Universidade Federal do Rio Grande do Sul

Mecanismos apoptóticos e o efeito antiproliferativo da combinação CPT-11/5-FU em linhagens celulares derivadas de carcinoma de cólon humano

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Mecanismos apoptóticos e o efeito antiproliferativo da combinação CPT-11/5-FU em linhagens celulares derivadas de carcinoma de cólon humano

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para obtenção do grau de Doutor em Ciências.

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SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

$\Delta\psi$	Colapso do potencial da membrana mitocondrial interna
Apaf-1	Fator de ativação de protease associada a apoptose 1
APC	Adenomatous polyposis coli
Cdk4	Quinase dependente da ciclina 4
CH_2-THF	5,10-metilenotetraidrofolato
CI	Índice de combinação
Cox-2	Ciclooxigenase-2
CPT-11	Irinotecan
Cu,Zn-SOD	Superóxido dismutase dependente de cobre e zinco
DCC	Deleted colon carcinoma
DD	Domínio de morte
DED	Efetor do domínio de morte
dNTP	Deoxinucleotídeo trifosfato
dTMP	Deoxitimidina monofosfato

DTT	Ditiotreitol
Dttp	Deoxitimidina trifosfato
dUMP	Deoxiuridina monofosfato
EDTA	Ácido etilenodiaminotetra-acético
EGTA	Ácido etilenoglicol bis(β-amino etil éter)-*N*,*N*,*N'*,*N'*, tetra-acético
ERK	“Extracellular-signal-regulated kinases”
ERO	Espécies reativas do oxigênio
DHF	Diidrofolato
DHFR	Diidrofolato redutase
FA	Fração afetada
FAP	Polipose adenomatosa familiar
FdUMP	Fluorodeoxiuridina monofosfato
FdUTP	Fluorodeoxiuridina trifosfato
FUTP	Fluorouridina trifosfato
5-FU	5-Fluorouracil
HBSS	Solução salina tamponada de Hank
HNPCC	Câncer colorretal hereditário não poliposo
L-OHP	Oxaliplatina
LV	Leucovorin
Mdm-2	“Murine double minute”

MEK	“Mitogen-activated protein kinase/ extracellular signal-regulated kinase 1”
MMR	Reparo de mal-emparelhamento
Mn-SOD	Superóxido dismutase dependente de manganês
MSI	Instabilidade de microssatélites
MTT	“Thiazolyl blue tetrazolium bromide”
Mu-MLV	“Murine Moloney leukemia vírus reverse transcriptase”
OD	Densidade óptica
PBS	Solução salina fosfato tamponada
PMSF	Fenil metil sulfonil fluoreto
PTP	Poro de transição de permeabilidade
rEGF	Receptor do fator de crescimento epidérmico
Rh123	Rodamina 123
ROS	“Reactive oxygen species”
rTNF	Receptor de fator de necrose tumoral
SF	Fração de Sobrevivência
SOD	Superóxido dismutase
SRB	Sulforodamina B
TGF-β	Fator de crescimento transformante β
THF	Tetraidrofolato
TK	Timidina quinase

Topo I	Topoisomerase I
TP	Timidina fosforilase
TPM	Transição da permeabilidade mitocondrial
Tris	Tris (hidroximetil) aminometano
TS	Timidilato sintase
VEGF	Fator de crescimento endotelial vascular

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RESUMO

O câncer de cólon é a terceira causa de morte relacionada ao câncer no mundo. São fatores de risco o histórico familiar, doenças inflamtórias e hábitos alimentares. Apesar de importantes progressos terapêuticos, as respostas na doença avançada permanecem insatisfatórias. A combinação Irinotecan (CPT-11) e 5-Fluorouracil (5-FU) é utilizada como tratamento de primeira linha no carcinoma avançado de cólon. Quando comparado com as drogas isoladas à utilização de CPT-11 2 h antes do 5-FU (CPT-11>5-FU) demonstrou um efeito sinérgico. Estas observações foram relacionadas com um aumento de danos ao DNA e apoptose. Neste estudo foi investigado qual a via de indução de apoptose é ativada após tratamento com CPT-11>5-FU. Também foi avaliado se a ativação da apoptose pode estar relacionada com alterações nas enzimas superóxido dismutase (SOD), timidilato sintase (TS) e topoisomerase I (Topo I). Com este objetivo, as linhagens celæ a

celular. Por outro lado, na linhagem HT-29, o tratamento com CPT-11>5-FU aumentou a atividade da Mn-SOD em 50% quando comparado com a exposição ao CPT-11 isolado. Estas observações indicam que o aumento da apoptose observado com a combinação CPT-11>5-FU na linhagem HT-29 pode ser explicado por um colapso no potencial da membrana mitocondrial levando a um aumento da atividade da Mn-SOD e indução das caspases. Na linhagem SNU-C4, a apoptose não é o principal mecanismo de citotoxicidade da combinação CPT-11>5-FU. Nossos achados sugerem que a toxicidade do tratamento com CPT-11>5-FU não está relacionada com as enzimas TS e Topo I, nas du

ABSTRACT

Cancer of the colon is both the third most common type of cancer and leading cause of cancer death. The incidence of colon cancer begins to rise at age 40 and peaks between 60 and 75 years. People with a family history of colon cancer have higher risk of developing the cancer themselves. A family history of familial polyposis or a similar disease also increases the risk of colon cancer. Diet affects the risk of colon cancer, but not fully elucidated mechanism. Notwithstanding important improvements in therapeutic strategies, results in advanced disease remain unsatisfactory. The combination of Irinotecan (CPT-11) and 5-Fluorouracil (5-FU) is currently used in the treatment of advanced colorectal carcinoma. When compared to both agents alone, the CPT-11 followed by 5-FU combination treatment (CPT-11>5-FU) demonstrated a synergistic effect. This observation can be related to an increased in apoptosis induction. In this study, we examined which pathways of apoptosis induction are activated after treatment with CPT-11>5-FU. Also, we investigated whether the activation of apoptosis can be associated to alterations in superoxide dismutase (SOD), thymidylate synthase (TS) and topoisomerase I (Topo I) activities. Thus, cells were exposed to each drug in varying concentrations and sequences, and assessed for cytotoxicity, apoptosis, changes in the mitochondrial membrane potential and involvement of caspases. We also evaluated the colony formation, Topo I activity, DNA-Topo I complex formation, cell cycle distribution, TS activity, TS mRNA, and SOD activity. Enhanced apoptosis was observed with all treatments containing 5-FU in SNU-C4 cells, however in HT-29 cells, only the CPT-11>5-FU enhanced apoptosis. In HT-29 cells, both caspase-3 and caspase-9 were involved in the activation of apoptosis after exposure to CPT-11>5-FU. Moreover, in these cells, a reduction of 50% in mitochondrial membr

HT-29 cell line, CPT-11>5-FU enhanced 50 % Mn-SOD activity when compared to cells treated with CPT-11 alone. These observation indicated that the increase in apoptosis observed with CPT-11>5-FU, in HT-29 cells, could be explained by a disruption in mitochondria membrane potential that enhanced Mn-SOD activity and induced caspases activation. In the SNU-C4 cells, apoptosis is not the most important mechanism of cytotoxicity of CPT-11>5-FU. Moreover, our findings suggest that CPT-11>5-FU cytotoxicity are not related to Topo I nor TS, in both HT-29 and SNU-C4 cell lines. In order to further elucidate these observations studies are ongoing in our laboratory.

INTRODUÇÃO

1 EPIDEMIOLOGIA E ETIOLOGIA

O câncer é um dos maiores problemas de saúde pública tanto nos países desenvolvidos como nos países em desenvolvimento. Na maioria dos países o câncer é a segunda causa de morte por enfermidade, logo depois das doenças cardiovasculares. No Brasil, as estimativas para o ano de 2005 apontam a ocorrência de 467.440 novos casos de câncer. Na região nordeste, diferente do restante do país, o câncer é a terceira causa de morte (6,3% dos óbitos), atrás das doenças infecciosas e parasitárias. Nas outras regiões, permanece como a segunda causa de 1584 Tc 3.83984 0 Td (i)Tj -0.06d.3983 Tc 120.4398 0 Td0 Td (15

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de câncer nos países industrializados (American Cancer Society Homepage, 2004). Apesar disso, as taxas de incidência desta neoplasia diminuíram numa razão de 1,8% ao ano no período de 1985-1995, se estabilizando nos últimos anos. Esse declínio é, provavelmente, resultado de um aumento nos exames e remoção de pólipos no intestino, o que previne a progressão para um câncer invasivo (Jemal *et al.*, 2003).

No Brasil, este quadro é diferente. Como sugerido pelo Instituto Nacional de Câncer (INCA), o carcinoma do cólon afeta, nos dias atuais, 70% mais brasileiros do que em 1979 (Ministério da Saúde. Instituto Nacional de Câncer, 2004). Segundo as estimativas de incidência e mortalidade por câncer no Brasil, publicadas pelo INCA, o número de novos casos de câncer de cólon previsto para o ano de 2005 é de 12.410 em homens e de 13.640 em mulheres. E os óbitos esperados, em homens e mulheres estão estimados em 3.700 e 4.270, respectivamente (Ministério da Saúde. Instituto Nacional de Câncer, 2004).

Apesar de diversos fatores contribuírem para a carcinogênese do câncer de cólon, as doenças associadas (retocolite ulcerativa e doença de Crohn) e a predisposição genética (polipose intestinal familiar e síndrome de Lynch) merecem especial atenção. De fato, aproximadamente 5-10% dos cânceres colorretais ocorrem devido à herança mendeliana (Jemal *et al.*, 2003).

Tem sido demonstrada uma forte associação entre pessoas com familiares em primeiro grau com câncer colorretal e o aumento do risco de desenvolver esta neoplasia (Slattery *et al.*, 2002). Além do mais, foi demonstrado que o aumento deste risco é diretamente proporcional ao número de familiares com este tipo de câncer (Johns and Houlston, 2001; Slattery *et al.*, 2002). Diversos estudos identificaram um aumento de 2 a 3 vezes no risco de desenvolver câncer de cólon em pessoas com parentes em primeiro grau com esta neoplasia (Tabela 1).

Embora apresente fatores de risco de natureza hereditária, a variabilidade na incidência e mortalidade deste tipo de câncer sugere a existência de causas ambientais (Potter, 1999; Fearon and Gruber, 2001). Aproximadamente 75% dos pacientes com câncer de cólon apresentam doença esporádica, sem evidências

de desordem herdada. Os 25% restantes dos pacientes possuem histórico familiar de câncer de cólon, o que sugere uma contribuição genética, exposição a fatores ambientais, ou a combinação de ambos (Cohen *et al*., 1997; Potter, 1999; Shields and Harris, 2000).

Tabela 1: Risco de desenvolvimento do câncer de cólon em relação ao histórico familiar.

Histórico familiar	**Risco**
Sem histórico familiar	4%
Um parente em primeiro grau com câncer de cólon	9%
Mais de um parente em primeiro grau com câncer de cólon	16%

Dados obtidos de Slattery *et al*., 2002.

O fator de risco primário para o câncer de cólon é a idade, com mais de 90% dos casos diagnosticados em pessoas com idade superior a 50 anos. Tal fato é resultante da associação de fatores genéticos com exposição prolongada a carcinógenos ambientais. O aparecimento da doença aumenta após os 40 anos de idade atingindo sua incidência máxima aos 70 anos, sendo muito raro em crianças (Cohen *et al*., 1997; Fearon and Gruber, 2001). Em pacientes jovens, ressalta-se a necessidade de pesquisar condições que possam estar associadas ao desenvolvimento do câncer de cólon como as doenças genéticas ou inflamatórias (Okuno *et al*., 1987).

Entre os possíveis fatores de risco ambientais estão: dieta alimentar

diferenciação e morte celular (Heitman and Cameron, 1990; Rupnarain *et al.*, 2004). Foi sugerido que o consumo de dietas hipercalóricas, podem desenvolver a resistência a insulina, aumentando os níveis de triglicerídeos e ácidos graxos circulantes. Este fato é um estímulo para a proliferação das células do cólon e acúmulo de intermediários tóxicos do oxigênio, propiciando um ambiente favorável para a promoção do câncer (Bruce *et al.*, 2000). Dois estudos recentes sugerem que a ingestão de uma dieta rica em fibras está inversamente relacionada com a incidência do câncer de cólon (Bingham *et al.*, 2003; Peters *et al.*, 2003). Por outro lado, outros autores não demonstraram relação entre o alto consumo de fibras nem suplementos de fibras com a redução do risco de desenvolver adenomas colorretais (revisado em Potter, 1999). Apesar da controvérsia, é sugerido que a ingestão de uma dieta rica em fibras protege contra o câncer colorretal, através da redução do tempo do trânsito intestinal, diminuindo a exposição da mucosa do colón a potenciais carcinógenos, como por exemplo, aminas heterocíclicas e nitrosaminas (Dashwood, 1999; Peters *et al.*, 2003; Mathew *et al.*, 2004). Em contraste, uma dieta rica em carne e gordura animal aumentaria os riscos por propiciar uma maior exposição a estes agentes (Potter, 1999; Mathew *et al.*, 2004). Além disso, a atividade física está relacionada com um decréscimo no risco do câncer de cólon, uma vez que reduz o tempo de trânsito dos alimentos no intestino (Potter, 1999). Também, foi demonstrado que o consumo excessivo de álcool associado à absorção reduzida de folato (encontrado nos vegetais) pode aumentar o risco de desenvolver câncer de cólon (Giovannucci *et al.*, 1995).

As infecções bacterianas também contribuem para a ocorrência do câncer de cólon, visto que, iniciam um processo inflamatório crônico e formam metabólitos carcinogênicos (Parsonnet, 1995). A inflamação causada pela bactéria *Helicobacter pylori*, gera radicais livres mutagênicos, além de induzir a proliferação das células intestinais, podendo iniciar a formação do câncer de cólon (Parsonnet, 1995). Neste sentido, Meucci *et al.* (1997) detectou anticorpos para *H. pylori* em 69,2% dos pacientes com câncer de cólon, comparado com 49% no grupo controle.

2 CLASSIFICAÇÃO E CARACTERÍSTICAS CLÍNICAS DOS TUMORES DE CÓLON

Do ponto de vista etiológico, podemos classificar os tumores de cólon como: associado a polipose adenomatosa familiar (FAP); câncer colorretal hereditário não poliposo (HNPCC) e câncer colorretal esporádico (Cohen *et al.*, 1997). Aproximadamente 1% dos casos de câncer de cólon provém da FAP, 3-5% da HNPCC, 30% de origem familial, 60% são cânceres esporádicos e os restantes são devido a síndromes raras como, por exemplo, síndrome de Gardner e de Turcot (Figura 1) (Liu *et al.*, 1996).

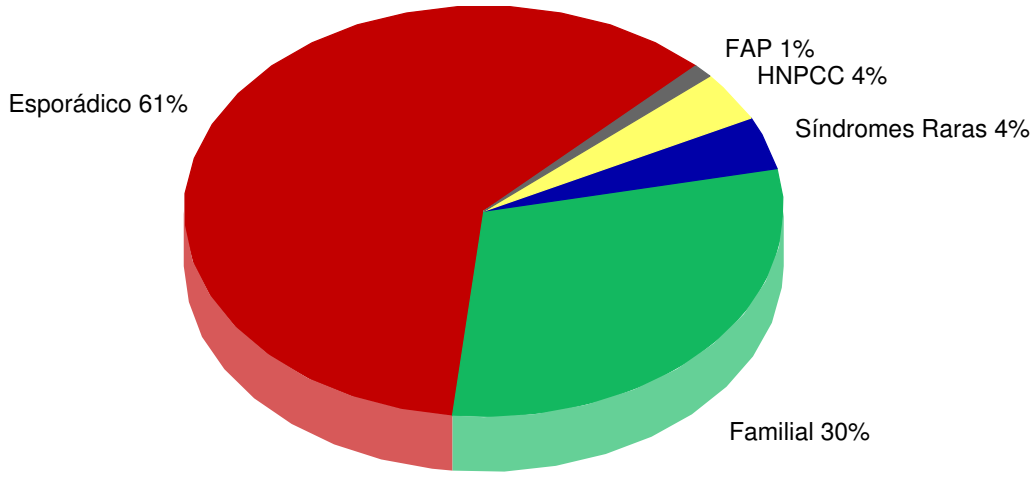


Figura 1: Freqüência dos tipos de tumores de cólon (FAP = polipose adenomatosa familiar; HNPCC = câncer colorretal hereditário não poliposo).

O câncer de cólon pode, também, ser classificado quanto a sua histologia em: carcinoma, adenocarcinoma e sarcoma. O adenocarcinoma é o tipo histológico mais freqüente, sendo responsável por 90% a 95% dos casos (Cohen *et al.*, 1997). A evolução e o prognóstico do câncer de cólon dependem

principalmente do estadiamento do tumor (Cohen *et al.*, 1997). O sistema TNM é um dos sistemas utilizados no estadiamento do câncer de cólon. Este sistema (Figura 2) se baseia na combinação de três características: T - tamanho do tumor primário e/ou grau de extensão local; N - presença ou não de metástases nos linfonodos regionais; M - presença ou não de metástases à distância. Além das letras, são utilizados números indicando a progressão da doença (American Joint Committee on Cancer, 2002).

TNM

Tumor Primário (T)

TX	Tumor primário não pode ser determinado
T0	Sem evidência de tumor primário
Tis	Carcinoma *in situ*: intraepitelial, ou invasão da lâmina basal
T1	Tumor invade submucosa
T2	Tumor invade muscular própria
T3	Tumor invade até a subserosa
T4	Tumor invade outros órgãos ou estruturas

Linfonodos Regionais (N)

NX	Linfonodos regionais não podem ser determinados
N0	Sem metástases em linfonodo regional
N1	Metástases em 1-3 linfonodos regionais
N2	Metástases em 4 ou mais linfonodos regionais

Metástases à Distância (M)

MX	Metástases à distância não podem ser determinadas
M0	Sem metástases à distância
M1	Metástases à distância

Figura 2: Sistema TNM para estadiamento clínico do câncer (Adaptado de American Joint Committee on Cancer, 2002).

O adenocarcinoma de cólon foi estadiado pelo sistema de Dukes' (Dukes, 1932), recebendo posteriormente diversas modificações. A mais importante foi proposta por Astler and Coller (1954). Este sistema ficou conhecido como sistema

de Dukes' modificado. O sistema de classificação mais utilizado atualmente é o proposto pela "American Joint Committee on Cancer" (AJCC), onde o câncer de cólon pode ser classificado em 4 estágios (American Joint Committee on Cancer, 2002) (Figura 3).

Estadiamento Clínico

AJCC	TNM	Dukes	Dukes' Modificado
Estágio 0	Tis,N0,M0		
Estágio I	T1,N0,M0	A	A
	T2,N0,M0	A	B1
Estágio IIA	T3,N0,M0	B	B2
Estágio IIB	T4,N0,M0	B	B3
Estágio IIIA	T1,N1,M0	C	C1
	T2,N1,M0	C	C1
Estágio IIIB	T3,N1,M0	C	C1
	T4,N1,M0	C	C1
Estágio IIIC	Qualquer T,N2,M0	C	C2
Estágio IV	Qualquer T,qualquer N,M1	D	D

Figura 3: Correlação entre os sistemas utilizados no estadiamento clínico dos tumores de cólon (Adaptado de American Joint Committee on Cancer, 2002).

Os tumores classificados como Estágio I (Dukes' A) apresentam sobrevida pós-operatória por mais de 5 anos em 90% dos casos. Os de Estágio II (Dukes' B) a sobrevida cai para 75% dos casos, já pacientes com estágio III (Dukes' C) a sobrevida é de 35-60% dos casos. Entretanto, menos de 10% dos casos de

pacientes com estágio IV (Dukes' D) apresentam sobrevida de 5 anos (American Joint Committee on Cancer, 2002).

A disseminação do tumor de cólon pode ocorrer por invasão direta para os órgãos vizinhos ou através de metástases venosa e linfática. As metástases são mais freqüentes nos nódulos linfáticos regionais, seguidos do fígado, pulmão, ossos, peritônio, bexiga e cérebro. O número de linfonodos aumenta proporcionalmente com o aumento do grau do tumor. De fato, linfonodos positivos estão associados a 30% dos tumores de baixo grau e com 81% dos tumores de alto grau (Dukes and Bussey, 1954; Cohen *et al.*, 1997).

3 CARACTERÍSTICAS MOLECULARES

O câncer colorretal pode ser dividido em dois grupos com base em padrões moleculares: (a) tumores com instabilidade cromossômica e (b) tumores com instabilidade de microssatélites (Lengauer *et al.*, 1998; Lindblom, 2001). Tumores com instabilidade cromossômica se desenvolvem preferencialmente no lado esquerdo do cólon, tem DNA aneuplóide e mutações nos genes *K-ras*, *APC* e *p53*. Por outro lado, tumores com instabilidade de microssatélites normalmente ocorrem no cólon direito, apresentam DNA diplóide e mutações no fator de crescimento transformante β (TGF-β), no gene *bax* e nos genes ligados ao sistema de reparo de mal-emparelhamento (“mismatch repair”; MMR) (Smyrk and Lynch, 1999). Aproximadamente 85% dos cânceres de cólon resultam de instabilidade cromossômica e 15% são devido à instabilidade de microssatélites (Lengauer *et al.*, 1998; Lindblom, 2001). Entre os tumores de instabilidade cromossômica encontramos os originados da FAP e entre os de instabilidade de microssatélites os HNPCC (Tabela 2).

Tabela 2: Características da Polipose Adenomatosa Familiar (FAP) e Síndrome do Câncer Colorretal Hereditário Não Poliposo (HNPCC).

Características	FAP	HNPCC
Padrão de Herança	Autossômica dominante	Autossômica dominante
Incidência	1:10.000	1:2.000
Genes[a]	*APC* (>90%)	MLH1 (59%)
		MSH2 (38%)
		MSH6 (1,3%)
Câncer de cólon[b]	39	44
Pólipos no cólon	> 100 pólipos em jovens	Poucos pólipos detectados por endoscopia
Variações das síndromes	Síndrome de Gardner (muitos pólipos, cistos epidermóides, osteomas)	Síndrome de Lynch Tipo I (câncer de cólon sem muitos pólipos)
	Síndrome de Turcot (pólipos e tumor cerebral)	Síndrome de Lynch Tipo II (câncer de colón sem muitos pólipos, câncer de estômago, câncer urotelial)

[a]Percentual de casos com mutações genéticas; [b]média de idade em anos (Adaptado de Grivicich, *et al*., 2005).

A FAP se caracteriza pelo desenvolvimento de múltiplos pólipos, que progridem para o câncer (Tabela 2). Quando não tratados, um ou mais pólipos poderão progredir para um câncer perto dos 45 anos, que corresponde a aproximadamente 20 anos antes da ocorrência do câncer de cólon em indivíduos não portadores da FAP (Nishisho *et al*., 1991).

A doença ocorre por uma mutação herdada no gene *adenomatous polyposis coli* (*APC*) localizado no cromossomo 5q, que induz a formação destes pólipos (Groden *et al*., 1991; Nishisho *et al*., 1991). Mutações no gene *APC* de células germinativas foram observadas na maioria dos pacientes com FAP. As mutações gênicas mais freqüentes são mutações sem sentido ou “frameshift mutation”, resultando na formação de uma proteína truncada com função alterada (Fearnhead *et al*., 2001). Diversas variantes da FAP foram descritas (Tabela 2). Entre estas, a síndrome de Gardner, onde os indivíduos afetados apresentam pólipos associados a cistos sebáceos, lipoma, fibromas, osteomas de face e dentes supranumerários impactados. E a síndrome de Turcot, na qual além dos pólipos, são freqüentes manchas café-com-leite, tumores papilares de tireóide, meduloblastomas, glioblastomas, cistos de pele e

defeitos neste sistema de reparo estão relacionados com os estágios iniciais do desenvolvimento de adenomas (De Jong *et al.*, 2004).

O processo de tumorigênese depende de um desequilíbrio nas funções de proto-oncogenes e genes supressores tumorais. Estes genes codificam proteínas reguladoras de vias de transdução de sinais para funções celulares essenciais, tais como: proliferação, diferenciação, apoptose, adesão, migração e angiogênese (Tronick and Aronson, 1995; Weinstein *et al.*, 1995; Weinberg and Hanahan, 1996).

Acredita-se que a transformação do adenoma em carcinoma é resultado de uma série de mudanças patológicas da mucosa normal para o carcinoma

estão relacionadas com o crescimento do tumor e o surgimento da displasia (Bos *et al.*, 1987; Fearon and Gruber, 2001). Os passos seguintes envolvem uma deleção no cromossomo 18 (18q) (Vogelstein *et al.*, 1988), sendo muito freqüente nos carcinomas (70%) e rara em adenomas iniciais (6%). Tal alteração leva à inativação do gene supressor de tumor *DCC* ("deleted in colon cancer"). Este gene está relacionado aos processos de adesão célula-célula e célula-matriz extracelular, apresentando papel importante no potencial metastático das neoplasias do cólon (Vogelstein *et al.*, 1988; Bos *et al.*, 1987; Fearon and Gruber, 2001).

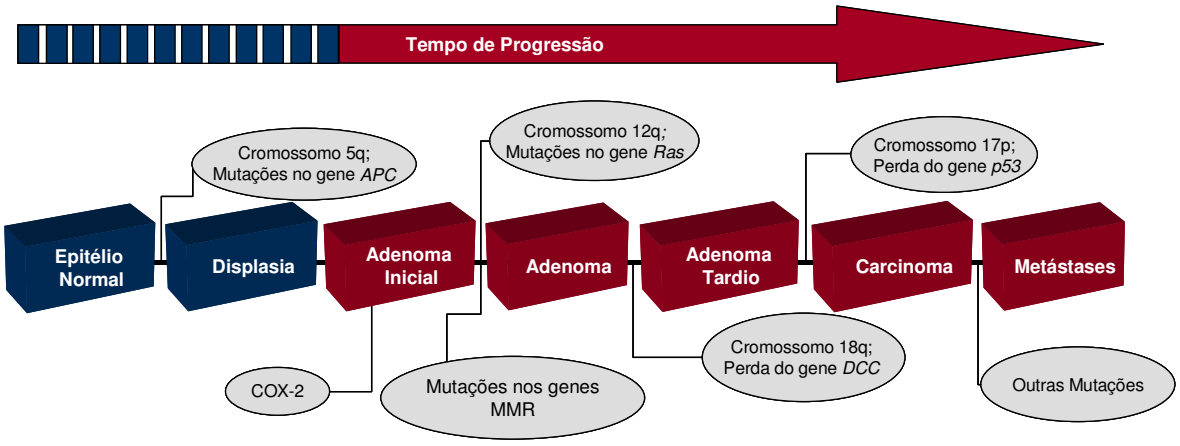


Figura 4: Modelo genético da progressão do câncer de cólon (Cox-2 = ciclooxigenase-2; MMR = reparo de mal-emparelhamento; *DCC* = "deleted in colon cancer"; *APC* = "Adenomatous poliposis coli").

As mutações mais tardias ocorrem no cromossomo 17 (17p) estando relacionada com a transição de adenomas para carcinomas (Vogelstein *et al.*, 1988). A região mutada inclui, entre outros, o gene supressor de tumor *p*53, que codifica uma fosfoproteína nuclear cuja disfunção contribui para tumorigênese e agressividade do tumor (Bos *et al.*, 1987; Fearon and Gruber, 2001). Mutações no *p53* estão presentes em aproximadamente 50% dos tumores colorretais. A proteína p53 (Figura 5) participa da regulação do ponto de checagem de G1, que

tem fundamental importância na manutenção da integridade do genoma, pois permite a ação dos mecanismos de reparo do DNA ou a remoção de células danificadas através do processo de apoptose (Lemoine, 1990). Danos no DNA promovem a superexpressão e conseqüente ativação da p53, resultando na parada do ciclo celular em G_1 e iniciando o reparo do DNA. Após o reparo, a p53 aumenta a transcrição da proteína mdm-2 ("murine double minute") que age como inibidora da p53 (Figura 5). A proteína mdm-2 se associa à p53 revertendo o bloqueio do ciclo celular e promovendo o avanço para a fase S (Sherr, 2000).

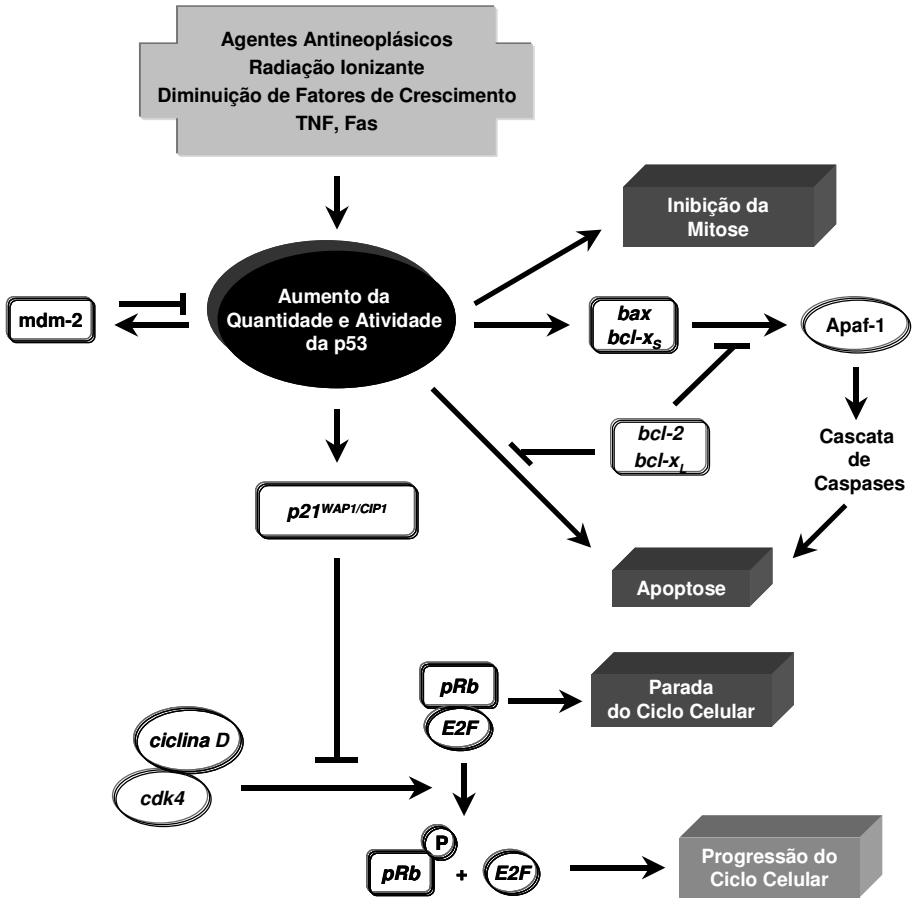


Figura 5: Componentes envolvidos na sinalização da p53 (Apaf-1 = "fator de ativação de protease associada a apoptose 1"; cdk4 = quinase dependente de ciclina 4; mdm-2 = "murine double minute") (Adaptado de Agarwal *et al.*, 1998).

Quando os danos no DNA não podem ser reparados, a ativação do ponto de checagem de G1 pode levar a célula a entrar em apoptose (Figura 5) (Steller, 1995; Ashkenazi and Dixit, 1998; Blagosklonny, 1999). Mutações no gene *p53* resultam em descontrole do ponto de checagem de G1, possibilitando que células danificadas entrem na fase S sem reparar as lesões. A probabilidade de estas alterações serem transmitidas para células-filha, é aumentada quando ocorrem simultaneamente mutações em elementos da cascata apoptótica, como por exemplo, nos genes *bcl-2* e *bax*. Esta instabilidade genômica cria condições para ocorrência de futuras mutações em oncogenes, além de induzir resistência às drogas citotóxicas (Agarwal *et al.*, 1998). De fato, mutações no *p*53 estão relacionadas com resistência a tratamentos com inibidores da timidilato sintase, como o 5-fluorouracil (Peters *et al.*, 2000). A p53 modula, também, a atividade de genes envolvidos com o controle da proliferação celular (Figura 5) (Fearon *et al.*, 1990).

Aproximadamente 15% dos cânceres colorretais esporádicos apresentam inserções e deleções de nucleotídeos com seqüências repetidas de DNA, chamadas Instabilidade de Microssatélites (MSI), devido ao defeituoso reparo de mal-emparelhamento (revisado em Grivicich *et al.*, 2005). Algumas destas alterações genéticas são utilizadas como marcadores prognósticos no câncer colorretal (McLeod and Murray, 1999). Por exemplo, a perda da heterozigosidade no cromossomo 18q indica um mau prognóstico (revisado em Grivicich *et al.*, 2005). Outras alterações que podem interferir no prognóstico incluem o aumento da expressão dos genes envolvidos no metabolismo das fluoropirimidinas como, por exemplo, as enzimas timidilato sintase e diidropirimidina desidrogenase (Macleod *et al.*, 1999; Salonga *et al.*, 2000). Além de alteração na expressão, o polimorfismo do gene *TS* é considerado uma ferramenta promissora para identificar o efeito clínico, toxicidade e risco de desenvolver o câncer de cólon (Kawakami *et al.*, 2001).

Vários outros mecanismos envolvidos na transformação adenoma-carcinoma foram sugeridos. Entre estes a participação da enzima ciclooxigenase (Cox). Esta enzima possui duas isoformas já identificadas: Cox-1 e Cox-2. Cox-1

é a forma constitutiva, enquanto que a Cox-2 somente se expressa após ser estimulada por fatores de crescimento, citoquinas e mitógenos. A participação da Cox na tumorigênese do carcinoma colorretal foi derivada de estudos com as prostaglandinas (Ferrandez *et al.*, 2003). Altos níveis de prostaglandinas especialmente PGE_2 foram encontrados em colônias e tumores colorretais humanos (Eberhart *et al.*, 1994). Consistente com essas observações, vários estudos demonstraram que o gene da *Cox-2* e a expressão desta proteína estão associados às neoplasias de cólon (Eberhart *et al.*, 1994; Hao *et al.*, 1999). A superexpressão da Cox-2 em tumores colorretais ocorre nos estágios iniciais (Figura 4) da tumorigênese (Hao *et al.*, 1999), e está associado com a invasão do tumor (Chen *et al.*, 2001).

4 MODALIDADES DE TRATAMENTO

Aproximadamente 70% dos pacientes com câncer de cólon se apresentam com doença aparentemente localizada no momento do diagnóstico. Uma vez confirmada a ausência de envolvimento metastático através de exame clínico, laboratorial e de imagem, estes pacientes são submetidos à ressecção cirúrgica com finalidade curativa (Andre *et al.*, 2004; revisado em Grivicich *et al.*, 2005). Entretanto, a curabilidade destes pacientes depende da presença ou não de células tumorais ocultas, não detectadas no momento do diagnóstico. Quando estes clones metastáticos persistem no organismo, após a retirada completa do tumor primário, levarão ao desenvolvimento de metástases à distância e à morte do paciente. Uma vez que a presença de metástases ocultas é maior à medida que aumenta o estadiamento do tumor, é de fundamental importância que o tratamento seja planejado de acordo com o estágio da doença (Skibber *et al.*, 2001). Sendo uma neoplasia de difícil diagnóstico inicial, a maioria dos pacientes

se apresenta com a doença em fase muito avançada, já com metástases nos linfonodos ou em outros órgãos (Haller, 1988).

Nos pacientes com estágios I e II, a cirurgia é curativa na maioria dos casos (aproximadamente 80%) (Plate, 2001). Entretanto, nos pacientes com estágio III, a presença de linfonodos regionais está associada a um maior risco de metástases ocultas à distância. Este fato reduz a chance de cura cirúrgica em 50% dos casos.

Esta é a base racional para o uso da quimioterapia sistêmica adjuvante após a cirurgia nos pacientes com metástases à distância. A quimioterapia sistêmica adjuvante tem sido capaz de reduzir cerca de 30-40% o risco de recidiva da doença em estudos prospectivos e randomizados (Sun and Haller, 2005). Ainda que sem a mesma comprovação científica, uma abordagem semelhante têm sido aplicada a pacientes com estágio II, que apresentam fatores prognósticos que sugiram maior risco de recidiva (Cohen *et al.*, 1997).

O tratamento padrão para pacientes com estágio III de câncer do cólon e de casos especiais de estágio II, consiste de cirurgia de ressecção tumoral completa e linfonodos regionais, seguida de quimioterapia adjuvante. O tratamento adjuvante, nestes casos, utiliza esquemas incluindo 5-fluorouracil (5-FU) e Leucovorin (LV) associados ou não a um terceiro agente (Tabela 3). A estimativa de sobrevida em 5 anos para pacientes com câncer de cólon no estágio I é de aproximadamente 85%, enquanto que para pacientes nos estágios II e III é de 70 a 80% e de 25 a 60%, respectivamente (Plate, 2001).

Com o objetivo de aumentar a eficácia do tratamento sistêmico dos pacientes portadores de câncer de cólon, vários estudos vêm sendo desenvolvidos no sentido de modular bioquimicamente a atividade do 5-FU (Peters and Van Groeningen, 1991), bem como identificar novos agentes e estratégias de tratamento (Grivicich *et al.*, 2001). Nas últimas décadas, além do irinotecan (CPT-11) e oxaliplatina (L-OHP), novos agentes com diferentes alvos ou diferentes formulações vêm sendo testados para incorporação no tratamento adjuvante. Estes agentes incluem as fluoropirimidinas orais, anticorpos

monoclonais e inibidores da ciclooxigenase-2 (Chau and Cunningham, 2002; Andre *et al.*, 2004).

Oxaliplatina (Eloxatin®; trans-*l*-diaminocyclohexane oxaloplatinum; L-OHP) é um composto platino de terceira geração e ativo em tumores colorretais, avaliada no estudo europeu de fase III denominado MOSAIC (De Gramont *et al.*, 2000; Giacchetti *et al.*, 2000). A L-OHP, através de conversão enzimática, se liga ao DNA resultando na inibição da replicação e transcrição (Raymond et al., 1998). A L-OHP demonstrou ser eficaz no tratamento de primeira linha bem como de segunda linha em pacientes com câncer de cólon refratários a terapia com 5-FU (Machover *et al.*, 1996). A associação da L-OHP com 5-FU/LV demonstrou importante atividade na terapia de primeira linha no câncer colo-retal (Tabela 3). Na Europa esta combinação foi aprovada no tratamento de segunda linha em 1999 e nos EUA em 2002 (De Gramont *et al.*, 2000; Giacchetti *et al.*, 2000).

A capecitabina (Xeloda

Tabela 3: Regimes quimioterápicos comumente utilizados no tratamento do câncer de cólon.

Regime	Esquema de Administração
NCCTG/ Mayo clinic	LV 20 mg/m^2 (i.v., bolo), 5-FU 425 mg/m^2 (i.v., bolo). Dias 1-5, repetido a cada 28 dias.
LV5FU2/ de Gramont	LV 200 mg/m^2 (i.v.i., 2 h), 5-FU 400mg/m^2 (i.v., bolo), 5-FU 600mg/m^2 (i.v.i., 22 h). Dias 1 e 2, repetido a cada 14 dias.
AIO	LV 500 mg/m^2 (i.v.i., 2 h), 5-FU 2600 mg/m^2 (i.v.i., 24 h). Uma vez por semana durante 6 semanas, repetido a cada 8 semanas.
Lokich	5-FU 300 mg/m^2/dia (i.v.i., 24 h). Infusão continua durante os dias 1-21, sem interrupção.
Capecitabina	2.500 mg/m^2/dia (oral, dividido em duas doses durante 14 dias). Intervalo de 7 dias, repetido a cada 21 dias.
Tegafur/uracil + folinato de cálcio	300 mg/m^2/dia tegafur + 672 mg/m^2/dia uracil + LV 90 mg/dia (oral, dividido em 3 doses durante 28 dias). Intervalo de 7 dias, repetido a cada 35 dias.
Raltitrexed	3 mg/m^2 (i.v.i., 15 min). Repetido a cada 21 dias.
Irinotecan	350 mg/m^2 (i.v.i., 30-90 min). Repetido a cada 21 dias.
FOLFIRI	LV 400 mg/m^2 (i.v.i., 2 h), CPT-11 180mg/m^2 (i.v.i., durante 90 min), 5-FU 400mg/m^2 (i.v., bolo), 5-FU 2.400 mg/m^2 (i.v.i., 46 h). Dia 1, repetido a cada 14 dias.
IFL	CPT-11 125 mg/m^2 (i.v.i., 90 min), LV 20 mg/m^2 (i.v., bolo), 5-FU 500 mg/m^2 (i.v., bolo). Uma vez por semana, durante 4 semanas, repetido a cada 6 semanas.
FOLFOX4	LV 200 mg/m^2 (i.v.i., 2 h), L-OHP 85mg/m^2 (i.v.i., 2 h), 5-FU 400mg/m^2 (i.v., bolo), 5-FU 600 mg/m^2 (i.v.i., 22 h). Dias 1 e 2 (L-OHP somente no dia 1), repetido a cada 14 dias.
FOLFOX6	LV 400 mg/m^2 (i.v.i., 2 h), L-OHP 100 mg/m^2 (i.v.i., 2 h), 5-FU 400mg/m^2 (i.v., bolo), 5-FU 2.400 mg/m^2 (i.v.i., 46 h). Dia 1, repetido a cada 14 dias.

CPT-11 = Irinotecan; 5-FU = 5-Fluorouracil; LV = Leucovorin; L-OHP = Oxaliplatina; i.v. = intravenoso; i.v.i. = infusão intravenosa.

O UFT (Orzel®), é outro exemplo de fluoropirimidina para uso oral, que consiste na combinação de uracil e tegafur (1-[2-tetrahydrofuranyl]-5-FU, ftorafur) na proporção de 4:1 (Hoff *et al.*, 1999) Tegafur é uma pró-droga do 5-FU que é rapidamente absorvida após administração oral. Após ser absorvido o Tegafur é hidroxilado e convertido em 5-FU através de enzimas hepáticas. O Uracil inibe a principal enzima do catabolismo do 5-FU, a diidropirimidina desidrogenase (DPD) A inibição da DPD, previne a degradação do 5-FU, permitindo a absorção do tegafur e aumentando as concentrações plasmáticas do 5-FU (Hoff *et al.*, 1999). Dois estudos de fase III em pacientes com carcinoma de colon avançado demonstraram que UFT/LV apresentou efeito equivalente aos esquemas com 5-FU/LV (Hoff *et al.*, 1999; Douillard *et al.*, 2002).

A angiogênese tem um papel importante no crescimento tumoral, invasão e metástase. O fator de crescimento endotelial vascular (VEGF) é de grande importância na promoção da angiogênese (Figura 6), tornando-se um alvo para as terapias anticâncer. Tumores colorretais que apresentam uma elevada expressão de VEGF estão relacionados com um prognóstico ruim (Cascinu *et al.*, 2000). O Bevacizumab (Avastin®), anticorpo monoclonal contra o VEGF recebeu em fevereiro de 2004, aprovação do FDA para uso na terapia de primeira linha em pacientes com câncer colorretal metastático. O Bevacizumab é o primeiro produto aprovado que previne a angiogênese (FDA – U.S. Food and Drug Administration, 2004). Este anticorpo demonstrou um aumento de sobrevida, quando adicionado ao esquema IFL (CPT-11/LV/5-FU em bolo) em estudos de fase II (Rothenberg, 2004).

O crescimento tumoral também depende da ativação de receptores de membrana como o receptor do fator de crescimento epidérmico (rEGF) que controlam a sinalização intracelular para proliferação, adesão e migração (Figura 6). A superexpressão do rEGF têm sido freqüentemente associada às baixas respostas ao tratamento e a progressão da doença (Baselga *et al.*, 2001). O cetuximab (Erbitux®), anticorpo monoclonal quimérico humano-murino, se liga seletivamente ao rEGF resultando na inibição da proliferação e angiogênese, além de estimular a apoptose (Baselga *et al.*, 2001). Em um estudo de fase II, o

cetuximab associado ao CPT-11, demonstrou maior atividade quando comparado com CPT-11 como agente único (Mendelsohn, 2001).

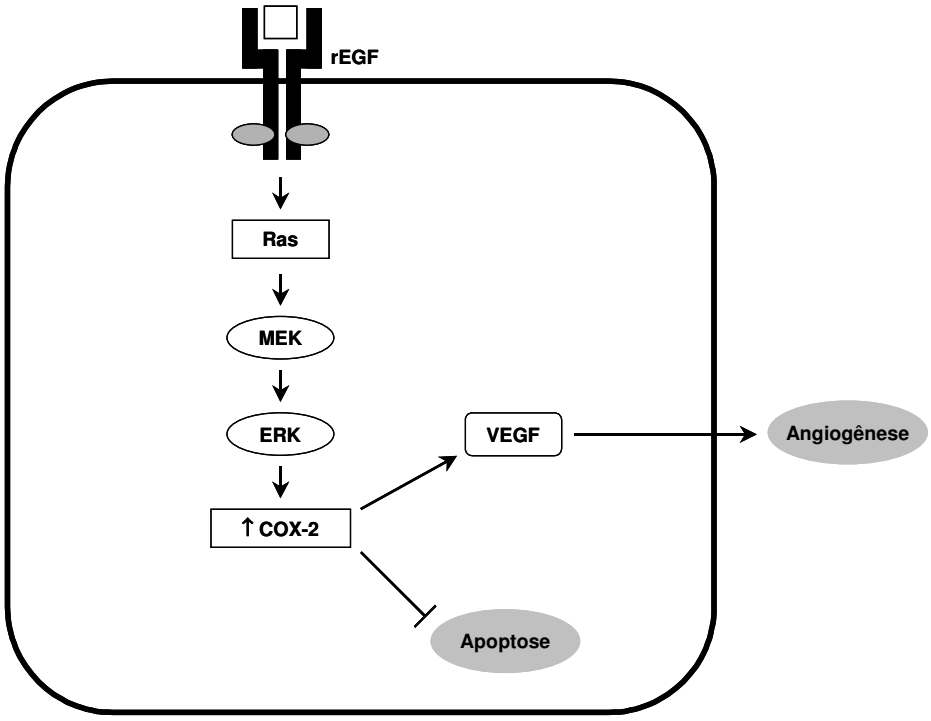


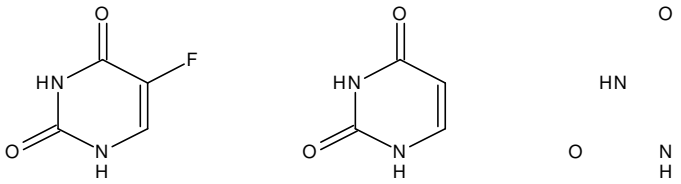
Figura 6: Papel do receptor do fator de crescimento epidérmico (rEGF), do fator de crescimento endotelial vascular (VEGF) e da enzima ciclooxigenase-2 (COX-2) na progressão tumoral (MEK = "mitogen-activated protein kinase/extracellular-signal-regulated kinase 1"; ERK = "extracellular-signal-regulated kinases").

Estudos recentes demonstraram que a ativação do rEGF pode induzir (Figura 6) a expressão da enzima ciclooxigenase-2 (Cox-2) (Kulkarni *et al*., 2001). A superexpressão da Cox-2 participa da proliferação celular, indução da angiogênese, aumento da invasão e metástase e inibição da apoptose (Gately, 2000; Church *et al*., 2003). Recentemente, foi demonstrado que o celecoxib (Celebrex®), um inibidor da Cox-2 pode aumentar as respostas obtidas com agentes quimioterápicos, sem induzir aumento de toxicidade (McMurray and Hardy, 2002). Novos inibidores da Cox-2 estão sendo avaliados no tratamento do câncer de cólon (Tortora *et al*., 2003).

4.1 5-FLUOROURACIL

Embora introduzido na clínica em 1957 e de apresentar respostas em no máximo 15% dos casos, o 5-Fluorouracil (5-FU) ainda é o agente mais utilizado no tratamento dos carcinomas colorretais (Heidelberger *et al.*, 1957; Andre *et al.*, 2004).

O 5-FU é um agente antimetabólito com peso molecular de 130.1 D que se caracteriza por apresentar semelhanças estruturais com intermediários da síntese dos ácidos nucléicos. Ele pode ser incorporado no lugar dos nucleotídeos do DNA ou do RNA causando alterações durante a replicação e transcrição (Grem, 1996). O 5-FU é um análogo estrutural do Uracil que apresenta um flúor substituindo o hidrogênio no carbono 6. Devido a esta semelhança estrutural, o 5-FU pode utilizar as mesmas rotas metabólicas que o Uracil e a Timina (Grem, 1996) (Figura 7).



URACIL

O 5-FU é uma pró-droga que para se tornar ativa necessita ser convertido no desoxirribonucleotídeo fluorodeoxiuridina monofosfato (FdUMP; Figura 7) pela ação da enzima Timidina Quinase (Figura 8) (Grem, 1996; Pinedo and Peters, 1998). O FdUMP, um desoxirribonucleotídeo com peso molecular de 326.2 D, interfere com a síntese do DNA através da inibição da enzima Timidilato Sintase (TS) (Grem, 1996). A TS catalisa a metilação da deoxiuridina monofosfato (dUMP; Figura 7) em deoxitimidina monofosfato (dTMP), que será, posteriormente, convertida em deoxitimidina trifosfato (dTTP) (Figura 8). O FdUMP compete com o dUMP ao ligar-se a TS, formando um complexo estável e de lenta dissociação, impedindo a formação do dTTP (Grem, 1996).

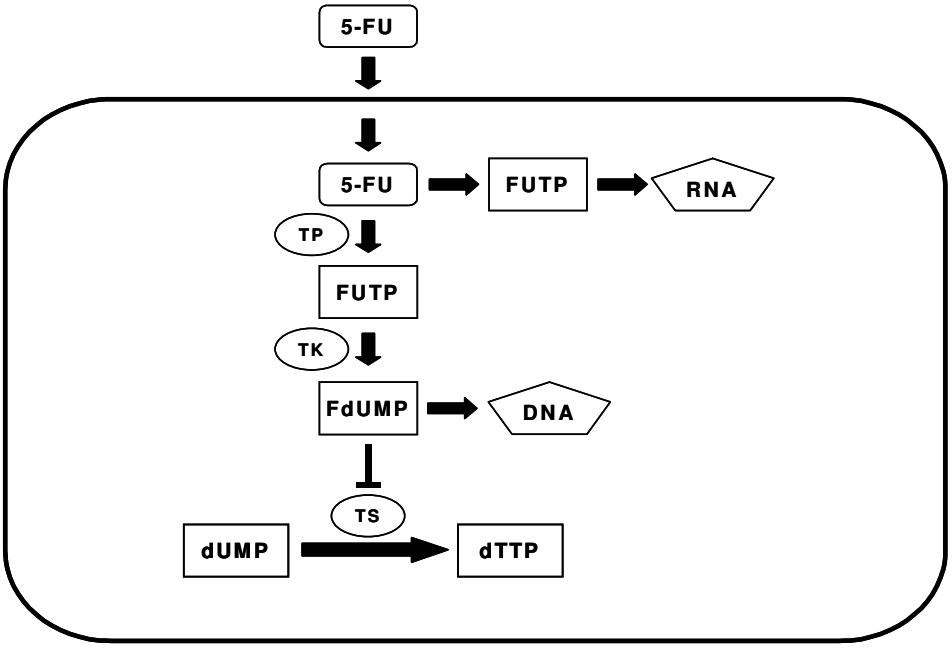


Figura 8: Mecanismos de ação do 5-Fluorouracil (5-FU = 5-Fluorouracil; TP = timidina fosforilase; TK = timidina quinase; TS = timidilato sintase; dUMP = deoxiuridina monofosfato; dTTP = deoxitimidina trifosfato; FdUMP = fluorodeoxiuridina monofosfato; FUTP = fluorouridina-5'-trifosfato).

Embora estas observações sustem que a inibição da TS seja o principal mecanismo de ação do 5-FU, o processamento defeituoso do RNA, após a

incorporação do ribonucleotídeo fluorouridina-5'-trifosfato (FUTP) (Mandel *et al.*, 1981), além de quebras no DNA induzidas pela incorporação do desoxirribonucleotídeo fluorodeoxiuridina-5'-trifosfato (FdUTP) no DNA (Schuetz and Diasio, 1985), podem contribuir com os diferentes níveis de citotoxicidade do 5-FU (Figura 8).

O 5-FU pode ser administrado em bolo ou em infusão contínua. É sugerido que após a administração em bolo, o mecanismo de ação do 5-FU ocorre através da interferência com o RNA. Enquanto que a inibição da TS é observada após infusão contínua (Sobrero *et al.*, 2000). Quando utilizado isolado, em bolo, o 5-FU apresenta taxas de respostas entre 10%-15%, quando administrado junto com leucovorin (LV) estas respostas podem chegar a 25%, (Skibber *et al.*, 2001). Este aumento significativo é devido a maior inibição da TS na presença do LV (Peters and Van Groeningen, 1991). O LV é um derivado do tetraidrofolato e serve como fonte intracelular de folato reduzido. O LV prolonga a estabilização do complexo entre FdUMP/TS (Peters and Van Groeningen, 1991; Pinedo and Peters, 1998).

4.2 IRINOTECAN

O Irinotecan (CPT-11; Camptosar®; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, Figura 9) é um análogo semi-sintético da Camptotecina, alcalóide isolado das folhas da árvore chinesa *Camptotheca acuminata* (Takimoto and Arbuk, 1996; Rothenberg, 2001). O CPT-11 tem peso molecular de 677 D e apresenta melhor solubilidade em soluções aquosas e toxicidade mais baixa quando comparado com a camptotecina (Takimoto and Arbuk, 1996).

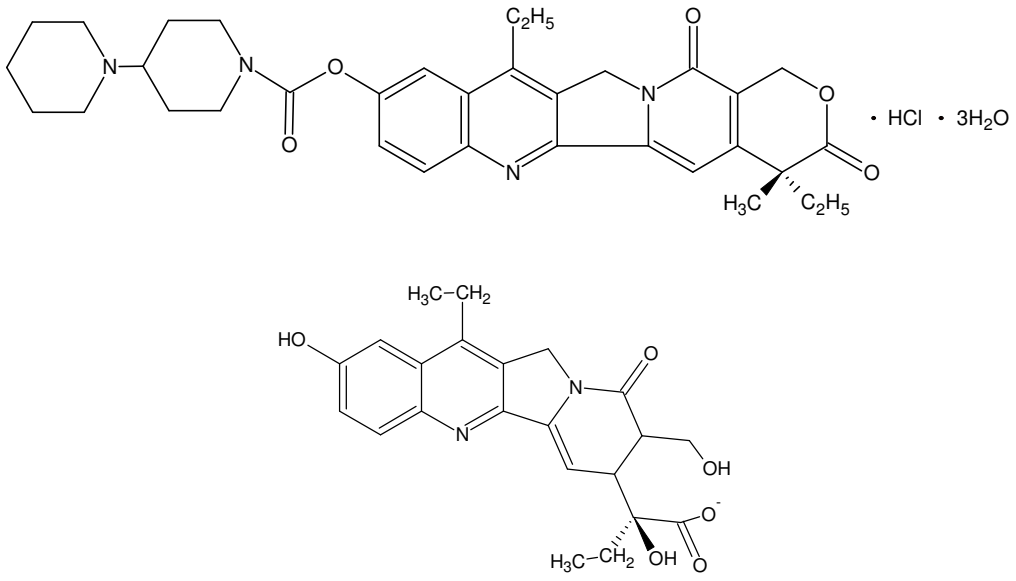


Figura 9: Estruturas do Irinotecan (CPT-11) e SN-38.

Por ser uma pró-droga, o CPT-11 deve ser convertido em SN-38 (7-ethyl-10-hydroxycamptothecin, Figura 9), que é 100-1000 vezes mai

em uma das cadeias do DNA, promovendo o relaxamento deste. Após a replicação a topo I se desliga do DNA e este é regenerado (Figura 10) (Vosberg, 1985; Takimoto *et al.*, 1997).

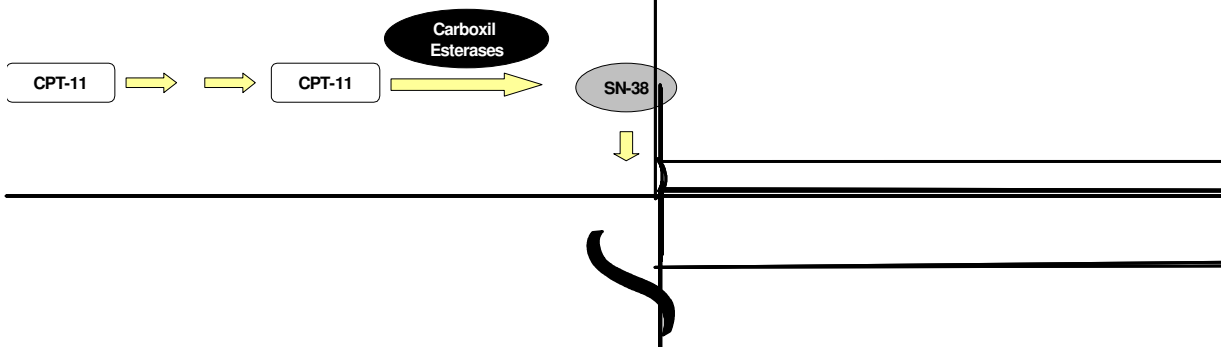


Figura 10: Mecanismo de ação do Irinotecan (CPT-11 = irinotecan; Topo I = Topoisomerase I).

A citotoxicidade do CPT-11 se baseia em manter estável o complexo DNA-topo I, formando um complexo ternário (droga + enzima + DNA). A maneira como a estabilização deste complexo leva a célula à morte não está bem elucidada. Foi demonstrado que este complexo impede a religação da fita de DNA feita pela topo I (Figura 10), resultando na formação de quebras no DNA (Kunimoto *et al.*, 1987; Stewart and Schutz, 1987; Takimoto *et al.*, 1997; Kerrigan and Pilch, 2001). Este processo induz a parada do ciclo celular em S/G2, causando a morte das células. Para que o CPT-11 exerça sua toxicidade é necessário que a síntese do DNA esteja em andamento, indicando que a citotoxicidade do CPT-11 é dependente da

fase S do ciclo celular (Kirstein *et al.*, 2002). Com base nestas observações, foi hipotetizado que a forquilha de replicação do DNA colide com este complexo DNA-topo I, levando à formação de quebras no DNA, bloqueio em G2 e morte celular (Avemann *et al.*, 1988; Hsiang *et al.*, 1989).

Em estudos pré-clínicos, o CPT-11 demonstrou atividade em vários tipos de tumores, incluindo os do trato gastrointestinal. Em subseqüentes estudos de fase I e fase II observou-se ampla ação antitumoral em vários tumores sólidos, incluindo carcinomas de cólon, além de leucemias e linfomas (revisado em Grivicich *et al.*, 2005). Devido a sua eficácia no câncer de cólon o FDA aprovou a utilização do CPT-11 como monoterapia no tratamento de segunda linha nesta doença (Sasaki *et al.*, 1994).

4.3 COMBINAÇÃO CPT-11/5-FU

Muitos estudos têm sido realizados no intuito de avaliar a combinação do CPT-11/5-FU/LV quanto a sua eficácia terapêutica no carcinoma de cólon. Evidências *in vivo* e *in vitro* sugerem uma interação de citotoxicidade dependente da seqüência de administração do 5-FU e CPT-11 (Grivicich *et al.*, 2001; Andre *et al.*, 2004).

Em estudos pré-clínicos a utilização do 5-FU junto com o CPT-11 ou o SN-38 demonstrou efeito aditivo na citotoxicidade do 5-FU em linhagens celulares de cólon, leucemia e pâncreas (revisado em Grivicich *et al.*, 2001). Em um estudo com seis linhagens celulares de cólon foi observado um sinergismo com a administração do SN-38 antes do 5-FU (Pavillard *et al.*, 1998). Também foi descrito um sinergismo quando o 5-FU foi utilizado antes do CPT-11 ou do SN-38 em linhagens de cólon (Guichard *et al.*, 1998b), enquanto que em outro estudo a mesma seqüência demonstrou ser antagônica (Mans *et al.*, 1999). Com base nos efeitos sinérgicos obtidos em estudos pré-clínicos, além dos resultados como agentes isolados, distintos mecanismos de ação e resistência e uma

sobreposição parcial de toxicidade, diferentes combinações com CPT-11 e 5-FU têm sido avaliadas (revisado em Grivicich *et al.*, 2001).

Diferentes protocolos combinando CPT-11 e 5-FU foram investigados em estudos de fase I na Europa, Estados Unidos e Japão (Comella *et al.*, 1999; Ducreux *et al.*, 1999; Vanhoefer *et al.*, 1999). Em subseqüentes estudos de fase II, a combinação foi avaliada como segunda ou primeira linha no tratamento do câncer de cólon. O chamado regime FOLFIRI (ácido FOLínico, 5-Fluorouracil, IRInotecan) foi eficaz como tratamento de segunda linha contra carcinoma colorretal metastático, produzindo uma citotoxicidade aceitável (Andre *et al.*, 1999). Dois estudos clínicos, randômicos, controlados (Douillard *et al.*, 2000; Saltz *et al.*, 2000) levaram a aprovação do regime CPT-11/5-FU/LV pelo FDA, nos EUA, em abril de 2000. Nestes estudos, a combinação de CPT-11 e 5-FU/LV resultou em um significativo retardo no tempo de progressão dos tumores e um aumento significativo na sobrevida, quando comparada com o protocolo 5-FU/LV (revisado em Grivicich *et al.*, 2001). Por esta razão, desde 2000, o regime CPT-11/5-FU/LV é o tratamento quimioterápico de primeira linha mais amplamente usado nos EUA para pacientes com câncer colorretal metastático (Andre *et al.*, 2004).

Entretanto, nem todos os pacientes respondem aos tratamentos quimioterápicos. A resistência às drogas antineoplásicas é um dos maiores problemas e causas do fracasso das terapias anticâncer (Gorlick *et al.*, 1999). Existem muitos mecanismos de resistência, como a superexpressão da glicoproteína P (pgp) (Beck, 1987), superexpressão do gene *bcl-2* (Fischer *et al.*, 1993) ou a supressão das DNA topoisomerases I e II (Beck, 1989), entre outros.

A resistência ao 5-FU parece ser determinada por alterações nas enzimas envolvidas em seu metabolismo, particularmente as enzimas responsáveis pela conversão do 5-FU em FdUMP (timidina fosforilase e timidina quinase). Além disso, alterações nos níveis da TS e afinidade desta com FdUMP também estão associados com a resistência ao 5-FU (Spears *et al.*, 1988; Peters *et al.*, 1995). Alterações na atividade da enzima topo I está relacionada à diminuição da capacidade do CPT-11 de introduzir quebras no DNA, sendo considerado o principal mecanismo de resistência a este agente (Schneider *et al.*, 1990;

Takimoto and Arbuk, 1996). Além disso, a enzima carboxil esterase (essencial para a conversão do CPT-11 no seu metabólito ativo SN-38) tem um papel importante na resposta ao tratamento com CPT-11. Neste sentido, Van Ark-Otte *et al*. (1998) demonstrou uma maior resistência ao CPT-11 em linhagens celulares com baixa expressão de carboxil esterase.

5 TIMIDILATO SINTASE

Significativa expressão da TS foi observada em melanomas, leucemias, tumores de ovário, cérvix, mama, pâncreas, pulmão, cabeça e pescoço, gástricos e colorretais. Tanto a atividade quanto à expressão desta enzima é maior (10-40%) em tecidos tumorais, quando comparados com tecidos normais (Parr *et al.*, 1998; Nishimura *et al.*, 1999; Otake *et al.*, 1999; Suzuki *et al.*, 1999). Neste sentido, foi demonstrado que a elevada expressão de TS em tumores colorretais está relacionada com resistência às terapias baseadas no 5-FU (Peters *et al.*, 1995). Além de ser considerada um alvo interessante para agentes antineoplásicos, a TS está envolvida com processos de morte e proliferação celular. A expressão da TS é dependente do ciclo celular, apresentando uma maior atividade durante a fase S (Johnson, 1992). Estudos mostraram que a expressão da TS pode ser inibida pela expressão de *p*53 e *p*21 através da supressão do E2F, o fator de transcrição desta enzima (Agarwal *et al.*, 1998; Van Triest *et al.*, 1999a).

A TS é uma enzima citosólica que catalisa a metilação não reversível do dUMP em dTMP, o precursor do dTTP. O co-substrato para a TS é o 5,10-metilenotetraidrofolato (CH_2THF), o qual forma um complexo ternário com o dUMP e a TS (Figura 11). Após transferir o grupo metil para o dUMP, o diidrofolato se desliga, seguido do dTMP (Takemura and Jackman, 1997). O 5-FU inibe a TS através da formação do FdUMP, que vai competir com o dUMP

ligando-se a TS e ao folato (Figura 11). Este novo complexo ternário é de difícil dissociação, porque o grupo metil não pode ser transferido (Lockshin *et al.*, 1984).

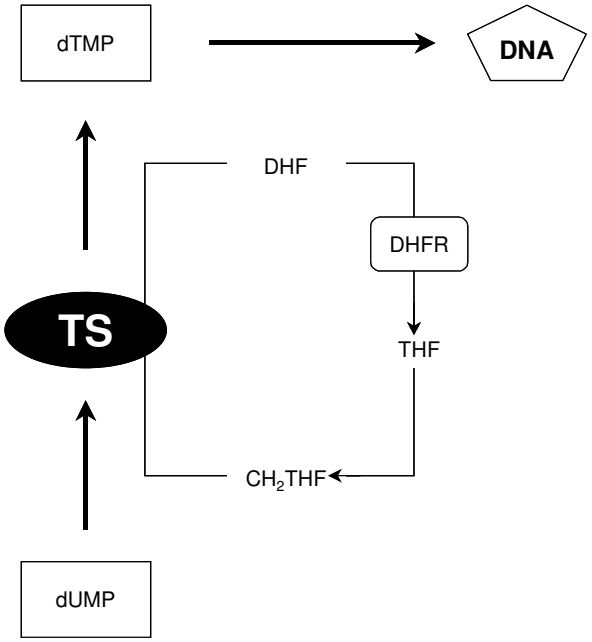


Figura 11: Mecanismo de ação da enzima timidilato sintase (TS = timidilato sintase; DHF = diidrofolato; DHFR = diidrofolato redutase; THF = tetraidrofolato; CH_2THF =5,10-metilenotetraidrofolato; dUMP = deoxiuridina monofosfato; dTMP = deoxitimidina monofosfato).

Assim como diversas proteínas envolvidas na síntese de DNA, a TS é regulada durante o ciclo celular (Chellappan *et al.*, 1991). A progressão do ciclo celular de G1 para S é determinada pela capacidade da proteína Rb (pRb) e proteínas associadas em inibir a atividade dos fatores de transcrição da família do E2F. Quando não fosforilada, a pRb se liga ao fator de transcrição E2F, impedindo que o DNA transcreva os genes necessários para o andamento do ciclo celular (Banerjje *et al.*, 1998). A fosforilação da pRb causa a separação do complexo pRb-E2F, liberando o fator de transcrição E2F (Figura 5). O E2F livre ativa a transcrição de genes necessários para a síntese do DNA, como, por exemplo, o da TS (Banerjje *et al.*, 2000). O aumento da expressão de E2F leva a

expressão aumentada da TS e, por conseqüência, a baixas respostas ao 5-FU (Van Triest *et al.*, 1999a). Foi demonstrado, também, que células com superexpressão de E2F são mais sensíveis ao tratamento com SN-38 (metabólito ativo do CPT-11) independente da sensibilidade ao 5-FU (Banerjje *et al.*, 2000). Além disso, a TS pode se ligar ao seu próprio RNAm e ao *p53*, reprimindo a sua transcrição (Ju *et al.*, 1999).

A inibição da TS causa danos no DNA (Spears *et al.*, 1988; Van Triest and Peters, 1999b), parada do ciclo celular (Tonkinson *et al.*, 1997) e apoptose (Backus *et al.*, 2001). O dano ao DNA induzido pela inibição da TS parece ativar proteínas que estimulam a apoptose (Backus *et al.*, 2001; Backus *et al.*, 2003). Por exemplo, foi demonstrado que a expressão de *p*53 e do receptor Fas aumentam após a inibição da TS pelo antifolato AG337, em linhagens celulares de carcinoma de cólon (Backus *et al.*, 2003). Também foi observado a existência de uma associação entre a redução na taxa *bcl-*x_L*/bax* e apoptose após exposição a inibidores da TS (Shimizu *et al.*, 1996).

6 TOPOISOMERASE I

A topo I é uma enzima nuclear de 100 kDa encontrada tanto em procariotos quanto em eucariotos (Takimoto *et al.*, 1997). Em mamíferos, esta enzima está diretamente envolvida com a manutenção da estrutura topográfica do DNA durante os processos de tradução, transcrição, reparação e divisão celular (Vosberg, 1985; Wang, 2002; Malik and Nitiss, 2004), promovendo a conversão do DNA superenrolado em DNA relaxado (Stewart and Schutz, 1987; Takimoto *et al.*, 1997). Para isto, a topo I introduz, no sítio de replicação de uma das cadeias do DNA, uma quebra reversível (Schneider *et al.*, 1990), formando um complexo covalente entre a enzima e o DNA, chamado de complexo clivável (Pommier, 1996; Takimoto *et al.*, 1997; Wang, 2002). Depois de se ligar na molécula de DNA

a topo I corta uma das fitas, gerando simultaneamente uma ligação fosfodiéster covalente entre o fosfato 5` liberado no DNA e um resíduo de tirosina na enzima. A extremidade hidroxila 3` do DNA é mantida em covalência pela enzima. A fita de DNA que não foi clivada passa por dentro do sítio de clivagem. A fita clivada é então liberada, formando uma estrutura com as mesmas ligações que o DNA no início, mas com uma super-hélice negativa a menos (Wang, 1996). A seguir, ocorre a religação da fita e dissociação da enzima, regenerando a dupla fita de DNA intacta (Figura 12) (Vosberg, 1985; Wang, 2002). A topo I não necessita de ATP ou NAD para a ligação, utilizando a energia armazenada na clivagem (Wang, 2002).

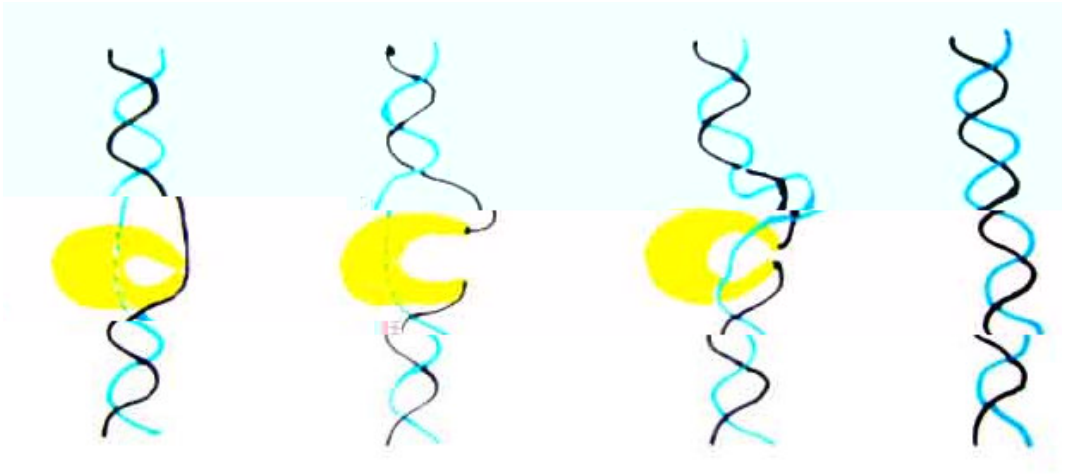


Figura 12: Mecanismo de ação da enzima topoisomerase I (Topo I) (Adaptado de Zaha, 1996).

A toxicidade dos agentes inibidores da topo I ocorre através da estabilização do complexo enzima-DNA-inibidor. Sabe-se que o acúmulo e a prolongada estabilização destes complexos resultam em defeitos irreversíveis na replicação do DNA, o que leva a morte celular (Pommier, 1996). Tumores com níveis elevados de topo I são alvos potenciais para os in 0 0 1584 Tc 5.63984 0 Td (i)Tj

inibidores da topo I depende do tempo de exposição e concentração do agente (Pommier, 1996).

7 APOPTOSE

O desenvolvimento e manutenção de organismos multicelulares dependem de uma interação entre as células que formam este organismo. Durante o desenvolvimento embrionário, muitas células produzidas em excesso são levadas à morte, contribuindo para a formação dos órgãos e tecidos (Meier *et al.*, 2000). Durante muito tempo à morte celular foi considerada um processo passivo de caráter degenerativo, que ocorre em situações de lesão celular, infecção e ausência de fatores de crescimento. Como conseqüência, a célula altera a integridade da membrana plasmática, incha e perde suas funções metabólicas (Yu and Choi, 2000). Entretanto nem todos os eventos de morte celular são processos passivos. Organismos multicelulares são capazes de induzir uma morte celular programada – Apoptose – como resposta a estímulos intra ou extracelulares (Hengartner, 2000). A apoptose é um processo fisiológico essencial para o desenvolvimento embrionário, diferenciação, proliferação, funcionamento do sistema imune e homeostasia dos tecidos (Rathmell and Thompson, 2002; Zuzarte-Luis and Hurle, 2002). Defeitos na apoptose podem levar a um aumento na proliferação celular, observado no câncer, em doenças autoimunes e infecções virais. Enquanto que, a apoptose em excesso é observada nas doenças neurodegenerativas ou isquêmicas e AIDS (Fadeel *et al.*, 1999).

A apoptose pode ser reconhecida por características morfológicas muito marcantes (Figura 13): retração da célula; perda de aderência com a matriz extracelular e células vizinhas, devido a um rearranjo do citoesqueleto; condensação da cromatina; fragmentação internucleossômica do DNA; formação de pequenas bolhas envoltas pela membrana plasmática; fragmentação celular e

formação dos corpos apoptóticos, sem liberação do conteúdo citoplasmático para o meio extracelular (Ziegler and Groscurth, 2004). Os corpos apoptóticos serão, a seguir, fagocitados por macrófagos e removidos sem causar processo inflamatório (Figura 13).

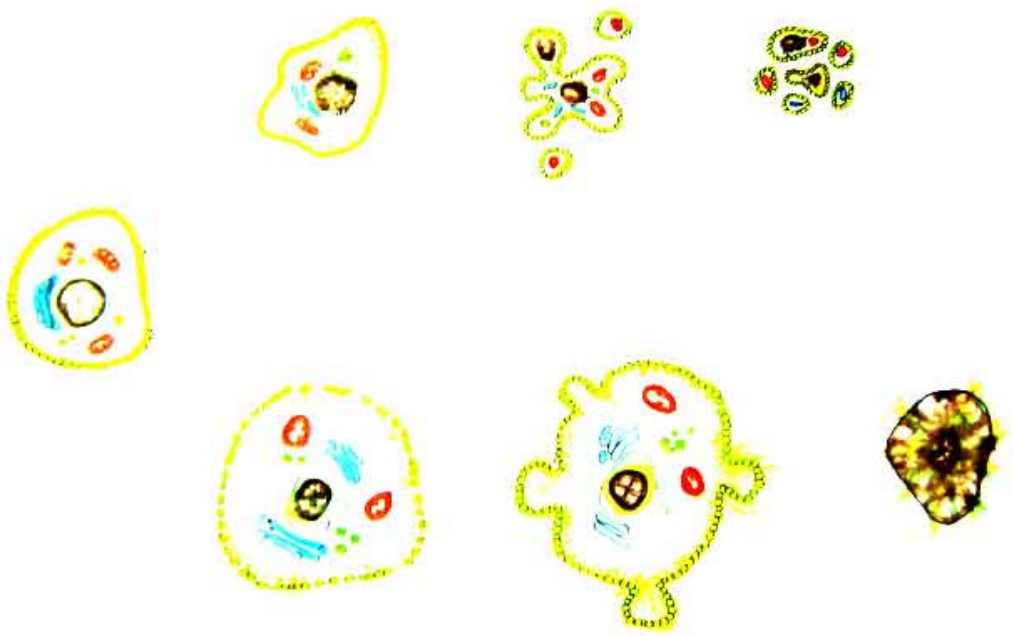


Figura 13: Características morfológicas da apoptose e necrose (Adaptado de van Cruchten, 2002).

Diferente da apoptose, a necrose é um tipo de morte onde as células sofrem um insulto irreversível que resulta na perda da integridade da membrana plasmática, inchaço da célula e ruptura da

7.1 MECANISMOS MOLECULARES DA SINALIZAÇÃO DA APOPTOSE

A apoptose pode ser desencadeada por uma variedade de estímulos que conduzem a subprogramas, que quando desencadeados, induzem complexas interações entre as proteínas supressoras (bcl-2, bax-1, bcl-x_L) e promotoras da apoptose (bax, bcl-x_S) (Hengartner, 2000; Borner, 2003).

7.1.1 Moléculas sinalizadoras

A apoptose é um programa de morte celular extremamente regulado e de grande eficiência, que requer a interação de inúmeros fatores. As alterações morfológicas observadas são conseqüência de uma cascata de eventos moleculares e bioquímicos específicos e geneticamente regulados (Saraste and Pulkki, 2000).

Quando células do sistema imune reconhecem células infectadas por antígenos não pertencentes ao organismo, acopla um ligante de sua membrana a um receptor na membrana da célula infectada que irá sinalizar a morte por apoptose. Estes receptores de superfície celular são denominados receptores de morte (“death receptor”), e induzem a ativação da apoptose após ligação com ligantes específicos. Os receptores de morte pertencem à superfamília dos receptores de fatores de necrose tumoral (“tumor necrosis factor receptor”, rTNF). Esta família inclui o rTNFR-1, Fas/CD95, Trail (Ashkenazi, 2002).

Todos os membros da família rTNF possuem um sub-domínio extracelular rico em cisteína, o qual permite que eles reconheçam seus ligantes. Tal fato resulta na trimerização e conseqüente ativação dos receptores de morte específicos (Figura 14) (Naismith and Sprang, 1998). A sinalização a seguir é mediada pela porção citoplasmática destes receptores de morte que contém uma

seqüência conservada chamada domínio de morte (“Death Domain”) (Naismith and Sprang, 1998).

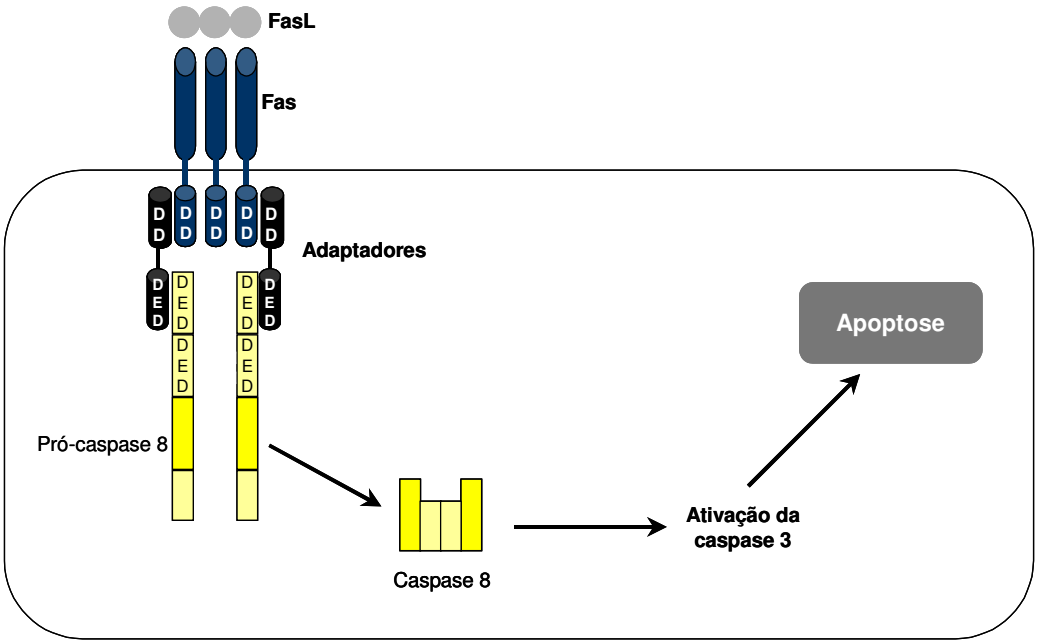


Figura 14: Indução da apoptose via Fas (DD = domínio de morte; DED = efetor do domínio de morte).

O receptor Fas é um membro da família dos receptores de morte com grande importância como mediador da apoptose (Krammer *et al.*, 1994; Nagata and Golstein, 1995). Este receptor é expresso constitutivamente em células normais do cólon bem como nas células de carcinoma de cólon (Moller *et al.*, 1994). O sistema Fas/FasL (receptor e ligante Fas) é essencial na sinalização da apoptose (Walczak and Krammer, 2000). Vários estudos sugerem que o sistema Fas/FasL pode estar envolvido na citotoxicidade do 5-FU (Friesen *et al.*, 1997; Tillman *et al.*, 1999; Eishorst *et al.*, 2001). A inibição da timidilato sintase pelo 5-FU parece ser o mecanismo através do qual o receptor Fas ativa a apoptose (Houghton *et al.*, 1997; Longley *et al.*, 2002).

Além dos receptores de membrana, diversos fatores podem desencadear a apoptose, entre estes: agentes quimioterápicos, radiação ionizante, danos no DNA, choque térmico, falta de fatores de crescimento, baixa quantidade de nutrientes e níveis aumentados d u sp i

Li and Oberley, 1997). Recentemente foi demonstrado que o 5-FU pode levar a um aumento de ERO na linhagem OSC-4 de carcinoma celular escamoso (Li *et al*., 2004). Da mesma forma, Kishida *et al.* (2004), observou que o SN-38 (metabólito do CPT-11) foi capaz de gerar ERO na linhagem AGS de câncer gástrico.

Além do seu papel como antioxidante, estudos recentes (Zhang *et al*., 2002; Cullen *et al*., 2003) sugerem que a atividade da SOD pode interferir na proliferação celular e induzir a apoptose (Hussain *et al*., 2004). Zhang *et al.* (2002) demonstrou que o aumento da atividade da SOD reduziu o crescimento em diferentes tumores, sugerindo que a SOD pode agir como um gene supressor de tumor. Recentemente, Hussain *et al.* (2004) mostrou que a superexpressão da Mn-SOD aumenta o estresse oxidativo e apoptose.

7.2 MECANISMOS REGULADORES DA SINALIZAÇÃO DA APOPTOSE

As vias de ativação da apoptose por agentes quimioterápicos estão relacionadas com receptores promotores de morte celular, ou mudanças na função mitocondrial. Tais processos levam a subprogramas envolvendo a liberação de fatores de ativação da cascata de caspases, responsáveis pelas alterações características da apoptose (mudanças na membrana plasmática, clivagem de proteínas do citoesqueleto, condensação nuclear e fragmentação de DNA) (Debatin *et al*., 2004; Kaufmann and Earnshaw, 2000). A caspase-8 é ativada pelo receptor promotor de morte celular Fas (Walczak and Krammer, 2000) e a caspase-9 pela liberação do citocromo c da mitocôndria (Crompton *et al*., 1999), ambas vão ativar a caspase-3 induzindo a apoptose (Kaufmann and Earnshaw, 2000).

A ativação da apoptose pode ser iniciada de duas diferentes maneiras: pela via intrínseca ou pela via extrínseca. A via intrínseca é ativada por estresse intra ou extracelular como a falta de fatores de crescimento, danos no DNA, hipóxia ou

ativação de oncogenes. Os sinais que são transduzidos em resposta a estes insultos convergem principalmente para a mitocôndria. A seguir diversos eventos bioquímicos ocorrem. Entre estes, a permeabilização da membrana mitocondrial externa (Crompton, 1999; Hengartner, 2000), liberação do citocromo c, formação do apoptossomo e ativação das caspases (Budihardjo *et al.*, 1999). Por outro lado, a via extrínseca é desencadeada pela ligação dos receptores de morte na superfície celular. Quando uma célula recebe estimulo de morte, como interação do Fas com seu ligante FasL ou do rTNF com TNF, é ativada a cascata das caspases (Budihardjo *et al.*, 1999).

7.2.1 Caspases como iniciadores e executores da apoptose

As caspases pertencem à família das cisteínas proteases que tem capacidade de reconhecer e clivar substratos que possuam resíduos aspartato (Nicholson and Thornberry, 1997). As caspases sinalizam para apoptose e clivam substratos proporcionando a condensação e fragmentação nuclear, externalização de fosfolipídios de membrana que irão sinalizar para estas células serem fagocitadas por macrófagos e a desmontagem do citoesqueleto para a futura formação dos corpos apoptóticos (Nicholson and Thornberry, 1997; Boatright and Salvesen, 2003).

Os estudos envolvendo ativação das caspases iniciaram com o nematódio *Caenorhabditis elegans* (Liu and Hengartner, 1999). Nestes organismos o gene supressor de apoptose CED-9 (homólogo ao gene humano bcl-2) sempre está associado com o gene CED-4, o que impede a ativação de CED-3. Quando ativada a apoptose, a proteína EGL-1 se associa a CED-9 induzindo a liberação de CED-4, ativando o CED-3 (Figura 15). Em humanos o processo é muito semelhante ao que ocorre com *C. elegans* (Figura 15) (Liu and Hengartner, 1999; Hengartner, 2000).

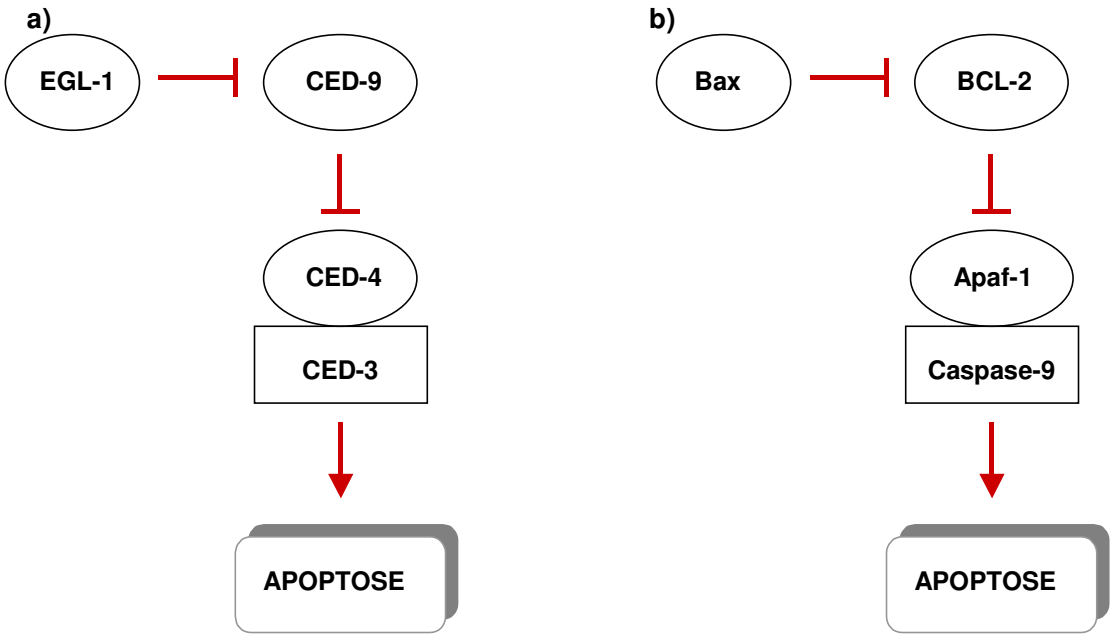


Figura 15: Modelo de ativação das caspases em *Caenorhabditis elegans* (a) e humanos (b).

As caspases são sintetizadas como precursores inativos chamados zimogênios (Hengartner, 2000). São conhecidas 12 caspases humanas, sendo que seis (caspases 3, 6, 7, 8, 9, 10) participam da apoptose. Após um sinal de morte celular, as caspases são ativadas por clivagem proteolítica. Estas enzimas podem interagir com receptores de membrana ou moléculas adaptadoras que contenham domínios de morte (Boatright and Salvesen, 2003), pois estes domínios também existem nas caspases e a presença deles permite esta interação (Nicholson and Thornberry, 1997).

As caspases podem ser classificadas de acordo com seu pró-domínio e seu papel na apoptose. Caspases iniciadoras: possuem pró-domínios longos, envolvidas na iniciação da cascata proteolítica. Caspases efetoras: com pró-domínios curtos ou inexistentes, responsáveis pela clivagem dos substratos (Rupnarain *et al.*, 2004). Entre os diversos substratos das caspases podemos citar o mdm-2, uma proteína que se liga ao p53, mantendo-a no citoplasma. Ao ser clivada pelas caspases esta proteína libera a p53 que se transloca para o

núcleo ativando a transcrição de genes pró-apoptóticos como o *bax* (Schuler *et al*., 2003).

As caspases possuem inibidores naturais e artificiais. Proteínas de baculovírus e Crma são os inibidores naturais mais conhecidos, atuando na inibição destas enzimas e evitando a morte da célula hospedeira para a reprodução do vírus. Existem muitos inibidores formados por tetrapeptídios sintéticos específicos para as diferentes combinações de aminoácidos relativas aos sítios de reconhecimento das caspases, entre estes o inibidor geral de caspases Z-VAD-fmk (Dotto and Silke, 2004).

7.2.2 Mitocôndria como reguladora da apoptose

Inúmeros estudos sobre apoptose apontam a mitocôndria como o principal mediador deste tipo de morte. Esta organela integra os sinais provenientes dos receptores de morte e sinais da

caspases 9 e 3 (Gottlieb *et al.*, 2000; Gottlieb, 2001)
(2001) demonstrou um aumento de bax e diminuiç
alta expressão de Mn-SOD, sugerindo que a M
está implicada na indução da apoptose.

Alguns estudos indicam que du
um megaporo (poro de transiçã
diversas proteínas e abrang
(Wetzel and Green, 199
juntamente com ATP
de
protease associad

16: Papel da mitocôndria na apoptose (Apaf-1 = fator de ativação de protease
iada a apoptose 1).

Os diferentes sinais indutores de apoptose são detectados pela mitocôndria fazendo com que ocorra um desacoplamento da cadeia respiratória e conseqüente liberação de citocromo c e proteínas ativadoras da apoptose para o citosol (Gupta, 2003). Uma vez liberado o citocromo c a ativação da cascata de caspases é irreversível (Okada and Mak, 2004). Quando no citosol, o citocromo c forma um complexo com Apaf-1, e caspase-9, o chamado apoptossomo, que promove a clivagem da pró-caspase-9, liberando a caspase-9 ativa (Figura 16) (Budihardjo *et al.*, 1999). Uma vez ativada a caspase-9, ativa a caspase-3 (Rupnarain *et al.*, 2004). Esta cascata de ativação de caspases é responsável pela desmontagem da célula durante a apoptose. A liberação do citocromo c pela mitocôndria é inibida pela proteína bcl-2 (Petros *et al.*, 2004).

A família bcl-2 é uma família de proteínas indutoras e repressoras de morte por apoptose que participam ativamente da regulação da apoptose (Borner, 2003). A homeostase é mantida pelo controle da quantidade de proteínas anti e pró-apoptóticas. Estímulos como dano no DNA levam ao aumento na expressão das proteínas pró-apoptóticas. Este desequilíbrio induz a apoptose (Petros *et al.*, 2004). Entre as proteínas mais estudadas desta família estão bax (pró-apoptótica) e bcl-2 (anti-apoptótica) que é superexpressa em adenomas e carcinomas colorretais (Bronner *et al.*, 1995). As proteínas bax e bcl-2 são capazes de formar homodímeros (bax-bax e bcl-2-bcl-2) e heterodímeros (bax-bcl-2), sendo que o equilíbrio entre estes homodímeros e heterodímeros pode definir o balanço pro ou anti-apoptótico da célula (Petros *et al.*, 2004). Após um estimulo de morte, bcl-2 inibe a permeabilização da membrana externa da mitocôndria, provavelmente pelo seqüestro de bax ou por competir por sítios que seriam ocupados por bax na membrana externa mitocondrial (Murphy *et al.*, 2000). A bax pode promover a apoptose através da interação com a mitocôndria, de forma independente da interação com proteínas anti-apoptóticas (Petros *et al.*, 2004).

OBJETIVOS

A combinação de 5-FU e CPT-11 como tratamento de primeira linha no câncer de cólon avançado tem sido justificada por diversos aspectos. A semelhança dentre as respostas clínicas aos tratamentos com 5-FU e CPT-11; a ausência de resistência cruzada dentre estas drogas, os alvos celulares distintos e a toxicidade tolerável representam as principais características que justificaram tal aprovação. Contudo, alguns pacientes desenvolvem resistência a esta terapia, não demonstrando respostas satisfatórias. Dentre os mecanismos associados a esta resistência, cabe ressaltar, a perda da capacidade das células neoplásicas de entrar em apoptose. Neste sentido, o presente estudo se propôs a investigar o efeito da combinação CPT-11/5-FU na indução de apoptose em linhagens celulares de adenocarcinoma de cólon.

Objetivos Gerais:

Investigar qual das vias de ativação da apoptose pode ser induzida pela combinação CPT-11 /5-FU nas linhagens celulares de adenocarcinoma de cólon HT-29 e SNU-C4.

Avaliar o envolvimento das enzimas superóxido dismutase, topoisomerase I e timidilato sintase na resposta das linhagens celulares de adenocarcinoma de cólon HT-29 e SNU-C4 aos tratamentos com CPT-11 /5-FU.

Objetivos Específicos:

Avaliar o efeito do tratamento combinado de CPT-11 e 5-FU quanto:

- a melhor seqüência de administração de ambas as drogas

- a distribuição das células nas fases do ciclo celular

- a ocorrência de apoptose

- a atividade das caspases na ativação da apoptose

- ao papel da mitocôndria na ativação da apoptose

- as atividades das enzimas superóxido dismutase, timidilato sintase e topoisomerase I

- a formação de complexo covalente DNA-topoisomerase I

CAPÍTULO I

Irinotecan/5-Fluorouracil combination induces apoptosis and enhances Manganese Superoxide Dismutase activity on HT-29 human colon carcinoma cells

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1. ABSTRACT

We examined whether induction of apoptosis and Mn-Superoxide Dismutase (Mn-SOD) and Cu,Zn-Superoxide Dismutase (Cu,Zn-SOD) activities were involved in the greater cytotoxicity of the irinotecan (CPT-11)/5-fluorouracil (5-FU) combination for human colon cancer cells when compared to both drugs alone. HT-29 and SNU-C4 human colon carcinoma cell lines were treated with 5-FU and CPT-11, then apoptosis was evaluated by flow cytometry and SOD activities were determined by polyacrylamide gel electrophoresis. Enhanced apoptosis was observed with all treatments containing 5-FU in SNU-C4 cells; however, in HT-29 cells, apoptosis was enhanced only with the CPT-11/5-FU combination. In the SNU-C4 cell line, none of the treatments exerted a significant effect on Cu, Zn- SOD or Mn-SOD activity. However, in HT-29 cells, the CPT-11/5-FU combination enhanced Mn-SOD activity when compared to cells treated with CPT-11 alone. Nevertheless, the combined treatment did not interfere with Cu,Zn-SOD activity. Treatment with the CPT-11/5-FU combination may promote in HT-29 cells apoptosis by enhancing Mn-SOD activity.

2. INTRODUCTION

5-fluorouracil (5-FU) and irinotecan (CPT-11) are the most important chemotherapeutic agents available for the treatment of advanced colorectal

cancer [1]. Several studies suggested that these agents may be synergistic in combination [1,2]. In fact, pre-clinical and clinical studies suggested that metastatic colon cancers do not generally manifest cross-resistance to these two agents when they are used serially [2-4]. These observations led to the approval of CPT-11 with 5-FU and leucovorin (LV) for first-line treatments of advanced colon cancer [5,6].

5-FU and CPT-11 act through distinct mechanisms. 5-FU is a fluoropyrimidine that demonstrated a dual mechanism of action: brief exposure to 5-FU induced an RNA-directed toxicity, while TS inhibition and DNA-directed toxicity occurred with prolonged exposure [7,8]. CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; irinotecan; CAMPTOSAR®) is a topoisomerase-I-inhibiting agent that has shown antitumor activity in patients with colon cancer when administered alone as first-line therapy or as second-line therapy after 5-FU failure [9,10]. CPT-11 is enzymatically converted by carboxylesterases to its most active metabolite, SN-38 (7-ethyl-10-hydroxyl-camptothecin) [11]. In this study, we used CPT-11 rather than SN-38, in order to achieve a better correlation with current treatment and ongoing clinical trials, in which a combination of CPT-11 and 5-FU was employed.

The combination of CPT-11 and 5-FU/LV increases the response rates up to 40% and leads to significant improvement in survival compared with 5-FU/LV alone [6]. The greater antitumor activity of this combination versus each agent alone, might be related to the synergistic antiproliferative effects seen in cultured human colon carcinoma cells treated with CPT-11 followed by 5-FU [12-14]. An *in vivo* study using a human tumor xenograft model demonstrated that administration of CPT-11 before 5-FU resulted in higher cure rates and sensitivity to this combination was associated to induction of apoptosis [15]. Despite research efforts, the mechanisms involved in this drug synergy remain unclear, yet apoptosis enhancement is one of the plausible mechanisms [16,17].

Apoptosis can be induced by diverse stimuli, and involves a conserved set of biochemical reactions ultimately leading to activation of caspases [18]. Reactive

oxygen species (ROS) are among the stimuli involved in apoptosis induction. Indeed, at milder concentrations, ROS may play subtle roles in the regulation of transcription factors [19] and cell signaling leading to proliferation [20-22] or apoptosis [21-23]. The superoxide dismutases (SODs) are antioxidant enzymes involved in defense systems against ROS [24]. The SODs catalyze the dismutation of superoxide anions to oxygen and hydrogen peroxide. There are two main isoforms of SOD in mammalian cells: (1) a copper- and zinc-containing superoxide dismutase isoform (Cu,Zn-SOD), found predominantly in the cytosol; and (2) a manganese-containing superoxide dismutase (Mn-SOD) localized in mitochondria. [25,26]. While Cu,Zn-SOD is a constitutive enzyme, Mn-SOD is an inducible one. The expression of the Mn-SOD gene is regulated by various stimuli, such as the redox state of the cell, cytokines, certain anticancer drugs, ionizing radiation and hyperthermia [25,26,28]. Besides the established antioxidant roles of SODs, recent studies [29-32] have suggested that SODs activities (both Cu,Zn- and Mn-SODs) can also influence the cellular proliferative status, and induce apoptosis [33]. Specifically, increased SODs activities have shown a suppressive action on the growth of several malignant tumors, leading to the proposal that SODs may act as tumor suppressors [31]. While many solid tumors have low antioxidant enzymes [29], high levels of Mn-SOD expression were observed in colorectal cancer, as well as in tumors of the central nervous system, and mesothelioma [34-37]. Furthermore, Hussain *et al.* [33] recently reported that up regulation of Mn-SOD increases oxidative stress and apoptosis in lymphobalst cell lines.

To our knowledge, the role of SOD activity on apoptosis induced by the CPT-11/5-FU combination on human colon cancer cells has not hitherto been investigated. Therefore, in this study we investigated the effects of treatment with CPT-11 followed by 5-FU on apoptosis induction and on Cu,Zn- and Mn-SODs activities in human colon cancer cells.

3. MATERIAL AND METHODS

3.1 DRUGS AND CHEMICALS

5-FU was from Hoffman-La Roche Inc (Nutley; NJ, USA), and CPT-11 (CAMPTOSAR®) from Pharmacia & Upjohn Company (Kalamazoo, Michigan, USA). The caspase inhibitor Z-VAD-fmk was obtained from Bachem AG (Voisins-le-Bretonneux, France). Unless otherwise specified, all other chemicals used were from our laboratory stock, and were of the highest grade available.

3.2 CELL CULTURE AND CELL LINE MAINTENANCE

The HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville; MD, USA). The SNU-C4 human colon carcinoma cell line was kindly supplied by Dr. GJ Peters (Department of Medical Oncology, Free University Hospital, Amsterdam, The Netherlands). The cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum at a temperature of 37 ^{o}C and in a humidified atmosphere of 5% CO_2 in air.

3.3 DRUG TREATMENTS

Cells were exposed to different drug treatments: (1) 24 h 5-FU or CPT-11 at IC_{50}; (2) 2 h with IC_{20} of CPT-11 followed by 22 h to IC_{50} of 5-FU; (3) 2 h with IC_{20} of 5-FU followed by 22 h to IC_{50} of CPT-11. Control cells were cultured during 24 h

without drug treatments. The cellular responses were assessed immediately after drug treatment.

3.4 CYTOTOXICITY STUDIES

Cells were collected by trypsinization, separated into single-cell suspensions in culture medium containing 50 µg/ml of gentamicin, and inoculated into 96-well microplates at densities of 5 x 10^3 cells per 100 µl per well. After stabilization for 24 h, triplicate cultures were treated in final volumes of 200 µl per well.

Cytotoxicity was assessed by means of the sulforhodamine B (SRB) assay [38], involving *in situ* fixation with trichloroacetic acid, staining with SRB, and solubilization of cell-bound SRB with Trizma base. The latter was colorimetrically assessed with a Model 750 Automatic Microplate Reader (Cambridge Technology, Inc, Watertown; MA, USA). Absorbances were read at a wavelength of 540 nm and semilogarithmically plotted against drug concentrations.

IC_{50} and IC_{20} values were derived from the resulting dose-response curves, *i.e.* drug concentrations leading to 50% and 20% inhibition of cell growth when compared to untreated controls. Data were corrected for background absorption, which was determined from wells containing medium alone, or drug-containing medium alone, but no cells.

3.5 MULTIPLE DRUG EFFECT ANALYSIS

Interactions between CPT-11 and 5-FU were assessed using a computer program for multiple drug effect analysis ([39]; Elsevier-Biosoft, Cambridge, UK).

The program enables calculation of combination indices (CIs) which, when smaller than 1, equal to 1, or greater than 1, indicate synergism, additivity, or antagonism, respectively, between two drugs.

CIs were calculated by the formula:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D_x)_1$ and $(D_x)_2$ are the concentrations of CPT-11 alone or 5-FU alone, giving x% growth inhibition, and $(D)_1$ and $(D)_2$ the drug concentrations in combination inhibiting cell growth also x%. $(D_x)_1$ and $(D_x)_2$ were calculated by the median-effect equation of Chou [39]:

$$D_x = D_m[FA/(1-FA)]^{1/m}$$

where D_m is the median-effect dose, FA is the fraction affected, and m the slope of the median-effect plot. Data were evaluated by means of CI *versus* FA plots, as well as by calculation of the means of the CIs at FAs 0.50, 0.75, 0.90, and 0.95, according to the latest update of the program.

3.6 FLOW CYTOMETRY ANALYSIS

After drug treatments, the cells were harvested and washed three times in ice-cold PBS. Then, samples of floating and adherent cells (5×10^5) were fixed in 70% ethanol at 4°C overnight [40]. The cell pellet was washed with ice-cold PBS, and resuspended in 0.5 mL PBS. After that, 0.5 mL of hypotonic fluorochrome solution (RNAse A 250 µg/mL, 100 µg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) was added to 0.5 mL cell sample. Then, after 30 min incubation in the dark at room temperature, cells were kept at 4°C until analysis. Cells (15,000) were assessed using the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The DNA content was analyzed using a ModFit 2.0 software. The number of cells having a hypodiploid DNA content (sub-G1

fraction) was taken as a measure of the number of apoptotic cells. Given that sub-G1 cells may derive in part from necrosis [41], the general caspase inhibitor z-VAD-fmk (50 μM was added together with the drug treatments for 24 h [42].

3.7 POLYACRYLAMIDE GEL ELECTROPHORESIS FOR SOD ACTIVITY ASSAY

Electrophoresis was carried out under non-denaturing conditions in gels containing 7% polyacrylamide with a 4 % stacking gel. A constant current of 30 mA^{-1} gel was applied for 4 h and the temperature set at 4^0C. Electrophoresis buffers and gels were prepared as described by Laemmli [43] except that SDS was excluded. Equal amounts (30μg) of protein (enzyme extracts) of all samples were loaded onto the gels.

3.8 SOD ACTIVITY STAINING

After electrophoretic separation, SOD activity was determined as described by Azevedo *et al*. [44]. The non-denaturing PAGE gel was rinsed in distilled-deionized water and incubated in the dark for 30 min at room temperature in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitro blue tetrazolium, and 0.3% N,N,N,N,-tetramethylenediamine. At the end of this period, the reaction mixture was poured off, the gel rinsed with distilled-deionized water and illuminated in water until the development of colorless bands of SOD activity on the dark-stained gel. Bovine SOD (Calbiochem Chemical Co., San Diego, CA, USA) was applied to all gels to be used as a positive control of SOD activity.

3.9 PROTEIN DETERMINATION

Protein concentration was determined spectrophotometrically at 590 nm as described by Bradford [45] using the Bio-Rad Protein Assay Dye Reagent with bovine serum albumin as a standard.

3.10 STATISTICAL ANALYSIS

Data presented are mean ± SD of at least three experiments performed in triplicate. One-way ANOVA followed by Tukey's test were used to indicate statistical significance. The differences were considered significant when $p < 0.05$ or $p < 0.001$.

4. RESULTS

4.1 CYTOTOXICITY STUDIES AND DRUG COMBINATION EFFECTS

The anti-proliferative effects of 5-FU and CPT-11 on the HT-29, and SNU-C4 human colon carcinoma cell lines were assessed after 24 h of drug exposure. Comparison of IC_{50} values (table 1) showed that the HT-29 cells (fig 1a) were relatively resistant to 5-FU (IC_{50} values of 8 µM), while the SNU-C4 cells (fig 1b) presented greater sensitivity to this drug (IC_{50} value of approximately 2 µM). The cell lines also demonstrated a significant difference in their sensitivity to CPT-11

(IC_{50} values approximately 2 µM for HT-29 cells, and 4 µM for SNU-C4 cells) (table 1).

Table 1: IC_{50} values (µM; means ± SD, n ≥ 3) of 5-FU or CPT-11 (alone

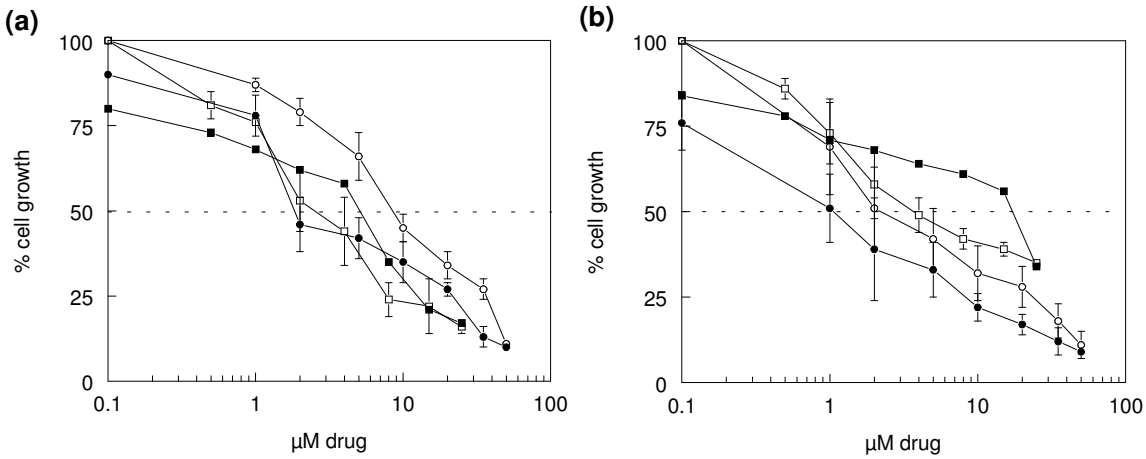

Figure 1: Inhibition of the proliferation of the human colon carcinoma cell lines HT-29 (a), and SNU-C4 (b) after 24 h of exposure to 5-FU alone (○); 2 h of exposure to IC_{20} CPT-11 followed by 22 h 5-FU (●);24 h of exposure to CPT-11 alone (□), and 2 h of exposure to IC_{20} 5-FU followed by 22 h CPT-11 (■). Data are means ± SD (vertical bars; n ≥ 3).

Multiple drug effect analysis (fig. 2) revealed CIs of about 0.5 in HT-29 and 1.0 in SNU-C4 cells, suggesting that prior treatment with CPT-11 at IC_{20} concentrations acted synergistically or additively with 5-FU. On the other hand, pretreatment with 5-FU at IC_{20} concentrations led to CIs about 2.7 in HT-29 and 1.7 in SNU-C4 cells, suggesting antagonistic interactions between the drugs when applied in this sequence (fig. 2). Indeed, prior treatment with IC_{20} of CPT-11 (1.5 µM and 1.0 µM for HT-29 and SNU-C4 cell lines, respectively) can sensitize 5-FU-resistant colon cancer cells (HT-29) to 5-FU. These phenomena seem to be mediated by the sequence-dependent induction of DNA lesions leading to different effects on cell viability [14].

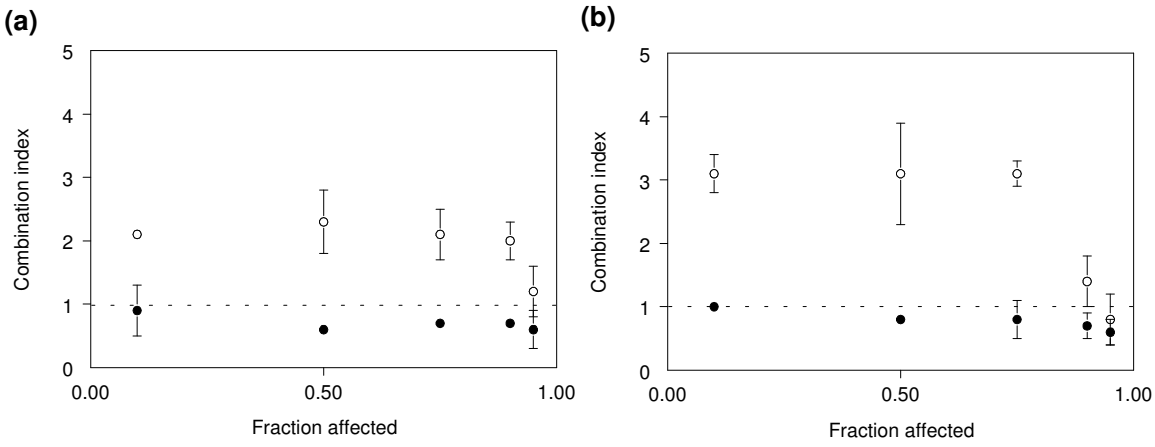

Figure 2: Multiple drug effect analyses of the interactions between CPT-11 and 5-FU in the human colon carcinoma cell lines HT-29 (a) and SNU-C4 (b) after 2 h of exposure to CPT-11 at IC_{20} followed by 22 h of exposure to 5-FU (○); or after 2 h of exposure to 5-FU at IC_{20} followed by 22 h of exposure to CPT-11 (●). Data were analyzed immediately after 24h of drug exposure. The analyses have been carried out three times in triplicate, and typical curves are presented. Combination indices < 1, = 1, or > 1 indicate synergism, additivity or antagonism, respectively.

4.2 INDUCTION OF APOPTOSIS

In order to determine whether the observed effects could be related to induction of apoptosis, cell cycle distribution analysis was performed. Cells with a hypodiploid DNA content (Sub-G1) were considered as apoptotic cells. As sub-G1 cells may derive in part from necrosis [41], we used the general caspase inhibitor z-VAD-fmk [42], to establish the extent of apoptosis in response to drug treatments.

As depicted in figure 3a, HT-29 cells treated with IC_{50} of either 5-FU or CPT-11 for 24 h exhibited a significant increase in the number of cells in sub-G1 phase (9.3% ± 0.1 for 5-FU and 8.4% ± 0.8 for CPT-11, respectively) when compared with the untreated controls (2.7 ± 0.36). Furthermore, treatment with IC_{20} of CPT-11 (for 2 h) followed by IC_{50} of 5-FU (22 h) induced a significant increase (17.45% ± 1.06) in hypodiploid cells. When compared to either 5-FU or

CPT-11 alone, this treatment (IC_{20} of CPT-11 followed by IC_{50} of 5-FU) significantly induced 1.8-fold more cells in Sub-G1 phase (fig. 3a; p = 0.0029). However, the sequence 5-FU followed by CPT-11 did not increase sub-G1 fraction (8.5% ± 1.4) when compared to 5-FU or CPT-11 alone (fig. 3a; p = 0.0029). Thus, while HT-29 presented sensitivity to either 5-FU and CPT-11 alone, the combination of CPT-11 followed by 5-FU significantly enhanced apoptosis, compared with both drugs alone.

Figure 3: Effect o r r rf r r ff dr 3f r

± SD. [a]significantly different from 5-FU alone; [b]significantly different from CPT-11 alone; $p < 0.001$.

In SNU-C4 cells (more sensitive to 5-FU than HT-29 cells; fig. 3b) treatment with 5-FU at IC_{50} for 24 h induced approximately 4.5-fold more cells (9.3% ± 0.35) in sub-G1 phase when compared to untreated control cells (1.95% ± 0.21). Comparable values of sub-G1 fraction were observed when SNU-C4 cells were treated with CPT-11 at IC_{20} followed by 5-FU at IC_{50} (10.3% ± 1.5). Conversely, CPT-11 alone or the sequence 5-FU IC_{20} followed by CPT-11 IC_{50} induced only 6.2% of cells in sub-G1 fraction (fig. 3b). Hence, for SNU-C4 cells, the combination of CPT-11 followed by 5-FU did not significantly enhance the apoptosis induction caused by 5-FU or CPT-11 alone.

The caspase inhibitor z-VAD-fmk prevented the hypodiploid peak induced by drug treatments in both cell lines (HT-29 and SNU-C4), corroborating that the majority of cells detected in sub-G1 phase were apoptotic. Thus, the differences observed between HT-29 and SNU-C4 cell lines in the amount of cells in sub-G1 might, at least in part, reflect differences in their susceptibility to undergo apoptosis in response to the distinct combinations of drug treatments. Noteworthy, the association that most efficiently induced apoptosis was the treatment with IC_{20} CPT-11 for 2 h followed by 22 h of IC_{50} 5-FU in HT-29 cells.

4.3 CHANGES IN SOD ACTIVITIES

SODs are involved in the defense against ROS [24], interference with cellular proliferation [29], tumor suppressor [31] and induction of apoptosis [33]. Then, Cu,Zn- and Mn-SOD activities were assessed to determine if the enhanced apoptosis induction observed in HT-29 cells treated with the sequence CPT-11/5-FU could be associated to variations in the activities of SOD isoforms.

After 24 h in culture, basal Cu, Zn-SOD activity (fig. 4b, c, 5b, c) was similar in both cell lines (68.0 units of SOD/mg protein ± 6.1 and 56.0 units of SOD/mg

protein ± 11 in HT-29 and SNU-C4, respectively). In contrast, the basal Mn-SOD activity of untreated control cells differed significantly between the cell lines (7,1 units of SOD/mg protein ± 1,1 and 19,7 units of SOD/mg protein ± 3,8 in HT-29 and SNU-C4, respectively; $p < 0.001$) (fig. 4a, c, 5a, c).

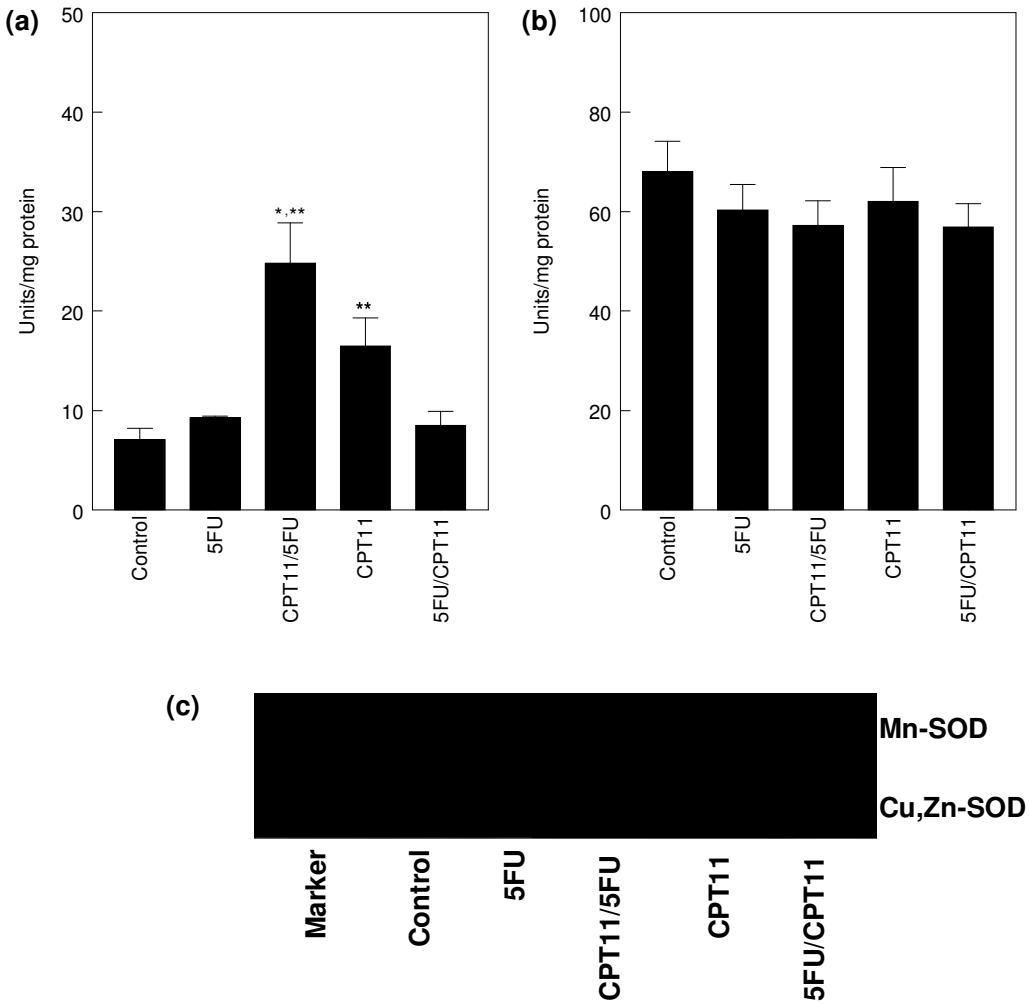

Figure 4: Mn-SOD (a) and Cu,Zn-SOD (b) activities in the HT-29 human colon carcinoma cell line. Cells were treated for 24 h with IC_{50} 5-FU alone (5FU); 2 h with IC_{20} CPT-11 followed by 22 h with 5-FU (CPT11/5FU), 24 h with IC_{50} CPT-11 alone (CPT11); and 2 h with IC_{20} 5-FU followed by 22 h CPT-11 (5FU/CPT11). Data were expressed as units of SOD/mg protein. Histograms represent Mn-SOD and Cu,Zn-SOD activities obtained after densitometric analysis performed on three gels of different activity. Representative gel (c) showed the enzymatic activities of Mn-SOD and Cu,Zn-SOD. [a]significantly different from 5-FU alone; [b]significantly different from CPT-11 alone; $p < 0.001$.

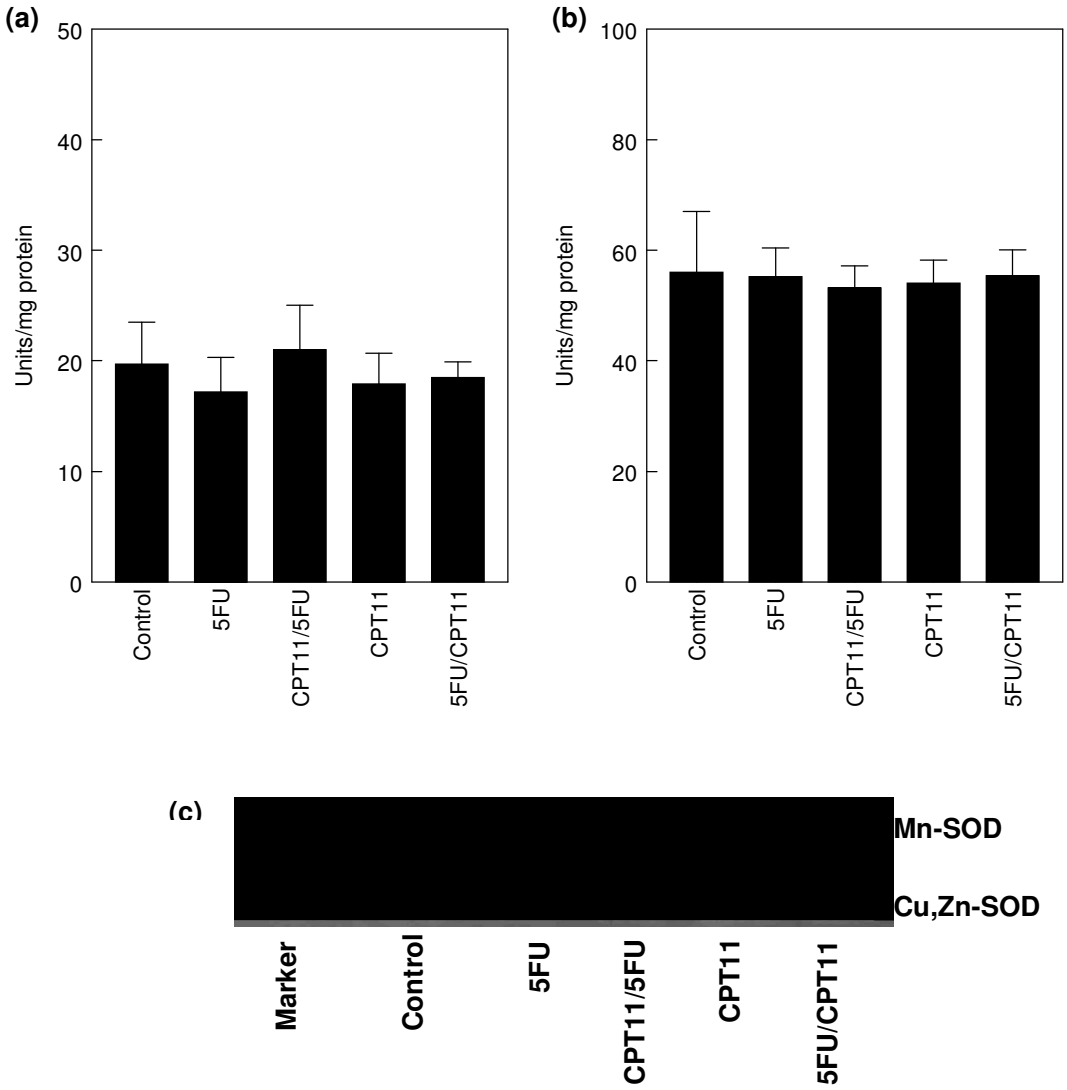

Figure 5: Mn-SOD (a) and Cu,Zn-SOD (b) activities in the SNU-C4 human colon carcinoma cell line. Cells were treated for 24 h IC_{50} 5-FU alone (5FU); 2 h IC_{20} CPT-11 followed by 22 h 5-FU (CPT11/5FU), 24 h IC_{50} CPT-11 alone (CPT11); and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (5FU/CPT11). Data were expressed as units of SOD/mg protein. Histograms represent Mn-SOD and Cu,Zn-SOD activities obtained after densitometric analysis performed on three different gels activity for these samples. Representative gel (c) showed the enzymatic activities of Mn-SOD and Cu/Zn-SOD.

In HT-29 cells, none of the treatments caused significant changes in Cu, Zn-SODs activities (fig. 4b, c). However, the sequence of CPT-11 followed by 5-FU treatment enhanced Mn-SOD activity in 3.5-fold relation to the untreated cells

and 5-FU alone and 1.5-fold in relation to the cells treated with CPT-11 alone (fig. 5a, c; $p < 0.001$). In fact, it should be emphasized that this combination (IC_{20} CPT-11 for 2 h followed by IC_{50} 5-FU for 22 h) in HT-29 cells was the drug sequence that most efficiently induced apoptosis among all treatment associations. Indeed, in SNU-C4 cells (in which combined CPT-11/5-FU treatment did not enhance apoptosis induction) none of the treatment combinations significantly affected Cu, Zn-SOD and Mn-SOD activities (fig. 5a-c).

5. DISCUSSION

Cancer of the colon is a highly treatable and curable disease when localized. In these cases, surgery is the primary treatment option. The prognosis of patients following surgery depends on the tumor stage. Systemic chemotherapy, therefore, plays an important role in patients with metastatic disease. Unfortunately, cytotoxic drug therapy generally produces partial clinical responses of short duration, making this form of treatment only of palliative value [46]. The most active cytotoxic drug in this malignancy, the antimetabolite 5-FU, produces responses in only 10 to 15% of patients as a single agent. Efforts to improve these results led to the 5-FU/LV combination, which enhances response rates about 2-fold, without, however, significantly improving survival rates. Several effective alternative treatment options became available for patients with colorectal cancer who relapsed after a 5-FU-based chemotherapy. The combination therapy with 5-FU/LV and CPT-11 or oxaliplatin demonstrated to be more active than 5-FU/LV as a first-line treatment. Oxaliplatin and CPT-11 combined, or not, with other agents are also active in the second-line treatment of colorectal carcinoma [2,47]. Hence, resistance to current therapies still remains one of the major problems in the treatment of colon cancers.

Therefore, efforts to clarify mechanisms involved in the resistance to anticancer treatments and to develop effective strategies to overcome this resistance are required. Since cancer cells that have acquired resistance to apoptosis seem to have a growth advantage [48,49] lowering the apoptotic threshold in the resistant cancer cells may be a promising strategy to enhance the effects of anti-cancer treatments. In the present study, we have attempted to elucidate the mechanism responsible for the greater cytotoxicity noted with CPT-11 followed by 5-FU combination. To study the combinations of drugs, a 24-hour exposure was chosen because 5-FU is dependent on time schedule as suggested by the TDI defined by Matsushima *et al.* [50].

We have previously [14] demonstrated that the combination of pretreatment with CPT-11 followed by treatment with 5-FU in cultured human colon carcinoma cells exerted anti-proliferative effects and caused DNA damage. When the cells are damaged by anticancer agents such as 5-FU and CPT-11, that cause DNA breaks, cell cycle progression will be transiently delayed to allow the cell to repair the damaged DNA. However, if the DNA is severely damaged, the cell will undergo apoptosis [51]. Accordingly, in the present study, we showed that the anti-proliferative effects of CPT-11 at IC_{20} followed by 5-FU at IC_{50} treatment in human colon cancer cell lines are, at least in part, related to the capacity of these drugs to induce apoptosis. However, we detected differences in the susceptibility of HT-29 and SNU-C4 colon cancer cell lines to the cytotoxic effects of CPT-11 and 5-FU treatments, reinforcing the conception that the heterogeneity of colon cancer cells is one of the factors that makes the development of effective chemotherapeutic protocols against all the subpopulations of tumor cells present in a malignant colon tumor so difficult.

Mitochondrial responses to oxidative stress play an important role in the early phase of apoptosis [52]. Oxidative stress caused by radiation and anticancer agents induces cell impair -0.01584 Tc 2 0 Td 1.81584 Tw (e e)Tj -0.0416 Tc 6.112.4 0 Td [(

3 is well known [55-57]. Furthermore, induction of apoptosis is regulated by the expression levels of proapoptotic and antiapoptotic proteins [58]. Ueta *et al* [59] showed the increase in Bax and Bak and the decrease of Bcl-2 and Bcl-X_L in tumors with high expression of Mn-SOD. Moreover, Hussain et al. [33] identified a novel mechanism of p53-dependent apoptosis in which p53 mediates the up-regulation of Mn-SOD produces an imbalance in antioxidant enzymes and oxidative stress. Thus, the Mn-SOD isoform, which is inducible and localized in the mitochondria has been implicated in apoptosis induction and could be involved in mechanisms of chemoresistance.

In general, Mn-SOD activity and/or expression is significantly lower in prostate [59,60] and some esophageal cancer cells [62], and higher in breast [63], ovarian [64], and central nervous system [34] cancer cells [37]. In this study we showed that treatment with the combination of CPT-11 at IC_{20} followed by 5-FU at IC_{50}, particularly in the HT-29 cell line, resulted in a significant increase in both apoptosis and Mn-SOD activity when compared to 5-FU treatment alone. In contrast, the combination treatment neither enhanced apoptosis induction, nor affected Mn-SOD activity in SNU-C4 cells. Therefore, in HT-29 cells, Mn-SOD could be possibly involved in the higher apoptosis induction observed with the combination CPT-11 followed by 5-FU when compared to other combinations of these agents. Indeed, the importance of mitochondrial Mn-SOD in mammals was demonstrated by gene-targeting experiments, which have shown that inactivation of both Mn-SOD alleles is incompatible with life [65]. Moreover, compelling evidence has shown that SODs may act as tumor suppressors [31]. Mn-SOD is decreased in primary pancreatic tumors and also in a variety of pancreatic cancer cell lines when compared with normal human pancreatic cells. Overexpression of Mn-SOD has been shown to decrease the rate of tumor cell growth in many tumors [66]. Specifically, transfection of human Mn-SOD cDNA into human melanoma cells [30], human glioma cell lines [31] and human oral squamous carcinoma cells [67] suppressed the malignant phenotype.

Thus, although the findings of this study cannot provide a mechanistic explanation, we suggest that the increased cytotoxicity/apoptosis observed with

CPT-11/5-FU treatment in HT-29 cells is associated with an increase in Mn-SOD activity that maybe amplifying the apoptotic pathway. To clarify these observations we are further investigating the role of Mn-SOD in colorectal cancers after exposure to CPT-11/5-FU.

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CAPÍTULO II

Irinotecan/5-Fluorouracil Combination Induces Alterations in Mitochondrial Membrane Potential and Caspases on Colon Cancer Cell Lines

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1. ABSTRACT

The combination of Irinotecan (CPT-11) and 5-Fluorour

2. INTRODUCTION

The combination of 5-fluorouracil (5-FU) and irinotecan (CPT-11) are current in use as a first line treatment of advanced colon cancer [1,2]. Several studies suggest that these agents may be synergistic in combination by increasing the amount of apoptotic cells [3-5]. 5-FU is a fluoropyrimidine that acts by inhibiting thymidylate synthase, a key enzyme in the de novo synthesis of thymidine nucleotides required for DNA synthesis [6]. While CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; CAMPTOSAR®) is a topoisomerase I-inhibiting agent that has shown antitumor activity in patients with colon cancer when administered alone as first-line therapy or as second-line therapy after 5-FU failure [7,8].

Many anticancer drugs in current use, such as 5-FU and CPT-11, have been shown to induce tumor cell death by apoptosis [9,10]. Great part of these drugs, seem to initiate apoptosis via mitochondria by activation of the cytochrome c/Apaf-1/caspase-9 pathway [11,12]. However, several studies suggest that the Fas system might also play an important role in drug-induced apoptosis [13-15]. Activation of the initiator caspase-8 by death receptors including Fas, or caspase-9 by mitochondria can activate the effector caspase-3 resulting in the cleavage of structural proteins and ultimately, in apoptosis [11,16]. Inhibition of caspases can prevent the acquisition of some hallmarks of the apoptosis [17].

Increasing evidence points to the central role of mitochondria in apoptosis [16,18-21]. This includes changes in mitochondrial membrane potential and the release of apoptosis-stimulating compounds from mitochondria [18,19]. During apoptosis two mechanisms of mitochondrial participation have been described. One involves opening of the mitochondrial permeability transition (PT) pore complex, swelling of the organelle and rupture of the outer membrane followed by release of apoptotic factors including cytocrome c [22]. The other relies on the direct release of these apoptotic factors without PT [21]. PT is characterized by a disruption of the inner transmembrane potential that precedes the nuclear signs of

apoptosis [22,23]. Furthermore, caspase activation has been reported to occur both upstream (Fas/caspase-8) and downstream (caspase-9) of these mitochondrial alterations [21].

In the present study, we investigated the involvement of the mitochondrial pathway of apoptosis in response to CPT-11 and 5-FU combination in human colon cancer cells.

3. MATERIAL AND METHODS

3.1 CELL CULTURE AND CELL LINE MAINTENANCE

The HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville; MD, USA). SNU-C4 human colon carcinoma cell line was gently supplied by Dr. GJ Peters (Department of Medical Oncology, Free University Hospital, Amsterdam, The Netherlands). The cells were maintained in RPMI 1640 medium (Invitrogen, Sao Paulo; SP, Brazil) containing 10% (v/v) fetal calf serum (Cultilab, Campinas; SP, Brazil) at a temperature of 37 ^{o}C and in a humidified atmosphere of 5% CO_2 in air.

3.2 DRUG TREATMENTS

Cells were exposed to different drug treatments: (1) during 24 h to IC_{50} of either 5-FU or CPT-11; (2) during 2 h with IC_{20} of CPT-11 followed by 22 h to IC_{50} of 5-FU; and, (3) during 2 h with $IC_{20\ of}$ 5-FU followed by 22 h to IC_{50} of CPT-11.

Control cells were cultured during 24 h without drug treatments. The cellular responses were assessed immediately after drug treatment.

3.3 GROWTH INHIBITION EXPERIMENTS

Cells were collected by trypsinisation, separated into single-cell suspensions in culture medium, and inoculated into 96-well microplates at densities of 5 x 10^3 cells per 100 µL per well. After stabilization for 24 h, triplicate cultures were treated in final volumes of 200 µL per well. Cellular responses were assessed by means of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5,diphenyl tetrazolium bromide) assay [24]. Thereafter, medium was removed and cells were incubated for 3 h at 37 ^oC in 50 µL MTT (final concentration 0,42 mg/mL). Formazan crystals were dissolved in 150 µL of dimethylsufoxide and the optical density (OD) was measured at 540 nm IC_{50} values were defined as the concentrations that correspond to a reduction of cellular growth by 50% when compared to values of untreated control cells.

3.4 FLOW CYTOMETRY ANALYSIS AND INDUCTION OF APOPTOSIS

To determine the involvement of caspases, the caspase-3 inhibitor, DEVD-CHO (10 µM; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, EUA), the caspase-8 inhibitor, Ac-IETD-CHO (30 µM; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, EUA) and the caspase-9 inhibitor, Ac-LEHD-CHO (40 µM; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, EUA) were added together with the drug treatments for 24 h [25]. After the drug treatments, the cells were harvested and washed in ice-cold PBS. Then, samples of floating and adherent cells (5 x 10^5) were washed with ice-cold PBS, and resuspended in 0.5 mL PBS. After that, 0.5 mL of hypotonic fluorochrome solution (RNAse A 250

µg/mL, 100 µg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) was added to a 0.5 mL of cell sample. Then, after 30 min incubation in the dark at room temperature, cells were kept at 4^{o}C until analysis. Cells (15,000) were assessed using the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The DNA content was analyzed using a ModFit 2.0 software [26]. The number of cells having a hypodiploid DNA content (sub-G1 fraction) was taken as a measure of the number of apoptotic cells. Given that sub-G1 cells may derive in part from necrosis [27], the addition of the caspases inhibitors was also performed, in order to confirm apoptosis in response to the drug treatments.

3.5 MITOCHONDRIAL MEMBRANE POTENTIAL ANALYSIS

After treatments, cells were collected by trypsinisation, separated into single-cell suspensions in culture medium, and counted in hemocytometer. After, 5 x 10^6 cells were stained with a final concentration of 10 µM of Rhodamine 123 (Rh 123) and incubated for 10 min in a 37 ^{o}C incubator. The cells were microcentrifuged at 200 x g for 6 min and washed twice with PBS. Then, the cells were resuspended in PBS containing 10 µM propidium iodide and incubated for 10 min [28]. Stained cells were analyzed on the flow cytometry within 15-30 min (excitation 488 nm, emission 530 nm)

3.6 STATISTICAL ANALYSIS

Data presented are means ± SDs of at least three experiments performed in triplicate. One-way ANOVA followed by Tukey's test were used to indicate statistical significance. The differences were considered significant when $P < 0.05$.

4. RESULTS

4.1 GROWTH INHIBITION STUDIES

The anti-proliferative effects of 5-FU and CPT-11 in the HT-29, and SNU-C4 human colon carcinoma cell lines were assessed after 24 h drug exposure. Comparison of IC_{50} values showed that the HT-29 cells (Fig 1a) were relatively resistant to 5-FU (IC_{50} values of 15.1 µM ± 2.6), while the SNU-C4 cells (Fig 1b) presented greater sensitivity to this drug (IC_{50} value of 7.6 µM ± 2.0). Further, the two cell lines also demonstrated significant difference in their sensitivity to CPT-11, since the IC_{50} values of this drug was 8.4 µM ± 1.6 for HT-29 cells, and 16.2 µM ± 2.8 for SNU-C4 cell line (Fig 1a and 1b).

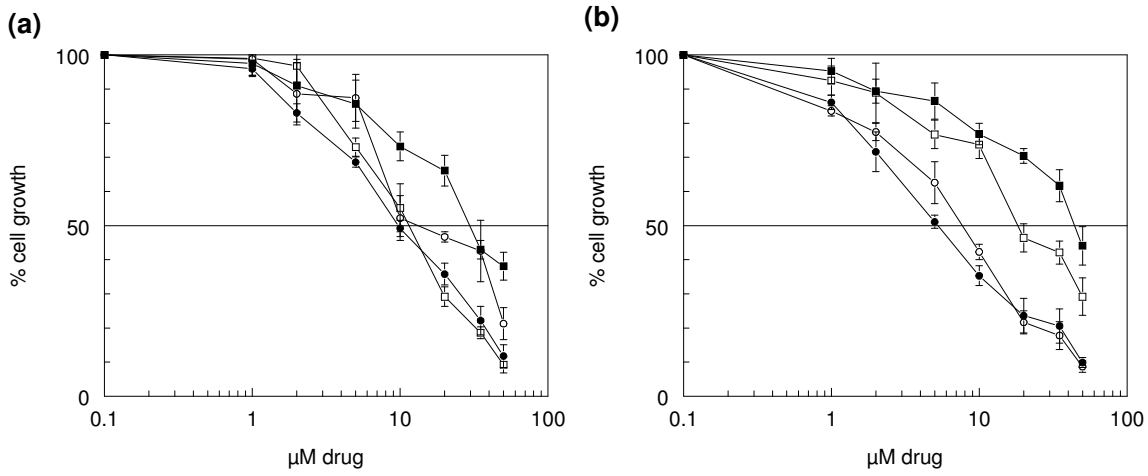

Figure 1: Inhibition of proliferation of the human colon carcinoma cell lines HT-29 (a), and SNU-C4 (b) after 24 h 5-FU alone (○); 2 h IC_{20} CPT-11 followed by 22 h 5-FU (●);24 h CPT-11 alone (□);and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (■). Data are means ± SD (vertical bars; n ≥ 3).

In order to verify whether one drug interferes with the cytotoxicity of the other, we further examined the effects of IC_{20} of one agent on the cell growth inhibition exerted by the other agent. Thus, cells were pretreated for 2 h with CPT-11 or 5-FU at IC_{20} concentrations followed by 22 h to serial dilutions of 5-FU or CPT-11, respectively. As shown in Fig. 1, treatment with CPT-11 potentiates 5-FU-mediated growth inhibition about 2-fold in HT-29 and in 1.4-fold in SNU-C4 cells. In contrast, pretreatment with IC_{20} of 5-FU decreased the effect of CPT-11 at IC_{50} (2.9-fold in HT-29 and 2.3-fold in SNU-C4 cells, respectively) (Fig. 1a and 1b).

4.2 INDUCTION OF APOPTOSIS AND CASPASES INVOLVEMENT

The induction of apoptosis was measured as the sub-G1 peak after iodide propidium staining using flow cytometry in the presence of caspases inhibitor. As shown in Fig 2a, less than 4% of apoptosis could be determined in untreated HT-29 and SNU-C4 cells. All treatments induced an increased in the number of cells in Sub-G1 fraction ($p < 0.05$) in both cell lines. In HT-29 cells treated with IC_{50} of either 5-FU or CPT-11 for 24 h caused a significant increase in the number of cells in sub-G1 phase (8.3 ± 0.2 for 5-FU and 7.9 ± 0.6 for CPT-11, respectively) when compared to untreated controls (2.7 ± 0.3). Furthermore, treatment with IC_{20} of CPT-11 (for 2 h) followed by IC_{50} of 5-FU (22 h) induced a significant increase (1.9-fold) in hypodiploid cells when compared to either of the drugs alone (Fig. 2a). On the other hand, the sequence 5-FU at IC_{20} followed by CPT-11 did not increase sub-G1 fraction (8.7 ± 0.9) when compared to 5-FU or CPT-11 alone (Fig. 2a). Thus, in HT-29 cells the combination of CPT-11 followed by 5-FU significantly enhanced apoptosis. In SNU-C4 cells, treatment with IC_{50} of 5-FU for 24 h displayed 10.3% of cells in sub-G1 phase when compared to untreated control cells (2.3 ± 0.2). Comparable values of sub-G1 fraction were observed when SNU-C4 cells were treated with CPT-11 IC_{20} followed by 5-FU IC_{50} (11.6 ± 0.8). Conversely, CPT-11 alone and the sequence 5-FU IC_{20} followed by CPT-11 IC_{50} induced only 7.1% of cells to sub-G1 fraction (Fig. 2b). Therefore, for SNU-C4

cells (the cell line more sensitive to 5-FU than the HT-29 cells), combination of CPT-11 followed by 5-FU did not enhance significantly apoptosis induction caused by 5-FU alone ($p > 0.05$).

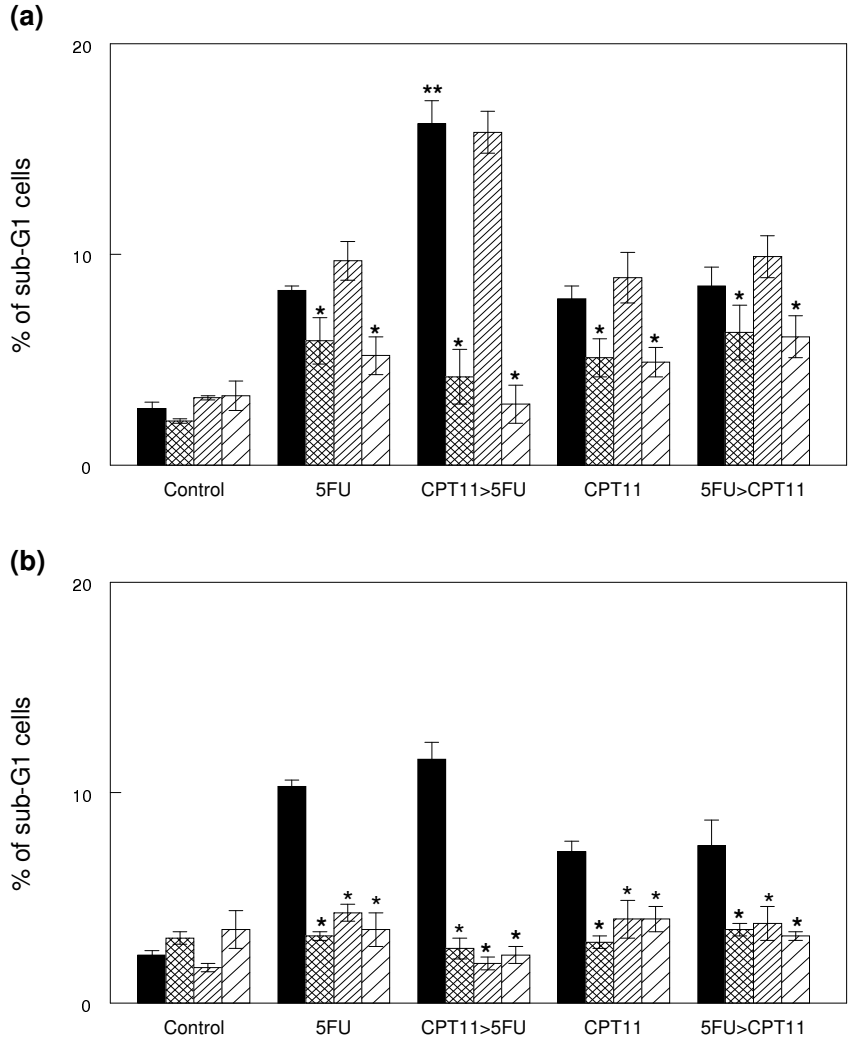

Figure 2: Effect of the caspases inhibitors on the sub-G1 fraction of adherent and floating treated HT-29 (a) and SNU-C4 (b) human colon carcinoma cell line. The cells were treated for 24 h IC_{50} 5-FU alone (5FU); 2 h IC_{20} CPT-11 followed by 22 h 5-FU (CPT11>5FU), 24 h IC_{50} CPT-11 alone (CPT11); and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (5FU>CPT11). Immediately after treatment cells were labeled with propidium iodide and analized in flow cytometry. The percentage of cells in Sub-G1 phase without caspase inhibitor (■), with the caspase-3 inhibitor DEVD-CHO (▩), with the caspase-8 inhibitor Ac-IETD-CHO (▨), with the caspase-9 inhibitor Ac-LEHD-CHO (◪) were plotted as mean ± SD. *significantly different from cells without caspase inhibitors; **significantly different from 5-FU alone; $p < 0.05$.

To assess the involvement of caspases in the observed apoptosis induced by CPT-11/5-Fu we measured the Sub-G1 peak after treatment with the caspases inhibitor. In HT-29 cells, the apoptosis induced after all the treatments by the caspase-3 inhibitor, DEVD-CHO, and the caspase-9 inhibitor, Ac-LEHD-CHO (Fig. 2a). Nevertheless, none of these treatments demonstrated reduction of Sub-G1 fraction in the presence of the caspase-8 inhibitor, Ac-IETD-CHO. The apoptosis induced by the combination CPT-11 IC_{20} followed by 5-FU IC_{50} was inhibited in 75% and 80% by the caspase-3 inhibitor, DEVD-CHO and the caspase-9 inhibitor, Ac-LEHD-CHO, respectively, in HT-29 cells (Fig. 2a). In the SNU-C4 cell line all three caspase inhibitors blocked the apoptosis in 50% to 60% after 5-FU at IC_{50}, CPT-11 at IC_{50} and 5-FU IC_{20} followed by CPT-11 IC_{50} treatments (Fig. 2b).

4.3 MITOCHONDRIAL MEMBRANE POTENTIAL

To analyze whether the mitochondria were involved in the activation of apoptosis pathway after the drug treatments, we measured mitochondrial membrane potential of the treated cells by staining with Rh123. The relative intensity of Rh123 in untreated cells differed significantly between the two cell lines (Fig.3). HT-29 cells showed 10-fold more Rh123 incorporation when compared to SNU-C4 cells ($p < 0.05$). In the HT-29 cell line the relative intensity of Rh123 was increased 1.5-fold after CPT-11 at IC_{50} and 5-FU at IC_{20} followed by CPT-11 IC_{50} exposure, suggesting an increased in the mitochondrial membrane potential. Nevertheless, 5-FU at IC_{50} and CPT-11 at IC_{20} followed by 5-FU IC_{50} treatments induced a decrease of 50% in the mitochondrial membrane potential (Fig.3). In contrast, in the SNU-C4 cell line none of the treatments altered the uptake of Rh123, suggesting that in this cell line the mitochondrial membrane potential were not significantly affected (Fig.3).

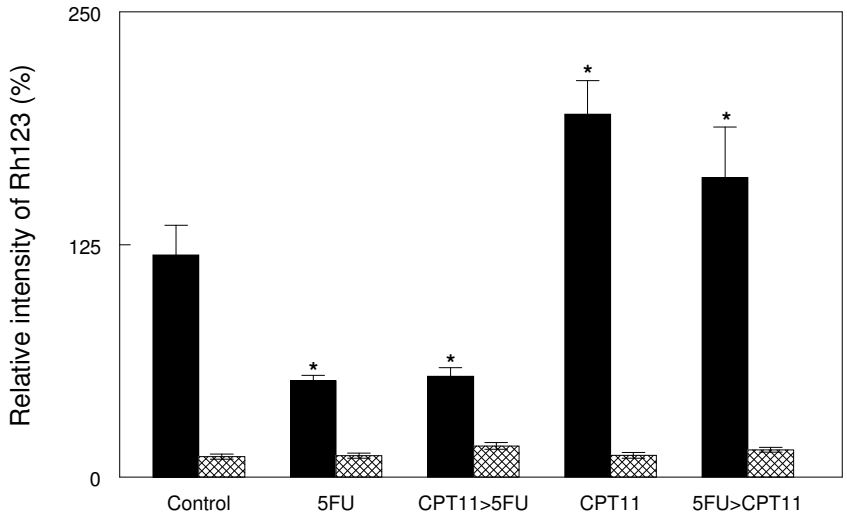

Figure 3: Mitochondrial membrane potential in the HT-29 (■) and SNU-C4 (▩) human colon carcinoma cell line. The cells were treated for 24 h IC_{50} 5-FU alone (5FU); 2 h IC_{20} CPT-11 followed by 22 h 5-FU (CPT11>5FU), 24 h IC_{50} CPT-11 alone (CPT11); and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (5FU>CPT11). Data were expressed as percentage of the relative intensity of Rh123 uptake. *significantly different from untreated controls; $p < 0.05$.

5. DISCUSSION

Cancer of the colon is a highly treatable and curable disease when localized. In these cases, surgery is the primary treatment option. Systemic chemotherapy, therefore, plays an important role in patients with advanced disease. Unfortunately, cytotoxic drug therapy generally produces partial clinical responses of short duration, making this form of treatment only of palliative value [29]. The most active cytotoxic drug in this malignancy, the antimetabolite 5-FU, produces responses in only 10 to 15% of patients as a single agent. The combination therapy with 5-FU/LV and CPT-11 demonstrated to be more active than 5-FU/LV as a first line treatment [reviewed in ref. 4]. Hence, resistance to

these therapies still remains one of the major problems in the treatment of colon cancers. Since cancer cells that have acquired the resistance to apoptosis seem to have growth advantage [30,31], lowering the apoptotic threshold in the resistant cancer cells may be a promising strategy to enhance the effects of anti-cancer treatments.

In the present study, we showed that the cytotoxic effect of CPT-11 at IC_{20} for 2 h followed by 22 h of 5-FU at IC_{50} treatment in human colon cancer cell lines are, at least in part, related to the capacity of these drugs to induce apoptosis. Indeed, prior treatment with IC_{20} of CPT-11 (1.5 µM and 1.0 µM for HT-29 and SNU-C4 cell lines, respectively) can sensitize 5-FU resistant colon cancer cells (HT-29) to 5-FU. These phenomena seem to be mediated by the sequence-dependent induction of DNA lesions leading to induction of apoptosis [32].

To elucidate the factors involved in the apoptosis induced by CPT-11 at IC_{20} followed by 5-FU at IC_{50} combination, we compared the responses on HT-29 cells (more resistant to 5-FU) with SNU-C4 cell line (more sensitive to 5-FU). In accordance with our previous study, we found that CPT-11 at IC_{20} followed by 5-FU at IC_{50} induced 2 times more apoptosis than 5-FU at IC_{50} in the HT-29 cell line [33]. On the other hand, we did not observe the same effect on the SNU-C4 cells. In this cell line this treatment induced the same amount of apoptosis than that observed with 5-FU at IC_{50}. This could be explained by the less sensitivity of this cell line to CPT-11. In fact, as reported previously [34] the CPT-11 exerts its toxicity in a dose-dependent manner. In SNU-C4 cells the dose of IC_{20} was not sufficient to induce apoptosis in these cells. Thus, in the SNU-C4 cells, the amount of cells in apoptosis observed with the CPT-11 at IC_{20} followed by 5-FU at IC_{50} was due to 5-FU action.

It was previously reported that 5-FU induces apoptosis through the activation of caspase-3 and caspase-8 [35]. Both caspase-8 and caspase-9 have been reported to exist upstream of caspase-3 [36]. In line with these observations we found that in HT-29 cells, both caspase-3 and caspase-9 inhibitors decreased the Sub-G1 fraction indicating that these two caspases were essential to activate apoptosis after exposure to CPT-11 at IC_{20} followed by 5-FU at IC_{50} treatment, in

this cell line. These observations were accompanied by a reduction of 50% in mitochondrial membrane potential. Moreover, several studies demonstrated that dissipation of the mitochondrial membrane potential marks the initiation of apoptotic cascade through the liberation of cytochrome c and subsequently activation of caspase-3 [37-39].

When the HT-29 cells were treated with the combination 5-FU at IC_{20} followed by CPT-11 at IC_{50}, that decreased the effect of CPT-11 at IC_{50} and induced less apoptosis, the same profile of sub-G1 cells treated with the caspases inhibitors was observed. However, the mitochondrial membrane potential was increased (1.5-fold). In support of these data, it was demonstrated that elevations in the mitochondrial membrane potential can result in escape from, or delayed, apoptosis [21,40].

The effect of caspases inhibitors in the SNU-C4 cells demonstrated a different profile. The three caspases inhibitors reduced the amount of cells in Sub-G1 cells treated with all treatments, suggesting that caspase-3, -8 and -9 clearly mediate the induction of apoptosis in this cell line. Caspase-8 has been reported to exist downstream of Fas system, and also it activates caspase-3 [41]. Moreover, in some colon cells, a Fas-dependent component was found in 5-FU induced cytotoxicity [42]. This could explain the participation of caspase-8 in the apoptosis in this cell line (more sensitive to 5-FU). Furthermore, in the SNU-C4 cell line, we did not observe changes in mitochondrial membrane potential, indicating that the apoptosis might be activated by other caspases, without mitochondrial involvement.

Thus, we suggest that the increase in apoptosis observed with CPT-11 followed by 5-FU treatment, in HT-29 cells, could be explained by a disruption in mitochondria membrane potential that induced caspases activation. Whether mitochondrial or Fas system were a prerequisite to apoptosis and how the mechanisms associated to their regulation remains to be investigated.

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CAPÍTULO III

Effects of irinotecan/5-fluorouracil combination on thymidylate synthase on human colon carcinoma cell lines

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1. ABSTRACT

Thymidylate synthase (TS) is responsible for the de novo synthesis of thymidylate, which is required for DNA synthesis and repair and is an important target for fluoropyrimidines such as 5-fluorouracil (5-FU). In this study we investigated the whether the greater cytotoxicity of irinotecan (CPT-11) followed by 5-fluorouracil (5-FU) in HT-29 and SNU-C4 cell lines was related to TS-mRNA expression and/or activity. Thus, cells were exposed for 24 h to each drug, in various concentrations and sequences, and assessed for colony formation, TS catalytic activity, and TS-mRNA expression. Pre-treatment with CPT-11 at IC_{20} concentration increased the 5-FU cytotoxicity in HT-29 and SNU-C4 cells. TS catalytic activity and TS-mRNA expression suggested that the differences in sensitivity to 5-FU among the cell lines were not correlated to TS. 5-FU treatment significantly induced a concentration dependent decreased TS-mRNA expression and TS catalytic activity in both cell lines. When we exposed cells to CPT-11 at IC_{20} and subsequently to 5-FU at IC_{50}, the impact on TS activity and mRNA expression were the same as observed with 5-FU alone. Our findings suggested that the greater cytotoxicity of CPT-11/5-FU combination in HT-29 and SNU-C4 cell lines are not related to interference with thymidylate synthase.

2. INTRODUCTION

The combination of irinotecan (CPT-11) with 5-Fluorouracil (5-FU) has been extensively investigated in view of its therapeutic efficacy against advanced colorectal carcinoma. *In vitro* and *in vivo* evidence suggests schedule-dependent cytotoxic interactions for the combination of thymidylate synthase (TS) inhibitors and CPT-11 or its active metabolite SN-38 [1-4]. Several studies with different schedules of CPT-11/5-FU are still under investigation to identify a more practical and tolerable treatment option [5]. The enhanced antitumor activity of this combination over that of each agent alone, might be related to the synergistic antiproliferative effects seen in cultured human colon carcinoma cells treated with CPT-11 followed by 5-FU [4-6]. However, the exact mechanism of interaction of these drugs still remains unclear. It has been suggested that the optimal response of this combination is dependent not only on the sequence of administration, but also on the duration of exposure to the drugs [3,4].

Even though it is proposed that inhibition of TS is the main mechanism of 5-FU cytotoxicity, abnormal RNA and protein processing after incorporation of fluorouridine-5'-triphosphate (FUTP) into various RNA species, and premature chain termination and/or DNA strand breakage upon incorporation of fluorodeoxyuridine-5'-triphosphate (FdUTP) into DNA, may also contribute to varying degrees to 5-FU cytotoxicity [7]. CPT-11 is a semi-synthetic derivative of the natural alkaloid camptothecin [8]. CPT-11 acts by introducing single-strand DNA breaks upon the stabilization of DNA-topoisomerase I complexes [9].

Thymidylate synthase is a critical target for cancer treatment and represent the major target for the widely used anticancer agent 5-fluorouracil (5-FU) and for new TS inhibiting folate antagonists, such as Raltitrexed and ALIMTA [10,11]. TS catalyzes the methylation of dUMP to dTMP for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyl donor [12,13]. The activity levels and amount of this enzyme fluctuates with the cell cycle progression with peak activity and maximum content occurring during the S phase [14]. Although the cell-cycle-

dependency of TS gene expression [15], some evidences demonstrated that the proliferation is an important factor for changes in TS levels [16]. The TS gene is located on chromosome 18q, for which a high percentage of alterations have been reported in human colon tumors [17]. Among others mechanisms of 5-FU resistance, the most frequent are TS gene amplification increased TS mRNA, insufficient TS inhibition by FdUMP, elevated levels of TS after treatment [18].

To elucidate the role of TS, we investigated whether the interaction of CPT-11/5-FU could be related to TS activity and mRNA expression in human colon carcinoma cell lines.

3. MATERIALS AND METHODS

3.1 DRUGS AND CHEMICALS

5-FU was obtained from Hoffman-La Roche Inc (Nutley; NJ, USA), and CPT-11 (CAMPTOSAR®) from Pharmacia & Upjohn Company (Kalamazoo, Michigan, USA). The [5-^{3}H]dUMP (specific activity 19 Ci/mmol) was obtained from Amersham International (Buckinghamshire, United Kingdom). Unless otherwise specified, all other chemicals used were of the highest grade available.

3.2 CELL CULTURE MAINTENANCE

The HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville; MD, USA). The SNU-C4 cell line was made

available by one of the authors (Dr. G.J. Peters). The cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum at a temperature of 37 ^{o}C in a humidified atmosphere of 5% CO_2 in air.

3.3 DRUG TREATMENTS

The cytotoxic effects and interference with thymidylate synthase were examined by exposing cells: (1) during 24 h to the IC_{20}, IC_{50} or 10 x IC_{50} of either 5-FU or CPT-11; (2) during 2 h with the IC_{20} of CPT-11 followed by 22 h to the IC_{20}, IC_{50} or 10 x IC_{50} of 5-FU; and (3) during 2 h with the $IC_{20\ of}$ 5-FU followed by 22 h to the IC_{20}, IC_{50} or 10 x IC_{50} of CPT-11. Control cells were cultured during 24 h without drug treatment.

3.4 COLONY FORMATION STUDIES

To investigate the cytotoxic effect of drug treatments we used a clonogenic assay as described previously [19]. Briefly, HT-29 and SNU-C4 cells were seeded into 6-well plates (300 cells/well) after treatment and incubated during 7 days. Then, the cells were fixed with 70% ethanol and counterstained with 0.5% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. The survival fraction (SF) was then calculated as:

$$SF = \frac{\text{Number of colonies in treated cells}}{\text{Number of colonies in untreated control}} \times 100$$

3.5 THYMIDYLATE SYNTHASE ACTIVITY

Thymidylate synthase activity was evaluated with a [^{3}H]-release assay to determine thymidylate synthase catalytic activity, *i.e.* the rate of conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTH

3.7 THYMIDYLATE SYNTHASE EXPRESSION

mRNA levels for TS were estimated by semiquantitative methods as previously described [23,24]. Briefly, simultaneous amplifications of TS and ß-actin as an internal standard were performed. The primers used were: the TS sense primer (5'-AGATCCAACACATCCTCCGCT-3'), the TS antisense primer (5'-CAGAACACACGTTTGGTTGTCA-3'), the ß-actin sense primer (5'-CGGGAAATCGTGCGTGACAT-3') and the ß-actin antisense primer (5'-GGAGTTGAAGGTAGTTTCGTG-3'), all of which were designed according to previous study [23]. Generally, 10 µl cDNA were amplified in a total volume of 50 µl containing, 50 mM $MgCl_2$, 8.5 mM dNTPs, 1.25 unit of Taq DNA Polymerase (Promega) and specific sense primers (50 pmol of each TS primer and 50 pmol ß-actin) using a 9600 thermal cycler (Perkin Elmer, CA). Initial heating at 94°C for 3 min was followed by 40 PCR cycles (95°C 1 min, 55°C 1 min and 30 seconds, 72°C 1 min). Amplification was terminated after a final extension step at 72°C of 5 min. The linear range of amplification was established for each cDNA. Amplified DNA fragments were separated on an ethidium bromide stained 2.0 % agarose gel. Gel pictures were scanned, and the density of each band was quantitated using an image analysis software. Relative amounts of mRNA for TS were calculated as TS/ß-actin ratios.

3.8 STATISTICS

Data presented are mean ± SD or mean ± SEM of at least three experiments. One-way ANOVA followed by Tukey's test were used to indicate statistical significance. The differences were considered significant when $P < 0.05$. For correlation analysis Pearson's coefficient were used.

4. RESULTS

4.1 CYTOTOXIC EFFECTS OF DRUGS COMBINATION

The cytotoxic effects of 5-FU and CPT-11 in the HT-29, and SNU-C4 human colon carcinoma cell lines were examined after 24 h drug treatments by colony formation assay. A concentration dependent cytotoxicity was observed with all treatments (Fig. 1). When HT-29 cells were exposed to 5-FU alone or CPT-11 alone for 24 h the dose necessary to inhibit 50% (IC_{50} values) the formation of colonies was 8.5 µM and 2.5 µM, respectively (Table 1). The inhibition of 50% of colony formation in SNU-C4 cells occurred when the cells were exposed to 2.5 µM and 4.0 µM of 5-FU and CPT-11, respectively.

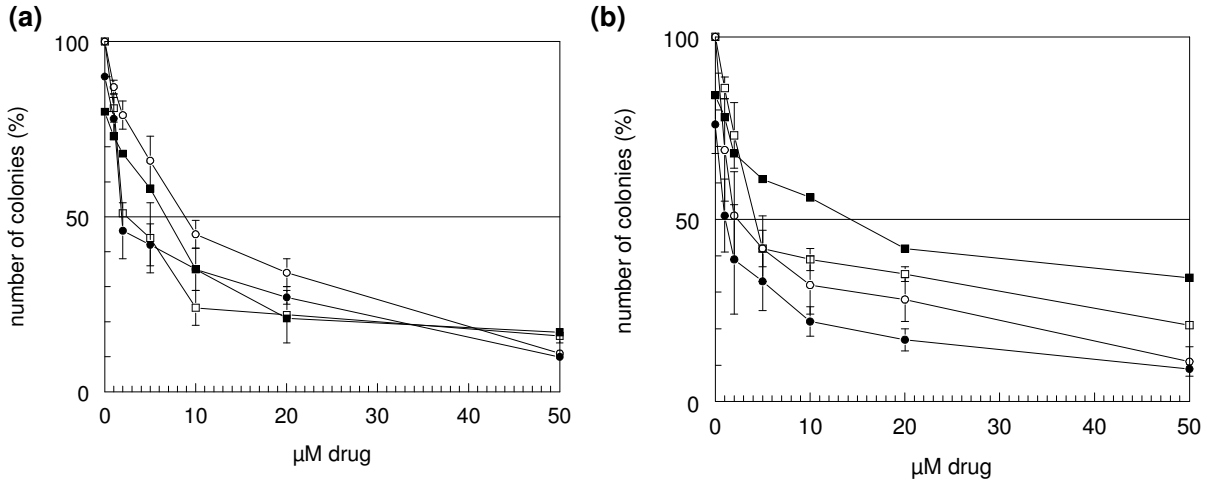


Figure 1: Effect of treatment with 5-FU alone (○); 2 h IC_{20} CPT-11 followed by 22 h 5-FU (●); 24 h CPT-11 alone (□); and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (■), on HT-29 and SNU-C4 human colon carcinoma cells survival fraction. Following treatment, cells were incubated during 10 days. Colonies containing $\geq$ 50 cells were scored. Data was expressed as the percentage of the survival fractions of cells versus the different treatments. Data was plotted as the mean $\pm$ SD of three different experiments. *Different from 5-FU alone, ** different from CPT-11 alone ($p < 0.05$).

When the cells were exposed to CPT-11 for 2 h at IC_{20} (1.5 and 1.0 µM, for HT-29 and SNU-C4 cell lines, respectively; Table 1) followed by 5-FU for 24 h at IC_{50}, the relative values of Survival Fraction (SF) decreased about 4-fold in HT-29 cells, and 1.6-fold in SNU-C4 cells when compared to 5-FU alone ($p < 0.05$; Fig. 1). Finally, when the cells were exposed to 5-FU for 2 h at IC_{20} (2.0 and 0.5 µM, for HT-29 and SNU-C4 cell lines, respectively; Table 1) followed by CPT-11 for 24 h at IC_{50} the relative values of SF increased 2-fold in HT-29 cells and about 3.5-fold in SNU-C4 cell line, when compared to cells treated with CPT-11 alone ($p < 0.05$; Fig. 1). Thus, as described previously [1-4], when cells were treated with sequential CPT-11 followed by 5-FU a greater effect was observed, when compared to the reverse sequence.

Table 1: IC_{50} values (µM; means ± SD, n = 3) of 5-FU or CPT-11 (alone, and after 2 h pre-treatment, as indicated) in HT-29 and SNU-C4 human colon carcinoma cell lines. Cellular responses were assessed immediately after 24 h treatment using the clonogenic assay.

	HT-29	**SNU-C4**
5-FU alone, 24 h	8.5 ± 1.3	2.5 ± 0.7
5-FU, after 2 h pre-treatment with CPT-11 IC_{20}	2.0 ± 0.5[I]	1.5 ± 0.2[I]
CPT-11 alone, 24 h	2.5 ± 0.5	4.0 ± 0.3
CPT-11, after 2 h pre-treatment with 5-FU IC_{20}	7.5 ± 0.7[II]	18.2 ± 0.6[II]

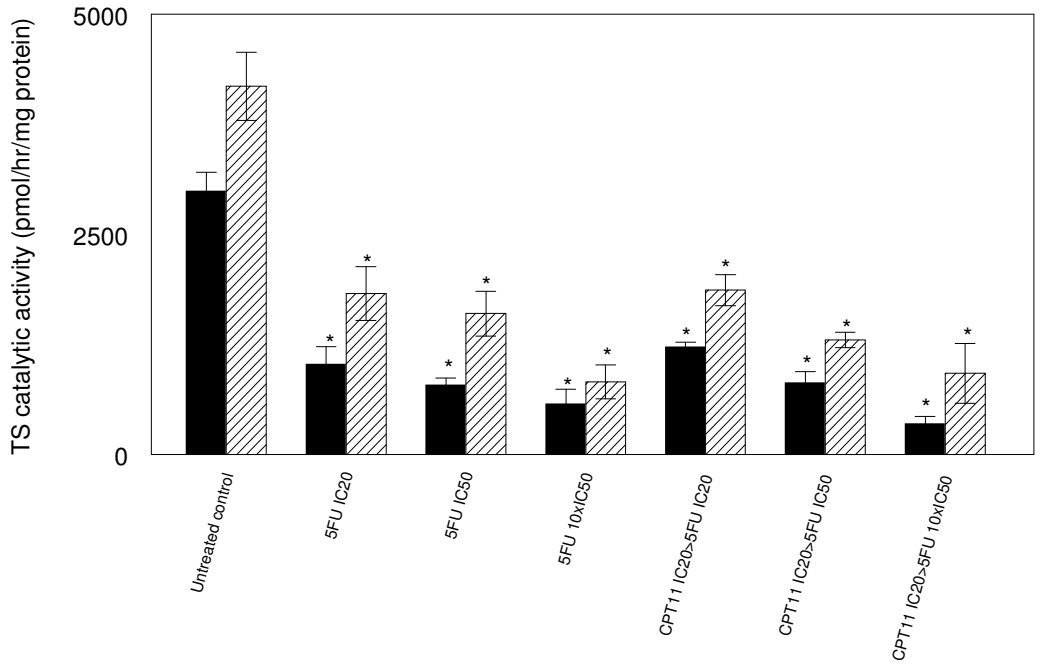
[I]Significantly different from 5-FU alone ($p < 0.05$)

[II]Significantly different from CPT-11 alone ($p < 0.05$)

4.2 THYMIDYLATE SYNTHASE ACTIVITY

We evaluated the TS activity in order to determine if the observed differences in the sensitivity of the HT-29 and SNU-C4 cell lines to the combination treatments were related to changes in TS activity. The basal level of TS was 1.4-fold higher in SNU-C4 cells when compared to the HT-29 cell line ($p < 0.05$; Fig 2a and 2b). When HT-29 and SNU-C4 cells were exposed to 5-FU alone, we observed a concentration dependent decrease of TS activity when compared to untreated controls (Fig. 2a). The HT-29 cell line demonstrated a more pronounced decrease than that observed in SNU-C4 ($p < 0.05$). When we exposed the cells to CPT-11 alone no significant modification on TS activity was observed in both cell lines with IC_{20} and IC_{50}. Treatment with CPT-11 at 10 x IC_{50} even led to an increased in TS activity of 1.7-fold in HT-29 cells and about 1.3-fold in SNU-C4 ($p < 0.05$; Fig. 2b).

(a)



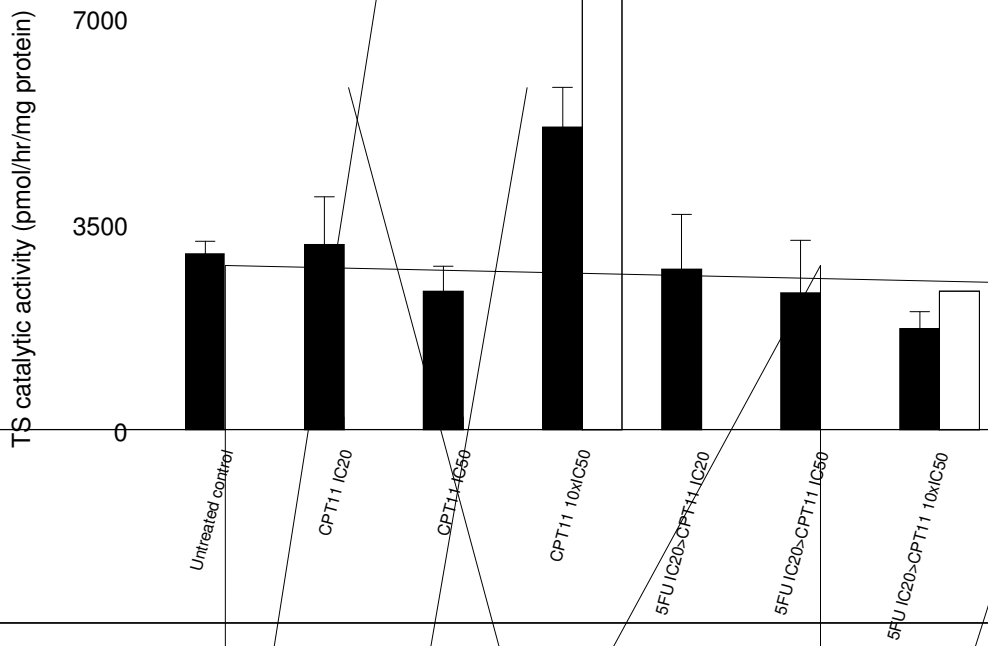


Figure 2: Effect of treatment with 5-FU alone and 2 h IC_{20} CPT-11 followed by 22 h 5-FU (a); 24 h CPT-11 alone and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (b) in HT-29 (■) and SNU-C4 (▨) human colon carcinoma cells on TS catalytic activity. TS catalytic activity in untreated and treated cells were expressed as pmol/hr/mg protein (mean ± SEM) of three different experiments. *Different from untreated control ($p < 0.05$).

The CPT-11 followed by 5-FU treatment showed a similar pattern than that with the drugs alone. When cells were preincubated with CPT-11 at IC_{20} followed by 5-FU, again, a significant concentration dependent decrease in TS was observed in both cell lines ($p < 0.05$; Fig 2a), but not more than for 5-FU alone. However, treatment with 5-FU at IC_{20} followed by CPT-11 at IC_{20} and IC_{50} had the same levels of the control cells in both cell lines. On the other hand, the 5-FU at IC_{20} followed by CPT-11 at 10 x IC_{50} decreased the TS activity to about 40% in HT-29 cells, but in SNU-C4 this treatment remained around the control levels of TS ($p < 0.05$; Fig.2b).

4.3 THYMIDYLATE SYNTHASE mRNA EXPRESSION

Taking into account that TS catalytic activity could be regulated by its mRNA [24], we determined whether the TS-mRNA levels were involved in the different responses to the combination treatments. The basal TS-mRNA levels demonstrated that SNU-C4 cells had 1.3-fold more TS-mRNA expression than the HT-29 cell line ($p < 0.05$; Fig. 3a and 3b). As shown in Fig. 3a, only 5-FU alone induced a concentration dependent reduction in TS-mRNA expression, in both cell lines.

In HT-29 cells the treatment with CPT-11 at IC_{20} followed by 5-FU IC_{20} did not differ from control levels of TS-mRNA. However, treatment with CPT-11 at IC_{20} followed by 5-FU IC_{50} decreased the TS-mRNA expression in about 20% when compared to untreated controls (Fig. 3a). When compared to 5-FU at IC_{50}, this treatment (CPT-11 at IC_{20} followed by 5-FU at IC_{50}) increased TS-mRNA expression in about 30% ($p < 0.05$). In SNU-C4 cells this combination did not induce significant differences in TS-mRNA expression when compared to untreated controls (Fig. 3a). Then again, CPT-11 at IC_{20} followed by 5-FU at 10 x IC_{50} decreased the TS-mRNA expression in approximately 30% in both cell lines ($p < 0.05$; Fig. 3a).

When we exposed the HT-29 cell line to CPT-11 alone at IC_{20} and IC_{50}, the mRNA expression were the same to that observed in untreated control, but with CPT-11 at 10 x IC_{50} we noticed an increased mRNA expression in approximately 30% ($p < 0.05$). In SNU-C4 cells this treatment did not differ from untreated controls (Fig. 3b). Treatment with 5-FU at IC_{20} followed by CPT-11 at IC_{20}, IC_{50} or 10 x IC_{50} showed the same TS-mRNA profile of the untreated controls, in both cell lines (Fig. 3b).

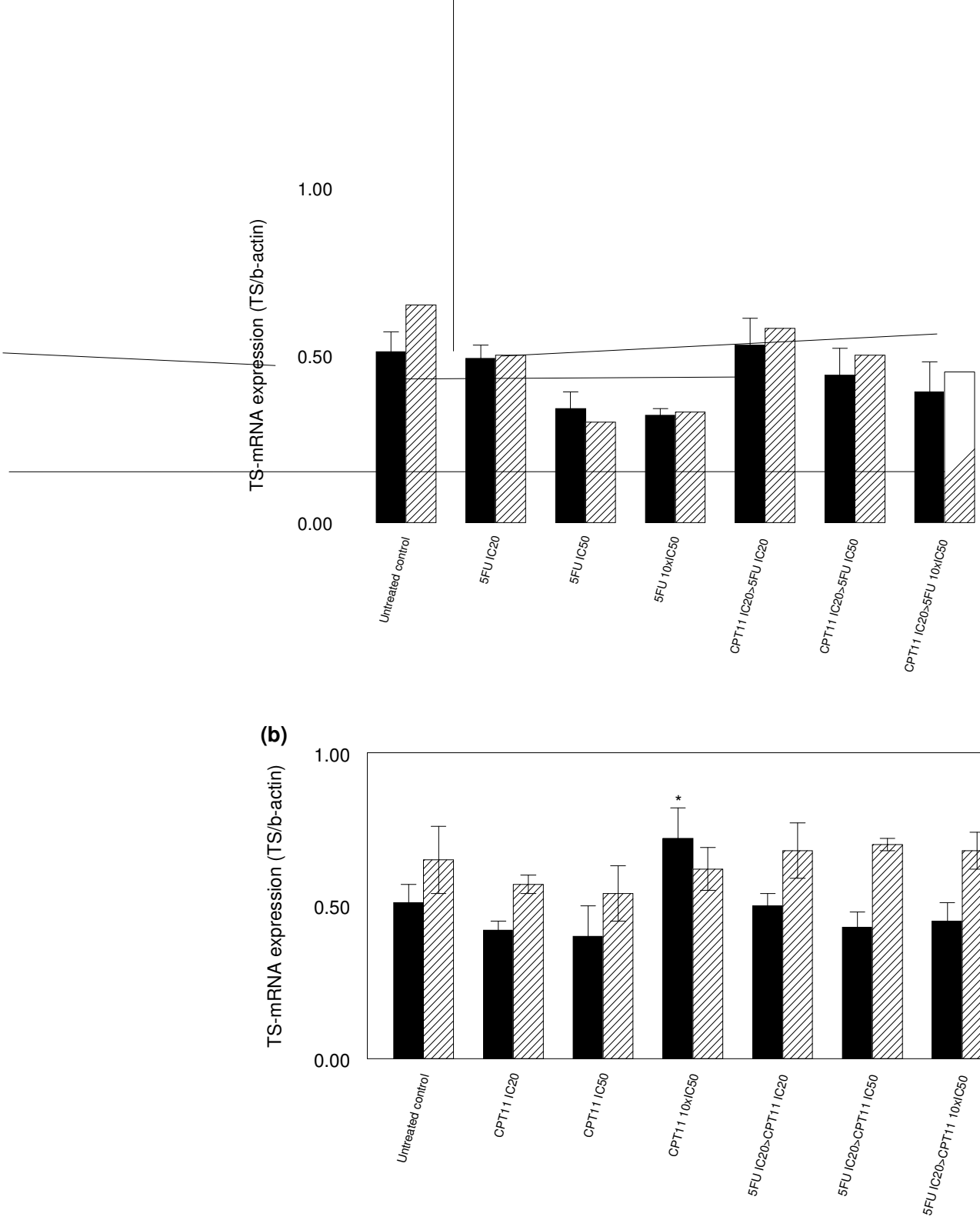


Figure 3: Effect of treatment with 5-FU alone and 2 h IC_{20} CPT-11 followed by 22 h 5-FU (a); 24 h CPT-11 alone and 2 h IC_{20} 5-FU followed by 22 h CPT-11 (b) in HT-29 (■) and SNU-C4 (▨) human colon carcinoma cells on on TS-mRNA. Data were expressed as TS/ ß-actin ratio (mean $\pm$ SD) of three different experiments. *Different from untreated control ($p < 0.05$).

TS activity and TS-mRNA expression were compared within the same samples to determine if the levels of TS-mRNA correlated to TS catalytic activity. A linear relation was noted between TS catalytic activity and TS-mRNA expression in SNU-C4 cells (r^2 = 0.6038), while HT-29 cell line did not show correlation (r^2 = 0.2503).

5. DISCUSSION

The combination of 5-FU with the topoisomerase I inhibitor CPT-11 is an important strategy in the treatment of advanced colon carcinoma. Previous studies have demonstrated that the synergy between TS and topoisomerase I inhibitors is greatest when the topoisomerase I inhibitors are administered prior to the TS inhibitors [1-4]. However, the mechanisms involved in this synergism, have not been elucidated. In the present study, we investigated the effects of the combination with CPT-11/5-FU on TS activity and TS-mRNA expression on the HT-29 and SNU-C4 cell lines. We demonstrated that prior treatment with CPT-11, at a low cytotoxic concentration (IC_{20}), potentiated 5-FU-mediated cytotoxicity, and that, conversely, 5-FU at IC_{20} prior to CPT-11, led to antagonistic interaction [1]. Although the two cell lines (HT-29 and SNU-C4) differed significantly (up to 8-fold) in their sensitivity to 5-FU, they showed less differences (approximately 2-fold) in their response to CPT-11. These findings are in agreement with the absence of cross-resistance between both drugs, as well as with the demonstrated efficacy of CPT-11 in 5-FU-resistant colon carcinoma [25-27].

The 5-FU treatment significantly decreased the TS-mRNA expression and TS catalytic activity in both cell lines. This is in agreement with Peters *et al* [28-30] who reported a decrease in TS activity in cell lines [29], and TS-mRNA in patients [30] after 23 h of 5-FU treatment, and with Xu *el al.* [31] that demonstrated a depletion of free TS after 24 h treatment in cell lines. In line with previous studies

[29,32,33], it was found that 5-FU induced a concentration dependent TS inhibition.

When HT-29 and SNU-C4 cells were treated with CPT-11 at IC_{20} followed by 5-FU we observed a similar TS activity inhibition as with 5-FU alone. These effects most probably are due only to 5-FU at IC_{50} concentration, and not associated with CPT-11 at IC_{20} interference on neither TS activity nor TS-mRNA expression. Our results corroborate a previous study [31], which suggested that SN-38 (a CPT-11 metabolite) does not interfere with TS expression. Indeed, CPT-11 at IC_{20} did not induce significant modification on TS activity or TS-mRNA expression. The same held true for CPT-11 at IC_{50}. However, when HT-29 cells were exposed to CPT-11 at 10 x IC_{50}, we noticed an increased in TS activity and TS-mRNA expression. In SNU-C4 cells, we observed an increase in TS activity but not in TS-mRNA expression when we treated these cells with CPT-11 at 10 x IC_{50}. These data suggested that at higher concentrations CPT-11 could interfere with TS regulation. In line with this, Guichard *et al*. [3], demonstrated that CPT-11 before 5-FU exposure induced a significant decrease in TS activity.

In summary, our data suggested that the synergistic response could not be attributed simply to an enhanced effect of CPT-11 on the expression or activity of thymidylate synthase. Therefore, additional investigations are required to monitor interference of CPT-11 in TS levels.

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CAPÍTULO IV

Interference of Topoisomerase I Activity in the Response of the Irinotecan/5-Fluorouracil Combination on Human Colorectal Cancer Cell Lines

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1. ABSTRACT

Irinotecan (CPT-11) is a topoisomerase I (Topo I) inhibitor that has proven activity in advanced colon cancer, with approximately 20% responses in untreated as well as in 5-fluorouracil (5-FU)-resistant tumors. Topo I is a nuclear enzyme involved in DNA replication transcription and repair. The work presented here intended to identify the involvement of Topo I on the greater cytotoxicity observed with combination of CPT-11/5-FU in HT-29 and SNU-C4 colon cancer cell lines. Thus, cells were exposed to each drug in varying concentrations and sequences, and assessed for colony formation, Topo I activity, DNA-Topo I complex formation and cell cycle distribution. Pre-treatment with CPT-11 at IC_{20} concentration increased the 5-FU cytotoxicity, while the pre-treatment with 5-FU at IC_{20} concentration decreased the CPT-11 cytotoxicity. Topo I activity was 2-fold higher in SNU-C4 cells than in the HT-29 cells, suggesting that the distinct sensitivity to CPT-11 among the cell lines were correlated to Topo I profile. The CPT-11 treatment significantly decreased the Topo I activity in both cell lines. Moreover, in the HT-29 cells, the sequence CPT-11/5-FU decreased Topo I activity in 20% compared to untreated control. In SNU-C4 cells the 5-Fu at IC_{20} could inhibit Topo I activity. The DNA-Topo I complex formation did not increase with the combination CPT-11/5-FU in any cell line studied. CPT-11 induced G2/M arrest, while 5-FU arrested cells in S-phase in both cell lines. This finding suggests that the CPT-11/5-FU cytotoxicity are not related to Topo I activity.

2. INTRODUCTION

Colorectal cancer is a common disease with high rate of mortality [1,2]. Although there is evidence of benefit of chemotherapy in adjuvant and metastatic disease, its use still remains unsatisfactory because of intrinsic or acquired drug resistance [2,3]. The most commonly used protocols in advanced colorectal carcinoma include the antimetabolite 5-fluorouracil (5-FU) [4]. 5-FU is a fluoropyrimidine that acts by inhibiting thymidylate synthase, a key enzyme in the *de novo* synthesis of thymidine nucleotides required for DNA synthesis [5]. The introduction of irinotecan (CPT-11) to the therapy with 5-FU has contributed to increase therapeutic options and to improve response rates and survival [6,7]. However, despite the promising results, only 40% of patients with advanced colorectal cancer respond to this combination [3,8]. One possible way to improve these results lies in the availability of biomarkers capable of identifying the patients who are more likely to benefit from this therapy, for instance topoisomerase I and thymidylate synthase [9].

Topoisomerase I (Topo I) is a nuclear enzyme essential for the conservation of DNA topology [10]. Topo I acts by nicking a single DNA strand, passing the intact strand through the nick and then resealing the nick, resulting in a decrease in the linking number by one. Thus, it unwinds supercoiled DNA and appears to function mainly during DNA transcription, replication, recombination and repair [10,11].

CPT-11 is a potent topoisomerase-I inhibitor that reversible stabilizes DNA-topo I cleavable complexes and blocks the progression of the DNA replication fork [10-13]. Precisely how this stabilization leads to cell death is not well understood. Since CPT-11 is S-phase specific drug, it is hypothesized that DNA replication forks collide with Topo I complexes resulting in fork arrest, topologically induced DNA double strand breaks, G2 cell cycle arrest, and cell death [10,13].

In vitro and *in vivo* evidences suggest a schedule-dependent cytotoxic interaction for the combination of 5-FU and CPT-11. These studies demonstrated that the sequence that presents the greatest cytotoxic effect is CPT-11 followed by 5-FU [14-17]. Despite research efforts, the mechanisms involved in this drug interaction still remain unclear, yet interference with Topo I activity is one of the plausible mechanisms [14-17].

Therefore, we examined in this study the *in vitro* effects of the treatments with CPT-11 and 5-FU at various combinations on Topo I activity. We used IC_{20} (hardly growth-inhibiting concentrations) of one drug in order to modulate the effect of the other drug.

3. MATERIALS AND METHODS

3. 1 CELL CULTURE MAINTENANCE

The HT-29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville; MD, USA). The SNU-C4 human colon carcinoma cell line was kindly supplied by Dr. G.J. Peters (Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands). The cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum at a temperature of 37 ^{o}C and in a humidified atmosphere of 5% CO_2 in air.

3.2 COLONY FORMATION STUDIES

The cytotoxic effects of 5-FU and CPT-11 were examined after treatments of HT-29 and SNU-C4 cells with: (a) 24 h to IC_{50} of either 5-FU or CPT-11; (b) 2 h with IC_{20} of CPT-11 followed by 22 h to IC_{50} of 5-FU; (c) 2 h with $IC_{20\ of}$ 5-FU followed by 22 h to IC_{50} of CPT-11. Control cells were cultured during 24 h without drug treatment. Clonogenic assay was performed as described previously [18]. Briefly, SNU-C4 and HT-29 cells were seeded into 6-well plates (300 cells/well) after treatment and incubated during 7 days. Then, the cells were fixed with 70% ethanol and counterstained with 0.5% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. The survival fraction (SF) was then calculated as:

$$SF = \frac{\text{Number of colonies in treated cells}}{\text{Number of colonies in untreated control}} \times 100$$

3.3 TOPOISOMERASE I ACTIVITY ASSAY

Topo I activity was assessed in nuclear extracts prepared from 5×10^6 cells as described previously [19]. In brief, cells were suspended in ice-cold 1 mM KH_2PO_4 pH 6.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF, and 0.3% Triton X-100, and nuclei were pelleted by centrifugation at 4 ^oC. The nuclear fractions were ressuspended with 10 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, followed by extraction at 4 ^oC with 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 M NaCl, 1 mM DTT, and 1 mM PMSF. The nuclear extracts were centrifuged and the supernatants were used to assess Topo I activity or protein contents.

Topo I catalytic activity was evaluated by relaxation of supercoiled pHOT1 plasmid DNA using a Topo I assay Kit from TopoGen (Columbus, OH, USA).

Relaxed DNA was separated from supercoiled DNA by electrophoresis at 20 V for 4 h in a 1% agarose gel containing 0.5 µg/ml of ethidium bromide, which was immediately photographed and analyzed.

3.4 ASSAY FOR DNA-TOPOISOMERASE I COMPLEXES

The *in vitro* formation of DNA-Topo I cleavable complexes was quantitated using the SDS precipitation assay described previously [20]. Briefly, 2 x 10^5 cells/mL were seeded into 6-well plates. After 24 h the culture medium was removed, the cells were labeled with [U-^{14}C]leucine (0,2 µCi/mL; specific activity 308 mCi/mmol; Amersham International, UK) and [methyl-^{3}H]thymidine (0,6 µCi/ml; specific activity 76 Ci/mmol; Amersham International, UK) and incubated for 12 h at 37 ^{o}C. Followed incubation time, the medium was removed and cultures were treated as described in colonony formation section. After 24 h treatments, the cells were washed and scrapped in ice-cold PBS and the pellets were lysed at 65 ºC in lysis solution (5 mM EDTA, 1.25% SDS and 0,4 mg/mL salmon sperm DNA). The lysates were transferred to an eppendorf tube containing 250 µL of 325 mM KCl solution. After vortexing, the samples were cooled on ice for 10 min and centrifuged for 10 min at 4 ºC. The pellets were resuspended in 1 mL of prewarmed wash solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM KCl 100 and 0,1 mg/mL salmon sperm DNA. After wash and centrifugation the pellets were resuspended in 200 µL H_20 (65 ºC) and 5 mL of scintillation liquid (Optiphase-Hisafe 3) was added. The radioactivity was determined in a scintillator counter.

3.5 FLOW CYTOMETRY ANALYSIS

After treatment, cells were harvested by trypsinization and washed in ice-cold PBS. Samples of floating and adherent cells (5 x 10^5) were then fixed in 70%

ethanol at 4^{o}C overnight. The 600 x g centrifuged cell pellet was washed in 1 mL ice-cold PBS, and resuspended in 0.5 mL PBS. To

formation in HT-29 cells occurred when the cells were exposed to 9.0 µM and 2.5 µM of 5-FU and CPT-11, respectively.

When the cells were exposed to CPT-11 for 2 h at IC_{20} (2.0 and 1.0 µM, for SNU-C4 and HT-29 cell lines, respectively) followed by 5-FU for 24 h at IC_{50}, the relative values of Survival Fraction (SF) decreased about 2.5-fold in both cell lines (16.6% and 19.6% for SNU-C4 and HT-29 cell lines, respectively; Fig 1) when compared to 5-FU or CPT-11 at IC_{50} ($p < 0.05$). Finally, when the cells were exposed to 5-FU for 2 h at IC_{20} (1.0 and 3.0 µM, for SNU-C4 and HT-29 cell lines, respectively) followed by CPT-11 for 24 h at IC_{50} the relative values of SF increased 1.8-fold in SNU-C4 cells and 1.3-fold in HT-29 cell line, when compared to cells treated with CPT-11 at IC_{50} ($p < 0.05$; Fig. 1). Thus, in accordance to previous studies [14-17], when cells were treated with sequential CPT-11/5-FU a greater cytotoxic effect was observed.

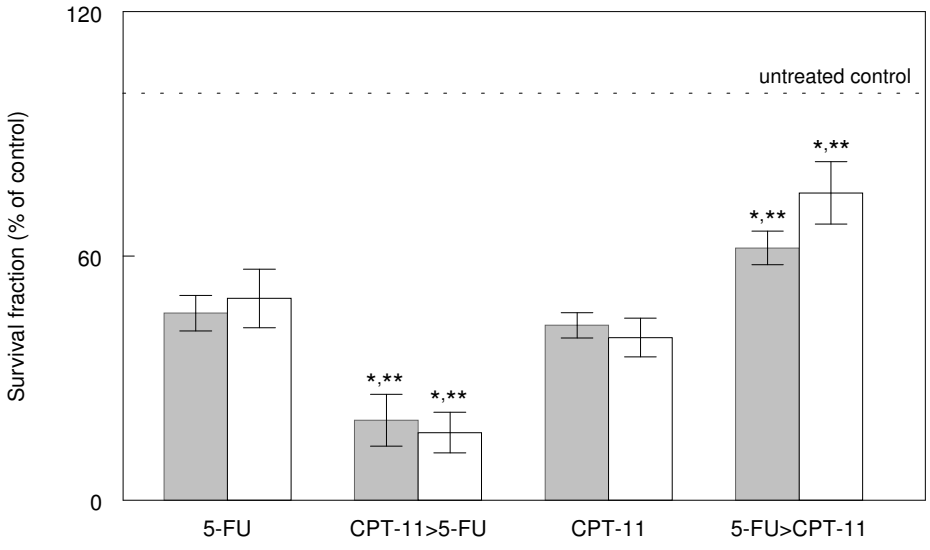

Figure 1: Effect of treatment with 5-FU alone; 2 h IC_{20} CPT-11 followed by 22 h 5-FU; 24 h CPT-11 alone and 2 h IC_{20} 5-FU followed by 22 h CPT-11 on HT-29 (▩) and SNU-C4 (□) human colon carcinoma cells survival fraction. Following treatment, cells were incubated during 10 days. Colonies containing $\geq$ 50 cells were scored. Data was expressed as the percentage of the survival fractions of cells versus the different treatments. Data was plotted as the mean $\pm$ SD of three different experiments. *Different from 5-FU alone, ** different from CPT-11 alone ($p < 0.05$).

4.2 EFFECTS OF DRUG TREATMENTS ON TOPOISOMERASE I ACTIVITY

To examine whether Topo I activity levels could account for the responses of the cell lines to drug treatments, catalytic activities of this enzyme were assessed in nuclear extracts and expressed as ng DNA relaxed/ng nuclear protein.

Table 1: Effect of treatments with 5-FU at IC_{50}; 2 h IC_{20} CPT-11 followed by 22 h 5-FU at IC_{50}; 24 h CPT-11 at IC_{50}; and 2 h IC_{20} 5-FU followed by 22 h CPT-11 at IC_{50} on HT-29 and SNU-C4 human colon carcinoma cells on Topo I catalytic activity. Topo I catalytic activities in untreated and treated cells were expressed as ng DNA relaxed per ng nuclear protein (means $\pm$ SD) of three different experiments. *Different from 5-FU alone, **Different from CPT-11 alone ($p < 0.05$).

	HT-29	SNU-C4
Untreated control	14.0 ± 0.1	30.2 ± 4.8
5-FU IC_{20}, 2 h	13.5 ± 0.5	22.2 ± 0.3[a]
5-FU IC_{50}, 24 h	12.5 ± 0.8	26.2 ± 1.2
CPT-11 IC_{20} 2 h, then 5-FU IC_{50} 22 h	7.7 ± 0.4[a]	27.1 ± 2.7
CPT-11 IC_{20}, 2 h	10.1 ± 0.3[a]	25.8 ± 0.9
CPT-11 IC_{50}, 24 h	8.2 ± 0.2[a]	17.4 ± 1.0[a]
5-FU IC_{20} 2 h, then CPT-11 IC_{50} 22 h	16.9 ± 1.0	29.8 ± 2.3

[a]Significantly different from untreated control ($p < 0.05$)

Topo I catalytic activity in the SNU-C4 cells was approximately 2-fold greater than that in the HT-29 cells (Table 1). These differences in Topo I activity

might explain the lesser sensitivity to CPT-11 observed in SNU-C4, when compared to HT-29 (4.5 µM and 2.5 µM, respectively). A linear relation was noted between Topo I activity and sensitivity to CPT-11 in both cell lines (r^2 = 0.8822, Fig. 2).

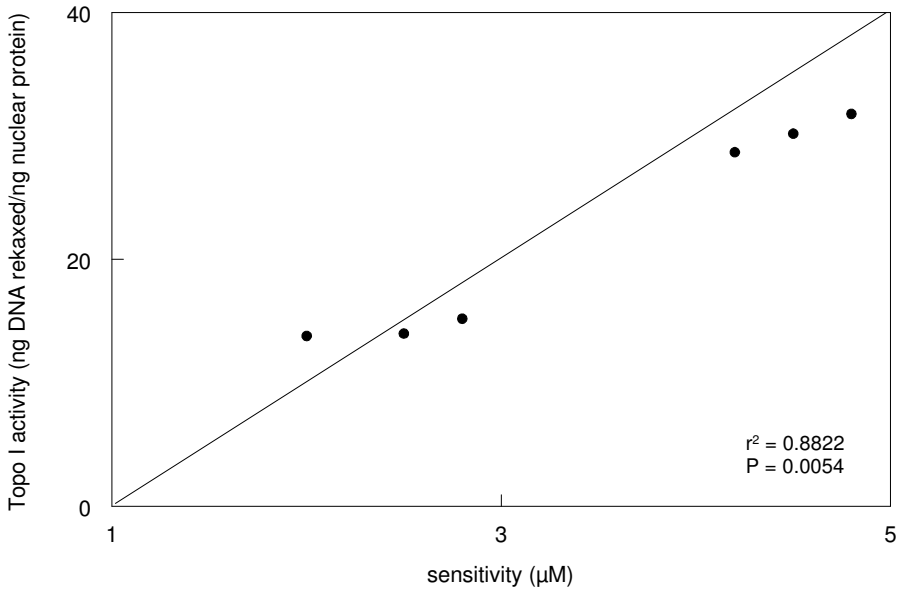

Figure 2: Correlation between Topo I activity and CPT-11 sensitivity in HT-29 and SNU-C4 cell lines. The analysis was carried out using a Pearson`s coefficient.

CPT-11 at IC_{50} decreased the Topo I activity in 1.7-fold in both cell lines ($p < 0.05$), while CPT-11 at IC_{20} induced a decrease of approximately 20% in Topo I activity only in HT-29 cells (Table 1). On the contrary, treatments with 5-FU at IC_{50} and 5-FU followed by CPT-11 did not alter significantly Topo I activity neither in SNU-C4 nor in HT-29 cells when compared to untreated controls ($p < 0.05$). However, in SNU-C4, 5-FU at IC_{20} inhibited Topo I activity (Table 1). When the SNU-C4 cells were exposed to CPT-11 followed by 5-FU the Topo I catalytic activity maintained levels comparable to those detected in the untreated control cells. However, in HT-29 cells the treatment with CPT-11 followed by 5-FU

decreased the Topo I activity to those in cells treated with CPT-11 at IC_{50} ($p < 0.05$; Table 1).

4.3 INTERFERENCE WITH DNA-TOPO I COMPLEXES FORMATION

Figure 3 summarizes the formation of DNA- topo I complexes in HT-29 and SNU-C4 cell lines after treatments with CPT-11 and 5-FU.

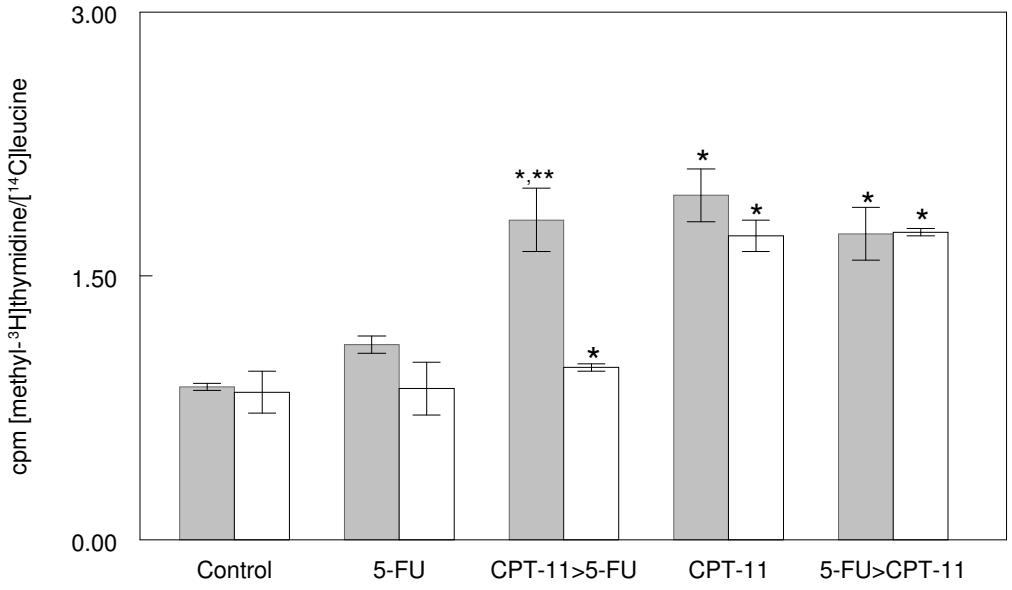

Figure 3: Effect of treatment with 5-FU alone; 2 h IC_{20} CPT-11 followed by 22 h 5-FU; 24 h CPT-11 alone and 2 h IC_{20} 5-FU followed by 22 h CPT-11 on DNA-Topo I complex formation on HT-29 (□) and SNU-C4 (■) human colon carcinoma cells. Data was expressed as the cpm [methyl-^{3}H]thymidine/[^{14}C]leucine ratio and plotted as the mean ± SD of three different experiments. *Different from untreated control, **Different from CPT-11 alone ($p < 0.05$).

When cells were exposed to CPT-11 at IC_{50}, a 2-fold increase in the DNA-Topo I complexes formation was observed in both cell lines. On the other hand,

after 5-FU at IC_{50} treatment, none of the cell lines increased the number of the DNA-Topo I complexes, when compared to untreated cells. When the cells were exposed to 5-FU at IC_{20} followed by CPT-11 at IC_{50} we observed the equal amounts of the DNA-Topo I complexes than the observed with CPT-11 at IC_{50} alone in both cell lines. However, when the cells were incubated with CPT-11 at IC_{20} followed by 5-FU at IC_{50} only in HT-29 cells an increased in the DNA-Topo I complexes was demonstrated, when compared to the untreated cells.

4.4 INFLUENCE OF TREATMENTS ON CELL CYCLE DISTRIBUTION

It is well known that CPT-11 acts through interference with Topo I during S phase [22]. Thus, we further investigated whether the interaction of CPT-11 with 5-FU, could alter the cell cycle distribution using a flow cytometry. In both untreated cell lines, 50-60 % of the cells were in G1 phase, 15-25 % in S phase and about 18 % in G2/M phase (Table 2).

The flow cytometry analysis demonstrated that, in SNU-C4 cells, all treatments containing 5-FU increased in 2.8-fold the number of cells in S-phase ($p < 0.05$; Table 2). On HT-29 cells we noticed an arrested in S-phase only after exposure to 5-FU at IC_{50} or CPT-11 at IC_{20} followed by 5-FU at IC_{50} (Table 2). When HT-29 cells were previously treated with 5-FU at IC_{20}, the cell cycle distribution was similar to untreated controls. In both cell lines, the treatment with CPT-11 at IC_{50} increased in 2-fold the number of cells in G2/M phase and the percentage of cells in G1 phase decreased relative to cells in S-phase. (Table 2).

Table 2: Effect of treatments with 5-FU at IC_{50}; 2 h IC_{20} CPT-11 followed by 22 h 5-FU at IC_{50}; 24 h CPT-11 at IC_{50}; and 2 h IC_{20} 5-FU followed by 22 h CPT-11 at IC_{50} on cell cycle distribution of HT-29 and SNU-C4 human colon carcinoma cells. Percentage (mean ± SD) of cell cycle phase observed in each cell line after treatments of three different experiments. *Different from 5-FU alone, **Different from CPT-11 alone ($p < 0.05$).

	G0/G1		S		G2/M	
	HT-29	**SNU-C4**	**HT-29**	**SNU-C4**	**HT-29**	**SNU-C4**
Control	56.6 ± 3.8	61.2 ± 1.3	25.6 ± 0.5	16.6 ± 3.8	16.9 ± 2.0	19.9 ± 4.3
5-FU	43.7 ± 0.4	42.4 ± 2.3	39.8 ± 1.0 [a]	44.6 ± 0.9[a]	13.1 ± 0.2	14.9 ± 1.3
CPT-11 > 5-FU	41.3 ± 3.4	43.7 ± 0.3	42.5 ± 0.3 [a,]	40.8 ± 2.3 [a]	15.7 ± 1.0	15.6 ± 2.3
CPT-11	37.5 ± 1.5	42.1 ± 7.1	26.7 ± 0.7	15.3 ± 0.7	36.2 ± 4.7[a]	40.8 ± 7.4[a]
5-FU > CPT-11	50.1 ± 1.4	34.4 ± 6.4	23.4 ± 3.3	41.7 ± 3.3 [a]	25.1 ± 3.8	26.2 ± 1.4

[a]signifficant different of untreated control ($p < 0.05$)

5. DISCUSSION

The combination therapy with 5-FU/LV and CPT-11 demonstrated to be more active than 5-FU/LV as a first line treatment in advanced colorectal cancer [23]. Previous studies have demonstrated that the synergism between 5-FU and CPT-11 is greatest when the CPT-11 is administered prior to 5-FU [14-17]. However, the mechanisms involved in this behavior, has not been elucidated. We have previously [14] demonstrated that the combination of pretreatment with CPT-11 at IC_{20} followed by treatment with 5-FU on cultured human colon carcinoma cells presented anti-proliferative effects and induced DNA damage [24]. Using the SNU-C4 and HT-29 colon carcinoma cell lines we investigated whether the greater

anti-proliferative effect of IC_{20} CPT-11 for 2 h followed by 22 h of IC_{50} of 5-FU treatment could be related to disturbances in Topo I activity.

The prior treatment with CPT-11 at IC_{20}, potentiated 5-FU-mediated cytotoxicity, and in contrast, 5-FU at IC_{20} prior to CPT-11, pointed to antagonistic interaction. The two cell lines (SNU-C4 and HT-29) differed significantly (3-fold) in their sensitivity to 5-FU, and in approximately 2-fold in their response to CPT-11. SNU-C4 cell line is 1.8-fold less sensitive to CPT-11 than HT-29 cells. This difference can be explained by the higher basal level of Topo I activity (2-fold) in SNU-C4 cells when compared to HT-29 cell line. Indeed, it has been suggested that Topo I in tumor cells is directly related to the to sensitivity to Topo I inhibitors [25,26].

As expected CPT-11 inhibited Topo I activity in both cell lines. This is in agreement with Ulukan *et al*. that demonstrated in the human intestinal cell line Caco-2 treated with CPT-11 a decrease in Topo I activity [27]. 5-FU alone did not interfere with Topo I activity in the cell lines studied. However, the 5-FU at IC_{20} followed by CPT-11 showed similar Topo I profiles to those observed in untreated cells. This data suggests that 5-FU at IC_{20} could reduce the inhibition of Topo I induced by CPT-11, since CPT-11 alone inhibited Topo I activity. There has been much speculation concerning the role of Topo I on DNA repair. Topo I has been localized in areas of the genome undergoing high rates of transcription [28]. These areas have also been shown to repair DNA damage at much faster rate than other non-transcription areas [29]. Therefore, Topo I maybe involved in DNA repair processes [30,31]. In a study associating camptothecin with X-ray, a correlation between synergistic effect of this combination and recruitment of Topo I for DNA repair was demonstrated [32]. In 5-FU followed by CPT-11 sequence a similar mechanism could be involved, since Topo I was implicated in the repair of abasic sites induced by 5-FU exposure [33]. Indeed, for SNU-C4 cell line this seems to be the case. We observed an inhibition of approximately 25% on Topo I activity when SNU-C4 cells were treated with IC_{20} of 5-FU for 2 h. When cells were treated with CPT-11 at IC_{20} followed by 5-FU only in HT-29 an inhibition of Topo I activity was observed. This could be explained by the greater sensitivity of this cell line to CPT-

11. Indeed IC_{20} is sufficient to inhibit the Topo I activity in this cell line. In line with this observation, we demonstrated that CPT-11 at IC_{20} is enough to increase the number of the DNA-Topo I complexes in HT-29 cell line, but not in SNU-C4 cell line.

It is well established that Topo I inhibitors induced an accumulation or prolongation of Topo I cleavable complexes resulting in irreversible DNA replication defects and subsequent cell cycle arrest and cell death [34]. The number of the DNA-Topo I complexes did not increase with the CPT-11 followed by 5-FU combination. These data were in accordance with Guichard *et al.* [16] that demonstrated that the this combination treatment did not increased the number of the DNA-Topo I complexes, but induced a greater stability of the cleavage sites formed.

The pattern of cell cycle distribution observed in this study demonstrated that in both cell lines CPT-11, at IC_{50} concentration, mainly induced G2/M arrest, while 5-FU preferentially induced arrest in S-phase. Previous studies [35,36], have found that 5-FU induced S-phase arrest. This arrest should be induced by inhibition of DNA synthesis in the middle of replication due to dTTP depletion [37]. In this study, the S-phase arrest was more pronounced in the SNU-C4 cell line compared to HT-29 cells. It is possible that this effect was due to a more specific inhibition of thymidylate synthase by 5-FU or its metabolite FdUMP in SNU-C4 cells. When cells were treated with CPT-11 followed by 5-FU we noticed an enhance in 5-FU-inducible S-phase arrest in both cell lines. This could explain the greater cell growth inhibition observed with this combination.

In summary, the results of the current study demonstrate that Topo I could be involved in the response of the HT-29 cell line to treatment with combination of CPT-11/5-FU. Although the findings of this study cannot provide a mechanistic explanation, it suggests that the role of Topo I in DNA repair after treatment with CPT-11/5-FU should be further investigated.

6 ACKNOWLEDGEMENTS

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DISCUSSÃO

O carcinoma de cólon é o responsável pela terceira causa de morte relacionada ao câncer no mundo (American Cancer Society Homepage, 2004. Este panorama pode ser explicado pela heterogeneidade encontrada neste tipo de tumor, o que dificulta o tratamento desta neoplasia. De fato, o câncer de cólon avançado não apresenta respostas satisfatórias com a terapia convencional (Skibber *et al.*, 2001). O agente quimioterápico mais ativo nesta neoplasia é o antimetabólito 5-FU associado ao LV, produzindo respostas de no máximo 30%. A combinação do 5-FU/LV com CPT-11 demonstrou ser mais ativa que o 5-FU/LV como tratamento de primeira linha no câncer de cólon avançado (Plate, 2001). Entretanto, somente 40% dos pacientes respondem a esta terapia (Chau and Cunningham, 2002). Tal fato ocorre, pois a resposta a esta terapia é comumente limitada pela resistência destas neoplasias. Neste sentido, diversos estudos têm sido desenvolvidos buscando identificar os mecanismos envolvidos na resistência a estas drogas antineoplásicas e desenvolver novas estratégias de tratamento (Pommier *et al.*, 2004). Uma vez que, as células tumorais que apresentam reconhecidos mecanismos anti-apoptose possuem vantagens sobre a manutenção do crescimento quando comparadas às demais, a identificação de uma forma de induzir a apoptose nestas células poderá representar uma estratégia de aumento da eficácia deste agentes antineoplásicos (Pommier *et al.*, 2004). Com base nestas considerações, no presente trabalho, foram investigados quais dos mecanismos moleculares estão envolvidos nas respostas obtidas com a

combinação CPT-11/5-FU, bem como quais das vias de apoptose são preferencialmente desencadeadas após o tratamento com estes agentes.

Com o emprego das linhagens celulares de adenocarcinoma de cólon humano HT-29 e SNU-C4, foi demonstrado significativa diferença entre as linhagens em relação à sensibilidade ao 5-FU e ao CPT-11. Nesta seqüência de experimentos foi verificado que a linhagem HT-29 é mais resistente ao 5-FU e mais sensível ao CPT-11, enquanto que a linhagem SNU-C4 apresenta maior sensibilidade ao 5-FU e maior resistência ao CPT-11 (Capítulo I). A inibição da TS é um importante mecanismo da citotoxicidade do 5-FU. Diversos autores mostraram que tumores com baixos níveis de expressão da TS apresentam uma maior resposta ao tratamento com o 5-FU (Paradiso *et al.*, 2000) Por outro lado, Grem *et al.* (2001) mostrou que não existe correlação entre sensibilidade ao 5-FU e atividade e RNAm da TS em um painel de 60 linhagens celulares. A avaliação da atividade e do RNAm da TS demonstraram que a diferença de sensibilidade ao 5-FU entre as linhagens celulares não está relacionada com a atividade da TS (Capítulo III). Contudo, devemos levar em consideração que outros processos, como transportes transmembrana e outras enzimas como a timidina quinase e diidropirimidina desidrogenase (Pinedo & Peters, 1988; Peters, 1995; van der Wilt *et al.*, 1997; van Kuilenburg, 2004) devem estar envolvidos na diferença de sensibilidade ao 5-FU observada. Em relação à sensibilidade das linhagens ao CPT-11, nossos resultados mostraram que a linhagem SNU-C4 apresenta maior atividade da topoisomerase I quando comparada com a linhagem HT-29 (Capítulo IV), sendo responsável pela maior resistência da linhagem SNU-C4 ao CPT-11. Ainda, estes achados estão de acordo com a ausência de resistência cruzada entre os dois agentes e com a eficácia do CPT-11 em carcinomas de cólon resistentes ao 5-FU (revisado em Grivicich *et al.*, 2001).

A combinação dos dois agentes demonstrou que o pré-tratamento com CPT-11 em uma baixa concentração (concentração de IC_{20}) e por um curto período de tempo (2 h) potencializou a inibição do crescimento celular induzida pelo 5-FU (4 x na linhagem HT-29 e 2 x na linhagem SNU-C4; Capitulo I). Entretanto, a exposição ao 5-FU, nas mesmas condições, antes do CPT-11 levou

a uma interação antagônica entre os agentes (2 x na linhagem HT-29 e 4 x na linhagem SNU-C4; Capítulo I). Portanto, somente foi observado sinergismo quando o CPT-11 precede o 5-FU. Nossos resultados estão de acordo com diversos estudos anteriores (revisado em Grivicich *et al.*, 2001) que demonstraram um efeito sinérgico ou aditivo da seqüência CPT-11>5-FU. Ainda que, os mecanismos envolvidos nestas respostas não estejam completamente elucidados.

Quando administrado em curtos períodos de tempo o 5-FU atua preferencialmente sobre o RNA através da incorporação do ribonucleotídeo FUTP a este, enquanto que em exposições prolongadas prevalece a toxicidade sobre o DNA (Grem, 1996). Este último efeito é decorrente da inibição da TS pelo metabólito ativo FdUMP bloqueando a síntese ou reparo do DNA (Peters *et al.*, 1986), ou ainda, através da interferência na elongação da cadeia de DNA após a incorporação do deoxiribonucleotídeo FdUTP, o que pode induzir a formação de quebras nas fitas do DNA (Yin & Rustum, 1991; Longley *et al.*, 2003). A citotoxicidade do CPT-11, por sua vez, resulta da inibição da atividade da topoisomerase I, igualmente levando á quebras nas cadeias do DNA (Takimoto & Arbuck, 1996). Em ambos os casos, as quebras do DNA podem levar a célula a apoptose ou ao reparo das lesões. Com o objetivo de identificar os mecanismos que levaram às diferentes respostas obtidas com as combinações do CPT-11 e 5-FU foi avaliada a capacidade destes agentes induzirem apoptose.

No capítulo I foi demonstrado que todos os tratamentos com CPT-11 e 5-FU como agentes únicos ou combinados induziram apoptose em ambas as linhagens. De acordo com o observado nos experimentos de citotoxicidade, a linhagem HT-29 demonstrou maior percentual de células em apoptose quando comparada com a linhagem SNU-C4. Na linhagem HT-29 este efeito foi mais marcante com a combinação CPT-11> 5-FU. Estas observações indicam que o efeito sinérgico (4 x mais citotóxico) observado com esta combinação, nesta linhagem, ocorre devido a maior ativação da apoptose. Desta forma, na linhagem HT-29, a exposição prévia a uma dose baixa de CPT-11 aumenta o dano celular

induzido pelo 5-FU, intensificando a apoptose comparativamente aos efeitos provocados pelo 5-FU ou CPT-11 como agentes únicos.

A participação da mitocôndria na resposta celular a apoptose está bem caracterizada (Desagher and Martinou, 2000). O estresse oxidativo é um dos fatores que participam da ativação da apoptose através da via mitocôndrial (Gottlieb, 2001). As espécies reativas do oxigênio (EROs) podem ser geradas de forma endógena durante o metabolismo celular, ou de forma exógena, por exposição a vários agentes como por exemplo à drogas antineoplásicas. Em ambos os casos causam danos à estrutura do DNA, lipídios, carboidratos e proteínas, além de outros componentes celulares (Salganik, 2001). Embora a associação das EROs com a sinalização apoptótica não esteja completamente esclarecida, sabe-se que a produção de ERO pode reduzir o potencial de membrana mitocondrial ativando a caspase-9 e em conseqüência a caspase-3 (Gottlieb *et al.*, 2000; Shimura *et al.*, 2000). Conforme apresentado no capítulo II, foi observado na linhagem HT-29, que a combinação CPT-11>5-FU reduziu em 50% o potencial de membrana mitocondrial e ativou as caspases 9 e 3, contribuindo assim para o maior efeito citotóxico observado nesta linhagem celular. Por outro lado, o tratamento 5-FU>CPT-11 (efeito antagônico) causou um aumento no potencial de membrana mitocondrial, que pode estar associado à tentativa da célula desviar ou retardar a entrada em apoptose (Zamzami *et al.*, 1996; Decaudin *et al.*, 1997).

Além disso, a indução da apoptose pode ser regulada pela expressão de genes pró- apoptóticos e anti-apoptóticos (Tsujimoto and Simizu, 2000). Ueta *et al.* (2001) demonstrou0.8376 voTc 6.35977 0 03168 Tc 3.71992 0 Td 1.81584 Td (u)Tj 0.1

examinado no presente trabalho, tal fato pode ser explicado por um aumento na produção de ERO pelo 5-FU (Li *et al.*, 2004) e pelo CPT-11 (Kishida *et al.*, 2004), ou ainda pela capacidade da Mn-SOD agir como gene supressor de tumor (Zhang et al., 2002).

Na linhagem SNU-C4 não se verificou diferenças no percentual de células em apoptose após tratamento com 5-FU ou com a combinação CPT-11>5-FU. Tal observação sugere que o efeito sinérgico observado com esta combinação nesta linhagem (2 x mais citotóxico), não ocorre devido a maior ativação da apoptose. Este fato pode ser justificado pela menor sensibilidade desta linhagem ao CPT-11, neste caso a dose de IC_{20} não seria necessária para interferir na indução de apoptose pelo 5-FU. A seqüência inversa (5-FU>CPT-11) demonstrou o mesmo percentual de células em apoptose que o observado com CPT-11, porém um menor percentual que os tratamentos com 5-FU e CPT-11 seguido do 5-FU. Assim, apesar da maior sensibilidade ao 5-FU, a dose de IC_{20} do 5-FU não parece ser suficiente para induzir apoptose nesta linhagem. Ou ainda, é possível sugerir que a linhagem SNU-C4 apresente um mecanismo de reparo bastante eficaz, e a dose de IC_{20} por 2 h seria suficiente para desencadear a reparação dos danos ao DNA.

Neste sentido, avaliamos a incorporação de [^{3}H-metil]timidina ao DNA, na presença de hidroxiuréia como um indicativo de reparo por excisão de bases. A hidroxiuréia inibe a enzima ribonucleotídeo redutase, impedindo a replicação do DNA e permitindo que a incorporação de [^{3}H-metil]timidina ocorra somente durante o reparo por excisão (Adams *et al.*, 1971). Resultados preliminares (Figura 17) demonstraram que a linhagem SNU-C4 possui uma maior capacidade de reparo por excisão (aproximadameente 2 vezes) quando comparada com a linhagem HT-29. A combinação 5-FU>CPT-11, na linhagem SNU-C4, levou a um aumento na incorporação de [^{3}H-metil]timidina de aproximadamente 30%, sugerindo um aumento do processo de reparo após este tratamento (Figura 17). Estas observações podem ser explicadas pelo reparo por excisão de sítios apirimídicos formados durante a clivagem da ligação glicosídica do FdUMP no DNA que ocorre em curtos períodos de exposição (2 h) (Peters, 1995). Sítios

apirimídicos são reparados preferencialmente pelo sistema de reparo de excisão de bases (BER), seguido do sistema de excisão de nucleotídeos (NER) (Boiteux and Guillet, 2004).

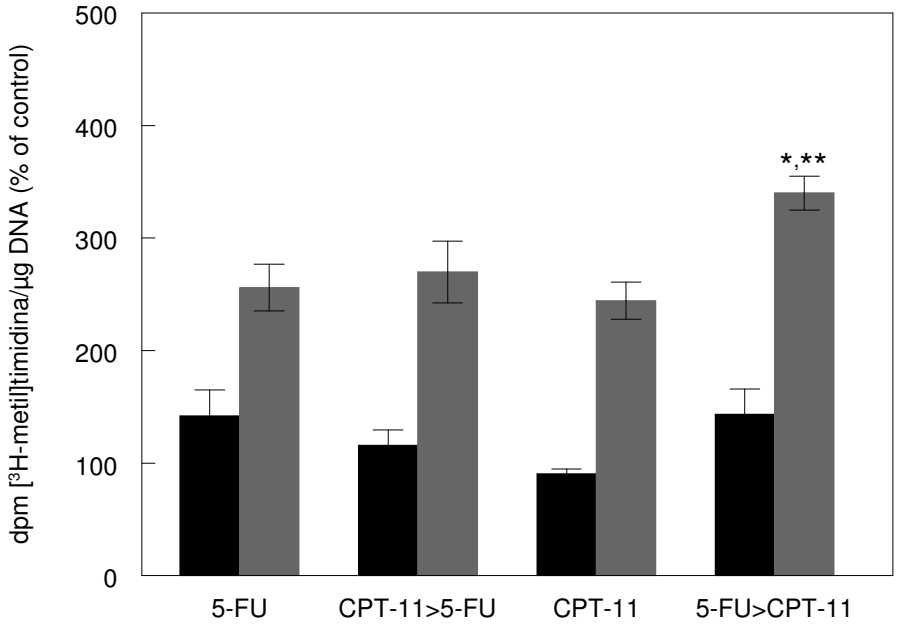

Figura 17: Incorporação de [^{3}H-metil]timidina nas linhagens celulares de carcinoma de cólon humano HT-29 (■) e SNU-C4 (▩) após tratamento com: IC_{50} 5-FU por 24 h; IC_{20} CPT-11 por 2 h seguido de IC_{50} 5-FU por 22 h; IC_{50} CPT-11 por 24 h; IC_{20} 5-FU por 2 h seguido de IC_{50} CPT-11 por 22 h (*significativamente diferente das células tratadas com 5-FU; **significativamente diferente das células tratadas com CPT-11; $p< 0,05$).

A avaliação do envolvimento mitocondrial nas respostas obtidas na linhagem SNU-C4 mostraram que nenhum tratamento alterou a permeabilidade de membrana mitocondrial nesta linhagem celular (capítulo II). Além disso, não foi observado alteração na atividade da Mn-SOD na linhagem SNU-C4 (capítulo I). Estes resultados indicam que, a apoptose causada pela combinação CPT-11>5-FU parece não ser desencadeada por sinalização mitocondrial (via intrínseca). Sabe-se que a apoptose pode ser iniciada por receptores de superfície celular (via extrínseca), como o receptor Fas (Nagata and Golstein, 1995). O receptor Fas é

expresso constitutivamente em células do carcinoma de cólon (Moller *et al.*, 1994) e parece estar envolvido na citotoxicidade do 5-FU (Eichorst *et al.*, 2001). Além disso, a caspase-8 é ativada pelo receptor promotor de morte celular Fas (Walczak and Krammer, 2000). Com o objetivo de verificar esta hipótese, a participação da caspase-8 nas respostas celulares com a combinação CPT-11>5-FU foi avaliada. Na linhagem HT-29, mais resistente ao 5-FU, aparentemente não existe a participação da caspase-8 (Capítulo II). Entretanto, na linhagem SNU-C4 (mais sensível ao 5-FU) esta caspase parece ser importante na indução da apoptose.

A inibição do crescimento celular observada com a combinação CPT-11>5-FU na linhagem SNU-C4, apesar do aparente aumento de apoptose, pode ter sido determinada por outras lesões que levam a outro tipo de morte. Por exemplo, a não excisão de sítios contendo fluorouracil ou uracil (Schuetz and Diasio 1985; Porquier *et al.*, 1997), e/ou dano ao RNA gerado pela incorporação do FUTP nos diversos tipos de RNA (Peters, 1995). Estas lesões podem levar à morte pós-mitótica ou clonogênica (Meyers *et al.*, 2001). A morte pós-mitótica também decorre da falta de reparo das lesões do DNA, mas ocorre tardiamente. Este tipo de morte decorre de uma sinalização de vias intracelulares não bem definidas ou simplesmente por impossibilidade de replicação, conduzindo à necrose, morte tardia ou senescência (Roninson *et al.*, 2001). A morte pós-mitótica resulta da perda da clonogenicidade, o que significa que aquela célula perdeu a capacidade de produzir novas gerações de descendentes a partir da sétima mitose. Em conseqüência, depois de determinado tempo, linhagem inicial deixará de existir. Assim, a célula está estéril, porém morfologicamente integra (Weltman and Salvajoli, 1996). Os resultados obtidos com ensaio clonogênico (Capítulos III e IV) demonstraram que existe morte pós-mitótica significativamente maior com este tratamento (CPT-11>5-FU) em ambas as linhagens. No caso da SNU-C4 este parece ser um mecanismo importante de morte celular.

O CPT-11 em concentração de IC_{50} precedido pela IC_{20} do 5-FU, não induziu mais apoptose que o CPT-11 isolado em nenhuma das linhagens. Não obstante, a exposição a IC_{20} do 5-FU antes do CPT-11 levou a uma diminuição na

inibição do crescimento celular (2 a 4 vezes) quando comparado com o observado com o CPT-11 isolado. Este aparente antagonismo pode ter sido causado pela inibição da síntese do DNA induzida pelo FdUMP e/ou FdUTP (Peters and Van Groeningen, 1991; Yin and Rustum, 1991). Isto estaria impedindo a ação do CPT-11, que necessita do processo de replicação do DNA em andamento (Burris III and Fields, 1994). De fato, na linhagem SNU-C4, o pré-tratamento com IC_{20} do 5-FU aumentou em 2,5 vezes o número de células na fase S quando comparada com as células não tratadas ou expostas ao CPT-11 (Capítulo IV). Uma vez que o 5-FU bloqueia a síntese do DNA o CPT-11 não tem capacidade de agir, diminuindo seu efeito sobre as células. O maior antagonismo, observado na linhagem SNU-C4 quando comparada com as linhagens HT-29, pode ser explicado pela maior sensibilidade da SNU-C4 ao 5-FU.

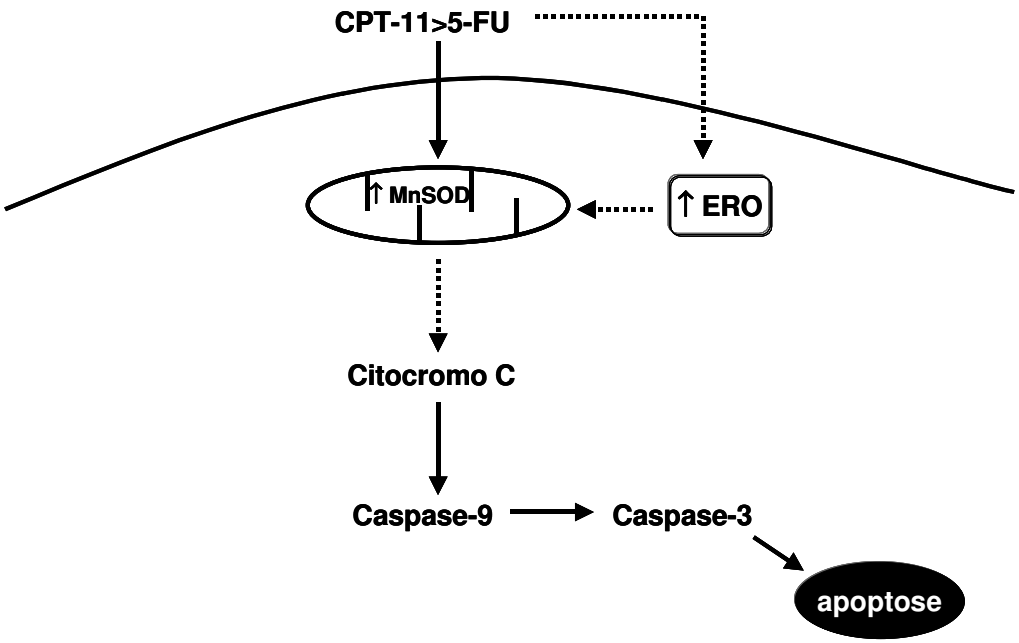

Figura 18: Modelo simplificado de indução da apoptose após tratamento com CPT-11>5-FU na linhagem HT-29. Linhas sólidas foram demonstradas neste estudo, enquanto que as linhas tracejadas se referem a processos que sugerimos serem desencadeados.

Com base nos resultados descritos nos capítulos I e II um modelo para indução da apoptose após tratamento com a combinação CPT-11>5-FU pode ser proposto para a linhagem HT-29 (Figura 18). De acordo com nossos resultados a combinação CPT-11 > 5-FU aumentou a atividade da Mn-SOD. Este aumento pode ter sido causado por um acréscimo na produção de ERO pela combinação dos dois agentes. Recentemente, o aumento da Mn-SOD foi relacionado com uma maior indução de apoptose (Hussain *et al.*, 2004). Em nosso estudo observou-se que na linhagem HT-29 ocorre uma ativação da apoptose via mitocôndria uma vez que a caspase-9 foi ativada. Estudos anteriores demonstraram que a liberação de citocromo c ativa a caspase-9 desencadeando a apoptose (Gupta, 2003). Na linhagem HT-29 parece ser este o principal mecanismo de citotoxicidade com a combinação CPT-11 > 5-FU.

Apesar da sinalização da apoptose via Fas por agentes neoplásicos ser considerada controversa (Revisado em Kaufmann and Earnshaw, 2000), foi demonstrado ativação da apoptose via fas após inibição da enzima TS (Houghton *et al.*, 1997). Da mesma forma, Backus *et al.* (2003) demonstraram que a ativação da apoptose via mitocondrial é importante após inibição da TS. Então, foi avaliado se as diferenças na indução de apoptose com as combinações do CPT-11 e 5-FU estão associadas a uma maior inibição da enzima TS (Capítulo III).

Nas duas linhagens estudadas não foi observado aumento significativo na inibição da TS com a combinação CPT-11>5-FU, quando comparado com o 5-FU isolado (Capítulo III). De acordo com nossos achados, Xu *et al.* (2002) mostraram que o SN-38 (metabólito do CPT-11) não causou alteração na expressão da TS. Mais recentemente, Azrak *et al.* (2004), não encontraram correlação entre os níveis de TS e as respostas obtidas com a combinação CPT-11>5-FU. Portanto, os resultados obtidos com a avaliação da atividade e expressão do RNAm da TS demonstraram que não existe interferência da IC_{20} do CPT-11 com a enzima TS.

O CPT-11 inibe a Topo I levando a uma parada do ciclo celular, e conseqüentemente reduzindo a proliferação celular (Takimoto *et al.*, 1997). Ainda, o CPT-11 forma um complexo entre Topo I e DNA, gerando quebras neste, que se não forem reparadas, podem iniciar o processo de apoptose (Pommier, 1996).

Com base nestas observações foi verificado se a combinação CPT-11>5-FU aumenta a inibição da Topo I ou induz maior numero de complexos cliváveis.

A dose de IC_{20} do 5-FU reduziu o efeito inibitório na atividade da Topo I causado pelo CPT-11 nas duas linhagens (Capítulo IV). Tal fato suscita a hipótese de o 5-FU estar interferindo com a enzima Topo I. A enzima Topo I está envolvida nos processo de reparação de sítios abásicos no DNA (Pouquier *et al.*, 1997; Mao and Muller, 2003). De fato, Mao and Muller (2003), utilizando RNA anti-senso, demonstraram que a repressão da Topo I causou uma redução no sistema NER, envolvido no reparo de sítios abásicos. Em outro estudo foi verificado que existe uma associação entre Topo I e PCNA (fator essencial para o sistema NER) (Loor *et al.*, 1997). Uma vez que o 5-FU pode levar a formação de sítios abásicos (Grem, 1996), a Topo I pode ter sido recrutada para resolver esta lesão, reduzindo o efeito do CPT-11. Por outro lado, a combinação CPT-11>5-FU não inibiu a atividade da Topo I na linhagem SNU-C4 (Capítulo IV). Já na linhagem HT-29 este tratamento inibiu a atividade da Topo I nos mesmos níveis que o CPT-11 isolado. Isto pode ser explicado pela maior sensibilidade da linhagem HT-29 ao CPT-11. Por outro lado, a combinação CPT-11>5-FU não levou a maior formação de complexos cliváveis entre DNA-Topo I, na linhagem HT-29 (Capítulo IV). Estes resultados estão de acordo com Guichard *et al.* (1998b), que demonstrou que este tratamento não aumentou o número de complexos cliváveis, mas prolonga a estabilização destes.

As diferentes respostas observadas após o tratamento com CPT-11>5-FU nas duas linhagens indicam que características intrínsecas das linhagens são importantes na resposta ao tratamento com CPT-11 e 5-FU. Tais características provavelmente incluem a expressão dos genes *p53*, *bcl-2*, *bax, cox-2* (Bukholm and Nesland, 2000; Violette *et al.*, 2002; Saikawa *et al.*, 2004), receptores de fatores de crescimento envolvidos na transdução de sinais como o rEGF (Levi *et al.*, 2004), ou ainda, proteínas associadas à proteção contra estresse celular como a Hsp70 (Rashmi *et al.*, 2003).

Em suma, os resultados do nosso estudo demonstraram que a citotoxicidade da combinação CPT-11 e 5-FU depende da seqüência de

administração das drogas e das características moleculares de cada linhagem celular. O efeito sinérgico observado com a combinação CPT-11>5-FU desencadeia uma sinalização mitocondrial da apoptose na linhagem HT-29. Por outro lado, na linhagem SNU-C4, apesar da combinação CPT-11>5-FU induzir apoptose, este não parece ser o efeito mais importante. Considerando a heterogeneidade dos tumores de cólon, este trabalho reitera a necessidade da caracterização molecular prévia de cada tumor, objetivando uma resposta terapêutica mais eficaz. Além disso, abre possibilidades para a busca de novos agentes capazes de interferir com alvos mais específicos.

CONCLUSÕES

Ainda que alguns pacientes demonstrem ser resistentes à combinação CPT-11>5-FU, grande parte dos pacientes com câncer de cólon avançado apresenta respostas satisfatórias com esta terapia. Entretanto, o mecanismo responsável pela maior atividade antitumoral observada com esta combinação permanece não totalmente elucidado. Neste sentido, foi investigado o efeito da combinação CPT-11>5-FU na indução de apoptose em linhagens celulares de adenocarcinoma de cólon.

Os estudos descritos neste trabalho geram informações que apóiam esta hipótese, cabendo ressaltar as seguintes conclusões gerais:

- A inibição do crescimento celular pela combinação CPT-11/5-FU e a indução de apoptose estão relacionadas, e dependem da seqüência de administração das drogas.

- A seqüência CPT-11>5-FU demonstrou efeito sinérgico, enquanto que a seqüência 5-FU>CPT-11 mostrou ser antagônica nas duas linhagens celulares estudadas.

- A linhagem celular HT-29 demonstrou uma maior citotoxicidade e indução de apoptose após tratamento com CPT-11>5-FU quando comparada com a linhagem SNU-C4.

- A caspase-3 e caspase-9 participam da ativação da apoptose nesta linhagem celular, sugerindo o envolvimento da mitocôndria neste evento.

- Na linhagem celular SNU-C4 existe participação da caspase-8, sugerindo que a via extrínseca da apoptose esteja ativada nesta linhagem após tratamento com a combinação CPT-11> 5-FU.

- O aumento da atividade da Mn-SOD induziu incremento na apoptose da linhagem celular HT-29 após tratamento com a combinação CPT-11> 5-FU.

- As enzimas timidilato sintase e topoisomerase I não são importantes para as respostas observadas após o tratamento com a combinação CPT-11> 5-FU em nenhuma das linhagens celulares estudadas.

- As alterações no ciclo celular não foram determinantes para a maior apoptose induzida pela combinação CPT-11> 5-FU em nenhuma das linhagens celulares estudadas.

PERSPECTIVAS

Tendo como ponto de partida os resultados obtidos com este estudo, alguns processos envolvidos nas respostas obtidas com a combinação CPT-11> 5-FU merecem especial atenção. No capítulo IV sugeriu-se que pode existir envolvimento de sistemas de reparação de DNA nos efeitos observados com combinação CPT-11> 5-FU. Dentre estes se destaca o sistema de excisão de bases (BER) e sistema de excisão de nucleotídeos (NER). A participação do dano oxidativo (Capítulo I) é outro ponto que deve ser investigado mais profundamente. Por exemplo, a identificação de aumento na produção de espécies reativas do oxigênio com a combinação CPT-11>5-FU, bem como participação de outras enzimas de defesa antioxidante como a catalase.

Considerando a existência da grande heterogeneidade entre os tumores de cólon, diversos alvos celulares devem estar relacionados com as respostas a agentes quimioterápicos, entre estes a participação de proteínas de estresse celular, como por exemplo, a Hsp70. Além disso, estratégias envolvendo combinações entre agentes com diferentes mecanismos de ação podem ser muito eficazes na terapia do câncer de cólon. A superexpressão do receptor do fator de crescimento epidérmico (rEGF) e da enzima ciclooxigenase 2 (Cox-2) têm sido freqüentemente associada à redução da resposta ao tratamento e a progressão da doença. Neste sentido, salienta-se a importância de avaliar a combinação do 5-FU ou CPT-11 com outros agentes, como inibidores de Cox-2 e rEGF.

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