic metastasis and adverse prognosis in Chinese gastric cancer. Int. J. Clin. Exp. Pathol. 8, 9264–9271.

Guerini, A.E., Triggiani, L., Maddalo, M., Bonù, M.L., Frassine, F., Baiguini, A., Alghisi, A., Tomasini, D., Borghetti, P., Pasinetti, N., Bresciani, R., Magrini, S.M., Buglione, M., 2019. Mebendazole as a Candidate for Drug Repurposing in Oncology: An Extensive Review of Current Literature. Cancers 11, 1284. https://doi.org/10.3390/cancers11091284

Guo, D., Meng, Y., Jiang, X., Lu, Z., 2023. Hexokinases in cancer and other pathologies. Cell Insight 2, 100077. https://doi.org/10.1016/j.cellin.2023.100077

Guo, G.F., Cai, Y.C., Zhang, B., Xu, R.H., Qiu, H.J., Xia, L.P., Jiang, W.Q., Hu, P.L., Chen, X.X., Zhou, F.F., Wang, F., 2011. Overexpression of SGLT1 and EGFR in colorectal cancer showing a correlation with the prognosis. Med. Oncol. 28, 197–203. https://doi.org/10.1007/s12032-010-9696-8

Halgren, T., 1996. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. J. Comput. Chem.

Han, J., Zhang, L., Guo, H., Wysham, W.Z., Roque, D.R., Willson, A.K., Sheng, X., Zhou, C., Bae-Jump, V.L., 2015. Glucose promotes cell proliferation, glucose uptake and invasion in endometrial cancer cells via AMPK/mTOR/S6 and MAPK signaling. Gynecol. Oncol. 138, 668–675. https://doi.org/10.1016/j.ygyno.2015.06.036

Han, L., Ma, Q., Li, J., Liu, H., Li, W., Ma, G., Xu, Q., Zhou, S., Wu, E., 2011. High Glucose Promotes Pancreatic Cancer Cell Proliferation via the Induction of EGF Expression and Transactivation of EGFR. PLOS ONE 6, e27074. https://doi.org/10.1371/journal.pone.0027074

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of Cancer: The Next Generation. Cell 144, 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hatami, H., Sajedi, A., Mir, S.M., Memar, M.Y., 2023. Importance of lactate dehydrogenase (LDH) and monocarboxylate transporters (MCTs) in cancer cells. Health Sci. Rep. 6, e996. https://doi.org/10.1002/hsr2.996

He, J., Qi, Z., Zhang, X., Yang, Y., Liu, F., Zhao, G., Wang, Z., 2019. Aurora kinase B inhibitor barasertib (AZD1152) inhibits glucose metabolism in gastric cancer cells. Anticancer. Drugs 30, 19–26. https://doi.org/10.1097/CAD.0000000000000684

Hirayama, A., Kami, K., Sugimoto, M., Sugawara, M., Toki, N., Onozuka, H., Kinoshita, T., Saito, N., Ochiai, A., Tomita, M., Esumi, H., Soga, T., 2009. Quantitative Metabolome

Profiling of Colon and Stomach Cancer Microenvironment by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. Cancer Res. 69, 4918–4925.

Hu, Z.-Y., Xiao, L., Bode, A.M., Dong, Z., Cao, Y., 2014. Glycolytic genes in cancer cells are more than glucose metabolic regulators. J. Mol. Med. 92, 837–845. https://doi.org/10.1007/s00109-014-1174-x

Joshi, S.S., Badgwell, B.D., 2021. Current treatment and recent progress in gastric cancer. CA. Cancer J. Clin. 71, 264–279. https://doi.org/10.3322/caac.21657

Karlsson, M., Ehinger, J.K., Piel, S., Sjövall, F., Henriksnäs, J., Höglund, U., Hansson, M.J., Elmér, E., 2016. Changes in energy metabolism due to acute rotenone-induced mitochondrial complex I dysfunction – An in vivo large animal model. Mitochondrion 31, 56–62. https://doi.org/10.1016/j.mito.2016.10.003

Karra, N., Cohen, R., Berlin, M., Dinavitser, N., Koren, G., Berkovitch, M., 2016. Safety of Mebendazole Use During Lactation: A Case Series Report. Drugs RD 16, 251–254. https://doi.org/10.1007/s40268-016-0142-z

Keystone, J.S., Murdoch, J.K., 1979. Mebendazole. Ann. Intern. Med. 91, 582–586. https://doi.org/10.7326/0003-4819-91-4-582

Kim, E.-Y., Chung, T.-W., Han, C.W., Park, S.Y., Park, K.H., Jang, S.B., Ha, K.-T., 2019. A Novel Lactate Dehydrogenase Inhibitor, 1-(Phenylseleno)-4-(Trifluoromethyl) Benzene, Suppresses Tumor Growth through Apoptotic Cell Death. Sci. Rep. 9, 3969. https://doi.org/10.1038/s41598-019-40617-3

Kralova, V., Hanušová, V., Caltová, K., Špaček, P., Hochmalová, M., Skálová, L., Rudolf, E., 2018. Flubendazole and mebendazole impair migration and epithelial to mesenchymal transition in oral cell lines. Chem. Biol. Interact. 293, 124–132. https://doi.org/10.1016/j.cbi.2018.07.026

Kulkarni, P., Makadia, V., Gondhale, P., Bhosale, R., Yewale, S., Padmanabhan, S., 2022. Novel Hexokinase Inhibition by Glycyrrhiza glabra: Therapeutic Uses in COVID. Journal of Medicine and Public Health 3, 50–58. https://doi.org/10.2174/138955752166621021016023 7

Laclette, J.P., Guerra, G., Zetina, C., 1980. Inhibition of tubulin polymerization by mebendazole. Biochem. Biophys. Res. Commun. 92, 417–423. https://doi.org/10.1016/0006-291x(80)90349-6

Lai, B., Xiao, Y., Pu, H., Cao, Q., Jing, H., Liu, X., 2012. Overexpression of SGLT1 is correlated with tumor development and poor prognosis of ovarian carcinoma. Arch. Gynecol. Obstet. 285, 1455–1461. https://doi.org/10.1007/s00404-011-2166-5

Lánczky, A., Győrffy, B., 2021. Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation. J. Med. Internet Res. 23, e27633. https://doi.org/10.2196/27633

Leal, M.F., Nascimento, J.L.M. do, Silva, C.E.A. da, Lamarão, M.F.V., Calcagno, D.Q., Khayat, A.S., Assumpção, P.P., Cabral, I.R., Smith, M. de A.C., Burbano, R.R., 2009. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. Cancer Genet. Cytogenet. 195, 85–91. https://doi.org/10.1016/j.cancergencyto.2009.04.020

Li, Q., Demir, S., Del Río-Álvarez, Á., Maxwell, R., Wagner, A., Carrillo-Reixach, J., Armengol, C., Vokuhl, C., Häberle, B., von Schweinitz, D., Schmid, I., Cairo, S., Kappler, R., 2022. Targeting the Unwindosome by Mebendazole Is a Vulnerability of Chemoresistant Hepatoblastoma. Cancers 14, 4196. https://doi.org/10.3390/cancers14174196

Li, T., Tan, X., Yang, R., Miao, Y., Zhang, M., Xi, Y., Guo, R., Zheng, M., Li, B., 2020. Discovery of novel glyceraldehyde-3-phosphate dehydrogenase inhibitor via docking-based virtual screening. Bioorganic Chem. 96, 103620. https://doi.org/10.1016/j.bioorg.2020.103620

Li, Y., Thomas, D., Deutzmann, A., Majeti, R., Felsher, D.W., Dill, D.L., 2019. Mebendazole for Differentiation Therapy of Acute Myeloid Leukemia Identified by a Lineage Maturation Index. Sci. Rep. 9, 16775. https://doi.org/10.1038/s41598-019-53290-3

Li, Y., Xu, C., Wang, B., Xu, F., Ma, F., Qu, Y., Jiang, D., Li, K., Feng, J., Tian, S., Wu, X., Wang, Y., Liu, Yang, Qin, Z., Liu, Yalan, Qin, J., Song, Q., Zhang, X., Sujie, A., Huang, J., Liu, T., Shen, K., Zhao, J.-Y., Hou, Y., Ding, C., 2022. Proteomic characterization of gastric cancer response to chemotherapy and targeted therapy reveals potential therapeutic strategies. Nat. Commun. 13, 5723. https://doi.org/10.1038/s41467-022-33282-0

Luengo, A., Gui, D.Y., Vander Heiden, M.G., 2017. Targeting Metabolism for Cancer Therapy. Cell Chem. Biol. 24, 1161–1180. https://doi.org/10.1016/j.chembiol.2017.08.028

Lugli, E., Roederer, M., Cossarizza, A., 2010. Data analysis in flow cytometry: the future just started. Cytom. Part J. Int. Soc. Anal. Cytol. 77, 705–713. https://doi.org/10.1002/cyto.a.20901

Machlowska, J., Baj, J., Sitarz, M., Maciejewski, R., Sitarz, R., 2020. Gastric Cancer: Epidemiology, Risk Factors, Classification, Genomic Characteristics and Treatment Strategies. Int. J. Mol. Sci. 21, 4012. https://doi.org/10.3390/ijms21114012

Martin, H.-J., Kornmann, F., Fuhrmann, G.F., 2003. The inhibitory effects of flavonoids and antiestrogens on the Glut1 glucose transporter in human erythrocytes. Chem. Biol. Interact. 146, 225–235. https://doi.org/10.1016/j.cbi.2003.06.001

Mesquita, F.P., Moreira-Nunes, C.A., da Silva, E.L., Lima, L.B., Daniel, J.P., Zuerker, W.J., Brayner, M., de Moraes, M.E.A., Montenegro, R.C., 2020. MAPK14 (p38α) inhibition effects against metastatic gastric cancer cells: A potential biomarker and pharmacological target. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 66, 104839. https://doi.org/10.1016/j.tiv.2020.104839

Mesquita, F.P., Pinto, L.C., Soares, B.M., de Sousa Portilho, A.J., da Silva, E.L., de Farias Ramos, I.N., Khayat, A.S., Moreira-Nunes, C.A., Bezerra, M.M., de Lucas Chazin, E., Vasconcelos, T.R.A., Burbano, R.M.R., de Moraes, M.E.A., Montenegro, R.C., 2018. Small benzothiazole molecule induces apoptosis and prevents metastasis through DNA interaction and c-MYC gene supression in diffuse-type gastric adenocarcinoma cell line. Chem. Biol. Interact. 294, 118–127. https://doi.org/10.1016/j.cbi.2018.08.006

Miao, Z.-F., Chen, H., Wang, Z.-N., Ji, J.-F., Liang, H., Xu, H.-M., Wang, J., 2022. Progress and remaining challenges in comprehensive gastric cancer treatment. Holist. Integr. Oncol. 1, 4. https://doi.org/10.1007/s44178-022-00002-z

Min, K.-W., Kim, D.-H., Son, B.K., Moon, K.M., Kim, S.M., Rahaman, M.I., Kim, S.W., Kim, E.-K., Kwon, M.J., Koh, Y.W., Oh, I.H., 2021. High SLC2A1 expression associated with

suppressing CD8 T cells and B cells promoted cancer survival in gastric cancer. PLOS ONE 16, e0245075. https://doi.org/10.1371/journal.pone.0245075

Moindjie, H., Rodrigues-Ferreira, S., Nahmias, C., 2021. Mitochondrial Metabolism in Carcinogenesis and Cancer Therapy. Cancers 13, 3311. https://doi.org/10.3390/cancers13133311

Mukhopadhyay, T., Sasaki, J., Ramesh, R., Roth, J.A., 2002. Mebendazole Elicits a Potent Antitumor Effect on Human Cancer Cell Lines Both in Vitro and in Vivo. Clin. Cancer Res. 8, 2963–2969.

Noh, M.A.A., Rahiman, S.S.F., Wahab, H.A., Gazzali, A.M., 2021. Discovery of new targeting agents against GAPDH receptor for antituberculosis drug delivery. J. Basic Clin. Physiol. Pharmacol. 32, 715–722. https://doi.org/10.1515/jbcpp-2020-0435

Nowak, N., Kulma, A., Gutowicz, J., 2018. Up-regulation of key glycolysis proteins in cancer development. Open Life Sci. 13, 569–581. https://doi.org/10.1515/biol-2018-0068

Nygren, P., Fryknäs, M., Agerup, B., Larsson, R., 2013. Repositioning of the anthelmintic drug mebendazole for the treatment for colon cancer. J. Cancer Res. Clin. Oncol. 139, 2133–2140. https://doi.org/10.1007/s00432-013-1539-5

Pacchiana, R., Mullappilly, N., Pinto, A., Bova, S., Forciniti, S., Cullia, G., Dalla Pozza, E., Bottani, E., Decimo, I., Dando, I., Bruno, S., Conti, P., Donadelli, M., 2022. 3-Bromo-Isoxazoline Derivatives Inhibit GAPDH Enzyme in PDAC Cells Triggering Autophagy and Apoptotic Cell Death. Cancers 14, 3153. https://doi.org/10.3390/cancers14133153

Pandey, S.K., Yadav, S., Goel, Y., Temre, M.K., Singh, V.K., Singh, S.M., 2019. Molecular docking of anti-inflammatory drug diclofenac with metabolic targets: Potential applications in cancer therapeutics. J. Theor. Biol. 465, 117–125. https://doi.org/10.1016/j.jtbi.2019.01.020

Pantziarka, P., Verbaanderd, C., Sukhatme, V., Rica Capistrano, I., Crispino, S., Gyawali, B., Rooman, I., Van Nuffel, A.M., Meheus, L., Sukhatme, V.P., Bouche, G., 2018. ReDO_DB: the repurposing drugs in oncology database. Ecancermedicalscience 12, 886. https://doi.org/10.3332/ecancer.2018.886

Patil, V.M., Menon, N., Chatterjee, A., Tonse, R., Choudhari, A., Mahajan, A., Puranik, A.D., Epari, S., Jadhav, M., Pathak, S., Peelay, Z., Walavalkar, R., Muthuluri, H.K., Krishna, M.R., Chandrasekharan, A., Pande, N., Gupta, T., Banavali, S., Jalali, R., 2022. Mebendazole plus lomustine or temozolomide in patients with recurrent glioblastoma: A randomised open-label phase II trial. eClinicalMedicine 49. https://doi.org/10.1016/j.eclinm.2022.101449

Pinto, L.C., de Fátima Aquino Moreira-Nunes, C., Soares, B.M., Burbano, R.M.R., de Lemos, J.A.R., Montenegro, R.C., 2017. Mebendazole, an antiparasitic drug, inhibits drug transporters expression in preclinical model of gastric peritoneal carcinomatosis. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 43, 87–91. https://doi.org/10.1016/j.tiv.2017.06.007

Pinto, L.C., Mesquita, F.P., Soares, B.M., da Silva, E.L., Puty, B., de Oliveira, E.H.C., Burbano, R.R., Montenegro, R.C., 2019. Mebendazole induces apoptosis via C-MYC inactivation in malignant ascites cell line (AGP01). Toxicol. In Vitro 60, 305–312. https://doi.org/10.1016/j.tiv.2019.06.010

Pinto, L.C., Soares, B.M., Pinheiro, J. de J.V., Riggins, G.J., Assumpção, P.P., Burbano, R.M.R., Montenegro, R.C., 2015. The anthelmintic drug mebendazole inhibits growth, migration and invasion in gastric cancer cell model. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 29, 2038–2044. https://doi.org/10.1016/j.tiv.2015.08.007

Qiao, T., Xiong, Y., Feng, Y., Guo, W., Zhou, Y., Zhao, J., Jiang, T., Shi, C., Han, Y., 2021. Inhibition of LDH-A by Oxamate Enhances the Efficacy of Anti-PD-1 Treatment in an NSCLC Humanized Mouse Model. Front. Oncol. 11.

Rampersad, S.N., 2012. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. Sensors 12, 12347–12360. https://doi.org/10.3390/s120912347

Razzak, R.A., Alshaiji, A.F., Qareeballa, A.A., Mohamed, M.W., Bagust, J., Docherty, S., 2018. High-normal blood glucose levels may be associated with decreased spatial perception in young healthy adults. PLoS ONE 13. https://doi.org/10.1371/journal.pone.0199051

San-Millán, I., Brooks, G.A., 2017. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. Carcinogenesis 38, 119–133. https://doi.org/10.1093/carcin/bgw127

Sasaki, J., Ramesh, R., Chada, S., Gomyo, Y., Roth, J.A., Mukhopadhyay, T., 2002. The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells. Mol. Cancer Ther. 1, 1201–1209.

Schiliro, C., Firestein, B.L., 2021. Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation. Cells 10, 1056. https://doi.org/10.3390/cells10051056

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73

Sheng, S.L., Liu, J.J., Dai, Y.H., Sun, X.G., Xiong, X.P., Huang, G., 2012. Knockdown of lactate dehydrogenase A suppresses tumor growth and metastasis of human hepatocellular carcinoma. FEBS J. 279, 3898–3910. https://doi.org/10.1111/j.1742-4658.2012.08748.x

Shibata, S., Sogabe, S., Miwa, M., Fujimoto, T., Takakura, N., Naotsuka, A., Kitamura, S., Kawamoto, T., Soga, T., 2021. Identification of the first highly selective inhibitor of human lactate dehydrogenase B. Sci. Rep. 11, 21353. https://doi.org/10.1038/s41598-021-00820-7

Shima, T., Taniguchi, K., Tokumaru, Y., Inomata, Y., Arima, J., Lee, S.-W., Takabe, K., Yoshida, K., Uchiyama, K., 2022. Glucose transporter-1 inhibition overcomes imatinib resistance in gastrointestinal stromal tumor cells. Oncol. Rep. 47, 1–13. https://doi.org/10.3892/or.2021.8218

Skibinski, C.G., Williamson, T., Riggins, G.J., 2018. Mebendazole and radiation in combination increase survival through anticancer mechanisms in an intracranial rodent model of malignant meningioma. J. Neurooncol. 140, 529–538. https://doi.org/10.1007/s11060-018-03009-7

Sobiepanek, A., Paone, A., Cutruzzolà, F., Kobiela, T., 2021. Biophysical characterization of melanoma cell phenotype markers during metastatic progression. Eur. Biophys. J. 50, 523–542. https://doi.org/10.1007/s00249-021-01514-8

Song, Z., Wu, Y., Yang, J., Yang, D., Fang, X., 2017. Progress in the treatment of advanced gastric cancer. Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med. 39, 1010428317714626. https://doi.org/10.1177/1010428317714626

Stine, Z.E., Schug, Z.T., Salvino, J.M., Dang, C.V., 2022. Targeting cancer metabolism in the era of precision oncology. Nat. Rev. Drug Discov. 21, 141–162. https://doi.org/10.1038/s41573-021-00339-6

Strober, W., 2015. Trypan Blue Exclusion Test of Cell Viability. Curr. Protoc. Immunol. 111, A3.B.1-A3.B.3. https://doi.org/10.1002/0471142735.ima03bs111

Sun, Y., Tao, C., Yu, F., Yang, W., Shan, Y., Yu, Z., Shi, H., Zhou, M., Zhang, Q., Wu, H., 2016. Discovery of a novel human lactate dehydrogenase A (LDHA) inhibitor as an anti-proliferation agent against MIA PaCa-2 pancreatic cancer cells. RSC Adv. 6, 23218–23222. https://doi.org/10.1039/C5RA27736A

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2021a. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer J. Clin. 71, 209–249. https://doi.org/10.3322/caac.21660

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2021b. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. https://doi.org/10.3322/caac.21660

Tang, Z., Li, Chenwei, Kang, B., Gao, G., Li, Cheng, Zhang, Z., 2017. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res. 45, W98–W102. https://doi.org/10.1093/nar/gkx247

Thakur, R.K., Patel, S.P., 2022. Mebendazole, StatPearls [Internet]. StatPearls Publishing.

Vakifahmetoglu-Norberg, H., Ouchida, A.T., Norberg, E., 2017. The role of mitochondria in metabolism and cell death. Biochem. Biophys. Res. Commun. 482, 426–431. https://doi.org/10.1016/j.bbrc.2016.11.088

Vasconcelos, Z.S., Ralph, A.C.L., Calcagno, D.Q., Dos Santos Barbosa, G., do Nascimento Pedrosa, T., Antony, L.P., de Arruda Cardoso Smith, M., de Lucas Chazin, E., Vasconcelos, T.R.A., Montenegro, R.C., de Vasconcellos, M.C., 2018. Anticancer potential of benzothiazolic derivative (E)-2-((2-(benzo[d]thiazol-2-yl)hydrazono)methyl)-4-nitrophenol against melanoma cells. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 50, 225–235. https://doi.org/10.1016/j.tiv.2018.03.001

Wang, J., Ying, X., Wang, L., 2017. Prognostic merit of glucose transporter GLUT1 expression status in gastric cancer. Ann. Oncol. 28, x14. https://doi.org/10.1093/annonc/mdx653.027

Warburg, O., 1956. On the origin of cancer cells. Science 123, 309–314. https://doi.org/10.1126/science.123.3191.309

Warburg, O., Dickens, F., 1931. The metabolism of tumours. Investigations from the Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Edited by Otto Warburg, Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Translated from the German edition, with accounts of additional recent researches, by Frank Dickens, M.A., Ph.D., whole-time worker for the Medical Research Council, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Demy 8vo. Pp. 327 + xxix. Illustrated. 1930. London: Constable & Co. Ltd. 40s. net. BJS Br. J. Surg. 19, 168–168. https://doi.org/10.1002/bjs.1800197331

Warburg, O., Wind, F., Negelein, E., 1927. THE METABOLISM OF TUMORS IN THE BODY. J. Gen. Physiol. 8, 519–530.

Williamson, T., Bai, R.-Y., Staedtke, V., Huso, D., Riggins, G.J., 2016. Mebendazole and a non-steroidal anti-inflammatory combine to reduce tumor initiation in a colon cancer preclinical model. Oncotarget 7, 68571–68584. https://doi.org/10.18632/oncotarget.11851

Williamson, T., de Abreu, M.C., Trembath, D.G., Brayton, C., Kang, B., Mendes, T.B., de Assumpção, P.P., Cerutti, J.M., Riggins, G.J., 2021. Mebendazole disrupts stromal desmoplasia and tumorigenesis in two models of pancreatic cancer. Oncotarget 12, 1326–1338. https://doi.org/10.18632/oncotarget.28014

Williamson, T., Mendes, T.B., Joe, N., Cerutti, J.M., Riggins, G.J., 2020. Mebendazole inhibits tumor growth and prevents lung metastasis in models of advanced thyroid cancer. Endocr. Relat. Cancer 27, 123–136. https://doi.org/10.1530/ERC-19-0341

World Health Organization, R.O. for E., 2021. Repurposing of medicines – the underrated champion of sustainable innovation. Copenhagen.

Xie, R.-F., Liu, S., Yang, M., Xu, J.-Q., Li, Z.-C., Zhou, X., 2019. Effects and possible mechanism of Ruyiping formula application to breast cancer based on network prediction. Sci. Rep. 9, 5249. https://doi.org/10.1038/s41598-019-41243-9

Xintaropoulou, C., Ward, C., Wise, A., Marston, H., Turnbull, A., Langdon, S.P., 2015. A comparative analysis of inhibitors of the glycolysis pathway in breast and ovarian cancer cell line models. Oncotarget 6, 25677–25695. https://doi.org/10.18632/oncotarget.4499

Xu, D., Zhou, Y., Xie, X., He, L., Ding, J., Pang, S., Shen, B., Zhou, C., 2020. Inhibitory effects of canagliflozin on pancreatic cancer are mediated via the downregulation of glucose transporter-1 and lactate dehydrogenase A. Int. J. Oncol. 57, 1223–1233. https://doi.org/10.3892/ijo.2020.5120

Yan, S., Wang, Y., Chen, M., Li, G., Fan, J., 2015. Deregulated SLC2A1 Promotes Tumor Cell Proliferation and Metastasis in Gastric Cancer. Int. J. Mol. Sci. 16, 16144–16157. https://doi.org/10.3390/ijms160716144

Yang, J., 2020. Identification of novel biomarkers, MUC5AC, MUC1, KRT7, GAPDH, CD44 for gastric cancer. Med. Oncol. Northwood Lond. Engl. 37, 34. https://doi.org/10.1007/s12032-020-01362-0

Zhang, F., Li, Y., Zhang, H., Huang, E., Gao, L., Luo, W., Wei, Q., Fan, J., Song, D., Liao, J., Zou, Y., Liu, F., Liu, J., Huang, J., Guo, D., Ma, C., Hu, X., Li, L., Qu, X., Chen, L., Yu, X., Zhang, Z., Wu, T., Luu, H.H., Haydon, R.C., Song, J., He, T.-C., Ji, P., 2017. Anthelmintic mebendazole enhances cisplatin's effect on suppressing cell proliferation and promotes

differentiation of head and neck squamous cell carcinoma (HNSCC). Oncotarget 8, 12968–12982. https://doi.org/10.18632/oncotarget.14673

Zhang, L., Li, Y., Dai, Y., Wang, D., Wang, X., Cao, Y., Liu, W., Tao, Z., 2021. Glycolysis-related gene expression profiling serves as a novel prognosis risk predictor for human hepatocellular carcinoma. Sci. Rep. 11, 18875. https://doi.org/10.1038/s41598-021-98381-2

Zhang, W., Wang, C., Hu, X., Lian, Y., Ding, C., Ming, L., 2022. Inhibition of LDHA suppresses cell proliferation and increases mitochondrial apoptosis via the JNK signaling pathway in cervical cancer cells. Oncol. Rep. 47, 1–11. https://doi.org/10.3892/or.2022.8288

Zheng, M., Wu, C., Yang, K., Yang, Y., Liu, Y., Gao, S., Wang, Q., Li, C., Chen, L., Li, H., 2021. Novel selective hexokinase 2 inhibitor Benitrobenrazide blocks cancer cells growth by targeting glycolysis. Pharmacol. Res. 164, 105367. https://doi.org/10.1016/j.phrs.2020.105367

Zhou, Y., Ding, B.-Z., Lin, Y.-P., Wang, H.-B., 2018. MiR-34a, as a suppressor, enhance the susceptibility of gastric cancer cell to luteolin by directly targeting HK1. Gene 644, 56–65. https://doi.org/10.1016/j.gene.2017.10.046

Zhu, X., Jin, C., Pan, Q., Hu, X., 2021. Determining the quantitative relationship between glycolysis and GAPDH in cancer cells exhibiting the Warburg effect. J. Biol. Chem. 296, 100369. https://doi.org/10.1016/j.jbc.2021.100369

Zhuang, Y., Chan, D.K., Haugrud, A.B., Miskimins, W.K., 2014. Mechanisms by Which Low Glucose Enhances the Cytotoxicity of Metformin to Cancer Cells Both In Vitro and In Vivo. PLOS ONE 9, e108444. https://doi.org/10.1371/journal.pone.0108444

Zou, T., Liu, X., Ding, S., Xing, J., 2010. Evaluation of sperm mitochondrial function using rh123/PI dual fluorescent staining in asthenospermia and oligoasthenozoospermia. J. Biomed. Res. 24, 404–410. https://doi.org/10.1016/S1674-8301(10)60054-1

SUPPLEMENTARY MATERIALS

Supplementary Table 1. Coordination and size of grid box used in the docking analysis on the DockThor server.

Proteins	PDB ID	Coordinations			Grid box
		X	Y	Z	
GLUT1	6THA	19.195	53.868	7.646	40 x 40 x 40
HK1	1CZA	34.852	49.673	21.177	40 x 40 x 40
GAPDH	6YND	-61.044	12.921	87.364	40 x 40 x 40
LDHA	5W8J	34.163	13.489	32.020	40 x 40 x 40

Supplementary Table 2. Docking Scores provided by the DockThor server for the interaction of MBZ and GLUT1 alone and with ligands.

Ligantes	Score (kcal/mol)			
	Docking Assays			
	Alone	Only native ligand	MBZ in the presence of ligands	Ligands in the presence of MBZ
MBZ	**-8.560**	*	**-8.601**	**-8.560**
P33	*	**-8.457**	-8.457	-7.362
BNG	*	-7.743	-7.743	-8.228
CL	*	-5.824	-5.824	-5.873

*Absent in the docking.
MBZ: Mebendazole. P33: Heptaethylene glycol. BNG: Nonyl beta-D-glucopyranosid. CL: chloride ion.

Supplementary Table 3. Docking Scores provided by the DockThor server for the interaction of MBZ and HK1 alone and with ligands.

Ligands	Score (kcal/mol)			
	Docking Assays			
	Alone	Only native ligand	MBZ in the presence of ligands	Ligands in the presence of MBZ
MBZ	**-7.457**	*	**-7.592**	**-7.457**
ADP	*	**-7.486**	-7.486	-6.932
G6P	*	-6.438	-6.989	-6.404
GLC	*	-5.770	-6.920	-5.776

*Absent in the docking.
MBZ: Mebendazole. ADP: Adenosine 5′-diphosphate. G6P: Glucose-6-phosphate dehydrogenase. GLC: α- D-Glucopyranose.

Supplementary Table 4. Docking Scores provided by the DockThor server for the interaction of MBZ and GAPDH alone and with ligands.

Score (kcal/mol)				
Ligands	**Docking Assay**			
	Alone	Only native ligand	MBZ in the presence of ligands	Ligands in the presence of MBZ
MBZ	**-7.195**	*	**-7.230**	**-7.195**
XPE	*	**-6.904**	-6.742	-6.862

*Absent in the docking.
MBZ: Mebendazole. XPE: Decaethylene glycol.

Supplementary Table 5. Docking Scores provided by the DockThor server for the interaction of MBZ and LDHA alone and with ligands.

Score (kcal/mol)				
Ligands	**Docking Assay**			
	Alone	Only native ligand	MBZ in the presence of ligands	Ligands in the presence of MBZ
MBZ	**-7.550**	*	-7.405	-7.550
DMSO	*	-6.441	-5.955	-6.180
9Y7	*	**-8.360**	**-8.360**	**-8.401**
GOL	*	-6.170	-6.436	-6.070
MLA	*	-6.226	-6.057	-5.556

*Absent in the docking.
MBZ: Mebendazole. DMSO: Dimethyl sulfoxide. GOL: Glycerol. MLA: Malonic acid. 9Y7: 2-[3-[3,4-bis(fluoranyl)phenyl]-5-oxidanyl-4-[(4-sulfamoylphenyl)methyl]pyrazol-1-yl]-1,3-thiazole-4-carboxylic acid.

CAPÍTULO III

NUCLEOTIDE SYNTHESIS AS A THERAPEUTIC TARGET OF MEBENDAZOLE IN METASTATIC GASTRIC CANCER

EMERSON LUCENA DA SILVA

NUCLEOTIDE SYNTHESIS AS A THERAPEUTIC TARGET OF MEBENDAZOLE IN METASTATIC GASTRIC CANCER

Emerson Lucena da Silva[a†]; Felipe Pantoja Mesquita[a]; Francisco Laio de Oliveira[a]; Maria Elisabete Moraes[a], Pedro Filho Noronha Souza[a,b,c*]; Raquel Carvalho Montenegro[a,d]*.

[a] *Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Cel. Nunes de Melo, Fortaleza, Brazil.*

[b] *Visiting Researcher at the Cearense Foundation to Support Scientific and Technological Development*

[c] *National Institute of Science and Technology in Human Pathogenic Fungi, Ribeirão Preto, Brazil*

[d] *Red Latinoamericana de Implementación y Validación de guias clinicas Farmacogenomicas (RELIVAF), Brazil*

* Address correspondence to this author: Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Street Cel. Nunes de Melo, 1000 – Rodolfo Teófilo, Fortaleza, Ceará, Brazil. E-mail addresses of corresponding authors: rcm.montenegro@gmail.com (Raquel Carvalho Montenegro) and pedrofilhobio@gmail.com (Pedro Filho Noronha Souza).

Artigo submetido a revista internacional *Chemico-Biological Interactions*
(Qualis CAPES Ciências Biológicas II: A2)

ABSTRACT

The nucleotide biosynthetic pathway has major importance in maintaining tumor replication through DNA and RNA synthesis. Therefore, drugs targeting the inhibition of these pathways are important to the clinic, Mebendazole (MBZ) is an anthelmintic with antitumor activity in gastric cancer (GC) metastatic cell line AGP-01, recent studies showed its effect in the glucose metabolic pathway, however, its influence in nucleotide synthesis remains poorly understood. Thus, this study aims to investigate the role of MBZ in nucleotide synthetic pathways and to evaluate whether modulation in the expression of key targets for DNA/RNA synthesis is associated with its antiproliferative effect. Results showed that MBZ has antitumor activity similar to chemotherapy 5-FU in the GC cell line, however, evidenced less toxicity to non-tumoral cells. Gene expression analysis attested that the nucleotide metabolism target genes *PRPS1*, *TYMS*, *MTHFD1,* and *HPRT1* are hyperregulated in tumor samples and that the high expression of *TYMS*, *MTHFD1*, and *HPRT1* is correlated to a reduction in overall survival rates in GC patients. Remarkably, MBZ can reduce the expression of metabolic targets that are overpressed, lead to cell cycle arrest to G0/G1, and suppress the proliferation of AGP-01, this effect was related to a lower expression of metabolic target genes. Altogether, data evidenced that MBZ has a role in modulating the nucleotide synthesis pathways, being associated with its antiproliferative and selective effect in the AGP-01 GC cell line. Thus, this work contributes to the exploration and validation of new important pharmacological metabolic targets for the treatment of metastatic GC.

Keywords: Chemotherapy; DNA synthesis; Metabolic Pathway; Proliferation; Toxicity.

1 INTRODUCTION

According to Hanahan and Weinberg [1], cellular energy dysregulation is among the 10 characteristics of cancer development. Energy reprogramming is essential for tumor development, cellular energy metabolism needs to adapt to the large energy demand necessary for the uncontrolled proliferation of neoplastic cells. In the 1920s, researcher Otto Warburg demonstrated that cancer cells exhibited changes in their metabolism when compared to normal cells. Cellular hyperproliferation depends on an increase in the number of replicative cycles, including the synthesis of new cellular macromolecules such as DNA and RNA that support uncontrolled replication [1]. Genes related to nucleotide synthesis pathways are overexpressed in several types of cancer and have shown interest as new pharmacological targets in antitumoral therapy [2,3].

The study of the relationship between nucleotide metabolism and its correlation to cancer development and progression has been highlighted in clinical research [4,5]. The cytotoxic drugs, known as antimetabolic chemotherapies, 5-fluorouracil (5-FU), methotrexate, capecitabine, and cytarabine are examples of antitumoral compounds with clinical success that target nucleotide metabolism, leading to direct or indirect inhibition of purine and pyrimidine synthesis pathways and thus preventing the formation of RNA and/or DNA and cell replication [6,7]. Moreover, the search and study of new molecules targeting cellular metabolism and, more specifically, nucleotide metabolism can help in the discovery of new molecular targets for cancer therapy [8].

Two pathways responsible for nucleotide synthesis are known, the *de novo* pathway and the salvage pathway. These pathways are not correlated and are independent of each other mainly in terms of their mechanisms and regulation. While the *de novo* pathway synthesizes nucleotides using metabolites as amino acids and glucose derivatives as precursors, the salvage pathway recovers intermediates (nucleosides) from the degradation of nucleic acids, using them as precursors for nucleotide synthesis [9]. Due to the high proliferative potential of tumor cells, nucleotide metabolism via the *de novo* pathway is hyperregulated, but unlike non-malignant cells, the tumor can switch to the salvage pathway to maintain replication efficiency in case of bioenergetic need [8–10].

Due to the importance of these pathways for tumor growth, some enzymes from these pathways have been chosen as pharmacological targets for drugs with antitumor potential. Genes that code for enzymes of the *de novo* nucleotide pathways such as Phosphoribosyl

Pyrophosphate Synthetase 1 (*PRPS1*), Thymidylate Synthetase (*TYMS*), the Methylenetetrahydrofolate dehydrogenase/cyclohydrolase/synthase (*MTHFD1*), Dihydroorotate Dehydrogenase (*DHODH*), and the salvage pathway such as Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) have been described as hyper-regulated in cancer, being associated with an increase in the proliferative, migratory, and chemoresistance profile in different types of neoplasms [11–18]. Based on that, the analysis of genes related to nucleotide metabolism can lead to the discovery of new pharmacological targets in cancer therapy [19,20].

Benzimidazoles are a class of heterocyclic compounds that are formed by the fusion of benzene and imidazole, their first derivative was synthesized by Hobrecker in 1872 and since then a wide range of pharmacological activities have been associated with these compounds [21]. Mebendazole (MBZ) is an approved benzimidazole used in the clinic for the treatment of a broad spectrum of helminth infections with an effect on tubulin polymerization [22]. Furthermore, MBZ has proven extensive antitumor activity against lung [23], colon [24], medulloblastoma [25], melanoma [26], and gastric cancers [27–30], among other neoplasms [31].

Gastric cancer (GC) is among the most common neoplasms in the world, being considered the fifth most common type of cancer, affecting more than 1 million people (5.6% of cases). Considered the fourth most lethal cancer in 2020 worldwide, with around 770 deaths reported [32]. Our previous studies evidenced that GC lineages have highly deregulated metabolism, which is associated with their replicative and metastatic potential [33]. Recent data showed that MBZ has a role in gastric tumor metabolic modulation and that this is related to its highly antitumoral activity *in vitro* in metastatic AGP-01 cell lone [30], however, the role of MBZ in the nucleotide synthesis pathways has not yet been elucidated.

Therefore, this study aims to analyze the potential of MBZ as a modulator of genes in the purine and pyrimidine pathways, as well as to evaluate whether this modulation is associated with a decrease in the proliferative profile of the metastatic gastric adenocarcinoma cell line (AGP-01) and with its selective pharmacological profile. Thus, proposing to bring new evidence of MBZ as a tumoral metabolic regulator and its relationship with its antitumoral activity, shedding light on new pharmacological targets for the therapy of GC.

2 METHODS

2.1 Chemicals

Mebendazole (MBZ) (Medley®, 500 mg) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM. MBZ and 5-Fluorouracil (5-FU) (Libbs®, 10 mL) were stored at -20 °C until use. 5-FU is chemotherapy widely used in cancer and GC treatment, Its mechanism of action relies primarily on the inhibition of Thymidylate Synthase enzyme or misincorporating its biosynthetic metabolite into RNA and DNA, leading to inhibition of nucleotide synthetic pathways and cell death [34]. Therefore, this compound was chosen as the positive control for the inhibition of nucleotide synthesis during further *in vitro* experiments.

2.2 Cell culture and conditions

The metastatic gastric adenocarcinoma cell line AGP-01 [35], the non-tumoral non-tumoral gastric cell line MNP-01 [36], and the non-tumoral cell line derived from lung fibroblast MRC-5 [37] were used in this study. For the metabolic studies, cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco ®) with Low Glucose (LG, 5.5 mM) [33]. For use media, were added 10% (v/v) fetal bovine serum (Gibco®), 1% (v/v) penicillin (100 U mL-1), and streptomycin (100 mg mL-1) (Invitrogen®), and cell lines were maintained in a 5% CO_2 air-humidified atmosphere at 37 °C.

2.3 Cytotoxic and Seletive Index (SI) analysis by Alamar Blue assay

For cytotoxic evaluation, the Alamar Blue assay was used to define the half-maximal inhibitory concentration (IC_{50}) of MBZ [38]. AGP-01 and MNP-01 cells were seeded in a 96-well plate with $3x10^3$ cells per well. After attachment, cells were treated in a concentration-response curve (20 µM – 0.3125 µM) of MBZ and 5-FU for 72 hours. Then, the Alamar Blue solution (0.2 mg mL-1) (Merck®) was added to each well for 3 hours, and the fluorescence intensity (Ex/Em: 530/590 nm) was measured in a microplate reader (Beckman Coulter Microplate Reader DTX 880) and IC_{50} calculated.

The Selective Index (SI) of each compound was calculated based on the average between the drug's IC_{50} in the non-tumoral cell lines and the IC_{50} in the tumoral strain AGP-01 [39], as shown in equation 1.

$$IS = \frac{IC_{50}\ Non-tumoral\ cell\ line}{IC_{50}\ \ Tumoral\ cell\ line} \quad (1)$$

2.4 Gene expression analysis of potential targets in online databases

Next, we sought to investigate the expression profile of genes related to nucleotide metabolism pathway as promising targets in GC therapy in clinical samples and study its correlation with disease outcome through global gene expression analysis in free access databases to transcriptome data from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and Genotype-Tissue Expression (GTEx). Expression patterns in tumor samples and the correlation of gene expression with the survival rate of *PRPS1*, *DHODH*, *TYMS*, *MTHFD1*, and *HPRT1* genes were assessed by GEPIA (http://gepia. cancer-pku.cn/) and Kaplan Meier plotter (https://kmplot.com/ analysis/) softwares, respectively [40,41].

2.5 Total RNA extraction and transcript analysis by qRT-PCR

To better comprehend the influence of metabolic targets of the nucleotide synthesis pathway *PRPS1*, *HPRT1*, *TYMS*, *MTHFD1*, and *DHODH* in gastric carcinogenesis and their modulation by MBZ, quantitative real-time PCR (qRT-PCR) was performed. Thus, gastric cell lines AGP-01 and MNP-01 were plated in a 6 plate at the concentration of 7 x 10^4 cells per well. After attachment, cells were treated with MBZ (0.1 µM) or 5-FU (1 µM) for 24 hours. Then, the total RNA was extracted using TRIzol® Reagent (Life Technologies®, USA). The RNA concentration and quality were determined using NanoDrop (Thermo Scientific), the reverse transcription was performed using a High-Capacity cDNA kit, and all assays followed the manufacturer's protocol (Life Technologies, USA).

The qRT-PCR was executed by the Fast SyberGreen kit (Applied Biosystems, USA). The Relative mRNA expression levels of *PRPS1* (NM_002764.4), *HPRT1* (NM_000194.3), *TYMS* (NM_001071.4), *MTHFD1* (NM_005956.4), and *DHODH* (NM_001361.5) were normalized and determined using *RPLP0* (NM_001002.4) gene as a normalizer control. Primer efficiency > 95% was determined for all genes described.

All requirements proposed in Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed [42]. The expression level was calculated using the $2^{-\Delta\Delta CT}$ method [43].

2.6 Cell cycle analysis

The nucleotide metabolism supports RNA synthesis and DNA replication, directly influencing cell growth and division [44], previous studies showed morphologic and apoptosis-inducing over time with MBZ's treatment with MBZ [30]. Thus, we sought to investigate the

role of MBZ in the AGP-01 cell cycle at different times and evaluate the relationship of this event with the inhibition of nucleotide pathways. AGP-01 cells were seeded in ($7x10^4$ cells/well) in a 24-well plate and, after 24 hours, for cell attachment, treated with MBZ (0.1 µM) or 5-FU (1 µM) for 24, 48 and 72 hours. Following, cells were fixed in 80% ethanol solution at 4 ºC overnight, then centrifugated, and the pellet was resuspended in a solution with propidium iodide (50 µg mL-1) for 30 minutes at 37 ºC. Cells were resuspended in PBS 1X, and DNA content was evaluated using flow cytometry (BD FACSVerse™). Ten thousand events were analyzed using FlowJo® software [36].

2.7 Clonogenic assay

To visualize the effect of MBZ treatment over time in AGP-01 cell proliferation, the clonogenic assay was performed [33,45,46]. AGP-01 cells ($1x10^2$ cells/well) cells were cultured in a 6-well plate and after 24 hours were treated with MBZ (0.1 µM) or 5-FU (1 µM) for 24, 48, and 72 hours. Then, cells were washed with PBS 1x and fixed in a solution with methanol, acetic acid, and water (1:1:8) for 30 minutes. Finally, colonies were stained with crystal violet (0.2%) for 30 minutes and photographed, the number of colonies was measured by the ImageJ software using the ColonyArea tool [47].

2.8 Statistical analyses

At least three independent experiments were performed in triplicate. Data are shown as mean ± standard deviation (SD). Normality distribution analysis was performed by the Kolmogorov- Smirnov test. The groups were compared by Student t-test or Analysis of Variance (ANOVA) followed by Bonferroni's post-test. Significant differences were considered with a confidence interval of 95% ($p<0.05$). GraphPad Prism 5.01 software was used for data analysis and graph design.

3 RESULTS

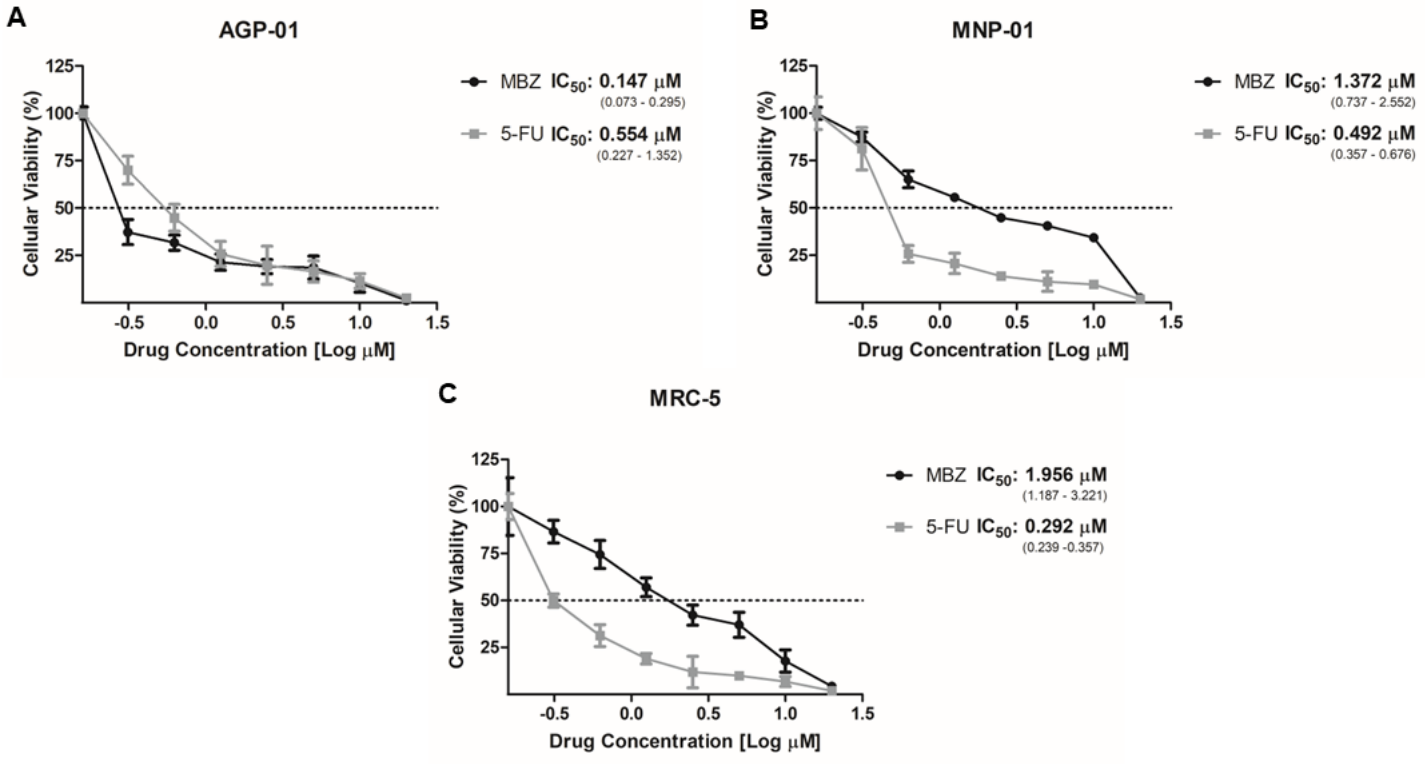
3.1 MBZ evidenced cytotoxic and selective effects in the AGP-01 GC cell

Firstly, we evaluated the cytotoxic potential of MBZ against GC cell line AGP-01 and compared its selectivity by using two non-tumoral cell lines, MNP-01 and MRC-5 after 72 hours of treatment. As shown in Figure 1, MBZ evidenced IC_{50} values of 0.147, 1.372, and 1.956 µM, in the AGP-01, MNP-01, and MRC5 respectively (Fig. 1A, 1B, and 1C).

Furthermore, the chemotherapy drug 5-FU also showed highly cytotoxic against AGP-01, with an IC_{50} of 0.554 µM against tumoral cell line AGP-01, however, 5-FU indicated to reduce the number of viable cells in low concentrations in non-tumoral cell lines MNP-01 and MRC-50, with IC_{50} values of 0.492 and 0.292 µM.

The selective index (SI) was calculated through the ratio between the IC_{50} of MBZ or 5-FU in the tumoral cell line AGP-01 and the IC_{50} in non-tumoral cell lines. Results evidenced that MBZ's SIs are 9.33 and 13.31 when compared to IC_{50} with MNP-01 and MRC-5, respectively (Fig. 1D). MBZ showed to be highly selective and that it needs a concentration around ten-fold higher to trigger any cytotoxic damage to non-tumor cells when compared to its activity in tumoral cell line. For the 5-FU drug, the SIs were below 1, evidencing that the drug is more cytotoxic to non-tumoral cell lines than to its tumoral target.

Figure 1. MBZ shows cytotoxic and selective effects on GC cell lines. Cells were treated in a concentration-response curve of MBZ or 5-FU in A) AGP-01, B) MNP-01, and C) MRC-5 cell lines for 72 hours, then Alamar Blue was added, and the half-maximal inhibitory concentration (IC_{50}) was estimated. D) The selective index was calculated based on the average between the drug's IC_{50} in the non-tumoral cell lines and the IC_{50} in the tumoral strain AGP-01. The IC_{50} values (µM) and confidence interval of 95% were obtained from three independent experiments. 5-FU: 5-fluorouracil. IC_{50}: Half-maximal inhibitory concentration. MBZ: Mebendazole.



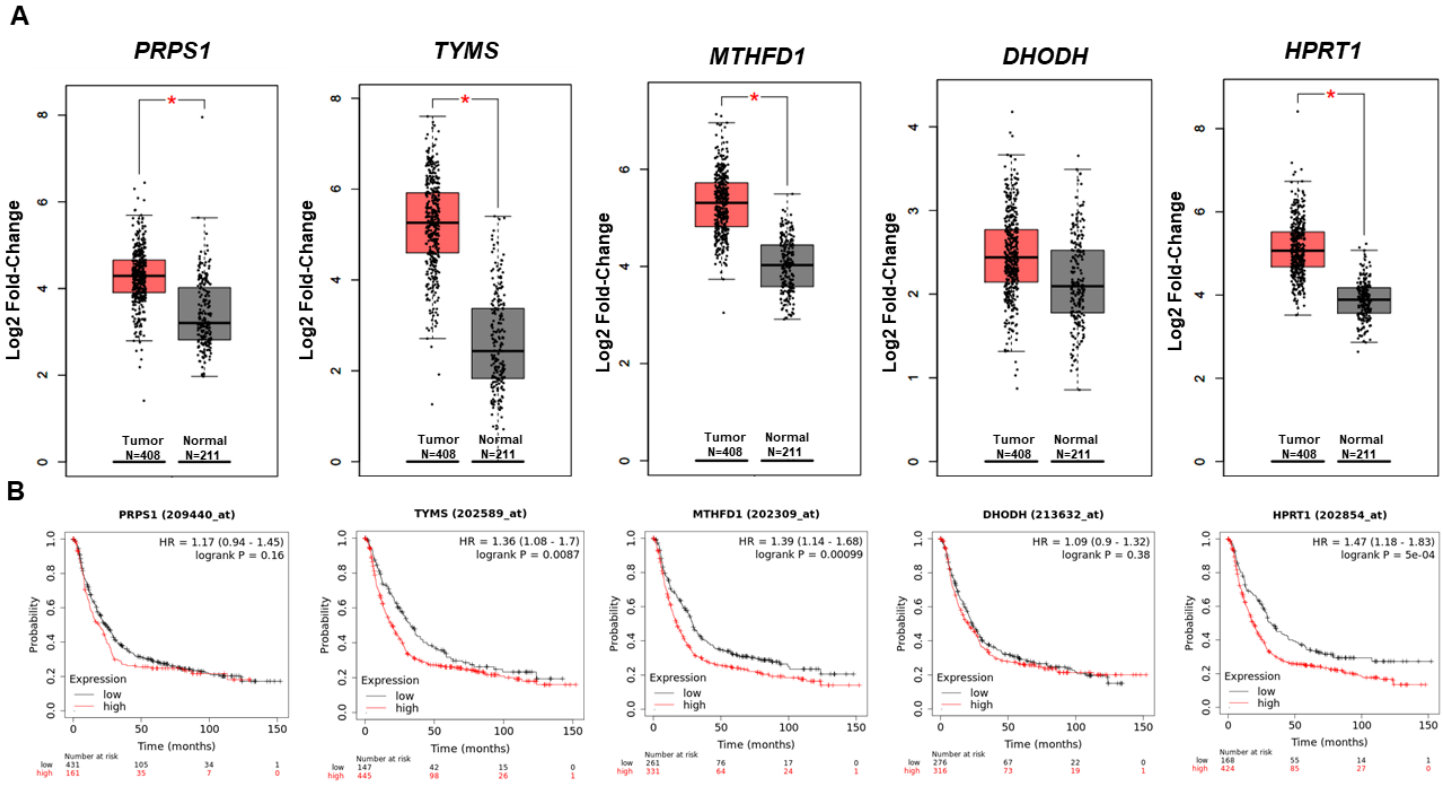
D

	Selective Index	
	MNP-01/AGP-01	MRC-5/AGP-01
MBZ	9.33	13.31
5-FU	0.88	0.53

3.2 Nucleotide metabolism pathway enzymes are targets of clinical interest for GC therapy

Then, we sought to investigate the expression of targets related to nucleotide metabolism to better understand their therapeutic potential in GC clinics. For that purpose, online databases that include expression data of clinical samples from TCGA, GTEx, GEO, and others were used. The analysis in the web server GEPIA showed that *PRPS1*, *TYMS*, *MTHFD1*, and *HPRT1* genes are significantly overexpressed in tumoral samples (N= 408) when compared to normal tissue (N= 211) (Fig. 2A). Furthermore, the Kaplan-Meier Plotter analysis in GC samples (N= 592) evidenced that the high expression of *TYMS*, *MTHFD1*, and *HRPT1* are correlated with reducing in the overall survival (OS) rate, indicating a worse prognosis in patients with GC (Fig. 2B). Therefore, the molecular targets analyzed have a substantial relevance in GC pathogenesis, showing that their pharmacological inhibition could improve the prognosis of the patients with GC.

Figure 2. Transcripts that codify for enzymes of the nucleotide metabolism pathway have a clinical interest in the pharmacological therapy for GC. The gene expression of targets related to nucleotide metabolism PRPS1, TYMS, MTHFD1, DHODH, and HPRT1 was analyzed by online gene expression databases. A) The GEPIA dataset shows the hyperexpression of genes in CG samples (red boxplot). B) Kaplan–Meier Plotter analysis evidenced reduced OS rate in patients with high gene expression of transcripts related to nucleotide metabolism pathway. Data are presented as boxplots, and statistical analysis was performed with ANOVA. Significant differences: *p < 0.05. HR: Hazard Ratio.

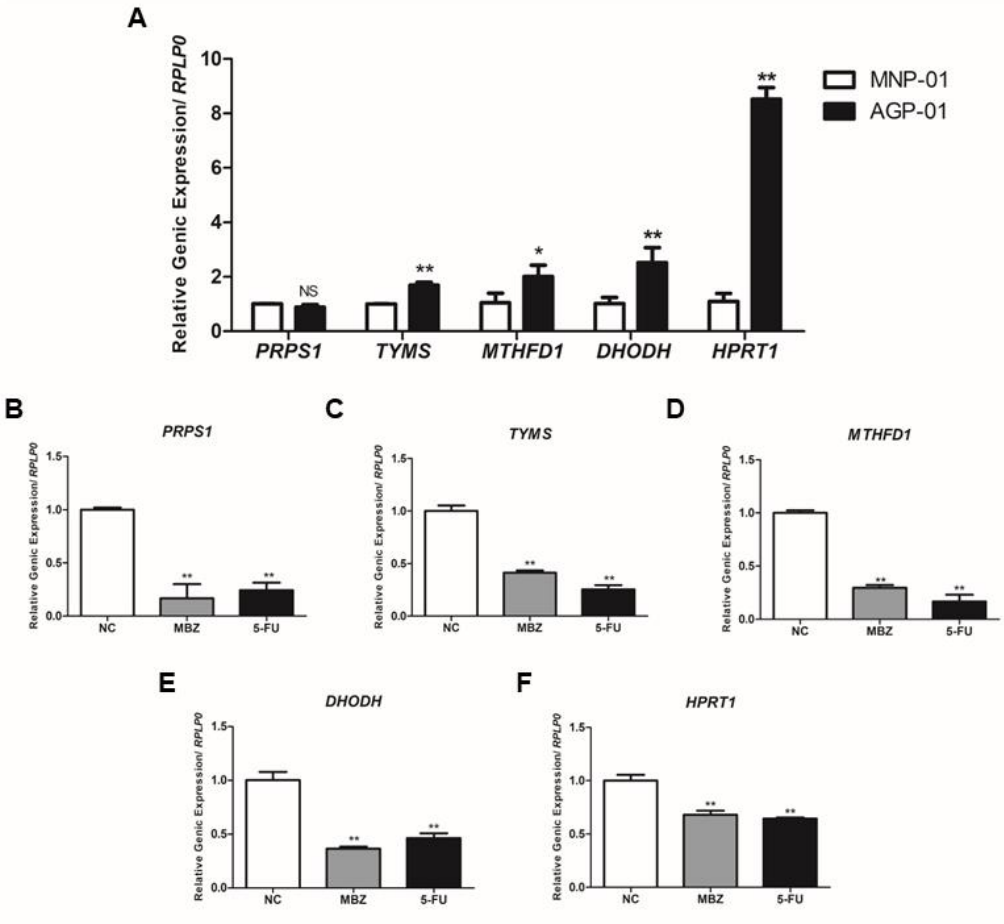


3.3 MBZ reduces gene expression of targets with clinical interest in the nucleotide metabolism

Next, we attempted to visualize the mRNA expression of *PRPS1*, *TYMS*, *MTHFD1*, *DHODH*, and *HPRT1* in the gastric cell lines used in this study. Then, the potential of MBZ in the modulation of the transcript levels of these genes *in vitro* was investigated after 24 hours of cell exposure.

As seen in Figure 3, the transcripts levels of *TYMS* ($p < 0.0001$), *MTHFD1* ($p < 0.001$), and *DHODH* ($p < 0.0001$) are overexpressed about two-fold and *HPRT1* ($p < 0.0001$) around eight-fold in AGP-01 when compared to non-tumoral gastric cell line MNP-01. Furthermore, MBZ reduces the mRNA levels of all targets evaluated significantly ($p < 0.0001$), at similar levels to those of the nucleotide synthesis inhibitor used in the clinic 5-FU.

Figure 3. MBZ reduces gene expression of overexpressed targets of nucleotide metabolism in the AGP-01 tumoral cell line. Total mRNA was extracted and the transcript levels of metabolic targets were compared between the A) non-tumoral cell line MNP-01 and GC cell line AGP-01. B) The effect in the transcript levels of B) *PRPS1*, C) *TYMS*, D) *MTHFD1*, E) *DHODH*, and F) *HPRT1* were also evaluated after 24 hours of AGP-01 cell exposure to MBZ. Gene expression was normalized by housekeeping gene RPLP0, and the MNP-01 (for A) or NC (for B-C) group was used as a calibrator of the experiment. Data are presented as the mean ± SD of three independent experiments, and statistical analysis was performed with the Student t-test (for A) and ANOVA followed by Bonferroni's post-test (for B-F). Significant differences: *$p < 0.001$, **$p < 0.0001$. 5-FU: 5-fluorouracil. MBZ: Mebendazole. NC: Negative control. NS: Not Significant.

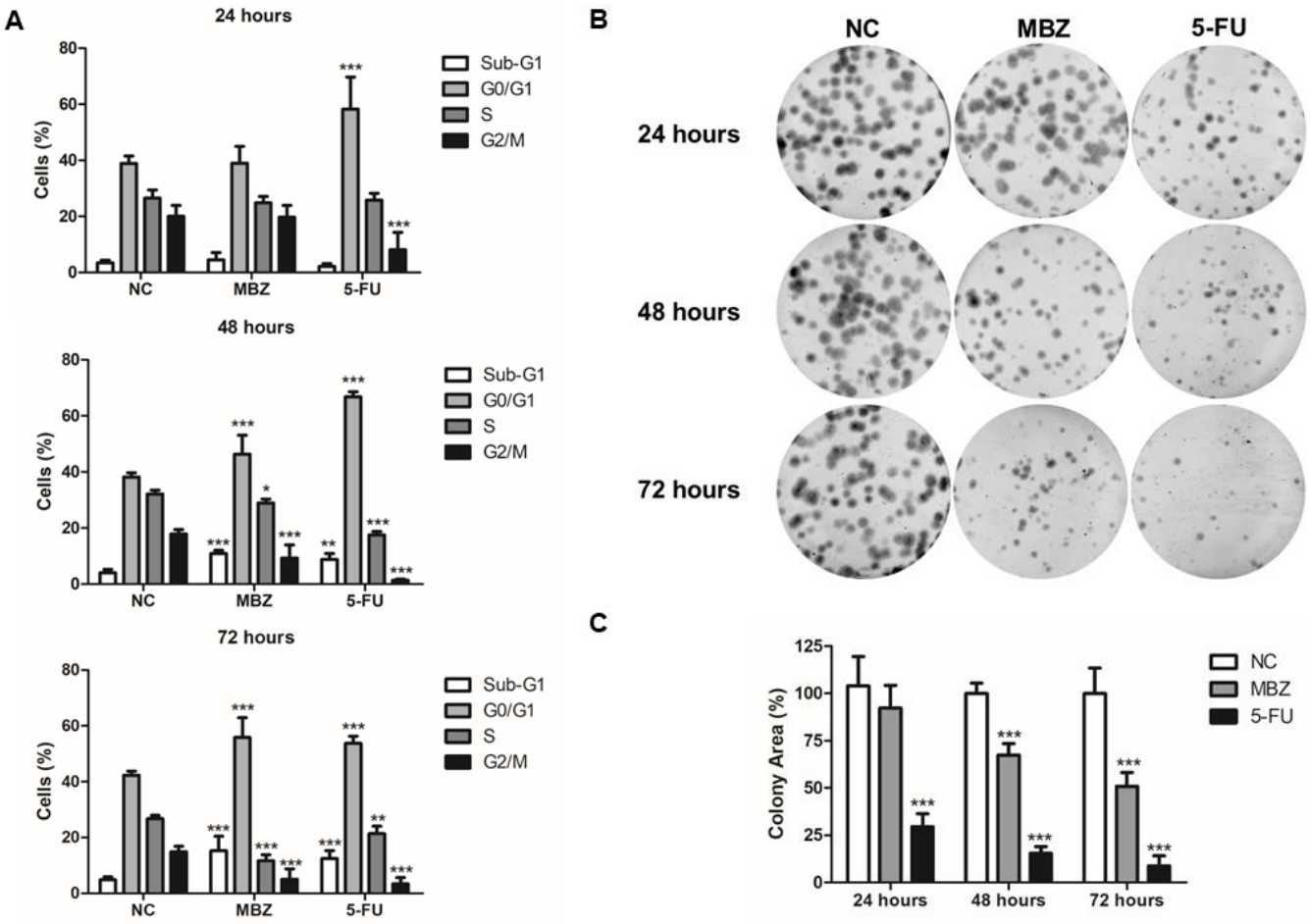


3.4 Treatment with MBZ over time leads to cell cycle arrest, increase in cell fragmentation, and reduces proliferation of AGP-01 cell line

The nucleotide metabolism pathway is highly related to cell growth and the hyperproliferative profile of cancer cell lines [8]. So far, results have shown the potential of MBZ in inhibiting the mRNA expression of genes related to nucleotide metabolism pathways after 24 hours of exposure, as well as, interacting with their coding enzymes and possibly modifying its structure and function. Last, we attempted to investigate the influence of MBZ treatment on the proliferative profile of AGP-01 for 24, 48, and 72 hours of treatment. Cell cycle analysis (Fig. 4A) evidenced that in the first 24 hours of treatment, MBZ did not alter cell cycle progression when compared to non-treated cells. Otherwise, MBZ leads to cell cycle arrest to the G0/G1 phase after 48 and 72 hours of treatment. Furthermore, was observed increasing levels of cells in Sub-G1 (characterized as cells with DNA fragmentation), after the same time of exposure.

Interestingly, the clonogenic assay revealed that MBZ leads to suppression in the colony formation, characterized by the reduction in the colony area, after 48 and 72 hours ($p < 0.001$) of cell treatment as well, as shown in Figure 4B. Altogether, the results showed that MBZ acts by reducing the mRNA expression of targets in the nucleotide metabolism pathway in the first 24 hours of treatment, and after 48 hours is possible to visualize its effects in the cell cycle progression and the proliferative profile of AGP-01 cell line.

Figure 4. MBZ triggers cell cycle arrest with cellular fragmentation and suppresses the proliferation of the AGP-01 cell line over time. The GC cell line cell line AGP-01 was treated with MBZ (0.1 µM) or 5-FU (1 µM) for 24, 48, and 72 h. A) The average percentage of the number of cells (%) in the Sub-G1, G0/G1, S, and G2/M phases was calculated using FlowJo® software. B) Pictures of cell colonies exposed to MBZ or 5-FU at different times, and then reincubated for colony formation assessment. C) The ImageJ software was used to calculate the colony area, and non-treated cells (NC) were set as 100%. Data are presented as the mean ± SD of three independent experiments. Statistical analysis was performed with 2way ANOVA followed by Bonferroni's posttest. Significant differences: *p < 0.05, ***p <0.001. 5-FU: 5-fluorouracil. MBZ: Mebendazole. NC: Negative control.



4 DISCUSSION

The study of molecular pathways that have a direct relationship with tumor growth and proliferation is essential in the oncology area, providing a source of new pharmacological targets that enable the development and validation of new drugs with antitumor potential [7]. Metabolic reprogramming has resurged as an emerging hallmark of cancer, once tumor cells need to reprogram their metabolism to maintain their unlimited replicative potential [48,49]. Among the metabolic pathways that are deregulated in cancer, the nucleotide metabolism

pathway is highlighted once it is closely linked to the synthesis of biosynthetic intermediates for RNA and DNA production, being greatly important for the cellular replicative process [50].

Chemotherapy drugs from the antimetabolite class are widely disseminated in the clinic and their activity is based on the inhibition of the precursors in the nucleotide syntheses. Drugs such as 5-FU, methotrexate, capacitabine, and cytarabine are largely used in the therapy of various types of cancer and are chosen as first-line drugs in the management of solid and hematological tumors [51–55]. However, this class of drugs still presents high toxicity and is likely to lead to the acquisition of intrinsic resistance during the time of treatment leading to therapeutic failure and cancer recurrence [52,56].

Therefore, the drug MBZ is an anthelmintic with proven antitumor potential against several types of cancer, including GC [27–29]. Our previous studies have shown that MBZ can inhibit the glycolytic pathway flow in the GC cell line and that the modulation in the tumor metabolism is related to MBZ cytotoxic activity and apoptosis triggering in the AGP-01 lineage [30]. Therefore, in this study, we seek to analyze the role of MBZ in modulating the nucleotide metabolism pathways and how this can contribute to its antiproliferative effect in the GC metastatic cell line AGP-01.

Our initial results demonstrated that MBZ has a cytotoxic effect on the AGP-01 lineage, with great selectivity being necessary to use around ten-fold its concentration to cause any type of cytotoxic damage in the non-tumoral cell lines evaluated. Furthermore, the expression profile of possible therapeutic targets in the nucleotide metabolism *PRPS1*, *TYMS*, *MTHD1*, *DHODH*, and *HPRT1* was screened showing its high expression in GC patients samples as well as in the GC *in vitro* cell line model AGP-01, the high expression of *TYMS*, *MTHFD1*, and *HPRT1* targets was also correlated with reduction of overall survival in patients with GC through *in silico* analysis, being good targets for therapy for this condition. MBZ evidenced to reduce the expression of the nucleotide metabolism targets after 24 hours of treatment, in addition, MBZ increased cell fragmentation, led to cell cycle arrest in G0/G1, and reduced the proliferative profile of the metastatic GC cell AGP-01 after 48 hours of treatment.

The cytotoxic potential of MBZ has already been described in other cancer models, with IC_{50} ranging from 0.07 µM – 4.3 µM, being the lowest results found in leukemic cell lines [57–59]. Our previous studies have shown that MBZ presents a higher cytotoxic in GC cell lines when they are cultivated at low glucose concentration (5,5 mM of glucose), this condition is more similar to normal glucose conditions in the human body, being validated as the ideal cell culture conditions [30,33]. Metabolic targets are upregulated in low glucose medium,

which could be correlated to the higher cytotoxic potential of MBZ in this condition, showing the potential of MBZ in modulating metabolic targets in GC [30].

It is worth highlighting that both MBZ and the 5-FU presented highly cytotoxic effects in the tumoral cell line AGP-01 (Fig. 1), however, MBZ was shown to be meaningful selective to the tumoral cell line, once it is necessary around ten times the current cytotoxic concentration of MBZ in the AGP-01 (0.147 μM) to cause any damage in the non-tumoral cell lines (1.372 μM for MNP-01 and 1.956 μM for MRC-5). In contrast, the chemotherapic drug 5-FU displayed a cytotoxic effect in lower concentrations to non-tumoral cell lines MNP-01 (0.492 μM) and MRC-5 (0.292 μM) as well, revealing to be extremely toxic to the non-tumoral models in this study.

5-FU is used as the main backbone chemotherapy agent for therapeutics of several types of cancer, such as colorectal (CRC) and advanced GC. 5-FU is metabolically transformed in tissues to its active form, 5-fluoro-deoxyuridine-monophosphate, and then acts as an inhibitor of the Thymidylate Synthase enzyme, modulating the nucleotide synthesis metabolism and jeopardizing DNA and RNA formation and tumor development [34]. Despite its known antitumoral activity, the use of 5-FU in clinics has faced certain challenges, its high toxicity has been proven to trigger gastrointestinal side effects such as mucositis, diarrhea, and stomatitis [60]. Myelosuppression is another of the most prevalent side effects caused by 5-FU in oncological patients, enhancing the risk of anemia, neutropenia, and thrombocytopenia development [61]. The aforementioned side effects, along with the limited efficacy and the acquired resistance to 5-FU can result in a reduced therapeutic index, cancer progression, and mortality [60,62].

The dysregulation in the tumoral metabolism has been extensively studied as a promising therapeutic target in cancer, the nucleotide metabolism pathways have significance since are essential to DNA and RNA formation used to sustain the tumoral proliferation and are a target of antitumoral compounds already used in the clinics [50]. The *de novo* and the *salvage* pathways comprehend a series of enzymatic reactions that conduct nucleotide formation, genes such as *PRPS1*, *TYMS*, *MTFHD1*, *DHODH*, and *HPRT1* encode for essential enzymes in both processes and were screened as potential targets in this study [8–10].

The enzyme Phosphoribosyl Pyrophosphate Synthetase 1, encoded by the *PRPS1* gene, is the first enzyme described in the *de novo* pathway of purine synthesis, it is responsible for the conversion of ribose 5-phosphate to phosphoribosyl pyrophosphate (PRPP). Studies have described the increased regulation of this enzyme along with the enchantment malignant profile of melanoma and colon cancer cells *in vitro* and *in vivo* [15,63], in addition, studies

emphasize that the increase in the expression of this enzyme is related to the acquisition of chemoresistance in breast cancer and in Acute Lymphoblastic Leukemia (ALL) to Cisplatin and 5-FU drugs [64,65].

Therefore, the enzyme Thymidylate Synthetase (*TYMS*) catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), supporting DNA synthesis. It is one of the targets of the 5-FU drug in chemotherapy, has high expression in solid tumors being related to an increase in the invasive profile, and is considered a biomarker of poor clinical prognosis in the clinic [66–70].

The *MTHFD1* gene codifies for a trifunctional enzyme, whith acitivy of dehydrogenase, cyclohydrolase and synthetase in the folic acid metabolic pathway, playing a key role in the nucleotide synthesis and indirect-acting in the methylation reaction thought the produce of 5,10-methyltetrahydrofolate, which provides single carbon units for methylation reactions [71]. The underexpression of *MTHFD1* was documented in renal carcinoma samples, and when overexpressed it showed a suppressive effect in the proliferation of the *in vitro* renal cancer model Caki-1, as well as triggering cell death [71]. Wang et al., [17] showed that there is no correlation between the expression of *MTHFD1* and a worse prognosis in oral squamous cell carcinoma. Otherwise, in concordance with our study, the high expression of *MTHFD1* seems to predict a poor prognosis in hepatocellular carcinoma [18], as well as the pharmacologic inhibition of *MTHFD1* leads to a reduction in cell viability in colon cancer cell lines, as well as, in the 3D tumor growth model due to the inhibition of *de novo* purine synthesis pathway [72].

Another enzyme of the *de novo* nucleotide synthesis pathway is Dihydroorotate Dehydrogenase (*DHODH*), this is a flavoenzyme that catalyzes the oxidation of dihydroorotate to orotate in the fourth step of *de novo* biosynthesis of pyrimidine nucleotides, this protein has demonstrated huge potential as a pharmacological target for several diseases, such against parasitic infections, in autoimmune diseases and cancer [73,74]. The overexpression of this enzyme has been widely investigated in different types of cancer and appears to play a role in increasing Reactive Oxygen Species (ROS) levels, favoring tumorigenesis [75,76]. Studies have evidence that oxidative stress contributes significantly to the development of GC, being a potential therapeutic target [77], our results evidenced that *DHODH* is overexpressed in AGP-01 GC cell lines and has its expression reduced by MBZ drug. Otherwise, there is no significant enhancement in the transcript levels in GC clinical models, and *DHODH* high expression does not seem to be correlated to a significant reduction in OS either.

The Hypoxanthine Phosphoribosyltransferase 1 (*HPRT1*) enzyme acts in the salvage pathway of nucleotide synthesis. It is a transferase classe enzyme, catalyzing the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate, this reaction transfers the 5-phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate (PRPP) to purine, works have related its increased expression in all tumor types dated in the TCGA [78,79]. Its enhancement in mRNA levels is associated with boosted proliferative and replicative potential in ALL and Burkitt's lymphoma and with the acquisition of pharmacological resistance in head and neck cancer as well as in oral squamous cell carcinoma [14,80,81].

As shown, these enzymes are usually overexpressed in different types of cancer, however, despite being promising targets, few studies have related the role of enzymes in the nucleotide synthesis pathway in GC [3,50]. Our results demonstrated that the *PRPS1*, *TYMS*, *MTHFD1*, and *HPRT1* genes have increased expression in clinical samples from patients with GC, when compared to normal samples, and that, when the *TYMS*, *MTHFD1*, and *HPRT1* genes are in high expression are related to a decrease in the overall survival of patients with GC. Data evidenced that the drug MBZ reduced significantly the mRNA levels of all genes evaluated in the AGP-01 cell line which showed hyperexpression of the gene targets. The effect of MBZ in the transcript modulation of the targets of the nucleotide metabolism pathways was similar to the one caused by the 5-FU drug.

Regulation of the cell cycle is an important event for the maintenance of tissue homeostasis and replicative control. Tumor cells tend to acquire mechanisms that bypass cell cycle checking and arrest events, leading to an unlimited replicative potential. This deregulation is linked to the reprogramming of tumor metabolism [82]. During the G1 phase, the cell prepares to replicate its genetic material and synthesize mRNA and proteins necessary for DNA synthesis to occur [83]. Arrest in G0/G1 may indicate metabolic stress and lack of energy demand since cells can only continue in the cell cycle if they have the necessary nutrients to produce energy and replicate their genetic material [33,84]. MBZ demonstrated the same cell cycle arrest profile as the drug 5-FU in the AGP-01 line after 48 hours of treatment, however after 72 hours the drug 5-FU has an increase in cells in S, while MBZ continues with more cells in G0/G1. The arrest in the S phase may indicate the incorporation of the drug into the DNA, adequately preventing its duplication to proceed to the G2/M phase. This mechanism of entrainment in S is common for DNA intercalators, as is the case of metabolites of 5-FU [85]

It is important to highlight that Sub-G1 cells can indicate a degree of cellular fragmentation (DNA content < 1N), which may be related to the activation of the apoptotic

pathway [86]. The drug MBZ only led to an increase in cells in Sub-G1 after 48 hours of treatment, this data corroborates our previous work where it was only possible to observe an increase in cells with an apoptotic profile (morphological change, activation of caspases 3/7 and increase in cell membrane fragmentation) after 48 hours of treatment with the drug [30]. Taken together, the data presented so far demonstrate that MBZ has a role in modulating genes in the nucleotide synthesis pathway, which may be targets of pharmacological interest for GC clinics.

5 CONCLUSION

Thus, the results demonstrate that the drug MBZ has a cytotoxic and selective effect on the AGP-01 gastric lineage. The AGP-01 lineage presents an increase in the expression of genes in the nucleotide synthesis pathway, which is associated with its malignant tumor and replicative potential. The effect of MBZ on the reduction in the expression of genes in the nucleotide synthesis pathways is related to its antiproliferative capacity in the AGP-01 lineage. The data show that targets in nucleotide metabolism may be of clinical interest since they are hyperexpressed in patients with GC and its high expression is linked to reduced life expectancy in these patients.

CRediT authorship contribution statement

Emerson Lucena da Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Felipe Pantoja Mesquita:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Francisco Laio de Oliveira:** Methodology, Formal analysis, Investigation. **Maria Elisabete Amaral de Moraes:** Resources, Supervision, Funding acquisition. **Pedro Filho Noronha Souza:** Conceptualization, Writing – original draft, Supervision, Methodology, Formal analysis, Investigation. **Raquel Carvalho Montenegro:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Council for Scientific and Technological Development (CNPq) for a research productivity grant to Raquel Carvalho Montenegro (305459/2019-8), Pedro Filho Noronha Souza (305003/2022-4), Felipe P. Mesquita (Process number: 421392/2023-1), and a graduate scholarship to Emerson Lucena da Silva (Process numbers: 140436/2020-0; 141236/2023-0). The authors are grateful to the Brazilian funding agencies: Coordination for Improvement of Higher Level Personnel (CAPES), and the Cearense Foundation of Scientific and Technological Support (FUNCAP) for additional financial assistance. Also, we thank the University Federal of Ceará and the multiuser unit of the Drug Research and Development Center (NPDM) for fellowships and structural support.

Ethics approval and consent to participate

Not applicable.

REFERENCES

[1] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674. https://doi.org/10.1016/j.cell.2011.02.013.

[2] H. Akhavan, S. Ramezani, Z. Shams, S. Hosseini-Asl, Revealing novel biomarkers involved in development and progression of gastric cancer by comprehensive bioinformatics analysis, Informatics in Medicine Unlocked 25 (2021) 100630. https://doi.org/10.1016/j.imu.2021.100630.

[3] Z. Wu, J. Tan, Y. Zhuang, M. Zhong, Y. Xiong, J. Ma, Y. Yang, Z. Gao, J. Zhao, Z. Ye, H. Zhou, Y. Zhu, H. Lu, X. Hong, Identification of crucial genes of pyrimidine metabolism as biomarkers for gastric cancer prognosis, Cancer Cell Int 21 (2021) 668. https://doi.org/10.1186/s12935-021-02385-x.

[4] K.M. Aird, R. Zhang, Nucleotide metabolism, oncogene-induced senescence and cancer, Cancer Letters 356 (2015) 204–210. https://doi.org/10.1016/j.canlet.2014.01.017.

[5] C.J. Halbrook, D.R. Wahl, C.A. Lyssiotis, Running the Light: Nucleotide Metabolism Drives Bypass of Senescence in Cancer, Trends in Biochemical Sciences 44 (2019) 991–993. https://doi.org/10.1016/j.tibs.2019.10.007.

[6] E.L. da Silva, R.C. Montenegro, C.A. Moreira-Nunes, Tumor Metabolism: Challenges and Future Perspectives, in: N. Rezaei (Ed.), Handbook of Cancer and Immunology, Springer International Publishing, Cham, 2022: pp. 1–27. https://doi.org/10.1007/978-3-030-80962-1_103-1.

[7] A. Luengo, D.Y. Gui, M.G. Vander Heiden, Targeting Metabolism for Cancer Therapy, Cell Chem Biol 24 (2017) 1161–1180. https://doi.org/10.1016/j.chembiol.2017.08.028.

[8] H. Wu, Y. Gong, P. Ji, Y. Xie, Y.-Z. Jiang, G. Liu, Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy, Journal of Hematology & Oncology 15 (2022) 45. https://doi.org/10.1186/s13045-022-01263-x.

[9] M. Walter, P. Herr, Re-Discovery of Pyrimidine Salvage as Target in Cancer Therapy, Cells 11 (2022) 739. https://doi.org/10.3390/cells11040739.

[10] M.G. Vander Heiden, R.J. DeBerardinis, Understanding the Intersections between Metabolism and Cancer Biology, Cell 168 (2017) 657–669. https://doi.org/10.1016/j.cell.2016.12.039.

[11] D.A. Bárcenas-López, D.K. Mendiola-Soto, J.C. Núñez-Enríquez, J.M. Mejía-Aranguré, A. Hidalgo-Miranda, S. Jiménez-Morales, Promising genes and variants to reduce chemotherapy adverse effects in acute lymphoblastic leukemia, Translational Oncology 14 (2021) 100978. https://doi.org/10.1016/j.tranon.2020.100978.

[12] T. Liu, Y. Han, C. Yu, Y. Ji, C. Wang, X. Chen, X. Wang, J. Shen, Y. Zhang, J.-Y. Lang, MYC predetermines the sensitivity of gastrointestinal cancer to antifolate drugs through regulating TYMS transcription, eBioMedicine 48 (2019) 289–300. https://doi.org/10.1016/j.ebiom.2019.10.003.

[13] T.K. Olsen, C. Dyberg, B.T. Embaie, A. Alchahin, J. Milosevic, J. Ding, J. Otte, C. Tümmler, I. Hed Myrberg, E.M. Westerhout, J. Koster, R. Versteeg, H.-F. Ding, P. Kogner, J.I. Johnsen, D.B. Sykes, N. Baryawno, DHODH is an independent prognostic marker and potent therapeutic target in neuroblastoma, JCI Insight 7 (2022) e153836. https://doi.org/10.1172/jci.insight.153836.

[14] T. Wu, Z. Jiao, Y. Li, X. Su, F. Yao, J. Peng, W. Chen, A. Yang, HPRT1 Promotes Chemoresistance in Oral Squamous Cell Carcinoma via Activating MMP1/PI3K/Akt Signaling Pathway, Cancers (Basel) 14 (2022) 855. https://doi.org/10.3390/cancers14040855.

[15] G. Xiong, Y. Feng, X. Yi, X. Zhang, X. Li, L. Yang, Z. Yi, B. Sai, Z. Yang, Q. Zhang, Y. Kuang, Y. Zhu, NRF2-directed PRPS1 upregulation to promote the progression and metastasis of melanoma, Front Immunol 13 (2022) 989263. https://doi.org/10.3389/fimmu.2022.989263.

[16] L. Yuan, Z. Xiao, R. Lu, Hypoxanthine guanine phosphoribosyltransferase 1, a target of miR-125b-5p, promotes cell proliferation and invasion in head and neck squamous cell carcinoma, Heliyon 9 (2023) e20174. https://doi.org/10.1016/j.heliyon.2023.e20174.

[17] W. Wang, W. Gu, H. Tang, Z. Mai, H. Xiao, J. Zhao, J. Han, The Emerging Role of MTHFD Family Genes in Regulating the Tumor Immunity of Oral Squamous Cell Carcinoma, J Oncol 2022 (2022) 4867730. https://doi.org/10.1155/2022/4867730.

[18] H. Yu, H. Wang, H.-R. Xu, Y.-C. Zhang, X.-B. Yu, M.-C. Wu, G.-Z. Jin, W.-M. Cong, Overexpression of MTHFD1 in hepatocellular carcinoma predicts poorer survival and recurrence, Future Oncol 15 (2019) 1771–1780. https://doi.org/10.2217/fon-2018-0606.

[19] X. Feng, D. Ma, J. Zhao, Y. Song, Y. Zhu, Q. Zhou, F. Ma, X. Liu, M. Zhong, Y. Liu, Y. Xiong, X. Qiu, Z. Zhang, H. Zhang, Y. Zhao, K. Zhang, X. Hong, Z. Zhang, UHMK1 promotes gastric cancer progression through reprogramming nucleotide metabolism, The EMBO Journal 39 (2020) e102541. https://doi.org/10.15252/embj.2019102541.

[20] J. Tan, S. Zhou, W. Zhang, B. Yang, G. Zhong, J. Huang, H. Hu, F. Han, M.-L. Luo, Long noncoding RNA OVAAL enhances nucleotide synthesis through pyruvate carboxylase to promote 5-fluorouracil resistance in gastric cancer, Cancer Science 113 (2022) 3055–3070. https://doi.org/10.1111/cas.15453.

[21] B. Pathare, T. Bansode, Review- biological active benzimidazole derivatives, Results in Chemistry 3 (2021) 100200. https://doi.org/10.1016/j.rechem.2021.100200.

[22] J.P. Laclette, G. Guerra, C. Zetina, Inhibition of tubulin polymerization by mebendazole, Biochemical and Biophysical Research Communications 92 (1980) 417–423. https://doi.org/10.1016/0006-291x(80)90349-6.

[23] J. Sasaki, R. Ramesh, S. Chada, Y. Gomyo, J.A. Roth, T. Mukhopadhyay, The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells, Molecular Cancer Therapeutics 1 (2002) 1201–1209.

[24] P. Nygren, M. Fryknäs, B. Agerup, R. Larsson, Repositioning of the anthelmintic drug mebendazole for the treatment for colon cancer, Journal of Cancer Research and Clinical Oncology 139 (2013) 2133–2140. https://doi.org/10.1007/s00432-013-1539-5.

[25] I. Bodhinayake, M. Symons, J.A. Boockvar, Repurposing mebendazole for the treatment of medulloblastoma, Neurosurgery 76 (2015) N15-16. https://doi.org/10.1227/01.neu.0000460594.93803.cb.

[26] N.A. Doudican, S.A. Byron, P.M. Pollock, S.J. Orlow, XIAP downregulation accompanies mebendazole growth inhibition in melanoma xenografts, Anti-Cancer Drugs 24 (2013) 181–188. https://doi.org/10.1097/CAD.0b013e32835a43f1.

[27] L.C. Pinto, B.M. Soares, J. de J.V. Pinheiro, G.J. Riggins, P.P. Assumpção, R.M.R. Burbano, R.C. Montenegro, The anthelmintic drug mebendazole inhibits growth, migration and invasion in gastric cancer cell model, Toxicol In Vitro 29 (2015) 2038–2044. https://doi.org/10.1016/j.tiv.2015.08.007.

[28] L.C. Pinto, C. de Fátima Aquino Moreira-Nunes, B.M. Soares, R.M.R. Burbano, J.A.R. de Lemos, R.C. Montenegro, Mebendazole, an antiparasitic drug, inhibits drug transporters expression in preclinical model of gastric peritoneal carcinomatosis, Toxicol In Vitro 43 (2017) 87–91. https://doi.org/10.1016/j.tiv.2017.06.007.

[29] L.C. Pinto, F.P. Mesquita, B.M. Soares, E.L. da Silva, B. Puty, E.H.C. de Oliveira, R.R. Burbano, R.C. Montenegro, Mebendazole induces apoptosis via C-MYC inactivation in malignant ascites cell line (AGP01), Toxicology in Vitro 60 (2019) 305–312. https://doi.org/10.1016/j.tiv.2019.06.010.

[30] E.L. da Silva, F.P. Mesquita, D.R. Aragão, A.J. de Sousa Portilho, A.D. Marinho, L.L.B. de Oliveira, L.B. Lima, M.E.A. de Moraes, P.F.N. Souza, R.C. Montenegro, Mebendazole targets essential proteins in glucose metabolism leading gastric cancer cells to death, Toxicology and Applied Pharmacology 475 (2023) 116630. https://doi.org/10.1016/j.taap.2023.116630.

[31] A.E. Guerini, L. Triggiani, M. Maddalo, M.L. Bonù, F. Frassine, A. Baiguini, A. Alghisi, D. Tomasini, P. Borghetti, N. Pasinetti, R. Bresciani, S.M. Magrini, M. Buglione, Mebendazole as a Candidate for Drug Repurposing in Oncology: An Extensive Review of Current Literature, Cancers (Basel) 11 (2019) 1284. https://doi.org/10.3390/cancers11091284.

[32] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J Clin (2021). https://doi.org/10.3322/caac.21660.

[33] E.L. da Silva, F.P. Mesquita, A.J. de S. Portilho, E.C.A. Bezerra, J.P. Daniel, E.S.P. Aranha, S. Farran, M.C. de Vasconcellos, M.E.A. de Moraes, C.A. Moreira-Nunes, R.C. Montenegro, Differences in glucose concentration shows new perspectives in gastric cancer metabolism, Toxicology in Vitro 82 (2022) 105357. https://doi.org/10.1016/j.tiv.2022.105357.

[34] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-Fluorouracil: mechanisms of action and clinical strategies, Nat Rev Cancer 3 (2003) 330–338. https://doi.org/10.1038/nrc1074.

[35] M.F. Leal, J.L.M. do Nascimento, C.E.A. da Silva, M.F.V. Lamarão, D.Q. Calcagno, A.S. Khayat, P.P. Assumpção, I.R. Cabral, M. de A.C. Smith, R.R. Burbano, Establishment and conventional cytogenetic characterization of three gastric cancer cell lines, Cancer Genetics and Cytogenetics 195 (2009) 85–91. https://doi.org/10.1016/j.cancergencyto.2009.04.020.

[36] F.P. Mesquita, L.C. Pinto, B.M. Soares, A.J. de Sousa Portilho, E.L. da Silva, I.N. de Farias Ramos, A.S. Khayat, C.A. Moreira-Nunes, M.M. Bezerra, E. de Lucas Chazin, T.R.A. Vasconcelos, R.M.R. Burbano, M.E.A. de Moraes, R.C. Montenegro, Small benzothiazole molecule induces apoptosis and prevents metastasis through DNA interaction and c-MYC gene supression in diffuse-type gastric adenocarcinoma cell line, Chem. Biol. Interact. 294 (2018) 118–127. https://doi.org/10.1016/j.cbi.2018.08.006.

[37] J.P. Jacobs, C.M. Jones, J.P. Baille, Characteristics of a Human Diploid Cell Designated MRC-5, Nature 227 (1970) 168–170. https://doi.org/10.1038/227168a0.

[38] S.N. Rampersad, Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays, Sensors (Basel) 12 (2012) 12347–12360. https://doi.org/10.3390/s120912347.

[39] J.M. Calderón-Montaño, S.M. Martínez-Sánchez, V. Jiménez-González, E. Burgos-Morón, E. Guillén-Mancina, J.J. Jiménez-Alonso, P. Díaz-Ortega, F. García, A. Aparicio, M. López-Lázaro, Screening for Selective Anticancer Activity of 65 Extracts of Plants Collected in Western Andalusia, Spain, Plants (Basel) 10 (2021) 2193. https://doi.org/10.3390/plants10102193.

[40] A. Lánczky, B. Győrffy, Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation, J Med Internet Res 23 (2021) e27633. https://doi.org/10.2196/27633.

[41] Z. Tang, C. Li, B. Kang, G. Gao, C. Li, Z. Zhang, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids Res 45 (2017) W98–W102. https://doi.org/10.1093/nar/gkx247.

[42] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622. https://doi.org/10.1373/clinchem.2008.112797.

[43] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat Protoc 3 (2008) 1101–1108. https://doi.org/10.1038/nprot.2008.73.

[44] F.F. Diehl, T.P. Miettinen, R. Elbashir, C.S. Nabel, A.M. Darnell, B.T. Do, S.R. Manalis, C.A. Lewis, M.G. Vander Heiden, Nucleotide imbalance decouples cell growth from cell proliferation, Nat Cell Biol 24 (2022) 1252–1264. https://doi.org/10.1038/s41556-022-00965-1.

[45] J.A. Plumb, Cell sensitivity assays: clonogenic assay, Methods Mol. Med. 88 (2004) 159–164.

[46] H. Rafehi, C. Orlowski, G.T. Georgiadis, K. Ververis, A. El-Osta, T.C. Karagiannis, Clonogenic Assay: Adherent Cells, JoVE (2011) 2573. https://doi.org/10.3791/2573.

[47] C. Guzmán, M. Bagga, A. Kaur, J. Westermarck, D. Abankwa, ColonyArea: An ImageJ Plugin to Automatically Quantify Colony Formation in Clonogenic Assays, PLoS One 9 (2014) e92444. https://doi.org/10.1371/journal.pone.0092444.

[48] R.J. DeBerardinis, J.J. Lum, G. Hatzivassiliou, C.B. Thompson, The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation, Cell Metabolism 7 (2008) 11–20. https://doi.org/10.1016/j.cmet.2007.10.002.

[49] D. Hanahan, R.A. Weinberg, Hallmarks of Cancer: The Next Generation, Cell 144 (2011) 646–674. https://doi.org/10.1016/j.cell.2011.02.013.

[50] H. Wu, Y. Gong, P. Ji, Y. Xie, Y.-Z. Jiang, G. Liu, Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy, Journal of Hematology & Oncology 15 (2022) 45. https://doi.org/10.1186/s13045-022-01263-x.

[51] S. Cascinu, M. Di Bartolomeo, S. Lonardi, G. Beretta, L. Fornaro, F. De Vita, The evolving strategies for the management of patients with metastatic gastric cancer: A narrative review and expert opinion, Frontiers in Medicine 9 (2022). https://www.frontiersin.org/articles/10.3389/fmed.2022.1002435 (accessed December 14, 2023).

[52] R. Di Francia, S. Crisci, A. De Monaco, C. Cafiero, A. Re, G. Iaccarino, R. De Filippi, F. Frigeri, G. Corazzelli, A. Micera, A. Pinto, Response and Toxicity to Cytarabine Therapy in Leukemia and Lymphoma: From Dose Puzzle to Pharmacogenomic Biomarkers, Cancers (Basel) 13 (2021) 966. https://doi.org/10.3390/cancers13050966.

[53] R. Guimbaud, C. Louvet, P. Ries, M. Ychou, E. Maillard, T. André, J.-M. Gornet, T. Aparicio, S. Nguyen, A. Azzedine, P.-L. Etienne, E. Boucher, C. Rebischung, P. Hammel, P. Rougier, L. Bedenne, O. Bouché, Prospective, randomized, multicenter, phase III study of fluorouracil, leucovorin, and irinotecan versus epirubicin, cisplatin, and capecitabine in advanced gastric adenocarcinoma: a French intergroup (Fédération Francophone de Cancérologie Digestive, Fédération Nationale des Centres de Lutte Contre le Cancer, and Groupe Coopérateur Multidisciplinaire en Oncologie) study, J Clin Oncol 32 (2014) 3520–3526. https://doi.org/10.1200/JCO.2013.54.1011.

[54] X.-B. Liang, S.-H. Hou, Y.-P. Li, L.-C. Wang, X. Zhang, J. Yang, Irinotecan or oxaliplatin combined with 5-fluorouracil and leucovorin as first-line therapy for advanced colorectal cancer: a meta-analysis, Chin. Med. J. 123 (2010) 3314–3318.

[55] V.K. Morris, E.B. Kennedy, N.N. Baxter, A.B. Benson, A. Cercek, M. Cho, K.K. Ciombor, C. Cremolini, A. Davis, D.A. Deming, M.G. Fakih, S. Gholami, T.S. Hong, I. Jaiyesimi, K. Klute, C. Lieu, H. Sanoff, J.H. Strickler, S. White, J.A. Willis, C. Eng, Treatment of Metastatic Colorectal Cancer: ASCO Guideline, J Clin Oncol 41 (2023) 678–700. https://doi.org/10.1200/JCO.22.01690.

[56] S. Azwar, H.F. Seow, M. Abdullah, M. Faisal Jabar, N. Mohtarrudin, Recent Updates on Mechanisms of Resistance to 5-Fluorouracil and Reversal Strategies in Colon Cancer Treatment, Biology 10 (2021) 854. https://doi.org/10.3390/biology10090854.

[57] R.-Y. Bai, V. Staedtke, C.M. Rudin, F. Bunz, G.J. Riggins, Effective treatment of diverse medulloblastoma models with mebendazole and its impact on tumor angiogenesis, Neuro Oncol 17 (2015) 545–554. https://doi.org/10.1093/neuonc/nou234.

[58] J.P. Daniel, F.P. Mesquita, E.L. Da Silva, P.F.N. de Souza, L.B. Lima, L.L.B. de Oliveira, M.E.A. de Moraes, C. de F.A. Moreira-Nunes, R.M.R. Burbano, G. Zanatta, R.C. Montenegro, Anticancer potential of mebendazole against chronic myeloid leukemia: in silico

and in vitro studies revealed new insights about the mechanism of action, Frontiers in Pharmacology 13 (2022). https://www.frontiersin.org/articles/10.3389/fphar.2022.952250 (accessed November 11, 2022).

[59] V. Walf-Vorderwülbecke, K. Pearce, T. Brooks, M. Hubank, M.M. van den Heuvel-Eibrink, C.M. Zwaan, S. Adams, D. Edwards, J. Bartram, S. Samarasinghe, P. Ancliff, A. Khwaja, N. Goulden, G. Williams, J. de Boer, O. Williams, Targeting acute myeloid leukemia by drug-induced c-MYB degradation, Leukemia 32 (2018) 882–889. https://doi.org/10.1038/leu.2017.317.

[60] A. Sharma, M.R. Chorawala, R.M. Rawal, N. Shrivastava, Integrated blood and organ profile analysis to evaluate ameliorative effects of kaempferol on 5-fluorouracil-induced toxicity, Sci Rep 14 (2024) 2363. https://doi.org/10.1038/s41598-024-52915-6.

[61] M. Ishibashi, M. Ishii, S. Yamamoto, Y. Mori, S. Shimizu, Possible involvement of TRPM2 activation in 5-fluorouracil-induced myelosuppression in mice, Eur J Pharmacol 891 (2021) 173671. https://doi.org/10.1016/j.ejphar.2020.173671.

[62] Q. Hu, J. Xu, J. Ke, Z. Zhang, T. Chu, S-1 and 5-Fluorouracil-related adverse events in patients with advanced gastric cancer: A meta-analysis, PLoS One 18 (2023) e0290003. https://doi.org/10.1371/journal.pone.0290003.

[63] X. Jing, X. Wang, T. Zhang, W. Zhu, Y. Fang, H. Wu, X. Liu, D. Ma, X. Ji, Y. Jiang, K. Liu, X. Chen, Y. Shi, Y. Zhang, M. Shi, W. Qiu, R. Zhao, Cell-Cycle–Dependent Phosphorylation of PRPS1 Fuels Nucleotide Synthesis and Promotes Tumorigenesis, Cancer Research 79 (2019) 4650–4664. https://doi.org/10.1158/0008-5472.CAN-18-2486.

[64] M. He, L. Chao, Y.-P. You, PRPS1 silencing reverses cisplatin resistance in human breast cancer cells, Biochem Cell Biol 95 (2017) 385–393. https://doi.org/10.1139/bcb-2016-0106.

[65] D. Wang, Y. Chen, H. Fang, L. Zheng, Y. Li, F. Yang, Y. Xu, L. Du, B.-B.S. Zhou, H. Li, Increase of PRPP enhances chemosensitivity of PRPS1 mutant acute lymphoblastic leukemia cells to 5-Fluorouracil, Journal of Cellular and Molecular Medicine 22 (2018) 6202–6212. https://doi.org/10.1111/jcmm.13907.

[66] C. Burdelski, C. Strauss, M.C. Tsourlakis, M. Kluth, C. Hube-Magg, N. Melling, P. Lebok, S. Minner, C. Koop, M. Graefen, H. Heinzer, C. Wittmer, T. Krech, G. Sauter, W. Wilczak, R. Simon, T. Schlomm, S. Steurer, Overexpression of thymidylate synthase (TYMS) is associated with aggressive tumor features and early PSA recurrence in prostate cancer, Oncotarget 6 (2015) 8377–8387.

[67] C. Chamizo, S. Zazo, M. Dómine, I. Cristóbal, J. García-Foncillas, F. Rojo, J. Madoz-Gúrpide, Thymidylate synthase expression as a predictive biomarker of pemetrexed sensitivity in advanced non-small cell lung cancer, BMC Pulmonary Medicine 15 (2015) 132. https://doi.org/10.1186/s12890-015-0132-x.

[68] W.M. Ciszewski, M. Chmielewska-Kassassir, L.A. Wozniak, K. Sobierajska, Thymidylate Synthase Overexpression Drives the Invasive Phenotype in Colon Cancer Cells, Biomedicines 10 (2022) 1267. https://doi.org/10.3390/biomedicines10061267.

[69] S.L. Showalter, T.N. Showalter, A. Witkiewicz, R. Havens, E.P. Kennedy, T. Hucl, S.E. Kern, C.J. Yeo, J.R. Brody, Evaluating the drug-target relationship between thymidylate synthase expression and tumor response to 5-fluorouracil, Cancer Biol Ther 7 (2008) 986–994.

[70] M.A. Siddiqui, P.N. Gollavilli, V. Ramesh, B. Parma, A. Schwab, M.E. Vazakidou, R. Natesan, O. Saatci, I. Rapa, P. Bironzo, H. Schuhwerk, I.A. Asangani, O. Sahin, M. Volante, P. Ceppi, Thymidylate synthase drives the phenotypes of epithelial-to-mesenchymal transition in non-small cell lung cancer, Br J Cancer 124 (2021) 281–289. https://doi.org/10.1038/s41416-020-01095-x.

[71] D. He, Z. Yu, S. Liu, H. Dai, Q. Xu, F. Li, Methylenetetrahydrofolate Dehydrogenase 1 (MTHFD1) is Underexpressed in Clear Cell Renal Cell Carcinoma Tissue and Transfection and Overexpression in Caki-1 Cells Inhibits Cell Proliferation and Increases Apoptosis, Med Sci Monit 24 (2018) 8391–8400. https://doi.org/10.12659/MSM.911124.

[72] A.C. Green, P. Marttila, N. Kiweler, C. Chalkiadaki, E. Wiita, V. Cookson, A. Lesur, K. Eiden, F. Bernardin, K.S.A. Vallin, S. Borhade, M. Long, E.K. Ghahe, J.J. Jiménez-Alonso, A.-S. Jemth, O. Loseva, O. Mortusewicz, M. Meyers, E. Viry, A.I. Johansson, O. Hodek, E. Homan, N. Bonagas, L. Ramos, L. Sandberg, M. Frödin, E. Moussay, A. Slipicevic, E. Letellier, J. Paggetti, C.S. Sørensen, T. Helleday, M. Henriksson, J. Meiser, Formate overflow drives toxic folate trapping in MTHFD1 inhibited cancer cells, Nat Metab 5 (2023) 642–659. https://doi.org/10.1038/s42255-023-00771-5.

[73] S. Christian, C. Merz, L. Evans, S. Gradl, H. Seidel, A. Friberg, A. Eheim, P. Lejeune, K. Brzezinka, K. Zimmermann, S. Ferrara, H. Meyer, R. Lesche, D. Stoeckigt, M. Bauser, A. Haegebarth, D.B. Sykes, D.T. Scadden, J.-A. Losman, A. Janzer, The novel dihydroorotate dehydrogenase (DHODH) inhibitor BAY 2402234 triggers differentiation and is effective in the treatment of myeloid malignancies, Leukemia 33 (2019) 2403–2415. https://doi.org/10.1038/s41375-019-0461-5.

[74] M.L. Lolli, S. Sainas, A.C. Pippione, M. Giorgis, D. Boschi, F. Dosio, Use of human Dihydroorotate Dehydrogenase (hDHODH) Inhibitors in Autoimmune Diseases and New Perspectives in Cancer Therapy, Recent Pat Anticancer Drug Discov 13 (2018) 86–105. https://doi.org/10.2174/1574892812666171108124218.

[75] M. Bajzikova, J. Kovarova, A.R. Coelho, S. Boukalova, S. Oh, K. Rohlenova, D. Svec, S. Hubackova, B. Endaya, K. Judasova, A. Bezawork-Geleta, K. Kluckova, L. Chatre, R. Zobalova, A. Novakova, K. Vanova, Z. Ezrova, G.J. Maghzal, S. Magalhaes Novais, M. Olsinova, L. Krobova, Y.J. An, E. Davidova, Z. Nahacka, M. Sobol, T. Cunha-Oliveira, C. Sandoval-Acuña, H. Strnad, T. Zhang, T. Huynh, T.L. Serafim, P. Hozak, V.A. Sardao, W.J.H. Koopman, M. Ricchetti, P.J. Oliveira, F. Kolar, M. Kubista, J. Truksa, K. Dvorakova-Hortova, K. Pacak, R. Gurlich, R. Stocker, Y. Zhou, M.V. Berridge, S. Park, L. Dong, J. Rohlena, J. Neuzil, Reactivation of Dihydroorotate Dehydrogenase-Driven Pyrimidine Biosynthesis Restores Tumor Growth of Respiration-Deficient Cancer Cells, Cell Metab 29 (2019) 399-416.e10. https://doi.org/10.1016/j.cmet.2018.10.014.

[76] Y. Zhou, L. Tao, X. Zhou, Z. Zuo, J. Gong, X. Liu, Y. Zhou, C. Liu, N. Sang, H. Liu, J. Zou, K. Gou, X. Yang, Y. Zhao, DHODH and cancer: promising prospects to be explored, Cancer & Metabolism 9 (2021) 22. https://doi.org/10.1186/s40170-021-00250-z.

[77] Y. Liu, Y. Shi, R. Han, C. Liu, X. Qin, P. Li, R. Gu, Signaling pathways of oxidative stress response: the potential therapeutic targets in gastric cancer, Front Immunol 14 (2023) 1139589. https://doi.org/10.3389/fimmu.2023.1139589.

[78] M.H. Passey, A.M. Felsted, Z.E. Ence, S.R. Piccolo, K.L. O'Neill, R.A. Robison, Unique HPRT1 upregulation in malignant tissue: Potential use as diagnostic biomarker, Cancer Research 78 (2018) 2536. https://doi.org/10.1158/1538-7445.AM2018-2536.

[79] M.J. Sedano, E. I. Ramos, R. Choudhari, A. L. Harrison, R. Subramani, R. Lakshmanaswamy, M. Zilaie, S.S. Gadad, Hypoxanthine Phosphoribosyl Transferase 1 Is Upregulated, Predicts Clinical Outcome and Controls Gene Expression in Breast Cancer, Cancers (Basel) 12 (2020) 1522. https://doi.org/10.3390/cancers12061522.

[80] M. Ahmadi, M. Eftekhari Kenzerki, S.M. Akrami, S. Pashangzadeh, F. Hajiesmaeili, S. Rahnavard, L. Habibipour, N. Saffarzadeh, P. Mousavi, Overexpression of HPRT1 is associated with poor prognosis in head and neck squamous cell carcinoma, FEBS Open Bio 11 (2021) 2525–2540. https://doi.org/10.1002/2211-5463.13250.

[81] M.H. Townsend, Z.E. Ence, T.P. Cox, J.E. Lattin, W. Burrup, M.K. Boyer, S.R. Piccolo, R.A. Robison, K.L. O'Neill, Evaluation of the upregulation and surface expression of hypoxanthine guanine phosphoribosyltransferase in acute lymphoblastic leukemia and Burkitt's B cell lymphoma, Cancer Cell International 20 (2020) 375. https://doi.org/10.1186/s12935-020-01457-8.

[82] L.C. Leal-Esteban, L. Fajas, Cell cycle regulators in cancer cell metabolism, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1866 (2020) 165715. https://doi.org/10.1016/j.bbadis.2020.165715.

[83] S. Bagga, M.J. Bouchard, Cell Cycle Regulation During Viral Infection, Cell Cycle Control 1170 (2014) 165–227. https://doi.org/10.1007/978-1-4939-0888-2_10.

[84] I. Roci, J.D. Watrous, K.A. Lagerborg, L. Lafranchi, A. Lindqvist, M. Jain, R. Nilsson, Mapping Metabolic Events in the Cancer Cell Cycle Reveals Arginine Catabolism in the Committed SG2M Phase, Cell Rep 26 (2019) 1691-1700.e5. https://doi.org/10.1016/j.celrep.2019.01.059.

[85] J. Bré, A.L. Dickson, O.J. Read, Y. Zhang, F.G. McKissock, P. Mullen, P. Tang, G.M. Zickuhr, C.M. Czekster, D.J. Harrison, The novel anti-cancer fluoropyrimidine NUC-3373 is a potent inhibitor of thymidylate synthase and an effective DNA-damaging agent, Cancer Chemother Pharmacol 91 (2023) 401–412. https://doi.org/10.1007/s00280-023-04528-5.

[86] B.-Y. Peng, A.K. Singh, C.-H. Chan, Y.-H. Deng, P.-Y. Li, C.-W. Su, C.-Y. Wu, W.-P. Deng, AGA induces sub-G1 cell cycle arrest and apoptosis in human colon cancer cells through p53-independent/p53-dependent pathway, BMC Cancer 23 (2023) 1. https://doi.org/10.1186/s12885-022-10466-x.

CAPÍTULO IV

TRANSCRIPTOME ANALYSIS REVEALED CHANGES IN THE MOLECULAR PROFILE OF GASTRIC CANCER CELLS TREATED WITH MEBENDAZOLE, DISPLAYING NEW INSIGHTS INTO THE MECHANISMS OF ACTION

EMERSON LUCENA DA SILVA

TRANSCRIPTOME ANALYSIS REVEALED CHANGES IN THE MOLECULAR PROFILE OF GASTRIC CANCER CELLS TREATED WITH MEBENDAZOLE, DISPLAYING NEW INSIGHTS INTO THE MECHANISMS OF ACTION

Emerson Lucena da Silva[a†], Felipe Pantoja Mesquita[a†], Laine Celestino Pinto[b†], Bruna Puty Silva Gomes[c], Edivaldo Herculano Correa de Oliveira[c], Rommel Mario Rodríguez Burbano[d,e], Maria Elisabete Moraes[a], Pedro Filho Noronha Souza[a,f,g,*], Raquel Carvalho Montenegro[a,h,*]

[a] *Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Cel. Nunes de Melo, Fortaleza, Brazil.*

[b] *Laboratory of Experimental Neuropathology, Biological Science Institute, Federal University of Pará, Mundurucus Street, Belém, Brazil*

[c] *Laboratory of Cytogenomics and Environmental Mutagenesis, Environment Section (SAMAM), Evandro Chagas Institute (IEC), Ananindeua, Brazil*

[d] *Molecular Biology Laboratory, Ophir Loyola Hospital, Av. Governador Magalhães Barata, Belém, Brazil*

[e] *Laboratory of Human Cytogenetics, Institute of Biological Sciences, Federal University of Pará, Augusto Correa Avenue, Belém, Brazil*

[f] *Visiting Researcher at the Cearense Foundation to Support Scientific and Technological Development*

[g] *National Institute of Science and Technology in Human Pathogenic Fungi, Ribeirão Preto, Brazil*

[h] *Red Latinoamericana de Implementación y Validación de guias clinicas Farmacogenomicas (RELIVAF), Brazil*

* Address correspondence to this author: Laboratory of Pharmacogenetics, Drug Research and Development Center (NPDM), Federal University of Ceará, Street Cel. Nunes de Melo, 1000 – Rodolfo Teófilo, Fortaleza, Ceará, Brazil. E-mail addresses of corresponding authors: rcm.montenegro@gmail.com (Raquel Carvalho Montenegro) and pedrofilhobio@gmail.com (Pedro Filho Noronha Souza).

† These authors share first authorship.

Artigo submetido a revista internacional Computers in Biology and Medicine

(Qualis CAPES Ciências Biológicas II: A2)

ABSTRACT

Gastric cancer (GC) is a common cancer worldwide. Therefore, searching for effective treatments is essential, and drug repositioning can be a promising strategy to find new potential drugs for GC therapy. For the first time, we sought to identify molecular alterations and validate new mechanisms related to Mebendazole (MBZ) treatment in GC cells through transcriptome analysis using microarray technology. Data revealed 1,066 differentially expressed genes (DEGs), of which 345 (2.41%) genes were upregulated, 721 (5.04%) genes were downregulated, and 13,231 (92.54%) genes remained unaltered after MBZ exposure. The overexpressed genes identified were *CCL2*, *IL1A*, and *CDKN1A*. In contrast, the *H3C7*, *H3C11*, and *H1-5* were the top 3 underexpressed genes. Gene set enrichment analysis (GSEA) identified 8 pathways significantly overexpressed in the treated group ($p<0.05$ and FDR<0.25). The validation of the expression of top desregulated genes by RT-qPCR confirmed the transcriptome results, where MBZ increased the *CCL2*, *IL1A*, and *CDKN1A* and reduced the *H3C7*, *H3C11*, and *H1-5* transcript levels. Expression analysis in samples from TCGA databases correlated that the lower *ILI1A* and higher *H3C11* and *H1-5* gene expression are associated with decreased overall survival rates in patients with GC, indicating that MBZ treatment can improve the prognosis of patients. Thus, the data demonstrated that the drug MBZ alters the transcriptome of the AGP-01 lineage, mainly modulating the expression of histone proteins and inflammatory cytokines, indicating a possible epigenetic and immunological effect on tumor cells, these findings highlight new mechanisms of action related to MBZ treatment. Additional studies are still needed to better clarify the epigenetic and immune mechanism of MBZ in the therapy of GC.

Keywords: Gastric cancer, Transcriptome, Mebendazole, Biomarkers.

1 INTRODUCTION

Gastric cancer (GC) is the fifth most common type of cancer, excluding non-melanoma skin cancer. It affects over 1 million people (5.6% of cases) worldwide. Considered the fourth most lethal cancer in the world, it is a serious global health problem, with around 768,000 deaths reported in 2020 [1]. Due to the complexity of GC diagnosis, the standard treatment procedure is primarily surgical excision of the tumor, and the technique used varies according to its characteristics [2]. Complete tumor removal and lymph node dissection are seen as essential for curing GC. Studies showed that the addition of the combination of chemotherapy drugs epirubicin, cisplatin, and infused fluorouracil or docetaxel, oxaliplatin, and fluorouracil/leucovorin to surgery enhanced the 5-year overall survival rate when compared to the surgery procedure alone [3,4]. Despite the advances in GC treatment, the development of drug resistance limits the efficacy of actual chemotherapy, leading to chemotherapy failure, tumoral progression, and/or high recurrence rates (14 - 60% of patients), which is frequently diagnosed in the first 2 years after resection in distant sites, and is one of the main factors associated with death in patients with GC [5–7].

Drug repositioning is a pharmaceutical strategy for discovering new therapeutic applications for a drug already approved for treating another disease [8]. The repositioning decreases the estimated time for approval by around 6 years, as initial studies have already been carried out, with development costs of around 300 million dollars [9]. Furthermore, repurposing studies can reveal new pharmacological mechanisms and molecular targets, enriching the discovery process [8,10].

Our group has demonstrated the anticancer activity of Mebendazole (MBZ), an antihelmintic clinically approved, in the GC cell lines. The anticancer effect of MBZ in GC cell line models has been extensively investigated, shedding light on its potential as a therapeutic agent in GC treatment. Results showed that MBZ can inhibit drug transporters' migration, invasion, and expression and reduce *MYC* expression and activity. These effects were linked to genotoxicity and apoptosis induction [11–13]. Tumor metabolism and the glycolytic pathway activity also changed in GC cell line AGP-01 after treatment with MBZ. The metabolic modulation was associated with the early effect of MBZ in the GC cell line and related to further cell death [14].

Based on that, this study aimed to analyze in-depth the effect of MBZ through transcriptome, the molecular signature of gastric adenocarcinoma cells after treatment with the drug MBZ, seeking to discover new mechanisms related to MBZ treatment and investigate

potential molecular targets for the treatment of patients with GC. This research could contribute to significant advances in the field of oncology and the search for more effective and personalized therapies for patients affected by this neoplasm.

2 METHODS

2.1 Chemicals

Mebendazole (MBZ) (Medley®, 500 mg) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM and stored at -20 °C until use.

2.2 Cell Culture and Conditions

The gastric adenocarcinoma cell line AGP-01 was established from malignant ascitic fluid [15]. The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco ®). Media were supplemented with 10% (v/v) fetal bovine serum (Gibco®), 1% (v/v) penicillin (100 U mL-1), and streptomycin (100 mg mL-1) (Invitrogen®), and cells were maintained in a 5% CO2 air-humidified atmosphere at 37 °C. In all *in vitro* experiments, cells were treated with MBZ at 1 μM for 14 hours; previous results show that it is a non-cytotoxic time [11].

2.3 RNA extraction

AGP-01 was seeded into a 6-well plate ($1x10^6$ cells/well) and then treated with MBZ (1 μM) for 14 hours. After that, the total mRNA extraction was performed by the AllPrep DNA/RNA/Protein Kit (Qiagen, Germany). RNA concentration and quality assessment were determined using a NanoDrop spectrophotometer (Kisker, Germany) and 1% agarose gels, respectively. Subsequently, the samples were stored at −80 °C until further use [16].

2.4 Microarray assay

The gene expression assay using microarray was conducted employing a one-color microarrays-based gene expression analysis kit (Agilent Technologies, USA), following the provided manufacturer's guidelines. Briefly, total RNA extracted from AGP-01 and AGP-01 cells treated with MBZ (1 μM for 14 hours) served as templates for cDNA synthesis with T7 RNA polymerase. The cDNA synthesis involved T7 RNA Polymerase Blend (red cap), which simultaneously amplified target material and incorporated Cyanine 3-CTP through a Low Input

Quick Amp Labeling kit (Agilent Technologies), adhering to the manufacturer's instructions. Labeled cRNA purification was carried out using an RNeasy mini-spin kit. CRNA was quantified using spectrophotometry (ng/mL) and analyzed based on the A260/280 parameter. For the hybridization step, 300 ng of Cy3 labeled cRNA, 5 mL of 10x Gene expression blocking agent, and 1 mL of 25x fragmentation buffer were used. Hybridization occurred for 17 hours at 65°C and 10 rpm on a SurePrint G3 Human Gene Expression 8 _ 60K microarray chip (G4851A, Agilent, USA). A microarray scanner (G4900DA, Agilent) was employed with the following settings: scan area (61 _ 21.6 mm), 5 mm resolution, and green channel. Microarray scan images were obtained using Feature Extraction v10.10 [17].

2.5 Differentially expressed genes and functional enrichment analysis

R studio with Limma package was used to evaluate differentially expressed genes (DEGs) [18] between the AGP-01 cell line treated with MBZ and the non-treated group (negative control, NC). We used an adjusted p-value <0.05 corresponding to differential expression tests using Log Fold Change < 2. The significantly enriched genes between MBZ treatment and untreated control were identified by employing the Gene Set Enrichment Analysis (GSEA) [19]. The H collection (hallmark gene set) available in the Molecular Signatures Database (MSigDB) 3.0 was chosen to perform the enrichment analysis [20]. The standard parameters defined by Subramanian et al. (2005) were used in this analysis [19]. One hundred permutations determined the GSEA analysis significance, corrected p-value <0.05, and False Discovery Rate (FDR) <0.25. The GraphPad Prism™ Software was also used to represent enrichment scores and signal-to-noise values of individual genes. Furthermore, the Venn diagram in InteractiVenn [21] was used to show the enriched genes among the significantly enriched gene sets.

2.6 Validation of gene expression by RT-qPCR

To validate the transcript expression of the top genes modulated by MBZ displayed by the transcriptome analyses, the AGP-01 was seeded into a 6-well plate ($1x10^6$ cells/well) and then treated with MBZ (1 μM) for 14 hours. The total RNA was extracted using TRIzol® Reagent (Life Technologies®, USA) based on the manufacturer's recommendations, and the RNA concentration and quality were determined using NanoDrop (Thermo Scientific). Further,

reverse transcription was performed using a High-Capacity cDNA kit (Life Technologies, USA).

Quantitative real-time PCR (qRT-PCR) was executed by the Fast SyberGreen kit (Applied Biosystems, USA). The Relative expression levels of *H3C7* (NM_021018.3), *H3C11* (NM_003533.3), *H1-5* (NM_005322.3), *CCL2* (NM_002982.4), *IL1A* (NM_000575.5) and *CDKN1A* (NM_000389.5) were normalized and determined using *RPLP0* (NM_001002.4) as reference gene. Primer efficiency > 95% was determined for all genes described.

All requirements proposed in Minimum Information for Publication of Quantitative Real-Time PCR Experiments - MIQE Guidelines were followed [22]. The expression level was calculated using the $2^{-\Delta\Delta CT}$ method [23], considering the non-treated group (Negative Control) as a calibrator of the experiments.

2.7 Gene expression analysis of genes regulated by MBZ in online databases

The expression of genes regulated by MBZ was also evaluated by an *in silico* global gene expression analysis in databases with free access to transcriptome data from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and Genotype-Tissue Expression (GTEx). Expression patterns in tumor samples and the correlation of gene expression with the survival rate of *H3C7*, *H3C11*, *H1-5*, *CCL2*, *IL1A*, and *CDKN1A* genes were assessed by GEPIA (http://gepia.cancer-pku.cn/) and Kaplan Meier plotter (https://kmplot.com/analysis/) software, respectively [24,25].

2.8 *In vitro* Statistical Analyses

All assays were performed in three independent experiments, in triplicates, and the results are expressed as mean ± standard deviation (SD). The Kolmogorov performed normality distribution analysis- Smirnov test. The NC group was compared with MBZ by t-test. Significant differences were considered with a confidence interval of 95% ($p<0.05$). GraphPad Prism 5.01 software was used for data analysis and graph design.

3 RESULTS

3.1 Identification of differentially expressed genes (DEGs)

We first analyzed the microarray transcriptome to compare the gene expression profile of GC cells treated with MBZ (1 µM) versus untreated cells (control). Figure 1 shows the volcano plot of differentially expressed genes (DEGs) in the dataset with > 2-fold change considering corrected *p*-value < 0.05, and the DEGs are shown as red dots. There were 345 (2.41%) up-regulated genes, 721 (5.04%) down-regulated genes, and 13,231 (92.54%) not significantly expressed genes in the MBZ-treated group compared to the control group, shown as black dots (Figure 1). The top ten most up-regulated genes and the top ten most down-regulated genes are presented in Table 1. Considering the fold change (FC), *CCL2* (C-C Motif Chemokine Ligand 2), *IL1A* (Interleukin 1 alpha) and *CDKN1A* (Cyclin Dependent Kinase Inhibitor 1A) were the three most up-regulated genes; and *H3C7* (H3 Clustered Histone 7), H3C11 (H3 Clustered Histone 11) and *H1-5* (H1.5 Linker Histone) were the three most down-regulated genes.

Figure 1. Volcano plot shows differentially expressed genes (DEGs) in the data set comparing the MBZ treatment in gastric cancer cell line AGP-01. Fold change (FC) as log with base 2 is plotted against adjusted p-value as log with base 10. The volcano plot shows the DEGs in red and the not-significantly expressed genes in black.

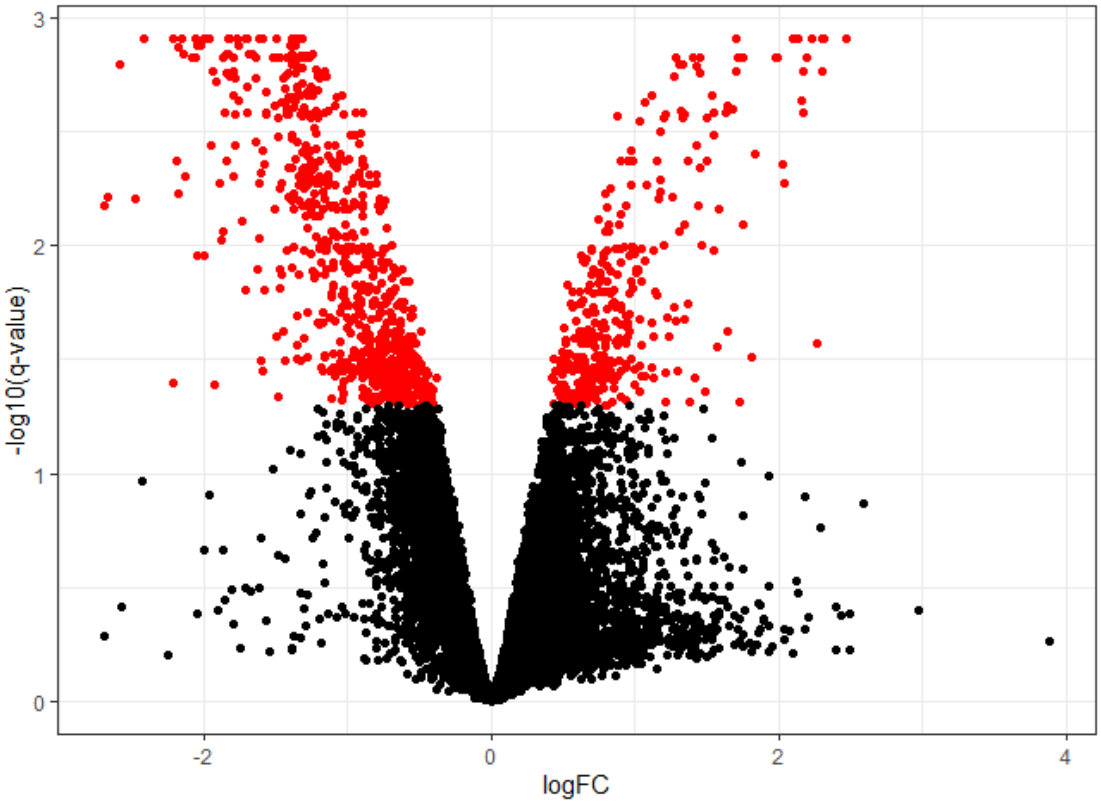


Table 1. Top 10 up- and down-regulated differentially expressed genes between MBZ-treated gastric cancer cells and untreated gastric cancer cells. The top 3 up-and down-regulated genes are highlighted.

Differently Expressed Genes (DEGs)							
Upregulated				**Downregulated**			
Gene Symbol	**ENTREZID**	**LogFC**	**Adj.P.Val**	**Gene Symbol**	**ENTREZID**	**LogFC**	**Adj.P.Val**
CCL2	6347	**2.47**	1.24×10^{-3}	***H3C7***	8968	**-2.69**	6.72×10^{-3}
IL1A	3552	**2.26**	2.68×10^{-2}	***H3C11***	8354	**-2.67**	6.15×10^{-3}
CDKN1A	1026	**2.16**	2.29×10^{-3}	***H1-5***	3009	**-2.59**	1.61×10^{-3}
BTG2	7832	2.13	1.24×10^{-3}	*H3C2*	8358	-2.48	6.27×10^{-3}
PSD2	84249	2.04	5.32×10^{-3}	*CDK1*	983	-2.42	1.24×10^{-3}
NEURL3	93082	2.02	4.38×10^{-3}	*H3C12*	8356	-2.21	4.00×10^{-2}
TP53INP1	94241	1.99	1.49×10^{-3}	*H3C6*	8353	-2.18	4.26×10^{-3}
H2BC12	85236	1.98	1.49×10^{-3}	*CKS1B*	1163	-2.18	5.90×10^{-3}
HHAT	55733	1.81	3.06×10^{-2}	*H4C3*	8364	-2.13	4.95×10^{-3}
DQX1	165545	1.75	1.49×10^{-3}	*H1-3*	3007	-2.08	1.49×10^{-3}

Adj.P.Val: adjusted *p*-value; ENTREZID: National Center for Biotechnology Information (NCBI) Gene ID; LogFC: Fold Change in logarithmic scale.

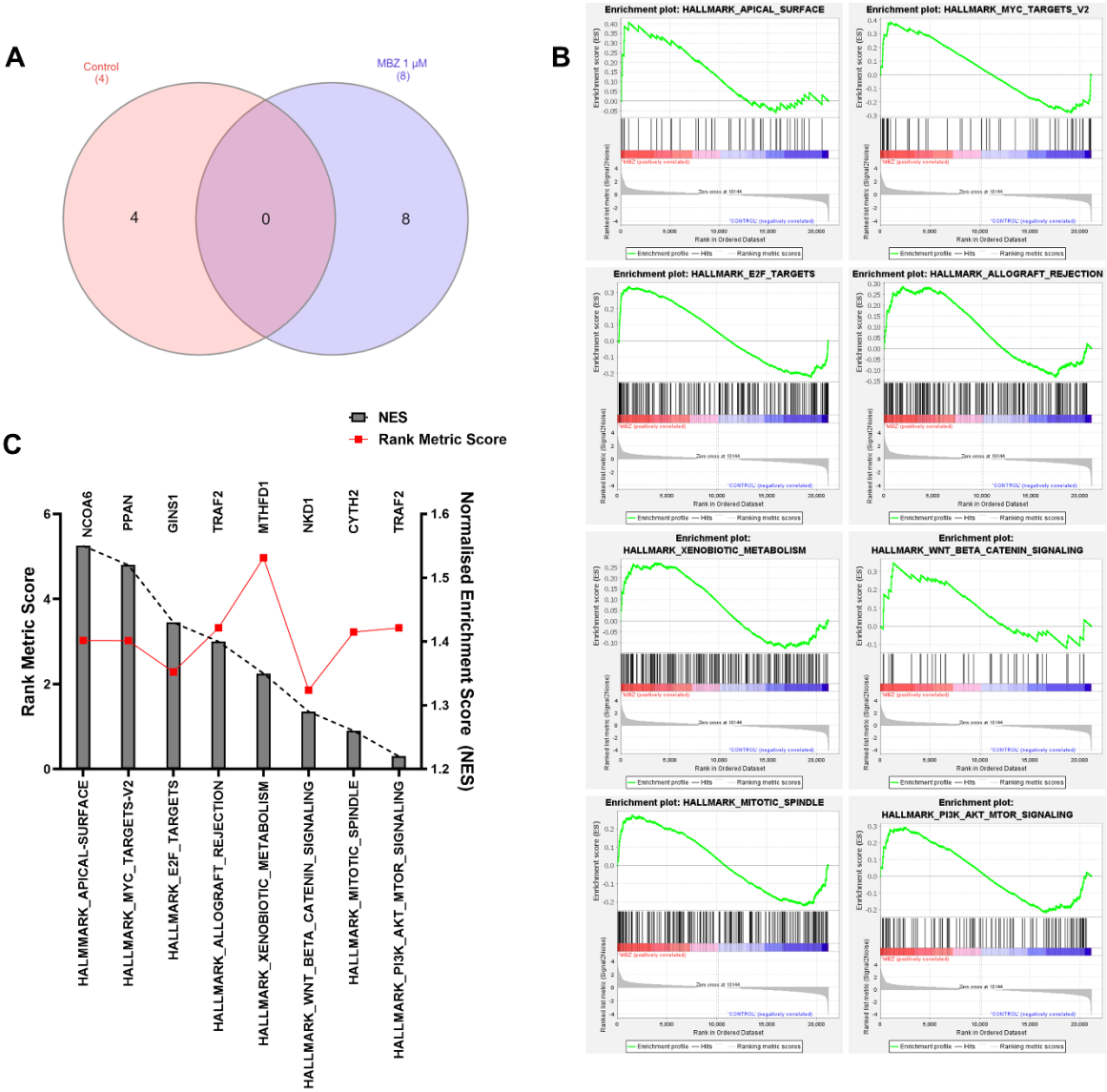
3.2 Hallmarks pathway enrichment analysis

The normalized dataset was used for enrichment comparing applying Gene Set Enrichment Analysis (GSEA). A total of 21,210 genes after collapsing features into gene symbols were analyzed. 10,144 (47.8%) enriched genes in the MBZ treatment group and 11,066 (52.2%) in the control group. Our analysis found 20 hallmark gene sets up-regulated in the treated group (Supplementary Table 1). However, only 8 gene sets are significantly enriched in the MBZ treatment group at a nominal p-value < 0.05 and FDR < 0.25. The hallmarks are: Apical surface (NES = 1.55, p = 0.00001), MYC Target V2 (NES = 1.52, p = 0.00001), E2F Targets (NES = 1.43, p= 0.00001), Allograft Rejection (NES = 1.40, p = 0.00001), Xenobiotic Metabolism (NES = 1.35, p = 0.00001), WNT Beta Catenin Signaling (NES = 1.29, p = 0.00001), Mitotic Spindle (NES = 1.26, p = 0.00001), and the PI3K AKT mTOR Signaling (NES = 1.22, p = 0.00001).

Each significant gene data set was plotted to demonstrate the ranked gene with enriched values positively correlated in the treatment group. It identified enriched DEGs to all eight-pathway datasets analyzed and represented as a Venn diagram (Figure 2A). Moreover, we

identified the enriched DEGs for each significantly enriched pathway for the treated group. In the GSEA analysis, genes were sorted by the rank list metric, which correlates with the gene measured by the signal-to-noise ratio and the running enrichment score (running ES) going down the ranked list of genes, increasing a running-sum statistic (Figure 2C). The genes *NCOA6*, *PPAN*, *GINS1*, *TRAF2* (top-ranked gene in two hallmarks), *MTHFD1*, *NKD1*, and *CYTH2* were the most correlated with the treatment group for the significant hallmarks.

Figure 2. GSEA analysis shows enriched pathways and genes related to MBZ treatment in the AGP-01 cell line. The A) Venn diagram shows the enriched hallmarks pathway in each group, B) the 8 significantly enriched pathways, and C) the enriched gene of each pathway are shown in cells treated with MBZ.

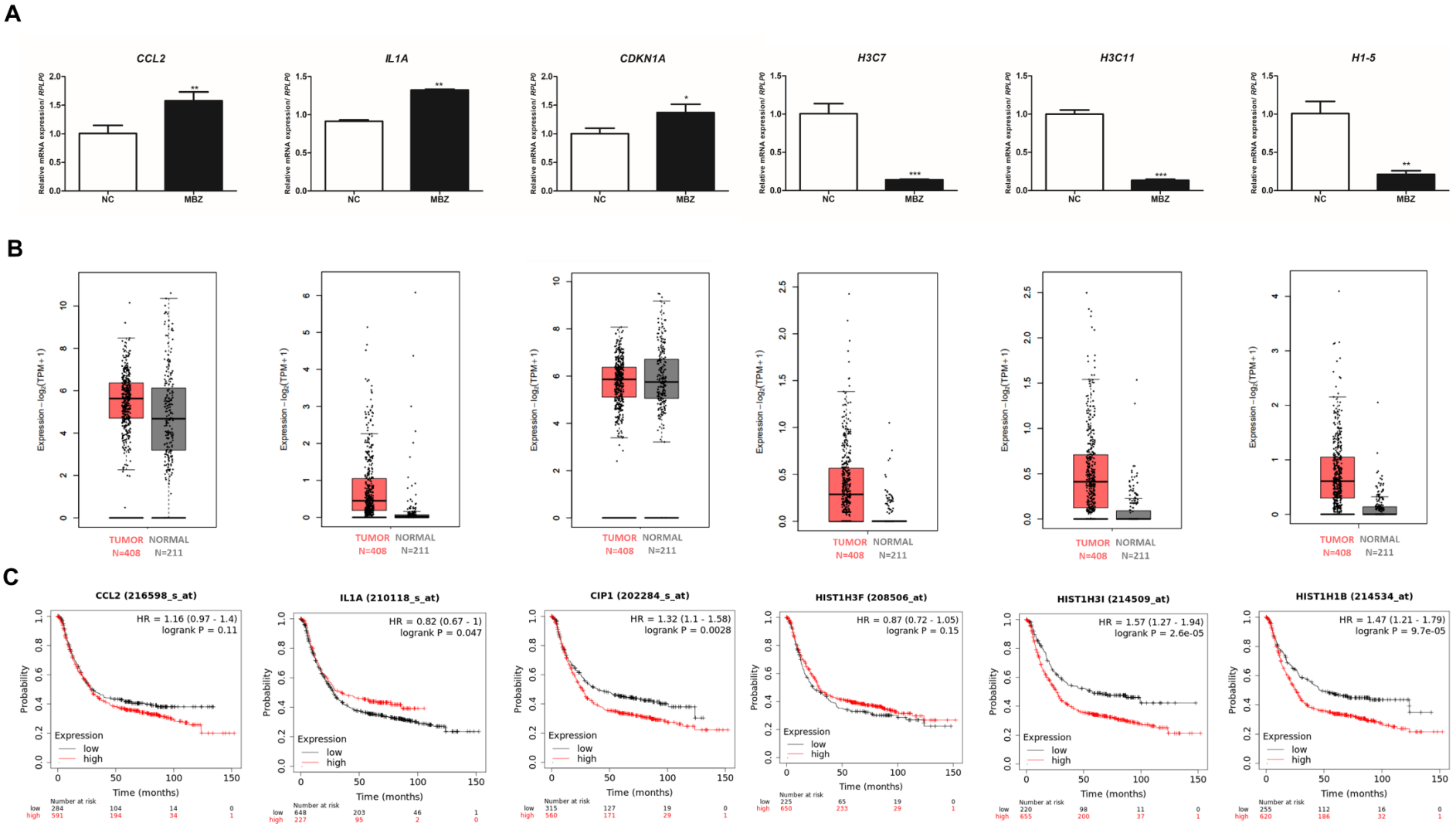


3.3 Transcriptome validation shows new MBZ potential targets of clinical interest

Then, the expression of the top 3 up-and down-regulated genes showed by the transcriptome was validated by RT-qPCR. Figure 3A shows evidence that the *CCL2* ($p<0.01$), *IL1A* ($p<0.01$), and *CDKN1A* ($p<0.05$) transcript levels were significantly enhanced after MBZ treatment. Otherwise, *H3C7* ($p<0.001$), *H3C11* ($p<0.001$), and *H1-5* ($p<0.01$) mRNA levels showed to be reduced after the treatment. Thus, the expression data validated the transcriptome analysis of the AGP-01 cell line after 14 hours of treatment with MBZ.

Next, to verify if the genes modulated by MBZ had clinical interest, we sought to analyze the pattern of gene expression in the GEPIA database, which contains the gene expression information of gastric tumor (N=408) and normal (N=211) samples of TCGA and GTEx. Results did not evidence a significant difference between the tumoral and normal expression of the MBZ target in clinical samples (Fig. 3B). Furthermore, the online database Kaplan–Meier Plotter showed that the low *IL1A* ($p<0.05$) expression and that the high transcript levels of *CDKN1A*/*CIP1* ($p<0.01$), *H3C11*/*HIST1H3I* ($p<0.0001$) and *H1-5*/*HIST1H1B* ($p<0.0001$) were highly correlated with the reduction in the overall survival (OS) rate in patients with CG (N= 875), as shown in figure 3C. Altogether, data suggest that MBZ may reduce the expression of *H3C11* and *H1-5* and enhance the expression of *ILI1A*, which could lead to an increase in the OS rate of patients with GC.

Figure 3. Genes regulated by MBZ have a clinical interest in GC therapy. (A) After 14 hours of cell exposure to MBZ, total mRNA was extracted and the top 3 up- (CCL2, ILI1A, and CDKN1A) and down- (H3C7, H3C11, and H1-5) regulated genes obtained by transcriptome analyses were validated by RT-qPCR. Gene expression was normalized by the reference gene RPLP0, and the NC group was used as a calibrator of the experiment. (B) Global expression of genes regulated by MBZ was analyzed by online gene expression databases. The GEPIA dataset shows the expression of genes in CG (red boxplot) and normal (grey boxplot) samples. (C) Kaplan–Meier Plotter analysis evidenced the relation of the high or low expression of genes regulated by MBZ in the OS rate of patients with GC. Data are presented as the mean ± SD of three independent experiments, and statistical analysis was performed with the t-test. Significant differences: *p<0.05, ***p<0.0001. MBZ: Mebendazole. NC: Negative control. HR: Hazard Ratio.



4 DISCUSSION

Gastric cancer (GC) is a human public health issue globally, responsible for more than one million new cases in 2020, and ranked as the fourth cause of death [1]. Several risk factors have been considered critical steps for carcinogenesis, such as *Helicobacter pylori* infection, alcohol consumption, smoking, salty food, and processed meat [26]. However, recent studies have proposed a molecular classification and stratification to understand the disease and find a better therapy strategy [27,28].

Studies associating genes with drugs and molecular targets (target-based drugs) are becoming more widespread in drug research and development [29]. The transcriptome analysis is one essential tool in the search, discovery, and/or validate new potential drug targets, providing a robust approach in the assessments of the mechanism of action of a new drug [30]. After that, we sought to adopt the drug repositioning strategy to validate new pharmacological biomarkers relevant to treating patients with GC. Although substantial results describing the cellular effects of MBZ in GC cell lines have been described by our group [11–14], the underlying molecular mechanism of the anti-cancer activities of MBZ remains elusive, and the transcriptome modulation by MBZ is poorly understood. Thus we south to investigate the effect of MBZ in the transcriptome of the GC cell line AGP-01 for the first time. Acknowledging the transcriptome level in the progression of MBZ-induced anti-cancer effects on the gastric cancer cell line can illustrate the fundamental mechanism for developing new therapeutic agents for GC.

As an efficient molecular biology tool, microarray delivers extraordinary details about the transcriptional machinery of an organism. We screened all the transcripts of AGP-01 cells exposed to MBZ. Microarray analysis found 1,066 DEGs between the MBZ and control groups, including 345 elevated genes and 721 down-regulated genes. Based on the obtained DEGs, the GSEA analysis was performed to investigate the effect of MBZ on the AGP-01 cells.

The Gene set enrichment analysis (GSEA) analysis aims to identify sets of genes in the huge list of significantly over-represented probes and group them based on their function in a known biological pathway [31]. In this study, the GSEA showed that the Apical Surface, MYC Targets V2, E2F Targets, Allograft Rejection, Xenobiotic Metabolism, Wnt/β-catenin Signaling, Mitotic Spindle, and PI3K/AKT/mTOR hallmarks were differentially expressed after MBZ treatment and that the genes *NCOA6*, *PPAN*, *GINS1*, *TRAF2*, *MTHFD1*, *NKD1*, and *CYTH2* were the most correlated with the treatment group for each significant hallmark, the

TRAF2 gene was strongly correlated with two different hallmarks, Allograft Rejection and PI3K/AKT/mTOR (Fig. 2), these targets are involved in signaling pathways related to tumor development, drug resistance, among others, and some of them still have an unknown role in gastric carcinogenesis.

The Nuclear Receptor Coactivator 6 (*NCOA6*) is a multifunctional coactivator of several transcription factors and nuclear receptors and modulates many critical cell functions, including cell migration/invasion [32,33]. The cytohesin-2 (*CYTH2*) is a member of the cytohesin family, which are key regulators of cytoskeletal dynamics, cell migration, and cellular signaling. The enhancement in *CYTH2* expression was associated with vascular invasion and reduction in OS rates in patients with hepatocellular carcinoma [34].

The GINS Complex Subunit 1 (*GINS1*) gene transcribes for PSF1 protein and participates in the DNA replication process. It is essential for initiating and elongating DNA by recruiting CDC45 and DNA polymerase enzymes. It also interacts directly with the Topoisomerase II alpha enzyme (TOP2A) assisting in the topography maintenance of DNA during replication [35,36]. The high expression of *GINS1* was linked to enhancement in proliferation and migration in glioma cell lines [35] and with a poor prognosis in GC [37]. Otherwise, its protein inhibition reduces proliferation in lung colon tumor models [38,39].

In agreement with the data, studies have shown that MBZ can reduce the expression of mesenchymal proteins and tumoral migration and invasion in different types of cancer [11,40,41]. Similarly, the cytotoxic effect of MBZ was shown to be related to the induction of DNA damage and mitotic arrest by depolymerizing tubulin and thus disrupting the functions of microtubules in lung, gastric, and brain cancer models with similar effects as anti-tubulin agents vincristine and paclitaxel [11,13,42,43].

Based on the recent advances in genome analysis, important signaling pathways and related biomarkers with clinical importance for GC have been identified [44]. The PI3K/AKT/mTOR signaling pathway is highly dysregulated in GC. Studies have shown that this pathway can lead to tumor progression by inhibiting apoptosis inducing drug resistance, metastasis, and angiogenesis [45].

Our analysis showed that the *TRAF2* (Tumor necrosis factor receptor-associated factor 2) gene was the most well-categorized into the PI3K/AKT/mTOR and Allograft Rejection signaling pathways after MBZ treatment. *TRAF2* is one member of the Tumor necrosis factor (TNF) superfamily and is mainly known for its role in the regulation and homeostasis of immune cells. Studies evidenced that this gene transcript for a dual-function protein, being a signal hub and acting as a ubiquitin E3 ligase that can mediate the TNFα-NFκB signal pathway

and regulate mTORC1/2 activity [46,47]. *TRAF2* is hyperregulated in GC and is associated with cell growth, migration, and invasion [48,49]. In addition, Liang et al. [50] showed that *TRAF2* can induce tumorigenesis by activating the mTORC1 pathway and that its inhibition leads to reducing the growth and survival profile of liver cancer cells both *in vitro* and *in vivo*, categorizing *TRAF2* as an interesting pharmacological target in cancer therapy.

Crosstalks between the PI3K-AKT and Wnt/β-catenin signaling pathway can occur once phosphorylated AKT can lead to indirect and/or direct activation of β-catenin, promoting tumorigenesis and drug resistance [51,52]. Interestingly, based on our analysis, the molecular target *NKD1* (*Naked Cuticle Homolog 1*) was also related to the Wnt/β-catenin signaling pathway after the treatment with MBZ. Several studies have shown that *NKD1* can deplete cancer metastatic profiles by inhibiting the Wnt signaling pathway by preventing the β-catenin nuclear accumulation [53]. In GC, using target-miRNAs to inhibit the *NKD1* expression enhanced the proliferation and invasiveness of GC cells, and the *NKD1* overexpression showed the opposite effect [54,55].

The *MYC* (also known as *c-MYC*) proto-oncogenes encode a family of transcription factors that are highly activated in many types of cancer. *MYC* acts as a regulator of multiple biological pathways, mediating its functions as a transcription factor by regulating the expression of thousands of genes, directly or indirectly [56]. *MYC* overexpression is observed in 40% - 77% of GC, being more observed in intestinal-type tumors and linked to a worse prognosis in patients with GC tumors [57–59].

Remarkably, the GC cell line AGP-01 used in this study was established from an intestinal-type metastatic gastric adenocarcinoma. These cell lines were cytogenetically characterized with a high frequency of *MYC* amplification and other alterations typically found in GC patients [15,60,61]. Our previous results showed that MBZ decreased signals of *MYC* amplification and its mRNA and protein levels in AGP-01. The *MYC* inactivation was strongly related to MBZ cytotoxic potential against the GC intestinal-type cell line [13].

MYC regulates the Peter Pan (PPAN) gene and is a key ribosome biogenesis regulator. Studies showed that the knockdown of *PPAN* triggers nucleolar stress, culminating in intrinsic apoptosis and stimulating autophagy [62,63]. In addition, the inhibition of *PPAN* expression impaired tumoral growth and induced cell cycle arrest in G0/G1 and later in G2/M. Also, the genetic silencing stabilized the expression of p53 and induced *CDNK1*/p21 expression, leading to cellular death [64]. Hence, since it seems difficult to inhibit the activity of the MYC protein pharmacologically due to the lack of available binding sites, PPAN has experimentally

demonstrated to be a potential druggable target to treat or prevent tumorigenesis downstream targets of *MYC* in cancer [65].

Recent studies have highlighted MBZ as a metabolic modulator in GC, initial data showed that MBZ can suppress glycolytic flow by reducing the mRNA expression and interacting with glycolic components, leading to a reduction in glucose uptake and ATP formation. This initial suppression was linked to MBZ's antitumoral effect in the AGP-01 cell line [14]. Surprisingly, this study evidenced that MBZ can regulate the trifunctional enzyme methylenetetrahydrofolate dehydrogenase1 (*MTHFD1*), which acts in the cell cytoplasm and catalyzes three sequential reactions for the biosynthesis of thymidylate, methionine, and purine nucleotides, being essential for nucleotide synthesis and tumoral growth [66], this finding adds new shreds of evidence to the hypothesis that the drug MBZ act a metabolic modulator in different biosynthetic pathways in GC. In turn, Piskounova et al. [67] showed that the folate pathway depletion by the chemotherapy drug Methotrexate (MTX) or by *MTHFD1* knockdown evidenced similar results by inhibiting tumor metastasis [11,14,68].

The analysis of the differentiated expressed genes showed that *CCL2*, *IL1A*, and *CDKN1A* genes were the top 3 ranked up-regulated ones after the MBZ treatment. I*n vitro* mRNA analysis by qRT-PCR validated the transcriptome analyses, evidencing that these genes were upregulated in AGP-01 after the treatment with MBZ (Fig. 3A).

The *CCL2* gene transcribes chemokine ligand 2, but the function of this protein remains contradictory in cancer. Some studies indicate that high expression of *CCL2* is associated with the initiation and progression of different types of cancer, including GC, having a non-favorable effect once the binding of CCL2 protein to its receptor, CCR2 (C-C chemokine receptor type 2), can activate tumor cell growth and proliferation, promoting migration and the recruitment of immunosuppressive cells into the tumor microenvironment, favoring cancer progression [69,70]. However, reports also show that CCL2 increases the antitumor capacity of specific immune cells, such as inflammatory monocytes and neutrophils, showing that the inflammatory state of the tumor may have a dual role in tumor development [71,72].

The *IL1A* gene plays a role in the transcription of interleukin 1α (IL-1α), an important cytokine known as alarmin, responsible for triggering and signaling an increase in the inflammatory response [73]. Like CCL2, IL-1α appears to have a dual role in tumor progression, as work shows that it triggers both pro-tumor and anti-tumor responses in different types of cancer [73]. Its antitumor role is related to the suppression of cell proliferation through binding with its receptors (IL-1R) on tumor cells, activation of T lymphocytes, Natural Killer (NK) cells, and dendritic cells as well as with the production of IL-12 [increasing the cytotoxic

activity of NK cells and T lymphocytes, inducing interferon-gamma (IFN-γ), TNF-α, among other pro-inflammatory molecules] and induction of apoptosis [74–80]. However, some studies with gastric specimens demonstrated a correlation between distant liver metastasis in GC patients with enhancement levels of IL-1α, highlighting the correlation between IL-1α expression and tumor progression and metastasis in GC [81]. Moreover, studies in mice showed that the up-regulation of IL-1α resulted in cell proliferation and acquisition of a pre-malignant profile, leading to further dysplasia [82].

Molecular signature analyses showed that treatment with MBZ in breast cancer (MCF-7) and leukemia (HL60) cell lines led to increased expression of genes that were related to the activation of the M1 phenotype of monocytes/macrophages, which have phagocytic and antigen-presenting activity, as well as the ability to produce activating cytokines of T Helper Lymphocytic 1 (Th-1) and, therefore, mediate antitumor response. The study also demonstrated the expression of cytokines such as TNF, IL8 and IL6, IL-1β, surface markers (CD80 and CD86), and other chemokines, favoring the antitumor response [83]. Thus, collected data suggests that the MBZ can induce an antitumor response in immune system cells, favoring its cytotoxic profile against GC.

The *CDKN1A* gene (*Cyclin Dependent Kinase Inhibitor 1A*), known as a tumor suppressor gene that transcribes for the p21 protein [84], was also found to be overexpressed after MBZ treatment in the gastric lineage AGP-01. This protein acts as a cell cycle regulator in the transition from the G1 to S phase. It can regulate tumor development by inducing G1 cell cycle arrest, thus decreasing the rate of tumor growth, cell proliferation, and differentiation [85,86]. Our group had shown previously that MBZ, in low concentrations, leads to G0/G1 cell cycle arrest in the AGP-01 cell line, being associated with its antitumoral mechanism [13].

The hypo-expression of p21 is well-reported in cancer. Some epigenetic modifications, such as methylation, can lead to *CDKN1A* gene silencing, which is related to a worse prognosis of patients with prostate and breast cancers and acute lymphoblastic leukemia (ALL) [87–89]. Interestingly, this study also showed that MBZ can alter *PPAN* expression. The *PPAN* genetic silencing induced the *CDNK1*/p21 expression [64], a similar effect attributed to MBZ treatment. Other studies have shown that the increase or the re-establishment of p21 expression can lead to the inhibition of tumor growth and increase the sensitivity of malignant cells to targeted chemotherapy drugs [90,91]. This evidence can point out MBZ as a promising new antitumoral drug.

Otherwise, transcriptome analysis revealed the *H3C7* (H3 histone cluster 7), *H3C11* (H3 histone cluster 11), and *H1-5* (H1 histone linker 5) genes as the top-3 transcripts down-

regulated after treatment with MBZ, which were validated by mRNA analysis (Fig. 3A). These genes encoding for histone proteins, and are linked to the DNA packaging processes in cells and can significantly control cellular transcription machinery and overall protein synthesis [92]. Studies have shown that the enhancement in transcript levels of these genes can assist in promoting cell transformation and the acquirement of a malignant phenotype, being related to the worst prognosis in different types of cancer [93–98].

Many epigenetic mechanisms can regulate chromatin packaging states, ranging from euchromatin (loosely compacted) to heterochromatin (highly compacted). Six epigenetic mechanisms that can alter the structure of chromatin have been described as 1) Nuclear dynamics; 2) DNA methylation; 3) Covalent modification of histones; 4) ATP-dependent chromatin remodeling complexes; 5) Histone variants; 6) Non-coding RNA (ncRNA), including microRNA (miRNA/miR) and long ncRNA (lncRNA) [99].

Moreover, benzimidazole derivatives have been shown to act as epigenetic regulators in cancer, being able to inhibit histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) enzymes [100–104]. Studies have shown the interaction between miRNAs and their role in modifying the gene expression of oncogenes such as *RAS*, *MYC*, *MYB*, and *ABL1* by altering their epigenetic status, favoring cancer development. Furthermore, miRNAs' downregulation of these genes has also proven to have an antitumoral effect [105–109]. Accordingly, MBZ proved to reduce mRNA expression or inhibit the same transcripts in different study models, showing that MBZ can act as an epigenetic modulator through different mechanisms and thus alter different cancer pathways [10,13,109–111]. However, more studies are needed to understand better the role of MBZ in altering the epigenetic machinery in cancer and its relationship with its antitumor activity.

5 CONCLUSION

Therefore, in this study, we evaluate the genes and molecular pathways modulated by MBZ in a metastatic GC line (AGP-01). Results showed that the top genes modulated by MBZ were *CCL2*, *IL1A*, *CDKN1A*, *H3C7*, *H3C11*, and *H1-5*, which had their mRNA expression validated by RT-qPCR.Gene set enrichment analysis (GSEA) identified 8 enriched pathways where *NCOA6*, *PPAN*, *GINS1*, *TRAF2*, *MTHFD1*, *NKD1*, and *CYTH2* genes were correlated. We also found that the low expression of *IL1A* and the high expression of *H3C11*, and *H15* are related to a poor prognosis in patients with GC, being pharmacological potential targets of interest for MBZ therapeutics in the clinic. By using transcriptome analysis and validation in

GC models, this study helped us to comprehend the mechanism of action of MBZ better and evidenced new mechanisms of action related to epigenetic modulation and the immune system's antitumor response, favoring MBZ cytotoxic potential, thus highlighting its potential as possible new chemotherapy in the treatment of GC. However, more studies are needed to understand better the role of MBZ in altering the epigenetic machinery and the immune system's antitumor response with its antitumoral activity.

CRediT authorship contribution statement

Emerson Lucena da Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Felipe Pantoja Mesquita:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Laine Celestino Pinto:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Bruna Puty Silva Gomes:** Methodology, Formal Analysis, Investigation. **Edivaldo Herculano Correa de Oliveira:** Resource. **Rommel Mario Rodríguez Burbano:** Resources, Supervision, Funding acquisition. **Maria Elisabete Amaral de Moraes:** Resources, Supervision, Funding acquisition.. **Pedro Filho Noronha Souza:** Conceptualization, Writing – original draft, Supervision, Methodology, Formal analysis, Investigation. **Raquel Carvalho Montenegro:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Council for Scientific and Technological Development (CNPq) for a research grant to Rommel Mario Rodrigues (403493/2021-8), for a research productivity grant to Raquel Carvalho Montenegro (305459/2019-8), Pedro Filho Noronha Souza (305003/2022-4), Felipe P. Mesquita (Process number: 421392/2023-1), and a graduate scholarship to Emerson Lucena da Silva (Process numbers: 140436/2020-0;

141236/2023-0). The authors are grateful to the Brazilian funding agencies Coordination for Improvement of Higher Level Personnel (CAPES) and the Cearense Foundation of Scientific and Technological Support (FUNCAP) for additional financial assistance. Pedro F. N. Souza also thanks FUNCAP for visiting research grant (process nº PVS-0215 00099.01.00/23). Also, we thank the Federal University of Ceará, the Federal University of Pará, and the Evandro Chagas Institute (IEC) for fellowships and structural support. Raquel Carvalho Montenegro also thanks Red Latinoamericana de Implementación y Validación de guias clínicas Farmacogenomicas (RELIVAF) for supporting this work.

Ethics approval and consent to participate

Not applicable.

REFERENCES

[1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, CA Cancer J Clin 71 (2021) 209–249. https://doi.org/10.3322/caac.21660.

[2] L. Mocan, Surgical Management of Gastric Cancer: A Systematic Review, J Clin Med 10 (2021) 2557. https://doi.org/10.3390/jcm10122557.

[3] S.-E. Al-Batran, N. Homann, H. Schmalenberg, H.-G. Kopp, G.M. Haag, K.B. Luley, W.H. Schmiegel, G. Folprecht, S. Probst, N. Prasnikar, P.C. Thuss-Patience, W. Fischbach, J. Trojan, M. Koenigsmann, C. Pauligk, T.O. Goetze, E. Jaeger, J. Meiler, M.H. Schuler, R. Hofheinz, Perioperative chemotherapy with docetaxel, oxaliplatin, and fluorouracil/leucovorin (FLOT) versus epirubicin, cisplatin, and fluorouracil or capecitabine (ECF/ECX) for resectable gastric or gastroesophageal junction (GEJ) adenocarcinoma (FLOT4-AIO): A multicenter, randomized phase 3 trial., JCO 35 (2017) 4004–4004. https://doi.org/10.1200/JCO.2017.35.15_suppl.4004.

[4] D. Cunningham, W.H. Allum, S.P. Stenning, J.N. Thompson, C.J.H. Van de Velde, M. Nicolson, J.H. Scarffe, F.J. Lofts, S.J. Falk, T.J. Iveson, D.B. Smith, R.E. Langley, M. Verma, S. Weeden, Y.J. Chua, null MAGIC Trial Participants, Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer, N Engl J Med 355 (2006) 11–20. https://doi.org/10.1056/NEJMoa055531.

[5] L. Jiang, Z. Ma, X. Ye, W. Kang, J. Yu, Clinicopathological factors affecting the effect of neoadjuvant chemotherapy in patients with gastric cancer, World Journal of Surgical Oncology 19 (2021) 44. https://doi.org/10.1186/s12957-021-02157-x.

[6] S. Hong, M.A. Pereira, C.R. Victor, J.V.A. Gregório, B. Zilberstein, U. Ribeiro Junior, L.A.C. D'albuquerque, M.F.K.P. Ramos, PREOPERATIVE CHEMOTHERAPY VERSUS UPFRONT SURGERY FOR ADVANCED GASTRIC CANCER: A PROPENSITY SCORE MATCHING ANALYSIS, ABCD, Arq. Bras. Cir. Dig. 36 (2023) e1736. https://doi.org/10.1590/0102-672020230018e1736.

[7] I. Mokadem, W.P.M. Dijksterhuis, M. van Putten, L. Heuthorst, J.M. de Vos-Geelen, N. Haj Mohammad, G.A.P. Nieuwenhuijzen, H.W.M. van Laarhoven, R.H.A. Verhoeven, Recurrence after preoperative chemotherapy and surgery for gastric adenocarcinoma: a multicenter study, Gastric Cancer 22 (2019) 1263–1273. https://doi.org/10.1007/s10120-019-00956-6.

[8] J. Nath, R. Paul, S.K. Ghosh, J. Paul, B. Singha, N. Debnath, Drug repurposing and relabeling for cancer therapy: Emerging benzimidazole antihelminthics with potent anticancer effects, Life Sciences 258 (2020) 118189. https://doi.org/10.1016/j.lfs.2020.118189.

[9] N. Nosengo, Can you teach old drugs new tricks?, Nature 534 (2016) 314–316. https://doi.org/10.1038/534314a.

[10] A.E. Guerini, L. Triggiani, M. Maddalo, M.L. Bonù, F. Frassine, A. Baiguini, A. Alghisi, D. Tomasini, P. Borghetti, N. Pasinetti, R. Bresciani, S.M. Magrini, M. Buglione,

Mebendazole as a Candidate for Drug Repurposing in Oncology: An Extensive Review of Current Literature, Cancers (Basel) 11 (2019) 1284. https://doi.org/10.3390/cancers11091284.

[11] L.C. Pinto, B.M. Soares, J. de J.V. Pinheiro, G.J. Riggins, P.P. Assumpção, R.M.R. Burbano, R.C. Montenegro, The anthelmintic drug mebendazole inhibits growth, migration and invasion in gastric cancer cell model, Toxicol In Vitro 29 (2015) 2038–2044. https://doi.org/10.1016/j.tiv.2015.08.007.

[12] L.C. Pinto, C. de Fátima Aquino Moreira-Nunes, B.M. Soares, R.M.R. Burbano, J.A.R. de Lemos, R.C. Montenegro, Mebendazole, an antiparasitic drug, inhibits drug transporters expression in preclinical model of gastric peritoneal carcinomatosis, Toxicol In Vitro 43 (2017) 87–91. https://doi.org/10.1016/j.tiv.2017.06.007.

[13] L.C. Pinto, F.P. Mesquita, B.M. Soares, E.L. da Silva, B. Puty, E.H.C. de Oliveira, R.R. Burbano, R.C. Montenegro, Mebendazole induces apoptosis via C-MYC inactivation in malignant ascites cell line (AGP01), Toxicology in Vitro 60 (2019) 305–312. https://doi.org/10.1016/j.tiv.2019.06.010.

[14] E.L. da Silva, F.P. Mesquita, D.R. Aragão, A.J. de Sousa Portilho, A.D. Marinho, L.L.B. de Oliveira, L.B. Lima, M.E.A. de Moraes, P.F.N. Souza, R.C. Montenegro, Mebendazole targets essential proteins in glucose metabolism leading gastric cancer cells to death, Toxicology and Applied Pharmacology 475 (2023) 116630. https://doi.org/10.1016/j.taap.2023.116630.

[15] M.F. Leal, J.L.M. do Nascimento, C.E.A. da Silva, M.F.V. Lamarão, D.Q. Calcagno, A.S. Khayat, P.P. Assumpção, I.R. Cabral, M. de A.C. Smith, R.R. Burbano, Establishment and conventional cytogenetic characterization of three gastric cancer cell lines, Cancer Genetics and Cytogenetics 195 (2009) 85–91. https://doi.org/10.1016/j.cancergencyto.2009.04.020.

[16] K.C.G. Berg, P.W. Eide, I.A. Eilertsen, B. Johannessen, J. Bruun, S.A. Danielsen, M. Bjørnslett, L.A. Meza-Zepeda, M. Eknæs, G.E. Lind, O. Myklebost, R.I. Skotheim, A. Sveen, R.A. Lothe, Multi-omics of 34 colorectal cancer cell lines - a resource for biomedical studies, Molecular Cancer 16 (2017) 116. https://doi.org/10.1186/s12943-017-0691-y.

[17] B. Puty, L.O. Bittencourt, L.A.O. Lima, J.R. Plaça, A. Dionizio, M.A.R. Buzalaf, B.D. Gomes, E.H.C. de Oliveira, R.R. Lima, Unraveling molecular characteristic of fluoride neurotoxicity on U87 glial-like cells: insights from transcriptomic and proteomic approach, Front. Cell. Neurosci. 17 (2023). https://doi.org/10.3389/fncel.2023.1153198.

[18] M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, G.K. Smyth, limma powers differential expression analyses for RNA-sequencing and microarray studies, Nucleic Acids Res 43 (2015) e47. https://doi.org/10.1093/nar/gkv007.

[19] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc Natl Acad Sci U S A 102 (2005) 15545–15550. https://doi.org/10.1073/pnas.0506580102.

[20] A. Liberzon, A. Subramanian, R. Pinchback, H. Thorvaldsdóttir, P. Tamayo, J.P. Mesirov, Molecular signatures database (MSigDB) 3.0, Bioinformatics 27 (2011) 1739–1740. https://doi.org/10.1093/bioinformatics/btr260.

[21] H. Heberle, G.V. Meirelles, F.R. da Silva, G.P. Telles, R. Minghim, InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams, BMC Bioinformatics 16 (2015) 169. https://doi.org/10.1186/s12859-015-0611-3.

[22] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611–622. https://doi.org/10.1373/clinchem.2008.112797.

[23] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat Protoc 3 (2008) 1101–1108. https://doi.org/10.1038/nprot.2008.73.

[24] A. Lánczky, B. Győrffy, Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation, J Med Internet Res 23 (2021) e27633. https://doi.org/10.2196/27633.

[25] Z. Tang, C. Li, B. Kang, G. Gao, C. Li, Z. Zhang, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids Res 45 (2017) W98–W102. https://doi.org/10.1093/nar/gkx247.

[26] J. Machlowska, J. Baj, M. Sitarz, R. Maciejewski, R. Sitarz, Gastric Cancer: Epidemiology, Risk Factors, Classification, Genomic Characteristics and Treatment Strategies, Int J Mol Sci 21 (2020) 4012. https://doi.org/10.3390/ijms21114012.

[27] Cancer Genome Atlas Research Network, Comprehensive molecular characterization of gastric adenocarcinoma, Nature 513 (2014) 202–209. https://doi.org/10.1038/nature13480.

[28] R. Cristescu, J. Lee, M. Nebozhyn, K.-M. Kim, J.C. Ting, S.S. Wong, J. Liu, Y.G. Yue, J. Wang, K. Yu, X.S. Ye, I.-G. Do, S. Liu, L. Gong, J. Fu, J.G. Jin, M.G. Choi, T.S. Sohn, J.H. Lee, J.M. Bae, S.T. Kim, S.H. Park, I. Sohn, S.-H. Jung, P. Tan, R. Chen, J. Hardwick, W.K. Kang, M. Ayers, D. Hongyue, C. Reinhard, A. Loboda, S. Kim, A. Aggarwal, Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes, Nat Med 21 (2015) 449–456. https://doi.org/10.1038/nm.3850.

[29] C.W. Lee, S.M. Kim, S. Sa, M. Hong, S.-M. Nam, H.W. Han, Relationship between drug targets and drug-signature networks: a network-based genome-wide landscape, BMC Medical Genomics 16 (2023) 17. https://doi.org/10.1186/s12920-023-01444-8.

[30] J. Paananen, V. Fortino, An omics perspective on drug target discovery platforms, Brief Bioinform 21 (2019) 1937–1953. https://doi.org/10.1093/bib/bbz122.

[31] C. Simillion, R. Liechti, H.E.L. Lischer, V. Ioannidis, R. Bruggmann, Avoiding the pitfalls of gene set enrichment analysis with SetRank, BMC Bioinformatics 18 (2017) 151. https://doi.org/10.1186/s12859-017-1571-6.

[32] X. Wang, Y. Jia, X. Xu, Y. Hu, G. Fan, D. Jing, Z. Zhang, C. Wang, C. Song, Y. Qin, L. Peng, Nuclear receptor coactivator 6 (NCoA6) promotes cell proliferation, migration, and invasion in pancreatic cancer, Cancer Med 12 (2023) 18425–18439. https://doi.org/10.1002/cam4.6427.

[33] L. Wu, K.-Q. Zhao, W. Wang, L.-N. Cui, L.-L. Hu, X.-X. Jiang, J. Shuai, Y.-P. Sun, Nuclear receptor coactivator 6 promotes HTR-8/SVneo cell invasion and migration by

activating NF-κB-mediated MMP9 transcription, Cell Prolif 53 (2020) e12876. https://doi.org/10.1111/cpr.12876.

[34] K. Xu, J. Gao, X. Yang, Y. Yao, Q. Liu, Cytohesin-2 as a novel prognostic marker for hepatocellular carcinoma, Oncology Reports 29 (2013) 2211–2218. https://doi.org/10.3892/or.2013.2366.

[35] H. Yang, X. Liu, X. Zhu, M. Zhang, Y. Wang, M. Ma, K. Lv, GINS1 promotes the proliferation and migration of glioma cells through USP15-mediated deubiquitination of TOP2A, iScience 25 (2022) 104952. https://doi.org/10.1016/j.isci.2022.104952.

[36] C. Zhou, Z. Chen, B. Xiao, C. Xiang, A. Li, Z. Zhao, H. Li, Comprehensive analysis of GINS subunits prognostic value and ceRNA network in sarcoma, Front. Cell Dev. Biol. 10 (2022). https://doi.org/10.3389/fcell.2022.951363.

[37] M. Huang, C. Liu, Y. Shao, S. Zhou, G. Hu, S. Yin, W. Pu, H. Yu, Anti-tumor pharmacology of natural products targeting mitosis, Cancer Biol Med 19 (2022) 774–801. https://doi.org/10.20892/j.issn.2095-3941.2022.0006.

[38] J. Wen, X. Han, B. Wei, S. Zhang, H. Wei, [Expression of PSF1 in colon cancer tissues and its effect on the proliferation of colon cancer cells], Zhonghua Wei Chang Wai Ke Za Zhi 16 (2013) 70–74.

[39] J. Zhang, Q. Wu, Z. Wang, Y. Zhang, G. Zhang, J. Fu, C. Liu, Knockdown of PSF1 expression inhibits cell proliferation in lung cancer cells in vitro, Tumour Biol 36 (2015) 2163–2168. https://doi.org/10.1007/s13277-014-2826-8.

[40] V. Kralova, V. Hanušová, K. Caltová, P. Špaček, M. Hochmalová, L. Skálová, E. Rudolf, Flubendazole and mebendazole impair migration and epithelial to mesenchymal transition in oral cell lines, Chem Biol Interact 293 (2018) 124–132. https://doi.org/10.1016/j.cbi.2018.07.026.

[41] Z. Liang, Q. Chen, L. Pan, X. She, T. Chen, Mebendazole induces apoptosis and inhibits migration via the reactive oxygen species-mediated STAT3 signaling downregulation in non-small cell lung cancer, J Thorac Dis 16 (2024) 1412–1423. https://doi.org/10.21037/jtd-23-1978.

[42] M. De Witt, A. Gamble, D. Hanson, D. Markowitz, C. Powell, S. Al Dimassi, M. Atlas, J. Boockvar, R. Ruggieri, M. Symons, Repurposing Mebendazole as a Replacement for Vincristine for the Treatment of Brain Tumors, Mol Med 23 (2017) 50–56. https://doi.org/10.2119/molmed.2017.00011.

[43] J. Sasaki, R. Ramesh, S. Chada, Y. Gomyo, J.A. Roth, T. Mukhopadhyay, The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells, Molecular Cancer Therapeutics 1 (2002) 1201–1209.

[44] Z.-N. Lei, Q.-X. Teng, Q. Tian, W. Chen, Y. Xie, K. Wu, Q. Zeng, L. Zeng, Y. Pan, Z.-S. Chen, Y. He, Signaling pathways and therapeutic interventions in gastric cancer, Signal Transduct Target Ther 7 (2022) 358. https://doi.org/10.1038/s41392-022-01190-w.

[45] A. Glaviano, A.S.C. Foo, H.Y. Lam, K.C.H. Yap, W. Jacot, R.H. Jones, H. Eng, M.G. Nair, P. Makvandi, B. Geoerger, M.H. Kulke, R.D. Baird, J.S. Prabhu, D. Carbone, C.

Pecoraro, D.B.L. Teh, G. Sethi, V. Cavalieri, K.H. Lin, N.R. Javidi-Sharifi, E. Toska, M.S. Davids, J.R. Brown, P. Diana, J. Stebbing, D.A. Fruman, A.P. Kumar, PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer, Molecular Cancer 22 (2023) 138. https://doi.org/10.1186/s12943-023-01827-6.

[46] N. Etemadi, M. Chopin, H. Anderton, M.C. Tanzer, J.A. Rickard, W. Abeysekera, C. Hall, S.K. Spall, B. Wang, Y. Xiong, T. Hla, S.M. Pitson, C.S. Bonder, W.W.-L. Wong, M. Ernst, G.K. Smyth, D.L. Vaux, S.L. Nutt, U. Nachbur, J. Silke, TRAF2 regulates TNF and NF-κB signalling to suppress apoptosis and skin inflammation independently of Sphingosine kinase 1, Elife 4 (2015) e10592. https://doi.org/10.7554/eLife.10592.

[47] Z. Zou, T. Tao, H. Li, X. Zhu, mTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges, Cell Biosci 10 (2020) 31. https://doi.org/10.1186/s13578-020-00396-1.

[48] H. Dai, H. Chen, J. Xu, J. Zhou, Z. Shan, H. Yang, X. Zhou, F. Guo, The ubiquitin ligase CHIP modulates cellular behaviors of gastric cancer cells by regulating TRAF2, Cancer Cell International 19 (2019) 132. https://doi.org/10.1186/s12935-019-0832-z.

[49] J. Zhang, W.-J. Dai, X.-Z. Yang, Methylation status of TRAF2 is associated with the diagnosis and prognosis of gastric cancer, Int J Clin Exp Pathol 8 (2015) 14228–14234.

[50] X. Liang, J. Yao, D. Cui, W. Zheng, Y. Liu, G. Lou, B. Ye, L. Shui, Y. Sun, Y. Zhao, M. Zheng, The TRAF2-p62 axis promotes proliferation and survival of liver cancer by activating mTORC1 pathway, Cell Death Differ 30 (2023) 1550–1562. https://doi.org/10.1038/s41418-023-01164-7.

[51] D. Fang, D. Hawke, Y. Zheng, Y. Xia, J. Meisenhelder, H. Nika, G.B. Mills, R. Kobayashi, T. Hunter, Z. Lu, PHOSPHORYLATION OF β-CATENIN BY AKT PROMOTES β -CATENIN TRANSCRIPTIONAL ACTIVITY, J Biol Chem 282 (2007) 11221–11229. https://doi.org/10.1074/jbc.M611871200.

[52] J.M. Perry, F. Tao, A. Roy, T. Lin, X.C. He, S. Chen, X. Lu, J. Nemechek, L. Ruan, X. Yu, D. Dukes, A. Moran, J. Pace, K. Schroeder, M. Zhao, A. Venkatraman, P. Qian, Z. Li, M. Hembree, A. Paulson, Z. He, D. Xu, T.-H. Tran, P. Deshmukh, C.T. Nguyen, R.M. Kasi, R. Ryan, M. Broward, S. Ding, E. Guest, K. August, A.S. Gamis, A. Godwin, G.S. Sittampalam, S.J. Weir, L. Li, Overcoming Wnt-β-catenin dependent anticancer therapy resistance in leukaemia stem cells, Nat Cell Biol 22 (2020) 689–700. https://doi.org/10.1038/s41556-020-0507-y.

[53] Y. Wang, C. Yang, W. Li, Y. Shen, J. Deng, W. Lu, J. Jin, Y. Liu, Q. Liu, Identification of colon tumor marker NKD1 via integrated bioinformatics analysis and experimental validation, Cancer Medicine 10 (2021) 7383–7394. https://doi.org/10.1002/cam4.4224.

[54] S. Hu, Q. Zheng, H. Wu, C. Wang, T. Liu, W. Zhou, miR-532 promoted gastric cancer migration and invasion by targeting NKD1, Life Sci 177 (2017) 15–19. https://doi.org/10.1016/j.lfs.2017.03.019.

[55] J. Huang, M. Shen, M. Yan, Y. Cui, Z. Gao, X. Meng, Exosome-mediated transfer of miR-1290 promotes cell proliferation and invasion in gastric cancer via NKD1, Acta Biochim Biophys Sin (Shanghai) 51 (2019) 900–907. https://doi.org/10.1093/abbs/gmz077.

[56] R. Dhanasekaran, A. Deutzmann, W.D. Mahauad-Fernandez, A.S. Hansen, A.M. Gouw, D.W. Felsher, The MYC oncogene — the grand orchestrator of cancer growth and immune evasion, Nat Rev Clin Oncol 19 (2022) 23–36. https://doi.org/10.1038/s41571-021-00549-2.

[57] R.R. Burbano, P.P. Assumpção, M.F. Leal, D.Q. Calcagno, A.C. Guimarães, A.S. Khayat, S.S. Takeno, E.S. Chen, M.D.A.C. Smith, C-MYC Locus Amplification as Metastasis Predictor in Intestinal-type Gastric Adenocarcinomas: CGH Study in Brazil, Anticancer Research 26 (2006) 2909–2914.

[58] C.R.T. de Souza, M.F. Leal, D.Q. Calcagno, E.K. Costa Sozinho, B. do N. Borges, R.C. Montenegro, Â.K.C.R. dos Santos, S.E.B. dos Santos, H.F. Ribeiro, P.P. Assumpção, M. de Arruda Cardoso Smith, R.R. Burbano, MYC Deregulation in Gastric Cancer and Its Clinicopathological Implications, PLoS One 8 (2013) e64420. https://doi.org/10.1371/journal.pone.0064420.

[59] A.D. Panani, Cytogenetic and molecular aspects of gastric cancer: Clinical implications, Cancer Letters 266 (2008) 99–115. https://doi.org/10.1016/j.canlet.2008.02.053.

[60] D.Q. Calcagno, V.M. Freitas, M.F. Leal, C.R.T. de Souza, S. Demachki, R. Montenegro, P.P. Assumpção, A.S. Khayat, M. de A.C. Smith, A.K.C.R. dos Santos, R.R. Burbano, MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer, BMC Gastroenterology 13 (2013) 141. https://doi.org/10.1186/1471-230X-13-141.

[61] M.F. Leal, D.Q. Calcagno, J. de F.F. Borges da Costa, T.C.R. Silva, A.S. Khayat, E.S. Chen, P.P. Assumpção, M. de Arruda Cardoso Smith, R.R. Burbano, MYC, TP53, and Chromosome 17 Copy-Number Alterations in Multiple Gastric Cancer Cell Lines and in Their Parental Primary Tumors, J Biomed Biotechnol 2011 (2011). https://doi.org/10.1155/2011/631268.

[62] D.P. Dannheisig, E. Beck, E. Calzia, P. Walther, C. Behrends, A.S. Pfister, Loss of Peter Pan (PPAN) Affects Mitochondrial Homeostasis and Autophagic Flux, Cells 8 (2019) 894. https://doi.org/10.3390/cells8080894.

[63] A.S. Pfister, M. Keil, M. Kühl, The Wnt Target Protein Peter Pan Defines a Novel p53-independent Nucleolar Stress-Response Pathway, J Biol Chem 290 (2015) 10905–10918. https://doi.org/10.1074/jbc.M114.634246.

[64] M. Keil, M.T. Meyer, D.P. Dannheisig, L.D. Maerz, M. Philipp, A.S. Pfister, Loss of Peter Pan protein is associated with cell cycle defects and apoptotic events, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1866 (2019) 882–895. https://doi.org/10.1016/j.bbamcr.2019.01.010.

[65] N. Zielke, A. Vähärautio, J. Liu, T. Kivioja, J. Taipale, Upregulation of ribosome biogenesis via canonical E-boxes is required for Myc-driven proliferation, Dev Cell 57 (2022) 1024-1036.e5. https://doi.org/10.1016/j.devcel.2022.03.018.

[66] W. Wang, W. Gu, H. Tang, Z. Mai, H. Xiao, J. Zhao, J. Han, The Emerging Role of MTHFD Family Genes in Regulating the Tumor Immunity of Oral Squamous Cell Carcinoma, J Oncol 2022 (2022) 4867730. https://doi.org/10.1155/2022/4867730.

[67] E. Piskounova, M. Agathocleous, M.M. Murphy, Z. Hu, S.E. Huddlestun, Z. Zhao, A.M. Leitch, T.M. Johnson, R.J. DeBerardinis, S.J. Morrison, Oxidative stress inhibits distant

metastasis by human melanoma cells, Nature 527 (2015) 186–191. https://doi.org/10.1038/nature15726.

[68] E.L. da Silva, F.P. Mesquita, A.J. de S. Portilho, E.C.A. Bezerra, J.P. Daniel, E.S.P. Aranha, S. Farran, M.C. de Vasconcellos, M.E.A. de Moraes, C.A. Moreira-Nunes, R.C. Montenegro, Differences in glucose concentration shows new perspectives in gastric cancer metabolism, Toxicology in Vitro 82 (2022) 105357. https://doi.org/10.1016/j.tiv.2022.105357.

[69] M. Xu, Y. Wang, R. Xia, Y. Wei, X. Wei, Role of the CCL2-CCR2 signalling axis in cancer: Mechanisms and therapeutic targeting, Cell Prolif 54 (2021) e13115. https://doi.org/10.1111/cpr.13115.

[70] J. Zhang, Y. Yan, X. Cui, J. Zhang, Y. Yang, H. Li, H. Wu, J. Li, L. Wang, M. Li, X. Liu, J. Wang, X. Duan, CCL2 expression correlates with Snail expression and affects the prognosis of patients with gastric cancer, Pathology - Research and Practice 213 (2017) 217–221. https://doi.org/10.1016/j.prp.2016.12.013.

[71] J. Jin, J. Lin, A. Xu, J. Lou, C. Qian, X. Li, Y. Wang, W. Yu, H. Tao, CCL2: An Important Mediator Between Tumor Cells and Host Cells in Tumor Microenvironment, Frontiers in Oncology 11 (2021). https://www.frontiersin.org/articles/10.3389/fonc.2021.722916 (accessed December 14, 2023).

[72] J.-B. Wang, Y.-X. Gao, Y.-H. Ye, T.-X. Lin, P. Li, J.-X. Lin, Q.-Y. Chen, L.-L. Cao, M. Lin, R.-H. Tu, J.-L. Lin, Z.-N. Huang, H.-L. Zheng, J.-W. Xie, C.-H. Zheng, C.-M. Huang, CDK5RAP3 acts as a tumour suppressor in gastric cancer through the infiltration and polarization of tumour-associated macrophages, Cancer Gene Ther 30 (2023) 22–37. https://doi.org/10.1038/s41417-022-00515-9.

[73] J.W. Chiu, Z.B. Hanafi, L.C.Y. Chew, Y. Mei, H. Liu, IL-1α Processing, Signaling and Its Role in Cancer Progression, Cells 10 (2021). https://doi.org/10.3390/cells10010092.

[74] M. Dagenais, J. Dupaul-Chicoine, T. Douglas, C. Champagne, A. Morizot, M. Saleh, The Interleukin (IL)-1R1 pathway is a critical negative regulator of PyMT-mediated mammary tumorigenesis and pulmonary metastasis, Oncoimmunology 6 (2017) e1287247. https://doi.org/10.1080/2162402X.2017.1287247.

[75] A. Douvdevani, M. Huleihel, M. Zöller, S. Segal, R.N. Apte, Reduced tumorigenicity of fibrosarcomas which constitutively generate IL-1 alpha either spontaneously or following IL-1 alpha gene transfer, Int J Cancer 51 (1992) 822–830. https://doi.org/10.1002/ijc.2910510526.

[76] T. Dvorkin, X. Song, S. Argov, R.M. White, M. Zoller, S. Segal, C.A. Dinarello, E. Voronov, R.N. Apte, Immune phenomena involved in the in vivo regression of fibrosarcoma cells expressing cell-associated IL-1alpha, J Leukoc Biol 80 (2006) 96–106. https://doi.org/10.1189/jlb.0905509.

[77] M. Espinosa-Cotton, S.N. Rodman Iii, K.A. Ross, I.J. Jensen, K. Sangodeyi-Miller, A.J. McLaren, R.A. Dahl, K.N. Gibson-Corley, A.T. Koch, Y.-X. Fu, V.P. Badovinac, D. Laux, B. Narasimhan, A.L. Simons, Interleukin-1 alpha increases anti-tumor efficacy of cetuximab in head and neck squamous cell carcinoma, J Immunother Cancer 7 (2019) 79. https://doi.org/10.1186/s40425-019-0550-z.

[78] M.K. Sgagias, A. Kasid, D.N. Danforth, Interleukin-1 alpha and tumor necrosis factor-alpha (TNF alpha) inhibit growth and induce TNF messenger RNA in MCF-7 human breast cancer cells, Mol Endocrinol 5 (1991) 1740–1747. https://doi.org/10.1210/mend-5-11-1740.

[79] T. Tian, S. Lofftus, Y. Pan, C.A. Stingley, S.L. King, J. Zhao, T.Y. Pan, R. Lock, J.W. Marglous, K. Liu, H.R. Widlund, R.C. Fuhlbrigge, K. Cichowski, T.S. Kupper, IL1α Antagonizes IL1β and Promotes Adaptive Immune Rejection of Malignant Tumors, Cancer Immunol Res 8 (2020) 660–671. https://doi.org/10.1158/2326-6066.CIR-19-0552.

[80] E. Voronov, Y. Weinstein, D. Benharroch, E. Cagnano, R. Ofir, M. Dobkin, R.M. White, M. Zoller, V. Barak, S. Segal, R.N. Apte, Antitumor and immunotherapeutic effects of activated invasive T lymphoma cells that display short-term interleukin 1alpha expression, Cancer Res 59 (1999) 1029–1035.

[81] S. Tomimatsu, T. Ichikura, H. Mochizuki, Significant correlation between expression of interleukin-1α and liver metastasis in gastric carcinoma, Cancer 91 (2001) 1272–1276. https://doi.org/10.1002/1097-0142(20010401)91:7<1272::AID-CNCR1128>3.0.CO;2-Z.

[82] K. Sakamoto, Y. Hikiba, H. Nakagawa, Y. Hayakawa, A. Yanai, M. Akanuma, K. Ogura, Y. Hirata, K.H. Kaestner, M. Omata, S. Maeda, Inhibitor of κB Kinase Beta Regulates Gastric Carcinogenesis via Interleukin-1α Expression, Gastroenterology 139 (2010) 226-238.e6. https://doi.org/10.1053/j.gastro.2010.03.047.

[83] K. Blom, W. Senkowski, M. Jarvius, M. Berglund, J. Rubin, L. Lenhammar, V. Parrow, C. Andersson, A. Loskog, M. Fryknäs, P. Nygren, R. Larsson, The anticancer effect of mebendazole may be due to M1 monocyte/macrophage activation via ERK1/2 and TLR8-dependent inflammasome activation, Immunopharmacol Immunotoxicol 39 (2017) 199–210. https://doi.org/10.1080/08923973.2017.1320671.

[84] N.-N. Kreis, F. Louwen, J. Yuan, The Multifaceted p21 (Cip1/Waf1/CDKN1A) in Cell Differentiation, Migration and Cancer Therapy, Cancers 11 (2019) 1220. https://doi.org/10.3390/cancers11091220.

[85] V. Andries, K. Vandepoele, K. Staes, G. Berx, P. Bogaert, G. Van Isterdael, D. Ginneberge, E. Parthoens, J. Vandenbussche, K. Gevaert, F. van Roy, NBPF1, a tumor suppressor candidate in neuroblastoma, exerts growth inhibitory effects by inducing a G1 cell cycle arrest, BMC Cancer 15 (2015) 391. https://doi.org/10.1186/s12885-015-1408-5.

[86] C. Park, N.Y. Jeong, G.-Y. Kim, M.H. Han, I.-M. Chung, W.-J. Kim, Y.H. Yoo, Y.H. Choi, Momilactone B induces apoptosis and G1 arrest of the cell cycle in human monocytic leukemia U937 cells through downregulation of pRB phosphorylation and induction of the cyclin-dependent kinase inhibitor p21Waf1/Cip1, Oncol Rep 31 (2014) 1653–1660. https://doi.org/10.3892/or.2014.3008.

[87] M. Askari, R.C. Sobti, M. Nikbakht, S.C. Sharma, Aberrant promoter hypermethylation of p21 (WAF1/CIP1) gene and its impact on expression and role of polymorphism in the risk of breast cancer, Mol Cell Biochem 382 (2013) 19–26. https://doi.org/10.1007/s11010-013-1696-5.

[88] S.R.J. Bott, M. Arya, R.S. Kirby, M. Williamson, p21WAF1/CIP1 gene is inactivated in metastatic prostatic cancer cell lines by promoter methylation, Prostate Cancer Prostatic Dis 8 (2005) 321–326. https://doi.org/10.1038/sj.pcan.4500822.

[89] J. Roman-Gomez, J.A. Castillejo, A. Jimenez, M.G. Gonzalez, F. Moreno, M. del C. Rodriguez, M. Barrios, J. Maldonado, A. Torres, 5′ CpG island hypermethylation is associated with transcriptional silencing of the p21CIP1/WAF1/SDI1 gene and confers poor prognosis in acute lymphoblastic leukemia, Blood 99 (2002) 2291–2296. https://doi.org/10.1182/blood.V99.7.2291.

[90] L.M. Fröhlich, E. Makino, T. Sinnberg, B. Schittek, Enhanced expression of p21 promotes sensitivity of melanoma cells towards targeted therapies, Exp Dermatol 31 (2022) 1243–1252. https://doi.org/10.1111/exd.14585.

[91] J. Sang, Y. Wang, Y. Ye, Effects of enhancing p21waf1on proliferation and expression of G1cyclins andCDKs in human breast cancer cells, Chin.Sci.Bull. 44 (1999) 1489–1493. https://doi.org/10.1007/BF03183569.

[92] M. Lawrence, S. Daujat, R. Schneider, Lateral Thinking: How Histone Modifications Regulate Gene Expression, Trends Genet 32 (2016) 42–56. https://doi.org/10.1016/j.tig.2015.10.007.

[93] V. Mirisola, R. Mora, A.I. Esposito, L. Guastini, F. Tabacchiera, L. Paleari, A. Amaro, G. Angelini, M. Dellepiane, U. Pfeffer, A. Salami, A prognostic multigene classifier for squamous cell carcinomas of the larynx, Cancer Letters 307 (2011) 37–46. https://doi.org/10.1016/j.canlet.2011.03.013.

[94] J.J. Milner, Z.-F. Chen, J. Grayson, S.-Y.P.K. Shiao, Obesity-Associated Differentially Methylated Regions in Colon Cancer, J Pers Med 12 (2022) 660. https://doi.org/10.3390/jpm12050660.

[95] V. Khachaturov, G.-Q. Xiao, Y. Kinoshita, P.D. Unger, D.E. Burstein, Histone H1.5, a novel prostatic cancer marker: an immunohistochemical study, Hum Pathol 45 (2014) 2115–2119. https://doi.org/10.1016/j.humpath.2014.06.015.

[96] R. Liao, X. Chen, Q. Cao, Y. Wang, Z. Miao, X. Lei, Q. Jiang, J. Chen, X. Wu, X. Li, J. Li, C. Dong, HIST1H1B Promotes Basal-Like Breast Cancer Progression by Modulating CSF2 Expression, Front. Oncol. 11 (2021). https://doi.org/10.3389/fonc.2021.780094.

[97] M. Momeni, T. Kalir, S. Farag, Y. Kinoshita, T.Y. Roman, L. Chuang, D.A. Fishman, D.E. Burstein, Immunohistochemical detection of promyelocytic leukemia zinc finger and histone 1.5 in uterine leiomyosarcoma and leiomyoma, Reprod Sci 21 (2014) 1171–1176. https://doi.org/10.1177/1933719114532845.

[98] W. Xie, J. Zhang, P. Zhong, S. Qin, H. Zhang, X. Fan, Y. Yin, R. Liang, Y. Han, Y. Liao, X. Yu, H. Long, Z. Lv, C. Ma, F. Yu, Expression and potential prognostic value of histone family gene signature in breast cancer, Experimental and Therapeutic Medicine 18 (2019) 4893–4903. https://doi.org/10.3892/etm.2019.8131.

[99] L.J. Castro-Muñoz, E.V. Ulloa, C. Sahlgren, M. Lizano, E. De La Cruz-Hernández, A. Contreras-Paredes, Modulating epigenetic modifications for cancer therapy (Review), Oncology Reports 49 (2023) 1–23. https://doi.org/10.3892/or.2023.8496.

[100] J.C. Bressi, R. de Jong, Y. Wu, A.J. Jennings, J.W. Brown, S. O'Connell, L.W. Tari, R.J. Skene, P. Vu, M. Navre, X. Cao, A.R. Gangloff, Benzimidazole and imidazole inhibitors

of histone deacetylases: Synthesis and biological activity, Bioorg Med Chem Lett 20 (2010) 3138–3141. https://doi.org/10.1016/j.bmcl.2010.03.092.

[101] D.M. Carter, E. Specker, P.H. Małecki, J. Przygodda, K. Dudaniec, M.S. Weiss, U. Heinemann, M. Nazaré, U. Gohlke, Enhanced Properties of a Benzimidazole Benzylpyrazole Lysine Demethylase Inhibitor: Mechanism-of-Action, Binding Site Analysis, and Activity in Cellular Models of Prostate Cancer, J Med Chem 64 (2021) 14266–14282. https://doi.org/10.1021/acs.jmedchem.1c00693.

[102] Y.T. Lee, Y.J. Tan, C.E. Oon, Benzimidazole and its derivatives as cancer therapeutics: The potential role from traditional to precision medicine, Acta Pharm Sin B 13 (2023) 478–497. https://doi.org/10.1016/j.apsb.2022.09.010.

[103] Y.J. Tan, Y.T. Lee, S.H. Petersen, G. Kaur, K. Kono, S.C. Tan, A.M.S.A. Majid, C.E. Oon, BZD9L1 sirtuin inhibitor as a potential adjuvant for sensitization of colorectal cancer cells to 5-fluorouracil, Ther Adv Med Oncol 11 (2019) 1758835919878977. https://doi.org/10.1177/1758835919878977.

[104] Y.J. Tan, Y.T. Lee, K.Y. Yeong, S.H. Petersen, K. Kono, S.C. Tan, C.E. Oon, Anticancer activities of a benzimidazole compound through sirtuin inhibition in colorectal cancer, Future Med Chem 10 (2018) 2039–2057. https://doi.org/10.4155/fmc-2018-0052.

[105] K.M.T. Arif, E.K. Elliott, L.M. Haupt, L.R. Griffiths, Regulatory Mechanisms of Epigenetic miRNA Relationships in Human Cancer and Potential as Therapeutic Targets, Cancers (Basel) 12 (2020) 2922. https://doi.org/10.3390/cancers12102922.

[106] V.J. Craig, S.B. Cogliatti, H. Rehrauer, T. Wündisch, A. Müller, Epigenetic Silencing of MicroRNA-203 Dysregulates ABL1 Expression and Drives Helicobacter-Associated Gastric Lymphomagenesis, Cancer Research 71 (2011) 3616–3624. https://doi.org/10.1158/0008-5472.CAN-10-3907.

[107] S.M. Johnson, H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K.L. Reinert, D. Brown, F.J. Slack, RAS is regulated by the let-7 microRNA family, Cell 120 (2005) 635–647. https://doi.org/10.1016/j.cell.2005.01.014.

[108] K.A. O'Donnell, E.A. Wentzel, K.I. Zeller, C.V. Dang, J.T. Mendell, c-Myc-regulated microRNAs modulate E2F1 expression, Nature 435 (2005) 839–843. https://doi.org/10.1038/nature03677.

[109] V. Walf-Vorderwülbecke, K. Pearce, T. Brooks, M. Hubank, M.M. van den Heuvel-Eibrink, C.M. Zwaan, S. Adams, D. Edwards, J. Bartram, S. Samarasinghe, P. Ancliff, A. Khwaja, N. Goulden, G. Williams, J. de Boer, O. Williams, Targeting acute myeloid leukemia by drug-induced c-MYB degradation, Leukemia 32 (2018) 882–889. https://doi.org/10.1038/leu.2017.317.

[110] J.P. Daniel, F.P. Mesquita, E.L. Da Silva, P.F.N. de Souza, L.B. Lima, L.L.B. de Oliveira, M.E.A. de Moraes, C. de F.A. Moreira-Nunes, R.M.R. Burbano, G. Zanatta, R.C. Montenegro, Anticancer potential of mebendazole against chronic myeloid leukemia: in silico and in vitro studies revealed new insights about the mechanism of action, Frontiers in Pharmacology 13 (2022). https://www.frontiersin.org/articles/10.3389/fphar.2022.952250 (accessed November 11, 2022).

[111] V. Staedtke, T. Gray-Bethke, G.J. Riggins, R.-Y. Bai, Preventative Effect of Mebendazole against Malignancies in Neurofibromatosis 1, Genes (Basel) 11 (2020) 762. https://doi.org/10.3390/genes11070762.

Supplementary Table 1. Hallmarks identified in the treated group by GSEA.

HALLMARK	SIZE	ES	NES	NOM p-value	FDR q-value	FWER p-value	RANK AT MAX
Apical Surface	43	0.41	**1.55**	**0.000**	0.086	0.061	802
MYC Targets V2	57	0.39	**1.52**	**0.000**	0.063	0.101	1024
E2F Targets	196	0.34	**1.43**	**0.000**	0.159	0.319	1165
Allograft Rejection	199	0.29	**1.40**	**0.000**	0.129	0.319	1931
Xenobiotic Metabolism	198	0.27	**1.35**	**0.000**	0.170	0.507	3480
WNT Beta Catenin Signaling	41	0.35	**1.29**	**0.000**	0.226	0.632	1293
Mitotic Spindle	195	0.27	**1.26**	**0.000**	0.261	0.747	1507
PI3K AKT mTOR Signaling	105	0.29	**1.22**	**0.000**	0.309	0.868	2480
Pancreas Beta Cells	39	0.32	1.18	0.131	0.339	0.868	1130
Unfolded Protein Response	110	0.26	1.17	0.078	0.341	0.868	880
UV Response UP	154	0.27	1.16	0.208	0.357	0.921	1949
TGF Beta Signaling	54	0.31	1.12	0.248	0.456	0.921	2045
Reactive Oxygen Species Pathway	49	0.33	1.09	0.203	0.503	1.000	808
G2M Checkpoint	197	0.26	1.09	0.073	0.474	1.000	1622
Estrogen Response Late	198	0.22	1.07	0.233	0.485	1.000	2346
Myogenesis	195	0.22	1.05	0.290	0.531	1.000	4611
Peroxisome	103	0.28	1.02	0.410	0.563	1.000	1786
Notch Signaling	31	0.28	1.01	0.398	0.573	1.000	1554
Estrogen Response Early	197	0.21	1.01	0.205	0.549	1.000	2173
Bile Acid Metabolism	112	0.23	1.00	0.425	0.546	1.000	2659

Size: Number of genes in the gene set; ES: Enrichment Score; NES: Normalized Enrichment Score; NOM p-value: Nominal p value. FDR q-value: False Discovery Rate; FWER p-value: Familywise-error; RANK AT MAX: The position in the ranked list at which the maximum enrichment score occurred.

CONSIDERAÇÕES FINAIS

O estudo de novos alvos farmacológicos é imprescindível na busca de melhor compreender o mecanismo de ação do fármaco, moléculas reposicionadas para novas terapias possuem a vantagem de já possuírem estudos iniciais descrevendo sua toxicidade e seguridade, sendo necessário ainda descrever seu mecanismo farmacológico e as rotas associadas a sua ação na nova terapia avaliada. No CG o estudo de novas moléculas antitumorais e de novos alvos terapêuticos é imprescindível para fornecer novas opções aos pacientes não responsivos as terapias atuais, podendo melhorar sua probabilidade de cura. Nesse sentido, esse trabalho teve como objetivo a identificação de novos alvos do fármaco MBZ para a terapia do CG, levando a uma melhor compreensão do seu mecanismo de ação *in vitro* e do seu potencial reposicionamento terapêutico.

Portanto, foi demonstrado que o MBZ possui atividade antitumoral proeminente nas linhagens tumorais gástricas, mais especificamente na linhagem metastática AGP-01. Dados ainda atestaram que o fármaco possui alta seletividade, sendo necessária uma concentração quase dez vezes superior desse para causar dano citotóxico em linhagens não tumorais. Em relação ao metabolismo tumoral, foi observado que o dano citotóxico e efeito antitumoral do fármaco estão relacionados a supressão da expressão de enzimas importantes para o metabolismo de glicose e de nucleotídeos, levando assim, a diminuição na produção enérgica celular e de moléculas importantes para a proliferação tumoral.

O estudo do transcriptoma da linhagem AGP-01 após o tratamento com o fármaco MBZ destacou novos alvos moleculares de interesse clínico na terapia do CG. A alteração na expressão de genes relacionados expressão de histonas e de proteínas do sistema imune levaram a uma melhor visualização do perfil farmacológico do fármaco, além disso, o estudo destacou alvos como *NCOA6*, *PPAN*, *GINS1*, *TRAF2*, *MTHFD1*, *NKD1* e *CYTH2* que estão relacionados a diversas vias de sinalização tumoral, sendo alvos de interesse terapêutico para a terapia do CG.

Portanto, o fármaco MBZ demonstrou potencial epigenético, levando a modulação de diferentes vias celulares no câncer, como as vias metabólicas que são essenciais para o crescimento e progressão tumoral. Além disso, também foi observada uma possível alteração na expressão gênica de transcritos relacionados a resposta antitumoral do sistema imunológico, podendo estar relacionado ao efeito citotóxico do MBZ. Ainda são necessários mais estudos para melhor elucidar seu efeito epigenético e imune, dando luz assim a novas possibilidades terapêuticas e possibilitando um aumento na probabilidade de cura aos pacientes com CG.