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TARGETING BRCAI-MEDIATED DNA REPAIR IN PANCREATIC DUCTAL ADENOCARCINOMA THERAPY

João Pedro da Costa Festa e Ventura Morais

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Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Resumo

O adenocarcinoma ductal pancreático (PDAC) é uma doença devastadora e com um prognóstico muito desfavorável. Devido à sua agressividade e à elevada propensão para adquirir resistência terapêutica, a taxa de sobrevivência ao fim de cinco anos é de aproximadamente 10%. Atualmente, a terapêutica de primeira linha consiste frequentemente em regimes que incluem gemcitabina ou FOLFIRINOX. No entanto, ambos apresentam benefícios limitados e induzem efeitos secundários graves. Para combater estas limitações, agentes terapêuticos mais direcionados e eficazes têm sido procurados para o tratamento do PDAC. Em particular, o inibidor de poli(ADP-ribose) polimerase (PARP), olaparib, tem sido usado no tratamento de cancros com mutações BRCA1/2, explorando deficiências na recombinação homóloga (RH) para desencadear letalidade sintética nas células cancerígenas . No entanto, a resistência do PDAC a esta terapêutica é frequentemente observada, representando uma grande limitação clínica. Recentemente, o nosso grupo descobriu o composto BBIT20, que inibe a RH devido à disrupção da interação entre as proteínas BRCA1/BARD1. Além disso, o BBIT20 também induziu morte celular, exibiu efeitos sinérgicos com a cisplatina e o olaparib em células de cancro de mama e ovário e inibiu a atividade da glicoproteína P, o que corrobora a sua capacidade de superar a resistência do PDAC às terapêuticas atuais.

Portanto, neste trabalho, estudamos o efeito anti-tumoral do BBIT20 em células de PDAC e a sua capacidade de combater a resistência terapêutica. Relativamente à primeira parte do trabalho, o BBIT20 mostrou um efeito inibitório de crescimento potente, superior ao observado com o olaparib, e também maior seletividade para as células cancerígenas. Devido à disrupção da interação BRCA1-BARD1, foi observada uma redução na expressão de proteínas cruciais na recombinação homóloga. Além disso, a exposição a concentrações crescentes de BBIT20, durante vários ciclos de tratamento, não induziu resistência e o BBIT20 interferiu na expressão de microRNAs associados à resistência terapêutica. A segunda parte do trabalho envolveu o uso de uma linha celular resistente à gemcitabina. Posteriormente, verificou-se a inexistência de resistência cruzada ao BBIT20. Foi observado um aumento na indução da apoptose, uma inibição da capacidade migratória da linha resistente e na atividade da glicoproteína P, bem como a redução da expressão de oncomiRNAs após o tratamento com BBIT20. Para finalizar, o BBIT20 “re-sensibilizou” as células à gemcitabina.

Em conclusão, o BBIT20 representa um agente terapêutico promissor para o tratamento do PDAC, pois possui propriedades antitumorais potentes, inibe a recombinação homóloga, não induz resistência e interfere com os microRNAs. Além disso,

também demonstramos que o BBIT20 pode combater a resistência terapêutica, inibindo os mecanismos de resistência adquirida.

Keywords: Resistência adquirida, Adenocarcinoma ductal pancreático, terapia direcionada, recombinação homóloga

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly devastating disease with poor prognosis and rising incidence. Due to its aggressiveness and high propensity to acquire therapeutic resistance, 5-year overall survival rate is approximately 10%. Nowadays, standard first-line therapy consists of regimens including gemcitabine or FOLFIRINOX (composed by 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin). However, this type of treatment has shown high toxicity and severe secondary effects to patients. Therefore, to counteract these limitations, effective targeted therapeutic agents have been pursued for PDAC. In particular, the poly(ADP-ribose) polymerase (PARP) inhibitor (PARPi), olaparib, exploits deficiencies in homologous recombination (HR), particularly mutations in *BRCA1/2* genes, to trigger synthetic lethality in cancer cells. However, PDAC resistance to this therapy is commonly observed, representing a major clinical limitation. Recently, our group has discovered the small molecule BBIT20 (nome quimico), which inhibits HR through disruption of the BRCA1 and BARD1 interaction, leading to a homologous recombination deficiency (HRD) phenotype. BBIT20 can also inhibit the activity of the multidrug resistance p-glycoprotein, which supports its potentiality to overcome PDAC resistance.

Therefore, in this work we studied the anti-tumor effect of BBIT20 on PDAC cells and its capability to counteract therapeutic resistance. Relatively to the first part of the work, BBIT20 showed a potent growth inhibitory effect, superior to the observed in Olaparib, and also selectivity for cancer cells. Due to the disruption of the BRCA1-BARD1 interaction a reduced expression of key regulators in HR was also observed. Furthermore, the exposition to increasing concentrations of BBIT20, during several rounds of treatment, did not induce resistance and BBIT20 stimulated the expression of tumor suppressor microRNAs. About the second part of the work, a cell line resistant to gemcitabine was used. Then, it was verified the inexistence of cross resistance to BBIT20. BBIT20 increased apoptosis, inhibited the migratory capacity of the resistant cell line and the activity of the multidrug resistance p-glycoprotein, and it reduced oncomirs. To finalize BBIT20 re-sensitized PDAC cells to GEM.

In conclusion, BBIT20 represents a promising therapeutic agent for PDAC treatment, having potent anti-tumor properties, inhibiting homologous recombination, not inducing resistance, and by interfering with microRNAs. Additionally, we also proved that BBIT20 can counteract therapeutic resistance by inhibiting mechanisms of acquired resistance.

Keywords: Acquired resistance, Pancreatic ductal adenocarcinoma, Targeted therapy, Homologous recombination

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List of abbreviations

53BP1 - p53-binding protein 1

5-FU - Fluorouracil

6–4PPs - 6–4 pyrimidine–pyrimidone photoproducts

ABC - ATP-binding cassette

ABRAXAS - BRCA1 A complex subunit

AKT - Protein kinase B

APE - Apurinic/apyrimidinic endonuclease

APTX - Aprataxin

AR - Androgen Receptor

ATR - Telangiectasia and Rad3-related protein

ATRIP- ATR-interacting protein

BARD1 - BRCA1-associated RING domain protein 1

BCA - Bicinchoninic acid protein assay

BER - Base excision repair

BIR - Break-induced DNA replication

BLM - Bloom syndrome protein

BRCA 1 - Breast cancer susceptibility gene 1

BRCA 2 - Breast cancer susceptibility gene 2

BRCT - BRCA1 C Terminus

BRIP1 - BRCA1-interacting protein C-terminal helicase 1

CC - Coiled coil

CDA - Cytidine deaminase

CDKN2A - Cyclin-dependent kinase inhibitor 2A

CDP - Cytidine diphosphate

CHD4 - Chromodomain-helicase-DNA-binding protein 4

CHK1 - Checkpoint kinase 1

CHK2 - Checkpoint kinase 2

CPDs - Cyclobutane–pyrimidine dimers

CR - Complete response

CtIP - C-terminal binding protein 1 interacting protein

DBD - DNA binding domain

dCDP - Deoxycytidine diphosphate

dCK - Deoxycytidine kinase

dCTP - deoxycytidine triphosphate

DDR - DNA damage repair

DFdC - 20,20-difluorodeoxycytidine

dFdCDP - Gemcitabine diphosphate

dFdCMP - Gemcitabine monophosphate

dFdCTP Gemcitabine triphosphate

dFdU - 2',2'-difluorodeoxyuridine

dFdUMP - 2'-deoxy-2',2'-difluorouridine monophosphate

D-loop - Displacement loop structure

DMEM - Dulbecco's Modified Eagle Medium

DMEM-F12 - Dulbecco's Modified Eagle Medium F12

DMSO - Dimethyl sulfoxide

DNA2 - DNA replication helicase/nuclease 2

DNA-PKcs - DNA-dependent protein kinase catalytic subunit

DSB - Double strand breaks

DSBR - DSBs repair

DsDNA - Double-stranded DNA

DSS1 - Split-hand/split-foot syndrome

ECL - Enhanced chemiluminescence

ECM - Extracellular matrix

EGFR - Epidermal growth factor receptor

EMT - Epithelial-mesenchymal transition

Erk - Extracellular signal-regulated kinase

ESPAC-4 - European Study Group for Pancreatic Cancer Trial 4

EXO1 - Exonuclease 1

GEM - Gemcitabine

GG-NER - Global Genome Nucleotide Excision Repair

GLUT 1/3 - Glucose transporters 1 and 3

GRIP1 - Glutamate receptor-interacting protein 1

H2AX - H2A histone family member X

hCNTs - Human concentrative nucleoside transporters

HDAC1/2 - Histones deacetylase 1 and 2

hENTs - Human equilibrative nucleoside transporters

HIF - Hypoxia inducible factor

Hj - Holliday junctions

HR - Homologous recombination

HRD - Homologous recombination deficiency

HRP - Horseradish peroxidase

IC_{50} - Half-maximal inhibitory concentration

IMDM - Iscove's Modified Dulbecco's Medium

IPMN - Cystic that englobe intraductal papillary mucinous neoplasm

ITPN - Intraductal tubule papillary neoplasm

KRAS - Kirsten rat sarcoma virus

LigI - DNA ligase I

LigIII - DNA ligase III

LigIV - DNA ligase IV

MAPK - mitogen-activated protein kinase

MCN - mucinous cystic neoplasm

miRNA - MicroRNA

MMR - Mismatch repair

MRN - MRE11-RAD50-NBS1

mTOR - Mammalian target of rapamycin

NAD - Nicotinamide adenine dinucleotide

NDPK - Nucleoside diphosphate kinase

NES - Nuclear export sequences

NETs - Neuroendocrine tumors

NHEJ - Non-homologous end joining

NLS - Nuclear localization signals

NTRK - Neurotrophic tyrosine receptor kinases

OB - Oligonucleotide binding

OS - Overall survival

PALB2 - Partner and Localizer of BRCA2

PanIN - Pancreatic intraepithelial neoplasia

PAR - Poly (ADP-ribose)

PARG - Poly(ADP-ribose) glycohydrolase

PARP - Poly (ADP-ribose) polymerase

PARPi - PARP inhibitors

PaSC - Pancreatic stellate cells

PBST - PBS containing tween

PC - Pancreatic cancer

PCNA - Proliferating cell nuclear antigen

PD-1 - Anti–programmed death-1

PDAC - Pancreatic ductais adenocarcinomas

PDGF - Platelet-derived growth factor

PD-L1/2 - Programmed death-ligand 1 and 2

PI3K - Phosphoinositide 3-kinase

PIKK - Phosphatidylinositol 3-kinase-like protein kinase

PNKP - Polynucleotide kinase 3'-phosphatase

PTIP - Pax2 transactivation domain-interacting protein

RFC - Replication factor C

RING - Really interesting new gene

ROS - Reactive oxygen species

RPA - Replication protein A

RPMI - Roswell Park Memorial Institute

RR - Relative risk

RT – Room temperature

SC - Serine cluster

SDSA - Synthesis-dependent strand annealing

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel

SMAD4 - Mothers against decapentaplegic homolog 4

SSA - Single-strand annealing

SSB - Single strand breaks

ssDNA - Single strand DNA

TAD - Transcriptional activation domain

TAMs - Tumor associated macrophages

TCA - Tricarboxylic acid

TC-NER - Transcription-Coupled Nucleotide Excision Repair

TD - Tower domain

TDP1/2 - Tyrosyl-DNA phosphodiesterases 1 and 2

TGF-β - Transforming growth factor-β

TMB - Tumor mutation burden

TME - Tumor microenvironment

TP53 - Tumor protein p53

TP53BP1 - Tumor suppressor p53-binding protein 1

UV - Ultraviolet radiation

XRCC1 - X-ray repair cross-complementing protein 1

XRCC4 - X-ray repair cross-complementing protein 4

ZEB 1/2 - E-box-binding homeobox 1 and 2

1. Introduction

1.1 Pancreatic cancer

1.1.1 Epidemiology & main characteristics

Cancer is considered a major public health intricacy at a global scale. Due to the complexity of the disease and the genetic population heterogeneity, early diagnostic tools and effective therapies are limited or inefficient despite the recent advances in medicine and research. Consequently, cancer ranks as one of the leading causes of death in a majority of countries [1]. Moreover, it is the first or second cause of premature death (occurring at the ages of 30-70 years) in several countries [2].

Regarding pancreatic cancer (PC), which is the focus of this work, it remains one of the deadliest cancers worldwide. It ranks seventh in cancer-related deaths and is normally associated with a low 5-year overall survival (OS) of approximately 12% [1,3]. Furthermore, its increasing incidence is a major public health concern, due to the population lifestyle in developed countries which includes physical inactivity, high-calorie/fat diet, high body mass index, and tobacco smoking [3,4]. Other risk factors associated to PC include diabetes mellitus, development of pancreatitis, and inherited genetic factors from familial history of cancers [4].

PC can be broadly categorized into two main types based on the cells of origin: exocrine tumors and endocrine tumors [5]. Most of the PC (about 95%) are exocrine tumors, specifically pancreatic ductal adenocarcinomas (PDAC), being the focus of this work [6].

Concerning PDAC, it occurs in the exocrine cells of the pancreas, typically in the ducts of the pancreas. Additionally, the process of tumorigenesis of these tumors arise mainly from 2 types of non-invasive precursors lesions, non-cystic including pancreatic intraepithelial neoplasia (PanIN) (most common type of precursor lesion) [7,8], and cystic that englobe intraductal papillary mucinous neoplasm (IPMN), intraductal tubule papillary neoplasm (ITPN), and mucinous cystic neoplasm (MCN) [8]. Moreover, these lesions act as a starting point for the development of PDAC and are accompanied by sequential genetic hits in several genes, including mutations in *Kirsten rat sarcoma virus (KRAS)*, *cyclin-dependent kinase inhibitor 2A (CDKN2A)*, *mothers against decapentaplegic homolog 4 (SMAD4)*, *breast cancer susceptibility gene 1 or 2 (BRCA 1/2)* and *tumor protein p53 (TP53)* [6].

Endocrine tumors, also known as neuroendocrine tumors (NETs), are a less common type of pancreatic cancer, accounting for about 5% of cases [9]. These tumors arise from the hormone-producing cells (islet cells) of the pancreas. Unlike exocrine tumors,

most pancreatic NETs are slow-growing and have a better prognosis [9]. Pancreatic NETs can be further classified into functioning or non-functioning tumors based on whether they produce hormones that cause specific symptoms [9].

1.1.2 Lethality & problematic of PDAC

PDAC low 5-year OS can be explained mainly by four factors: localization of the pancreas, aggressive behavior, physiologic effects and exhibition of high resistance levels to the therapies used in the clinic [10].

Regarding the first challenge, pancreas is located in the upper abdomen behind the stomach and between the aorta and its main branches (Figure 1). This particular site not only shields growing tumors from detection but can incapacitate tumor resection due to the encasement of the vessels by the high tumor aggressiveness [10]. Consequently, only 15% to 20% of the patients undergo tumor resection, which is the most viable option in a curative treatment perspective [11]

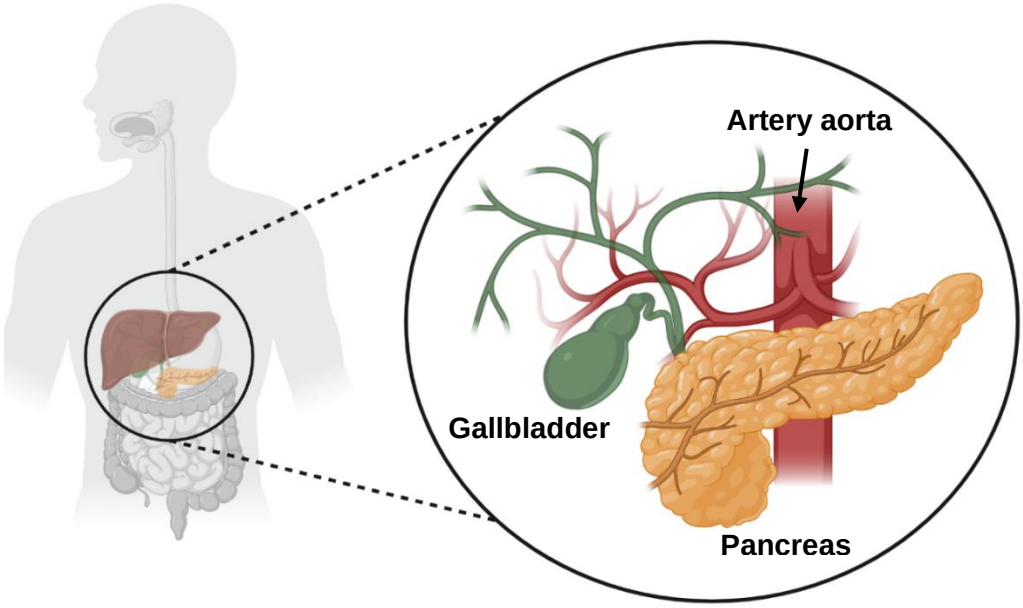


Figure 1. **Schematic representation of the pancreas in the human body and its surroundings including organs and vascularization.**

Secondly, PDAC is characterized by an aggressive biology resulting in early metastasis and higher tumor dissemination throughout the body. More than 50% of the patients at the time of the diagnostic have a distant metastatic disease, even the patients that undergo tumor resection complemented with adjuvant chemotherapeutic agents have a high risk of developing metastases 4 years post-surgery, suggesting the presence of micro metastases in patients with apparently localized tumors and proving the aggressive behaviour of PDAC [10,12].

Thirdly, PDAC causes major concerning physiological effects, weakening the patients, and restricting their capacity to withstand any aggressive treatment [10]. At the diagnostic and during the disease the most common debilitating symptoms include pain, asthaenia, anorexia, and cachexia, worsening if there is the presence exocrine and endocrine pancreatic dysfunction [11,13,14]. Moreover, there is a decreased survival rate in patients presenting cachexia, after pancreatectomy or chemotherapy, evidencing patients' poor treatment tolerance [10,15,16].

Lastly, PDAC resistance to the standard-of-care therapies is commonly observed, representing a major clinical limitation [10]. Indeed, even with the most effective systemic agents or radiotherapy, the chances of complete response (CR) are low [17]. Due to the rapid tumor progression, high heterogeneous cell niches and tumor microenvironment (TME), different resistance mechanisms can occur, which will be reviewed in more detail posteriorly [18].

1.1.3 Prevalent mutations in PDAC

The occurrence of PDAC is associated with several mutations in specific genes that play crucial roles in the development and progression of the disease. Throughout the years with the advancements in technology and medicine, the genetic landscape of PDAC has been extensively unveiled by researchers, revealing new targets for more efficient therapies, better diagnosis and prognosis tools, and extensive knowledge in PDAC genesis and progression including gene expression changes, copy number aberrations, chromosomal rearrangements, and epigenetic alterations [19].

Mutations can occur spontaneously, usually referred as somatic mutations, and are commonly caused by the exposition to environmental or occupational risk factors; or can be inherited mutations, generally named germline mutations, and normally happen due to the existence of familial history of cancers, transferring the mutations throughout the following generations increasing the risk to develop the cancers associated to the mutations.

Concerning somatic mutations, the proto-oncogene *KRAS* appears to be the most frequent activating mutation (approximately 90% of the patients) in early stages, leading to the development of precursor lesions mainly low grade PanIN (Figure 2) [6,20]. Additionally, in PDAC, the gene is constitutional active resulting in the overexpression of the protein and an exacerbated and abnormal activation of a plethora of signaling pathways due to the intrinsic GTPase activity of *KRAS* [21,22]. These signaling pathways include cell-autonomous phosphoinositide 3-kinase (PI3K)/ Protein kinase B (AKT)/ mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (Erk), the small GTPases Rho, Rac and Ral, and phospholipase C;

altogether these signaling pathways regulate cell growth, proliferation, survival, metabolism and motility [21,23]. In later stages and higher-grade lesions, the most frequent mutations occur in the genes *CDKN2A*, *SMAD4* and *TP53* (Figure 2) [19,24,25]. Moreover, mutations in *CDKN2A* lead to the loss of regulation of the cyclin dependent kinase (CDK) 4 and 6 cell cycle checkpoints resulting in the dysregulation of the cell cycle and consequentially carcinogenesis [19,26]; mutations in *SMAD4* lead to a decreasing SMAD4-dependent inhibition of transforming growth factor-β (TGF-β) resulting in the promotion of the non-canonical TGF-β signaling pathway, thus facilitating pro-tumorigenic responses [19,27]; mutations in *TP53* difficult DNA damage recognition, blocking cell cycle arrest, allowing cells to avoid cell cycle checkpoints and apoptotic signals [12,19]. Altogether these 3 genes act as tumor suppressors, and their inactivation has a major importance for the development of PDAC, contributing to multifaceted defects in tumor suppressor mechanisms resulting in dysregulated growth signaling and inflammation [21].

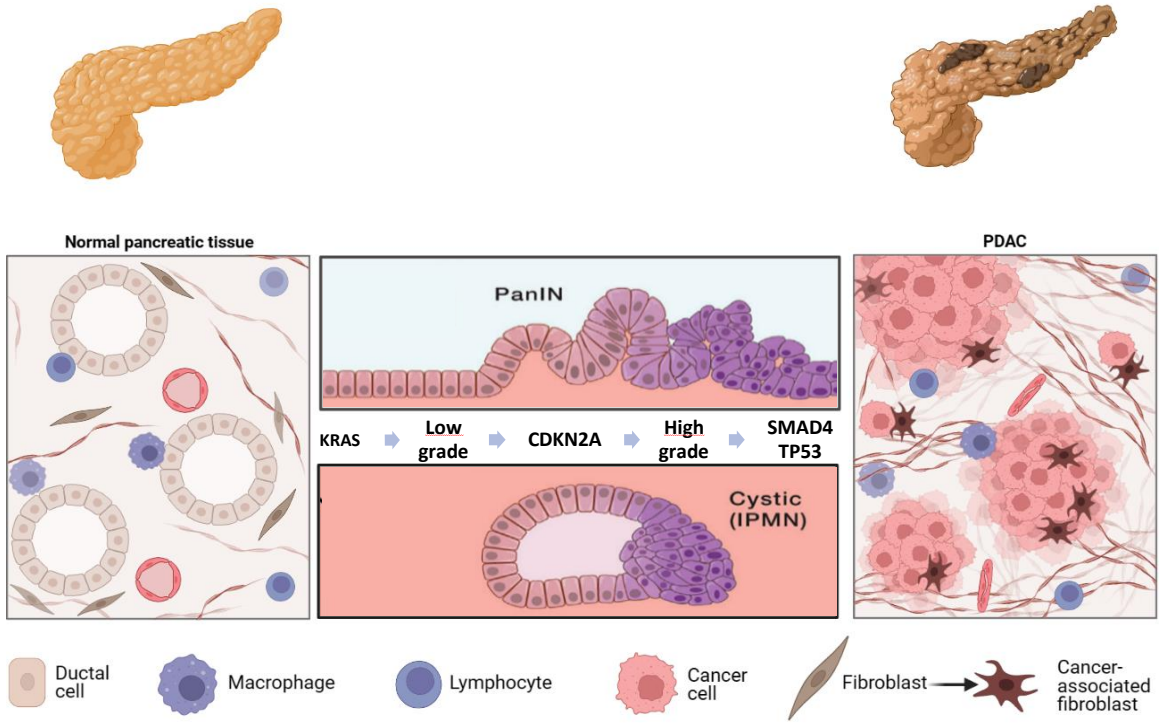


Figure 2. **Pancreatic cancer progression accompanied by the mutations that generally occur for each grade and also cellular and non-cellular surroundings.** Kirsten rat sarcoma virus (*KRAS*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), mothers against decapentaplegic homolog 4 (SMAD4) and tumor protein p53 (*TP53*).

Regarding germline mutations, approximately 5-10% of the cases have pathogenic germline mutations mainly in genes relevant to DNA damage repair (DDR) mechanisms [28]. The most recurrent mutations in hereditary PDAC occur in *BRCA1/2* followed by *Partner and Localizer of BRCA2 (PALB2)* and *Ataxia-Telangiectasia Mutated (ATM)*, representing core genes for the correct functioning of homologous recombination (HR)

[10,29-31]. Moreover, this mechanism is vital for the repair of double strand breaks (DSB), and it will be revised with more detail posteriorly. Additionally, the presence of mutations in these DNA repair mechanisms that mimic BRCA1 or BRCA2 loss are usually referred to induce a "BRCAness" phenotype. Altogether these mutations increase the relative risk (RR) of developing cancer being strongly associated to other types of cancers mainly breast, ovarian and prostate cancers [32]. For this reason patients must undergo regular surveillance to ensure that tumors are detected early and their families must be subjected to genetic testing [31].

Despite the carriers of the germline mutations being affected by increased RR of developing cancer, clinicians have taken advantage of the deficient DDR pathway, discovering targeted therapies that inhibit other major repair mechanisms inducing synthetic lethality and increasing consequently cancer cells death with decreased secondary effects and high efficacy [33,34]. We have the example of olaparib, that is an inhibitor of the protein poly (ADP-ribose) polymerase (PARP), recently approved in the clinic for the treatment of pancreatic cancer, specifically for patients with *BRCA 1/2* mutations [10,29].

1.2 DNA damage repair mechanisms

1.2.1 Types of DNA repair mechanisms

Conservation of the integrity of DNA is an essential condition to prevent genomic instability, a hallmark of cancer. DNA damage represents a major concern if not repaired rapidly and correctly, since the accumulation of these defects can lead to genetic errors, increasing the probability of malignant transformation and cancer progression [29]. Therefore, DDR mechanisms, accurate and effective activity, are pivotal for maintaining the integrity of our genome and ensure the correct transfer of genetic information during cell division [29,35].

DNA damages are triggered by endogenous and exogenous agents like chemicals, radiation, free radicals, chemotherapeutic agents, and others [35]. These errors can occur in the form of single strand breaks (SSB), DSB, base mismatch and interstrand crosslinks, having each one a specialized DDR mechanism for the effective repair (Figure 3) [35,36] .

Regarding SSB, they appear to be the most common type of DNA damage, and are frequently associated to the exposition to ionizing radiations and reactive oxygen species (ROS) (Figure 3) [37]. Additionally, the DDR mechanism of choice is base excision repair (BER), responsible for the loss or removal of damaged bases [38]. Briefly, the process starts with the detection of the damage by the proteins DNA glycosylases or PARP. Then, the SSB is subjected to an incision and end trimming process that is performed by the enzymes apurinic/apyrimidinic endonuclease (APE1), aprataxin (APTX), polynucleotide kinase 3'-

phosphatase (PNKP) and the tyrosyl-DNA phosphodiesterases 1 and 2 (TDP1 and TDP2), being consequentially generated a clean SSB that finally enables gap filling and ligation. This last step is mediated by a complex comprised of scaffold X-ray repair cross-complementing protein 1 (XRCC1) and DNA ligase III (LigIII), or DNA ligase I (LigI) [39,40].

About DSB, they not only occur by the exposition to ionizing radiations and ROS, but also if SSB aren't repaired correctly (Figure 3) [40]. These types of errors represent a greatest risk for provoking genomic instability and are mainly repaired by two major pathways, HR and non-homologous end joining (NHEJ), with the addition of single-strand annealing (SSA) (Figure 3) [29,41,42]. Additionally, NHEJ and SSA are prone to introduce errors in the DNA sequence increasing the chances of tumorigeneses, while HR repairs DSB with high fidelity, highlighting its importance in the repair of these type of defects [42-44]. Regarding to HR, which is part of the focus of this work, it is a process that mainly occurs during the G2 and S phases of the cell cycle and has several critical steps associated with it that will be reviewed in more detail in the next chapter [43]. Concerning NHEJ, it occurs throughout the cell cycle especially in the G1 phase when a sister chromatid might not be available for HR, resulting in the direct rejoining of the broken DNA ends without the need for a homologous template [33,45]. Furthermore, the canonical pathway can be subdivided into three vital steps, recognition and binding performed by the proteins Ku70 and Ku80, end processing executed by the proteins DNA-dependent protein kinase catalytic subunit (DNA-PKcs) that have a high affinity for Ku proteins and can form complexes with other proteins like artemis. To finalize, ligation is accomplished by the proteins DNA ligase IV and X-ray repair cross-complementing protein 4 (XRCC4) [45,46]. To conclude DSB repair, SSA is a pathway that is rarely active due to its mutagenic properties, even though there are present some key proteins that participate in HR [42,47]. Additionally, it happens in the G2 and S phases of the cell cycle and is usually activated when NHEJ is dysfunctional [47].

For the repair of mismatched bases, the DDR mechanism of choice is mismatch repair (MMR), composed by two protein heterodimer complexes: MSH2 and MSH6 and MLH1 and PMS2 (Figure 3) [48]. Mutations in this pathway are used in clinic for diagnostic, prediction, and prognosis due to its association to the hereditary lynch syndrome and/or microsatellite instability [48]. This hereditary syndrome increases the chances of cancer development, especially colorectal cancer in men and endometrial cancer in women, other type of tumors that are in Lynch Syndrome tumor spectrum include ovarian, gastric, small bowel, pancreatic, hepatobiliary, brain and urothelial [49,50]. The most frequent mutations occur in the genes of the proteins MLH1 and MSH2 and in approximately 10% of the cases in the MSH6 [48,50]. This DDR mechanism is subdivided into distinct critical steps: recognition and recruitment performed by the heterodimer MSH2-MSH6; excision executed by the two

heterodimers, proliferating cell nuclear antigen (PCNA), exonuclease 1 (EXO1) and replication factor C (RFC); and finally, synthesis and repair done by the proteins DNA ligase I, PCNA and replication protein A (RPA) [48].

At last, NER represents the DDR mechanism to repair bulky lesions, interstrand crosslinks or a distorted DNA helix (Figure 3) [51]. These lesions occur mainly by the exposition to ultraviolet radiation (UV) and are usually present in the form of cyclobutane–pyrimidine dimers (CPDs) and 6–4 pyrimidine–pyrimidone photoproducts (6–4PPs); other causes for this type of defects can be chemotherapeutics, like cisplatin, and chemicals [52]. Furthermore, NER can be classified in two subtypes, Global Genome Nucleotide Excision Repair (GG-NER) and Transcription-Coupled Nucleotide Excision Repair (TC-NER) [51]. Additionally, as the name suggests, GC-NER acts in the totality of the genome it is not restricted to any particular region of the DNA and is the primary mechanism that deals with bulky DNA adducts. Conversely, TC-NER is specifically focused on repairing DNA lesions that block transcription, primarily in actively transcribed regions of the genome [52,53]. Focusing on the GG-NER pathway it is constituted by 4 different phases: damage recognition, lesion verification, excision of the oligonucleotides, gap filling and ligation [52] (Figure 3).

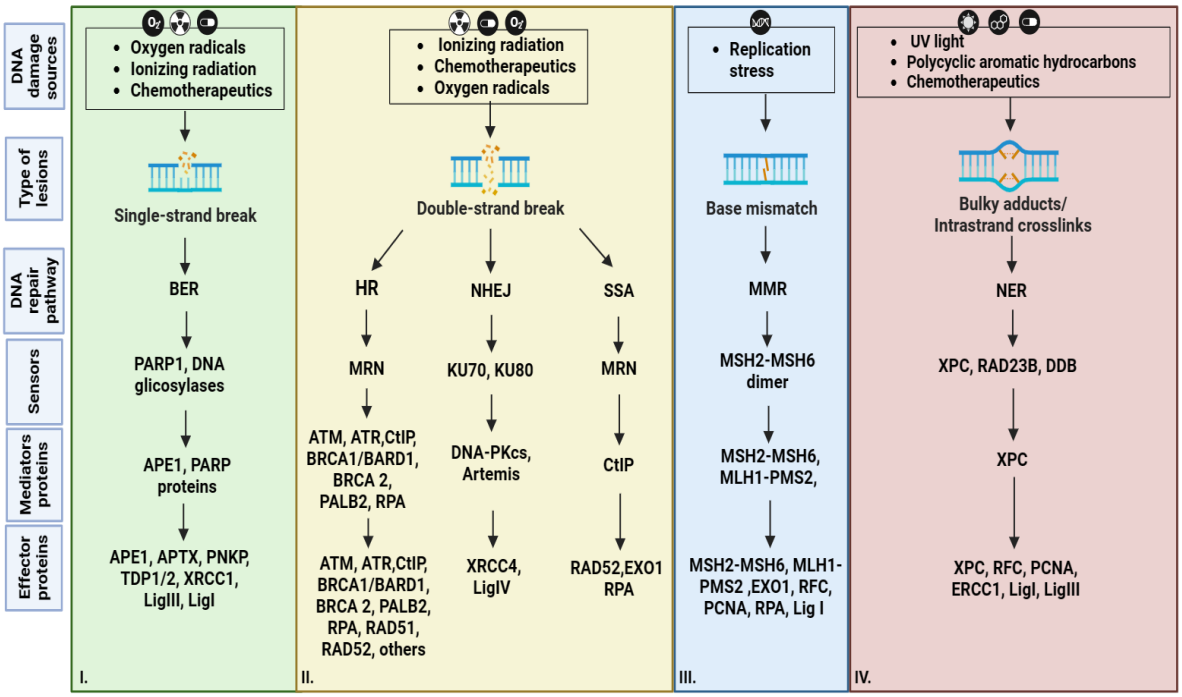


Figure 3. **Overview of DNA lesions and respective damage repair pathways. I.** Single-strand break repair by base excision repair (BER), **II.** double-strand break repair by homologous recombination (HR), non-homologous end joining (NHEJ) and single strand annealing (SSA), **III.** base mismatch repair by mismatch repair and **IV**. DNA adducts repair by nucleotide excision repair (NER).

1.2.2 Homologous recombination

As mentioned previously, HR is a DDR pathway significantly affected in PDAC with prevalent mutations in key proteins that are indispensable and usually associated to the clinic, allowing the use of targeted therapies like PARP inhibitors (PARPi). This high-fidelity pathway is specialized in the precise reparation of DSB, not losing information and accurately mirroring the DNA strand that is used as template [33]. In addition to the DNA repair, this DDR mechanism also has three other functions: DNA replication-fork rescue, meiotic chromosome segregation and telomere maintenance [54]. Giving emphasis on the DNA repair function, this pathway it is massively dependent on the template provided by the sister chromatid, restricting its action to the G2 and S phase of the cell cycle [54]. In addition, this pathway is generally mediated by Serine/Threonine protein kinases belonging to the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family, which include ATM and ataxia telangiectasia and Rad3-related protein (ATR) [55].

The first event that occurs in HR is the recognition and end resection of the DSB, initiated by the MRE11-RAD50-NBS1 (MRN) complex and the phosphorylated C-terminal binding protein 1 interacting protein (CtIP) (Figure 4) [56,57]. The MRN complex not only does damage recognition, but is also responsible for several steps: endonucleolytic cleavage to generate 3′ overhangs (critical step in the commitment to repair DSBs trough HR), recruitment of endonucleases (like EXO1, DNA replication helicase/nuclease 2 (DNA2) and Bloom syndrome protein (BLM) to perform bulk 5′–3′ DNA resection), load of RPA complexes on the single strand DNA (ssDNA) to prevent self-annealing and stabilization, and activation of ATM (Figure 4) [54,57-59]. This last interaction between the MRN complex and ATM, specifically between NBS1 with ATM, leads to the phosphorylation of the H2A histone family member X (H2AX) producing its phosphorylated form yH2AX, increasing DNA accessibility, and initiation of a phosphorylation cascade started by the checkpoint kinase 2 (CHK2), affecting posteriorly effector proteins that are pivotal for the HR repair process [60,61]. These effector proteins include p53, CDC25C and CDC25A, and BRCA1, contributing for cell cycle delay and initiation of DNA repair [54,60]. Additionally, with the loading of RPA to the ssDNA there is also a dependent ATR-interacting protein (ATRIP) activation of the protein ATR, resulting in the phosphorylation of the checkpoint kinase 1 (CHK1) and consequently phosphorylation of effector proteins, that as CHK2 support cell cycle delay and recruitment of DDR proteins to initiate the repair process [29,55,62].

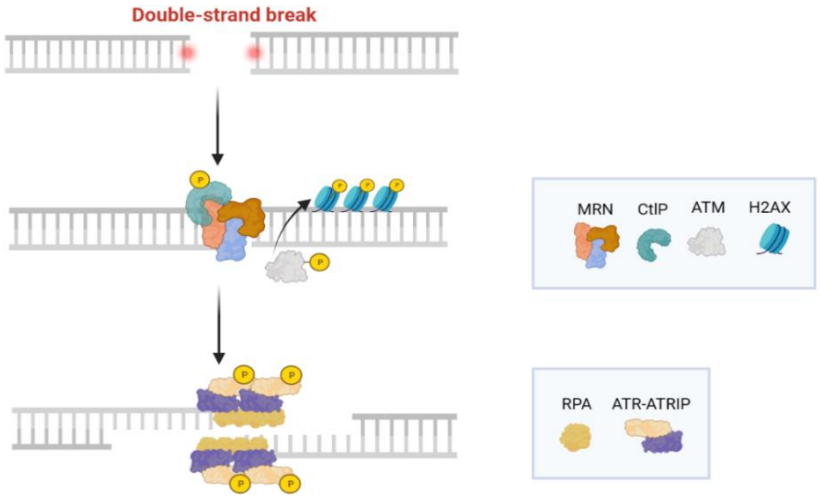


Figure 4**. Representation of the first events in HR and some of the main associated proteins.** Sensing of DSB and end resection by the MRN complex, and ATM or ATR dependent-recruitment of proteins important for the progression of HR.

After end resection of the DSB and activation of effector proteins, DDR factors such as BRCA2/PALB2, RAD51/RAD52, and BRCA1 are recruited to initiate homologous pairing and strand invasion. This process usually starts with the formation of pre-synaptic filaments composed by RAD51 monomers, and the mediators are BRCA2 and PALB2 [55,63]. Afterwards, alongside BRCA1 and PALB2, BRCA2 provides RAD51 monomers to the ssDNA, resulting in RPA heterodimer removal [55,63] (Figure 5). Posteriorly, an important factor for the success of this process is the invasion of ssDNA coated with RAD51 into homologous sequences and consequent formation of the displacement loop structure (D-loop) facilitated by the presence of the BRCA1 and BRCA1-associated RING domain protein 1 (BARD1) heterodimer [55].

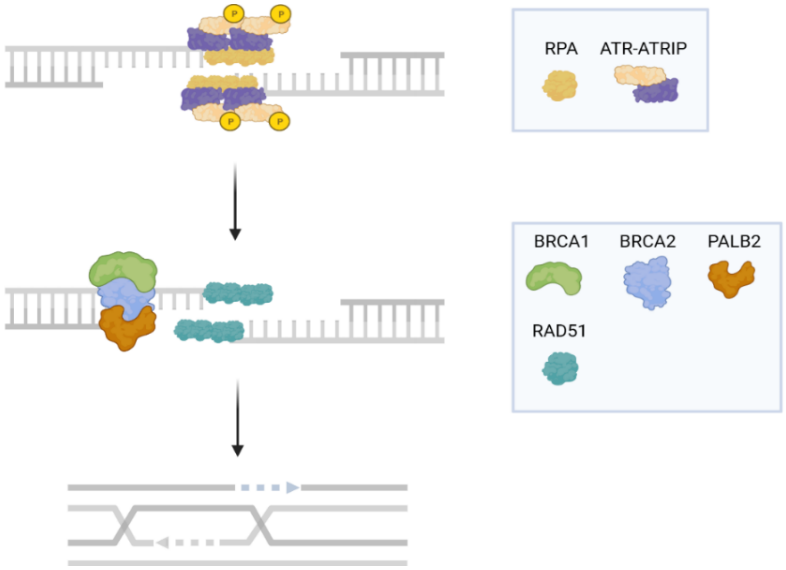


Figure 5. **Representation of following events in HR and some of the main associated proteins.** Interaction of BRCA1 and BRCA2 mediated by PALB2 and consequent exchange of RPA for RAD51 monomers in ssDNA promoting alignment of homologous sequences and formation of D-loop structures.

At last, after strand invasion and interaction with the intact homologous templates supplied by the sister chromatid, extension of the D-loop structure and complete resolution can be achieved in three different mechanisms: DSBs repair (DSBR), synthesis-dependent strand annealing (SDSA), and break-induced DNA replication (BIR) (Figure 6) [64]. Regarding the first mechanism, two Holliday junctions (Hj) (alignment of two DNA molecules and exchange genetic information) are formed and posteriorly are resolved by the catalytic function of resolvases or endonucleases, which cleave the DNA at specific points within the Hj ensuring that the DNA strands are correctly cut and resealed to create either crossover or non-crossover products [64]. Different from DSBR, SDSA does not form a stable Hj, instead, after strand invasion, the invading single-stranded DNA anneals with the complementary strand in the homologous DNA molecule [64]. Following this annealing, the single-stranded DNA is displaced, and the gaps are filled by DNA synthesis using the homologous DNA molecule as a template. Once the gaps are filled, the newly synthesized DNA dissociates from the homologous template resulting in a non-crossover product. Lastly, this pathway is different from DSBR and SDSA because it involves the repair of a one-sided double-strand break, where only one end of the break has a homologous template available for repair [65]. BIR is particularly important for large DNA lesions, such as collapsed replication forks or eroded telomeres and it may result in the complete restoration of the damaged DNA sequence but can also lead to the loss of genetic material in some cases [65].

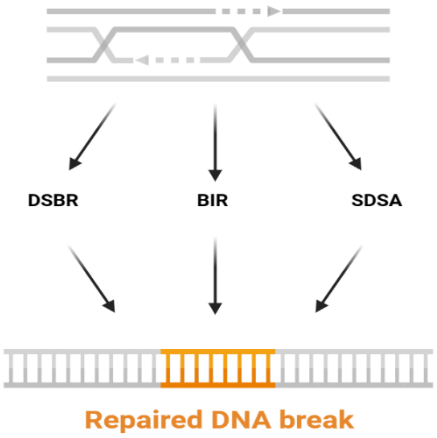


Figure 6. **Final step of HR with the three distinct mechanism that can perform D-loop structure complete resolution.**

1.2.3 BRCA 1/2 proteins structure and function

Tumor suppressor BRCA1 and 2 proteins are pivotal for the process of HR, displaying essential functions as mediators and effector proteins, demonstrated in the previous chapter. For this reason, mutations in the genes of these proteins lead to a homologous recombination deficiency (HRD) phenotype consequently increasing

susceptibility for PDAC [32]. Moreover, the RR and cumulative incidence of PC are 3.5–10% and 2–7% for germline BRCA2 pathogenic variants and 2.26 and 1–3% for germline BRCA1 pathogenic variants, respectively [32].

Regarding the structure and function of the two caretaker proteins that ensure the error-free resolution of DNA damages they are well-defined and distinct. Regarding BRCA1, it is constituted by 1863 amino acids, contains 4 domains: a structured zinc-binding finger really interesting new gene (RING) domain, a Coiled coil (CC) domain, a serine cluster (SC) domain and 2 BRCA1 C Terminus (BRCT) domain; and Nuclear localization signals (NLS) and Nuclear export sequences (NES) sites that control subcellular localization of BRCA1 between the cytoplasm and nucleus [66] (Figure 7). Beginning upstream the sequence near the N-terminus of the protein, the RING domain grants the interaction with BARD1, conferring the activity of E3 ubiquitin ligase that mediates the formation of Lys-6-linked polyubiquitin chains [67,68]. This property not only promotes ubiquitination of specific proteins and their proteasomal degradation, like H2AX linked to the DNA end resection step of HR, but also auto-ubiquitination of the BRCA1-BARD1 heterodimer, increasing its own activity levels and stability [67,69]. Additionally, this self-ubiquitination of the heterodimer has been broadly described to have an important tumor suppression activity by increasing DNA binding and BRCA1 nuclear confinement, supporting cells DDR mechanisms in case of a genotoxic event [69-71]. Furthermore, the functions and nuclear retention of BRCA1 are closely dependent and determined by its RING-binding partner BARD1, as the NES sites are mutually masked in the BRCA1-BARD1 heterodimer, supporting an active site for BRCA1 and consequently efficient DDR, facilitating end resection and presynaptic complex formation in HR [67,71,72]. Conversely, in case of BRCA1 shuttling to the cytoplasm, previous studies have demonstrated an association with an abnormal DNA repair, apoptosis and chemosensitivity [73,74].

The following domain, CC, is responsible for mediating the interaction with BRCA2 and PALB2 that acts as the linker to form BRCA1-BRCA2 complex [75,76]. Briefly, it has been shown that CC domains of both BRCA1 and PALB2 interact, recruiting posteriorly BRCA2 to form the complex, enabling the assimilation of RAD51 filaments onto the ssDNA in HR [75,77]. The SC domain comes next, and is commonly considered a target of post transcriptional modifications made by the kinases ATM and ATR [78]. The phosphorylations mediated by these two kinases causes the recruitment of BRCA1 to sites of DSB which facilitates DDR [78].

Reaching to the BRCT domain at a position more downstream the sequence near the C-terminus of the protein, it allows the binding of BRCA1 to proteins that are phosphorylated by ATM or ATR on serine in SXXF motifs and are responsible for regulating cell cycle, transcriptional activity and DNA repair [70,79]. Also, some BRCT-binding proteins

like CtIP, BRCA1 A complex subunit (ABRAXAS), BRCA1-interacting protein C-terminal helicase 1 (BRIP1; also known as BACH1) and others, play a major role in DDR mechanisms, forming unique macro-protein complexes that assist in the responses to DNA damage [70,77]. The protein P53 can also interact with BRCA1 through the BRCT domain, not only in a transcription modulation manner but in protein-protein binding, disrupting the interaction with BARD1, leading consequently to nuclear export of BRCA1 to the cytoplasm [80]. Conversely, in case of a dysfunctional p53, that is common in PDAC, nuclear confinement of BRCA1 stays stable, reducing the susceptibility of cells to ionizing radiations [80]. Other proteins that can be targeted by the BRCT domains are responsible for epigenetic regulation of genes, especially the histones deacetylase 1 and 2 (HDAC1/2), supporting that BRCA1 can also participate in the regulation of genes transcription [81].

Figure 7. **Functional structure of BRCA1 with relevant interacting proteins.** Really Interesting New Gene (RING); BRCA1-associated RING domain protein 1 (BARD1); nuclear export sequences (NES); nuclear localization signals (NLS) coiled-coil (CC); serine cluster domain (SCD); BRCA1 C Terminus (BRCT).

Regarding BRCA2, in contrast to the multifunction activity of BRCA1, its primary functions occur in HR [70]. It is a protein composed by a transcriptional activation domain (TAD), a DNA binding domain (DBD) that binds ssDNA and double-stranded DNA (dsDNA), a TR2 domain, eight BRC repeats and NES and NLS sites (Figure 8) [70,82]. In the N-terminus of the protein, the TAD allows the binding of PALB2, which is an essential linker to assist in the BRCA1-BRCA2 complex formation, aiding BRCA2 in the recruitment of RAD51 recombinase to perform in HR [76,83].

Regarding BRC repeats, they comprise eight conserved sequence motifs, with varying affinities for RAD51 between them [84]. The motifs specialized in the binding of RAD51 include BRC3 and BRC4 and both achieve this for having a structure that mimics an adjacent RAD51 monomer, enabling the binding to the RAD51 core [84,85]. Besides facilitating recruitment of RAD51 the BRC repeats also accelerate the exchange of RPA in the ssDNA by RAD51 and contribute to the RAD51 filament formation on ssDNA, maintaining the active ATP-bound form of RAD51 on ssDNA [70,85].

Concerning the DBD, it is constituted by five main components: a 190-amino-acid α-helical domain, three oligonucleotide binding (OB) motifs and a tower domain (TD)

[70,86]. The OB motifs are recognized to be ssDNA-binding modules and the TD that protrudes from OB2 for binding to dsDNA [70]. Additionally, the helical domain, OB1 and OB2 can also associate with the small acidic protein deleted in split-hand/split-foot syndrome (DSS1), contributing for the stability of BRCA2 and loading of BRCA2-RAD51 complex to RPA-coated ssDNA through its ability to bind to RPA, attenuating its affinity for ssDNA, which is a crucial step HR [70,87,88]. Other DBD domain interacting protein is p53, and interestingly the overexpression of BRCA2 inhibits its apoptotic and transcriptional activity, reducing the expression levels of its targets genes such as p21 and Bax [89].

Finally, the last domain closer to the C-terminus of the protein, the TR2 domain, facilitates the interaction of BRCA2 with multimeric forms of RAD51, supporting, for example the stabilization of the RAD51 filaments in HR [90]. Furthermore, this BRCA2-RAD51 interaction seems to be tightly regulated during the cell cycle and in responses to DNA damage, being activated by a CDK mediated phosphorylation site at S3291 [90]. Despite the importance of BRCA2 in HR by intervening in the nuclear foci of RAD51 in response to DNA damage, BRCA2 can also participate in the transcriptional control of certain genes [89]. One example is the inhibition of the proliferative activity of the Androgen Receptor (AR) due to the cooperation between BRCA2, BRCA1 and the histone acetyltransferase P/CAF. This leads to the enhancement of AR and Glutamate receptor-interacting protein 1 (GRIP1) (coactivator of AR)-mediated transactivation, promoting the anti-proliferative effect of AR [89,91].

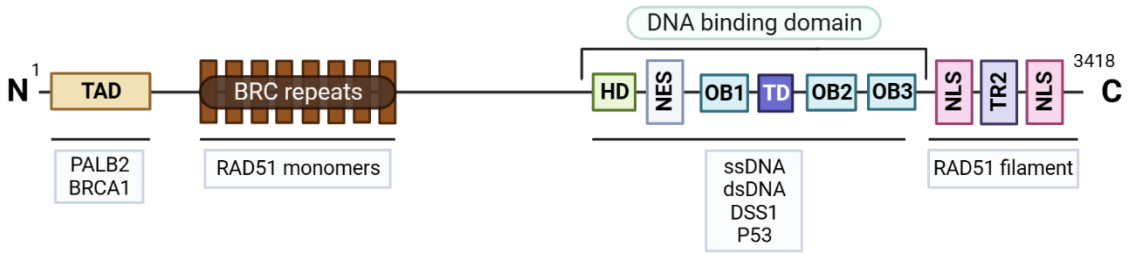


Figure 8. **Functional structure of BRCA2 with relevant interacting proteins.** Transcriptional activation domain (TAD), BRC repeats; the DNA binding domain (DBD) incorporating a helical domain (HD), nuclear export sequences (NES), oligonucleotide binding (OB) and tower domain (TD); nuclear localization signals (NLS) and TR2 domain.

1.3 Current Treatment for PDAC

1.3.1 Standard of care with conventional chemotherapeutics

As mentioned previously, early detection of PDAC still remains a challenge and metastatic disease perseveres as the most common clinical presentation, limiting the possibility of tumor resection complemented with adjuvant chemotherapy [10,24]. Currently, this multimodal care regimen with surgical resection followed by adjuvant chemotherapy is acknowledged to be the best available potentially curative therapy for

patients with a localized disease and a clinical staging defined as resectable and borderline resectable, with neoadjuvant therapy being necessary in some cases and further resection assessment by a multidisciplinary team [10,24]. The exception are patients with locally advanced PDAC, representing approximately 30% of the cases at diagnostic in PC, and being commonly inoperable due to tumor major vessels invasion and low response to neoadjuvant therapy [10,24,92,93].

Regarding the chemotherapeutics used in the clinic for adjuvant purposes in patients eligible for tumor resection, they include modified FOLFIRINOX (combination of folinic acid (leucovorin), fluorouracil (5-FU), irinotecan and oxaliplatin, and in which the term ”modified” refers to the reduction of irinotecan and the exclusion of the 5-FU bolus due to premature adverse effects associated to FOLFIRINOX) for patients with high functional status and gemcitabine (GEM) plus capecitabine or GEM alone for patients with a poor functional status [24,93-95]. According to the phase 3 clinical trial European Study Group for Pancreatic Cancer Trial 4 (ESPAC-4), the combination of GEM with capecitabine benefits patients OS in comparison to GEM alone, and the PRODIGE-24 trial demonstrates that GEM alone has a lower efficacy when compared to the modified FOLFIRINOX that increases the median disease-free survival by 9 months [96,97]. However, due to the high toxicity of this therapies accompanied by a variety of secondary effects, patients tolerance to adjuvant therapy is low, with less than 50% of the planned dose being received in most of the cases, contributing for the ineffectiveness of the treatment and high probability of relapse [10,98].

So, other pre-operative strategies like introducing neoadjuvant therapies have been broadly explored since surgery can debilitate patients to undergo posteriorly aggressive regimens of chemotherapy [10]. Based on this premise, the possible goals of this procedure can include improvement of chemotherapy response compared with post-resection adjuvant therapy, increasement of the likelihood of margin-negative resection, upfront treatment of occult micro-metastases avoiding unnecessary resection for rapidly progressing tumors and expansion of the pool of patients that can be eligible for tumor resection especially borderline resectable pancreatic tumors [10,99]. Nonetheless, neoadjuvant strategies in pancreatic cancer are seen by oncologists as a “double edge sword” as it may limit the possibility of surgical resection [99,100]. With this paradigm pre-operative trials like the phase 3 PREOPANC trials urge to try discovering the best combinations of neoadjuvant chemotherapy or chemoradiotherapy and the advantages of a correct timing for surgery and regimens of treatment in resectable and borderline resectable patients [101,102]. For these trials, the treatment regimens being tested commonly include the multi-agent FOLFIRINOX, GEM, nab-Paclitaxel with possible combination with radiotherapy [93,99].

For patients with a locally advanced disease, resection of the tumor in the majority of the cases is an unattainable procedure, even with the advancements in surgery techniques and improvement of neoadjuvant therapies [10,93,99]. Standard of care for these patients is not well established and the recommendations are to do a clinical trial evaluation and possible enrolment [93,99]. Additionally, owing to the uncurable nature of this stage is very common to apply the same regimens of chemotherapy used in patients with metastatic disease and palliative radiotherapy can be offered in some cases if certain criteria is accomplished [10,99,103].

Concerning the chemotherapeutics used as first line treatment options for patients who have metastatic disease, they include FOLFIRINOX, GEM + nab-Paclitaxel or GEM alone. Their usage is dependent on the functional status of the patient, with FOLFIRINOX being the less tolerable and used in fit patients [92,93]. Nevertheless, high cases of resistance and toxicity are observed being necessary to apply second line treatment options to mitigate the levels of toxicity and elevate clinical efficacy [92,93]. About second line treatment options, patients with good performance status and previously treated with FOLFIRINOX, subsequent treatment includes GEM + nab-Paclitaxel and patients treated with GEM-based therapy succeeding treatments include 5-FU+nanoliposomal irinotecan, 5-FU alone, or the combination of 5-FU, oxaliplatin and folinic acid/leucovorin (OFF or FOLFOX) [92]. Nevertheless, the goal of the administration of this chemotherapeutics in these patients still remains palliative, not being potentially curative due to the high levels of resistance and toxicity [10].

1.3.2 Targeted therapy

About the mechanism of action of the following discussed conventional chemotherapeutics used in the clinic to tackle PDAC, they mainly affect DNA repair and synthesis, provoking also severe damage to normal cells hence an elevated toxicity [10,99]. So, to counteract conventional chemotherapeutics undesirable effects and lack of efficiency, targeted therapies capable of having high antitumor activity and selectivity for tumor cells, while decreasing secondary effects, are needed.

With the discovery of the genetic landscape of PDAC and the identification of multiple targets, the conception of therapies capable of inhibiting specific signaling pathways with oncogenic character and cancer selective strategies to induce apoptosis in PDAC have increased [20]. One good example are PARPi that explore the concept of synthetic lethality and were firstly introduced in the therapy of breast and ovarian cancer patients with germline mutBRCA1/2 genes (Figure 9) [104]. Briefly, this concept implies that simultaneous disruption or inactivation of two or more genes or pathways results in cell

death or impairment of cell survival, even though disruption of each individual gene or pathway alone is not lethal. Applying this to PDAC, since it is also affected by mutations in BRCA1 and BRCA2, cancer cells can be selectively targeted by PARPi due to their dysfunctional HR mechanism (Figure 9) [105]. Particularly, PARPi can bind to the catalytic site of PARP proteins promoting conformational changes that increase stabilization of PARP-DNA complexes and inhibition of the cleavage of nicotinamide adenine dinucleotide (NAD)+ to nicotinamide and ADP-ribose (Figure 9) [105]. Without the capacity to cleavage, PARP proteins are unable to form poly (ADP-ribose) (PAR) chains that are important for the poly(ADP-ribosyl)ation (PARylation) of proteins, which is a post-transcriptional modification that regulates interaction, function, degradation and recruitment of proteins [105,106]. Consequently, with the stabilization of PARP-DNA complexes the catalytic cycle is broken, with PARP proteins not being able to return to an inactive state resulting in a dysfunctional PARP, and PARylation is compromised blocking the recruitment of effector enzymes involved in BER machinery which leads to the accumulation of unrepaired SSB that progress to cytotoxic DSB [105]. Finally, cells with a defective process of HR, namely with mutBRCA1/2, are unable to accurately repair DSB, leading to selective chromosome instability, cell cycle arrest and apoptosis (Figure 9) [105].

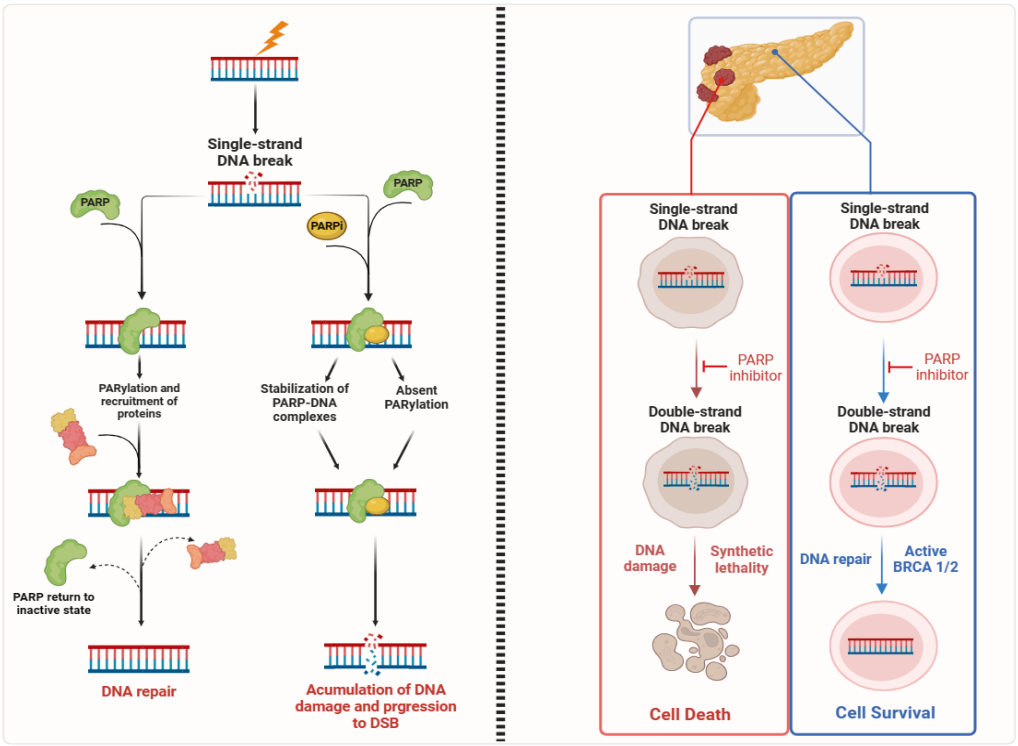


Figure 9. **Schematic representation of the mechanism of action of PARP inhibitors (PARPi) and concept of synthetic lethality.** PARPi bind to cataltytic domain of PARP proteins competing with co-factor NAD+ and supporting PARP-DNA complexes leading to a dysfunctional PARP protein **(Left).** Inhibition of PARP proteins leads to DSB breaks which need to be repaired by HR, however cancer cells have this mechanism compromised since BRCA1/2 are mutated leading to synthetic lethality **(Right).**

Currently, olaparib is considered as the standard PARPi therapy used as first-line maintenance treatment for metastatic PC patients with mutBRCA1/2, due to an increased disease progression-free survival observed in the Pancreas Cancer Olaparib Ongoing (POLO) trial, that granted its FDA approval in 2019 [107,108]. However, other PARPi have been developed, mainly targeted for breast and ovarian cancer, with a tendency to expand for PC treatment [104]. These include niraparib, rucaparib, talazoparib, veliparib and pamiparib [104].

Nevertheless, the promising results of these PARPi therapies are commonly oppressed by the numerous mechanisms of acquired resistance described in pre-clinical and clinical studies [104,108,109]. Therefore, new strategies to neutralize the clinical inefficiency of PARPi, like combining with conventional chemotherapy or other targeted agents that can induce HR deficiency, would be beneficial for the treatment of PDAC. In fact, the combination of olaparib with GEM and cisplatin has shown to be effective, however patients' tolerability to the triple combination was low, highlighting the importance of new clinical trials to evaluate beneficial combinations and correct dose reduction for major effects [107,110].

Targeting epidermal growth factor receptors (EGFRs) is also another promising targeted therapy, due to their increased expression and high prevalence in PDAC [10,95]. These receptors are tyrosine receptors and their activation can trigger signalling pathways that are involved in cell proliferation and survival, and metastases, being their abnormal expression commonly associated with worst prognosis [95,111]. Currently, erlotinib is the only EGFR inhibitor used in the clinic to treat patients with metastatic PC, and its administration is usually combined with GEM, by blocking GEM-induced MAPK signalling activation [95,112-114]. In addition, inhibition of EGFRs by erlotinib seems to compromise HR, interfering in the recruitment of RAD51 to DNA damage sites and in the nuclear localization of BRCA1, emphasizing its combination with other DNA-targeting therapies like olaparib [115].

Apart from EGFRS, other types of tyrosine kinases like Neurotrophic tyrosine receptor kinases (NTRK) can be targeted in PC, even though only a minority of cases are affected [95]. Their oncogenic behaviour is the result of the fusion of genes that result in the constitutive activation of their respective signalling pathways [116]. To selectively target these tyrosine receptors the inhibitors that are used to treat PC patients include larotrectinib or entrectinib [93].

Regarding immunotherapy, it is also considered a high potential targeted method to treat PDAC. Currently, the anti–programmed death-1 (PD-1) monoclonal antibody pembrolizumab is used in the clinic to treat PC patients with tumors that have a deficient MMR mechanism [117,118]. Usually, neoplasias with this dysfunctional DDR mechanism

harbour an abnormal quantity of somatic mutations that encode potential neoantigens, therefore increasing their likelihood to be immunogenic (tumors that can be effectively detected and attacked by the immune system) [117,119]. So, with the administration of pembrolizumab, the interaction between PD-1, present in immune cells like T-cells, and its ligands, programmed death-ligand 1 and 2 (PD-L1/2), which are often expressed on the surface of cancer cells is inhibited [120]. Consequently, there is an augmentation of the immune response against the tumors by repressing the inhibitory signal made by the interaction between PD-1 and PD-L1/2 and activated T-cells can now efficiently recognize cancer cells due to the presence of cancer-specific antigens or neoantigens [120]. Additionally, the combination of pembrolizumab plus olaparib is being explored in the phase 2 clinical trial SWOG S2001, and unconnected to the trial, a case report where this combination was administrated in a patient with metastatic PDAC harboring germline mutBRCA1 and a high tumor mutation burden (TMB) a complete radiologic response was observed [121,122].

1.3.3 Acquired therapeutic resistance in PDAC

Regardless of the improvements and new strategies in both conventional and targeted treatment regimens, PDAC ability to acquire therapeutic resistance represents a major clinical limitation in PC care [10]. As previously discussed, the aggressiveness and heterogeneity nature of these type of tumors contribute to a high capacity of modulation and adaption, antagonizing the anti-tumor effect of therapies through multiple resistance mechanisms.

The TME is a complex dynamic multifaceted topic of major concern in therapeutic failure, being usually responsible for both primary and acquired resistance to therapies, extending beyond conventional chemotherapies to targeted therapies and immunotherapies [18,123]. Its composition fluctuates for the benefit of the tumor, being constituted by a variety of cell populations and non-cellular surrounds, in which all of them interact with the tumor that is constituted by heterogeneous niches of cancer cells [18]. Additionally all the interactions work in syntony to promote tumor survival and progression, metastases and response to therapies [18].

One of the most significant resistance mechanism that is a histopathological hallmark of PDAC, and is associated with TME, is its abundance in extracellular matrix (ECM) usually termed as desmoplasia [124,125]. This characteristic is the result of the paracrine interaction between the inflammatory conditions in the TME by the release of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), and myofibroblastic cancer-associated fibroblasts (or pancreatic stellate cells (PaSC)), which

liberates them from a quiescent form, promoting the production of a fibrotic stroma that surrounds the tumor (Figure 10) [126,127]. The creation of this desmoplastic tissue leads to the formation of a chemoresistance environment with a physical barrier that prevents appropriate vascularization, blocking the delivery of chemotherapeutics and immune cells infiltration [124]. In fact, the abundant fibrotic stroma in PDAC have been widely considered as a physical barrier to deliver GEM, other chemotherapies and targeted therapies contributing to treatment failure [128].

Also, due to the constriction of the blood vessels, the flow of oxygen is compromised, causing a hypoxic environment, therefore creating conditions that can potentiate therapeutic resistance [129]. Moreover, several downstream effects caused by hypoxia are well described and are mediated by the transcription factors called hypoxia inducible factors (HIFs), especially the variant HIF-1α, being the most commonly studied and attributed to a variety of tumorigenic characteristics [129,130]. For instance, the metabolic reprogramming of glycolysis to produce lactate is mediated by HIF-1α, becoming for cancer cells the primary mean of obtaining energy, and also a characteristic of great importance to sustain tumor progression in a hypoxic environment (Figure 10) [130-132]. Briefly, HIF-1α upregulates the transcription of glucose transporters 1 and 3 (GLUT 1/3) enhancing glucose uptake, and also glycolytic enzymes like lactate dehydrogenase helping cancer cells to convert pyruvate in lactate, instead of being oxidized via the tricarboxylic acid (TCA) cycle (Warburg effect or aerobic glycolysis) [129-131]. Consequently, with the accumulation of lactate a beneficial environment for cancer cells is created, promoting a easier access to a source of energy, impairment of T-cell cytokine production and acidification of the environment, conferring epithelial-mesenchymal transition (EMT) that results in resistance against GEM [129,133].

Additionally, expression of HIF-1α supports cancer cells stemness by interacting with the NOTCH signaling pathway and mediating the increasement of autophagy, favoring undifferentiated cells that are associated with a worst prognosis factor due to their capacity to acquire resistance to therapies (Figure 10) [129,134,135]. Furthermore, cancer stem cells are a particular niche of cells with the capability of indefinite self-renewability, differentiation in various types of cells, a low mitotic rate, high EMT, improved DDR mechanisms in comparison to other cancer cells and expression of multi-drug resistant transporters, supporting tumor formation, metastasis and resistance against conventional chemotherapeutics and radiotherapies used in the clinic [136,137].

The immunosuppressive capability of the TME is also an important characteristic to mention, and can also be associated to the hypoxic environment in some cases, resulting in a manner for cancer cells acquiring resistance to current immunotherapies [129]. Indeed, the recruitment of cells, liberation of specific cytokines and expression of certain receptors by cancer cells that inhibit immune responses are main mechanisms that contribute to

immune evasion [129,138]. The release of TGF-β and IL-10 by cancer cells is a good example, leading to the recruitment of tumor associated macrophages (TAMs), specifically by conducting the polarization of M1 to the M2 phenotype of macrophages (Figure 10) [129,139]. Moreover, this specific phenotype of macrophages, conversely to the M1, is acknowledged to have anti-inflammatory and pro-tumorigenic properties, increasing angiogenesis, tumor invasion and causing depression of immunity, denying the effectiveness of immunotherapies (Figure 10) [129,139,140]. Furthermore, recently it was discovered other mechanism of acquired resistance against anti-PD1 therapies like pembrolizumab, suggesting that cancer cells and dendritic cells could co-express both PD1 and its ligands PDL-1/2, and the result being that tumor cell-derived PD-1 could exhaust the exogenous anti-PD-1 and consequently guarantee the combination of PD-L1 and PD-1 on T cell, inhibiting T-cell activation and cytotoxicity (Figure 10) [139,141].

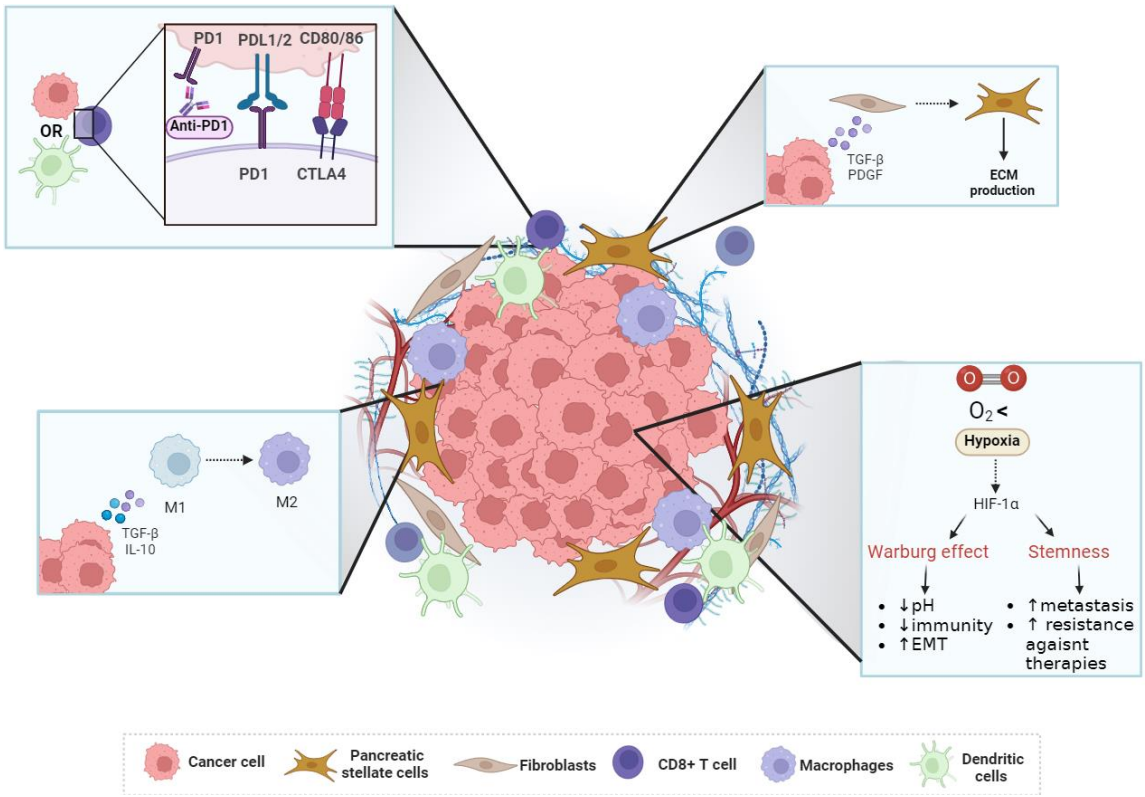


Figure 10. **Tumor microenvironment of PDAC with some of the main acquired resistance mechanisms**, including contribution of pancreatic stellate cell to the formation of fibrotic stroma trough the paracrine interaction with cancer cells. Distinct effects of hypoxia in cancer cells mediated by hypoxia inducible factors (HIFs), especially the variant HIF-1α, being the glycolysis metabolism reprogrammed to lactate production (Warburg effect) and stemness of cancer cells sustained. Immune escape systems like polarization of pro-inflammatory M1 to anti-inflammatory M2 macrophages and also co-expression of PD1/PDL1 in cancer cells.

Apart from the TME, genetic and epigenetic aberrant alterations can also beneficiate tumor survival against therapies. One particular case is the altered expression of enzymes and transporters in the metabolism of GEM (also known as dFdC: 20,20-difluorodeoxycytidine) that are indispensable for the efficient mechanism of action of this

therapy [128]. To better understand the metabolism of GEM, firstly this nucleoside analog has to enter the cell trough specific transporters due to its hydrophilic nature, and then to exert its effects after intracellular uptake, it has to undergo multiple phosphorylation's performed by several enzymes, enabling DNA sequence incorporation and inhibition of DNA synthesis that result in cell death (Figure 11.) [128].

Regarding the specialized transporters they can be subdivided into two families, the solute carriers SLC28 family of cation-dependent human concentrative nucleoside transporters (hCNTs) that only have a unidirectional transport, and the solute carriers SLC29 family of energy-independent human equilibrative nucleoside transporters (hENTs) having bidirectional nucleoside transport according to a concentration gradient [128]. In addition, previous studies demonstrated that the most influential variants of nucleoside transporters for the uptake of GEM include hENT1, hCNT1 and hCNT3, being their reduced or null expression associated to a worst prognosis and acquired resistance for PDAC patients that have been administrated with GEM [142,143].

Following intracellular uptake, GEM is phosphorylated by the enzyme deoxycytidine kinase (dCK) into GEM monophosphate (or dFdCMP) due to a high affinity interaction between them (Figure 11) [128,144]. However, the phosphorylation performed by this enzyme is a rate-limiting step for the consequential production of active metabolites of GEM, dictating its expression levels generally GEM resistance ability in PDAC patients [128,144]. Consequently, most of the administrated GEM that was not phosphorylated by dCK, is deactivated by a rapid deamination process performed by the enzyme cytidine deaminase (CDA), resulting in the synthesis of the less active form of GEM, 2',2'-difluorodeoxyuridine (dFdU), that can be expelled by multidrug resistant transporters or turn into 2'-deoxy-2',2'-difluorouridine monophosphate (dFdUMP) (Figure 11) [128,144].

Subsequently, the GEM phosphorylated by dCK is also subjected to enzymes that can either deactivate this active metabolite or mediate the synthesis of gemcitabine triphosphate (dFdCTP) that is the major active metabolite of GEM [128,144]. Concerning the enzymes that contribute for the mode of action of GEM they include pyrimidine nucleoside monophosphate kinase (NMPK, also known as UMP/CMP) inducing the phosphorylation of dFdCMP to gemcitabine diphosphate (dFdCDP) and nucleoside diphosphate kinase (NDPK) transforming dFdCDP into dFdCTP (Figure 11) [128,144]. With the synthesis of dFdCTP, that acts as a competitive substrate of deoxycytidine triphosphate (dCTP) and is a major active metabolite of GEM, incorporation of dFdCTP to the DNA sequence during replication increases [144]. Consequently, DNA synthesis is inhibited due to the disabled DNA polymerases and proof-reading exonuclease that aren't capable of removing the GEM metabolite that has the capability of mimicking chain termination not

allowing elongation of the DNA strand, generating DNA damages and inducing cell death (Figure 11) [144].

In addition, GEM has a mechanism that can potentiate its effect by enhancing its activation and incorporation in the DNA sequence [128,144]. Briefly, the metabolite dFdCDP is capable of inhibiting ribonucleoside reductase (RR) enzymes that are responsible for converting cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) (Figure 11) [128,144]. As a result, a decreased competitive pool of dCTP is available for DNA synthesis, thus facilitating the incorporation of dFdCTP into the DNA sequences [128]. Nevertheless, in PDAC the overexpression of RR enzymes especially the variant RRM1 is correlated with a low OS representing a major mechanism of resistance against GEM [128].

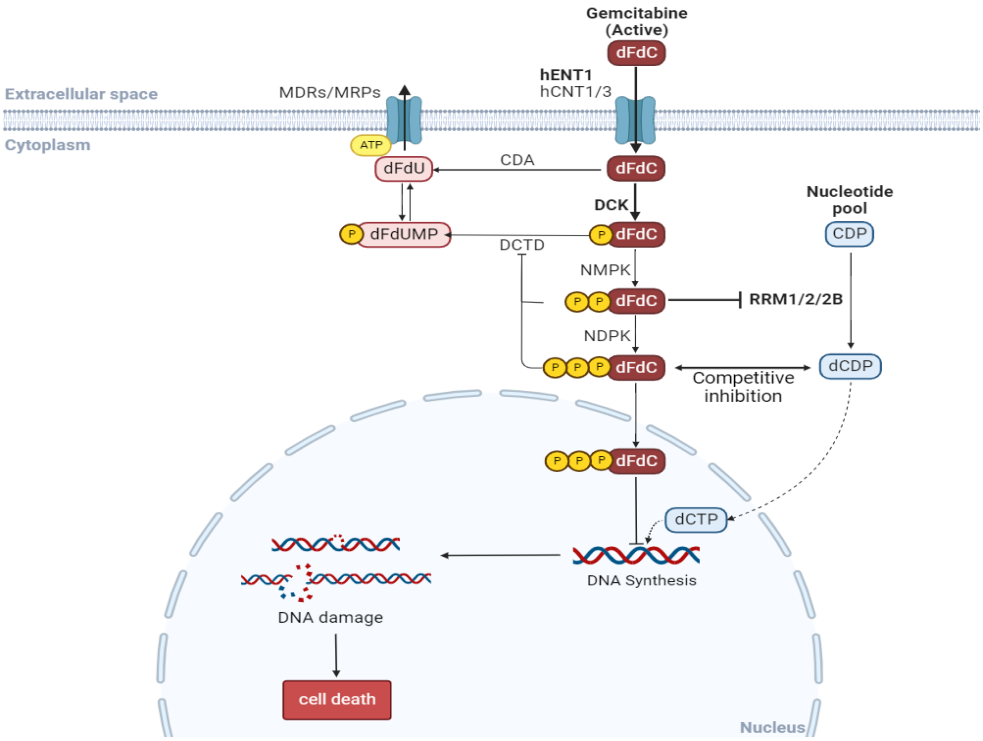


Figure 11. **Representation of the metabolism of gemcitabine, including the major steps for the success of its mechanism of action**. CDA: cytidine deaminase, dCK: deoxycytidine kinase, DCTD: deoxycytidylate deaminase, dFdC: 2′,2′-difluorodeoxycytidine, dFdU: 2′,2′-difluorodeoxyuridine, hENTs and hCNTs: human nucleoside transporters, NDPK: nucleoside diphosphate kinase, NMPK: nucleoside monophosphate kinase, RR(M1/M2), ribonucleotide reductase, 5′-NT: 5′-nucleotidase.

The high expression of members that belong to the family of ATP-binding cassette (ABC) transporters, especially multidrug resistance protein 1 (MDR1) (also known as P-glycoprotein), have also contributed to the resistance of PDAC tumors against current therapies, leading to treatment failure [145,146]. These molecules are responsible for the transport of metabolites according to a concentration gradient and are energy dependent, being also capable of inhibiting the activity of certain chemotherapies and targeted therapies

by conducting efficiently their efflux [145,146]. In addition to MDR1, more types of transporters were identified in PDAC, and this include BCRP, MRP1 and MRP4 [145].

Altered expression of microRNAs (miRNA) that modulate epigenetically different characteristics of tumor cells promoting certain pro-tumorigenic responses are also an area of interest when it comes to acquired resistance and clinical prognostics values [147,148]. This small non-coding RNAs can be subdivided into two groups, oncomiRs that usually are up-regulated in tumors exerting certain effects that support tumor progression and survival, or tumor suppressor miRNAs that have anti-tumor properties and are commonly suppressed in tumors [147].

The oncomiRs that are predominantly overexpressed in PDAC and can be responsible for acquired resistance in this type of tumors include the miR-21 and miR-155 [149,150]. Consequently, overexpression of miR-21 supports cancer stem cells survival against therapies, proliferation by up-regulating the signaling pathway Wnt and expression of transcription factors associated to EMT. The miR-155 intervenes in the metabolism of GEM, inhibiting the activity of the enzyme dCK, and also in pathways associated with apoptosis and inflammation [149,151]. Additionally, despite its lower presence in PC cases and pro-tumorigenic characteristics, the oncomiR miR-20a can be serving as a useful tool in the clinic, enabling to differentiate between healthy patients and PC cases, since in blood samples its expression is up-regulated, however more studies are needed [152]. Due to its oncogenic characteristics miR-20a could also potentially contribute for tumor proliferation and acquired resistance, activating cellular signal pathways, such as PTEN/PI3K/AKT even though its incidence in PDAC is low [152,153].

Regarding tumor suppressor miRNAs in PDAC, the family of the miR-200s, that usually have a low expression in this tumors, play a major role in controlling transcription factors associated to EMT and stemness, being excellent targets for the creation of therapies that could possibly increase their expression levels in order to mitigate metastases and resistance against therapies [154,155]. To better understand their intervention in EMT, this process is recognized for the repression of proteins responsible for cell adhesion like E-cadherin and increased expression of mesenchymal markers such as Vimentin, N-cadherin, Snail, and E-box-binding homeobox 1 and 2 (ZEB 1/2), therefore, inhibiting one of these markers with a member of the miR-200 family could potentially restore an epithelial phenotype [156]. The miR-200c is a great candidate due its multifaceted anti-tumor activities and more importantly by the capability of repressing EMT by directly targeting the transcription factors ZEB 1/2, leading to the restoration of the expression levels of E-cadherin and possible polarization of mesenchymal cells to epithelial [155]. Moreover, in previous studies, the miR-200c have demonstrated to interact with the transporter MDR1, contributing also for impeding the efflux of chemotherapies and targeted

therapies, showing even more its potential in retarding or decreasing levels of acquired resistance in PDAC [155]

PARPi have revolutionized the treatment of mutBRCA1/2 carriers in PDAC, having promising results at the start, however, as a long-term therapy they turned out to be obsolete, since numerous mechanisms of acquired resistance described in pre-clinical and clinical studies have appeared [104,109]. Furthermore, these resistance mechanisms can occur mainly by three distinct forms: drug or target related alterations, restoration of HR and restoration of replication fork stability (Figure 12) [157,158].

Within the first form of acquire resistance to PARPi, one of the methods is mainly defined by the disruption of intra-cellular availability of PARPi because of the overexpression of multi-drug resistance transporters like MDR1, triggering resistance since PARPi are not on their active site (Figure 12) [157,158]. Furthermore, given that PARPi target specifically the catalytic domain of PARP proteins by competing with the cofactor NAD+, several mutations at this site have arisen, resulting in an alteration that could possibly disrupt the interaction between PARPi and its target (Figure 12) [157,158]. In addition to this type of alterations, previous studies have also identified mutations at other sites, namely in the domains necessary for trapping PARP proteins onto DNA, counteracting other effect of PARPi and conferring resistance against them (Figure 12) [158]. Mutations in enzymes involved in the remotion of PAR chains of PARP proteins like poly(ADP-ribose) glycohydrolase (PARG) have also been associated to determine the levels of resistance to PARPi since their repression can potentially restore the capability of PARylation (Figure 12) [158].

Regarding mechanisms that can restore HR, carriers of epigenetic alterations in the promoters of BRCA1/2 genes can suffer demethylation that will lead to the re-expression of *BRCA1/2* genes leading to the resistance to PARPi (Figure 12) [157,158]. Due to the high genomic instability in mutBRCA1/2 carriers, which can be increased by PARPi and cisplatin, the chances of reversion mutations that conduct the exchange of mutBRCA1/2 to wild-type genes or hypomorphic variants can occur, having cancer cells higher chances of a new restored proficient HR mechanism (Figure 12) [157,158]. Inactivating mutations in the gene tumor suppressor p53-binding protein 1 (*TP53BP1*) suppresses the expression levels of the encoding protein p53-binding protein 1 (53BP1) that is important for the choice of DSB repair pathways, namely NHEJ or HR, and represents an antagonist of HR by blocking ATM-dependent resection [159,160]. Consequently, by repressing 53BP1 or its downstream factors REV7/MAD2L2 and RIF1, there is a restoration of ATM-dependent resection of DNA in mutBRCA1/2 carriers, promoting the production of recombinogenic ssDNA competent for HR, oppressing sensitivity to PARPi by restoring partial HR activity (Figure 12) [157,159,160].

Finally, restoration of fork replication stability can be attained by the loss Pax2 transactivation domain-interacting protein (PTIP) or nucleosome remodeling factor chromodomain-helicase-DNA-binding protein 4 (CHD4) (Figure 12) [157,158]. Briefly, in case of BRCA1/2 deficiency, the replications forks are commonly affected by nucleases like MRE11 leading to fork collapse, and chromosomal aberrations. However being both PTIP and CHD4 responsible for the recruitment of MRE11, their loss of expression results in fork protection and consequently PARPi resistance [157,158]. Other mechanism that ensures replication fork stability is the upregulation of RAD51 expression and consequent increased foci formation (Figure 12) [161,162].

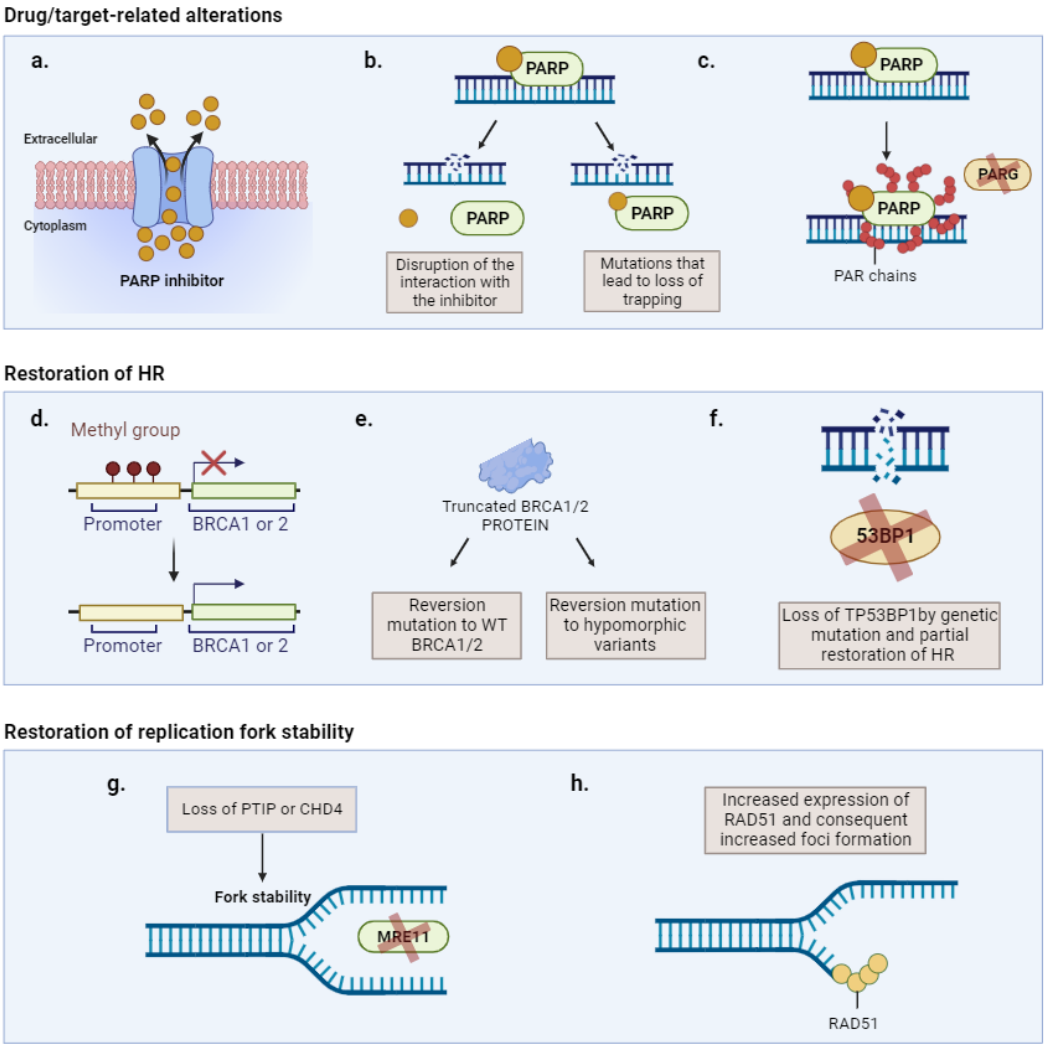


Figure 12. **Mechanisms of resistance to PARP inhibitors**. Alterations related to drug or target, such as overexpression of the efflux transporter MDR1 **(a)**, mutations in PARP that lead to disruption of the interaction with PARPi or DNA **(b)** and loss of poly(ADP-ribose) glycohydrolase (PARG) **(c)**. Restoration of homologous recombination (HR): re-expression of BRCA1/2 function due to epigenetic alterations or reversion mutations **(d,e)** and loss factor 53BP1 restoring partially HR **(f)**. Restoration of replication fork stability: loss of PTIP or CHD4 **(f)** **(g**) and increased RAD51 foci formation **(h)**.

1.3.4 Innovative approaches to overcome PDAC resistance

Since several acquired resistant mechanism exist in PDAC, new innovative approaches to counteract them have arisen. Since KRAS mutations are ubiquitous in PDAC, targeting it or its downstream proteins could be a promising option for targeted therapy [163]. Therapies that could reestablish the wt function of mutp53 protein could also be explored, namely with the compound MANIO, since PDAC is also highly affected by p53 mutations and this compound was never tested as PC therapy option [164].

For mediating PARPi restoration of sensitivity or even cause apoptosis by themselves, multiple small molecules that target HR key players, generating a HRD phenotype have been explored in both clinical and pre-clinical trials. Their targets have included ATM, ATR, CHK1, CHK2, RAD51, RAD52 and the BRCA2-RAD51 interaction, with only a minority of inhibitors reaching clinic trials to treat PDAC [164-167] . However, a new promising and innovative compound that is capable of inhibiting a protein-protein interaction and targets BRCA1 creating also a HRD phenotype has been discovered by our group [164,168]. The natural monoterpene indole alkaloid derivative dregamine 5-bromo-pyridin-2-ylhydrazone, BBIT20 (International Patent), which is the focus of this work is a compound capable of disrupting the interaction between BRCA1-BARD1 (Figure 13) and of inhibiting the activity of MDR1, which has been proven to have promising results in breast and ovarian cancers [164,168,169]. Additionally, in previous studies in breast and ovarian cancer cell lines, BBIT20 inhibited HR by interfering with key proteins that participate in this pathway, showing a potent anti-tumor activity by inducing cell death [168]. BBIT20 also exhibited synergistic effects with cisplatin and olaparib, not inducing cell resistance and showing high selectivity to cancer cells [168]. Altogether, these results showed that BBIT20 could be an excellent drug candidate for PC therapy.

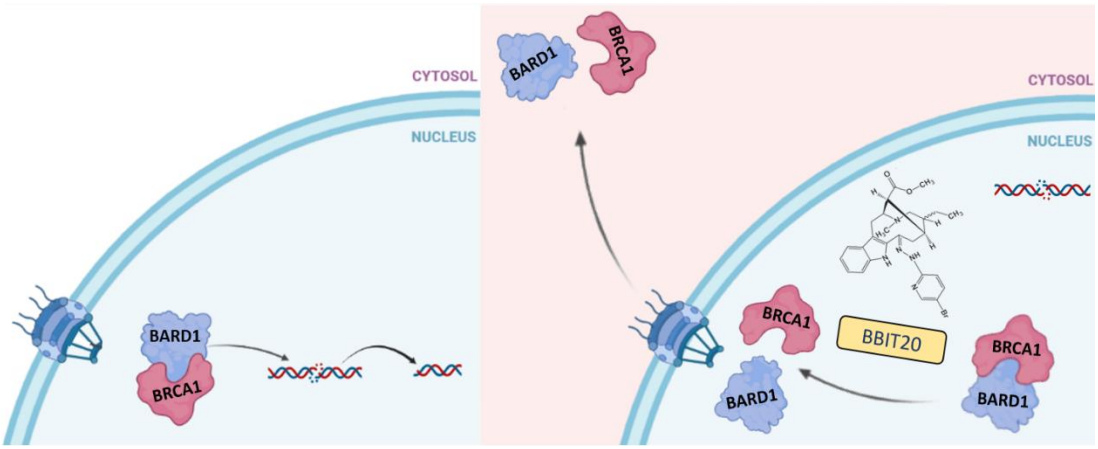


Figure 13. **Mechanism of action of BBIT20.** Presence of BBIT20 in cancer cells results in apoptosis due to the induction of homologous recombination deficiency and consequent genomic instability.

1.4 Aims of this work

As previously mentioned, BBIT20 showed to have promising *in vitro* and *in vivo* antitumor activity against breast and ovarian cancer, and its mechanism of action has been unveiled. In the present work it was aimed to:

-Evaluate the antitumor activity of BBIT20 as a single agent, and in combination therapy with conventional chemotherapeutic agents, in PDAC.

-Elucidate the mechanism of action of BBIT20 in PDAC.

-Study the ability of BBIT20 to counteract therapeutic resistance in PDAC.

2. Matherial & methods

2.1. Compounds

BBIT20 was synthesized by Prof Maria José Ferreira, from Faculty of Pharmacy of University of Porto and iMed.ULisboa, using the method previously described [169]. Gemcitabine was purchased from Sigma-aldrich and Olaparib from Santa Cruz Biotecnology.

All the compounds were dissolved in DMSO (Sigma-Aldrich) and in all the experiments, the solvent was included as a control in a concentration that did not affect cell proliferation (maximum concentration used 0.5%).

2.2 Cell Culture

A non-cancer cell line and several cancer cell lines from PDAC were used in this study (Table s1). Cells were maintained in T25 cm^2 or T75 cm^2 flasks at 37 °C, with an atmosphere of 5% of CO2 and with the respective culture mediums and supplements (Table s1).

Culture medium was replaced every week and high confluent cells were submitted to tryple express treatment to maintain cell culture. The culture mediums used were the Roswell Park Memorial Institute (RPMI), Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium F12 (DMEM-F12) and Iscove's Modified Dulbecco's Medium (IMDM); (VWR). Relevant characteristics of each cell line and respective culture medium are summarized in Table 1.

2.3 Sulfarhodamine B (SRB) assay

2.3.1 Cell proliferation assay

Cell lines were seeded in 96-well plates at a final density of 5.0×10^3 cells/well and allowed to adhere for 24h. Afterwards cells were treated with serial dilutions of BBIT20 for an additional 48h. Cells passed then through a series of essential steps for determination of the half-maximal inhibitory concentration (IC_{50}) through a SRB assay described in [170]. IC_{50} values were determined using the GraphPad Prism software, Version 7.0 (La Jolla, CA, USA).

2.3.2 Acquired resistance studies

Cells were plated in T25 cm^2 flasks with a concentration of $5.0x10^5$ and were allowed to adhere. Next, cells were treated twice a week, for five rounds, with increasing

concentrations of BBIT20 (6, 9, 13, 21 and 30 µM) each round, which were added to the culture medium for 24 h, followed by a recovery time of 2 to 3 days. Then cells were collected, seeded in 96-well plates, and treated twice for each concentration. IC_{50} were determined with an SRB assay as described in [171].

2.3.3 Combined therapy assays

Cells were cultured in in 96-well plates at a final density of 5.0×10^3 cells/well and allowed to adhere for 24h. Then cells were treated for 48h with a fixed concentration of BBIT20 and/or increasing concentrations of therapeutic agent of interest, in this case gemcitabine. The effect of combined treatments was analysed by SRB assay with the Dose reduction index and combination index calculated and analyzed as described in [171].

2.4 Flow cytometry

Cells were seeded in 6-well plates at a final density of 1.5×10^5 cells/well and were allowed to adhere for 24h. Afterwards, cells were exposed to a 48h treatment with BBIT20 or a combination with gemcitabine. To assess apoptosis the Annexin-V-Fluos staining kit (Sigma-Aldrich) was used according to the manufacturer's instructions. The AccuriTM C6 flow cytometer and the BD Accuri C6 software (BD Biosciences) were used.

2.5 Western Blot

Cells were seeded in 6-well plates overnight allowing them to adhere, followed by a treatment with BBIT20 for 48H. For protein extraction, cells were incubated with RIPA buffer (Sigma-Aldrich) to obtain total protein lysates. Afterwards, protein concentration was determined using a bicinchoninic acid protein assay (BCA) (Thermo scientific).

Protein samples were combined with sample buffer and denatured at 95ºc for 5 min. Denatured protein samples were then loaded into sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) to be resolved and afterwards transferred to a nitrocellulose membrane.

Ponceau solution was used to confirm protein transfer, and membranes were blocked with 2% bovine serum albumin (BSA) or 5% milk in PBS containing 0.05% tween (PBST). Incubation with the primary antibody was done at 4°C overnight proceeded by incubation with a horseradish peroxidase (HRP) conjugated secondary antibody for one hour at room temperature (RT). Between incubations, membranes were washed using PBST. GAPDH was used as a loading control. The analysis was obtained using enhanced chemiluminescence (ECL) Amersham kit from GE Healthcare (VWR), and detection of bands with ChemiDoc™ XRS Imaging System (Bio-Rad). Band intensity quantification was

achieved by the mage Lab software (version 5.2.1; Bio-Rad). Primary and secondary antibodies information's are summarized in Table s2.

2.6 Reverse transcription and real-time polymerase chain reaction (RT-qPCR)

Cells were seeded in 6-well plates and were allowed to adhere overnight, being posteriorly treated for 24h and 48h with BBIT20. The RNA was extracted using TRIzol reagent (Merck) protocol according to the manufacturer and as it is described in [172]. RNA concentration and purity were determined by using a NanoDrop Spectrophotometer ND1000 (Thermo Fisher Scientific), measuring the absorbance at 260nm.

For analysis of miRNA expression levels, TaqMan MicroRNA Reverse Transcription Kit (Alfagene) and gene specific stem-loop reverse transcription primers (has-miR-200c-3p and has-miR-20a-5p (Invitrogen)) were used according to manufacturer instructions and the reverse transcription for the obtention of the cDNA was performed as described in [172]. The qPCR reaction was performed in a CFX Real-Time PCR Detection System with the conditions as described in [172]. Small nuclear RNA U6 was used as a reference gene.

Relative expression levels were calculated using the quantification cycle (Cq) method, according to MIQE guidelines. Data was analyzed using Bio-Rad CFX Manager software and all the reactions were performed in duplicate.

2.7 Wound healing assay

The artificial wound was created with a 2 well silicone insert (IBIDI) in 6-well plates. Cells were seeded and cultured inside the 2 wells compartments, and when they reached high confluence, the insert was removed, creating an artificial wound to assess the migratory capacity of cells. The migratory capacity was evaluated by measuring at regular intervals until the wound closuring. Images were acquired using an inverted Nikon TE 2000-U microscope from Nikon Instruments Inc. (Izasa) at 100× magnification with a DXM1200F digital camera (Nikon Instruments Inc.) and NIS-Elements microscope imaging software (version 4; Nikon Instruments Inc.) and "wound healing" measured using ImageJ.

2.8 MDR1 activity screening assay

Cells were cultured overnight in a clear-bottom 96-well plate, and analysis of the activity of MDR1 was performed using the Multidrug Efflux Transporter P Glycoprotein (MDR1/P-gp) Ligand Screening Kit (abcam) following the instructions of the manufacturer.

3. Results

3.1 Anti-tumor properties of BBIT20 and the effects of its mechanism of action in homologous recombination DNA repair

3.1.1 BBIT20 has potent growth inhibitory effect on PDAC cell lines and high selectivity to cancer cells

In the present work, we tested the anti-tumor properties of BBIT20 in several PDAC cell lines and its selectivity to cancer cells. According to the IC_{50} values obtained for BBIT20 through the SRB assay (Table 1), it was possible to verify that BBIT20 had a potent anti-tumor effect, particularly when compared to Olaparib that is the therapeutic agent approved for advanced mutBRCA1-related PDAC patients (figure 14A and B). Additionally, a much higher IC_{50} value was obtained for the non-malignant, proving its high selectivity to cancer cells (figure 14B).

Table 1. Antiproliferative effect of BBIT20 on pancreatic ductal adenocarcinoma cells and non-malignant cells.

Cell Lines		**IC_{50} (μM)**	
		BBIT20	**Olaparib**
wtBRCA1/2 expressing cells	Panc-1	5.63±0.26	29.88±2.30
	Miapaca-2	6.18±0.22	52.40±3.20
	AsPC1	2.47±0.80	52.67±8.17
	BxPC3	3.93±0.43	45.80±4.12
	Hs766T	5.98±0.81	48.50±0.50
	HPAF-II	6.22±0.49	62.67±4.54
MutBRCA2 expressing cells	Capan-1	10.05±2.07	13.40±7.96
Non-malignant cells	HFF-1	33.6±3.20	36.0±1.10

Growth obtained with control (DMSO) was set as 100%; data are mean ± SEM (n=6). IC_{50} values of BBIT20 and olaparib in PDAC and non-malignant cells was determined by sulforhodamine B assay after 48h treatment. Data are mean ± SEM (n=6).

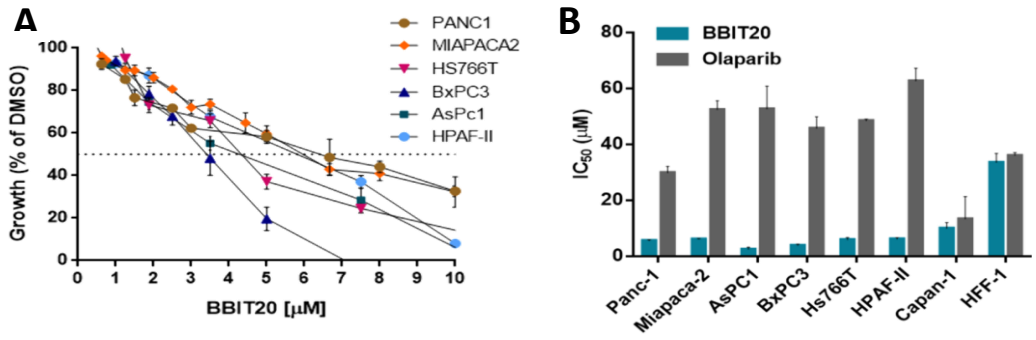
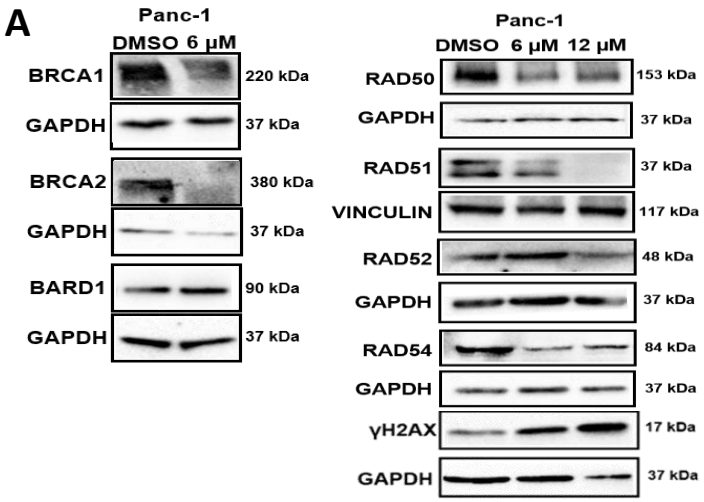


Figure 14. **Growth inhibitory effect of BBIT20 on PDAC cell lines and a non-malignant cell line.** In **A** Dose-response curves for BBIT20 in PDAC cells, determined by sulforhodamine B assay, after 48h treatment. Growth obtained with control (DMSO) was set as 100%; data are mean ± SEM (n=6); **B** IC_{50} values of BBIT20 and olaparib in PDAC and non-malignant cells, determined by sulforhodamine B assay after 48h treatment. Data are mean ± SEM (n=6).

3.1.2 BBIT20 affects the expression of key players in Homologous recombination

Preliminary results of BBIT20 in MiaPaca-2 and Panc-1 cell lines have proven it´s mechanism of action by disrupting the interaction between BRCA1-BARD1 trough co-immunoprecipitation (data not shown). Additionally, they showed that the compound induces cell cycle arrest and apoptosis, and it has genotoxic properties (Data not shown). Together these results indicated that, like in breast and ovarian cancers [168], BBIT20 may possibly abolish HR, in PDAC cell lines. So, to further asses the effect of the compound on key players of HR, we verified their expression in PANC-1 and MIA-PaCa-2 cell lines by western blotting. The results showed that, in both cell lines, the proteins BRCA1, BRCA2, RAD50, Rad51, Rad52 and Rad54 are downregulated (figure 14A and B). Interestingly, BARD1 that is responsible for ensuring the active site of BRCA1 maintained its expression levels (figure 15 and B). Additionally, the phosphorylated form of H2AX (γH2AX) besides facilitating the access to the DNA in HR, it also is considered a marker for DNA damage, which is consistent with its upregulation in both cell lines (figure 15A and B).



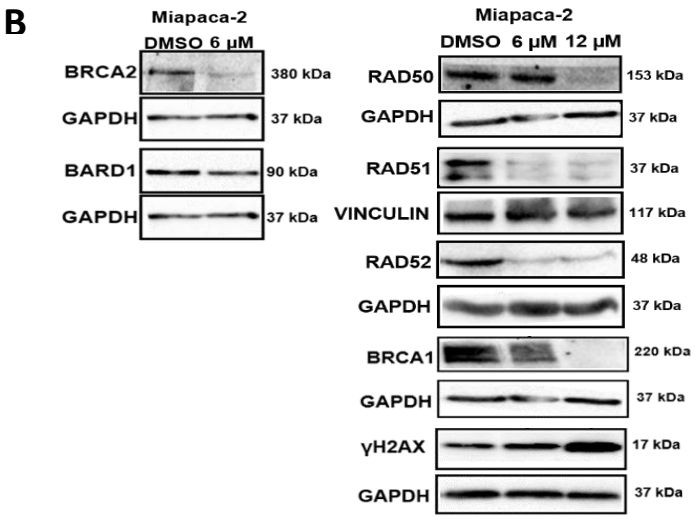


Figure 15 BBIT20 regulates the expression levels of proteins involved in HR DNA repair in PANC-1 and MiaPaCa-2 cell lines. **A** PANC-1 and **B** MIA-PaCa-2 **cancer cells** were exposed to 6 and 12 µM BBIT20 and the effect on protein expression levels were evaluated a after 48h treatment. Representative immunoblots are shown; GAPDH served as a loading control.

3.1.3 BBIT20 does not induce acquired-resistance

A major concern related to the treatment of PDAC is its capacity to tackle therapeutic approaches due to several mechanisms of acquired resistance that reduce its susceptibility to either conventional or targeted therapies. Based on this premise, we evaluated if BBIT20 induces resistance in Panc-1 and MiaPaCa-2 cell lines. Based on the IC_{50} values obtained with the SRB assay, it was possible to verify that consistent IC_{50} values (with no statistic difference between each round) were maintained in both cell lines, after the treatments with increased concentrations of BBIT20 for each round (figure 15A and B). Even in comparison to the not treated parental cells, no statistic variations were observed (figure 16A and B).

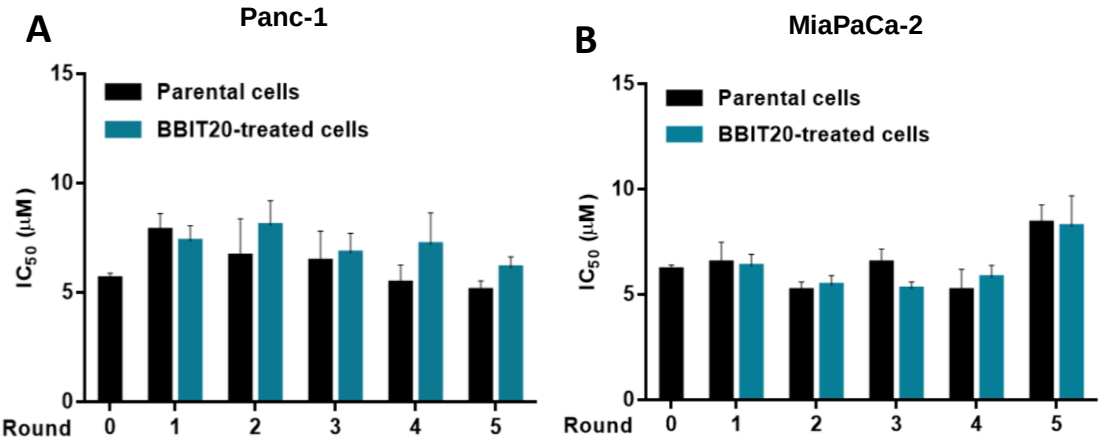


Figure 16. PANC-1 and MIA-PaCa-2 cancer cells did not develop resistance to BBIT20. A PANC-1 and **B** MIA-PaCa-2 **cancer cells** were exposed to five rounds of treatment with increasing concentrations of BBIT20 (6, 9, 13, 21, and 30 µM, twice-a-week). IC_{50} values were determined at the end of each round by SRB assay, after 48h of treatment. Data were normalized to DMSO and correspond to mean ± SEM, n=3; values not significantly different from parental cells: p>0.05, two-way ANOVA followed by Sidak's test.

3.1.4 BBIT20 can affect the expression of miRNA´s that are associated with therapeutic resistance

Several acquired resistance mechanisms could be attributable to the altered expression of miRNA´s, leading to the dysregulation of cellular pathways that may contribute to tumor progression. To better understand the influence of BBIT20 on the expression pattern of miRNA´s, cells were treated for 24h and 48h with the compound and then analyzed by a RT-qPCR. The results showed that the oncomir miR-20 was significantly downregulated after treatment with BBIT20 at 12 µM in Panc-1 (for 24h) and MiaPaca-2 (for 48h) cell lines (figure 17A and B). Additionally, the tumor suppressor miR-200c was only significantly upregulated in Panc-1 cells after 24h of treatment with BBIT20 at 12 µM (figure 17A).

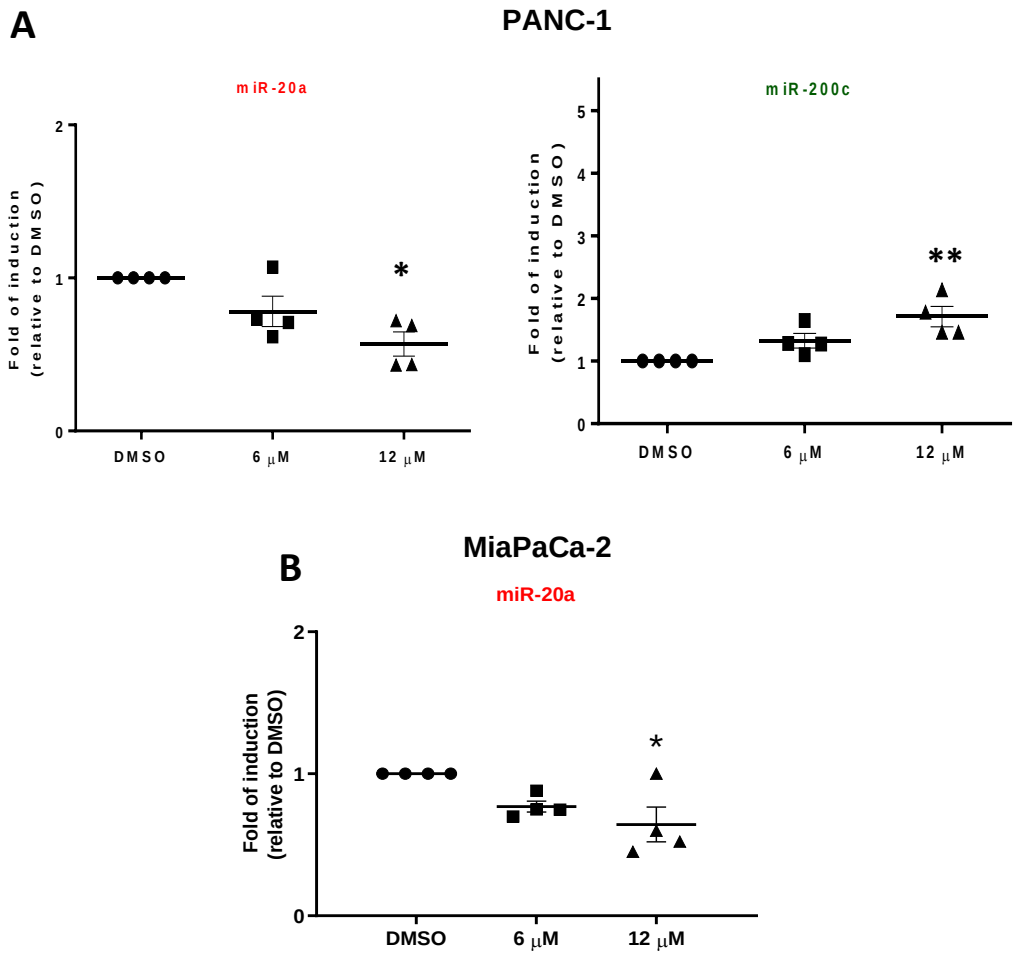


Figure 17. **BBIT20 can interfere with the expression levels of miRNA´s, which are associated with therapeutic resistance.** In **A,** a RT-qPCR was performed to assess the expression levels of miR-200c and miR-20a in Panc-1 cancer cells, after 24h of treatment with 6 and 12 µM BBIT20. Fold change is relative to DMSO; data are mean ± SEM (*n*=4); values significantly different from DMSO: * $p < 0.05$, one-way ANOVA followed by Dunett´s test; **B** a RT-qPCR was performed to assess the expression levels of miR-20a in MiaPaCa-2 cancer cells, after 48h of treatment with 6 and 12 µM BBIT20. Fold change is relative to DMSO; data are mean ± SEM (*n*=4); values significantly different from DMSO: * $p < 0.05$, one-way ANOVA followed by Dunett´s test.

Since the tumor suppressor Mir-200c directly targets the transcription factor ZEB1 by controlling it´s expression and consequently avoiding EMT, we evaluated its expression by western blot to verify if it is in agreement with the results of the RT-qPCR [155]. Analyzing the blots, it´s possible to observe that in both timepoints, 24h and 48h, at 12 µM, there is a substantial decreased expression of the transcription factor ZEB1 (figure 18).

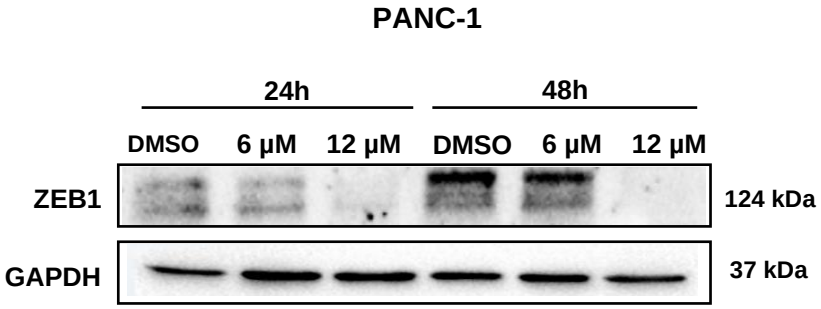


Figure 18**. BBIT20 decreases the expression of ZEB1.** Immunoblotting to verify the expression leves of the transcription factor ZEB1 in PANC-1 cell line treated with BBIT20 for 24h and 48h; GAPDH served as a loading control.

3.2 Evaluation of the ability of BBIT20 to counteract therapeutic resistance

3.2.1 Characterization of gemcitabine resistant MIA-Paca-2 cell line

Gemcitabine (GEM) is a therapeutic agent frequently used in the treatment of PDAC as a single agent or in combination depending on the genetic landscape and the fitness of the patient [10]. However, gemcitabine usually is very susceptible to acquired resistance mechanisms [10]. With this in mind, we wanted to assess if BBIT20 retained its antitumor effect in a MiaPaca-2 cell line that is resistant to GEM and whether it had the capacity to reestablish the sensibility of this cell line to gemcitabine. The first step of this study was to characterize the resistant cell line in order to proceed future experiments. Starting with the morphology it was possible to observe that the resistant cells, in comparison to the correspondent parental cells (not exposed to GEM) had a more rounded morphology (figure 19A). Regarding the growth inhibitory effect, the IC_{50} value obtained for GEM in the resistant cell line was 9.82 µM, while the IC_{50} value in the correspondent parental cell line was 0.73 µM. The increase of over 14 times in the IC_{50} value confirmed the resistance profile of this cell line to GEM. Conversely, BBIT20 showed a potent growth inhibitory effect in the resistant and parental cell lines with IC_{50} values of 4.67 µM and 4.17 µM, respectively, which indicated the inexistence of cross-resistance to BBIT20 (figure 19B). The expression of key

proteins that participate in the metabolism of GEM that are responsible to ensure its anti-tumor effect were also evaluated by western blot. A decreased expression of the enzyme dCk (involved in the phosphorylation of GEM) and the transporter hENT1 (involved in the intracellular uptake of GEM) in the resistant cell line, were in agreement with the obtained IC_{50} values and further corroborated the resistance properties of the MiaPaca-2 cell line to GEM (figure 19C).

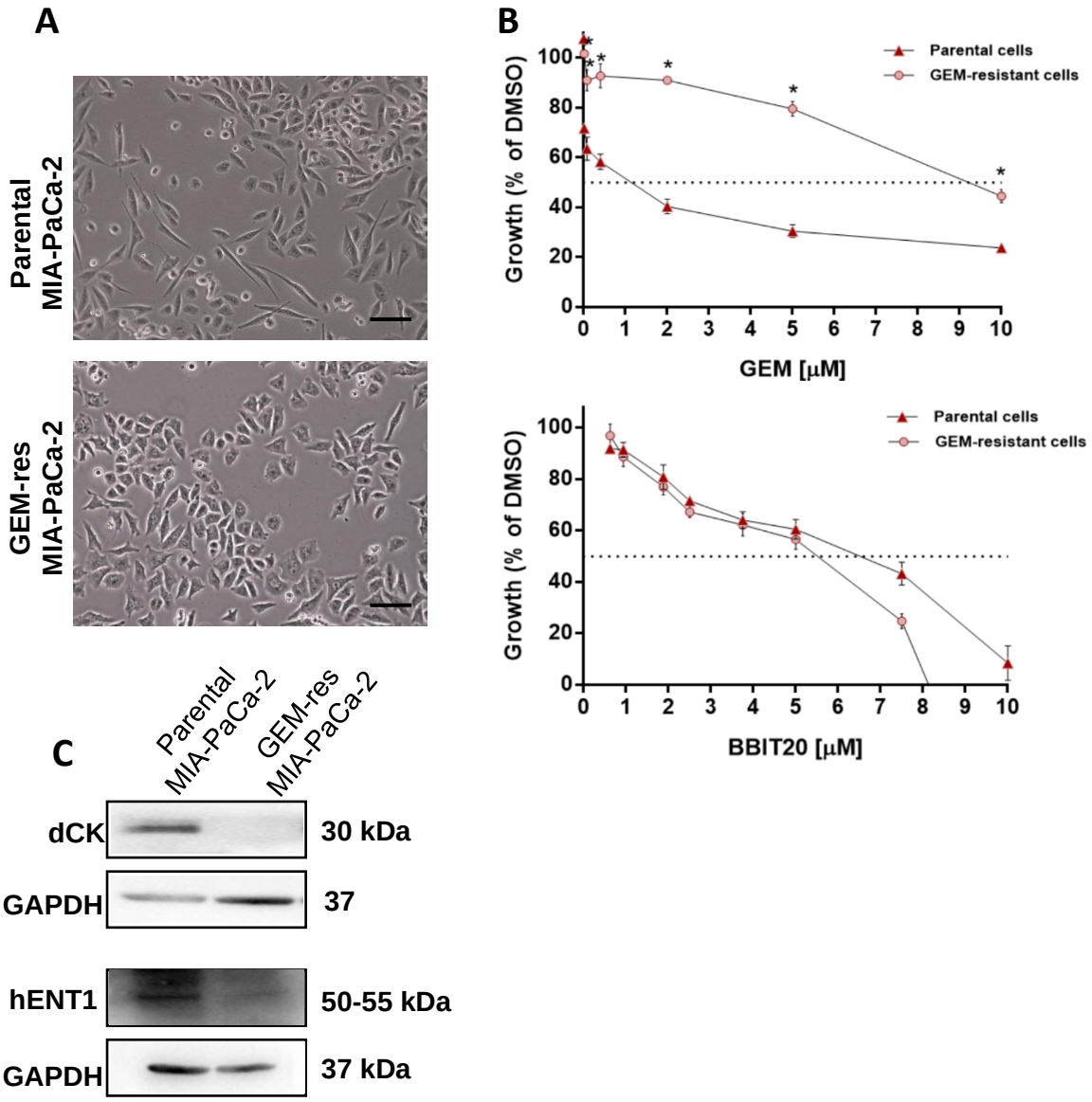


Figure 19. **Characterization of Gem resistant MIA-Paca-2 cell line**. In **A** morphologic changes that resulted from the prolonged treatment of MIA-PaCa-2 cells with GEM with more plump, rounded cell morphology in MIA-PaCa-2 GEM-res cell clones compared with parental cells that exhibit spindle-shaped character and enhanced formation of pseudopodia. Representative images are shown; scale bar = 100 μm; magnification 100x; **B** Concentration–response curves for gemcitabine (GEM; A) and BBIT20 (B) in control (non-resistant, parental) and gemcitabine-resistant (GEM-res) MIA-PaCa-2 cells, after 48 h treatment by SRB assay. Growth obtained with control (DMSO) was set as 100%. Data shown are mean ± SEM, n = 4 (two replicates each); values of GEM-res cell growth significantly different from parental cells: * $p < 0.05$; two-way ANOVA followed by Sidak's test; **C** immunoblotting representing decreased expression in gemcitabine metabolism-associated entities, such as deoxycytidine kinase (dCK) and nucleoside transportes as ENT1, in GEM-res MIA-PaCa-2 cells. GAPDH served as a loading control.

3.2.2 BBIT20 induces an antiproliferative effect in MIA PaCa-2 GEM-res associated with apoptosis

After the characterization of the resistant cell line, the induction of apoptosis by BBIT20 was assessed. After a 48h treatment with BBIT20, the percentage of Annexin-V-positive cells at 5 µM and 10 µM is significantly increased, further demonstrating that the cells did not show cross-resistance to BBIT20 and still retains its anti-tumor properties (figure 20B). Additionally, the morphology exchanged to similar of the correspondent parental cell line with spindle-shaped character and enhanced formation of pseudopodia (figure 20A).

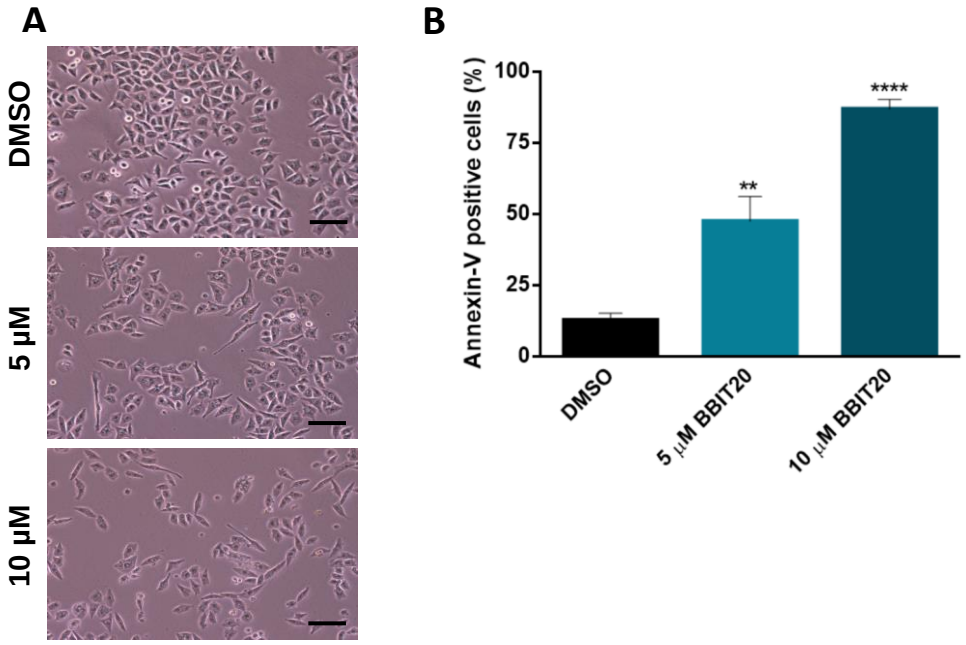


Figure 20. **BBIT20 induces apoptosis in MIA-PaCa-2 GEM-res cell line.** In **A** morphological changes (spindle-shaped character and enhanced formation of pseudopodia) in MIA-PaCa-2 GEM-res cells after 48h treatment with BBIT20. Representative images are shown; scale bar = 100 µm; magnification 100x; B Annexin V-FITC apoptosis: 5 and 10 µM BBIT20 effect, after 48h treatment in MIA-PaCa-2 GEM-res cells. Mean±SEM (n=4), values significantly different from DMSO: * p<0.05 (one-way ANOVA, Dunnett's test)

3.2.3 BBIT20 inhibits MDR1 activity in MIA PaCa-2 GEM-resistant cells

The increased expression of ABC transporters, specially MDR1 (also known as P-glycoprotein) is considered a mechanism of acquired resistance that is well described in PDAC [145]. In a previous work, the ability of BBIT20 to inhibit the activity of MDR1 was reported [169]. In the present work, we evaluated if this feature of the compound was also maintained in PDAC cells.

For testing the activity of the MDR1 transporter, a lipophilic non-fluorescent P-glycoprotein substrate that readily diffuses through the plasma membrane, where it is hydrolyzed to an active fluorophore by cytosolic esterases, was used. If there is an increased activity of MDR1, the esterase-cleaved dye is pumped out of the cell reducing the fluorescence observed; however, if the activity of MDR1 is reduced the dye is not expelled and the fluorescence increases. The results showed that 5 µM of BBIT20 significantly increased the fluorescence in comparison to the control, which indicated a reduction of the MDR1 activity (figure 21A and B).

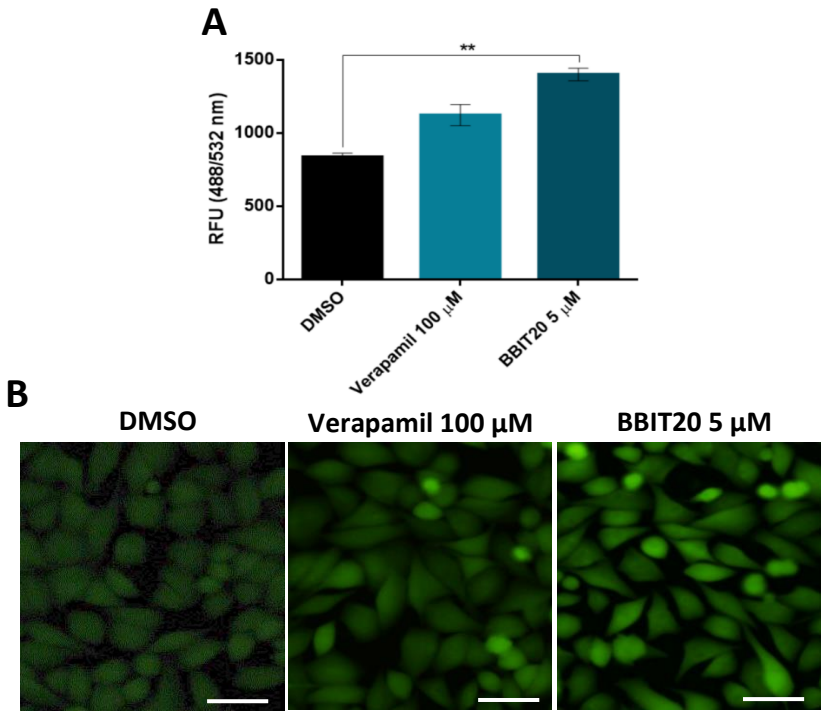


Figure 21. **BBIT20 inhibits the activity of MDR1.** In **A,** 5 µM BBIT20 significantly inhibits p-gp activity in MIA-PaCa-2 GEM-res cells using the ligand screening kit. Cells were cultured overnight in 96-well plate, preincubated with either vehicle (1% DMSO, negative control), 100 µM verapamil (positive control) or 5 µM BBIT20 and exposed to p-gp substrate for 30 minutes to measure the fluorescence intensity (Ex/Em = 488/532 nm). Data are mean of fluorescence intensity ± SEM (n=3), values significantly different from DMSO: * $p<0.05$ (one-way ANOVA followed by Tukey's test); **B**, representative images of intracellular accumulation are shown. scale bar = 50 µm; magnification 400x.

3.2.4 BBIT20 impacts the motility of GEM-resistant MIA PaCa-2 cells

To evaluate the anti-migratory capacity of BBIT20 in the resistant cell line a wound-healing assay was performed. For that, a concentration of compound with no significant effect on cancer cell growth of 2.5 µM of BBIT20 was used. Along the different timepoints, we could infer that BBIT20 significantly reduced wound closer in comparison to the untreated cells (figure 22A and B).

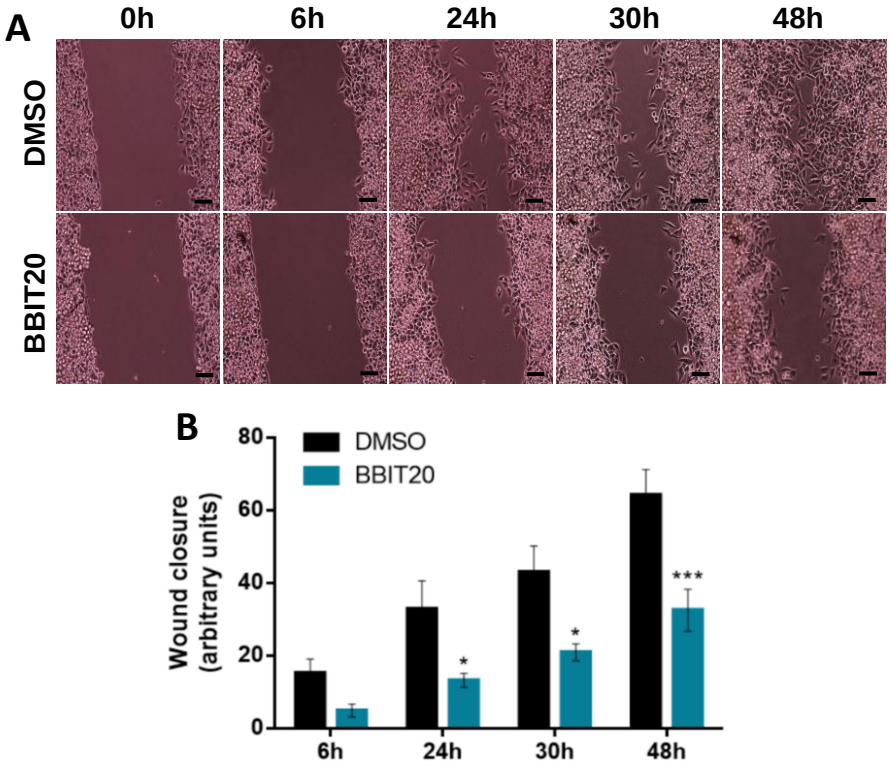


Figure 22. **BBIT20 inhibits GEM-resistant MIA PaCa-2 migration. Representative figures** are shown; scale bar = 100 µm; magnification = 100x. **B.** Effect of 2.5 µM BBIT20 (or DMSO only) on confluent MIA-PaCa-2 GEM-res cells migration after 6h, 24h, 30h and 48h treatment. Quantification of wound closure using randomly selected microscopic fields (six fields per sample). Data are mean ± SEM (n=4), values significantly different from DMSO: * $p < 0.05$ (two-way ANOVA followed by Sidak's test)

3.2.5 BBIT20 can affect the expression of miRNAs associated with resistance in GEM-resistant MIA PaCa-2 cells

Likewise in parental PDAC cells, we assessed if BBIT20 could interfere with the expression of miRNA´s in this cell line resistant to GEM. The results showed that the oncomir miR-20 was significantly downregulated after 24h of treatment at 10 µM (figure 23).

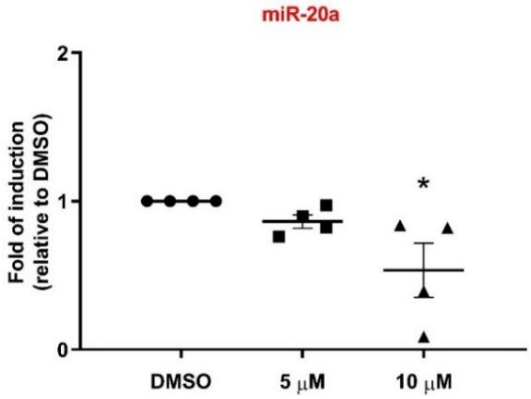


Figure 23. **BBIT20 can interfere in the expression levels of miRNA´s that are associated with resistance against therapies.** a RT-qPCR was performed to assess the expression levels of miR-20a in MIA-PaCa-2 GEM-res cancer cells, after 24h of treatment with 6 and 12 µM BBIT20. Fold change is relative to DMSO; data are mean ± SEM (n=4); values significantly different from DMSO: * $p < 0.05$, one-way ANOVA followed by Dunett´s test.

3.2.6 BBIT20 synergizes with gemcitabine and re-sensitizes GEM-resistant MIA PaCa-2 cells to its effect

To further investigate the anti-tumor properties of BBIT20 in the GEM resistant cell line, markers associated with cell proliferation, survival and division were assessed by western blot. BBIT20 reduced the expression of β-catenin that is associated with cell proliferation, survivin that is involved in cell survival, and CDC20 that is associated with cell division. We next evaluated if it could re-sensitize the resistant cell line to GEM, by assessing the expression of key players that participate in the metabolism of GEM by western blot. The results evidenced an up-regulation of the expression levels of the transporter hENT1, which potentially allow a higher intracellular uptake of GEM, and a decreased expression of the RRM2 enzyme, decreasing the competitive pool of dCTP available for DNA synthesis thus facilitating the incorporation of the active metabolite of GEM into the DNA sequence (figure 24B).

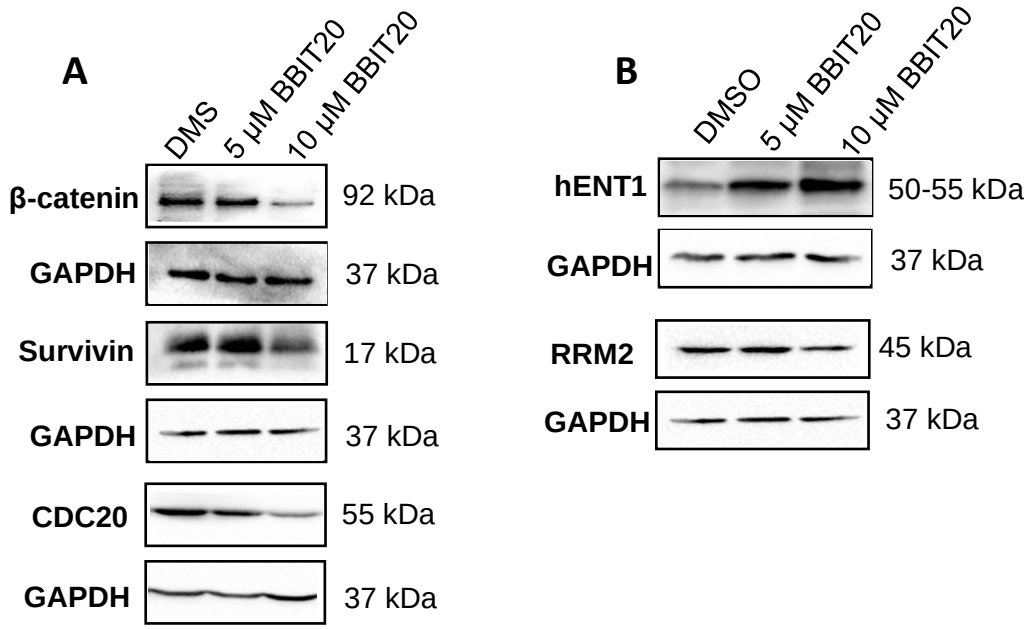


Figure 24. **BBIT20 downregulates proteins associated to cell proliferation, survival and division and can re-sensitize GEM-resistant MIA PaCa-2 cells to GEM again.** Immunoblotting with protein expression levels in **A of** ENT1, R2 and in **B of** β-catenin, survivin and CDC20 in GEM-res MIA-PaCa-2 cells, after 48h treatment with BBIT20. GAPDH served as a loading control.

Thereafter, since the standard therapeutic options for PDAC commonly includes the combination of therapeutic agents, we assessed if BBIT20 in combination with GEM could enhance the sensitivity of the resistant cell line to GEM [10]. Therefore, cells were treated with a constant concentration of BBIT20 at 2.5 µM and increasing concentrations of GEM. Afterwards, the combination index (C.I), which corresponds to the

value that determines the type of interaction between two or more drugs when used in combination, and the dose reduction index (D.R.I), which corresponds to the value that quantifies the degree to which the dose of a drug can be reduced when it is used in combination with another drug to attain a particular level of therapeutic efficacy. The concentrations of 1.25 µM and 2 µM of GEM demonstrated synergistic effects with BBIT (C.I. <1) (figure 25A). However, the most promising concentration of GEM was 1.25 µM for which a lower C.I was obtained and a 10-fold reduction of the effective dose of GEM was achieved (figure 25A). Moreover, BBIT20 significantly enhanced the growth inhibitory effect of GEM (figure 25A). After determining the most efficient concentration, the induction of apoptosis was assessed. After 48h of treatment, the percentage of Annexin-V-positive cells in the combination of BBIT20 and GEM was significantly higher compared to the GEM alone , which corroborated the synergistic effects of BBIT20 with GEM (figure 25B).

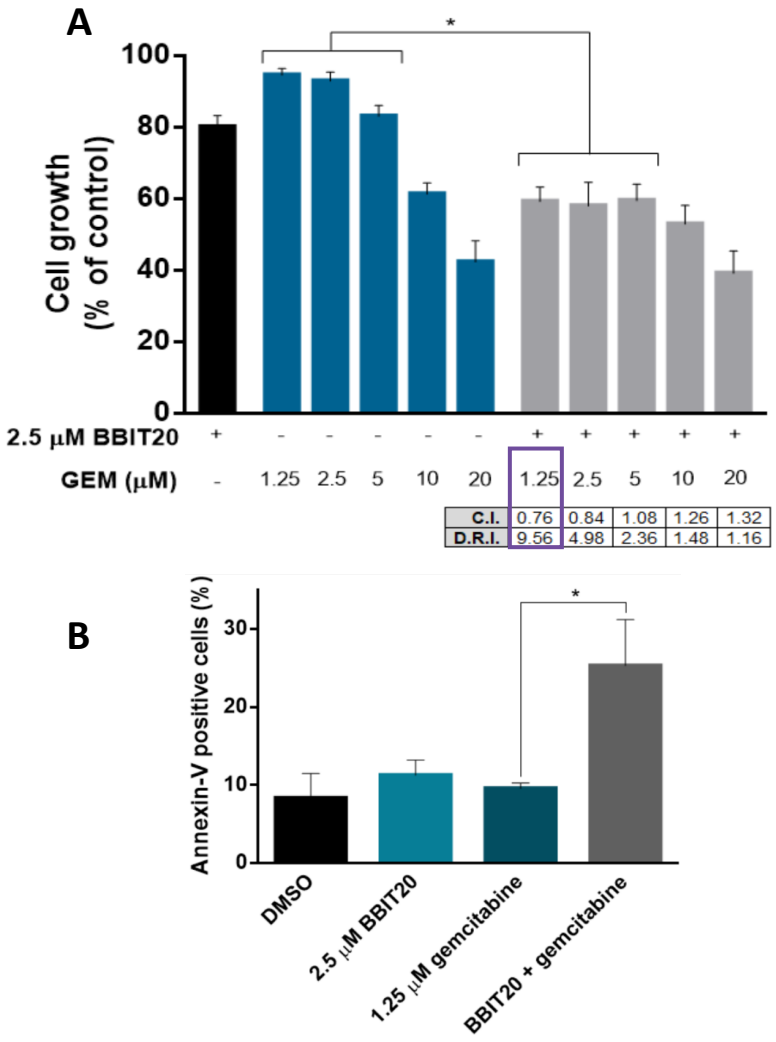


Figure 25. **BBIT20 has a synergistic effect with GEM and can enhance its effects in GEM-resistant MIA PaCa-2 cells.** In **A** Drug combination using Sulforhodamine B assay. Increasing doses of GEM with 2.5 µM BBIT20 in MIA-PaCa-2 GEM-res cells, after 48h treatment. Mean ± SEM (n=6), Cell growth significantly different than GEM alone: * $p < 0.05$ (Two-way ANOVA, Sidak's test). CI, combination index; D.R.I. dose reduction index. CI<1, synergy; 1<CI<1.1 additive effect; CI>1.1, antagonism (calculated in Compusyn); **B** Annexin V-FITC apoptosis: 2.5 µM BBIT20 and 1.25 µM gemcitabine effect, after 48h treatment. Mean±SEM (n=3), values significantly different from gemcitabine: * $p<0.05$ (one-way ANOVA, Dunnett's test)

4. Discussion

Currently the treatment of PDAC still remains a challenge in the field of both conventional and targeted therapies, being tumor resection complemented with adjuvant chemotherapy considered the best potentially therapeutic option [10]. Nonetheless, owing to the aggressive nature of this type of tumor most of the patients reach the clinic with a locally advanced or metastatic disease hampering tumor resection [10,11,24]. Based on this, these patients highly rely on conventional chemotherapy or targeted therapies to reduce the size of the tumor to possibly make it resectable or maintain the disease controlled [10,24]. However, the aggressive secondary effects of conventional chemotherapy and the debilities that PDAC imposes on the patients results in low tolerance to this type of therapy, with less than 50% of the planned dose being received in most of the cases [10]. So, targeted therapies that are more selective and have lower chances of adverse secondary effects are a more promising option, assuring a potent anti-tumor activity, while maintaining the quality of life of the patients [10,98].

Regarding targeted therapies, the PARP inhibitor olaparib has demonstrated promising results and currently is on the frontline for the treatment of PDAC, having received an approval by the FDA as a first-line treatment option for metastatic PC patients with mutBRCA1/2 [104]. Nevertheless, only a particular niche of patients beneficiates from it and is highly susceptible to several mechanism of acquired resistance [105].

This plasticity that PDAC has to acquire resistance to therapies, affects both targeted therapies and conventional chemotherapy, and it is also considered a major recurrent problem in PDAC, hence the importance to discover innovative therapeutic options [164]. Based on this premise, new treatment approaches have emerged like the inhibitors of HR that can possibly re-sensitize cancer cells to DNA-damaging agents like GEM that is frequently used in treatment regimens, and extend the pool of patients eligible to a treatment already established in the clinic like olaparib [164].

In this work we explored the anti-tumor properties of the indole alkaloid derivative BBIT20 as a possible future HR inhibitor used in the clinic and its capability to counteract therapeutic resistance, since it demonstrated promising results in breast and ovarian cancers by disrupting the BRCA1-BARD1 interaction [168].

Firstly, BBIT20 demonstrated an efficient growth inhibitory effect when compared to the targeted therapy olaparib in a panel of several PDAC cell lines with different BRCA1/2 status. Moreover, this result supported that BBIT20 can be used in an extended pool of patients, beneficiating even those who don't have mutBRCA1/2. Additionally, this analysis also allowed us to infer about the high selectivity of BBIT20 to tumor cells, which is a feature

highly pursued to reduced undesirable secondary effects. A possible explanation for this could be related to the nature of PDAC cells, that in contrast to the non-malignant cells, are frequently characterized to have complex rearrangement patterns and mitotic errors being highly dependent on functional DNA repair mechanisms [29]. Therefore, by inhibiting HR, cells opt for the compensatory pathway that is NHEJ, however this DDR mechanism doesn´t repair with the same accuracy, introducing more genetic errors consequently generating genomic instability that promotes apoptosis [29]. Since non-malignant cells rely on intact DNA repair mechanisms and don't have the same amount of genetic errors they can escape the cytotoxic effects of BBIT20 and other HR inhibitors [29,173]. So, testing the growth inhibitory effect of other HR inhibitors that disrupt protein-protein interactions or inhibit specific proteins of HR, at the same conditions and comparing it to the values obtained with BBIT20 would be interesting to verify if it has a higher efficacy.

Afterwards, since our group already had preliminary results on the PANC-1 and MiaPaCa-2 cell lines, by proving the mechanism of action of BBIT20, the induction of cell cycle arrest and apoptosis, and its genotoxic properties by abolishing HR, all the following experiments related to this first part of the work were performed in these two cell lines.

The assessment of the effect of BBIT20 in key proteins that participate in the HR repair mechanism were performed by western blot. As expected, in both cell lines, BBIT20 induced down-regulation of the proteins BRCA1, BRCA2, RAD50, Rad51, Rad52 and Rad54, which all play major roles throughout the HR mechanism, proving that BBIT20 affects the HR process in all stages. Furthermore, interestingly it was observed that BARD1 maintains its expression levels in both cell lines, potentially due to its apoptotic activity stimulated by the nuclear export that has been reported in previous studies where the downregulation or mutation of BRCA1 were observed [174]. Additionally, as a result of an impaired HR mechanism, cells probably use other less efficient compensatory mechanism like NHEJ resulting in more DNA damages that can be confirmed by the upregulation of the phosphorylated γH2AX, which has been identified as a marker of genotoxicity [175].

As it was previously mentioned, the capacity of PDAC to acquire therapeutic resistance is considered a major obstacle, however in the performed acquired resistance assays, BBIT20 did not induced therapeutic resistance in both cell lines. Therefore, doing a more prolonged evaluation of acquired resistance assays and recurring to a broader panel of immortalized cell lines or cancer models that simulate better the TME, like more complex in vitro 3D models, could be a great approach to discover the full potential of BBIT20. This behavior could be possibly justified by the inhibitory activity that BBIT20 has on the efflux pump MDR1, that is widely described as one of the most prevalent mechanism of acquired resistance [146,169]. Additionally, since this feature of BBIT20 of not inducing acquired resistance is highly relevant for PDAC treatment and the combination of therapies is

frequently used in the clinic, exploring the combination of BBIT20 with other drugs can allow the enhancement of their effects and reduce their dose, making them more tolerable for patients ensuring quality of life.

The study of the influence that BBIT20 has on other mechanism of acquired resistance is also a promising field to explore, unveiling more information about how it can counteract therapeutic resistance. So, since the altered expression of miRNA´s is associated with acquired resistance mechanisms, we evaluated if BBIT20 can affect their expression and if it can revert their expression patterns linked to tumor progression for anti-tumor expression levels [147]. One of the miRNAs that had consistent results in both cell lines was the oncomir mir-20a, being its expression downregulated after the treatment with BBIT20. This miRNA apart from stimulating tumor proliferation and acquired resistance by activating cellular signal pathways, such as PTEN/PI3K/AKT it can also be used as a clinical diagnostic indicator tool [152,153]. However, its incidence and information are low in PDAC, so more studies are needed to verify if this miRNA can be used as a diagnostic tool in this type of tumor and if it has more roles in acquired resistance mechanisms [152,153]. Doing in silico predictions could be a great alternative to confirm the targets and testing it in a broader panel of cells could better determine its incidence in PDAC. The other miRNA upregulated after the treatment with BBIT20 was the tumor suppressor miR-200C. This miRNA directly targets the transcription factor ZEB1 which is an inhibitor of E-cadherin , inhibiting EMT transition [155]. Consistently, the expression levels of this transcription factor were reduced by BBIT20. Additionally *in silico* predictions are required to validate this regulatory mechanism.

In this second part of the study, all the following experiments were conducted in a MiaPaCa-2 cell line, where it was induced resistance to GEM. Moreover, we addressed the capability of BBIT20 to counteract therapeutic acquired resistance in this case to GEM.

In the first task we did a characterization of the resistant cell line proving its resistance properties to GEM. Starting with the morphology of the cells, it was possible to observe that resistant cells had a more plump and round morphology, and the parental cells exhibited a spindle-shaped character and enhanced formation of pseudopodia. This characteristic is frequently associated to therapeutic resistance however more studies need to be performed to confirm it [176]. Subsequently, the dose response curves demonstrated that GEM-res MiaPaca-2 cell line has a higher IC_{50} for GEM compared to the parental cell, confirming its resistance to this therapy. Additionally, since the induced resistance to GEM can make molecular alterations that can confer resistance to other therapies, we confirmed also the inexistence of cross-resistance to BBIT20, by verifying similar IC_{50} values in the resistant and parental cell line. Furthermore, the decreased expression of the enzyme dCk that phosphorylates GEM metabolites to an active form, and also the transporter hENT1

that is responsible for the intracellular uptake of GEM, evidence even more the resistant properties [128].

Thereafter to the characterization of the resistant cell line and the confirmation of the resistance, several functional experiments were conducted to assess the anti-tumor properties of BBIT20 with the IC50 values previously obtained. Consistently, the capacity of BBIT20 to significantly increase apoptosis was confirmed, corroborating the inexistence of cross resistance to BBIT20.

Furthermore, since BBIT20 was described to have an inhibitory effect on the efflux pump MDR1 we used a kit that trough an enzymatic reaction it is produced a fluorescent dye [169]. After the treatment with BBIT20 in the resistant cell line, it was observed an increased fluorescence inside the cells. In addition, this feature of BBIT20 must be explored in more detail and in more complex cancer models in order to evidence even more its potential in reverting therapeutic resistance.

EMT transition is considered a negative event that promotes malignant progression of cancer and highly prejudices the treatment of PDAC being considered a mechanism that confers acquired resistance [155]. Therefore, we demonstrated that BBIT20 inhibits the migratory capacity of the cells. However, expression levels of proteins related to EMT, or other markers must be assessed to strengthening even more this property of BBIT20.

As performed in the non-resistant cell lines in the first part of this work, we also evaluated the capability of BBIT20 to interfere in the expression levels of miRNAs. Similar to the results non-resistant cell lines, after the treatment with BBIT20 interestingly, it was observed a significant decreased of the expression level of the oncomir mir-20a. As previously mentioned, in silico predictions are needed to be performed and also more cell lines need to be tested.

To finalize, we assessed the expression of markers associated with cell proliferation, survival and division, and also if it can re-sensitize the resistant cell line to GEM again. After the treatment with BBIT20 it was demonstrated that BBIT20 reduces the expression of the proteins β-catenin that is associated with cell proliferation, survivin that is involved in cell survival and CDC20 associated with cell division, evidencing even more the anti-tumor properties of BBIT20 and the inexistence of cross resistance.

Posteriorly, we revisited the expression of proteins that are involved in the metabolism of GEM and it was possible to observe an increased expression of the transporter hENT1 and decreased expression of the enzyme RRM2, reducing the competitive pool of dCTP available for DNA synthesis, suggesting that the resistant cell line may be susceptible to GEM again. To confirm this, we evaluated the growth inhibitory effect of the combination of GEM with BBIT20, and it was possible to infer that they had a synergistic effect and BBIT20 significantly enhanced the effect of GEM. After determining

the most efficient concentration of GEM to combine with BBIT20 the induction of apoptosis was evaluated, and as expected, the levels of apoptotic cells increased, strengthening the great potential of BBIT20 on the reversion of therapeutic resistance.

5. Conclusions

To conclude, in the present work, we showed that BBIT20 has potent anti-tumor properties, it inhibits homologous recombination, it does not induce resistance and it interferes with microRNAs. Additionally, we also prove that BBIT20 can counteract therapeutic resistance by inhibiting mechanism of acquired resistance.

Altogether, these results demonstrate that BBIT20 represents a promising therapeutic agent for PDAC treatment, not inducing resistance, and potentially overcoming acquired resistance in PDAC**.**

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Supplements

Table s1. **Characteristics of human immortalized normal and cancer cells.**

Cell Line	Type of tissue & disease	BRAC1/2 status	Culture medium & supplements
Miapaca-2	Pancreas PDAC	BRCA1/2 Wt	DMEM + 10% FBS
Panc-1		BRCA1/2 Wt	DMEM + 10% FBS
AsPc1		BRCA1/2 Wt	RPMI + 10% FBS
BxPc 3		BRCA1/2 Wt	DMEM + 10% FBS
HPAF-II		BRCA1/2 Wt	RPMI + 10% FBS
Capan-1		Hemizygous for BRCA2 p. Ser1982Argfs*22 (c.5946delT) (6174delT)wt	IMDM + 20%
Miapaca-2 GEM-res		BRCA1/2 Wt	DMEM + 10% FBS
HFF-1	Skin, foreskin Non-malignant	BRCA1/2 Wt	DMEM F12 + 10%

MIA-PaCa-2 GEM-resistant cell line was generated and kindly supplied by Dr Luigi Sapio from [177].

Table s2. **List of antibodies used in western blot.**

Antigen	Blocking buffer	Dilution	Source
Primary antibodies			
Rad-50	5% non-fat milk	WB 1:200	Santa Cruz Biotechnology
Rad-51		WB 1:1000	
Rad-52		WB 1:200	
Rad-54			
BRCA1		WB 1:1000	Cell Signaling
BRCA2	1% BSA	WB 1:600	
BARD1	5% non-fat milk	WB 1:500	Santa Cruz Biotechnology
γH2AX		WB 1:1000	Cell Signaling
hENT1		WB 1:200	Santa Cruz Biotechnology
dCk			
RRM2			

B-catenin			Abcam
Survivin	5% non-fat milk	WB 1:1000	Abcam
CDC20		WB 1:200	Santa Cruz Biotechnology
ZEB1		WB 1:200	
GAPDH		WB: 1:5000	
Secondary antibodies			
Anti-mouse HRP-conjugated	5% non-fat milk	1:5000	Santa Cruz Biotechnology
Anti-rabbit HRP-conjugated			