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AVALIAÇÃO DOS MECANISMOS ENVOLVIDOS NA RESPOSTA AOS DANOS NO DNA INDUZIDOS PELO AGENTE ANTITUMORAL 5-FLUOROURACIL

Tese de Doutorado

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LISTA DE ABREVIATURAS E SIGLAS

5-FU: 5-Fluorouracil

ATM: *Ataxia telangiectasia* mutada

ATR: *Ataxia telangiectasia* mutada e relacionada a Rad3

BER: “Base Excision Repair”, Reparação por Excisão de Bases

dCK: desoxicitidina cinase

dCMP: desoxicitidilato desaminase

DNA-PK: proteína cinase dependente de DNA

dFdC: Gemcitabina, 2′,2′-difluoro-2′-desoxicitidina

dFdCMP: gemcitabina monofosfatada

dFdCDP: gemcitabina difosfatada

dFdCTP: gemcitabina trifosfatada

DNMT: DNA Metil-transferase de histonas

DSBs: “Double-strand breaks”, quebras de fita dupla de DNA

FdUMP: 5-Fluoro-2’-desoxiuridina-5’-monofosfato

GGR: “Global Genome Repair”, reparo global do genoma pelo NER

HAT: Acetil-transferase de histonas

HDAC: Desacetilase de histonas

HR: “Homologous Recombination”, Reparação por Recombinação Homóloga

MMR: “Mismatch Repair”, Reparação de bases mal-emparelhadas

MRN: complexo MRE11/RAD50/NBS1

MRX: complexo MRE11/RAD50/XRS2

NER: “Nucleotide Excision Repair”, Reparação por Excisão de Nucleotídeos

NHEJ: “Non-homologous end joining”, Reparação por Recombinação Não-Homóloga ou ilegítima

PI3K: Fosfatidilinositol 3-cinase

PRR: “Post-replication Repair”, reparo pós-replicativo

RPA: “Replication protein A”, proteína de replicação A

RR: Ribonucleotídeo Redutase

RTX: Raltitrexato

SAHA: ácido Suberoilanide Hidroxâmico

Sítios AP: sítios apurínicos/apirimídicos

SSB: “Single-strand breaks”, quebras de fita simples

ssDNA: “single-stranded DNA”, DNA de fita simples

TCR: “Transcription coupled repair”, reparo por NER acoplado à transcrição

TK: Timidina cinase

TLS: “Translesion synthesis”, Reparação por Síntese translesão

TS: Timidilato sintase

UV: luz ultra-violeta

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triphosphate; dUTP = deoxyuridine triphosphate. Enzymes: TK = thymidine kinase; K = kinases; RNR: ribonucleotide reductase.

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CAPITULO III

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S1: *Mitotic cells distribution.* HeLa cells treated with IC_{50} antimetabolites in the presence or absence of 50nM AZD7762 for 24h. Legend: (A) Untreated cells, (B) AZD7762, (C) 5-FU, (D) 5-FU + AZD7762, (E) FdUMP, (F) FdUMP + AZD7762, (G) Gemcitabine, (H) Gemcitabine + AZD7762, (I) RTX, (J) RTX + AZD7762.

RESUMO

O 5-Fluorouracil (5-FU) é um agente antitumoral amplamente empregado no tratamento de diversos tipos de cânceres. Embora haja muitos trabalhos publicados com este antineoplásico, estudos sobre seus mecanismos de ação ainda tornam-se necessários a fim de se obter protocolos clínicos mais eficazes. Desta forma, novos aspectos sobre seu mecanismo de citotoxicidade foram investigados neste trabalho. Na primeira parte foram avaliadas as vias de reparação de DNA que participam em resposta a danos induzidos pelo 5-FU e seus efeitos comparados com o seu metabólito ativo FdUMP em *Saccharomyces cerevisiae*. Os resultados apontam que as lesões induzidas pelo 5-FU podem ser processadas pela vias de reparo por excisão de bases (BER), reparação de bases mal-emparelhadas (MMR), reparo pós-replicativo (PRR) e recombinação homóloga (HR), enquanto que os danos induzidos pelo FdUMP seriam reconhecidos e removidos apenas pelas vias BER e MMR. Estas diferenças no recrutamento das vias de reparo relacionam-se aos diferentes tipos de lesões que são geradas pelos antimetabólitos: o 5-FU induz quebras de fita simples (SSBs) e duplas (DSBs), enquanto que o FdUMP forma principalmente SSBs. Na segunda parte foi investigada a participação de modeladores da cromatina na citotoxicidade do 5-FU em *S. cerevisiae*. Os resultados em conjunto sugerem que os remodeladores da cromatina dependentes de ATP e algumas acetiltransferases de histona podem influenciar na citotoxicidade do agente 5-FU, possivelmente atuando no relaxamento da cromatina, e assim facilitando os processos de reparação de DNA por HR e PRR. Na terceira parte foi avaliada a

resposta ao dano no DNA por ATR/Chk1 e ATM/Chk2 em células tumorais humanas. Os resultados mostraram que o 5-FU ativa principalmente a via ATR/Chk1. Ao se investigar os efeitos do 5-FU em combinação com o AZD7762, um inibidor de Chk1/2, observou-se aumento de sensibilidade ao agente antitumoral, diretamente relacionado ao aumento de células em sub-G1, diminuição em G2/M e indução de mitose prematura. Os resultados em conjunto obtidos neste trabalho nos permitem uma melhor compreensão destes mecanismos de ação do 5-FU, e fornece subsídios para melhora de protocolos terapêuticos.

ABSTRACT

5-Fluorouracil (5-FU) is an antitumor drug employed in the treatment of several cancer types. Despite many studies have been conduced with this antineoplasic drug, investigations concerning on its action mechanism become necessary in order to obtain more efficient clinical protocols. Therefore, new aspects about its cytotoxicity mechanism were investigated in this work. First, DNA repair pathways involved in the repair of 5-FU-induced lesions were evaluated and compared to the effects of its active metabolite FdUMP in *Saccharomyces cerevisiae*. Our data showed that lesions induced by 5-FU may be processed by base excision repair (BER), mismatch repair (MMR), post-replication repair (PRR) and homologous recombination (HR), while FdUMP lesions are recognized and removed only by BER and MMR. These differences in repair pathways recruitment are related to the different lesion types induced by the antimetabolites: 5-FU induces single- and double stranded breaks (SSBs and DSBs), while FdUMP induces mainly SSBs. In the second part we investigated the participation of chromatin remodeling in the 5-FU cytotoxicity in *S. cerevisiae.* Together, our data suggest that ATP-dependent chromatin remodeling and some histone acetyltransferases may influence 5-FU cytotoxicity, probably acting in chromatin relaxation and facilitating DNA repair process by HR and PRR. In the third part, the DNA damage response by ATR/Chk1 and ATM/Chk2 were evaluated in human tumor cells. The data showed that 5-FU activates mainly ATR/Chk1 pathway. When we investigate the effects of 5-FU in combination with AZD7762, the Chk1/2 inhibitor, we observed increased sensitivity to this antitumor agent, directly related to enhanced number of sub-G1

cells, decrease in the G2/M cells, and premature mitose induction. Our data permit a better comprehension of 5-FU action mechanisms and provide clues to improve the current therapeutics protocols.

INTRODUÇÃO

1. Introdução Geral

O câncer é uma das principais causas de mortalidade no mundo inteiro e pode ser definido como uma coleção de doenças nas quais ocorre multiplicação e disseminação incontrolada de formas anormais das próprias células corporais formando tumores (RANG *et al.*, 2006). Trata-se de uma doença complexa que é muito variável na sua apresentação e desenvolvimento, inclusive diferindo entre pacientes. A mesma heterogeneidade existe tanto em nível celular quanto molecular, como resultado de profundas mudanças metabólicas em programas genéticos que controlam a proliferação celular e suas relações com as células vizinhas (WHO, 2009). A maioria dos cânceres é decorrente do acúmulo de mutações espontâneas nos tecidos somáticos, porém, em alguns casos a predisposição é herdada. A incidência de câncer tem aumentado nos últimos anos devido ao envelhecimento da população e de fatores como tabagismo, sedentarismo e dieta (GREENWALD & DUNN, 2009; JEMAL *et al.*, 2011).

As células tumorais podem apresentar múltiplas alterações genéticas, tais como deleções, inserções e translocações (SALK *et al.*, 2010), perda de heterozigosidade e instabilidade de microsatélites em cânceres de cólon hereditários não-poliposos (FEARON, 2011). Estas alterações são responsáveis pela conversão de células normais à células cancerosas, através de modificações em oncogenes e supressores tumorais que as tornarão capazes de proliferar além do limite normal (WHO, 2009). As células normais são progressivamente convertidas para o estado neoplásico através da aquisição de capacidades características que as tornam tumorigênicas e malignas. Os sistemas envolvidos

na manutenção da estabilidade genômica são os primeiros a serem afetados nesta transformação (HANAHAN & WEINBERG, 2000). Diversas mudanças fisiológicas podem ocorrer no processo de tumorigênese, tais como:

(i) *Auto-suficiência em sinais de crescimento*: As células normais necessitam de sinais de crescimento para que possam passar do estado quiescente para o proliferativo. No entanto, as células neoplásicas desenvolveram a capacidade da auto-suficiência em sinais de crescimento que interfere na função dos pontos de checagem de DNA e na progressão do ciclo celular, levando uma proliferação incontrolada (WHO, 2009).

(ii) *Falta de sensibilidade a sinais antiproliferativos*: Os sinais antiproliferativos são responsáveis pela manutenção das células em estado quiescente. Entretanto, as células cancerosas são capazes de contornar estes sinais, permitindo a continuidade da proliferação (HANAHAN & WEINBERG, 2011).

(iii) *Evasão da morte celular programada*: A apoptose é um importante tipo de morte celular que controla a homeostase celular e atua como barreira antitumoral. A evasão da apoptose é resultado de mutações nas vias de sinalização, e permite que as células que apresentam muitos danos persistam sob condições impróprias (WHO, 2009; HANAHAN & WEINBERG, 2011).

(iv) *Capacidade replicativa ilimitada*: A células normais podem se replicar por um número finito de vezes, devido a uma estrutura terminal em cada cromossomo denominada de telômero. A cada divisão celular, o telômero é encurtado e ao final de sucessivas divisões, as células tornam-se senescentes. No entanto, as células cancerosas possuem capacidade replicativa ilimitada, pois possuem a enzima

telomerase, que evita o encurtamento dos telômeros (WHO, 2009; HANAHAN & WEINBERG, 2011).

(v) *Indução de angiogênese*: Todas as células necessitam de oxigênio e nutrientes para desenvolver suas atividades, que são fornecidos pelos vasos sangüíneos. O processo de formação de novos vasos sangüíneos em tecidos, denominado de angiogênese, é extremamente regulado. Entretanto, as células tumorais adquiriram a capacidade de induzir a angiogênese para promover a vascularização dos tumores (WHO, 2009; HANAHAN & WEINBERG, 2011), através do aumento da expressão de sinais estimulantes como o VEGF ("vascular endothelial growth factor") e FGF1/2 ("acid and basic fibroblast growth factors") (FERRARA, 2009).

(vi) *Poder invasivo e metástases:* As células cancerígenas podem apresentar alterações na regulação da aderência e motilidade celular, que possibilitam a invasão da matriz extracelular e migração através dela. Estas células cancerosas podem formar tumores secundários denominados de *metástases*, originados de células liberadas do tumor primário que chegaram a outros locais através de vasos sangüíneos ou linfáticos (RANG *et al.*, 2006; MENDELSOHN *et al.*, 2008; HANAHAN & WEINBERG, 2011).

A Figura 1 resume o processo de carcinogênese a partir de mutações que não foram reparadas de modo adequado. A maneira como uma célula normal é transformada em tumoral pode ser variável, ou seja, a aquisição de vantagens fisiológicas como a evasão da morte celular programada, o controle da angiogênese e o potencial replicativo ilimitado podem ocorrer em diferentes etapas do desenvolvimento tumoral e não necessariamente nesta ordem. A

seqüência das alterações fisiológicas nas células cancerosas pode variar entre tumores do mesmo tipo e entre tumores de tecidos diferentes (HANAHAN & WEINBERG, 2000).

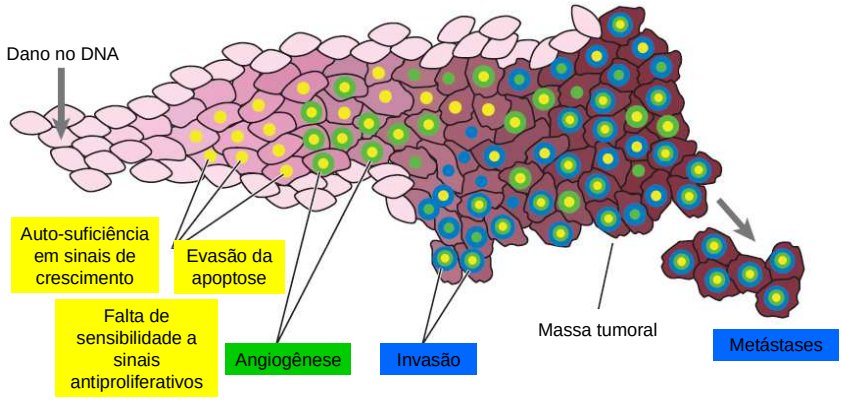

Figura 1: A evolução do câncer. Os danos no DNA que não foram reparados podem desencadear diversas modificações que culminam na formação de tumores e metástases. Adaptado de SALK *et al.* (2010).

2. Agentes antineoplásicos

Existem quatro abordagens para o tratamento do câncer: a excisão cirúrgica, a irradiação, a quimioterapia e as terapias alvo tumoral. Dependendo do tipo de tumor e de seu estágio de desenvolvimento, é determinada a melhor estratégia de tratamento, que pode associar o procedimento cirúrgico com radioterapia e/ou quimioterapia (RANG *et al.*, 2006). De maneira geral, a radioterapia é empregada no tratamento de tumores localizados, enquanto a quimioterapia é voltada para o tratamento sistêmico de cânceres com metástases

ou de difícil acesso (CHABNER & ROBERTS Jr., 2005; HENNEQUIN *et al.*, 2009; WHO, 2009).

Os tratamentos antitumorais atuais baseiam-se no emprego de substâncias tóxicas que induzem diversos tipos de danos no DNA e que levam a morte celular. Uma vez que as células tumorais dividem-se mais rapidamente que as normais, acumulam mais lesões e por isso são mais suscetíveis aos tratamentos com antineoplásicos (WHO, 2009). Existem diferentes classes de fármacos citotóxicos empregados como agentes antitumorais que atuam causando lesões no DNA de maneira direta ou indireta formando quebras, pontes inter e intra-cadeia no DNA ou com proteínas, ou que tem como alvo proteínas relacionadas a replicação e síntese de ácidos nucléicos (Tabela 1) (HURLEY, 2002; DING et al., 2006; RANG *et al.*, 2006; HENNEQUIN *et al.*, 2009; POMMIER, 2009; DEANS & WEST, 2011). Estes agentes podem atuar ou não em fases específicas do ciclo celular: os alcalóides da vinca e os antimetabólitos atuam em fases específicas (mitose e fase S, respectivamente), porém, as bleomicinas e nitrosouréias atuam sem esta especificidade (RANG *et al.*, 2006).

Em geral, os tratamentos com antineoplásicos empregam combinações de medicamentos com diferentes mecanismos de ação, a fim de se obter respostas mais eficazes quando comparado aos efeitos obtidos em monoterapias. Os protocolos utilizados consistem na associação de dois ou mais agentes antitumorais, como por exemplo o uso de 5-fluorouracil associado ao irinotecan para tratamentos de câncer de colon (GRIVICICH *et al.*, 2005), docetaxel, cisplatina e 5-fluorouracil para câncer de esôfago (EMI *et al.*, 2012), entre outros.

Inúmeros estudos vem sendo conduzidos a fim de se descobrir terapias mais eficazes no tratamento do câncer, tais como: a identificação de mutações em tumores que os tornam mais sensíveis ou resistentes a determinados agentes (IYER *et al.*, 2006; MODRICH, 2006; SALK *et al.*, 2010); a combinação de agentes antineoplásicos com diferentes mecanismos de ação (GRIVICICH *et al.*, 2005; ILSON *et al.*, 2011) e o desenvolvimento de inibidores de vias celulares, como os inibidores de "checkpoint" cinases (ASHWELL *et al.*, 2008; ASHWELL & ZABLUDOFF, 2008, LAINCHBURY & COLLINS, 2011) ou de reparação de DNA (DING *et al.*, 2006).

Recentemente têm sido desenvolvidas terapias-alvo que se baseiam em agentes que inibem a proliferação de células tumorais, sem afetar as células saudáveis. Esta especificidade contribui tanto para maior eficácia do tratamento quanto para a redução de efeitos colaterais (WU *et al.*, 2006). Compreendem substâncias que agem destruindo as células tumorais através de moléculas específicas envolvidas no crescimento e progressão tumoral, tais como (i) os anticorpos monoclonais, que se ligam especificamente nas células cancerosas; (ii) os inibidores de angiogênese, que previnem a formação de novos vasos sanguineos (KOUKOURAKIS & SOTIROPOULOU-LONTOU, 2011); e (iii) os bloqueadores de tirosina cinase (DEININGER & MANLEY, 2012). Estas terapias-alvo podem ser empregadas em monoterapias, em associação com a quimioterapia convencional ou associadas a outras terapias-alvo (WU *et al.*, 2006).

Tabela 1: Agentes antineoplásicos citotóxicos empregados na clínica.

Classe	Mecanismo de ação	Exemplos
Agentes alquilantes	Formam pontes intra- ou inter-cadeia no DNA, que podem interferir na transcrição e replicação. Podem ser mono ou bi-funcionais, caso possuam um ou dois grupos alquilantes. Os efeitos ocorrem principalmente durante a fase S do ciclo celular e leva a um bloqueio em G2/M, seguido por morte celular.	Mostardas nitrogenadas (ciclofosfamida, ifosfamida e clorambucil), nitrosouréias, bussulfan e compostos platinados (cisplatina, oxaliplatina e carboplatina)
Antimetabólitos	Interferem no metabolismo dos ácidos nucléicos, podendo ser incorporados de maneira errônea no DNA ou RNA. Podem levar ao bloqueio da replicação. Os efeitos ocorrem principalmente durante a fase S do ciclo celular e podem desencadear morte celular.	Antagonistas fólicos (raltitrexato, metotrexato), análogos pirimídicos (5-Fluorouracil, gemcitabine e citarabina), análogos purínicos (mercaptopurina e tioguanina)
Inibidores de topoisomerase	Estabilizam o complexo de clivagem entre a enzima topoisomerase I ou II com o DNA, formando quebras de fita simples e duplas, o que resulta em elevada citotoxicidade.	Inibidores de top I (camptotecinas), inibidores de top II (doxorrubicina, etoposideo)
Inibidores da formação de microtúbulos	Ligam-se a tubulina impedindo a formação do fuso nas células em mitose. Causam paradas de ciclo celular em metáfase.	Alcalóides da vinca (vincristina, vimblastina e vindesina) e taxol
Antibióticos citotóxicos	Atuam por ação direta no DNA e não são dependentes do ciclo celular para serem citotóxicos.	Bleomicina

2.1. 5-Fluorouracil

O 5-Fluorouracil (5-FU) é um agente antitumoral pertencente à classe dos antimetabólitos. O 5-FU difere da base uracil pela substituição de um átomo de flúor na posição 5 do carbono no anel pirimídico (Figura 2) (LONGLEY *et al.*, 2003). Este agente vem sendo empregado nos tratamentos de adenocarcinomas de mama, ovários e trato gastrointestinal, e carcinomas de cabeça e pescoço (MALET-MARTINO *et al.*, 2002), geralmente em terapias combinadas com outros agentes antineoplásicos como leucovorin (KUEBLER & GRAMONT, 2003; LONGLEY *et al.*, 2003; LANSIAUX, 2011), irinotecan (GRIVICICH *et al.*, 2005), metotrexato (LONGLEY *et al.*, 2003) e compostos platinados como cisplatina (GREM, 1997) e oxaliplatina (KUEBLER & GRAMONT, 2003; PERA *et al.*, 2011), ou à radioterapia (RICH *et al.,* 2004). 5-FU é administrado intravenoso e durante as primeiras 24h, cerca de 90% da droga é excretada (CUNNINGHAM *et al.*, 2002).

Para tornar-se ativo, o 5-FU precisa ser convertido aos seus metabólitos. Estes metabólitos precisam ser fosforilados para que sejam erroneamente incorporados no DNA e RNA, ou atuar inibindo a enzima timidilato sintase (TS) (LONGLEY *et al.*, 2003; RICH *et al.,* 2004). A TS é responsável pela conversão de desoxiuridina monofosfato (dUMP) a desoxitimidina monofosfato (dTMP) com um doador de metil (5,10-metileno-tetrahidrofolato) e é a única fonte de timidilato intracelular na via da síntese de pirimidinas. O 5-FU quando convertido ao seu metabólito ativo 5-fluoro-desoxiuridina monofosfato (FdUMP) (Figura 2) inibe a TS, formando um complexo ternário covalente entre a TS e o 5,10-metileno-

tetrahidrofolato, aumentando assim a concentração de dUMP intracelular (LONGLEY *et al.*, 2003; WILSON & TAYLOR, 2009). Tanto o dUMP quanto o FdUMP podem ainda ser fosforilados, produzindo dUTP e FdUTP, respectivamente, os quais podem ser incorporados no DNA (SHEWACH & LAWRENCE, 2007).

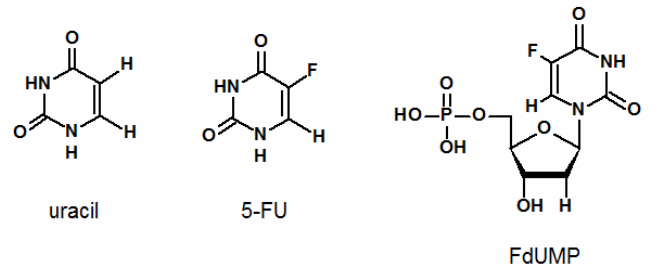

Figura 2: Estrutura química do uracil, 5-FU e FdUMP.

A citotoxicidade do 5-FU pode ser atribuída aos erros de incorporação de fluoronucleotídeos no DNA e RNA, e/ou inibição da enzima TS. A incorporação de 5-FU no DNA resulta em diversos efeitos celulares, tais como a inibição de síntese pela depleção de desoxiribonucleotídeos, alteração na estabilidade e indução de quebras de fita simples e duplas de DNA. Já a incorporação no RNA pode levar a diminuição da sua síntese, a inibição da poliadenilação do RNA mensageiro e alterações na sua estrutura secundária. A inibição da enzima TS pelo metabólito FdUMP resulta no aumento de incorporação de uracil no DNA, bloqueio de replicação e quebras de fita simples de DNA (Figura 3) (KUFE & MAJOR, 1981a; KUFE & MAJOR, 1981b; GREM, 1997; LONGLEY *et al.*, 2003; MATUO *et al.,* 2009).

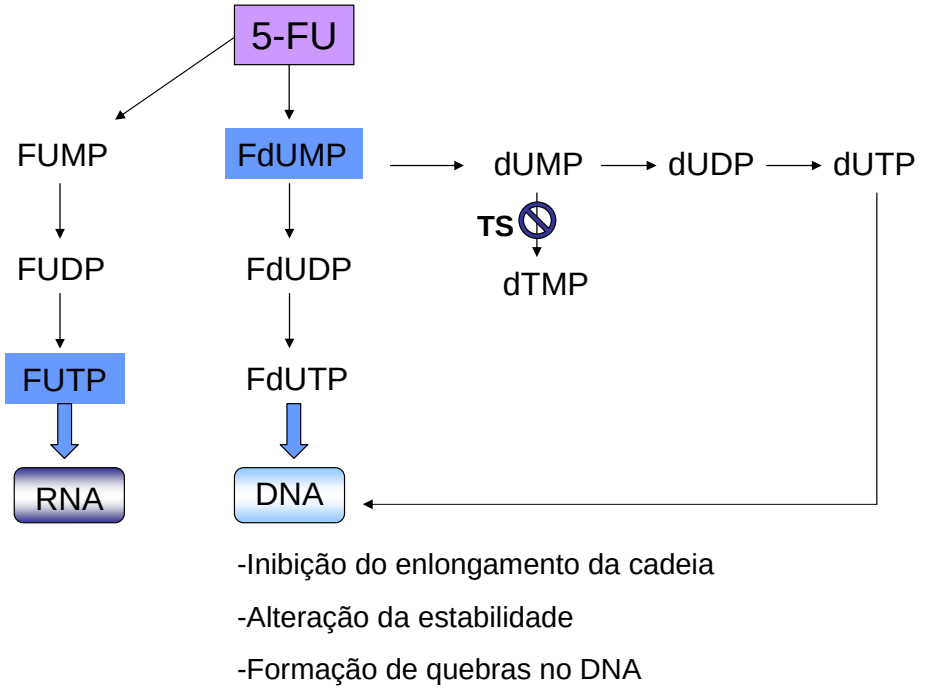

Figura 3: Metabolismo do 5-FU. Dentro das células, o 5-FU é convertido aos seus metabólitos, que por sua vez podem ser incorporados erroneamente no DNA ou RNA. O FdUMP, considerado o metabólito ativo, atua principalmente inibindo a timidilato sintase (TS), que leva a incorporação de uracil no DNA. O FdUMP também pode ser fosforilado e incorporado no DNA, o que resulta em diferentes efeitos, tais como a inibição do enlongamento da cadeia, alteração da estabilidade e formação de quebras.

Diversos mecanismos de resistência ao 5-FU têm sido descritos (CRUZ-MORCILLO *et al.,* 2005; UCHIBORI *et al.*, 2011). A Tabela 2 resume alguns destes (GREM, 1997; PETERS *et al.*, 2002, LONGLEY *et al.*, 2003; WANG *et al.,* 2007).

Tabela 2: Mecanismos de resistência ao 5-FU.

Mecanismos de resistência ao 5-FU
1. Diminuição do acúmulo de metabólitos ativos
(a) Diminuição da ativação
(b) Aumento da inativação
2. Resistência associada a alvos
(a) Diminuição do efeito no RNA
(b) Efeito alterado na TS
3. Resistência farmacocinética
(a) A droga não chega ao tumor
(b) Problemas na distribuição da droga
(c) Aumento da eliminação da droga
4. Diminuição da incorporação de fluoronucleotídeos no DNA
(a) Aumento da atividade da enzima dUTPase, que diminui a concentração de dUTP
(b) Elevada taxa de reparação das lesões geradas por 5-FU

3. Modeladores da Cromatina

Durante décadas, acreditou-se que apenas as alterações genéticas eram fundamentais para o início e o desenvolvimento de cânceres. No entanto, estudos recentes sugerem que mudanças epigenéticas podem ser igualmente importantes para a progressão tumoral e para a resposta a agentes antineoplásicos (ESCARGUEIL *et al.*, 2008; WHO, 2009; SHARMA *et al.*, 2010). O termo "Epigenético" refere-se às mudanças herdáveis na expressão gênica em células somáticas que ocorrem sem a alteração da seqüência do DNA, na qual a estrutura da cromatina exerce controle na regulação transcricional (CORTEZ &

JONES, 2008; ELLIS *et al.*, 2009). A seqüência primária do DNA especifica a seqüência do RNA transcrito e a correspondente seqüência de aminoácidos nas proteínas codificadas. Entretanto, apenas a seqüência de DNA não é capaz de determinar quais regiões do genoma serão expressas. O perfil da expressão gênica é determinado pela compactação do DNA nuclear e pela disponibilidade das regiões do DNA à maquinaria transcricional (ELLIS *et al.*, 2009).

Uma vez que o espaço nuclear é relativamente pequeno e as moléculas de DNA grandes, a compactação do DNA permitiu acomodar enormes quantidades de DNA dentro do núcleo, mas mantendo-o em uma conformação adequada ao acesso de fatores regulatórios (BECKER & HÖRZ, 2002; ALTAF *et al.*, 2007). A compactação nuclear ocorre pela interação do DNA com proteínas histonas e não-histonas, cuja unidade molecular básica da compactação do DNA é o nucleossomo. O nucleossomo é composto por um octâmero de histonas, contendo duas cópias de cada histona canônica (H2A, H2B, H3 e H4), envolvido por aproximadamente 146 pares de base de DNA (ALTAF *et al.*, 2007, PALOMERA-SANCHEZ & ZURITA, 2011). Cada cerne de histonas no nucleossomo contém um domínio globular e uma cauda amino-terminal (N-terminal), que se projeta para fora do cerne, rica em resíduos básicos e é altamente dinâmica. Esta cauda contribui para a manutenção da estrutura e estabilidade dos nucleossomos, bem como para a compactação da cromatina de ordem superior (BECKER & HÖRZ, 2002), além de ser o local de interação com diversos tipos de maquinarias enzimáticas capazes de modificá-las covalentemente (SHILATIFARD, 2006). Além das histonas canônicas, existem variantes de histonas em menor abundância que podem ser incorporadas nos

nucleossomos, como por exemplo, a histona H2AZ em mamíferos (Htz1 em *Saccharomyces cerevisiae*) que está presente em regiões que flanqueiam o sítio de início da transcrição (CLAPIER & CAIRNS, 2009) e contribui para ativação gênica por proteger contra a metilação de DNA (SHARMA *et al.*, 2010).

Além de solucionar o problema da compactação do DNA, estrutura da cromatina regula também diversos processos celulares como a replicação de DNA, transcrição, recombinação e reparação, uma vez que controla o acesso das maquinarias macromoleculares ao seu substrato (BECKER & HÖRZ, 2002; ATAIAN & KREBS, 2006). Para que tais processos possam atuar, a cromatina deve ser remodelada, de modo que o seu estado de compactação seja modificado (ESCARGUEIL *et al.*, 2008). A cromatina pode ser alterada por remodeladores dependentes de ATP e modificadores pós-traducionais. Dentre as modificações pós-traducionais covalentes das caudas N-terminais das histonas, destacam-se: (i) acetilação, (ii) metilação, (iii) fosforilação e (iv) ubiquitinação, que podem alterar a carga de resíduos específicos e afetar as interações histona-histona e histona-DNA, e, por conseguinte, sinalizar e recrutar outros complexos protéicos (ALTAF *et al.*, 2007; SHARMA *et al.*, 2010). À combinação destas intrincadas modificações pós-traducionais dá-se o nome de código de histonas, o qual é responsável por variadas e específicas respostas biológicas (ALTAF *et al.*, 2007; CORTEZ & JONES, 2008). Frente às lesões no DNA, diferentes tipos de modificadores da estrutura da cromatina são recrutados, a fim de que o dano seja sinalizado e reparado de maneira adequada (LUIJSTERBURG & VAN ATTIKUM, 2011).

3.1. Remodeladores dependentes de ATP

Os remodeladores dependentes de ATP são grandes complexos multi-protéicos que podem alterar a organização dos nucleossomos dentro da cromatina utilizando a energia da hidrólise do ATP (ESCARGUEIL *et al.*, 2008). Estes complexos podem mover octâmeros intactos de nucleossomos para regiões adjacentes no DNA, alterando o grau de compactação da cromatina, ou inserindo variantes de histonas dentro do nucleossomo. As variantes de histonas possuem propriedades distintas daquelas do cerne de histonas canônicas, e a sua substituição resulta em mudanças importantes na estrutura de ordem superior da cromatina (HENIKOFF & AHMAD, 2005; CLAPIER & CAIRNS, 2009). Modificadores pós-traducionais de histona podem alterar a interação das histonas com o DNA, levando a uma distorção na estrutura da cromatina, e assim permitindo o recrutamento de proteínas não-histônicas (ALTAF *et al.*, 2007; ESCARGUEIL *et al.*, 2008).

Além de atuar na re-locação dos nucleossomos para tornar o DNA mais acessível às proteínas de interação, os remodeladores da cromatina dependentes de ATP são importantes na ativação e repressão da transcrição que afetam o controle do ciclo celular, a diferenciação celular e o desenvolvimento de organismos multicelulares (BECKER & HÖRZ, 2002), além de atuar em respostas a danos no DNA (LUIJSTERBURG & VAN ATTIKUM, 2011). Deficiências nestes remodeladores estão relacionadas com o desenvolvimento de algumas doenças como a síndrome de Cockayne do grupo B (CSB) e a α-talassemia ligada ao X (CLAPIER & CAIRNS, 2009).

3.2. Acetilação

A acetilação ocorre pela atividade de enzimas acetiltransferase de histonas (HAT) que transferem grupamentos acetil, provenientes do acetil-CoA, para resíduos de lisina nas caudas N-terminais do cerne de histonas. Desta forma, as cargas das lisinas são neutralizadas, o que aumenta o acesso das proteínas ao DNA e por fim leva à ativação gênica (ROTH *et al.*, 2001; ESCARGUEIL *et al.*, 2008). O processo inverso a acetilação, a desacetilação, ocorre pela atividade de enzimas desacetilase de histonas (HDAC) e está relacionado à repressão transcricional. As HATs e HDACs regulam a expressão de muitos genes, inclusive os envolvidos em apoptose e proliferação celular (KRISTENSEN *et al.*, 2009), progressão do ciclo celular, recombinação e reparação de DNA, e é de extrema importância para a embriogênese em mamíferos (ROTH *et al.*, 2001). Existem algumas HATs, tais como Gcn5 e p300, que atuam em resposta a danos no DNA, aumentando a acessibilidade ao DNA (DINANT *et al.*, 2008; LUIJSTERBURG & VAN ATTIKUM, 2011).

As HATs podem ser classificadas em 2 grupos, baseados na origem celular e função: (i) *HATs do Tipo A*, nucleares, catalizam eventos de acetilação relacionados à transcrição (ex: Gcn5) e atuam em histonas associadas a nucleossomos da cromatina; (ii) *HATs do Tipo B*, citoplasmáticas, que catalizam eventos de acetilação em histonas não associadas a cromatina que se encontram no citoplasma e que serão transportadas para o núcleo posteriormente (ex: Hat1) (ROTH *et al.*, 2001; QIN & PARTHUM, 2002).

3.3. Metilação

O processo de metilação ocorre imediatamente após a replicação pela atividade das enzimas DNA metiltransferases (DNMT) que transferem grupamentos metil, provenientes da S-adenosilmetionina ou AdoMet, para os aminoácidos arginina e lisina, e para a base nitrogenada citosina (SHILATIFARD, 2006; SZYF, 2009). A metilação atua no controle da expressão gênica tanto na ativação quanto no silenciamento, dependendo do substrato. A metilação de citosinas em sítios de início de transcrição leva ao silenciamento. No entanto, a metilação de lisinas pode estar relacionada à ativação (lisinas 4, 36 e 79 da histona H3) ou inativação gênica (lisinas 9 e 27 da histona H3, e lisina 20 da histona H4) (DINANT *et al.*, 2008). O silenciamento da expressão gênica pela metilação pode ser resultado de interferência na ligação de fatores de transcrição ou pela atração de proteínas metiladas que se ligam ao DNA (SZYF, 2009).

As DNMT podem ser classificadas como: (i) enzimas *de novo* (DNMT3a e DNMT3b), que metilam o DNA não-metilado; (ii) enzimas de *manutenção* (DNMT1), que atuam preferencialmente em sítios CpG hemimetilados (CORTEZ & JONES, 2008).

A metilação de citosinas nos dinucleotídeos de CG no DNA genômico assegura o padrão de repressão transcricional herdável, de maneira a evitar a expressão de elementos indesejáveis como inserções virais, elementos repetitivos e outras seqüências deletérias (CORTEZ & JONES, 2008; SZYF, 2009). O processo de metilação é essencial para diversas funções, tais como: (i) o desenvolvimento embrionário, uma vez que camundongos com deficiências

nestas enzimas morrem em estágios embrionários ou logo após o nascimento, (ii) a repressão transcricional de regiões repetitivas e centroméricas, (iii) a inativação do cromossomo X em fêmeas e (iv) o “imprinting” genômico (GROTH *et al.*, 2007; GAL-YAM *et al.*, 2008).

Estudos reportaram que padrões aberrantes da metilação do DNA podem atuar na tumorigênese: as células tumorais apresentavam conteúdo de 5-metilcitosina diminuído, assim como uma hipometilação global de elementos de DNA repetitivos, que contribui para a instabilidade genética e super-expressão de proteínas oncogênicas. Além disso, a hipermetilação de promotores de supressores tumorais, que resulta em silenciamento, também atua no desenvolvimento tumoral, tais como no retinoblastoma, no gene *VHL* (von Hippel-Lindau) em câncer renal, *CDKN2 A/p16* em câncer de bexiga, e no gene *MLH1* em câncer de cólon (GAL-YAM *et al.*, 2008).

Adicionalmente, o processo de metilação pode atuar em resposta a danos no DNA. Deficiências na metilação da lisina 79 da histona H3 pela Dot1p ou mutações nesta lisina resultam em hipersensibilidade a luz ultravioleta (UV) e problemas no ponto de checagem intra-S. A metilação da lisina 20 da histona H4 também é importante na sinalização para os pontos de checagem frente aos danos de quebra de fita dupla de DNA geradas pela UV (DINANT *et al.*, 2008).

3.4. Fosforilação

O processo de fosforilação ocorre pela atividade de enzimas cinases, que transferem grupamentos fosfato para as serinas, utilizando a energia do ATP. Dentre os eventos de fosforilação de histonas, o mais conhecido é o da serina-139 da histona H2AX em mamíferos (ser-129 em *S. cerevisiae*) em resposta à formação de quebras de fita dupla de DNA (DSBs). Após a formação das DSBs, as cinases da família da fosfatidilinositol 3-cinase (PI3K) (ATM, ATR ou DNA-PK) são ativadas e fosforilam H2AX, bem como outras proteínas de reparo e pontos de checagem. A fosforilação da histona H2AX é de extrema importância para que a lesão seja sinalizada e processada corretamente (ESCARGUEIL *et al.*, 2008; LUIJSTERBURG & VAN ATTIKUM, 2011).

Existem ainda outros resíduos da histona H3, como a serina 10 e a treonina 11, que quando fosforilados, atuam em resposta ao dano no DNA levando a ativação transcricional (DINANT *et al.*, 2008). A histona H2B é fosforilada na serina 14 antes da formação de γH2AX, porém sua função é desconhecida (LUIJSTERBURG & VAN ATTIKUM, 2011).

3.5. Ubiquitinização

A ubiquitinização é uma modificação pós traducional de histonas na qual a ubiquitina é conjugada a resíduos de lisina. A ubiquitina é um pequeno peptídeo de 8.5 kDa que pode marcar proteínas conjugadas para a degradação pelos

proteassomos ou servir como modificador de funções de proteínas. O processo de ubiquitinização ocorre pela reação das enzimas de ativação (E1), conjugação (E2) e ligação (E3) (DINANT *et al.*, 2008; MÉNDEZ-ACUÑA *et al.*, 2010). Cerca de 10 a 15% de todas moléculas de H2A nucleares em células humanas encontram-se ubiquinadas na lisina 119, e este processo está relacionado à repressão transcricional. Apenas 1% das moléculas de H2B apresentam ubiquitinização na lisina 120, que resulta em ativação transcricional. As histonas H3 e H4 também podem ser ubiquitinadas, porém em níveis muito baixos (~0.1%) (LUIJSTERBURG & VAN ATTIKUM, 2011).

A ubiquitinização vem sendo apontada como uma modificação chave na resposta ao dano no DNA, principalmente do tipo DSBs. As histonas H2A e H2AX (lisina 119) são ubiquitiladas em resposta ao dano pelas ubiquitinas ligases RNF8 e RNF168, juntamente com enzimas de conjugação UBC13. A ubiquitinização da histona H2A é importante na sinalização e recrutamento de fatores como BRCA1, 53BP1 nos locais de quebras de fita dupla, e é dependente de ATM/DNA-Pk, ATR e γH2AX (LUIJSTERBURG & VAN ATTIKUM, 2011). Além disso, as histonas H3 e H4 podem ser ubiquitinizadas temporariamente em resposta ao dano, dependendo do tipo da lesão (DINANT *et al.*, 2008).

Em leveduras, a ubiquitinização da histona H2B na lisina 123 pelo complexo Rad6/Bre1 E2/E3 é necessária para o reparo de diferentes tipos de lesões, incluindo as derivadas de radiação ultra-violeta (UV). Na ausência desta modificação, a ativação de Rad53 é afetada e outra proteína regulatória de ponto de checagem, a Rad9, é ativada (GAME & CHERNIKOVA, 2009).

3.6. A Epigenética e o Câncer

Alterações epigenéticas podem resultar na ativação de oncogenes, no silenciamento de supressores tumorais e até na proliferação descontrolada em cânceres. Estas mudanças podem estar relacionadas com alterações nos níveis de enzimas que modificam a cromatina e com a distribuição da metilação do DNA pelo genoma (ELLIS *et al.*, 2009), e caso ocorram em células em divisão, podem ser transmitidas para as células-filhas (WHO, 2009). O conhecimento de como estas anomalias ocorrem pode auxiliar no desenvolvimento e na melhoria de agentes que atuem nestes fatores durante a tumorigênese (CORTEZ & JONES, 2008).

Em alguns cânceres humanos observou-se que os remodeladores de cromatina dependentes de ATP da família SWI/SNF encontravam-se inativados, sugerindo que eles possam funcionar como proteínas supressoras de tumor (LUIJSTERBURG & VAN ATTIKUM, 2011). O padrão de metilação do DNA também pode ser alterado durante a progressão tumoral: tanto a hipometilação quanto a hipermetilação em diferentes regiões do genoma contribuem para a tumorigênese. Durante este processo, a desmetilação do genoma pode promover a instabilidade genômica pela ativação de retrotransposons silenciados ou pelo aumento de rearranjos cromossômicos (CORTEZ & JONES, 2008). A atividade alterada de HATs, tanto pela inativação por mutação quanto por oncoproteinas virais, está presente em cânceres sólidos e hematológicos: mutações em p300 foram identificadas em tumores de cólon, gástricos, mama e pâncreas, e as proteínas dos adenovírus E1A e SV40T podem interagir com p300/CBP, levando

a transformação celular e alteração da expressão gênica (ELLIS *et al.*, 2009). Adicionalmente, a HAT Tip60 atua na modulação da sinalização via ATM, p53 e Myc, e quando ocorre redução na expressão desta HAT, a hipoacetilação leva a defeitos na indução de apoptose (ELLIS *et al.*, 2009).

Diversos tipos de câncer apresentam alta freqüência de silenciamento gênico. No entanto, o silenciamento epigenético é potencialmente reversível e por isso é um importante alvo terapêutico (KWA *et al.*, 2011). Atualmente, existem agentes capazes de inibir a metilação DNA, como a 5-azacitidina (5-Aza-CR ou Vidaza®) e 5-aza-2-desoxicitidina (5-Aza-CdR ou decitabine), ou inibidores de desacetilases, tais como a tricostatina A e o ácido suberoilanide hidroxâmico (SAHA) para o tratamento da síndrome mielodisplástica, leucemias mielóide aguda e mielóide crônica (KRISTENSEN *et al.*, 2009). A 5-Aza-CR difere-se da citosina pela substituição de um nitrogênio na posição 5 do carbono. Durante a replicação, esta droga é incorporada no DNA e o anel modificado da citosina inibe a metilação por bloquear as DNMTs. Logo, a depleção das DNMTs resulta na redução da metilação da citosina no DNA sintetizado (SZYF, 2009). Estas drogas atuam principalmente por restaurar funções celulares normais por re-expressar genes supressores tumorais que foram hipermetilados. Desta forma, é possível corrigir vias e restaurar funções celulares que foram afetadas durante a tumorigênese, como o controle do ciclo celular, apoptose, adesão celular e migração celular (MENDELSOHN *et al.*, 2008; KWA *et al.*, 2011).

4. Vias de reparação de DNA

O DNA pode sofrer alterações por diferentes agentes, tanto endógenos quanto exógenos, e frente a estas lesões, uma complexa resposta celular é ativada com a finalidade de se preservar a estabilidade genômica. A resposta ao dano no DNA envolve a detecção do sítio lesionado, a amplificação do sinal através de uma cascata de proteínas cinases e a ativação de uma série de efetores que promovem a parada de ciclo celular e reparo de DNA (MÉNDEZ-ACUÑA *et al.*, 2010). As vias de reparação de DNA são responsáveis pelo processamento de diferentes tipos de danos induzidos no DNA e são essenciais para evitar o acúmulo de mutações. O acúmulo de mutações em organismos unicelulares pode resultar em morte e em multicelulares em cânceres. Dependendo do tipo da lesão no DNA, diferentes proteínas são recrutadas para reconhecer e processar ou tolerar o dano. Existem 6 principais vias de reparação DNA: (i) o reparo por excisão de bases (BER); (ii) reparo por excisão de nucleotídeos (NER); (iii) reparo de bases mal-emparelhadas (MMR); (iv) reparo por síntese translesão (TLS); (v) reparo por recombinação homóloga (HR) e (vi) reparo por recombinação não-homóloga ou ilegítima (NHEJ) (HSIEH, 2001; BERNSTEIN *et al.*, 2002; DUDÁS & CHOVANEC, 2004; DUDÁSOVÁ *et al.*, 2004; IYER *et al.*, 2006).

4.1. Reparação por excisão de bases (BER)

O reparo por BER é uma via livre de erro que processa lesões que causam pouca distorção na dupla hélice de DNA, tais como bases danificadas pela metilação, desaminação, alquilação e oxidação, ou ainda bases que foram erroneamente incorporadas durante a replicação (ATAIAN & KREBS, 2006; MÉNDEZ-ACUÑA *et al.*, 2010). Nesta via, os danos são reconhecidos por uma DNA glicosilase, que remove a base errônea do DNA ao hidrolisar a ligação glicosídica entre a base e a cadeia desoxirribose-fosfato, formando um sítio apurínico/apirimídico ou abásico (AP). Em seguida, uma AP endonuclease ou DNA glicosilase/AP liase cliva a ligação fosfodiéster do DNA e uma DNA polimerase livre de erro insere o nucleotídeo correto. Por fim, as extremidades são seladas pela enzima ligase (BOITEUX & GUILLET, 2004; DIZDAROGLU, 2005; FRIEDBERG *et al.*, 2006). O reparo por BER possui duas vias, uma curta que remove apenas 1 nucleotídeo e outra longa, na qual são substituídos de 2 a 13 nucleotídeos (MEMISOGLU & SAMSON, 2000; BROZMANOVÁ *et al.*, 2001; ATAIAN & KREBS, 2006). A Figura 4 mostra o esquema de reparo por BER em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 3.

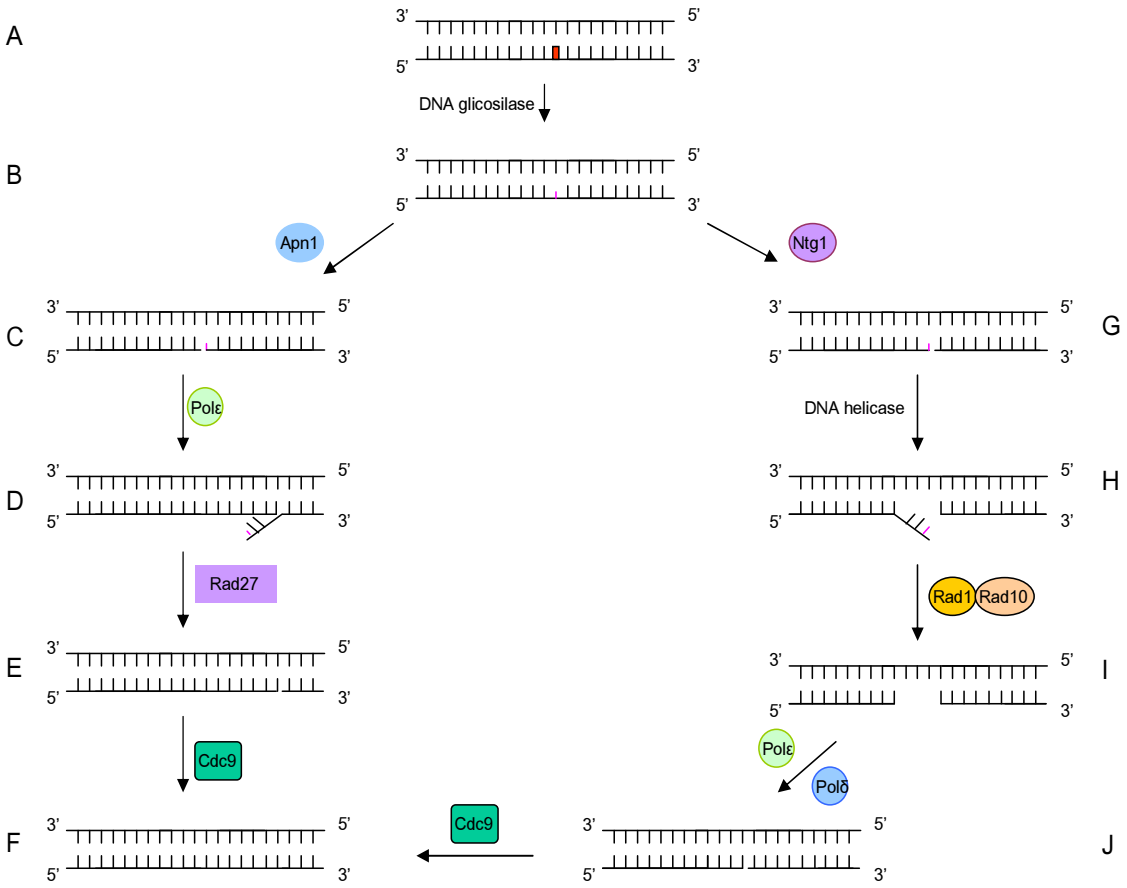

Figura 4: Esquema da reparação por BER em *S. cerevisiae*. A base danificada (A) é removida pela DNA glicosilase formando um sítio AP (B). A maior parte dos sítios AP são reparados pela AP endonuclease (Apn1), DNA polimerase (Polε), 5'-flap endonuclease (Rad27) e uma DNA ligase (Cdc9) (C-F). Uma fração menor de sítios AP é clivada por AP liases (Ntg1, Ntg2 ou Ogg1), seguida pela clivagem por Rad1-Rad10 (G-J) (Adaptado de BOITEUX & GUILLET, 2004).

Tabela 3: Principais proteínas do BER em *S. cerevisiae.*

Proteína	Função
Ogg1	DNA glicosilase e AP liase que excisa danos do tipo 8-oxoguanina
Ung1	DNA glicosilase que excisa uracil do DNA
Mag1	DNA glicosilase que excisa danos do tipo 3-metil adenina
Ntg1	DNA glicosilase e AP liase que atua no núcleo e mitocôndria
Ntg2	DNA glicosilase e AP liase que atua no núcleo
Apn1	Principal AP endonuclease que repara danos oxidativos e alquilações
Apn2	AP endonuclease
Rad27	"Flap" endonuclease com atividade 5´- 3´ exonuclease
Cdc9	DNA ligase presente no núcleo e mitocôndria

4.2. Reparação por excisão de nucleotídeos (NER)

A via de reparação por NER é uma via de reparo livre de erros que processa lesões que causam grandes distorções da dupla hélice de DNA, tais como fotoadutos gerados pela luz UV (dímeros de pirimidina ciclobutano – CPD e 6-4-pirimidina pirimidona – 6-4-PP), adutos gerados por espécies reativas de oxigênio, e pontes inter e intra-cadeia de DNA (JEPPESEN *et al.*, 2011). Nesta via, o complexo de excisão reconhece a lesão e remove um fragmento de DNA de fita simples de 24 a 30 nucleotídeos que contém o dano. A DNA polimerase re-sintetiza a cadeia de DNA e a ligase sela as extremidades restaurando a fita (PRAKASH & PRAKASH, 2000; FRIEDBERG *et al.*, 2006). O NER é subdividido em duas vias: a reparação acoplada à transcrição (TCR: "transcription coupled repair") e a reparação global do genoma (GGR: "global genome repair"). A TCR

repara lesões presentes em fitas transcritas de genes expressos, enquanto a GGR atua no restante do genoma (para revisão ver PRAKASH & PRAKASH, 2000; SARASIN & STARY, 2007; PALOMERA-SANCHEZ & ZURITA, 2011) e diferem-se nas proteínas que reconhecem as lesões (VAN HOFFEN *et al.,* 2003; JEPPESEN *et al.*, 2011). A Figura 5 mostra o esquema de reparo por NER em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 4.

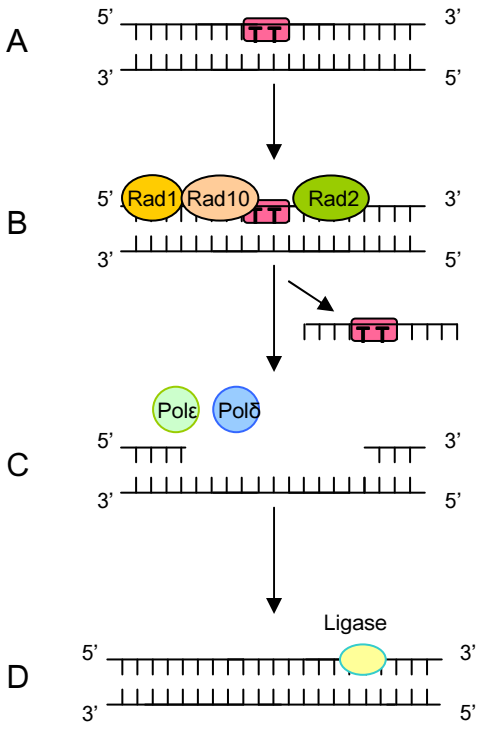

Figura 5: Esquema da reparação por NER em *S. cerevisiae*. A lesão (A) é reconhecida e o complexo de excisão formado por Rad1-Rad10 e Rad2 cliva as extremidades do oligonucleotídeo que contém o dano (B). As DNAs polimerases ε e δ preenchem a lacuna e a DNA ligase sela as extremidades (Adaptado de SWANSON *et al.*, 1999).

Tabela 4: Principais proteínas do NER em *S. cerevisiae.*

Proteína	Função
Rad1	Nuclease que juntamente com Rad10 cliva a extremidade 5´ do oligonucleotídeo contendo a lesão
Rad2	Nuclease que cliva a extremidade 3´ do oligonucleotídeo contendo a lesão
Rad3	DNA helicase 5´→ 3´
Rad4	Atua junto com a Rad23 no reconhecimento e ligação ao dano
Rad10	Nuclease que juntamente com Rad1 cliva a extremidade 5´ do oligonucleotídeo contendo a lesão
Rad14	Proteína que reconhece e se liga ao dano
Rad25	DNA helicase 3´ → 5´
Rad26	ATPase dependente de DNA envolvida no TCR

4.3. Reparação de bases mal-emparelhadas (MMR)

A reparação por MMR é uma via de reparo por excisão livre de erros que corrige mal-emparelhamentos de pares de base no DNA gerados durante a replicação ou recombinação homóloga, ou que foram causados por agentes genotóxicos (ATAIAN & KREBS, 2006; JEPPESEN *et al.*, 2011). Falhas nesta via de reparação aumentam significativamente as taxas de mutagênese e de instabilidade genômica, e está associada com o desenvolvimento de cânceres em humanos (LI, 2008). O mal-emparelhamento é reconhecido pela distorção na dupla fita de DNA por proteínas específicas, que recrutam outros fatores que levam a mudanças conformacionais e clivagem de um fragmento contendo a lesão na fita-filha. O oligonucleotídeo é removido por uma exonuclease, a DNA

polimerase re-sintetiza a fita-filha e as extremidades são seladas pela ligase (JUN *et al.*, 2006; IYER *et al.*, 2006; JIRICNY, 2006). A Figura 6 mostra o esquema de reparo por MMR em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 5.

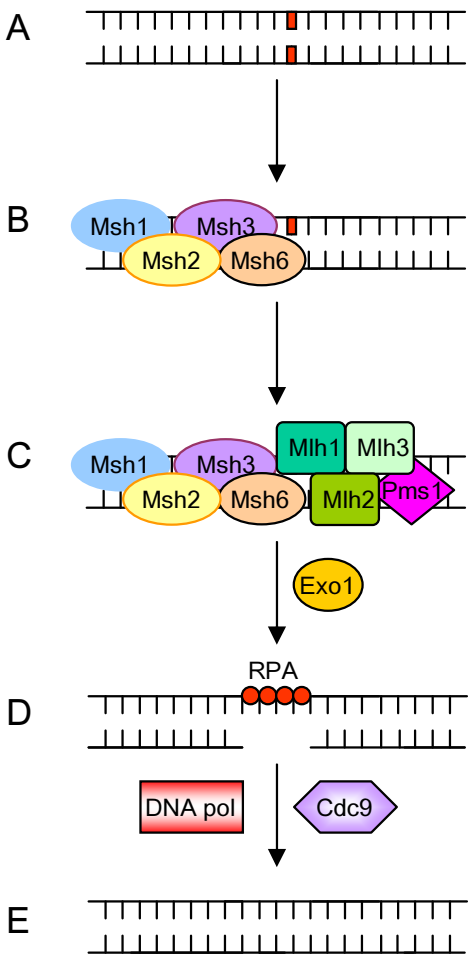

Figura 6: Esquema da reparação por MMR em leveduras. As bases mal-emparelhadas (A) são reconhecidas pelas proteínas Msh1, Msh2, Msh3 e Msh6 (B). Em seguida, ligam-se Mlh1, Mlh2, Mlh3 e Pms1 às proteínas de reconhecimento, e, juntas promovem a clivagem do oligonucleotídeo que contém a base mal-emparelhada (C). A exonuclease Exo1 remove o fragmento de DNA e a RPA liga-se às regiões do DNA de fita simples (D). A enzima DNA polimerase re-sintetiza o fragmento e a ligase Cdc9 sela as extremidades (Adaptado de ATAIAN & KREBS, 2006).

Tabela 5: Principais proteínas do MMR em *S. cerevisiae.*

Proteína	Função
Msh1	Liga-se a base mal-emparelhada. Homólogo ao MutS em *E.coli*
Mlh1	Forma complexo com Pms1 e Msh2-Msh3. Homólogo ao MutL em *E.coli*
Pms1	Liga-se a DNA de fita simples ou dupla. Homólogo ao MutL em *E.coli*

4.4. Reparação por síntese translesão (TLS)

As lesões no DNA capazes de bloquear a replicação, geralmente consideradas letais, são reparadas principalmente por BER e NER. No entanto, caso estas vias estejam saturadas ou sejam incapazes de reparar tais danos por serem muito extensos para serem eficientemente removidos, as vias de tolerância podem ser ativadas a fim de se evitar a morte celular. A via de reparo por TLS é um mecanismo de tolerância ao dano, pós-replicativa, na qual uma DNA polimerase especial é capaz de ultrapassar a lesão inserindo um nucleotídeo no lado oposto à base lesada reiniciando a replicação (FRIEDBERG *et al.*, 2006; LEHMANN, 2005; ACHARYA *et al.*, 2006; GAN *et al.*, 2008). Porém, este nucleotídeo que é adicionado, pode não ser correspondente ao original e, desta forma, são introduzidas mutações como substituição de pares de bases e deslocamento do quadro de leitura no DNA que está sendo reparado (PRAKASH & PRAKASH, 2000; GAN *et al.*, 2008). O reparo por TLS pode ser efetuado por dois tipos de DNA polimerases, uma passível de erro pela DNA pol zeta (ζ) ou por uma livre de erros pela DNA pol eta (η) (FRIEDBERG *et al.*, 2006). A Figura 7

mostra o esquema de reparo por TLS em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 6.

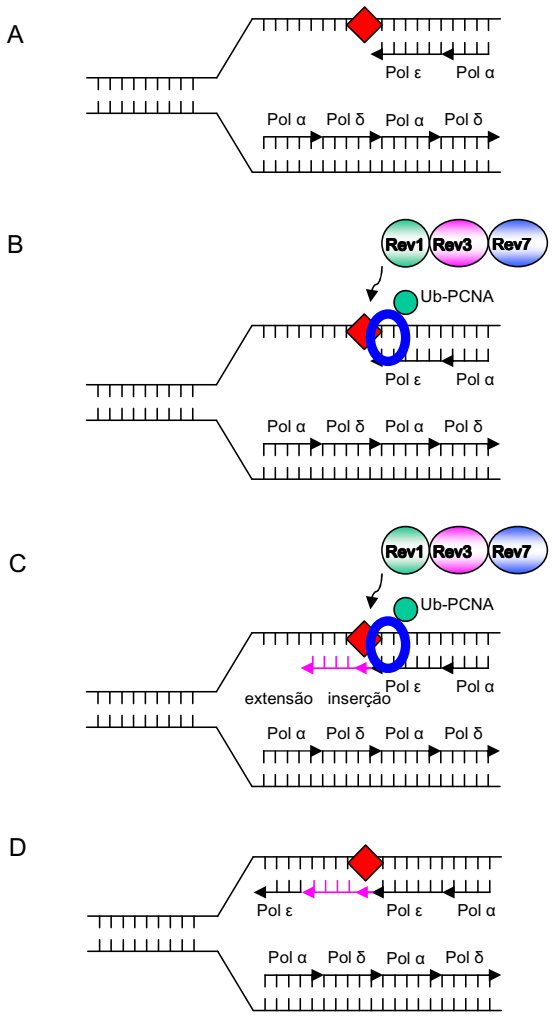

Figura 7: Reparação por TLS em leveduras. O losango vermelho representa uma lesão no DNA. As DNA polimerases replicativas, polδ e polε, são responsáveis pela replicação do DNA genômico, porém não são capazes de ultrapassar lesões e param a replicação (A). Perante a parada da forquilha de replicação, o complexo ubiquitina – PCNA dissocia as polimerases de replicação e recruta polimerases que são capazes de ultrapassar lesões: pol zeta (Rev3-Rev7) associadas a Rev1 (B). A Rev3-Rev7 ou possivelmente Rev1, insere um nucleotídeo no filamento oposto ao que contém a lesão, e pol zeta realiza a extensão (C). Após ultrapassar a lesão, o complexo envolvendo Rev3-Rev7 e Rev1 é dissociado e as polimerases de replicação são re-associadas, dando

continuidade à síntese de DNA (D). Adaptado de GAN *et al.* (2008).

Tabela 6: Principais proteínas do TLS em *S. cerevisiae.*

Proteína	Função
Rev1	Atividade de desoxicitidil transferase capaz de ultrapassar lesões do tipo sítios AP. Atua juntamente com Rev3 e Rev7.
Rev3	Subunidade catalítica da DNA pol zeta (ζ). Ultrapassa lesões do tipo dímeros de pirimidina e sítios AP. Repara DSBs.
Rev7	Atua em conjunto com a Rev3. Repara DSBs.
Rad30	Codifica a DNA pol eta (η) que é capaz de reparar danos gerados por UV por uma via livre de erros, mas passível de erro quando repara lesões do tipo O^6-metilguanosina

4.5. Reparação por recombinação homóloga (HR)

A via de reparo por HR processa lesões do tipo DSBs e pontes inter-cadeia, resgatando a informação genética de uma fita homóloga não danificada (cromossomo homólogo ou cromátide-irmã). É uma via livre de erro que atua nas fases S e G2/M do ciclo celular (LISBY & ROTHSTEIN, 2009; MISTELI & SOUTOGLU, 2009). A reparação por HR é iniciada pelo reconhecimento e ligação do complexo Mre11/Rad50/Nbs1 (Mre11/Rad50/Xrs2 em *S. cerevisiae*) na extremidade da DSB, seguido pela remoção de extremidades 5´ nas DSBs pelas nucleases, formando caudas de DNA de fita simples com extremidade 3´. Em seguida, uma fita simples de DNA com extremidade 3´ livre invade o duplex e gera uma estrutura *D-loop*. As duas extremidades 3´ servem então de primer para nova síntese de DNA utilizando a fita intacta como molde. Este processo, seguido

pela ligação, leva à formação de duas junções de *Holliday*, que podem se mover ao longo do DNA e que são clivadas por uma resolvase, gerando produtos com ou sem *crossing-over* (PASTWA & BLASIAK, 2003; SAFFI & HENRIQUES, 2003; DUDÁS & CHOVANEC, 2004; SHUMAN & GLICKMAN, 2007; LI & HEYER, 2008). A Figura 8 mostra o esquema de reparo por HR em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 7.

Tabela 7: Principais proteínas do HR em *S. cerevisiae.*

Proteína	Função
Rad50	Atua juntamente com Mre11 e Xrs2 na ligação ao DNA e possui atividade de endo e exonuclease.
Rad51	Proteína que realiza a troca entre as fitas.
Rad52	Facilita a ligação da Rad51 ao DNA de fita simples e estimula o anelamento e a troca entre as fitas.
Mre11	Atua juntamente com Rad50 e Xrs2 na ligação ao DNA e possui atividade de endo e exonuclease.
Xrs2	Atua juntamente com Mre11 e Rad50 na ligação ao DNA e possui atividade de endo e exonuclease.

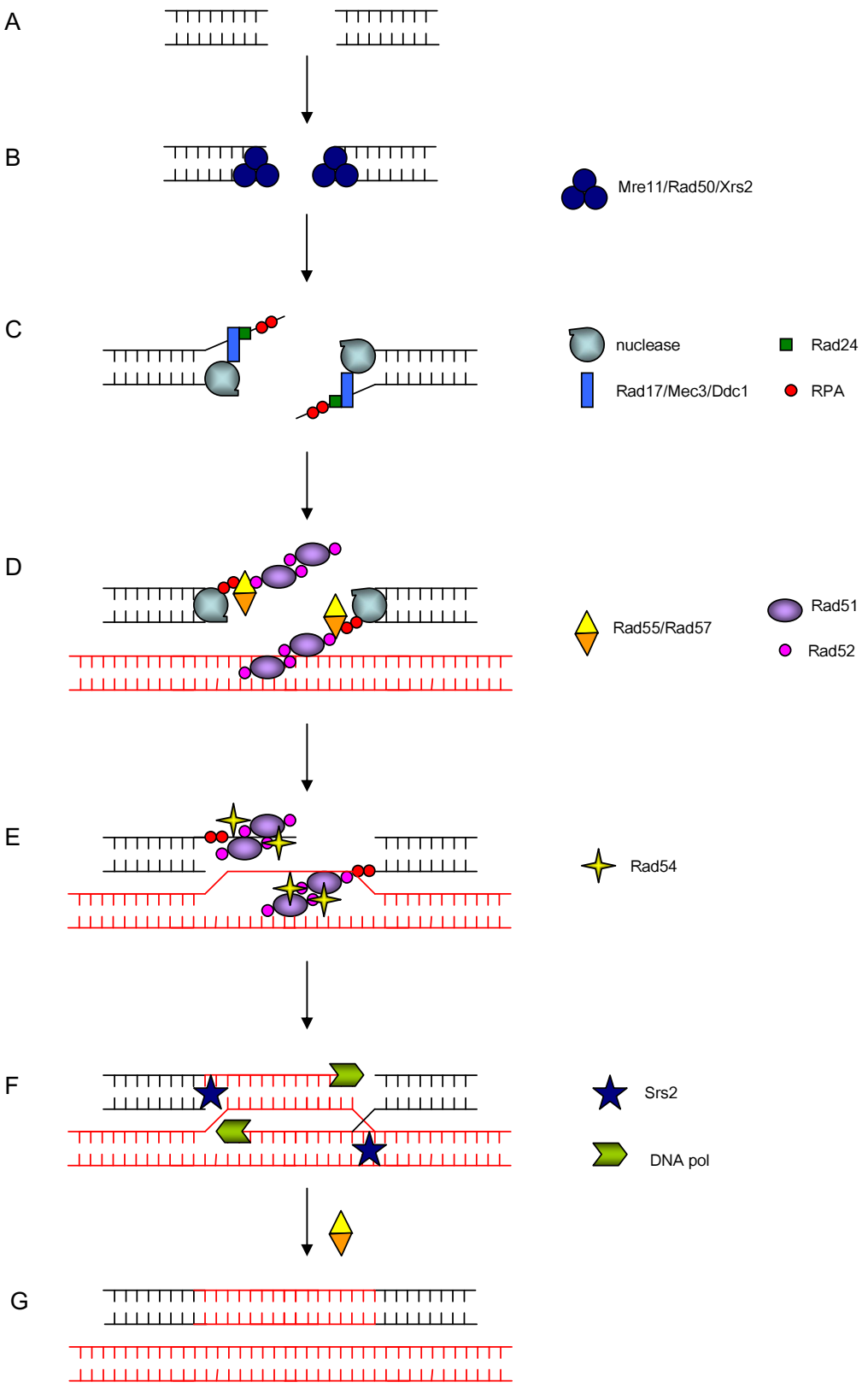

Figura 8: Esquema da reparação por HR em *S. cerevisiae.* Após a formação da DSB (A), o complexo Mre11/Rad50/Xrs2 reconhece e liga-se as extremidades (B),

seguido pelas nucleases que formam caudas de DNA de fita simples, que por sua vez são cobertas pelas RPAs (C). Em seguida, o filamento de Rad51 é formado, ocupando o lugar das RPAs. A Rad52 e o complexo Rad55/Rad57 mediam a formação do filamento (D), podendo haver a participação da Rad54. Concomitantemente ocorre a invasão do filamento na busca por seqüências homólogas, e uma vez encontrada, o processo de re-secção cessa e Rad51 dissocia-se (E). A helicase Srs2 media a dissociação da Rad51 e a DNA polimerase sintetiza as fitas (F). Por fim, as extremidades são religadas (G) (Adaptado de AYLON & KUPIEC, 2004).

4.6. Reparação por recombinação não-homóloga ou ilegítima (NHEJ)

A via de reparação por NHEJ processa DSBs pela ligação direta das extremidades sem necessidade de homologia de seqüência, e conseqüentemente, é uma via altamente mutagênica. Esta via de reparo ocorre preferencialmente na fase G1 do ciclo celular (LISBY & ROTHSTEIN, 2009; MISTELI & SOUTOGLU, 2009). Em mamíferos, na presença de DSBs, o heterodímero Ku (Yku70 e Yku80 em leveduras) se liga às extremidades do DNA, recrutando a subunidade catalítica de proteína cinase dependente de DNA (DNA-PKcs) ao sítio da lesão e desencadeando mudanças na estrutura da cromatina, semelhante a heterocromatina condensada em volta da lesão. O processo de ligação das extremidades envolve a DNA ligase IV (PASTWA & BLASIAK, 2003; BRUGMANS *et al.*, 2007; MLADENOV & ILIAKIS, 2011). O reparo de DSB em *S. cerevisiae* ocorre preferencialmente pela via HR, porém, quando esta se encontra inativada, a via NHEJ é recrutada (HEFFERIN & TOMKINSON, 2005). Os complexos Yku70/Yku80 e Dnl4/Lif1 de leveduras são homólogos funcionais das

proteínas Ku e DNA ligase IV/XRCC4 de mamíferos. No entanto, não existem homólogos à DNA-PKcs em leveduras, o que sugere que o complexo Rad50/Mre11/Xrs2 atue como fator que aproxime as extremidades rompidas (HEFFERIN & TOMKINSON, 2005). A Figura 9 mostra o esquema de reparo por NHEJ em *S. cerevisiae* e as principais proteínas envolvidas nesta via estão apresentadas na Tabela 8.

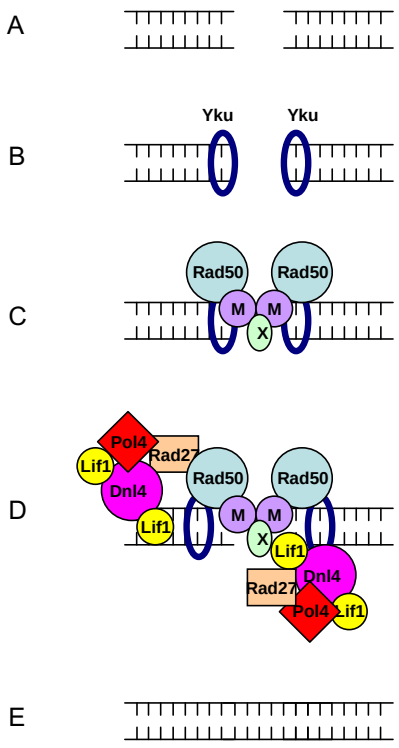

Figura 9: Modelo da reparação por NHEJ em *S. cerevisiae*. Após a indução de DSB (A), as proteínas Yku ligam-se às extremidades (B). O complexo Mre11(M)/Rad50/Xrs2(X) se associa ao Yku ligado ao DNA formando uma ponte (C). A DNA ligase Dnl4 associada com Lif1 é recrutada ao local da quebra, promovendo o processamento e preenchimento da lacuna pela Rad27 e Pol4 (D, E). Adaptado de HEFFERIN & TOMKINSON (2005).

Tabela 8: Principais proteínas do NHEJ em *S. cerevisiae.*

Proteína	Função
Yku70	Atua juntamente com Yku80 na manutenção do comprimento e estrutura de telômeros, e é realocado para locais de DSBs para promover o reparo por NHEJ
Yku80	Atua juntamente com Yku70 e é realocado para locais de DSBs para promover o reparo por NHEJ
Dnl4	DNA ligase
Lif1	Componente da DNA ligase IV

4.7. A Reparação de DNA e o Câncer

Uma vez que as vias de reparação de DNA mostram-se de extrema importância na homeostase celular, defeitos nestas vias podem levar ao desenvolvimento de diversas doenças, tais como a ataxia telangiectasia, anemia de Fanconi, *Xeroderma pigmentosum*, síndrome de Cockayne e tricotiodistrofia, câncer de cólon não-poliposo hereditário, entre outros (ANDRESSOO & HOEIJMAKERS, 2005; JEPPESEN *et al.*, 2011). Estas doenças apresentam aumento à predisposição ao câncer, radiosensibilidade, neurodegeneração e envelhecimento precoce, como conseqüência de problemas no processamento de quebras de fitas simples e duplas de DNA (ANDRESSOO & HOEIJMAKERS, 2005; CLEAVER, 2005; DAVID *et al.*, 2007; KRWAWICZ *et al.*, 2007; MCKINNON & CALDECOTT, 2007; RASS *et al.*, 2007; WILSON & BOHR, 2007; SALK et al., 2010).

A reparação de DNA é um importante fator que influencia na resposta antitumoral de terapias baseadas em drogas que induzem danos no DNA. Muitos pacientes com tumores resistentes à quimioterapia podem apresentar alta expressão e/ou elevada atividade das proteínas de reparo de DNA, que removem as lesões antes delas se tornarem tóxicas (DING *et al.*, 2006; HELLEDAY *et al.*, 2008). No entanto, muitos tipos de câncer possuem deficiências em vias de reparo que podem torná-los mais sensíveis ou resistentes aos agentes anticâncer. Desta forma, os mecanismos de reparo apresentam-se como alvos promissores no tratamento do câncer, pois sua exploração pode levar ao desenvolvimento protocolos mais específicos e com menos efeitos colaterais (HELLEDAY *et al.*, 2008), além de identificar pacientes que melhor responderão a um determinado medicamento.

Neste sentido, os cânceres de mama e ovário que apresentam defeitos em genes *BRCA1* e *BRCA2*, da via de reparação por HR (SALK *et al.*, 2010), respondem de maneira muito eficaz à agentes indutores de DSBs (HELLEDAY *et al.*, 2008; SMITH *et al.*, 2010). O câncer colorretal hereditário não-poliposo pode apresentar deficiências em genes do reparo por MMR (*MLH1* e *MSH2)* importantes no processamento de lesões que persistiram à replicação, na sinalização de parada de ciclo celular e na indução de apoptose em resposta a agentes que causam danos no DNA, e podem levar a resistência a alguns agentes antitumorais como cisplatina e 5-FU (HSIEH *et al.*, 2001; MASSEY *et al.*, 2003; IYER *et al.*, 2006; MODRICH, 2006; O´BRIEN & BROWN, 2006).

A expressão de genes de reparação também influencia na resposta aos agentes antineoplásicos. O câncer de testículo apresenta resposta clínica

favorável à cisplatina quando os pacientes exibem baixos níveis de *XPA* e *ERCC1-XPF,* relacionada à baixa capacidade de reparo dos adutos cisplatina-DNA (WANG & LIPPARD, 2005). No tratamento de sarcomas com o agente antitumoral ecteinascidina-743, verificou-se que a alta expressão de *BRCA1/BRCA2* combinada com a baixa expressão de *ERCC1* (da via NER) resultava em uma resposta clínica desfavorável (SOARES *et al.*, 2007).

Recentemente, diferentes tipos de inibidores de reparo de DNA têm sido desenvolvidos com a finalidade de se aumentar a gama de medicamentos disponíveis para terapias e melhorar a eficácia clínica. Estes inibidores podem ser utilizados como agentes único ou combinados. Dentre eles, destacam-se os inibidores de PARP, que vêm sendo empregados como monoterapia no tratamento de pacientes com cânceres deficientes em *BRCA1*/*BRCA2*. Os inibidores de PARP induzem quebras de fita simples no DNA que podem ser convertidas em DSBs, as quais são normalmente reparadas pela via de HR. Desta forma, tumores com deficiências em *BRCA1/BRCA2* mostram-se mais sensíveis aos inibidores de PARP quando comparado com tumores sem estes defeitos (HELLEDAY *et al.*, 2008; SMITH *et al.*, 2010). Outros tipos de inibidores de reparo também vem sendo desenvolvidos, tais como inibidores da APE1 endonuclease da via BER (metoxiamina, ácido 7-nitroindol 2-carboxílico e lucantona), que são capazes de potencializar a atividade citotóxica de diferentes tipos de agentes alquilantes e antimetabólitos, aumentando a apoptose e diminuindo a proliferação celular em linhagens de câncer de ovário, mama, cólon e fibrosarcoma (KELLEY & FISHEL, 2008; MCNEILL *et al.,* 2009). Além disso, os inibidores de DNA-PK da via de reparo por NHEJ (tais como o IC87361, NU7026

e a vanilina) mostraram-se interessantes agentes antitumorais, uma vez que potencializam a atividade antiproliferativa da doxorrubicina, etoposideo, mitroxantrona e cisplatina (DING *et al.*, 2006).

5. Reparação de DNA e Modeladores da Cromatina

As células desenvolveram uma complexa rede de sinalização para monitorar cuidadosamente a integridade genômica durante a replicação do DNA e efetuar paradas de ciclo celular, reparo e indução de apoptose em resposta a danos no DNA. Para que a reparação de DNA seja bem sucedida, é necessária a atuação coordenada de diferentes vias celulares. Recentemente, as modificações na estrutura da cromatina têm se mostrado de extrema importância na reparação de DNA, uma vez que certas alterações atuam como plataformas necessárias à sinalização de proteínas e recrutamento de maquinarias de reparo (ATAIAN & KREBS, 2006; ALTAF *et al.*, 2007). Estudos relatam que em todas as vias de reparação, as modificações da cromatina parecem ser necessárias (ATAIAN & KREBS, 2006; ESCARGUEIL *et al.*, 2008). O envolvimento de modeladores da cromatina no reparo de DSBs é o que mais tem sido investigado.

Frente a lesões no DNA, as enzimas da família PI3K ativam os pontos de checagem através da indução de uma cascata de fosforilação em diferentes substratos, tais como mediadores, transdutores e efetores. Pertencem à classe de PI3K as cinases: ATM (*Ataxia-telangiectasia* mutada), ATR (*Ataxia-telangiectasia* relacionada a Rad3) e DNA-PK (proteína cinase dependente de DNA) em

mamíferos (Tel1 e Mec1 em leveduras). Em resposta a danos do tipo DSBs, a variante de histona H2AX é fosforilada na serina-139 em mamíferos por PI3K cinases (BONNER *et al.*, 2008; DINANT *et al.*, 2008). A histona H2AX fosforilada (γH2AX) atua em estágios iniciais da resposta ao dano no DNA e facilita o acesso de diferentes proteínas de reparo às quebras, bem como a sinalização celular. γH2AX está envolvida no reparo de DSBs tanto por NHEJ quanto por HR, e atua regulando a recombinação homóloga entre as cromátides-irmãs, suprimindo o anelamento do DNA de fita simples, prevenindo a formação de quebras no DNA (ALTAF *et al.*, 2007) e auxiliando no recrutamento de proteínas de reparo como Nbs1 e Brca1, e de reguladoras de ciclo celular como MDC1 e 53BP1 ao local do dano (DINANT *et al.*, 2008). γH2AX também interage com complexos de HAT (NuA4) e remodeladores da cromatina dependentes de ATP, como Ino80 e Swr1. NuA4 é necessário para o recrutamento eficiente de fatores que reparam DSBs e sinalizam o dano. Ino80 parece atuar na reparação por HR, promovendo a remoção dos nucleossomos para facilitar o processamento das extremidades quebradas pelo complexo MRN (MRX em *S. cerevisiae*) (VAN ATTIKUM *et al.,* 2004). E o remodelador Swr1 é responsável pela incorporação da variante de histona H2AZ (Htz1 em leveduras) na cromatina (ALTAF *et al.*, 2007).

OBJETIVOS

1. Objetivo Geral

O objetivo geral deste trabalho foi investigar os mecanismos de ação do agente antitumoral 5-fluorouracil em *Saccharomyces cerevisiae* e em células tumorais humanas, visando compreender como as lesões no DNA induzidas por esta droga são reparadas, o envolvimento de modeladores da cromatina na citotoxicidade deste agente e a resposta a danos no DNA.

1.1. Objetivos Específicos

- Avaliar a participação das vias de reparação de DNA nas lesões induzidas pelo 5-FU, em *S. cerevisiae,* e comparar seus efeitos com o seu metabólito ativo FdUMP;

- Investigar a participação de modeladores da cromatina na citotoxicidade do 5-FU, empregando linhagens de *S. cerevisiae* deficientes em remodeladores dependentes de ATP e modificações covalentes pós-traducionais, tais como acetilação, metilação e ubiquitinização, bem como verificar a interação entre remodeladores da cromatina dependentes de ATP e acetiltransferase de histonas empregando duplos mutantes;

- Estudar o envolvimento de modeladores da cromatina na reparação das lesões geradas pelo 5-FU pelas vias de recombinação homóloga e reparo pós-replicativo;

- Avaliar a participação das vias de sinalização celular ATR/Chk1 e ATM/Chk2, frente a danos induzidos pelo 5-FU e comparar seus efeitos com o FdUMP, raltitrexato e gemcitabina em células tumorais humanas HeLa;

- Investigar os efeitos da inibição de Chk1/Chk2, empregando AZD7762, na citotoxicidade do 5-FU, FdUMP, raltitrexato e gemcitabina, na progressão do ciclo celular e entrada em mitose;

CAPÍTULO I

DNA repair pathways involved in repair of lesions induced by 5-fluorouracil and its active metabolite FdUMP

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DNA repair pathways involved in repair of lesions induced by 5-fluorouracil and its active metabolite FdUMP

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ABSTRACT

5-Fluorouracil (5-FU) is an antitumor antimetabolite that can be converted into fluoronucleotides and FdUMP. Fluoronucleotides are incorporated into DNA and RNA, while FdUMP results in nucleotide pool imbalance. *Saccharomyces cerevisiae* is unable to convert 5-FU into FdUMP, making yeast a unique model system to study the cellular effects of 5-FU and FdUMP independently. A panel of repair-deficient yeast strains was used to identify the DNA repair pathways needed for repair of lesions generated by 5-FU or FdUMP. This included yeast deficient in base excision repair (BER), nucleotide excision repair (NER), translesion synthesis (TLS), mismatch repair (MMR), post-replication repair (PRR), homologous recombination (HR) and non-homologous end-joining (NHEJ). The results revealed an important role of BER, since BER-mutants (ntg1, ntg2, apn1, apn2) showed pronounced sensitivity to both 5-FU and FdUMP. MMR mutants also showed high sensitivity to both compounds. In contrast, deficiencies in NER, NHEJ and TLS repair had only minor influence on the sensitivity to FU and FdUMP. Interestingly, deficiencies in HR (rad52) and PPR (rad6, rad18) were associated with increased sensitivity to 5-FU, but not to FdUMP. Taken together, our study reveals an important contribution of DNA repair pathways on the sensitivity to 5-FU and its active metabolite FdUMP. Importantly, the repair mechanisms differed for the 2 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.

1. Introduction

5-Fluorouracil (5-FU) is a pyrimidine analogue widely used as a chemotherapeutic agent, in particular for colorectal cancer [1,2]. 5-FU is an uracil analog with a fluorine atom at the fifth position and is metabolized like uracil [3].

For cytotoxic activity, 5-FU requires uptake and metabolic activation by cellular phosphorylases and kinases [1,4]. As outlined in Fig. 1, 5-FU activation involves its conversion into fluoronucleotides and to FdUMP. The fluoronucleotides are incorporated into DNA and RNA during macromolecular synthesis [5,6] leading to a wide range of biological effects which can act as a trigger for apoptotic cell death [7]. This includes DNA mutations and protein miscoding [8], inhibition of pre-rRNA processing [9], inhibition of post-transcriptional modification of tRNAs [10] as well as polyadenylation and splicing of mRNA [1]. The FdUMP metabolite inhibits thymidylate synthase (TS) resulting in nucleotide pool imbalance with decreased levels of dTMP and increased concentrations of dUMP. Since most DNA polymerases have poor discrimination for dTTP and dUTP, a strong increase in dUMP concentrations may result in a significant incorporation of uracil into DNA [1,3,11,12]. It has also been reported that FdUMP can be further phosphorylated to FdUTP followed by DNA incorporation [13]. For these reasons, FdUMP is believed to be the major active metabolite of 5-FU [14,15].

During DNA repair, mismatched bases are removed from DNA by uracil glycosylase and the DNA backbone is nicked by an abasic endonuclease. Then, the 5′-deoxyribose phosphate is removed by a flap endonuclease and the resulting gap is filled with another trinucleotide through the action of a repair DNA polymerase

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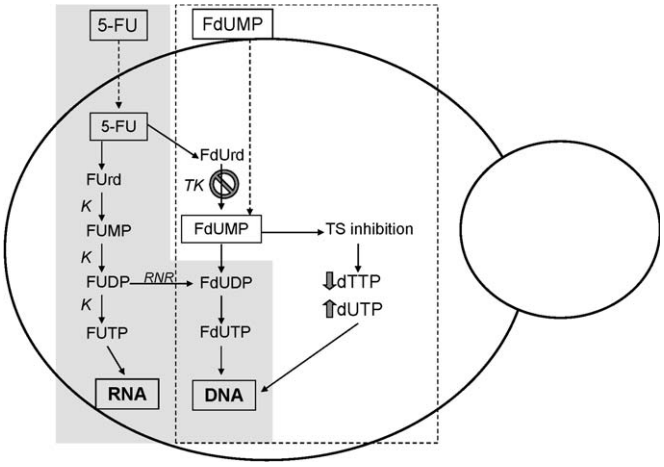

Fig. 1. 5-FU metabolism in *Saccharomyces cerevisiae*. 5-FU shares the same facilitated transport system as uracil, adenine and hypoxanthine, whereas derivatives of 5-fluoro-2′-deoxyuridine enters the cell by a distinct facilitated membrane transport mechanism used by purine and pyrimidine nucleosides [40]. Fluoropyrimidines are converted into fluorinated ribonucleotides and deoxyribonucleotides by the same pathways as uracil and thymine. The absence of thymidine kinase in yeast does not allow the direct conversion of 5-FU into FdUMP, suggesting that the toxicity of 5-FU treatment is due to misincorporation of fluoropyrimidines into DNA and RNA (grey box), while the FdUMP-mediated toxicity is principally related to TS inhibition (box outlined by dashed lines). FUrd = 5-fluorouridine; FUMP, FUDP and FUTPs = 5-fluorouridine-5′-mono-, di- and triphosphate; FdUDP = 5′-fluoro-2′-deoxyuridine diphosphate; FdUTP = 5′-fluoro-2′-deoxyuridine triphosphate; dUTP = deoxyuridine triphosphate. Enzymes: TK = thymidine kinase; K = kinases; RNR: ribonucleotide reductase.

followed by DNA ligation [16]. Although uracil excision is not essential for the cytotoxic activity of 5-FU [16,17], an incomplete/futile repair process may lead to accumulation of toxic repair intermediates including abasic sites and DNA strand breaks which can provoke cell death [18]. Accordingly, studies with repair-deficient yeast strains showed that deletion of *UNG1*, which codes for uracil DNA glycosylase had a protective effect against 5-FU in contrast to deletion of *APN1*, the major abasic site endonuclease in yeast, which was accompanied by pronounced sensitivity to the drug [16].

Yeast is unable to convert 5-FU into FdUMP due to the absence of thymidine kinase [19], making yeast a unique cellular model to study the cellular effects of 5-FU and FdUMP independently. Specifically, treatment with 5-FU will lead to its conversion into fluoronucleotides (but not into FdUMP) that can be incorporated into DNA during replication, while treatment with FdUMP allows us to study the impact of TS inhibition and the nucleotide pool imbalance (Fig. 1).

In the present study, we have compared the sensitivity of *Saccharomyces cerevisiae* strains deficient in the major repair proteins and repair pathways to 5-FU and FdUMP. Our results show that base excision repair and mismatch repair play an important role in the sensitivity to both 5-FU and FdUMP. Homologous recombination and post-replication repair are also needed for repair of lesions induced by 5-FU, but seem to play a minor role for FdUMP. These data emphasize the importance of DNA repair in the response to 5-FU and identify differences in the biological response to the major 5-FU metabolites.

2. Materials and methods

2.1. Chemicals

5-Fluorouracil (5-FU) was purchased from ICN Pharmaceutics (Valeant Pharmaceuticals International, USA). 5-Fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) was bought from Sigma–Aldrich (St. Louis, MO, USA). Reagents for culture medium (yeast extract, bacto-peptone, bacto-agar and glucose) were acquired from Merck.

2.2. Yeast strains and media

The relevant genotypes of the *S. cerevisiae* strains used in this work are indicated in Table 1. Mutants strains were obtained from

Table 1
Saccharomyces cerevisiae strains used in this study. BER: base excision repair; NER: nucleotide excision repair; MMR: mismatch repair; HR: homologous recombination; NHEJ: non-homologous end-joining; PRR: post-replication repair; TLS: translesion synthesis.

Strains	Relevant genotypes	DNA repair pathway affected	Source
BY4742 (WT)	*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	–	Euroscarf
ung1Δ	BY4742; with *ung1::kanMX4*	BER	Euroscarf
apn1Δ	BY4742; with *apn1::kanMX4*	BER	Euroscarf
rad27Δ	BY4742; with *rad27::kanMX4*	BER	Euroscarf
rad1Δ	BY4742; with *rad1::kanMX4*	NER	Euroscarf
rad10Δ	BY4742; with *rad10::kanMX4*	NER	Euroscarf
rad6Δ	BY4742; with *rad6::kanMX4*	PRR	Euroscarf
rad18Δ	BY4742; with *rad18::kanMX4*	PRR	Euroscarf
rad50Δ	BY4742; with *rad50::kanMX4*	NHEJ	Euroscarf
rad52Δ	BY4742; with *rad52::kanMX4*	HR	Euroscarf
rev1Δ	BY4742; with *rev1::kanMX4*	TLS	Euroscarf
rev3Δ	BY4742; with *rev3::kanMX4*	TLS	Euroscarf
BY4741 (WT)	*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	–	Euroscarf
mlh1Δ	BY4741; with *mlh1::kanMX4*	MMR	L Meira
pms1Δ	BY4741; with *pms1::kanMX4*	MMR	L Meira
FF18733 (WT)	MATa; *leu2-1*, *trp1-289*; *his7-3*; *ura3-52*; *lys1-1*	–	R Medina-Silva
apn1Δ	FF18733; with *apn1::URA*	BER	R Medina-Silva
apn2Δ	FF18733; with *apn2::kanMX*	BER	R Medina-Silva
apn1Δ apn2Δ	FF18733, with *apn1::URA apn2::kanMX*	BER	R Medina-Silva
SJR751 (WT)	*MATα*; *ade2-101$_{oc}$*; *his3Δ200*; *ura3ΔNco*; *lys2ΔBgl*; *leu2-R*	–	RL Swanson[a]
ntg1Δ	SJR751; with *ntg1::LEU2*	BER	RL Swanson[a]
ntg2Δ	SJR751; with *ntg2::hisG*	BER	RL Swanson[a]
ntg1Δ ntg2Δ	SJR751; with *ntg1::LEU2 ntg2::hisG*	BER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3*	BER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rad1Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rad1::hisG*	BER/NER	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rev3Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rev3::kanMX4*	BER/TLS	RL Swanson[a]
ntg1Δ ntg2Δ apn1Δ rad52Δ	SJR751; with *ntg1::LEU2 ntg2::hisG apn1::HIS3 rad52::URA3*	BER/HR	RL Swanson[a]

[a] Swanson et al. (1999).

the parental wild-type strains BY4741, BY4742, FF18733 and SJR751 by gene disruption. Yeast strains deficient in MMR were kindly provided from Dr. Lisiane Meira (Biological Engineering Division, MIT, Cambridge, USA) and BER pathway mutants were kind gifts from Dr. Renata Medina-Silva (Pontifícia Universidade Católica, PUC, Brazil). Complete liquid medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose was employed for routine growth. Medium containing 2% (w/v) bacto-agar was used for plates.

2.3. Yeast growth conditions

Exponential phase (Log) cultures were obtained by inoculation of 5×10^5 cells/mL of YPD culture in stationary phase into 5 mL of fresh YPD medium. After 14 h incubation, at 30 °C with aeration, the cultures contained $1–2 \times 10^7$ cells/mL with 20–30% budding cells. The number of cells was determined by counting in Neubauer chamber.

2.4. Survival assays

The survival after treatment with 5-FU and FdUMP was measured by preparing cell suspensions containing 5×10^6 Log - cells/mL and incubated in culture medium at 30 °C for 4 h with agitation. After incubation, samples were diluted in saline solution, plated onto YPD agar, and incubated at 30 °C for 2–3 days. 5-FU concentrations employed were 18.75, 37.5, 75 and 150 μM; and FdUMP concentrations were 37.5, 75, 150 and 300 μM. All assays were performed at least twice with each dose in triplicate.

2.5. Drop tests

Logarithmic cultures were serially diluted by 1:10 steps and 6 μL aliquots spotted onto rich media plates with or without 5-FU (150 μM) or FdUMP (300 μM). Plates were incubated at 30 °C for 2 days. Experiments were performed at least twice with each dose in triplicate.

3. Results and discussion

Understanding the repair of genotoxic anticancer agents should facilitate the identification of predictive markers for response prediction and help to identify tumors with intrinsic or acquired drug resistance.

5-FU is widely used for the treatment of solid tumors and many studies have been conduced to elucidate its mechanism of action [1,6–10,20]. In mammalian cells, 5-FU is converted into fluoronucleotides, that are incorporated into nucleic acids, thereby altering their function and stability. Alternatively, 5-FU may be converted into FdUMP, a thymidylate synthase (TS) inhibitor leading to nucleotide pool imbalance and uridine incorporation into DNA [1,21]. Both fluoronucleotides and uracil are recognized by DNA repair proteins such as glycosylases, followed by the recruitment of other enzymes in order to eliminate the lesion and restore the integrity of the DNA. However, many DNA repair-related intermediates are in themselves toxic, including the AP sites, which are generated by glycosylases [16,22].

In the present work, all cytotoxicity experiments were carried out with cells in the logarithmic growth phase because 5-FU and FdUMP depend upon ongoing DNA synthesis for incorporation into

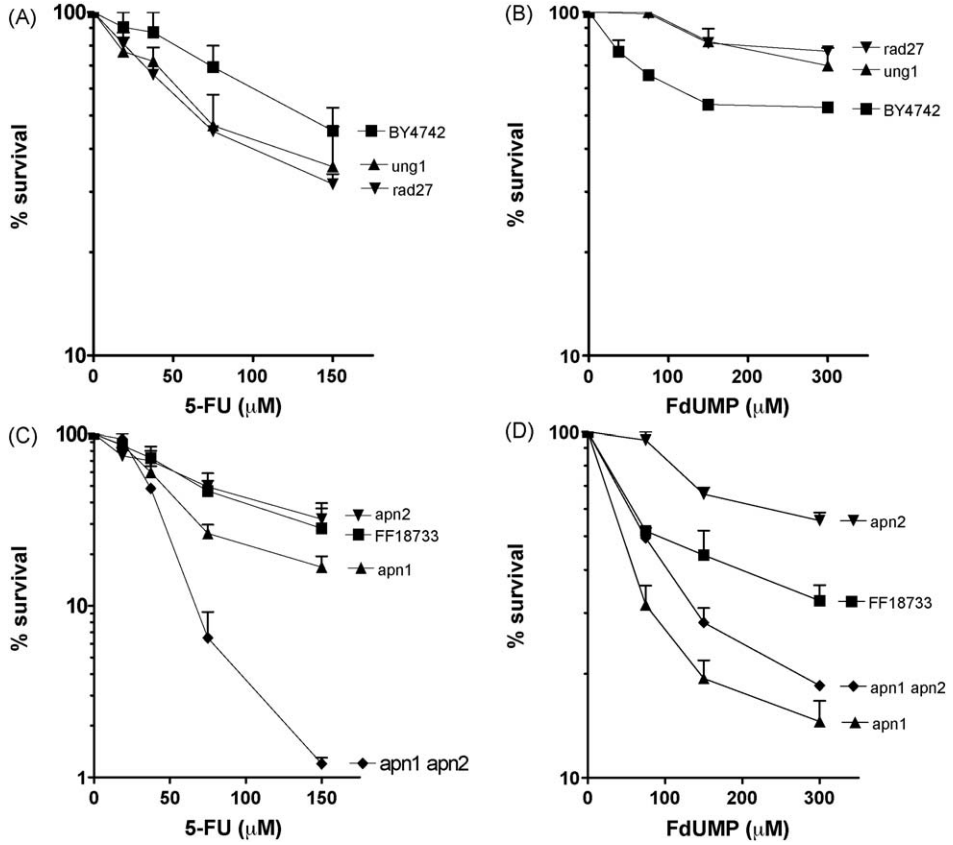

Fig. 2. Survival of *S. cerevisiae* strains single (A and B) and double (C and D) mutants deficient in BER proteins after treatment with 5-FU and FdUMP. The survival of parental strains (BY4742 and FF18733) is compared with that of variants defective in the Ung1 glycoysylase, the Rad27 structure-specific flap endonuclease, and the Apn1 and Apn2 AP endonucleases.

DNA [6]. *S. cerevisiae* strains deficient in the BER proteins *ung1* and *rad27* showed basically unchanged sensitivity to 5-FU, and modest resistance to FdUMP, in comparison with wild-type cells (compare Fig. 2A and B). Our findings are in agreement with previous studies suggesting that deletion of Ung1 is accompanied by either unchanged [17,23] or decreased [16] sensitivity to 5-FU. Ung1 is a glycosylase involved in the removal of uracil bases in DNA resulting in the formation of an AP site [16]. It is believed that Ung1 removes uracil from U:G, 5-FU:G, U:A and 5-FU:A mispairs, as well as uracil from single-stranded DNA [3]. In the absence of Ung1, uracil remains in the DNA. Uracil lesions are better tolerated by the cells than the Ung1 repair intermediates, the AP sites, which can be converted into DNA strand breaks [16,18]. Rad27, a structure-specific flap endonuclease in long-patch base excision repair, is needed to remove 5′deoxyribose phosphate (dRP)-blocked ends, thereby generating a nucleotide gap. Loss of Rad27 is accompanied by accumulation of 5′dRP-blocked sites in the DNA which, apparently, are less toxic than a nucleotide gap [16].

5-FU exposure was accompanied by pronounced sensitivity of *apn1*Δ, *apn1*Δ*apn2*Δ double-, *ntg2*Δ, *ntg1*Δ*ntg2*Δ double-, *ntg1*Δ*ntg2*Δ*apn1*Δ triple- and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ quadruple mutants (Figs. 2C, 3A and C). In comparison, FdUMP only showed enhanced toxicity in *apn1*Δ, *apn1*Δ*apn2*Δ double-, *ntg1*Δ*ntg2*Δ double-, and *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutants (Figs. 2D and 3B). Apn1, Ntg1 and Ntg2 are important BER components. Apn1 is the major AP endonuclease in *S. cerevisiae* while Ntg1 and Ntg2 are N-glycosylase/AP lyases. The absence of Apn1 will lead to an accumulation of AP sites, which are potentially deleterious lesions that can be converted into toxic DNA strand breaks. Ntg1 and Ntg2 recognize and remove damaged bases, generating AP sites that can be repaired either by the lyase activity of glycosylases or by the Apn1 endonuclease [22].

Our results showed that the triple mutant *ntg1*Δ*ntg2*Δ*apn1*Δ is very sensitive to 5-FU and FdUMP (Fig. 3A and B). It is important to note that the single mutant *apn1*Δ shows also significant sensitivity to both compounds (Figs. 2C, D, 3A and B). For 5-FU, the sensitivity of the double mutant *ntg1*Δ*ntg2*Δ *is due to* the deletion of *NTG2*, since the double mutant shows the same sensitivity as the *ntg2*Δ single mutant (Fig. 3A). Ntg1 and Ntg2 remove oxidized purines and pyrimidines with different substrate specificities. Furthermore, they have different localization, since Ntg1 is present in both the nucleus and the mitochondria while Ntg2 only is present in the nucleus [22,24,25]. In addition, *NTG1* is damage-inducible, whereas *NTG2* is expressed constitutively [24]. The important differences in survival suggest that Ntg2 may be more important than Ntg1 for the removal of fluoronucleotides. In contrast, only the *ntg1*Δ*ntg2*Δ double mutant was sensitive to FdUMP (Fig. 3B) indicating that both Ntg1 and Ntg2 proteins recognize FdUMP-induced lesions and can replace each other. Deletion of apn1 was more important for the sensitivity than deletion of apn2, while the double mutant *apn1*Δ*apn2*Δ was very sensitive to both drugs (Fig. 2C and D).

Both *mlh1*Δ and *pms1* showed increased sensitivity toward 5-FU and FdUMP (Fig. 4A and B), indicating a role for MMR in lesion processing of the two drugs. MMR is also important in the response of mammalian cells to 5-FU. However, in mammalian cancer cells, as well as in colon cancer patients, MMR deficiency is associated with increased resistance, rather than increased sensitivity, to 5-FU [26–29]. This difference may be explained by the additional cellular functions of the MMR system in mammalian cells, in particular with respect to induction of apoptotic signaling [30]. Interestingly, the MED1/MBD4 protein is absent in *Saccharomyces cerivisiae*. MED1 is a BER protein that is required for integrity of the MMR system [31] and which also influence the induction of

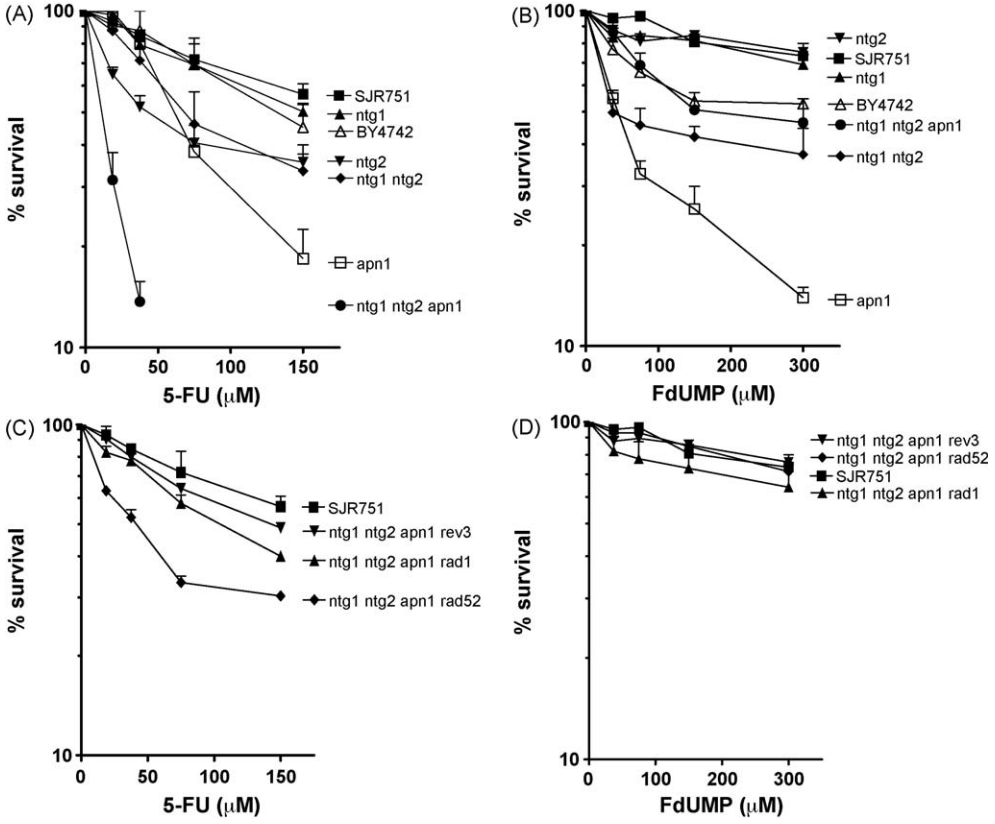

Fig. 3. Survival of *S. cerevisiae* strains single-, double-, triple- and quadruple mutants deficient in BER, HR, NER and TLS, after treatment with 5-FU and FdUMP. The survival of parental strains (BY4742 and SJR751) is compared with that of variants defective in the BER proteins Ntg1, Ntg2 and Apn1, the HR protein Rad52, the NER endonuclease Rad1 and the TLS protein Rev3.

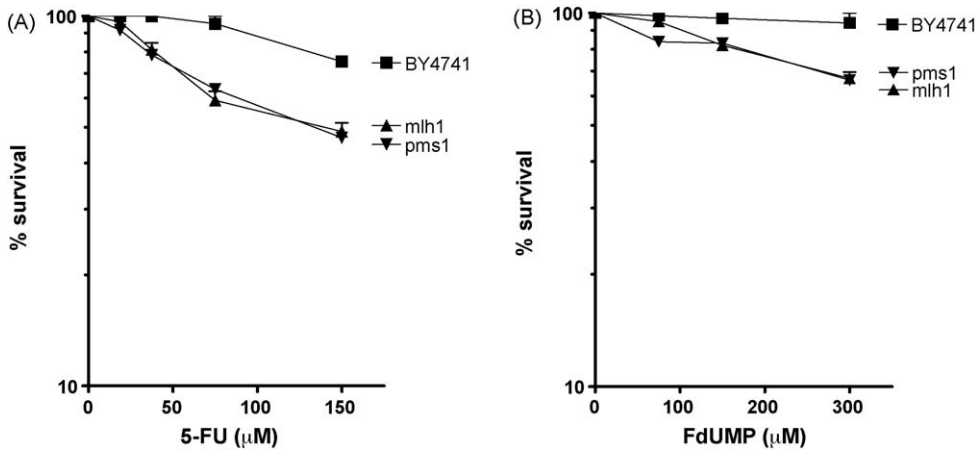

Fig. 4. Survival of *S. cerevisiae* strains deficient in MMR after treatment with 5-FU and FdUMP. The survival of the parental strain (BY4741) is compared to that of variants defective in the MMR proteins Mlh1 and Pms1.

apoptotic signaling [32]. Therefore, differential expression of important MMR-related proteins in yeast and human may explain the observed discrepancy in the sensitivity to 5-FU.

Next, the influence of proteins involved in NER, TLS and NHEJ was determined. Deletion of the NER endonucleases Rad1 and Rad10 had marginal influence on the sensitivity to 5-FU and FdUMP (Fig. 5A and B). The same result was observed for *rev3*Δ, which is deficient in the catalytic subunit of the TLS protein DNA polymerase zeta and for *rev1*Δ, which is deficient in the deoxycytidyl transferase, that forms a complex with DNA polymerase ζ [33] (Fig. 5C and D). In addition, deletion of *rad50*, that is deficient for a subunit of the MRX complex needed for NHEJ [34], had also little effect on the sensitivity to the two drugs (Fig. 6). These data suggest that deficiencies in the NER, NHEJ and TLS pathways have little influence on the sensitivity to 5-FU and FdUMP.

Interestingly, mutants deficient in *rad52*Δ, *rad6*Δ and *rad18*Δ showed increased sensitivity to 5-FU, but not to FdUMP (Fig. 6). Rad52 is the major protein involved in double-strand break repair by HR in *S. cerevisiae* [35–37] while Rad6 and Rad18 act in PRR.

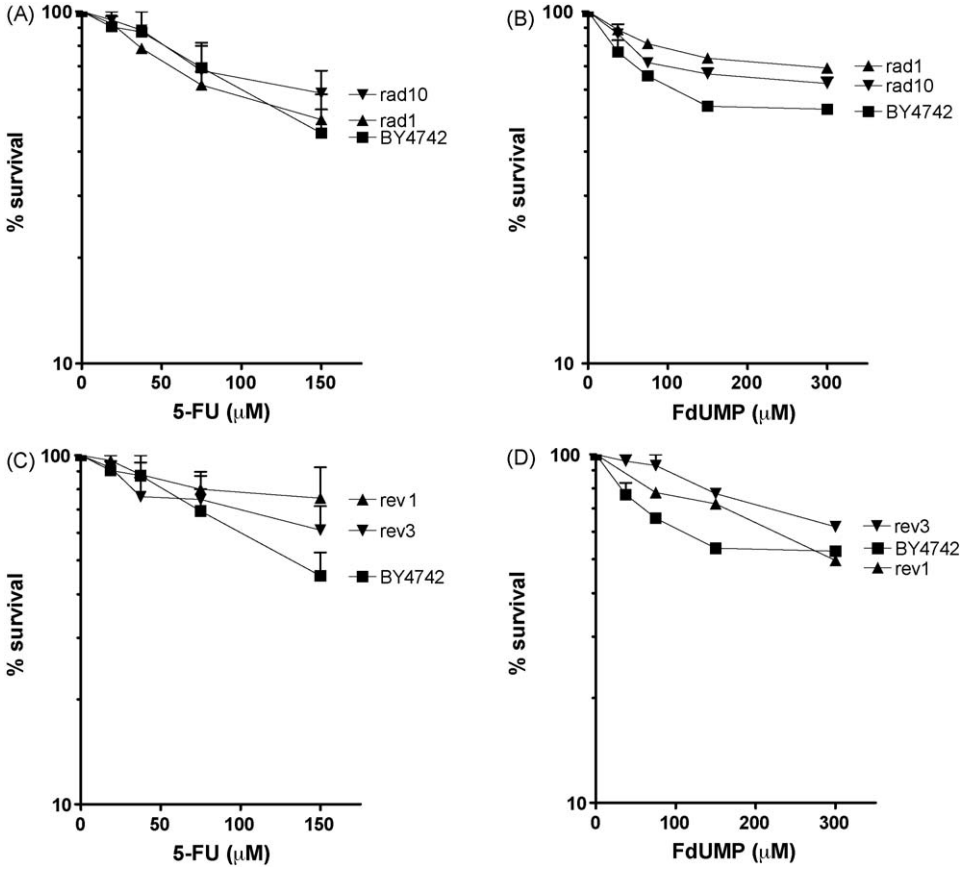

Fig. 5. Survival of *S. cerevisiae* strains deficient in NER (A and B), and TLS (C and D) after treatment with 5-FU and FdUMP. The survival of the parental strain (BY4742) is compared to that of variants defective for the NER endonucleases Rad1 and Rad10 and the TLS proteins Rev1 and Rev3.

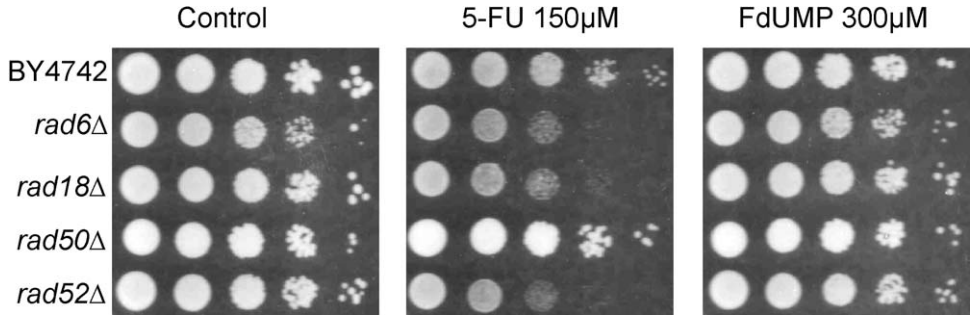

Fig. 6. Sensitivity of *S. cerevisiae* strains deficient in PRR and recombination repair to 5-FU and FdUMP Logarithmic cultures were serially diluted 10-fold, and were spotted onto YPD media plates with 5-FU (150 μM) or FdUMP (300 μM). The growth of the parental strain (BY4742) is compared to that of variants defective for the PPR proteins Rad6 and Rad18, the NHEJ protein Rad50, and the HR protein Rad 52.

Rad6 is an ubiquitin-conjugating enzyme needed for replication across DNA lesions [38], while Rad18 is maintaining the integrity of single-stranded DNA [39].

In conclusion, the BER pathway would initiate the repair of fluoronucleotide lesions, recognizing and removing the mismatched bases derived from 5-FU lesions mainly by the Apn1, Ntg1 and Ntg2, glycosylases and endonucleases. Although the Ung1 glycosylase and the Rad27 flap endonuclease likely participate in the processing of 5-FU lesions, their absence have no strong impact on the cellular survival. Failure of the BER process could result in the formation of DNA single (SSBs) and double (DSBs) strands breaks, which are recognized by the HR pathway (*RAD52*). Alternatively, MMR or PRR could be involved in processing the fluoronucleotide lesions that were not removed during replication (Fig. 7).

In contrast, for FdUMP, only BER and MMR play an important role (Fig. 7). DNA damage caused by FdUMP is processed mainly by Ntg1, Ntg2 and Apn1. Failure of this process could generate toxic single-strand breaks while mispairs, that were not repaired by BER, might be repaired by MMR (Mlh1 and Pms1). FdUMP cytotoxicity is principally attributed to TS inhibition, suggesting that the proportion of uracil misincorporation would be higher than for the FdUTPs. This is in agreement with recent results showing the formation of double-strand breaks after treatment with 5-FU, but not with FdUMP in human adenocarcinoma cells [20].

Taken together, the availability of a large panel of isogenic *S. cerevisiae* strains differing in defined repair proteins provides a powerful tool for identification of relevant repair processes. Since some repair pathways have additional functions in higher eukaryotes, major findings would need confirmation in mammalian models. A better understanding of the relevant repair processes is needed for personalized treatment with genotoxic anticancer agents and for the design of novel therapeutic reagents and strategies with better efficacy and/or less toxicity.

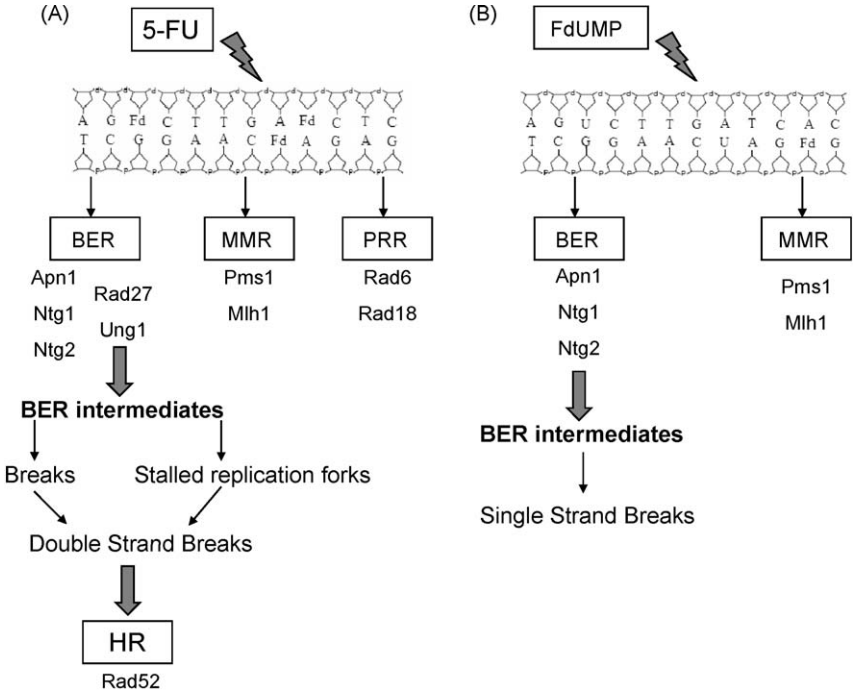

Fig. 7. Processing of 5-FU and FdUMP-induced-induced DNA lesions. (A) Fluoronucleotides misincorporated into DNA during S phase can be repaired by the BER, NER, HR or PRR. BER glycosylases and endonucleases would start repairing the damage and failure in this process could lead to formation of DNA single and double-strand breaks. Double-strands breaks are substrates for HR, that repairs the DNA with high fidelity. An other possibility is the involvement of MMR in the removal of fluoronucleotides from the DNA. The lesions that persisted in the DNA after replication can be processed by PPR. (B) Processing of FdUMP-induced DNA lesions. Misincorporation of uracil into DNA as a consequence of TS inhibition is mainly repaired by BER. If FdUMP has undergone further phosphorylation, FdUTP might be incorporated into DNA followed by subsequent removal by the MMR pathway. Fd = fluoronucleotides.

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CAPÍTULO II

Chromatin remodeling and histone acetyltransferases involvement in 5-FU cytotoxicity in Saccharomyces cerevisiae

Chromatin remodeling and histone acetyltransferases involvement in 5-FU cytotoxicity in *Saccharomyces cerevisiae*

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Abstract

Chromatin structure is well known to influence many biological processes including DNA repair. It is suggested that it modulates the access of repair proteins to DNA lesions, and that it may be altered by ATP-dependent chromatin remodelers (CR) and covalent post-translational modifications to facilitate repair. This work aimed to investigate the participation of chromatin remodelers and DNA repair proteins in 5-Fluorouracil (5-FU) cytotoxicity employing a panel of CR mutant *Saccharomyces cerevisiae* strains. 5-FU is an antimetabolite, antineoplasic drug that has been largely employed in the clinic. Among the several strains defective in chromatin remodelers tested, only those with deficiencies in CR and some histone acetyltransferases (HAT) exhibited asensitivity to 5-FU. The effect of 5-FU exposure in yeast double mutant strains for CR and HATs was also investigated. Interestingly, these double mutant cells displayed an increased resistance to 5-FU in comparison to wild type, but they still arrested in G2/M like the sensitive single mutants. The participation of the histone variant Htz1p in 5-FU toxicity was also evaluated in single and double mutants involving CR and HATs. The most significant effect was on the cell cycle distribution. 5-FU lesions are repaired by different DNA repair machineries, including homologous recombination (HR) and post-replication repair (PRR), so we investigated the potential role of CR and HATs in these DNA repair pathways. Our data showed that deficiencies in Nhp10, Ino80 and Swr1 combined with deficiencies in Xrs2 or Rad52 (HR) or Rad6 (PRR) increased the sensitivity to 5-FU. However, the combined deficiencies in Gcn5 and Hat1 (HATs) with Xrs2, Rad52 and Rad6 did not altered the 5-FU sensitivity. Ino80

and Swr1, both CR, are directly recruited to DNA damage and lead to chromatin relaxation, which facilitate the access of HR and PRR proteins to 5-FU lesions. On the other hand, combined deficiencies in Gcn5 and Hat1 with defects in HR and PRR did not potentiate 5-FU cytotoxicity, possibly because they work in a common pathway.

Key words: 5-FU, ATP-depedent chromatin remodelers, HAT, HR and PRR

1. Introduction

Most antineoplasic drugs target the DNA of cancer cells to create cytotoxic effects. This cytotoxicity may be related to DNA damage induction as single- (SSBs) and double strand breaks (DSBs), inter- and intra-strands crosslinks, and interference in purine and pyrimidine metabolism (DING *et al.,* 2006). Since the DNA damages may be repaired by different cellular DNA repair machineries, an efficient repair of these lesions could result in drug resistance and chemotherapy failure (WYATT & WILSON III, 2008). Therefore, therapies targeting DNA repair have emerged as promising approaches in anticancer research.

Efficient DNA repair depends on many factors, including how the DNA is packaged with histones and non-histones proteins into chromatin. Highly condensed structures likely do not allow the DNA-associated repair factors accessibility to the lesions (ATAIAN & KREBS, 2006; ESCARGUEIL *et al.*, 2008; HUERTAS *et al.*, 2009). The first step in chromatin packaging consists of 146 pb of DNA wrapped around a histone octamer containing two copies of each core histones H2A, H2B, H3 and H4. This structure may be modified by chromatin modifying enzymes that either act as (i) covalent post-translational histone tails modifiers (acetylation, methylation, phosphorylation, and ubiquitylation) or (ii) ATP-dependent remodelers (ALTAF *et al.,* 2007; OSLEY *et al.,* 2007). Covalent modifications alter the charge of specific residues, affecting the histone-histone and histone-DNA interactions, and signalling to other protein complexes. Chromatin remodeling depends on multi-protein complexes which employs ATP hydrolysis energy to alter the interaction histone-DNA. These complexes act by

sliding nucleosomes in the DNA molecule, regulating the access to specific sequences (for review, see ESCARGUEIL *et al.,* 2008; GANGARAJU & BARTHOLOMEW, 2007), or inserting the histone variants into nucleossomes, which may alter the higher order chromatin structure (HENIKOFF & AHMAD, 2005; ALTAF *et al.*, 2007).

Our work was designed to investigate the involvement of DNA repair and chromatin structure modifiers in response to the antineoplasic drug 5-fluorouracil (5-FU). We employed *S. cerevisiae* as a genetically tractable model organism that has many features of chromatin and DNA repair that are common to human cells. 5-FU is an antimetabolite analog of uracil base that needs to be converted to its active metabolites to exert its cytotoxic effect (for review, see GREM, 1997; WYATT & WILSON III, 2008). These metabolites could be misincorporated into DNA (KUFE *et al.*, 1981a) and RNA (KUFE & MAJOR, 1981b) or result in nucleotide pool imbalance (NOORDHUIS *et al.,* 2004). Many aspects of 5-FU action mechanism have already been reported (PETERS *et al.,* 2000; NOORDHUIS *et al.,* 2004; MATUO *et al.,* 2009), however the involvement of DNA repair associated with chromatin remodelers for this drugs' cytotoxicity has never been described.

2. Materials and Methods

2.1. Yeast strains and growth conditions

Yeast deficient strains in chromatin remodelers and post-translational chromatin modificators were kindly provided from Dr. Lisiane Meira (Biological

Engineering Division, MIT, Cambridge, USA), acquired from Euroscarf (European *Saccharomyces cerevisiae* Archive for Functional Analysis) or constructed by gene replacement. Relevant genotypes of *S. cerevisiae* strains used in this work are indicated in Table 1. For routine growth, complete liquid medium (YPD) containing 1% (w/v) yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose was employed. Medium containing 2% (w/v) bacto-agar was used for plates. Synthetic media containing 1.7g/L yeast nitrogen base, 5g/L ammonium sulfate, 20g/L glucose, supplemented with the appropriate amino acids (40g/mL) was employed for selection of transformants.

Experiments were performed in exponential phase (Log). Log cultures were obtained by inoculation of 5×10^6cells/mL of YPD overnight cultures into 5 mL of fresh YPD medium. After 3 h incubation at 30 ℃ with aeration, the cultures contained 1-2×10^7 cells/mL. The number of cells was determined by counting in Neubauer chamber.

Table 1: *Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotypes	Pathway affected	Source
BY4741 (WT)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	-	Euroscarf
hho1Δ	BY4741; with *hho1::kanMX4*	H1 histone	Euroscarf
hta1Δ	BY4741; with *hta1::kanMX4*	H2A histone	Euroscarf
htb2Δ	BY4741; with *htb2::kanMX4*	H2B histone	Euroscarf
hht1Δ	BY4741; with *hht1::kanMX4*	H3 histone	Euroscarf
hhf1Δ	BY4741; with *hhf1::kanMX4*	H4 histone	Euroscarf
nhp10Δ	BY4741; with *nhp10::kanMX4*	HMG	L. Meira
arp4Δ	BY4741; with *arp4::kanMX4*	CR	L. Meira
ino80Δ	BY4741; with *ino80::kanMX4*	CR	L. Meira
swr1Δ	BY4741; with *swr1::kanMX4*	CR	L. Meira
elp3Δ	BY4741; with *elp3::kanMX4*	HAT	L. Meira
esa1Δ	BY4741; with *esa1::kanMX4*	HAT	L. Meira
gcn5Δ	BY4741; with *gcn5::kanMX4*	HAT	L. Meira
hat1Δ	BY4741; with *hat1::kanMX4*	HAT	L. Meira
hat2Δ	BY4741; with *hat2::kanMX4*	HAT	L. Meira
hpa2Δ	BY4741; with *hpa2::kanMX4*	HAT	L. Meira
hpa3Δ	BY4741; with *hpa3::kanMX4*	HAT	L. Meira
sas2Δ	BY4741; with *sas2::kanMX4*	HAT	L. Meira
sas3Δ	BY4741; with *sas3::kanMX4*	HAT	L. Meira
ubc4Δ	BY4741; with *ubc4::kanMX4*	UB	Euroscarf
ubc5Δ	BY4741; with *ubc5::kanMX4*	UB	Euroscarf
hda1Δ	BY4741; with *hda1::kanMX4*	HDAC	L. Meira
sin3Δ	BY4741; with *sin3::kanMX4*	HDAC	L. Meira
sir2Δ	BY4741; with *sir2::kanMX4*	HDAC	L. Meira
hos1Δ	BY4741; with *hos1::kanMX4*	HDAC	L. Meira
hos2Δ	BY4741; with *hos2::kanMX4*	HDAC	L. Meira
hos3Δ	BY4741; with *hos3::kanMX4*	HDAC	L. Meira
hst1Δ	BY4741; with *hst1::kanMX4*	HDAC	L. Meira
hst2Δ	BY4741; with *hst2::kanMX4*	HDAC	L. Meira
hst3Δ	BY4741; with *hst3::kanMX4*	HDAC	L. Meira
hst4Δ	BY4741; with *hst4::kanMX4*	HDAC	L. Meira
rpd3Δ	BY4741; with *rpd3::kanMX4*	HDAC	L. Meira
dot1Δ	BY4741; with *dot1::kanMX4*	HML	L. Meira
msi1Δ	BY4741; with *msi1::kanMX4*	HMT	Euroscarf
set2Δ	BY4741, with *set2::kanMX4*	HMT	Euroscarf
rph1Δ	BY4741; with *rph1::kanMX4*	HDML	Euroscarf
htz1Δ	BY4741; with *htz1::LEU2*	HV	This study
arp4Δhtz1Δ	BY4741; with *arp4::kanMX4, htz1::LEU2*	CR/HV	This study
hat1Δarp4Δ	BY4741; with *hat1::kanMX4, arp4::URA3*	HAT/CR	This study
hat1Δesa1Δ	BY4741; with *hat1::kanMX4, esa1::URA3*	HAT	This study
hat1Δhtz1Δ	BY4741; with *hat1::kanMX4, htz1::LEU2*	HAT/HV	This study
esa1Δhtz1Δ	BY4741; with *esa1::kanMX4, htz1::LEU2*	HAT/HV	This study
rad6Δ	BY4741; with *rad6::LEU2*	PRR	This study
rad52Δ	BY4741; with *rad52::LEU2*	HR	This study
xrsΔ	BY4741; with *xrs2::LEU2*	HR	This study
nhp10Δrad6Δ	BY4741; with *nhp10::kanMX4, rad6::LEU2*	HMG/PRR	This study
nhp10Δrad52Δ	BY4741; with *nhp10::kanMX4, rad52::LEU2*	HMG/HR	This study
nhp10Δxrs2Δ	BY4741; with *nhp10::kanMX4, xrs2::LEU2*	HMG/HR	This study
hat1Δrad6Δ	BY4741; with *hat1::kanMX4, rad6::LEU2*	HAT/PRR	This study
hat1Δrad52Δ	BY4741; with *hat1::kanMX4, rad52::LEU2*	HAT/HR	This study
hat1Δxrs2Δ	BY4741; with *hat1::kanMX4, xrs2::LEU2*	HAT/HR	This study
gcn5Δrad6Δ	BY4741; with *gcn5::kanMX4, rad6::LEU2*	HAT/PRR	This study
gcn5Δrad52Δ	BY4741; with *gcn5::kanMX4, rad52::LEU2*	HAT/HR	This study
gcn5Δxrs2Δ	BY4741; with *gcn5::kanMX4, xrs2::LEU2*	HAT/HR	This study
ino80Δrad6Δ	BY4741; with *ino80::kanMX4, rad6::LEU2*	CR/PRR	This study
ino80Δrad52Δ	BY4741; with *ino80::kanMX4, rad52::LEU2*	CR/HR	This study
ino80Δxrs2Δ	BY4741; with *ino80::kanMX4, xrs2::LEU2*	CR/HR	This study
swr1Δrad6Δ	BY4741; with *swr1::kanMX4, rad6::LEU2*	CR/PRR	This study
swr1Δrad52Δ	BY4741; with *swr1::kanMX4, rad52::LEU2*	CR/HR	This study
swr1Δxrs2Δ	BY4741; with *swr1::kanMX4, xrs2::LEU2*	CR/HR	This study

HMG: High Mobility Group non-histone protein; CR: ATP-dependent Chromatin Remodeling; HAT: Histone Acetyltransferase; UB: Ubiquitin; HDAC: Histone Deacetylase; HML: Histone Methylase; HMT: Histone Methyltransferase; HDML: Histone Demethylase; HV: Histone Variant; PRR: Post-Replication Repair; HR: Homologous Recombination.

2.2. 5-FU sensitivity assays

In order to pre-determinate 5-FU sensitivity and optimal drug range, logarithmic cultures were serially diluted by 1:10 steps between 10^7-10^3 cells/mL and 4 µL aliquots spotted onto rich media plates with or without 5-FU. Plates were incubated at 30 ºC for 2 days. Experiments were performed at least twice for each dose and on independent days.

2.3. Cytotoxicity and cytostatic effect evaluation by Poissoner Quantitative Drop Test (PQDT)

PQDT Protocol was previous described by POLETTO *et al.* (2008) and it was employed with minor modifications. Cytotoxicity was measured by survival assays after 5-FU treatments in YPD plates and cytostatic activity was evaluated by colony area measurement estimated from scanned images from Petri plates, using ImageJ Analysis Software (version 1.39; National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Graphics present average of three independent experiments.

2.4. Construction of double mutants

Double mutants were obtained by disruption of *ARP4, ESA1, HTZ1, RAD6, RAD52* and *XRS2* genes by homologous recombination. The bifunctional yeast -

E. coli vector YcpLac33 was used as template for amplification of *arp4*::URA3 and *esa1*::URA3 disruption cassetes. pGadT7 vector was the template for *htz1*::LEU2, *rad6*::LEU2, *rad52*::LEU2 and *xrs2*::LEU2 cassetes. Primers are described in Table 2. Cassetes were amplified with Platinum´s Taq DNA polymerase High Fidelity (Invitrogen), purified with PureLink^{TM} gel extraction kit (Invitrogen) and used for yeast transformation by LiAc/PEG protocol (GIETZ & WOODS, 2002). Disruption was confirmed by PCR and restriction analysis performed with purified genomic DNA from yeast transformant colonies selected in synthetic media lacking uracil or leucine.

2.5. Cell cycle analysis

Cells were monitored by distribution in G1/S (unbudded), S (small-budded) and G2/M (large-budded). About 200-300 cells, treated or not with 5-FU, were counted in a Neubauer chamber. Large-budded cells were defined as those in which the bud was >50% of the size of the mother cell (CARDONE *et al.,* 2006). Graphics present the average of three independent experiments.

2.6. Analysis of 5-FU in DNA

Cells were lysed with lyticase (Sigma) and genomic DNA was isolated using 10% SDS, 5M KOAc and 70% ethanol. 1.5 µg was digested at 37ºC for 3h with 1U uracil DNA glycosylase (UDG) and 1U human AP endonuclease (Ape1) in buffer containing 50mM Tris-HCl (pH 7.5), 1mM EDTA, 50mM NaCl and 10mM $MgCl_2$. Each reaction was run on a 0.8% agarose gel and stained with ethidium bromide. Graphics present average of three independent experiments. Images were

acquired with a Kodak Gel Logic 200 Imaging System and the DNA band intensity was measured with Kodak Molecular Imaging Software.

Table 2: Primers used in this study.

Name and Sequence	Product Length
***arp4*::URA3** 5'-ATGTCCAATGCTGCTTTGCAAGTTTATGGCGGCGACGAAGGCAGTTGGACGATCGATGAT-3' 3'-CTATCTAAACCTATCGTTAAGCAATCTTTCGACGCCCACC CAGGGTTATTGTCTCATGAG-5'	1404 bp
***esa1*::URA3** 5´-ATGTCCCATGACGGAAAAGAAGAACCTGGTATTGCCAAAAGCAGTTGGACGATCGATGAT-3' 5'-TTACCAGGCAAAGCGTAACTGAGAGGCAGTAAATACCGGTCAGGGTTATTGTCTCATGAG-3'	1404 bp
***htz1*::LEU2** 5'- ATGTCAGGAAAAGCTCATGGAGGTAAAGGTAAATCCGGCG GGCCGGTCGAAATTCCCCTA-3' 5'-TTATTTCTTACTTCCCTTTTTTTCCACTTTCAATAATAAT GCCGGAACCGGCTTTTCATA-3'	1489 bp
***rad6*::LEU2** 5´-ATGTCCACACCAGCTAGAAGAAGGTTGATGAGAGATTTTACTTAACTTCTTCGGCGACAG–3´ 5´-TCAGTCTGCTTCGTCGTCGTCGTCGTCGTCATCATCATCA TAGCAACCATTATTTTTTTC–3´	1489 bp
***rad52*::LEU2** 5´-ATGAATGAAATTATGGATATGGATGAGAAGAAGCCCGTTT CTTAACTTCTTCGGCGACAG–3´ 5´-TCAAGTAGGCTTGCGTGCATGCAGGGGATTGATCTTTGGT CACAGGAAACAGCTATGACC–3´	1489 bp
***xrs2*::LEU2** 5´-ATGTGGGTAGTACGATACCAGAATACATTGGAAGATGGCTCTTAACTTCTTCGGCGACAG-3´ 5´-TTATCCTTTTCTTCTTTTGAACGTAAACTTCGGACCGTCG ATGTCTGCCCCTAAGAAGAT-3´	

3. Results and Discussion

Chromatin structure is well known to affect DNA-related processes such as replication, transcription, and recombination (ALTAF *et al.,* 2007). More recently its influence on DNA repair has been evidenced (ATAIAN & KREBS, 2006; ESCARGUEIL *et al.,* 2008). Since most antineoplasic drugs act by inducing DNA damage the efficient repair of these lesions may influence the clinical response (DING *et al.,* 2006), and considering that chromatin structure modulates the access of repair proteins to the damage, chromatin remodelers are emerging as a promising target in cancer therapy (ESCARGUEIL *et al.,* 2008).

5-FU is an antimetabolite antitumor drug whose cytotoxicity is largely based on creating DNA damage and it has been employed to treat carcinomas arising in the gastrointestinal tract, ovary, breast, head, neck and esophagus. Several potential modes of action have been proposed, but the participation of chromatin modifiers in 5-FU toxicity has never been examined.

3.1. Strains deficient in chromatin remodeling and histone acetyltransferases presented sensitivity towards 5-FU

In order to investigate if chromatin remodeling factors are important for 5-FU cytotoxicity, we first screened a large panel of *S. cerevisiae* strains deleted for genes with roles in canonical histones, non-histone protein related to the high-mobility group (HMG), ATP-dependent chromatin remodeling (CR), and covalent post-translational modifications by histone acetyltransferases (HAT), histone deacetylases (HDAC), histone ubiquitinization (UB), histone methylation (HML, HMT) and histone demethylation (HDML). Strains deficient in HMG, CR and some HATs were more sensitive to 5-FU in comparison to the wild-type (WT) (Figure 1).

The data presented in Figure 1 showed that strains deficient in Nhp10 (a HMG-1 like protein), Arp4, Ino80 or Swr1 (ATP-dependent CR proteins), were more sensitive towards 5-FU when compared to the wild type control. Interestingly, all of these proteins belong to the INO80 subfamily, which is composed of the INO80 and SWR1 complexes. The INO80 complex acts to slide nucleosomes into DNA regions to promote chromatin relaxation and it includes several proteins, such as Ino80, Arp4 and Nhp10 (for review, see BAO & SHEN, 2007). The Ino80 subunit is a functional ATPase and a nucleosome spacing factor with a 3'-5' DNA

helicase activity *in vitro* (VAN ATTIKUM & GASSER, 2005a). Arp4 (actin-related protein 4) is present in chromatin modifying complexes such as INO80, SWR1 and NuA4, and this has the ability to bind histones, modified histones and nucleosomes, as well as an ATP-binding activity (HARGREAVES & CRABTREE, 2011). Nhp10 is a subunit of the INO80 complex that can bind to DNA or nucleosomes; its absence leads to reduced binding activity by the INO80 complex, but it is still able to mobilize nucleosomes (BAO & SHEN, 2007). Nhp10 is also necessary for the specific interaction of INO80 at DSB sites in response to the DNA damage (MORRINSON *et al.,* 2004). The SWR1 complex is composed of fourteen polypeptides, among them Swr1, Vps71, Act1 and Arp4 (for review, see BAO & SHEN, 2007), and the major function of this complex is to exchange histone H2A in nucleosomes for its variant Htz1 (H2AZ in mammals), replacing the preexisting H2A-H2B dimer for Htz1-H2B (MIZUGUCHI *et al.,* 2004). This substitution of canonical histones with histone variants generates a structurally and functionally distinct region in the chromatin (HENIKOFF & AHMAD, 2005).

5-FU also sensitized strains deficient in Esa1, Gcn5 and Hat1 (Figure 1). All of these proteins are involved in DSB recombinational repair (TAMBURINI & TYLER, 2005; ATAIAN & KREBS, 2006). Esa1 is the catalytic subunit of the NuA4 HAT complex and mutations in this protein are related to reduced recruitment of INO80 and SWR1 to damaged sites (DOWNS *et al.,* 2004). It is required for cell cycle progression (DOYON & CÔTÉ, 2004); preferentially and it acetylates H4 histone (CLARKE *et al.,* 1999) within coding regions, which indicates its role in global acetylation (KURDISTANI & GRUNSTEIN, 2003). Gcn5 is a nuclear HAT that belongs to Ada and SAGA complexes, that preferentially acetylates H3

histone, and plays important roles in histone acetylation during transcription activation (GRANT *et al.,* 1997; CLARKE *et al.,* 1999). Gcn5 also has a separate and independent role in nucleotide excision repair and it is essential for the efficient repair of UV damage to DNA at some repressed loci (YU *et al.*, 2005). It is responsible for most of the post-UV increase in hisonte acetylation at lysines 9/14 seen throughout much of the yeast genome and which are not related to transcriptional activation. Hat1 is a HAT Type B, and it acetylates cytoplasmatic non-chromatin-associated histones that will be transported to the nucleus. At the nucleus, it may play role in chromatin assembly with Hif1, a histone H3/H4 chaperone (QIN & PARTHUM, 2006; BENSON *et al.,* 2007); it is also important for the proper telomeric silencing and a Hat1 mutant has decreased level of recombinational repair (QIN & PARTHUM, 2002; BENSON *et al.,* 2007).

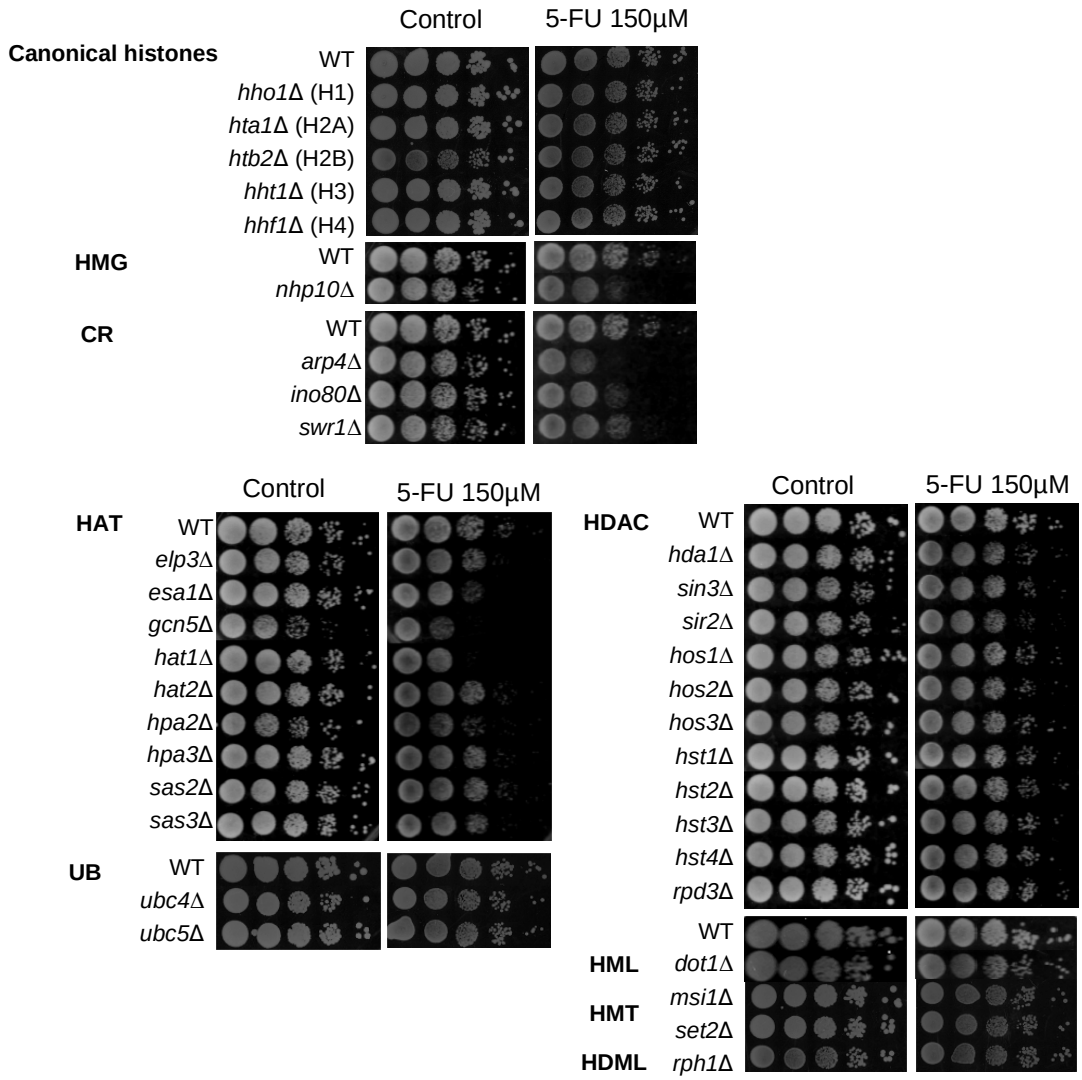

Figure 1: 5-FU sensitivity in *S. cerevisiae* deficient strains. Logarithmic cultures were serially dilluted 10-fold and spotted onto YPD media plates with 5-FU. Abbreviations: HMG: high mobility group; CR: ATP-dependent chromatin remodeling; HAT: histone acetyltransferase; UB: ubiquitin; HDAC: histone deacetylase; HML: histone methylase; HMT: histone methyltransferase; HDML: histone demethylase.

3.2. 5-FU effects on the interaction between chromatin remodeling factors

Based on the preliminary data from 5-FU sensitivity screening (Figure 1) the most sensitive strains were selected to investigate the cellular effects of these deletions. The cytotoxic and cytostatic activities of 5-FU were evaluted by the

PQDT method. The *arp4Δ* and *ino80Δ* CR single mutants were sensitive to 5-FU, while the deletion of Arp4 resulted in a more pronounced toxicity (Figure 2A). The WT strain presented a potent and similar cytostatic activity for all 5-FU concentrations tested. Contrarily strains defective in Arp4 or Ino80 presented a stronger cytostatic activity and in a dose-responsive manner (Figure 2B). The *esa1Δ*, *hat1Δ* and *gcn5Δ* HATs single mutants, showed sensitivity towards 5-FU treatment (Figure 2C) and strong cytostatic dose-response in *esa1Δ* and *hat1Δ* (Figure 2D).

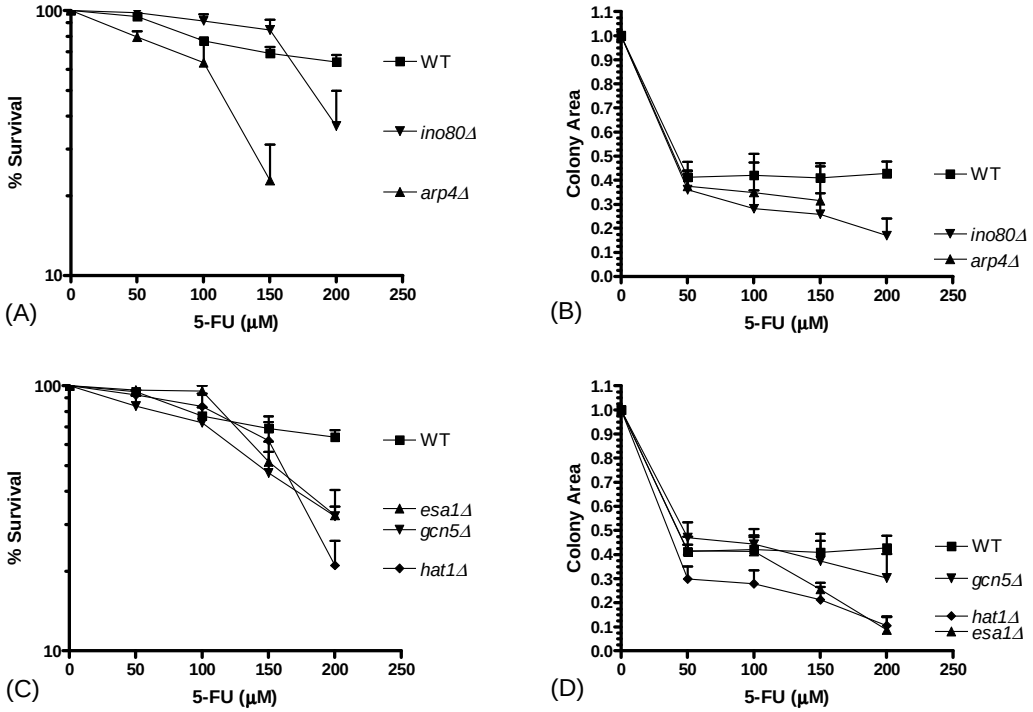

Figure 2: Cytotoxicity (A, C) and cytostatic (B, D) effect of 5-FU in *S. cerevisiae* deficient strains. Data are presented as mean ± standard deviation from three independendent experiments.

The possible interaction between chromatin remodeling factors in 5-FU toxicity was evaluated by constructing double-mutants. Deficiencies in CR/HAT, such as *arp4Δesa1Δ* and *hat1Δarp4Δ,* did not exhibit sensitivity to 5-FU (Figures

3A, 3C) and showed a cytostatic effect similar to the WT (Figures 3B, 3D). Surprisingly the double HAT mutant *hat1Δesa1Δ* was not sensitive towards 5-FU (Figure 3E) and it presented a cytostatic effect similar to the WT (Figure 3F).

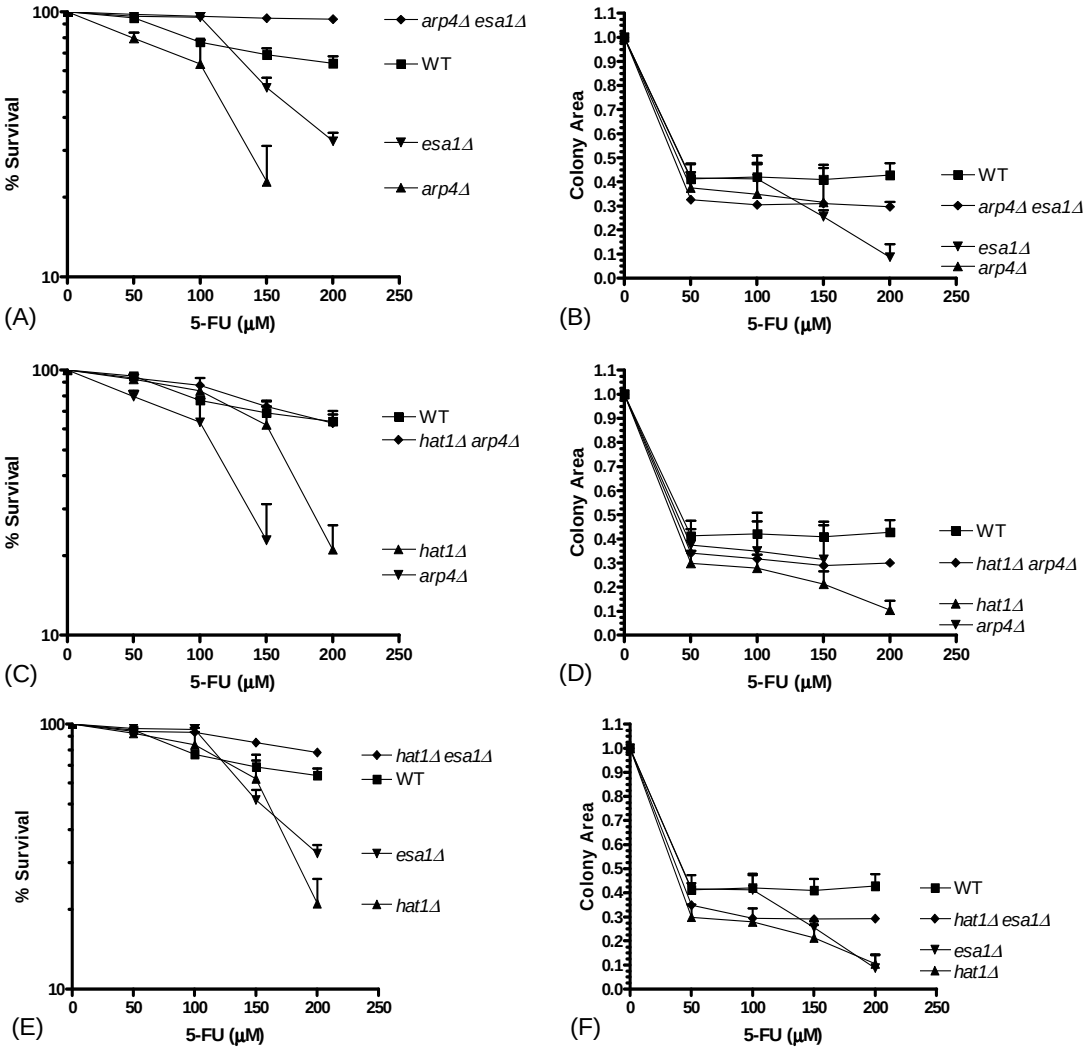

Figure 3: 5-FU sensitivity (A, C, E) and cytostatic (B, D, F) activity in yeast defective strains in chromatin remodeling double mutants. Data are presented as mean ± standard deviation from three independent experiments.

Since we observed that Swr1 was important for 5-FU cytotoxicity, we investigated whether or not there was a role for the histone variant Htz1 and its interactions with CRs and HATs. Htz1 incorporation into nucleosomes prevents the spreading of silent chromatin into euchromatin regions (RAISNER &

MADHANI, 2006). It also ensures efficient initiation of transcription and cooperates with other components to repel silencing factors (CAMPOS & REINBERG, 2009). The data presented in the Figures 4A, 4C, 4E showed that defects in Htz1 did not result in significant sensitivity to 5-FU, and that the double mutants involving *htz1Δ* and *arp4Δ, esa1Δ* or *hat1Δ* showed no sensitivity to this drug in comparison to WT. The single and all double-mutants involving Htz1 showed cytostatic effect similar to WT (Figures 4B, 4D, 4F).

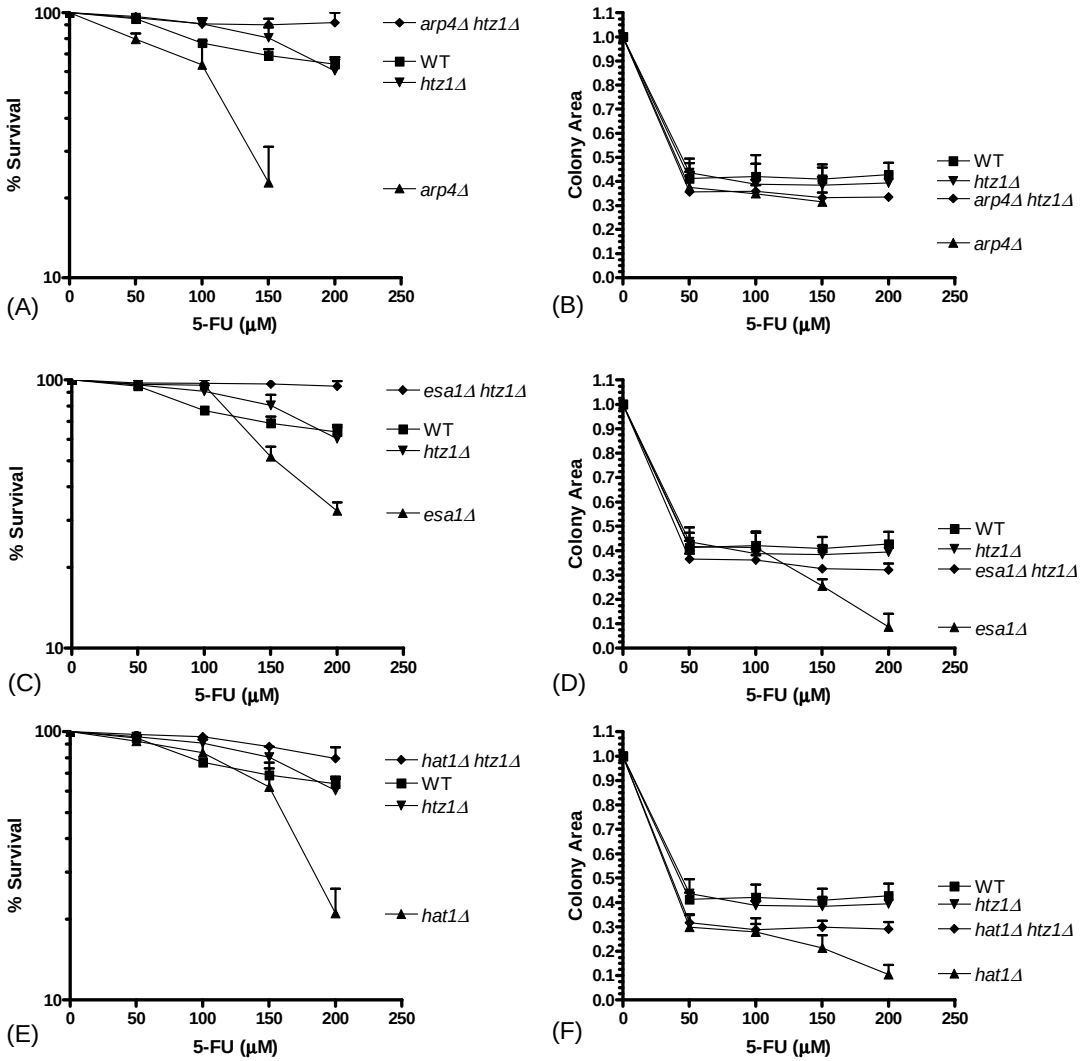

Figure 4: Cytotoxicity (A, C, E) and cytostatic (B, D, F) effect of 5-FU in *S. cerevisiae* deficient strains involving the histone variant Htz1. Data are presented as mean ± standard deviation from three independent experiments.

3.3. 5-FU induces cell cycle arrest in chromatin remodeling deficient strains

Since the data presented in Figures 2, 3 and 4 indicated that 5-FU has a potent cytostatic activity, the cell cycle distribution in chromatin remodeling deficient strains was investigated. After 5-FU exposure, WT, *esa1Δ* and *htz1Δ* strains showed an increasing in the S phase population, while strains defective in Arp4, Hat1, Ino80 and Swr1 as well as the double mutants *arp4Δesa1Δ, hat1Δarp4Δ, hat1Δesa1Δ, arp4Δhtz1Δ, esa1Δhtz1Δ* and *hat1Δhtz1Δ* displayed a G2/M arrest, (Figure 5). The single mutants were more sensitive and presented pronounced cytostatic activity to 5-FU showing G2/M arrest, except *esa1Δ,* probably because of its role in cell cycle progression. Interestingly, although the double mutants did not present cytotoxic and cytostatic effect towards 5-FU, they displayed G2/M arrest.

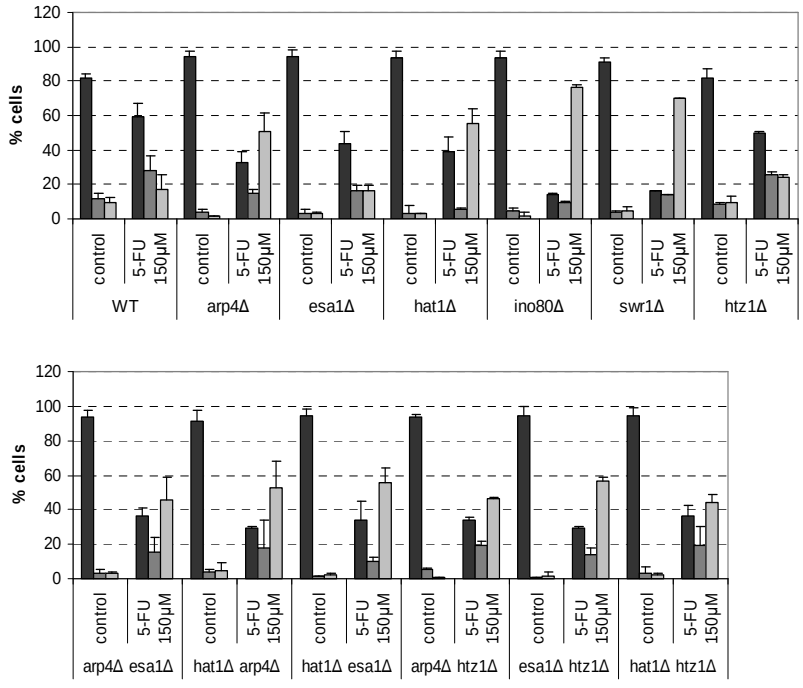

Figure 5: Cell cycle analysis. Distribution of G1/S (■), S (■) and G2/M (■) cells in single and double mutants after 5-FU exposure. Data are presented as mean ± standard deviation from three independent experiments.

3.4. Deficiencies in chromatin remodeling did not interfer in the excision of 5-FU misincorporated into DNA

Considering that chromatin remodelers are important factors that alter chromatin structure to allow the access of repair proteins, this aspect of our work aimed to investigate if strains deficient in CR, HATs and Htz1 were able to repair 5-FU lesions. 5-FU is an antimetabolite that may be misincorporated into DNA, and it is removed from DNA by the uracil glycosylase Ung1 (UDG in human) via base excision repair (BER) (MATUO *et al.*, 2010). The resulting AP site is processed by Apn1 (APE1 in humans) (SEIPLE *et al.,* 2006), and if not properly repaired, it is converted to SSBs and DSBs (BOITEUX & GUILLET, 2004). Several chromatin remodeling mutant strains were exposed to 5-FU, the DNA extracted and digested with the UDG and APE1 enzymes. In the presence of misincorporated 5-FU, DNA breaks are generated, so decreasing the molecular weight of DNA in denaturing agarose gels. Our data showed that defects in CR, HATs and Htz1 did not influence 5-FU excision from the DNA, since there is no difference between the migration of DNA digested with both enzymes or that of the undigested DNA migration (Figure 6). Therefore, 5-FU misincorporated into DNA is effectively removed by BER enzymes in chromatin remodeling defective strains, including the CR/HAT and HATs double mutants.

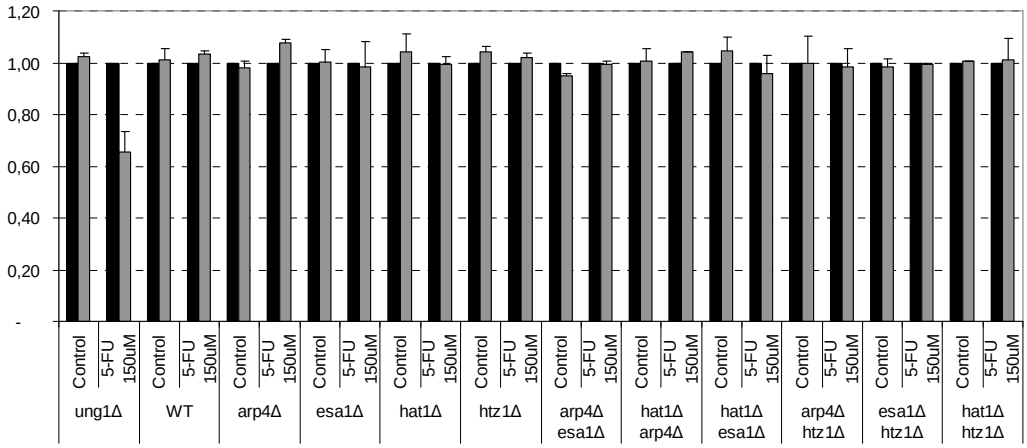

Figure 6: Presence of 5-FU and abasic sites in yeast genome DNA. DNA from strains treated with or without 5-FU were digested with UDG and Ape1, and fractionated by agarose gel electrophoresis. The strain *ung1Δ* exposed to 5-FU was employed as a positive control, since this strain accumulates fluoronucleotides, and when its DNA is digested with both BER enzymes, 5-FU is removed and DSBs may be formed, which decrease the amount of DNA band in comparison to control without enzymes. The graph represents the relative value of DNA band quantified by Kodak Molecular Imaging Software. ■ = DNA without digestion, ■ = DNA digested with enzymes. Data are presented as mean ± standard deviation from three independent experiments.

3.5. Chromatin modifiers influence the repair of 5-FU by HR

Since we observed that deficiencies in CR, HATs and Htz1 did not interfere in 5-FU removal from the DNA by BER, the influence of chromatin remodeling on other DNA repair pathways was investigated. 5-FU lesions are processed by several DNA repair pathways, such as BER, mismatch repair (MMR), post-replication repair (PRR) and homologous recombination (HR) as previously described (MATUO *et al.,* 2010). HR repairs DSBs and is the prevalent mechanism in yeast (AYLON & KUPIEC, 2004). Considering the observed G2/M arrest in strains deficient in chromatin modifiers, the participation of chromatin

remodelers in HR repair was investigated by employing double mutants. Double mutants involving Rad52 with HMG, CR and HATs, as well as Xrs2 with the same chromatin modifiers were constructed. Xrs2 acts with Mre11 and Rad50 (MRX complex) at the initial steps of HR, recognizing and processing the broken ends, meanwhile Rad52 works in the intermediate steps, stimulating strand exchange by facilitating Rad51 binding to single-stranded DNA (ATAIAN & KREBS, 2006). Results showed that the deletion of *RAD52* or *XRS2* in *nhp10*Δ, *ino80*Δ and *swr1*Δ increased the sensitivity to 5-FU in comparison to the respective single mutants, but not in the HAT deficient strains *gcn5*Δ and *hat1*Δ (Figures 7 and 8). Thissuggests that both HATs may work together within the HR pathway. These data suggest that HMG and CRs are important factors to promote chromatin relaxation at DSB damaged sites during HR repair, and HATs may also contribute to this process.

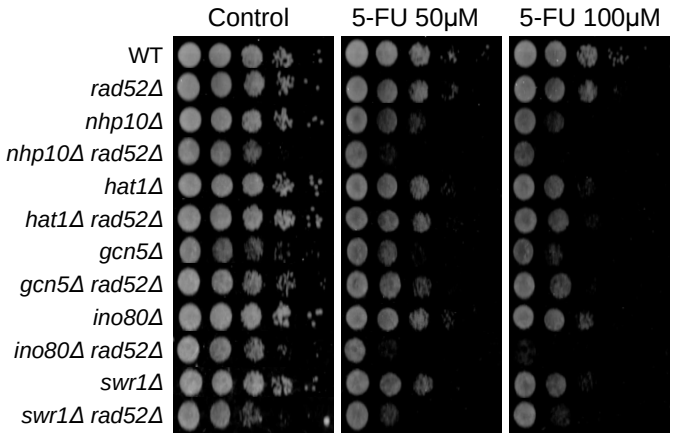

Figure 7: Participation of chromatin remodelers in HR repair of 5-FU lesions. Sensitivity was evaluated in single and double mutants involving *rad52*Δ and HMG, CRs or HATs.

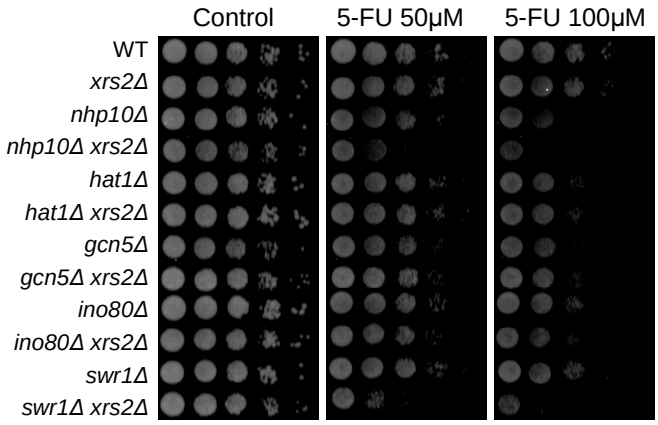

Figure 8: Participation of chromatin remodelers in HR repair of 5-FU lesions. Sensitivity was evaluated in single and double mutants involving *xrs2*Δ and HMG, CRs or HATs.

Indeed, previous studies have reported that several chromatin modifiers are involved in DSB repair by HR (TAMBURINI & TYLER, 2005; ATAIAN & KREBS, 2006). At the lesion site, the Mec1 and Tel1 enzymes phosphorylate histone H2AX at serine-129 (referred as γH2AX) (SHIMADA, 2008). Mec1, through its binding partner Ddc2 and Tel1 via interaction with the Mre11/Rad50/Xrs2 complex, results in the recruitment of multiple chromatin-modifying complexes that are able to alter the chromatin structure to allow DNA repair factors access to the lesion (VAN ATTIKUM & GASSER, 2005b). The γH2AX recruits the HAT complex NuA4 (yeast homologue of mammalian TIP60) via its Arp4 subunit and Nhp10 facilitates Arp4-γH2AX interaction (VAN ATTIKUM & GASSER, 2005a). Esa1 (catalytic component of NuA4) acetylates the N-terminal tail of H4 histone surrounding the break site (VAN ATTIKUM & GASSER, 2005a; LOIZOU *et al.,* 2006); then, INO80 and SWR1 CR complexes, as well as HATs Hat1 and Gcn5, are recruited to facilitate efficient repair of DNA damage (DOWNS *et al.,* 2004; QIN & PARTHUM, 2006; BAO & SHEN, 2007). The INO80 complex promotes the removal or

nucleosome slide to facilitate the processing of newly broken DNA ends, which enables the conversion of double-strand DNA ends into 3' single-strands overhangs by MRX complex (VAN ATTIKUM *et al.,* 2004), and controls the rate at which Rad51 displaces RPA during HR (ATAIAN & KREBS, 2006; BAO & SHEN, 2007). The SWR1 complex is also recruited for DNA repair and it possess ATP-dependent CR activities, which deposit the histone variant Htz1 (homolog of H2AZ) in specific locations *in vivo,* and exchange modified histones after the repair has been completed (LOIZOU *et al.,* 2006). The histone variant Htz1 has roles in transcriptional activation, antagonization of gene-silencing and chromosome stability (RAISNER & MADHANI, 2006), and when incorporated to DSB sites, it is also believed that contributes to local chromatin relaxation. Moreover, other histone modifications are also important for DNA repair by HR (VAN ATTIKUM & GASSER, 2005b), such as acetylation by Hat1 and Gcn5 (ATAIAN & KREBS, 2006). Hat1 is recruited at DSB after H2AX phosphorylation concomitant with Rad52, which suggests that Hat1 may act facilitating DNA repair by HR and/or act in the restoration of chromatin structure that followed the recombinational repair, since Hat1 catalyze the acetylation of amino-terminal tails of newly synthesized histones (QIN & PARTHUM, 2006). Gcn5 is required to transcriptional activation and it separately participates in the nucleotide excision repair of ultraviolet light-induced DNA lesions (YU *et al.*, 2005; LEE & WORKMAN, 2007).

3.6. Chromatin remodeling interferes in 5-FU repair by PRR

5-FU lesions are also repaired by PRR (MATUO *et al.*, 2010). In order to investigate the influence of chromatin modifiers in PRR, we constructed double

mutants involving Rad6 with HMG, CR and HATs. Rad6p is an ubiquitin-conjugating enzyme that exists in a complex with Rad18. Rad6-Rad18 form a stable complex with single-stranded DNA-binding (BROOMFIELD *et al.,* 2001) and mediate ubiquitin conjugation of the DNA polymerase processivity factor PCNA (Proliferating Cell Nuclear Antigen) which promotes replication through DNA lesions by mutagenic or error-free translesion synthesis (TLS) (MINESINGER & JINKS-ROBERTSON, 2005; PRAKASH *et al.,* 2005). Monoubiquitylation of PCNA mediates error-prone TLS, while polyubiquitylation triggers the error-free pathway. TLS occurs in S-phase in order to ensure replication completion, but it also operates in G2/M (KARRAS & JENTSCH, 2010). Our data demonstrated that the deletion of *RAD6* in *nhp10*Δ, *ino80*Δ and *swr1*Δ increased the sensitivity to 5-FU in comparison to their respective single mutants, but the same was not observed for strains defective in the HATs such as *hat1*Δ and *gcn5*Δ (Figure 9).

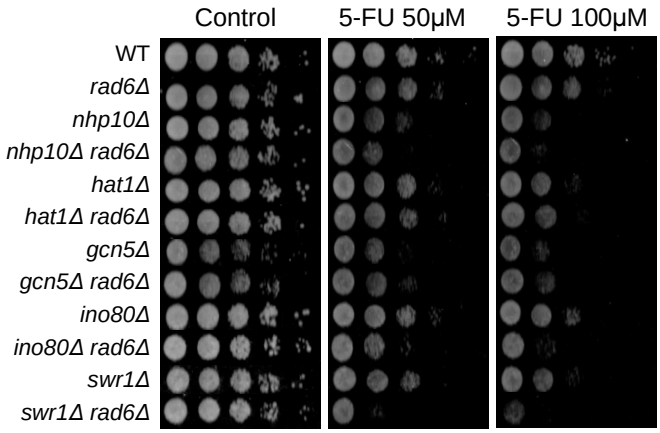

Figure 9: Involvement of chromatin modifiers in PRR repair of 5-FU. Sensitivity was investigated in single and double mutants combining *rad6Δ* and HMG, CR or HATs.

Few studies have been conducted in order to investigate the participation of chromatin modifiers in PRR. Besides the INO80 action in HR, it also play roles in DNA damage tolerance during replication: this complex binds to replication forks during S phase and allows the access of RAD6 and RAD51 pathways to process obstructed replication forks. INO80 regulates ubiquitination of PCNA and Rad51-mediated processing of recombination intermediates at blocked replication forks by allowing proper recruitment of Rad18 and Rad51 (FALBO, 2009).

Concluding remarks

The data presented here demonstrated that some ATP-dependent CR factors and specific HATs may influence 5-FU cytotoxicity, probably due to their interference with DNA repair. Ino80 and Swr1 CR are directly recruited to DNA damage and they lead to chromatin relaxation, which facilitate the access of HR and PRR proteins to 5-FU lesions, as well as the Nhp10. Deficiencies in Rad52 and Xrs2 (HR) or Rad6 (PRR) combined with Ino80, Swr1 and Nhp10 presented increased sensitivity to 5-FU. However, deficiencies in Gcn5 and Hat1 combinated with defects in HR and PRR, did not potentiate 5-FU cytotoxicity, possibly because they work in a common pathway. Figure 10 summarize the participation of chromatin remodeling in 5-FU lesions repair by HR and PRR.

Chromatin structure influences many biological processes and it may modulate DNA repair, which then directly interfers in the activity of antineoplasic drugs. Thus the identification of new targets that improve the efficacy of anticancer agents can provide new possibilities for cancer treatment and possibly overcome drug resistance and drug side effects.

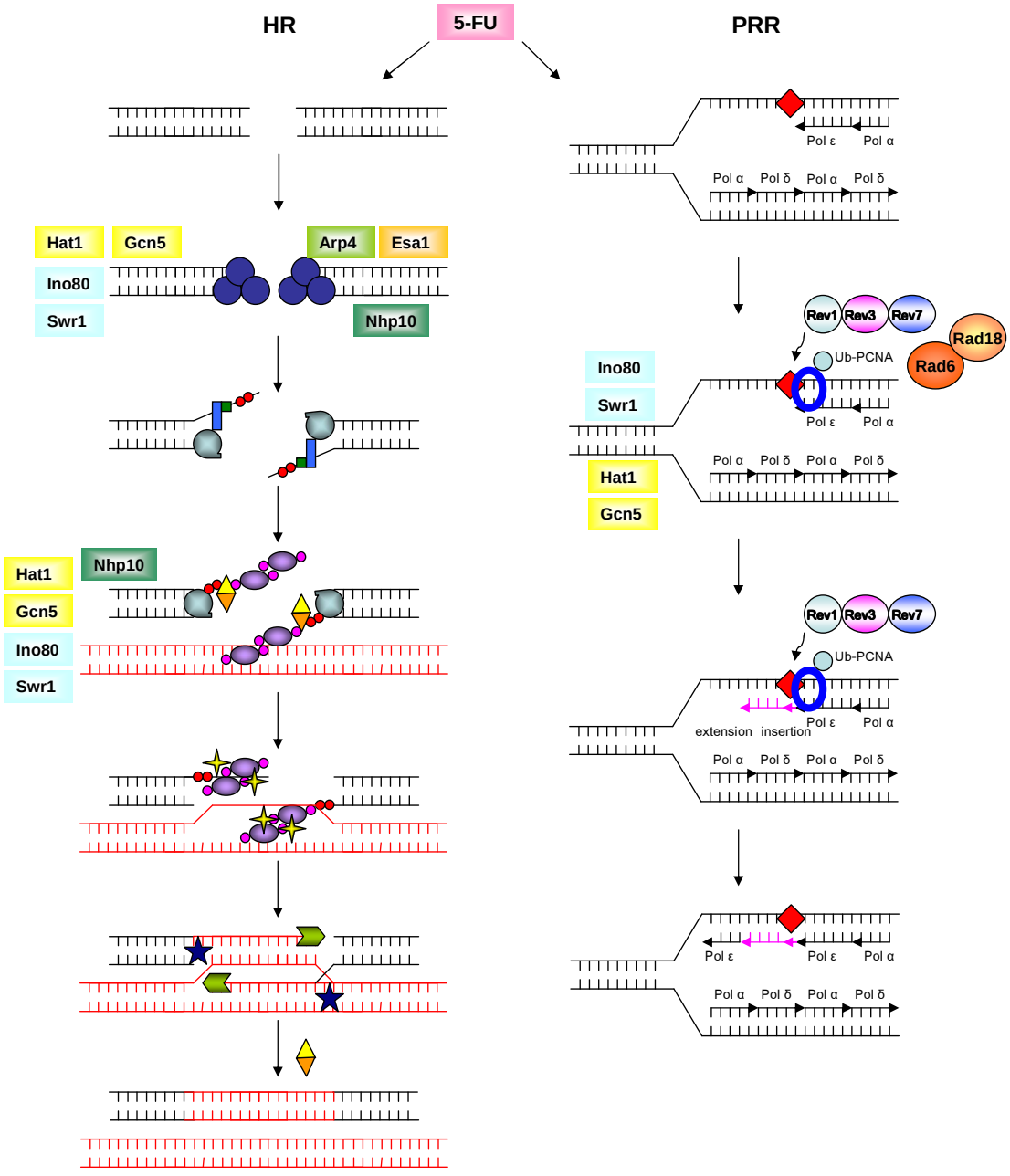

Figure 10: Chromatin remodeling and DNA repair of 5-FU lesions. 5-FU may induce different types of DNA damage, such as DSBs and replication inhibition, which are repaired by HR or PRR respectively. Several types of HMG, CRs and HATs participate in different steps of HR and PRR. In HR pathway (*left side*): after DSB formation, the MRX complex ( ) recognize the damaged DNA. At this step, several chromatin modifiers (Arp4, Esa1, Nhp10, Ino80, Swr1, Gcn5 and Hat1) may act. Nucleases ( ) bind to DNA and generate single-stranded DNA tails, which are coated by RPAs ( ). Then, Rad51 ( ) filament is formed, displacing

RPA from resected DNA. Rad52 (●) and Rad55/Rad57 (▲) complex mediate filament formation, and Rad54 (✦) may also participate at this step. During this step, chromatin remodelers may work. Concomitantly, the filament search for homologous sequences and when they are found, the resection ceases and Rad51 filament is dissassembled, mediated by Srs2 helicase (★). DNA polymerase (▰) restore the DNA sequency followed by religation. ■ Rad24; ▮ Rad17/Mec3/Ddc1 (HR pathway modified from AYLON & KUPIEC, 2004). In PRR pathway (*right side*): 5-FU lesions (◆) may stall replication forks. DNA polδ and polε replicate genomic DNA, but they are unable to bypass lesions. In response to stalled replication fork, Rad6/Rad18 complex ubiquitinates PCNA (O), which causes the dissociation of DNA replication polymerases and the association of damage bypass polymerases: pol zeta (Rev3/Rev7) associated to Rev1. At this step, chromatin modifiers (Nhp10, Ino80, Swr1, Hat1 and Gcn5) may act. Pol zeta or Rev1 inserts a nucleotide in opposite to the lesion and then pol zeta extends the DNA. The lesion bypass complex (Rev3/Rev7 and Rev1) dissociates from the template and normal replication polymerases reassociate to continue replication (PRR pathway adapted from GAN *et al.,* 2008).

Abbreviations

5-FU: 5-Fluorouracil

BER: Base Excision Repair

CR: ATP-dependent Chromatin Remodeling

DSB: Double-Strand Break

HAT: Histone Acetyltransferase

HDAC: Histone Deacetylase

HDML: Histone Demethylase

HMG: High-Mobility Group non-histone protein

HML: Histone Methylase

HMT: Histone Methyltransferase

HR: Homologous Recombination Repair

HV: Histone Variant

MRX: Mre11/Rad50/Xrs2 complex

PCNA: Proliferating Cell Nuclear Antigen

PRR: Post-Replication Repair

RPA: Replication Protein A

SSB: Single-Strand Break

TLS: Translesion synthesis

UB: Ubiquitin

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CAPÍTULO III

Antimetabolites cytotoxicity is potentialized by checkpoint kinases inhibition

Antimetabolites cytotoxicity is potentialized by checkpoint kinases inhibition

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Abstract

The effectiveness of antitumor agents that target the DNA may be modulated by many factors, including the DNA damage response (DDR) pathways. Thus, the inhibition of DDR components has been emerging as a promising approach to improve the current anticancer therapies. Indeed, the recently developed checkpoint kinase inhibitors (Chki) are able to sensitize cancer cells to treatment with several DNA damaging agents. However, the effect of Chki association with DNA damaging inducers is only known for a reduced number of antineoplastic agents and conditions. Herein, the combined effect of four antimetabolite drugs with a Chki was investigated, as well as the participation of ATR/Chk1 and ATM/Chk2 pathways in response to antimetabolites lesions. Our data showed that 5-fluorouracil, its active metabolite FdUMP, raltitrexed and gemcitabine are able to activate ATR/Chk1 pathway, but only gemcitabine triggers ATM/Chk2. The associations of AZD7762 (Chk1/2 inhibitor) with antimetabolites result in a remarkable sensitivity increase, which was related to differential cell cycle checkpoints activation. Single treatments with antimetabolites led S-phase accumulation, while its combinations with AZD7762 increased Sub-G1 and S-phase populations with a considerable decrease of G2/M. Accordingly to the sub-G1 increasing, AZD7762 may induce cell death by cell cycle acceleration, abrogation of G2/M arrest and promoting premature mitosis in antimetabolite-treated cells.

Key words: Antimetabolites, AZD7762, ATM and ATR.

1. Introduction

To assure genome integrity, cells have developed complex signaling networks which include the coordinated action of cell cycle arrest, DNA repair and apoptosis induction proteins. DNA damage is recognized by multiprotein complexes which recruit and activate signaling transducers that, in turn, will induce the activity of cellular response effectors (SMITH *et al.*, 2010). ATR (*ataxia telangiectasia and Rad3 related*) and ATM (*ataxia telangiectasia mutated*) belong to phosphoinositide 3-kinase-like family and are DNA damage responsive kinases (ZABLUDOFF *et al.*, 2008; MCNEELY *et al.*, 2010; FLYNN & ZOU, 2011). ATR is activated in response to stalled replication forks with its partner ATRIP (ATR interacting protein) (ZOU & ELLEDGE, 2003; SMITH *et al.*, 2010). On the other hand, ATM, in conjunction with MRE11/RAD50/NBS1 (MRN) sensor complex, is activated mainly by double-strand breaks (DSBs) (ASHWELL & ZABLUDOFF, 2008; MCNEELY *et al.*, 2010). When activated, both ATR and ATM may initiate a signaling cascade by phosphorylating common and distinct substrates, which final result is the appropriated cell cycle arrest and DNA repair machinery recruitment. The checkpoint kinases Chk1 and Chk2 are pivotal downstream substrates of ATR and ATM, respectively (ZABLUDOFF *et al.*, 2008; REINHARDT & YAFFE, 2009). Chk1 phosphorylates several proteins, which result in S and G2/M phase arrests, while Chk2 activates a different set of proteins, leading to G1/S arrest (for review, see HURLEY & BUNZ, 2007; SMITH *et al.*, 2010).

Widely employed in anticancer therapy protocols, antimetabolites are inhibitors of macromolecular biosynthesis that block cell replication by competitive

starvation (KINSELLA & SMITH, 1998). 5-Fluorouracil (5-FU) is one of the most employed antimetabolite to treat carcinomas arising in the gastrointestinal tract, ovary, breast, head, neck and esophagus. 5-FU is a DNA base analog that differs from uracil by a substitution of a fluorine atom in the place of hydrogen at the carbon-5 position of the pyrimidine ring (GREM, 1997). Inside the cells, 5-FU is converted to several metabolites that may be misincorporated into DNA and RNA, or induce to anti-proliferative effects through inhibition of thymidylate syntase (TS) enzyme (LONGLEY *et al.*, 2003; XIAO *et al.*, 2005). All of these mechanisms may lead directly or indirectly to several DNA lesions as SSBs and DSBs and consequently to cytotoxicity (ROBINSON *et al.*, 2006; MATUO *et al.*, 2009). The most studied active metabolite that may derivate from 5-FU conversion, 5-fluoro-deoxyuridine monophosphate (FdUMP) acts inhibiting TS activity by covalent binding. Once TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to dTMP and is the only intracellular source of thymidylate in the pyrimidine nucleotide synthesis pathway, FdUMP exposure may lead to nucleotide pool imbalance which results in uracil misincorporation into DNA and DNA SSBs formation (GREM, 1997; MATUO *et al.*, 2009; WILSON & TAYLOR, 2009).

Raltitrexed (RTX, Tomudex^{TM}, AstraZeneca) is an antimetabolite analogue of tetrahydrofolate cofactor that may be employed to treat colorectal cancer in pacients with 5-FU intolerance (JACKMAN *et al.*, 1999). Unlike 5-FU or FdUMP, RTX can not be incorporated into DNA, but inhibits TS directly and specifically without requiring any conversion or modulation (BLACKLEDGE, 1998; WILSON & TAYLOR, 2009). As a TS inhibitor, RTX induces nucleotide pool imbalance and uracil misincorporation into DNA, sister chromatid exchanges and DNA strand

breaks (VAN TRIEST *et al.*, 2000; LI *et al.*, 2005; YANG *et al.*, 2008; WALDMAN *et al.*, 2008). The antimetabolite gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdCyd) is the standard chemotherapy for pancreatic cancer (MORGAN *et al.*, 2010). Inside the cells, gemcitabine is phosphorylated and converted to its active metabolites, diphosphorylated (dFdCDP) and triphosphorylated (dFdCTP). dFdCDP inhibits the enzyme ribonucleotide reductase (RR), which leads to depletion of deoxynucleotide triphosphate pools, primary dATP, while dFdCTP competes with endogenous dCTP, resulting in misincorporation of dFdCTP into DNA and polymerase stalling one base beyond the site of addition (MORGAN *et al.*, 2005; MINI *et al.*, 2006). These perturbations may lead to replication stress, DNA synthesis inhibition, DNA breaks and activate the DNA damage response pathway (MCNEELY *et al.*, 2010; MORGAN *et al.*, 2010).

Finally, the modulation of checkpoint pathways employing inhibitors has been recently proposed as a promising approach to potentiate the cytotoxicity of antineoplasic drugs (ASHWELL & ZABLUDOFF, 2008; PARSELS *et al.*, 2011). Remarkable, the abrogation of cell cycle checkpoints pathways by Chk1 and Chk2 inhibition may provide important alternatives to improve the effectiveness of numerous anticancer therapies (ASHWELL *et al.*, 2008; ASHWELL & ZABLUDOFF, 2008; MORGAN *et al.*, 2010; ZABLUDOFF *et al.*, 2008). However, few studies have been conduced employing antimetabolites and checkpoint inhibition. Therefore, this work aimed to investigate the role of ATR and ATM pathways towards cytotoxicity induced by 5-FU, FdUMP, RTX and gemcitabine, as well as the cellular effects from combination of these antimetabolites and

AZD7762, an ATP competitive Chk1/2 inhibitor (ASHWELL *et al.*, 2008; LAPENNA & GIORDANO, 2009; MCNEELY *et al.*, 2010).

2. Materials and Methods

2.1. Cell culture and chemicals

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, InVitrogen, Cergy-Pontoise, France) supplemented with 10% (v/v) fetal calf serum (Perbio Science, Brebières, France), 100 units/mL penicillin and 100 µg/mL streptomycin (PanPharma, Fougères, France). Cells were cultivated at 37ºC and 10% of CO_2. 5-FU was obtained from Teva-Pharma (Courbevoie, France). 5-Fluoro-2'-Deoxyuridine-5'-Monophosphate (FdUMP) and raltitrexed were acquired from Sigma-Aldrich (Saint-Quentin Fallavier, France). Gemcitabine was obtained from Eli Lilly and Company (Indianapolis, IN, U.S.A.). AZD7762 was purchased from Axon Medchem (Groningen, Netherlands).

2.2. Cell Viability Assays

Viability was determined by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay, as previous described by POINDESSOUS *et al.* (2003). 5000 cells per well were seeded onto 24-well plate 24h before drug treatment. Treatments with antimetabolites in the presence or absence of 50nM AZD7762 were performed for 120h. AZD7762 concentration was definied by MTT (data not shown). Graphics present the average of at least three independent experiments, each one done in duplicate.

2.3. Imunofluorescence, Microscopy and Antibodies

Imunofluorescence was carried out as previous described by ESCARGUEIL *et al.* (2008). For RPA32 and RPA70 marcages, coverslips were washed in PBS, resuspended in ice-cold CSK-lysis buffer (150 mM NaCl, 3 mM $MgCl_2$, 1% Triton X-100, 50 mM HEPES pH 7.4, 30 mM sucrose and protease inhibitors) and kept at 4℃ for 5 minutes before fixation in 4% paraformald ehyde (Electron Microscopy Sciences, Hatfield, PA, U.S.A.). Antigens were revealed by using the indicated primary antibodies and DNA was counterstained with DAPI. For analysis, at least 200 cells were analyzed per sample employing a BX61 microscope and Cell F Imaging Software (Olympus) to collect the images. Fluorescence intensity was measured by Metamorph Software (Universal Imaging Corporation, Downingtown, PA) and the background fluorescence over non-cellular regions was subtracted. Graphics present average of at least two independent experiments. The RPA32 (clone 4E4, #2208), RPA70 (# 2267), phospho-Ser317-Chk1 (# 2344), phospho-Thr68-Chk2 (# 2661) and phospho-Ser1981-ATM (clone 10H11.E12, # 4526) antibodies were purchased from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, France). Fluorescent dye-conjugated antibodies (Cy3 and Alexa 488) were acquired from Jackson Immunoresearch (Bar Harbor, ME).

2.4. Cell Cycle Analysis and Marcage of Mitotic Cells

Cell cycle assays were performed as previously described by SKLADANOWSKI *et al.* (2005). Treatments were carried out for 24 and 48h with antimetabolites in the presence or absence of 50nM AZD7762. Cells were washed in PBS and $1x10^6$ cells per sample were fixed in 70% ethanol overnight. Then,

cells were resuspended in PBS, and incubated with 2µg/mL propidium iodide (Sigma-Aldrich) and 100µg/mL RNase (Sigma-Aldrich). Samples were analysed with LSR II flow cytometer and DIVA Software (BD Biosciences, France). For mitotic marcage, cells were treated with antimetabolites in the presence or absence of 50nM AZD7762 for 24h, and then harvested with trypsin and fixed in ethanol 70% overnight. Cells were washed with cold PBS and incubated with primary antibody directed against Ser10-phosphorylation histone H3 (Cell Signaling Technology, Ozyme, Saint Quentin en Yvelines, France) overnight. The secondary antibody employed was Cy5-conjugated (Jackson Immunoreasearch, Bar Harbor, ME) for 1h. The fluorescence emitted by Cy5 was measured by flow cytometry and the DNA was counterstained with propidium iodide.

3. Results

In order to properly understand the cellular effects of antimetabolites combination with AZD7762, the main DNA damage signaling pathways activated by single exposure to 5-FU, FdUMP, RTX and gemcitabine were investigated, as well as cell cycle checkpoints induced by those anticancer drugs.

3.1. Antimetabolites treatment induced ATR/Chk1 pathway activation

Concerning on ATR/Chk1 signaling pathway, it is well established that ATR is recruited by ATRIP to regions of single-stranded DNA (ssDNA) coated with RPA (Replication Protein A) (SMITH *et al.*, 2010). The, stable recruitment of ATR-ATRIP to damaged sites depends on direct binding of ATRIP to RPA that is a

heterotrimeric protein composed by three subunits, RPA70, RPA32 and RPA14 (ZOU *et al.*, 2006; FLYNN & ZOU, 2011). In response to DNA damage, ATR activates Chk1 by phosphorylating it on Ser317 and Ser345. This signaling cascade normally results in S and G2/M arrests (MCNEELY *et al.*, 2010). However, once ATR activation does not necessarily involve autophosphorylation or other posttranslational modification, thus ATR activity needs to be presumed by evaluation of its partners activities (*ex.*: RPA subunits expression, TopBP1 activity) (SMITH *et al.*, 2010).

In this sense, Figure 1 presented the results for quantification of RPA32 and RPA70 expression and Chk1 phosphorylation (Ser317) employing single treatments with pre-established toxic-equivalent doses (IC_{50}) of 5-FU, FdUMP, RTX and gemcitabine. Treatments with gemcitabine or FdUMP lead to a notably increase of RPA32/70 expression with 6h of exposure and a pronounced effect with 24h (Figure 1A and B). On the other hand, RTX or 5-FU effect on RPA32/70 expression was observed only with 24h of exposure (Figure 1A and B). Additionally, a considerable phosphorylation of Chk1 was induced by antimetabolites in a similar pattern (Figure 1C). Together, these data indicates that DNA damage induced by antimetabolites may activate the ATR/Chk1 pathway with different intensities, which is likely to be a result of the different types and severity of DNA lesions produced.

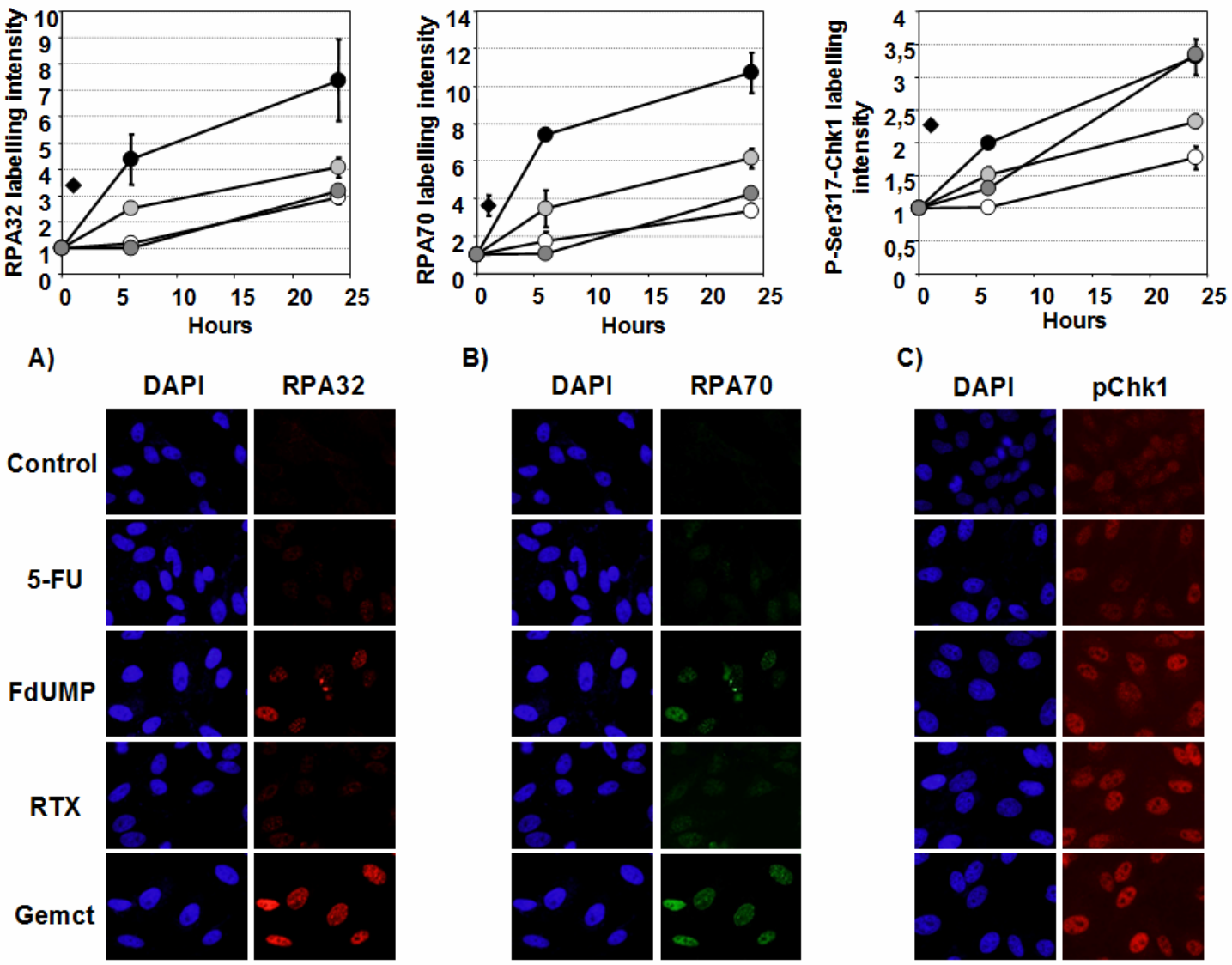
RPA32 labelling intensity
Hours
RPA70 labelling intensity
Hours
P-Ser317-Chk1 labelling intensity
Hours
A)
B)
C)
DAPI
RPA32
DAPI
RPA70
DAPI
pChk1
Control
5-FU
FdUMP
RTX
Gemct

Figure 1: *Antimetabolites effect on ATR/Chk1 pathway activation.* HeLa cells were incubated with IC_{50} concentration of 5-FU, FdUMP, Gemcitabine and RTX, for 6 and 24h. Soluble proteins were extracted with ice-cold CSK-lysis buffer for 5 minutes before fixation and the cells were labeled with: (A) RPA32-directed antibody and Cy3 secondary antibody; (B) RPA70-directed antibody and Alexa 488 secondary antibody and (C) phospho-Ser317 Chk1 and Cy3 secondary antibody. Fluorescence intensities were quantified by Metamorph Software. At least 400 cells were analyzed for each condition. Lines indicate the average intensity obtained for each condition and standard deviations are indicated by error bars. Treatment with 200ng/mL diflomotecan for 1h was employed as positive control. Cells were fixed and processed for RPA32, RPA70 and pChk1 staining and the DNA was counterstained with DAPI (images from 24h of drug exposure).
○: 5-FU; ●: FdUMP; ●: RTX; ●: Gemcitabine; ◆: Diflomotecan.

3.2. Gemcitabine exposure leads to activation of ATM/Chk2 pathway

ATM is a homodimer that dissociates through autophosphorylation to form catalytically active monomers (REINHARDT & YAFFE, 2009; SOARES *et al.*, 2011). These monomers are recruited to DSBs via interactions with MRN sensor complex, which also stimulate its full activation at the damaged site (SMITH *et al.*, 2010). In response to DNA damage, the ATM monomers phosphorylate Chk2 on Thr-68, triggering a chain of additional Chk2-autophosphorylation events on Thr-383 and Thr-387, which result in Chk2 activation (REINHARDT & YAFFE, 2009). In turn, Chk2 may phosphorylate cell cycle arrest signaling transducers, including p53, which final result is G1/S checkpoints (LAPENNA & GIORDANO, 2009; SMITH *et al.*, 2010).

The Figure 2 presented the results for ATM (Ser1981) quantification and Chk2 (Thr68) phosphorylation employing single treatments with pre-established toxic-equivalent doses (IC_{50}) of 5-FU, FdUMP, RTX and gemcitabine. Interestingly,

gemcitabine exposure leads to a remarkable increase in ATM and Chk2 phosphorylation, while the others antimetabolites presented a discrete effect on ATM/Chk2 pathway activation (Figure 2A and B). These results are in agreement with KARNITZ *et al.* (2005), which observed that gemcitabine, but not cytarabine (an antimetabolite based on cytosine structure), exposure for 24h lead to a pronounced phosphorylation of Chk2. This intriguing difference between gemcitabine and the other antimetabolites in DNA damage signaling pathways activation has been proposed to be a result of the different DNA lesions induced by their metabolites. Indeed, the incorporation of dFdCTPs into DNA may lead to DNA synthesis inhibition and replication stress due to polymerase stalling, which result in accumulation of dangerous DNA lesions (KARNITZ *et al.*, 2005; MINI *et al.*, 2006).

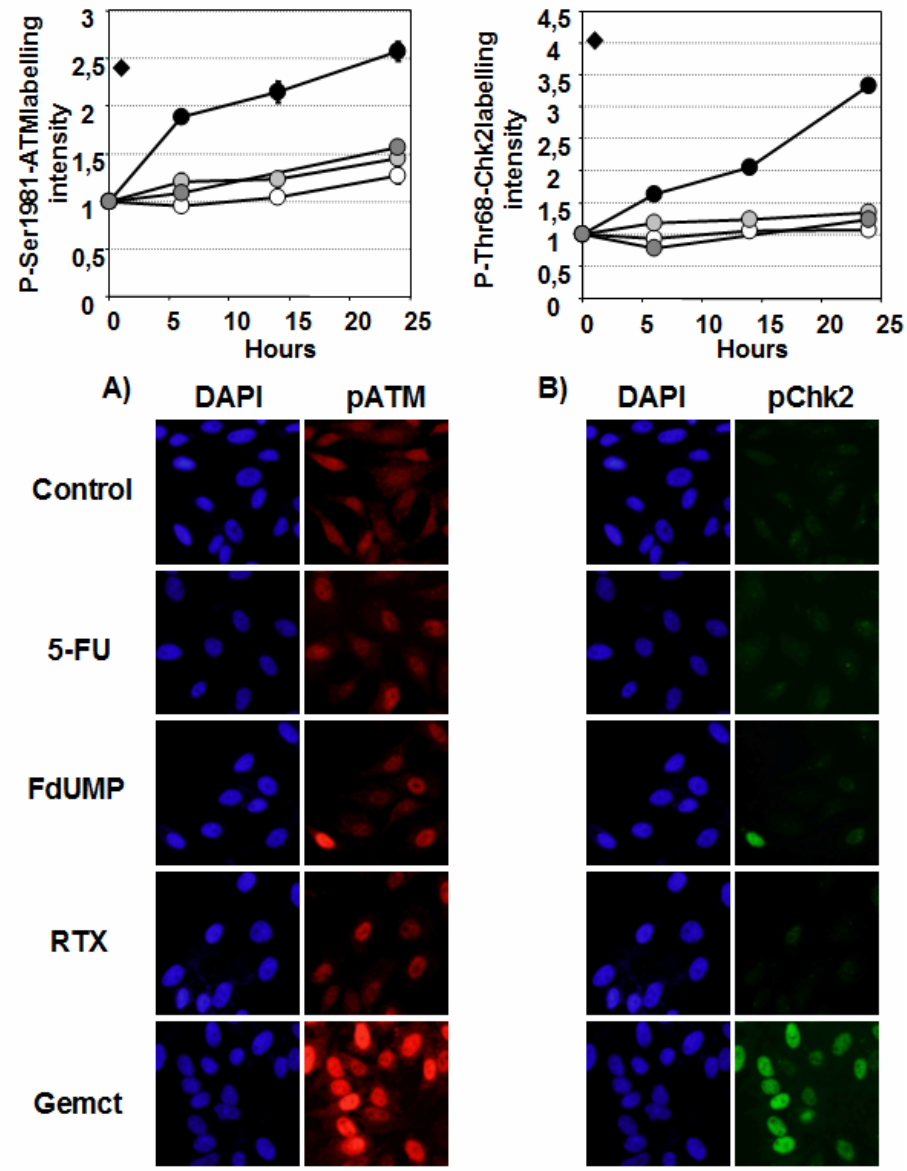

Figure 2: *Antimetabolites effect on ATM/Chk2 pathway.* HeLa cells were exposed to IC_{50} antimetabolites for 6, 14 and 24h, fixed and processed for immunolabeling with antibody directed against: (A) Ser1981-phosphorylated ATM and Cy3 secondary antibody and (B) Thr68-phosphorylated Chk2 and Alexa 488 secondary antibody. The fluorescence intensities were quantified by Metamorph analysis. At least 400 cells were analyzed for each condition. Treatment with 200ng/mL diflomotecan for 1h was employed as positive control. Cells were fixed and processed for pATM and pChk2 staining and the DNA was counterstained with DAPI (images from 24h of drug exposure). ○: 5-FU; ●: FdUMP; ●: RTX; ●: Gemcitabine; ◆: Diflomotecan.

3.3. Antimetabolites cytotoxicity is potentialized by AZD7762

AZD7762 is a potent and selective ATP-competitive inhibitor of Chk1/2 which structure is based on thiophene urea carboxamides (ASHWELL *et al.*, 2008; ASHWELL & ZABLUDOFF, 2008). This Chki has been shown to potentiate the effects of a variety of different DNA-damaging agents across a number of different cell lines, with the precise degree of potentiation depending upon both the cell line and the DNA-damaging agent used (ASHWELL *et al.*, 2008; ASHWELL & ZABLUDOFF, 2008). The compound abrogated S and G2 DNA damage checkpoints *in vivo*, and augmented the cell killing induced by gemcitabine and irinotecan in rodent tumor models. These promising results lead AZD7762 to entered Phase I clinical trials in combination with gemcitabine or irinotecan in patients with advanced solid tumours (LAPENNA & GIORDANO, 2009).

To determine the influence of Chk1/2 inhibition in antimetabolites cytotoxicity, the cell viability was evaluated by MTT assay for a concentration range of 5-FU, FdUMP, RTX and gemcitabine, in association or not with a fixed dose of AZD7762 (50nM). Interestingly, AZD7762 combinations were shown to increase HeLa sensitivity to all antimetabolites, but with different intensities (Figure 3). The association with AZD7762 increased the antimetabolites sensitivity by approximately 2-fold for 5-FU, 10-fold for FdUMP, 15-fold for RTX and 20-fold for gemcitabine. Notably, the pattern of sensitivity increasing conferred by AZD7762 association is quite similar to the pattern of increasing in Chk1 phosphorylation (Ser317) induced by single treatment with antimetabolites for 24h (Figure 1C and Figure 3). Therefore, indicating that Chk1 inhibition is more effective in

combination with the antimetabolites which exposure lead to high activation of ATR/Chk1 pathway.

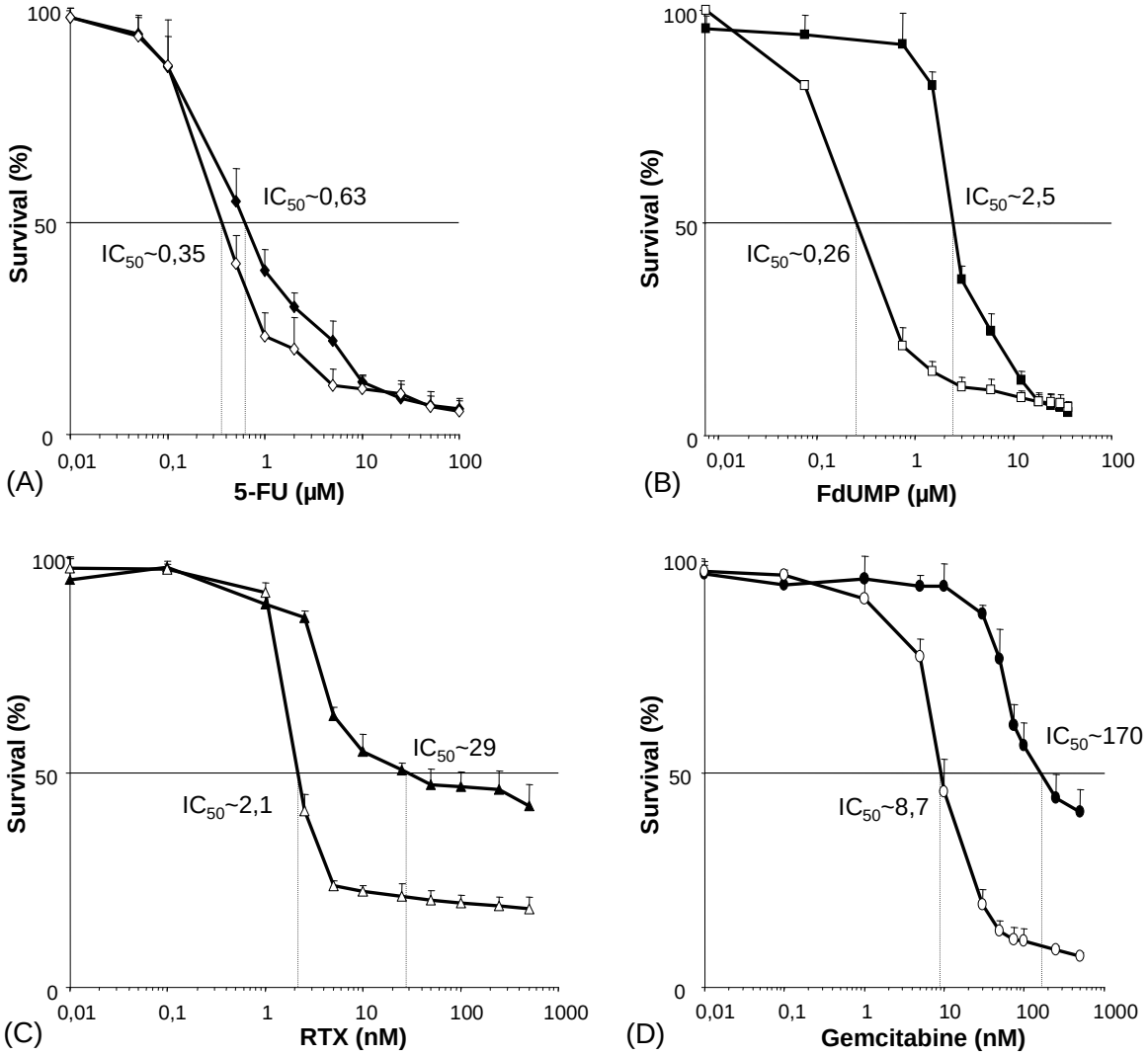

Figure 3: *Cytotoxic effect of AZD7762 combination with antimetabolites*. HeLa cells were exposed to the indicated concentrations for 120h and the growth inhibitory effect was determined by the MTT viability assay. Sensitivity of (A) (♦) 5-FU and (◊) 5-FU + 50nM AZD7762; (B) (■) FdUMP and (□) FdUMP + 50nM AZD7762; (C) (▲) RTX and (Δ) RTX + 50nM AZD7762; (D) (●) Gemcitabine and (○) Gemcitabine + 50nM AZD7762. All values are averages of at least three independent experiments, each done in duplicate. Standard deviation are indicated by error bars when they exceed symbol size.

3.4. The combination of AZD7762 with antimetabolites result in acceleration of cell cycle and premature mitosis

The effect of AZD7762 combination with antimetabolites in cell cycle progression was evaluated employing flow cytometry. HeLa cells were treated with toxic-equivalent doses (IC_{50}) of 5-FU, FdUMP, RTX and gemcitabine in presence or absence of 50nM AZD7762 for 24 or 48h (Figure 4A and B, respectively). As an expected consequence of ATR/Chk1 pathway activation (Figure 1), the single exposure to antimetabolites was shown to increase the S phase cell populations for all treatments within 24h (Figure 4A). This tendency was also observed for 48h of exposure, but with a considerable increase in sub-G1 populations. The combinations of AZD7762 with antimetabolites for 24h lead to an outstanding increase in sub-G1 cell populations for 5-FU, RTX and gemcitabine treatments, while a similar effect was also observed for 48h of exposure to 5-FU and gemcitabine associations. In addition, combined treatments with AZD7762 also decreased G2/M population for all antimetabolites after 24 and 48h of exposure (Figure 4).

AZD7762 is expected to abrogate cell cycle checkpoints and increase the sub-G1 population, which may result in cell death induction (ASHWELL & ZABLUDOFF, 2008). AZD7762 combinations with antimetabolites lead to cell cycle acceleration and premature mitosis entry, which in turn, may result in mitotic catastrophe and cell death depending on the extent of DNA damage accumulated. To test this possibility, the mitotic cells accumulation was quantified using flow cytometry for phospho-Ser10-histone H3. Phosphorylation at Ser10 of histone H3

is tightly correlated with chromosome condensation during mitosis and thus, is a direct indicative o mitotic entry.

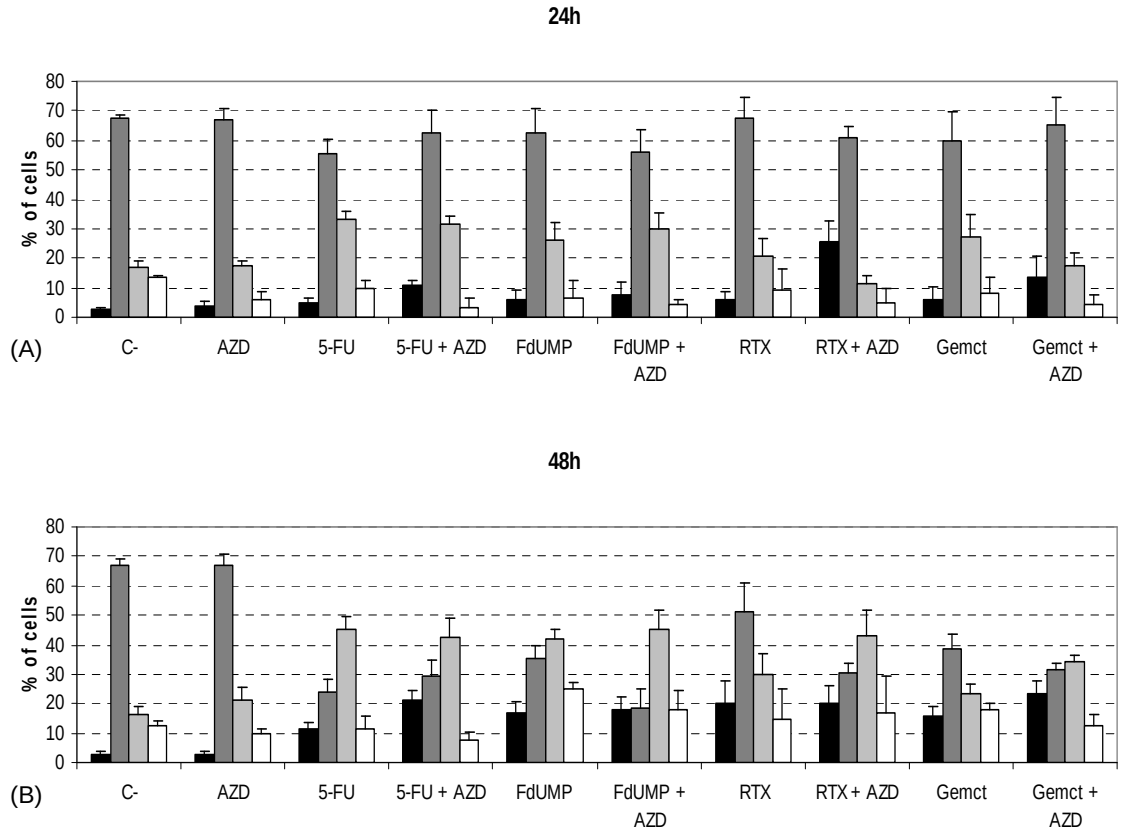

Figure 4: *Cell cycle distribution for AZD7762 combination with antimetabolites.* HeLa cells were treated with for (A) 24h and (B) 48h, in the presence or absence of IC_{50} antimetabolites and 50nM AZD7762, and then prepared for cell cycle analysis. Bars represent the mean value of four independent experiments. ■: Sub-G1; ■: G1/S; ■: S; ❒: G2/M.

The data presented in the Figure 5 demonstrated that single treatments with 5-FU, FdUMP, RTX and gemcitabine lead to a reduction in number of cells entering mitosis, which is an expected result of the cytostatic activity of antimetabolites. However, the association of antimetabolites with AZD7762 restored p-Ser10-H3 marker, indicating that Chk1/2 inhibition induced premature

mitosis in antimetabolite-treated cells. The premature mitosis was observed in G1/S, S and G2/M phases of cell cycle for all antimetabolites combinations with AZD7762 (Figure 5 and Supplementary data S1), indicating that cells from all phases of cell cycle entered in mitosis despite incomplete DNA synthesis. Once premature mitosis may result in cell death, these results are in agreement with in the Sub-G1 population increasing observed at Figure 4.

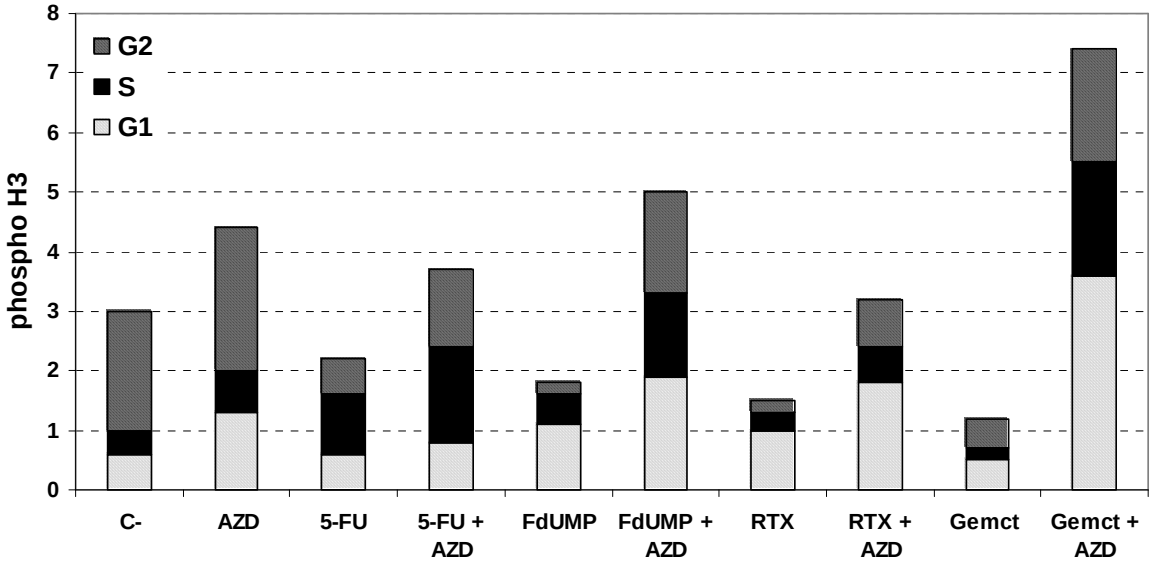

Figure 5: *Mitotic cells distribution for AZD7762 combination with antimetabolites.* HeLa cells were treated with IC_{50} antimetabolites in the presence or absence of 50nM AZD7762 for 24h, incubated with primary antibody directed against Ser10-phosphorylation histone H3 and Cy5 as secondary antibody. Bars represent the mitotic cells labeled with Cy5 measured by flow cytometry, divided in G1/S, S and G2/M cell cycle phases.

4. Discussion

In theory, the modulation of checkpoints employing Chk1/2 inhibitors may potentiate the cytotoxicity of a variety of antineoplastic drugs. However, in practice, few drugs associations with Chkis have been studied and little is know about the

mechanisms involved in those combinations. AZD7762 has been reported to abrogated cell cycle arrest and induced apoptosis in gemcitabine-treated cells (ZABLUDOFF *et al.*, 2008; MCNEELY *et al.*, 2010), but nothing was described for 5-FU, FdUMP and RTX. Herein, it was demonstrated that 5-FU, FdUMP, RTX and gemcitabine exposure lead to activation of ATR/Chk1 pathway, but only gemcitabine treatment activates ATM/Chk2 pathway (Figures 1 and 2). These differences are possibly a result of the varied DNA lesions induced by these drugs. Indeed, 5-FU, FdUMP and RTX employs different cytotoxicity mechanisms based on TS inhibition. 5-FU needs to be converted to its metabolites to exert its cytotoxicity, which includes DNA/RNA misincorporation and TS inhibition. The only fluoronucleotide derivate from 5-FU conversion that can inhibit TS is the FdUMP, which forms a covalent ternary complex with TS (LONGLEY *et al.*, 2003). While RTX inhibits TS directly and specifically, and differently from other antimetabolites, is active in its parent form, which is rapidly converted into polyglytamated forms (BLACKLEDGE, 1998; VAN CUTSEN *et al.*, 2002; WILSON & TAYLOR, 2009). TS inhibition leads to nucleotide pool imbalance and consequently to replication blocks, bases missincorporation and SSBs (WALDMAN *et al.*, 2008), which lead to ATR/Chk1 activation (Figure 1). On the other hand, gemcitabine acts inhibiting RR and stalling replication forks, which may lead to replication blocks and severe DNA damages as DSBs that result in ATR/Chk1 and ATM/Chk2 activation (Figure 2) (KARNITZ *et al.*, 2005; MCNEELY *et al.*, 2010).

Despite our data showed that 5-FU (IC_{50}) activated mainly ATR/Chk1 pathway in HeLa cells, GENG *et al.* (2011) have reported that this antimetabolite may also induce Chk2 phosphorylation (Thr68) in HT-29 colon cancer cells. This

can be explained as a result of the different experimental conditions employed, such as a higher 5-FU concentration (IC_{90}) and the differences in genetic backgrounds between HeLa and HT-29.

Accordingly to the type and severity of DNA lesion, different DNA damage signaling pathways are activated. These pathways are important cellular features that allow the coordinated action of various cellular processes in response to DNA damage, including cell cycle arrest, recruitment of DNA repair proteins and repair of DNA lesions before entering in mitosis. The differences in ATR/Chk1 activation intensities observed at the Figure 1 presented a direct correlation with the sensitivity increasing conferred by AZD7762 combination with the antimetabolites (Figure 3). In agreement, some authors have proposed that sensitization by AZD7762 is mediated by Chk1 inhibition rather than Chk2 inhibition (ASHWELL *et al.,* 2008; MORGAN *et al.,* 2010). Chk1 inhibition allows firing of suppressed replication origins after replicative stress, and the abrogation of cell cycle arrest, allowing S-phase cells to progress directly into mitosis with incompletely replicated DNA, which results in cell death (XIAO *et al.*, 2005; MCNEELY *et al.,* 2010). Indeed, the results presented at the Figure 4 demonstrated that AZD7762 combination with antimetabolites lead a considerable increase in sub-G1 population, which may be related to extensive cell death induction. This effect is associated with cell cycle acceleration and premature mitosis observed at the Figure 5.

In summary, the results presented here indicated that the combinations of AZD7762 and antimetabolites may potentiate the cytotoxicity of all antimetabolites tested by acceleration of cell cycle and premature mitosis induction, which may

lead to cell death induction. Additionally, the degree of cytotoxicity increasing conferred by AZD7762 combination was shown to be a direct result of ATR/Chk1 activation intensity induced by single treatments with 5-FU, FdUMP, RTX and gemcitabine. These results may be employed to improve the conventional anticancer protocols, as well as to provide important clues on antimetabolites and AZD7762 basic mechanisms, which certainly may result in development of more efficient clinical treatments and side effects reduction. Finally, the DNA damage response pathways are emerging as interesting targets that should be more investigated and exploited for future applications in oncology treatment.

Abbreviations

5-FU: 5-Fluorouracil

ATM: *Ataxia telangiectasia* mutated

ATR: *Ataxia telangiectasia* mutated and Rad3 related

ATRIP: ATR interacting protein

DDR: DNA damage response

dFdCDP: gemcitabine diphosphorylated

dFdCTP: gemcitabine triphosphorylated

dFdCyd; Gemcitabine: 2′,2′-difluoro-2′-deoxycytidine

DSBs: Double-strand breaks

dUMP: deoxyuridine monophosphate

FdUMP: 5-Fluoro-2’-deoxyuridine-5’-monophosphate

MRN: MRE11/RAD50/NBS1

RPA: Replication Protein A

RR: Ribonucleotide Reductase

RTX: Raltitrexed

ssDNA: single-stranded DNA

TS: Thymidylate Syntase

Acknowledgments

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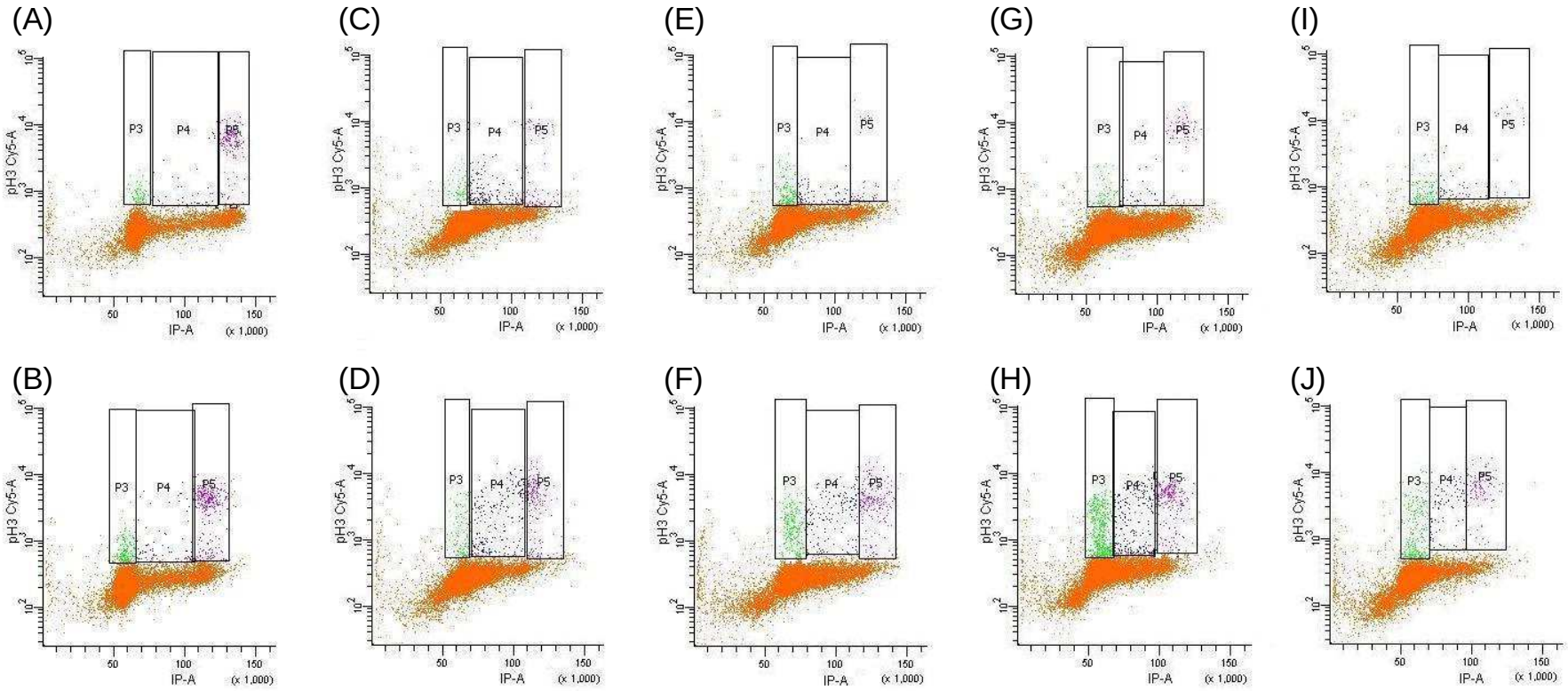
Supplementary Data

S1: *Mitotic cells distribution.* HeLa cells treated with IC_{50} antimetabolites in the presence or absence of 50nM AZD7762 for 24h. Legend: (A) Untreated cells, (B) AZD7762, (C) 5-FU, (D) 5-FU + AZD7762, (E) FdUMP, (F) FdUMP + AZD7762, (G) Gemcitabine, (H) Gemcitabine + AZD7762, (I) RTX, (J) RTX + AZD7762.



DISCUSSÃO

A compreensão detalhada de como os agentes antineoplásicos atuam a nível molecular, inibindo a progressão tumoral e levando a morte celular, bem como a identificação de fatores capazes de potencializar a atividade citotóxica destas drogas, são de extrema importância, uma vez que podem contribuir para o desenvolvimento de terapias mais eficazes. Entretanto, os mecanismos de ação de diversos agentes antitumorais empregados na clínica atual ainda não foram completamente elucidados, de maneira que investigar tais mecanismos é necessário para o desenvolvimento de protocolos mais eficientes.

O agente antineoplásico 5-FU vem sendo empregado no tratamento de diversos tipos de tumores desde 1957 (NOORDHUIS *et al.*, 2004). O 5-FU possui diferentes mecanismos de citotoxicidade, tais como a incorporação errônea de nucleotídeos análogos ao uracil contendo um átomo de flúor no DNA e/ou RNA, ou levando a inibição da enzima TS pelo seu metabólito FdUMP (LONGLEY *et al.*, 2003). Diversos estudos vêm sendo conduzidos a fim de se elucidar seus mecanismos de ação (LONGLEY *et al.*, 2004; SEIPLE *et al.,* 2006; MATUO *et al.*, 2009), propondo combinações com outros agentes antitumorais a fim de se potencializar sua atividade citotóxica (GRIVICICH *et al.*, 2005; MORITA *et al.*, 2007; PERA *et al.*, 2011), investigando mecanismos de resistência (PETERS *et al.*, 2002; LONGLEY *et al.*, 2003) e identificando genes que contribuem ou não para melhor resposta terapêutica (CARETHERS *et al.*, 1999; LONGLEY *et al.*, 2003). No entanto, muitos aspectos sobre seu mecanismo de ação ainda não foram investigados. Neste trabalho foram estudados diferentes aspectos relacionados às vias de reparação de DNA envolvidas nas lesões induzidas pelo 5-FU, a participação de modeladores da cromatina e a sinalização celular via

ATR/Chk1 e ATM/Chk1, em linhagens de *S. cerevisiae* e células tumorais humanas.

Na primeira parte deste trabalho foi avaliado o envolvimento das vias de reparação de DNA em resposta as lesões induzidas pelo 5-FU e pelo seu metabólito ativo FdUMP, empregando diferentes linhagens de *S. cerevisiae* deficientes em genes de reparo. A levedura *S. cerevisiae* não possui a enzima timidina cinase, o que a torna incapaz de converter o 5-FU a FdUMP (LADNER, 2001). Desta forma, este modelo biológico nos permite estudar os efeitos dos erros de incorporação gerados pelo 5-FU e a atividade do metabólito FdUMP de maneira independente. Os resultados obtidos demonstraram que a via BER é importante no reparo de danos induzidos tanto pelo 5-FU quanto pelo o FdUMP, uma vez que linhagens deficientes em *NTG1, NTG2* e *APN1* apresentaram alta sensibilidade a estes agentes (Capítulo l, Figura 3). Linhagens deficientes em AP endonuclease (*apn1*Δ) ou liases (*ntg1*Δ e *ntg2*Δ), podem acumular sítios abásicos no DNA, que são altamente nocivos, uma vez que podem ser convertidos a quebras no DNA (BOITEUX & GUILLET, 2004). Desta forma, quando estas linhagens são expostas ao 5-FU, a uracil glicosilase inicia a excisão destes fluoronucleotídeos que foram erroneamente incorporados, porém, os sítios AP permanecem, uma vez que as enzimas capazes de processá-las adequadamente não se encontram disponíveis. Conseqüentemente, estes sítios podem ser convertidos em quebras.

Os mutantes em MMR (*MLH1* e *PMS1*) também foram sensíveis a ambas as drogas. Dados da literatura reportaram que tumores humanos deficientes em MMR apresentam resistência ao 5-FU e relacionam isto ao fato que em

mamíferos, este sistema de reparação também atua na sinalização para a apoptose (WYATT & WILSON, 2009). Entretanto, as leveduras não possuem tais enzimas responsáveis pela sinalização para apoptose como as de mamíferos e isto pode explicar a diferença de resultados obtidos entre *S. cerevisiae* e os encontrados em tumores humanos. As linhagens deficientes em NER (*RAD1* e *RAD10*) e TLS (*REV1* e *REV3*) foram pouco sensíveis ao 5-FU e ao FdUMP, o que sugere que estas vias tenham pouca participação no processamento destas lesões (Capítulo l, Figuras 4 e 5). Interessantemente, mutantes em *RAD52* (da via HR), *RAD6* e *RAD18* (via PRR) apresentaram sensibilidade apenas ao 5-FU (Capítulo l, Figura 6). Esta diferença pode ser explicada pelo mecanismo de ação de cada droga: o 5-FU pode levar a formação de quebras de fita simples (SSBs) e duplas (DSBs), enquanto que o FdUMP apenas SSBs. As DSBs induzidas pelo 5-FU podem ainda ser reparadas pela HR, por isso a sensibilidade da linhagem *rad52*Δ. Além disso, o 5-FU, que leva a erros de incorporação, pode induzir danos capazes de bloquear a replicação, que recrutam a via de PRR, ao contrário das lesões geradas pelo FdUMP, que seriam principalmente de incorporação de uracil no DNA. Ao investigar a sobreposição das vias de reparação de DNA das lesões induzidas pelo 5-FU e FdUMP empregando quádruplos mutantes, observou-se que apenas a linhagem *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ foi sensível ao 5-FU. Este resultado sugere que os danos provenientes da incorporação de fluoronucleotideos podem ser reparados inicialmente pela via BER e canalizados para a via HR.

Assim, as lesões induzidas pelo 5-FU podem ser reparadas pela via BER, através das Ntg1/Ntg2 glicosilases e Apn1 endonuclease que reconhecem e

removem as bases que foram erroneamente incorporadas. Embora a Ung1 glicosilase e a Rad27 “flap” endonuclease participem da via BER, deficiências nestas proteínas não aumentam a morte celular frente a lesões induzidas pelo 5-FU (SEIPLE *et al.*, 2006). Falhas durante o BER podem resultar na formação de SSBs e DSBs, as quais são reconhecidas pela via HR. Outra possibilidade é o envolvimento do MMR e PRR no reparo das lesões por 5-FU que não foram removidos durante a replicação. Entretanto, os danos gerados pelo FdUMP podem ser processado pelas Ntg1/Ntg2 e Apn1, e deficiências neste processo podem gerar SSBs. Os erros de emparelhamento que não foram processados pelo BER podem ser reparados pela via MMR. As diferenças nas vias de reparo recrutadas no processamento das lesões induzidas pelo 5-FU e FdUMP podem ser atribuídas ao mecanismo de ação de cada droga e aos diferentes tipos de danos induzidos pelos agentes. Em *S. cerevisiae,* o 5-FU leva a incorporação errônea de fluoronucleotídeos no DNA e RNA, enquanto o FdUMP, que atua inibindo a enzima TS, leva a incorporação de uracil no DNA..

Os resultados obtidos neste capítulo estão de acordo com MATUO *et al.* (2009), que estudou o efeito de ambos antimetabólitos em células de câncer de cólon SW620 (Anexo I). Nesta linhagem celular, o 5-FU induz SSBs e DSBs, enquanto que o FdUMP forma principalmente SSBs. Além disso, observou-se que a formação de DSBs também coincidia com a indução de apoptose. Desta forma, sugere-se que as DSBs provocadas pelo 5-FU são resultado da indução de apoptose e/ou possíveis falhas durante o processo de reparação de DNA.

Estudos que visam compreender os mecanismos de reparação das lesões de DNA induzidas por agentes antitumorais têm apresentado resultados muito

interessantes, pois o eficiente reparo destes danos pode modular o efeito citotóxico dos agentes antitumorais. Além disso, muitos tipos de câncer apresentam deficiências em vias de reparo, que os tornam mais sensíveis ou resistentes aos antineoplásicos. Logo, a identificação de mutações que conferem aumento de sensibilidade a determinados agentes apresenta-se como uma importante ferramenta para protocolos mais eficazes. Da mesma forma, a caracterização de deficiências que conferem resistência a certas drogas permite que estas combinações possam ser evitadas (HELLEDAY *et al.,* 2008). Baseado nestas observações, inibidores de enzimas de reparo de DNA vêm sendo desenvolvidos com o propósito de potencializar a atividade citotóxica de agentes antitumorais. Os resultados obtidos no Capítulo I sugerem que as AP endonucleases e liases poderiam ser importantes alvos para terapias com fluoronucleotideos.

No Capítulo II foi estudada a participação de modeladores da cromatina e da reparação de DNA na citotoxicidade do 5-FU em *S. cerevisiae*. A estrutura da cromatina é responsável pela regulação de diferentes processos biológicos, como replicação, transcrição e recombinação (BECKER & HÖRZ, 2002; ALTAF *et al.,* 2007). Recentemente tem se proposto que reparação de DNA também é regulada pela estrutura da cromatina, uma vez que controla o acesso das proteínas ao DNA danificado (ATAIAN & KREBS, 2006; ESCARGUEIL *et al.,* 2008). A compactação da cromatina pode ser alterada por remodeladores dependentes de ATP (CR) ou por modificações covalentes pós-traducionais de histonas, tais como acetilação (HATs e HDACs), metilação, fosforilação e ubiquitinização (OSLEY *et al.,* 2007). Primeiramente, a sensibilidade do 5-FU foi avaliada em um amplo

painel de linhagens deficientes em modeladores da cromatina e observou-se que mutantes em Nhp10 (proteína não-histônica relacionada ao grupo de alta mobilidade HMG), Ino80, Swr1 e Arp4 (CR), e Hat1, Esa1 e Gcn5 (HATs) apresentaram alta sensibilidade ao 5-FU (Capítulo ll, Figura 1). Nestas linhagens, o 5-FU foi altamente citotóxico e citostático, e induziu parada de ciclo celular em G2/M (Capítulo ll, Figuras 2 e 5).

Ao investigar duplos mutantes em CR/HAT ou HATs, não foram observadas diferenças na citoxicidade e citostaticidade quando comparados com a linhagem selvagem, o que sugere que outra via possivelmente compense estas deficiências e permita o reparo das lesões induzidas pelo 5-FU, uma vez que estas linhagens não acumulam 5-FU no seu DNA (Capítulo ll, Figuras 3 e 6). Além disso, o efeito mais significativo do 5-FU nestes duplos mutantes foi em relação à distribuição do ciclo celular que foi similar à observada nos simples mutantes sensíveis (G2/M). A participação da variante de histona Htz1 também foi investigada. Estudos reportaram que esta variante de histona é introduzida nos nucleossomos pelo complexo SWR de remodeladores da cromatina dependentes de ATP, e a sua incorporação evita o silenciamento de regiões de eucromatina (RAISNER & MADHANI, 2006; CAMPOS & REINBERG, 2009). A linhagem *htz1*Δ apresentou o mesmo padrão de citotoxicidade, o mesmo efeito citostático e resposta ao ciclo celular que a selvagem (parada em S), mas quando associada a defeitos em Arp4, Esa1 e Hat1, mostrou diferenças na distribuição do ciclo celular (G2/M) (Capítulo ll, Figuras 4 e 5). Os resultados sugerem que embora esta variante de histona pareça ser importante na citotoxicidade do 5-FU, uma vez que o simples mutante *swr1*Δ mostrou-se sensível, linhagens deficientes em HTZ1

(simples e duplos mutantes) não apresentam aumento de morte celular, mas um efeito na progressão do ciclo celular.

Considerando que foi observada uma importante parada de ciclo celular em G2/M nas linhagens deficientes em modeladores da cromatina, fase em que ocorre a reparação de DNA pelas vias HR e PRR, foi avaliado o envolvimento destes modificadores nestas vias de reparo. As deficiências em Ino80, Swr1 e Nhp10 associadas a defeitos em HR (Xrs2 e Rad52) e PRR (Rad6) aumentaram significativamente a sensibilidade ao 5-FU, o que sugere que na ausência destes modeladores, os danos causados pelo 5-FU não estejam sendo adequadamente reparados, o que pode resultar em aumento de morte celular. Enquanto que, defeitos em Gcn5 e Hat1 combinados com deficiências nestas mesmas vias de reparo não potencializaram a sensibilidade ao 5-FU, provavelmente porque ambas as HATs atuam em conjunto com as vias de reparo por HR e PRR. Em resumo, os resultados deste capítulo sugerem que diferentes remodeladores da cromatina dependentes de ATP e HATs podem atuar facilitando o processo de reparação de DSBs.

Estudos recentes demonstraram que muitos modificadores da estrutura da cromatina estão envolvidos nos processos de reparação de DNA (ATAIAN & KREBS, 2006; ALTAF *et al.*, 2007). Durante o reparo de DSBs por HR, a histona H2AX é fosforilada na serina-129 (γH2AX) pelas cinases Mec1 e Tel1 (VAN ATTIKUM & GASSER, 2005b). A γH2AX recruta complexos de modeladores da cromatina como INO80, SWR1 e NuA4, que possui Arp4 e Esa1 como subunidades (VAN ATTIKUM & GASSER, 2005a). O complexo INO80 promove a remoção de nucleossomos para facilitar o processamento de extremidades de

DNA quebradas, o qual permite a conversão de DSBs em extremidades 3’ de fita simples pelo complexo MRX (VAN ATTIKUM *et al.,* 2004), além de controlar a taxa de remoção de RPA pela Rad51 durante a HR (ATAIAN & KREBS, 2006). O complexo SWR1 também é recrutado no reparo de DNA e possui atividade de remodelador da cromatina dependente de ATP, o qual deposita a variante de histona Htz1 em locais específicos na cromatina. Esta variante de histona atua na ativação transcricional e estabilidade do cromossomo, e possivelmente contribui para o relaxamento da cromatina quando incorporado em regiões próximas a quebras de DNA (RAISNER & MADHANI, 2006). As HATs Hat1 e Gcn5 também atuam de maneira a facilitar o reparo de danos no DNA (DOWNS *et al.,* 2004; QIN & PARTHUM, 2006; BAO & SHEN, 2007). A Hat1 também é recrutada a locais de DSBs juntamente com a Rad52, o que sugere que Hat1 possa facilitar o reparo por HR e/ou atuar na restauração da estrutura da cromatina após a HR, uma vez que é responsável pela acetilação de caudas amino-terminais de histonas recém sintetizadas (QIN & PARTHUM, 2006). E a Gcn5 atua na ativação transcricional, possivelmente levando ao relaxamento da cromatina, e sabe-se que atua no reparo por NER de lesões induzidas pela luz UV (YU *et al*., 2005; LEE & WORKMAN, 2007).

Poucos se sabe sobre a participação de modeladores da cromatina no PRR. O complexo INO80 parece atuar no processo de tolerância ao dano, ligando-se às forquilhas de replicação e permitindo o acesso da RAD6 para a desobstrução das mesmas. INO80 também regula a ubiquitinização da PCNA, permitindo o recrutamento da Rad18 (FALBO, 2009).

Uma vez que as alterações epigenéticas também são importantes para a progressão de tumoral e estas podem ser potencialmente revertidas pelo uso de agentes tais como inibidores de metilação ou desacetilases de histonas, o desenvolvimento de drogas capazes de atuar sobre estas alterações podem corrigir vias e restaurar funções celulares que foram afetadas pelo processo de tumorigênese. Desta forma, a compreensão de como as mudanças epigenéticas contribuem para a carcinogênese, assim como a descoberta de agentes capazes de reverter estes efeitos, são imprescindíveis para melhora de terapias clinicas. Além disso, a identificação de alvos que possam conferir aumento de sensibilidade a determinados agentes antitumorais é de suma importância para possíveis aplicações terapêuticas. Os resultados obtidos no Capítulo ll demonstraram que CRs e algumas HATs podem atuar na citotoxicidade do 5-FU juntamente com proteínas das vias de reparação por HR e PRR. Assim, nossos resultados sugerem que estes modificadores da cromatina são alvos importantes que poderiam ser quimicamente modulados, a fim de se potencializar a atividade antitumoral do 5-FU. No entanto, estudos complementares em outros modelos biológicos são necessários para estas confirmações.

Nos Capítulos l e ll a levedura *S. cerevisiae* foi empregada como modelo biológico para o estudo de mecanismos de ação do agente antitumoral 5-FU. Considerando que a levedura é uma eficiente ferramenta na investigação de processos celulares determinantes na manutenção genômica e do desenvolvimento do câncer, tais como a reparação de DNA, regulação de ciclo celular e controles epigenéticos, no Anexo ll propomos uma revisão baseada no uso de diferentes linhagens de leveduras mutantes para estudar fenótipos

observados em cânceres, triagem de novos compostos com potencial atividade antitumoral e investigar interações sintético-letais. O emprego de leveduras no estudo de respostas a agentes antineoplásicos apresenta diversos benefícios como o baixo custo, a facilidade de manipulação e de se construir mutantes, a possibilidade de se realizar grandes “screenings” e a alta taxa de conservação das principais vias de sinalização celular com células humanas. No entanto, por ser um eucarioto inferior, a levedura apresenta algumas limitações, como a baixa permeabilidade a determinados agentes, ausência de algumas enzimas envolvidas na metabolização de drogas, supressão tumoral e apoptose, e impossibilidade de se investigar respostas tecido-específico e aspectos avançados do câncer, como metástases, invasão de tecidos e angiogênese.

No Capítulo lll foi investigada a resposta ao dano no DNA pelas vias ATR/Chk1 e ATM/Chk2 frente a tratamentos com antimetabólitos em células tumorais humanas HeLa, e os efeitos destas drogas combinados ao AZD7762, um inibidor de Chk1/2. ATR e ATM atuam na sinalização celular de lesões no DNA. ATR é ativada principalmente em resposta a bloqueios na forquilha de replicação, enquanto que ATM atua em resposta a DSBs (MCNEELY *et al.*, 2010). Chk1 e Chk2 são “checkpoint” cinases efetoras de ATR e ATM, respectivamente, que possuem papel determinante na resposta celular a danos no DNA (ASHWELL & ZABLUDOFF, 2008). Neste trabalho foram estudados os antimetabólitos 5-FU, FdUMP, raltitrexato (RTX) e gemcitabina, que possuem diferentes mecanismos de ação. 5-FU atua levando a erros de incorporação no DNA e RNA, além de inibir a enzima TS. Tanto o FdUMP quanto o RTX atuam inibindo a TS, porém de maneira diferente, uma vez que ocupam diferentes sítios de ligação com a enzima

e possuem especificidades distintas (VAN CUTSEN *et al.,* 2002*)*. A gemcitabina inibe a enzima ribonucleotídeo redutase (RR), que resulta na depleção dos nucleotídeos, além de levar a erros de incorporação e bloqueio de replicação (MINI *et al.*, 2006).

Nossos resultados mostraram que o 5-FU, o FdUMP, o RTX e a gemcitabina ativaram principalmente a via ATR/Chk1, e apenas a gemcitabina recrutou a via ATM/Chk2 (Capítulo lll, Figuras 1 e 2). A fim de se investigar o efeito da inibição de Chk1/2 combinado aos antimetabólitos, foi empregado o AZD7762, um inibidor competitivo destas "checkpoint" cinases. Ao estudar a atividade dos antimetabólitos associados ao AZD7762, observou-se um aumento na sensibilidade de aproximadamente 2 vezes para o 5-FU, 10 vezes para o FdUMP, 15 vezes para o RTX e 20 vezes para a gemcitabina (Capítulo lll, Figura 3). Estas diferenças de sensibilidade dos antimetabólitos frente à inibição de Chk1/2 pode estar relacionada ao mecanismo de ação de cada droga. O FdUMP, o RTX e a gemcitabina apresentaram elevada citotoxicidade quando associados ao AZD7762, provavelmente porque estas drogas induzem forte bloqueio de replicação. O 5-FU também induz inibição de replicação, porém possui outras atividades como erros de incorporação em ácidos nucléicos relacionados à formação de SSBs e DSBs (MATUO *et al.*, 2010). A inibição da TS leva ao bloqueio das forquilhas de replicação. Tanto o FdUMP quanto o RTX atuam inibindo TS, no entanto, o RTX apresenta um efeito mais pronunciado na sensibilidade em combinação com AZD7762 quando comparado ao FdUMP, possivelmente porque o RTX atua especificamente inibindo a síntese de DNA, enquanto que o FdUMP pode atuar também levando a erros de incorporação no

DNA. A gemcitabina inibe a RR, que depleta o pool de nucleotídeos, e leva a erros de incorporação que contribuem para um eficiente bloqueio da replicação, que podem levar à terminação de cadeia, e que parece ser mais eficiente que a inibição da TS.

Embora nossos resultados tenham mostrado que o 5-FU ativa principalmente a via ATR/Chk1, um estudo conduzido por GENG *et al.* (2011) reportou a participação da via ATM/Chk2 na linhagem de câncer de cólon HT-29. Entretanto, isto pode ser explicado pelas diferenças das condições experimentais: (i) GENG *et al.* (2011) empregou concentrações de 5-FU muito superiores (mais de 100 vezes) que as utilizadas nos experimentos do Capitulo lll, que possivelmente levava a formação de DSBs, e assim recrutava ATM/Chk2; e (ii) diferentes “backgrounds” genéticos entre as linhagens celulares HT-29 e HeLa.

Os tratamentos com antimetabólitos induziram o acúmulo em fase S, enquanto que combinados com AZD7762, aumentaram Sub-G1 e diminuíram G2/M, o que sugere que a inibição de Chk1/2 é capaz de aumentar o potencial de induzir morte celular pelos antimetabólitos (Capítulo lll, Figura 4). Além disso, o AZD7762 aumenta a população em fase S para o FdUMP, RTX e gemcitabina quando comparado com o respectivo tratamento com antimetabólito após 48h de exposição, o que indica que a inibição de Chk1/2 pode acelerar o ciclo celular e contribuir para a ultrapassagem do “checkpoint” de G2/M. A inibição de Chk1/2 pelo AZD7762 também induz mitose prematura nas células tratadas com antimetabólitos, o que resulta em morte celular, como observado pelo aumento do número de células em Sub-G1 (Capítulo lll, Figura 5).

Interessantemente, observou-se que as diferenças na ativação da via ATR/Chk1 (Capítulo lll, Figura 1) apresentavam uma correlação direta com o aumento de sensibilidade conferido pelo inibidor AZD7762 combinado com os antimetabólitos (Capítulo lll, Figura 3). Estes resultados estão de acordo com trabalhos anteriores que mostraram que a sensibilização pelo AZD7762 é mediada principalmente pela inibição de Chk1 ao invés de Chk2 (ASHWELL *et al.,* 2008; MORGAN *et al.,* 2010; GARRETT & COLLINS, 2011). A inibição de Chk1 permite a ativação de origens de replicação que se encontram bloqueadas, indução de mitose prematura e morte celular (XIAO *et al.*, 2005; JANETKA & ASHWELL, 2009; MCNEELY *et al.,* 2010; LAINCHBURY & COLLINS, 2011).

Atualmente, inibidores de “checkpoint” cinases têm sido desenvolvidos com a finalidade de melhorar os protocolos empregados no tratamento do câncer (JANETKA & ASHWELL, 2009; LAINCHBURY & COLLINS, 2011). Estudos reportaram que estes inibidores são capazes de potencializar a atividade citotóxica de alguns agentes antitumorais, tais como irinotecan, gemcitabina, topotecan, cisplatina, docetaxel e radiação ionizante, e alguns encontram-se em fases de estudo clínicos (ASHWELL *et al.*, 2008; ASHWELL & ZABLUDOFF, 2008; ZABLUDOFF *et al.*, 2008, GARRETT & COLLINS, 2011; MA *et al.*, 2011).

Os resultados obtidos nesta tese contribuem para a compreensão de diversos aspectos do mecanismo de ação do agente antitumoral 5-FU, tais como o envolvimento das vias de reparação de DNA, a participação de modeladores da cromatina e a resposta celular via ATR/Chk1 e ATM/Chk2. Embora haja muitos estudos sobre este agente antitumoral, esta tese investigou aspectos inéditos como: (i) a reparação de DNA, utilizando o 5-FU e seu metabólito ativo FdUMP no

modelo biológico *S. cerevisiae*, que permite compreender o efeito dos erros de incorporação gerados pelo 5-FU e a inibição da TS pelo FdUMP de maneira independente; (ii) a participação de modeladores da cromatina na citotoxicidade do 5-FU; (iii) o envolvimento de modeladores da cromatina e reparo de DNA; (iv) a participação das vias de resposta ao dano no DNA e o efeito da inibição de Chk1/2 empregando o inibidor AZD7762. A compreensão de todos estes mecanismos de ação contribui para o desenvolvimento de protocolos que combinam diferentes agentes capazes de potencializar a atividade citotóxica deste agente antineoplásico, cuja finalidade é melhorar a resposta clínica.

Embora os ensaios em *S. cerevisiae* forneçam importantes subsídios para o estudo de mecanismos de ação de agentes antitumorais, estes necessitam ser confirmados empregando modelos murinos e em células humanas antes de serem conduzidos a estudos clínicos.

CONCLUSÕES

1. Conclusão Geral

Os resultados deste trabalho mostraram que as lesões induzidas pelo agente antitumoral 5-FU podem ser removidas por enzimas glicosilases e endonucleases da via de reparação por BER, e caso ocorram erros durante estes processos, podem ser formadas SSBs. Caso estas SSBs não sejam reparadas, elas podem ser convertidas a DSBs, que por sua vez, podem recrutar a via de reparo HR. Os danos provocados pelo 5-FU que persistiram a replicação podem ainda ser reparados pela RAD6/RAD18 da via pós-replicativa ou pelo MMR. Os remodeladores da cromatina dependentes de ATP e algumas acetiltransferases de histonas também participam da citotoxicidade do 5-FU, o que sugere que possam atuar no relaxamento da cromatina, facilitando os processos de reparação por HR e PRR. Além disso, o 5-FU ativa principalmente a via de resposta ao dano no DNA ATR/Chk1. O 5-FU quando combinado ao AZD7762, inibidor de Chk1/2, apresenta aumento de citotoxicidade, leva ao aumento de células em sub-G1 e diminuição de G2/M, e indução de catástrofe mitótica.

1.1. Conclusões Específicas

- As lesões induzidas pelo 5-FU são reparadas principalmente pelas vias BER, MMR, HR e PRR, enquanto que seu metabólito ativo FdUMP recruta apenas as vias BER e MMR;

- Remodeladores da cromatina dependentes de ATP (Ino80, Swr1, Arp4) e algumas acetiltransferases de histonas (Hat1, Gcn5, Esa1) participam da citotoxicidade do 5-FU. Linhagens deficientes nestes modeladores da cromatina apresentam alta sensibilidade, efeito citostático e parada de ciclo celular em G2/M frente à exposição com 5-FU;

- Os modeladores da cromatina Nhp10, Ino80, Swr1, Hat1 e Gcn5 atuam no relaxamento da cromatina em locais contendo lesões induzidas pelo 5-FU e possivelmente facilitam a reparação pelas vias HR (Rad52 e Xrs2) e PRR (Rad6);

- O 5-FU ativa principalmente a via ATR/Chk1 em resposta ao estresse replicativo gerado pela inibição da replicação;

- A inibição de Chk1/2 pelo AZD7762 potencializa a citotoxicidade do 5-FU em duas vezes, aumenta a população em Sub-G1 e diminui G2/M, e aumenta a passagem de células para a mitose.

PERSPECTIVAS

Em função dos resultados obtidos neste trabalho propõem-se as seguintes perspectivas para continuidade deste estudo:

- Avaliar a participação das vias de reparação de DNA das lesões geradas pelo 5-FU em linhagens de células de mamíferos defectivas em genes de reparo;

- Investigar a participação de modeladores da cromatina na via de reparação de DNA por BER de lesões induzidas pelo 5-FU, em *S. cerevisiae,* construindo duplos mutantes;

- Avaliar a proliferação celular por BrdU e a velocidade do ciclo celular das linhagens deficientes em modeladores da cromatina expostas ao 5-FU;

- Analisar o genoma total das linhagens de *S. cerevisiae* deficientes em modeladores da cromatina expostas ao 5-FU através de microarray, a fim de comparar o efeito do reparo de DNA com as mudanças epigenéticas, e poder distinguir a contribuição de vários modificadores da cromatina no reparo;

- Avaliar o tipo de morte celular (apoptose, necrose) e a indução de quebras no DNA (ensaio Cometa e fosforilação da histona H2AX) em resposta aos antimetabólitos 5-FU, FdUMP, RTX e gemcitabina associados ao AZD7762;

- ❖ Avaliar a citotoxicidade dos antimetabólitos combinado com um inibidor específico de Chk1 (PF-477736).

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ANEXOS

ANEXO I

5-Fluorouracil and its active metabolite FdUMP cause DNA damage in human SW620 colon adenocarcinoma cell line

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5-Fluorouracil and its active metabolite FdUMP cause DNA damage in human SW620 colon adenocarcinoma cell line

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ABSTRACT: **5-Fluorouracil (5-FU) is an antineoplasic drug widely used to treat cancer. Its cytotoxic effect has been principally ascribed to the misincorporation of fluoronucleotides into DNA and RNA during their synthesis, and the inhibition of thymidylate synthase (TS) by FdUMP (one of the 5-FU active metabolites), which leads to nucleotide pool imbalance. In the present study, we compared the ability of 5-FU and FdUMP to induce apoptosis and to influence the cell cycle progression in human colon SW620 adenocarcinoma cells in regards to their genotoxic and clastogenic activities. Our study demonstrates that 5-FU induces SSB, DSB and apoptosis earlier than FdUMP. Interestingly, while both drugs are able to induce apoptosis, their effect on the cell cycle progression differed. Indeed, 5-FU induces an arrest in G1/S while FdUMP causes an arrest in G2/M. Independently of the temporal difference in strand breaks and apoptosis induction, as well as the differential cell cycle modulation, both drugs presented similar clastogenic effects. The different pattern of cell cycle arrest suggests that the two drugs induce different types of primary DNA lesions that could lead to the activation of different checkpoints and recruit different DNA repair pathways.**

Keywords: 5-fluorouracil; FdUMP; cytotoxicity; genotoxicity; cell cycle arrest; apoptosis

Introduction

5-Fluorouracil (5-FU) is a prodrug used to treat several malignancies, including colorectal, ovary, breast, head and neck cancers (Rahman *et al*., 2006; Seiple *et al*., 2006). 5-FU differs from uracil by virtue of a fluorine atom in place of hydrogen at the carbon-5 position of the pyrimidine ring. 5-FU enters cells by nonfacilitated diffusion and facilitated nucleobase transport system; meanwhile fluoronucleosides (FdUrd) enter by a facilitated membrane transport mechanism used by purine and pyrimidine nucleosides (Grem, 1997; Meyers *et al*., 2003).

5-FU requires uptake and conversion to its active forms before exerting its cytotoxic effects. This effect has been principally ascribed to misincorporation of fluoronucleotides into (i) DNA and (ii) RNA, and (iii) inhibition of thymidylate syntase (TS) by fluorodeoxyuridine monophosphate (FdUMP). When 5-FU is converted to 5-fluorouridine-5′-triphosphate (FUTP) and 5′-fluoro-2′ deoxyuridine-5′-trifosfato (FdUTP), they can be incorporated into RNA or DNA respectively, and several altered phenomena can be observed in the synthesis and stability of these molecules (Meyers *et al*., 2003). The active metabolite FdUMP forms a stable complex with TS that catalyzes the conversion of dUMP to dTMP, leading to enzyme inhibition which results in nucleotide pool imbalances (Meyers *et al*., 2003; Longley *et al*., 2004; Fisher *et al*., 2007).

Two of the three mechanisms involved in 5-FU cytotoxicity act at the level of the DNA: misincorporation of fluoronucleotides and inhibition of TS. Consequently, 5-FU directly interferes with DNA through: (i) direct incorporation of deoxyribonucleotide analogue FdUTP into DNA; (ii) incorporation of dUTP into DNA as a consequence of TS inhibition; (iii) enhanced misincorporation of deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) due to decreased fidelity of DNA polymerase in response to dNTP pool imbalances; (iv) inhibition of DNA synthesis as consequence of DNA polymerase stalling; and (v) interference with DNA repair enzymes. Important consequences of such events are the

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potential mutagenic effects of deoxyribonucleotides misincorporation and the appearance of DNA breaks in the cell's attempts to repair these lesions (Meyers *et al*., 2003). However, since both the disruption of dNTP pools, as a consequence of TS inhibition, and the direct incorporation of fluoropyrimidine (FP) into DNA occur concomitantly, it is difficult to distinguish the relative contribution of both mechanisms in the cytotoxic activity of 5-FU.

FdUMP has been suggested to be the active metabolite of 5-FU responsible for specifically inhibiting TS. This inhibition results in nucleotide pool imbalance that increases the concentration of dUTP and consequently induces uracil misincorporation into DNA. This results in a futile DNA repair cycle in which uracil is excised. However, as the cell tries to repair the excised base, more dUTP is incorporated (Welsh *et al*., 2003). Additional phosphorylation of FdUMP to its 5′-triphosphate counterpart, FdUTP, can also result in the incorporation of this nucleotide into DNA (Shewach and Lawrence, 2007). Finally, DNA replication can be inhibited in response to the dNTP pool imbalance, which could result in cell death by apoptosis (Meyers *et al*., 2003; Noordhuis *et al*., 2004; Li *et al*., 2005).

Many studies have been conducted to better understand the mechanism of action of 5-FU concerning the interference with cell signaling and apoptosis induction (Longley *et al*., 2004; Grivicich *et al*., 2005), alterations in cell cycle progression (Pizzorno *et al*., 1995), DNA repair pathways involved in processing 5-FU lesions (Carethers *et al*., 1999; Seiple *et al*., 2006; Fisher *et al*., 2007), genotoxicity and clastogenicity (Oliver *et al*., 2006). The clastogenic activity of 5-FU was also observed in human lymphoblastoid WTK-1 cells (Oka *et al*., 2006) and human lymphocytes (Lorge *et al*., 2006). Moreover, Tokunaga *et al*. (2000) evidenced the induction of single-stranded DNA regions after treatment with 5-FU in human colon cells using Klenow/3′-exonuclease enzyme and related this to delays in S phase progression. Other reports showed that the inhibition of TS by different compounds, such as FdUMP[10] (Liao *et al*., 2005), CB3717 (Curtin *et al*., 1991), ZD9331 and raltitrexed (Webley *et al*., 2000; Webley *et al*., 2001; Welsh *et al*., 2003), causes DNA single-strand breaks detected by comet assay and alkaline elution. However, the precise mechanism of FdUMP cytotoxicity is still not completely understood. Thus, the aim of our work was to compare the ability of 5-FU and FdUMP to induce apoptosis and to influence the cell cycle progression in human colon SW620 adenocarcinoma cell line with regards to their genotoxic and clastogenic activities.

Materials and Methods

Chemicals

5-Fluorouracil was purchased from ICN Pharmaceutics. 5-fluoro-2′-deoxyuridine-5′-monophosphate, methyl methane sulfonate (MMS), trichloroacetic acid (TCA), trizma base and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 culture medium, fetal calf serum, trypsin-EDTA and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Agarose and low-melting point agarose were obtained from Invitrogen (Carlsbad, CA, USA). Giemsa stain was purchased from Merck (Darmstadt, Germany). BCA protein assay reagent (Pierce) and caspase 3/7 substrate (Peptide Institute) were kindly provided by Dr Fabiana Horn from the Biophysics Department, Federal University of Rio Grande do Sul State. Primary and secondary antibodies for γH2AX (Upstate Biotecnology and Jackson Immuno Research) were supplied from the Group of Molecular and Clinical Cancer Therapeutics, Hôpital Saint-Antoine, Paris.

Cell Line and Culture

SW620 human colon cancer cell line was acquired from American Type Culture Collection, Rockville, MD, USA. Cells were cultured in complete medium consisting of RPMI 1640 containing 10% (v/v) fetal calf serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

Cell Viability Assay

For the cell viability assay, 2.5×10^4 cells per well were seeded onto 96 well plates 24 h before drug treatment. After this, the cells were treated with various concentrations of 5-FU and FdUMP for 24 h. Cytotoxicity was assessed by means of the sulforhodamine B (SRB) assay described previous by (Skehan *et al*., 1990), involving *in situ* fixation with TCA, staining with SRB and solubilization of cell-bound SRB with Trizma base. The latter was colorimetrically assessed with microplate reader at a wavelength of 540 nm. Three independent experiments were performed for each dose. The 5-FU and FdUMP concentrations causing a 50% growth inhibition (IC_{50}) compared with the controls were calculated from a semilogarithmic dose–response curve by linear interpolation. 5-FU and FdUMP concentrations employed in apoptosis, cell cycle, comet, H2AX phosphorylation and micronucleus assay were based on IC_{50} values in order to compare the action of both drugs in the same toxic concentrations.

Apoptosis Analysis

Apoptosis was evaluated by enzymatic caspase 3/7 assay and morphological analysis. For both assays, 5×10^5 cells were treated with 15.4 μM of 5-FU and 12.3 μM of FdUMP for 3, 6, 12, 24 and 48 h. Protocols for conducting the apoptosis analysis by measure the caspase 3/7 activity were described previous by (Bastiani *et al*., 2005). The culture medium was removed after treatments, cells were washed with PBS and lysed with Triton X-100 0.2% in PBS for 10 min on ice. After centrifugation at 12 000 rpm for 10 min, protein concentration in cell extracts was estimated using BCA Protein Assay Reagent. A 40 μg aliquot of protein was incubated with 20 μM caspase synthetic substrates in 100 mM HEPES-NaOH, pH 7.5, 10% sucrose, 0.1% CHAPS, 0.1 mg ml^{-1} BSA and 10 mM DDT. The substrate tested was Ac–Asp–Glu–Val–Asp–MCA. Substrate hydrolysis was monitored for 1.5 h at 370 nm excitation/460 nm emission in a microplate fluorescence reader; substrate hydrolysis was quantified by comparison with a standard curve for MCA substrates. Experiments were performed in triplicate. Morphological analysis was based on Amarante-Mendes *et al*. (1998). After treatment, cells were harvested, centrifuged and ressuspended in 20 μl of PBS buffer. A 2 μl aliquot of acridine orange (100 μg ml^{-1}) and ethidium bromide (100 μg ml^{-1}) were added in PBS buffer. Cells were analyzed in fluorescence microscope with 60 × objective.

Cell Cycle Analysis

For cell cycle evaluation, 1×10^6 cells were treated with 15.4 μM of 5-FU and 12.3 μM of FdUMP for 6, 24 and 48 h. After treatments,

cells were harvested and fixed in ethanol 70% overnight. The samples were washed in PBS, resuspended in 0.5 ml PBS and incubated with RNAse A 100 μg ml^{-1} and propidium iodide 50 μg ml^{-1} for 20 min in the dark at room temperature, based on Skladanowski *et al*. (2005). Data from 15,000 cells were collected by flow cytometry in a FACS Calibur (Becton Dickinson) equipped with an argon laser 488 nm and analyzed by WinMDI Software. Three independent experiments were performed for each concentration.

Comet Assay

For genotoxicity assays, 1.0×10^6 cells were seeded on six-well plates and incubated for 24 h. Treatments were performed with concentrations based on IC_{50} defined by the cell viability assay; MMS 4.0×10^{-5} M was used as positive control. Three different treatment times were employed: 3, 6 and 12 h. Each dose was evaluated by three independent experiments.

Protocols of conducing comet assay were based on Tice *et al*. (2000). Cells were harvested with trypsin, centrifuged at 1000 rpm for 5 min and re-suspended in medium. Slides were prepared with 20 μl of cellular suspension and 120 μl of low-melting point agarose at 37 °C. Slides were immersed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) for 1 h. Slides were placed in an electrophoresis chamber with cold alkaline buffer (300 mM NaOH and 1 mM de EDTA, pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was performed at 25 V and 300 mA for 20 min. Slides were then neutralized with Tris–HCl buffer (pH 7.5) for 15 min and stored at room temperature.

Slides were stained with silver and analyzed in 400 × magnification using an optical microscope. International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis (Burlinson *et al*., 2007). Migration of DNA fragments was determined according to comet class as described by (Collins, 2004): class 0, intact nuclei, without tail; class 1, nuclei with tail less than the diameter of the nucleus; class 2, tail size varying between one and two times the diameter of the nucleus; class 3, tail size varying between two and three times the diameter of the nucleus; class 4, tail size more than three times the diameter of the nucleus but with the head and tail of the comet still distinguishable.

γH2AX Evaluation

In order to investigate if 5-FU and FdUMP induce DSBs, we performed the γH2AX evaluation. The variant histone H2AX is known to be phosphorylated on carboxyl-terminal tail at position ser139 in mammalian cells, following the introduction of double-strand breaks (DSBs) in DNA genome (Harvey *et al*., 2005). Aliquots of 1×10^6 cells were treated with 5-FU and FdUMP for 3, 6 and 12 h. MMS was employed as positive control. After treatment, cells were harvested with trypsin and fixed in ethanol 70%. Cells were washed with cold PBS and incubated with primary mouse antibody directed against γH2AX (1 : 100 dilution) for 2 h. The secondary antibody employed was Cy5-conjugated donkey anti-mouse IgG (1:100 dilution) for 1 h. The fluorescence emitted by Cy5 was measured by flow cytometry. Experiments were performed in triplicate.

Micronucleus Assay

For the clastogenicity assay, 5×10^5 cells were seeded on six-well plates and incubated for 24 h. Treatments were performed for 48 h. Procedures of harvesting and fixing cells were based on Oliver *et al*. (2006). Cells were harvested with trypsin, hypotonized with sodium citrate 1% and fixed with methanol acetic acid (3 : 1) prior to transferring to slides.

For micronucleus counts, cells were Giemsa-stained and analyzed in 1000 × magnification using optical microscope. A total of 6000 cells were examined per treatment.

Statistical Analysis

Dose–response curves from cell viability assay were plotted and IC_{50} values (50% inhibitory concentration) were estimated by regression analysis. Results of the comet assay, morphological apoptosis and the micronucleus test were evaluated by statistical tests analysis of variance — one-way ANOVA, to compare DNA damage between treatments within groups (3, 6 and 12 h). Bonferroni's *post-hoc* test was used to compare treatment groups.

Results

Cytotoxicity of 5-FU and FdUMP Towards Human SW620 Adenocarcinoma Cell Line

The cell growth inhibition activity of both compounds was assessed by the SRB assay. Figure 1 shows that FdUMP was more potent than 5-FU at low concentrations. However, at higher concentrations (above 25 μM), both drugs showed a similar pattern of cell growth inhibition.

Apoptosis Induction

To determine if the cell growth inhibition activity of both compounds was correlated with the induction of apoptosis, we measured the caspase 3/7 activation (Wang *et al*., 2005; Werner and Steinfelder, 2008) and further performed a morphological analysis (McGahon *et al*., 1995; Amarante-Mendes *et al*., 1998) after different treatment times. Our results show that both drugs induce apoptosis (Fig. 2 and Table 1). However, 5-FU acted more quickly than FdUMP. Indeed, apoptosis induction was observed after only 12 h of treatment with 5-FU, while FdUMP needed at

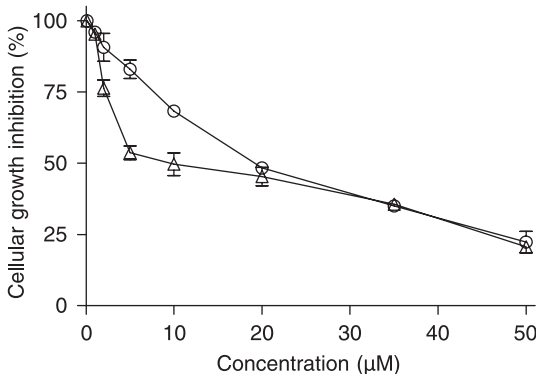

Figure 1. Cytotoxicity of 5-FU and FdUMP in SW620 human colon adenocarcinoma cell line employing the sulforhodamine B assay. The percentage of cellular growth inhibition after treatment with different concentrations of (○) 5-FU and (△) FdUMP.

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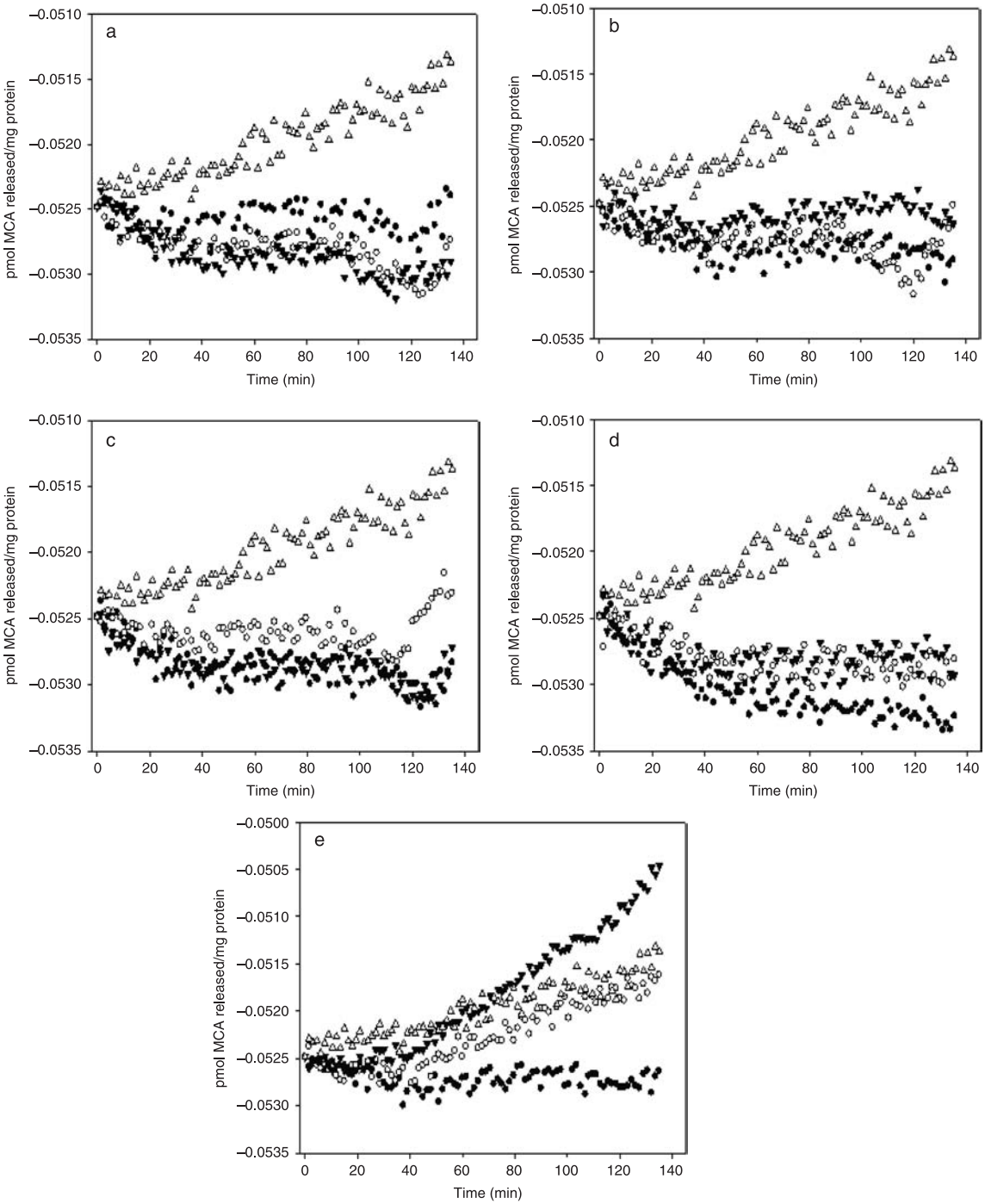

Figure 2. Apoptosis evalution by caspase 3/7 activation in SW620 treated with 5-FU and FdUMP. The quantity of pMol MCA released per mg of protein after treatment with: (●) negative control; (○) 5-FU, 15.40 μM; (▼) FdUMP, 12.30 μM; and (Δ) positive control; culture medium with serum starvation for 72 h. Treatments were performed for: (a) 3 h; (b) 6 h; (c) 12 h; (d) 24 h; and (e) 48 h.

least 24 h to induce apoptosis at a similar level to 5-FU (Table 1 and Fig. 2).

Cell Cycle Analysis

To determine if both drugs mediated cell death through an identical process, we determined their effect on the cell cycle progression (Fig. 3). Surprisingly, our flow cytometry data revealed pronounced differences between cells treated with 5-FU or FdUMP. Indeed, while 5-FU induced a strong G1/S cell cycle arrest after 24 h of treatment [Fig. 3(b)], FdUMP treatment led to a G2/M arrest, suggesting that both drugs induce lesions that affect differentially the cell cycle checkpoints [Fig. 3(c)]. The cell cycle distribution of non-treated cells is presented in Fig. 3(a).

Table 1. Apoptosis evaluation of 5-FU and FdUMP in SW620 cells by morphological assay

Substances	Dose (μM)	Apoptosis (%)[a]/treatment time				
		3 h[b]	6 h	12 h	24 h	48 h
5-FU	0	0.75 ± 0.35	0.50 ± 0.02	1.25 ± 0.33	1.50 ± 0.03	1.50 ± 0.70
	15.40	0.35 ± 0.30	1.25 ± 0.35	4.50 ± 0.72*	5.25 ± 0.35*	6.00 ± 0.03*
FdUMP	0	0.75 ± 0.35	0.50 ± 0.02	1.25 ± 0.33	1.50 ± 0.03	1.50 ± 0.70
	12.30	1.50 ± 0.70	1.06 ± 0.71	1.50 ± 0.70	5.75 ± 1.00*	6.75 ± 1.06*

[a]Mean values and standard deviation obtained from average of 200 cells per experiment — total of three experiments for each substance.
[b]SW620 cells were exposed to different times of treatment with 5-FU and FdUMP.
*Significance: *$P < 0.05$; (ANOVA, Bonferroni's *post-hoc* test).

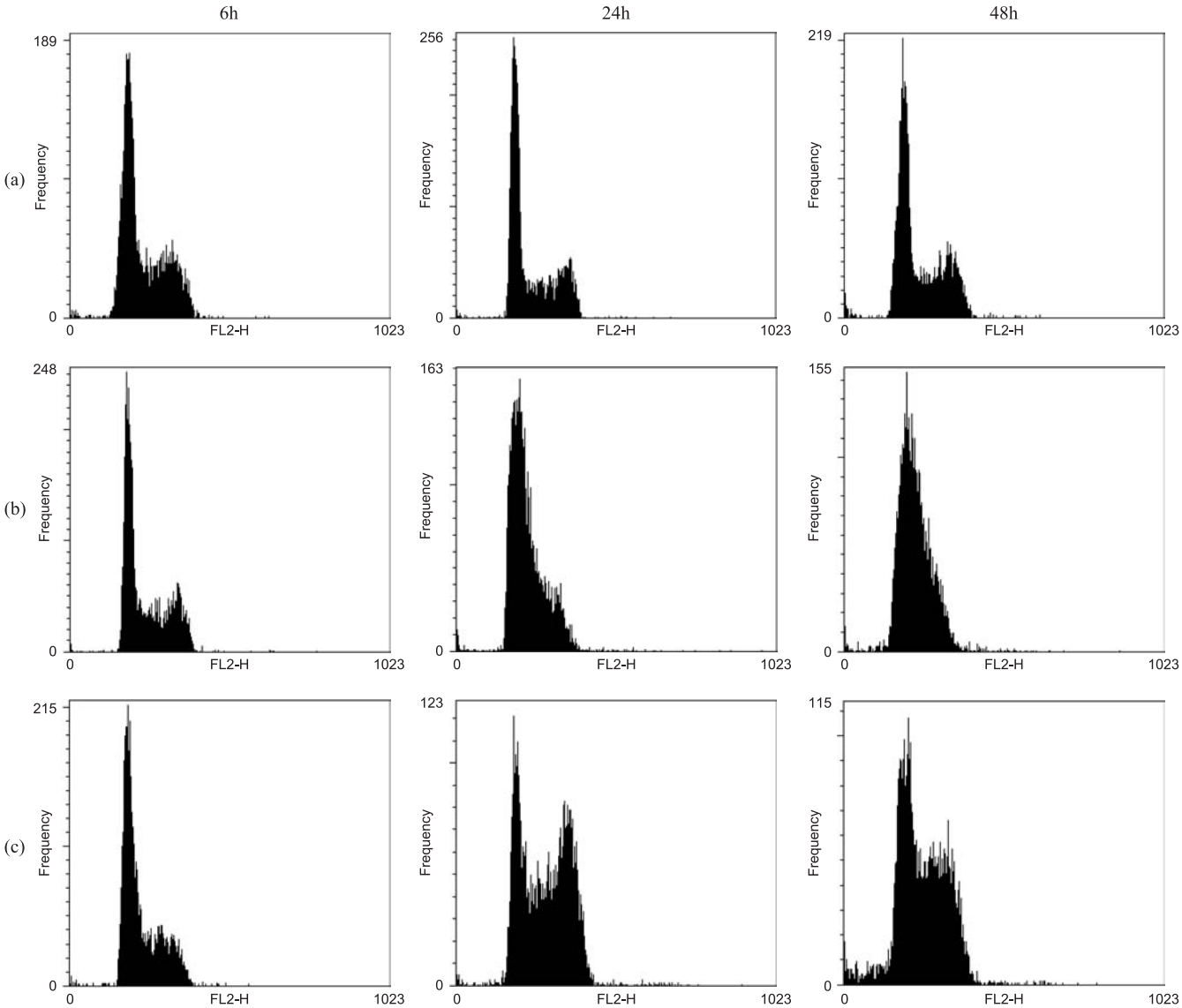

Figure 3. Cell cycle progression in human colon adenocarcinoma cells evaluated by flow cytometry after treatments with 5-FU and FdUMP. Treatments were performed during 6, 24 and 48 h. Lane (a), negative control; lane (b), 5-FU 15.4 μM; and lane (c), FdUMP 12.30 μM.

Single Cell Electrophoresis Gel/Comet Assay and γH2AX Evaluation

To determine whether the differential effects of both compounds on the cell cycle progression could result from different types of DNA lesions, we used the alkaline version of the comet assay to detect DNA strand breaks, induced by either 5-FU or FdUMP. This assay allows the detection of a broad spectrum of DNA damage including double- and single-strand breaks, alkali-labile sites that are expressed as single-strand breaks, and DNA–DNA and

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Table 2. Genotoxicity of 5-FU and FdUMP in SW620 employing alkaline comet assay

Substances	Dose (μM)	Treatment time and comet assay parameters		
		3 h[a]	6 h	12 h
		Damage index[b] (DI)		
5-FU	0	5.75 ± 3.2	5.00 ± 4.2	5.00 ± 0.8
	3.85	37.25 ± 5.6*	49.00 ± 3.7**	69.75 ± 14.38***
	7.70	31.00 ± 8.4	71.25 ± 9.5***	98.25 ± 20.5***
	15.40	48.25 ± 12.6**	79.00 ± 9.2***	94.00 ± 13.1***
	30.80	38.00 ± 14.4*	73.75 ± 14.3**	81.75 ± 9.97***
FdUMP	0	7.50 ± 0.9	8.00 ± 1.8	7.25 ± 2.9
	3.07	6.50 ± 3.7	31.25 ± 23.2	64.75 ± 7.8***
	6.15	2.00 ± 0.8	34.50 ± 12.6	77.75 ± 6.7***
	12.30	4.25 ± 3.7	32.50 ± 14.8	87.75 ± 3.8***
	24.60	2.00 ± 3.36	36.50 ± 16.5*	93.00 ± 3.9***
MMS[c]	40	334.75 ± 42.5***	341.50 ± 45.1***	386.00 ± 5.8***

[a] SW620 cells were exposure to different times of treatment with 5-FU and FdUMP.
[b]Mean values and standard deviation obtained from average of 100 cells per experimental — total of three experiments for each substance.
[c]MMS was employed as positive control.
Significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (ANOVA, Bonferroni's *post-hoc* test).

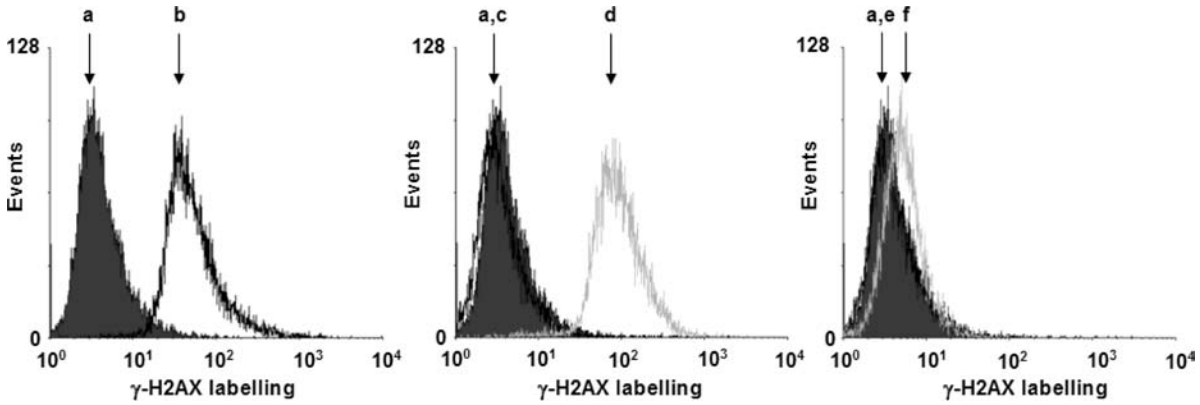

Figure 4. Time course of γH2AX fluorescence after 5-FU and FdUMP treatment in SW620 cells. Legend for figure: (a) untreated cells; (b) positive control employing MMS (40 μM); (c) 5-FU, 15.40 μM for 3 h; (d) 5-FU, 15.40 μM for 12 h; (e) FdUMP, 12.30 μM for 3 h; (f) FdUMP, 12.30 μM for 12 h.

DNA–protein crosslinking (Burlinson *et al*., 2007). Table 2 presents the genotoxicity of 5-FU and FdUMP by alkaline comet assay. Cells were scored by visual analysis as described by (Collins, 2004). Our data showed that both 5-FU and FdUMP induced time-dependent DNA damages. The most significant changes were observed after 6 h for 5-FU and 12 h for FdUMP.

To further characterize the type of damages that were generated by either 5-FU or FdUMP, we measured, by flow cytometry, the appearance of the γH2AX phosphoepitope. This phosphorylated form of the histone H2AX (γH2AX) is indeed considered as a specific marker for the detection of DNA double-strand breaks (Soares *et al*., 2007). Our data clearly show that only the 5-FU was able to induce a strong γH2AX labeling. However, this phenomenon was only observed after 12 h of treatment (Fig. 4), time that corresponds to the induction of the apoptosis (Fig. 2). Interestingly, at the same time point, FdUMP induced a much slighter γH2AX labeling, which is in agreement with a later induction of the apoptosis. At drug concentrations lower than the IC50, no γH2AX labeling could be detected for both compounds (data not shown), while damages could be observed by alkaline comet assay (Table 1). Thus, it is likely that the formation of DNA double-strand breaks is not due to a direct effect of either 5-FU or FdUMP, but corresponds to DNA repair and/or induction of the programmed cell death.

Micronucleus Assay

It has been shown that the deoxyribonucleotides misincorporation induced by exposure to drugs such as 5-FU can result in potent mutagenic activity if not repaired efficiently (Meyers *et al*., 2003). Thus, to determine if the DNA lesions induced by either 5-FU or FdUMP are efficiently repaired, we performed the micronucleus assay in both cases, since the presence of micronuclei is an indicator of chromosomal damage that persisted after DNA replication. Cells were treated for 48 h, to allow them to divide twice during the drug exposure time (Matsushima *et al*., 1999). The results, shown in Fig. 5, revealed a clear dose-dependent clastogenic effect of both 5-FU and FdUMP. Interestingly, while at high

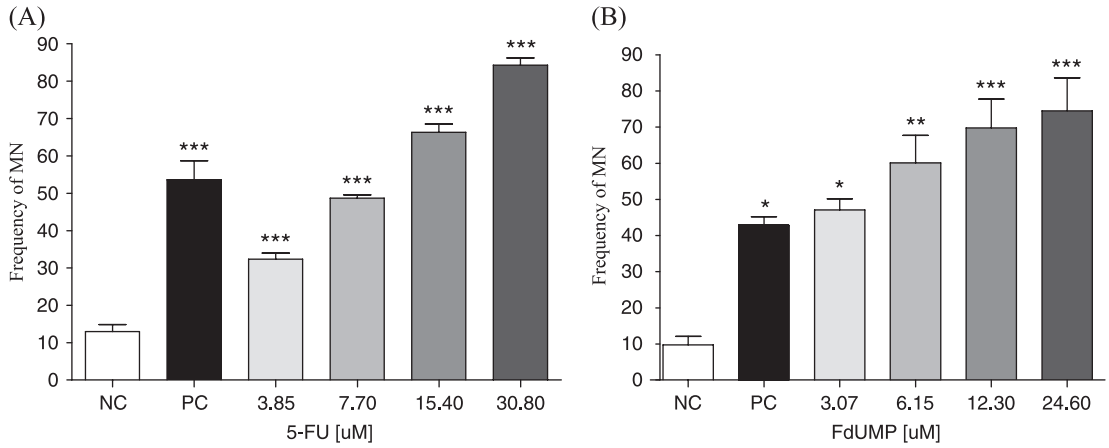

Figure 5. Frequency of micronucleus in treatment with different concentrations of 5-FU (A) and FdUMP (B) in human colon adenocarcinoma cells. NC, negative control (culture medium); PC, positive control employing MMS (4×10^{-5} M). The symbols represent: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, as tested by one-way ANOVA (Bonferroni's *post-hoc* test): MMS, 5-FU and FdUMP compared with NC.

drug concentrations, the effects of both compounds appear to be identical, the clastogenic activity of FdUMP seems to be stronger at low drug concentration. Together, these data strongly suggest that both drugs are able to induce DNA lesions that are poorly repaired, leading to a higher mutation rate.

Discussion

The pro-drug 5-FU has been largely employed in the clinic for over 40 years (Grem, 1997; Rahman *et al*., 2006); however, many aspects of its mechanisms in cell biology were not completely understood. Although one of the mechanisms of 5-FU action was attributed to thymidylate synthase inhibition by its active metabolite FdUMP, the activity profile of 5-FU was shown to differ from those of FdUMP, FdUMP[10] (10mer of FdUMP) and FdUrd (Liao *et al*., 2005). Thus, to clarify possible differences in FdUMP and 5-FU cytotoxic effects, we compared how both compounds were able to affect the cell cycle progression and apoptosis induction, as well as their genotoxic and clastogenic potential in SW620 human colon carcinoma cell line (SW620 cell line).

Our data demonstrated that FdUMP is more toxic than 5-FU at low drug concentrations in SW620 cell line (Fig. 1). However, 5-FU seems to act more quickly than FdUMP especially to induce apoptosis (Table 1 and Fig. 2). Interestingly, the relatively low apoptotic response observed after 24 h suggests a strong cell growth inhibition rather than a cell death induction. This hypothesis is corroborated by the cell cycle analysis that shows a strong cell cycle block induced at 24 and 48 h. However, both compounds influenced differently the cell cycle progression. Indeed, 5-FU induced a G1/S arrest [Fig. 3(b)], while FdUMP led to a G2/M arrest [Fig. 3(c)]. This different pattern of cell cycle arrest suggests that the two drugs induce different types of primary DNA lesions, leading to the activation of different checkpoints and to the recruitment of different DNA repair pathways. To characterize the type of damage, we thus performed alkaline comet assay (Table 2) and γH2AX labeling (Fig. 4). In both cases, 5-FU acts faster than FdUMP. The formation of γH2AX-foci after 5-FU treatment did not seem to be a direct effect of the drug since it appeared concomitantly with the S-phase arrest and could reflect collapsed replication forks (Berger *et al*., 2008). DSB, detected after 12 h treatment with 5-FU, could also result from apoptosis induction, which is observed earlier for 5-FU (12 h) than for FdUMP (24 h). To determine if the damages visualized by the comet assay were prone to be repaired, we performed the micronucleus assay. In both cases, the frequency of micronucleus increased in a dose–response manner suggesting that the induced DNA lesions persisted after replication (Fig. 5). The clastogenic effect of 5-FU could be related to different mechanisms: (i) fluoropyrimidines and uracil incorporated into DNA would be recognized by repair machinery and the failure of repair could result in strand breaks (Seiple *et al*., 2006; Luo *et al*., 2008); (ii) the enzyme Top1 could be trapped by uracil and fluoropyrimidines misincorporations, abasic sites and nicks, resulting in DNA strand breaks (for review, see Liao *et al*., 2005); (iii) the nucleotide pool imbalance as a result of TS inhibition and accumulation of BER intermediates could result in collapsed replication forks (Berger *et al*., 2008).

The DNA-directed toxicity of 5-FU causes an immediate growth inhibition and arrest of the cells in S-phase, whereas the RNA-directed toxicity of 5-FU blocks the cells in G1 (Maybaum *et al*., 1980; Pizzorno *et al*., 1995). It has been shown that after exposure to 5-FU, dUMP increased approximately 100-fold within 3 h in human and murine tumor cells (reviewed in Berger *et al*., 2008; Meyers *et al*., 2003). In the context of this elevation of dUMP (and dUTP) and dTTP depletion caused by TS inhibition, an increased incorporation of dUTP in DNA occurs, as the latter is good substrate for the DNA polymerases. Genomic uracil is a substrate for uracil DNA glycosylases (UDG), but the resynthesis step of BER becomes problematic because of the dTTP depletion, thus, leading to accumulation of BER strand break intermediates that are clastogenic and more toxic than the initial damaged base. DNA double-strand breaks generated from persistent intermediates and/or collapsed replication forks are processed by homologous recombination (HR) activating the S-phase checkpoint (reviewed in Berger *et al*., 2008). If one supposes that, in the first hours of 5-FU treatment, dUTP is preferentially incorporated into DNA, this could trigger the activation of BER and HR, resulting in S-phase arrest. On the other hand, a greater extent of FdUTP incorporation into DNA after FdUMP treatment could fail to recruit BER, and thus escape the S-phase checkpoint, as the affinity of human UDG for 5-FU moieties in DNA is 17-fold lower than for uracil (reviewed in Meyers *et al*., 2003). The lesions that remained unrepaired can be further substrates for BER and MMR during G2. Interestingly, it has recently been proposed that MMR

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specifically detects FU:G in the first round of DNA replication, signaling a sustained G2 arrest and lethality (Meyers *et al*., 2003, 2005). Thus, we can suggest that the lesions induced by FdUMP in our test system could be primarily processed by this repair pathway, leading to G2 arrest.

Our results demonstrate that 5-FU induces SSB, DSB and apoptosis earlier than FdUMP. Moreover 5-FU treatment leads to S-phase cell cycle arrest in SW620 human colon adenocarcinoma cells, while FdUMP causes G2 arrest. Independently of the temporal difference in strand breaks and apoptosis induction, as well as the differential cell cycle modulation, both drugs presented similar clastogenic effects. Thus, we propose that the distinct mechanism of toxicity of both drugs cannot be correlated to their clastogenic potential but, instead, could reflect the induction of different types of primary lesions that trigger different checkpoints and recruit different DNA repair pathways.

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ANEXO II

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

Sumetido à "Cancer Chemotherapy and Pharmacology"

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 for the improvement of standard protocols and the creation of 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 drug sensitivity or resistance. The budding yeast *Saccharomyces cerevisiae* presents conserved cellular processes with high homology to humans and it is a rapid, inexpensive 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 the important determinants in genomic maintenance and cancer development such as DNA repair, cell cycle control and epigenetics, this review proposes 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 the yeast model are 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 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 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 that is 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 genomic maintenance systems including DNA repair, cell cycle and epigenetic control.

Because 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. In this way, isogenic model systems have been contributing to the generation of basic biological knowledge as well as medicine applications. These models allow 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 in the investigation of the 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 the 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 a small genome (approximately 200 times smaller than human), fast doubling time (approximately 2 h), easy manipulation, inexpensive cultivation and the potential to monitor cell cycle progression by cellular and nuclear morphology. In addition, yeast exists in a haploid or diploid state during its life cycle, which allows the investigation of recessive mutations that can be masked in the diploid state by the wild-type allele (PEREGO *et al.,* 2000). Finally, yeast growth can be controlled by adjusting the environmental conditions and it is an organism that is amenable to modification such as 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 reveal important aspects of diseases such as Type 2 diabetes, 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, such as chromosome instability, aneuploidy and genes involved in cancer susceptibility (STROME & PLON, 2010; STIRLING *et al.,* 2011). Phenotypic screening based on marker stability provides a unique approach to identify genes that act to preserve genomic structures such as the 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 the production of human medicines such as insulin, hydrocortisone, artemisinin, hirudin and vaccines employed in hepatitis B and cancer prevention (ARDIANI *et al.,* 2010). However, despite all of 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 the important aspects of cancer development, including 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 by carcinogenesis may provide a window of therapeutic advantage designated as synthetic lethality. First discovered and described in yeast, synthetic lethality principles have encouraged the development of therapeutics that specifically kills 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 synthetic inhibition, which is induced by the anticancer drug, with preexisting oncogenic mutations, which will selectively kill the tumor cells. The final observed result is an increase of the therapeutic index and a reduction of the side effects. However, there are still only a few synthetic lethal interactions described and exploited in anticancer treatments, which signal for the urgent necessity to identify new lethal interactions.

Yeast strain panels carrying specific mutations have been employed in large-scale drug screenings for the identification and characterization of new compounds with mutant-selective cytotoxicity (SPRADLING *et al.,* 2006). The Seattle Project conducted by the 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 potential therapeutic agents for tumors that are defective in post-replication repair (PRR) and DSB repair, respectively (HARTWELL *et al.*, 1997;

BROOMFIELD *et al.,* 2001; FOX, 2004; MOORE *et al.,* 2009). Furthermore, 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, it is possible to use yeast models 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 have shown that bleomycin cytotoxicity may be altered by intercalating agents such as 9-aminoacridine, which can potentialize its activity, or heat shock treatment which induces a resistance to potentially lethal and mutagenic effects (KESZENMAN *et al.,* 2000; KESZENMAN *et al.,* 2005; HOFFMANN *et al.,* 2011). Furthermore, mutant yeast strains may be employed to study specific genetic pathways in carcinogenesis. Although there are combinations of double, triple, or 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 of the 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

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 injury, cells have evolved several DNA repair systems, each one responsible for the repair of specific subsets of DNA damage. However, this creates a dilemma for incipient tumor cells because mutations in the DNA repair proteins are needed to generate the variability in cancer cells and select cells with physiological advantages (HANAHAN & WEINBERG, 2000; HANAHAN & WEINBERG, 2011). Although DNA repair disabling provides an advantage to incipient tumors, it also may simultaneously represent an opportunity to differentiate them from normal cells. Therefore, targeting DNA repair systems is a promising field of anticancer therapy that is only beginning to be intelligently explored.

The budding yeast *S. cerevisiae* DNA repair systems are remarkable well understood and are similar to the 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). A 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 cancer types are characterized by the high incidence of specific DNA repair deficiencies. This is especially the case for hereditary non-polyposis colorectal cancer, which presents high frequency spontaneous mutations as microsatellite instability, which results 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 the Werner syndrome protein (WRN), which is one of five families of human RecQ helicases that have been implicated in genomic stability maintenance, presents premature aging and increased cancer susceptibility (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 an AP endonuclease, is involved in glioma and melanoma pathogenesis (MOHAMMED *et al.,* 2011). In contrast, breast and ovary cancers frequently present deficiencies in *BRCA1* and *BRCA2,* both of which are involved in the HR pathway (HELLEDAY *et al.,* 2008; SMITH *et al.,* 2010).

The primary component of anticancer therapies based on DNA damage induction presents a low therapeutic index and a variety of collateral effects, which result from the low selectivity of these agents. The majority of cancer cells carrying DNA repair deficiencies present increased sensitivity to DNA damage based on these treatments. A recent reported synthetic lethal approach has been

demonstrated that explores DNA repair deficiencies in a more selective manner. The best studied 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 damage in cells where PARP activity is inhibited. This may cause lesions to be converted into harmful DSBs. Normal cells, without DSB repair defects, are able to correctly repair the DSBs that result from PARP inhibition. However, in tumor cells with *BRCA1* or *BRCA2* mutations, the accumulation of non-repaired DSBs results in high and selective cytotoxicity (CARDEN *et al.,* 2010). Therefore, therapies based on DSB induction for breast and ovary cancers deficiencies in HR genes are also promising strategies (HELLEDAY *et al.,* 2008; SMITH *et al.,* 2010). Because *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 a powerful tool in the investigation of the role of DNA repair in lesions induced by antineoplastic drugs. SIMON *et al.* (2000) characterized the DNA damage and repair profiles of several antineoplastic agents against a panel of isogenic yeast strains, each of which were defective at a specific checkpoint in a DNA damage repair or cell cycle signaling pathway. The identification of drugs that are selectively toxic to one specific pathway contributes towards future clinical approaches because it would be conducive to select patients that are most responsive towards the treatment with a specific agent. Furthermore, large-scale chemical screenings are feasible in a yeast model for the discovery of new toxic compounds (CANAANI, 2009). DUNSTAN *et al.* (2002)

employed yeast *S. cerevisiae*-based assays to identify anticancer agents that were selectively cytotoxic to cells with defined mutations. From 85,000 compounds, they identified 126 compounds that were selectively toxic to yeast cells defective in DSB repair (*rad50* and *rad52*). Of the 126 compounds, 87 of these were structurally related to known topoisomerase poisons and 39 were not. Among these 39 agents, they had characterized 8 compounds: two of which were identified as novel topoisomerase ll poisons that were equipotent to etoposide, five exhibited topoisomerase l-dependent toxicity and one directly bound to DNA and induced strand breaks in both yeast and mammalian cells.

Studies using different mutants from the same DNA repair pathway enabled a better understanding of the relative contributions of each protein in the DNA damage process. 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, a deletion of *APN1*, the major abasic site (AP) endonuclease in *S. cerevisiae*, results in a strong sensitivity. This suggests that the AP sites are the major progenitors that give rise to the DNA-mediated toxic effects of 5-FU. Considering that the accumulation of AP sites are a potent target for anticancer chemotherapy, some studies have proposed the use of *APE1* inhibitors (human AP endonucleases) in association with agents that induce base damage and can be repaired via BER, to potentiate the 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 provide a promising strategy for cancer treatment (ABBOTTS & MADHUSUDAN, 2010; BAPAT *et al.,* 2010; MOHAMMED *et al.,* 2011).

Finally, the investigation of overlapping DNA repair pathways can also be studied using *S. cerevisiae* as a biological model system by constructing double, triple and quadruple mutants that contribute towards a better understanding of the mechanistic actions of a drug (SWANSON *et al.,* 1999). SOARES *et al.* (2005) have characterized the mechanism of action of ecteinascidin-743 (ET-743) by employing a panel of yeast-deleted strains for DNA repair. This study showed that yeast strains lacking endonucleases of NER and BER are resistant towards 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 the DNA repair pathways in lesions induced by the antineoplastic drug 5-FU and its active metabolite FdUMP in yeast cells. These results revealed that the repair mechanisms differed for the both antimetabolites because 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 by the identification of G2/M arrest after X-ray irradiation in the budding yeast *S. cerevisiae* (WEINERT & HARTWELL, 1988). The eukaryotic cell cycle consists of

a collection of ordered events in which the initiation of late processes depends upon the completion of earlier ones (FOIANI *et al.*, 2000). Surveillance mechanisms as checkpoints assure that these cell cycle events occur in the proper sequence to avoid replication and segregation of damaged DNA (POEHLMANN & ROESSNER, 2010). Failure in the proper response 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 a consequence of mutations in the mitotic checkpoint genes including *MAD2, BUB2* or *BUBR1*, and *S. cerevisiae* has been shown to be one important model for the study of 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 damage including arrest or the slowing of the cell cycle, and activation or repression of cellular pathways. Some DNA repair proteins may act as sensors and effectors, as well as checkpoint proteins that are a part of the replication complex sensors and transducers, and even effectors (PUTNAM *et al.*, 2009). The yeast checkpoint pathways are well defined and share similar features with the mammalian cell cycle checkpoint proteins. Accordingly, Table 2 presents the proteins involved in checkpoints that are 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 for the study of anticancer drug responses. As an example, the cytotoxic DNA-damaging agent adozelesin was examined 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, these findings revealed 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 to 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 the Rad17 yeast checkpoint protein towards camptothecin derivatives. They observed that Rad17p appears to be dispensable for cell cycle arrest and phosphorylation following treatment with camptothecin. Other studies employing hydroxyurea, one antineoplastic drug that causes nucleotide depletion, demonstrated 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. The *S. cerevisiae pso9/mec3* (human homolog *HUS1*) mutant was isolated and was subsequently, molecularly and phenotypically characterized. This mutant failed to arrest its cell cycle after treatment with the

bifunctional agent methoxypsoralen (8-MOP) + UVA, confirming its role in response to interstrand crosslinkers (BRENDEL *et al.*, 2003; CARDONE *et al.,* 2006).

In addition to antitumor drugs that interfere with the 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). Moreover, PARP inhibitors have been shown to exhibit strong and specific cytotoxic effects in ATM defective cells, which suggest that targeting checkpoint proteins may also result in synthetic lethality and reveals new therapeutic possibilities. However, the checkpoint protein interactions that may result in synthetic lethality, as well as the cellular outcomes of checkpoint targeting drugs are still poorly understood. Therefore, employing yeast to evaluate the molecular response of these new drugs and interactions may represent a critical step towards confirming direct targets and effects *in vivo*.

2.3. Budding yeast as a 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 throughout cell cycle division. The 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 do involve gene transcription regulation (PLASS, 2002). Acetylation of lysine (K) residues of the N-terminal tails neutralizes the histones' positive charge and decreases its interactions with the negatively charged DNA, leading to an open chromatin structure that is 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 the genome as well as histone modifications (EGGER *et al.,* 2004; LAFON-HUGHES *et al.,* 2008). Epigenetic changes may inactivate tumor-suppressor genes and/or activate genes that lead to cancer when overexpressed, such as oncogenes (SIMON & BEDALOV, 2004). Global DNA hypomethylated and hypermethylated tumor suppressor gene promoters can be observed in almost all cancers. Patients with sporadic colorectal cancers often present a microsatellite instability phenotype that is related to methylation and silencing of *MLH1*. In addition, many malignancies are associated with aberrant histone deacetylase (HDAC) expression and activity (KRISTENSEN *et al.,* 2009). One example is the oncogenic fusion of promyelocytic leukemia with the retinoic acid receptor, which recruits HDAC to repress genes that are necessary for hematopoietic cell differentiation. Furthermore, deficiencies in ATP-dependent

chromatin remodeling complexes such as the highly conserved SWI-SNF complex, have also been implicated in cancers. The loss of *SNF5* is found in pediatric 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 such as *HMGB1* may contribute towards cancer development, because this protein, which is expressed on the surface of specific cells, may contribute to cell migration and tumor invasion (ANDERSSON *et al.,* 2002; BREZNICEANU *et al.,* 2003).

Because epigenetic changes may be reversed by employing drugs that inhibit chromatin-modifying enzymes, epigenetic modifications have emerged as potential targets for therapeutic interventions in cancer treatment. For example, CpG methylation and histone hypoacetylation can be reversed by inhibiting enzymes such as DNMTs or HDACs. Altering the epigenetic regulation of gene expression shows great promise for resetting the chromatin changes in cancer cells; however, its effects in normal cells are difficult to predict (SIMON & BEDALOV, 2004). Some nucleoside analogues such as 5-azacytidine (azacitidine) and 5-aza-2´-deoxycytidine (decitabine), functions in the demethylation of tumor suppressor genes at non-cytotoxic concentrations, and they present cytotoxic effects at high concentrations related to enzyme-DNA adduct formation. Applied studies employing the agent decitabine combined with cisplatin or carboplatin, showed that drug resistance caused by the hypermethylation of MMR genes 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 such as trichostatin A, belinostat and vorinostat also exhibited synergistic responses when combined with conventional chemotherapeutic agents such as paclitaxel, gemcitabine, cisplatin, etoposide and doxorubicin in cell culture. The administration of HDAC inhibitors before chemotherapy also appears to be a promising strategy used to overcome multidrug resistance because histone acetylation results in an opened chromatin that is more accessible to drug treatment. In contrast, these treatments did not present the same efficacy when applied in reverse (KRISTENSEN *et al.,* 2009). Using *S. cerevisiae*, KAISER *et al.* (2011) observed that sodium phenylbutyrate (PBA) dramatically reduced H4 K8 acetylation, suppressed 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 was redirected to a non-recombinogenic pathway without a loss of cell viability; however, for MMS, this combination was accompanied by a dramatic loss in cell viability.

Unfortunately, there are few effective drugs available to investigate the epigenetic changes that occur in cancer therapy. However, because the main component of these cellular processes are affected by cancer-associated epigenetic alterations and are conserved among eukaryotes, the employment of budding yeast in chromatin-modifying agent screenings is a powerful tool used in the identification of new targets and drugs for anticancer research. In addition, some chromatin-modifying inhibitors including trichostatin A and depsipeptide are also active in yeast (SIMON & BEDALOV, 2004). Accordingly, yeast-based systems have proved to be a useful tool in small molecules screening for HDAC

activity (BEDALOV *et al.,* 2001; HIRAO *et al.*, 2003). HIRAO *et al.* (2003), used whole-genome DNA microarray analysis to identify compounds that exhibited a higher degree of selectivity towards NAD^+-dependent deacetylases involved in transcriptional repression in yeast. The identified compounds were splitomicin, which improved selectivity for Sir2 and dehydrosplitomicin, which were specifically effective in Hst1 defective yeast strains. WEERASINGHE *et al.* (2010) also developed a yeast-based gene reporter that centered on the class l yeast homolog Rpd3. Yeast Rpd3 deacetylase shares a 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 for Rpd3 mutants and inhibitors of class l HDAC proteins.

Nevertheless, *S. cerevisiae* mutant strains provide a better understanding of the complex interplay between chromatin remodeling mechanisms and other 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 damage and also participates in the recruitment of DNA repair factors to the damaged site. For example, the phosphorylation of histone H2A is directly related to DSB signaling and repair. Other chromatin modifiers involved in DSB repair include HATs, HDACs, ATP-dependent remodeling proteins, and histone kinases and phosphatases (for review, see ATAIAN & KREBS, 2006; VAN ATTIKUM & GASSER, 2005). In addition, LABAZI *et al.* (2009) proposed that *NHP6* (the

closest HMGB1 homolog) can also influence MMR activity by dissociation of *MSH2-MSH6* in the absence of mismatched DNA.

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

The data accumulated thus far clearly support yeast models as important tools in the identification and study of 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 of the use *S. cerevisiae* as a biological model system in anticancer research are summarized in Box 1. The most remarkable limitations of this model are the relative impermeability of the cell wall and the lack of important human proteins. These proteins belong to relevant categories in anticancer research such as tumor-suppression, apoptosis, and drug metabolism, among others (KOLACZKOWSKI & GOFFEAU, 1997). The strategies used to overcome such limitations require case-by-case planning and experimental adaptations as exemplified.

The *S. cerevisiae* cell wall has been reported as an important factor that permits reductions in drug sensitivities of several anticancer compounds such as DNA topoisomerase poisons (NITISS & WANG, 1988). In this case, the relative impermeability of the yeast cell wall may be overcome through the use of mutant strains that are defective in *ISE1*, *PDR1* and *SNQ2,* which has been reported to

increase drug sensitivity to several anticancer agents, including camptothecin, a potent antitumor drug that specifically targets topoisomerase I (NITISS & WANG, 1988; REID *et al.,* 1997). Other factors that increase yeast cell permeability to small molecules is the use of the lytic zymolyase enzyme in association with drugs because zymolyase acts to digest the cell wall. This enzyme has been shown to enhance the permeability of some HDAC inhibitors, such 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 using a yeast promoter (BJORNSTI, 2002). In these cases, the phenotype of the 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 is absent in yeast (FLAMAN *et al.,* 1995). By employing a random mutagenesis screen for this gene, p53 yeast mutants were isolated. The mutants demonstrated increased growth inhibition or even lethality. These toxic p53 variants might be useful for the dissection of p53-regulated cellular responses (INGA & RESNIK, 2001). Furthermore, yeast engineered to express apoptotic 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 ectopic expression of Bax in yeast produced a lethal phenotype by inducing mitochondrial cytochrome C release (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 require metabolizing by cytochrome P450 to become active (LYNCH & PRICE, 2007). The drug-metabolizing cytochrome P450 and glucuronosyl-transferase, both of which are absent in yeast, can be heterologously expressed and may be employed to study the mutagenic effects of oxidative metabolites of xenobiotics, such as N-alkylformamides, aflatoxin 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 also possible to use drugs analogs or metabolites rather than expressing the metabolizing enzyme. This is particularly true for 5-FU, topotecan, irinotecan, cytarabine, and 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 inappropriately integrate into DNA or RNA or inhibit the thymidylate synthase (TS) enzyme. Once TS is inhibited by FdUMP, the main 5-FU cytotoxic active metabolite, and because yeast does not possess thymidine kinase to convert 5-FU into FdUMP (LADNER, 2001), it is possible to directly administer FdUMP on to the yeast cells, which make *S. cerevisiae* a unique model system to independently investigate the cellular effects of 5-FU or FdUMP (MATUO *et al.,* 2010).

4. Concluding remarks

This review discussed important aspects and applications of yeast in anticancer research. Aside from contributing in the determination of the basic

mechanistic actions of anticancer agents, *S. cerevisiae* has also proven to be an extraordinary tool for drug screenings. Here, we discuss the evidence for the high degree of similarity between yeast and human DNA repair, checkpoint and epigenetic control systems. These basic cellular processes are directly involved in genomic maintenance and their improper regulation has a critical outcome at all stages of carcinogenesis. Therefore, these genomic maintenance systems have emerged as promising targets in cancer therapy. However, exploration of these complex safeguard systems in therapy are still challenging, particularly because of the high heterogeneity of the genetic alterations and background in tumor cells, as well as the longstanding and elevated cost of screenings in mammalian systems.

S. cerevisiae is an organism that is easy to manipulate, exhibits a rapid doubling time, is experimentally inexpensive, is amenable to gene disruptions and demonstrates conserved signaling pathways between eukaryotes. Despite these advantages, yeast also presents limitations such as their low cellular permeability to several compounds and the absence of diverse tumor suppressors and metabolizing enzymes. These limitations may be overcome using numerous case-by-case strategies as previously discussed. However, it is important to note that *S. cerevisiae* do not completely substitute for mammalian models in anticancer research. Due to the yeast's unicellularity, some tumor aspects such as angiogenesis, tissue invasion and metastasis cannot be evaluated in this model organism. However, yeast screenings are fast and powerful tools for the screening of compounds and basic mechanisms in anticancer research.

Nevertheless, for decades, scientists worldwide have generated yeast mutant strains. As a result, the *S. cerevisiae* strains collection include all viable

single gene mutation strains. These mutant yeast strains may be obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF) or directly from yeast specialized laboratories. The use of this extraordinary *S. cerevisiae* panel could represent a faster and cheaper solution to screen anticancer drugs, cytotoxicity, and also provide an easier and manipulating system to mimic the numerous combinations of genetic alterations in cancer cells that may be explored using the synthetic lethal approaches.

Abbreviations:

5-FU: 5-Fluorouracil

Azacitidine: 5-azacytidine

ATR: *Ataxia telangiectasia* and *Rad3* related

ATRIP: ATR-interacting protein

ATM: *Ataxia telangiectasia* mutated

BER: Base Excision Repair

Decitabine: 5-aza-2'-deoxycytidine

DNMT: DNA Methyl Transferase

DSB: Double-Strand Break

ET-743: Ecteinascidin-743

FdUMP: 5-Fluoro-2'-deoxyuridine 5'-monophosphate

HAT: Histone Acetyl Transferase

HDAC: Histone Deacetylase

HMGB: High Mobility Group Box-1

HR: Homologous Recombination

K: lysine

MMR: Mismatch Repair

MMS: Methyl Methane Sulfonate

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End-Joining

PARP: Poli (ADP-Ribose) Polymerase

PBA: Sodium Phenylbutyrate

PCNA: Proliferating Cell Nuclear Antigen

PI3K: phosphatidylinositol 3-kinase

PRR: Post-Replication Repair

RPA: Replication Protein A

SAHA: Suberoylanilide Hydroxamic Acid

TLS: Translesion synthesis

TopBP1: topoisomerase-binding protein-1

ssDNA: single-strand DNA

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fellowship from CNPq and CAPES respectively, and are a graduate student at UFRGS.

Figure Captions:

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

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

Box 1: Positive and negative aspects of employing *S. cerevisiae* as a biological model in anticancer research

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Table 1

Pathway	Function	*S. cerevisiae*	*H. sapiens*	*Reference*
BER	Excises damaged single DNA bases or a short strand containing the damaged base, DNA polymerase fills the gap and ligase connects the ends.	Apn1, Rad27	APE1, FEN1	BERNSTEIN *et al.,* 2002
NER	Excises the 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 the post-replicative repair and corrects the DNA mismatches that have escaped the proofreading function of replicative polymerases, recognizes the non-canonical base pair and replaces the offending nucleotide on the newly strand by excision repair mechanism.	Mlh1, Pms1	MLH1, PMS2	HSIEH, 2001; IYER *et al.,* 2006
TLS	A damage tolerance mechanism in which the DNA polymerase (Polζ) and a complex of proteins (Rev3 and Rev7) bypass the DNA lesions to allow cell survival 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

Table 2

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

Box 1:

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.

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2001 – 2005 - Licenciatura e Bacharelado em Ciências Biológicas pela Universidade Estadual de Londrina - UEL;

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3. ESTÁGIOS:

- Estágio de Doutorado Sanduíche. Período: 01 de novembro de 2009 a 31 de outubro de 2010. Université Pierre et Marie Curie - Paris 6, Laboratory of Cancer Biology and Therapeutics Centre de Recherche Saint-Antoine do Institut National de la Santé et de la Recherche Médicale (INSERM) U893, Paris, França. Fonte de financiamento: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior -

CAPES. Orientação: Dra. Annette K. Larsen e Dr. Alexandre E. Escargueil. Atuação no projeto de avaliação dos mecanismos de ação de antimetabólitos como parte da tese, e participação no trabalho sobre S23906 de autoria de D. G. Soares.

- Estágio Curricular. Monografia para obtenção do título de Bacharel. Período: 01 de fevereiro a 01 de dezembro de 2005. Universidade Estadual de Londrina. Fonte de financiamento: Conselho Nacional de Auxílio à Pesquisa Científica - CNPq. Orientação: Dr. Mário Sérgio Mantovani. Atuação no projeto intitulado: "Avaliação da atividade clastogênica e anticlastogênica do extrato aquoso de *Agaricus blazei*, em células metabolizadoras, linhagem HTC, em diferentes fases do ciclo celular".

- Estágio Curricular. Estágio Supervisionado: Metodologia e Prática do Ensino de Ciências Biológicas. Período: 01 de fevereiro a 01 de dezembro de 2004. Universidade Estadual de Londrina. Dr. Álvaro Lorencini Junior. Atividade didática de ensino médio.

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- Bolsa de Iniciação Científica. Período: 01 de abril de 2003 a 31 de janeiro de 2005. Universidade Estadual de Londrina. Fonte de financiamento: Conselho Nacional de Auxílio à Pesquisa Científica - CNPq. Dr. Mário Sérgio Mantovani. Atuação no projeto intitulado: Efeito Antigenotóxico de *Agaricus blazei*, em células CHO-k1, perante inibição de reparo de DNA.

- Estagio Voluntário. Período: 16 de agosto de 2002 a 30 de abril de 2003. Laboratório de Mutagênese, Universidade Estadual de Londrina. Dr. Mário Sérgio Mantovani. Noções básicas de cultivo de células de mamíferos e análise de aberrações cromossômicas.

- Estagio Voluntário. Período: 01 de março a 20 de dezembro de 2002. Laboratório de Genética de Microorganismos, Universidade Estadual de Londrina. Dra. Gisele Maria de Andrade Nóbrega. Noções básicas de cultivo de microorganismos e técnicas de biologia molecular (transformação, extração e purificação de DNA).

4. PRÊMIOS E DISTINÇÕES

Prêmio Pós-Graduação / 51º Congresso Brasileiro de Genética / co-autoria no trabalho de Rodrigo Juliano Oliveira, Sociedade Brasileira de Genética - SBG.

5. ARTIGOS COMPLETOS PUBLICADOS

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8. MANTOVANI, M. S.; MATUO, R.; BELLINI, M. F.; OLIVEIRA, R. J. & RIBEIRO, L. R. Atividade clastogênica e genotóxica de altas concentrações do extrato aquoso de Agaricus brasiliensis e diferentes respostas quando associado aos inibidores de reparo de DNA, Ara-C e 3DeoT, in vitro. *Semina*, 27: 13-22, 2006.

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11. SILVA, A. F.; OLIVEIRA, R. J.; MATUO, R.; RIBEIRO, L. R.; MANTOVANI, M. S. Efeitos do *Agaricus blazei* na clastogenicidade induzida pela radiação ultravioleta em cultura de células CHO-k1. *Semina*, 26: 131-138, 2005.

6. ARTIGOS COMPLETOS ÀCEITOS PARA PUBLICAÇÃO

1. SOUSA, F. G.; MATUO, R.; SOARES, D. G.; ESCARGUEIL, A. E.; HENRIQUES, J. A.P.; LARSEN, A. K.; SAFFI, J. PARPs and the DNA damage response. *Carcinogenesis*.

7. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

1. MATUO, R.; SOUSA, F. G.; SOARES, D. G.; SAFFI, J.; HENRIQUES, J. A. P. & ESCARGUEIL, A. E. Citotoxicidade de antimetabólitos é potencializada com a inibição de checkpoint kinases. *XII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS*. 2010.

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7. OLIVEIRA, R. J.; BARBOZA, M. Z.; MIGUEL, R. B.; MATUO, R.; SILVA, A. F.; RIBEIRO, L. R. & MANTOVANI, M. S. Avaliação da atividade anticlastogenica da suplementação enteral com glutamina em danos induzidos por cisplatina em camundongos. *52º Congresso Brasileiro de Genética,* 2006.

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