Anticancer effect of SN-38 on colon cancer cell lines with different metastatic potential

ÅSA WALLIN[1], JOAR SVANVIK[2], BIRGITTA HOLMLUND[1], LILLIANNE FERREUD[1] and XIAO-FENG SUN[1]

Divisions of [1]Oncology and [2]Surgery, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

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Abstract. SN-38 is an active metabolite of the topoisomerase I inhibitor irinotecan. The mechanism behind its antitumor effect in colorectal cancer is not fully understood. In this study, we examined the response of colon cancer cell lines with different metastatic potential to SN-38. The parental human colon cancer cell line KM12C and its two highly metastatic derivatives KM12SM and KM12L4a were cultivated in 5% CO_2 at 37˚C for 24 h and then exposed to SN-38 (2.5 μg/ml) at 37˚C for 4, 24 and 48 h, respectively. The cell cycle was measured by flow cytometry, apoptotic activity was determined by flow cytometry and immunocytochemistry and the expression of topoisomerase I, Bax and survivin proteins were examined by Western blot. The exposure of the cells to SN-38 induced S-phase and G2 arrest (P<0.0001) and the KM12L4a cells had the highest response in a time-dependent manner (P<0.0001). The rates of apoptosis in the KM12SM (P=0.001) and KM12L4a cell lines (P=0.01) were increased time-dependently, though there was no such change in the KM12C cells. The expression of topoisomerase I protein was decreased in each cell line tested and the expression of Bax protein was increased, especially in KM12L4a. In conclusion, the effect of SN-38 on the colon cancer cell lines was mediated via conducting S-phase and G2 arrest and apoptosis. This effect was found in the cell lines with higher metastatic potentials, indicating that SN-38 can be used to treat advanced colon cancers.

Introduction

Irinotecan (CPT-11), a camptothecin derivative, exerts an antitumor effect in many types of cancers including colorectal, lung, breast, cervical and testicular cancers (1). In Sweden, irinotecan is the only topoisomerase inhibitor registered for treatment of advanced stages of colorectal cancer (CRC) and is approved for second-line treatment in metastatic CRC. Camptothecin is a toxic alkaloid isolated from the Chinese tree, *Camptotheca acuminta*, a drug whose antitumor activity was discovered in the 1960s (2). Irinotecan is a water soluble camptothecin analogue with reduced side effects, due to water solubility (3). Irinotecan is a prodrug that requires carboxylesterases for conversion to the active metabolite 7-ethyl-10-hydroxy-camptothecin, i.e. SN-38, which is 100- to 1000-fold more active compared to irinotecan (4,5).

Irinotecan is a DNA topoisomerase I (Topo-I) inhibitor. Topo-I is an enzyme involved in DNA replication and transcription and thus the inhibitor possesses the ability to induce DNA damage and transient S-phase arrest through the stabilisation of cleavable complexes, which leads to single strand DNA breaks that subsequently are transformed into double strand (ds) DNA breakages. This step becomes irreversible in the presence of irinotecan treatment and via dsDNA breakages a cascade of different apoptotic-related signalling pathways are activated, resulting in apoptotic cell death (6). The role of apoptosis in cancer treatment is still being debated (7). Other mechanisms, i.e. long-term cell cycle arrest (cell senescence) and mitotic catastrophe, are also potent ways of controlling cell proliferation and, eventually, inducing cell death (8,9). Apoptosis is nevertheless one way of measuring the response of a drug. Since the apoptotic pathway is very complex, there are several proteins involved in the execution of apoptosis, i.e. the pro-apoptotic Bax protein or the anti-apoptotic protein survivin, both operating in the mitochondria-associated pathway of apoptosis (10,11).

In this study, we examined the effect of SN-38 on three colon cancer cell lines with different metastatic potential by analysing cell cycle, apoptosis and apoptosis-related proteins using flow cytometry, immunocytochemistry (ICC) and Western blot.

Materials and methods

Material and drug treatments. Three human colon cancer cell lines, KM12C, KM12SM and KM12L4a, kindly provided by Professor I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX), were used as a model in the present study. Originally, the

Correspondence to: Åsa Wallin, KEF, Faculty of Health Sciences, University Hospital, SE-581 85 Linköping, Sweden
E-mail: asawa@ibk.liu.se

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parental cell line KM12C was established by cells from a primary colon cancer of Dukes' B2. KM12C was poorly metastatic and KM12SM and KM12L4a were highly metastatic cell lines, derived from KM12C by repeatedly injecting cells into the cecum and spleen respectively in athymic mice (12).

The cell lines were maintained in Eagle's MEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), sodium pyruvate, vitamins and a cocktail of penicillin and streptomycin at 37˚C in 5% carbon dioxide (Gibco, Invitrogen, Paisley, UK). Cells growing exponentially were harvested when a confluency of 80% was achieved.

The prodrug irinotecan is converted by carboxylesterases to its active metabolite SN-38. We used the metabolite SN-38 (Aventis Pharma, Paris, France) for drug treatment, which was diluted to a stock solution of 50 mg/ml in dimethyl sulfoxide (DMSO). The stock solution was kept at -20˚C and diluted in a medium of appropriate concentrations before use. For each of the experiments cells were trypsinized, counted, plated in duplicates and then incubated for 24 h to allow the cells to adhere. The cells were then treated with SN-38 (2.5 μg/ml) and harvested after 4, 24 and 48 h, respectively. Untreated cells were used as a control for each experiment and were also harvested after 4, 24 and 48 h, respectively. Each experiment was repeated three times.

Cytotoxic assay. The IC_{50} value was estimated using a cytotoxic assay, cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany). Cells were plated in a 96-well plate in triplicates and treated with five different concentrations (0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml) of SN-38. After 24 h, the 48-h cells were harvested and incubated for 1.5 h at 37˚C with WST-1. The optical density (O.D.) was measured with a spectrophotometer (Anthos htIII, Anthos Labtec Instruments) and based on the O.D. value, IC_{50} was calculated. Because of the inability of proliferation tests to detect exclusively apoptotic cell death, the IC_{50} value was multiplied by five to ensure the induction of apoptosis. WST-1 is a tetrazolium salt that converts to formazan when cleaved by mitochondrial dehydrogenases. Hence the formation of formazan is evidence of metabolic activity and viable cells.

Cell cycle analysis. Cells grown in a 3-cm petri dish were scraped in 100 μl of citric buffer and prepared for cell cycle analysis using the Vindelöv protocol with propidium iodine for DNA staining (13). Cell cycle analysis was performed using a FACScan (Becton-Dickinson, San José, CA). Cell cycle data, S-phase fraction and G2 were analysed and calculated by ModFit LT 3.0 (Verity Software House, Inc, Topsham, ME).

Apoptotic assay. The apoptotic marker M30 CytoDeath (Roche Applied Science, Mannheim, Germany), which detects a cytokeratin 18 (CK18) neo-epitope as an early indication of apoptosis, was used for ICC and flow cytometry. The expression of CK18 is an indication of cleaved, hence active, caspase-3, -6, -7 and -9.

Immunocytochemistry. Cells were plated in 3-cm petri dishes (Cornstar) and incubated for 24 h to allow cells to adhere.

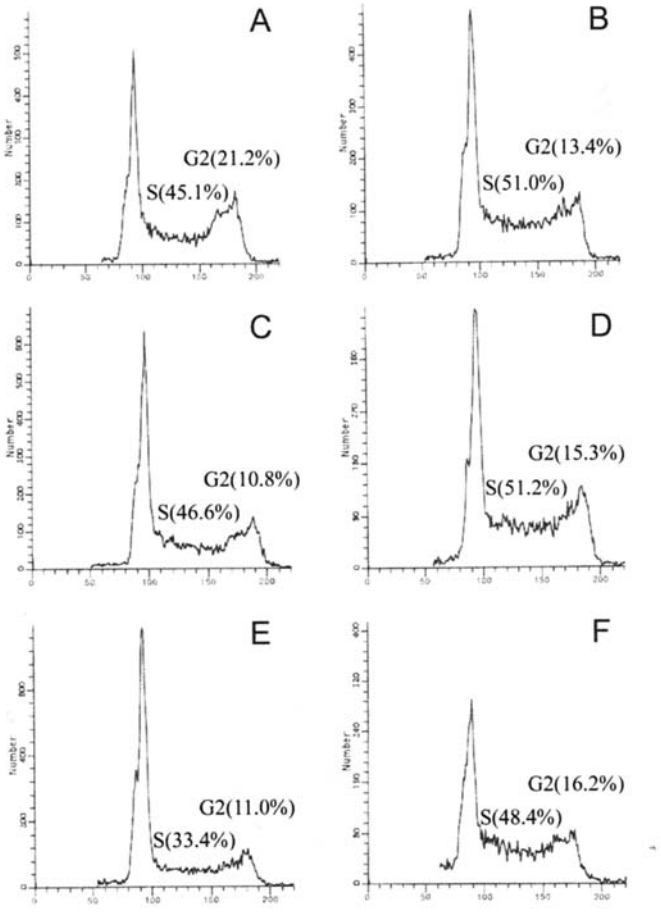

Figure 1. Cell cycle analysis by flow cytometry showing the distribution of cells for the tetraploid cell line KM12L4a. (A) 4 h, untreated control; (B) 4 h, SN-38; (C) 24 h, untreated control; (D) 24 h, SN-38; (E) 48 h, untreated control and (F) 48 h, SN-38.

After treatment with SN-38, cells were trypsinized and transferred to slides using cytospin (300 rpm, 5 min) and fixated in ice-cold pure methanol at -20˚C for 30 min. Slides were stained using the standard protocol for M30 CytoDeath (Roche Applied Science). Primary antibody M30 and secondary rabbit anti-mouse biotin-labelled antibody (DakoCytomation, Glostrup, Denmark) were diluted at 1:250 in an incubation buffer and the cells were incubated for 1 h respectively in a humid chamber at room temperature for 30 min. The cells were incubated with Streptavidin-HRP (1:500, DakoCytomation) for 30 min at room temperature and then counterstained with hematoxylin. Untreated cells were used as reference and all samples (reference and treated) were counted twice by two independent researchers. For every slide, five different areas and ~1,000 cells in total were counted. The mean value derived from this process was the basic data for the statistical analysis.

Flow cytometry. After growth and treatment in 6-well plate cells were trypsinated, re-suspended in 0.5 ml phosphate-buffered saline (PBS) and prepared following the standard protocol for M30 CytoDeath, Fluorescein (Roche Applied Science). The cells were fixated in ice-cold pure methanol at -20˚C for 30 min. After washing the cells with a buffer (0.1% Tween-20/PBS), the cells were incubated with M30

Table I. S-phase and G2 of KM12C, KM12SM and KM12L4a cells measured by flow cytometry.

Cell line	S-phase (%)		P	G2 (%)		P
	Untreated	SN-38		Untreated	SN-38	
KM12C						
4 h	47.8	46.5	n.s.	21.6	12.1	<0.0001
24 h	41.0	47.7	0.0048	17.9	12.6	<0.0001
48 h	34.1	45.6	0.0001	12.2	12.8	n.s.
KM12SM						
4 h	48.9	50.6	n.s.	20.475	12.6	0.0001
24 h	41.3	48.1	0.0001	17.73	12.9	0.0002
48 h	33.0	46.3	0.0001	11.73	12.6	n.s.
KM12L4a						
4 h	45.1	51.0	0.0098	21.2	13.4	0.0001
24 h	46.6	51.2	0.0322	10.8	15.3	0.0003
48 h	33.4	48.4	0.0001	11.0	16.2	<0.0001

CytoDeath, Fluorescein antibody diluted at 1:250 in an incubation buffer (1% BSA/0.1% Tween-20/PBS) for 1 h at room temperature. Cells were washed, re-suspended in 0.5 ml PBS and stored in darkness until analysis. The quantitative analysis was performed on a FacsCalibur (Becton-Dickinson, San José, CA).

Western blot. Cells were plated in a 10-cm petri dish and scraped after treatment with SN-38 in an RIPA buffer (150 mM NaCl, 1% Triton X, 0.1% SDS, 50 mM Tris pH 8.0) supplemented with protease inhibitor (Complete Mini, Roche Applied Science, Penzberg, Germany) and centrifuged 20,800 rpm for 10 min at 4˚C to isolate the proteins. The supernatant containing the proteins was transferred to new tubes and the protein concentration was measured (BCA™ Protein Assay kit, Pierce, Rockford, IL). The proteins (40 μg/well) were separated by a 4-20% gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad Laboratories, Hercules, CA) and transferred by electroblotting to a polyvinylidine diflouride membrane (PVDF membrane, Amersham Biosciences). To block non-specific binding sites, membranes were incubated in 1X TBS (Trisbase, NaCl) containing 0.1% Tween-20 and 5% non-fat milk for 1 h. After incubation overnight at 4˚C with a primary antibody against Topo-I at 1:500 (BD Biosciences, San Diego, CA), Bax at 1:1000 (Cell Signaling Technology, Inc., Beverly, MA), or survivin at 1:100 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) membranes were rinsed and incubated with horseradish peroxidase-conjugated secondary antibody (IgG Mouse, Cell Signalling; rabbit immunoglobulins, DakoCytomation) diluted at 1:2000 in milk-buffer for 1 h at room temperature. Immunoreactive proteins were visualised with an enhanced chemiluminescense system (Amersham Biosciences, Little Chalfont, UK). Each membrane was washed and rinsed before incubation with ß-actin as a reference.

Statistical analysis. A pooled t-test was used for testing the significance between the treated and untreated cells within the same cell line, examining any time-dependency for the drug response and differences between the cell lines. Each P-value <0.05 was considered significant. The statistical software SPSS (SPSS Inc., Chicago, IL) was used for the calculations.

Results

SN-38 in relation to cell cycle. The distribution of cell cycle stages was examined by DNA flow cytometry and the results are presented in Table I. Cell line KM12L4a shows tetraploidy, meanwhile KM12C and KM12SM show diploidy. KM12C and KM12SM showed a significantly increased fraction of cells in the S-phase compared to the untreated ones after 24 and 48 h, though not after 4 h. KM12L4a showed a significantly increased S-phase fraction for all three time points (Fig. 1). Regarding the G2 fraction in the three cell lines, after treatment with SN-38, KM12C and KM12SM showed decreased G2 fractions after 4 and 24 h when compared to the untreated ones. After 48 h, the two treated cell lines showed similar G2 fractions to the untreated ones. KM12L4a showed a significantly increased G2 fraction, compared to the untreated one and to the other two cell lines, after 24 and 48 h, although there was a decreased G2 fraction after 4 h.

SN-38 in relation to apoptosis. We examined the apoptotic cells by using ICC with the apoptotic marker, M30. The nucleus was stained exclusively and the typical characteristics of an apoptotic cell, i.e. cell shrinkage, nuclear condensation and apoptotic bodies were visualised (Fig. 2). As shown in Table II, KM12SM and KM12L4a had significantly increased apoptotic rates after 48 h compared to the untreated ones.

Apoptotic cells were also examined with flow cytometry with the apoptotic marker, M30. Each of the three treated cell lines had significantly increased apoptotic rates after 24 and 48 h compared to the untreated ones (Table III).

SN-38 in relation to protein expression. Fig. 3 presents protein expression of Topo-I, Bax and survivin determined by using Western blot. For each of the treated cell lines, the expression

Table II. Apoptosis of KM12C, KM12SM and KM12L4a cells detected by ICC.

Cell line	Apoptotic cells (%)		P
	Untreated	SN-38	
KM12C			
4 h	0.1	0.3	n.s.
24 h	0.2	3.3	n.s.
48 h	1.3	20.9	n.s.
KM12SM			
4 h	0.3	1.3	n.s.
24 h	1.7	7.1	n.s.
48 h	1.9	22.2	0.0012
KM12L4a			
4 h	0.2	0.8	n.s.
24 h	0.9	5.9	n.s.
48 h	4.3	19.0	0.0107

Table III. Apoptosis of KM12C, KM12SM and KM12L4a cells detected by flow cytometry.

Cell line	Apoptotic cells (%)		P
	Untreated	SN-38	
KM12C			
4 h	0.39	0.54	n.s.
24 h	0.60	7.73	<0.0001
48 h	0.26	8.13	<0.0001
KM12SM			
4 h	0.64	1.73	n.s.
24 h	0.48	6.15	0.0002
48 h	1.04	9.34	<0.0001
KM12L4a			
4 h	0.45	1.4	n.s.
24 h	0.15	4.33	0.0019
48 h	0.65	6.81	0.0001

of Topo-1 was decreased compared to the untreated ones at all three time points. Bax showed a trend towards a higher expression for treated KM12L4a after 24 and 48 h. The expression of survivin was strongly decreased for treated KM12L4a compared to the untreated one, though a minor decrease of the expression was also seen in KM12C. KM12SM did not show a significant difference in the expression of either Bax or survivin.

Discussion

In this study, SN-38 was administrated to poorly (KM12C) and highly (KM12SM and KM12L4a) metastatic colon cancer cell lines in order to see whether the metastatic potential had an impact on the outcome of the treatment. The cells treated with SN-38 tended to arrest in the S-phase and

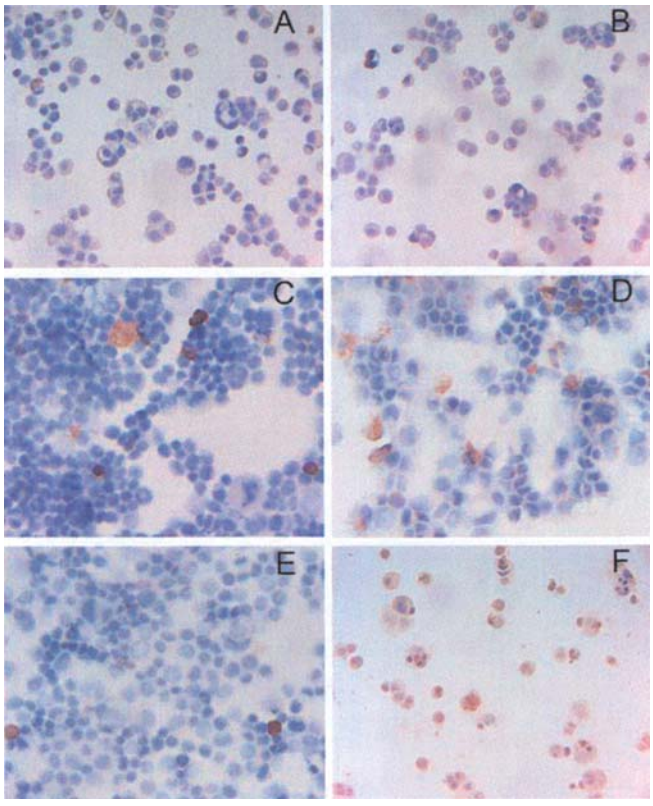

Figure 2. Immunocytochemistry with an apoptotic marker, M30, in the KM12L4a cell line. (A) 4 h, untreated control; (B) 4 h, SN-38; (C) 24 h, untreated control; (D) 24 h, SN-38; (E) 48 h, untreated control and (F) 48 h, SN-38.

G2 compared to the untreated cells. KM12L4a especially showed a potential accumulation of cells in S-phase and G2. It is postulated that SN-38 stabilizes Topo-I cleavable complexes during the transcription and by this introduces single strand breakages in the DNA, which subsequently leads to double strand breakages during the S-phase (14). As a consequence, cells arrest in the S-phase and later halt in G2 due to the induced DNA damage. The higher sensitivity in KM12L4a could be partly explained by the fact that KM12L4a was tetraploid, in contrast to the KM12C and KM12SM that were diploid. The tetraploidy would contribute to a larger amount of damaged DNA, hence an increased risk of cell cycle failure.

Furthermore, S-phase and G2 arrest could be a preceding indication of apoptosis. ICC and flow cytometry employed in the present study point towards the consistent results regarding the distribution of apoptotic cells. We found that SN-38 induced apoptosis in a time-dependent manner in each of the three cell lines. The apoptotic rate was increased when the incubation was extended and this was seen for each of the three cell lines, regardless of the detection methods. A previous study has indicated that SN-38 is able to induce apoptosis in a testicular carcinoma cell line (15). A study by Motwani *et al* demonstrated that when p21 deficient and p21 intact HCT116 cells were exposed to SN-38 apoptosis was induced only in the p21 deficient cells, although p21 intact cells underwent G2 arrest (16). The p21 status of the cell lines used in our study is unknown, however, since p21 is a

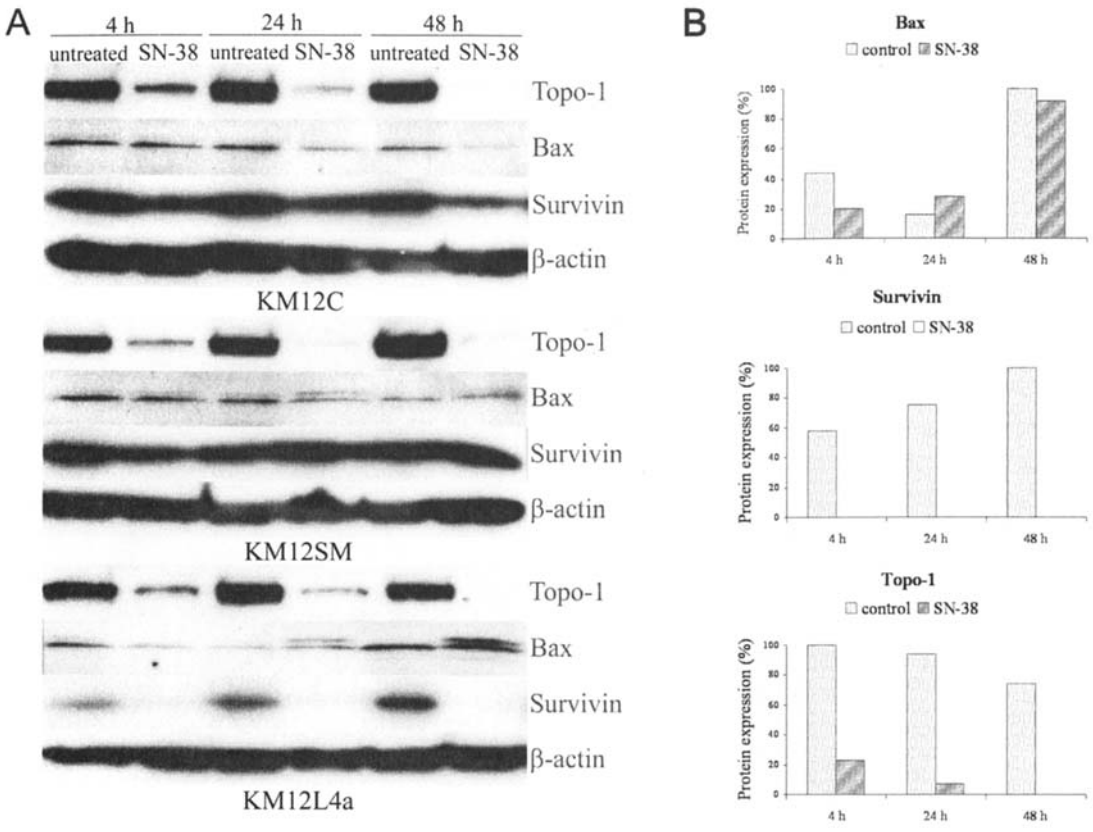

Figure 3. Protein expression of topoisomerase I (Topo-1), Bax and survivin detected by Western blot, using ß-actin as an internal control (A). The histogram shows the levels of protein expression adjusted for differences in ß-actin expression (B).

downstream effector of p53 certain similarities in the SN-38 response could probably be expected between p53-negative cells and the p21-negative HCT116 cells.

It is also postulated that the status of p53 is essential for the type of cell death induced after treatment with irinotecan (17). Wild-type p53 can lead to long-term cell cycle arrest, i.e. cellular senescence (18). The mutation of p53 results in premature mitosis due to transient G2/M arrest, which leads to mitotic catastrophe and later apoptosis (19,20). The KM12 cell lines have a p53 mutation at amino residue 179 resulting in a shift, His→Arg (21). In this study, we observed a prominent shift of cells in the cell cycle after treatment with SN-38. Untreated controls showed a decrease of cells in the S-phase over a period of time. This decrease was not so pronounced for cells treated with SN-38 and this was seen for each of the three cell lines, indicating that SN-38 interferes with the DNA synthesis as expected. Since our cells are p53 deficient, we would expect a transient G2-arrest. For KM12C and KM12SM, cells were kept in G2 at a constant level for each of the three time points. Compared to the untreated cells, however, the proportion of cells in G2 were already much lower after a 4-h treatment and then kept at this level even after 48 h. For the tetraploid cell line KM12L4a, the number of cells in G2 increased along with time, showing an accumulation of cells rather than an arrest. These results taken together show that SN-38 induced a transient arrest for KM12C and KM12SM in the way that the cells after G2 stop re-entered the cell cycle and continued to proliferate, although with DNA damage. For the accumulated KM12L4a cells in G2, the accumulation instead lead to a more permanent arrest, but we do not know the behaviour of the cells after 48 h, whether they stayed in G2 arrest or also re-enter the cell cycle.

We examined the protein expression of Topo-I, Bax and survivin by using Western blot. We observed a decreased expression of Topo-I in a reversed time-dependent manner, indicating that Topo-I, as a target protein by SN-38, was inhibited. Previous findings further confirmed an intimate link between the halt of Topo-I and the efficacy of SN-38 (22-24). The pro-apoptotic protein Bax, a member of the Bcl-2 family, was demonstrated to have a central role in the mitochondria-mediated apoptotic pathway. Bax promotes apoptosis through cytochome c release, which subsequently activates caspase-9 and -3, respectively (25). We found that the expression of Bax was increased in KM12l4a after 24 and 48 h of treatment. It has been reported from previous *in vitro* studies that treatment with SN-38 leads to overexpression of Bax in both head and neck squamous cell carcinoma and erythroleukemia cells. Therefore the pro-apoptotic Bax could be a potential target for sensitization against SN-38 (26,27).

Survivin is a member of the inhibitor of the apoptosis protein (IAP) family and is rarely expressed in normal adult tissue. In tumor tissue, however, the expression of survivin is found. Therefore, survivin is considered as a molecular target for the development of chemotherapeutics. In this study, survivin displayed a significantly decreased expression in KM12l4a cells and a minor decreased expression in KM12C cells, after SN-38 treatment. In KM12L4a, the expression after treatment was almost depleted. Since survivin, is an anti-apoptotic protein it is logical that a decrease in survivin expression will lead to an increase in apoptosis as a

consequence. Increased survivin in cancer was related to decreased apoptosis due to the aberrant progression of transformed cells and their ability to overcome the apoptotic checkpoint (11). Survivin is expressed during the G2/M-phase and interacts with the microtubule of the mitotic spindle. When this interaction is disrupted, the inhibitory effect on apoptosis is lost and caspase-3 activity is increased (28). Our results show that the cell line, KM12L4a, is most sensitive to apoptosis and demonstrates an increase of Bax expression, though also a decrease of survivin.

In conclusion, the effect of SN-38 on the colon cancer cell lines was mediated via conducting S-phase and G2 arrest and apoptosis. The most potential effect was found in the cell lines with higher metastatic potential, indicating that SN-38 can be used to treat advanced colon cancers.

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