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Efeito citotóxico do Olaparib em células de câncer colorretal: Estudo da influência de defeitos genéticos

Tese de Doutorado

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Lista de abreviaturas e siglas

5-Fu: 5-Fluorouracil

6,4PP: 6-4-primidina-pirimidona

Abcb1a/b: ATP-binding cassette sub-family B, member 1a/b

Abcg2: ATP-binding cassette sub-family G, member 2

ADPr: ADP-ribose

AIF: Apoptosis Inducing Factor

AM: Activity modulation

AMD: automodification domain

ARH3: poly(ADP-ribose) hydrolase 3

ART: ADP-ribosyltransferase

ATCC: American type culture collection

BER: *Base excision repair*

BRCA1: Breast Cancer 1

BRCA2: Breast Cancer 2

CD: Catalytic domain

CDI: Cell death induction

CFA: Colony formation assay

CI: Combination index

CIP: Cellular interplay

CP: checkpoint

CPD: Ciclobutanos de pirimidina

CRC: *Colorectal cancer*

CSM: Chromatin structure modulation

DBD: DNA-binding domain

DDR: *DNA damage response*

DNA: Ácido desoxirribonucléico

DNAP: DNA polimerase

DSB: *Double-strand break*

DSBR: *Double-strand break repair*

F_a*: Fraction affected*

FGF1/2: Acid and Basic Fibroblast Growth Factors 1 and 2

FI: Functional interaction

GDDR: General DNA damage response

GG-NER: Reparo global do genoma por excisão de nucleotídeo

HR: *Homologous recombination repair*

IARC: International Agency for Resarch on Cancer

INCA: Instituto Nacional de Câncer

IO: Interaction outcome

Lig: DNA ligase

MART: mono(ADP-ribose) polymerase

MMR: *Mismatch repair*

MN: *Micronucleus*

MSI: *Microsatellite instability*

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

NAD^+: Nicotinamida adenina dinucleotídeo

NAM: *Nicotinamide*

NER: *Nucleotide excision repair*

NHEJ: *Non-homologous end-joining repair*

NLS: Nuclear localization signal

NoLS: nucleolar localization signal

NuRD: Nucleosome remodeling and deacetylase

PcG: Polycomb group

PI: Physical interaction

PSM: PARP signature motif

Oxp: Oxaliplatina

PAR: poli(ADP-ribose) polymer

PARP: poli(ADP-ribose) polimerase

PARPi: Inibidor de PARP

PARG: poly(ADP-ribose) glycohydrolase

PBZ: PAR-binding zinc finger

pRb: Retinoblastoma protein

PTEN: Phosphatase and tensin homolog

p53: Protein 53

ras: Rat sarcoma

RTK: Receptor tyrosine kinases

SSB: *Single-strand break*

SSBR: *Single-strand break repair*

ST: Synthesis

TC-NER: Reparo por excisão de nucleotídeos acoplado a transcrição

TDP1: Tyrosil-DNA Phosphodiesterase 1

VEGF: Vascular Endothelial Growth Factor

VHL: Von Hippel-Lindau

WHO: World Cancer Organization

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Resumo

O câncer é a principal causa de morte nos paises economicamente desenvolvidos e a segunda em paises em desenvolvimento, resultado, em parte, da grande falta de especificidade dos tratamentos atualmente disponíveis. Por outro lado, uma aplicação clínica muito específica, denominada letalidade sintética, foi recentemente proposta. Nesta abordagem terapêutica os inibidores de poli(ADP-ribose) polimerases (PARP), também conhecidos como PARPis, mostraram-se capazes de induzir a morte celular seletiva em células tumorais com defeitos em BRCA1 e BRCA2 (ambas envolvidas no reparo de quebras duplas - DSBR). Assim, a excitante possibilidade de eliminar as células cancerígenas de maneira seletiva fez com que os PARPis passassem de interessantes ferramentas moleculares às mais promissoras drogas anticâncer da atualidade. Contudo, os mecanismos básicos envolvidos na citotoxicidade dos PARPis continuam pouco conhecidos e suas aplicações restritas a um pequeno grupo de cânceres. Por este motivo, neste trabalho, a citotoxicidade do Olaparib (um inibidor de PARP) foi investigada em um painel de linhagens de câncer colorretal (CRC). Os resultados demonstraram que o Olaparib é uma droga de ação lenta, cuja citotoxicidade pode ser modulada por defeitos genéticos em *MLH1* (envolvido no reparo de bases mal-emparelhadas) e no supressor tumoral *PTEN*. Por outro lado, observou-se que o fenótipo MSI (Instabilidade de microssatélites) e os defeitos genéticos em p53 não influenciaram a citotoxicidade do Olaparib. Além

disso, linhagens com resistência adquirida a Oxaliplatina (Oxp) e a 5-Fluorouracil (5-Fu) não apresentaram efeito refratário ao Olaparib, enquanto que linhagens com resistência adquirida a SN-38 (metabólito ativo do Irinotecano) apresentaram um forte efeito refratário. Finalmente, as associações de Oxp ou 5-Fu com Olaparib foram capazes de sensibilizar células com resistência relativa e adquirida. Juntos, estes resultados sugerem uma série de novas possibilidades para o emprego de inibidores de PARP no tratamento de CRC.

Palavras-chave: Olaparib; Câncer colorretal; PARP; PTEN; MLH1.

Abstract

Cancer is the main cause of death in developed countries and the second in less-developed countries, that results in part from the low specific treatments available. However, a very specific therapeutic approach, called synthetic lethality, was recently proposed. The best documented synthetic lethal interaction was reported between poly(ADP-ribose) polymerases inhibitors (PARPis) and defects in BRCA1 and BRCA2 (both involved in double-strand break repair - DSBR), which may induce selective cancer cells death. Therefore, the exciting possibility to selectively kill cancer cells has been moving PARPis from interesting molecular tools to the forefront of cancer therapy research. However, the basic mechanisms involved in PARPis cytotoxicity are still poorly studied and its clinical applications are restricted to a small number of malignances. Herein, the Olaparib (PARPi) cytotoxicity was investigated in a colorectal cancer (CRC) cell line panel. The results demonstrated that Olaparib is a slow action drug, which may have its effects increased in cells with *MLH1 (involved in mismatch repair)* and *PTEN (tumor supressor)* defects. On the other hand, neither the MSI (microsatellite instability) phenotype nor the p53 defects were observed to influence on Olaparib cytotoxicity. Further, neither Oxp nor 5-Fu resistant cell lines presented cross-resistance to Olaparib, whereas a pronounced cross-resistance was observed for SN-38 (Irinotecan metabolite) resistant cell line. Finally, Olaparib associations with Oxaliplatin or 5-Fluorouracil were shown to sensitize cells with both

relative and acquired resistances. Together, these results suggest a series of new possible uses for PARP inhibitors in CRC treatment.

Keywords: Olaparib; Colorectal cancer; PARP; PTEN; MLH1.

Introdução

1.1. Câncer: Incidência

O câncer é a principal causa de morte nos paises economicamente desenvolvidos e a segunda em paises em desenvolvimento (JEMAL *et al.*, 2011). Atualmente, a incidência de câncer vem aumentando nos paises em desenvolvimento devido ao crescimento e envelhecimento da população e, também, como resultado da adoção de estilos de vida que propiciam o surgimento de câncer, como por exemplo, o tabagismo, a "dieta ocidentalizada" e o sedentarismo (GREENWALD & DUNN, 2009; INCA, 2009; JEMAL *et al.*, 2011). No mundo, mais de 12,7 milhões de novos casos de câncer são diagnosticados todos os anos e mais de 7,6 milhões de mortes são causadas por esta doença (WHO, 2009; JEMAL *et al.*, 2011). No Brasil, estima-se que são mais de 490 mil novos casos de câncer e mais de 163 mil mortes por ano (INCA, 2009).

Segundo estimativas globais da Agência Internacional para Pesquisa em Câncer (IARC), os tipos tumorais mais frequentes em mulheres são os de mama, cólon e pulmão, enquanto que, em homens, o de pulmão é o mais freqüente seguido pelos cânceres de cólon e próstata (WHO, 2009). Acompanhando o mesmo perfil da magnitude observada para a América Latina, no Brasil, os tipos mais incidentes são os cânceres de próstata e de pulmão no sexo masculino e os cânceres de mama e do colo do útero no sexo feminino (INCA, 2009). A Figura 1 apresenta os percentuais de incidência de cânceres por sexo no Brasil e no mundo.

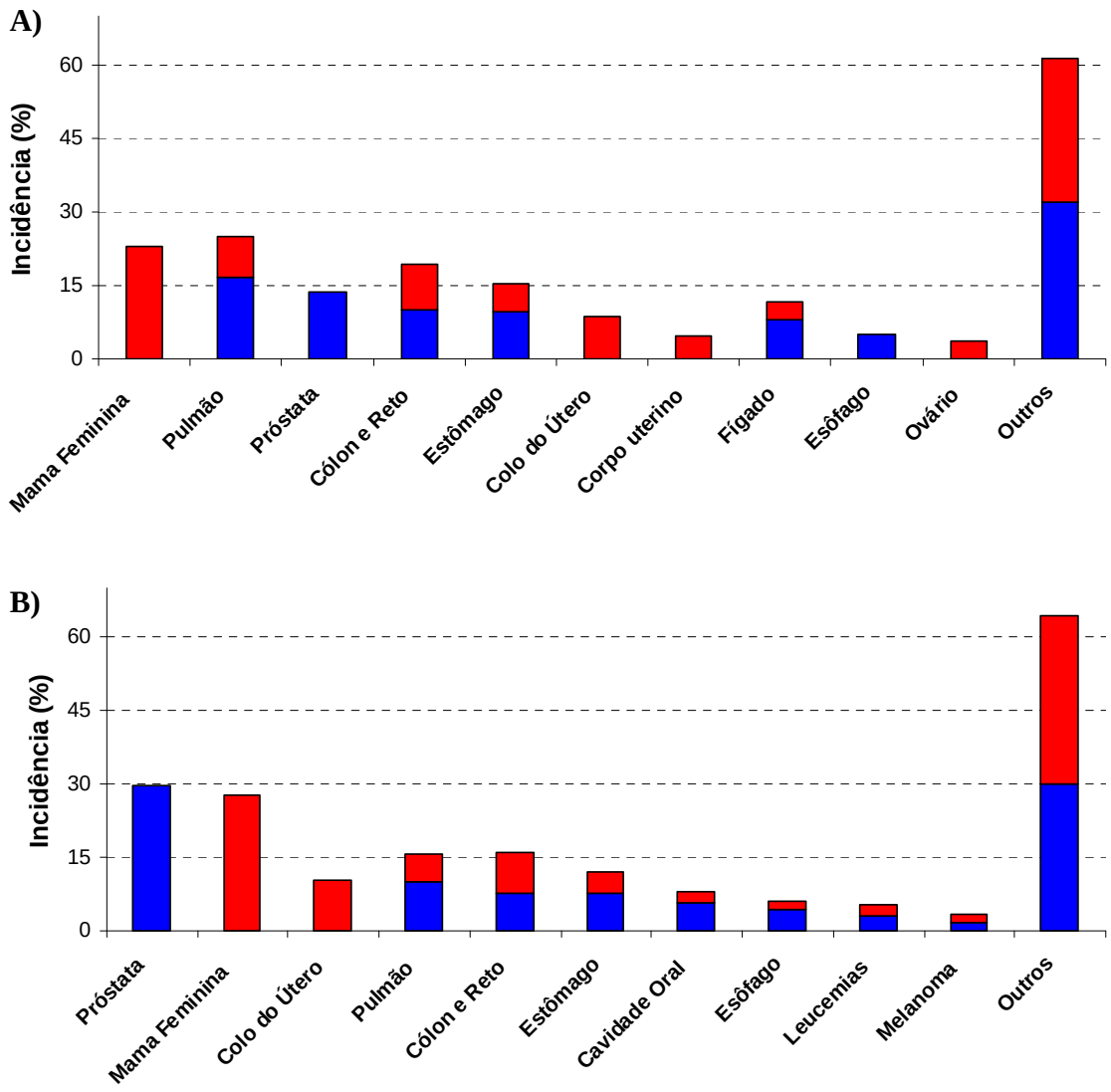

Figura 1. Percentual de incidência de cânceres por sexo. A) Incidência no mundo; B) Incidência no Brasil. (■) homens, (■) mulheres. Os dados foram obtidos e adaptados com base no *World Cancer Report* 2008 (WHO, 2009) e na Estimativa 2010 – Incidência de câncer no Brasil (INCA, 2009).

1.2. Bases moleculares do câncer

O entendimento sobre as bases moleculares do câncer mudou drasticamente nas últimas décadas, devido a uma verdadeira revolução nos métodos e conceitos empregados em biologia molecular. O emprego de novas ferramentas moleculares tornou possível a dissecação de maquinarias moleculares complexas, bem como nos permitiu caracterizar as diferenças entre as células normais e neoplásicas (MENDELSOHN *et al.*, 2008). Como resultado, atualmente a tumorigênese em humanos é vista como um processo dinâmico que envolve múltiplas etapas, as quais refletem alterações genéticas que, progressivamente, transformam células normais em malignas (MENDELSOHN *et al.*, 2008; SALK *et al.*, 2010; HANAHAN & WEINBERG, 2011).

Contudo, para que as células normais tornem-se neoplásicas, é necessário que ocorram mudanças fisiológicas importantes, como o acúmulo de mutações, as quais conferem às células pré-malignas a instabilidade e a variabilidade genética necessárias para a seleção de características capazes de lhes fornecer vantagens proliferativas em relação às células normais. Desta forma, os sistemas de reparo de DNA (Ácido desoxirribonucleico) e a manutenção da integridade genômica constituem os primeiros de muitos sistemas que são afetados na progressão tumoral (HANAHAN & WEINBERG, 2000). Neste sentido, diversas evidências científicas sugerem que o processo de tumorigênese ocorre de maneira análoga à evolução darwiniana, de modo que a sucessão de alterações genéticas que conferem algum

tipo de vantagem proliferativa leva à progressiva conversão de células normais em células cancerígenas (MENDELSOHN *et al.*, 2008; HANAHAN & WEINBERG, 2011).

As alterações genéticas encontradas nas neoplasias são tão diversas quanto numerosas e resultam em um vasto catálogo de genótipos tumorais. Contudo, apesar da imensa complexidade de alterações genéticas que podem dar origem a células cancerígenas, seis são as principais mudanças fisiológicas que ditam o processo de tumorigênese: 1) Autosuficiência em sinais de crescimento; 2) Insensibilidade a sinais antiproliferativos; 3) Evasão da morte celular programada; 4) Potencial replicativo ilimitado; 5) Controle da angiogênese; 6) Invasão de tecidos e metástase (HANAHAN & WEINBERG, 2000). O desenvolvimento de cada uma destas mudanças fisiológicas, além de conferir vantagens proliferativas as células tumorais, representa a quebra de um complexo mecanismo anticâncer nas células e tecidos do corpo humano (MENDELSOHN *et al.*, 2008; HANAHAN & WEINBERG, 2011).

1.2.1. Autosuficiência em sinais de crescimento

As células normais necessitam de sinais mitogênicos ou fatores de crescimento para passarem do estado quiescente para o proliferativo. Desta maneira, a autosuficiência em fatores de crescimento mostra-se uma característica fundamental para o desenvolvimento tumoral. Os eventos moleculares que estão envolvidos na aquisição da autonomia mitótica incluem alterações dos sinais de crescimento extracelulares, dos transdutores de sinais mitogênicos e/ou dos circuitos

intracelulares que transformam os sinais em ação (HANAHAN & WEINBERG, 2011). Estas alterações celulares podem resultar da atividade de oncogenes que mimetizam o efeito dos sinais mitogênicos (ex.: *ras* – *rat sarcoma*), de alterações na expressão ou sensibilidade dos receptores destes fatores de crescimento (ex.: *RTK* – *receptor tyrosine kinases*), ou ainda da capacidade de manufaturar sinais mitogênicos (ex.: glioblastomas e sarcomas) (HANAHAN & WEINBERG, 2011).

1.2.2. Insensibilidade a sinais antiproliferativos Ø

Em tecidos normais, múltiplos sinais antiproliferativos operam para manter a quiescência celular e a homeostasia. Estes sinais antiproliferativos podem forçar as células a entrar em estado quiescente (G_0), do qual podem re-emergir no futuro quando sinais extracelulares o permitirem (HANAHAN & WEINBERG, 2000), ou, alternativamente, as células podem abandonar permanentemente o seu potencial proliferativo quando forçadas a entrar em estados pós-mitóticos. As células tumorais incipientes precisam sair do estado quiescente interrompendo o efeito dos sinais antiproliferativos (HANAHAN & WEINBERG, 2011). Este é resultado da seleção de mutações que afetam o funcionamento de proteínas envolvidas na transição de fases do ciclo celular (especialmente G_1) ou do ciclo circadiano (principalmente proteínas da via pRb – *Retinoblastoma proteins*) (BURKHART & SAGE, 2008). Em contrapartida, não são conhecidos mecanismos capazes de reverter efeitos antiproliferativos de estados pós-mitóticos. Desta forma, as células tumorais empregam diversas estratégias para evitar esta diferenciação terminal, tais como a

superexpressão do oncogene *c-myc*, que codifica fatores de transcrição capazes de promover o crescimento e impedir a diferenciação em estados pós-mitóticos (HANAHAN & WEINBERG, 2000).

1.2.3. Evasão da morte celular programada 

A habilidade de expandir numericamente a população de células tumorais não se restringe apenas à capacidade de proliferação das células cancerígenas, mas também depende de morte celular programada (MENDELSOHN *et al.*, 2008). Dentre os tipos de morte celular programada destaca-se a apoptose, que é um processo inerente a todas as células do corpo humano e que uma vez ativado, desencadeia uma série de passos moleculares que culminam na fagocitose dos corpos apoptóticos resultantes. A morte celular programada é uma importante barreira antitumoral que pode ser ativada por diversos sinais, que incluem danos excessivos ao DNA, sinalização celular anormal provocada por oncogenes, hipóxia, entre outros (HANAHAN & WEINBERG, 2000). Contudo, como a apoptose depende de vários passos, as células tumorais podem evadir-se deste processo de morte quando são selecionadas mutações que inativam sensores, transdutores ou efetores da via (HANAHAN & WEINBERG, 2011). Dentre as várias proteínas que podem ter sua atividade afetada pelas mutações antiapoptóticas, o supressor tumoral p53 destaca-se por encontrar-se mutado em pelo menos 50% dos tipos tumorais (JUNTTILA & EVAN, 2009).

1.2.4. Potencial replicativo ilimitado 

A autosuficiência em sinais de crescimento, a insensibilidade a sinais antiproliferativos e a evasão da morte celular programada são condições fisiológicas que permitem as células cancerígenas replicarem-se de maneira independente das demais células. Contudo, as células de mamíferos portam sistemas autônomos que limitam o número de vezes que cada célula pode se dividir e que se encontram desativados no processo tumoral (MENDELSOHN *et al.*, 2008). Um destes sistemas é conhecido por senescência, e é caracterizada pela parada de crescimento e morte celular massiva após vários processos de divisão (HANAHAN & WEINBERG, 2011). Entretanto, a inatividade dos supressores tumorais p53 e pRb é uma das maneiras pela qual as células tumorais podem contornar a senescência (JUNTTILA & EVAN, 2009; HANAHAN & WEINBERG, 2011).

Adicionalmente, devido a uma característica intrínseca das DNA polimerases (DNAP), cada ciclo de replicação celular leva a diminuição de 50-100 pares de bases do DNA telomérico, de forma que sucessivos ciclos de replicação resultam no progressivo encurtamento dos telômeros. Uma vez que os telômeros desempenham um papel fundamental na proteção das extremidades dos cromossomos, a excessiva perda deste DNA leva a instabilidade genômica e a inevitável morte celular (HANAHAN & WEINBERG, 2011). Porém, diversos estudos demonstram que 85-90% das células tumorais são capazes de regular a atividade ou a expressão das telomerases. Assim, a superexpressão ou a superatividade das telomerases culmina

na manutenção do comprimento telomérico e, consequentemente, pode conferir às células tumorais potencial replicativo ilimitado (SHAY & WRIGHT, 2000; BLASCO, 2005).

1.2.5. Controle da angiogênese

O sistema vascular fornece o oxigênio e os nutrientes cruciais para o funcionamento celular e a sobrevivência, obrigando todas as células no tecido a residirem muito próximas aos capilares sanguíneos. Em tecidos já formados, o crescimento de novos vasos sanguíneos (angiogênese) é um processo transitório e rigidamente regulado, o que constitui uma das principais barreiras para o desenvolvimento macroscópico das neoplasias. Desta forma, os tumores incipientes precisam adquirir a habilidade de formar novos vasos sangüíneos (MENDELSOHN *et al.*, 2008). Assim, para o êxito na etapa macroscópica da progressão tumoral, as células cancerígenas precisam ser capazes de contrabalançar os sinais positivos e negativos para amplificar o processo angiogênico, tais como o aumento da expressão de sinais estimulantes como VEGF (*vascular endothelial growth factor*) e FGF1/2 (*acid and basic fibroblast growth factors*), e/ou afetando a expressão/atividade de repressores angiogênicos como a trombospondina-1 (FERRARA, 2009). Apesar dos mecanismos envolvidos no processo angiogênico tumoral serem pouco compreendidos, alguns exemplos foram documentados. Por exemplo, mutações no supressor tumoral p53 levam ao decréscimo nos níveis celulares de trombospondina-1, ou a ativação do oncogene *ras* ou, ainda, a perda da

atividade do supressor tumoral VHL (*Von Hippel-Lindau tumor supressor*) resultando, em alguns casos, no aumento da expressão de VEGF (HANAHAN & WEINBERG, 2011).

1.2.6. Invasão de tecidos e metástase

Durante o desenvolvimento da maioria dos tipos de cânceres humanos, massas tumorais geram células pioneiras capazes de se movimentar para outros tecidos onde podem originar novos tumores. Este processo é conhecido por metástase e é responsável por 90% das mortes por câncer (MENDELSOHN *et al.*, 2008). A capacidade de invadir e metastizar outros tecidos permite às células cancerígenas evadir-se da massa tumoral primária e colonizar novas regiões no corpo humano onde, pelo menos inicialmente, os nutrientes e espaço não são limitados. Contudo, assim como na formação do tumor primário, o sucesso na invasão e metástase depende de todas as outras cinco características previamente discutidas (HANAHAN & WEINBERG, 2000).

Invasão e metástase são processos extremamente complexos cuja base molecular ainda não é totalmente compreendida. Ambos os processos empregam estratégias operacionais similares que envolvem a junção física das células ao seu microambiente e a ativação de proteases extracelulares. Do ponto de vista molecular, as células com características invasivas e metastáticas são capazes de alterar várias classes de proteínas envolvidas em adesão celular, como imunoglobulinas, caderinas e integrinas, além de regularem a expressão de

proteases, tanto para evadirem-se do tumor primário quanto para fixarem-se no tecido invadido (HANAHAN & WEINBERG, 2011).

1.3. A complexidade molecular do câncer

O modo como as células normais transformam-se em células malignas é altamente variado, uma vez que as mutações consideradas essenciais para o desenvolvimento cancerígeno podem diferir enormemente na fase do processo tumoral em que surgem (HANAHAN & WEINBERG, 2000; MENDELSOHN *et al.*, 2008). Consequentemente, a aquisição de algumas vantagens fisiológicas como evasão da morte celular programada, controle da angiogênese e potencial replicativo ilimitado podem aparecer em diferentes etapas do desenvolvimento tumoral. A sequência de mudanças fisiológicas nas neoplasias varia muito em tumores do mesmo tipo e amplamente entre tumores de tecidos diferentes, como exemplificado na Figura 2. Além disso, uma alteração genética pontual pode contribuir para a aquisição de apenas uma ou mais vantagens fisiológicas. Mais ainda, as células malignas da mesma massa tumoral podem apresentar diferentes alterações genéticas, adicionando mais complexidade à massa tumoral (HANAHAN & WEINBERG, 2000).

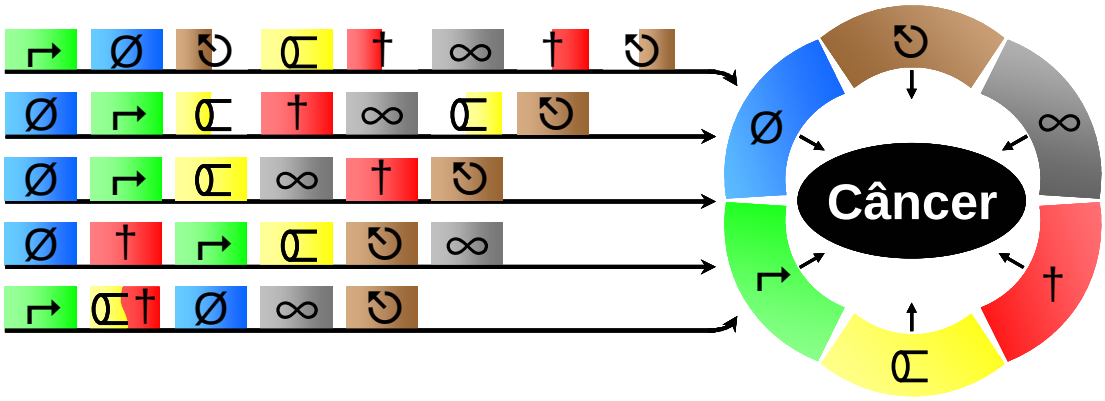

Figura 2. Seqüências mais comuns de alterações fisiológicas adquiridas pelas neoplasias ao longo do processo tumoral. Apesar da maioria dos cânceres desenvolverem as seis alterações fisiológicas em seus estágios mais avançados, a maneira como este processo ocorre varia mecanisticamente e cronologicamente. A figura exemplifica que algumas alterações fisiológicas necessitam de mais de uma alteração genética ( ), enquanto que outras alterações genéticas podem levar a aquisição de mais de uma vantagem fisiológica ( ). :auto-suficiência em sinais de crescimento; : insensibilidade a sinais antiproliferativos; :evasão da morte celular programada; :potencial replicativo ilimitado; : controle da angiogênese; :invasão de tecidos e metástase. Adaptado do esquema publicado por HANAHAN & WEINBERG (2000).

1.4. Câncer: Tratamento

Acompanhando a vasta complexidade molecular dos cânceres, os métodos empregados para tratar os diferentes tipos tumorais variam consideravelmente de

caso para caso. Os principais tipos de tratamentos antineoplásicos estão incluídos nas três grandes categorias a seguir: remoção cirúrgica, radioterapia e quimioterapia. A remoção cirúrgica é a primeira linha de tratamento para grande parte dos cânceres, sendo geralmente acompanhada pelo tratamento adjuvante, pré- e/ou pós-operatório, com radiação ionizante ou quimioterápicos (RANG *et al.*, 1997). No entanto, em alguns casos, os tumores não podem ser cirurgicamente removidos, de modo que a radioterapia e a quimioterapia são os únicos tratamentos possíveis. Por sua vez, a radioterapia e a quimioterapia podem ser empregadas isoladamente ou em conjunto, de acordo com o tipo e estágio de desenvolvimento neoplásico, sendo a radioterapia comumente empregada para o tratamento local de tumores sólidos ou que afetem a circulação sanguínea, enquanto a quimioterapia é geralmente empregada para o tratamento sistêmico de tumores em fase metastática ou de difícil acesso (RANG *et al.*, 1997; CHABNER & ROBERTS, 2005).

1.4.1. Terapias baseadas na indução de danos no DNA

Na radioterapia são empregados feixes de radiação ionizante que, ao interagirem com os componentes celulares, são capazes de ejetar elétrons dos orbitais dos átomos de carbono, hidrogênio, oxigênio e nitrogênio. Por sua vez, estes elétrons livres podem transferir a sua energia diretamente para moléculas de DNA das células, danificando a sua estrutura (RANG *et al.*, 1997). Alternativamente, a energia dos elétrons pode ser transferida para uma molécula intermediária (*e.x.* água), cuja radiólise acarreta a formação de produtos altamente reativos capazes de lesionar o DNA (Figura 3). Na quimioterapia são empregados compostos químicos

tóxicos para as células (citotóxicos) que são capazes de induzir a morte celular ou impedir o funcionamento normal das células (Figura 3). Estes agentes citotóxicos apresentam uma variada gama de estruturas químicas e possuem diferentes mecanismos de ação, mas em geral, atuam induzindo danos no DNA das células (RANG *et al.*, 1997; HURLEY, 2002).

Desta forma, apesar de possuírem mecanismos de ação distintos, tanto a radioterapia quanto a quimioterapia baseiam-se na indução direta ou indireta de danos no DNA (Figura 3). Uma vez que os excessivos danos no DNA podem levar a paradas de ciclo celular e a indução de morte celular, as células que se encontram em divisão rápida, tais como as células cancerígenas, são as mais afetadas pelos tratamentos com radio- e quimioterápicos. Contudo, as células do sistema imunológico também se dividem rapidamente, e por este motivo, as terapias baseadas em agentes indutores de danos no DNA são frequentemente acompanhadas de supressão imunológica (RANG *et al.*, 1997). Adicionalmente, as lesões de DNA induzidas tanto pela radioterapia quanto pela quimioterapia podem levar à conversão de células normais em células tumorais ou à seleção de novas características tumorais nas células cancerígenas pré-existentes.

1.4.2. Letalidade sintética

Tendo-se em vista a vasta gama de efeitos colaterais associados tanto à radioterapia quanto à quimioterapia, a busca por novas abordagens terapêuticas menos nocivas vem se mostrando fundamental para a redução destes efeitos, bem como o aumento da eficiência dos tratamentos atuais. De fato, uma recente

aplicação clínica denominada letalidade sintética começou a explorar de maneira seletiva as mutações existentes nas células cancerígenas. De acordo com o conceito de letalidade sintética, a inibição ou a deleção de um determinado gene é tolerável, contudo a combinação da deleção ou inibição de ambos os genes leva a morte celular (ROULEAU *et al.*, 2010; BANERJEE *et al.*, 2010). A aplicação clínica deste conceito alia a inibição sintética induzida por um agente antineoplásico com mutações oncogênicas pré-existentes nas células tumorais para induzir a citotoxicidade seletiva (ROULEAU *et al.*, 2010; BANERJEE *et al.*, 2010). Como resultado, o índice terapêutico é aumentado juntamente com a redução dos efeitos colaterais.

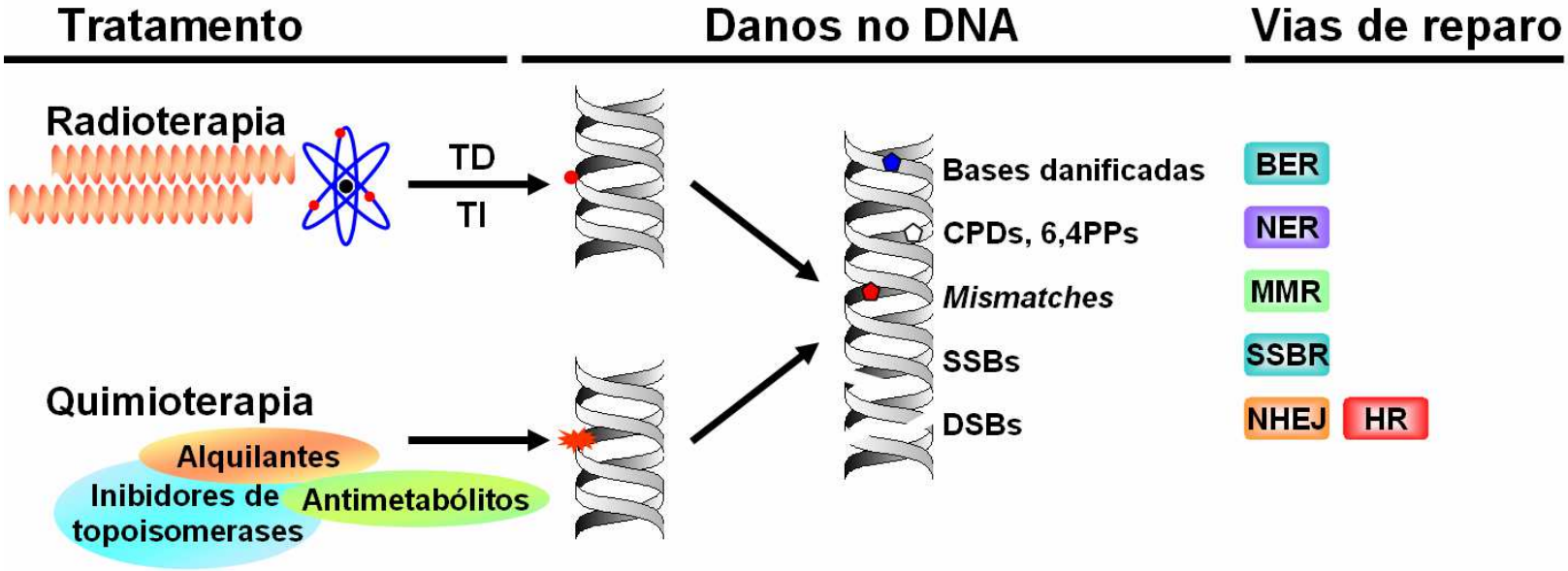

Figura 3. A radio- e quimioterapia induzem diferentes tipos de lesões no DNA que, por sua vez, recrutam diversas vias de reparação de DNA em resposta ao dano. Na radioterapia, as ondas de radiação ionizante podem levar a ejeção de elétrons dos átomos de diversos componentes celulares, que podem transferir diretamente (TD) ou indiretamente (TI) a sua energia para moléculas de DNA. Na quimioterapia são empregadas várias classes de agentes antineoplásicos, geralmente indutores de lesões de DNA. Os danos mais comuns foram exemplificados, bem como as vias de reparação de DNA envolvidas na sua resposta. CPDs: ciclobutanos de pirimidina, 6,4PPs: 6-4-pirimidina-pirimidona, *mismatches*: bases mal-emparelhadas, SSBs: quebras de fita simples, DSBs: quebras de fita dupla, BER: reparo por excisão de bases, NER: reparo por excisão de nucleotídeos, MMR: reparo de bases mal-emparelhadas, SSBR: reparo de quebras de fita simples, NHEJ: recombinação não homóloga e HR: recombinação homóloga.

1.4.2.1. Inibidores de PARP

Os atuais inibidores das poli(ADP-ribose) polimerases (PARPs) são promissores agentes antitumorais cuja estrutura química é largamente baseada em benzamidas ou purinas (JAGTAP & SZABÓ, 2005). Estes inibidores vêm sendo projetados para competir com NAD^+ (Nicotinamida adenina dinucleotídeo) no domínio catalítico PARP e são capazes de aumentar a sensibilidade de células cancerígenas frente a tratamentos com agentes indutores de danos no DNA ou mesmo induzir a letalidade sintética em células com deficiências no reparo de quebras duplas (*DSB*) (LORD & ASHWORTH, 2008). Alguns estudos têm demonstrado que os inibidores de PARP (PARPis) são capazes de induzir a letalidade sintética em células com defeitos nos genes *BRCA1* ou *BRCA2* (JAGTAP & SZABÓ, 2005). Desta forma, os cânceres de mama e ovário são alvos preferenciais para o tratamento letal com PARPis, pois apresentam alta frequência de defeitos nestes genes (LORD & ASHWORTH, 2008). Contudo, pouco se sabe sobre o efeito destes inibidores em células com outros tipos de deficiências em sistemas de reparo de DNA.

Dado o grande percentual de homologia entre os sítios catalíticos das diversas PARPs já identificadas, pressupõe-se que os inibidores de PARP possam inibir a atividade enzimática de grande parte de membros da família PARP (JAGTAP & SZABÓ, 2005; ROULEAU *et al.*, 2010). Contudo, apenas as PARPs1-3 foram reportadas como diretamente envolvidas na resposta aos danos no DNA, de forma

que a sua inibição pode levar ao acúmulo de variadas lesões de DNA[1] (HOTTIGER *et al.*, 2010). Por sua vez, BRCA1 e BRCA2 são enzimas fundamentais para o reparo de DSBs por recombinação homóloga (*HRR*) (LORD & ASHWORTH, 2008). Desta forma, acredita-se que a administração de PARPis leve ao acúmulo de lesões de DNA geradas espontaneamente pelo metabolismo celular. Estas lesões primárias alteram a estabilidade do DNA e podem dar origem a lesões secundárias ainda mais tóxicas, como por exemplo, as DSBs. As células normais são capazes de reparar corretamente as DSBs, enquanto que as células tumorais com defeitos em *BRCA1* ou *BRCA2* acumulam estas lesões, as quais induzem forte e seletiva citotoxicidade (CARDEN *et al.*, 2010). Porém, ainda não está claro se a hipersensibilidade aos PARPis apresentada pelas células deficientes em *BRCA1* e *BRCA2* restringe-se a apenas este mecanismo.

Dentre os atuais inibidores de PARP, o Olaparib (AZD2281 - AstraZeneca) (Figura 4), vem se destacando por exibir um proeminente efeito letal em células com defeitos em *BRCA1* ou *BRCA2*. Este PARPi liga-se de maneira não-covalente ao sítio catalítico das PARPs1-2 e inibe as suas atividades enzimáticas. Atualmente, o Olaparib encontra-se em fases I e II[2] de testes clínicos tanto como agente simples, quanto em combinação com diversas drogas antineoplásicas (SANDHU *et al.*, 2010). Porém, apesar do notável potencial deste agente, grande parte das pesquisas com Olaparib encontram-se restritas a tumores de mama, ovário e endométrio. Desta forma, estudar o Olaparib em outros tipos tumorais mostra-se essencial para ampliar

[1] Para uma revisão detalhada sobre o envolvimento das PARPs na resposta aos danos no DNA *vide* o artigo de revisão no capítulo I "*PARPs and the DNA damage response*"
[2] http://clinicaltrials.gov/ct2/results?term=olaparib acessado em janeiro de 2012

o espectro de aplicações clínicas deste promissor agente antineoplásico, bem como para entender os seus mecanismos de ação.

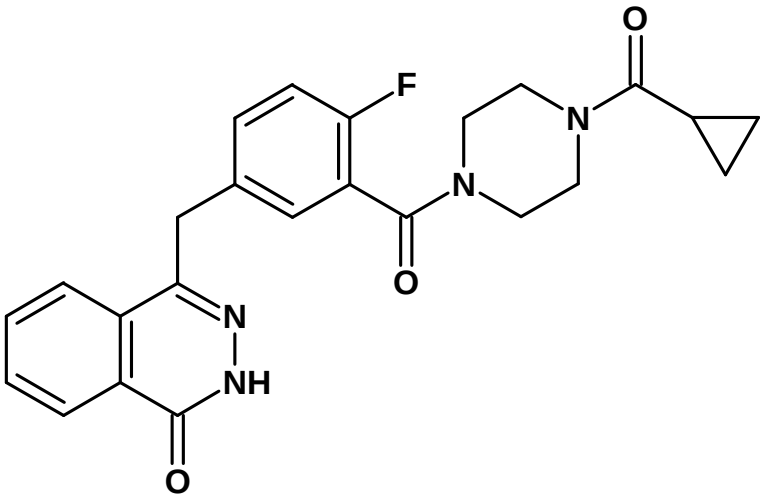

Figura 4. Estrutura química do Olaparib. Estrutura obtida com o emprego do programa ACD/ChemSketch 12.0.

1.5. Câncer e os sistemas de reparo de DNA

As lesões no DNA podem ser processadas por diversos complexos protéicos denominados sistemas de reparação de DNA. Cada um destes complexos é responsável pelo reparo de tipos específicos de lesões de DNA. Contudo, existe uma grande sobreposição de funções entre os diferentes sistemas de reparo, sendo que, lesões tipicamente reparáveis por um dado sistema também podem, em alguns casos, ser processadas por outros sistemas (HELLEDAY *et al.*,

2008). Isto é especialmente válido para os diversos tipos de danos de DNA induzidos pelos agentes antineoplásicos.

Dependendo do contexto, os sistemas de reparo de DNA podem ser considerados inimigos ou aliados das células neoplásicas. Como previamente discutido, a inativação de algumas proteínas de reparo pode facilitar o acúmulo de mutações e, assim, garantir a variabilidade necessária para o desenvolvimento das características cancerígenas nos estágios iniciais do desenvolvimento tumoral (HANAHAN & WEINBERG, 2000; HANAHAN & WEINBERG, 2011). Contudo, quando as células cancerígenas são tratadas com agentes indutores de danos de DNA, o eficiente reparo destas lesões pode garantir a sobrevivência das células neoplásicas, levando à resistência aos tratamentos antitumorais (HELLEDAY *et al.*, 2008). Desta forma, existe consenso no meio científico de que a modulação dos sistemas de reparo desempenha um papel fundamental no desenvolvimento e na sobrevivência das células tumorais.

1.5.1. Reparo de bases danificadas, mal-emparelhadas e fotoprodutos

O sistema de reparo por excisão de bases, também conhecido como BER, é responsável pela remoção de pequenas lesões no DNA que pouco distorcem a dupla hélice. Este sistema de reparo é especialmente importante para o processamento de bases danificadas por oxidação, metilação ou desaminação, bases que foram erroneamente incorporadas durante a replicação ou incompatíveis (tais como gerados por antimetabólitos análogos de bases – *e.x.* 5-Fluorouracil, citarabine) (FORTINI & DOGLIOTTI, 2007; MÉNDEZ-ACUÑA *et al.*, 2010). O BER é iniciado pela atividade de DNA glicosilases, as quais reconhecem

e removem a base danificada/incompatível, formando um sítio AP (Apurínico/Apirimídico). Por sua vez, os sítios APs são clivados por uma AP endonuclease, resultando em uma quebra de fita simples (Figura 5). Finalmente, esta quebra pode ser processada por duas vias; a via curta que substitui apenas um nucleotídeo ou a via longa que substitui de 2-10 nucleotídeos (BOITEUX & GUILLET, 2004; FORTINI & DOGLIOTTI, 2007).

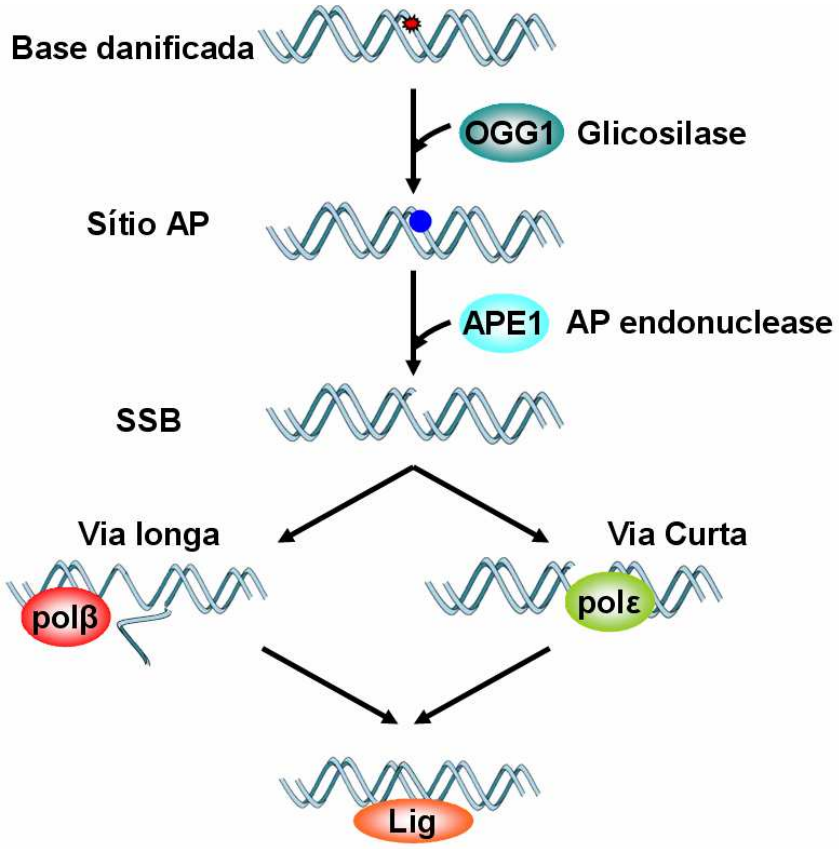

Figura 5. Representação esquemática do BER. A base oxidada é reconhecida por OGG1 (ou outra DNA glicosilase), que remove a base e cria um sítio AP. Os sítos APs são geralmente clivados por APE1 (endonuclease), a qual produz uma quebra de fita simples. A quebra de fita simples pode ser processada tanto pela via longa, na qual a polimerase β atua sintetizado de 2-10 nucleotideos, quanto pela via curta, na qual a polimerase ε insere apenas um nucleotídeo. Finalmente, as extremidades são seladas pela ação de uma DNA ligase.

O reparo por excisão de nucleotídeos, comumente conhecido por NER, diferentemente do BER, reconhece e remove uma vasta gama de lesões associadas a grandes distorções na dupla hélice de DNA (HANAWALT & SPIVAK, 2008). Este sistema de reparo remove principalmente, mas não exclusivamente, lesões induzidas por radiação ultravioleta (UV – *e.x.* ciclobutanos de pirimidina e 6-4-pirimidina-pirimidona) (HANAWALT & SPIVAK, 2008) e pontes inter- ou intra-cadeia de DNA (FRIEDBERG *et al.*, 2006). Após o reconhecimento da lesão/distorção no DNA (Figura 6), os componentes protéicos do NER promovem a remoção de um fragmento de DNA onde o dano se encontra, criando uma região de fita simples (HANAWALT & SPIVAK, 2008; LEHMANN, 2011). Esta região de fita simples é posteriormente preenchida por uma DNA polimerase, a qual utiliza a fita remanescente como molde. Adicionalmente, o NER também pode ser dividido em dois subsistemas: o acoplado a transcrição (TC-NER), que repara os danos das regiões transcricionalmente ativas, e o que repara globalmente o genoma (GG-NER), o qual promove o reparo nas regiões transcricionalmente inativas (HANAWALT & SPIVAK, 2008; LEHMANN, 2011).

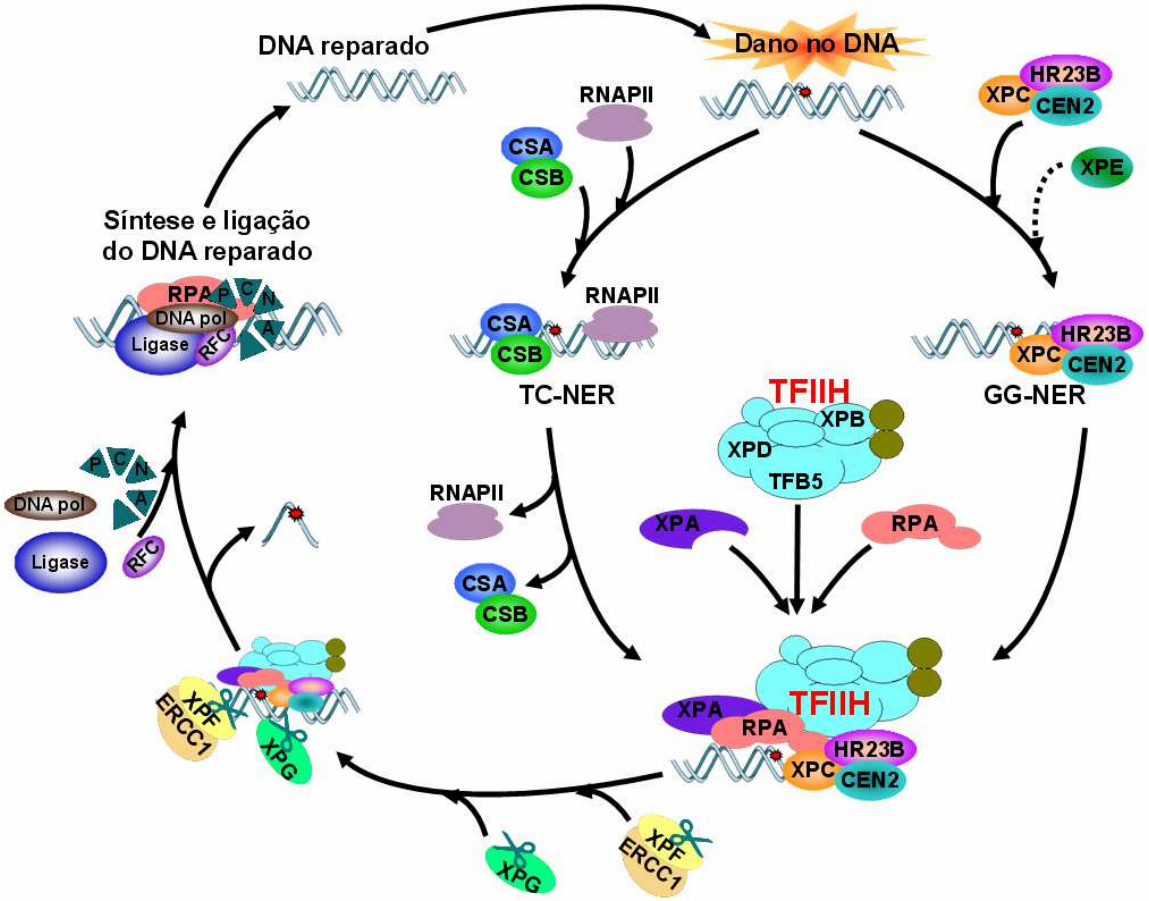

Figura 6. Representação esquemática do NER. No TC-NER, as lesões são inicialmente reconhecidas pela RNAPII, a qual recruta CSA e CSB. No GG-NER, as lesões são reconhecidas pelo complexo formado por XPC, CEN2, HR23B e eventualmente XPE. Ambos os subsistemas recrutam o complexo TFIIH, XPA e RPA, que por sua vez, abre a dupla fita de DNA e recruta as endonucleases XPF-ERCC1 e XPG. O fragmento com a lesão é excisado pelas endonucleases, uma nova sequência de DNA é sintetizada por uma DNA polimerase (com o auxílio de PCNA e RFC) e selado por uma DNA ligase.

Finalmente, o sistema de reparo de bases mal-emparelhadas (também conhecido por MMR) reconhece, excisa e repara erros derivados de fontes

exógenas ou da recombinação e replicação de DNA, como por exemplo, bases erroneamente inseridas ou pareadas, deleções e substituições (MARTIN *et al.*, 2010; VILAR & GRUBER, 2010). As proteínas do MMR reconhecem as bases mal emparelhadas pela distorção na dupla fita de DNA e recrutam outros fatores envolvidos na clivagem do fragmento que contém a lesão (Figura 7). Posteriormente uma DNA polimerase (DNAP) resintetiza uma nova sequência de DNA no sítio antes lesado. Como último passo, uma DNA ligase (Lig) sela o fragmento recém sintetizado (LAHUE *et al.*, 1989; AQUILINA & BIGNAMI, 2001; JIRICNY, 2006).

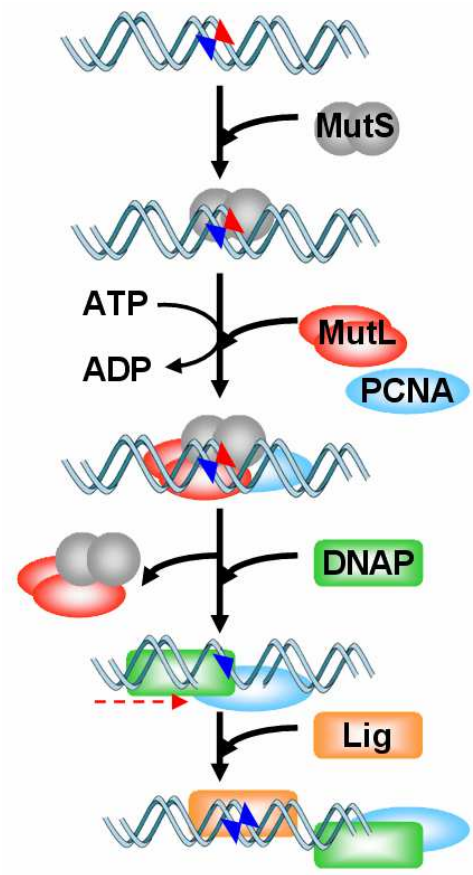

Figura 7. Representação esquemática do MMR. As bases erroneamente incorporadas (▼) são reconhecidas pelo complexo MutS. Por sua vez, MutS recruta o complexo MutL e PCNA. O fragmento contendo a base errônea é excisado com o consumo de ATP e uma DNA polimerase re-sintetiza uma nova sequência de DNA complementar a fita parental. MutS é um heterodímero que pode existir na forma α (MSH2 e MSH6) ou β (MSH2 e MSH3). MutL também é um heterodímero que pode existir nas formas α (MLH1 e PMS2), β (MLH1 e PMS1) ou γ (MLH1 e MLH3).

1.5.2. Reparo de quebras simples – SSBR

As quebras de fita simples (SSBs) podem resultar de intermediários do reparo mal-resolvidos, de danos oxidativos que levam à desintegração dos anéis de desoxirribose do DNA, da atividade de topoisomerases e de uma variedade de danos exógenos. Caso não sejam reparadas, as SSBs podem levar ao colapso de forquilhas de replicação e/ou originar as tóxicas DSBs (HEGDE *et al.*, 2008). Uma vez que as SSBs são muito frequentes e perigosas, espera-se uma grande sobreposição de vias de reparação de DNA envolvidas no reparo deste tipo de lesão. De fato, as SSBs são reparadas por diferentes complexos protéicos de acordo com o processo genotóxico que lhe deu origem (Figura 8). Assim, as SSBs resultantes de intermediários de BER mal-resolvidos são reparadas pelos próprios componentes do BER em subsequentes ciclos de reparo (CALDECOTT, 2008). As SSBs resultantes da atividade de topoisomerases são reparadas pela *Tyrosil-DNA Phosphodiesterase 1* (TDP1) em conjunto com alguns componentes do BER (CALDECOTT, 2008). Enquanto que as SSBs resultantes de danos oxidativos ou exógenos são detectadas por PARP-1 e reparadas pelos componentes da via longa do BER (CALDECOTT, 2008).

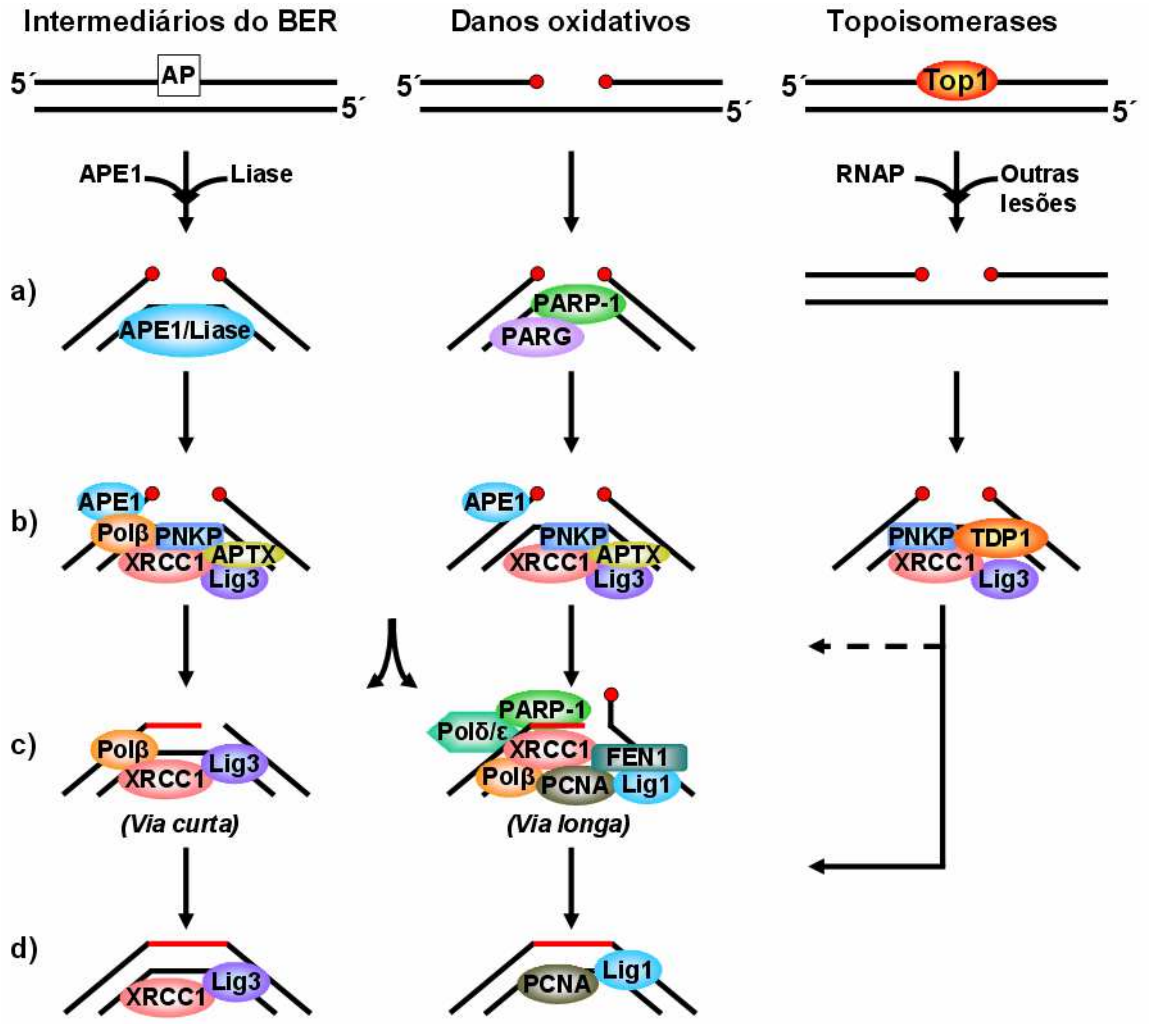

Figura 8. Representação esquemática do SSBR. As SSBs são reparadas por diferentes complexos protéicos de acordo com o tipo de lesão que lhes deu origem. Desta forma, as SSBs podem ser detectadas por APE1/Liase, PARP-1/PARG ou RNAPs (a). O processamento das lesões normalmente envolve XRCC1, Lig3 e PNKP (b). Dependendo do contexto, o processamento das lesões também pode envolver APE1, Polβ, APTX e TDP1 (b). Nas lesões derivadas de intermediários do BER, a síntese da nova sequência de DNA é realizada pela maquinaria da via curta do BER (*short-patch*) e requer a ação da Polβ em conjunto com XRCC1 e Lig3 (c). O processo de síntese de DNA nas lesões derivadas de processos oxidativos ou da ação de topoisomerases necessita de proteínas envolvidas na via longa do BER (*long-patch*), tais como PARP-1, PCNA, Lig1,

FEN1 e Polδ/ε. Finalmente, o selamento da nova sequência de DNA pode ser realizado por XRCC1/Lig3 (via curta) ou PCNA/Lig1 (via longa).

1.5.3. Reparo de quebras duplas – DSBR

As DSBs são lesões de DNA extremamente tóxicas que podem levar a grandes rearranjos genômicos, bem como a perda de grandes sequências de DNA (HEYER *et al.*, 2010). Este tipo de dano pode surgir quando outras lesões menos severas não são apropriadamente reparadas. Adicionalmente, diversos agentes indutores de danos (*e.x.* Oxaliplatina, 5-Fluorouracil e SN-38) podem levar, direta ou indiretamente, à formação de DSBs (HOLTHAUSEN *et al.*, 2010; MATUO *et al.*, 2009). Em células eucarióticas superiores existem dois mecanismos principais de reparo de DSBs (apresentados na figura 9): 1) O reparo por recombinação homóloga (HR), que é livre de erros por utilizar um cromossomo ou cromátide homóloga; e 2) O reparo por recombinação não-homóloga (NHEJ), que por apenas unir as extremidades de DNA rompidas, pode levar à perda de material genético, assim como a rearranjos e translocações cromossômicas (HEYER *et al.*, 2010; HOLTHAUSEN *et al.*, 2010).

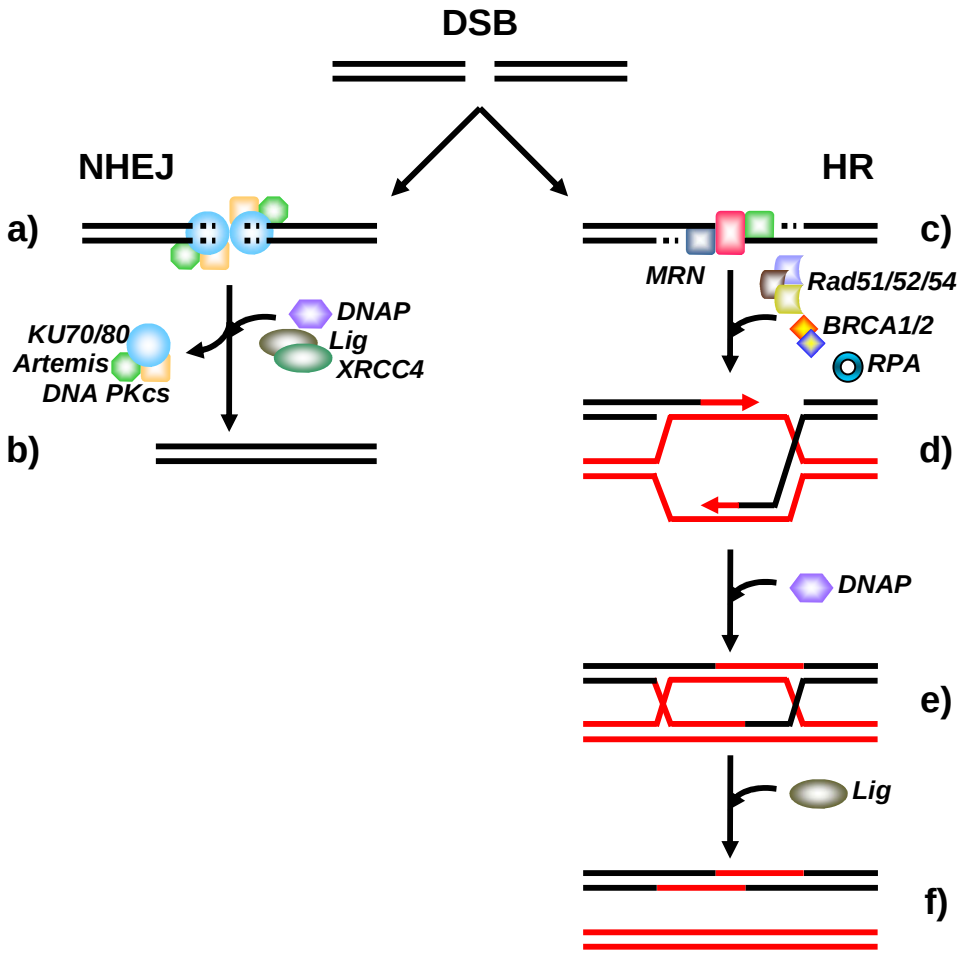

Figura 9. Representação esquemática do DSBR. No NHEJ, KU70/80 atuam detectando as DSBs e recrutando Artemis e DNA PKcs (a). Por sua vez, este complexo processa as extremidades do DNA rompido e recruta DNA polimerase, ligase e XRCC4, as quais são responsáveis pela junção das extremidades de DNA (b). Na HR, o complexo MRN (MRE11/Rad50/Nbs1) atua detectando as DSBs, excisando as extremidades danificadas e recrutando fatores adicionais (c). Dentre as proteínas recrutadas pelo complexo MRN, destacam-se as Rads 51, 52 e 54, bem como BRCA1/2 e RPA. Estas proteínas, juntamente com uma DNAP, atuam formando junções do tipo Holliday com as cromátides irmãs ou cromossomos homólogos (d). As junções de Holliday permitem que a DNAP utilize a sequência complementar de DNA para sintetizar uma nova sequência na região onde ocorreu

o dano. Quando as junções de Holliday são finalmente resolvidas, uma DNA ligase sela a nova sequência sintetizada (f).

1.6. Câncer colorretal

O câncer colorretal (CRC) é um alvo em potencial para a terapia letal pois apresenta alta incidência de defeitos genéticos, como instabilidade de microssatélites (MSI) e mutações em diversos supressores tumorais. O fenótipo MSI é observado em 10-15% de todos os CRCs e está associado a mutações em genes de reparo de bases mal-emparelhadas (MMR) (HAMPEL *et al.*, 2005). Os defeitos em genes do MMR podem levar a mutações em unidades repetitivas de microssatélites em vários outros genes como, por exemplo, o supressor tumoral PTEN (*Phosphatase and tensin homolog*) que se encontra mutado em aproximadamente 18% dos CRC-MSI (NASSIF *et al.*, 2004). Adicionalmente, o importante supressor tumoral p53 encontra-se mutado em cerca de 50% dos CRCs (RODRIGUES *et al.*, 1990).

Apesar das numerosas variações procedimentais que existem na terapia de CRCs, a remoção cirúrgica é a primeira linha de tratamento para grande parte destas enfermidades, sendo geralmente acompanhada pelo tratamento quimioterápico adjuvante com Oxaliplatina (Oxp), 5-Fluorouracil (5-Fu) ou Irinotecano. Quando comparado com os tratamentos para outros tipos tumorais, este procedimento clínico apresenta bons resultados (WHO, 2009). Contudo, todos os anos pelo menos 600 mil pessoas morrem de CRC no mundo (WHO,

2009). Grande parte destas mortes pode ser atribuída a metastização e a reincidência tumoral, que por sua vez estão diretamente associadas a inespecificidade dos agentes quimioterápicos atuais (WHO, 2009).

Os agentes quimioterápicos utilizados no tratamento de CRC induzem danos diretos e/ou indiretos no DNA das células por diferentes mecanismos. A Oxaliplatina (Figura 10A) é um agente platinado que forma ligações intra- e intercadeia com o DNA que, por sua vez, induzem uma variedade de lesões, incluindo SSBs e DSBs (FAIVRE *et al.*, 2003). O 5-Fluorouracil (Figura 10B) é um análogo de pirimidina cujos metabólitos podem inibir a atividade da enzima timidilato sintase ou podem ser incorporados aos ácidos nucléicos, levando a inibição da síntese de DNA e a variados danos de DNA que incluem SSBs e DSBs (MATUO *et al.*, 2009). Finalmente, o Irinotecano (Figura 10C) é um análogo sintético do alcalóide camptotecina cujo metabólito ativo (SN-38 - Figura 5D) atua inibindo a atividade da topoisomerase I e, desta forma, leva a inibição da replicação e transcrição, bem como a formação de SSBs e DSBs (ILLUM, 2011).

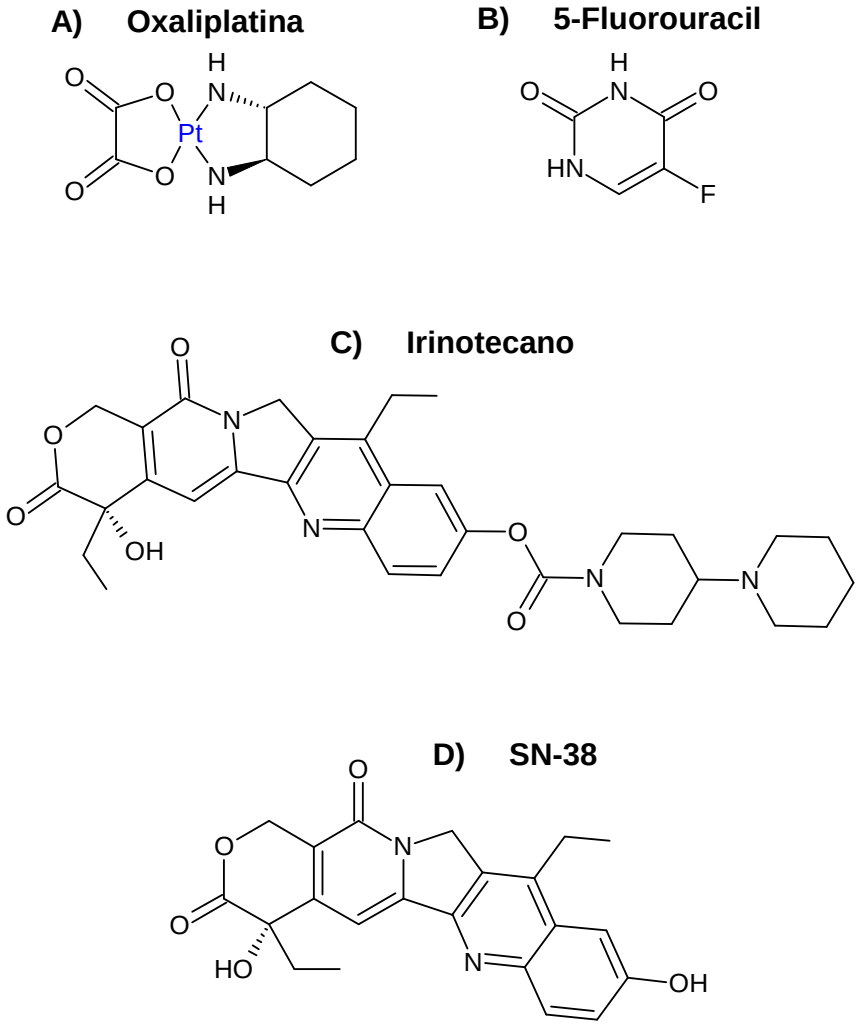

Figura 10. Estruturas químicas dos antineoplásicos comumente empregados no tratamento de CRCs. A) Oxaliplatina; B) 5-Fluorouracil; C) Irinotecan; SN-38. As estruturas químicas foram obtidas com o emprego do programa ACD/ChemSketch 12.0.

Objetivos

2.1. Objetivo geral

O objetivo geral deste trabalho foi estudar os mecanismos envolvidos no efeito citotóxico do inibidor de PARP Olaparib em um painel de células humanas derivadas de câncer colorretal (CRC). Adicionalmente, buscou-se investigar a influência de variados defeitos genéticos na citotoxicidade do Olaparib, bem como o resultado da combinação deste inibidor de PARP com agentes antineoplásicos comumente empregados no tratamento de CRC.

2.2. Objetivos específicos

- Estudar a influência exercida pelo tempo de exposição ao Olaparib na indução de citotoxicidade em células de CRC;

- Verificar se diferentes mutações associadas ao fenótipo MSI podem sensibilizar células de CRC ao tratamento com Olaparib;

- Investigar se mutações em p53 podem alterar a sensibilidade das células de CRC para o tratamento com Olaparib;

- Analisar a possível influência dos defeitos em PTEN na citotoxicidade do Olaparib;

- Analisar a possível resistência cruzada entre Olaparib e Oxaliplatina, 5-Fluorouracil ou SN-38;

- Determinar que tipos de interações podem resultar da combinação do Olaparib com Oxaliplatin e 5-Fluorouracil em células de CRC;

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REVIEW

PARPs and the DNA damage response

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Adenosine diphosphate (ADP)-ribosylation is an important post-translational modification catalyzed by a variety of enzymes, including poly (ADP ribose) polymerases (PARPs), which use nicotinamide adenine dinucleotide (NAD^+) as a substrate to synthesize and transfer ADP-ribose units to acceptor proteins. The PARP family members possess a variety of structural domains, span a wide range of functions and localize to various cellular compartments. Among the molecular actions attributed to PARPs, their role in the DNA damage response (DDR) has been widely documented. In particular, PARPs 1–3 are involved in several cellular processes that respond to DNA lesions, which include DNA damage recognition, signaling and repair as well as local transcriptional blockage, chromatin remodeling and cell death induction. However, how these enzymes are able to participate in such numerous and diverse mechanisms in response to DNA damage is not fully understood. Herein, the DDR functions of PARPs 1–3 and the emerging roles of poly (ADP ribose) polymers in DNA damage are reviewed. The development of PARP inhibitors, their applications and mechanisms of action are also discussed in the context of the DDR.

Introduction

Cells of multicellular organisms are continuously exposed to both endogenous and exogenous DNA-damaging insults. Cells have evolved intricate mechanisms to protect their genomes, collectively termed the DNA damage response (DDR) (1). The DDR involves multiple cellular processes, including DNA damage sensing, signaling and repair as well as checkpoint activation, local transcriptional blockage, chromatin remodeling and cell death induction (1,2). These cellular responses to DNA damage act in concert to prevent cells from accumulating mutations, which may be lethal or promote carcinogenesis (2,3). Therefore, in addition to ensuring genomic integrity, the DDR processes may contain useful therapeutic targets for anticancer therapy. However, how these complex responses to DNA damage are interconnected and regulated remain to be elucidated.

Abbreviations: ADP, adenosine diphosphate; AIF, apoptosis-inducing factor; BER, base excision repair; CD, catalytic domain; DBD, DNA-binding domain; DDR, DNA damage response; DSB, double-strand break; DSBR, double-strand break repair; NAD^+, nicotinamide adenine dinucleotide; PARP, poly (ADP ribose) polymerase; PARPi, PARP inhibitor; SSB, single-strand break.

[†]These authors contributed equally to this work.

Adenosine diphosphate (ADP)-ribosylation, a posttranslational modification, is a transferase reaction mainly catalyzed by poly (ADP ribose) polymerases (PARPs) in which ADP-ribose units are synthesized and transferred to acceptor proteins using nicotinamide adenine dinucleotide (NAD^+) as a substrate (4). PARPs have been linked to diverse cellular processes, including DDR, chromatin remodeling, genomic imprinting, transcriptional regulation, intracellular trafficking, telomere cohesion, energy metabolism and mitotic spindle formation (4–6). Among the PARP family members, PARPs 1–3 have been reported to be DNA damage responsive and their inhibition is emerging as an innovative approach to cancer therapy (7). However, the involvement of PARPs in the DDR is incompletely understood. A comprehensive understanding of the contribution of PARPs to DNA damage processes is likely to promote further advances in the development and application of PARP inhibitors (PARPis). In this review, we aim to review recent data on the role of PARPs 1–3 in the mammalian DDR.

The PARP family, structure and biochemical activities

ADP-ribosylation reactions and PARP-like genes have been identified in a wide variety of unicellular and multicellular eukaryotes as well as in eubacteria, archaebacteria and double-stranded DNA viruses (8). PARP family members possess a variety of structural domains, span a wide range of functions and localize to various cellular compartments (9). Although the detailed biochemical profiling of each PARP member has not yet been undertaken, recent reviews by Schreiber *et al.* (10) and Hottiger *et al.* (11) describe the different PARP members along with their respective domains, functions and cellular localizations. Because this review focuses on the DDR, the majority of this text will be dedicated to mammalian PARPs 1–3. However, we draw parallels to other cellular processes and PARP members when applicable.

PARP family and structure

For decades, PARP-1 was the only protein known to catalyze the transfer and polymerization of ADP-ribose units (PARylation) from NAD^+ to form a ramified polymer (PAR), which can be covalently linked to a variety of protein targets, including PARP-1 itself (8–11). However, studies over the past decade have identified up to 20 proteins that share homology with the catalytic domain (CD) of PARP-1 (12). These PARP family members were recently grouped under a new unified nomenclature proposed by Hottiger *et al.* (11). According to this new classification based on the CD structure and the ADP-ribosylation activity, the PARP family members (ADP-ribosyltransferases or ARTs) are divided into three groups: (i) PARPs 1–5, which are bona fide PARPs that possess the conserved glutamate residue (Glu 988) required for poly (ADP-ribose) polymerase activity (PARTs); (ii) PARPs 6–8, 10–12 and 14–16, which are confirmed or putative mono (ADP-ribose) polymerases (MARTs) and (iii) PARPs 9 and 13, which are likely to be inactive because they lack key NAD^+-binding residues and the catalytic glutamate (9–11).

The founding member of the PARP family, PARP-1 (ARTD1, ~116 kDa), is a highly conserved nuclear protein with a modular structure that can be divided into three major functional units (Figure 1): (i) an amino-terminal DNA-binding domain (DBD), (ii) a central automodification domain and (iii) a carboxy-terminal CD (9). The DBD is composed of the following: two zinc fingers (FI/Zn1 and FII/Zn2) and one zinc-binding domain (FIII/Zn3) which mediates DNA-binding and DNA-dependent enzyme activation; a nuclear localization signal and a caspase-3 cleavage site (13). The automodification domain contains a BRCT (BRCA1 C-terminal) fold that mediates protein–protein interactions (10–14). The CD is the most conserved domain across the PARP family and contains the following: a PARP signature motif (β–α-loop-β–α NAD^+ fold) that binds NAD^+; a conserved glutamic acid residue and a 'WGR' motif that is named after

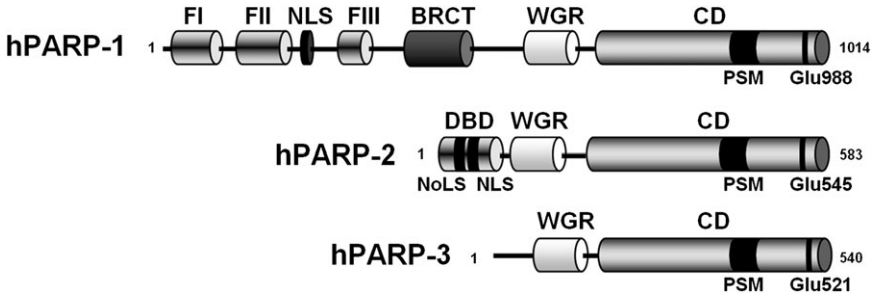

Fig. 1. Schematic representation of hPARPs 1–3 domains. The following domains are indicated: zinc fingers (FI and FII), zinc-binding domain (FIII), nuclear localization signal (NLS), BRCA1 C terminus domain (BRCT), WGR motif (WGR), nucleolar localization signal (NoLS), the PARP signature motif (PSM) and the conserved glutamic acids residues are indicated as darkened regions on CDs. The functional aspects of the domains are noted in text. Protein domains were defined according to the Pfam 25.0 database.

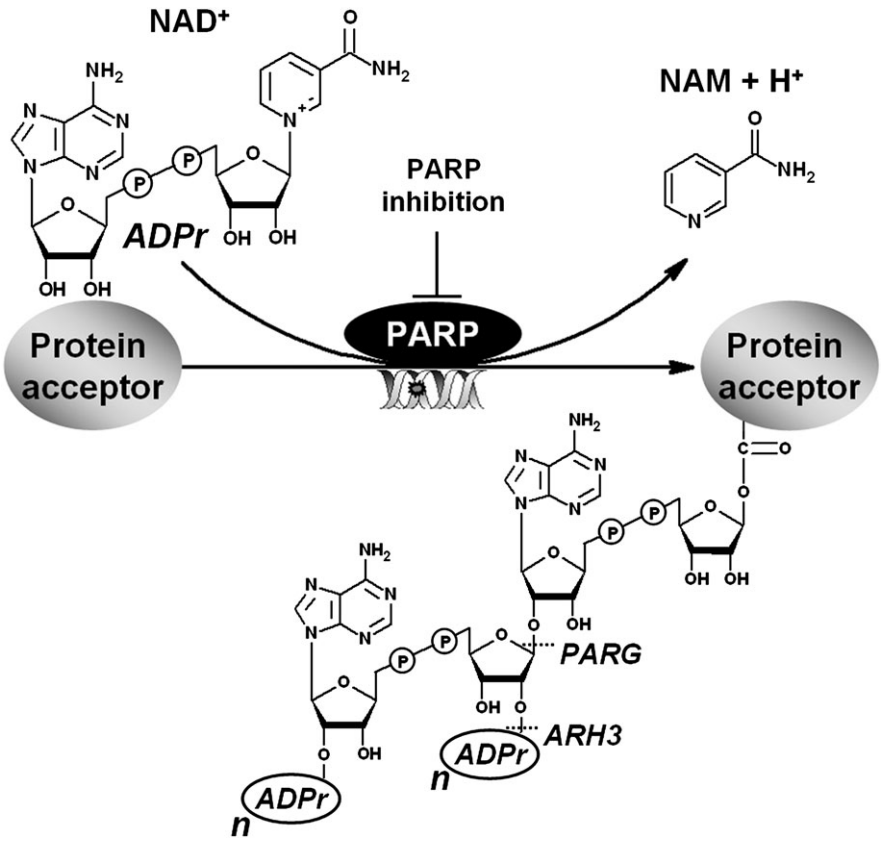

Fig. 2. Poly(ADP-ribosyl)ation reaction. In response to DNA damage or other cellular stimulus, PARPs 1–3 hydrolyse NAD^+ and catalyse the successive transfer of the ADP-ribose moiety to protein acceptors, releasing nicotinamide (NAM) and one proton (H^+). The ADPr units are linked to each other via glycosidic ribose–ribose bonds resulting in linear or multibranched poly(ADP-ribose) polyanions. These PAR polymers are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) and poly(ADP-ribose) hydrolase 3 (ARH3) enzymes (the chemical structures were based on REF. 9 and plotted using ACD/ChemSketch 12.0).

the most conserved amino acid sequence in the motif (Trp, Gly and Arg) and has an unknown function (14).

PARP-2 (ARTD2, ~62 kDa) is the closest PARP-1 paralog and a nuclear protein composed of three functional domains (Figure 1): (i) a basic amino-terminal DBD without zinc fingers that recognizes different DNA structures than PARP-1 and contains an nuclear localization signal and a caspase-8 cleavage site, (ii) a central WGR motif that may interact with protein partners and is subject to automodification and (iii) a conserved carboxy-terminal CD with high homology to the PARP-1 CD that contains a PARP signature motif, which binds NAD^+ and has a glutamic acid residue (15,16).

Finally, PARP-3 (ARTD3, ~60 kDa) is a nuclear protein and is highly related to PARPs 1–2 but lacks a DBD (Figure 1); therefore, it has unclear mechanisms for its activation. PARP-3 possesses an amino-terminal WGR motif of unknown function and a carboxy-terminal CD, which has a conserved PARP signature motif and a glutamic acid residue (12,17,18).

ADP-ribosylation activity and dynamics

ADP-ribosylation is a protein modification that involves the addition of ADP-ribose (ADPr) unit(s) to a target protein and occurs preferentially on glutamate or lysine residues (8). The ADPr attachment is a unique posttranslational modification that alters the activity of target proteins through both steric and electrostatic effects (8). If one or more than one ADPr moiety is added, the transfer reaction is characterized as mono- or poly (ADP-ribosyl)ation, respectively (19,20). The catalysis of ADPr units involves NAD^+ hydrolysis, release of nicotinamide (Nam) and a proton (H^+) and transfer of single or successive ADPr moieties to acceptor proteins (Figure 2) (10). In PAR polymers, the ADPr units are linked to each other via glycosidic

ribose–ribose bonds resulting in linear or multibranched poly (ADP-ribose) polyanions (21). These polymers are large and negatively charged, and they function as posttranslational modifications as well as free polymers (9). Finally, PAR polymers are rapidly degraded by poly (ADP-ribose) glycohydrolase (PARG) and poly (ADP-ribose) hydrolase 3 (ARH3) enzymes, which account for their transient nature (20,21).

The basal levels of PAR polymers are usually low in non-stimulated cells (22). However, in the presence of DNA strand breaks, PARP activity and the levels of PAR polymers are rapidly increased by 10- to 500-fold (22). Most of the PAR produced in response to DNA damage is catalyzed by PARP-1, where its catalytic activity is regulated through different mechanisms, including various posttranslational modifications, allosteric mechanisms, NAD^+ availability and degradation dynamics (22,23). In addition, PARP-2 catalytic activity is also regulated by allosteric mechanisms, posttranslational modifications and cellular NAD^+ levels (16,23). Although the poly (ADP-ribosyl)ation activity of PARP-3 has been recently reported, the mechanisms involved in its activation remain to be determined (12,17).

The roles of PARPs in DDR processes

DNA damage elicits immediate cellular responses in which diverse mechanisms are orchestrated to repair the DNA lesions or induce cell death. As discussed in the following sections, PARPs 1–3 have been reported to modulate various DDR processes to ensure genomic integrity. These mechanisms are strictly regulated by a dynamic feedback of PAR production, which occurs through cycles of PARP binding to DNA damage, PAR synthesis and chromatin dissociation (24,25). This mechanism contributes to the amplification of DNA damage signals as well as the modulation of the DNA lesion environment and the switch between DNA repair or cell death induction.

Despite the most part of PARP literature has been devoted to PARP-1 function in DDR processes, recent results are demonstrating that PARP-2, PARP-3 and PAR polymers may also play decisive roles in response to DNA lesions (12,17–19). The numerous cellular outcomes described for PARPs interplay with DDR proteins are fundamental pieces to understand their roles in DDR. Therefore, a comprehensive summarization of these interactions is presented at Table I, which compares the processes and proteins involved in DDR along with PARPs 1–3 and PAR, including base excision repair (BER; APE-1, DNA polβ, FEN-1, XRCC1 and DNA ligIIIα), single-strand break (SSB) repair (Aprataxin, Condensin I and Xip1), DNA damage signaling (ATR, p53, p21 and ATM), homologous recombination repair (MRE11 and NBS), non-homologous end joining repair (Ku70, K80, DNA ligase IV and DNAPK), chromatin structure modulation (PgC, nucleosome remodeling and deacetylase, Spt16, macroH2A1.1 and ALC1), checkpoint (CHFR and APLF) and cell death induction [apoptosis-inducing factor (AIF), caspase-3 and caspase-8].

DNA damage recognition by PARPs

PARP-1 has been reported to bind to a variety of DNA structures, including cyclobutane pyrimidine dimers, 6,4-photoproducts, apurinic and apyrimidinic sites, cruciforms, SSBs and double-strand breaks (DSBs) (26,58–60). These aberrant DNA structures are recognized by PARP-1's zinc finger FI/Zn1 and FII/Zn2 motifs, and the

Table I. PARPs 1–3, PAR and theirs partners in response to DNA damage

DDR protein	PARP-1		PARP-2		PARP-3		PAR		References
	Interplay	Outcome	Interplay	Outcome	Interplay	Outcome	Interplay	Outcome	
APE-1	Func.	Mod.							(26)
DNA polβ	Func.	Mod.	Func.	Mod.					(27,28)
FEN-1	Func.	Mod.							(27)
XRCC1[a]	Func./phys.	Recr./mod.	Func./phys.	Mod.			Func./phys.	Recr./mod.	(28–30)
DNA ligIIIα	Func./phys.	Recr./mod.	Func./phys.	Recr./mod.			Func./phys.	Recr./mod.	(28,30,31)
Aprataxin	Func./phys.	Recr./mod.			Func./phys.	Recr./mod.	Func./phys.	Recr./mod.	(12,30,32)
Condensin I	Func./phys.	Recr./mod.					Func./phys.	Recr./mod.	(30,33)
Xip1	Func.	Recr./mod.							(34)
ATR[a]	Func./phys.	Mod.							(35)
p53	Func./phys.	Mod.	Func./phys.	Mod.			Func./phys.	Mod.	(30,36,37)
p21	Func./phys.	Mod.					Func./phys.	Mod.	(30,38)
ATM	Func./phys.	Recr./mod.	Func.	NA			Func./phys.	Recr./mod.	(30,39,40)
MRE11	Func./phys.	Recr./mod.					Func./phys.	Recr./mod.	(30,41)
NBS	Func./phys.	Recr./mod.					Func./phys.	Recr./mod.	(41)
Ku70	Func./phys.	Mod.			Phys.	NA	Func./phys.	Recr./mod.	(30,42,43)
Ku80	Func.	Mod.			Phys.	NA			(42,44)
DNA ligase IV	Func.	Mod.			Phys.	NA			(42,43)
DNAPK[a]	Func./phys.	Mod.			Phys.	NA	Func.	Mod.	(30,42,45)
PgC	Func.	Recr./TB			Phys.	NA			(42,46)
Nucleosome remodeling and deacetylase	Func.	Recr./TB							(46)
Spt16[a]	Func.	TB							(47)
macroH2A1.1							Func./phys.	Recr./mod.	(48)
ALC1							Func./phys.	Recr./mod.	(49)
CHFR							Func./phys.	Recr./mod.	(30,50)
APLF	Func.	Mod.			Func./phys.	Recr./mod.	Func./phys.	Recr./mod.	(12,30,51)
AIF							Func.	Cell death	(30,52,53)
Caspase-3	Func./phys.	Cleavage							(54)
Caspase-8			Func./phys.	Cleavage					(55)
PARP-1[a]	Func./phys.	Mod.	Func./phys.	Mod.	Func./phys.	Mod.	Synthesis	Various	(42,56,57)
PARP-2[a]	Func./phys.	Mod.					Synthesis	Various	(56)
PARP-3[a]	Func./phys.	Mod.					Synthesis	Various	(57)

The types of cellular interplays (functional and/or physical) and the resulting interaction outcomes (activity modulation, DDR protein recruitment, PAR synthesis, cell death induction, PARP-cleavage and transcriptional blockage) between PARPs 1–3, PAR and the DDR proteins are presented. Func., functional interplay; phys., physical interplay; mod., activity modulation; recr., DDR protein recruitment; TB, transcriptional blockage; NA, not analyzed.
[a]Proteins PARylated in response to DNA damage.

binding status is relayed to the CD by the FIII/Zn3 motif (61). As a result, the CD of PARP-1 is immediately activated, and its PAR production is estimated to represent 90% of the total PAR synthesis in response to DNA damage (61).

The extent and type of DNA damage recognized by PARP-1 seem to dictate the PARP-1–DNA-binding stoichiometry and activity. Severe DNA damage is linked with high and rapid PARylation activity due to PARP-1 dimerization, where one protein acts as donor of ADPr and the other acts as the acceptor (62). In contrast, less severe DNA damage is accompanied by PARP-1–DNA-binding in a 1:1 stoichiometry and slower PARylation activity generated by automodification (62). Additionally, PAR polymers may also differ in length and branching. The short and branched polymers are degraded more slowly than longer and more linear polymers (63). However, it is still unclear if different DNA lesions are accompanied by the synthesis of different types of PARs and how the intensity of PAR synthesis is dependent on the kind of lesions.

Like PARP-1, PARP-2 is activated by allosteric interaction with DNA lesions. However, due to the structural differences between the PARP-2 and PARP-1 DBDs, PARP-2 was shown to bind less efficiently to SSBs (16,23). Instead, the available data suggest that PARP-2 recognizes gap and flap structures (16,23). The activity of the CD of PARP-2 accounts for 5–10% of the total PAR production in response to DNA damage (56). Interestingly, PARP-2 was reported to homodimerize or heterodimerize with PARP-1 in response to DNA damage. However, the biological implications of these protein complexes remain to be determined (28). Finally, recent studies indicate that PARP-3 is activated *in vitro* by DSBs and plays a role in nonhomologous end joining repair (64). However, the dynamics of PARP-3 activation in response to DSBs remains elusive.

PAR polymers as signaling molecules

PARP-1 is one of the first proteins to recognize damaged DNA and its interaction with DNA lesions triggers the PARylation of a variety of proteins, with PARP-1 itself being the main PAR acceptor (39). Therefore, PARP-1 activation immediately creates long negatively charged PAR polymers attached to PARP-1 at DNA lesion sites (22). Although it has long been recognized that local PAR synthesis is implicated in the recruitment of diverse DDR factors to DNA lesion sites, it was only recently that the molecular mechanisms behind this process have been elucidated. Studies over the past few years have described PAR polymers not only as covalent protein modifications but also as protein-binding matrices (65). Accordingly, a variety of proteins involved in different cellular processes possess motifs or domains that are able to bind PAR and NAD^+ metabolites (65).

Three different PAR-binding motifs/domains have been identified: (i) short conserved motifs, $[HKR]_1$-X_2-X_3-$[AIQVY]_4$-$[KR]_5$-$[KR]_6$-$[AILV]_7$-$[FILPV]_8$, which were identified by experimental and computational approaches and are found in a large set of proteins involved in various cellular processes, including DNA damage signaling and repair, chromatin structural modification and cell death (66); (ii) PAR-binding zinc fingers (PBZ), which are C2H2 zinc finger motifs found in the checkpoint proteins CHFR and APLF and are required to target these proteins to damaged DNA where they act as suppressors of PAR synthesis (50,51) and (iii) macrodomains, which were recently identified in macroH2A1.1 (involved in chromatin compaction and DDR) and ALC1 (involved in nucleosome remodeling). These ancient and highly conserved structures act by targeting proteins to the sites of PAR synthesis (48,49,67).

The PAR-binding motifs/domains target the proteins that contain them to sites of PAR synthesis and regulate their activity upon PAR binding (65). Thus, PAR polymers serve both as posttranslational modifications and as targeting signals required for the recruitment of DDR factors to the sites of DNA lesions. Whether the differences in PAR length and branching influence its targeting properties is currently unknown; however, it could be an elegant explanation for the differential recruitment of DDR factors to different types of DNA lesions. Finally, the list of proteins known to contain PAR-binding motifs/domains is not likely to be exhaustive, and the functional contribution of these motifs/domains to biological processes is still incompletely understood.

PARP involvement in DNA damage signaling and checkpoint activation

In response to DNA damage, several signaling pathways are activated to arrest cell cycle progression and allow the repair of DNA lesions. The cellular response to DNA damage is coordinated primarily by two distinct kinase signaling cascades: (i) the ATR–Chk1 pathway, where ATR is recruited and activated in response to the formation of single-stranded DNA and (ii) the ATM–Chk2 pathway, where ATM is recruited and activated in conjunction with MRE11-Rad50-NBS1 (MRN) sensor complex in response to DNA DSBs (1–3). ATR and Chk1 are crucial for the G_2 and intra-S checkpoints, whereas ATM is an important determinant for G_1 checkpoint induction through p53 activation and p21 expression (1–3).

It is well established that PARP-1 is implicated in the cellular response to SSBs and DSBs. However, it was only recently that PARP activity has been connected to the signaling kinases ATR and ATM. Haince *et al.* (39) showed that ATM and PAR interact via PAR-binding domains and suggested that such interaction is involved in the modulation of DSB signaling and repair (Table I). In agreement with this notion, the disruption of the ATM–PAR interaction prevents the proper localization of ATM to DNA breaks and significantly reduces the phosphorylation of several ATM targets, such as p53, SMC1 and γ-H2AX (39,68). Moreover, experimental evidence suggests that ATM-deficient cells are sensitized to treatment with PARPis (69–72). Altogether, these results indicate that PAR polymers play a fundamental role in both ATM recruitment and activation upon DNA damage (Figure 3). However, a direct relationship between ATM–PAR interaction and checkpoint induction has not yet been established.

Interestingly, PARP-1 was recently reported to physically interact with and PARylate ATR in response to alkylating DNA damage (35). This interaction prevented the ATR-mediated S-phase checkpoint (35,73). Furthermore, PARP-$1^{-/-}$ cells exhibited a stronger Chk1-dependent G_2 checkpoint response following ionizing radiation compared with wild-type cells (74). Collectively, these results indicate that PARP-1 activity may act as a negative regulator of the ATR–Chk1 pathway under certain conditions. Additionally, PARP-1 was shown to directly interact with and activate p53 in response to DNA damage, which in turn is required for the expression of p21 and MDM2 (44,75). Unfortunately, little is known about the involvement of PARP-2 and PARP-3 in DNA damage signaling and checkpoint activation. In particular, PARP-$2^{-/-}$ATM$^{-/-}$ mice exhibit embryonic lethality although a functional interaction between PARP-2 and ATM was not revealed (40).

PARP functions in DNA repair

Although none of the PARP family members have any known DNA repair enzymatic activity, PARP activity has been historically linked to DNA repair (76). This is based on three main observations: (i) DNA damage is the main activator of PAR synthesis, (ii) the depletion or inhibition of PARPs 1–3 sensitizes cells to DNA-damaging agents and (iii) PARPs 1–3 have been reported to interact physically and/or functionally with diverse DNA repair proteins. Additionally, studies in mouse models demonstrated that although PARPs 1–3 are not required for viability, PARP-$1^{-/-}$ or PARP-$2^{-/-}$ animals exhibit a variety of DNA repair defects and chromosomal aberrations (56). Interestingly, PARP-$1^{-/-}$PARP-$2^{-/-}$ double-knockout mice display embryonic lethality along with genomic instability, whereas PARP-$1^{-/-}$PARP-$3^{-/-}$ animals have an increased sensitivity to X-irradiation (56,57). Together, these data indicate both overlapping and non-redundant functions between PARPs 1–3 in DNA repair and genome maintenance.

PARP-1 has been reported to be involved in different DNA repair systems, including BER, single-strand break repair and double-strand break repair (DSBR) (14). Although the precise role of PARP-1 in DNA repair is still under debate, the data accumulated so far clearly indicate that PARP-1 plays an important role in the early steps of

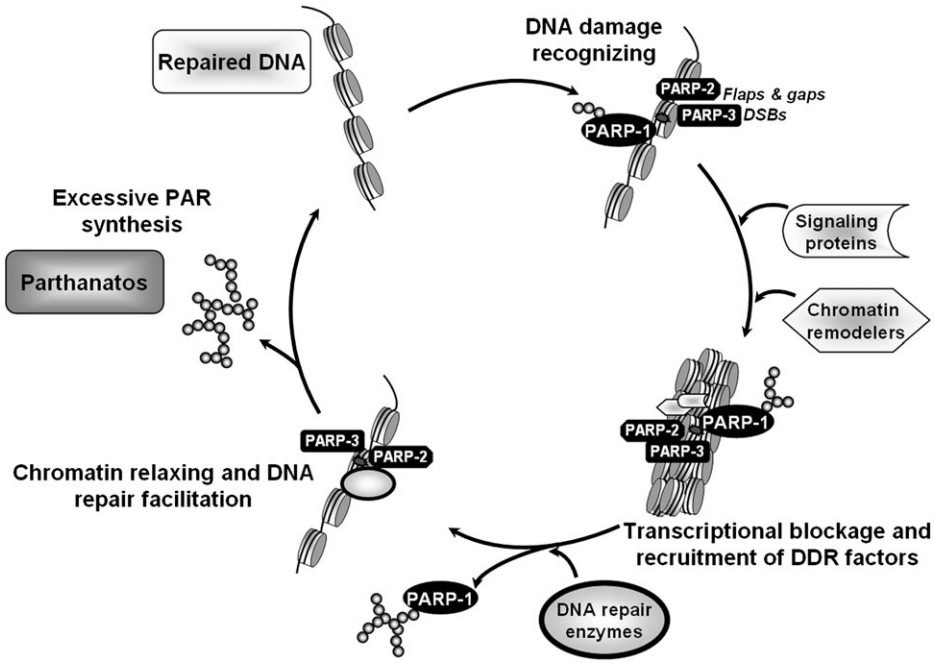

Fig. 3. PARP response to DNA damage. PARP-1 recognizes various DNA lesion types, targets DDR factors to the damage site, prevents transcription and facilitates DNA repair through chromatin remodeling and protein–protein interactions and switches DNA repair to programmed cell death when DNA damage levels are beyond the DNA repair capacity. PARP-2 is involved in flap and gap structure recognition, whereas PARP-3 responds to DSBs. Both PARP-2 and PARP-3 interact with and modulate the activity of various proteins involved in DDR, including PARP-1.

DNA repair targeting and in modulating the DNA repair proteins at the sites of DNA lesions (Table I). Furthermore, PARP-1-dependent chromatin remodeling was shown to facilitate the access of DNA repair proteins to DNA damage (discussed below). Moreover, PARP-1 may protect DNA ends and apyrimidinic sites until DNA repair proteins become available to repair the DNA lesions (26,77).

PARP-2 was also shown to interact with BER/single-strand break repair proteins (Table I). Since PARP-2 accumulation at repair sites is not immediate, PARP-2 may preferentially be involved in the late steps of BER/single-strand break repair (14). Additionally, PARP-$2^{-/-}$ATM$^{-/-}$ and PARP-$2^{-/-}$p53$^{-/-}$ mice exhibit total or partial embryonic lethality, which suggests the involvement of PARP-2 in DSBR (37,40). In contrast, PARP-3 is recruited to DSB sites and may modulate DSBR through physical interaction with non-homologous end joining repair proteins as summarized in Table I (42,64). Intriguingly, both PARP-2 and PARP-3 were shown to interact with and modulate PARP-1 activity in response to DNA damage (42). Nevertheless, several other PARP members as tankyrases 1 and 2 and PARP-14 are implicated in genomic instability and therefore may also be involved in DNA repair (9–11). However, the detailed contributions of PARPs to DNA repair have not yet been determined.

PARPs and chromatin remodeling in response to DNA damage

The modulation of chromatin structure in response to DNA damage has been shown to play a fundamental role in DNA damage detection and repair (78). Evidence supports PARP-1's involvement in chromatin modulation under both stressed and non-stressed conditions (79). In response to DNA damage, PARP-1 establishes a transient repressive chromatin structure at the sites of DNA lesions, thus blocking transcription and facilitating DNA repair (41,46). This transcriptional blockage was shown to result from the activities of polycomb group (PcG) and nucleosome remodeling and deacetylase complexes, which are recruited to DNA damage sites in a PARP-1-dependent manner (46). Interestingly, PARP-3 was found to be part of the PcG complex; however, its functional role has yet to be elucidated (42). The recruitment of PcG and nucleosome remodeling and deacetylase to DNA damage sites is accompanied by the removal of nascent RNA and elongating RNA polymerase II from sites of DNA damage, thereby preventing active RNA polymerase II complexes from interfering with the recruitment and activity of repair proteins (46).

Additionally, the PARylation of Spt16 (a component of the histone chaperone, FACT) in response to DNA damage was reported to facilitate DNA repair (47). Because the FACT complex is involved in the exchange of histone H2A for H2AX during DNA repair, the PARylation of Spt16 could prevent the H2AX exchange and lead to the stabilization of nucleosomal H2AX (80). Furthermore, it has been observed that due to the accumulation of negatively charged PAR units, PARP-1 concomitantly loses its affinity for DNA strand breaks. This mechanism has been proposed to relax the structure of chromatin thereby resulting in more efficient DNA repair (81–83). Thus, the accumulated data suggest that PARP-1 may modulate chromatin structure to facilitate DNA repair processes.

PAR-dependent cell death induction—parthanatos

When the levels of DNA damage are beyond the cellular repair capacity, programmed cell death is activated to prevent cells from accumulating mutations that may lead to carcinogenesis. Because PARylation is a DNA damage-dependent enzymatic activity, extensive DNA damage is accompanied by large-scale PAR polymer synthesis (84). However, excessive PAR production may leads to a unique form of caspase-independent cell death, termed parthanatos (85). The morphological aspects of parthanatos were observed in neuronal cells and include the following: shrunken and condensed nuclei, membrane disintegration and rapid propidium iodide staining (52,53,86). This type of cell death is associated with rapid PARP-1 activation, early PAR accumulation, mitochondrial depolarization, early AIF translocation, loss of cellular NAD and adenosine triphosphate and late caspase activation (52,53,86). In addition, the point at which a cell commits to parthanatos is when AIF translocation activates the cell death process; therefore, caspase activation has been reported to act as a bystander in PAR-dependent cell death (85).

The mechanistic aspects of parthanatos involve the nuclear synthesis of PAR and its translocation to the cytosol, where the polymers colocalize and interact with mitochondria (52,53). This interaction

triggers AIF release, which in turn translocates to nucleus, binds to DNA and mediates large-scale DNA fragmentation and cell death (52,53). Although the main events in parthanatos have been identified, the exact mechanism by which PAR is transported to the cytosol and induces AIF release remains largely unknown. Interestingly, Andrabi *et al.* (53) demonstrated that PAR toxicity is dose and structure dependent. Highly complex and long-chain polymers are more toxic than simpler and shorter polymers (53). Therefore, PAR polymers act as cell death signaling molecules, which may deliver different death messages depending on their length and branching characteristics. These results support PARP activity as an elegant switch between DNA repair and programmed cell death induction.

PARP inhibition in therapeutics and the DDR

PARPis are generally based on benzamide or purine structures designed to compete with NAD^+ in the CD of PARP-1 and/or PARP-2 (Figure 2) (87). The current PARPis are emerging as promising anticancer agents, which are able to sensitize tumor cells to DNA-damaging agents or induce synthetic lethality as single drugs in cancer cells with DSBR defects (88). According to the concept of synthetic lethality, the inhibition or deletion of either of two genes is tolerable, whereas the combined deletion and inhibition of both genes leads to death (89,90). Therefore, this therapeutic approach combines the synthetic inhibition by an anticancer drug with a pre-existent oncogenic mutation to selectively kill the tumor cells. The final result is an increase in the therapeutic index coupled with a reduction of the toxic side effects.

The best documented synthetic lethal interaction is the one observed between BRCA1 or BRCA2 defects and PARP inhibition (91). This lethal condition has been attributed to the accumulation of spontaneous DNA damage in cells with decreased PARP activity, which facilitates the conversion of these lesions into harmful DSBs. Normal cells that lack DSB repair defects are able to correctly repair the DSBs resulting from PARP inhibition, whereas tumor cells harboring a BRCA1 or BRCA2 defects accumulate highly toxic DSBs, which lead to potent and selective cytotoxicity (92). However, it is possible that additional factors may contribute to the hypersensitivity of BRCA1- or BRCA2-deficient tumors to PARPis (89). Indeed, an alternative non-exclusive model argues that since PARylation is needed to dissociate PARP-1 from DNA damage, its chemical inhibition could trap PARP-1 at the lesion site, which may cause the obstruction of replication forks and consequently toxic one-ended DSBs (93).

In agreement with both models, some authors have been demonstrating that PARP inhibition in cells harboring BRCA1/2 deficiency elicit rapid and profound arrest of cells in G_2 or M phase of cell cycle followed by apoptosis induction (94–96). These results have been attributed to the accumulation of chromosome aberrations, which may arise from inefficient DSBR capacity of BRCA1/2-defective cells (94–96). However, the mechanisms involved in the potential toxicity of PARPis in cells with normal DSBR activity are incompletely understood. Some authors observed DNA damage accumulation and apoptosis induction in cells with normal BRCA1/2 activity but due to the variability of experimental conditions is difficult to compare the available data (97–99). Therefore, further analysis is necessary to understand the potential cytotoxicity of PARPis in cells with normal DSBR activity.

Olaparib (AZD2281 - AstraZeneca) was the first PARPi tested in clinical trials using the synthetic lethal approach for BRCA1- or BRCA2-deficient tumors and has been undergoing several phase I and II studies, both as a single agent and in combination with DNA-damaging agents (100). Olaparib has been mainly administered to ovarian and breast cancer patients, and its clinical benefit rate may reach 66% for high doses with mild toxicity (mainly grade 2 or less) (90). Other promising PARPis include the following: Veliparib(ABT-888—Abbott) tested in BRCA1- or BRCA2-deficient tumors, glioblastoma and leukemia (101) and AGO 14699, tested in advanced solid tumors and melanomas (102).

These and other exciting findings have been moving the PARP family members from interesting subjects of molecular analysis to the forefront of cancer therapy as clinical targets (9). However, because the primary focus of PARPi research has been on their therapeutics aspects, relatively little is known about their biological effects. As discussed herein, PARPs 1–3 modulate diverse processes in response to DNA damage, which may not all be equally inhibited by PARPi treatment. Moreover, given the high level of conservation across PARP CDs, these inhibitors may affect the other PARP family members, mono-ADP-ribosyl transferases and sirtuins, although the extent of this inhibition remains to be determined (89). Additionally, the influence of long-term exposure of both normal and tumor cells to PARPis is also largely unknown (89). Finally, the characterization of the PARP family members' roles in DDR processes is likely to provide new targets for synthetic lethal strategies as well as accelerate the development of more effective inhibitors to target specific interactions and increase their therapeutic index.

Concluding remarks

The research discussed in this review demonstrates that PARPs 1–3 are multifunctional DNA damage sensors, which recognize different types of DNA lesions, target DDR factors to damage sites, prevent transcription and facilitate DNA repair through chromatin remodeling and protein–protein interactions. They can also switch a cell from DNA repair to programmed cell death, when the levels of DNA damage are beyond its DNA repair capacity (Figure 3). These mechanisms are strictly regulated by dynamic PARP-1 feedback through cycles of DNA damage-binding, PAR synthesis and chromatin dissociation (24). Thus, PARPs 1–3 are central players in the DDR by modulating and integrating diverse processes, which ensure the repair of DNA damage or induce cell death. The roles of PARP-2 and PARP-3 in response to DNA damage are beginning to be established (Figure 3). Although PARP-2 is involved in flap and gap structure recognition, PARP-3 responds to DSBs. Moreover, both PARP-2 and PARP-3 are able to interact with and modulate the activity of various proteins involved in the DDR, including PARP-1. These data indicate both overlapping and non-redundant functions between PARPs 1–3 in response to DNA damage.

Although the mechanisms by which PARPs orchestrate so many DDR processes are not well established, recent studies support an important role for PAR as signaling molecules involved in protein targeting and apoptosis induction. As it was shown that PAR with different lengths and branching characteristics can differentially influence cell death induction, one may speculate that the chemical structure of these polymers may act as a code in PAR-dependent processes. Although the real significance for the differences in PAR structure is still unclear, this could be an elegant explanation for PARP-specific activities. Further studies of PARP functions in the DDR will certainly provide clues and perhaps novel targets to improve cancer therapy in the coming years.

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Capítulo II

Olaparib cytotoxicity toward colorectal cell lines

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Abstract

The exciting possibility to selectively kill cancer cells has been moving poly(ADP-ribose) polymerase (PARP) inhibitors (PARPis) from interesting molecular tools to the forefront of cancer therapy research. However, the basic mechanisms involved in PARPis cytotoxicity are still poorly studied and its clinical applications are restricted to a small number of malignances. With the aim to study basic and applied aspects of PARPis, Olaparib cytotoxicity was investigated in a CRC cell line panel. The results demonstrated that Olaparib is a slow action drug, which cytotoxicity is significantly increased with long term exposure. Additionally, defects or mutations in *MLH-1* and *PTEN* are shown to sensitize CRC cells to Olaparib treatment, while p53 status does not influence on its effects. Further, neither Oxaliplatin (Oxp) nor 5- Fluorouracil (5-Fu) resistant cell lines present cross-resistance to Olaparib, whereas a pronounced cross-resistance was observed for SN-38 resistant cell line. Finally, Olaparib associations with Oxp or 5-Fu were shown to sensitize cells with both relative and acquired resistances. The data discussed here amplifies the applications to Olaparib in cancer therapy, as well as points to the necessity of identification of basic and applied mechanisms by which PARPis exert its effects on CRC cells.

Keywords: Olaparib; Colorectal cancer; microsatellite instability; PTEN; p53.

1. Introduction

Carcinogenesis is a dynamic multistep process analogous to Darwinian evolution, in which a succession of genetic alterations, each conferring one or another type of growth advantage, leads to the progressive selection and conversion of normal human cells into cancer cells (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). An important part of these physiological advantages selected in cancer cells are associated with mutations in genes involved in genomic maintenance, tumor suppression and cell signaling (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). These genetic alterations have been studied for decades; however, just recently, they became intelligently explored by a therapeutic approach called synthetic lethality. According to the concept of synthetic lethality, the inhibition or deletion in either of two genes is tolerable, but the combined deletion or inhibition of both genes leads to cell death (Rouleau *et al.*, 2010; Banerjee *et al.*, 2010). The clinical application of this promising approach combines the synthetic inhibition induced by the anticancer drug with the preexistent oncogenic mutations to selectively kill tumor cells, thus increasing the therapeutic index coupled with the reduction of side effects (Rouleau *et al.*, 2010; Banerjee *et al.*, 2010).

The current poly(ADP-ribose) polymerase (PARP) inhibitors (PARPis) are emerging anticancer drugs able to sensitize tumor cells to DNA damaging agents or to induce synthetic lethality as single drugs in cancers with double strand-break (DSB) repair defects (Lord & Ashworth, 2008). Under developing by AstraZeneca, Olaparib (AZD2281) specifically binds and inhibits the nuclear enzymes PARP-1

and -2, and has been subject to several phase I and II trials, either as a single drug or in combination with DNA damaging agents (Sandhu *et al.*, 2010). The administration of Olaparib as a single drug was shown to induce synthetic lethality in cancers with defects in *BRCA1* or *BRCA2*. This lethal condition has been attributed to the accumulation of spontaneous DNA damages in cells with inhibited PARP activity, which lesions may be converted to the harmful DSBs (Carden *et al.*, 2010). Normal cells are able to correctly repair DSBs resulting from PARP inhibition, whereas tumor cells defective in *BRCA1/2* accumulate the non-repaired DSBs since both proteins are essential for DNA repair by homologous recombination, which leads to high and selective cytotoxicity (Carden *et al.*, 2010).

At the moment, only the lethal interaction between PARP inhibition and *BRCA1/2* mutations was probed and applied in clinical trials (Helleday, 2011). However, given the vast list of genetic mutations related with tumor development, new lethal interactions are expected to be discovered in the next years (Helleday, 2011). Indeed, colorectal cancers (CRC) are potential targets to selective-chemotherapeutic approaches due to its high incidence of mismatch repair (MMR), PTEN and p53 defects. Mutations in MMR genes are associated with microsatellite instability (MSI) phenotype, which are observed in 10-15% of all CRCs and may affect the function of numerous downstream genes as a consequence of microsatellite repeat track mutations (Hampel *et al.*, 2005). The tumor suppressor PTEN is one of those downstream genes and is mutated in approximately 18% of MSI-CRCs (Nassif *et al.*, 2004). Additionally, mutations in the tumor suppressor p53 account up to 50% of all CRCs and are associated with poor prognosis (Rodrigues *et al.*, 1990).

The exciting possibility to selectively kill cancer cells has been moving PARPis from interesting molecular tools to the forefront of cancer therapy research (Krishnakumar & Kraus, 2010). However, the exact mechanisms involved in PARPis cytotoxicity are still under intense debate (Rouleau *et al.*, 2010; Helleday, 2011). Additionally, the result of PARPis treatment into cells with other DNA repair defects than *BRCA1/2* defects is largely unknown. Finally, the effect of PARPi combinations with established anticancer agents as well as the possible cross-resistance between them is still poorly characterized. Therefore, the aim of this study was to investigate the effect and cytotoxicity mechanisms of the PARP inhibitor Olaparib in a CRC panel cell lines with different genetic backgrounds and characteristics, including MSI, p53 and PTEN deficiencies. Moreover, the combination of Olaparib with Oxaliplatin (Oxp) and 5-Fluorouracil (5-Fu) as well as the possible cross-resistance between these antineoplasic drugs were also evaluated.

2. Material and Methods

2.1. Cell culture and chemicals

The HCT15, KM12, Lim2405 and DLD-1 cell lines were kindly provided by Dr. Richard Hamelin. HT-29 was donated by Institut Gustave-Roussy (IGR), Lovo92 was a gift from Dr. Amélie Lansiaux and SW620 was provided by American Type Culture Collection (ATCC). The HCT116, as well as the HCT116 p53 null, were provided by Dr. Bert Vogelstein. The HCT116 were employed to develop the drug resistant cell lines HCT116 5-Fu resistant, HCT116 Oxp resistant and HCT116 SN-38 resistant were developed in the laboratory of Annette K Larsen. All the cell lines were cultivated accordingly to the provider recommendations. The culture media DMEM, RPMI and McCoy, as well as the antibiotics, were obtained from PAA cell culture company (Pashing, Austria). The bovine fetal serum was purchased from Eurobio (Courtaboeuf, France). Olaparib (AZD2281) was obtained from Axon MedChem (Groningen, Netherlands), 5-Fu was purchased from Teva-Pharma (Courbevoie, France), SN-38 (*7-Ehyl-10-Hydroxy-20(s)-Camptothecin*) was acquired from Abatra technology (Xi'An, China) and Oxaliplatin (Eloxatin, *oxalato-(trans-L-1,2-diaminocyclohexane*) platinum(II) was purchased from Sanofi-Aventis.

2.2. Growth inhibitory effect evaluation

The growth inhibitory effect of Olaparib as a single agent was determined by both colony formation assay and MTT (*methylthiazolyldiphenyl-tetrazolium bromide*), while the combinations of Olaparib and 5-Fu or Oxp were accessed only by MTT. For the clonogenic assay, cells in logarithmic phase were washed, counted and

plated (300-400 cells). The cells were seeded 4 hours before the administration of an Olaparib range. After 12 days of continuous treatment the media was apirated, the cell colonies were fixed with absolute ethanol, stained with giemsa 5% (Sigma-Aldrich) and visually scored. All curves represent an average of at least three independent experiments, each done in triplicate (treatments) or quintuplicate (controls). The MTT assay was performed as previously described by Poindessous *et al*. (2003). Cells were continuously exposed to ranges of 5-Fu, Oxp or olaparib as single drugs, or to combinations of 5-Fu or Oxp ranges with 5μM of Olaparib for five generation times (120 h). All curves represent an average of at least three independent experiments, each done in duplicate. The dose-effect curves of Olaparib combinations with Oxp and 5-Fu were analyzed by the median-effect method of Chou and Talalay (Chou, 2010) using the Calcusyn Software (Biosoft, Cambridge, UK).

2.3. Micronucleus test

The cells ($2x10^5$) were plated in 60mm Petri dishes 24 hours prior to treatments. In order to observe the long term effect of PARP inhibition in micronucleus induction, cells were continuously exposed to treatments for 5 days (120 hours). Based on the MTT results, Olaparib was employed in a range with 1μM as the higher dose. Diflomotecan (5μM) was used as a positive control. To obtain the binucleated cells, 1μg/mL of cytochalasin-B was added 30 hours before the end of treatments. The procedures for harvesting, fixation and staining were carried out as previously described by Matsushima and colleagues (1999). A total of 4000 cells were analyzed per treatment for the presence of micronucleus in optical microscopy with

1000× of magnification. The micronucleus frequencies were evaluated by the statistical test analysis of variance (one way ANOVA) and Bonferroni´s post-test was used to compare treatment groups.

3. Results and discussion

3.1. Olaparib is a slow drug

A CRC cell line panel, presented in supplementary data (Table S1[1]), was continuously exposed to an Olaparib range and the percent of survival was accessed after 5 days of treatment using MTT or after 12 days of treatment using CFA (Colony Formation Assay). Surprisingly, remarkable cytotoxicity differences between MTT and CFA assays were observed for the most sensitive cell lines (Supplementary data - Table S2[2]). Since MTT assay is based on the enzymatic reduction of MTT to formazan and CFA assay is based on the clonogenic capability of cells, the differences observed would be due to the time of exposure or to the assay used. To address this question a 12 days MTT was performed employing only the most sensitive and resistant cell lines, respectively HCT116 and HT-29. Interestingly, the differences observed between CFA and MTT were shown to be drastically minimized in 12 days MTT (Figure 1A).

[1] Tabela apresentada em anexos como A - I.
[2] Tabela apresentada em anexos como A - II.

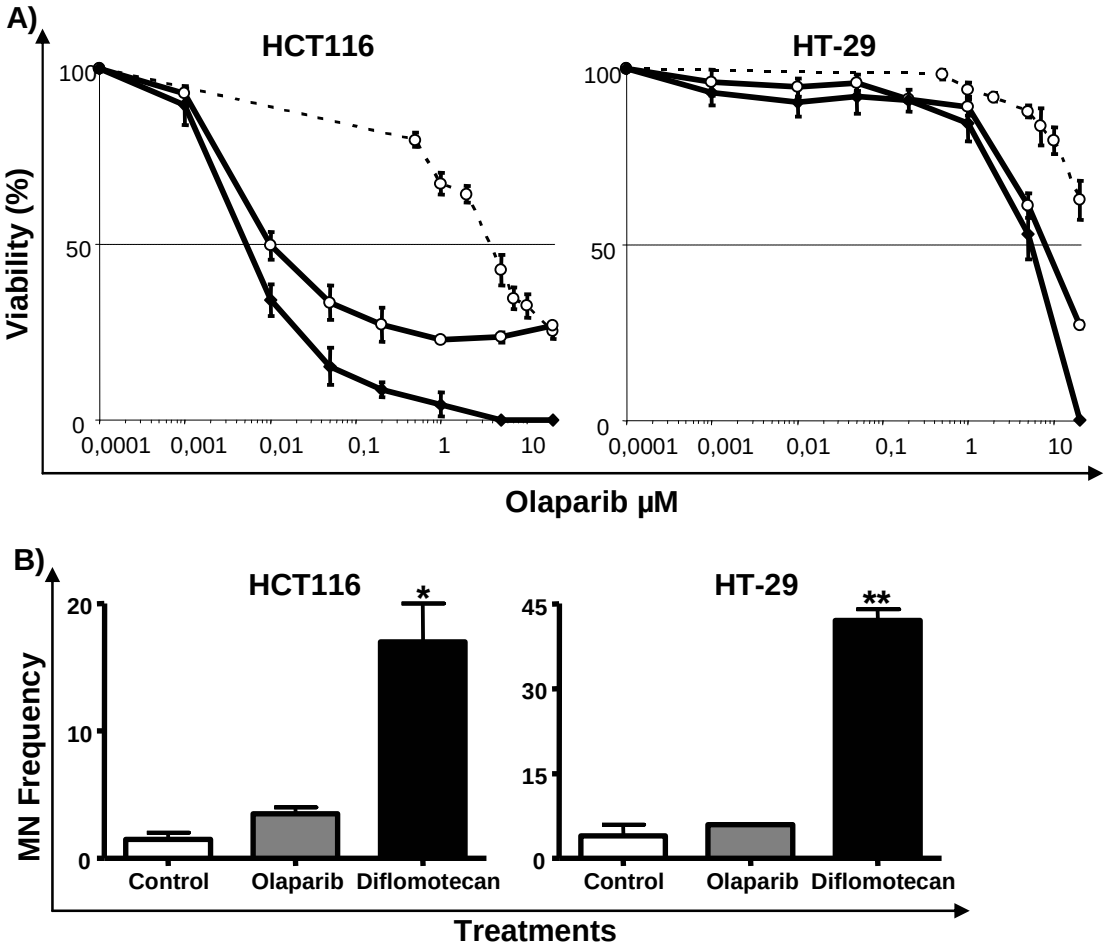

Figure 1. Olaparib effect: comparison between assay, time of exposure and DNA damage induction. A) Comparison between 5 days MTT (▪ ○ ▪), 12 days MTT (—○—) and 12 days CFA (—◆—). Cells were exposed to a fixed range of Olaparib for different times of exposure (5 or 12 days) and using different assays (MTT and CFA). The means represent at least three independent experiments; B) Micronucleus frequencies were evaluated after 5 days of continuous treatment. Cells ($2x10^5$) were exposed to an Olaparib range with 1µM as the higher dose. Diflomotecan (5µM) was used as a positive control. Statistic significance of $p<0.05$ (*) and $p<0.01$ (**) were accessed by ANOVA-one way and Bonferroni's post test employing GraphPad Prism 5.

These results are in agreement with the DNA damage accumulation model for PARPi cytotoxicity (Helleday, 2011). According to this model, PARP inhibition may lead to impaired DNA repair and consequently to accumulation of DNA lesions. Since the most part of PARPi-resulting DNA lesions are assumed to arise from endogenous sources, one may expect PARPi-cytotoxicity for long term exposures (Helleday, 2011). Indeed, no statistically significant micronucleus frequencies were observed for 5 days of continuously exposure to Olaparib (Figure 1B). Therefore, the time of exposure to Olaparib is a crucial factor to achieve its maximum cytotoxic effect, which is likely to result from long-term DNA damage accumulation.

3.2. Influence of MSI, PTEN, p53 and drug-resistance phenotypes status on sensitivity to Olaparib

Once the cancer cells of different patients may be similar in genetic mutations but different in genetic backgrounds, the comparison between cell lines with similar mutations and different genomic backgrounds is expected to be a more realistic approach in comparison to the use of only isogenic cell lines. Therefore, Olaparib effect on MSI, PTEN, p53 and drug-resistance phenotypes status was accessed by colony formation assay using a non-isogenic CRC cell line panel and confirmed employing isogenic cell lines when applicable (Figure 2). CRC panel cell lines were continuously exposed to an Olaparib range for 12 days. The most resistant and the most sensitive cell lines, respectively HT-29 and HCT116, are presented in all figures to compare the relative-resistance/sensitivity.

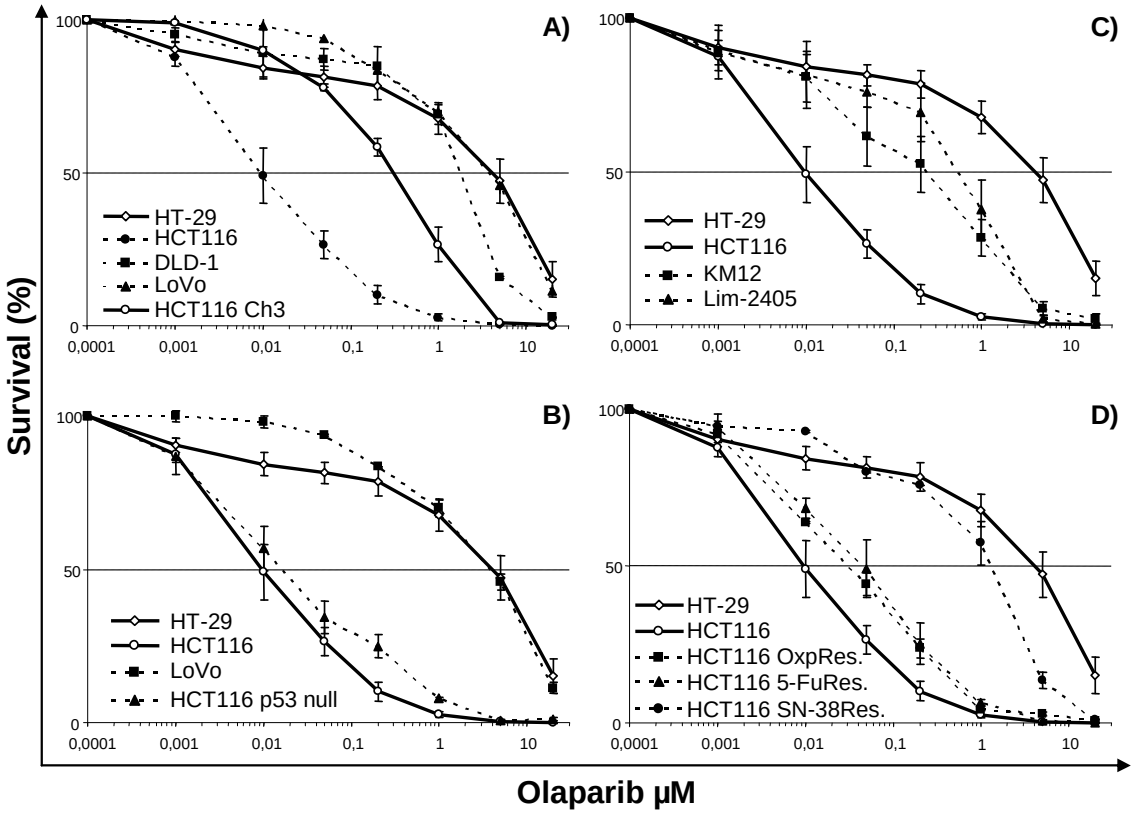

Figure 2. Influence of MSI, PTEN, p53 and resistance phenotypes status on the sensitivity to Olaparib. A) Comparison between MSI cell lines (HCT116, DLD-1 and LoVo) and non-MSI cell lines (HT-29 and HCT116 Ch3); B) Comparison between p53 mutant cell lines (HTC116 $p53^{-/-}$ and LoVo) and normal p53 (HCT116 and HT-29); C) Comparison between PTEN null cell lines (KM-12 and Lim2405) and (HT-29 and HCT116) and D) Comparison between acquired drug resistance phenotypes (HCT116 resistant to Oxaliplatin, 5-Fluorouracil and SN-38) and normal cell lines (HT-29 and HCT116). Cells (300-400) were continuously exposed to a range of Olaparib for 12 days and the percentage of survival was obtained by means of three independent colony formation assays.

3.2.1. Olaparib presents high cytotoxicity toward MLH1-MSI cell line

To study the influence of the MSI phenotype on Olaparib cytotoxicity three CRC cell lines harbouring MMR mutations in *MLH1* (HCT-116), *MSH2* (LoVo) and *MSH6* (DLD-1) were compared to HT-29, a non-MSI cell line (Figure 2A). Interestingly, HCT-116 was the only MSI cell line to show a pronounced sensitivity to PARP inhibition, thus suggesting *MLH1* as an important determinant on Olaparib sensitivity. To confirm *MLH1* importance on Olaparib-induced cytotoxicity, a HCT-116 cell line carrying an exogenous chromosome 3 with a functional copy of *MLH1* was employed. This non-MSI cell line presented a partial sensitivity to Olaparib when compared to the parental HCT116, which indicate *MLH1* as an important, but not exclusive, determinant on Olaparib sensitivity in this cell line.

The results presented at Figure 2A indicated that just specific mutations associated to the MSI phenotype may influence Olaparib cytotoxicity toward CRC cell lines. In agreement, Vilar and colleagues (2011) showed that *MRE11*-mutant MSI cell lines were more sensitive to the PARPi ABT-888 than others CRC-MSI cell lines. Together, these results suggest that other DNA repair deficiencies than *BRCA1/2* defects may also sensitize tumor cells to PARPis, thus broadening the applications to PARPis in cancer therapy. Moreover, since *MLH1* encodes an essential protein (Mlh1) to MMR and its defects are associated with a variety of malignances including hereditary nonpolyposis colon cancer (HNPCC), it is possible that PARPis may help to treat other *MLH1*-associated diseases (Wimmer & Etzler, 2008).

3.2.2. p53 does not interfere on CRC cell lines sensitivity to Olaparib

p53 is a central tumor suppression protein involved in a wide range of cellular processes in response to DNA damage, which include DNA damage signaling, regulation of DNA repair enzymes expression and activity, and apoptosis induction (Bernstein *et al.*, 2002). This genomic guardian protein is a difficult obstacle to tumoral development of incipient cancer cells and, therefore, is frequently mutated or inactivated in a variety of cancer cells (Bernstein *et al.*, 2002). To access p53 influence on Olaparib cytotoxicity, a p53 mutant cell line (HT-29) was compared to a normal p53 cell line (LoVo). Additionally, the results were confirmed by comparing a HCT116 $p53^{+/+}$ to a HCT116 $p53^{-/-}$. Surprisingly, both p53 mutant and null cell lines presented a similar sensitivity to Olaparib in comparison to the normal p53 cell lines (Figure 2B). Thus, indicating that p53 status does not interfere on CRC cell lines sensitivity to Olaparib.

Although p53 do not have any direct enzymatic repair role, the activity of this tumor suppressor is directly correlated to DNA damage levels (Bernstein *et al.*, 2002). Thus, one may expect an increased sensitivity of p53 mutant/null cells to a variety of antineoplasic drugs, including PARPis. However, as single drugs, PARPis cytotoxicity seems to be dependent on the accumulation of endogenous DNA damages, which may arise from oxidative metabolism and replication errors, among others (Yélamos *et al.,* 2011). Once PARPi-derived DNA lesions levels are low and constant, perhaps p53 activity is not elicited by them, but at the long term the accumulated mutations are likely to compromise cell viability.

3.2.3 PTEN null cell lines were sensitize by Olaparib

PTEN is a tumor suppressor which acts as a negative regulator of AKT and consequently as a cell cycle and survival repressor (Bunney & Katan, 2010). Recently, PTEN mutations were shown to sensitize ovarian and endometrial cancer cells to PARPis, which have been associated to a newly described secondary role of PTEN in RAD51 transcription regulation (Mendes-Pereira *et al.*, 2009; Dedes *et al.*, 2010). To verify PTEN status influence on Olaparib cytotoxicity, two PTEN null CRC cell lines (KM12 and Lim2405) were compared to two normal PTEN cell lines (HT-29 and HCT116) (Figure 2C). The PTEN status of these cell lines was confirmed by western blot (Supplementary data - Figure S1[3]). Interestingly, PTEN deficient cell lines showed an intermediate cytotoxicity profile, which indicates that PTEN status may also sensitize CRC cells to Olaparib.

Despite its important functions in cell cycle and survival processes, it has been recently demonstrated that PTEN defective cells present significant fewer RAD51 foci upon DNA damage (Shen *et al.*, 2007; Dedes *et al.*, 2010). Once RAD51 is directly involved in homologous recombination (HR) repair, PTEN defects were proposed to compromise DSB repair and sensitized cells to PARP inhibition (Shen *et al.*, 2007; Dedes *et al.*, 2010). In agreement, the results presented at the Figure 2C indicated that, when compared to relative-resistant cells, PTEN null CRC cells were sensitize by Olaparib treatment. However, the modest cytotoxicity observed in CRC cells suggests that other factors may modulate PARPis effect on cell lines from different tissues.

[3] Figura apresentada, nesta tese, em anexos como A - III.

3.2.4. Olaparib present cross-resistance with SN-38

Drug resistance, either pre-existing (intrinsic resistance) or induced by therapeutic drugs (acquired resistance), is associated with high rates of chemotherapy failure (Gottesman, 2002). The strategies frequently employed to overcome resistant phenotypes are based on changes in antineoplasic drugs treatments (Gottesman, 2002). However, frequently the new drug shares resistance mechanisms with the original antineoplasic agent, which is known as cross-resistance (Gottesman, 2002). To investigate the possible cross-resistance between Olaparib and common antineoplasic drugs used to treat CRC, three resistant cell lines derivate from HCT116 were employed. These cell lines were generated by long time of chronic exposure to 5-Fu, Oxp and SN-38 (the active metabolite of Irinotecan). Interestingly, the results presented at the Figure 2D indicated that only the HCT116 SN-38 resistant cell line showed cross-resistance to Olaparib.

Resistance to synthetic lethal interaction was observed in *BRCA1/2* mutant breast cancer cells, in which reverse mutations able to restore *BRCA1/2* activity have shown to inhibit the lethal effect of PARPis administration (Ashworth, 2008). Additionally, the long term exposure to Olaparib was reported to induce acquired resistance through up-regulation of *Abcb1a/b* and *Abcg2* genes encoding P-glycoprotein efflux pumps (Rottenberg *et al.*, 2008). Interestingly, the acquired resistance to Irinotecan and its metabolite SN-38 was also associated to polymorphisms at *Abcb1a/b* and *Abcg2* gene promoters, which regulate their expression (Han *et al.*, 2007). Therefore, the cross-resistance between Olaparib

and SN-38 is likely to be the result of common P-glycoprotein efflux pumps up-regulation.

3.3. Olaparib synergizes with Oxaliplatin and 5-Fluorouracil

Therapeutic approaches associating anticancer agents are frequently employed in clinic to reduce drug toxicity and resistance. This result is achieved when the combined drugs effect is stronger than the addition effect of both agents separately (Chou, 2010). This effect, designated synergism, represents an improved manner to diminish side effects and the amount of administered drug, which in turn increases the therapeutic index and treatment effectiveness (Chou, 2010). As a single drug, Olaparib requires long term exposure to induce cytotoxicity (Figure 1). However, in combination with other antineoplasic agents, a synergistic effect is observed at shorter exposure times. The Figures 3 and 4 present the combination indexes for Olaparib associations with Oxp and 5-Fu, respectively. Cell lines were exposed to a range of Oxaliplatin or 5-Fu with or without a fixed Olaparib dose (5µM). The viability was accessed by MTT assay after five days of continuous treatments and the drug interactions were analysed using the mediam-effect equation of Chou & Talalay (Chou, 2010). The antineoplasic associations were expressed as Combination Index (CI) values, which indicate different degrees of addition (CI 0.8-1.2), synergism (CI<0.8) or antagonism (CI>1.2).

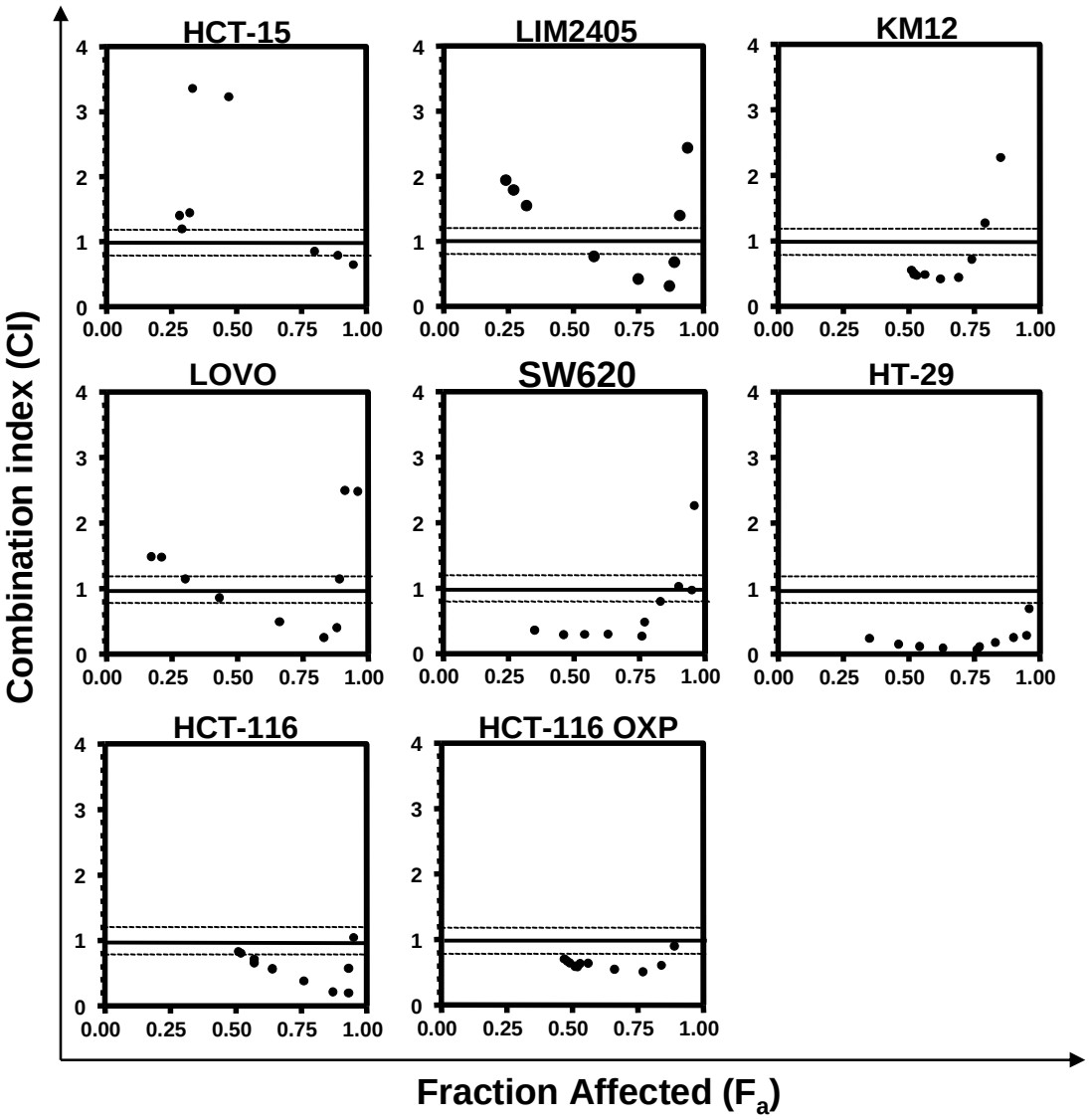

Figure 3. The effect of Olaparib combination with Oxaliplatin. Cells were continuously exposed (120h) to Oxaliplatin ranges with or without 5µM of Olaparib. The means, obtained by at least three independent MTT assays, were analyzed with the mediam-effect equation of Chou & Talalay and plotted using GraphPad Prism 5. Combination Index (CI) values were calculated using assumptions for the drug mutually exclusive. The dotted area indicates the addition area based on a null interval of 0.8-1.2, CIs above and bellow indicate antagonism and synergism degrees, respectively. The Fraction Affected (F_a) indicates the toxicity of combinations.

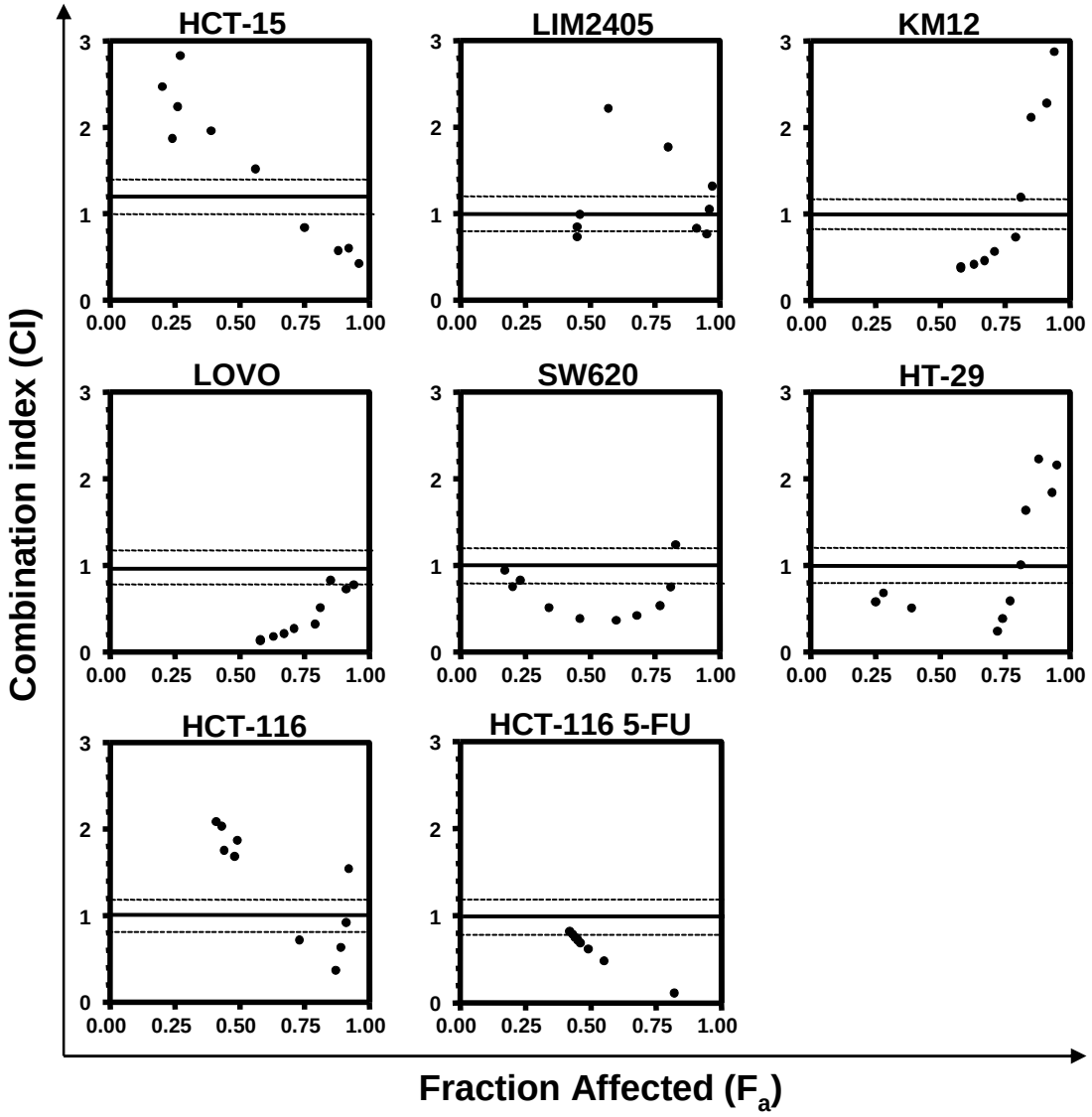

Figure 4. The effect of Olaparib combination with 5-Fluorouracil. Cells were continuously exposed (120h) to 5-Fluorouracil ranges with or without 5µM of Olaparib. The means, obtained by at least three independent MTT assays, were analyzed with the mediam-effect equation of Chou & Talalay and plotted using GraphPad Prism 5. Combination Index (CI) values were calculated using assumptions for the drug mutually exclusive. The dotted area indicates the addition area based on a null interval of 0.8-1.2, CIs above and bellow indicate antagonism and synergism degrees, respectively. The Fraction Affected (F_a) indicates the toxicity of combinations.

For both Oxp and 5-Fu, the Olaparib combinations may be either synergistic, additive or antagonist in an apparently cell-type specific manner. Strikingly, the cell lines relative resistant to Olaparib (HT-29, LoVo and SW620) showed a remarkable additive to synergistic effect for both Oxp and 5-Fu combinations (Figure 3 and 4). Additionally, additive to synergistic interactions were also observed for HCT116 cell lines with acquired resistance to Oxp and 5-Fu (Figure 3 and 4). These results suggest that Olaparib associations with Oxp or 5-Fu may sensitize cells with both relative and acquired resistances. On the other hand, the associations on PTEN null cell lines LIM-2405 and KM12, as well as DLD-1, showed a modest antagonist to synergistic effect (Figure 3 and 4).

Interestingly, Olaparib synergizes in a similar pattern with both Oxaliplatin and 5-Fu combinations (Figure 3 and 4). In fact, Oxp and 5-Fu are DNA damaging inducers with different mechanisms of action but similar effects into cells. Oxp is a platinum-based drug that form intrastrand and interstrand crosslinks with DNA, which induces a variety of DNA lesions including SSBs and DSBs (Faivre *et al.*, 2003). Whereas 5-Fu is a pyrimidine analog that acts by thymidylate synthase inhibition or misincorporation into nucleic acids, which inhibits DNA synthesis and leads to several DNA lesions as SSBs and DSBs (Matuo *et al.*, 2009). Once PARPis have been recently reported to compromise SSB/DSB DNA repair processes by trapping PARP-1 with DNA repair complex (Helleday, 2011), it is possible that the DNA lesions induced by Oxp/5-Fu have their repair affected by PARP inhibition, which in turn is expected to enhance the effects of both Olaparib and the antineoplasic drug. Further, PARPis exposure may also lead to SSBs and

DSBs accumulation, which is likely to synergize with the DNA lesions induced by Oxp/5-Fu treatment.

4. Concluding Remarks

The results presented here demonstrated that Olaparib is a slow drug, which cytotoxicity is significantly increased with long term exposure (Figure 1). Additionally, *MLH-1* and *PTEN* defects are able to sensitize CRC cells to Olaparib treatment, while p53 status does not influence on its effects (Figure 2). On the other hand, it was also demonstrated that Olaparib may synergize with Oxp and 5-Fu in short exposure times (Figure 3 and 4). These data suggest that, as a single agent, Olaparib cytotoxicity is dependent on endogenous DNA damages accumulation, while in combination, the DNA lesions induced by Oxp and 5-Fu accelerate Olaparib cytotoxicity. Supporting this promising association, neither Oxp nor 5-Fu resistant cell lines present cross-resistance to Olaparib, whereas a pronounced cross-resistance was observed for SN-38 resistant cell line (Figure 2D). The data discussed here expands the applications of Olaparib in cancer therapy, as well as points to the necessity of identification of basic and applied mechanisms by which PARPis exert their effects on CRC cells.

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Discussão geral

Durante décadas, pesquisas na área de câncer que buscam estratégias para eliminar as células cancerígenas sem comprometer a viabilidade das células normais vêm sendo conduzidas a fim de melhorar as respostas terapêuticas, reduzindo os efeitos colaterais e melhorando a qualidade de vida dos pacientes. Contudo, uma vez que os tipos e as frequências de mutações incidentes nos tumores pode variar largamente em um mesmo paciente e ainda mais entre pacientes diferentes, encontrar um tratamento que afete apenas as células tumorais vem se mostrando uma tarefa estafante (HANAHAN & WEINBERG, 2011).

Recentemente, uma aplicação clínica denominada letalidade sintética demonstrou que, em alguns casos, é possível eliminar as células cancerígenas sem comprometer a viabilidade das normais. Neste tipo de tratamento, as mutações preexistentes nas células tumorais podem ser exploradas de modo a induzir a letalidade seletiva na presença de um determinado composto. Teoricamente, este procedimento pode beneficiar uma vasta gama de pacientes, mas na prática a interação entre defeitos em *BRCA1/BRCA2* e a inibição de PARP é a única interação letal conhecida e explorável na terapia antitumoral (ROULEAU *et al.*, 2010; SANDHU *et al.*, 2010). Desta forma, apenas tumores com alta incidência de defeitos em *BRCA1/BRCA2*, como os de mama e ovário, vêm sendo beneficiados pelo tratamento sintético letal (ROULEAU *et al.*, 2010; SANDHU *et al.*, 2010). Contudo, considerando o grande repertório de mutações que podem estar presentes nas células cancerígenas, possivelmente outras interações letais serão descobertas nos próximos anos.

O Olaparib é um dos mais promissores inibidores de PARP. Estudos têm demonstrado que, além de apresentar o efeito letal em células deficientes em *BRCA1/BRCA2*, este PARPi também exibe um forte efeito citotóxico em células de câncer de endométrio com defeitos em PTEN (MENDES-PEREIRA *et al.*, 2009; DEDES *et al.*, 2010). Porém, muitos aspectos relacionados aos mecanismos básicos da sua citotoxicidade, bem como seu efeito nos demais tipos tumorais, continuam pouco conhecidos (ROULEAU *et al.*, 2010).

Os agentes antineoplásicos, além de possuírem diferentes mecanismos de ação, apresentam atividade citotóxica em diferentes espaços de tempo: alguns agentes agem rapidamente e seu pico de atividade pode ser observado em poucos minutos ou horas, enquanto que outros levam dias e até semanas para agir (RANG *et al.*, 1997). Desta forma, determinar o tempo necessário para se obter o efeito terapêutico é essencial para atingir o potencial máximo de qualquer droga antitumoral (MENDELSOHN *et al.*, 2008). Neste sentido, este trabalho demonstrou que o Olaparib, quando empregado isoladamente, é uma droga lenta que necessita de pelo menos 12 dias de exposição crônica para exibir a sua notável citotoxicidade em células de CRC (Capítulo II, Figura 1A).

Este aspecto inusitado vem sendo largamente reportado para os diversos inibidores de PARP e pode estar relacionado, principalmente, a duas hipóteses não necessariamente excludentes. A primeira explicação para os mecanismos envolvidos na citotoxicidade dos PARPis propõe que, como PARP-1 participa do reparo de DNA por BER, a sua inibição poderia levar ao acúmulo de lesões provenientes de estruturas intermediárias de reparo que não foram adequadamente processadas (D'AMOURS *et al.*, 1999; SCHREIBER *et al.*, 2006).

Considerando-se que, na ausência de indutores de danos externos, as lesões reparáveis por BER derivam principalmente de fontes endógenas, longos tempos de exposição aos PARPis seriam necessários para o acúmulo suficiente de lesões tóxicas capazes de comprometer a viabilidade celular (D'AMOURS *et al.*, 1999; SCHREIBER *et al.*, 2006). A segunda hipótese tenta explicar os longos períodos de exposição aos PARPis como resultado da não-dissociação da PARP-1 do DNA lesionado. De acordo com dados recentes, discutidos no capítulo I, PARP-1 pode atuar como um sensor de diversos tipos de danos no DNA que incluem SSBs e DSBs. Ao encontrar uma lesão no DNA, PARP-1 é capaz de modificar a si mesma em um processo conhecido como auto-PARilação. Por sua vez, a auto-PARilação produz longas cadeias de PAR capazes de recrutar diversos fatores protéicos envolvidos no reparo do DNA lesado. Além disso, a medida em que as longas cadeias de PAR são adicionadas, PARP-1 concomitantemente perde a sua afinidade pelo DNA e eventualmente acaba dissociando-se do sítio da lesão e, assim, facilitando o acesso das proteínas de reparo ao sítio do DNA danificado. Contudo, na presença de PARPis a auto-PARilação é inibida e pode levar a não-dissociação da PARP-1 do DNA lesado, que por sua vez poderia comprometer o reparo do DNA e levar ao acúmulo de danos (HELLEDAY, 2011).

Ambas as hipóteses sugerem que os PARPis não são capazes de induzir a danos no DNA por conta própria, sendo que os danos de origem endógena ou exógena são sempre necessários para a indução de citotoxicidade. Apesar de plausíveis, nenhuma das hipóteses foi definitivamente comprovada. Isso se deve em parte ao maior interesse científico pelas promissoras questões clínicas que envolvem os PARPis e em parte pela ausência de protocolos experimentais

estabelecidos para detectar danos no DNA em longos períodos de exposição. Em acordo com ambas as hipóteses, os resultados apresentados neste trabalho demonstraram que a exposição crônica ao Olaparib por cinco dias não é suficiente para induzir danos no DNA das células de CRC (Capítulo II, Figura 1B). Contudo, o efeito do Olaparib no acúmulo de lesões de DNA em períodos mais longos ainda é desconhecido.

Em contrapartida, foi observado no presente trabalho que o fenótipo MSI não é um determinante na sensibilidade ao Olaparib em células de CRC (Capítulo II, Figura 2A). Estes resultados estão de acordo com os encontrados por Vilar e seus colegas (2011), cujo painel de células derivadas de CRCs respondeu de maneira semelhante ao tratamento com o inibidor de PARP ABT-888, independentemente da presença ou ausência do fenótipo MSI. Contudo, surpreendentemente, os defeitos em *MLH1* podem conferir uma proeminente sensibilidade ao Olaparib (Capítulo II, Figura 2A). Estes resultados sugerem que a atividade de MLH1 pode modular a sensibilidade das células de CRC ao Olaparib de maneira independente do fenótipo MSI e abrem um leque de novas possíveis aplicações para o Olaparib.

MLH1 é conhecida por ser uma enzima fundamental para o reparo pós-replicativo de DNA. Esta proteína é parte dos complexos MutLα (quando complexada com PMS2) e do MutLβ (quando complexada com PMS1), os quais são essenciais no reparo por MMR (Introdução - Figura 7) de lesões derivadas de erros ocorridos durante a replicação (HEWISH *et al.*, 2010; MARTIN *et al.*, 2010). Contudo, alguns estudos sugerem que MLH1 também pode estar envolvida em outros processos fisiológicos em resposta aos danos no DNA, como por exemplo, a indução de morte celular dependente de PARP-1 (MCDAID *et al.*, 2009). De fato,

foi observado que a indução de morte celular dependente de PARP-1/AIF (descrita em detalhes no capítulo I) pode ser inibida pela depleção de MLH1 (MCDAID *et al.*, 2009). Assim, os defeitos em MLH1 possivelmente sensibilizam as células de CRC para o tratamento com Olaparib por diversos mecanismos. Adicionalmente, devido à alta correlação entre defeitos em MLH1 e a Síndrome de Lynch[4], terapias baseadas na inibição de PARP poderiam beneficiar o tratamento desta doença (HEWISH *et al.*, 2010).

Intrigantemente, defeitos no supressor tumoral p53 não alteraram a sensibilidade das células de CRC para o tratamento com Olaparib (Capítulo II, Figura 2B). Este importante supressor tumoral encontra-se mutado em 50% de todos os tipos tumorais e sua atividade está diretamente relacionada à sinalização e resposta a vários tipos de danos no DNA, incluindo DSBs (BERNSTEIN *et al.*, 2002). Apesar da p53 não participar enzimaticamente de nenhum dos processos celulares envolvidos na sinalização de danos ao DNA, esta proteína atua modulando a expressão e a atividade de diversas enzimas que participam da resposta aos danos de DNA. De modo que, em geral, a atividade deste supressor tumoral pode ser proporcionalmente correlacionada aos níveis de danos no DNA (BERNSTEIN *et al.*, 2002). Contudo, uma vez que o acúmulo de danos endógenos no DNA ocorre de maneira lenta e constante, provavelmente as lesões de DNA provenientes da inibição de PARP não induzem a ativação de p53. Mas, a longo prazo, estas lesões podem levar à instabilidade e perda do DNA genômico e, assim, comprometer a viabilidade celular.

[4] Também conhecida como câncer de colorretal hereditário não polipóide (HNPCC)

A sensibilidade das células deficientes em *PTEN* é outro achado interessante apresentado neste trabalho (Capítulo II, Figura 2C). Os dados obtidos nas células de CRCs suportam os recentes resultados de DEDES *et al.* (2010) e MENDES-PEREIRA *et al.* (2009), cujos estudos demonstraram que células de endométrio com defeitos em PTEN também podem exibir um efeito letal, semelhante ao observado em células com defeitos em *BRCA1/2*, quando tratadas com PARPis. Esta curiosa sensibilidade aos PARPis apresentada pelas células com defeitos em *PTEN* vem sendo atribuída a um papel secundário que este supressor tumoral desempenha na regulação da transcrição da enzima RAD51. Segundo algumas linhas de evidências, defeitos em *PTEN* podem levar à diminuição na expressão de RAD51 (SHEN et al., 2007; DEDES *et al.*, 2010). Uma vez que RAD51 interage com BRCA1/2 no reparo de DNA por HRR, a diminuição na sua expressão pode comprometer o reparo de DSBs e desta maneira induzir ao efeito letal na presença de PARPis (DEDES *et al.* 2010).

Desta forma, os resultados aqui apresentados indicam que *PTEN* é um determinante na citotoxicidade do Olaparib. Contudo, o grau em que as deficiências em *PTEN* influenciam na sensibilidade aos PARPis parece ser dependente de fatores adicionais que ainda não são conhecidos. De qualquer maneira, a excitante possibilidade de explorar os defeitos em *PTEN* abre um leque de novas potenciais aplicações clínicas para os PARPis. Dentre estas prováveis aplicações, vale a pena destacar o tratamento de glioblastomas, câncer de próstata, câncer de endométrio e câncer de colorretal. Todos estes com alta incidência de defeitos em *PTEN* (LIU *et al.*, 2008).

Finalmente, a combinação de PARPis com agentes antineoplásicos já estabelecidos pode ser uma solução eficiente para o tratamento de tumores refratários ou que não possuam marcadores genéticos de sensibilidade a drogas antitumorais. Porém, os efeitos da combinação de PARPis com outros agentes antineoplásicos continuam pouco explorados. Adicionalmente, os mecanismos envolvidos na resistência aos PARPis, bem como a sua possível resistência cruzada com outros agentes antineoplásicos, são praticamente desconhecidos.

Neste estudo foi demonstrado que o Olaparib não apresenta resistência cruzada em células resistentes a Oxaliplatina ou 5-Fluorouracil, contudo, um forte efeito refratário foi observado em células resistentes a SN-38 (Capítulo II, Figura 2D). De acordo com Rottenberg e co-autores (2008) a regulação transcricional dos genes Abcb1a/b e Abcg2 pode levar a resistência adquirida ao Olaparib. Marcantemente, a resistência ao Irinotecano e ao seu metabólito SN-38 também foi relacionada a polimorfismos nos promotores dos mesmos genes (Han *et al.*, 2007). Desta maneira, é muito provável que a regulação transcricional das bombas de efluxo, codificadas pelos genes Abcb1a/b e Abcg2, seja responsável pela resistência adquirida tanto ao Olaparib quanto ao Irinotecano/SN-38.

Uma vez que não foi observado efeito refratário ao Olaparib em células resistentes a Oxaliplatina e a 5-Fluorouracil, as associações destes agentes apresentam-se como grandes promessas para o tratamento de CRC. De fato, as combinações de tanto Oxaliplatina quanto 5-Fluorouracil com Olaparib mostraram-se sinergísticas, aditivas ou antagonistas dependendo do tipo celular (Capítulo II, Figuras 3 e 4). Pode-se observar, nas Figuras 3 e 4 do Capítulo II, que as linhagens relativamente resistentes ao Olaparib apresentaram um proeminente

efeito aditivo para sinergístico. Adicionalmente, interações aditivas/sinergísticas também foram observadas nas células com resistência adquirida a Oxaliplatina e a 5-Fluorouracil (Capítulo II, Figuras 3 e 4). Estes interessantes resultados sugerem que as associações de Oxaliplatina ou 5-Fluorouracil com Olaparib podem sensibilizar tanto as células relativamente resistentes quanto às com resistência adquirida. Desta forma, é possível que estes tipos de combinações possam ser empregadas no tratamento de tumores não responsivos tanto para PARPis quanto para Oxaliplatina e 5-Fluorouracil.

Conclusões

6.1 Conclusão Geral

O emprego dos inibidores de PARP para o tratamento sintético letal de cânceres com defeitos em *BRCA1/BRCA2* vem se mostrado extremamente eficiente e promissor. Contudo, pouco é sabido a respeito dos efeitos de tais substâncias em células com outros tipos de defeitos genéticos associados aos fenótipos tumorais. Neste sentido, o presente trabalho foi o primeiro a apresentar aspectos relacionados aos mecanismos e aplicações do Olaparib em células de câncer colorretal. Demonstrou-se que, como agente simples, a citotoxicidade do Olaparib é dependente de longos períodos de exposição. Porém, em combinação, o Olaparib é capaz de potencializar o efeito da Oxaliplatina e do 5-Fluorouracil em curtos períodos de exposição. Adicionalmente, os dados gerados neste estudo indicaram que defeitos nos genes *MLH1* do MMR e no supressor tumoral *PTEN* podem sensibilizar as células de CRC para o tratamento com o Olaparib. Desta forma, além de agregar conhecimentos básicos sobre os mecanismos envolvidos na citotoxicidade do Olaparib, este trabalho também ampliou o leque de possíveis aplicações para os inibidores de PARP.

6.2. Conclusões específicas

- A citotoxicidade do Olaparib é marcantemente aumentada de maneira tempo dependente em células de CRC. Estes dados sugerem que a inibição de PARP leva ao acúmulo de danos no DNA que, por sua vez, podem vir a comprometer a viabilidade celular a longo prazo. De acordo com está hipótese, não foi observado um aumento na frequência de micronúcleos com apenas cinco dias de tratamento.

- Em acordo com dados previamente publicados para outros inibidores de PARP, células de CRC exibindo o fenótipo MSI não apresentaram aumento na sensibilidade ao Olaparib em relação às células não-MSI.

- Interessantemente, células de CRC com defeitos em *MLH1* apresentaram uma grande sensibilidade ao Olaparib. Uma vez que MLH1 pode participar de diversos processos celulares em resposta aos danos no DNA, é impossível precisar quais são os mecanismos envolvidos neste efeito. Contudo, estes dados sugerem que podem ser elaboradas novas aplicações clínicas visando à inibição de PARP em células com defeitos em *MLH1*.

- Reforçando os achados para células de câncer de endométrio e ovário, os defeitos em *PTEN* também podem sensibilizar as células de CRC para o

tratamento com Olaparib. Contudo, o modesto efeito observado nas células de CRC sugere que fatores adicionais podem modular a sensibilidade conferida pelos defeitos em *PTEN*.

- Intrigantemente, defeitos no supressor tumoral p53 não influenciaram na citotoxicidade do Olaparib em células de CRC.

- Células de CRC com resistência adquirida a Oxaliplatina ou a 5-Fluorouracil não compartilharam efeito refratário ao Olaparib, enquanto que células de CRC com resistência adquirida a SN-38 apresentaram um forte efeito refratário ao Olaparib.

- As combinações de tanto Oxaliplatina (Oxp) quanto 5-Fluorouracil (5-Fu) com Olaparib mostraram-se sinergísticas, aditivas ou antagonistas de maneira dependente do tipo celular. Desta forma, o Olaparib apresenta-se como um promissor agente para ser usado em combinação com Oxp ou 5-Fu no tratamento de CRC.

Perspectivas

De maneira geral, os dados apresentados neste trabalho levantam muitas questões importantes. Parte destas questões poderia ser explorada em novos experimentos complementares aos já realizados, enquanto que outras questões são tão complexas que para serem adequadamente respondidas dariam origem a novos trabalhos totalmente independentes do atual. Desta maneira, as principais perspectivas para a continuidade deste trabalho, bem como para o desenvolvimento de outros estudos são apresentadas a seguir:

- Uma vez que a citotoxicidade do Olaparib é aumentada de maneira dependente do tempo de exposição, seria muito importante investigar a possibilidade do acúmulo de danos no DNA em longos períodos de exposição.

- Estudar os processos celulares pelos quais os defeitos em *MLH1* podem modular a sensibilidade das células de CRC ao Olaparib é um excitante desafio que certamente permitirá novas aplicações clínicas aos inibidores de PARP.

- A surpreendente resistência cruzada entre o Olaparib e o SN-38 em células de CRC pode estar relacionada à expressão ou a atividade das proteínas codificadas pelos genes *Abcb1a/b* e *Abcg2*. Desta forma, analisar a expressão e a atividade destas bombas de efluxo poderia esclarecer este interessante efeito refratário.

- Aparentemente, a maneira como os defeitos em PTEN podem influenciar na sensibilidade das células aos inibidores de PARP é dependente de variados fatores ainda não compreendidos. Desta forma, investigar como os defeitos em PTEN podem modular a citotoxicidade dos inibidores de PARP é um passo imprescindível para garantir a segurança desta promissora aplicação clínica.

- Os promissores resultados obtidos com a combinação do Olaparib com a Oxaliplatina ou o 5-Fluorouracil em células de CRC servem de base para trabalhos subsequentes que visem determinar como e em que condições ocorre o sinergismo, antagonismo e/ou aditivismo. Seria particularmente proveitoso estudar diferentes sequências e tempos de incubação entre as associações.

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Anexos

Anexo I – Dados suplementares (Tabela S1)

Supplementary data

Table S1. Main somatic mutations of the CRC panel were confirmed by COSMIC[1] and western blot (supp. Figure S3)

	Genes					
Cell line	**TP53**	**PI3KCA**	**PTEN**	**KRAS**	**APC**	**MSI related**
HT-29	*MT*	*MT*	**WT**	**WT**	*MT*	**WT**
LoVo	**WT**	**WT**	**WT**	*MT*	*MT*	*MSH2*
HCT-15	*MT*	*MT*	**WT**	*MT*	*MT*	*MSH6*
Lim2405	NA	**WT**	Null	**WT**	*MT*	NA
KM12	*MT*	**WT**	Null	**WT**	**WT**	**WT**
DLD-1	NA	NA	**WT**	*MT*	*MT*	NA
SW620	*MT*	**WT**	**WT**	*MT*	*MT*	**WT**
HCT-116	**WT**	*MT*	**WT**	*MT*	**WT**	*MLH1*

WT = Wild Type *MT = Mutant* NA = Not Analysed

[1] http://www.sanger.ac.uk/genetics/CGP/cosmic/

Anexo II – Dados suplementares (Tabela S2)

Supplementary data

Table S2. Comparison of the IC50 as determined by colony formation and by MTT.

The CRC cell line panel was continuously exposed to an Olaparib range and the percentage of survive was accessed after 5 days of treatment using MTT or after 12 days of treatment using CFA.

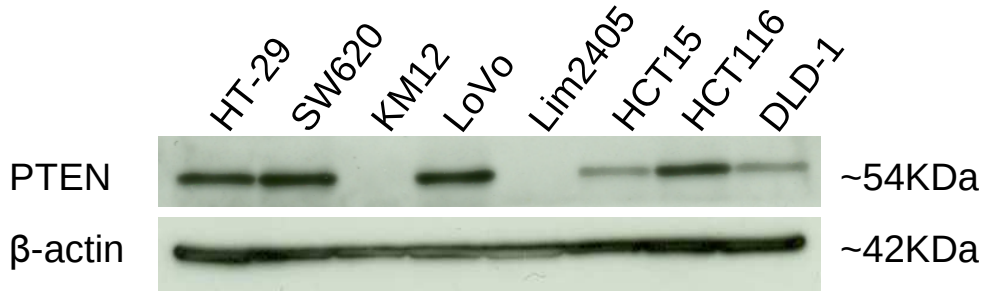
	MTT	CFA	FD*
HT-29	**>20**	**4**	**>5**
LoVo	**>20**	**3,6**	**>5**
DLD-1	**>20**	**1,8**	**>10**
HCT15	**14**	**1,7**	**8**
HCT116 SN-38 Res.	**>20**	**1,3**	**>15**
Lim-2405	**8,7**	**0,55**	**16**
HCT116 Ch3	**5,9**	**0,3**	**20**
KM12	**7**	**0,24**	**29**
HCT116 5-Fu Res.	**7,1**	**0,049**	**145**
HCT116 Oxp Res.	**7,8**	**0,033**	**236**
HCT116 p53 null	**4,5**	**0,018**	**250**
HCT116	**5,1**	**0,01**	**510**
SW620	**>20**	**N.A.**	**-**

Results expressed in µM of Olaparib
****Fold Difference***

Anexo III – Dados suplementares (Figura S1)

Supplementary data

Figure S1. PTEN status confirmation by immunoblotting



Proteins were resolved on a SDS-polyacrylamide gel (12%) and blotted onto nitrocellulose membranes (Biorad). Membranes were saturated by TBST-milk (50mM Tris pH8.0, 150mM NaCl, 0.5% Tween 20 and 5% dehydrated skimmed milk) and the antigens were revealed by immunolabeling. Antigens were detected using an enhanced chemiluminescence kit (Amershan Biosciences). The primary antibody to PTEN (#9559) was acquired from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, France), while β-actin (A-5441) was obtained from Sigma-Aldrich (L'Isle d'Abeau Chesnes, Saint Quentin en Fallavier, France). The appropriated secondary antibodies were purchased from Jackson Research.

Anexo IV – Artigo de revisão relacionado

The yeast system: a cellular approach to study anticancer drug responses

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Abstract

The development of new strategies for cancer therapeutics is indispensable to improve standard protocols and create other possibilities in cancer treatment. Yeast models have been employed to study numerous molecular aspects directly related to cancer development, as well as to determine the genetic contexts associated with anticancer drugs sensitivity or resistance. The budding yeast *Saccharomyces cerevisiae* presents conserved cellular processes with high homology to humans and it is a fast, cheap and efficient compound screening tool. However, yeast models are still underused in cancer study and antineoplasic drug screenings. Here the employment of *S. cerevisiae* as a model system to anticancer research is discussed and exemplified. Focusing on important determinants in genomic maintenance and cancer development as DNA repair, cell cycle control and epigenetics, this review propose the use of mutant yeast panels to mimic cancer phenotypes, screen and study tumor features and synthetic lethal interactions. Finally, the benefits and limitations of yeast model were highlighted, as well as the strategies to overcome *S. cerevisiae* model limitations.

Key words: *Saccharomyces cerevisiae,* anticancer drugs, DNA repair, epigenetics, synthetic lethality.

1. Introduction

Carcinogenesis is understood as a dynamic multistep processes analogous to Darwinian evolution, in which a succession of genetic alterations, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (HANAHAN & WEINBERG, 2000). The physiological advantages that may be selected in cancer cells are so varied and each novel tumor capabilities represent the successful breaching of an anticancer defense mechanism hardwired into cells and tissues (HANAHAN & WEINBERG, 2000; HANAHAN & WEINBERG, 2011). However, it also means that incipient cancer cells are dependent on genetic alterations from the early until the late steps of carcinogenesis. Therefore, the life span of cancer cells is marked by an incessant conflict with the genome maintenance systems including DNA repair, cell cycle and epigenetic control.

Since tumor cells present concomitant multiple alterations, it is difficult to understand the specific contribution of each single alteration to the drug sensitive or resistant phenotype. This way, isogenic model systems have been contributing to generate basic biological knowledge as well as medicine applications. These models allowed the identification and dissection of key genetic pathways, such transcription control, DNA repair and cell cycle regulation, which play critical roles in numerous diseases (SPRADLING *et al.*, 2006). Accordingly, yeast has been providing excellent clues on investigation of determinants of sensitivity or resistance to anticancer drugs. The characteristics which make yeast an important tool in cancer research include its lower complexity in comparison to tumor cells (PEREGO *et al.*, 2000; KURTZ *et al.*, 2004), the high degree of similarity between yeast and mammalian cellular processes (CARR & HOESKSTRA, 1995; FREIRE *et al.,* 1998; SPRADLING *et al*., 2006) and the possibility to focus on the effect of a single gene alteration (SIMON & BEDALOV, 2004).

The budding yeast *Saccharomyces cerevisiae* was the first eukaryote to have its genome fully sequenced and it has been successfully used as an efficient tool and model organism to study cell cycle control, DNA repair, aging, gene

expression, autophagy and molecular and cellular pathways of human diseases, including cancer (LONGHESE *et al.,* 1998; KLIONSKY *et al.,* 2003; KURTZ *et al.,* 2004; SUZUKI & OHSUMI, 2007; ALMEIDA *et al.,* 2008; KARATHIA *et al.,* 2011). Moreover, *S. cerevisiae* presents many advantages as small genome (about 200 times less than human), fast doubling time (approximately 2h), easy manipulation, cheap cultivation and the possibility of monitoring cell cycle progression by cellular and nuclear morphology. Besides, yeast exists in a haploid or diploid state during its life cycle, what allows the investigation of recessive mutations, which are masked in diploid state by the wild-type allele (PEREGO *et al.,* 2000). Finally, yeast growth can be controlled by adjusting environmental conditions and it is an organism amenable to modification such gene disruption (MAGER & WINDERICHX, 2005).

Approximately 30% of known genes involved in human diseases have yeast orthologs (i.e. functional homologs) and hundreds of yeast genes exhibit a link to human disease genes (KARATHIA *et al.*, 2011). *S. cerevisiae* studies are helping to discover important aspects of diseases as diabetes-2, hereditary non-polyposis colon cancer, neurofibromatosis type l, ataxia telangiectasia and Werner's syndrome (BOTSTEIN *et al.,* 1997; BOLOTIN-FUKUHARA *et al.,* 2010). In addition, yeast may be employed to study important features in cancer development, as chromosome instability, aneuploidy and genes involved in cancer susceptibility (STROME & PLON, 2010; STIRLING *et al.,* 2011). The phenotype screening based on marker stability provides a unique approach to investigate genes that act in preserving genome structures, as origins of replication, cell cycle control, microtubule dynamics, DNA replication, repair and condensation (YUEN *et al.*, 2007).

Finally, yeast is also an important tool for the identification and production of new drugs and targets (BOLOTIN-FUKUHARA *et al.,* 2010). Accordingly, *S. cerevisiae* strains has been employed for production of human medicines, as insulin, hydrocortisone, artemisinin, hirudin and vaccines employed in hepatitis B and cancer prevention (ARDIANI *et al.,* 2010). However, despite all the potential uses of yeast in cancer study, this extraordinary tool is still underused. Therefore,

this review proposes to describe how yeast can be employed in anticancer research, highlighting the benefits and limitations of this model system. We focused this review on important aspects of cancer development, as DNA repair, cell cycle control and epigenetics, which have also been reported as important targets in anticancer therapy.

2. Yeast mutants to study drug sensitivity, resistance and synthetic lethal interactions

It is becoming evident that the genetic changes accompanied the carcinogenesis may provide a window of therapeutic advantage designated synthetic lethality. First discovered and described in yeast, the synthetic lethality principles have encouraging the development of therapeutics that specifically kills the cancer cells (MEYSKENS & GERNER, 2011). According to the concept of synthetic lethality, the inhibition or deletion in either of two genes is tolerable, but the combined deletion or inhibition of both genes leads to cell death (BANERJEE *et al.*, 2010; ROULEAU *et al.,* 2010). The clinical application of this promising approach combines the synthetic inhibition induced by the anticancer drug with the preexistent oncogenic mutations to selective kill the tumor cells. The final result observed is an increase of the therapeutic index and a reduction of the side effects. However, there still only few synthetic lethal interactions described and exploited in anticancer treatments, which signal for the urgent necessity to search for new lethal interactions.

Yeast strain panels carrying specific mutations have been employed in large scale drug screening for the identification and characterization of new compounds with mutant-selective cytotoxicity (SPRADLING *et al.,* 2006). In this sense, The Seattle Project conduced by National Cancer Institute (NCI), screened a panel of several compounds against *S. cerevisiae* mutant strains that have alterations in DNA damage repair or cell cycle control. Additional examples of successful employments of yeast screenings in anticancer research include the identification

of the bifunctional alkylating agent cisplatin and the topoisomerase II poison mitoxantrone as possible therapeutic agents for tumors defective in post-replication repair (PRR) and DSB repair, respectively (HARTWELL *et al.*, 1997; BROOMFIELD *et al.,* 2001; FOX, 2004; MOORE *et al.,* 2009). Further, the *S. cerevisiae* screenings were also used to characterize the doxorubicin and bleomycin resistant profiles (KULE *et al.,* 1994; CHEN & CLARK-WALKER, 2000; CONTAMINE & PICARD, 2000; BUSCHINI *et al.,* 2003; AOUIDA *et al.,* 2004; HOFFMANN *et al.,* 2011).

Moreover, using yeast models, it is possible to investigate the interactions between different antitumor agents and/or genetic mutations. LILLO and colleagues (2011) determined the maximal lethal and mutagenic synergism of the combination of γ-rays, cisplatin and etoposide. Other studies showed that bleomycin cytotoxicity may be altered by intercalating agents as 9-aminoacridine, that potentialize its activity, or heat shock treatment, which induces resistance to potentially lethal and mutagenic effects (KESZENMAN *et al.,* 2000; KESZENMAN *et al.,* 2005; HOFFMANN *et al.,* 2011). Further, mutant yeast strains may be employed to study specific genetic pathways in carcinogenesis. Thought the combinations of double, triple, quadruple mutant genes (SWANSON *et al.,* 1999) it is possible to mimic a variety of cancer phenotypes in the yeast conserved genetic background. Between all aspects of carcinogenesis which may be studied in *S. cerevisiae*, the genomic maintenance systems are distinguished by its high degree of conservation from yeast to human and by its critical importance in all stages of carcinogenesis. The next sections discuss and exemplify how *S. cerevisiae* may be employed to study and screen the major genomic maintenance systems in anticancer research.

2.1. DNA Repair Pathways as important targets in cancer therapy

The cellular DNA is constantly injured by both endogenous and exogenous DNA damage sources which may result in cell death or genomic instability if not

repaired. To counteract this danger, cells have evolved several DNA repair systems, each one responsible for repair specific subsets of DNA damages. However, this creates a dilemma for incipient tumor cells, since mutations in DNA repair proteins are needed to generate the variability in cancer cells and select cells with physiological advantages (HANAHAN & WEINBERG, 2000; HANAHAN & WEINBERG, 2011). But at the same time that DNA repair disabling gives an advantage to incipient tumors, it also may represent an opportunity to differentiate them from normal cells. Therefore, targeting DNA repair systems is a promising field of anticancer therapy that begins to be intelligently explored.

The budding yeast *S. cerevisiae* DNA repair systems are remarkable well understood and similar to human DNA repair systems. The six main DNA repair pathways conserved from yeast to mammalian cells are: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), translesion synthesis (TLS), homologous recombination (HR) and non-homologous end joining (NHEJ) (HSIEH, 2001; BERNSTEIN *et al.*, 2002; DUDÁS & CHOVANEC, 2004; DUDÁSOVÁ *et al.*, 2004; IYER *et al.,* 2006). The brief description of each process, as well as the main proteins involved in *S. cerevisiae* and *Homo sapiens* DNA repair systems are summarized in Table 1 (SWANSON *et al.,* 1999; HSIEH, 2001; PELTOMÄKI, 2001; BERNSTEIN *et al.,* 2002; DUDÁS & CHOVANEC, 2004; DUDÁSOVÁ *et al.,* 2004; IYER *et al.,* 2006).

Numerous cancers types are characterized by the high incidence of specific DNA repair deficiencies. This is the case of hereditary non-polyposis colorectal cancer, which present high frequency of spontaneous mutations as microsatellite instability resulting from mutations in the MMR genes such *MSH2* and *MLH1* (HSIEH *et al.,* 2001). NER deficiencies are also related to cancer development and/or aging syndromes, such as *Xeroderma pigmentosum* skin cancer, Cockayne syndrome and Trichothiodystrophy (ANDRESSOO *et al.,* 2006). Deficiencies in Werner syndrome protein (WRN), one family of five human RecQ helicases implicated in genome stability maintenance, present premature aging and increased cancer susceptilibity (BOTSTEIN *et al.,* 1997, SAFFI *et al.,* 2000; SAFFI *et al.,* 2001). WRN proteins interact with cell cycle regulators and DNA repair

factors (for review, see ROSSI *et al.,* 2010). The increased activity of *APE1*, a critical BER protein that acts as AP endonuclease, is involved in glioma and melanoma pathogenesis (MOHAMMED *et al.,* 2011). Whereas breast and ovary cancers frequently present deficiencies in *BRCA1* and *BRCA2,* both involved in HR pathway (HELLEDAY *et al.,* 2008; SMITH *et al.,* 2010).

Table 1: The main DNA repair pathways and proteins involved in processing DNA damage in *Saccharomyces cerevisiae* and *Homo sapiens*.

Pathway	Function	*S. cerevisiae*	*H. sapiens*	*Reference*
BER	Excises single damage DNA base or a short strand containing the damaged base, DNA polymerase fills the gap and ligase joins the ends.	Apn1, Rad27	APE1, FEN1	BERNSTEIN *et al.,* 2002
NER	Excises single-stranded DNA molecule of 24-30 nucleotides containing the lesion, DNA polymerase fills the gap and ligase joins the ends.	Rad1, Rad10	RAD1, ERCC1	BERNSTEIN *et al.,* 2002
MMR	Acts in post-replicative repair and corrects DNA mismatches that have escaped the proofreading function of replicative polymerases, recognizing the non-canonical base pair and replacing the offending nucleotide on newly strand by excision repair mechanism.	Mlh1, Pms1	MLH1, PMS2	HSIEH, 2001; IYER *et al.,* 2006
TLS	Damage tolerance mechanism in which DNA polymerase (Polζ) and a complex of proteins (Rev3 and Rev7) bypass DNA lesions to allow cell survivor when the damage is too extensive to be removed efficiently. However, it may increase the mutation rate.	Rev1, Rev3	Rev1, hRev3	SWANSON *et al.,* 1999
HR	Repair DSBs by retrieving genetic information from an undamaged homologue (sister-chromatid or homologous chromosome). Accurate repair.	Rad52, Mre11-Rad50-Xrs2	RAD52, MRE11-RAD50-NBS1	DUDÁS & CHOVANEC, 2004
NHEJ	Repair DSBs by direct ligation of DNA ends without any requirement for sequence homology. Mutagenic process.	Yku70, Yku80, Lif1	Ku70, Ku80, DNA ligase IV	DUDÁSOVÁ *et al.,* 2004

The most part of anticancer therapies based on DNA damage induction present low therapeutic index and a variety of collateral effects which result from the low selectivity of these agents. The majority cancer cells carrying DNA repair deficiencies present increased sensitivity to DNA damage based treatments. The recent reported synthetic lethal approach has been demonstrated that it is possible to explore DNA repair deficiencies in a more selective way. The most documented synthetic lethal interaction is the peculiar lethality between *BRCA1* or *BRCA2* mutations and PARP inhibition (ANDERS *et al.,* 2010). This lethal condition has been attributed to the accumulation of spontaneous DNA damages in cells with PARP activity inhibited, which lesions maybe converted to the harmful DSBs. Normal cells, without DSB repair defects, are able to correctly repair the DSBs resulting from PARP inhibition. However, in tumor cells with *BRCA1* or *BRCA2* mutations the accumulation of non-repaired DSBs lead to high and selective cytotoxicity (CARDEN *et al.,* 2010). Therefore, therapies based on DSBs induction for breast and ovary cancers deficients in HR genes are also promising strategies (HELLEDAY *et al.,* 2008; SMITH *et al.,* 2010). Since *S. cerevisiae* DNA repair systems are closely related to human, the screening of lethal DNA repair interactions using yeast may provide substantial advances in anticancer research.

S. cerevisiae has been shown to be an important tool to investigate the role of DNA repair in lesions induced by antineoplasic drugs. SIMON *et al.* (2000) characterized the DNA damage and repair profiles of several antineoplasic agents towards a panel of isogenic yeast strains, each defective in a particular DNA damage repair or cell cycle checkpoint pathway. The identification of drugs selectively toxic to one specific pathway contributes for future clinical approaches, since it would be possible to select pacients that most respond to the treatment with that agent. Further, large-scale chemical screenings are feasible in yeast model in order to discover new toxic compounds (CANAANI, 2009). DUNSTAN *et al.* (2002) employed yeast *S. cerevisiae* based assays to identify anticancer agents that are selectively cytotoxic to cells with defined mutations. From 85.000 compounds, they identified 126 compounds selectively toxic to yeast cells defective in DSB repair (*rad50* and *rad52*). 87 of these 126 compounds were

structurally related to known topoisomerase poisons and 39 were not. Among these 39 agents, they characterized 8 compounds: two of them as novel topoisomerase ll poisons equipotent to etoposide, five with topoisomerase l-dependent toxicity and one that directly bound to DNA and induced strand breaks, in yeast and mammalian cells.

Studies using different mutants from the same DNA repair pathway allow us to better understand the contribution of each protein in the process of DNA damage. SEIPLE *et al.* (2006) investigated the role of BER in 5-fluorouracil (5-FU) toxicity in yeast model with mutants in DNA glycosylases and endonucleases. Interestingly, deficiencies in *UNG1* and *RAD27*, uracil DNA glycosylase and flap endonuclease, respectively, presented resistance towards 5-FU, however the deletion of *APN1*, the major abasic site (AP) endonuclease in *S. cerevisiae*, results in a strong sensitivity. It suggests that the AP sites are the major progenitor giving rise to the DNA-mediated toxic effects of 5-FU. Considering the accumulation of AP sites as a potent target for anticancer chemotherapy, some authors have proposed the use of inhibitors of *APE1* (human AP endonuclease) in association to agents that induce base damage and repaired through BER, to potentiation cytotoxicity in cancer cells (LUO & KELLEY, 2004; MADHUSUDAN *et al.*, 2005; BAPAT *et al.,* 2010; WILSON III & SIMEONOV, 2010). These studies indicated that *APE1* inhibitors either alone or in combination with chemotherapy may be a promising strategy in cancer (ABBOTTS & MADHUSUDAN, 2010; BAPAT *et al.,* 2010; MOHAMMED *et al.,* 2011).

Finally, the investigation of DNA repair pathways overlapping is also avaliable by employing *S. cerevisiae* as biological model by constructing double, triple and quadruple mutants, that contribute to better understand the action mechanism of a drug (SWANSON *et al.,* 1999). SOARES *et al.* (2005) have characterized the ecteinascidin-743 (ET-743) molecular mechanism of action, employing a pannel of yeast deleted strains for DNA repair. This study showed that yeast strains lacking endonucleases of NER and BER are resistant for ET-743, and suggest that this resistance results from the damage tolerance by TLS activation (error-prone) or its combination with HR (error-free) pathways. MATUO *et al.*

(2010) investigated the differences in DNA repair pathways in lesions induced by the antineoplasic drug 5-FU and its active metabolite FdUMP in yeast cells. The results revealed that the repair mechanisms differed for the both antimetabolites, since lesions induced by 5-FU were repaired by BER, MMR, HR and PRR, while only BER and MMR were required for repair of FdUMP-induced lesions.

2.2. A model to study cell cycle checkpoints

The concept of DNA damage checkpoints was first characterized through the identification of G2/M arrest after X-ray irradiation in the budding yeast *S. cerevisiae* (WEINERT & HARTWELL, 1988). The eukaryotic cell cycle comprehends a collection of ordered events in which the initiation of late processes depends upon the completion of early ones (FOIANI *et al.*, 2000). Surveillance mechanisms as checkpoints assure that cell cycle events occur in the proper sequence in order to avoid replication and segregation of damaged DNA (POEHLMANN & ROESSNER, 2010). Failure in properly respond to DNA alterations may lead to increased genomic instability, which is one of the most prominent hallmarks of cancer cells (HANAHAN & WEINBERG, 2011). Tumor cells often present chromosomal instability caused by gross chromosomal rearrangements and aneuploidy, as consequence of mutations in mitotic checkpoint genes such as *MAD2, BUB2* or *BUBR1*, and *S. cerevisiae* has been shown to be one important model to study chromosomal instability mechanisms and their effects on cellular physiology (JUNG *et al.,* 2011).

In summary, DNA damage is recognized by sensors, and this information is communicated through signal transducers to effectors that mediate the response to the damage, including arrest or slow the cell cycle, activation or repression of cellular pathways. Some DNA repair proteins may act as sensors and effectors, as well as checkpoint proteins that are replication complexes components sensors and transducers, and might even effectors (PUTNAM *et al.*, 2009). The yeast checkpoint pathways are well defined and share similar features with mammalian

cell cycle checkpoints. Accordingly, the Table 2 presents the proteins involved in checkpoints conserved in *S. cerevisiae* and *Homo sapiens* (for review, see LUCCA *et al.,* 2004; PELLICIOLI & FOIANI, 2005; MORDES *et al.,* 2008a; MORDES *et al.,* 2008b; NAVADGI-PATIL & BURGERS, 2009; PUTNAM *et al.*, 2009; MURAKAMI-SEKIMATA *et al.,* 2010; RUPNIK *et al.,* 2010).

This high degree of conservation between human and yeast checkpoint pathways makes *S. cerevisiae* an excellent tool to study anticancer drug responses. As an example, the cytotoxic DNA-damaging agent adozelesin was evaluated in yeast cells defective in *RAD53* and *MEC1* (WANG *et al.,* 2001). Although this anticancer agent inhibited activation and fork progression at a replication origin in a chromosome, WANG *et al.* (2001) showed that mutations in *RAD53* and *MEC1* checkpoint genes did not abrogate these responses. Thus, the findings reveal that inhibitory effects of adozelesin on replication origin activation and fork progression are independent of the mutations in the intra-S phase checkpoint genes *RAD53* and *MEC1*. The anticancer drug camptothecin, one topoisomerase poison, leads G2 accumulation, and *RAD9* is important for this response. ZHANG & SIEDE (2003) have developed a two-hybrid based plate assay to visualize DNA damage-induced homomeric complex formation of Rad17 yeast checkpoint protein towards camptothecin derivatives. They observed that Rad17p appears to be dispensable for cell cycle arrest and for Rad53p phosphorylation following treatment with camptothecin. Other study employing hydroxyurea, one antineoplasic drug that cause nucleotide depletion, showed that mutants lacking *RAD53* and *MEC1* are highly sensitive towards this agent (PUTNAM *et al.*, 2009). Recently, a model for checkpoint activation generated by photo-induced-DNA adducts was proposed. *S. cerevisiae pso9/mec3* (human homolog *HUS1*) mutant was isolated, and molecular and phenotipically characterized. This mutant fails to arrest its cell cycle after treatment with the bi-functional agent methoxypsoralen (8-MOP) + UVA confirming its role in responding to interstrand crosslinkers (BRENDEL *et al.*, 2003; CARDONE *et al.,* 2006).

In addition to antitumor drugs that interfere with cell cycle, checkpoint abrogators, such as Chk1/Chk2 inhibitors, are currently emerging as a new class of

anticancer agents that can enhance cytotoxic responses to existing chemotherapy drugs (MCNEELY *et al.,* 2010). Further, PARP inhibitors have been shown a strong and specific cytotoxic effect in ATM defective cells, which suggest that targeting checkpoint proteins may also result in synthetic lethality and indicate new therapeutic possibilities. However, the checkpoint proteins interactions that may result in synthetic lethality, as well as the cellular outcomes of checkpoint targeting drugs, still poorly understood. Therefore, employing yeast to evaluate the molecular response of these new drugs and interactions may represent a critical step to confirm their intended targets and effects *in vivo*.

Table 2: Conserved *Saccharomyces cerevisiae* and *Homo sapiens* checkpoint proteins and their functions.

S. cerevisiae	***H. sapiens***	**Function**	**Reference**
RFA	RPA	Responsible to coat stretches of ssDNA that are generated by decoupling of helicase and polymerase activities at stalled replication forks	LUCCA *et al.,* 2004
Mec1	ATR	PIKK acts as a damage sensor and signal transducer	PUTNAM *et al.*, 2009; MORDES *et al.,* 2008b
Tel1	ATM	PIKK acts as a damage sensor and signal transducer	PUTNAM et al., 2009
Ddc2	ATRIP	Recruits Mec1 (ATR) to regions of RFA (RPA)-coated of ssDNA	LUCCA *et al*., 2004; MORDES *et al*., 2008b
Dpb11	TOPBP1	Involved in activation of Mec1-Ddc2 (ATR-ATRIP) complex	MORDES *et al.,* 2008a
Rad24	Rad17	Sensor (RFC-like complex)	MURAKAMI-SEKIMATA *et al.,* 2010
Ddc1- Rad17-Mec3/Pso9	Rad9- Rad1-Hus1	Damage sensor (PCNA-like protein), involved in activation of PIKK family members	LUCCA *et al.,* 2004; PUTNAM *et al.*, 2009
Mre11-Rad50-Xrs2	Mre11-Rad50-NBS1	Damage sensor (MRX/MRN complex), recruits Tel1 (ATM) to damage sites via its interaction interact with its terminal end-binding domain	PUTNAM *et al.,* 2009; RUPNIK *et al*., 2010
Rad9	BRCA1/53BP1	Mediator, involved in Rad53 (CHK2) activation	PUTNAM *et al*., 2009
Mrc1	Claspin	Mediator, a component of the replication fork that seems specifically signal replication stress	PUTNAM *et al.,* 2009
Rad53	CHK2	Downstream kinase activated by PIKK proteins	PELLICIOLI & FOIANI, 2005
Chk1	CHK1	Downstream kinase activated by PIKK proteins	PUTNAM *et al*., 2009

2.3. Budding yeast as model system to study epigenetic effects

Epigenetic effects are defined as heritable changes in gene expression that occur independent of changes in the primary DNA sequence. These heritable changes are established during differentiation and are maintained through cell cycle division. DNA epigenetic effects are mediated through DNA modifications (CpG residues methylation), post-translational modifications of histones (phosphorylation, acetylation, methylation and ubiquitylation), and the positioning of nucleosomes along the DNA (SHARMA *et al*., 2010). These modifications do not alter the sequence code, but they involve gene transcription regulation (PLASS, 2002). Acetylation of lysine (K) residues of N-terminal tails neutralizes the histones positive charge and decrease the interaction with the negative charged DNA, leading to an open chromatin structure more accessible for DNA repair and transcriptional machinery. Methylation of histone H3 at K4 is associated with transcriptional activation, while methylation of H3 at K9/K27 and H4 at K20 is related to transcriptional repression (KRISTENSEN *et al.,* 2009). The high mobility group box-1 protein (HMGB) is a non-histone protein that stabilizes nucleosomes and facilitates gene transcription, DNA repair and V(D)J recombination (ANDERSSON *et al.,* 2002; BREZNICEANU *et al.,* 2003).

Recent studies have shown that several diseases including cancer present changes in genome and histone modifications (EGGER *et al.,* 2004; LAFON-HUGHES *et al.,* 2008). Epigenetics changes may inactivate tumour-suppressor genes and/or activate genes that lead to cancer when overexpressed, as oncogenes (SIMON & BEDALOV, 2004). The global DNA hypomethylated and hypermethylated tumor suppressor gene promoters can be observed in almost all cancers. Patients with sporadic colorectal cancers often present microsatellite instability phenotype related to methylation and silencing of *MLH1*. In addition, many malignancies are associated to aberrant histone deacetylase (HDAC) expression and activity (KRISTENSEN *et al.,* 2009). One example is the oncogenic fusion of promyelocytic leukaemia with retinoic acid receptor, which recruits HDAC to repress genes necessary for the hematopoietic cells differentiation. Further,

deficiencies in ATP-dependent chromatin remodelling complexes, such as the highly conserved SWI-SNF complex have been implicated in cancers also. The loss of *SNF5* is found in paediatric cancers, and mutations in *BRM* and *BRG1* (ATPase) are related to a variety of cancer cells (EGGER *et al.,* 2004). In addition, high mobility group proteins as *HMGB1* may contribute in cancer development, since this protein at the surface of certain cells may contribute to cellular migration and tumor invasion (ANDERSSON *et al.,* 2002; BREZNICEANU *et al.,* 2003).

Since the epigenetic changes may be reversed employing drugs that inhibit the chromatin modifying enzymes, epigenetic modifications have emerging as potential targets for therapeutic interventions in cancer treatment. For example, CpG methylation and histone hypo-acetylation can be reversed by inhibiting enzymes such as DNMTs or HDACs. Altering the epigenetic regulation of gene expression is a great promise for re-setting the chromatin changes in cancer cells; however, the effect in normal cells is difficult to predict (SIMON & BEDALOV, 2004). Some nucleoside analogues as 5-azacytidine (azacitidine) and 5-aza-2´-deoxycytidine (decitabine) act in demethylation of tumor suppressor genes at non-cytotoxic concentrations, and they present cytotoxic effects at high concentrations related to enzyme-DNA adducts formation. Applied studies employing the agent decitabine combined with cisplatin or carboplatin, showed that drug resistance caused by hypermethylation MMR genes silenced could be reversed using this demethylating drug. Decitabine is currently being tested in combination with carboplatin in a phase ll clinical trial in patients with ovarian cancer (HELLEDAY *et al.,* 2008), hematological malignancies or solid tumors (PLASS, 2002). Many HDAC inhibitors as trichostatin A, belinostat and vorinostat also presented synergism when associated to conventional chemotherapeutic agents as paclitaxel, gemcitabine, cisplatin, etoposide and doxorubicin in cell culture. The administration of HDAC inhibitors before chemotherapy appears to be a promising strategy to overcome multidrug resistance, since histone acetylation results in opened chromatin more accessible to the drugs. However, applying the reversed order, the treatments did not present the same efficacy (KRISTENSEN *et al.,* 2009). Using *S. cerevisiae*, KAISER *et al.* (2011) observed that sodium

phenylbutyrate (PBA), that dramatic reduces H4 K8 acetylation, suppresses camptothecin and methyl methane sulfonate (MMS)-induced genetic recombination as well as DSB repair during mating-type interconversion. In the presence of PBA, camptothecin-induced damage is redirected to a non-recombinogenic pathway without loss of cell viability, however, for MMS, this combination is accompanied by a dramatic loss in cell viability.

Unfortunately, there are few effective drugs available to investigate epigenetic changes in cancer therapy. However, since the most part of processes affected by cancer-associated epigenetic alterations are conserved among eukaryotes, the employment of budding yeast in chromatin-modifying agent screenings is a potent tool to identify new targets and drugs for anticancer research. Further, some chromatin-modifying inhibitors, including trichostatin A, and depsipeptide are also active in yeast (SIMON & BEDALOV, 2004). Accordingly, yeast-based systems have proving a useful tool for small molecules screening with HDAC activity (BEDALOV *et al.,* 2001; HIRAO *et al.*, 2003). HIRAO *et al.* (2003), using whole-genome DNA microarray analysis, identified compounds that exhibit a higher degree of selectivity toward NAD^+-dependent deacetylases involved in transcriptional repression in yeast. The compounds identified by authors were splitomicin with improved selectivity for Sir2 and dehydrosplitomicin specifically effectively in Hst1 defective yeast strains. WEERASINGHE *et al.* (2010) developed a yeast-based gene reporter centered on class l yeast homolog Rpd3. Yeast Rpd3 deacetylase shares 60% identity to human class l HDAC proteins. The screening was dependent on HDAC activity, sensitivity to trichostatin A, apicidin and suberoylanilide hydroxamic acid (SAHA), and it was validated in qualitative and quantitative formats, making it an important tool to screen Rpd3 mutants and inhibitors of class l HDAC proteins.

Nevertheless, *S. cerevisiae* mutant strains may aid us to better understand the complex interplay between chromatin remodeling mechanisms and others cellular processes. Recently, it has been proposed that chromatin remodeling is an important factor in DNA repair (ATAIAN & KREBS, 2006; ESCARGUEIL *et al.,* 2008). In fact, chromatin structure controls the access of proteins to DNA damaged

and also participates on recruitment of DNA repair factors to damage site. For example, the phosphorylation of histone H2A is directly related to DSBs signaling and repair. Others chromatin modifiers involved in DSBs repair include HATs, HDACs, ATP-dependent remodelers, histone kinases and phosphatases (for review, see ATAIAN & KREBS, 2006; VAN ATTIKUM & GASSER, 2005). In addition, LABAZI *et al.* (2009) propose that *NHP6* (closest HMGB1 homologue) may also influence the MMR activity by dissociation of *MSH2-MSH6* in absence of mismatch DNA.

3. Limitations to the use yeast for pharmacological studies and strategies to overcome them

The data accumulated so far clearly support yeast models as important tools to identify and study new compounds, mechanisms and applications in anticancer research. However, compared to the multicellular mammalian tissues and cells, this versatile unicellular organism also presents limitations which need to be considered (RESNICK & COX, 2000). The positive and negative aspects to the use *S. cerevisiae* as a biological model in anticancer research are summarized in Table 3. The most remarkable limitations of this model are the relative impermeability of cell wall and the lack of important human proteins. These proteins belong to relevant categories in anticancer research as tumor-suppression, apoptosis, drug metabolizing, among others (KOLACZKOWSKI & GOFFEAU, 1997). The strategies to overcome these limitations demands case-to-case planning and experimental adaptations as following exemplified.

Table 3: Positive and negative aspects of employing *S. cerevisiae* as a biological model in anticancer research

Positive aspects
- Low experimental cost;
- Easy to manipulate and construct mutants;
- Small genome;
- High degree of conservation of major signaling pathways with human cells;
- Fast doubling time;
- Cell cycle progression may be monitored by cell and nuclear morphology;
- It allows to understand the contribution of a specific single alteration or the combination of diverse mutations to a drug sensitive/resistant phenotype;
- Easy to perform screenings;
Negative aspects
- Low permeability to some agents;
- Absence of some enzymes involved in drug metabolizing, tumor suppression and apoptosis;
- Lack tissue-specific response, observed in mammalians towards anticancer treatments;
- Impossibility to study some advanced aspects of cancer as metastasis, tissue invasion and angiogenesis.

The *S. cerevisiae* cell wall has been reported as an important factor able to decrease the drug sensibility of several anticancer compounds as DNA topoisomerase poisons (NITISS & WANG, 1988). In this case, the relative impermeability of yeast cell wall could be overcome through the use of mutant strains defective in *ISE1*, *PDR1* and *SNQ2* which has been reported to increase sensitivity to several anticancer agents, including camptothecin, a potent antitumor drug that specifically targets topoisomerase I (NITISS & WANG, 1988; REID *et al.,* 1997). Other possibility to increase the yeast cell permeability to small molecules is the use of lytic zymolyase enzyme in association with the drugs, since zymolyase acts in digestion of cell wall. This enzyme has shown to enhanced the permeability of some HDAC inhibitors, as apicidin and SAHA (WEERASINGHE *et al.,* 2010).

When a pathway or gene function is completely lacking in yeast, it may be possible to express a human cDNA from a yeast promoter (BJORNSTI, 2002). In these cases, the phenotype of a yeast mutant can be complemented by the expression of a human protein (MAGER & WIDERICKX, 2005). One example is the protein p53, a key regulator of cell cycle and apoptosis in mammalian cells that lacks in yeast (FLAMAN *et al.,* 1995). Employing random mutagenesis screening for this gene, p53 yeast mutants were isolated and they presented increased growth inhibition or even lethality. These toxic p53 variants might be useful for dissection of p53-regulated cellular responses (INGA & RESNIK, 2001). Further, yeast engineered to express apoptosis target proteins provides an important source to identify new genes and chemical compounds that modulate the cell-death pathways of humans and other organisms (JIN & REED, 2002). Previous studies have reported that the ectopic expression of Bax in yeast produced a lethal phenotype by inducing a cytochrome C release from mytochondria (XU & REED, 1998). Accordingly, the Bax-induced death of budding yeast was suppressed by Bcl-2 and other anti-apoptotic members (SATO *et al.,* 1994; HANADA *et al.,* 1995).

Additionally, it is well known that some drugs demand metabolizing by cytochrome P450 to become active (LYNCH & PRICE, 2007). Drug-metabolizing cytochrome P450 and glucuronosyl-transferase, both absent in yeast, can be heterologously expressed and may be employed to study the mutagenic effects of oxidative metabolites of xenobiotics, as N-alkylformamides, aflatoxine B1, paclitaxel and diclofenac, or for the synthesis of drug metabolites (GUO *et al.,* 2005; PURNAPATRE *et al.,* 2008; DEL CARRATORE *et al.,* 2000; MASIMIREMBWA *et al.,* 1999; PETERS *et al.,* 2009). Moreover, in some cases it is possible to use drugs analogues or metabolites instead of express the metabolizing enzyme. This is particularly true for 5-FU, topotecan, irinotecan, cytarabine, gemcitabine, among others (SIMON *et al.,* 2000; KURTZ *et al.,* 2004; LONGLEY *et al.,* 2003). 5-FU, for example, is metabolized by thymidine kinase and its resulting metabolites may missincorporate into DNA or RNA, or inhibit the thymidilate synthase (TS) enzyme. Once TS is inhibited by FdUMP, the main 5-FU cytotoxic active metabolite, and yeast does not possess the enzyme thymidine

kinase to convert 5-FU into FdUMP (LADNER, 2001), it is possible to administrate FdUMP directly on yeast cells, which make *S. cerevisiae* an unique model system to investigate cellular effects of 5-FU or FdUMP independently (MATUO *et al.,* 2010).

4. Concluding remarks

This review discussed important aspects and applications of yeast in anticancer research. More than contribute to determine basic action mechanisms of anticancer agents, *S. cerevisiae* has been proving an extraordinary tool for drug screenings. Here we evidenced the high degree of similarity between yeast and human DNA repair, checkpoint and epigenetic control systems. These basics cellular processes are directly involved in genomic maintenance and their improperly regulation have a critical outcome in all stages of carcinogenesis. Therefore, the genomic maintenance systems have been emerging as promising targets in cancer therapy. However, explore these complex safeguard systems in therapy are still a challenging goal, especially due to the high heterogeneity of genetic alterations and backgrounds in tumor cells, as well as the longstanding and elevated cost screenings in mammalian systems.

S. cerevisiae is an organism easy to manipulate, with fast doubling time, low experimental costs, amenable to gene disruptions and conserved signaling pathways between eukaryotes. Although all these facilities, yeast also present limitations as the low cellular permeability to several compounds and the absence of diverse tumor suppressors and metabolizing enzymes. These limitations may be overcome by numerous case-to-case strategies as previously discussed. However, it is important to note that *S. cerevisiae* do not completely substitute mammalian models in anticancer research. Due to yeast unicellular characteristic, some tumor aspects as angiogenesis, tissue invasion and metastasis can not be evaluated in this model organism. Although, yeast screenings are fast and powerful tools for screening of compounds and basic mechanisms in anticancer research.

Nevertheless, scientists all over the world have generated yeast mutant strains for decades. As a result, the actual *S. cerevisiae* strains collection have include all viable single gene mutation strains. These mutant yeast strains may be obtained from European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF) or directly from yeast specialized laboratories. The use of this extraordinary *S. cerevisiae* panel could represent the faster and cheaper solution to screen anticancer drugs cytotoxicity and also the easier way to mimic the numerous combinations of genetic alterations in cancer cells that may be explored by the synthetic lethal approaches.

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4. Mutagênese
5. Toxicologia

Idiomas

Inglês: Compreende bem, fala bem, escreve bem, lê bem

Francês: Compreende bem, fala razoavelmente, escreve pouco, lê razoavelmente

Produção científica

Artigos completos publicados em periódicos

1. MATUO, R.; SOUSA, F.G. *et al*. DNA repair pathways involved in repair of lesions induced by 5-fluorouracil and its active metabolite FdUMP. **Biochemical Pharmacology**, v.79, p.147-153, 2010.

2. VIAU, C.M.; SOUSA, F.G. *et al*. SnCl2-induced DNA damage and repair inhibition of MMS-caused lesions in V79 Chinese hamster fibroblasts. **Archives of Toxicology**, v.83, p.769-775, 2009.

3. MATUO, R.; SOUSA, F.G. *et al*. 5-Fluorouracil and its active metabolite FdUMP cause DNA damage in human SW620 colon adenocarcinoma cell line. **Journal of Applied Toxicology**, v.29, p.308-316, 2009.

4. SOUSA, F.G. *et al.* Clastogenic and anticlastogenic effect of the volatile oil from *Casearia sylvestris* Swart. **The Journal of Essential Oil Research**, v.19, p.376-378, 2007.

5. SOUSA, F.G. *et al*. Evaluation of allelopathic and genotoxic potential of the crude extract from *Casearia sylvestris* Sw. **Allelopathy Journal**, v.20, p.195-202, 2007.

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1. MATUO, R.; SOUSA, F.G. *et al*. Participação de modeladores da cromatina e da reparação de dna na citotoxicidade do 5-fluorouracil em *Saccharomyces cerevisiae* In: XIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2011, Porto Alegre. **Livro de Resumos**, 2011.

2. MATUO, R.; SOUSA, F.G. *et al.* Citotoxicidade de antimetabolitos é potencializada com a inibição de checkpoint kinases In: XII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2010, Porto Alegre. **Livro de Resumos**, 2010.

3. SOUSA, F.G. *et al*. p38MAPK-dependent anti-mutagenesis and cytotoxicity induced by *Agaricus blazei* beta-glucan In: VIII Congresso Brasileiro de Mutagênese, Carcinogênese e Teratogênese Ambiental, 2007, Mangaratiba. **Brazilian Journal of Toxicology**, 2007.

4. MATUO, R.; SOUSA, F.G. *et al*. 5-Fluorouracil and its active metabolite FDUMP cause DNA damage in human colon adenocarcinoma cells In: VIII Congresso Brasileiro de Mutagênese, Carcinogênese e Teratogênese Ambiental, 2007, Mangaratiba. **Brazilian Journal of Toxicology**, 2007.

5. SOUSA, F.G. *et al*. Atividade anti-tumoral do polisacarideo β-(1-6)-D-glucan extraido de Agaricus blazei e sua possivel relação com p53 In: VIII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2006, Porto Alegre. **Livro de Resumos**, 2006.

6. SOUSA, F.G. *et al*. Avaliação da clastogenicidade e anticlastogenicidade do óleo de *Casearia sylvestris in vitro*, pelo ensaio de aberração cromossômica In: IX

Seminário de Iniciação Científica, 2005, Chapecó - SC. **Resumos IX Seminário de Iniciação Científica**. Chapecó-SC: Argos, 2005.

7. SOUSA, F.G. *et al.* Avaliação da toxicidade do extrato bruto de *Casearia sylvestris* pela inibição do crescimento radicular em *Allium cepa* In: 56º Congresso Nacional de Botânica, 2005, Curitiba - PR. **Resumos Congresso Nacional de Botânica**, 2005.

8. SOUSA, F.G. *et al.* Concepções dos alunos e professores do ensino médio sobre o tema câncer em escolas de Chapecó In: Curso de Verão: I Genética e Biologia Molecular nas Férias, 2005, Londrina - PR. **Curso de Verão: I Genética e Biologia Molecular nas Férias**, 2005.

9. SOUSA, F.G. *et al*. Estudo da clastogenicidade e anticlastogenicidade do óleo de *Casearia sylvestris*, em células metabolizadoras In: 51º Congresso Brasileiro de Genética, 2005, Águas de Lindóia - SP. **Resumos 51º Congresso Brasileiro de Genética**, 2005.

10. SOUSA, F.G. *et al*. Estudo da genotoxicidade do extrato bruto de *Casearia sylvetris* usando o teste de aberração cromossômica em *Allium cepa* In: IX Seminário de Iniciação Científica, 2005, Chapecó - SC. **Resumos IX Seminário de Iniciação Científica**. Chapecó-SC: Argos, 2005.

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Cursos ministrados

1. SOUSA, F.G. **Microorganismos: O mundo que não enxergamos**, 2009 (Aperfeiçoamento, Curso de curta duração ministrado).

2. SOUSA, F.G. **Genotoxicidade em *Allium Cepa***, 2006 (Aperfeiçoamento, Curso de curta duração ministrado).

3. SOUSA, F.G. **Avaliação da genotoxicidade de amostras complexas pelo teste de aberrações cromossômicas em *Allium cepa* L.**, 2005 (Aperfeiçoamento, Curso de curta duração ministrado).