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Repair of DNA-double-strand breaks induced by the chemotherapeutic drug Topotecan in mamma carcinoma cells with genetic defects in the BRCA1 or BRCA2 genes

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1. Central question and Hypothesis

The tumour suppressor genes BRCA1 and BRCA2 play an essential role for homologous recombination during embryonic development and for maintenance of genomic stability during the adult life. The central question of this doctoral thesis is: Do cells with defects in both alleles of either BRCA1 or BRCA2, such as tumour cells of inheritable breast cancer, have deficiency in repairing DNA-double-strand breaks induced by the chemotherapeutic drug Topotecan when compared with BRCA1- and BRCA2-proficient cells?

2. Introduction

Every year, about 400,000 persons in Germany are diagnosed with cancer, 200,000 men and 195,000 women. 47,500 of these women are diagnosed with breast cancer which is the most frequent cancer among women followed by cancer of the colon with 34,200, lung cancer with 10,400 and cancers of the female sexual organs (corpus uteri 10,000, ovaries 9700, cervix uteri 6600). In all regions of the world, breast cancer is one of the three most often diagnosed cancers for women. The mean age for breast cancer in Germany is 63 years but more than 40% of all diseased women are younger than 60 years. Concerning the most common causes of death for women, breast cancer is high at rank on position five with 17,173 women dying from breast cancer in 2003 which corresponds to 3.8% of all female death causes in 2003. For men and women, breast cancer is ranked to position nine with 17,437, about 2% of all deaths. This means in detail: not only women die of breast cancer but also 264 men. These data illustrate the great importance of intensive breast cancer research.

Breast cancer can be divided into two forms different in origin, sporadic and hereditary. Hereditary breast cancer can be diagnosed in 5% to 10% and of these, 75% can be ascribed to mutations in the proteins BRCA1 or BRCA2.[1]

BRCA1 and BRCA2 are both involved in DNA-repair. BRCA1 is localized to chromosome 17q21 and it consists of 5589 bases in 22 exons which result in a 220 kD in size protein of 1863 amino acids. Two active domains can be found in the protein: the N-terminal RING domain from amino acids 8 to 96[2] and the C-terminal BRCT (= BRCA1 C-terminal repeats) domain from amino acids 1646 to 1859[3]. For both domains, special functions are known: The ring domain forms heterodimeric complexes with the protein BARD1 (= BRCA1 associated RING domain) and has Zn^{2+} dependent Ubiquitin-protein-ligase-activity[4]. The BRCT domain has transcriptional activation activity and plays a role in chromatin unfolding. But its main task is nuclear interaction with DNA-damage-response proteins and cell-cycle-involved proteins[5]. BRCA1 becomes phosphorylated by the kinases ATM (= Ataxia teleangiectasia mutated) at Serin 1387, 1423 or 1524 or Chk2 (= Checkpoint kinase 2) at Serin 988[6]. Thereafter, the

[1] Statistisches Bundesamt Deutschland; Deutsche Krebshilfe e.V.
[2] Wu et al., 1996
[3] Koonin et al., 1996
[4] Meza et al., 1999
[5] Jasin, 2002
[6] Cortez et al., 1999; Lee et al., 2000

activated Protein can interact with DNA-damage repair proteins, especially those necessary for homologous recombination, an error-free way of DNA-double-strand break repair. The involvement of BRCA1 in response to DNA-damage has been well documented. This involvement includes hypersensitivity to ionizing radiation and a defective repair of the arisen damage[7], a higher base-level of spontaneous DNA-double-strand breaks, a lower level of homologous recombination and a higher level of non-homologous repair in cells with defects in both alleles of BRCA1[8]. In addition, there is a linkage between BRCA1 and Fanconi Anemia, a cancer susceptibility syndrome supporting the evidence for a tumour suppressor function of BRCA1: Fanconi Anemia is a rare autosomal recessive disease (incidence: 1/300,000 life births) characterized by chromosome instability, congenital abnormalities, increased predisposition to cancers, mainly leukemias and squamous cell carcinomas of the head, neck and gynaecologic system, and hypersensitivity to ionizing radiation and DNA-cross-linking agents such as Mitomycin C or Cisplatin. Although the precise functions of the Fanconi Anemia genes (FANC A, B, C, D1, D2, E, F, G, L) is largely unknown, the genes cooperate with BRCA1 in a common cellular pathway: Following DNA-damage, FANC A, C, F, G and L form a nuclear complex which monoubiquitinates FANC D2 to FANC D2-L. FANC D2-L colocalizes with BRCA1 which can start its function in DNA-repair. After a successful repair of the damage, FANC D2-L is de-ubiquitinated which is followed by a progress of the cell-cycle[9].

In knockout mice, early embryonic lethality has shown that a lack of functional BRCA1 protein can not be compensated and leads to death[10].

The tumour cell lines studied here carry mutations in both alleles of BRCA1: the individual human donors might have had a germline mutation in one of the two alleles and an accidental mutation in the second BRCA1 allele which then promotes tumourigenesis. The incidence for a single allele germline mutation ranges between 1:800 and 1:500 for the whole population. For women with cases of breast cancer in their families, the risk to bear a BRCA1 mutation is 15% to 20% and it increases up to 60% to 80% if both, breast and ovarian cancer occurred in the family history. In case of a BRCA1 mutation, the lifetime risk for breast cancer for women is about 50% to 85%, for ovarian cancer about 20% to 40% and for men, an increased risk for prostate or

[7] Scully et al., 1999
[8] Moynahan et al., 1999; Snouwaert et al., 1999
[9] Garcia-Higuera et al., 2001
[10] Moynahan et al., 2002

colon carcinoma is reported. The average manifestation age of BRCA1-related breast cancer is 42 years, 20 years earlier than sporadic breast cancer[11]. These data underscore an important role of BRCA1 for maintenance of genomic and cellular stability.

The second gene responsible for familial breast cancer, BRCA2, is localized on chromosome 13q12-13. It consists of ~10,300 bases which are translated into a 384 kD protein of 3418 amino acids. Two active domains have been identified: the BRC-repeat region between amino acids 990 and 3418, an eight-fold repeat of a 30 amino acids sequence[12]. Its function is precisely described: Rad51, the principal protein of homologous recombination, binds with its RecA-homology domain to the BRC-repeat region of BRCA2, especially to BRC1, 2, 3, 4, 7 and 8. BRCA2 then helps to hook up Rad51 onto the damaged DNA and to form helical Rad51-filaments and by that to initiate the cascade of homologous recombination steps[13]. For binding of the BRCA2-Rad51-complex to the DNA-damage, the C-terminal 800 amino acids of BRCA2 are needed: three oligonucleotide binding folds, OB1, OB2 and OB3, can link directly to the damage-associated DNA-single-strand at its transition to the undamaged DNA-double-strand[14].

BRCA2 and its importance for DNA-repair has been demonstrated: BRCA2 is required for homologous recombination of DNA-double-strand breaks[15] and both, BRCA1 and BRCA2, are needed to restart broken replication forks through the process of homologous recombination[16]. Like BRCA1, BRCA2 also has an important task in the pathway of Fanconi Anemia proteins as it has been found that BRCA2 and FANC D1 are identical[17]. Defects in the FANC D1 genes lead to the symptoms described above for Fanconi Anemia, illustrating also the role of BRCA2 for chromosomal stability.

The incidence for a mutation in one allele of the BRCA2 genes has not yet been investigated but it is expected to be smaller for BRCA1. A BRCA2 mutation is associated with a 50% to 80% lifetime risk for breast cancer comparable to BRCA1. For ovarian cancer, the risk is 10% to 20%, not as high as for BRCA1 mutations. Interestingly, the male lifetime risk for breast cancer among BRCA2 mutation carriers is 6%, a 100-fold increase compared with the normal population.

[11] Nathanson et al., 2001
[12] Pellegrini et al., 2002
[13] Sung et al., 1995; Baumann et al., 1996
[14] Yang et al., 2005
[15] Moynahan et al., 2001
[16] Scully et al., 2000; Venkitaraman et al., 2002
[17] Howlett et al., 2002

The cell lines used in this study have a defect in both alleles of either BRCA1 or BRCA2 and it was speculated that they are deficient in homologous recombination. To test this, the cells were treated with the chemotherapeutic drug Topotecan which leads to DNA-double-strand-breaks in replicating cells. This kind of damage is believed to require homologous recombination for repair.

The chemotherapeutic drug Topotecan is a derivative of Camptothecin, a component of fruit and bark of the Chinese tree "Camptotheca acuminata". It had already been used in traditional Chinese medicine for treatment of malign tumours. Western medicine rediscovered this active agent in 1966. Today, two derivatives of Camptothecin are clinically used, Topotecan and Irinotecan.

Fig. 1 A

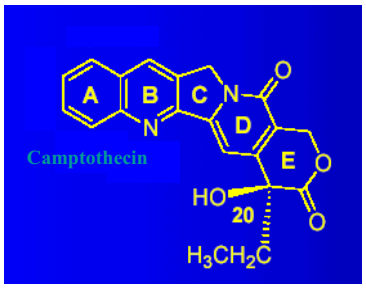

Fig. 1 B

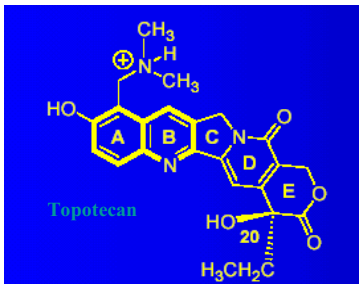

Fig. 1 C

Fig. 1 A: The Chinese tree "Camptotheca acuminata"

Fig. 1 B: Chemical structure of Camptothecin

Fig. 1 C: Chemical structure of Topotecan

Topotecan´s mode of action was elucidated in 1985 and can be described as follows: Topotecan interacts with Topoisomerase I. Topoisomerases are nuclear enzymes which regulate DNA-topology through breakage and rejoining of DNA-strands. This is necessary during many cellular processes such as transcription, replication or repair. Topoisomerase I acts by (i) forming a single-strand break to achieve DNA-relaxation and (ii) subsequent closure of the single-strand gap. Topoisomerase I has both nuclease and ligase activities and is not ATP-dependent.

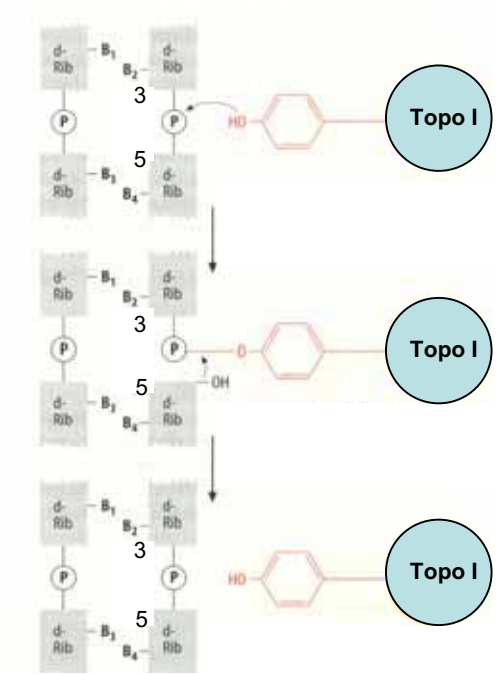

Fig. 2: Topoisomerase I – mode of action

Eukaryotic topoisomerase I produces DNA-single-strand breaks by interaction of its tyrosyl-OH with a DNA-phosphate group which leads to breakage of one DNA-strand and the formation of a covalent enzyme-DNA-intermediate at the 3´ DNA-terminus. After relaxing of both, positively or negatively supercoiled DNA, the break is resealed and the topoisomerase I releases from the intact DNA.

Topotecan covalently binds the complex of DNA and Topoisomerase I, the so called cleavable complex, and by this, prevents rejoining of the DNA-strands. If a DNA-replication-fork collides with this open single-strand break, a so called "one-sided" double-strand break will result which has to be repaired or will lead to cell death. Hence, the toxicity of Topotecan is largely restricted to S-phase cells.

The central questions for this work are the following: (i) Are cells with a defect in homologous recombination deficient in repairing Topotecan-induced DNA-double-strand breaks compared with "normal" cells expressing BRCA1 or BRCA2 wild-type? (ii) Are those cells hypersensitive against Topotecan?
Given these hypotheses prove to be true, a topoisomerase inhibitor might be a promising agent towards BRCA1- and BRCA2-related breast cancer as it might be more effective in those cases compared to tumours with normal recombination capacity.

To assess DNA-double-strand breaks in this study specific damage-associated chromatin alterations were visualized and quantified by means of a fluorescent antibody against the phosphorylated histone protein H2AX.

In eukaryotes, the basal unit of chromatin is the nucleosome leading to a 4-fold condensation of the DNA. Each nucleosome is composed of 146 base pairs of DNA and eight histone proteins, two of each of the four core histones H2A, H2B, H3 and H4. The H2A family includes three subfamilies, H2A1-H2A2, H2AX and H2AZ, which all differ in characteristic sequence elements that have developed independently throughout eukaryotic evolution. H2AX represents about 2-25% of all H2A in mammalian cells. The domain discriminating H2AX from the other H2A isoforms is the C-terminal region which is longer in H2AX than in H2A1-H2A2. After DNA-double-strand breakage thousands of H2AX molecules flanking this break become phosphorylated on serine 139 within minutes[18]. The phosphorylated form is called γ-H2AX because it was firstly discovered after γ-irradiation. γ-H2AX can be labelled by an antibody. The resulting γ-H2AX-antibody complex can be detected by a fluorescence-marked second antibody which allows an evaluation under the fluorescence microscope. Each DNA-double-strand break leads to numerous phosphorylated γ-H2AX molecules which together appear as one so called “focus”. Every γ-H2AX-focus corresponds hence to one DNA-double-strand break.

Taken together, the experimental idea of this thesis is the following: Different cell lines are incubated with the chemotherapeutic drug Topotecan which leads to DNA-double-strand breaks. After drug removal the cells get time to repair their DNA-damage. During the time of the experiments the number of γ-H2AX-foci is monitored. A difference in the ability to repair those DNA-double-strand breaks is expected between the cells with defects in either BRCA1 or BRCA2 and their control cells.

[18] Rogakou et al. 1998

3. Material and methods

3.1. The cells and cell culture

3.1.1. The cells

Two human cell lines were used, HCC1937 and VU423. HCC1937 is isolated from a mamma carcinoma of a woman with familial history of breast-cancer. VU423 is a Fanconi Anemia D1 cell line.

VU423

The VU423 cell line has two different mutations in its BRCA2 alleles, both insertions (paternal 7691insAT, exon 15 and maternal 9900insA, exon 27) which lead to C-terminal truncated proteins. VU423 cells were stably transfected with either one of two plasmid vectors, one of which expresses the wild type BRCA2, the other one an empty vector. Those cells are called VU423B, transfected with BRCA2, or VU423F, transfected with an empty vector, respectively[19]. VU423B has about 69.6 ± 2.8 chromosomes, VU423F has about 70.5 ± 2.3 chromosomes per cell.

HCC1937

The HCC1937 cell line is characterized by a biallelic insertion mutation in the BRCA1 gene. A single inserted cytosine at position 5382 (5382insC) causes a frameshift resulting in a C-terminal truncated BRCA1 protein. Similar to VU423, HCC1937 cells were also stably transfected with vectors carrying either the wild-type or no BRCA1. These cells were named HCC1937/BRCA1wt expressing normal level of wild-type BRCA1 and HCC1937/pcDNA3[20]. HCC1937/BRCA1wt has about 62.8 ± 5.3 chromosomes, HCC1937/pcDNA3 has about 62.7 ± 4.0 chromosomes per cell.

[19] Howlett et al. 2002
[20] Scully et al. 1999

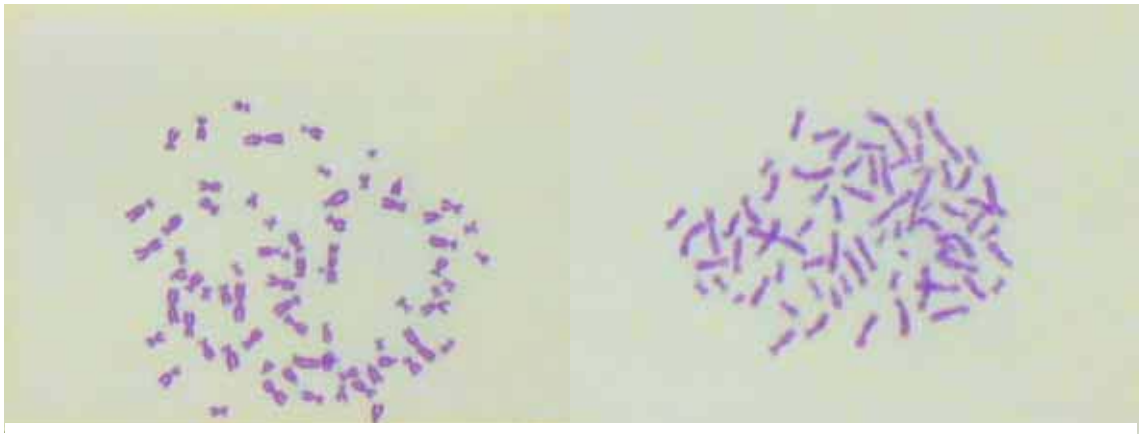

Fig. 3 A *Fig. 3 B*

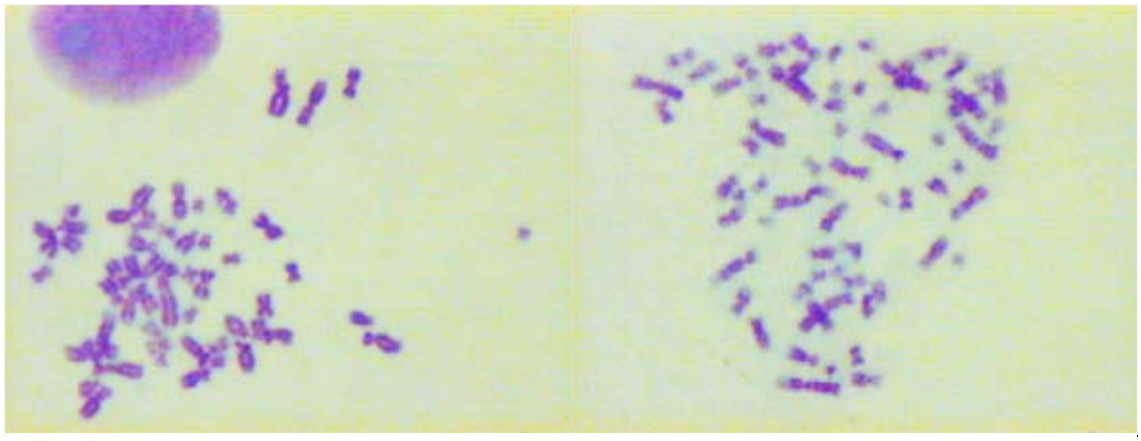

Fig. 3 C *Fig. 3 D*

Fig. 3: Chromosomal status

A : VU423B, 69.6 ± 2.8 chromosomes per cell

B : VU423F, 70.5 ± 2.3 chromosomes per cell

C : HCC1937/BRCA1wt, 62.8 ± 5.3 chromosomes per cell

D : HCC1937/pcDNA3, 62.7 ± 4.0 chromosomes per cell

3.1.2. Cell cultivation and passaging

All cells were cultivated in tissue culture flasks[21]. They were kept at 37°C and nearly 100% humidity and 5% CO_2 in an incubator[22]. The culture media were the following:

HCC1937/BRCA1wt and HCC1937/pcDNA3:

DMEM (Dulbecco´s Minimal Essential Medium)[23]

10%	FCS (Fetal calf serum)[24]
2%	1M Hepes[25]
2%	200mM L-Glutamin[24]
1%	10.000 U/ml Penicillin / 10.000 µg/ml Streptomycin[24]
+ 150µg/ml	Geneticin[26]

VU423F:

DMEM (Dulbecco´s Minimal Essential Medium)

10%	FCS (Fetal calf serum)
2%	1M Hepes
1%	Penicillin / Streptomycin

VU423B:

DMEM (Dulbecco´s Minimal Essential Medium)

10%	FCS (Fetal calf serum)
2%	1M Hepes
1%	Penicillin / Streptomycin
+ 500µg/ml	Geneticin

Before reaching confluence, every third day cells were trypsinized for passaging. The medium was removed, cells were washed with 1xPBS and incubated with 0.25% EDTA-Trypsin[24] for 3 minutes at 37°C. Fresh medium was added and the cells were reseeded at lower density.

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[21] Sarstedt
[22] Heraeus Instruments
[23] Invitrogen
[24] Gibco
[25] Sigma
[26] G 418 sulfate, Gibco, stock solution of 50 mg/ml kept in 4°C

3.1.3. Freezing and thawing

Stock cultures were kept in liquid nitrogen tanks. For freezing, cells were trypsinized and centrifuged[27] $1x10^6$ cells for 3 minutes at 200 x g at 4°C. The pellet was resuspended in a mixture of medium and 10% DMSO[28] and transferred into cryotubes which were kept at -20°C for two hours, subsequently at -80°C for 24 hours and then permanently in liquid nitrogen at -196°C. For thawing, the cryotubes were placed into a water bath of 37°C for 2 min. Cells were immediately centrifuged to remove the toxic DMSO and resuspended in fresh medium.

3.1.4. Cell counting

To determine the cell number, cells were trypsinized and measured at a 1:50 dilution (0.9% NaCl) in an automatic cell counter[29].

3.2. Preparation of the cellular chromosomes

Confluent cells were seeded at lower density into T75 flasks. After 24 hours, medium was renewed, now containing 0.2 µg/ml Colcemid[30], a methylated derivative of colchicine which blocks microtubule assembly by binding to the tubulin heterodimer. Mitosis is blocked at metaphase. After 5 hours of incubation, the cells were trypsinized and centrifuged for 5 minutes at 200 x g. For washing, the pellet was resuspended in 10 ml 1xPBS and centrifuged again for 5 minutes at 200 x g. 9ml of the supernatant were removed and 10 ml 0.56 % KCl-solution were dropwise added whilst vortexing. The cells were pelleted again and the supernatant was removed except from 1 ml. 10 ml Carnoy´s Fixative[31] were added dropwise whilst vortexing[32]. The cells were centrifuged for 5 minutes at 200 x g, the fixing process was repeated and thereafter, the cell suspension was diluted to $1x10^6$ cells / ml. 30 µl of the cell suspension were dropped on a wet slide. After drying, the slides were transferred into a suitable glass container for staining with 4% Giemsa[33] for 10 minutes. The slides were shortly washed 6 times in water and were kept overnight for drying. The next day, synthetic resin[34] was

[27] Centrifuge by Eppendorf
[28] DiMethyl SulphOxide, Sigma
[29] Casy I, Schärfe Systems
[30] Demecolcine, Sigma
[31] pure methanol : 100% acetic acid = 4 : 1
[32] Vortex, Scientific Industries
[33] Giemsa stain, Sigma
[34] Entellan, Merck

dropped on the slides and covered with glass cover slips. After drying, the cellular chromosomes were counted under the microscope[35].

3.3. Preparation of Topotecan

Topotecan hydrochloride[36] is delivered as 1 mg of a yellow powder. Its molecular weight is 457.9. To receive a dilution of 1 mMol, 2.184 ml sterile distilled water was added. Stock solutions of 0.1 mMol were prepared and stored at -20°C.

3.4. Colony formation

To evaluate different toxicities of Topotecan, colony forming tests for the different cells were performed. Colony formation was enhanced by a factor of 5 when using lethally irradiated feeder cells of the same strain. Those were tested separately to be uncapable of forming colonies. Feeder cells were lethally irradiated with 20 Gy with an X-ray tube[37] at 20 mA with an additional 0.5 mm Cu filter. The dose rate was 1 Gy/min.
For the colony forming tests, normally growing cells were removed from a T75 flask, quantified and seeded at the wanted concentration in T25 tissue culture flasks together with 10^5 feeder cells. After 24h, the medium was renewed now containing Topotecan. After incubation of seven days, medium was replaced with drug-free medium. After 12-16 days colonies were fixed in 70% ethanol for 5 minutes after washing in 0.9% NaCl solution. The following table shows the numbers of cells seeded for each concentration of Topotecan:

Concentration of TPT	**Seeded cells**			**Seeded feeder cells**
no TPT	200	500	1000	10^5
0.001 µM	200	500	1000	10^5
0.0025 µM	200	500	1000	10^5
0.005 µM	200	500	1000	10^5
0.0075 µM	500	1000	1500	10^5
0.025 µM	2000	3000	4000	10^5
0.05 µM	4000	6000	8000	10^5
0.075 µM	8000	10.000	12.000	10^5
0.1 µM	10.000	20.000	30.000	10^5

[35] Axioplan, Zeiss
[36] Calbiochem
[37] Seifert, Ahrensburg

3.5. Detection of γ-H2AX-foci after incubation with Topotecan

$3.5x10^5$ cells were seeded in a 10 ml Petri dish[38] into which a sterile slide was placed before. After 24 hours, medium was changed against fresh one containing 0.05 µM Topotecan. After 4 hours, medium was replaced with drug-free medium and the cells on the slides were fixed after 0, 2, 4, 6, 8, 16, 24 hours of repair time. For each experiment a mock-treated sample was analysed in parallel. For fixation, the slides were washed in 1xPBS and fixed for 15 minutes in 2% formaldehyde, washed three times in 1xPBS and stored at -20°C until further treatment. From this point on, the slides were processed in the dark to avoid bleaching of the fluorescent label. The cells were

- permeabilized on ice for 5 minutes

  1xPBS + 1% BSA[39] + 0.2% Triton X-100[40]
- washed

  1xPBS + 1% BSA
- blocked for one hour

  1xPBS + 3% BSA
- incubated with γ-H2AX antibody for one hour in a humid atmosphere chamber

  1xPBS + 1%BSA + 0.5% Tween 20[41]

  + 1:100 dilution of Anti-phospho-Histone H2AX mouse monoclonal antibody [42]

  50 µl each object slide, covered with plastic coverslips
- washed three times for 10 minutes

  1xPBS + 1% BSA + 0.5% Tween 20
- incubated with a fluorescent anti-mouse-antibody for one hour in a humid atmosphere chamber

  1xPBS + 1% BSA + 0.5 % Tween 20

  + 1:600 dilution of Alexa Fluor 594 goat anti-mouse[43]

  50 µl each object slide, covered with plastic coverslips
- washed four times for 10 minutes

  1xPBS + 0.5% Tween 20
- faded with DAPI/Antifade[44]
- covered with glass coverslips
- compressed in a paper drying block

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[38] Sarstedt
[39] Albumin bovine fraction, Serva
[40] t-Octylphenoxypolyethoxyethanol, Sigma
[41] Sigma
[42] Upstate
[43] Molecular Probes
[44] 4,6-DiAmidine-2-PhenylIndole hydrochloride, Qbiogene

The coverslips were sealed with clear nail polish and stored at 4°C in a shaded box until microscopy. Of each slide, foci of about 50 to 70 cells were counted under a fluorescent microscope[45].

3.6. Western Blot

3.6.1. Extraction of the cellular proteins

Non-confluent growing cells were trypsinized, washed twice in 4°C cold 1xPBS and the pellet was stored at -20°C until further treatment.

The frozen cell pellet was resuspended in lysis buffer[46] (volume of the pellet : volume of lysis buffer = 1 : 1), three times alternately frozen in liquid nitrogen and immediately thawed in a heating block[47] and then centrifuged[48] for 10 minutes at 11.000 x g at 4°C. The supernatant now contains the proteins. The protein extract was transferred into a new tube and stored at -20°C until further treatment. For protein quantification, three dilutions of the extracted protein were prepared: 1:500, 1:1000, 1:2000. 800µl of each dilution were transferred into a photometer cuvette[49] and mixed each with 200µl Protein assay Dye reagent concentrate[50]. After 10 minutes of incubation at room temperature, the optical density was measured with a photometer[51]. Those values were compared to a calibration curve, generated by known concentrations of BSA from which the protein concentrations were calculated.

3.6.2. Separation of the proteins by gel electrophoresis

First, the proteins were prepared. 40 µg of extracted proteins together with 10 µl loading buffer[46] in a total volume of 18µl distilled water were denatured in a boiling water bath for 8 minutes, cooled on ice, and kept at -80°C until further use. The unpacked gel[52] was kept in running buffer[53] for five minutes. The gel was set into an electrophoresis chamber[54] which was filled up with running buffer. After that, the prepared proteins and

[45] Axioplan, Zeiss
[46] Ref. Solutions
[47] Thermomixer comfort, Eppendorf
[48] Microfuge, Beckman
[49] Greiner
[50] BioRad
[51] Uvicon Spectro Photometer, Kontron Instruments
[52] NuPAGE 3-8% Tris-Acetate Gel, 1 mm, Invitrogen
[53] Novex Tris-Acetate SDS running buffer 20x, Invitrogen
[54] Invitrogen

the markers, a mixture of 10 µl bench marker[55] and 5 µl magic marker[56] were applied to the slots of the gel which were rinsed with running-buffer before. The chamber was attached to a Power Pack[57] and gel electrophoresis was started with a collection phase for 10 minutes at 100V followed by a separation phase for 60 minutes at 200V.

3.6.3. Performance of the Western Blot

A 9 cm x 7 cm in size PVDF-Transfer Membrane[58] was activated 30 seconds in Methanol, washed five minutes in distilled water and 10 minutes in transfer buffer[59]. Two sponges[60], four pieces of gel blotting paper[58] and the gel were kept in transfer buffer for five minutes. Sponge, two pieces of gel blotting paper, gel, membrane, two pieces of gel blotting paper, sponge, were piled and this sandwich was set into a blotting chamber[60], filled with transfer buffer and attached to a Power Pack. The blot was started at 100 V for 60 minutes at room temperature.

3.6.4. Staining of the gel

After the blot, the gel was washed in distilled water, stained in Simply Blue Safe Stain[61] for one hour, decoloured for 1 minute in methanol and washed in distilled water which was renewed every 30 minutes.

[55] Bench Mark Pre-Stained Protein Ladder, Invitrogen
[56] Magic Mark XP Western Protein Standard, Invitrogen
[57] Consort
[58] Schleicher & Schuell
[59] Ref. solutions
[60] BioRad
[61] Invitrogen

3.6.5. Processing of the membrane

The membrane was:

- washed for three minutes in 1xPBS
- blocked for two hours

  1xPBS + 10% skimmed milk powder[62] + 0.3% Tween 20
- incubated with the first antibody over night at 4°C

  1xPBS + 5% skimmed milk powder + 0.3% Tween 20

  + a wanted dilution of the wanted first antibody
- washed once for 15 minutes and three times for five minutes

  1xPBS + 0.3% Tween
- incubated with the second antibody for one hour

  1xPBS + 5% skimmed milk powder + 0.3% Tween 20

  + a wanted dilution of the wanted second antibody
- washed once for 15 minutes and three times for five minutes

  1xPBS + 0.3% Tween.

After detection, this process was repeated with different antibodies.

3.6.6. Detection by chemiluminescence

For detection, ECL Western Blotting detection reagents[63] was prepared and dropped on the dripped membrane. After one minute of incubation, the membrane was shrink-wrapped, taken to the darkroom and exposed to the film[63]. The film was developed for two minutes, washed in tap water and fixed for two minutes.

3.6.7. Antibodies

Anti-BRCA1 N-terminal mouse monoclonal[64]	2 µg / ml
Anti-BRCA2 N-terminal mouse monoclonal[65]	2 µg / ml
Anti-Topoisomerase I mouse monoclonal[66]	2µg / ml
Anti-p53 mouse monoclonal[67]	1 µl / ml
Anti-β-Actin mouse monoclonal[68]	0.1 µl / ml
Anti-Mouse (sheep)[63]	1 µl / ml
Anti-Rabbit (donkey)[63]	1 µl / ml

[62] Naturaflor, Töpfer
[63] Amersham Biosciences
[64] Oncogene
[65] Calbiochem
[66] Novus Biologicals
[67] Novocastra
[68] Sigma

3.7. Flow cytometry

Flow cytometry is the measurement of characteristics of suspended single cells. They are passed through a laser beam of 488 nm blue light by continuous flow of a fine stream of the suspension in a capillary. Each cell scatters some of the laser light. The scattered 488 nm blue light detected about 180° to the incidence of light is called forward scatter (FSC). Its intensity is approximately proportional to cell diameter. The scattered 488 nm blue light detected about 90° to the incidence of light is called sideward scatter (SSC). Its intensity is approximately proportional to the quantity of granular structures within the cell. Furthermore, you can detect green about 530 nm (Fl 1), yellow about 585 nm (Fl 2) and red about 650 nm (Fl 3) light 90° to the incidence of light which gives an account of the cellular fluorescence. For my measurements, I used a FACScan[69] flow cytometer.

3.7.1. Cell-cycle analysis

To assess the impact of Topotecan on the cell-cycle, cells were stained with Propidium Iodide and the DNA-content per cell was measured. The more DNA the cells contain, the more Propidium Iodide can be detected.

The cells were trypsinized and taken up in 5 ml 1xPBS, centrifuged for 3 minutes at 200 x g and the pellet was resuspended in 10 ml 1xPBS. They were again centrifuged for 3 minutes at 200 x g and the pellet was retaken up in 1 ml 1xPBS. Cells were fixed by adding 1 ml 70% ethanol and 4.5 ml 96% ethanol in drops whilst vortexing. The fixed cells were kept for 10 minutes at 4°C and stored at -20°C until analysis at the flow cytometer.

Directly before measurement at the flow cytometer, the fixed cells were centrifuged for 3 minutes at 200 x g at 4°C and the pellet was resuspended in 1 ml 4°C cold 1xPBS. The cells were centrifuged for 3 minutes at 200 x g at 4°C and to the pellet, 0.5 ml of a 1 : 10 mixture of RNAse[70] : Propidium Iodide solution[71] was added. The tubes were immediately wrapped in aluminium foil and kept for 20 minutes in the dark until analysis at the flow cytometer. To avoid cell aggregates, the cell suspension was filtered through a finely woven gauze.

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[69] Fluorescence-Activated Cell Sorter, Becton Dickinson
[70] 1xPBS + 10 mg/ml Ribonuclease A from bovine pancreas, Serva
[71] 1xPBS + 10 µg/ml Propidium Iodide

The cell-cycle was analysed at the flow cytometer by measuring Propidium Iodide at Fl 2. The data were charted with ModFit LT[72] in DNA-histograms.

3.7.2. Apoptosis

Apoptotic cells can be distinguished from non-apoptotic cells by changes in the position of phosphatidylserine (PS) in the cell membrane. In non-apoptotic cells, most PS molecules are localized at the inner layer of the plasma membrane, but soon after inducing apoptosis, PS redistributes to the outer layer. This translocation to the extracellular environment precedes other apoptotic events and can be used as an early and long persisting apoptotic marker[73].

Externalized PS can be detected with annexin V, a protein with strong affinity for PS. Annexin V-FITC is marked with FITC (=Fluorescein IsoThioCyanate), a small fluorescent organic molecule which is typically excited by 488 nm laser light.

For analyzing the apoptotic fraction, the cells were trypsinized, taken up in medium, centrifuged for 3 minutes at 200 x g and the pellet was resuspended in 200µl binding buffer[74]. Again they were centrifuged for 3 minutes at 200 x g and the pellet was resuspended in 200µl binding buffer. 5µl Annexin V-FITC[75] and 10 µl Propidium Iodide[76] were added, the cells were kept in the dark for 15 minutes and then fixed by adding 1 ml 70% ethanol and 4.5 ml 96% ethanol in drops whilst vortexing. The fixed cells were kept for 10 minutes at 4°C and stored at -20°C until analysis at the flow cytometer. Directly before measuring, the fixed cells were centrifuged for 3 minutes at 200 x g and the pellet was resuspended in 5 ml 1xPBS. Again the cells were centrifuged for 3 minutes at 200 x g and the pellet was resuspended in 500µl binding buffer. To avoid cell aggregates, the cell suspension was filtered through a finely woven gauze.

The apoptotic fraction of the cells was analyzed at the flow cytometer by measuring FITC at Fl 1 and Propidium Iodide at Fl 2. The data were charted with CellQuest Pro[77] in dot-blot diagrams.

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[72] Verity Software House
[73] Martin et al. 1995, Fadok et al. 1992 / 1993
[74] BD ApoAlert, BD Biosciences
[75] 20 µg/ml in Tris-NaCl, BD ApoAlert, BD Biosciences
[76] 50µg/ml in Binding buffer, BD ApoAlert, BD Biosciences
[77] BD Biosciences

3.7.3. Apoptosis inhibition by Caspase Inhibitor Z-VAD-FMK

During the process of apoptosis, a cascade of caspases (= cystein-containing aspartate-specific proteases) leads the cell-death signal induced by interaction of specific ligands with cell surface receptors into the nucleus. Caspase inhibitor Z-VAD-FMK[78] acts by binding to the active sites of caspases 1, 3, 4, 7 and forms reversible linkages. The apoptotic signal can not be transmitted and apoptosis will not proceed.

The caspase inhibitor Z-VAD-FMK is delivered as 1 mg powder. Its molecular weight is 468. To receive a dilution of 50 mM, 42.8 µl DMSO was added. These solutions were thinned with sterile 1xPBS at 1 mM stock solutions which were stored at -20°C.

For regulating down the apoptotic rate, cells were incubated with caspase inhibitor at a concentration of 50 µM during the whole time of the experiments.

[78] Calbiochem

3.8. Solutions

10xPBS (Phospate Buffered Saline)

80g NaCl

27.2g $Na_2HPO_4 \circ 7H2O$

2.4g KH_2PO_4

2g KCl

ad 1l a.dest

10xTG-buffer

144g Glycin

30.3g Trizma-base (Tris[hydroxymethyl]aminomethane)

ad 1l a.dest

Lysis buffer

Distilled water

\+ 200 mM NaOH

\+ 1% SDS (Sodium Dodecyl Sulfate)

Loading buffer

Distilled water

\+ 50 mM Trizma-base

\+ 100 mM DTT (DiThioThreitol)

\+ 2% SDS (Sodium Dodecyl Sulfate)

\+ 0.1% BPB (Bromine Phenol Blue)

\+ 20% 99% Glycerol

Transfer buffer

1xTG-buffer

\+ 20% methanol

\+ 0.067% SDS (Sodium Dodecyl Sulfate)

4. Results

4.1. Cell Growth

A growth test of all used cell lines was performed to identify growth differences that might impact upon subsequent survival and repair experiments.

Fig. 4 shows comparable growth rates for all cells between days two and six. The mean population doubling time calculated from the exponential part of the growth curve ranged between 1.2 days (VU423) and 2.3 days (HCC1937). The close agreement between the respective cell pairs means that the growth characteristics per se will not differently affect further experiments.

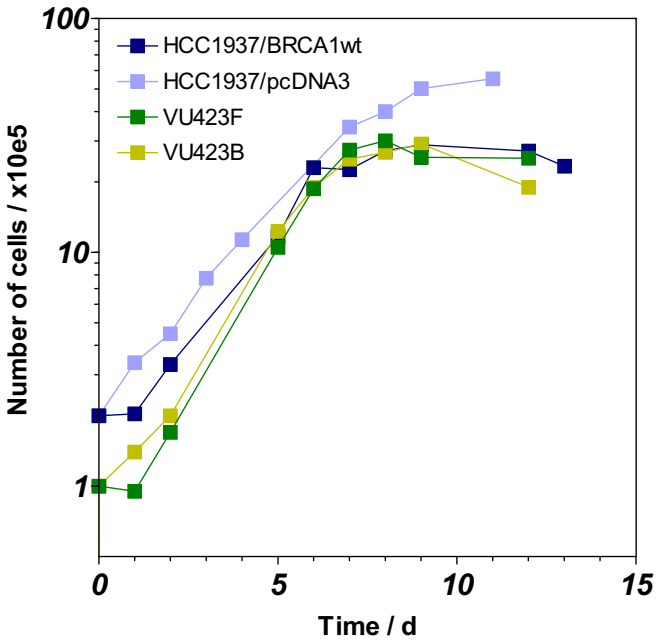


Fig. 4: Cell growth kinetics

For VU423, 1x10^5 cells and for HCC1937, 2x10^5 cells were seeded into T-25 culture flasks. Every day the cell number of one flask was determined up to 13 consecutive days. Between days two and six, an exponential growth rate was measured, thereafter cells grew into plateau. Growth characteristics per se will not differently affect further experiments.

4.2. Protein Expression

Protein expression in normally growing cells was analyzed by Western Blot for all cell lines used in this doctoral thesis. The proteins analysed were BRCA1 and BRCA2, Topoisomerase I as the target protein for the chemotherapeutic drug Topotecan and p53, an important DNA-repair protein.

For analyzing the BRCA1 and BRCA2 expression, N-terminal antibodies were favoured because they show positive expression results independent of any mutations which lead to C-terminal truncated proteins. Additional experiments with C-terminal antibodies would proof the difference between the mutated cell line and its control. Although numerous C-terminal antibodies of different producers were tested, none of them showed specific signals for any of the two proteins and thus did not produce further results.

Fig. 5A shows the protein expression of BRCA1 (220 kDa) in VU423 and HCC1937 cell lines. All cell lines showed analogue levels of BRCA1 expression irrespective of any mutations in BRCA1. Fig. 5B shows the BRCA2 (384 kDa) protein expression in the same cell lines and again, they showed analogue protein expression levels for each mutated cell line compared to its control. Under the conditions applied both BRCA1 and BRCA2 were detected at similar position on the blots slightly above the 220 kDa marker protein. The similar position is presumably due to the poor resolution for extremely large proteins.

To control for equal protein loading the blot was probed for β-Actin (42 kDa) (Fig. 5C). β-Actin is a structural protein equally expressed in all cells. The β-Actin signal was comparable indicating that the same amount of protein was applied.

Fig. 5D shows the protein expression for Topoisomerase I (100 kDa). All cell lines had comparable Topoisomerase I levels indicating that a putative difference in the sensitivity to Topotecan can not be ascribed to a different amount of cellular Topoisomerase I. β-Actin levels are shown for comparison (Fig. 5E).

In addition, the most important tumour suppressor protein p53 was analysed. p53 or its downstream pathway responsible for cell cycle regulation is disturbed in the majority of human tumours. Most of the p53 mutations lead to increased protein stability. High protein levels in tumour cells compared to normal cells are hence indicative of a p53 mutation. Fig. 5F shows a much higher p53 expression level in both, VU423B and VU423F than in HCC193. β-Actin was probed for comparison (Fig.5G). Comparison with other cell lines like the human tumour cell line FaDu and the human fibroblasts MR1 and Ki66 (Fig. 5H) revealed that only VU423 but not HCC1937 showed enhanced p53 levels indicative of a p53 mutation.

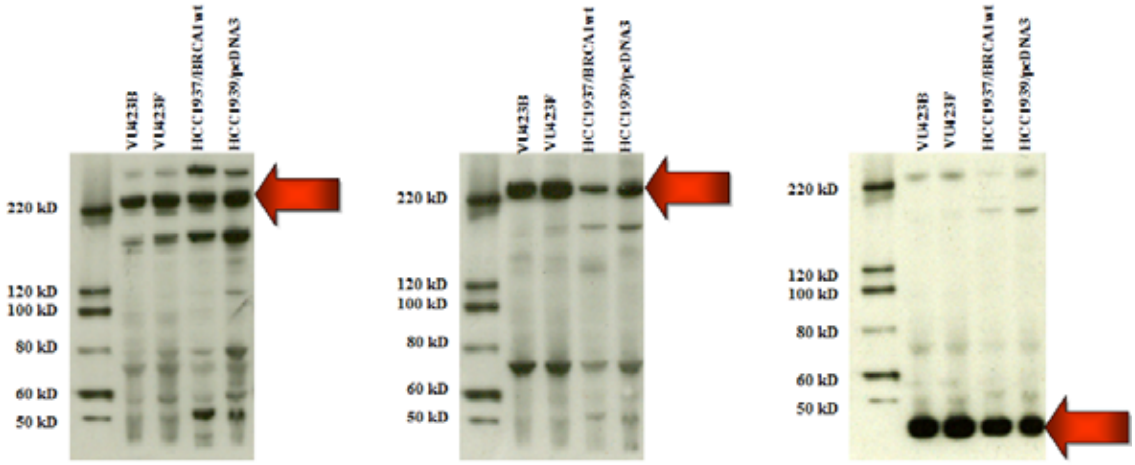


Fig. 5 A *Fig. 5 B* *Fig. 5 C*

Fig. 5: Protein expression levels for VU423 and HCC1937 cell lines

A: BRCA1 (220 kDa)

B: BRCA2 (384 kDa)

C: β-Actin (42 kDa)

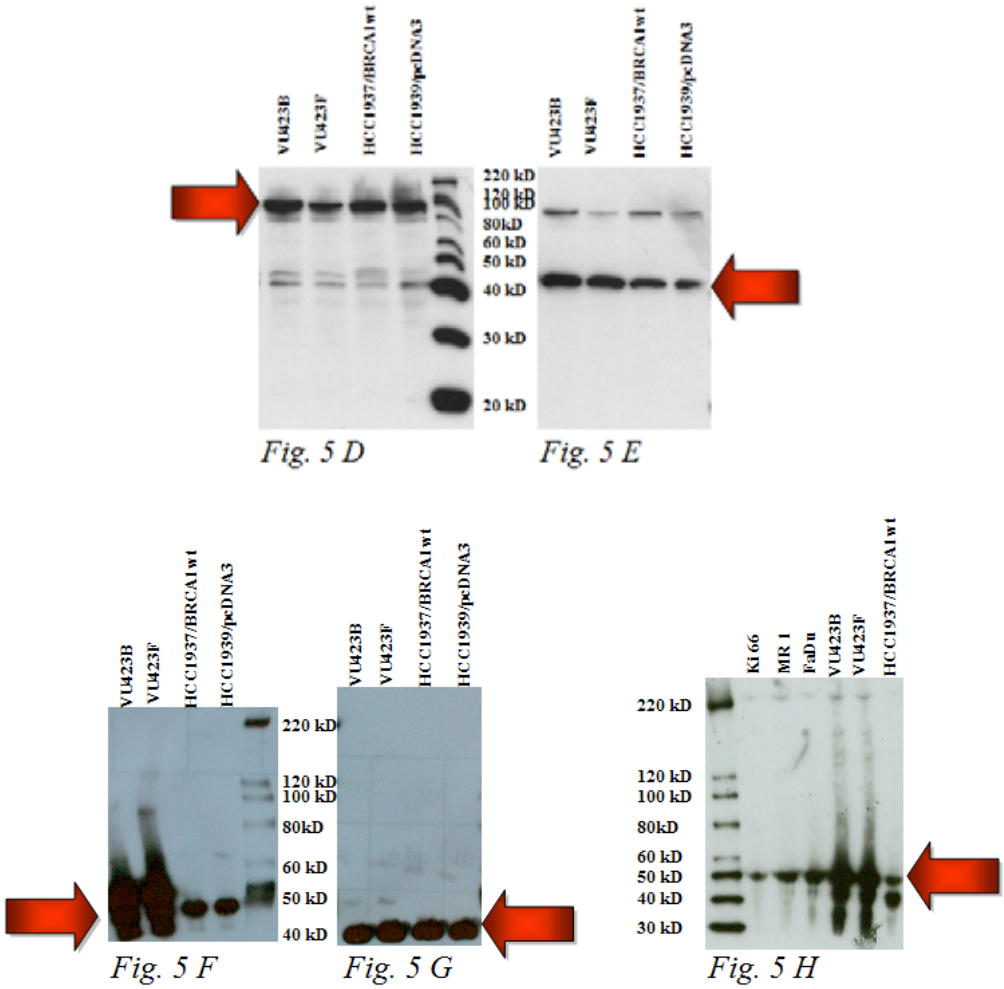


Fig. 5: Protein expression of VU423 and HCC1937 cell lines (cont.)

D: Topoisomerase I (100 kDa)

E: β-Actin (42 kDa)

F: p53 (53 kDa)

G: β-Actin (42 kDa)

H: p53 expression in comparison with other human cell lines

4.3. Colony formation

Fig. 6A-D show the colony formation for different concentrations of Topotecan. For all cell lines, increasing Topoptecan concentrations resulted in a continuous decrease of the surviving fraction. Differences in Topotecan sensitivity between the defective cell line and its complemented control cells could be detected for both VU423 and HCC1937 strains. The BRCA2-deficient VU423F cells were slightly but reproducibly more sensitive than the VU423B cells.

Similarly HCC1937/BRCA1wt cells were slightly more resistant than the BRCA1-deficient HCC1937/pcDNA3 cells (Fig. 6C). Depiction of the dose response to linear scale is suitable to judge sensitivities at high but not at low survival levels. In order to better illustrate differences at higher doses, the survival curves are also given on logarithmic scale. This depiction shows that HCC1937/BRCA1wt cells were more resistant than HCC1937/pcDNA3 at all dose levels studied (Fig. 6D).

As expected, both deficient strains were more sensitive than their proficient counterparts. Furthermore, comparing both sets of survival curves it can also be seen that the VU423 cells were more sensitive than HCC1937 cells with a $C_{0.1\ VU423} \approx 0.02$ μM and $C_{0.1\ HCC1937} \approx 0.05$ μM respectively[79].

[79] $C_{0.1}$ = concentration that reduces the surviving fraction to 10%

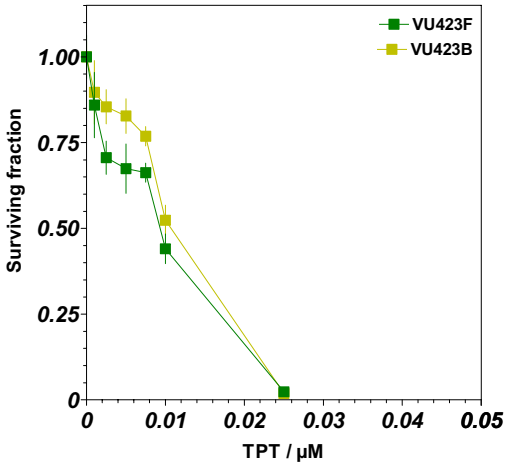


Fig. 6 A

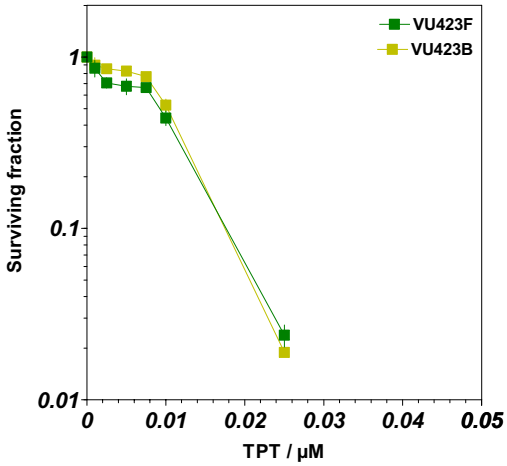


Fig. 6 B

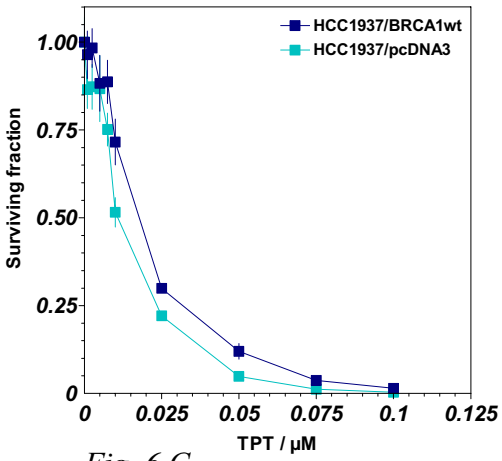


Fig. 6 C

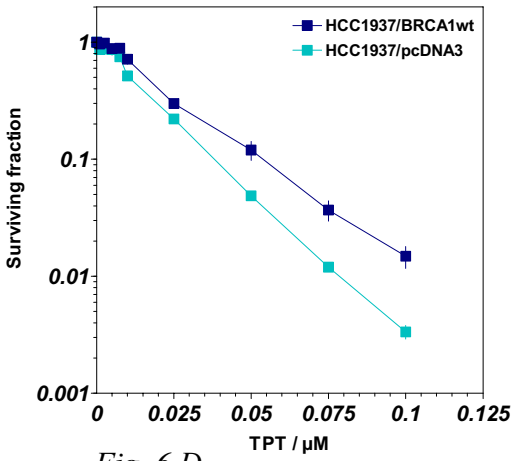


Fig. 6 D

Fig. 6: Colony formation

A: VU423 colony formation, linear scale

B: VU423 colony formation, logarithmic scale

C: HCC1937 colony formation, linear scale

D: HCC1937 colony formation, logarithmic scale

The colony forming tests were all performed with $1x10^5$ feeder cells per T25-flask as described. Cells were incubated with Topotecan for seven days, thereafter medium was replaced with drug-free medium and colonies were fixed after 12-16 days. In both cell lines, the cells with a defect either in BRCA1 or BRCA2 were more sensitive than their control cells. Furthermore, VU423 cells were even more sensitive than HCC1937 cells.

4.4. Repair of DNA-double-strand breaks

4.4.1. Induction of γ-H2AX-foci by irradiation

It is generally assumed that γ-H2AX-foci represent DNA-double-strand breaks. For ionising radiation, it has been shown that in fibroblasts each focus corresponds to a single DNA-double-strand break. For the present study, it was firstly tested whether γ-H2AX-foci are properly formed in VU423B and VU423F tumour cells and whether the number of induced foci is equal in both cell lines.

VU423 cells were irradiated with 2 Gy. They were fixed 15 minutes after irradiation to allow time for foci formation. Fig. 7 gives the percentages of cells containing different numbers of γ-H2AX-foci. The cells were ascribed to one of four classes showing either 0, 1-5, 6-10 or >10 foci per cell. The red bars indicate untreated controls with a main fraction of cells without foci, several cells with 1-5 foci and hardly any cells with >10 foci. This was similarly observed for both VU423B and VU423F cells. Irradiation with 2 Gy induced γ-H2AX-foci in both cells (green bars). The fraction of cells without γ-H2AX-foci decreased and with more than ten foci increased. The frequency of induced γ-H2AX-foci was comparable for both VU423B and VU423F.

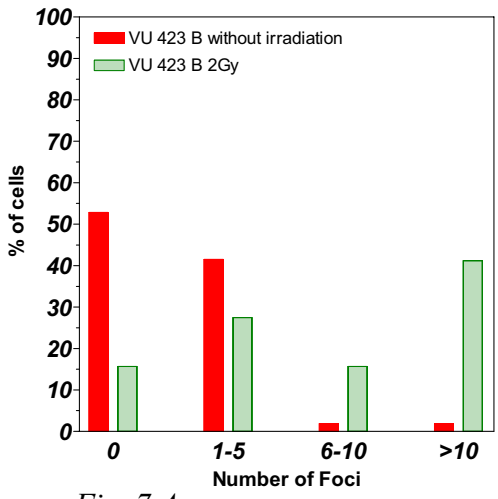


Fig. 7 A

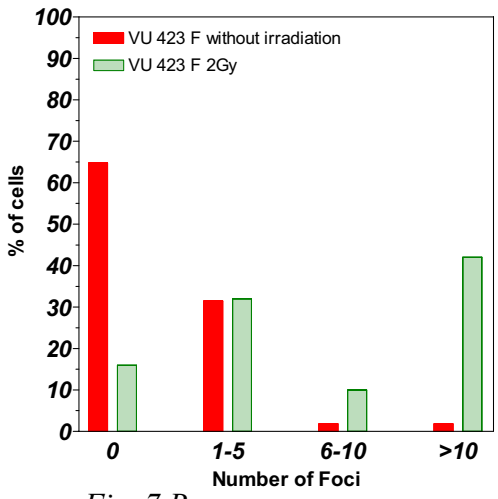


Fig. 7 B

Fig. 7: VU423 - Induction of γ-H2AX-foci by irradiation

A: VU423B

B: VU423F

To allow time for focus formation the cells were fixed 15 minutes after irradiation and prepared for immunofluorescence microscopy as described above. Both cell lines showed comparable results.

4.4.2. Effects of different Topotecan-concentrations on induction of DNA-double-strand breaks

For finding the appropriate Topotecan dose for further repair experiments, VU423B and VU423F cells were incubated for 16 h with Topotecan concentrations ranging between 0.001 µM and 0.07 µM. Immediately thereafter cells were fixed and prepared for detection of γ-H2AX-foci. In fig. 8, only cells without and with more than ten foci are presented as these groups show most obviously the effect of increasing Topotecan concentrations. For both strains, the number of cells without foci declined continuously with increasing Topotecan concentrations while, conversely, the number of cells with more than ten foci increased. At doses above 0.007 µM Topotecan, the number of foci in VU423B did not significantly increase and reached almost a plateau. In VU423F, the dose response curve was similar but the number of DNA-double-strand breaks appeared to increase slightly also beyond 0.007 µM Topotecan. A concentration of 0.05 µM Topotecan was chosen for further repair experiments.

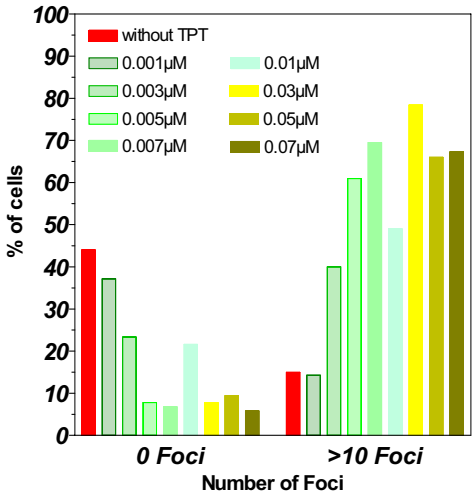


Fig. 8 A

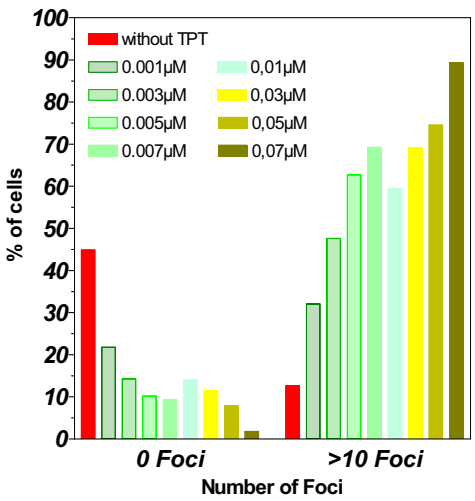


Fig. 8 B

Fig. 8: Incubation with different Topotecan concentrations

A: VU423B, concentrations from 0.001 µM up to 0.07 µM for 16 hours

B: VU423F, concentrations from 0.001 µM up to 0.07 µM for 16 hours

Immediately after 16 hours of incubation with different concentrations of Topotecan, VU423 cells were fixed for γ-H2AX-foci preparation. A Topotecan concentration of 0.05 µM was selected for further experiments.

In order to confirm that the Topotecan concentration chosen for VU423 cells also suits the other strains, HCC1937/BRCA1wt cells were incubated for 16 hours with either 0.03 µM (Fig. 9A) or 0.07 µM Topotecan (Fig. 9B). The untreated control cells contain a main fraction of cells without foci, several cells with 1-5 foci and only small fractions of cells with more than 5 foci. Similar to VU423, for HCC1937/BRCA1wt, the percentage of cells without foci decreased and the percentage of cells with more than 10 foci increased directly after incubation time. Also resembling VU423 cells, there was no difference between 0.03 and 0.07 µM Topotecan. It could be concluded that a Topotecan concentration of 0.05 µM should suit for both strains of HCC1937 cells, in these experiments represented by HCC1937/BRCA1wt.

Fig. 9 does not only show the number of foci measuered immediately after Topotecan incubation but also after a 4 h drug free interval. It was aimed to test whether repair could be measured after the concentrations chosen. For these experiments, all groups of cells with 0, 1-5, 6-10 and >10 foci are presented in fig. 9 because all data are necessary to see the repair capability. After 4 h of repair time, the cells with more than 10 foci were reduced and the cells without foci increased. The fractions of the other groups nearly stayed at constant levels which proofs the repair effect seen for both doses, 0.03 µM and 0.07 µM. It is reasonable to assume that a dose of 0.05 µM chosen previously for VU423 cells is also suitable to investigate the Topotecan effects in HCC1937 cells.

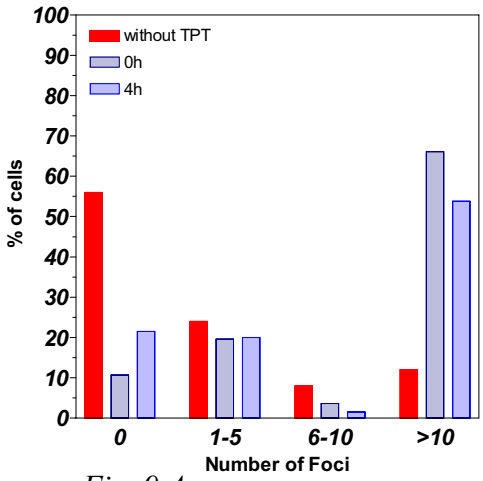


Fig. 9 A

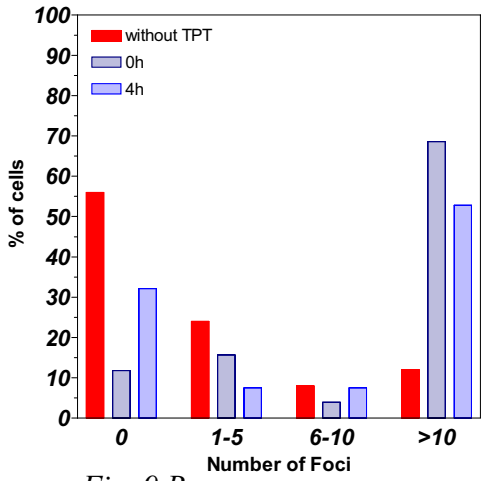


Fig. 9 B

Fig. 9: Incubation with different Topotecan concentrations and first repair experiments

A: HCC1937/BRCA1wt, 0.03 µM Topotecan for 16 hours

B: HCC1937/BRCA1wt, 0.07 µM Topotecan for 16 hours

HCC1937/BRCA1wt cells were incubated either with 0.03 µM or 0.07 µM Topotecan for 16 hours. Thereafter, cells were immediately fixed or medium was replaced with drug-free medium and cells were fixed after 4 hours for γ-H2AX-foci preparation. As for VU423 cells, also for HCC1937 cells a concentration of 0.05 µM was chosen for further experiments. Comparable Topotecan effects and DNA-double-strand break repair could be seen for both investigated doses.

4.4.3. Effects of different Topotecan exposure times on induction of DNA-double-strand breaks

The purpose of these experiments was to define the Topotecan exposure time for further repair experiments. VU423B cells were incubated with 0.05 µM Topotecan for 2 h, 4 h, 8 h or 12 h. Directly after drug exposure, the cells were fixed and γ-H2AX-foci were counted. In fig. 10, only cells without or with more than ten foci are presented. The percentage of cells with more than 10 foci continuously increased with incubation time and correspondingly, the percentage of cells without foci decreased. After 4 h of incubation, a clear effect of Topotecan could be seen which seems to be sufficient for repair experiments. A longer incubation time might have strong influence on other parameters such as cell cycle or apoptosis (see below).

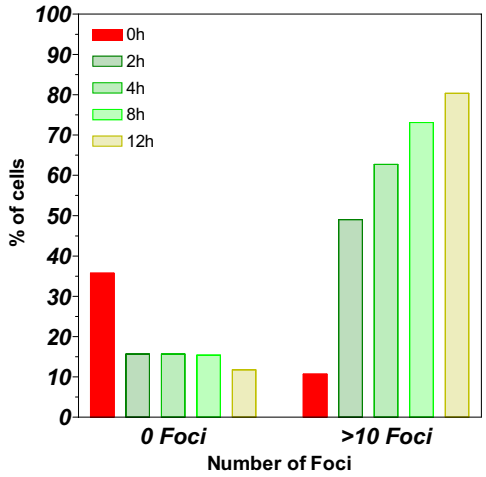


Fig. 10: Different incubation times for 0.05 µM Topotecan

VU423B cells were incubated with 0.05 µM Topotecan and fixed for γ-H2AX-foci preparation directly after different incubation times. An incubation time of 4 hours was selected for further repair experiments because a clear effect of Topotecan could be seen which is sufficient for repair experiments and might have no influence on other parameters such as cell cycle or apoptosis.

HCC1937/BRCA1wt and HCC1937/pcDNA3 were also incubated for 4 h with 0.05 μM Topotecan and γ-H2AX-foci were prepared directly after drug removal and after 4 h and 24 h of repair time. In fig. 11, all groups of cells with 0, 1-5, 6-10 and >10 foci are presented in order to illustrate the difference in repair capability between HCC1937/BRCA1wt and HCC1937/pcDNA3. The untreated control cells were comparable between both cell lines. Directly after drug exposure (0h), both cell lines showed a strong Topotecan effect as the percentage of cells without foci decreased and the percentage of cells with more than 10 foci increased. This effect was even stronger for HCC1937/pcDNA3 than for the complemented counterpart.

After 4 h of repair time, a repair effect can be seen for HCC1937/BRCA1wt as the fraction of cells with more than 10 γ-H2AX-foci decreased nearly back to the value of untreated control cells. For HCC1937/pcDNA3, this repair effect was smaller in the fraction of cells with more than 10 foci although the percentage of cells without foci increased, similar to HCC1937/BRCA1wt. Constant levels of cells containing 1-5 or 6-10 foci could be seen in both cell lines during repair time. Interestingly, when more repair time was allowed, the fractions of cells showing more than 10 γ-H2AX-foci increased again in both cell lines, reaching the levels observed immediately after drug removal before repair started (see Fig. 9A and B, last columns).

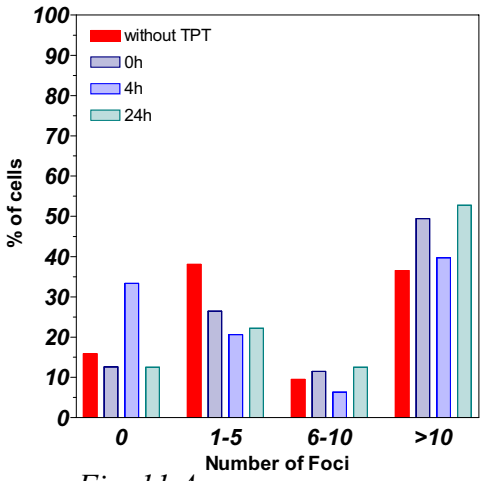


Fig. 11 A

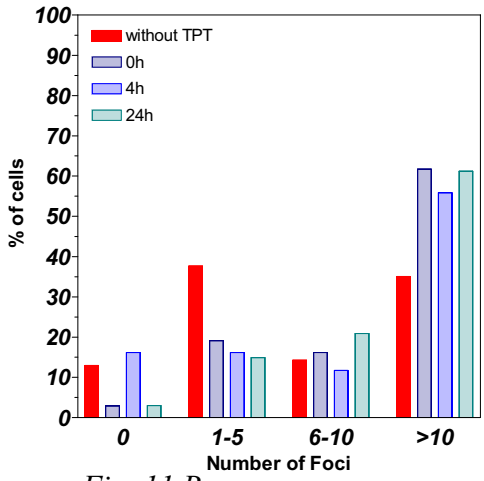


Fig. 11 B

Fig. 11: Repair effects after 4 hours incubation time with 0.05 µM Topotecan

A: HCC1937/BRCA1wt, 0.05 µM Topotecan for 4 hours

B: HCC1937/pcDNA3, 0.05 µM Topotecan for 4 hours

HCC1937 cell lines were both incubated with 0.05 µM Topotecan for 4 hours. Thereafter, cells were immediately fixed or medium was replaced with drug-free medium and cells were fixed after 4 h and 24 h of repair time for γ-H2AX-foci preparation. After 4 h of repair time, HCC1937/BRCA1wt showed a repair effect and HCC1937/pcDNA3 did not. For both cell lines, an increase of cells with >10 γ-H2AX-foci could be observed after 24 hours of repair time. The incubation time of four hours with 0.05 µM Topotecan fits for HCC1937 cells as for VU423 cells for which this had already been shown.

4.4.4. Repair of Topotecan induced DNA-double-strand breaks

For investigating the repair of DNA-double-strand breaks in detail, VU423B and VU423F cells were incubated with 0.05 µM Topotecan for four hours. The subsequent repair process was followed for up to 24 h after drug removal.

All classes of cells with either 0, 1-5, 6-10 or more than 10 foci are shown in Fig. 12A and B. The untreated control cells were comparable for both cell lines. Directly after exposure to Topotecan, both cell lines showed an increased level of cells with more than 10 foci while the levels of cells in all other classes declined. With ongoing repair time, the numbers of cells ascribed to the various classes did not consistently change any more. At best, for both strains there were slightly more cells after 16 h in the classes with more than 10 foci. The constant level of γ-H2AX-foci during repair time becomes even more obvious in fig. 12C and D in which only a difference is made between cells with or without foci. Taken together, neither the repair deficient VU423F nor the proficient VU423B cells show any repair of Topotecan-induced DNA-double-strand breaks.

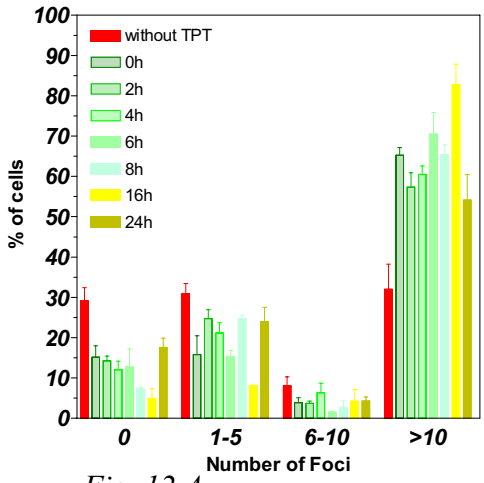


Fig. 12 A

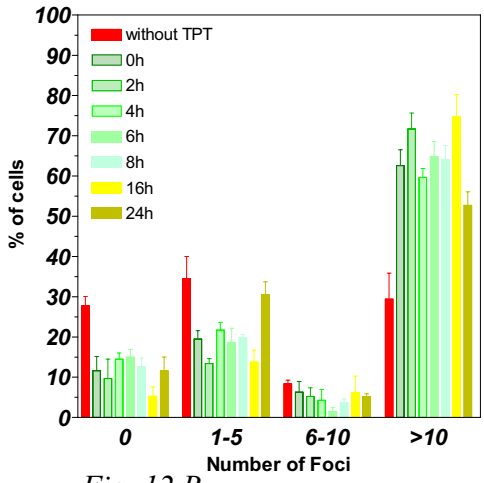


Fig. 12 B

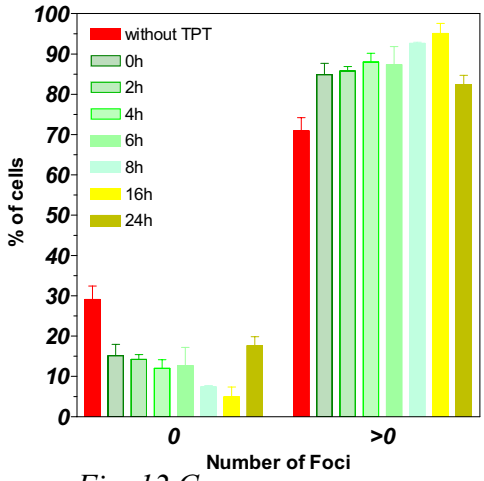


Fig. 12 C

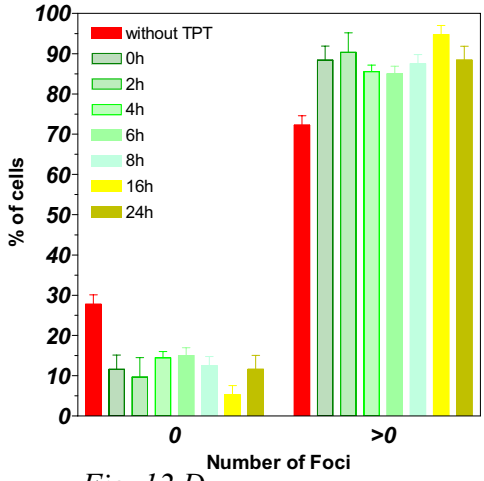


Fig. 12 D

Fig. 12: Repair of Topotecan induced DNA-double-strand breaks for VU423

A: VU423B, 0.05 µM Topotecan for 4 hours (0, 1-5, 6-10, >10 foci)

B: VU423F, 0.05 µM Topotecan for 4 hours (0, 1-5, 6-10, >10 foci)

C: VU423B, 0.05 µM Topotecan for 4 hours (0, >0 foci)

D: VU423F, 0.05 µM Topotecan for 4 hours (0, >0 foci)

VU423 cell lines were incubated with 0.05 µM Topotecan for four hours. Immediately thereafter, medium was replaced with drug-free medium and a repair process was followed up for 24 hours after drug removal. The percentages of the classes of cells with different numbers of γ-H2AX-foci nearly stayed at constant levels during the first 8 hours. Thereafter, both cell lines showed an increasing fraction of cells with more than 10 foci. Neither the repair deficient VU423F nor the proficient VU423B cells showed any repair effect.

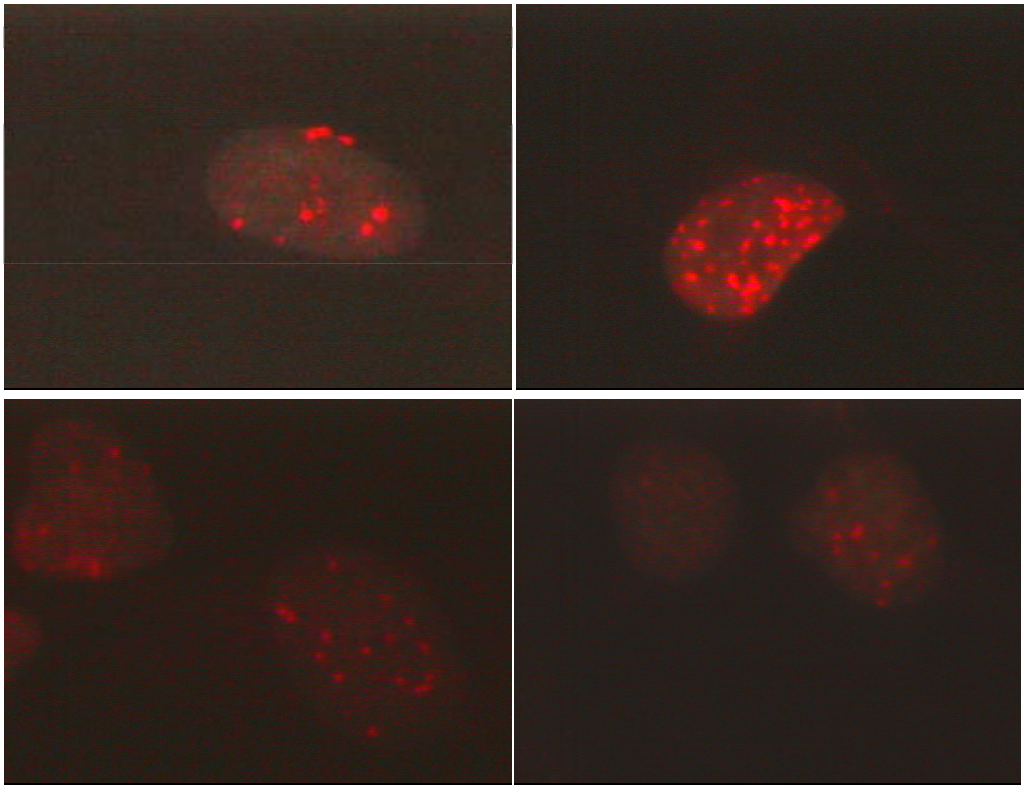


Fig. 13 A: Pictures of γ-H2AX-Foci in VU423B

upper left: 9 foci

upper right: >10 foci

down left: 6 Foci and >10 foci

down right: 10 foci and 0 foci

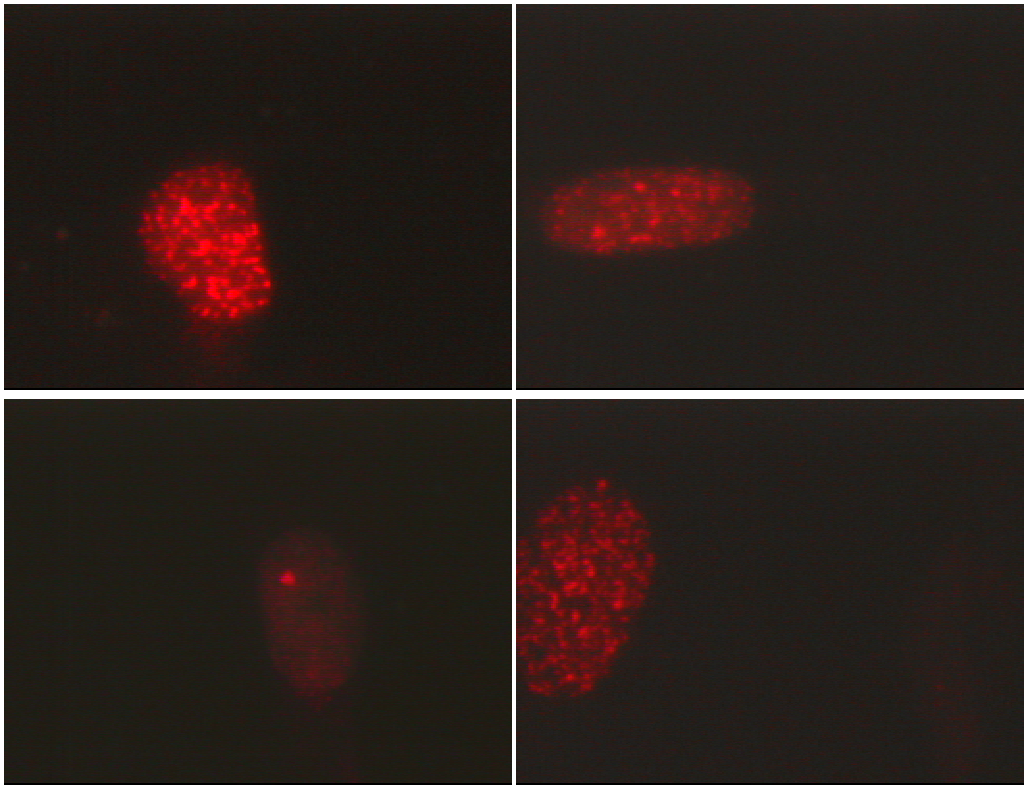


Fig. 13 B: Pictures of γ-H2AX-Foci in VU423F

upper left: >10 foci

upper right: >10 foci

down left: 1 focus

down right: >10 foci

HCC1937/BRCA1wt and HCC1937/pcDNA3 cells were treated exactly as described above for VU423 cell lines. In fig. 14A and B, all classes of cells with 0, 1-5, 6-10 and more than 10 foci are shown.

For HCC1937/BRCA1wt, a damage inducing effect of Topotecan could be seen in an increasing percentage of cells with more than 10 foci while a decreasing percentage of cells could be observed in all other classes. During the first 6 hours of repair time, the numbers of γ-H2AX-foci decreased continuously and straight proportional nearly back to base level which is obvious at an increasing fraction of cells without and a decreasing fraction of cells with more than 10 foci. This observation can be interpreted as proceeding DNA-double-strand break repair. Eight hours after drug removal, an increase of DNA-damage became obvious at a strong increase of cells with more than 10 and a decrease of cells without any γ-H2AX-foci.

Unless a higher base level of DNA-double-strand breaks in HCC1937/pcDNA3 and accordingly only relatively few additional cells which were severely damaged after Topotecan incubation, the percentage of cells with more than 10 foci was higher for HCC1937/pcDNA3 than for HCC1937/BRCA1wt directly after incubation with Topotecan. This might indicate that HCC1937/pcDNA3 cells repaired less damage than HCC1937/BRCA1wt while being exposed to the drug. During the first six hours of repair time, all classes of cells nearly stayed at constant levels. Especially the constant level of cells without foci and only little increasing fractions of cells with 1-5 or 6-10 foci hint at the absence of any repair effect although the decreasing fraction of cells with more than ten foci could mislead to a repair effect. Comparable to HCC1937/BRCA1wt, after eight hours of repair time HCC1937/pcDNA3 showed an increasing percentage of cells with more than 10 foci and a decreasing percentage of cells without any foci.

For clarity, fig. 14C and D only make a difference between cells with or without foci. The repair effect for HCC1937/BRCA1wt can be seen in both groups of cells and the constant level of DNA-double-strand breaks for HCC1937/pcDNA3 is even more obvious than in fig. 14B.

The repair kinetics in HCC1937/BRCA1wt cells is depicted separately in fig. 15 in logarithmic scale for pointing at the decrease of Topotecan-induced DNA-double-strand breaks. The percentage of cells with more than 10 foci seen directly after incubation minus the base level was assigned to 100% which stands for the DNA-double-strand breaks induced by Topotecan. Most of these DNA-double-strand breaks could be

repaired during the first six hours of repair time with a half-life of about 5 hours. After six hours of repair time, an increasing percentage of cells with more than 10 foci could be seen. This can be explained either by cell-cycle abnormalities or apoptosis which is both investigated in further experiments.

In conclusion, there is a difference in the ability to repair Topotecan induced DNA-double-strand breaks between BRCA1 deficient and proficient cells. The cells lacking wild-type BRCA1 were completely unable to repair the induced DNA-double-strand breaks while the cells expressing BRCA1 show almost complete repair within the first 6 hours after drug removal.

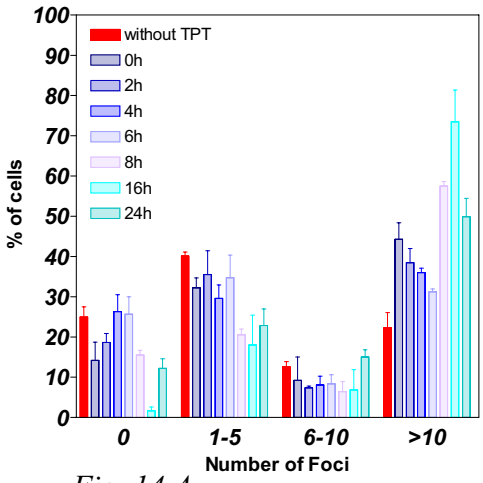


Fig. 14 A

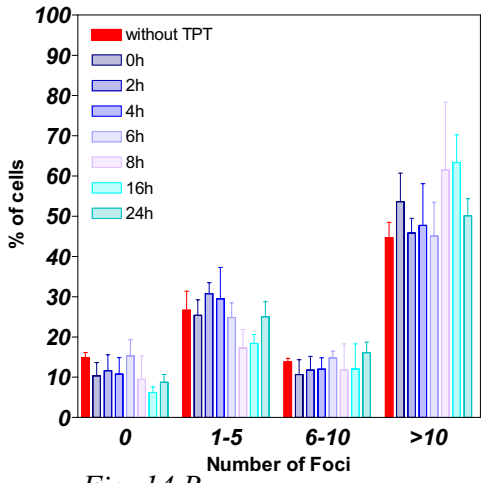


Fig. 14 B

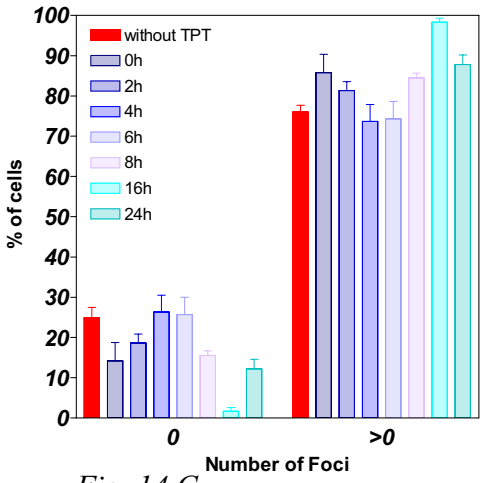


Fig. 14 C

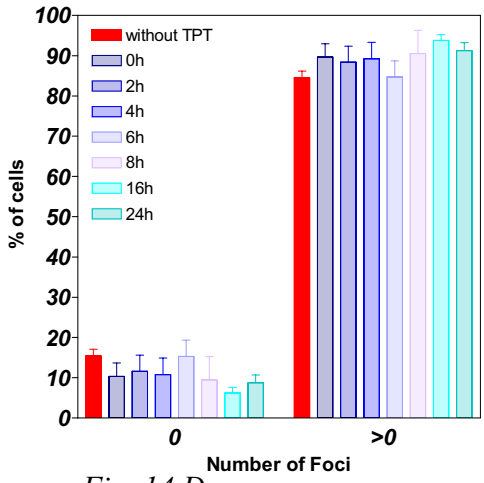


Fig. 14 D

Fig. 14: Repair of Topotecan induced DNA-double-strand breaks for HCC1937

A: HCC1937/BRCA1wt, 0.05 µM Topotecan for 4 hours (0, 1-5, 6-10, >10)

B: HCC1937/pcDNA3, 0.05 µM Topotecan for 4 hours (0, 1-5, 6-10, >10)

C: HCC1937/BRCA1wt, 0.05 µM Topotecan for 4 hours (0, >0 foci)

D: HCC1937/pcDNA3, 0.05 µM Topotecan for 4 hours (0, >0 foci)

HCC1937 cell lines were incubated with 0.05 µM Topotecan for four hours. Immediately thereafter, medium was replaced with drug-free medium and a repair process was followed up for 24 hours after drug removal. For HCC1937/BRCA1wt, a repair effect could be seen during the first 6 hours while no repair effect was observable for HCC1937/pcDNA3. Thereafter, for both cell lines a strongly increasing level of cells with more than 10 γ-H2AX-foci could be seen which will be investigated in further experiments.

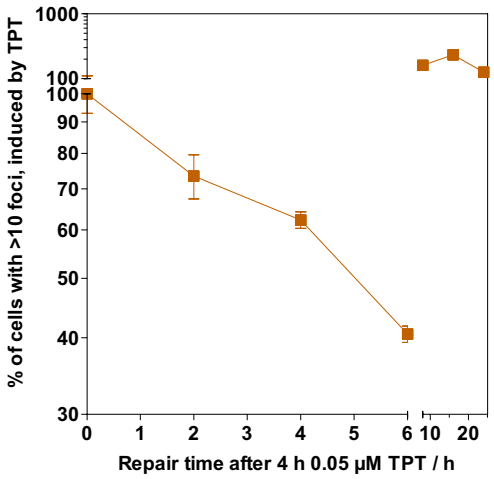


Fig. 15: Repair kinetics for cells with more than ten foci, reference 0h

A logarithmic scale is chosen for illustrating the repair process for HCC1937/BRCA1wt. During the first six hours of repair time, the fraction of cells with more than ten foci could be reduced to 40% of the initial value. After that, a strong increase of DNA-double-strand breaks could be seen which can be explained by cell-cycle abnormalities or apoptosis. Both theories are observed in further experiments.

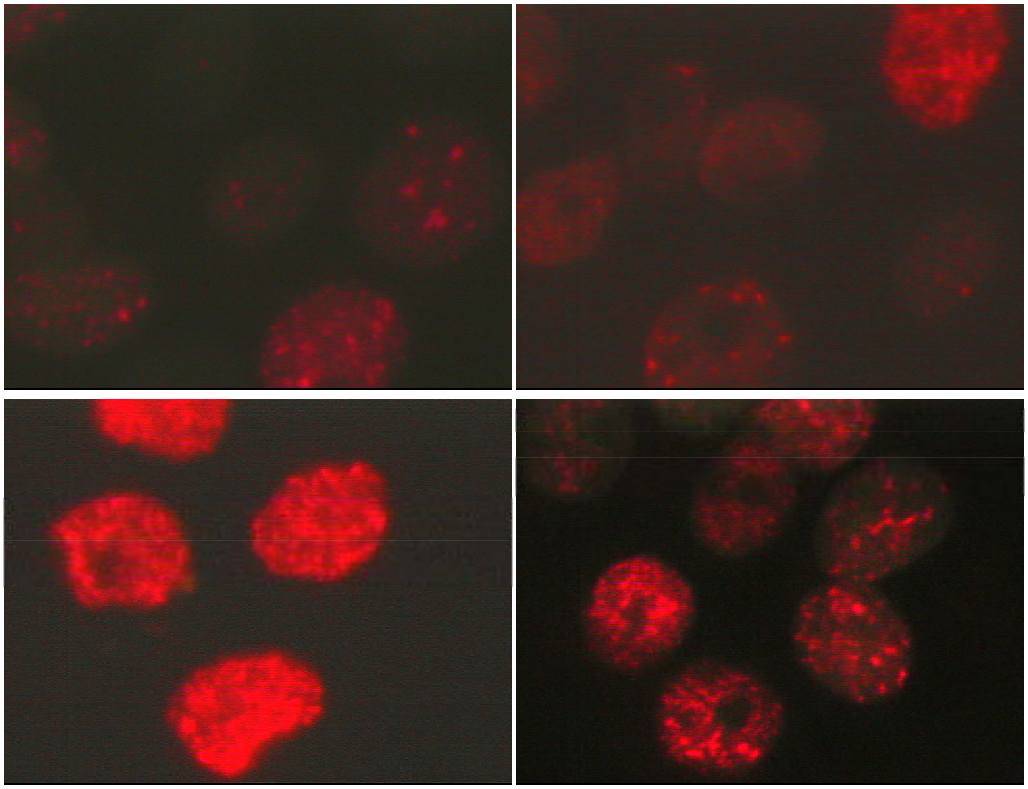


Fig. 16 A: Pictures of γ-H2AX-foci in HCC1937/BRCA1wt

upper left: 1 focus, 2 foci, 9 foci and >10 foci

upper right: 0 foci, 1 focus, 2 foci and >10 foci

down left: all >10 foci

down right: all >10 foci

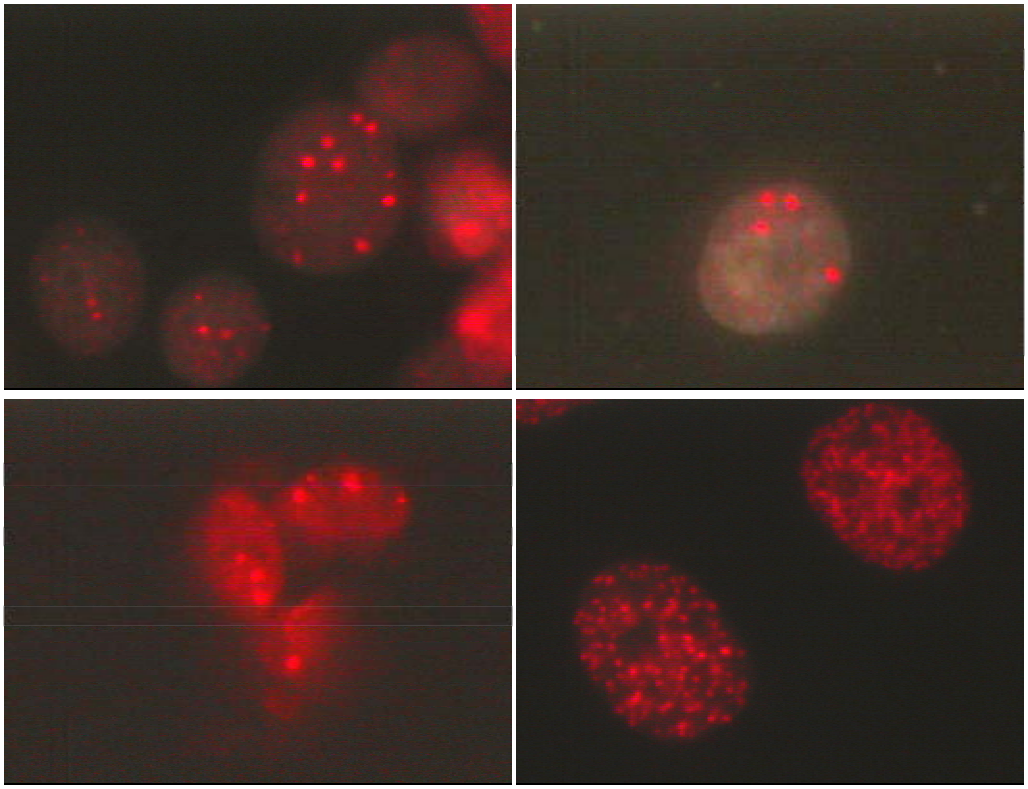


Fig. 16 B: Pictures of γ-H2AX-foci in HCC1937/pcDNA3

upper left: 0 foci, 5 foci, 6 foci and >10 foci

upper right: 4 foci

down left: 3 foci, 4 foci and 5 foci

down right: all >10 foci

4.5. Cell cycle analysis

The repair of DNA-double-strand breaks might eventually be affected by cell cycle progression as the possibility to use one or the other pathway for repair changes during the cell cycle. For VU423B and VU423F cells, the absence of any repair effect could be explained by a lack of S-phase progress. If cells were unable to proceed in cell cycle after incubation with Topotecan, they would not be able to repair the induced DNA-double-strand breaks by homologous recombination which is required for this kind of DNA-damage because homologous recombination shows the highest rate in late S-phase.

For evaluating this theory, VU423B and VU423F cells were incubated for four hours with 0.05 µM Topotecan, medium was renewed and cells were fixed at different intervals and processed for FACS analysis.

Fig. 17 shows examples of DNA-histograms measured by flow cytometry. On the x-axis, Fl 2 is plotted which detects propidium iodide representing the DNA-content per cell. Two peaks could be seen, one corresponding to cells in G1 (2n DNA-content, left peak) and the other to cells in G2 (4n DNA-content, right peak). All cells between these two peaks can be interpreted as S-phase cells because they have a DNA-content between 2n and 4n while they are synthesizing DNA for mitosis.

For both, VU423B and VU423F, a certain fraction of cells showed a reduced DNA-content (Sub-G1) which can be interpreted as dying G1-cells. An analogous fraction was also calculated for S and G2 cells (blue areas). In contrast to VU423B cells, untreated VU423F did not show dying cells. However, in other experiments VU423F controls showed also evidence for cell death comparable with VU423B.

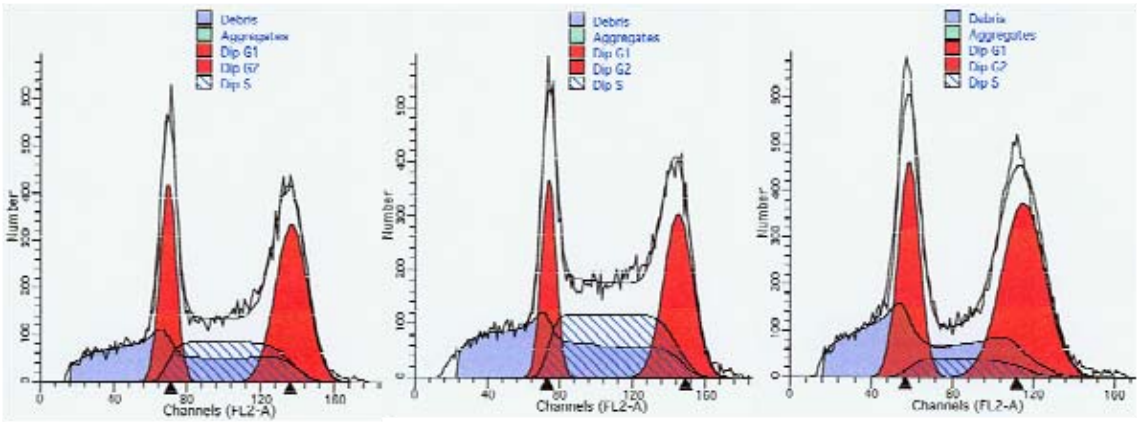
It was next studied whether exposure to Topotecan affects cell cycle progression. Therefore, the cells were incubated for 4 h with Topotecan and the cell cycle distribution was measured either directly or after 24 h incubation in drug free medium (Fig.17A and B middle and right hand chart). It can be seen that the percentage of dying cells increases over the time analyzed for both VU423B and VU423F.

Figure 18 shows the kinetics of cell cycle progression measured in the same way over the entire repair period after incubation with Topotecan. The time point -4 hours corresponds to results for untreated cells before Topotecan was added. The 4 hours of incubation with Topotecan lead to increasing percentages of cells in S-phase, comparable for both cell lines. The increase of the S-phase fraction continued for VU423F cells for another 4-6 h after Topotecan was removed. During this period, the

fraction of VU423B cells in S-phase remained constant. For both cell lines, this can be interpreted as a Topotecan-induced S-phase delay or transient arrest. After these six hours of repair time, the levels of S-phase cells decreased similarly for both cells which indicates a release from the S-phase block. In parallel, the fractions of G2-phase cells increased and remained constantly high (50-60%) for another 18 h indicating a subsequent sustained G2-arrest.

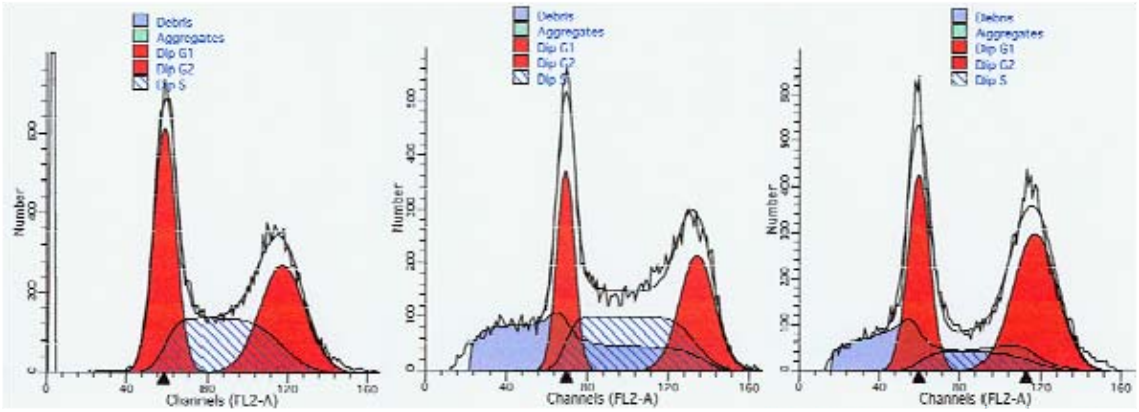
In conclusion, cell cycle progression was parallel for both, VU423B and VU423F cells. Topotecan induced a transient S-phase arrest and a subsequent G2-arrest.

As the cells can proceed in cell cycle, they should also be able to repair the Topotecan induced DNA-double-strand breaks by homologous recombination in late S-phase. The absence of any repair effect can not be explained by a disability of process in cell-cycle.



Control cells *0 h* *24 h*

Fig. 17 A



Control cells *0 h* *24 h*

Fig. 17 B

Fig. 17: Cell cycle analysis at the flow cytometer

A: VU423B

B: VU423F

Cells were incubated with 0.05 µM Topotecan for four hours and either fixed directly or after a repair interval of 24h. The DNA-histograms were measured after propidium iodide staining by the flow cytometer.

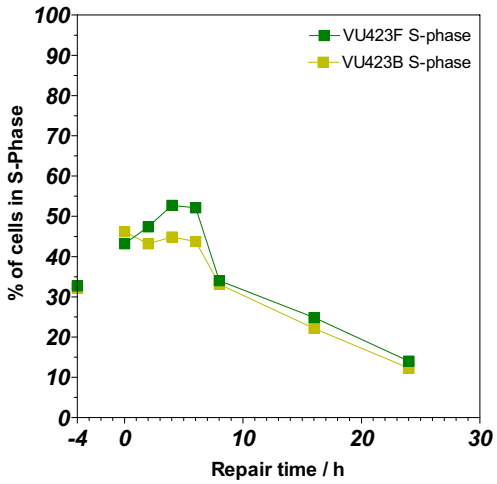


Fig. 18 A

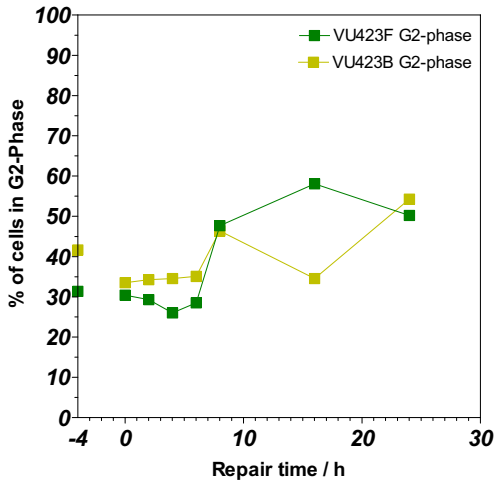


Fig. 18 B

Fig. 18: Summary of the cell cycle analysis

A: Fraction of cells in S-phase

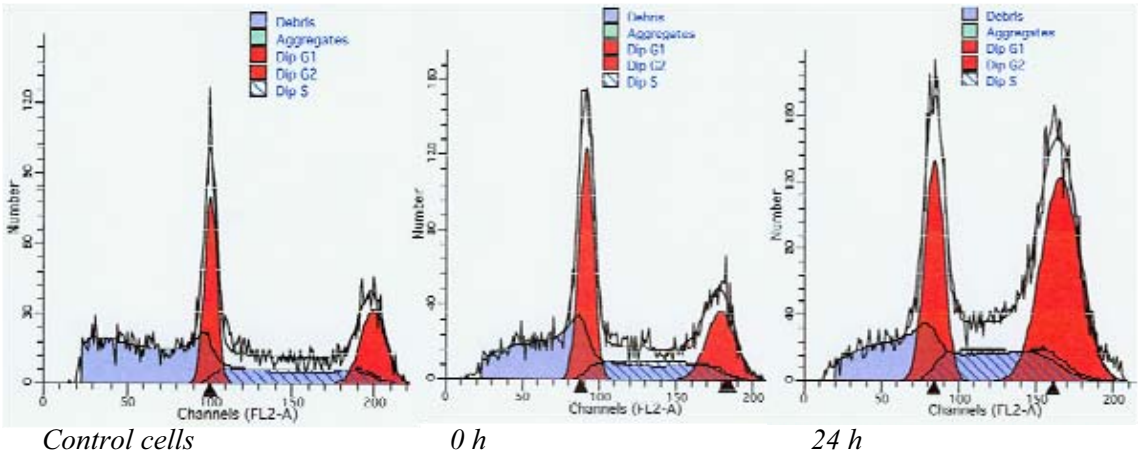
B: Fraction of cells in G2-phase

Kinetics of cell cycle progression measured over the entire repair period after incubation with Topotecan. The time point -4 hours corresponds to results for untreated cells before Topotecan was added. A Topotecan-induced S-Phase delay and subsequent sustained G2-arrest can be seen for both cell lines. Cell cycle process is parallel for both, VU423B and VU423F.

For HCC1937 cell lines, a decreasing number of replication-associated DNA-double-strand breaks (Fig. 14 / 15) might not only be due to the repair capability but might be also affected by cell cycle progression. It is assumed that cells need to stop proliferation in order to restore the stalled replication forks after Topotecan treatment. Thus, an S-phase delay might be necessary for repairing the Topotecan-induced DNA-damage by homologous recombination whilst ongoing replication could result in a disadvantage for the repair machinery. In order to test whether HCC1937/BRCA1wt and HCC1937/pcDNA3 show differences in proliferation, cell cycle analysis was performed as described above for VU423 cells by means of flow cytometry.

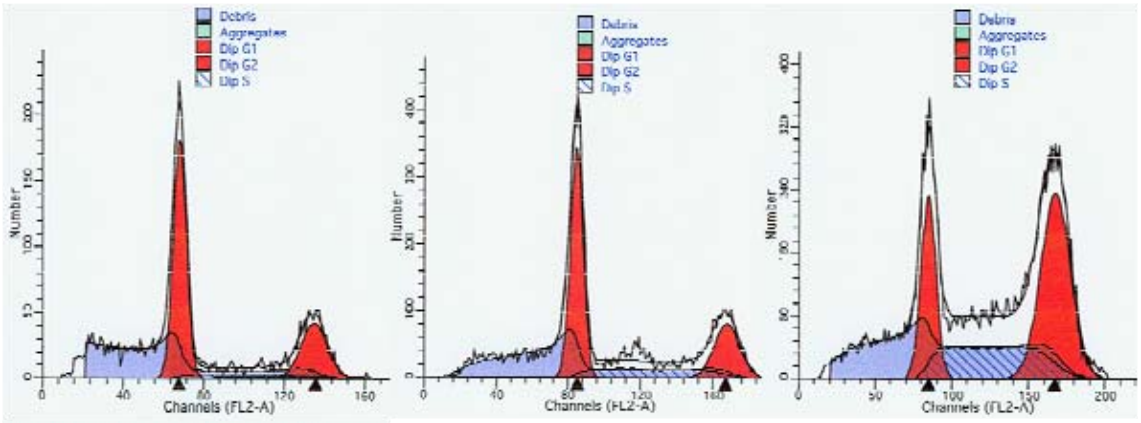
Fig. 19 shows DNA histograms as already described for VU423B and VU423F. Furthermore, cell-cycle progress was influenced by Toptecan which is illustrated separately in fig. 20.

After incubation with Topotecan an increased rate of cells in S-phase could be seen for both cell lines which can be interpreted as Topotecan-induced S-phase delays. The delay was even stronger for the repair-proficient HCC1937/BRCA1wt. For eight hours of repair time, the levels of S-phase cells increased constantly and thereafter, the S-phase fraction decreased similarly for both cells which indicates a release from the S-phase block. In parallel, the fraction of G2-phase cells increased and remained constantly high for another 16 hours indicating a subsequent sustained G2-arrest. Temporally, the cell cycle progression was parallel for both cell lines although the repair-proficient HCC1937/BRCA1wt cells showed a stronger S-phase delay. The detected differences in repair capability between the two cell lines can be explained by the Topotecan-induced S-phase delay which was much stronger in the cells with functional repair machinery.



Control cells *0 h* *24 h*

Fig. 19 A



Control cells *0 h* *24 h*

Fig. 19 B

Fig. 19: Cell cycle analysis at the flow cytometer

A: HCC1937/BRCA1wt, control, 0 h, 24 h

B: HCC1937/pcDNA3, control, 0h, 24 h

Cells were incubated with 0.05 µM Topotecan for four hours and thereafter fixed at different intervals. The DNA-histograms were measured after propidium iodide staining at the flow cytometer.

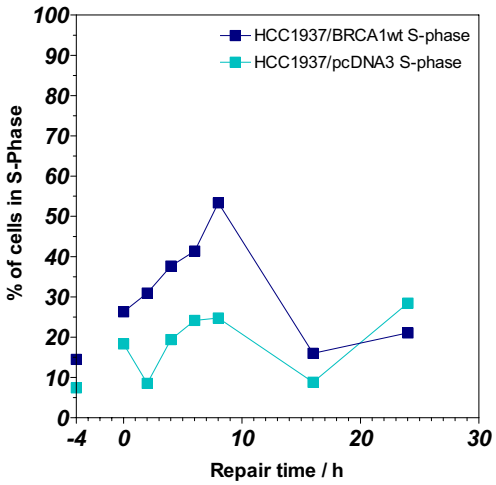


Fig. 20 A

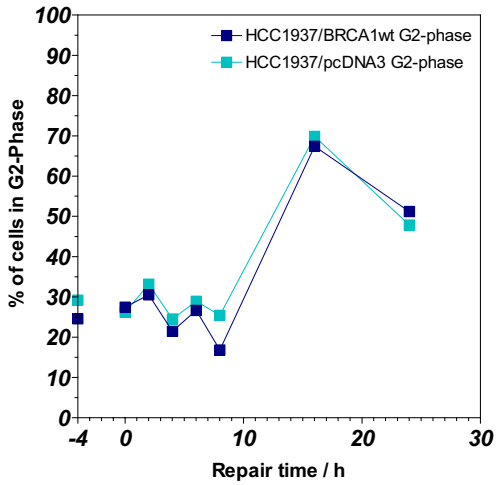


Fig. 20 B

Fig. 20: Summary of the cell cycle analysis

A: Fraction of cells in S-phase

B: Fraction of cells in G2-phase

Kinetics of cell cycle progression measured over the entire repair period after incubation with Topotecan. The time point -4 hours corresponds to results for untreated cells before Topotecan was added. Temporally, cell-cycle process is parallel for both HCC1937/BRCA1wt and HCC1937/pcDNA but the Topotecan-induced S-Phase delay was even stronger for HCC1937/BRCA1wt. After eight hours of repair time, both cell lines showed a subsequent sustained G2-arrest.

4.6. Analysis of the apoptotic fractions

The high base level of γ-H2AX-foci, especially in VU423B and VU423F cells, could be explained by apoptosis. Apoptosis causes DNA-double-strand breaks which induce γ-H2AX-foci formation[80]. Thus, a high base level of apoptotic γ-H2AX-foci could cover repair effects. Furthermore, a strong apoptotic effect of Topotecan could cover the typical Topotecan-induced DNA-double-strand breaks and thereby, no repair effect could be observed.

Therefore, the analysis of the apoptotic fractions in VU423 cells was performed. VU423B and VU423F were incubated for four hours with 0.05 µM Topotecan, medium was renewed and cells were fixed directly thereafter and after 6 h and 24 h of repair time. Cells were stained with Annexin V as described in material and methods and analyzed by flow cytometry. Fig. 21 shows dot-blot-diagrams from FACS-analysis, half logarithmic on the left hand, double logarithmic on the right hand. For each cell, the Annexin fluorescent signal is plotted against the PI signal. Two populations can be seen which are separated by gating the majority of cells (green dots (R1), mean Annexin V of 10 relative units) considered Annexin-negative and a small population considered Annexin-positive and apoptotic (red dots (R2), mean Annexin V of 100 relative units). For the decision whether a cell is positive for Annexin or not, two regions are defined by a border line. Cells lying over this borderline are defined as Annexin-positive apoptotic cells, cells lying under this border are Annexin-negative. The more Annexin is measured for a cell, the more its position is displaced to the left upper corner of the diagram.

Fig. 22 shows the fractions of apoptotic cells, -4 h stands for the base level of apoptosis from a flask of untreated control cells. For both, VU423B and VU423F, a low level of apoptotic cells comparable to fibroblasts could be found. Base levels were 6.3 % for VU423B and 5.2 % for VU423F which is, compared with the apoptotic fraction of MR1 human fibroblasts of 4.3 %, not extremely high. During the 4 h Topotecan incubation, an increase of the level of Annexin-positive cells appeared for both cell lines which can be explained by an apoptotic effect of Topotecan. This effect was even stronger for VU423F cells. After that, the apoptotic fractions slowly decreased for both cell lines. Analogous experiments were performed with parallel incubation of the cells with caspase inhibitor Z-VAD-FMK for the whole time as described in material and

[80] Rogakou et al., 1999

methods. The caspase inhibitor was used to suppress Topotecan-induced apoptosis. It actually reduced the apoptotic effect of Topotecan from 9.2 % to 8.4 % for VU423B and from 11.6 % to 8.9 % for VU423F. Even during repair time, the level of apoptotic cells was always a little below the apoptotic fraction without caspase inhibitor.

The base level of apoptotic cells and even the apoptotic effect of Topotecan are not as high as expected. These experiments confirm that in VU423B and VU423F cells only little apoptosis is detectable by Annexin V.

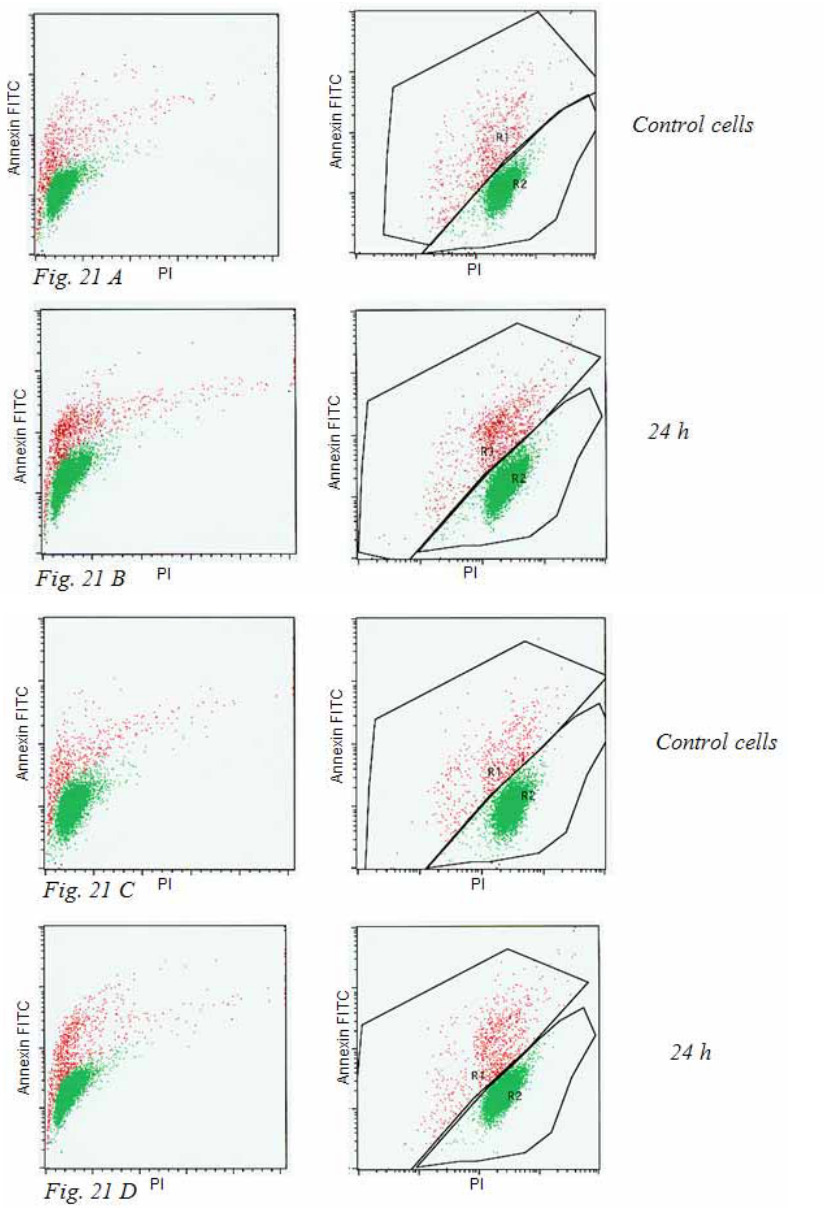


Fig. 21: Apoptosis measured at the flow cytometer

A: VU423B, untreated control

B: VU423B, after 24 hours of repair time

C: VU423F, untreated control

D: VU423F, after 24 hours of repair time

Cells were incubated with either 0.05 µM Topotecan or with 0.05 µM Topotecan plus 50 µM Caspase Inhibitor for four hours and thereafter fixed at different intervals. Cells were stained with Annexin V and Propidium Iodide and dot-blot diagrams were made from the data measured at the flow cytometer. The left diagrams present the data half-logarithmic, the diagrams on the right hand are double-logarithmic diagrams. The green fraction represents Annexin-negative and the smaller red fraction Annexin-positive, apoptotic cells.

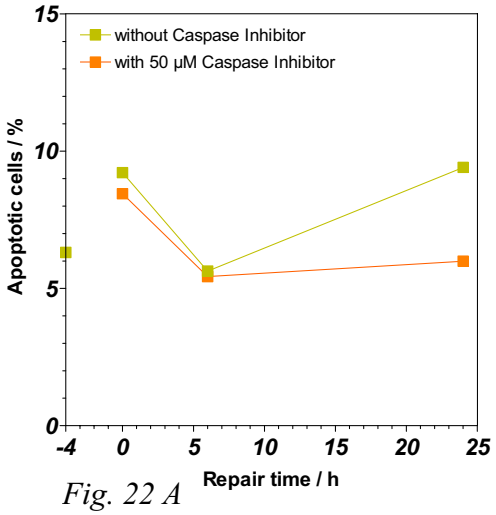


Fig. 22 A

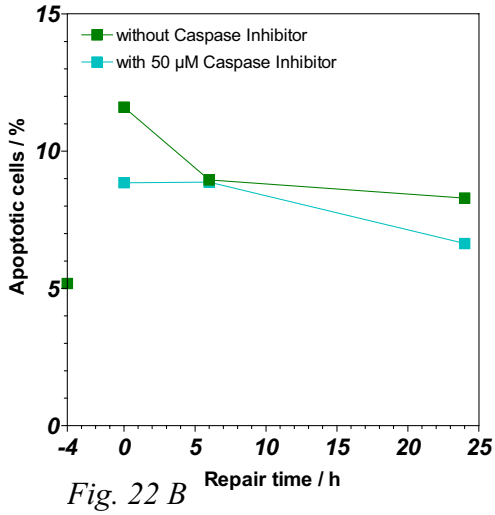


Fig. 22 B

Fig. 22: Analyzing the apoptotic fractions with or without Caspase Inhibitor

A: VU423B, Apoptosis after 0.05 µM Topotecan for 4 hours

B: VU423F, Apoptosis after 0.05 µM Topotecan for 4 hours

The apoptotic fractions of VU423B and VU423F cells after incubation with Topotecan and during repair time were measured with or without addition of Caspase Inhibitor Z-VAD-FMK. An apoptotic effect of Topotecan can be seen for both cell lines, even stronger for VU423F. The caspase inhibitor reduces the apoptotic effect of Topotecan and even during repair time, the level of apoptotic cells is always a little below the apoptotic fraction without caspase inhibitor.

5. Discussion

Differences in sensitivity against Topotecan were discovered for both cell lines. The BRCA2 defective cell line VU423F showed an increased sensitivity against Topotecan compared to the control cell line VU423B expressing wild-type BRCA2.
This suits to other results: For another cell line with only one allele of BRCA2 leading to a truncated protein, the pancreatic carcinoma cell line CAPAN 1, an increased sensitivity could be found against ionizing radiation, the DNA-intercalator Mitoxantrone which leads to DNA-damage by interaction with Topoisomerases, Amsacrine which is a DNA-intercalator as well as a Topoisomerase II inhibitor and the Topoisomerase II inhibitor Etoposide. No increased sensitivity was found for the mitotic spindle poison Paclitaxel or the ribonucleotide-reductase inhibitor Hydroxyurea which blocks DNA-synthesis[81]. Only for ionizing radiation and chemotherapeutic drugs which lead to DNA-damage, an increased sensitivity could be observed. Compared with the results of increased sensitivity against Topotecan, all results mentioned here support the hypothesis that BRCA2 defective cells are defective in repair of DNA-double-strand breaks.

The BRCA1 defective cell line HCC1937/pcDNA3 also showed a higher sensitivity against Topotecan than the BRCA1 wild-type cell line HCC1937/BRCA1wt. For these cell lines, the differences in sensitivity were more obvious than for VU423 and even at higher concentrations of Topotecan, the differences could be seen clearly.
This result can be compared with the high sensitivity against Cisplatin discovered by Tassone et al., 2003: They found that BRCA1 defective HCC1937 was very sensitive against Cisplatin, a chemotherapeutic drug which leads to DNA-interstrand-crosslinks. Furthermore, BRCA1 deficient HCC1937 cells did not show an increased sensitivity against other chemotherapeutic drugs like Paclitaxel, a mitotic spindle poison which suppresses depolymerisation of tubulin-dimers. HCC1937/BRCA1wt cells did not show an increased sensitivity against any of the drugs mentioned as expected for this control cell line. Taken to a conclusion, increased sensitivities could be demonstrated for chemotherapeutic drugs which generate DNA-double-strand breaks directly or in an indirect way. This can be explained by a defective DNA-repair in cells without wild-type BRCA1. Conflicting results are the increased sensitivity of BRCA1-inhibited

[81] Abbott et al., 1998

cells against Etoposide[82], a Topoisomerase II inhibitor and no increased sensitivity against Doxorubicin[83], a DNA-helix-intercalator inducing DNA-damage and also an inhibitor of the Topoisomerase II. Therefore, no convincing explanation has been found yet. Nevertheless, the observed differences in sensitivity against DNA-damaging drugs might hint at DNA-repair deficiencies in the cell lines either without BRCA1 or BRCA2 although conflicting results can not be explained yet.

In addition to differences in sensitivity against Topotecan, HCC1937 did show a difference in DNA-double-strand break repair. HCC1937/pcDNA3 did not show any repair effect during the analyzed period of time but cells stably transfected with BRCA1 were able to repair the Topotecan-induced damage. The C-terminal truncated BRCA1 proteins in HCC1937/pcDNA3 can not fulfil their tasks for DNA-double-strand break repair and furthermore it has been shown that BRCT-mutated BRCA1 proteins are mislocated to the cytoplasm instead of the nucleus where they have to work[84]. This suggests that functional BRCA1 protein is necessary for an efficient repair of Topotecan induced DNA-double-strand breaks.
The observed repair effects in HCC1937/BRCA1wt might be due to cell-cycle effects and therefore, analysis of the cell-cycle was performed. For both HCC1937 cell lines, cell cycle progression was parallel and the observed repair effects were not only due to cell-cycle abnormalities. But the Topotecan induced S-phase delay was more distinct at HCC1937/BRCA1wt cells which only gives these cells the possibility to repair the arisen damage. This hints at the hypothesis that BRCA1 might not only be necessary for DNA-double-strand break repair itself but also for the induction of Topotecan-induced S-phase delays. It has already been shown that BRCA1 seems to be responsible for cell-cycle checkpoints: normal BRCA1 function is needed for the ionizing radiation induced checkpoints during S-phase and G2/M[85]. Contrary to the results mentioned here, it has been described that BRCA1 defective HCC1937 does not have a functional S-phase checkpoint after irradiation. Cell-cycle analysis after incubation with Topotecan in this doctoral thesis did show a strong S-phase delay for both HCC1937/BRCA1wt and HCC1937/pcDNA3 cell lines. Moreover, the base levels of cells in G2 phase were even higher than the base levels of cells in S-phase which is not typical but has been

[82] Lafarge et al., 2001
[83] Tassone et al., 2003
[84] Rodriguez et al., 2004
[85] Xu et al., 2001

described before by Zhang et al., 2004. This gives a hint at a functional G2/M checkpoint activated by the high base level of DNA-double-strand breaks investigated for the HCC1937 cell lines. Maybe the discrepancy between the lack of any S-phase delay after irradiation and the strong S-phase delay after incubation with Topotecan can be explained by a difference in cellular reaction upon spontaneous DNA-double-strand breaks, DNA-double-strand breaks induced by ionizing radiation or by Topotecan.

In contrast to HCC1937, for both VU423B and VU423F no repair of Topotecan-induced DNA-double-strand breaks could be seen. For VU423F, the cell line with functional defects in both alleles of BRCA2, this result was expected: truncated BRCA2 proteins can not fulfil their tasks in repair. Furthermore, a mislocalization of the truncated BRCA2 proteins to the cytoplasm instead of the nucleus could inhibit their function. This has also been described for the pancreas carcinoma cell line CAPAN 1[86]. But what is the reason for a lack of DNA-repair in VU423B, the cell line which expresses wild-type BRCA2?

It was necessary to demonstrate that the absence of any repair effect is independent of the cell-cycle. VU423 cell lines showed base level distributions of the cell-cycle different from most other cells but comparable with HCC1937 cells: In VU423, G2-phase levels are as high as or even higher than the levels of cells in S-phase although the base levels of cells in G2-phase should not be higher than S-phase levels. After incubation with Topotecan, a strong S-phase delay could be observed. Both results hint at functional S-phase or G2/M checkpoints in the VU423 cell lines. The Topotecan induced DNA-double-strand breaks or the high base levels of DNA-double-strand breaks activate these checkpoints. Nevertheless, cell cycle is parallel for both VU423B and VU423F and there is no reason why cell-cycle should inhibit DNA-repair.

Furthermore, at concentrations above 0.01 µM, a concentration which many other human tumour cell lines survive, both VU423B and VU423F showed hardly any surviving colonies. Compared to HCC1937 cell lines, the VU423 cells were much more sensitive against Topotecan with a $C_{0.1\ VU423} \approx 0.02$ µM and $C_{0.1\ HCC1937} \approx 0.05$ µM respectively. This can be interpreted as hypersensitivity against Topotecan for both VU423 cell lines, either with or without wild-type BRCA2. Hypersensitivity against

[86] Howlett et al., 2002

Topotecan as seen for VU423B and VU423F could already be seen in previous experiments at colony forming tests with XRCC3-deficient cell lines (fig. 23 A[87]).

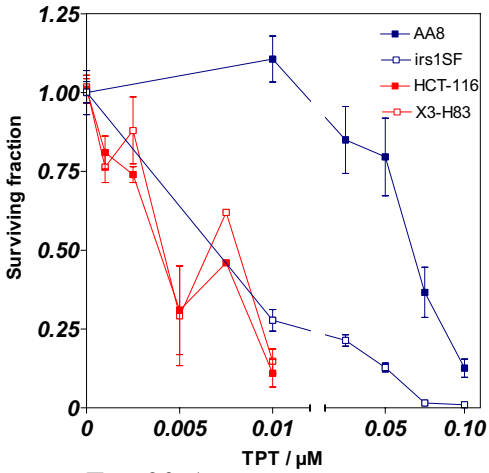


Fig. 23 A

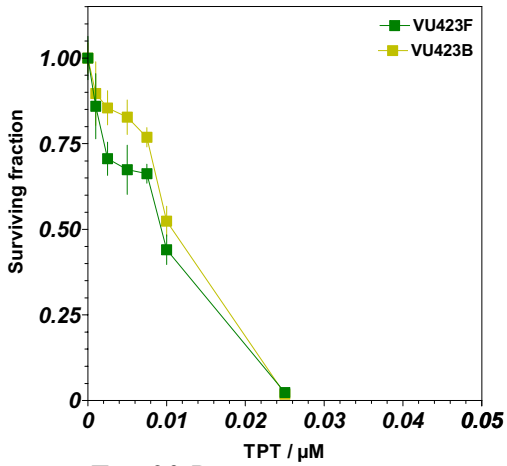


Fig. 23 B

Fig.23: Comparison of colony formation between XRCC3$^{-/-}$ and BRCA2$^{-/-}$ cells

A: AA8 (wild-type XRCC3) / irs1SF (XRCC3$^{-/-}$) hamster cell lines

HCT-116 (wild-type XRCC3) / X3-H83 (XRCC3$^{-/-}$) human cell line

Colony formation, linear scale

B: VU423B (wild-type BRCA1) / VU423F (BRCA1$^{-/-}$)

Colony formation, linear scale

Cells were incubated with Topotecan for seven days, thereafter medium was replaced with drug-free medium and colonies were fixed after 12-16 days.

For the XRCC3 hamster cell lines, a strong difference in Topotecan sensitivity can be seen. The XRCC3 human cell lines are hypersensitive and no difference is obvious. In comparison: The human BRCA1 cell lines VU423B and VU423F are also hypersensitive and only a small difference can be seen.

The protein XRCC3 is essential for homologous recombination, too. Two hamster cell lines were used, AA8 with a normal expression of wild-type XRCC3 and irs1SF, an XRCC3$^{-/-}$ hamster cell line. The XRCC3$^{-/-}$ hamster cell line is much more sensitive than the XRCC3 wild-type hamster cell line which shows that there is a difference in Topotecan sensitivity between cells with or without normal function of homologous repair. For the two human cell lines, HCF-116 with normal expression of wild-type

[87] Original data from El-Awady, 2004

XRCC3 and X3-H83, an XRCC3$^{-/-}$ human cell line, no difference in sensitivity against Topotecan could be seen. At concentrations of more than 0.01 µM Topotecan, no colonies were found: the cells can be called hypersensitive compared with the hamster cell lines. This hypersensitivity is comparable with VU423B and VU423F for which only a small difference could be seen at low concentrations of Topotecan and at higher concentrations, colony formation was impossible.

Another thing to be explained is the high base level of DNA-double-strand breaks in both cell lines. For VU423F, a reason for DNA-damage could be the absence of any BRCA2 wild-type protein which might lead to deficiency in repairing any kind of DNA-damage, appearing spontaneously, necessary for replication or induced by any kind of stress. But for VU423B, this is not a meaningful explanation as it expresses wild-type BRCA2 at a normal level.

The high base level of DNA-double-strand breaks, hypersensitivity against Topotecan and even the lack of any repair effect in either VU423B or VU423F could be explained by a defect in any other repair protein which has not yet been observed but prevents both cell lines from DNA-repair. This could even explain the observed abnormalities in cell-cycle distribution if the defective protein was involved in cell-cycle, too.

Overexpression of the tumour suppressor p53 might also lead to chromosomal instability: Studies have shown that mutations in p53 usually lead to p53 overexpression which may result in chromosomal amplification, increased proliferation and chromosomal instability[88]. The cells tolerate DNA-damage because the errorprone DNA-repair increases. Chromosomal amplification as found for VU423 cell lines could be responsible for a high base level of DNA-double-strand breaks. When lots of copies of the genome are present, the cells do not necessarily have to repair DNA-damage because copies of the needed DNA-region are available from undamaged chromosomes. For VU423 cell lines, p53 overexpression could be shown by Western Blot analysis. This could further imply that the cells express mutated p53 which can not fulfil its task: stopping cell cycle progress and initiating DNA-repair. Even a reduction of apoptosis by mutated p53 could result in numerous persistent DNA-double-strand breaks.

But a much more convincing explanation for all, the hypersensitivity against Topotecan, the high base level of DNA-double-strand-breaks and the absence of any repair effect would be cell death like apoptosis which could take place independent of any kind of cellular stress or after incubation with Topotecan. It generates DNA-double-strand

[88] Garrity et al., 2004

breaks which lead to γ-H2AX-foci. Analysis of the apoptotic base level by Annexin showed results which were comparable with normal human fibroblasts and therefore not high enough to serve as an explanation for a high base level of DNA-double-strand breaks.

If, after incubation with Topotecan, much more γ-H2AX-foci were induced by apoptosis than by Topotecan, this could cover the repair effect. Or Topotecan induced apoptosis could be an explanation for the absence of any repair effect as both, DNA-degeneration and DNA-repair would appear contemporaneously and therefore compensate their influences on the γ-H2AX-level.

For evaluating these ideas, the caspase inhibitor Z-VAD-FMK was used to suppress apoptotic effects of Topotecan. By Annexin staining, it could be seen that Topotecan itself only induced a small level of apoptosis. And although the caspase inhibitor reduced the apoptotic fractions, either the base level or the Topotecan induced fraction, it was not expected that the investigation of apoptosis by Annexin would lead to further results. The influence of Annexin-mediated apoptosis on the absence of repair effects can be neglected. But many results argue for apoptosis: the high base level of DNA-double-strand breaks, hypersensitivity against Topotecan and a high percentage of dying cells when analyzing the cells at the flow cytometer.

Maybe the cells suffer from a kind of apoptosis which is different from Annexin-mediated apoptosis. It has already been shown that Topotecan induces cell death but different from typical apoptosis[89]. This could be the reason why Annexin was not able to detect cells during their process of cell death. And caspase inhibition might not have a strong effect on the level of γ-H2AX-foci. Maybe the cells also die in a manner different from apoptosis independent of Topotecan which could explain the high base level of DNA-double-strand breaks even in untreated cells.

Taken together, the high base levels of DNA-double-strand breaks in VU423B and VU423F could be explained by cell death which is different from Annexin-mediated apoptosis. A high base level of DNA-double-strand breaks coupled with an induced DNA degradation by Topotecan could also be responsible for the absence of any repair effect which together constitutes the hypersensitivity against Topotecan.

To definitely exclude that BRCA2 might play a role in repair of Topotecan-induced DNA-damage, further experiments should be performed with cells less sensitive than VU423B and VU423F. BRCA2-proficient and BRCA2-deficient cells might then show

[89] Alexandre et al., 2000; Morris et al., 1996

pronounced differences in Topotecan sensitivity. Although the experiments in this doctoral thesis could not show a difference in repair, this does not necessarily imply that all BRCA2-deficient cells will show the same and therefore are not promising targets for Topotecan chemotherapies. Maybe in other tumour cell lines, the repair effect would be more obvious and it seems worth to continue research in this field with other BRCA2-deficient cell lines.

The last thing to be observed is the protein expression of the investigated cell lines. For detection of the truncated BRCA1 or BRCA2 proteins by western blot, N-terminal and C-terminal antibodies should be used to make differences between the cell lines stably transfected with wild-type BRCA1 or BRCA2 and the cell lines with two alleles resulting in truncated proteins of either BRCA1 or BRCA2.
In this doctoral thesis, it was only possible to show protein expression by N-terminal antibodies. Neither a specific BRCA1 nor BRCA2 C-terminal antibody could be found although numerous different antibodies were tested.
For VU423 cell lines, Howlett et al., 2002 were able to detect a truncated BRCA2 protein in the VU423F cell line while VU423B cells showed a normal expression level for wild-type BRCA2. Nevertheless it is mystifying how Howlett et al. were able to detect this truncated BRCA2 protein by an antibody raised against amino acids 3245 to 3418 since the BRCA2-mutation described for VU423F cells is located proximal to this position and the frameshift resulting from this mutation completely changes the C-terminal aminoacid sequence.
HCC1937 cell lines have been described before by Scully et al. 1999 and therefore, it will be referred to the paper in which they showed that HCC1937/BRCA1wt has a normal protein expression level for BRCA1 while HCC1937/pcDNA3 expresses a truncated BRCA1 protein.

Taken together, both BRCA1- or BRCA2-defective cell lines showed differences in Topotecan sensitivity compared to their complemented counterparts.

With the results in this doctoral thesis, it could be shown for BRCA1 that cells with a defect in this protein are highly sensitive against Topotecan because they have deficiency in repairing the arisen DNA-double-strand breaks after Topotecan treatment. Cell-cycle is also influenced by BRCA1 as for the cells with defective proteins, a stronger S-phase delay could be seen than for their control cells. For BRCA2, it was not possible to show comparable results which does not necessarily mean that the cells with a defect in BRCA2 will not be more sensitive against Topotecan compared with control cells expressing wild-type BRCA2. Maybe only the cells used for these experiments were inappropriate. The clinical use of Topotecan as a chemotherapeutic agent against tumours with defects in BRCA1 (and maybe even in BRCA2) could be a promising attempt of specific treatment of tumours concerning their genetic characters. For the nearer future, animal experiments might lead to further information and maybe in several years, breast cancer might be treated differently concerning its genetic origin.

6. Summary

The aim of this doctoral thesis was to investigate the sensitivity of cells which have a defect in the breast cancer related tumour suppressor genes BRCA1 or BRCA2 against the chemotherapeutic drug Topotecan. Both genes have been previously shown to be involved in homologous recombination. It was thus hypothesized that BRCA1- and BRCA2-deficient cells have a repair defect for DNA-double-strand breaks which should enhance their sensitivity towards Topotecan, an inhibitor of the enzyme topoisomerase I. Two human breast cancer cell lines were used carrying either a defect in the BRCA1 or BRCA2 gene. Both defects had been previously complemented by adding back the respective human wild-type c-DNA. Hence, two pairs of cell strains were available for this study.

It was found out that the treatment with Topotecan induced DNA-double-strand breaks as measured by the formation of fluorescent γ-H2AX-foci. BRCA1 deficient HCC1937/pcDNA3 cells accumulated more DNA-double-strand breaks upon Topotecan treatment. They showed almost no repair of those breaks after drug removal. In contrast, the BRCA1 proficient cells, HCC1937/BRCA1wt, almost completely repaired the double-strand breaks within 6 hours. In parallel, these cells showed an accumulation in the S-phase of the cell-cycle, which was less pronounced in the deficient cell line. These results suggest that activation of the S-phase checkpoint by Topotecan is coupled to a repair proficiency which was similarly shown previously by investigating the XRCC3 gene. According to those results, the BRCA1 deficient HCC1937/pcDNA3 cells were more sensitive against Topotecan.

In contrast, neither BRCA2-deficient VU423B cells nor their complemented counterparts VU423F showed appreciable DNA-double-strand break repair after incubation with Topotecan which could be best explained by early activation of apoptosis. However, this programmed cell death was not reflected by relocalisation of AnnexinV, a frequently observed marker of apoptosis. Similar to the BRCA1 cells, Topotecan induced an S-phase delay which was nearly identical in the BRCA2 proficient VU423B cells compared to the deficient VU423F cells.

Taken together, these results suggest that at least BRCA1 deficient cells may be in particular sensitive to the action of Topotecan which opens the clinical perspective to effectively and specifically targeted breast cancer with BRCA1-deficiencies by treatment with topoisomerase I inhibitors. In contrast, such a beneficial effect could not

be shown for BRCA2 defects. Further studies with cells less susceptible towards apoptosis are required.

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9. Curriculum vitae

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Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfaßt, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich übernommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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Hamburg, den 07.05.2007