6. MATERIALS AND METHODS

6.1 Chemicals, solutions, kits, and instruments

6.1.1 Chemicals

All chemicals, if not explicitly mentioned, were obtained from Merck (Darmstadt, Germany) and Sigma (Steinheim, Germany).

Chemical	Company
30% Acrylamide/bisacrylamide 37.5:1	Bio-Rad, Hercules, CA, USA
Agarose (for DNA gels)	Appligene, Illkirch, France
Agarose Qualex Gold Agarose (for RNA gels)	AGS, Heidelberg, Germany
AmpliTaq Gold™ DNA polymerase	Roche Diagnostics, Basel, Switzerland
[γ-^{32}P]ATP	Amersham, Freiburg, Germany
Bovine serum albumin standard (BSA)	Pierce, Rockford, IL, USA
Bradford assay	Bio-Rad, Hercules, CA, USA
[α-^{32}P]dATP	Amersham, Freiburg, Germany
4',6-Diamidino-2-phenylindole-2HCl (DAPI)	Serva, Heidelberg, Germany
Dithiothreitol (DTT) for PCR	Invitrogen, Karlsruhe, Germany
Dulbecco' s MEM medium (DMEM)	Invitrogen, Karlsruhe, Germany
DNA-ladder 10 kb (#SM0331)	MBI Fermentas, St. Leon-Rot, Germany
DNase I	Roche Diagnostics, Basel, Switzerland
Ethanol	Baker, Deventer, Holland
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
5X First strand buffer	Invitrogen, Karlsruhe, Germany
Fluoromount G	Southern Biotechnology Associates, Birmingham, Alabama, USA

Glycin	Roth, Karlsruhe, Germany
Herring sperm	Promega, Madison, WI, USA
7-Hydroxystaurosporine (UCN-01)	Kyowa Hakko Kogyo, Tokyo, Japan
Hygromycin B	Roche Diagnostics, Basel, Switzerland
Irinotecan hydrochloride x $3H_2O$ (irinotecan, CPT-11)	Aventis, Frankfurt, Germany
Isopropanol	Baker, Deventer, Holland
Methanol	Baker, Deventer, Holland
$MgCl_2$	Roche Diagnostics, Basel, Switzerland
Non-fat dry milk	Sucofin, Zeven, Germany
Nucleotides (dATP, dCTP, dGTP, dTTP)	Amersham, Freiburg, Germany
10X PCR buffer	Roche Diagnostics, Basel, Switzerland
PCR primers	TibMolBiol, Berlin, Germany
PD98059	Calbiochem, San Diego, CA, USA
Random hexanucleotide primers	Invitrogen, Karlsruhe, Germany
RNase inhibitor	Promega, Madison, WI, USA
6X Sample buffer (for DNA electrophoresis)	MBI Fermentas, St. Leon-Rot, Germany
Scintillation Cocktail Optiphase Supermix	Wallac, Turku, Finland
SuperScript reverse transcriptase II	Invitrogen, Karlsruhe, Germany
Super Signal West Pico	Pierce, Rockford, IL, USA
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Serva, Heidelberg, Germany
1,3,7-Trimethylxanthine hydrate (caffeine)	ICN, Aurora, Ohio, USA
Tris hydrochloride	Roth, Karlsruhe, Germany
Tris ultrapure	ICN, Aurora, Ohio, USA
Trypsin (1:250, 2.5% w/v)	Biochrom, Berlin, Germany
U0126	Calbiochem, San Diego, CA, USA

6.1.2 Solutions

Solution	Components
1X Kinase buffer	20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT
1X Lysis buffer for protein extraction	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF, 10 μg/ml of aprotinin, pepstatin A and leupeptin
1X Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$, 1.47 mM KH_2PO_4, pH 7.4
1X PBS-EDTA buffer	137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.2H_2O$, 1.47 mM KH_2PO_4, 0.53 mM EDTA, pH 7.4
5X SDS gel-loading buffer (for protein electrophoresis)	SDS 5% w/v, sucrose 25% w/v, 49.5 mM Tris, 5 mM EDTA, bromophenol blue 0.025%, pH 6.8, 0.194 M DTT
1X Sodium chloride/sodium citrate (SSC)	3M NaCl, 0.3 M sodium citrate, pH 7.0
1X Transfer buffer for Western blot	25 mM Tris, 192 mM glycin, 20% methanol (v/v), pH 8-8.3
1X Tris acetate-EDTA buffer (TAE)	40 mM Tris-acetate, 1mM EDTA, pH 8.0
1X Tris buffered saline (TBS)	7.4 mM Tris, 43.5 mM Tris-HCl, 150.5 mM NaCl, pH 7.4-7.6
1X Tris glycin electrophoresis buffer	0.025 M Tris, 0.192 M glycin, 0.1% SDS w/v

6.1.3 Kits

Kit	Company
Atlas™ Human Cell Cycle cDNA Expression Array (# 7748-1)	BD Bioscience, Clontech, Heidelberg, Germany
Cell Death Detection ELISA (# 1544675)	Roche Diagnostics, Basel, Switzerland
Dynabeads® Oligo(dT)$_{25}$	Dynal, Hamburg, Germany
RNA-Clean LS™ System Kit	Hybaid-AGS, Heidelberg, Germany

6.1.4 Instruments

Instrument	Company
Agarose-gel electrophoresis chambers:	
Mini-Sub™ DNA Cell	Bio-Rad, Hercules, CA, USA
Wide Mini-Sub™ DNA Cell	Bio-Rad, Hercules, CA, USA
Balances	Sartorius (Göttingen, Germany), Kern (Albstadt, Germany)
Cell scrapers (23- and 32-cm length)	Nunc, Roskilde, Denmark
Centrifuges:	
Table centrifuges	Heraeus Sepatech (Hanau, Germany), Jouan (Winchester, Virginia, USA), Eppendorf (Hamburg, Germany)
Cytospin 3 centrifuge	Shandon, Pittsbugh, PA, USA
Cooling centrifuge	Sigma, Steinheim, Germany
β-counter 1450 Microbeta Trilux	Wallac, Turku, Finland
Cover slips	Menzel, Braunschweig, Germany
Cryo tubes (1 ml)	Nunc, Roskilde, Denmark
Disposable conical tube (15 ml)	Nunc, Roskilde, Denmark
Disposable conical tube (50 ml)	Sarstedt, Nümbrecht, Germany
Disposable cuvettes	Pharmacia, Erlangen, Germany

Disposable plastic cuvettes for photometrical determination of protein concentration	Brand, Wertheim, Germany
ELISA Reader MR5000	Dynatech, Denkendorf, Germany
FACScalibur	Becton Dickinson, Heidelberg, Germany
Film exposition cassettes	Kodak, Stuttgart, Germany
Films:	
BioMax MR 18x24 cm	Kodak, Stuttgart, Germany
T-MAT Plus DG 13x18 cm	Kodak, Stuttgart, Germany
Flasks for cell culture (25-, 80-, 185- cm^2 area)	Nunc, Roskilde, Denmark
Fluorescence microscope BX60	Olympus, Hamburg, Germany
Gassed incubator	Heraeus Sepatech, Hanau, Germany
Haemocytometer	Brand, Wertheim, Germany
Hybridization bottle	Amersham, Freiburg, Germany
Laboratory glasses	Brand, Wertheim, Germany
Laminar airflow Bio 48	Faster, Ferrara, Italy
Light microscope binocular type CK2	Olympus, Hamburg, Germany
Microliter 710 syringe	Hamilton, Bonaduz, Switzerland
Microscope slides	Menzel, Braunschweig, Germany
Microwave oven	Bosch, Berlin, Germany
ModFit LT2 software	Verity Software House, Topsham, ME, USA
Multiple pipettor	Eppendorf, Hamburg, Germany
Non-steril latex examination gloves	Ansell, München, Germany
Paper cuts for cytospins (25 x 75 mm/ two holes)	Schleicher and Schuell, Dassel, Germany
Pasteur pipettes	Brand, Wertheim, Germany

Petri-dishes for cell culture (8.8-, 22-, and 56.7-cm^2 area)	Nunc, Roskilde, Denmark
Phosphoimager 1500	FUJI, Düsseldorf, Germany
Pipetboy	Integra Bioscience, Fernwald, Germany
Pipettes (10-, 20-, 100-, 250-, 1000- µl)	Eppendorf, Hamburg, Germany
Pipettes (1-, 5-, 10-, 25- ml)	BD Bioscience, Falcon, Le Pont De Claix, France
Pipette tips (10 µl)	Eppendorf, Hamburg, Germany
Pipette tips (100 µl)	Sarstedt, Nümbrecht, Germany
Pipette tips (1000 µl)	Brand, Wertheim, Germany
Polaroid Imaging system	Polaroid, Offenbach, Germany
Polystyrene round-bottom tubes (5 ml)	BD Bioscience, Falcon, Le Pont De Claix, France
Polyvinylidene fluoride (PVDF) microporous membrane	Immobilon-P, Millipore, Eschborn, Germany
Power supply models 200/2.0 and 200/2.5	Bio-Rad, Hercules, CA, USA
Reaction cups 0.2 ml	Rapidozym, Luckenwalde, Germany
Reaction cups 0.5 ml	Eppendorf, Hamburg, Germany
Reaction cups 1.5 ml	Brand, Wertheim, Germany
Reaction cups 2 ml	Eppendorf, Hamburg, Germany
Rotating oven	Heraeus Sepatech, Hanau, Germany
SDS-gel electrophoresis and blotting:	
Mini Protean™ II	Bio-Rad, Hercules, CA, USA
Mini Trans-Blot electrophoretic transfer cell	Bio-Rad, Hercules, CA, USA
Scanner GS-700 Imaging Densitometer	Bio-Rad, Hercules, CA, USA

Shakers	Köttermann (Uetze/Hänigsen, Germany), Ika (Staufen, Germany)
Spectrophotometer Ultrospec 2000	Pharmacia, Erlangen, Germany
Stirrers	Heidolph (Kelheim, Germany), Ika (Staufen, Germany)
Superpolyethylen scintillation vials (20 ml)	Packard, Groningen, Germany
Thermal cycler	Primus, MWG-Biotech, Ebersberg, Germany
Thermo block	Eppendorf, Hamburg, Germany
TINA software	DesignWare, Meriden, CT, USA
Transilluminator	Desaga, Heidelberg, Germany
UV-Transilluminators	Konrad Benda (Wiesloch, Germany), UVP (San Gabriel, CA, USA)
Vacuum centrifuge (Speed vac RC.10.10)	Jouan, Saint Herblain, France
Vortex	Heidolph, Kelheim, Germany
Water bath	Julabo, Seelbach, Germany
Water boiler	Patz, Wankendorf, Germany
96-Well plates	Nunc, Roskilde, Denmark

6.2 Molecular biology

6.2.1 Isolation of total RNA

For isolation of total RNA from cell lines, the RNA-Clean LS™ System Kit, based on extraction with phenol and chloroform, was employed. For handling and extraction of RNA, RNAse free material has been used. This is achieved by sterilizing glasses for 1 hr at 180°C and by adding DEPC to water solutions (to a final concentration of 0.1%) before autoclaving them [153].

A cell number varying from 1- to 10-x 10^6 was seeded in 80-cm^2 flasks, to obtain a confluency of 50-70% at the time of harvesting (corresponding to 10-50 x 10^6 cells in a 80-cm^2 flask). One day after seeding, cells were treated with UCN-01 (0.5-1 μM) for 2 days. Cells were washed with PBS and harvested on ice with a cell scraper by adding 0.9 ml per 80-cm^2 area of RNA-Clean LS™-containing phenol. The lysates were collected in aliquots of 1 ml in 1.5 ml Eppendorf cups. After adding 0.2 ml chloroform, the samples were shaked vigorously for 15 seconds and then kept on ice for 5 min. Then, the samples were centrifuged for 15 min at 12 000 x g at 4°C. The upper aqueous RNA-containing phase (about 500 μl) was transferred into a new Eppendorf cup. An equal volume of isopropanol was added, mixed, and a precipitation followed for 15 min at -20°C. The precipitate was centrifuged for 15 min at 12 000 x g at 4°C, the supernatant was removed, and the white pellet was subsequently washed two times with 1 ml of 70% ethanol, followed by 10 min centrifugation at 12 000 x g at 4°C. Ethanol was removed and the pellet was dried for 15 min at 37°C. The RNA from 10 to 50 x 10^6 cells was dissolved in 20-50 μl of DEPC-H_2O at 56°C for 5-10 min. Photometrical determination of RNA concentration followed; 1 μl of the RNA solution was added to 100 μl of H_2O and measured at 280 and at 260 nm. The 260/280 ratio (indicating the purity of the extracted RNA) was around 2.1. Since a solution of RNA whose OD_{260} = 1 contains approximately 40 μg/ml of RNA [153], the RNA concentration in the sample (μg/μl) is obtained by multiplicating the OD_{260} for a factor 4. To control the quality of the extracted RNA, 1 μg of the RNA was run on a 1.0% TAE gel. RNA was stored at -70°C. Eventually, the RNA solution was concentrated by drying the RNA in a vacuum centrifuge and resuspending the pellet in a proper volume of DEPC-H_2O.

6.2.2 Isolation of polyA$^+$ RNA

PolyA$^+$ RNA was isolated from cellular total RNA with Dynabeads® Oligo(dT)$_{25}$. This method is based on the base pairing between the polyA$^+$ residues at the 3'-end of messenger RNAs and the oligo(dT) residues covalently coupled to the surface of the Dynabeads® Oligo(dT)$_{25}$. The mRNA is captured by the Dynabeads® Oligo(dT)$_{25}$ and washed thoroughly using a Magnetic Particle Concentrator (Dynal MPC®). The mRNA

is eluted from the solid phase by using a buffer without salt, thus destabilizing the dT:A hybrid.

The volume containing 500 µg of total RNA was adjusted to 500 µl with DEPC-H_2O. 5 mg Dynabeads® Oligo$(dT)_{25}$ (1 ml of suspension) were transferred from the stock suspension to an Eppendorf cup placed in a Dynal MPC®. When the solution was clear, the supernatant was removed and the Dynabeads® Oligo$(dT)_{25}$ were washed twice in 2X binding buffer (20 mM Tris-HCl pH 7.5, 1.0 M LiCl, 2 mM EDTA). After mixing, the cup was replaced in the magnet, the supernatant removed, and the Dynabeads® Oligo$(dT)_{25}$ again resuspended in 500 µl of 2X binding buffer. The RNA was then denatured for 2 min at 65°C, added to the resuspended Dynabeads® Oligo$(dT)_{25}$, and incubated for 10 min at room temperature. The cup was placed in the Dynal MPC® for 30 seconds and the supernatant removed. Afterwards, the Dynabeads® Oligo$(dT)_{25}$ were washed two times with 1 ml of washing buffer (10 mM Tris-HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA) using the Dynal MPC®.

Finally, polyA$^+$ RNA was eluted in 100 µl of DEPC-H_2O and subsequently dried in a vacuum centrifuge. Obtained mRNA was dissolved in 10 µl of DEPC-H_2O, and concentration was determined photometrically as described in paragraph 6.2.1.

6.2.3 Differential hybridization (Atlas™ Array)

Clontech's Atlas™ cDNA Expression Arrays were the first commercially available large-scale nucleic acid arrays. This technology has a wide range of applications, including differential gene expression. The method is based on the hybridization of entire cDNA populations to nucleic acid probes, bound to the membranes. Atlas™ Human Cell Cycle cDNA Expression Array (# 7748-1) used in this study comprises 111 human cell cycle-related cDNAs.

The procedure consists of the following steps: Reverse transcription of each polyA$^+$ RNA population using [α-^{32}P]dATP, hybridization of the radio-labelled cDNA to each Atlas™ Array, and analysis of the hybridization pattern on each Array. The relative expression levels of a given cDNA in two different RNA sources can be assessed by comparing the signal obtained with a sample from one RNA source to that obtained with a sample from another source.

a) Radioactive cDNA synthesis

The radioactive cDNA synthesis was performed according to the protocol of the manufacturer.

A master-mix was prepared with the following amounts of components (provided by the kit):

Component	**Volume per sample**
5X reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)	2.0 µl
10X dNTP mix (5mM dCTP, dGTP, dTTP, 50 µM dATP)	1.0 µl
[α-^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml)	3.5 µl
DTT (100 mM)	0.5 µl
Superscript reverse transcriptase II	0.5 µl
Final volume	7.5 µl

For each mRNA probe, the following mixture was prepared:

Component	**Volume per sample**
mRNA	2 µg
10X primers mix (0.02 µM each)	1 µl
Final volume	3µl

This mixture was briefly vortexed and centrifuged. Then, the mRNA-primer mix was incubated for denaturation for 2 min at 70°C and additionally for 2 min at 50°C. The master-mix was added, followed by incubation for 20 min at 50°C to allow cDNA synthesis. For stopping synthesis reaction, 1 µl of 10X termination mix (0.1 M EDTA pH 8.0, 1 mg/ml glycogen) was added.

b) Column chromatography

Column chromatography was used to purify the ^{32}P-labeled cDNA from unincorporated ^{32}P-labeled nucleotides and small cDNA fragments. The provided Chroma Spin-200 columns were prewarmed for 1 hr at room temperature and mixed gently until the Sepharose was completely dissolved. The bottom and the top of the cup were removed carefully from the column, which then was placed in a 1.5 ml Eppendorf tube.

The H_2O was drained through the column by gravity flow, until the surface of the gel beads was recognizable on the column matrix. The collected H_2O was discarded. The sample was applied to the centre of the gel bed's flat surface, and it was allowed to be fully adsorbed into the matrix. 40 µl followed by 250 µl of deionized H_2O were applied to the column, let completely drain out, and discarded. Then, five fractions were collected into five tubes by sequentially adding 100 µl of deionized H_2O. The incorporation of ^{32}P into the probe was calculated by counting the incorporated activity of 1 µl of each fraction with a scintillation counter. An elution profile was plotted. The first peak, usually in the second fraction, contained the purified labelled probe and had a total of 1-5 x 10^6 cpm. The second peak (fourth or fifth fraction) contained the unincorporated nucleotides.

c) Hybridization

15 ml of provided ExpressHyb hybridization solution per membrane were prewarmed at 68°C. 0.1 mg of herring sperm per 1 ml of hybridization solution were denatured for 5 min at 95°C and then mixed with the hybridization solution and kept at 68°C. The Atlas™ Array was placed in a hybridization bottle, washed with deionized H_2O, and pre-hybridized with 10 ml of the ExpressHyb-containing herring sperm solution for 30 min at 68°C in a rotating oven.

In the meantime, 2-5 x 10^6 cpm of the labelled cDNA probe was mixed with 10% of the total volume of 10X denaturing solution (1 M NaOH, 10 mM EDTA) and incubated for 20 min at 68°C. Then, an equal volume of 2X neutralization solution (1 M NaH_2PO_4 pH 7.0) was added and incubated for 10 min at 68°C. This mixture, containing the labelled cDNA, was added to the remaining 5 ml of ExpressHyb-containing herring sperm solution. The pre-hybridization solution was discarded from the hybridization tube and replaced with the 5 ml solution containing the labelled cDNA. Hybridization was performed overnight at 68°C in a rotating oven.

d) Washing of hybridized membranes

Initially, membranes were washed within the hybridization tube for 3 times, 20 min each, with prewarmed washing solution I (2X SSC, 1% SDS) at 68°C. Then, membranes were washed two times, 20 min each, with prewarmed washing solution II (0.1X SSC, 0.5% SDS) at 68°C. Membranes were carefully removed from the

hybridization tubes, covered with a plastic foil, and exposed overnight on a phosphoimager plate. Alternatively, the hybridization was visualised by autoradiography, by overnight exposition to an x-ray film at -70°C.

e) Evaluation of gene expression

The autoradiography films were scanned, and the densitometrical evaluation of gene expression was carried out by means of TINA software. Gene expression was normalized to the median expression value of all nine housekeeping genes detected on the membrane.

f) Stripping of membranes

150 ml of a 0.5% SDS-solution were boiled and membranes were added. Stripping occurred for 10 min at 100°C and then for further 10 min in the same, cooled SDS-solution. Then, membranes were stored at -20°C and eventually reprobed from 3 to 4 further times.

6.2.4 Reverse-transcription polymerase chain-reaction (RT-PCR)

This technique was performed for the amplification of genes found differentially expressed by using Atlas™ Array technique.

To eliminate genomic DNA contamination, RNA was DNase-digested before cDNA synthesis as follows:

Component	Volume	Final concentration
Total RNA	17.0 µl	20.0 µg
Tris-HCl pH 6.5	2.5 µl	50.0 mM
$MgCl_2$	5.0 µl	10.0 mM
DTT	5.0 µl	10.0 mM
RNase inhibitor	0.5 µl	0.4 U/µl
RNase free DNase I	20.0 µl	4.0 U/µl
DEPC-H_2O	x	
Total volume	50.0 µl	

The mixture was first incubated at 37°C for 2 hr and then, to inactivate DNase, at 100°C for 5 min and kept in ice.

6.2.4.1 cDNA synthesis

The random primers were left to anneal with the RNA:

Component	Volume
DNase-digested total RNA	6.25 µl
Random hexanucleotide primers	2.50 µl
DEPC-H_2O	13.25 µl
Total volume	22.00 µl

The solution was incubated for 10 min at 70°C and kept in ice.

During incubation, the master-mix for the cDNA synthesis was prepared as follows:

Component	Volume	Final concentration
5X First strand buffer	10.0 µl	1X
dNTP mix (2.5 mM each nucleotides)	10.0 µl	0.5 mM each
DTT	5.0 µl	10.0 mM
RNase inhibitor	0.5 µl	0.4 U/µl
SuperScript reverse transcriptase II	2.5 µl	10.0 U/µl
Total volume	28.0 µl	

This master-mix was added to the RNA-containing random primers solution. The reaction was kept for 1 hr at 37°C for cDNA synthesis, then 10 min at 100°C to denature the reverse transcriptase and stop the reaction, and finally kept in ice.

For control of complete DNase digestion, a cDNA synthesis reaction without reverse transcriptase was prepared.

6.2.4.2 Polymerase chain reaction (PCR)

This reaction comprises of different phases: First the DNA strands are separated through incubation at 95°C, then the oligonucleotides primers anneal to the DNA strands through lowering the temperature, and finally Taq polymerase (in the presence of dNTP and at the temperature of 72°C) synthesizes the complementary strand using the oligo-primers as starting point. This reaction is repeated in every amplification cycle, thus resulting in an exponential accumulation of the target fragment of DNA.

A modified form of AmpliTaq® DNA polymerase, AmpliTaq Gold™ DNA polymerase, was used. It is activated by 10 min pre-PCR incubation at 95°C. This

modification renders the reaction more specific, since any low-stringency misprimings that may have occurred at low temperature will dissolve at 95°C and will not lead to DNA amplification.

A master-mix was prepared as follows:

Component	Volume	Final concentration
10X PCR buffer II	2.5 µl	1X
$MgCl_2$	2.0 µl	2.00 mM
dNTP mix	2.5 µl	0.25 mM each
Primer forward (FWD) (10 mM)	1.0 µl	0.40 µM
Primer reverse (REV) (10 mM)	1.0 µl	0.40 µM
AmpliTaq Gold™ DNA polymerase	0.1 µl	0.02 U
Deionized H_2O	10.9 µl	
Final volume	20.0 µl	

For each PCR reaction 5 µl of a dilution 1/5 of the cDNA was added to 20 µl of master-mix. For control of contamination of the PCR reagents, a reaction was set with 5 µl deionized H_2O instead of cDNA.

6.2.4.3 PCR programs

PCR oligonucleotides primers for amplification of target genes were designed with the following rules:

- 18-25 nucleotides in length;
- GC content of about 40-60%;
- Avoidance of stretch of G or C nucleotides at the 3'-end of the primers;
- No complementation between or within the two primers, to avoid primer dimer and hairpin formation;
- Melting temperature of the primers differing by not more than 5°C (so the GC content and length were chosen accordingly);
- Annealing temperature of at least 5°C lower than melting temperature.

The oligonucleotides were synthesised by TibMolBiol, Berlin, Germany.

Characteristics of the chosen primers were as follows:

Gene	**Primers (5'-3')**	**Position in the gene**	**Length of the amplified fragment (bp)**
Pyruvate dehydrogenase (lipoamide) (PDH)	FWD: GGTATGGATGAGGAGCTGGA REV: CTTCACAGCCCTCGACTAA	142-161 224-239	97 bp
MAP kinase kinase 2 (MEK2)	FWD: GATCAGCATTTGCATGGAACA REV: GTCCGACTGCACCGAGTAATG	679-700 981-1002	323 bp

PCR conditions for synthesis of target DNA fragments:

Gene	**Number of cycles**	**Denaturing temperature (sec)**	**Annealing temperature (sec)**	**Extension temperature (sec)**
PDH	32	95°C (35'')	57°C (45'')	72°C (60'')
MEK2	28	95°C (60'')	64°C (60'')	72°C (60'')

All templates were initially denatured for 10 min at 95°C. An extension step at 72°C for 10 min followed each amplification reaction.

6.2.5 Agarose gel electrophoresis

Separation of DNA fragments occurred, according to the length of the fragment amplified, in 0.8-1.5% TAE-agarose gels. All gels contained ethidium bromide at a final concentration of 10 µg/µl. Before loading, 6X sample buffer was added to the samples. To determine the length of the DNA fragments, a 10 kb DNA-ladder was used. Electrophoresis followed at 100 Volts for 20-45 min. The ethidium bromide-stained DNA bands were visualized on a UV-transilluminator, and were photographed with a Polaroid camera.

6.3 Cellular biology

6.3.1 Culture of established human colorectal cell lines

The following table is indicating the human colorectal adenocarcinoma cell lines used in this study. The *p53* mutation status and the mismatch repair status are indicated:

Cell line	***p53* mutation status**	**Mismatch repair status**
HCT116	Wild type [154]	Deficient through homozygous nonsense mutation in the *hMLH1* gene located on chromosome 3, resulting in absence of hMLH1 protein [155]; homozygous inactivation of *hMSH3* gene [156]
HCT116+ch2	Wild type	Deficient, *hMLH1* mutant. HCT116 subline complemented with chromosome 2 [157]
HCT116+ch3	Wild type [158]	Proficient. HCT116 subline complemented with chromosome 3 [157], carrying the *hMLH1* gene
HCT116 $p53^{-/-}$	Knocked out, obtained by homologous recombination from the parental HCT116 cell line [159]	Deficient, *hMLH1* mutant

HCT116, HCT116+ch2, HCT116+ch3 (generously provided by C.R. Boland, Department of Medicine and Cancer Center, University of California, San Diego, La Jolla, California, CA, USA), and HCT116 $p53^{-/-}$ (kindly provided by B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, MD, USA) were maintained in DMEM-medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The HCT116+ch2 and HCT116+ch3 cell lines were maintained in medium containing 400 µg/ml geneticin, and the HCT116 $p53^{-/-}$ cells were propagated in medium

supplemented with 400 µg/ml geneticin and 100 µg/ml hygromycin B. During all experiments performed, cells were grown without geneticin and hygromycin.

The phenotype of the cell lines was checked on the protein level with regard to the presence of the p53 and hMLH1 proteins (**Fig. 6**).

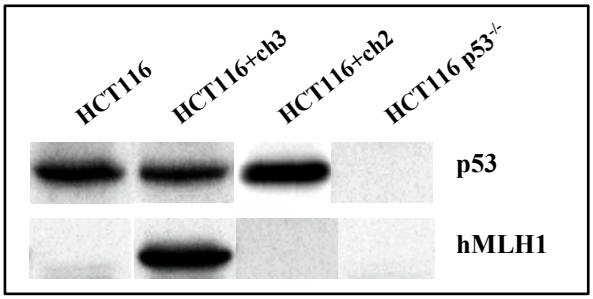

Fig. 6: Detection of p53 and hMLH1 proteins by Western blot in the cell lines HCT116 ($p53^{+/+}$,$hMLH1^{-}$), HCT116+ch3 ($p53^{+/+}$,$hMLH1^{+}$), HCT116+ch2 ($p53^{+/+}$,$hMLH1^{-}$), and HCT116 $p53^{-/-}$ ($p53^{-/-}$,$hMLH1^{-}$).

6.3.1.1 Maintenance and passaging of the cultured cell lines

Cells were stored in 1 ml of a mixture 80% FCS and 20% dimethylesulfoxide (DMSO), placed in Nunc Cryo tubes in liquid nitrogen. Cells were cultured in strictly sterile conditions without antibodies in the media. Cells were thawed and propagated in their respective medium in cell culture flasks of different areas (25-, 80-, 185- cm^2), and grown in an incubator at 37°C in 5% CO_2 at 100% humidity. They were maintained in culture for not more than 15 passages.

Since the cell lines used were all adherent cell lines, every two or three days they reached a confluency (defined as percentage of the area occupied) of 60-80%. To propagate them further, they were detached from the flask surface through trypsinization. For this, the medium was discarded and cells were washed with 1X PBS-EDTA buffer. 1 or 2 ml of 0.025-0.5 % (w/v) trypsin were added and distributed on the flask bottom. Trypsinization occurred at 37°C for 10-20 min until the cells were detached from the surface. This suspension was then rinsed with medium. For some experiments (i.e. clonogenic assay, FACS analysis), a single-cells suspension had to be obtained. For this, after trypsinization cells were rinsed first with 1X PBS-EDTA buffer

and thoroughly resuspended with a 1 ml pipette to eliminate cell clumps, then medium was added to this suspension.

Cells suspensions were collected in 50 ml Falcon tubes and centrifuged for 5 min at 1 500 x g. The supernatant was discarded and the cells were resuspended in the corresponding medium. A portion of the resuspended cells was placed in the original flask for propagation.

In case of further experiments, an aliquot (50 µl) of the cells suspension was counted to obtain the number of cells/ml. To the 50 µl of cells suspension, 50 µl of trypan blue were added. 10 µl of this suspension were loaded into a haemocytometer, covered with a cover slip and examined by light microscopy under x 20 magnification. Cells number was determined by counting cells present in the four squares (64 sub-squares) of the haemocytometer chamber. The mean value (cells counted/4) was multiplied by 2 x 10^4 to account for dilution of the cell sample and for the volume of the chamber. This represented the number of cells in 1 ml of solution.

From 10^4 to 10^5 cells/cm^2, considering the experimental conditions at which the cells were subsequently subjected (treatment with chemotherapeutic agents and length of the treatment), were spread onto Petri-dishes or flask. The number of cells seeded was selected to have a confluency of 50-70% at the time of harvesting.

6.3.2 Treatment of cultured cells

Cells were seeded 1 day before treatment with each chemotherapeutic drug.

CPT-11 was stored at 4°C in infusion-solution at a concentration of 30 mM and diluted in FCS-containing medium immediately before use.

UCN-01 (kindly provided by E. A. Sausville, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Rockville, MD, USA) was dissolved in DMSO to a final concentration of 8 mM and stored aliquoted at –20°C. Immediately before use, it was diluted in FCS-containing culture media.

In all experiments, cells were treated in FCS-containing medium for 2 days with the drugs at given concentration, unless the cells were investigated at earlier time points.

After washing with PBS, the cells were incubated in drug-free media for the periods indicated.

6.3.3 Assays of cell growth and cytotoxicity

6.3.3.1 Measurement of viable cells by trypan blue exclusion

At different time points after treatment with chemotherapeutic agents or vehicle, medium-containing detached cells was collected. Adherent cells were trypsinized as described in paragraph 6.3.1.1. Trypsinized cells were pooled with the medium-containing detached cells. The mixture was centrifuged at 1 500 x g for 5 min, the pellet resuspended in a proper volume of PBS and an aliquot of the suspension was mixed 1:1 with trypan blue. The density of viable, trypan blue-excluding cells was evaluated (paragraph 6.3.1.1) and the total number of viable cells per sample was calculated multiplying the density obtained with the volume of the suspension.

6.3.3.2 MTT assay

This assay is a colorimetric test employed to detect living, metabolizing cells present in a cell population. The reaction of the yellow-coloured tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) with NADH and NADPH reduces water-soluble tetrazolium salts into a purple-coloured and insoluble formazan which precipitates out of solution. Production of the formazan is therefore proportional to living cells in a cell population.

Cells were trypsinized and their density was evaluated (paragraph 6.3.1.1). To ensure an equal distribution of cells between the wells, cells were diluted to the desired density in a Falcon tube, and 50 µl of this suspension was distributed equally into a 96-well flat-surface plate, to have five thousand cells/well. Triplicates for each sample were done. After 1 day, 50 µl of drugs-containing medium were added, while 50 µl medium were added in the non-treated control wells. After 2 days, 25 µl of MTT (1 mg/ml) were pipetted into each well, and the plate was incubated for 2 hr at 37^{o}C. 100 µl/well of an extraction buffer, composed of 20% SDS in dimethylformamid previously diluted

1:1 (v/v) in H_2O (to a final pH 4.7 obtained adding a solution 1:1 (v/v) of 80 % acetic acid : 1 M HCl), were added and pipetted. After 1 hr incubation at 37°C, the absorbance was measured at 550 nm in an ELISA-reader taking as background 100 µl medium to which 50 µl MTT and 100 µl extraction buffer were added. Alternatively, after 2 days of treatment medium was removed, cells were washed with PBS and incubated in drug-free medium for another 3 to 4 days, and, at the end of this time, assayed with MTT. The percentage of viable cells at each concentration was then calculated as 100 x (average value of the absorbance of the treated wells) / (average value of the absorbance of the non-treated wells).

6.3.3.3 Clonogenic assay

This test has been employed to evaluate the capacity of single cells to form colonies after treatment with chemotherapeutic agents.

Adherent cells were trypsinized to obtain a single-cells suspension, and cell density in the suspension was determined (paragraph 6.3.1.1). A stock suspension with the desired cell density was made and distributed equally into 22-cm^2 Petri-dishes. The proper seeding density was empirically determined for each cell line, so that the final end-of-assay colony numbers were in the range of 100-300 colonies per 22-cm^2 Petri-dish. Optimal seeding density depends on growth rate and cloning efficiency, this latter defined as 100 x (number of colonies in the non-treated sample) / (number of cell seeded).

The number of cells seeded and the cloning efficiency for each cell line were as follows:

Cell line	Cells seeded in 22-cm^2 Petri-dishes	Cloning efficiency
HCT116	150	64-80%
HCT116+ch2	200	43-61%
HCT116+ch3	300	50%
HCT116 p53$^{-/-}$	200	40-50 %

One day after seeding, the chemotherapeutic agents were added at given concentrations for the successive 2 days. After this time, drug-containing medium was removed, the cells were carefully washed with PBS, and 4 ml of drug-free medium were added. After 10-12 days the medium was removed, cells were washed three times with cold PBS and fixed from 10 to 30 min with ice-cold methanol. After washing with PBS, cells were stained for 10-30 min with a dilution of 1:800 v/v of 10% crystal violet (dissolved in ethanol) in PBS. Cells were washed with PBS, and colonies of at least 50 cells were scored visually on a transilluminator. Experiments were performed each time using triplicate cultures for each drug concentration. The percentage of surviving colonies was defined as 100 x (average value of the number of colonies in the treated group) / (average value of the number of colonies in the non-treated group).

6.3.4 Analyses of cell death and apoptosis

6.3.4.1 Measurement of dead cells by trypan blue exclusion

One of the morphological features associated with cell death is loss of membrane integrity with the concomitant inability to exclude dyes such as trypan blue. This method is useful as a preliminary estimation of cell death because of the speed and ease with which it can be performed. However, it does not give an indication of the mode of cell death, since both necrotic and apoptotic cells undergoing secondary necrosis permit dye uptake.

To determine the percentage of dead cells, the total number of viable, trypan blue-excluding cells as well as the total number of trypan blue-stained cells was evaluated as follows:

Total number of viable cells = Density of trypan blue-excluding cells x volume of the cells suspension;

Total number of dead cells = Density of trypan blue-stained cells x volume of the cells suspension.

6.3.4.2 Detection of apoptosis: Cell Death Detection ELISA

Cell Death Detection ELISA Kit is an assay quantifying DNA fragments generated during apoptosis.

Cells were seeded, and they were treated after one day with the chemotherapeutic drugs at different concentrations or for different laps of time after removal of the drug-containing medium. Adherent cells were trypsinized and pooled with the floating cells. Cells suspension was centrifuged 5 min at 1 500 x g, the supernatant was discarded, and the pellet was resuspended in PBS. The total cell density (viable and not viable cells) was evaluated (paragraph 6.3.1.1). For each sample, 0.1 x 10^6 cells were collected and centrifuged 5 min at 1 500 x g. The supernatant was removed, and the pellet was resuspended thoroughly in 500 µl of the incubation buffer (provided in the kit), and cells were lysed 30 min at 4°C. After centrifugation for 10 min at 12 000 x g at 4°C, 300 µl of the supernatant (containing the cytoplasmic fraction) were carefully removed. This fraction was aliquoted and stored at -20°C.

The ELISA assay was performed as follows: 100 µl of coating solution (provided anti-histone antibody diluted 1:10 in coating buffer, previously diluted 1:10 in deionized water) were pipetted into each well of the microtiter plate module (MTP, provided in the kit) and incubated for 1 hr at room temperature or overnight at 4°C. Then, the coating solution was removed by tapping, and non-specific binding sites were saturated by treatment with 200 µl of incubation buffer for 30 min at room temperature. The solution was removed by tapping, and the wells were washed three times with 250 µl washing solution (provided washing buffer was prewarmed and diluted 1:10 in deionized water). Washing solution was carefully removed, and 100 µl of sample

solution (diluted 1:10 or 1:20 in incubation buffer) were added into each well of the MTP-module and incubated for 90 min at room temperature. For determination of the background staining, 100 µl of incubation buffer per well were pipetted into two wells. During this second incubation step the nucleosomes contained in the sample bind via their histone components to the immobilized anti-histone antibody. Three washing steps followed. Then, 100 µl of conjugate solution (reconstituted peroxidase-conjugated anti-DNA antibody diluted 1:10 in incubation buffer, provided in the kit) were pipetted into each well and incubated 90 min at room temperature; in this third incubation step, peroxidase-conjugated anti-DNA antibody binds to the DNA-part of the nucleosome. After removal of unbound peroxidase-conjugated by a washing step, 100 µl of substrate solution, composed of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) dissolved in substrate buffer (both of them provided in the kit), were added. The MTP-modules were incubated on a shaker until the color was developed (usually after 10-20 min). The amount of peroxidase retained in the immunocomplex was determined photometrically, measuring the absorbance at 405 nm in an ELISA-reader against substrate solution as blank.

The occurrence of apoptosis (enrichment of mono- and oligonucleosomes released into the cytoplasm) was estimated by averaging the values from the triple absorbance measurements of each samples and applying the following formula:

Enrichment factor = (Absorbance of sample) / (Absorbance of non-treated cells – Absorbance of background wells)

6.3.4.3 Detection of apoptosis: Flow cytometry

Because of activation of an endonuclease, which preferentially cleaves DNA at the internucleosomal sections, a fraction of DNA in apoptotic cells is of low molecular mass [160]. This DNA is detectable in FACS as subG1 peak and is considered a marker of cell death by apoptosis [161].

Preparation of the samples and flow cytometric analysis are described in paragraph 6.3.5.5. The percentage of cells in subG1 has been assessed by ModFit LT2 software.

6.3.4.4 Detection of apoptosis: PARP cleavage

The prominent biochemical events occurring during apoptosis are the proteolytic activation of caspases, as the critical executioners of apoptosis [162], and the proteolytic cleavage of PARP protein. Caspases-8 and -10 cleave the 32 kDa form of caspase-3 to its 17 kDa active form, which cleaves PARP protein from the 116 kDa form to 85- and 24-kDa fragments [163]. An antibody detecting the entire and the fragmented 85-kDa PARP polypeptides (BD Pharmingen, Lexington, Kentucky, USA) was used in Western blot experiments. Intensity of the bands was densitometrically evaluated with TINA software. The percentage of PARP cleavage was estimated as follows: 100 x (intensity of 85-kDa PARP band) / (Intensity of 85-kDa PARP band + Intensity of 116-kDa PARP band).

6.3.4.5 Detection of apoptosis: DAPI nuclear staining

The fluorescent dye 4',6-diamidino-2-phenylindole-2HCl (DAPI) has been shown to bind specifically to adenine-thymine-rich DNA of various origins. This dye was used in this study to identify cells that exhibit morphological features of apoptosis e.g. cell shrinkage, nuclear and chromatin condensation, extensive formation of membrane blebs, and apoptotic bodies [164].

Cells were treated with the chemotherapeutic drugs for the time indicated in the figures legends. After trypsinization of the adherent cells, floating and adherent cells were collected together, centrifuged at 1 500 x g for 5 min, and the pellet was resuspended in PBS. Cell density was evaluated as described in paragraph 6.3.1.1. From each sample, 0.2-0.4 x 10^6 cells were transferred into a new Eppendorf cup and centrifuged at 1 500 x g for 5 min. The pellet was resuspended in 1 ml of PBS, to have a cellular density of 0.2-0.4 x 10^6 cells/ml. Cytospins were prepared as follows: 50 µl of PBS were loaded into a cytospin chamber and centrifuged for 3 min at 300 rpm. Afterwards, 50 µl of the PBS-containing cells suspension (corresponding to 1-2 x 10^4 cells) were loaded into the cytospin chamber and centrifuged for 5 min at 500 rpm. The microscope glass slides were left to air-dry overnight. Afterwards, they were stored at -70°C or fixed 10 min in ice-cold methanol. Then, cells were washed in PBS and incubated in a solution of 1 µg/ml DAPI for 10 min. They were then washed 3 times in PBS. Fluoromount G was

used as mounting medium. Slides were analysed and photographed under a fluorescence microscope.

Alternatively, cells were grown in 22-cm^2 Petri-dishes and treated with chemotherapeutic drugs. At different time points after start of treatment, medium was removed, and the adherent cells were rinsed in PBS and fixed 10 min in ice-cold methanol. The borders of the Petri-dishes were cut to obtain a flat surface. Staining with DAPI and microscope analysis were performed as described.

6.3.4.5 Detection of apoptosis: Immunocytochemistry with M30 CytoDeath antibody

M30 CytoDeath monoclonal antibody (Roche Diagnostics, Basel, Switzerland, #2140322) detects a specific formalin-resistant caspases cleavage site within cytokeratin 18. Cytokeratins, in particular cytokeratin 18 that is cleaved by caspase-3 or -7, are affected in the very early events of apoptosis [165, 166]. Therefore, this epitope is present before disruption of membrane asymmetry and before DNA strand-break occurs [167].

Immunocytochemistry was performed as follows: Cells were grown and treated with 1 μM UCN-01 on 22-cm^2 Petri-dishes. At different time points after treatment, medium was removed and adherent cells were rinsed in PBS and fixed 30 min in ice-cold methanol. The borders of Petri-dishes were cut to obtain a flat surface. After washing in 0.1% Tween-20 in PBS, cells were blocked for 10 min with 1% albumin bovine (BSA) diluted in 0.1% Tween-20 in PBS. After removal of the blocking solution, they were incubated 1 hr with M30 CytoDeath antibody diluted 1:10 in 1% BSA in 0.1% Tween-20 in PBS. Cells were washed 3 times in 0.1% Tween-20 in PBS and incubated 30 min in a solution of tetramethylrhodamine isothiocyanate isomer R (TRITC)-conjugated anti-mouse immunoglobulins (Dako Diagnostika, Hamburg, Germany) at a concentration of 0.01 μg/μl in 1% BSA in 0.1% Tween-20 in PBS. After three washes with 0.1% Tween-20 in PBS, cells were incubated for 10 min in a solution of 1 μg/ml DAPI. Afterwards they were washed 3 times in PBS. Fluoromount G was used as mounting medium. DAPI-associated fluorescent staining and M30 antibody-associated red staining were detected by fluorescent microscopy.

6.3.5 Cell cycle analyses

6.3.5.1 Synchronization of cells in S-phase of the cell cycle

HCT116 and HCT116+ch3 cells were synchronized in S-phase by treatment with aphidicolin, a mycotoxic agent specifically inhibiting DNA synthesis by interacting with DNA polymerase α [168].

Aphidicolin was stored at -20°C dissolved in DMSO at a final concentration of 5.7 mM. It was used at a final concentration of 1 μM. Cells were pretreated for 1 day with aphidicolin, then UCN-01 was added for the successive day in the presence of aphidicolin, or vehicle alone was added. Samples were thereafter collected for FACS analysis (6.3.5.5).

6.3.5.2 Synchronization of cells in mitotic-phase of the cell cycle

HCT116, HCT116+ch3 and HCT116 $p53^{-/-}$ cell lines were synchronized in M-phase by treatment with nocodazole and colcemid, two compounds that similarly bind to tubulin and prevent the polymerization of microtubules, thus inhibiting mitosis [169].

Nocodazole was dissolved in DMSO at a concentration of 13.2 mM and stored at -20°C. It was employed at a final concentration of 166 nM (0.05 μg/ml), with different protocols: In the experiment represented in **Fig. 15**, HCT116 and HCT116+ch3 were treated 24 hr with 1 μM UCN-01, then nocodazole was added in the presence of UCN-01 for the successive 17 hr; in the experiment represented in **Fig. 17**, HCT116 and HCT116+ch3 cells were pretreated 3 hr with nocodazole, then 1 μM UCN-01 was added for the successive 24 hr or vehicle alone was added; in the experiment outlined in **Fig. 29**, HCT116 $p53^{-/-}$ cells were simultaneously exposed to CPT-11 (4.5 μM) and to nocodazole for 2 days.

Colcemid was stored at -20°C diluted in PBS at a final concentration of 2.7 mM. It was used at a final concentration of 54 nM (2.0 ng/ml). Cells were pretreated 24 hr with colcemid, then colcemid-containing medium was removed, the cells were washed with PBS, and UCN-01-containig medium was added for the successive 24 hr or vehicle alone was added.

It is shown in the present work that aphidicolin, an agent arresting cells preferentially in S-phase (6.3.5.1) [168], was inducing a G2/M-phase arrest when cells were treated with this agent at 1 μM for 2 days. After this period, UCN-01 was added in the presence of aphidicolin for the successive day or vehicle alone was added.

Samples were collected for FACS analysis (6.3.5.5) and for Western blot (6.4.1-6.4.2).

6.3.5.3 Mitotic index measurement

HCT116 and HCT116+ch3 cells were subjected to treatment with UCN-01 (1 μM) for 24 hr. After this period, nocodazole (166 nM) was added for the successive 17 hr in the presence of UCN-01. At the end of this period, total cell density was determined. From 0.2 to 0.4 x 10^6 cells were transferred into a new Eppendorf cup, centrifuged for 5 min at 1 500 x g and the supernatant was removed. Cells were washed in PBS and centrifugated for 5 min at 1 500 x g. The supernatant was removed and cells were resuspended and incubated for 10 min in 75 mM KCl [170]. After centrifugation for 5 min at 1 500 x g, cells were resuspended in 1 ml of a fixative solution composed of acetic acid:methanol 1:3 (v/v). From 1 to 2 x 10^4 cells, corresponding to 50 μl of the cell mixture in acetic acid:methanol, were centrifuged onto slides and allowed to air-dry. Cells were rinsed in PBS and stained with 1 μg/ml DAPI for 5 min. Slides were washed in PBS, then mounted with fluoromount G and evaluated under a fluorescence microscope. Cells arrested in mitosis by nocodazole were detectable, as their chromosomes are aligned in the metaphase plate (metaphase-anaphase stage of mitosis) [169]. 500 nuclei were counted for each sample. The percentage of mitotic cells was defined as 100 x (n° of mitotic nuclei) / (n° total nuclei).

6.3.5.4 Abrogation of G2/M-phase arrest

Caffeine is an agent capable of override the DNA-damage-induced G2/M-phase arrest, through its ability to inhibit DNA-damage-induced chk2 protein and ATM/ATR kinases [171]. Caffeine was dissolved in deionized H_2O to a final concentration of 210 mM and kept at 4°C. Before use, this solution was thawed at 37°C for about 10 min.

Cells were treated for 2 days with 1.5 mM caffeine, added simultaneously with CPT-11. Thereafter, cells were allowed to grow in drug-free medium up to 6 days from the start of treatment. Samples were collected for FACS analysis (6.3.5.5).

6.3.5.5 Flow cytometric analysis (FACS)

A common application of flow cytometry, a more generic term to indicate FACS (fluorescence activated cell sorter), is measurement of DNA content to obtain the cell cycle distribution of cells sub-populations. Cycling cells undergo four distinct phases of the cell cycle (G1, S, G2 and M) characterized by a different DNA content. Measuring DNA content allows determining whether a cell is in G0/G1- (2N), S- (from 2- to 4-N), or G2/M-phase (4N) of the cell cycle. DNA content < 2N (subG1 peak), indicates fragmented DNA and reflects apoptosis [161]. DNA content > 4N indicates endoreduplication and reflects hyperploidy.

Cells were seeded on Petri-dishes for 1 day and then treated with the chemotherapeutic drugs at the concentrations and time indicated. After trypsinization of the adherent cells, adherent and floating cells were collected, and total cell density was determined as described in paragraph 6.3.1.1. 1 x 10^6 cells were placed in a new Eppendorf tube and centrifuged 5 min at 1 500 x g. Supernatant was discarded, and the pellet was resuspended in cold PBS and centrifuged. After removal of the supernatant, the pellet was resuspended in 1 ml of an ice-cold solution of 70% ethanol in PBS (v/v). This cells solution was kept at -20°C for at least 2 hr, this improving the quality of the DNA histogram. Fixed cells were centrifuged 5 min at 1 500 x g, resuspended in 1 ml PBS, and the suspension was transferred to polystyrene round-bottom tubes. A further step of centrifugation followed, for 5 min at 1 500 x g. The supernatant was discarded and fixed cells were stained by suspension in 1 ml of a solution composed of the propidium iodide dye (20 μg/ml) and RNase A (200 μg/ml, made DNase free by boiling for 5 min) dissolved in 0.1% Triton X-100 in PBS. Incubation followed for 30 min in the dark. Propidium iodide (PI) intercalates into double-stranded nucleic acids and stains DNA. Since it also binds to double-stranded RNA, this latter was removed by treatment of the samples with RNase A. Flow cytometry analysis was performed not more than three hours later. Stained nuclei were analyzed using a Becton Dickinson FACScalibur,

equipped with a doublet discrimination module. ModFit LT2 software (Verity Software House, Topsham, ME, USA) was used to assess the cell cycle status. Marie-Luise Hanski performed flow cytometry analyses routinely.

6.4 Protein biochemistry

6.4.1 SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gels allow separation of the proteins into their individual polypeptide subunits. The SDS-bound negatively-charged polypeptides migrate through polyacrylamide gels in accordance with their molecular size. By using marker of known molecular weight, is therefore possible to estimate the molecular weight of the polypeptides.

At different time points after treatment, adherent cells were scraped off and pooled with the floating cells. After two washes in cold PBS, pellets were resuspended in one volume of ice-cold lysis buffer for 15 min at 4°C. Lysates were precleared by centrifugation at 13 000 x g for 15 min at 4°C. The supernatant was separated in two aliquots. One was employed for the determination of proteins concentration, while to the other 5X SDS gel-loading buffer was added and the sample immediately boiled for 5 min. This step ensures the dissociation of proteins, and binding of the denatured polypeptides to SDS confers them a negative charge.

Proteins concentration was determined using the Bradford assay. This assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. It is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs [172]. A calibration curve was prepared choosing as standard BSA. As blank, lysis buffer was added instead of protein lysate. After 15 min, absorbance was photometrically measured at 595 nm against the blank sample. The standard curve (μg protein/OD_{595}) was plotted and resulted in a linear function. Sample protein concentrations were determined extrapolating with the standard curve from the OD_{595} values the corresponding μg. This number was corrected for the volume and for the dilution in 5X SDS gel-loading buffer to obtain the protein concentration (μg/μl) of

the samples diluted in 5X SDS gel-loading buffer. Samples were further diluted (in lysis buffer + 5X SDS gel-loading buffer 4+1 v/v) to have a final protein concentration of 1 μg/μl, in order to load on the gel same volumes of samples.

SDS-polyacrylamide electrophoresis was carried out in vertical apparatus, in electrophoresis tanks and plates designed by Bio-Rad, Hercules, CA, USA. The effective range of separation of SDS-polyacrylamide gels depends on the concentration of polyacrylamide used in the resolving gel and on the amount of cross-linking, this being dependent on the molar ratio of acrylamide/bisacrylamide. In dependence of the molecular weight of the protein to be separated, resolving SDS-polyacrylamide gels were designed with different acrylamide concentrations, from 7.5 to 15.0%. After polymerization of the resolving gel (30 min), the stacking gel was poured (3% acrylamide/bis, 0.124 M Tris pH 6.8, 0.1% SDS, 0.1% TEMED, 0.1% APS). A 10-well comb was inserted, and the gel was left to polymerize for 30 min. After this time, the gel was mounted in the electrophoresis apparatus. Tris-glycin electrophoresis buffer was added to the top and bottom reservoirs. Samples were loaded into the bottom of the wells with a microliter syringe. As marker, 10 μl of Color Marker Wide Range (#C3437, Sigma) was loaded. Separation followed at 100 V for 60-90 min. Afterwards, the gel was detached from the glass slides and Western blot followed.

6.4.2 Western blot

With this technique, proteins electrophoretically separated are transferred to a solid support that is probed with antibodies that react specifically with antigenic epitopes displayed by the target proteins attached to the solid support. This method has been used in order to identify and quantify specific proteins in complex mixtures of proteins.

Western blot was carried out by electrophoretic transfer of proteins from the gel to a PVDF membrane. To decrease lateral diffusion due to warming, the transfer was accomplished in an ice-cooled chamber on a stirrer at a constant current of 0.2 mA for a period of 2 hr.

After this period, the non-specific binding sites were saturated by incubation for 1 hr on a shaker in 5% non-fat dried milk in 0.1% Tween-20 dissolved in PBS or TBS buffers. TBS buffer was used for analysis of phospho-proteins. Afterwards, membranes were

washed once in 0.1% Tween-20 in PBS or TBS and incubated overnight at 4°C with gentle agitation on a platform shaker with an antibody directed against the target protein. The first antibodies were diluted in 0.1% Tween-20 in PBS or TBS. The following first antibodies were employed:

Antibody specificity	**Mr of the antigen (kDa)**	**Species and antibody class**	**Dilution and/or concentration (μg/ml)**	**Purchased from**	**Clone number and catalogue number**
β-actin	42	Mouse IgG (from ascites)	1:200 000	Sigma, Steinheim, Germany	Clone AC-74, #A5316
Bax	21	Rabbit IgG (from antiserum)	1:10 000 (0.02 μg/ml)	Santa Cruz Biotechnology, Heidelberg, Germany	Clone N-20, #sc-493
Bcl-2	29	Rabbit IgG (from antiserum)	1:100 (2 μg/ml)	Santa Cruz Biotechnology, Heidelberg, Germany	Clone N-19, #sc-492
Bcl-X_L	28	Mouse IgG (monoclonal)	1:500 (1 μg/ml)	BD Pharmingen, Lexington, Kentucky, USA	Clone 2H12, #556499
cdc2	34	Rabbit IgG (from antiserum)	1: 10 000 (0.02 μg/ml)	Santa Cruz Biotechnology, Heidelberg, Germany	Clone H-297, #sc-747
cdc2-P (Tyr-15)	33	Rabbit IgG (from antiserum)	1: 5 000 (0.034 μg/ml)	Cell Signaling, Frankfurt, Germany	#9111
cdk2	33	Mouse IgG (monoclonal)	1: 5 000 (0.05 μg/ml)	BD Transduction Laboratories, Lexington, KY, USA	Clone 55, #610145
cdk4	33	Mouse IgG (monoclonal)	1:500 (0.5 μg/ml)	BD Transduction Laboratories, Lexington, KY, USA	Clone 97, #610147

cyclin B1	62	Rabbit IgG (from antiserum)	1: 10 000 (0.02 µg/ml)	Santa Cruz Biotechnology, Heidelberg, Germany	Clone H-433, #sc-752
cyclin D1	36	Rabbit IgG (from antiserum)	1:3 000 (0.3 µg/ml)	NeoMarkers, Fremont, CA, USA	Clone Ab-3, #RB-010-PO
ERK-P	42 - 44	Rabbit IgG (from antiserum)	1:3 000 (0.016 µg/ml)	Cell Signaling, Frankfurt, Germany	#9101
hMLH1	80	Mouse IgG (monoclonal)	1: 3 000 (0.166 µg/ml)	BD Pharmingen, Lexington, Kentucky, USA	Clone G168-15, #554072
hMSH2	102	Mouse IgG (monoclonal)	1: 500 (0.5 µg/ml)	BD Transduction Laboratories, Lexington, KY, USA	Clone 27, #610360
$p21^{CIP1}$	21	Rabbit IgG (from antiserum)	1: 3 000 (0.066 µg/ml)	Santa Cruz Biotechnology, Heidelberg, Germany	Clone C-19, #sc-397
$p27^{KIP1}$	27	Mouse IgG (monoclonal)	1: 2 500 (0.1 µg/ml)	BD Transduction Laboratories, Lexington, KY, USA	Clone 57, #610241
p53	53	Mouse IgG (monoclonal)	1: 3 000 (0.083 µg/ml)	Dako, Glostrup, Denmark	Clone DO-7, #M7001
PARP	116 and 85	Mouse IgG (from ascites)	1: 4 000	BD Pharmingen, Lexington, Kentucky, USA	Clone C2-10, #556362
Rb	105-116	Mouse IgG (monoclonal)	1: 500 (1 µg/ml)	BD Pharmingen, Lexington, Kentucky, USA	Clone G3-245, #554136

Membranes were subsequently washed 3 times with 0.1% Tween-20 in PBS or TBS for 30 min (10 min each wash) on a platform shaker at room temperature. Tween-20 PBS/TBS solution was discarded and the second antibody (diluted in 0.1% Tween-20 in PBS or TBS) was applied for 1 hr at room temperature with gentle agitation on a

platform shaker. The following second antibodies, both of them conjugated to horseradish peroxidase, were employed:

Antibody specificity	Antibody class	Dilution or concentration (μg/ml)	Purchased from	Catalogue number
Rabbit IgG (H+L)	Goat	1:1 500 (0.5 μg/ml)	Dianova, Hamburg, Germany	#111-035-003
Mouse IgG+IgM (H+L)	Goat	1:3 000 (0.25 μg/ml)	Dianova, Hamburg, Germany	#115-035-044

Then, the second antibody solution was discarded and membranes were washed three times, 10 min each time, in 0.1% Tween-20 in PBS or TBS at room temperature with agitation on a platform shaker. The membrane was thereafter incubated for 5 min with the chemiluminescent substrate Super Signal West Pico. Membranes were wrapped in plastic bags and a luminescent ruler was applied at one corner of the bags. Membranes were exposed to T-Mat Plus DG autoradiographic films from 1 second to 5 min, depending on the intensity of the signal. To assess the relative protein expression, films were scanned, and the band density was evaluated with TINA software.

6.4.3 Immunoprecipitation of cdk2 and cdc2/cyclin B1 complexes and determination of their kinase activities

In this procedure, mouse antibody directed against the target protein is added to aliquots of cell lysates and the immunecomplexes are sedimented with an anti-mouse antibody coupled to agarose.

Cells were treated with UCN-01 or CPT-11 for 2 days and were lysed at the indicated time points in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF, 10 μg/ml of aprotinin, pepstatin A and leupeptin). 500 μg of protein lysate was used for each immunoprecipitation. For cdk2 immunoprecipitation, the lysates were precleared by incubation with 20 μl of rabbit serum-agarose (Sigma, #R6755) while for cdc2/cyclin B1 immunoprecipitation

lysates were precleared by incubation with 60 µl of mouse IgG-agarose (Sigma, #A6531) for 1 hr at 4°C with gentle agitation. The suspensions were centrifuged 1 min at 13 000 x g, then the supernatants were transferred to new Eppendorf cups. For cdk2 immunoprecipitation, 20 µl of agarose-conjugated anti-cdk2 antibody (Santa Cruz, #sc-163AC) were added to the supernatant, while for cdc2/cyclin B1 immunoprecipitation 3 µg of anti-cyclin B1 antibody (Santa Cruz, #sc-245) were added to the supernatants and left to react overnight at 4°C with gentle agitation. The cdk2 immunoprecipitate was recovered by centrifugation. The cdc2/cyclin B1 immunoprecipitate was recovered by adding anti-mouse IgG-agarose (Sigma, A6531) for 1 hr at 4°C. The supernatant was discarded and the immunocomplexes were washed 2 times with lysis buffer and 3 times with 1X kinase buffer.

The kinase reaction was carried out in a volume of 20 µl of kinase buffer containing 10 µg of histone H1 (Roche Molecular Biochemicals, #223549), 5 µCi [γ-^{32}P]ATP and 25 µM nonradioactive ATP. The samples were incubated at 30°C for 30 min. The reactions were stopped by adding an equal volume of 2X SDS gel-loading buffer. The samples were boiled for 5 min, centrifuged, the supernatant was recovered and counted for radioactivity. Equal numbers of counts per lane were applied to a 12.5% SDS-PAGE. The gel was dried and autoradiographed. M. Bhonde and M.-L. Hanski carried out immunoprecipitations.

6.4.3.1 Detection of cdk2, p27^{KIP1}, p21^{CIP1}, cyclin B1, and cdc2 in the immunoprecipitates

The cdk2 or cdc2/cyclin B1 immunoprecipitates were subjected to 12.5% SDS-PAGE, blotted and detected with anti-cdk2- (BD Transduction Laboratories, #610145), anti-p27^{KIP1}- (BD Transduction Laboratories, #610241), anti-p21^{CIP1}- (Santa Cruz Biotechnology, #sc-397), anti-cyclin B1- (Santa Cruz Biotechnology, #sc-752) and anti-cdc2- (Santa Cruz Biotechnology, #sc-747) antibodies.

6.4.4 Inhibition of the MAP kinase pathway

The two MEK1/2 inhibitors PD98059 and U0126 were stored at -20°C under light-protection in DMSO at a concentration of, respectively, 18.5 mM and 10 mM. PD98059 was used at a final concentration of 5 μM; U0126 was used at a final concentration of 20 μM. Cells were pretreated with PD98059 and U0126 for 30 min. After this lap of time, UCN-01 (1 μM) was added and the cells were harvested 24 hr after addition of UCN-01.