2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Common solutions and chemicals.

Reagent	Manufacturer
Aqua redistilata	DeltaSelect, Dreieich
Bromphenolblue	Sigma, Deisenhofen
DMSO	Sigma, Deisenhofen
Ethanol	Sigma, Deisenhofen
Fiocoll-Hypaque separating solution	Seromed, Berlin
PBS Dulbecco w/o Ca2+ and Mg2+	Biochrom AG, Berlin
Serum physiologicum	Baxter, Heidelberg
Sodium butyrate	DeltaSelect, Dreieich

2.1.2. RNA isolation solutions and buffers

Name	Ingredient	Manufacturer
GTC buffer	4mM Guanedinethiocyanate	Sigma, Deisenhofen
	25mM Natrium citrate	Sigma, Deisenhofen
	0.5% Laoryl sacrosine	Sigma, Deisenhofen
	DEPC water	Sigma, Deisenhofen
RW1 buffer		Quiagen, Hilden
RPE buffer		Quiagen, Hilden

2.1.3. Complementary DNA synthesis solutions and buffers

Name	Manufacturer
Dnase	Quiagen, Hilden
dNTP mix	Quiagen, Hilden
Omniscript buffer	Quiagen, Hilden
Primer oligo p (DT) 15	Roche, Mannheim
Primer random p (dN) 6	Roche, Mannheim
Protector Rnase Inhibitor	Quiagen, Hilden
RDD Buffer	Quiagen, Hilden
RNase free water	Quiagen, Hilden
RT buffer	Quiagen, Hilden

2.1.4. Polymerase chain reaction solutions and buffers

Name	Manufacturer
PCR-graded water	Roche, Mannheim
Magnesium dichloride 25mM	Roche, Mannheim
LC-FastStart Enzyme 1a	Roche, Mannheim
LC-FastStart Enzyme 1b	Roche, Mannheim
pCR2.1-topoisomerase	Invitrogen, Karlsruhe
Polyadenylic acid	Sigma, Deisenhofen

2.1.5. Solution, antibodies and reagents used in multidrug resistant protein staining and caspase-propidium iodide assay

Name	Manufacturer
CaspACE FITC-VAD-FMK	Promega, USA
Cisplatin-ribosepharm	Ribosepharm, Muenchen
FACS lysing solution	Becton Dickenson, Heidelberg
FACS permeabilising solution 2(10x)	Becton Dickenson, Heidelberg
FACS Flow	Becton Dickenson, Heidelberg
Flebogamma/endoglobulin	Grifols, Langen
Goat-Anti-Mouse-PE MAB	Becton Dickenson, Heidelberg
Goat-Anti-Mouse-FITC MAB	Dianova, Hamburg
MouseIg2a-PE	Beckman Coulter, France
Mouse-Anti-MRP1 MAB, clone QCR1	Chemicon, USA
Mouse-Anti-LRP MAB, clone LRP56	Chemicon, USA
Mouse-Anti-P-glycoprotein MAB,clone UIC2	Immunotech, France
Propidium iodide (PI)	Sigma, Deisenhofen
Phycoerythin (PE)	Beckton Dickenson, Heidelberg
Ribodoxo (doxorubicin)	Ribosepharm, Muenchen
Ribomustine (bendamustine)	Ribosepharm, Muenchen
Verapamil	Knoll, Wiesbaden

2.1.6. Other chemicals

Name	Manufacturer
SeaKem LE Agarose	Cambrex, USA
Ethidium bromide	Sigma, Deisenhofen
Mops-EDTA-Acetat (MESA)	Sigma, Deisenhofen

2.2. Cell culture and cell mediums

2.2.1. Cell mediums

Name	Manufacturer
RPMI 1640	Biochrom AG, Berlin
w Phenolrot- 5mg/l	Biochrom AG, Berlin
w NaHCO3- 2g/l	Biochrom AG, Berlin
w FCS	GibcoBRL, Karlsruhe
w Penicillin 10 000 U/ml	GibcoBRL, Karlsruhe
w Streptomycin 10mg/ml	GibcoBRL, Karlsruhe
w Glutamax 4 mM/l	GibcoBRL, Karlsruhe

2.2.2. Tumor cell lines

Cell line	Origin	Provider
Caco2	colon cancer	DMSZ, Heidelberg
Colo205	colon cancer	ATCC, USA
Colo206F	colon cancer	ATCC, USA
Colo320	colon cancer	DMSZ, Heidelberg
Cx94	colon cancer	ATCC, USA
DLD1	colon cancer	DMSZ, Heidelberg
HBL100	breast cancer	ATCC, USA
HCT116	colon cancer	ATCC, USA
MDAMB435s	breast cancer	DSMZ, Heidelberg
MDAMB436	breast cancer	DSMZ, Heidelberg
MCF7	breast cancer	DSMZ, Heidelberg
Mx1	breast cancer	DSMZ, Heidelberg
T47D	breast cancer	ATCC, USA
SW403	colon cancer	DSMZ, Heidelberg
SW480	colon cancer	ATCC, USA
SW620	colon cancer	ATCC, USA

2.2.3. Laboratory inventory

Product	Name	Manufacturer
Accelarator	Clinac2100	Varian, Darmstadt
Agarose gel chamber	Horizon58	GibcoBRL, Karlsruhe
Camera	Polaroid	Polaroid, Offenbach
Centrifuge	Eppendorf 5402	Eppendorf, Hamburg
Electrical pipette	Pipetus-accu	Hirschmann, Hamburg
Flow cytometer	FACScan	Beckton Dickenson, Heidelberg
Incubator	Steri-cult2000	Labortect,Goettingen
Microscope	Zeiss	Zeiss, Goettingen
Microwave oven	Moulinex	Moulinex, Solingen
Pipettes	Eppendorf	Eppendorf, Hamburg
Quevette	Ultrospect	Pharmacia Biotech, GB
Safety bench	Clean air	Woerden, Holland
Scale	Universal	Sartorium, Goettingen
Spectrophotometer	GeneQuantII	Pharmacia Bio, Freiburg
Thermocycler	LightCycler	Roche, Mannheim
Thermoblock	Eppendorf 5436	Eppendorf, Hamburg
Vortexer	VF2	IKA, Staufen

2.2.4. Laboratory materials

Material	Manufacturer
Culture flasks	NUNC, Wiesbaden
Cell scrapers	Sarstedt, Nuembrecht
6-well-multidishes	NUNC, Wiesbaden
24-well-multidishes	NUNC, Wiesbaden
FACS tubes	BDFalcon, Heidelberg
Falcon tubes (15 ml and 50 ml)	BDFalcon, Heidelberg
Neubauer chamber	Sigma, Deisenhofen
RNA isolation column RNeasy Mini Spin	Qiuagen, Hilden
Serological pipettes	BDFalcon, Heidelberg

2.3. Methods

2.3.1. Cultivation of tumor cells

Only sterile mediums, medium additives, cell scrapers and serological pipettes were used in the cultivation of tumor cell lines. Medium change and all other procedures with cell culture flasks, except irradiation, were carried out under a clean air bench. The frozen cells were thawed in water bath at 37 C° and dissolved in warm medium. Then the cells were washed twice in a medium to remove DMSO (final concentration < 0.01%) and sowed in flasks at the recommended by the provider density. Every three days the cell count was checked and cells were repetitively repopulated at the recommended density.
All cell lines, irradiated as well as non-irradiated with gamma-radiation, were cultured in doubles in flasks at 37 C°, 5 % CO2 and 95 % humidified sterile air. All cell lines were cultured in RPMI 1640 medium supplemented with 10 % FCS, 1 % Glutamax, 1 % penicillin/streptomycin. Only the Caco2 cell line was cultured in RPMI1640 supplemented with 20 % FCS, 1 % Glutamax and 1 % penicillin/streptomycin.

2.3.2. Cell irradiation

The cell lines were grown at least for two weeks in flasks before the irradiation began. The flasks of the control cell cultures were brought each time along with the treated ones to the radiotherapy clinic. Cells growing exponentially in cell flasks were repetitively irradiated with a total dose of 27.0 Gy, five fractions of 1.8 Gy per week, Monday to Friday.

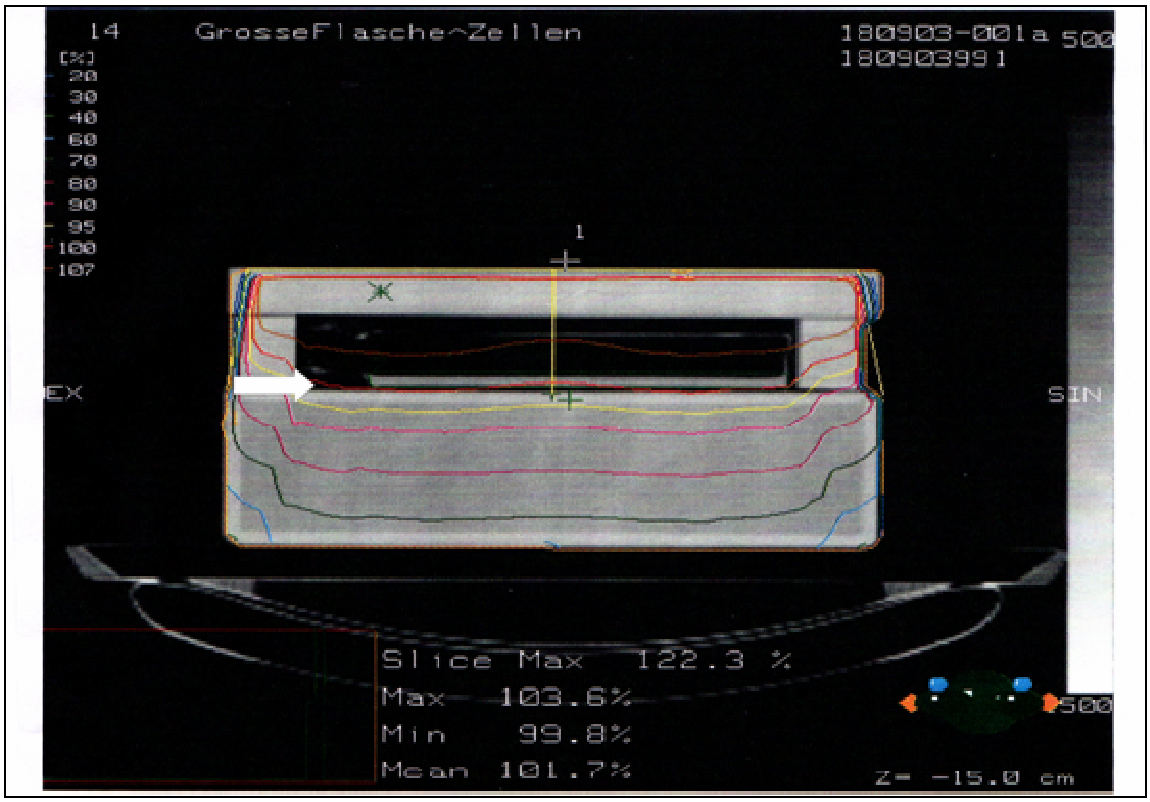


Fig. 2.3.2.1. Computer planning scheme of a cell culture flask placed on a water barrel before starting the irradiation procedure. The red line (indicated with an arrow) represents the isodose at the bottom of the flask. The minimum dose at any point of the flask bottom is above 99.8%. The maximal dose does not exceed 103.6%.

Irradiation was performed with a Varian Clinac 2100C linear accelerator (Varian, Darmstadt, Germany) using 6 MeV photons at a dose rate of 3 Gy/min. The vials were placed under a water bolus to achieve the 100 % isodose at the bottom of the flasks because of the adherent cell lines. A field size of 30 x 30 cm² was used.

2.3.3. Cell harvest and viable cell separation

Cells were harvested on day 3, after the last irradiation procedure. However, in five colon cancer cell lines cells the harvest was done on day 3 as well as on day 18 after the last irradiation procedure. Viable cells were obtained using a density Ficoll-Hypaque gradient centrifugation. The cells were washed from the medium, resuspended in 20 ml of PBS

Dulbecco and carefully pipetted in 50 ml tubes on 15 ml Ficoll-Hypaque separating solution possessing 1.0077 density. The tubes were centrifuged at 2400 rpm for 20 min without a brake. The pellet of viable cells on the Ficoll-Hypaque solution was separated in another 50 ml tube, washed twice in PBS at 3000 rpm for 5 min and resuspended in the medium.

2.3.4. RNA isolation according to the Qiagen protocol

Viable cell count was obtained after staining 1:1 with trypan blue, and 1,000,000 cells were washed twice in PBS and lysed in 700 µl GTC for total RNA extraction. Total RNA of cell pellet was isolated by RNeasy Mini Kit (Roche Diagnostics, Mannheim, Germany), including RNase-FreeDNase Set (Qiagen, Hilden, Germany). The cell samples were then homogenized in 1 ml 70 % ethanol and the mixture was centrifuged at 14000 rpm for 15 sec in the RNeasy spin column. After being washed with 350 µl of RW1 buffer the samples were incubated with 80 µl dissolved in RDD buffer desoxyribonuclease (DNase) for 15 min at room temperature and washed again with 350 µl RW1 buffer. Then samples were washed twice with RPE buffer for 15 sec and 2 min, respectively. The total RNA was eluted in a new tube adding 53µl RNase-free water to the column and centrifuged for a minute. The eluted total RNA was stored at -80 C°.

2.3.5. Quantitative and qualitative determination of total RNA

Following the RNA elution, RNA was diluted 1:1 with bromphenolblue for gel electrophoresis and 1:10 with RNase-free water for spectrophotometry.

2.3.5.1. Spectrophotometry

Spectrophotometry is based on the fact that RNA absorbs light in proportion to its quantity. Absorption is usually is measured at 260 nm and 280 nm. Absorption of 1 at 260 nm means 40 µg/l of RNA. The ratio of absorption at 260 nm and 280 nm (A260/A280) is the level of RNA purity or quality, and should be between 1.8 and 2.1, a lower level indicates protein contamination.

2.3.5.2. Gel electrophoresis

The gel electrophoresis uses the phenomenon that in direct current (DC) the RNA fragments move with different speed to the anode, forming two clear bands - 18S (1.9 kb) and 28S (5 kb), depending on their molecular weight. RNA is made visible by means of ethidium bromide, which intercalates with RNA and at UV light with 302 nm length eludes fluorescence. There had been used 0.3 % agar in MESA buffer, which was boiled in microwave at 700 W for 1 min. Then ethidium bromide was added in concentration 1 µl per 20 ml gel and the mixture was poured in a gel chamber, cooled down at room temperature and the gel was covered with MESA buffer. The wells, formed by a gel comb, were filled with the probes and exposed to 50 V direct current for 30 min. The RNA gel was analyzed and documented using AlphaEaseFC software Version 4.0 (Alpha Innotech, CA, USA). Only probes with clear 18 S and 28 S bands were taken into consideration in this study.

2.3.6. Complementary DNA synthesis

The transcription is the process of overwriting genomic DNA in mRNA. There is a group of viruses called retroviruses (i.e. HIV), which contain an enzyme called reverse transcriptase and can transcribe mRNA in complementary DNA (cDNA). This natural phenomenon is used in biology to produce cDNA from extracted RNA. In this study, cDNA was synthesized by the means of the Omniscript Reverse Transcriptase Kit (Qiagen, Hilden, Germany) according to the manufacture's instructions (Korfhage et al.,

2000). Frozen total RNA was thawed on ice, then 2 µg of RNA was diluted in 15 µl RNase-free water, incubated for 5 min at 65 C° for destruction of RNA`s second structure, thereafter chilled on ice for 5 min. A 7.5 µl mixture containing 1 µl of oligonucleotides p(dT)15 primer (0.8 µg/ml) and 1 µl random p(pT)6 primer (0.2 µg/ml), 2 µl of desoxynucleotide triphosphate (5 mM), 0.5 µl of RNasin (40 units/ml), 1 µl of Omniscript reverse transcriptase (4.5 units/µl) and 2 µl reverse transcriptase buffer (x10) was prepared and added to the diluted RNA. After incubation at 37 C° for 1 hour, Omniscript reverse transcriptase was inactivated at 95 C° for 5 min and cDNA was frozen at -20 C°. All reverse transcriptase agents except oligo and random primers (Roche Diagnostics, Mannheim, Germany) were purchased from Qiagen.

2.3.7. Real-time quantitative polymerase chain reaction

The polymerase chain reaction (PCR) is a process of exponential amplification of DNA material using artificial oligonucleotides (Meuer et al., 2001). Once all reactions components are combined, amplification can proceed automatically by temperature cycling of the sample. PCR consists of three general steps, denaturation of DNA template, annealing of primers and extension. Conventionally, analysis of PCR products is a separate step performed after PCR is completed. Analyzing of PCR probes during amplification has become popular as real-time PCR. In the LightCycler System (Roche Diagnostics, Mannheim), PCR temperature cycling is achieved using air and temperature transitions can be reached within a few seconds with up to 20 °C/sec rate. PCR occurs in specially designed borosilicate glass capillaries, which have a high surface-to-volume ratio to ensure rapid equilibration between air and the reaction components (Fig. 2.3.7.1). This allows a single PCR cycle to be completed in less than 30 seconds. The high accuracy and sensitivity is due to fluorescence measurements at pre-programmed intervals during PCR, in which the intensity of the signal produced by a fluorophore is proportional to the amount of PCR product (Wittwer et al., 1997-1+2). The analytical possibilities offered by the LightCycler System are extensive. Certainly one of the most important features is the cycle-by-cycle monitoring of PCR, which is a prerequisite for

accurate quantification of target sequences. The amount of PCR product increases logarithmically in the first few PCR cycles before reaching plateau. To ensure accurate quantification of sample concentration, the first cycles in which the PCR is in the exponential log-linear phase are considered. In this phase the signal is easily distinguished from the background signal, providing very accurate information about the starting concentration of the target sequence.

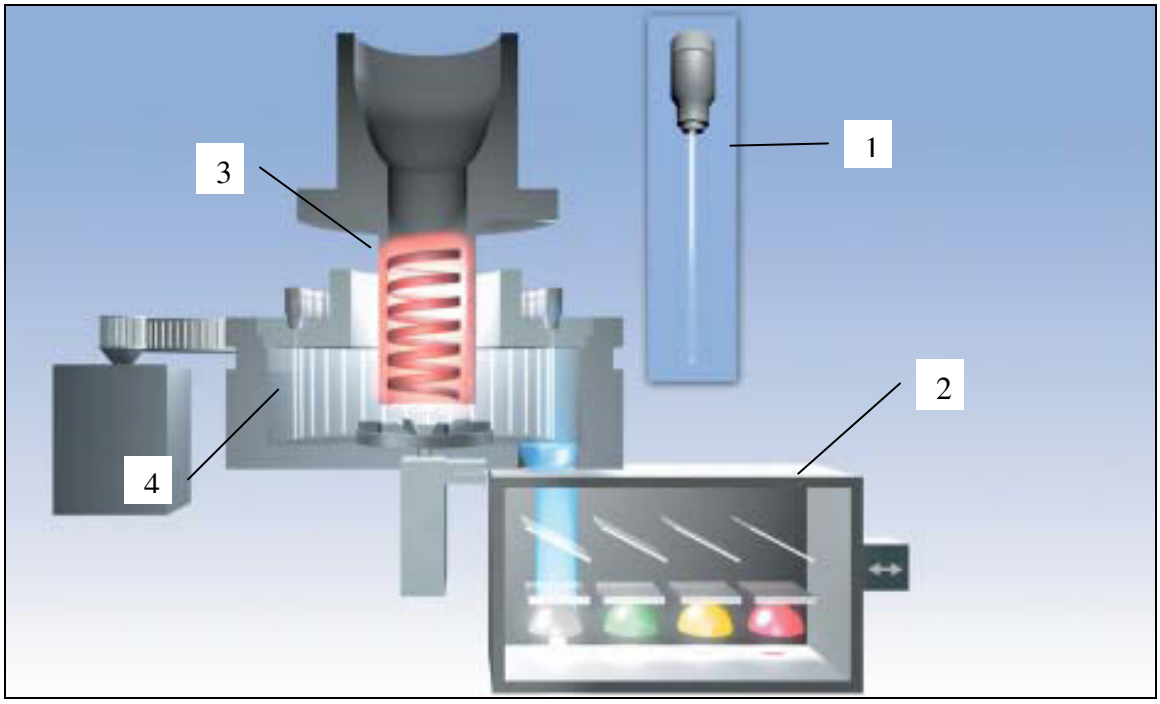


Fig. 2.3.7.1. Schematic view of a LightCycler System and its components. Capillaries (1) are placed in a thermal chamber (4), in which the temperature is regulated by a thermal coil (3) and a ventilator. The fluorescence is measured by a light detector (2).

This study uses LightCycler FastStart DNA MasterHybProbes Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacture's instructions. Each cDNA probe was diluted to a volume of 20 µl of PCR mix, containing 0.5 pmol of primer and 0.2 pmol of probe DNA and a final magnesium dichloride concentration as listed bellow (Tabl.2.3.7.1). For amplification, an initial denaturation step at 95 C° for 10 min was used. For the subsequent 54 cycles the conditions were: denaturation for 10 sec at 95 C°, annealing for 12 sec at 65 C° and elongation for 10 sec at 72 C°. This was followed by a final extension of 2 min at 72 C°. Sequence of primers and fluoroflores as well as

annealing temperatures and magnesium dichloride ($MgCl_2$) concentrations are listed below.

Primer and probe sequences	Amplicone (bp)	Spanned cDNA sequence	Annealing temperature (°C)	MgCl2 concentration (mM)
PBGD[1] 5´-TGC AGG CTA CCA TCC ATG TCC CTG C	187	914-1100	65°C	4mM
PBGD[2] 5´-AGC TGC CGT GCA ACA TCC AGG ATG T				
PBGD FL 5´-CGT GGA ATG TTA CGA CGA GCA GTG ATG CCT ACC-Fluo				
PBGD LC 5´-Y[3] TGT GGG TCA TCC TCA GGG CCA TCT TC-Pho				
LRP[1] 5´-CCC CCA TAC CAC TAT ATC CAT GTG	385	136-521	65°C	4mM
LRP[2] 5´-TCG AAA AGC CAC TCA TCT CCT G				
LRP FL 5´-GCA AGT TCG GCT TCG CCA CGC TGA-Fluo				
LRP LC 5´ Y[3] CGA GAT CCG GCT GGC CCA GGA CC-Pho				
MRP1[1] 5´-TTC CGG AAC TAC TGC CTG CGC TAC	248	4079-4102	65°C	4mM
MRP1[2] 5´-AGG GTC CTG GGG GAT GAT GGT GAT				
MRP1 FL 5´-CTC GTT GAT CCG AAA TAA GCC CAG GGT CA-Fluo				
MRP1 LC 5´Y[3] ACG ACT TCC CAG CTC CCG TCC GCC-Pho				
MDR1[1] 5´-CCG GTT TGG AGC CTA CTT G	272	2834-3106	65°C	3mM
MDR1[2] 5´-GGT CGG GTG GGA TAG TTG				
MDR1 FL 5´-CTG ATA TTT TGG CTT TGG CAT AGT CAG GAG GAG C-Fluo				
MDR1 LC 5´ Y[3] ATG AAC TGA TGA				

[1] Sense

[2] Antisense

[3] Y LC Red640

Table 2.3.7.1. Sequence of primers and probes as well as conditions used in the real-time reverse transcriptase PCR. Pho - phosphorylated; Fluo – fluorescin.

All samples were analyzed for PBGD, MRP1, MDR1 and LRP mRNA at least in duplicate. The average of both duplicates was used as a quantitative value. If just one had a positive value, the positive was taken into consideration. For the generation of plasmid controls, PCR products of mdr1, mrp1, lrp and PBGD were cloned into a vector pCR2.1-topoisomerase (Invitrogen, Karlsruhe, Germany). Recombinant vectors, linearized with EcoRV, were serially diluted in water containing 0.4 mg/ml polyadenylic acid. A standard curve with three dilutions of the respective plasmid in duplicates (1, 0.1 and 0.001 pg/ml) was included in each PCR run. To reduce the risk of contamination, RNA isolation, cDNA synthesis and thermocycling were performed in separate laboratories. PCR mixtures were set in a template tamer (Oncor Appligene, Heidelberg, Germany). All reagents for cDNA synthesis were prepared in RNase-free water. Negative controls were performed for all RT-PCR steps, including a reverse transcriptase-negative sample control for every sample and a water control for each PCR run. Data was analyzed using the LightCycler software Version 3.0 (Roche Diagnostics). Crossing points were assessed using the Second Derivate Maximum algorithm and plotted versus the concentrations of the standards. PBGD (porphobilirubin deaminase) was used as a housekeeping gene. The results from the markers were plotted against the PBGD values in order to be avoided the variations of all pre- PCR steps.

2.3.8. Fluorescent flow cytometry (FACS)

Flow cytometry is a method by which more than 5000 particles per second can simultaneously be evaluated for up to five parameters in a liquid flow (Robinson et al., 1999). This is realized as follows: diluted cells are adsorbed from a FACS tube and by the means of hydrodynamic forces focused on an argon-ionic laser. The scattered light is measured by five detectors. The forward scatter (FSC) detects waves scattered along the primer beam and in that way gives information about the cell size. The side scatter (SSC) detects waves scattered perpendicularly to the primer source of light and depicts cell granularity. In this study three fluorescent dyes were used - FITC, detected at fluorescent channel 1 with emission maximum at 520 nm; PE, detected at fluorescent channel 2 with

emission maximum at 580 nm and propidium iodide, detected at fluorescent channel 3 with emission maximum at 650 nm. Each dye emits light with a definite spectrum after being excited by an argon laser. However, the spectrums overlap each other and thus have to be compensated. At each measurement we counted at least 5000 events.

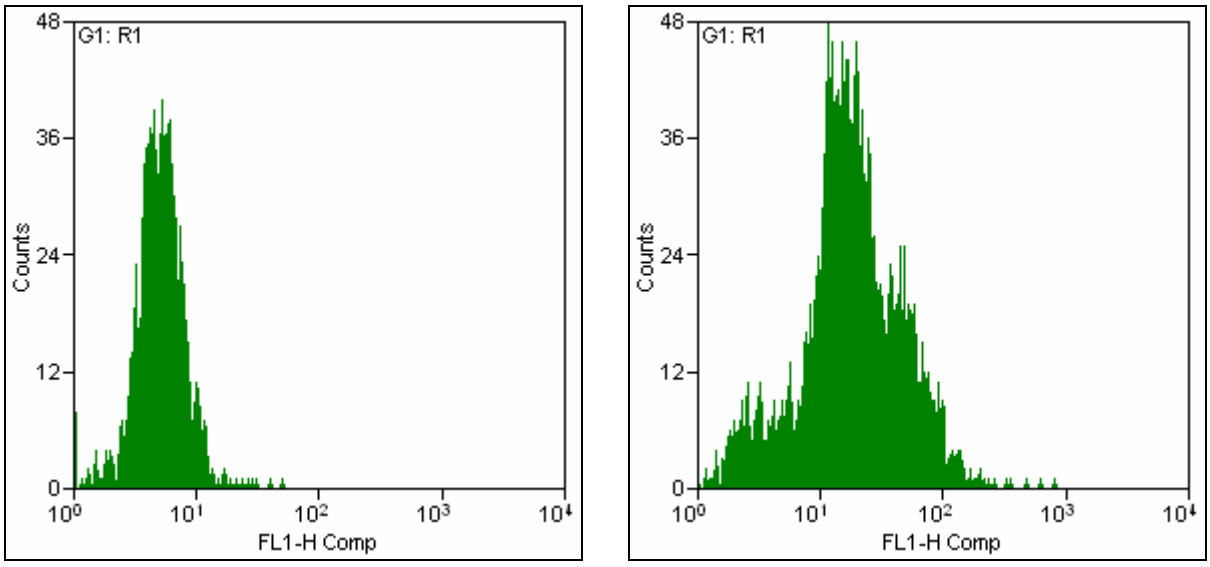
2.3.9. FACScan analyses and quantification of P-glycoprotein, multidrug-associated protein 1 and lung resistance protein expression

P-glycoprotein and MRP1 are ABC transport proteins and as such are placed on the cell membrane, on the other hand LRP is an intracellular protein. The most accurate detection technique includes blotting with fluorescin-labeled antibodies and fluorescence quantification (Beck et al., 1996). The evaluation of P-glycoprotein, MRP1 and LRP expression was performed as previously described (Hipfner et al., 1999; Mossink et al., 2003; Pavelic et al., 1993; Schroijers et al., 1998) and modified as follows.

2.3.9.1. Protocol for MRP1 and P-glycoprotein staining (Hipfner et al., 1999)

After harvest, cells both of irradiated and the control groups, were centrifuged at 1700 rpm for 5 min in PBS. The cell pellet was resuspended in 2 ml PBS Dulbecco (PBS/Endo) supplemented with 2 % solution of endoglobulin. After 10 min of incubation on ice the aliquots were washed, resuspended in 100 µl PBS/Endo and 3 µl Mouse-Anti-MRP1 MAB (monoclonal antibody), clone QCR1, was added according to the manufacturer's instruction. After 30 min of incubation on ice in the dark, cells were washed and resuspended in 100 µl PBS/Endo. Then 5 µl Goat-Anti-Mouse-FITC was added, diluted ex tempore following manufacturers' instructions and the cells were incubated for 15 min in the dark. After a washing and resuspension in 100 µl PBS/Endo 7 µl Mouse-Anti-P-glycoprotein MAB, clone UIC2 was added and incubation in the dark for 30 min followed. The cell samples were finally washed and the mean fluorescence was measured on a FACScan flow cytometer (Fig. 2.3.9.1.1). The background

fluorescence was subtracted using the relevant IgG isotype control in order to compensate antibodies unspecific binding. A sample, not treated with Mouse-Anti-MRP1 MAB, was used as an isotype control for MRP1 staining. For isotype control of the P-glycoprotein staining, IgG2a isotype antibody was added instead of Anti-P-glycoprotein MAB.



A **B**

Fig. 2.3.9.1.1. Flow cytometric assessment of MDR proteins expression before (A) and after irradiation (B). Arbitrary units were achieved by excluding the non-specific fluorescence of the respective isotype control (not shown) for non-irradiated and irradiated samples (see text).

2.3.9.2. Protocol for LRP staining (Mossink et al., 2003)

After harvest, cell samples of both irradiated and non-irradiated groups were centrifuged at 1700 rpm for 5 min, medium was decantated and cell pellet resuspended in 2 ml PBS/Endo. After 10 min of incubation on ice, the aliquots were washed, resuspended in FACS Lysing solution (x 1), centrifuged and pellet resuspended again in 250 µl FACS Permeabilising solution (x 1) and then incubated for 10 min at room temperature. After a washing and resuspention in 100 µl of PBS/Endo, 7 µl Mouse-Anti-LRP MAB was

added following manufacturers` instructions. The cells were incubated in the dark, on ice for 30 min before being washed and resuspended in 100 µl PBS/Endo and incubated with 5 µl Goat-Anti-Mouse-PE for 15 min. Finally, the cell samples were washed and direct at FACScan flow cytometer assessed as mentioned above (2.3.9.1). A sample which was not treated with Mouse-Anti-LRP MAB was used as an isotype control for LRP staining.

2.3.10. Incubation with cytotoxic drugs

To analyze the ability of P-glycoprotein, MRP1 and LRP to induce a multidrug resistance phenotype and so to evade the cytotoxic drugs provoked cell apoptosis and cell death, suspension aliquots of each cell line, both irradiated and non-irradiated, were incubated with cytotoxic drugs. In order to select the most suitable cell line as well as endpoint for the cytotoxic drug incubation assay, we incubated six breast cancer cell lines, namely - HBL100, MCF7, MDAMB435s, MDAMB436, Mx1, T47D and ten colon cancer cell lines, namely - Caco2, Colo205, Colo206F, Colo320, Cx94, DLD-1, HCT116, SW403, SW480 and SW620 in bendamustine, cisplatin and doxorubicin for 12, 24 and 48 hours. In the following studies only cell lines, sensitive to physiologically relevant concentrations of bendamustine, cisplatin and doxorubicin after 48 h of incubation were used, in particular: two breast - MCF7 and Mx1 and six colon cancer cell lines - Caco2, Colo320, Cx94, HCT116, SW403 and SW620. The assays were done 3 days after the last irradiation procedure. A consecutive investigation was done 18 days after the last irradiation procedure of the following five colon cancer cell lines - Caco2, Colo320, HCT116, SW403, SW620, allowing the irradiated cells to regain a logarithmic growth. The drug cytotoxity assays were carried out as follows. About 2.5 x 10000/ml cells were exposed in vitro for 48 hours to either medium or increasing concentrations of bendamustine - 1 µg/ml, 10 µg/ml and 100 µg/ml, cisplatin - 0.1 µg/ml, 1 µg/ml, 10 µg/ml and doxorubicin - 0.1 µg/ml, 1 µg/ml and 10 µg/ml.

Bendamustine was dissolved in aqua bidestillata in a concentration of 5 mg/ml and frozen at - 20 C° to prevent rapid hydrolysis. Doxorubicin and cisplatin were obtained as ready-to-use solutions in 2 mg/ml and 0.5 mg/ml concentrations, respectively and were stored at

4 C°. Immediately before incubation, all three cytotoxic drugs were diluted with serum physiologicum to the appropriate concentrations. The cell samples were incubated with 2 ml of appropriate medium in 24-well plates a t 37 C°, 95 % humidified air with 5 % CO_2 in a sterile incubator.

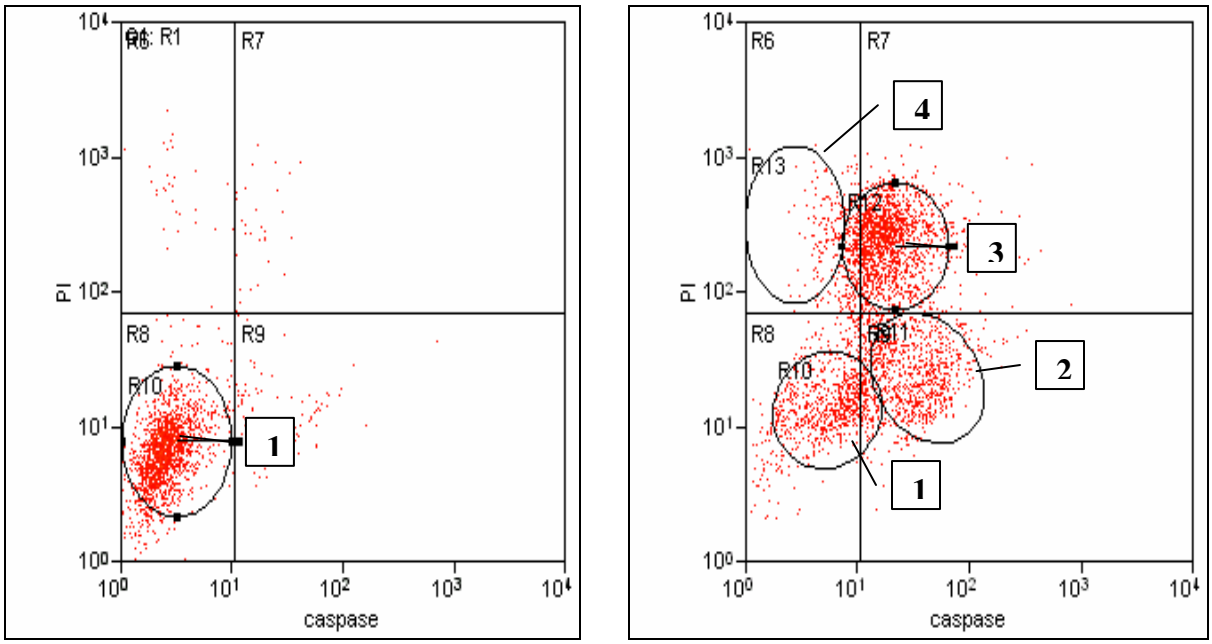
2.3.11. Evaluation of the calcium channel blocker verapamil as a P-glycoprotein and MRP1 activity modulator

The multidrug resistance-reversing agent verapamil is known to change the uptake characteristics of resistant cells and to reverse their resistance to P-glycoprotein and MRP1 substrates. In order to elucidate to what extent P-glycoprotein and MRP1 overexpression after radiation therapy leads to the formation of the multidrug resistant phenotype, we incubated irradiated cells with bendamustine, doxorubicin and cisplatin (2.3.10) and verapamil in rising concentrations. It is known that approximately 13.5 mM verapamil blocks 50 % of the P-glycoprotein efflux activity, just as 40 mM verapamil blocks 50 % of the MRP1 efflux activity (Takara et al., 2002). A concentration of 25 mM verapamil was used to be overcome the multidrug resistant phenotype caused by P-glycoprotein and 75 mM verapamil to overcome the resistance caused by both P-glycoprotein and MRP1.

2.3.12. Detection of cell apoptosis and cell death

The method is based on the principle that apoptotic cells activate the caspase pathway of programmed cell death and dead cells are stained by propidium iodide (PI). The cells are divided into four groups depending on their staining - viable, which neither activate caspase nor are stained by propidium iodide; early apoptotic, activating only caspase; late apoptotic, which activate caspase and are stained by propidium iodide and dead cells, which are stained only by propidium iodide (Fig.2.3.12.1). As already mentioned above, cell suspensions from both irradiated and non-irradiated control groups of cell lines were

incubated in 24-well plates, with bendamustine, cisplatin and doxorubicin. After 48 hours, cells were harvested by gentle up and down pipetting and washed in PBS Dulbecco. The cell samples were incubated at 37 C° for 20 min protected from light with 150 µl medium and 0.4 µl caspACE FITC-VAD-FMK following the manufacturer's instructions. Then cells were washed twice in PBS and resuspended in 100 µl PBS Dulbecco with 1 µg propidium iodide, vortexed and evaluated with a FACScan flow cytometer. In all cell lines, for both irradiated and non-irradiated (control) cell groups were used the same settings of forward and side scatters as well as channel 1, 2 and 3. Before starting the experiments for each cell line had been determined the most suitable settings at flow cytometer and then throughout the experiments used.



A **B**

Fig. 2.3.12.1. Flow cytometric assessment of drug induced cell apoptosis and cell death. Cell debris was excluded by setting a gate (not shown). The cells are divided in four groups depending their propidium iodide (PI), (1) caspase staining – viable, (2) early apoptotic, (3) late apoptotic and (4) dead. **A.** *Cell sample incubated in absence of cytotoxic drugs.* **B.** *Cell sample following incubation with cytotoxic drugs.*

The number of apoptotic and dead cells was determined on the basis of the fluorescence intensity on a logarithmic scale using the FACScan. The data were analyzed using Cell-Quest Software version 3.0 (BD Biosciences, Heidelberg). At least 5000 events of each sample were analyzed. Cell debris could easily be differentiated and excluded by setting a gate.

2.3.13. Positive control for lrp, mrp1, mdr1 mRNA as well as LRP, MRP1 and P-glycoprotein expression

Recent studies reported that sodium butyrate induced upregulation of lrp, mrp1 and mdr1 mRNA in SW620 colon cancer cell line and this phenomenon was preserved over 14 days. Moreover, overexpression of P-glycoprotein, MRP1 and LRP was detected as long as sodium butyrate was present in the medium and the drug resistance was partially reversed by verapamil (Kitazono et al, 1999). We incubated SW620 cells in medium containing 2 mM sodium butyrate (SW620NaBr) for 7 days as previously described. The level of overexpression of multidrug resistance-related genes and proteins was used for calibrating the methods used for their measurement (2.3.7 and 2.3.8). SW620NaBr cells were incubated with bendamustine, cisplatin and doxorubicin for 48 hours. As sodium butyrate is known to reduce the growth rate and hence to attenuate the cell sensitivity to cytotoxic drugs, we used a SW620 cell line grown in 5 % FCS as a control. Under such conditions SW620 cell line possess the same growth rate as SW620NaBr (Kitazono et al., 1999). We induced a partial reversal of multidrug resistance using 25 mM and 75 mM solutions of verapamil as previously described (Kitazono et al, 2001).

2.4. Statistical analysis

The statistical analysis was performed using SPSS Version 12.0 software. Descriptive statistics were attained and value for every studied parameter of each cell line was achieved. The overexpression of genes and proteins and the appearance of resistance were calculated separately for each cell line and then the median was presented. The normality of distribution was evaluated using the Kolmogorov-Smirnov test with the Lilliefors significance correction. To attain the p-value by normal distribution the Student's paired sample t-test (t) was used, in case of an abnormal distribution the non-parametric Wilcoxon's matched pairs signed test (z) was used. To evaluate the correlation were used the Pearson method or, where mentioned - the non-parametric Spearman method.