From the Department of Radiotherapy and Radiation Oncology
(Prof. Dr. med. Dr. rer. nat. C.-F. Hess)
in the Center for Radiology of the
Faculty of Medicine, University of Göttingen

Experimental study of the combined effect of irradiation, lovastatin, and monoclonal antibodies on tumour and normal tissue cell lines. Its genesis and mechanisms of action

INAUGURAL – DISSERTATION
for the degree of Doctor
of the Faculty of Medicine
Georg-August-University of Göttingen

presented by
Petra Miglierini

from
Bratislava, Slovakia

Göttingen 2014

Dean: Prof. Dr. rer. nat. H. K. Kroemer

I. Correspondent: PD Dr. med. H. A. Wolff
II. Correspondent: Prof. Dr. R. P. Virsik-Köpp
III. Correspondent: Prof. Dr. R. Mausberg

Day of examination: August 20, 2014

Table of Contents

1 Introduction

1.1 Purpose of the present work

The main aim of the following study was to assess whether statins, known for their lipid-lowering potential, do indeed possess also an anticancer and, eventually radiosensitizing/radioprotective effects on different tumour and normal tissue cell lines. Impact of statin addition to concomitant radiochemotherapy based on monoclonal antibodies (cetuximab and bevacizumab) has been evaluated as well. This has been examined using methods of cell survival, cell viability, and several methods of molecular biology (Western blot, reporter assay, caspase activity).

1.2 Statins

1.2.1 Definition of statins

Statins are 3 - hydroxyl - 3 methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors that are widely used to lower levels of serum cholesterol in primary and secondary prevention of cardiovascular diseases (Zhou and Liao 2010; Wang et al. 2008). Recent clinical and experimental evidence suggests that the beneficial effects of statins may extend beyond their cholesterol-lowering effect and exert the pleiotropic effects.

Statins were initially isolated and identified as secondary metabolites of fungi. In 1980, Alberts et al. isolated an active fungal inhibitor of HMG-CoA reductase inhibitor named lovastatin (mevinolin) from *Aspergillus terreus* (Alberts et al. 1980; Alberts 1988). Statins in general inhibit the rate-limiting step of cholesterol biosynthesis, the conversion of HMG-CoA to l-mevalonic acid, through binding to HMG-CoA reductase's active site and blocking the substrate product transition state of the enzyme (Istvan and Deisenhofer 2001). This leads to decreased hepatic cholesterol synthesis, upregulation of low-density lipoprotein (LDL) receptor, and increased clearance of plasma LDL-cholesterol. In addition, by inhibiting HMG-CoA reductase, statins could also inhibit the synthesis of important isoprenoid intermediates, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) that lie downstream from l-mevalonic acid (Goldstein and Brown 1990). These intermediates serve as important lipid attachments for the post-translational modification of intracellular proteins such as nuclear lamins, Ras, Rho, Rac and Rap (Van Aelst and D'Souza-Schorey 1997). Thus, it is possible that, in addition to cholesterol lowering, the inhibition of these intracellular isoprenoid-dependent proteins may contribute to some of the biological effects of statins (see figure 1.1).

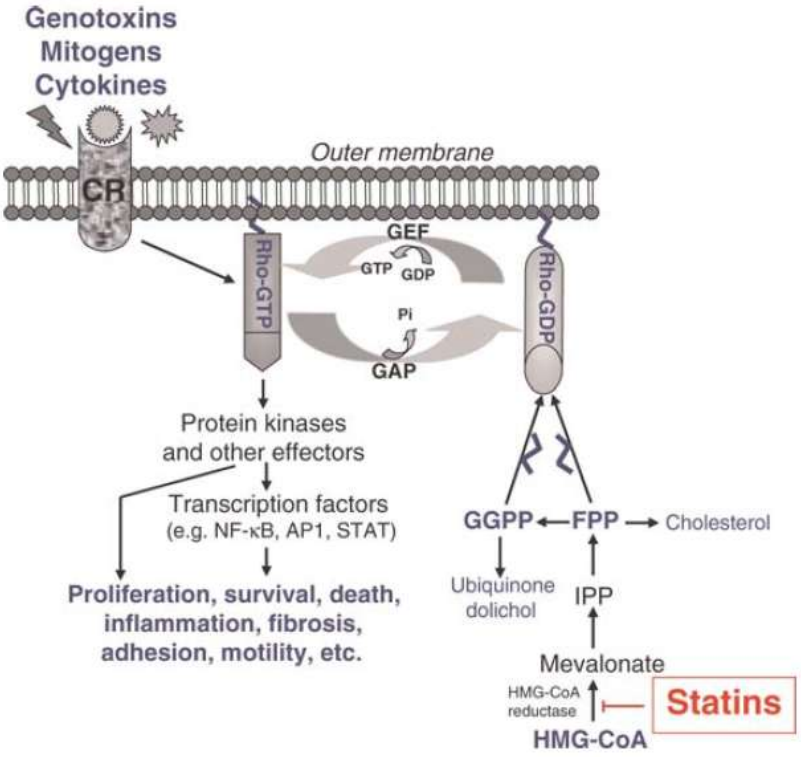


Fig. 1.1: Mechanisms of action of statins (from Fritz et al. 2011, p. 18).

Indeed, HMG-CoA reductase is a rate-limiting enzyme involved in the so-called mevalonate pathway that aims at generating several fundamental end-products such as cholesterol and isoprenoids. Apart of all, dysregulation of this mevalonate pathway seems to have certain oncogenic potential to drive tumorigenesis. HMG-CoA itself appears to be capable of promoting the transformation of transformed, nontransformed, and normal cells, too (Clendening et al. 2010). How this dysregulation occurs is not yet well defined. The overlap between essential mevalonate pathway dependent functions and many processes involved in tumour cell metabolism and oncogenesis elucidates how statins may exhibit tumour-selective anticancer activity (Clendening and Penn 2012).

Lovastatin

Within all existing statins, lovastatin is probably the most widely studied one. This one, being the first available statin on market since 1987 (Grundy 1998), has demonstrated anticancer properties *in vitro* and *in vivo* (Chan et al. 2003). Preclinical data of lovastatin on animals (including mouse, rat, rabbit and dog) revealed linear pharmacokinetics. Doses close to 200 mg/kg/day would produce serum concentrations in the range of 2 – 20 μM. Circulating serum concentrations of 2 – 4 μM were well tolerated for months in all animal models whereas

levels of 20 – 25 μM were associated with progressive anorexia and death in rabbits (Kornbrust et al. 1989).

The therapeutic dose for the treatment of hypercholesterolemia is approximately 1 mg/kg/day which yields serum concentration of 0.1 μM.

A phase I study aimed to define the highest tolerable dose of lovastatin in men (Thibault et al. 1996). This HMG-CoA reductase inhibitor was administered in patients with a confirmed solid tumour at different concentration, ranging from 2 to 45 mg/kg/day (2, 4, 6, 8, 10, 25, and 45 mg/kg/day) over 7 consecutive days in monthly cycles. Lovastatin serum levels were examined as well throughout the treatment course. These have been revealed to be in the range of 0.1 to 3.92 μM regardless the dose of lovastatin administered. Regarding the toxicity, most commonly described side effects were gastrointestinal problems (nausea, anorexia, and diarrhoea) which represented 56 % of all episodes. The most severe side effects were related to musculoskeletal system (muscle weakness, myalgia). They have occurred at lovastatin doses starting at 25 mg/kg/day and did not differ in higher doses.

The cytostatic effect of lovastatin has been evaluated on various tumour cell lines (adenocarcinoma, melanoma, neuroblastoma) and a half maximal inhibitory concentration (IC_{50}) in the range of 0.3 – 2 μM has been found (Prasanna et al. 1996). This could make lovastatin a promising drug. However, studies on other cell lines (glial tumour, prostate cancer) demonstrated that the levels of lovastatin required to induce apoptosis may be as high as 30 – 100 μM that is not life compatible in animal models and could be related to important toxicity in man.

1.2.2 Anticancer activity of statins

During the past 20 years, a large amount of studies have demonstrated the antiproliferative and proapoptotic effect of statins both *in vitro* and *in vivo* models of cancer (Sanli et al. 2011). Growth inhibition, cell cycle arrest, and induction of apoptosis in cancer cells have been demonstrated (Sassano and Platanias 2007). The interest in these drugs was enhanced by epidemiological studies indicating that patients being treated by statins may have lower risk of development of colorectal carcinoma (Broughton et al. 2012; Poyntner et al. 2005) or lung cancer (Khurana et al. 2007). Promising results were as well observed among men taking metformin for type 2 diabetes associated to statins intake (Lehman et al. 2012). This combination showed to be beneficial in the term of reduction of prostate cancer incidence in comparison to those taking neither statin nor other medication (statins or oral antidiabetics).

Furthermore, once a prostate cancer is diagnosed, statin use is suggested to decrease the risk for advanced and metastatic cancer in epidemiological studies (Platz et al. 2006) to slow the disease progression after radical prostatectomy (Hamilton et al. 2010), and to reduce the disease recurrence in patients treated with curative radiotherapy (Kollmeier et al. 2011; Gutt et al. 2010).

Statin use, and especially lipophilic statin (simvastatin), in women with stage I – III breast carcinoma was associated with a reduced risk of breast cancer recurrence (Ahern et al. 2011). In this population-based prospective cohort study, no association between hydrophilic statin use and breast cancer relapse was observed.

Under *in vitro* conditions, HMG–CoA reductase inhibitors have been shown to synchronize tumour cells by blocking the transition of G1-S in the cell cycle and thereby exerting their antiproliferative effect (Keyomarsi et al. 1991). Apoptosis induced by statins appears to be mediated predominantly through depletion of geranylgeranylated proteins (Xia et al. 2001) and lovastatin itself seems to decrease the expression of the antiapoptotic protein Bcl-2 and increase the expression of the proapoptotic protein Bax (Agarwal et al. 1999).

1.2.3 Statins and irradiation

1.2.3.1 Radiosensitizing effect of statins

The potential radiosensitizing effect of statins could be explained by the arrest of cells in the late G1 phase of the cell cycle in which cells are more sensitive to radiation-induced cell death as in the S-phase (Chan et al. 2003).

However, cell sensitivity to irradiation is determined by other numerous factors. The most important are DNA repair and radiation-induced signalling mechanisms that cause changes in gene expression, cell cycle progression, and apoptosis (Cortez et al. 2001). DNA damage caused by irradiation causes activation of DNA damage-specific kinases ATM/ATR and DNA-PKcs (Iliakis et al. 2003; Cortez et al. 2001; Yang et al. 2003). Subsequently, downstream functions such as p53 and checkpoint kinases are activated and result in changes in repair and cell cycle progression and, probably, induction of cell death (Sancar et al. 2004). Apart from DNA damage–triggered functions, irradiation also causes activation of cell surface receptors that eventually lead to the activation of mitogen-activated protein kinases (MAPK) and transcription factors, e.g. activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). Similar to DNA damage–triggered stress responses, signal mechanisms originating from activated cell receptors also affect the cellular susceptibility to irradiation (Chen et al., 1996).

A pharmacologic approach for intervening with radiation-induced stress responses is based on the fact that Ras and Rho GTPases, which are required for genotoxic stress-stimulated activation of MAPK and NF-κB, are subject to COOH-terminal prenylation. Attachment of a C15 or C20 lipid moiety to the cystein of the COOH terminal– located CAAX box is essential for the physiologic activity of Ras/Rho because it is required for their correct localization at the cell membrane. Statins cause depletion of the cellular pool of isoprene precursor molecules. Thereby, statins eventually lead to a down-modulation of Ras/Rho-regulated signal mechanisms (Walker and Olson 2005). The Ras-related GTPase RhoB affects the susceptibility of cells to killing by γ-rays and Ras dependent mechanisms interfere with γ-ray-triggered cellular stress responses and cell survival as well. Furthermore, inhibitors of farnesylation, which affect Ras- and RhoB regulated signalling, modulate cellular resistance to tumour-therapeutic drugs and irradiation (McKenna et al. 2002). This is also the case of statins. Therefore, the combination of inhibitors of the Ras/Rho pathways with radiotherapy appears to be a promising experimental strategy in cancer treatment (Gabryś et al. 2008). However, because of a not very tumour specific cytotoxic effect of statins, apoptosis has been observed in normal tissue cells as well, e.g. in HUVEC (Li et al. 2002).

Beneficial antiproliferative and radiosensitizing effects of statins have been already documented in various *in vitro* studies on different tumour cell lines. Gabryś et al. (Gabryś et al. 2008) studied U87MG glioblastoma cell line and FaDu squamous cell head and neck carcinoma associated with lovastatin. Using *in vitro* models, they documented an accumulation of the cells in G0-G1 phase of the cell cycle *in vitro* associated with a significant decrease of tumour cell proliferation. Nevertheless, the described combination of lovastatin and irradiation had similar antiproliferative effect as the lovastatin alone.

Sensitizing a cervical carcinoma cell line, HeLa cells, to irradiation by lovastatin has been shown. This could be related to an abrogation of the radiation-induced G2 block and an increase in apoptotic and necrotic cell death (Fritz et al. 2003). Cell death through apoptosis has been observed as a mechanism of radiosensitizing effects demonstrated on lung cancer cells (A 549) if a combination of lovastatin and irradiation (Sanli et al. 2011) was used.

1.2.3.2 Radioprotective effect of statins

Despite improved radiation techniques (e.g. intensity modulated radiation therapy (IMRT)) aiming at reducing the radiation-induced side effects, the latter are still clinically highly relevant as it is for acute or for chronic effects. These are mostly driven by the production and release of

pro-inflammatory cytokines from death or differentiated cells as well as the upregulation of the endothelial cell adhesion molecules (e.g. E-selectin) which promote inflammatory processes (Hallahan et al. 1996). As a consequence of reactive and reparative processes of normal tissue, fibrotic tissue remodelling occurs. This results in severe and irreversible damaged tissue architecture that may lead to important organ dysfunctions. Hence, different strategies of radioprotection are being explored.

As for pharmacological approach, reduction of radiation-induced DNA damage and inhibition of pro-apoptotic DNA-damage repair systems seem to be a desired therapeutic target. However, 'non-target' (i.e. DNA damage independent) effects of radiation therapy are supposed to be strongly harmful as well. Thus, inhibition of pro-inflammatory and pro-fibrotic stress responses regulated by such pathways as Rho/NF-κB and Rho/ROCK, respectively (where Rho GTPases are localized in the outer cell membrane) is a very tempting therapeutic way, too.

Statins have anti-inflammatory properties and therefore appear to be ideal candidates for protecting normal tissue from the acute and chronic toxicity provoked by radiotherapy (Fritz et al. 2011).

In vitro data showed that statins abolish radiation-induced activation of NF-κB (Nübel et al. 2006; Ostrau et al. 2009) which is the key transcription factor required for the expression of interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α). Furthermore, statins diminish radiation-induced expression of transforming growth factor beta (TGF-β) and its downstream effector connective tissue growth factor (CTGF) where both play a role in fibrosis (Haydont et al. 2005).

Pre-clinical *in vitro* and *in vivo* studies published up to now consider a pleiotropic effect of statins (apart of all anti-inflammatory and anti-fibrotic) to be beneficial in terms of protection against radiation-induced tissue harms.

1.3 Targeted therapy and irradiation

Recently, several new targeted drugs appeared and tend to be promising therapeutic candidates with comparatively low toxicity profile due to their targeted action (Niyazi et al. 2011). However, beside few exceptions, the possible toxicity of the targeted treatment and radiotherapy has not been studied into details yet. Along with the action of targeted therapy on neoplastic signaling pathway we should not omit the fact that there exists a considerable overlap between cancer and normal cell signaling pathways. Therefore, in a case of association of these to

irradiation this may result in worsening of the already present radiation induced side effects on normal tissue (Mangoni et al. 2012).

The idea of combining targeted treatment instead of standard chemotherapy and radiotherapy is very seductive. Since at present we do not have enough information about their real interactions within normal cells (and also possible negative side effects) one of the solutions could be to define an agent that would potentially enhance the cytotoxic effect of the targeted drug without further harm on normal tissue and permit to reduce the effective dose to a minimum. Or, otherwise, to find an agent that would be able to provide a radioprotection to in-field organs at risk. If such an agent would possess a relatively good radiosensitizing effect this could be the reason for its use instead of any other chemotherapy or even targeted therapy.

1.3.1 Anti-EGFR and anti-VEGF therapy

The epidermal growth factor receptor (EGFR, HER-1, c-erbB-1) is a 170 kDa transmembrane protein consisting of an extracellular EGF-binding domain, a short transmembrane region, and an intracellular domain with ligand-activated tyrosine kinase activity (Cohen et al. 1982). Two ligands can activate EGFR: EGF and transforming growth factor alpha (TGF-α). Once ligands are attached to a receptor, an increased synthesis of DNA is triggered as well as the proliferation and differentiation of target cells (Chen et al. 1989). ErbB-1 is a member of the EGFR family that consists of different oncogenes ranging from erbB-1 to erbB-4 (Barnea et al. 2013).

EGFRs are expressed in various normal epithelial tissues and can be detected using antibody staining in such tissues like epidermal cells of skin, oesophagus, kidney, testis, placenta, and prostate.

Overexpression of EGFR is present in many neoplasias (endometrial carcinoma, squamous cell carcinoma, adenocarcinoma or neuroendocrine lung tumour, head and neck squamous cell carcinoma, or glioblastoma multiforme). High expression is often, at least in head and neck cancer, correlated with worse prognosis of the disease.

Anti-erbB-1 (cetuximab) is a chimeric human/mouse monoclonal antibody that binds specifically to the extracellular domain of the receptor and prevents ligand binding and activation of downstream signalling pathway. The radiosensitizing effect of cetuximab has been explained by several potential mechanisms including regulation of cell cycle progression, blockage of radiation-induced EGFR transport into the nucleus, and interference with DNA repair mechanisms (Saki et al. 2012).

The vascular endothelial growth factor (VEGF) belongs to a family of five related mammalian growth factors: VEGFA (the prototype), VEGFB, VEGFC, VEGFD, and PlGF (placental growth factor). They are homodimeric polypeptides although naturally occurring heterodimers of VEGFA and PlGF have been described (Koch et al. 2011).

The VEGF receptors play a pivotal role in the maintenance of vascular integrity, endothelial cell survival, and angiogenesis (O'Reilly 2006). Radiotherapy *per se* may have a systemic/local effect on angiogenesis since increased expression of pro-angiogenic factors such as VEGF have been observed after irradiation (Gorski et al. 1999). In this context, drugs targeting VEGF (e.g. anti-VEGFA = bevacizumab) have been developed aiming at inhibiting angiogenesis and act as anticancer treatment. Unfortunately, angiogenesis is not tumour restricted but it is also found in many other physiological and pathological conditions (e.g. normal growth, wound healing, inflammation, etc.). Hence anti-angiogenic therapy alone or combined with other treatment approaches (such as radiotherapy) may increase normal tissue toxicity (Mangoni et al. 2012).

1.3.2 Target therapy and statins

As it was observed in the study of Sanli and his co-workers (Sanli et al. 2011), lovastatin possesses an ability to selectively abrogate EGF-induced phosphorylation of EGFR as well as that of its downstream effector protein Akt. This information could be very promising as far as irradiation itself activates the downstream effector pathway of EGFR such as PI3k – Akt – mTOR (mammalian target of rapamycin) and the Raf – MEK 1 – ERK (Park et al. 2006; Zimmermann et al. 2006). These are known to mediate cell survival and radiation resistance, gene expression, and protein synthesis (Nakamura et al. 2005; Le Tourneau and Siu 2007). EGFR is also involved in the development and progression of cancers derived from these tissues including squamous cell carcinomas of the head and neck and of the cervix, non–small cell lung carcinomas (NSCLC), and colon cancer (Mantha et al. 2005). Thus, blocking the radiation-induced EGFR activation process and/or its downstream pathways would possibly enhance cell death and render cells more radiosensitive. This is nowadays the role of so called anti-EGFR molecular antibodies or inhibitors of tyrosin-kinase receptors.

However, response to these molecules is strongly attributed to the presence versus absence of mutations affecting residues contributing to the ATP binding site of the EGFR (Lynch et al. 2004; Paez et al. 2004). Nevertheless, in a study that dealt with different squamous head and neck, and cervix cancer cell lines, lung, colon and breast cancer cell lines it was concluded that *in vitro* lovastatin inhibits the function of the EGFR and PI3k – Akt pathway and that this is

independent of the mutational status of the ATP binding site of EGFR (Mantha et al. 2005). The only condition for lovastatin to act in this way is the expression of EGFR at the cellular surface. Furthermore, a combination of statin and the thyrosin-kinase inhibitor gefitinib yielded a synergistic effect of these both.

Regarding all these statements about an anti-cancer potential of statins we assumed that *in vitro* research on HMG-CoA reductase inhibitors in combination with irradiation and/or targeted therapies on various human tumour and normal tissue cell lines could be of interest. For this purpose we have chosen to work with lovastatin and two well known monoclonal antibodies, cetuximab and bevacizumab.

2 Materials and methods

2.1 Cell culture

2.1.1 Cell lines

In all performed experiments, only human cell lines have been used. We worked with four tumour and three normal tissue cell lines. For experiments, all were taken from stocks maintained in a liquid nitrogenous tank in the property of the Department of Radiotherapy and Radiation Oncology in Göttingen.

ZMK-1 cell line

This cell line represents a poorly differentiated (grade 2) squamous cell carcinoma of an oropharyngeal tumour from a 47-year-old female patient obtained through the tumour resection performed in 1996 at the Department of Maxillofacial surgery (Klinik für Zahn-Mund-Kiefer-Chirurgie) in Universitätsklinikum Göttingen. Cells from this tumour were then isolated and cultivated at the Department of Radiotherapy and Radiation Oncology in Göttingen (Rave-Frank et al. 1996).

A 549 cell line

A 549 cells are adenocarcinomic human alveolar basal epithelial cells. They were for the first time described and developed in 1972 by D. J. Giard (Giard et al. 1973) from the cancerous lung tissue in an explanted tumour of a 58-year-old Caucasian man. Cells were obtained from the American Type Culture Collection (ATCC).

MO59K and MO59J

Both of these cell lines have been isolated concurrently from the same tumor specimen from a glioblastoma brain tumor of a 33-year old man. The difference between these two lines is the fact that MO59J cells lack DNA-dependent protein kinase (DNA-PKcs) activity while MO59K cells express normal levels of DNA-PKcs. This causes that MO59J are approximately 30-fold more sensitive to irradiation than MO59K. MO59J are DNA double strand break repair deficient (Allalumnis-Turner et al. 1993). The cells were obtained from the ATCC.

Human fibroblasts

These not immortalized cells were obtained from a skin spindle coming from two different women, a 55-year and an 85-year-old. Cells were isolated and cultivated in November 2012 by Ms. Bitter, a laboratory and research assistant (Department of Radiotherapy and Radiation Oncology, UMG, Göttingen).

HUVEC-VI and HUVEC-VII cell lines

HUVECs are not immortalized human umbilical vein endothelial cells. These have been isolated in laboratory conditions within the Department of Radiotherapy and Radiation Oncology by Mrs. Kasten-Krapp, laboratory and research assistant in July 1998 from two different umbilical cords. After few days of cultivation cells were frozen and hold in liquide nitrogenous tank as a reserve.

HaCaT cell line

This line represents an immortalized but highly differentiated human keratinocyte cell line. It is a spontaneously transformed human epithelial cell line that was obtained in 1988 from a histologically normal skin specimen from a distant periphery of a melanoma of a 62-year old male patient (Boukamp et al. 1988). HaCaT cells present a heteroploid stemline with specific stable marker chromosomes but remain not tumorigenic.

2.1.2 Culture conditions and media

The origins of all materials i. e. chemicals and pharmaceuticals, devices, experimental and detection kits, software tools, and accessories that were used during the experiments are described within the chapter Appendix in tables 6.1, 6.2, 6.3, 6.4, and 6.5 respectively.

In order to avoid all possible contaminations all manipulations with cells were performed under strict sterile conditions using a clean bench. All materials coming to clean bench were decontaminated by a 70 % ethanol impregnated towel before use.

Prior to any experiments, all cell lines have been tested for presence of Mycoplasma infection using a MycoAlert™ mycoplasma detection kit. Cells that have revealed to be Mycoplasma positive have been treated by a combination of antibiotics comprised in Mynox®Gold and once the treatment was over they have been re-tested after 4 weeks again. In our condition, all tumor cell lines (except A 549) were initially contaminated by this intracellular bacterium. The treatment has been successful in all instances except for the ZMK-1 cell line where eradication of Mycoplasma could not be achieved despite numerous attempts.

Cells from each cell line serving as a reserve were maintained at -80 °C in freezing resistant plastic vials in quantity of 1 million cells per vial. Each vial contained 1.8 ml of solution of culture medium and 9 % dimethyl sulphoxide (DMSO) that permitted a protection against potential freezing associated cell damages. Thawing and seeding procedure is described in chapter 2.1.3.

During the use, cells were maintained in sterile 50 and 200 ml flasks at the temperature of 37 °C and at CO_2 concentration of 5 % in an incubator. Cells were growing attached to the bottom of the flasks so the mean volume of medium present in 50 ml flask was 10 ml and in 250 ml flask was 20 ml in order to cover correctly the whole surface of the flask wall.

The cell medium was changed every 2-3 days. Once the cell confluence in monolayer was 90 % or more, cells were trypsinized and seeded in new flasks at different cell densities. For the HUVEC cell line and fibroblasts, the passage number was strictly marked on each flask so that only the same passage cells were used for the same experiment and that the number of passages for these cell lines did not exceed 15. The passage number for all tumor cell lines and HaCaT cells was not noted since all of these cell lines are immortalized.

All reagents and mediums in use were stored at 4 to 8 °C in a refrigerator. Long term storage of fetal calf serum, trypsin and antibiotics was insured by placement into a -20 °C freezer. Just before use, reagents and mediums were carefully warmed to 37 °C. Compositions of each cell line-related medium are listed in table 2.1.

Tab. 2.1: Culture mediums used for the cell lines.

Cell line	Medium description and contents
ZMK-1	Dulbecco's Minimum Essential Medium (DMEM) and RPMI 1640 in ratio 1:1 including 10 % inactivated fetal calf serum (FCS) and 1 ml Ampicillin (Ampicillin 0.5 g/10 ml)
A 549	Idem as for ZMK-1 cell line
MO59K	DMEM including 10 % inactivated FCS, 2.5 ml Penicillin/Streptomycin (10 000 U/ml / 10 000 µg/ml) and 1 ml Ampicillin
MO59J	DMEM including 15 % inactivated FCS, 2.5 ml Penicillin/Streptomycin and 1 ml Ampicillin
Fibroblasts	DMEM incl. 10 % active FKS
HUVEC VI, HUVEC VII	Endothelial Cell Growth Medium (ECGM) + supplement mix Supplements within the supplement mix and their concentration after addition to the medium: FCS 0,02 ml/ml; Endothelial Cell Growth Supplement 0.004 ml/ml; Epidermal Growth Factor 0.1 ng/ml; Basic Fibroblast Growth Factor 1 ng/ml; Heparin 90 µg/ml; Hydrocortisone 1 µg/ml
HaCaT	DMEM including 10 % FCS

2.1.3 Cell culturing

Cell trypsinization

Because all cultured cells were in normal conditions firmly attached to the flask's bottom, they needed to be detached from it before an experiment in order to gain a mass of freely swimming cells, i. e. a cellular suspension. This was ensured by the process of trypsinization. This was also inevitable in case of high cell confluence with the aim to create new passaged cells reserve flasks.

In the first step, after the removal of the medium from the flask, cell layer was washed by 2-3 ml of Phosphate Buffered Saline (PBS) without calcium and magnesium.

Afterwards, 1 ml (for 50 ml flasks) or 2 ml (for 250 ml flasks) of trypsine (0.5 % Trypsin; 0.2 % ethylenediaminetetraacetic acid in PBS) was added and the flask was placed for 5 - 10 minutes into an incubator so that the trypsine effect was increased.

The above described procedure was suitable for all cell lines except HUVEC and HaCaT. They turned out to be more strongly attached to the flask bottom. Because of that a reinforced procedure was applied.

The procedure consisted of a prior addition of 1 ml of EDTA (ethylenediaminetetraacetic acid) solution into the flask after the previous washing by PBS. One to three minutes later, EDTA was removed and 1 ml of trypsine was added and maintained in a flask in an incubator for 5-10 minutes as in the procedure mentioned above.

After 5 – 10 minutes, the flask was removed from the incubator and slightly shaken by hand. That has permitted to detach the cells mechanically. The cells were controlled under the microscope whether all of them were correctly freed from the flask's bottom.

Then, a fresh medium was added in the amount of 9 ml and 19 ml for 50 ml and 250 ml flask, respectively, in order to obtain a cell suspension.

Cell counting using a Neubauer counting chamber

A Neubauer chamber is a thick crystal slide with a size of a glass slide. Its dimensions are 30 x 70 mm and thickness of 4 mm. There exist either single or double chamber slides. For our purposes, only double chamber slides were used. There are two separate counting areas: the upper and the lower chamber.

A small quantity of previously prepared cell solution after trypsinization is aspirated into a sterile Pasteur pipette and loaded into both chambers.

Once both chambers are filled, the slide is placed under the light microscope. Cells laying in each of the 25 small squares of the big central square are counted. The counted number of cells is registered and the same procedure is repeated for the second chamber.

Two different numbers are obtained which permits us to calculate the mean value. Multiplying this value by 1 x 10^4 will give us a number of cells present in 1 ml of our cell suspension. From this obtained value, volume containing the desired amount of cells for new suspension can be calculated using a simple cross-multiplication equation.

2.1.4 Cell irradiation

For all performed experiments, a X-ray tube was used. The parameters of the accelerator during use were as follows: voltage 200 kV, current 15 mA, 0.5 mm thick copper filter, temperature of 22-24 °C. The table high, defined as a distance between the table and the radiation source, was modified according to the desired dose rate. This high was 500 mm and 351 mm for a dose rate of 1 Gy/min and 2 Gy/min, respectively.

The irradiation was performed at different time points according to the cell seeding following various treatment regimes and procedures (see appropriate chapters describing each experimental procedure).

2.2 Treatment molecules

2.2.1 Statins

Lovastatin

Lovastatin was donated by courtesy of Dr. Corcos (INSERM U1078-ECLA, Brest, France) in a powder form. Thirteen milligrams of this powder were dissolved in 1.3 ml of DMSO and a solution with a molar concentration of 25 mM was obtained. This one was later diluted once again in DMSO in order to gain stock dilutions of 12.5 and 2.5 mM. Because concentrations used in the experiment were much smaller, the necessary dilutions with DMSO have been performed short before each use. All solutions were held in sterile plastic 2 ml vials in a freezer at -20 °C. Before use, the vials were decontaminated by a 70 % ethanol impregnated paper towel, placed under the airflow and maintained at room temperature few minutes to defreeze spontaneously.

2.2.2 Targeted therapies

Cetuximab, bevacizumab

These monoclonal antibodies were kindly donated by the Department of Oncological Pharmacy, Regional University Hospital Morvan in Brest, and UMG Göttingen.

Cetuximab (Erbitux®) was obtained in its clinical presentation of 5 mg/ml (molar concentration of 32.89 µM) and stored at 4 °C. The solution also contained the following inactive ingredients: sodium chloride, glycine, polysorbate 80, citric acid monohydrate, sodium hydroxide, and water for injections.

Bevacizumab (Avastin®) was also obtained in its clinical presentation of 25 mg/ml (molar concentration of 168 µM) and stored at 4 °C. Except of the active agent, the solution contained also α,α-trehalose dihydrate, monobasic monohydrate sodium phosphate, dibasic sodium phosphate, polysorbate 20, and water for injections.

The desired final concentrations of both molecules were obtained by diluting the primary solutions in a cell medium (in ECGM for HUVEC cell line and in DMEM for the other cell lines). The used concentrations were 0.1 µM and 16.8 µM for cetuximab and bevacizumab, respectively. Theses concentrations have been chosen without any previous particular dose determination according to the values used in already published *in vitro* studies (Lee et al. 2011; Kil et al. 2012).

Incubation time for cetuximab has been defined to be 1 hour prior to irradiation. This decision was supported by the experiments performed in the study of Saki and co-workers (Saki et al. 2012). Concerning bevacizumab, a decision for 24-hour incubation interval before irradiation was taken (Mangoni et al. 2012).

2.3 Preliminary experiments

2.3.1 Assays of cell survival and viability

2.3.1.1 Colony formation unit assay

Clonony formation unit assays (CFU assays) represent the basis of cellular response studies in tumours, and in some normal tissues, and have a central role in tumour radiobiology. They aim to detect the presence of clonogenic cells (either of a tumour or normal tissue) by their ability to form a colony within a defined growth environment (Joiner and van der Kogel 2009).

In general, a certain known number of cells is seeded into two or more sterile plates or Petri dishes where one part is irradiated and the other one is kept as a non-irradiated reference.

Assuming that irradiation will kill some cells, a larger number of cells are plated within the irradiated plates. After a suitable period of incubation, the colonies are counted and the plating efficiency (PE) is determined by dividing the number of colonies through the number of seeded cells. In order to get the information about colony formation capacity and treatment sensitivity of our cell line the surviving fraction is calculated:

$$\text{Surviving fraction} = \frac{\text{PE}_{\text{treated}}}{\text{PE}_{\text{control}}}$$

Thus we take into consideration the correction for the efficiency of undamaged clonogenic cells and for the different number of cells plated. The obtained values of the surviving fraction for different treatment conditions permit thereafter to create the so-called survival curves (see below).

Seeding schemes

Different seeding and cell culture preparation procedures for this assay are available and possible. Within this work, two approaches have been performed. The choice of the particular approach depended on the cell line behaviour and its characteristics.

In the first method, a single-cell suspension of cells was prepared according to the procedure described above (chapter 2.1.3) and divided into various parts according to the number of different radiation doses tested. As already mentioned, one part of cells was kept without irradiation (eventually without any particular treatment) as a control of the assay.

Suspensions with the desired total number of cells were prepared in plastic sterile tubes and carefully marked in order to avoid an exchange of the cell numerated suspensions. Tubes were subsequently irradiated using the X-ray tube within the laboratory at doses that were previously defined. These used to be 1, 2, 3, 4, and 6 Gy while the dose rate was 2 Gy/min and the radiation parameters of the X-ray tube were as defined in chapter 2.1.4. During the irradiation the control cells were held in a tube under the clean bench.

After the irradiation, the cell suspensions were dispensed on sterile 6-well plates in triplicate for each treatment condition. Usually, the total volume of cell suspension per well was 3 ml except for two cell lines (HUVEC and MO59J) in which this volume was raised to 4 ml per well because of the prolonged incubation time of these lines.

The number of cells plated per well and per treatment condition varied according to the expected empirically obtained information about each cell line. In general 100 – 3500 cells/well have been seeded taking into consideration proliferative capacity of each line. If this number turned out to

be not sufficient and colonies have been detected with difficulty, the number of cells per well has been increased in the subsequent experiments. For more precise details about seeding cell number per cell line and condition see chapter 3.2.2.2 below.

If there was another treatment except irradiation, this was added into cell suspensions before their plating into wells. The interval between addition of the treatment and irradiation has been strictly obeyed with respect to the treatment molecule and its defined procedure, e. g. for lovastatin and cetuximab the time intervals of 4 hours and 1 hour, respectively were preserved. All treatment molecules have been added into cell suspensions using pipette with sterile pipette tips of different volume according to the desired final volume and concentration of molecules. Cells that were supposed to serve as a control within the lovastatin experiment were treated with DMSO alone which volume corresponded to the volume of lovastatin needed to obtain the highest concentration. In cetuximab and bevacizumab treatment schemes, only a cell medium was added to the control cells since both antibodies have been diluted in it. Volume of the medium was equal to the volume of the antibodies used.

For this seeding procedure, treatment and irradiation were performed on the same day.

After seeding into wells, plates were placed into an incubator under standard conditions (see chapter 2.1.2) for a certain period which has varied between 8 to 14 days. This inhomogeneous incubation duration was influenced by different cell growth capacity of each line. This one was very slow for MO59J and HUVEC cells.

The second seeding procedure consisted of preparation of a cell suspension in the first step followed by direct plating of the desired number of cells per well into sterile 6-well plates on day 0 (D0). The prepared plates were placed into an incubator for 24 hours so that the cells had time to attach to the plate bottom before any treatment was applied.

Cells were seeded in such a way that all cells that were meant to be irradiated at the same dose were placed in the same plate which was subsequently irradiated not sooner than 24 hours after seeding, i. e. on day 1 (D1) or on day 2 (D2) if a treatment by bevacizumab has preceded the irradiation by 24 hours. All other treatments associated have been added into wells in the desired moment according to the later irradiation. As for the incubation time and conditions these were similar to those described previously.

All plates were continuously observed under the microscope with evaluation of colony growth. If these were considered to be sufficiently large, a fixation and staining of the colonies has been done as described in the following chapter.

Colony fixation and crystal violet staining

In the first step, the cell culture medium was removed from all wells of the plates using a pipette. This procedure as well as the process of fixation and staining did not require sterile conditions.

Two millilitres of 70 % ethanol have been pipetted into each well and maintained for 20 minutes. Afterwards, ethanol has been removed and empty plates have been placed into an incubator to dry overnight. On the next day, staining of the plates with crystal violet solution was performed. Once 2 ml of the solution has been added into each well they have rested for 20 minutes and then the solution was removed by washing with water. Using this procedure, the colonies became visible because the crystal violet has stained the cell membranes and they can be now easily counted.

Plates with stained colonies were placed under the light microscope and only colonies formed of 50 cells or more were taken into consideration with the exception of the colonies formed by MO59J and fibroblasts. These two cell lines had a poor growing capacity and have formed only very small colonies that consisted in general of 30 - 40 cells in maximum. For this reason, in these two cell lines the colonies that contained more than 30 cells (more than 10 cells for MO59J) were considered significant. The total number of colonies per well was later on related to the number of initially seeded cells as described above.

Cell survival curves

After the fixation and staining of colonies, these were counted and values of the surviving fraction per each treatment condition were defined as described above. Since every treatment condition has been performed in triplicate within each experiment and each experiment was repeated on average 3 times the mean values (expressed as points in curves) and the standard errors (SE) were determined and are plotted as error bars. If the error bars are not visible within the points this means that they are smaller than the size of the point. The obtained results of surviving fractions are transformed into a semi-logarithmic scale representation using a Kaleidagraph® software version 4.1 (Synergy Software, Reading, USA) and OriginPro® software version 7.5 (OriginLab Corporation, USA). The radiation dose forms the abscissa and the y-axis presents the respective quantities in a logarithmic scale. The curves are helpful for evaluation of the cell behaviour under the irradiation conditions associated or not with some other treatment regime. The form of the survival curves is unique for every cell line.

Two types of survival curves were created for each cell line and experimental condition. First, without normalization of the results, i. e. only the control colonies (non-irradiated and untreated)

had SF equal to 1 (=100%). All other SF values were calculated by putting into relation (ratio) the plating efficiencies of irradiation and/or treatment with the plating efficiency of the control. These curves were plotted by a simple interpolation between obtained mean values of surviving fractions. The second ones were normalized curves where non-irradiated but pre-treated colonies were given the SF value of 1, i. e. all curves started at the same point of the y-axis and thus real impact of treatment caused only by irradiation could be observed. For these curves the normalized radiation survival was fitted using a linear quadratic model.

Calculation of Sensitizer Enhancement Ratio

Determination of Sensitizer Enhancement Ratio (SER) allows us to postulate whether certain molecules, if associated with irradiation, enhance the radiation effect or not. In practice, within normalized survival curves of all cell lines tested, a radiation dose (in gray, Gy) corresponding to a 50 % surviving fraction of untreated cells and cells treated with sensitizer (i. e. with lovastatin, cetuximab, bevacizumab or combinations) are put into relation as follows:

$$\mathrm{SER} = \frac{\text{Radiation dose without sensitizer}}{\text{Radiation dose with sensitizer}}$$

SER > 1 is characteristic for a radiosensitizing agent because higher radiation dose without sensitizer is necessary to achieve the same effect as if the sensitizer (agent) is present. Values of SER below 1 describe a radioprotective effect of the agent.

2.3.1.2 Cell viability determination

In order to complete the CFU assay when testing cell viability and survival under different treatment conditions, the so-called CellTiter-Blue® Cell Viability Assay (CTB assay) procedure was performed several times.

This assay provides a homogenous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurine to measure the metabolic capacity of cells. Viable cells retain the ability to reduce resazurine into resorufin which is highly fluorescent (Promega, Technical Bulletin, revised 6/09).

Non-viable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Resazurine is dark blue in colour and has little intrinsic fluorescence until it is reduced to resorufin which is pink and highly fluorescent.

In practice, sterile 96-well black plates with clear bottom were used for the test. In the initial step, the quantity of seeded cells as well as the time of incubation before the CTB analysis

performance needed to be defined. Afterwards, assay on different cell lines with chosen treatment regimens could be done.

Determination of optimal cell quantity and optimal incubation time within CTB assay

Each cell line (tumour and normal tissue) has been tested separately. For each assay, three 96-well plates were needed, i. e. for three different incubation times (48, 72, and 96 hours). First, a cellular suspension containing the necessary quantity of cells was prepared and maintained in a plastic 15 ml tube as described in chapter 2.1.3. One tube contained cells for control group (no irradiation, no treatment), the second tube was irradiated when the cellular suspension was ready. Irradiation was done according to the description in chapter 2.1.4. The total dose of 4 Gy was delivered with the dose rate of 2 Gy/min to all cell lines except of MO59J that received only 2 Gy since this cell line is supposed to be more radiosensitive. After the irradiation, the cells were seeded into plates in such a manner that the final quantity of cells per each well was that as shown in figure 2.1 below. The green area represents non-irradiated cells (negative control) and the blue one irradiated cells. As seen, for each cell number and condition the test was done in triplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
C	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
D	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
E	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
F	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
G	Medium	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	Medium
H	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium

Fig. 2.1: Schematic representation of a 96-well plate seeded with cells and medium (see text).

In order to complete and homogenize the volume per well, a serum-supplemented medium appropriate for the particular cell line was added into each well to obtain a total volume of 100 µl per well. The most peripheral wells on all four sides were maintained free of cells. They were filled with 100 µl of pure serum-supplemented medium. It should be noted that for further analysis, the contents of the wells in the first column (column 1), except the wells A1 and H1, were included into the analysis because they served as blank samples to determine a possible background fluorescence.

The prepared plates were placed into an incubator and maintained at standard incubation conditions (37 °C, 5 % CO_2) for 48, 72, and 96 hours. One plate served for one defined incubation time. After the corresponding incubation time, the plate was removed from incubator and a CellTiter-Blue® Reagent was added to cells in culture. Each cell-containing well as well as 6 wells in the column 1 received 20 µl of reagent. After the reagent addition the plate was slightly shaken during 10 seconds. According to the general recommendation of the CTB protocol, once the reagent is added, the plate should be left for incubation at 37 °C for 1 – 4 hours. In our conditions, the incubation time was empirically defined to be of 1 hour.

Data analysis

One hour after the above-mentioned procedure, a fluorescence analysis was performed using a Wallec1420 VICTOR™ plate reader. The wavelength of the recorded fluorescence was 560/590 nm. The obtained data were imported into Microsoft Office Excel (Microsoft, Albuquerque, USA) and calculations were done as follows. Fluorescence of the control wells (cell-free wells) was measured to obtain the fluorescence of the serum-supplemented medium. Mean values of these results were calculated and subsequently substracted from the raw fluorescence results of all experimental wells. Because each experimental condition was done in triplicate, the mean values and their standard deviations of these background corrected results were calculated as well. For better and easier visualisation, the results are plotted in graphic curves with strict differentiation between the irradiated and non-irradiated cells. The calculations were performed using the Microsoft Excel program and graphic outputs were plotted in OriginPro® software version 7.5. The same procedure was repeated for all 3 plates after the defined interval of incubation.

To determine the optimal time of incubation, the final curves of all three results were compared. As an optimal time for further explorations we took the incubation time after which it was possible to observe differences between the curves corresponding to the irradiated and non-irradiated cells. When the curves overlapped each other it was an indication that longer incubation time is needed. An optimal quantity of seeded cells corresponded to cell counts located shortly before a "plateau" of the curve. This represents saturation of the test´s capacity to distinguish a difference in the fluorescence under the examined conditions.

It is noteworthy that in majority of the experimental conditions the fluorescent signal for CellTiter-Blue® Reagent is proportional to the number of viable cells. There is a linear relationship between the cell number and the fluorescence.

Use of CTB assay for various treatment regimens

The notion of optimal cell quantity and incubation time permits to optimize the experimental process. Although using still a 96-well plate, the wells can be charged more effectively so that various treatment conditions can be applied on the same plate. An example of a different treatment distribution within the plate is shown in figure 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
B	Medium											Medium
C	Medium											Medium
D	Medium											Medium
E	Medium											Medium
F	Medium											Medium
G	Medium											Medium
H	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium

Fig. 2.2: An alternative well seed using the same 96-well plate as in figure. 2.1 (see text).

The green area corresponds to non-irradiated and untreated cells (negative control), the blue area is exposed only to irradiation, the yellow area contains chemical molecules (statin or monoclonal antibody), and the pink area represents a combination of irradiation and treatment molecules.

In case when more treatment combinations are needed within the same experiment, e. g. irradiation with or without lovastatin and/or bevacizumab, one plate is not irradiated and another one is irradiated as designed. So each treatment condition can be examined with and without additional irradiation.

In figure 2.2, each column corresponds to different cell numbers seeded per well. Thus, for each cell number the defined experiment is performed three times as seen in the figure. The final results represent mean fluorescence unit values obtained for each treatment condition and for each cell line normalised to values obtained for every 1000 cells/well fluorescence units. These mean values are plotted as columns and standard deviations (SD) are shown within each graphic representation as bars.

2.3.1.3 Determination of appropriate lovastatin concentration for further experiments

Prior to any experiment based on lovastatin, a search for its suitable concentration was performed using colony formation assay. We searched for a dose that would permit a surviving fraction of approximately 80 % regardless the irradiation effect.

For this purpose, two tumour cell lines have been tested, ZMK-1 and A 549, since these two possess rather high proliferation activity and thus easy manipulation. Cells were seeded into 6-well plates as described in chapter 2.3.1.1 and cell number per well varied from 100 to 1000 cells. Before the seeding itself cell suspensions have been pre-treated with different concentrations of lovastatin (0; 2.5; 5; 10; and 25 μM). One set of plates was left without irradiation, the other one was irradiated 4 hours after lovastatin addition and plates were left for incubation as long as needed (8-13 days).

Colonies were subsequently fixed in alcohol and stained as described in chapter 2.3.1.1.

2.3.2 Determination of the epidermal growth factor receptors (EGFR) expression status of the studied cells

Since several experimental conditions included treatment with cetuximab, anti-EGRF monoclonal antibody, immunohistochemistry staining has been performed to determine EGFR expression within all studied cells. The detection procedure consisted of a cytocentrifugation prior to the staining itself.

Cytocentrifugation

A cellular suspension of each cell line was prepared and cells were counted according to the procedure described in chapter 2.1.3. For our purpose, 1×10^4 cells were needed for the preparation of each glass slide.

Once the cell suspension was prepared, construction of glass slide porters of the Cytospin 4 cytocentrifuge was done. Each porter consisted of a stainless steel clip, glass microscopic slide, filter card, and a re-usable sample chamber as seen in figure 2.3.

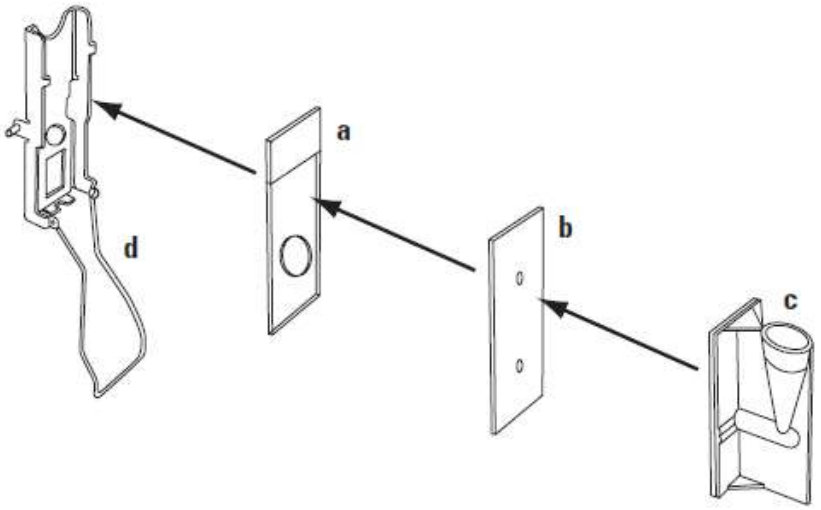


Fig. 2.3: Composition of a glass slide porter: a – glass slide, b- filter card, c – re-usable sample chamber, d- slide clip (from Thermo Scientific, Instructions for use, revised 6/12, p. 1).

For each glass slide, 100 µl of cellular suspension was inserted using a pipette inside the sample chamber when the whole porter was prepared and placed into a cytocentrifuge. The cells were centrifuged during 5 minutes at 1500 rpm using the program 1 (predefined for this purpose according to the manufacturer protocol). After 5 minutes, the slides were removed from the centrifuge and out of the clips and a cellular monolayer could already be observed on each glass slide. These were left to dry at room temperature overnight and were afterwards ready for staining.

Immunohistochemistry EGFR staining method

The procedure of staining was performed by Mrs. Jünemann (Department of General and Visceral Surgery, UMG, Göttingen).

In the first step, A 549 cells were stained to confirm the reliability and feasibility of this test since A 549 is known to overexpress the EGF-receptors. Afterwards, all other cells lines were tested as well.

Once the glass slides with cellular monolayer were ready, these were fixed in -20 °C acetone for 8 minutes then dried at room temperature. The samples were subsequently placed in a Wash Buffer for few minutes and then the procedure of staining was ready to start.

First, the samples were incubated for 28 minutes at 37 °C with the primary mouse anti-EGFR antibody diluted in 1:100 ratio. Next, an indirect detection of primary antibodies using OptiView DAB IHC Detection Kit was done. The system was based on the principle of peroxidase

inhibition. The samples were left to stain first at haematoxylin II during 8 minutes, then in bluing reagent for 4 minutes using automated slide stainer.

After the staining, the samples were fixed once again in 75 % alcohol then in xylene and finally the cover glass was put on the glass slide with the samples and fixed using xylene-containing Vitro-Clud.

For the final visualization, a light microscope with a magnification factor of 40 coupled with a camera was used and permitted to create photographic documentation of the observed images.

If cells expressed EGFR this was seen as a brown staining either of the cell surface, cytoplasm or both in comparison to cells without EFGR expression or negative control that could be seen as blue ones.

2.4 Molecular biology experiments

2.4.1 Reporter assay

2.4.1.1 Multi-pathway activity assay

For this purpose, a Cancer Cignal Finder 10-Pathway Reporter Array kit was used. This array enables to pinpoint the pathways regulated by the gene products or chemical compounds which is very helpful for determination of the effects of lovastatin on cell lines examined in order to facilitate further experiments, i. e. Western blot assay. This kit contains of 10 different Cignal reporter assays (see table 2.2).

Each reporter was a mixture of an inducible transcription factor responsive construct and consecutively expressing *Renilla* luciferase construct (40:1). The inducible transcription factor-responsive construct encoded the firefly luciferase reporter gene under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific Transcriptional Response Element (TRE). This construct monitored both increases and decreases in the activity of a key transcription factor which is a downstream target of a specific signalling pathway. The consecutively expressing *Renilla* construct encoded the *Renilla* luciferase reporter gene under the control of a CMV (cytomegalovirus) immediate early enhancer/promoter and acted as an internal control for normalizing transfection efficiencies and monitoring the cell viability.

Tab. 2.2: Cancer Cignal Finder 10-Pathway Reporter Array kit.

	Pathway	Transcription Factor
1	Wnt	TCF/LEF
2	Notch	RBP-Jκ
3	p53/DNA Damage	p53
4	TGF-β	SMAD2/3/4
5	Cell cycle/pRb-E2F	E2F/DP1
6	NF-κB	NF-κB
7	Myc/Max	Myc/Max
8	Hypoxia	HIF-1A
9	MAPR/ERK	Elk-1/SRF
10	MAPK/JNK	AP-1
11	Negative Control	
12	Positive Control	

The negative control was a mixture of non-inducible reporter construct and consecutively expressing *Renilla* luciferase construct. The non-inducible reporter construct encoded firefly luciferase under control of a basal promoter element (TATA box) without any additional transcriptional response elements. It served to determine a background reporter activity.

The positive control was a constitutively expressing GFP (green fluorescent protein) construct pre-mixed with a constitutively expressing firefly luciferase construct and a constitutively expressing *Renilla* luciferase construct. It was necessary for visual confirmation of transfection (Qiagen, Format Handbook, revised 01/2011).

For our purpose, the transfection and treatment protocol for reporter assay with small molecule/organic compound was followed.

Assay procedure

In practice, the procedure was performed under sterile conditions. First, all compounds and solution were prepared in advance in sufficient quantities and maintained in 5 ml polysterene test

tubes. Transfection as well as revelation was performed using sterile white flat bottom 96-well plates.

For experimental transfections based on per well basis, 1 µl of each Cignal reporter needed to be diluted in 25 µl of Opti-MEM® serum-free culture medium. It is noteworthy that for each examined reporter, 4 wells were necessary in order to have 4 different lovastatin concentrations for examination (0; 0.25; 2.5; and 25 µM). For each experiment, a negative control was needed consisting equally of at least 4 wells (1 well per lovastatin concentration). At least one positive control was always associated to each experiment as well.

In the next step, Attractene solution was prepared by dilution of 0.6 µl of Attractene in 25 µl of Opti-MEM®serum-free medium for every well. This dilution was let for incubation at room temperature for 5 minutes and added afterwards to the previously prepared reporter solutions so that there was at the end 25 µl of Attractene dilution per well. In order to allow occurrence of complex formation, this mixture was immediately shaken after addition of Attractene using a Vortex and incubated for 20 minutes at room temperature.

Meanwhile, a cellular suspension needed to be prepared. Since various cell lines are believed to show a great deal of variation in the levels of signalling proteins, three different cell lines (two tumour, A 549, ZMK-1, and one normal tissue, HaCaT) were used in this experiment.

This preparation aimed at obtaining a suspension that contained 4 x 10^5 cells/ml. The final volume of suspension depended upon the number of wells needed. The cells were obtained by a process of trypsinization as described in chapter 2.1.3. The only difference was that once trypsinized the cells were resuspended in Opti-MEM® medium containing 5 % of fetal calf serum. After the cell counting, as described in 2.1.3, the suspension was centrifuged in a centrifuge with 1200 rpm during 10 minutes at room temperature (22 °C). Afterwards, the supernatant was removed and the cells were resuspended this time in Opti-MEM® containing 5 % of FCS and 1 % of non-essential amino acids (NEAA).

After 20 minutes of incubation for complex formation, 50 µl of specific complexes were aliquoted into appropriate wells and 100 µl of cell suspension was added into each well already containing constructs-Attractene complexes.

The plate with all prepared and filled wells was maintained for incubation at 37 °C and 5 % CO_2 for 6 hours.

Six hours after transfection, the medium had to be changed to assay medium consisting of Opti-MEM® containing 0.5 % FBS, 1 % NEAA, 100 U/ml Penicillin, and 100 µg/ml Streptomycin. This time, 75 µl of the assay medium was added per well.

Treatment with lovastatin was done 24 hours after transfection. The volume of the diluted lovastatin that was added into appropriate wells depended upon the desired final concentration in each well. In case of control wells without lovastatin, DMSO was used instead.

Six to eighteen hours after the experimental treatment, a revelation using Dual Luciferase Assay was performed.

2.4.1.2 Dual-Luciferase reporter assay

For the revelation of the results Dual-Glo®Luciferase Assay System was used 6-18 hours after treatment according to the manufacturer's protocol (Promega, Technical Manual, revised 9/11) as follows.

Because of its high sensitivity, the firefly luciferase assay permits a simple detection of upregulation of genetic elements. In order to be able to determine gene downregulation as well, a normalization of the expression of an experimental reporter (firefly luciferase in this case) to the expression of a control reporter (*Renilla* luciferase) needs to be done. This will allow the differentiation between nonspecific cellular response (cell death) and specific cellular response (gene downregulation).

Both of these luciferases are widely used as co-reporters permitting quick and sensitive normalized studies. Firefly luciferase is a 61 kDa and Renilla luciferase a 36 kDa protein. None of them requires post-translational processing so that they can be used as genetic reporters immediately after translation.

To generate luminescence firefly luciferase requires beetle luciferin, ATP, magnesium, and molecular oxygen. Renilla luciferase requires only coelenterate luciferin (coelenterazine) and molecular oxygen. Both luciferases undergo spontaneous inactivation after generating luminescence.

Assay procedure

In practice, in the first step of the revelation, a volume of Dual-Glo®Luciferase Reagent equal to the culture medium volume (75 µl) was added into each well of the plate. This reagent induced cell lysis and acted as a substrate for firefly luciferase. After the first reagent was added, this was left for an incubation of approximately 15 – 45 minutes at room temperature.

After 15-45 minutes of incubation, firefly luminescence was measured using a luminometer and expressed in RLU (relative luminescence units). The measurement of firefly luminescence was performed at room temperature without prior shaking. It was repeated 3 times in 10-minute intervals. In the second step, Dual-Glo®Stop & Glo®Reagent of the same volume as the first reagent (75 µl) was added into each well and left for an incubation of 15 – 45 minutes at room temperature. This reagent quenched the luminescence from the firefly reaction and provided substrate for *Renilla* luciferase. Measurement of *Renilla* luminescence was done in the same way as the firefly luminescence, i. e. after 15 – 45 of incubation and repeated 3 times in 10-minute intervals.

Normalizing ratio, Relative Response Ratios, and data analysis

Since firefly luciferase is immediately functional upon translation, as described above, if the amount of luminescence from an experimental sample (firefly luciferase) is greater than the luminescence from a control sample (*Renilla* luciferase) an increase in transcription or translation occurs. A decrease in experimental reporter response can be due to specific effect on the reporter or due to global effect, e. g. cell death. Normalization of an experimental reporter (firefly) with a control reporter (*Renilla*) from the same sample permits a distinction between specific and global effects. For this reason, a ratio of firefly : *Renilla* luminescence has to be calculated for each well. Since in our conditions each measurement (firefly and *Renilla* luciferase) was performed in triplicate a mean value of these three results was determined and then the ratio of both luminescence outcomes was calculated. All calculations were performed using the Microsoft Excel program.

In order to determine a quantitative impact of an experimental treatment (lovastatin at different concentrations in this case) on reporter gene expression a Relative Response Ratio (RRR) calculation is very helpful.

This calculation requires the presence of both positive and negative controls on each plate and for each cell line tested. In this case, positive control was without any treatment and the negative one was treated with lovastatin.

At first, the experimental reporter luminescence/control reporter luminescence (firefly : *Renilla* luminescence) was calculated for all wells as already explained above. So we obtained the experimental sample ratio, negative and positive control ratios and calculated the RRR for each experimental treatment using this formula:

$$\mathrm{RRR} = \frac{(\text{experimental sample ratio}) - (\text{negative control ratio})}{(\text{positive control ratio}) - (\text{negative control ratio})}$$

Once this relative response ratios were calculated, a relation was done in a manner that no treatment (i. e. lovastatin 0 μM = DMSO, in these condition) RRR was considered to represent 100 % and subsequently all other RRR for experimental treatments were put into relation with it. A negative control would generate a RRR of 0 %. Those experimental treatments that would give negative RRR will be the most significant ones because they would be more effective inhibitors than the negative control. The more this negativity increases with increasing concentration of lovastatin the higher inhibitory effect is observed. On the other hand, positive and increasing values of RRR of the experimental treatment mean that this therapeutic molecule activates the specific cellular pathway.

2.4.2 Determination of apoptosis through caspase-3 and caspase -7 activity

Apoptosis is a highly regulated form of cell death that can be either a result of conditions occurring within the cell itself or from signals generated externally such as those from a surrounding tissue or immune cells (Taylor RC et al. 2008). Members of the cysteine aspartic acid-specific protease (caspase) family play a key role in apoptosis in mammalian cells. Detection of caspase-3 and/or -7 activity in the treated cells (and control) permits to obtain the information about apoptotic potential of different treatment approaches used.

2.4.2.1 Principles of the caspase-3 and caspase -7 activity assay

The Caspase-Glo®3/7 Assay provided a luminogenic caspase-3/7 substrate which contained the DEVD tetrapeptide sequence in a reagent optimized for caspase activity, luciferase activity, and cell lyses. Adding of this substrate resulted in cell lyses followed by caspase cleavage and generation of a “glow-type” luminescent signal produced by luciferase. The measured

luminescence is proportional to the amount of caspase activity present (Promega, Technical Bulletin, revised 12/12).

2.4.2.2 Assay procedure

At first, a cellular suspension of 1 x 10^5 cells/ml for each examined cell line and for every treatment condition (lovastatin 2.5 μM, irradiation = 4 Gy, lovastatin + irradiation, and control) was prepared under sterile conditions as described above.

If a combined treatment should be done, the cells had to be first pre-treated with a 2.5 μM lovastatin and then irradiated in 4 hours with 4 Gy. The cells were subsequently seeded into a white 96-well plate with flat bottom, 100 μl in each well. Within each experiment, every treatment condition (and control) was seeded in triplicate. Afterwards, the cells were left for incubation at standard conditions (chapter 2.1.2) for 6 – 48 hours depending on the cell line.

After the defined incubation time and before starting the assay, the cells were removed from the incubator and left to equilibrate to room temperature. A Caspase-Glo®3/7 Reagent (reconstituted of Caspase-Glo®Substrate and Caspase-Glo®Buffer) was added into each well in a volume of 100 μl and the contents of the wells were mixed using a plate shaker at 400 rpm for 30 seconds. The plate was then incubated at room temperature for 1 hour.

Each plate consisted, except of the assay wells, also of a blank and negative control. For blank reaction the cell medium was mixed with DMSO (solvent used to dissolve lovastatin) and Caspase-Glo®3/7 Reagent whereas negative control contained DMSO-treated cells in medium and Caspase-Glo®3/7 Reagent. Blank reaction was used to measure the background luminescence which value was subsequently subtracted from the experimental and negative control values.

After the first hour of reagent incubation, luminescence in RLU was performed using a luminometer in the same way as in the assay described in chapter 2.4.1.2. The second measurement, still for the same plate, was repeated one hour later in order to assess whether there was a difference in luminescence performance with time. As mentioned in the manufacturer's protocol, the peak of luminescence should be reached within 1 – 2 hours of reagent incubation.

Data analysis

As already mentioned, once the results (expressed in RLU) were obtained, the mean of the three blank values within each experiment was calculated and subsequently subtracted from each experimental value in order to remove a potential influence of the background. The mean values from these experiments and their standard deviations could be calculated and expressed in graphs as columns and error bars, respectively. The calculations were accomplished with the Microsoft Excel program and graphs plotted with OriginPro® software version 7.5 (OriginLab Corporation, USA).

2.4.3 Western blot assay

Also known as protein immunoblot, this technique is used to detect specific proteins in different tissues or cell culture samples. Gel electrophoresis is used to separate proteins with respect to the length of the polypeptide. These proteins are subsequently transferred to a nitrocellulose membrane where they are stained with antibodies specific to the target protein (Tobwin et al. 1979).

2.4.3.1 Preparation of samples and cell lyses

Since a large quantity of cells is required for this kind of experiment, ZMK-1, A 549, and HaCaT cell lines have been chosen to be explored due to their high proliferation activity. The initial cell suspension was prepared under sterile conditions as described in chapter 2.1.3 and appropriate quantities of cells (10×10^6 and 20×10^6) were resuspended in 20 ml of medium and seeded into 250 ml flasks.

Seeding was performed on day 1 and the experimental treatments on day 2 (approximately 20 hours later). For assays with lovastatin, 4 flasks needed to be prepared: control (no lovastatin, no irradiation, addition of DMSO exclusively), lovastatin 2.5 μM alone, irradiation alone (4 Gy), and combination of lovastatin and irradiation. In the case of combined treatments, the cells were first pre-treated with lovastatin 2.5 μM and irradiated 4 hours later by 4 Gy (dose rate = 1 Gy/min). Lovastatin or DMSO was added into a cell appropriate medium which replaced the medium given on day 1 during the seeding.

Two hours after the irradiation, the medium was removed under unsterile conditions from each flask and the cell layer was washed by 2-3 ml of PBS in order to remove all residual medium using unsterile 10 ml pipettes. Once the cell layer was free of medium, the cells were scraped using a cell scraper inside the flask. PBS was used to create a suspension that was subsequently

transported into unsterile 50 ml plastic tubes (one tube per experimental condition, i. e. 4 tubes in total). These cell suspensions were centrifuged at 1200 rpm for 10 minutes at room temperature. After the centrifugation, the PBS supernatant was removed. Prior to the cell harvesting, a lyses buffer was prepared that consisted from the substances listed in table 2.3.

Tab. 2.3: Composition of the lyses buffer.

Substrate	Molecular weight	Dilutions
20 mM Tris HCl (ph=7.5)	157.60	0.0315 g/100 ml water
150 mM NaCl	58.60	0.0876 g/100 ml water
1 mM $MgCl_2$	203.30	0.002 g/100 ml water
1 mM $CaCl_2$	147.02	0.0014 g/100 ml water
1 % NP-40	--	1 ml
10 % glycerol	--	10 ml

For our purpose and volumes, 1 pill of Mini, EDTA-free, a protein inhibitor, was dissolved in 8 ml of the lyses buffer. Note that both the lyses buffer solution and the tubes with cell suspension/sediment were maintained over the whole duration of the preparation process in an ice-filled box (ice was produced in an ice machine).

When the cell sediment was obtained, 500 µl of our lyses buffer was added into each tube with sediment using unsterile pipette and mixed together within the tube. In order to facilitate the cell lyses and protein liberation, ultrasound was used with the following parameters: duration 15 seconds; intensity 42 %.

Afterwards, the cells – buffer suspension was left for 10 minutes without any action in order to let the foam descend, still in an ice box. To enhance the protein liberation, a series of 5 – 6 passages through a tiny insulin injection was done manually and repeated after 10 minutes once again. For each experimental condition/tube, the same injection was strictly used. After the last passage, the whole content of each tube was transferred into unsterile plastic 1.5 ml cups and these were centrifuged at 6000 rpm for 10 minutes at 4 °C.

Supernatant (lysate) from each cup was removed into a new separate 1.5 ml cup and served for protein assay and Western blot. If not used immediately, aliquots containing 20 µl of supernatant per cup were prepared and stored at -20 °C.

2.4.3.2 Bradford protein assay

In order to determine the adequate amount of protein required for further steps of Western blot analysis a Bradford colorimetric protein assay based on standard protein concentrations and their standard curve were done (Bradford, 1976).

Data analysis

A standard curve was created by plotting the standard absorbance values on the y – axis and their concentrations in mg/ml on the x – axis. The experimental sample concentration was determined using the standard curve. Because two independent measurements were performed per experimental sample, the mean value was calculated. It corresponds to the protein concentration within 20 µl of lysate. In order to determine the amount of lysate necessary to obtain 20 µg of proteins, 20 (volume in µl) was divided by the mean concentration value. We obtained the volume of lysate to be loaded into gel. In addition, we calculated the volume of SDS (sodium dodecyl sulphate) buffer (see the chapter below) that needed to be added to obtain a final volume of 20 µl.

2.4.3.3 Gel electrophoresis

This procedure serves to separate proteins of the samples using electric charge according to their molecular weight. In general, a polyacrylamide gel is used for migration and separation of these proteins.

Before the electrophoresis can start, all samples that are supposed to be loaded had to be prepared following the volumes determined according to standard curve calculations as described in the previous chapter. If protein lysates have not been used immediately for migration, they had to be frozen at -20 °C. For this reason, in the first step, the lysates needed to be thawed at room temperature. SDS buffer (cleavage buffer) should already be ready in advance. Its consistence is the following: 30 mM Tris-Base, 9 % sodium dodecyl sulphate (SDS), 15 % glycerine, 0.04 % bromphenol blue Na-salt. Immediately before use, 10 % of 2-mercaptoethanol was added. These samples and solutions were prepared under an unsterile clean bench because of the characteristic and very intensive smell of 2-mercaptoethanol.

Once the experimental lysate samples were ready for use, adequate volumes of these and SDS buffer – mercaptoetahnol solution were mixed together in unsterile cups and centrifuged for few seconds by rapid acceleration. Afterwards, these mixtures were incubated for 5 minutes at a temperature of 95 °C.

Meanwhile, a montage of migration device was done. For our purposes, precast 10-well polyacrylamide gels were used. These gels were placed within the migration set that consisted of a tank, lid, and an electrode assembly (two assemblies could enter into one tank) while two gels could be fixed onto one electrode assembly simultaneously.

Once the montage was finished, the combs serving as spacers for wells of each gel were carefully removed and 20 μl of our sample-SDS buffer solution was loaded into each well according to the previously defined loading design. The whole volume was transferred onto wells using an unsterile pipette. Note that one of the wells needed to be loaded by a marker, i. e. a commercially available mixture of proteins having the defined molecular weights and stained so as to form visible, coloured bands.

Once all wells were filled with samples and a marker, the migration tank and space between two gels was filled with a migration buffer to facilitate the electrophoresis.

The tank was covered with the lid and the electrodes placed at its top were bond to the electricity source. The following parameters were chosen for migration of 2 gels: current of 30 mA, voltage of 200 V. The samples were left for migration and were frequently observed until they descended to the bottom of the gel. This process lasted approximately 2 hours. Once the migration was over, a transfer of the proteins from gel onto a membrane was executed as the next step.

2.4.3.4 Transfer of the proteins from gel onto membrane

In order to proceed subsequently to antibody detection of our proteins, these need to be moved from the polyacrylamide gels onto a membrane. This membrane can be either made of nitrocellulose or polyvinylidene difluoride (PVDF). The procedure of protein transfer is based on the principle of electroblotting, i. e. using an electric current that pulls the proteins from the gel onto the membrane while their organisation as it was within the gel is maintained.

Electroblotting was executed using a Trans-Blot Turbo^{TM} Transfer System. This consisted of the device itself and two cassettes that consisted of two electrodes (cathode and anode). Between these two electrodes, a "blotting sandwich" was assembled. The sandwich was formed of two ion reservoir stacks one at the top and the other at the bottom of the sandwich; 0.2 μm nitrocellulose membrane (being a part of one of two transfer packs) and gel after the electrophoresis. When the sandwich was ready, it was placed on the anode, covered with the lid of the cassette (electrode) and inserted into the device. One gel was inserted into one cassette. Maximum two cassettes

could be blotted simultaneously. Following the manufacturer's manual, an appropriate transfer protocol was chosen with respect to the type of the gel. The transfer time was 3 minutes.

After the transfer, the membranes were removed from the sandwiches and stained using a sodium salt of a diazo dye to assess the loading and transfer efficiency. This was later on reversed by simple water washing and membranes were ready for blocking and incubation with antibodies.

2.4.3.5 Membrane blocking and antibodies incubation

For the purpose of membrane blocking and antibody probing, a WesternBreez® Chromogenic Western blot immunodetection kit was used.

In the first step, it is essential to prevent non – specific binding of antibodies to the membrane by blocking the spaces of the membrane that are not already occupied by proteins. By preventing the interactions between the membrane and the antibodies, the target proteins can be detected without any confusion.

In practice, once the membrane was stained in Ponceau S solution and presence of protein bands was verified, it was subsequently washed in pure water, placed into 10 ml of blocking solution in a covered plastic dish and incubated for 30 minutes on a rotary shaker. Then the solution was decanted and the membrane was rinsed with 10 ml of distilled water for 5 minutes. This procedure was repeated once. Prior to the following step, a solution of our primary antibody needed to be prepared. For the study, mouse anti-ERK IgG2a antibody, rabbit anti-caspase 3, rabbit anti-p-ERK1/2 phosphorylated at Thr202/Tyr204, and rabbit anti-p-Akt antibody phosphorylated at Thr308 were used. A part of specific antibodies, a control of appropriate protein presence within bands was ensured using monoclonal mouse anti-β-actin antibody that is in normal conditions always detectable since actin is a structural protein. Its molecular weight is 42 kDa.

The primary antibodies were diluted in 10 ml of blocking solution in order to obtain dilutions recommended by the manufacturers. For anti-ERK and actin, a dilution 1:4000 was performed. Anti-p-Akt was diluted 1:1000, anti-p-ERK 1:2000 and anti-caspse -3 1:200. For anti-p-ERK1/2, anti-p-Akt and anti-caspase 3 antibodies, no combination with actine was possible because of incompatible hosts (rabbit and mouse respectively).

Thus, in the next step, the membrane was left for incubation in 10 ml of primary antibody solution for one hour still placed on a rotary shaker. After one hour, the membrane was washed

for 5 minutes in 10 ml of antibody wash that consisted of distilled water and antibody wash solution diluted 15:1. This was repeated three times. Subsequently, the membrane was incubated for 30 minutes in 10 ml of secondary antibody solution adapted according to the primary antibody, i. e. mouse or rabbit, than washed again for 5 minutes in 10 ml of antibody wash three times. After the last antibody wash, the membrane was rinsed again in 10 ml of distilled water for three times 2 minutes.

The last step was the incubation with 5 ml of chromogenic substrate, this time without shaking. The incubation time was supposed to vary from one antibody to another. The development was completed in general in 1 to 60 minutes. For our antibodies, 10 minutes were enough for visualisation of the bands.

Finally, the membrane needed to be washed three times in 10 ml of distilled water for 2 minutes and was then left to dry on a clean piece of filter paper at open air at room temperature.

Data analysis

After all washing procedures, the unbound probes were washed away and only the probes labelled and bound to proteins of interest were detected. Size approximations were ensured by visual comparison of the stained bands to that of the marker loaded during electrophoresis. Another way of confirmation was the position of structural protein (actin) that should not change between samples. Actin served also to normalize the amount of the target protein in order to detect eventual errors or an incomplete transfer.

2.5 Statistical analysis of results

All results obtained in CFU, CTB, and caspase-3 and caspase -7 activity assays were tested in order to determine if these were statistically significant or not. For this purpose, a Student t-test in Kaleidagraph® software version 4.1 was used. Appropriate values for pre-treated cells, i. e. lovastatin, cetuximab, bevacizumab, were compared to untreated cells irradiated at the same dose.

The p-values inferior to 0.05 were considered as statistically significant. A star sign (*) was placed within the figures and curves over the points or columns that were significant. To determine the p-values, results for experimental treatment molecule (i .e. lovastatin, cetuximab, bevacizumab or their combinations) were compared with the result obtained for the same cell line irradiated with the same dose without experimental molecule.

3 Results

3.1 Preliminary experiments

3.1.1 Appropriate lovastatin concentration for further experiments

In order to detect the most suitable lovastatin concentration for all upcoming experiments, a CFU assay procedure as described in chapter 2.3.1.2 was adopted.

As seen in figure 3.1, 25 μM lovastatin even alone exhibits a highly toxic effect in both cell lines. However, this concentration has been used in few experiments of cell viability to prove its toxicity. On the other hand, the 2.5 μM lovastatin exerted slight but sufficient decrease of clonogenic cell potential and thus turned out to be the most appropriate for further studies. Since 5 and 10 μM lovastatin did not seem to sensitize particularly the A 549 cells and seemed quite harmful in ZMK-1 these concentrations were almost completely omitted in this work.

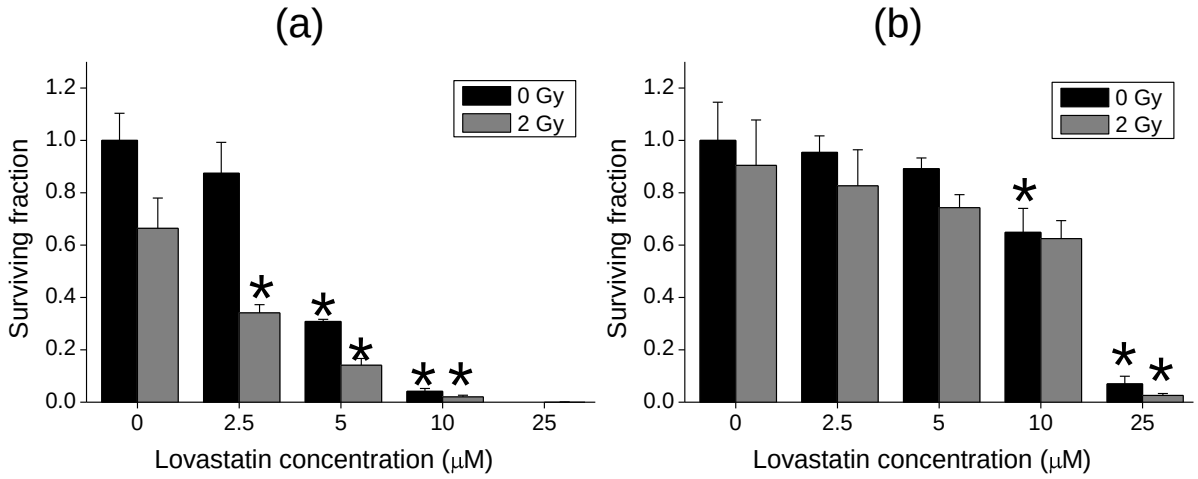


Fig. 3.1: Mean value of surviving fraction plotted against concentration of lovastatin for two different cell lines (figure (a) for ZMK-1, figure (b) for A 549) exposed to irradiation with the dose of 2 Gy and without irradiation. Pre-treatment by lovastatin was applied 4 hours before the irradiation. Experiments were performed once in triplicate.

3.1.2 Optimal incubation time and cell number within cell viability assay

According to the procedure described above (chapter 2.3.1.3) the determination of sufficient incubation time as well as of optimal cell quantity per well could be achieved as documented in table 3.1 below.

Thus all experiments using CTB assay were performed with an incubation time of 72 and 96 hours for all examined cell lines.

Tab. 3.1: Review of optimal cell number to seed, incubation time, and radiation dose for CTB assay according to each cell line.

Cell line	Nb cells to seed	Incubation time [h]	Radiation dose [Gy]
HGT 1	4000 – 5000	72; 96	4
ZMK – 1	6000	72; 96	4
MO59J	4000-5000	72; 96	2
MO59K	3000 – 4000	72; 96	4
A 549	4000 - 5000	72; 96	4
HaCaT	4000-5000	72; 96	4
HUVEC	3000-5000	72; 96	4
fibroblasts	3000-5000	72; 96	4

3.2 Imapct of lovastatin on all cell lines examined

3.2.1 Lovastatin alone

Surviving fraction representation

Using Kaleidagraph® software, all surviving fraction (SF) values of non-irradiated and untreated cells obtained within all performed experiments were gathered and mean SF and standard deviations were calculated. The same procedure was applied for the cells treated with 2.5 μM lovastatin. As already mentioned above, lovastatin 25 μM had a very toxic effect on ZMK-1 and A 549 cells in colony formation tests and that is why the results presented concern uniquely lovastatin 2.5 μM association.

Graphic representation of all cell lines shown in figure 3.2 except fibroblasts permits to observe a differential effect of low dose lovastatin in comparison with the untreated cells. Fibroblasts turned out to be very lovastatin responsive and for this reason few complementary CFU assays were performed using 0.25 μM lovastatin. However, even this concentration attenuated the cell survival in a very important manner, thus SF outcomes for this cell line are not presented in this work.

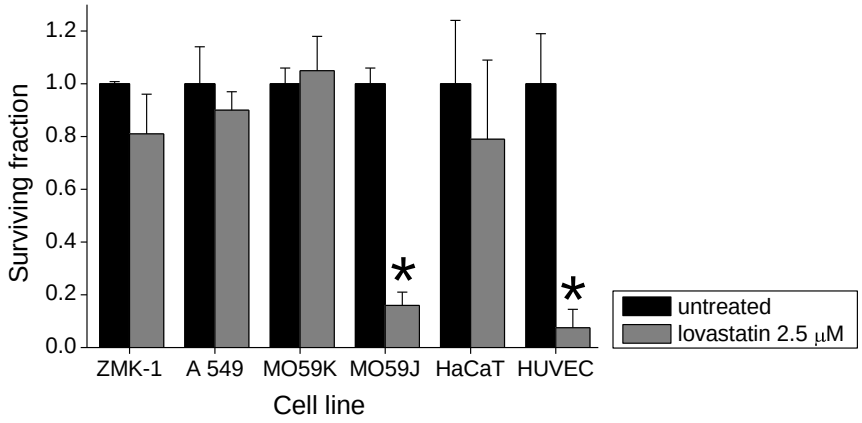


Fig. 3.2: Mean survival fraction values of untreated (i.e. non-irradiated, no lovastatin) and lovastatin 2.5 μM pre-treated cell lines. Each experiment has been done three times, always in triplicate.

Cell viability representation

Similar to the previous procedure, also the cell viability results were assembled for the untreated (non-irradiated) cells and for the cells that were pre-treated with lovastatin 2.5 and 25 μM. Subsequently, the mean values of fluorescence that correlate directly with the cell viability (metabolic activity), and their standard deviations were calculated. The scheme of the experiments that permitted to obtain these results was already mentioned in chapter 2.3.1.2. The seeding and incubation conditions were adapted according to the results shown in chapter 3.1.2.

Figures 3.3 and 6.1 show that for the lovastatin 2.5 μM treatment, a clear difference in cell viability can be observed regarding tumour and normal tissue cell lines. The latter are noticeably less active. This could be also seen in CFU assays where normal tissue cell lines (especially HUVEC cells) and MO59J as tumour cell line needed more time to create sufficiently large colonies.

As for the incubation time, the viability seems to be slightly higher if longer incubation is applied although the tendencies within the cell lines are maintained similar.

Of all tumour cell lines, A 549, MO59K, and MO59J are rather lovastatin-sensible. However, even ZMK-1 presents significant decrease in cell viability if pre-treated with lovastatin. Activity of HaCaT and HUVEC cells remains unchanged with 2.5 μM lovastatin. Lovastatin induced decreased viability of fibroblasts is in accordance with the CFU assay results.

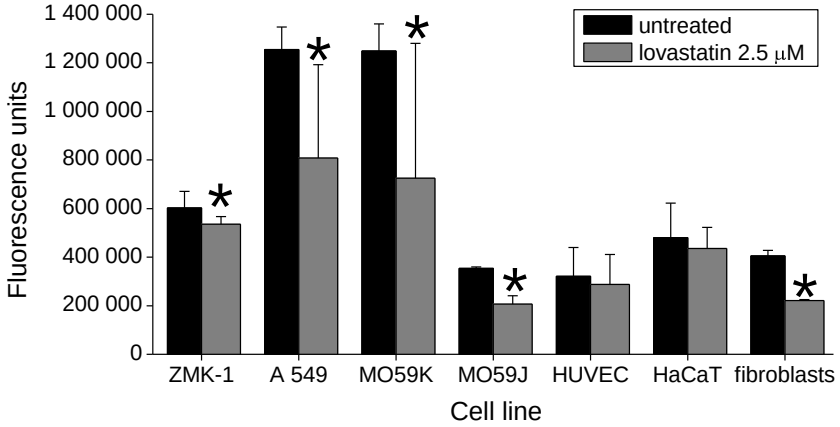


Fig. 3.3: Cell viability expressed in fluorescence units per cell line without treatment and with lovastatin 2.5 μM pre-treatment. Incubation time was 96 hours. Each experiment has been performed at least once, always in triplicate.

For 25 μM lovastatin treatment (figures 3.4 and 6.2), MO59J and fibroblasts were excluded from the experiments because of already very strong sensitizing effect at even low doses of lovastatin within CFU assays as well as because of extremely slow cell growth (permanently insufficient quantity of cells for the CTB assay). It is obvious that high dose lovastatin causes significant cell viability decrease for all other cell lines similar as in the CFU assay.

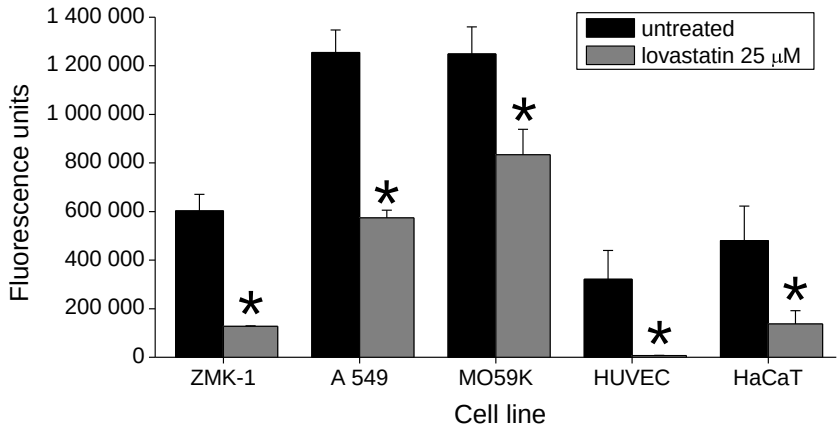


Fig. 3.4: Cell viability expressed in fluorescence units per cell line without treatment and with lovastatin 25 μM pre-treatment. Incubation time was 96 hours. Each experiment has been performed at least once, always in triplicate.

3.2.2 Lovastatin associated with irradiation

3.2.2.1 Comparison of overall impact

In order to better visualize graphically the impact of lovastatin (2.5 and 25 μM) on cells together with irradiation, the mean values of fluorescence obtained in cell viability assays were calculated

in the same way as in the previous cases (chapter 3.2.1) together with SD bars. This allows us to determine whether there exists a radiosensitizing effect of statin or not.

As for the 2.5 μM lovastatin (figures 3.5 and 6.3), a slight but significant decrease in cell viability can be observed in ZMK-1. Lovastatin 2.5 μM alone lowers activity in A 549, MO59K, and HaCaT and association with irradiation does even enhance this lowering tendency. HaCaT cells do not respond to lovastatin even if combined with irradiation. HUVEC cells seem to respond mostly if combined effect of irradiation and lovastatin and fibroblasts are highly sensible to lovastatin with or without additional irradiation.

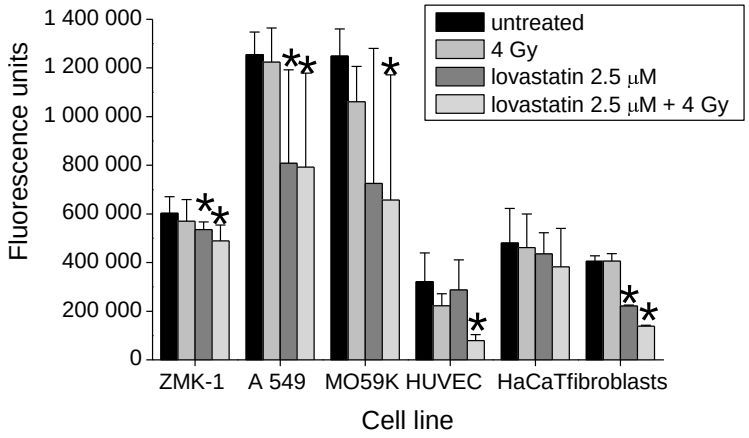


Fig. 3.5: Mean values of cell viability expressed in fluorescence units per cell line without any treatment, with irradiation alone (4 Gy), with lovastatin 2.5 μM pre-treatment alone or combination of lovastatin 2.5 μM pre-treatment 4 hours prior to irradiation (4 Gy). Incubation time was 96 hours. Each experiment has been done at least once, always in triplicate.

While MO59J cell line is considered to be highly radiosensitive, a dose of 2 Gy of irradiation was delivered and the obtained results appear separately below in figure 3.6.

As expected, already 2 Gy of irradiation lowered the cell viability in a very important manner. Furthermore, an addition of lovastatin 2.5 μM sensitized the cell even more.

For the association of irradiation and lovastatin 25 μM the following results, as seen in figures 3.7 and 6.4, were achieved.

As for CFU assay, in CTB test treatment with highly concentrated lovastatin abolishes significantly activity of all cell lines without exception coupled with a strong radiosensitizing effect. Effect over HUVEC cells seems to be the most toxic one. As well as in other CTB tests, difference in incubation time does not play any role.

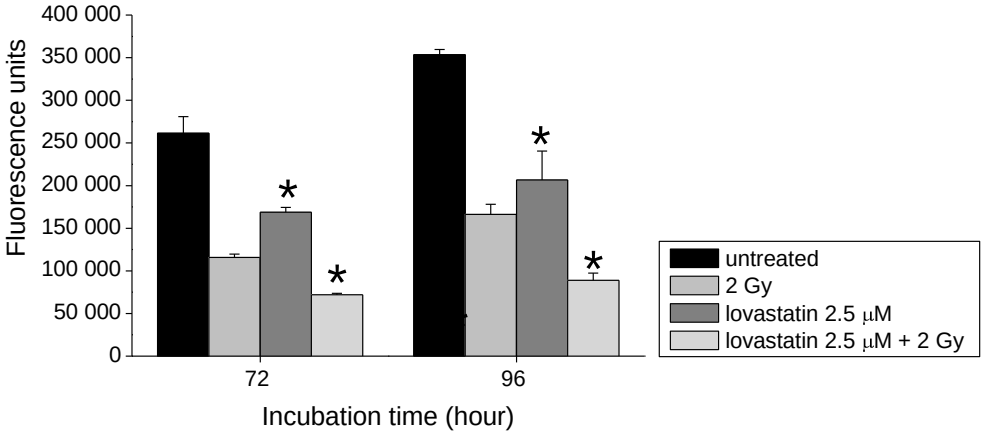


Fig. 3.6: Mean cell viability values in fluorescence units exclusively for MO 59 J cell line without any treatment, with irradiation alone (2 Gy), with lovastatin 2.5 μM pre-treatment alone or combination of lovastatin 2.5 μM pre-treatment 4 hours prior to irradiation (2 Gy). Incubation times 72 and 96 hours are presented. Experiment was performed once in triplicate.

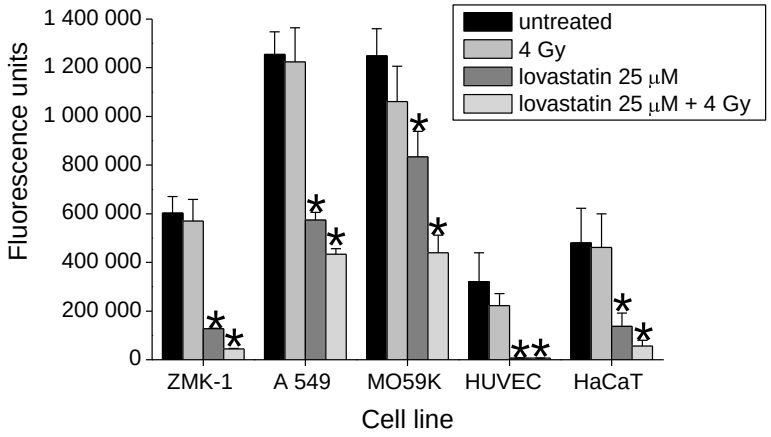


Fig. 3.7: Mean values of cell viability expressed in fluorescence units per cell line without any treatment, with irradiation alone (4 Gy), with lovastatin 25 μM pre-treatment alone or combination of lovastatin 25 μM pre-treatment 4 hours prior to irradiation (4 Gy). Incubation time was 96 hours. Each experiment has been done once always in triplicate.

3.2.2.2 Impact according to cell lines

Here, using the results of CFU assays, surviving fraction curves that have been created as described in chapter 2.3.1.1, are presented for each cell line separately. Furthermore, survival curves with and without correction for effects induced by lovastatin are given in order to compare how important the differences are if the results are normalized, i. e whether irradiation related lovastatin pre-treatment affects the cell survival.

The sensitizer enhancement ratios of lovastatin 2.5 and 0.25 μM (for HUVEC exclusively) at a survival fraction of 50 % were calculated as mentioned in chapter 2.3.1.1 and are listed in table 3.2.

The SER could not be determined for fibroblasts since in CFU assay no colonies could be observed after the pre-treatment with lovastatin at 2.5 μM. No other concentrations of lovastatin were tested because of slow cell growth and uneasy culturing conditions of this cell line. Cell lines that turned out to be radiosensitized by the effect of lovastatin are mostly the normal tissue cells (especially HaCaT) and MO59J cell line that is already known for its high intrinsic sensitivity to IR. All other tumour cell lines were protected by addition of lovastatin.

Tab. 3.2: Sensitizer enhancement ratio of two lovastatin concentrations (2.5 and 0.25 μM) on 6 cell lines.

Cell line	Lovastatin [μM]	SER (50%)	Cell line	Lovastatin [μM]	SER (50%)
ZMK-1	2.5	1	MO59J	2.5	1.36
A 549	2.5	0.86	HUVEC	0.25	1.06
MO59K	2.5	1	HaCaT	2.5	1.53

ZMK-1

Seeding and treatment procedure for this cell line corresponds to the first seeding method mentioned in chapter 2.3.1.1. Since ZMK-1 cells grew quickly the number of cells that were seeded per well varied between 100 and 1000. Colonies were fixed and stained 8 – 10 days after seeding.

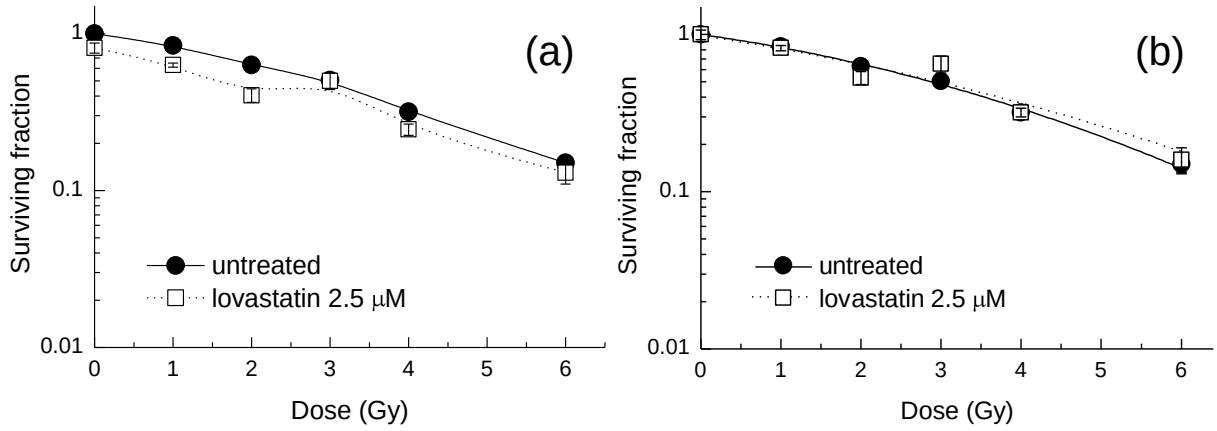


Fig. 3.8: Surviving fraction curves of ZKM-1 cell line with irradiation alone (full lines) and pre-treated with 2.5 μM lovastatin 4 hours before irradiation (dotted lines): SF curves before correction for lovastatin-induced effects on colony formation (a);SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b).

The curves in figure 3.8 show that lovastatin alone, without normalizing the outcomes, leads to decrease of cell survival however the tendency remains similar to irradiation alone at different radiation doses. Lovastatin normalized to irradiation has even radioprotective effect on this cell line.

A 549

For this cell line, the seeding and treatment scheme is equivalent to that of ZMK-1. For the experiments, 100 to 1000 cells were seeded per well and left for incubation during 8 – 12 days. Similar results should be concluded in the case of A 549 as for ZMK-1, i. e. lovastatin 2.5 μM alone lowers slightly the cell survival without sensitizing to the associated irradiation. On contrary, the same aspect of radioprotection is observed. The obtained data are given in figure 3.9.

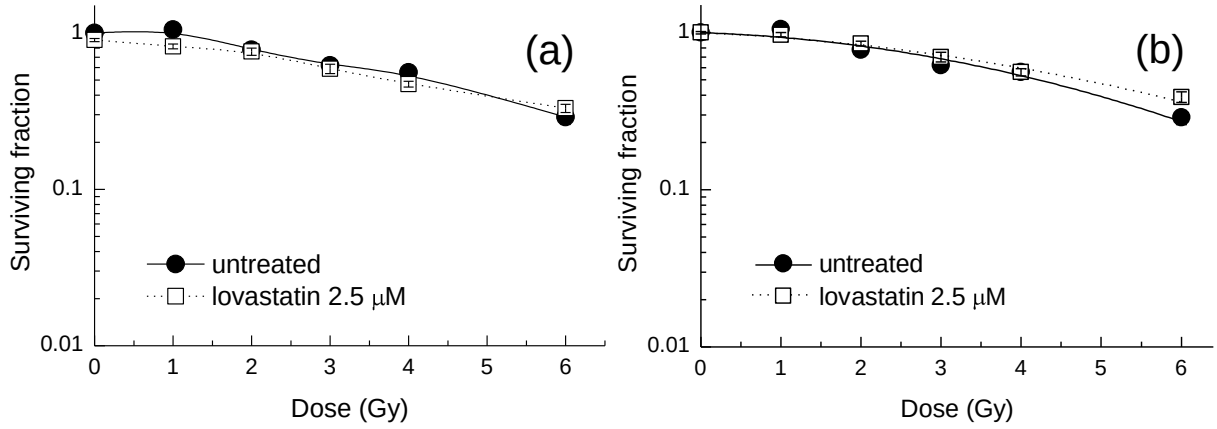


Fig. 3.9: Surviving fraction curves of A 549 cell line with irradiation alone (full lines) and pre-treated with 2.5 μM lovastatin 4 hours before irradiation (dotted lines): SF curves before correction for lovastatin-induced effects on colony formation (a); SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b).

MO59K

MO59K and HaCaT cell lines were exceptions within the CFU assays since a part of lovastatin 2.5 μM and lovastatin 5 μM was tested, too. Concerning seeding and treatment procedures, these were designed as described in chapter 2.3.1.1 (second detailed method, i. e. treatment by lovastatin was done 24 hours after seeding followed by irradiation 4 hours later). In general, 200 – 2000 cells/well were seeded and 12 – 14 days were left for colony formation.

Interestingly, there is almost no difference between normalized and non normalized curves in figure 3.10. Furthermore, in both considerations (with or without normalization) a

radiosensitizing tendency of lovastatin can be observed. However, SER of 2.5 μM lovastatin does not confirm this theory (SER 50 % = 1). As supposed, higher concentration of lovastatin is more toxic. The only significant outcomes were obtained for cells pre-treated with 5 μM lovastatin and irradiated by a dose of 1 Gy.

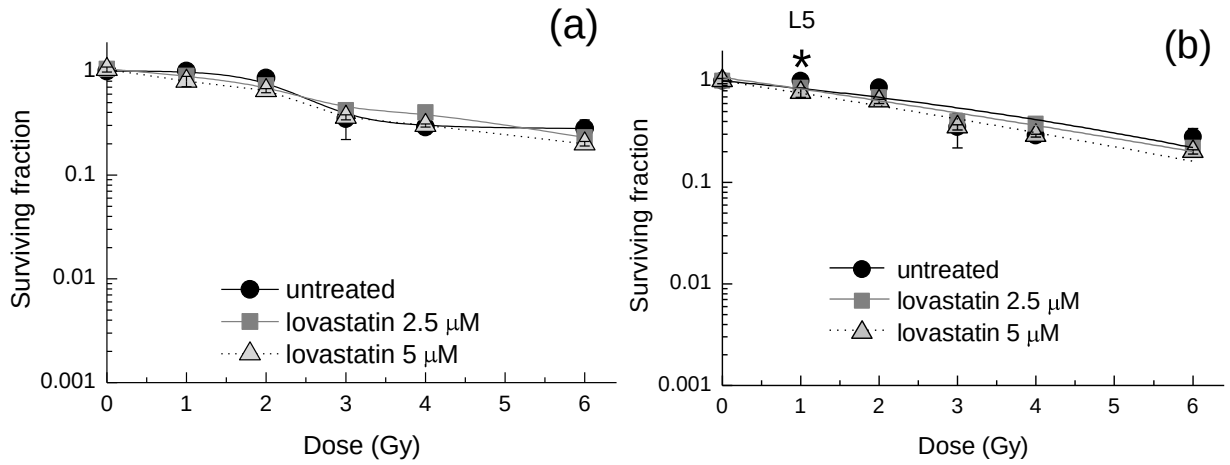


Fig. 3.10: Surviving fraction curves of MO59K cell line with irradiation alone (full black lines), pre-treated with 2.5 μM lovastatin (full gray lines) or 5 μM lovastatin (dotted lines) 4 hours before irradiation (dashed lines): SF curves before correction for lovastatin-induced effects on colony formation (a); SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b). L5 = lovastatin 5 μM.

MO59J

The seeding and treatment scheme was similar to the scheme for MO59K except for the fact, that only 2.5 μM lovastatin was added and that the irradiation consisted of only 1 and 2 Gy (plus control). As it was already mentioned above, MO59J cells revealed an extremely slow growth thus the incubation time before colony fixation and staining was at least 14 days long. Furthermore, high cell mortality even in conditions of standard cultivation obliged us to seed relatively high number of cells per well, in this case 1000 – 3000 cells. Despite such elevated cell quantity, the final character of colonies was very unsatisfactory.

Numerous single-cell or very few-cell colonies could be visualized. Since almost no colonies of 50 or more cells were present, all colonies formed of up to 10 cells were taken into consideration. As far as survival curves in figure 3.11 with or without normalization are concerned, both show strong sensitization of cells to lovastatin with a proved radiosensitizing effect already at small radiation doses.

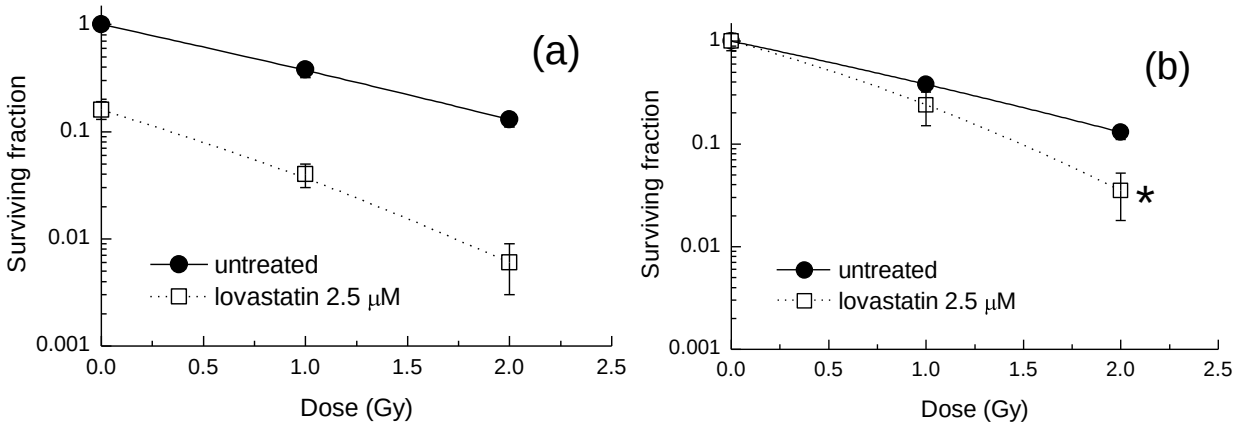


Fig. 3.11: Surviving fraction curves of MO59J cell line with irradiation alone (full lines) and pre-treated with 2.5 μM lovastatin 4 hours before irradiation (dotted lines):. SF curves before correction for lovastatin-induced effects on colony formation (a); SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b).

HUVEC

For colony formation assays on HUVEC, the cells were seeded according to the second method described in chapter 2.3.1.1, i. e. pre-treatment with lovastatin was done 24 hours after seeding and 4 hours before irradiation. In the very first experiments, lovastatin 2.5 μM was used. This concentration demonstrated extremely high cell toxicity within CFU assays with no detectable colonies after even long incubation time and for this reason 10-times lower dose of 0.25 μM was chosen for the further experiments. One thousand to three thousand five hundred cells were seeded per well and the incubation time needed was 15 days.

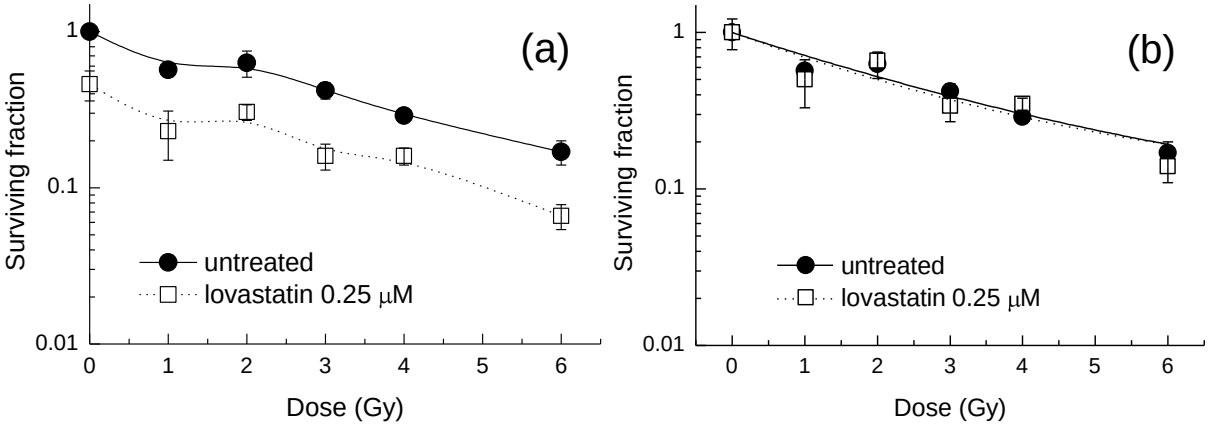


Fig. 3.12: Surviving fraction curves of HUVEC cell line with irradiation alone (full lines) and pre-treated with 0.25 μM lovastatin 4 hours before irradiation (dotted lines): SF curves before correction for lovastatin-induced effects on colony formation (a); SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b).

As demonstrated in figure 3.12, even a low concentration of lovastatin induces decreased cell survival, however in comparison with the irradiation alone the addition of lovastatin does not have such toxic effect as expected.

HaCaT

As it was already mentioned for the MO59K cells, even HaCaT were exceptionally treated during the initial experiments with two different low concentrations of lovastatin, i. e. 2.5 and 5 μM according to the scheme similar to HUVEC, MO59K, and MO59J. In average, 500 – 3000 cells were seeded per well and these well growing cells were maintained for incubation during 11 – 15 days. Both concentrations of lovastatin sensitize HaCaT cells as seen in figure 3.13. It is noteworthy that in association with irradiation the 2.5 μM lovastatin seems to radiosensitize the cells more than the 5 μM lovastatin although these results are not significant. The pure effect of statins should be logically higher for elevated concentrations of lovastatin.

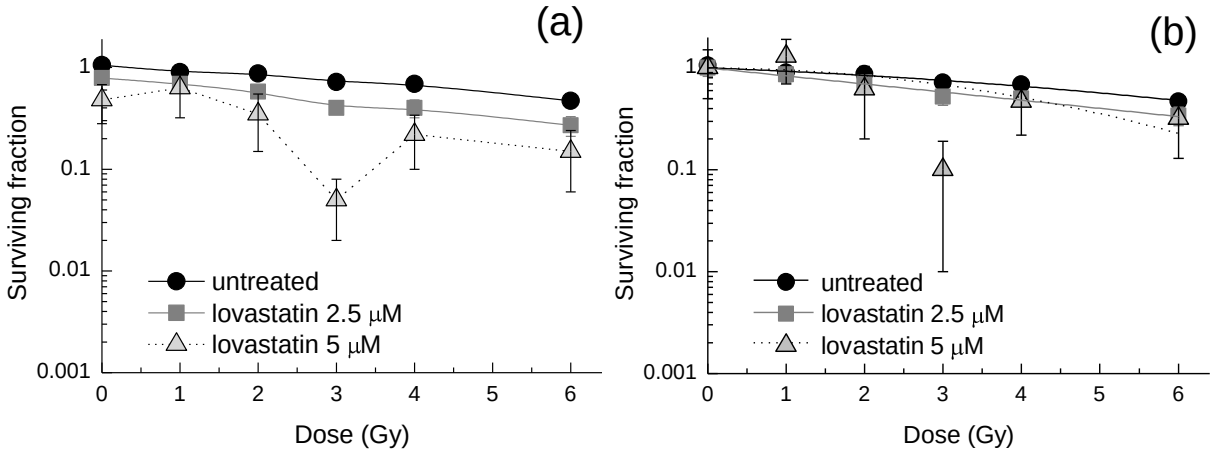


Fig. 3.13: Surviving fraction curves of HaCaT cell line with irradiation alone (full black lines), pre-treated with 2.5 μM lovastatin (full gray lines) or 5 μM lovastatin (dotted lines) 4 hours before irradiation: SF curves before correction for lovastatin-induced effects on colony formation (a); SF curves after correction for lovastatin-induced effects on colony formation (normalized survival) (b).

Fibroblasts

Within the initial experiments, fibroblasts were tested using lovastatin 2.5 μM in the same manner as in the four previous cell lines. However, these cells exhibited very slow growth potential and even after 16 days of incubation no relevant colonies could be observed. Furthermore, lovastatin at this concentration acted very toxically even without concurrent

irradiation. Thus, the outcomes received for this cell line could not be presented. Fibroblasts were subsequently excluded from all CFU assays and only cell viability test were maintained.

3.3 Association with the targeted therapies

Since seven different cell lines and two monoclonal antibodies were investigated within this study, combinations of them would expect a large number of experiments and material. For this reason and in order to follow relevant clinical indications, only three cell lines were tested per monoclonal antibody, namely ZMK-1, A 549, and HaCaT for cetuximab and A 549, MO59K, and HUVEC for bevacizumab.

Association of cetuximab in head and neck cancers as well as in lung tumours is known and performed in every day clinical practice. The main side effect of cetuximab, acne, is related to the fact that keratinocytes of human skin express high EGF receptors and thus become targets of this treatment as well.

Bevacizumab is often indicated in lung tumours and as the second line treatment of malignant glioblastoma. Because of acting on VEGF, the choice of the HUVEC cells as the third cell line to be tested seemed relevant.

3.3.1 Cetuximab

3.3.1.1 EGFR status of all cell lines

Presence of EGFRs on cell membrane and in cytoplasm was explored as described in chapter 2.3.2.

The following pictures in figure 3.14 show the results obtained for each cell line that was studied in this work including negative control.

Brown staining correlates with the presence of EGF receptors. The more receptors are expressed the browner the cells are. Receptor-free cells remain blue in colour.

Thus, when compared to the negative control it can be seen that all cell lines except HUVEC express more or less these receptors both on the membrane and in cytoplasm. Remarkable high expression status within HaCaT cells could be correlated with high response to cetuximab-based treatment as it will be discussed later in chapter 3.3.1.3.

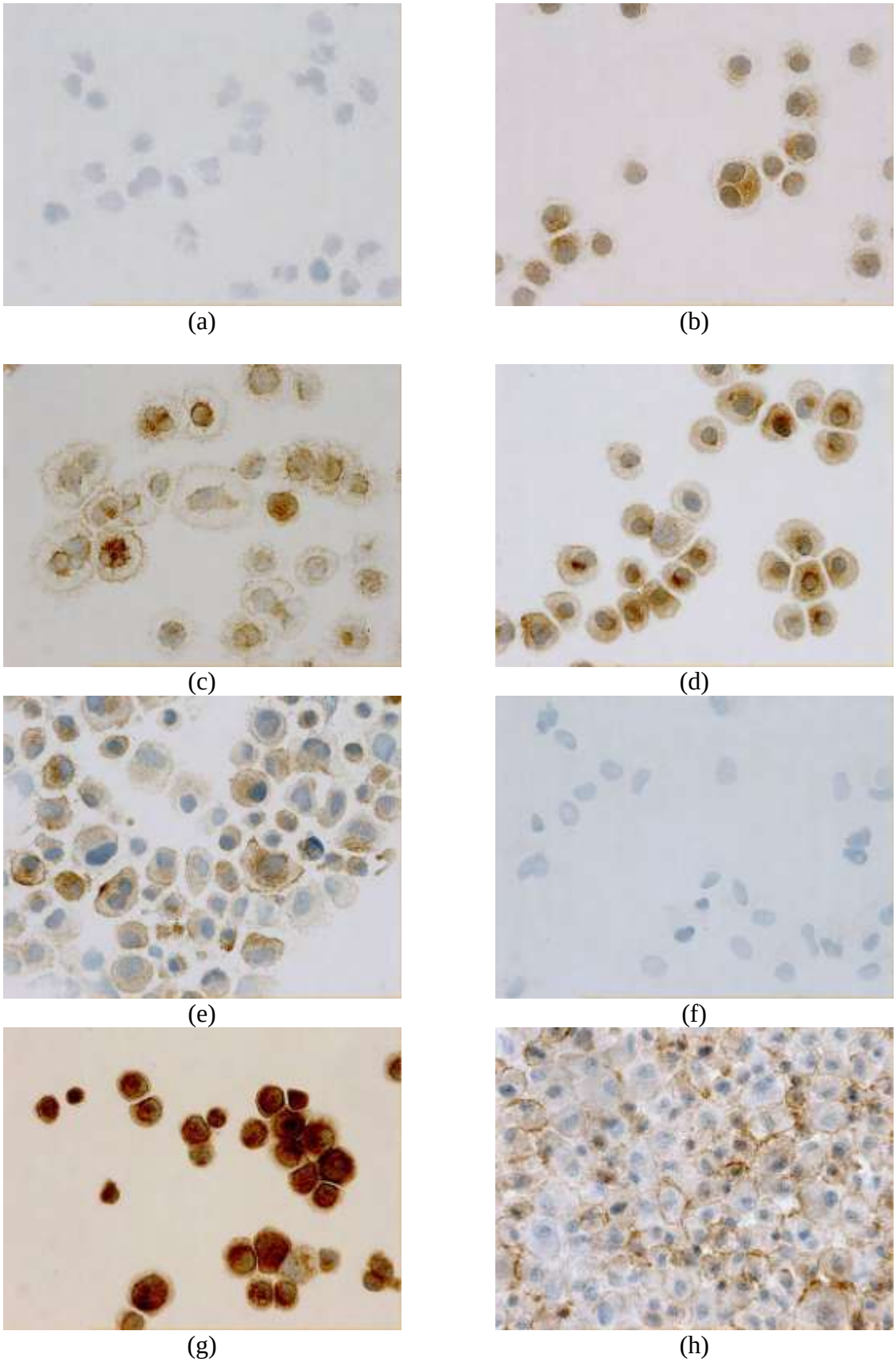


Fig. 3.14: Cells stained using IHC method (anti-EGFR antibody) to detect the outer membrane and cytoplasm EGF receptor in different cell lines. 40 x magnifications in light microscope. Cell lines: a – negative control on A 549, b – ZMK-1, c – A 549, d – MO59K, e – MO59J, f – HUVEC, g – HaCaT, h – fibroblasts.

3.3.1.2 Impact of cetuximab alone in three cell lines

Here, the results of cell viability assay on 3 cell lines (ZMK-1, A 549 and HaCaT) treated with cetuximab 0.1 μM and untreated control are presented. The seeding procedure was mentioned in chapter 2.3.1.1. The choice of the following concentration of cetuximab as well as the interval between treatment and irradiation were based upon the results of the recently published works (Lee et al. 2011; Saki et al. 2012).

Within this viability test cetuximab seems to hardly affect the activity of tumour cells as demonstrated in figures 3.15 and 6.5. On the other hand, it induces rather strong and significant decrease in keratinocytes. This could be explained probably by the fact that HaCaT cells express highly EGF receptors as seen in figure 3.14.

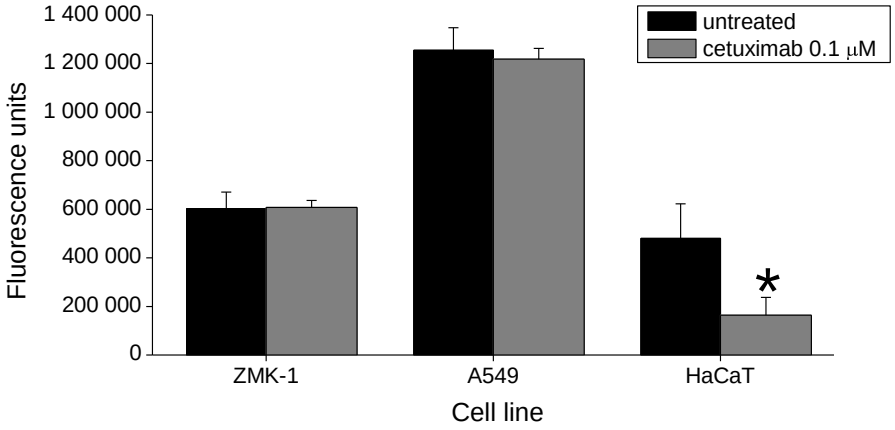


Fig. 3.15: Cell viability expressed in fluorescence units per cell line without treatment and with cetuximab 0.1 μM pre-treatment. Incubation time was 96 hours. Each experiment has been performed twice, always in triplicate.

3.3.1.3 Combination of cetuximab, lovastatin, and irradiation according to cell lines

Survival curves obtained from CFU assays and outcomes of cell viability tests are shown in order to compare the impact of cetuximab, lovastatin or irradiation alone and of their combination on the three cell lines.

For colony formation assays, the results were corrected for both treatments preformed and are shown together with the uncorrected ones. It should be noted that a difference in seeding procedure was adopted if all treatment steps (i. e. lovastatin, cetuximab and irradiation) were used together. The cells were seeded on day 0 (D0) and further treatments were planed in order

to perform irradiation 24 hours after seeding (on D1). Lovastatin was administered 4 hours and cetuximab one hour prior to irradiation. This was similar for ZMK-1, A 549, and HaCaT as well.

In the cell viability assays, the seeding scheme followed the method described in chapter 2.3.1.2. The cell quantity corresponded to the results shown in chapter 3.1.2. Similar to the CFU assay, the lovastatin was administered 4 hours and cetuximab 1 hour prior to irradiation.

Survival curves obtained for each cell line permitted to determine the SER of cetuximab 0.1 µM as well as of the combination cetuximab – lovastatin at a survival fraction of 50 % as seen in table 3.3.

Treatment by cetuximab alone sensitizes tumour cells to irradiation whereas further association with lovastatin renders cells resistant to this sensitization. This can be explained by the fact that lovastatin alone possesses a radioprotective effect on ZMK-1 and A 549 cell lines as seen in table 3.2. No outcomes could be revealed for HaCaT cells because of a highly toxic impact of cetuximab 0.1 µM that allowed no colonies to be grown up under this experimental condition.

Tab. 3.3: Sensitizer enhancement ratio of cetuximab 0.1 µM +/- lovastatin 2.5 µM on 3 cell lines.

Cell line	SER (50%) cetuximab 0.1 µM	SER (50%) cetuximab 0.1 µM + lovastatin 2.5 µM
ZMK-1	1.2	0.95
A 549	1.2	1.04
HaCaT	--	--

ZMK-1

Cells for the CFU assay were seeded as described above. In general, 100 – 2000 cells per well were plated and incubated during 10 days before alcohol fixation and staining.

Figure 3.16 shows that cetuximab itself is less toxic for ZMK-1 cells than lovastatin alone however, after normalization to irradiation cetuximab sensitizes cells better. No experimental point was significant. In these conditions, lovastatin acts rather as a protector against IR-induced toxicity and cancels toxic effect of cetuximab if administered concurrently.

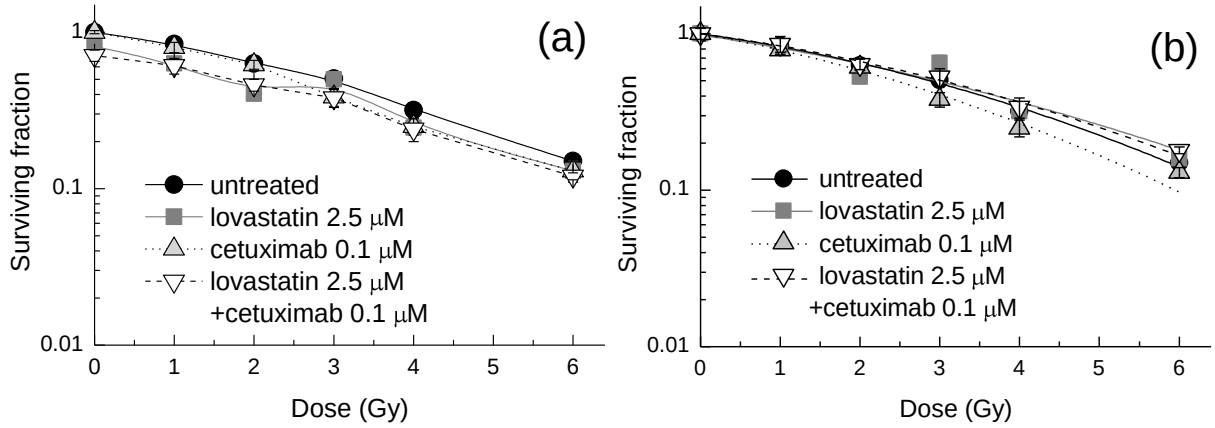


Fig. 3.16: Surviving fraction curves of ZKM-1 cell line with irradiation alone (full black lines), pre-treated with 0.1 μM cetuximab 1 hour before irradiation (dotted lines), pre-treatment by 2.5 μM lovastatin 4 hours before irradiation (full gray lines) and combination of both molecules (dashed lines): SF curves before correction (a); SF curves after correction for cetuximab- and lovastatin-induced effects on colony formation (normalized survival) (b).

Results within the test of cellular viability (figures 3.17 and 6.6) are comparable to the CFU results since cetuximab alone has no particular toxic impact in comparison with lovastatin. Treatment with lovastatin associated with cetuximab or not decreases cell viability in comparison to untreated cells even without irradiation. A significant radiosensitizing effect can be seen if irradiation is associated with lovastatin and cetuximab pre-treatment.

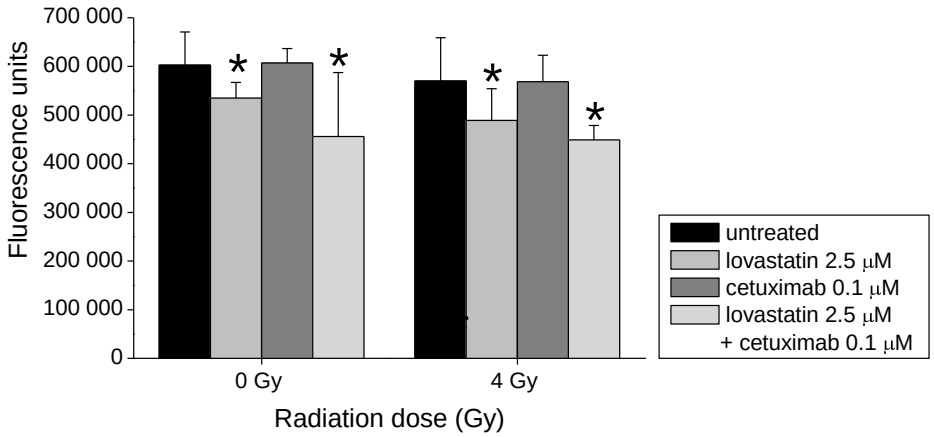


Fig. 3.17: Cell viability expressed in fluorescence units in ZMK-1 pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 96 hours. Experiment has been performed at least once, always in triplicate.

A 549

For this cell line, the same seeding scheme is applied as for the ZMK-1 cells with the same quantity of the seeding used. The incubation was of 10 – 12 days.

In figure 3.18, comparable results can be observed as in the case of ZMK-1, i. e. more toxic effect of lovastatin than cetuximab if given alone. However, once results are normalized according to the radiation itself cetuximab lowers slightly the clonogenic potential of A 549 (SER 50 % = 1.2) whereas if combined with lovastatin this sensitizing effect disappears.

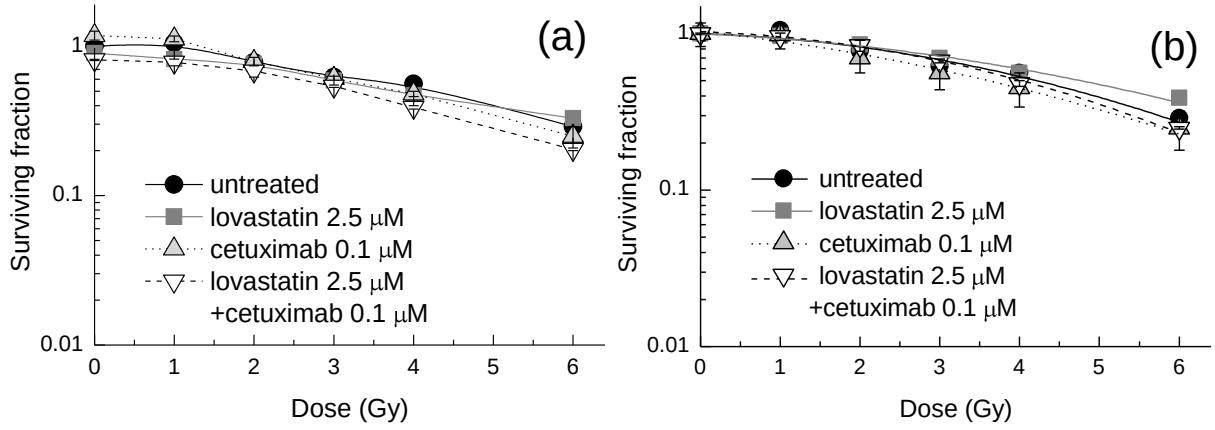


Fig. 3.18: Surviving fraction curves of A 549 cell line with irradiation alone (full black lines), pre-treated with 0.1 μM cetuximab 1 hour before irradiation (dotted lines), pre-treatment by 2.5 μM lovastatin 4 hours before irradiation (full gray lines), and combination of both molecules (dashed lines): SF curves before correction (a); SF curves after correction for cetuximab- and lovastatin-induced effects on colony formation (normalized survival) (b).

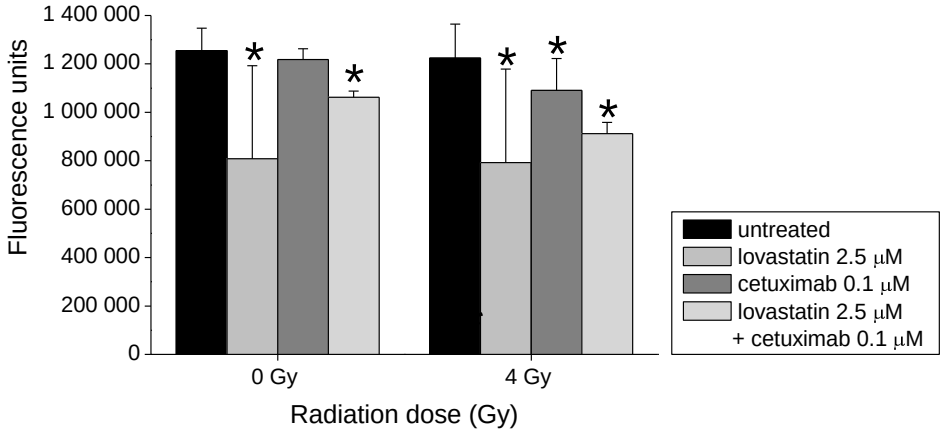


Fig. 3.19: Cell viability expressed in fluorescence units in A 549 pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was of 96 hours. Experiment has been performed at least once, always in triplicate.

As presented in figures 3.19 and 6.7, the effect of cetuximab, lovastatin and irradiation alone or in combination one with another gives approximately the same results as in the case of ZMK-1, i. e. lovastatin seems more harmful than cetuximab both in non-irradiated or irradiated cells. Combination of all three therapeutic modalities demonstrates a significant decrease in cell activity in comparison with the untreated cells although the final effect is less prominent than the effect of lovastatin alone.

HaCaT

In contrary to the previously described cetuximab treated cell lines, HaCaT cells exhibited an extreme toxicity induced by cetuximab 0.1 μM within CFU assays. For this reason, the formation of clonogenic curves was impossible. Thus, a CFU assay aiming at exploring different rising cetuximab concentrations (0.001; 0.01; and 0.1 μM) upon HaCaT was performed according to the same seeding procedure as described above with exception of irradiation. In each well 500 – 2000 cells were plated.

Figure 3.20 demonstrates that all tested concentrations of cetuximab act very toxically even without concurrent irradiation. No further colony formation tests were performed in this field.

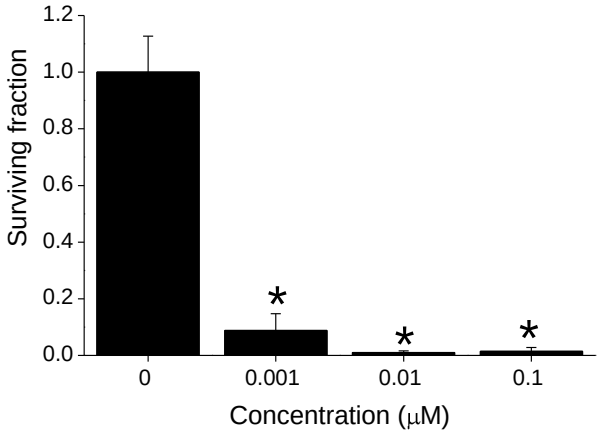


Fig. 3.20: Dose-effect relation of cetuximab at different concentrations on HaCaT cells. Experiment has been performed once in triplicate.

Combination of irradiation, cetuximab, and lovastatin was evaluated in the cell viability assay as it was for ZMK-1 and A 549 described above and the results are presented in figures 3.21 and 6.8.

Similar observations of an important cetuximab-induced decrease in cell viability can be mentioned within this test, too. Moreover, combination of lovastatin and cetuximab promote a significant radiosensitizing effect upon this cell line.

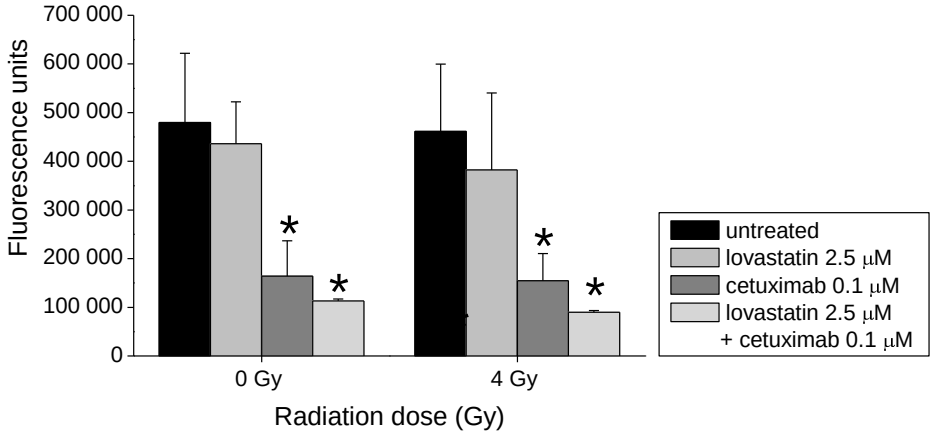


Fig. 3.21: Cell viability expressed in fluorescence units in HaCaT pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 96 hours. Experiment has been performed at least once, always in triplicate.

3.3.2 Bevacizumab

3.3.2.1 Impact of bevacizumab alone in three cell lines

Here, the results of cell viability essay upon the 3 cell lines (A 549, MO59K and HUVEC) treated with bevacizumab 16.8 μM and untreated controls are presented. The seeding procedure was already mentioned in chapter 2.3.1.1.

In figures 3.22 and 6.9 we can see that bevacizumab alone decreases significantly the viability exclusively in normal tissue cell line, HUVEC, whereas two tumour cell lines seem completely untouched and even a significant protective effect can be observed for A 549 cell line.

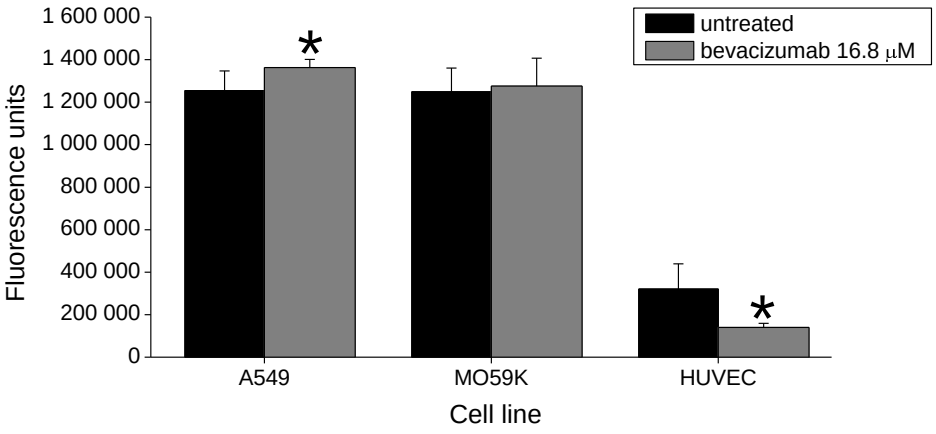


Fig. 3.22: Cell viability expressed in fluorescence units per cell line without treatment and with 16.8 μM bevacizumab pre-treatment. Incubation time was 96 hours. Each experiment has been performed twice, always in triplicate.

3.3.2.2 Combination of bevacizumab, lovastatin, and irradiation according to cell lines

As it was for cetuximab, the survival curves obtained in colony formation assays and the results of the cell viability tests are shown here in order to compare the impact of bevacizumab, lovastatin or irradiation alone and of their combination on the three cell lines.

For CFU assays, the results were corrected for both treatments preformed and they are shown together with the uncorrected ones. A difference in seeding procedure was observed if all treatment steps (i. e. lovastatin, bevacizumab and irradiation) were applied all together. The cells were seeded on day 0 (D0), bevacizumab was administered approximately 24 hours after the seeding (D1), and 24 hours before irradiation (D2). Lovastatin was added 4 hours prior to irradiation on day 2. This was similar for A 549, MO59K and HUVEC except the fact that HUVEC cells were treated with 10-times lower lovastatin concentration, i. e. 0.25 µM.

In the cell viability assays, the seeding scheme followed the method described in chapter 2.3.1.2. The cell quantity corresponded to the results shown in chapter 3.1.2. Similar as in the CFU assay, bevacizumab was administered 24 hours and lovastatin 4 hours prior to the irradiation. Like in the combination with cetuximab, even for bevacizumab the SER was calculated for all three cell lines (see table 3.4).

No radiosensitizing potential was found in these cell lines either for bevacizumab or for lovastatin co-association. Inversely, for HUVEC cells lovastatin promoted a protective effect against irradiation. Because of almost complete absence of colonies in plates pre-treated with association lovastatin – bevacizumab SER could not be calculated.

Tab. 3.4: Sensitizer enhancement ratio of bevacizumab 16.8 µM +/- lovastatin 2.5 µM (lovastatin 0.25 µM for HUVEC) on 3 cell lines.

Cell line	SER (50%) bevacizumab 16.8 µM	SER (50%) bevacizumab 16.8 µM + lovastatin 2.5 or 0.25 µM
A 549	0.86	1
MO59K	1.03	1
HUVEC	0.36	--

A 549

The seeding procedure was described above. In principle, 100 – 2000 cells were plated per well and incubated during 11 days before alcohol fixation and staining.

Pure bevacizumab and lovastatin administration decreases the clonogenic potential accompanied by more pronounced toxicity if combined together. However, if normalized to irradiation, radioprotective rather than radiosensitizing potential of both, lovastatin and bevacizumab, can be noted as seen in figure 3.23.

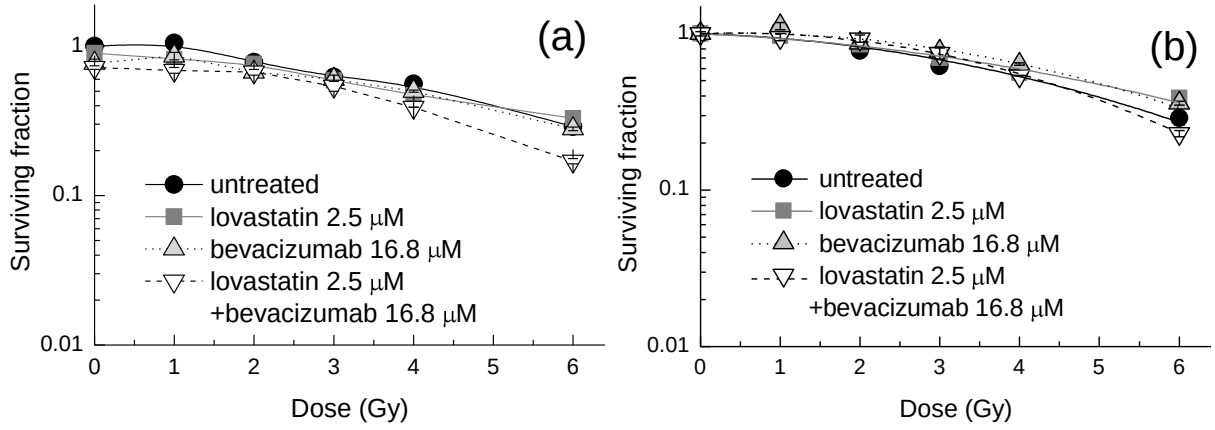


Fig. 3.23: Surviving fraction curves of A 549 cell line with irradiation alone (full black lines), pre-treated with 16.8 μM bevacizumab 24 hours before irradiation (dotted lines), pre-treatment by 2.5 μM lovastatin 4 hours before irradiation (full gray lines), and combination of both molecules (dashed lines): SF curves before correction (a); SF curves after correction for bevacizumab- and lovastatin-induced effects on colony formation (normalized survival) (b).

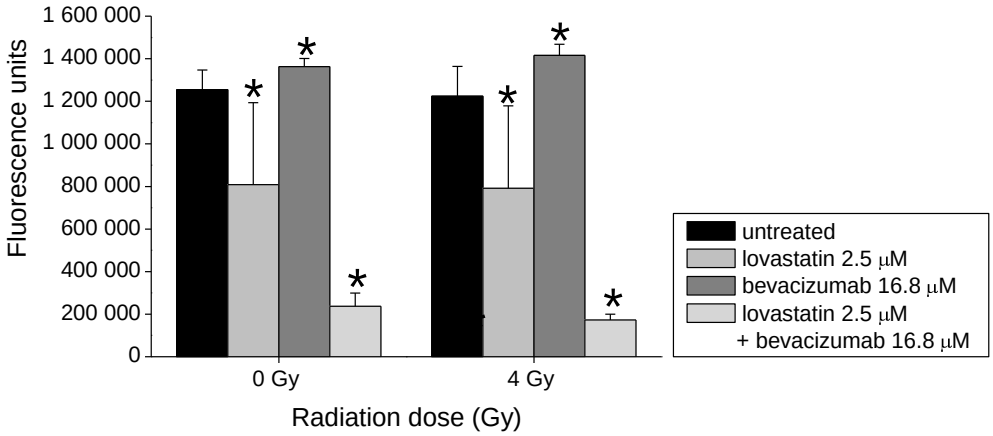


Fig. 3.24: Cell viability expressed in fluorescence units in A 549 pre-treated with 16.8 μM bevacizumab and 2.5 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 96 hours. Experiment has been performed at least once, always in triplicate.

Within the cell viability assay (figures 3.24 and 6.10), there exists a strong and significant sensitizing effect of the association of lovastatin and bevacizumab whereas bevacizumab alone has no particular impact upon A 549 cells. Irradiation does not seem to modify the effect of these two molecules.

MO59K

The seeding procedure was the one as described above with 200 – 2000 cells plated per well and incubated during 14 days. Figure 3.25 demonstrates that bevacizumab alone initiates higher survival rates of MO59K however lovastatin decreases slightly its clonogenic potential. If the results are normalized according to the irradiation alone there is a mild radiosensitization of the cells if pre-treated with bevacizumab (SER = 1.03). The only significant decrease of clonogenicity is present in cells pre-treated with lovastatin and bevacizumab and irradiated by a dose of 1 and 4 Gy.

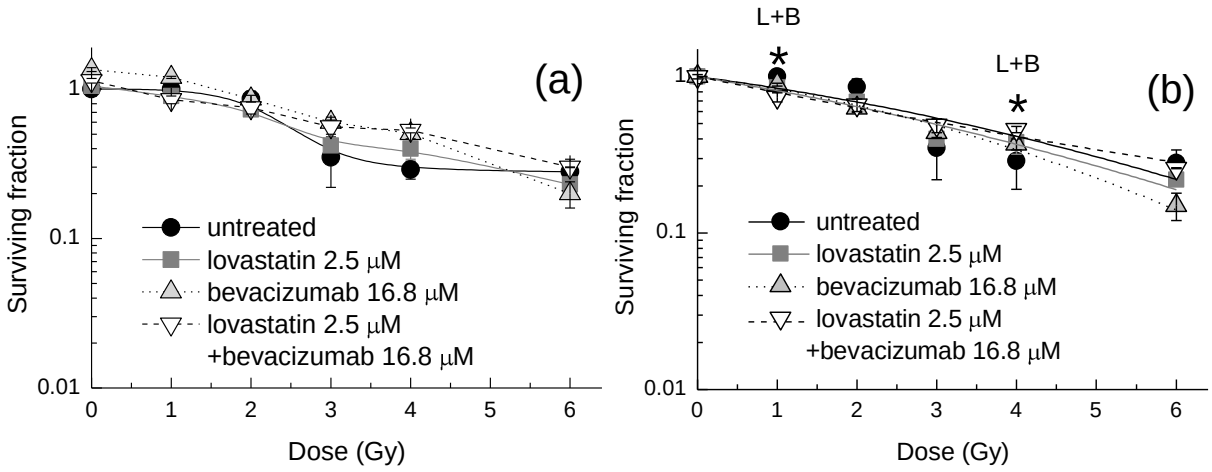


Fig. 3.25: Surviving fraction curves of MO59K cell line with irradiation alone (full black lines), pre-treated with 16.8 μM bevacizumab 24 hours before irradiation (dotted lines), pre-treatment by 2.5 μM lovastatin 4 hours before irradiation (full gray lines) and combination of both molecules (dashed lines): SF curves before correction (a); SF curves after correction for bevacizumab- and lovastatin-induced effects on colony formation (normalized survival) (b). L+B = 2.5 μM lovastatin and 16.8 μM bevacizumab.

A similar strong toxic effect of combination of lovastatin and bevacizumab exists within MO59K cells as in A 549. This can be noted in figures 3.26 and 6.11. Interestingly, although not significant, untreated and bevacizumab pre-treated cells show comparable cell viability.

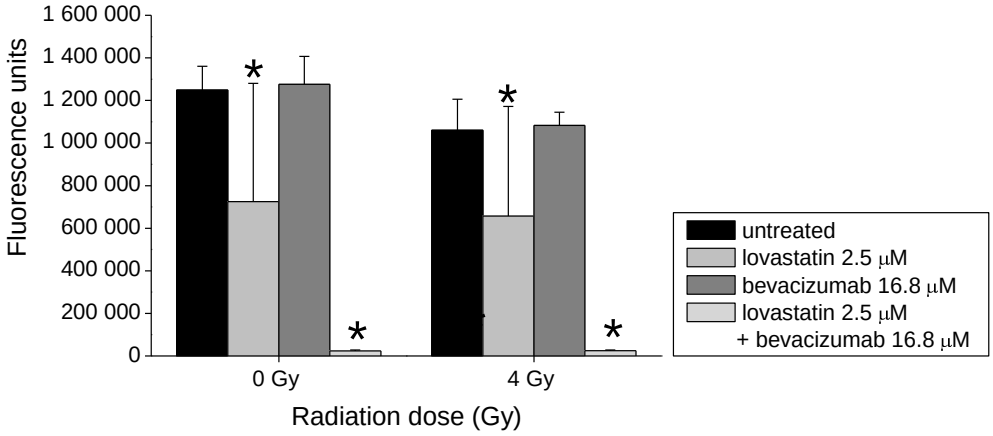


Fig. 3.26: Cell viability expressed in fluorescence units in MO59K pre-treated with 16.8 μM bevacizumab and 2.5 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 96 hours. Experiment has been performed at least once, always in triplicate.

HUVEC

Even HUVEC cells were seeded according to the procedure described at the beginning of this chapter. For CFU assay, 1000 – 3500 cells were plated in each well and incubation lasted 15 days. As already noticed, since 2.5 μM lovastatin turned out to be highly toxic in colony formation assay, for this purpose and for the purpose of the cell viability test 0.25 μM lovastatin was implemented.

Concurrent administration of bevacizumab and lovastatin caused an extremely important decrease of clonogenic potential (figure 3.27). Almost no colonies were observed within the plates. That is the reason, why these results are not presented within the survival curves.

Bevacizumab alone is highly harmful but in contrast to tumour cells, if this is related to irradiation, it does not lead to cell toxicity. Besides, a possible radioprotective effect can be described (SER = 0.36) with a significant difference for bevacizumab-pre-treated cells and irradiated by 4 Gy in comparison to irradiated bevacizumab-free cells.

On the contrary, as seen in figures 3.28 and 6.12, cell viability is significantly lowered after pre-treatment with 0.25 μM lovastatin, 16.8 μM bevacizumab and their combination. Because the activity is almost similar after lovastatin-pre-treatment and lovastatin + bevacizumab-pre-treatment we can assume that the toxic effect depends on lovastatin-induced cell toxicity.

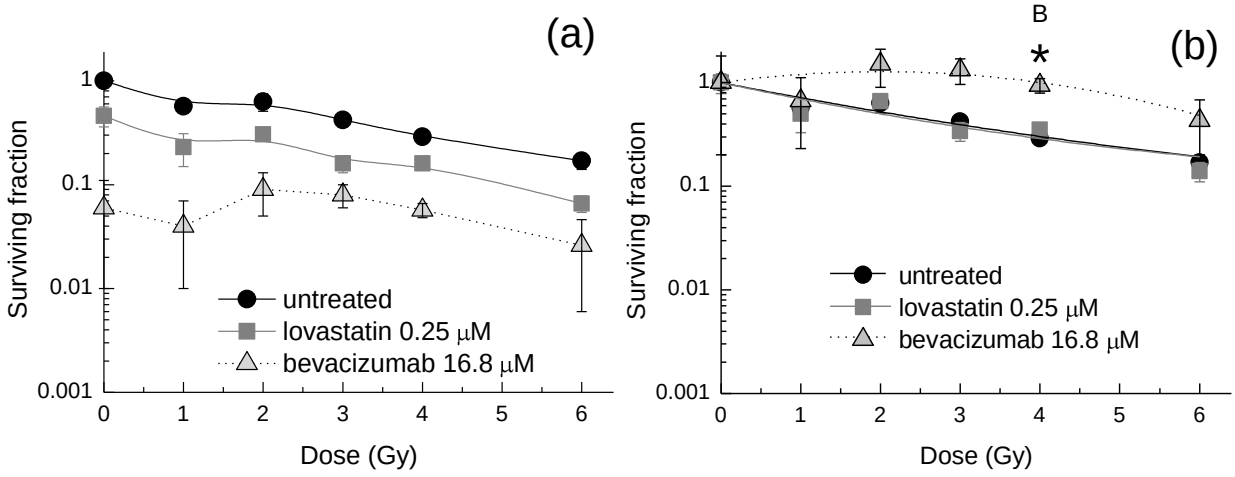


Fig. 3.27: Surviving fraction curves of HUVEC cell line with irradiation alone (full black lines), pre-treated with 16.8 μM bevacizumab 24 hours before irradiation (dotted lines) and pre-treatment by 0.25 μM lovastatin 4 hours before irradiation (full gray lines): SF curves before correction (a); SF curves after correction for bevacizumab- and lovastatin-induced effects on colony formation (normalized survival).

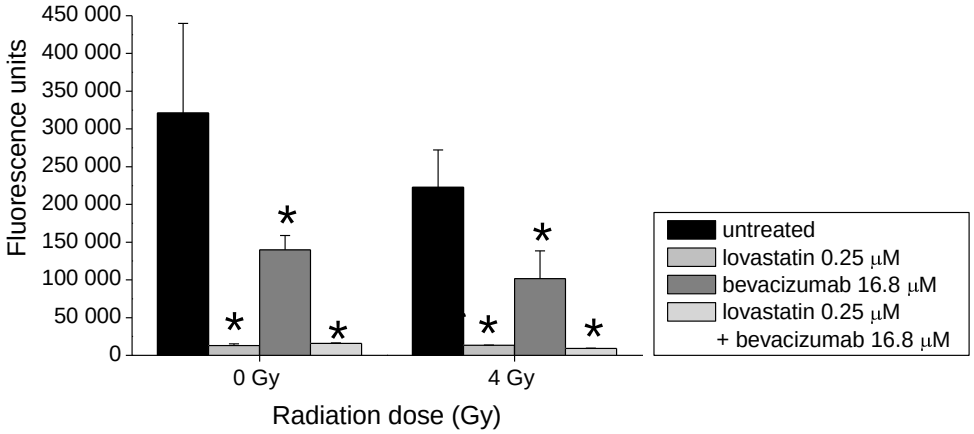


Fig. 3.28: Cell viability expressed in fluorescence units in HUVEC pre-treated with 16.8 μM bevacizumab and 0.25 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 96 hours. Experiment has been performed once and in triplicate.

3.4 Molecular biology experiments

3.4.1 Multi-pathway reporter assay in three cell lines (A 549, ZMK-1, HaCaT)

With the purpose to determine the major molecular pathways being modified by lovastatin, a reporter assay was performed as described in chapter 2.4.1. The advantage of this assay was that multiple various pathways could be explored at the same time using 10 different transcription

factors. Only three cell lines were used within this method. The choice of ZMK-1, A 549, and HaCaT was supported by the fact that these 3 lines had a high growth potential. Three different concentrations of lovastatin were examined (0.25; 2.5; and 25 μM) with a control (DMSO).

The results of the assay (expressed in relative response ratios, RRR) permitted to define the final effect of lovastatin and its different concentration that could be either activating (positive or increasing values of RRR) or inhibiting (negative or decreasing values of RRR).

In the very first experiments, all reporters were tested (i. e. 10). Unfortunately, repeated experiments within each cell line turned out to be not reproducible for certain reporters and thus, in the end, 6 pathways were maintained for further assays. The ultimate lovastatin-induced effects on each cell line and on pathways are shown in table 3.5.

Lovastatin itself, regardless of concentration, activates 6 pathways implicated in cell functioning in all the three cell lines examined. HaCaT cells have strongly reacted to the treatment and thus several results were difficult to interpret.

Tab. 3.5: General effect of lovastatin on cell lines and 6 pathways in reporter assay. A = activation, Tox = toxic effect. Three concentration of lovastatin were explored. Each experiment has been done twice for ZMK-1 and A 549, once for HaCaT.

	Cell line		
Pathway	ZMK-1	A 549	HaCaT
Wnt	A	A	A
Notch	A	A	Tox
TGF-β	A	A	Tox
NF-κB	A	A	A
MAPK/ERK	A	A	A
MAPK/JNK	A	A	A

By observing more particularly the impact of increasing concentrations of lovastatin, the following tendencies can be noticed as shown in table 3.6.

The more the concentration of lovastatin rises, the more inhibitory the effect of this statin seems to be, particularly for HaCaT and partially for certain pathways of ZMK-1 and A 549. MAPK pathways seem to be generally the mostly inhibited pathways that are related to the increasing dose of lovastatin.

In majority of the explored pathways, lovastatin 25 μM has revealed to be rather toxic on all three cell lines tested since both firefly and *Renilla* luminescence values were abolished in comparison to the untreated control and this was due to global impact, i. e. the cell death.

Tab. 3.6: Tendency of lovastatin-induced effect on cell lines and 6 pathways in reporter assay with increasing lovastatin concentration (from 0 to 25 μM). A = activation, I = inhibition, Tox = toxic effect. Each experiment has been done twice for ZMK-1 and A 549, once for HaCaT.

	Cell line		
Pathway	ZMK-1	A 549	HaCaT
Wnt	I	A	I
Notch	A	A	A
TGF-β	A	A	I
NF-κB	A	A	I
MAPK/ERK	I	I	I
MAPK/JNK	I	I	I

3.4.2 Caspse-3 and caspase -7 activity in three cell lines (ZMK-1, A 549, HUVEC)

As well as in the previous experimental method, the choice of cell lines for the caspase activity assay was based on the growth potential, i. e. ZMK-1 and A 549 cells were chosen. The third cell line, HUVEC, seemed to be an interesting candidate for this assay because of its high sensitivity to lovastatin.

The procedure was performed as described in chapter 2.4.2.2. One unique concentration of lovastatin and only one radiation dose were used because of technical reasons.

For each experiment, luminescence measurement was done 1 hour after the incubation with Caspase-Glo®3/7 Reagent and this was repeated another hour later (i. e. after 2 hours of reagent

incubation) in order to compare the performance of the measurement. It should be noted, that no particular difference in luminescence can be observed at hour 1 and 2 in all three cell lines. Both these incubation times were maintained in future experiments within this assay.

ZMK-1

For this cell line, the measurement of caspase- 3 and -7 activity was performed 24, 48, and 72 hours after the treatment with lovastatin 2.5 μM and/or irradiation by 4 Gy.

Figure 3.29 shows that lovastatin alone does not increase the caspase activity neither after 24 nor after 48 hours of incubation whereas the irradiation alone starts to promote caspase-3 and -7 associated apoptosis only after 48 hours. The combination of both treatment regimens induced significant elevation of caspase-3 and -7 levels 24 and 48 hours after the treatment. Interestingly, caspase-3 and -7 activities decreased on the third day after lovastatin pre-treatment and irradiation. These outcomes were significant when compared to irradiated cells without statin.

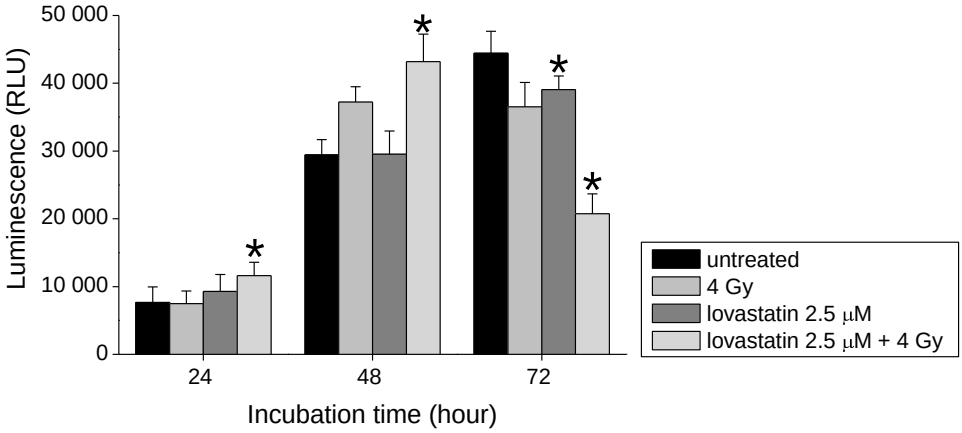


Fig. 3.29: Caspse-3 and -7 activity expressed in RLU with background subtraction in ZMK-1 cell line. In case of association lovastatin – irradiation, pre-treatment by lovastatin 2.5 μM 4 hours prior to irradiation (4 Gy). Each experiment has been done once, in triplicate.

A 549

This cell line was tested for caspase activity after 24 and 48 hours. The therapeutic scheme is similar to ZMK-1.

As seen in figure 3.30, after 24 hours only 2.5 μM lovastatin pre-treatment and concurrent irradiation caused a significant increase in caspase-3 and -7 activities. Equally, 48 hours after treatment, lovastatin together with 4 Gy activated significantly both caspase-3 and caspase-7.

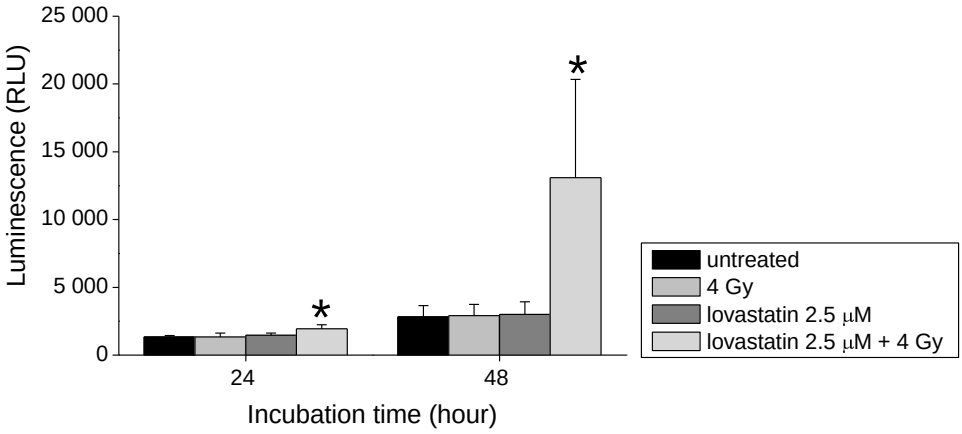


Fig. 3.30: Caspse-3 and -7 activity expressed in RLU with background subtraction in A 549 cell line. In case of association lovastatin – irradiation, pre-treatment by lovastatin 2.5 μM 4 hours prior to irradiation (4 Gy). Each experiment has been done once, in triplicate.

HUVEC

Concerning the HUVEC cells, the fact that these are very responsive already to lovastatin alone, no association with irradiation was adopted in this case. Treatment by lovastatin 0 and 2.5 μM alone was followed by the luminescence measurement 6, 24, and 48 hours later.

An increased caspase activity can be noticed even after lovastatin alone which remains in accordance with the results of the cell viability and colony formation assays upon this cell line. The first peak appeared 24 hours after treatment but significant increase was achieved only after 48 hours (figure 3.31).

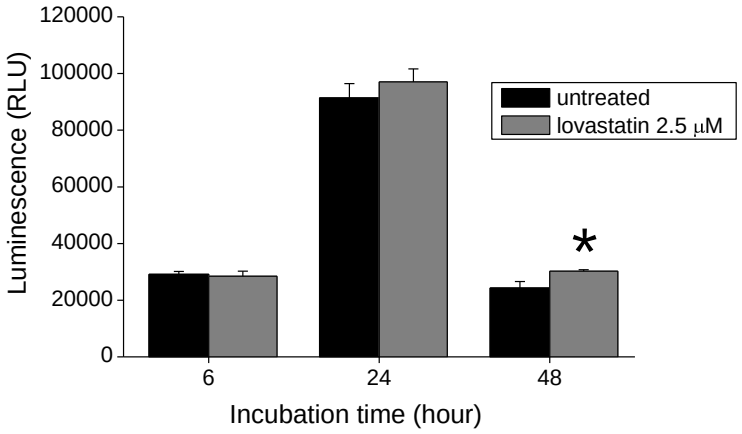


Fig. 3.31: Caspse-3 and -7 activity expressed in RLU with background subtraction in HUVEC cell line. Each experiment has been done once, in triplicate.

3.4.3 Western blot analysis

Following the results obtained in multipathway reporter assays (see chapter 3.4.1), we decided to confirm the overexpression or not of proteins that are involved in pathways of MAPK/ERK and Akt. These experiments were performed on three cell lines exclusively. Namely, ZMK-1, A 549, and HaCaT were chosen for this purpose because of their capacity of rapid cell growth and because they have already been tested within other molecular biology assays as well.

The procedure of Western blot analysis was already described in chapter 2.4.3.

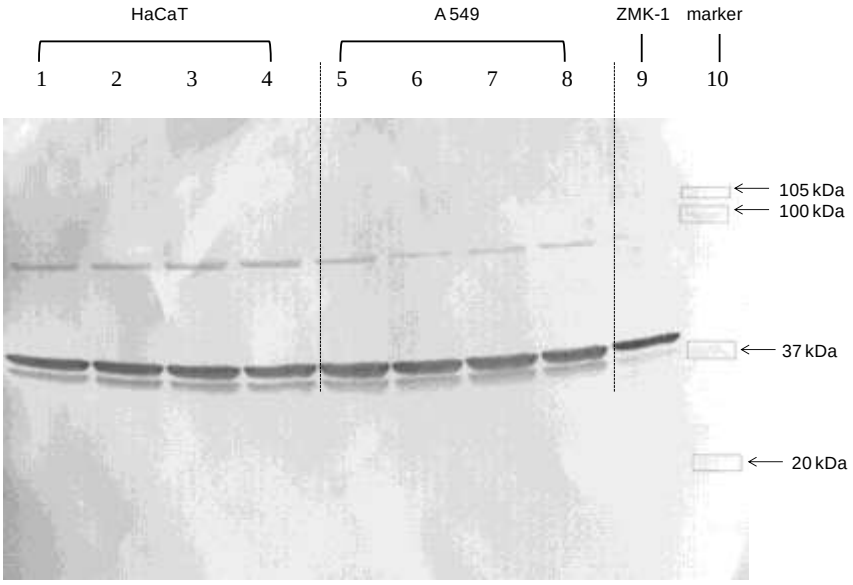


Fig. 3.32: Western blot performed on nitrocellulose membrane using mouse anti-ERK and anti-β-actin antibodies on three different cell lines. Columns 1, 5, 9 – control; 2, 6 – 4 Gy alone; 3, 7 – lovastatin 2.5 μM alone; 4, 8 – 4 Gy + lovastatin 2.5 μM; 10 – marker. Corresponding protein weights are visualized on the right side of the membrane.

In the first step, we searched for expression of ERK protein using mouse anti-ERK antibody. Two separate membranes were created, one being made of nitrocellulose (figure 3.32) and the other one of PVDF (figure 3.33). On each of these two membranes, two cells lines were explored. Thus, results concerning one cell line (A 549) were obtained twice. This served as a control of reproducibility. Furthermore, in order to benefit of all available spaces on membranes, the last band (corresponding to position number 9) represents the non-irradiated and lovastatin free control of the third cell line left.

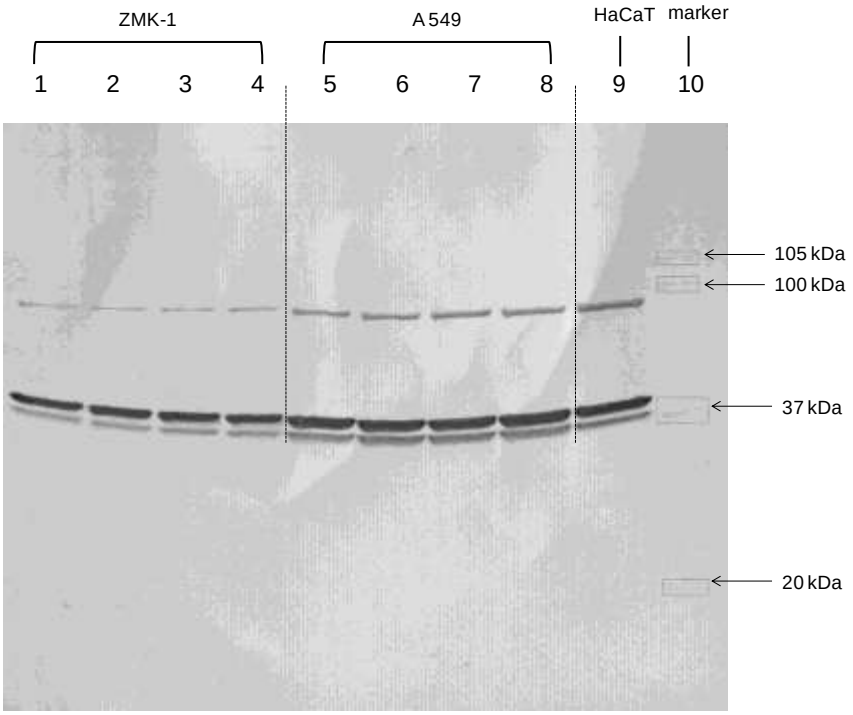


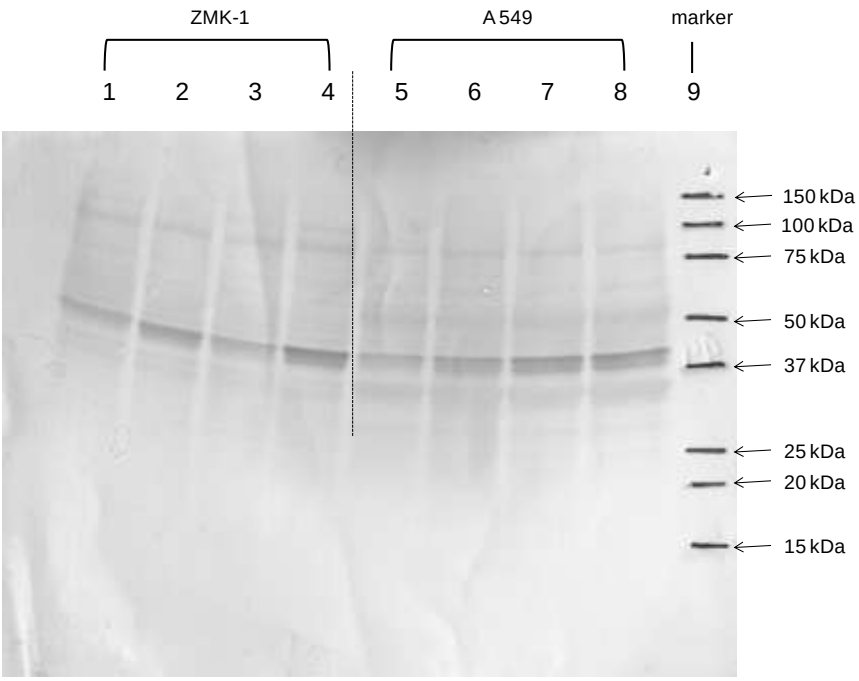
Fig. 3.33: Western blot performed on PVDF membrane using mouse anti-ERK and anti-β-actin antibodies on three different cell lines. Columns: 1, 5, 9 – control; 2, 6 – 4 Gy alone; 3, 7 – lovastatin 2.5 μM alone; 4, 8 – 4 Gy + lovastatin 2.5 μM; 10 – marker. Corresponding protein weights are visualized on the right side of the membrane.

Because anti-ERK antibody was of mouse origin, as well as anti-β-actin antibody, both of these antibodies were applied on both membranes simultaneously. For this reason, various bands can be distinguished within the membranes.

Bands contributed to presence of actin located at the proximity of marker band of protein weight equal to 37 kDa are found on both membranes which means that transfer as well as protein amounts were sufficient. Actin molecular weight is 42 kDa. Thinness of bands corresponding to ZMK-1 cells may be explained by smaller amount of protein contents in experimental samples.

Bands located below actin bands should be the bands corresponding to ERK 2 (molecular weight of 42 kDa) and bands in the proximity of marker band of protein weight 100 kDa represent MAPK kinase which molecular weight is 85/90 kDa.

Despite different treatment conditions within membrane columns, no difference can be observed either concerning MAPK kinase or ERK 2 expression. This conclusion is however desirable since all cells are supposed to express both of these proteins in unphosphorylated form regardless of additional treatment or irradiation.



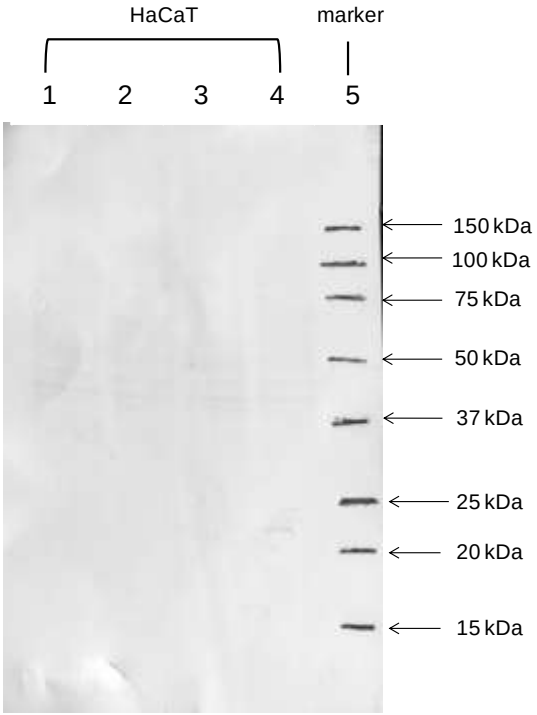


Fig. 3.34: Western blot performed on PVDF membrane using rabbit anti-p-ERK antibody on three different cell lines. Top figure: columns: 1, 5 – control; 2, 6 – 4 Gy alone; 3, 7 – lovastatin 2.5 μM alone; 4, 8 – 4 Gy + lovastatin 2.5 μM; 9 – marker. Bottom figure: columns: 1 – control; 2 – 4 Gy alone; 3 – lovastatin 2.5 μM alone; 4 – 4 Gy + lovastatin 2.5 μM; 5 – marker. Corresponding protein weights are visualized on the right side of the membrane.

In the second step, phosphorylated forms of ERK (p-ERK) and Akt (p-Akt) were determined in all three cell lines.

As for p-ERK (figure 3.34), which molecular weight is equal to 44/42 kDa, expression is to be noticed in A 549 and ZMK-1 cell line whereas no bands appeared in HaCaT cells.

Concerning A 549 cells, p-ERK expression increases in irradiated cells, lovastatin pre-treated cells and attain maximum if concurrent irradiation-lovastatin treatment was executed.

In ZMK-1 cells, no particular difference in expression is seen in cells pre-treated with lovastatin only. On the other hand, irradiation by 4 Gy induced higher protein expression which is even enhanced if combined with lovastatin.

Explanations of lack of p-ERK expression in HaCaT cells are numerous. Anti-p-ERK antibody was targeting only ERK phosphorylated at epitopes Thr202/Tyr204. Therefore, if phosphorylation was induced this might have occurred on different epitope. Otherwise, there may be no phosphorylation induction at all.

Figure 3.35 demonstrates the results obtained using 60 kDa anti-p-Akt antibody. No expression of the protein in ZMK-1 cells may be explained in the same way as the lack of protein p-ERK in HaCaT cells as mentioned above, i.e. phosphorylation occurred on epitope other than Thr308 or no phosphorylation is induced.

As supposed, expressions of this protein in the control group (position columns 1 and 5) are low whereas irradiation induces an overexpression in both A 549 and HaCaT cells. Interestingly, lower expression is seen in lovastatin pre-treated HaCaT cells in comparison to control ones. Moreover, this expression decrease is even more pronounced after additional irradiation.

Contrariwise, A 549 cells express high p-Akt after stimulation with lovastatin as well as with irradiation and both treatments reinforce the production of phosphorylated form of Akt.

Finally, caspase-3 (45 kDa full length, 32-35 kDa partial intermediate, and cleaved 17/20 kDa fragment) expression was detected. We decided to examine this protein despite the fact that cells were harvested already 2 hours after irradiation. This short incubation period may explain why no bands could be seen in ZMK-1 in figure 3.36. Bands in A 549 cells remain difficult to interpret because of no difference between various treatment conditions.

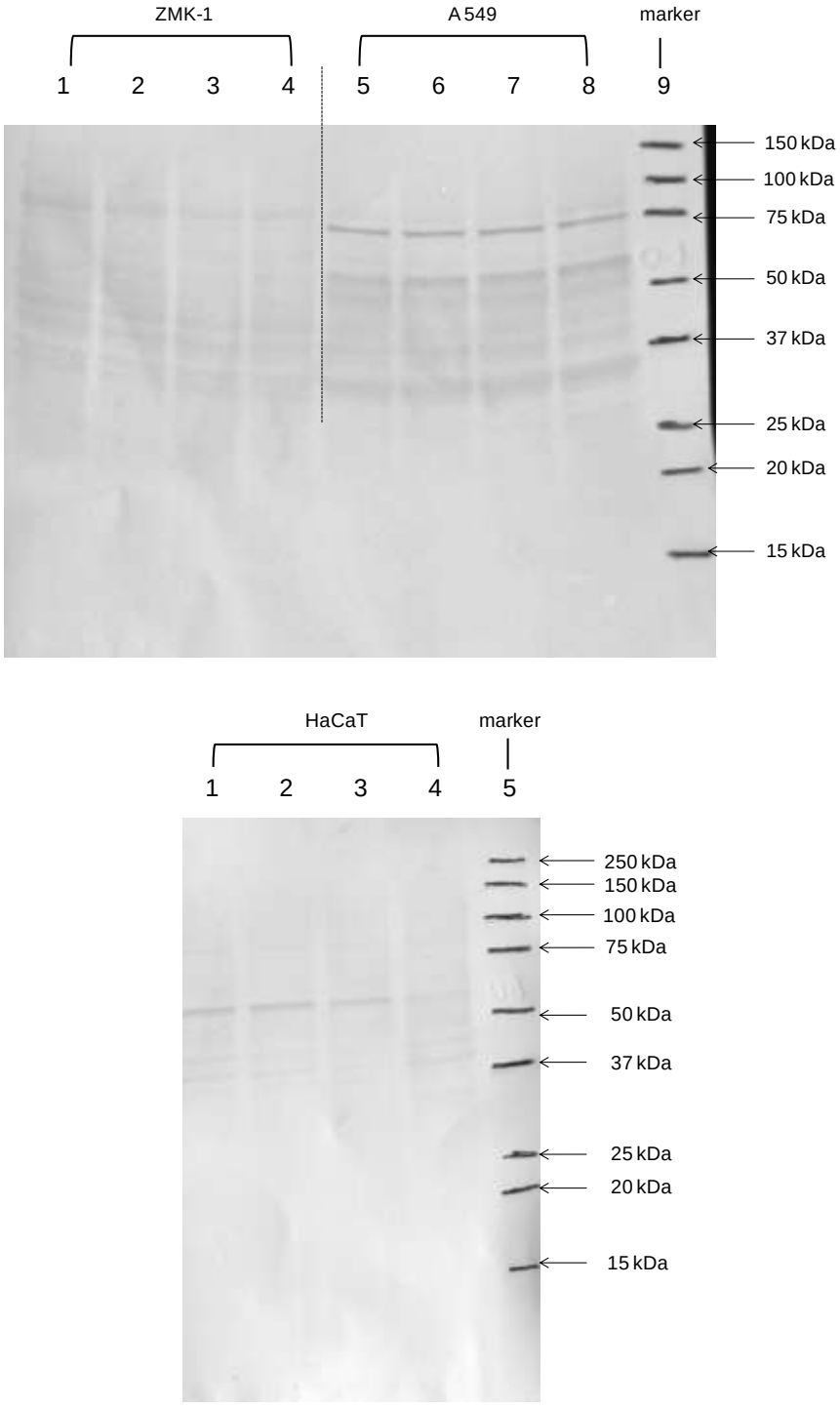


Fig. 3.35: Western blot performed on PVDF membrane using rabbit anti-p-Akt antibody on three different cell lines. Top figure: columns: 1, 5 – control; 2, 6 – 4 Gy alone; 3, 7 – lovastatin 2.5 μM alone; 4, 8 – 4 Gy + lovastatin 2.5 μM; 9 – marker. Bottom figure: columns: 1 – control; 2 – 4 Gy alone; 3 – lovastatin 2.5 μM alone; 4 – 4 Gy + lovastatin 2.5 μM; 5 – marker. Corresponding protein weights are visualized on the right side of the membrane.

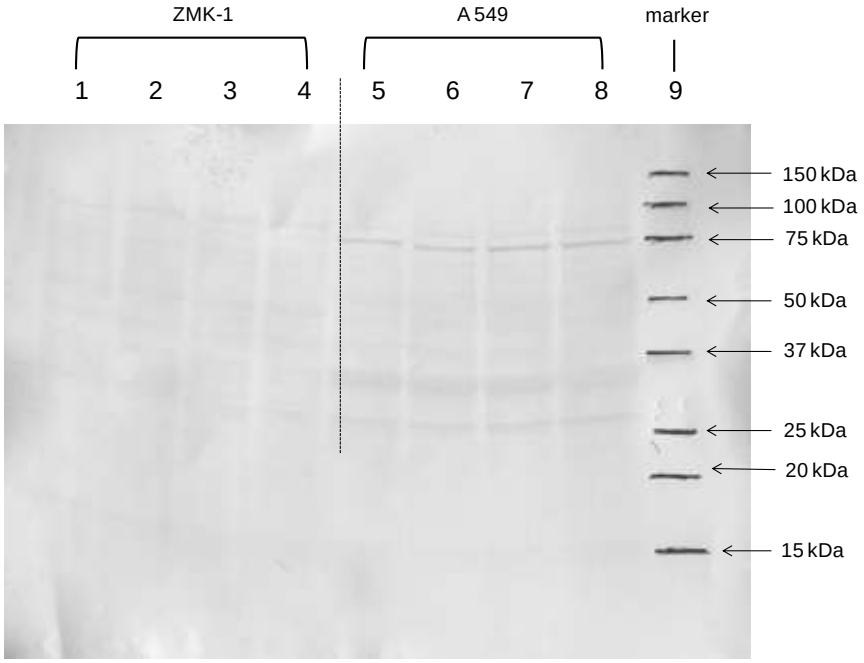


Fig. 3.36: Western blot performed on PVDF membrane using rabbit anti-caspase-3 antibody on two different cell lines. Columns: 1, 5 – control; 2, 6 – 4 Gy alone; 3, 7 – lovastatin 2.5 μM alone; 4, 8 – 4 Gy + lovastatin 2.5 μM; 9 – marker. Corresponding protein weights are visualized on the right side of the membrane.

4 Discussion

In this study we intended to explore *in vitro* effects of a combination of lovastatin and irradiation on various human tumour and normal tissue cell lines as well as its potential mechanism of action. The main reason that motivated this exploration was the fact that statins in general are known for their anti-cancer potential. Several *in vitro, in vivo*, as well as clinical studies dealing with concurrent statins intake and irradiation have already been published.

The aim was to determine whether lovastatin, as one of the members of a statin family, has a radiosensitizing and/or radioprotective effect *in vitro* and whether these results correlate with those published.

In the second part, we associated lovastatin into concurrent treatment regimen consisting of irradiation and monoclonal antibody based targeted therapies, bevacizumab, and cetuximab.

In our preliminary experiments we observed that the cytotoxic effect of lovastatin is strongly dose-dependent, i. e. the higher the concentration was the more cytotoxicity was achieved. For further assays we decided to focus on a dose that permitted an 80 % survival fraction in tumour cells. This dose was at the same time supposed to be clinically relevant. Indeed, as already mentioned, plasma concentration of lovastatin in patients being treated by this molecule for a lipid-lowering purpose varies in the range of 0.1 to 3.92 μM (Thibault et al. 1996). For this reason, confirmation of a possible radiosensitizing potential of low dose lovastatin could be an interesting and achievable therapeutic approach in human.

In our initial experiments, we decided to determine clonogenic cell survival capacity and cell viability and, interestingly, different results were obtained.

Regardless the modifying impact of concurrent irradiation, lovastatin induced a decrease of clonogenic cell survival while the tendency of this decrease at different radiation doses followed the tendency of irradiation alone. However, if the survival curves were normalized for the effect of lovastatin alone, no radiosensitization was observed. On the contrary, we could conclude that it protected tumour cells (except MO59J) and destroyed the normal tissue ones.

On the other hand, assays conducted on cell viability showed that even 2.5 μM lovastatin alone was significantly cell toxic and the toxicity increased if lovastatin pre-treated cells were

irradiated. The only exception was the HaCaT cell line where no significant difference was observed.

The question that arose was what caused this evident difference in results obtained within these two experimental methods.

We assume that lovastatin has a strong cell toxic potential and therefore induces an important decrease of cell viability. However, cells that survive, even not too many, may be those described as clonogenic ones, i.e. those which give subsequently birth to new colonies. Hence, survival curves are in favour of maintained survival despite pre-treatment by lovastatin. Yet, we assume that both of these results are not comparable since endpoints of these two experimental methods are not similar at all. Still, they give a prediction of cell behaviour in different treatment conditions.

The association with targeted therapies has demonstrated that neither cetuximab nor bevacizumab had a particular cytotoxic effect on tumour cell lines with a SER almost equal to 1 if used in concentrations of 0.1 μM for cetuximab and 16.8 μM for bevacizumab. Contrariwise, normal tissue cells suffered significantly because of their administration. This may correlate with various side effects clinically observed in patients being treated by these molecules, e. g. skin rash after cetuximab treatment.

As supposed, addition of lovastatin to this combined treatment renders tumour cells even more resistant to irradiation and abolishes even their smallest cell toxicity.

As for cell viability, on contrast to the above-mentioned outcomes, lovastatin acted highly toxically either alone or in combination with monoclonal antibodies. Furthermore, monoclonal antibodies alone potentiated in certain tumour cell lines the cell viability, e. g. cetuximab in ZMK-1 and bevacizumab in A 549 and MO59K.

More detailed explication of particular effects of these treatment molecules and lovastatin is described below separately for each cell line explored.

The literature describes various cell signalling pathways that are modified by statins explaining their pleiotropic effects (Wang et al. 2008; Fritz et al. 2011). Assays conducted with lovastatin alone without irradiation in our conditions permitted to explore six different pathways on three different cell lines. Generally taken, lovastatin has an activating tendency, however the more its concentration rises, the more this tendency turns towards an inhibitory effect.

Regardless of their anti-cancer potential, *in vitro* and *in vivo* statins were denoted to prevent from fibrosis formation as well as from epithelial-mesenchymal transition of various forms of fibroblasts or epithelial cells (Park et al. 2012; Urakami et al. 2012; Schaafsma et al. 2011; Rodrigues-Díez et al. 2010; Meyer-Ter-Vehn et al. 2008). In these studies, the goals were achieved by inhibiting effects of statins on the expression of transforming growth factor β (TGF-β). The reporter assay performed in our condition using only lovastatin treated cells without irradiation showed that in ZMK-1 as well as in A 549 cell lines this treatment induced an activation of TGF-β what was in discordance with the above mentioned properties. One of the reasons for this difference could be the fact that the studies that we mention worked with higher concentrations of statins. Thereby we tested also the impact of higher concentrations but still the overall tendency was rather in favour of TGF- β activation in tumour cells and inhibition was exclusively observed in HaCaT cells.

Another interesting pathway that was explored was the Wnt signalling pathway which is implicated, except of genesis of various cancers, also in metabolism of glucose. An *in vitro* study carried out by Lin et al. in 2008 revealed that simvastatin permitted to restore high glucose-induced downregulation of Wnt in mesangial cells (Lin et al. 2008). At this point our results correlate with those of Lin since lovastatin had an activating impact on all three cell lines. However, rising concentrations of lovastatin seem to have an inhibitory tendency in our ZMK-1 and HaCaT cells.

Notch pathway is an intracellular signalling pathway involved in the process of angiogenesis, arteriogenesis as well as in cell differentiation, proliferation, and apoptosis. *In vivo* (Zacharek et al. 2009) and *in vitro* studies (Xu et al. 2009) demonstrated that statins, especially simvastatin, are able to induce the activation of this pathway and subsequently by overexpression of various proteins (Notch, presenilin 1, NCID-Notch Intracellular Domain) permit a better endothelial cell differentiation and proliferation. Our reporter assay on lovastatin is in accordance since activation of Notch was noted in all three cell lines. The question remains whether this effect is of a positive value regarding tumour cells whose proliferation may be similarly enhanced in this way.

We explored equally the status of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and its activation or inhibition after lovastatin treatment. This regulator of gens may be often altered, i. e. constitutively active, in tumour cells where it leads to uncontrolled cell proliferation, survival, and protects against apoptosis. Hence, its inhibition would be desirable. In

a study conducted on breast cancer cells, addition of simvastatin inhibited activity of NF-κB (Ghosh-Choudhury et al. 2010). Our results were rather in favour of activation (except of HaCaT cell line pre-treated with low-dose lovastatin).

Pathways that preoccupied our interest the most were pathways being regulated through mitogen-activated protein kinases (MAPK). Indeed, this family of serine/threonine protein kinases is involved in various cellular programs such as cell proliferation, differentiation, motility, and death. One of these is also the p44/42 MAPK (ERK1/2) signalling pathway that may be activated as a response to diverse extracellular stimuli.

Initial reporter assays demonstrated that 2.5 μM lovastatin had an activating impact on MAPK/ERK pathway whereas increasing concentration of lovastatin had a more inhibiting effect on gene expression. In complementary experiments based on Western blots we found that the expression of the phosphorylated form of ERK increased in two of three cell lines tested (ZMK-1 and A 549) after previous irradiation, lovastatin pre-treatment, and especially after combination of both of these. These findings are in correlation with the results obtained in colony formation assays where irradiated cells survived as well or even better with lovastatin in comparison to irradiation alone.

Another important protein kinase aiming to control the cell survival and apoptosis is Akt that may be activated by different growth factors via phosphatidylinositol 3-phosphate kinase (PI3-kinase) pathway. Iradiation alone has been described to induce activation of Akt (Nakamura et al. 2005; Le Tourneau and Siu 2008). For this reason a molecule that could act here as an inhibitor would be of high interest to ensure a radiosensitization. Unfortunately, in our treatment conditions pre-treatment by 2.5 μM lovastatin enhanced expression of p-Akt in A 549 cell line if used alone and even potentiated p-Akt expression if associated to irradiation. This is contradictory to various published studies (Sanli et al. 2011). On the other side, it is noteworthy that in HaCaT cells the p-Akt expression decreased after lovastatin treatment also after additional irradiation. Hence, the beneficial radiosensitizing effect of lovastatin on tumour cells could not be concluded. Furthermore, toxicity was remarkably high in normal tissue cell lines. The impact of lovastatin and/or irradiation on p-ERK and p-Akt expression will be discussed more specifically in chapter below for each cell line separately.

Both of these protein kinases, ERK and Akt are activated not only via radiation but also through epidermal growth factor receptors (EGFRs). We consider interesting and worth of search to understand this mechanism more deeply, possibly by associating not only lovastatin to

irradiation but targeted therapies (anti-EGFR) as well. Further studies could be carried out for this purpose.

Finally, we aimed at determining whether caspase-3 and caspase-7 activation participate in cell death processes induced by lovastatin or not, i. e. whether apoptosis plays a role in cell destruction. Assessment of these activities after lovastatin treatment or irradiation or combination of both showed that already after 24 hours of incubation lovastatin increases caspase-3 and -7 activities and thus induces death by apoptosis. Caspase-3 expression was tested also using Western blot. However, no bands were observed because of very short incubation time after irradiation.

4.1 Individual work outcomes according to cell lines

4.1.1 ZMK-1 cell line

This cell line, being cultivated at the Department of Radiotherapy and Radiation Oncology in Göttingen, possess a high proliferation potential and therefore was used in all experiments, including molecular biology research.

As already mentioned, cell growth or viability inhibition induced by lovastatin was dose-dependent. In colony formation assays, lovastatin at the concentration of 2.5 μM, as a reference dose, did not induce any radiosensitization if administered prior to irradiation. However, cell viability was lowered either by lovastatin alone or in combination with irradiation at 4 Gy. A staining method permitted to detect that this cell line strongly expresses intracellular and membrane EGF receptors. Unfortunately, experiments with 0.1 μM cetuximab showed no sensitization.

Regarding the molecular biology, all 6 signal pathways mentioned above were activated by lovastatin. This is in accordance with the results of Western blot were lovastatin and radiation-induced p-ERK overexpression was noticed. Furthermore, it may provide the explication of cell resistance to this statin.

Finally, activation of caspase-3 and caspase-7 in process of death of ZMK-1 cells was shown in particular assay with their increased activity 48 hours after concurrent lovastatin and irradiation effect.

Various studies on head and neck squamous cell carcinoma cells already demonstrated that statins have a dose-dependent cytotoxic effect (Takeda et al. 2007; Mantha et al. 2003). For

example, according to Takeda et al., simvastatin exerted an important cell growth inhibition with already low doses. In contrast to our results, in the previous study, expression of p-ERK was reduced after simvastatin pre-treatment that explained its toxic effect. Mantha et al. reported a potential of statins to target EGFR downstream signalling and thus question of synergistic effects of these and anti-EGFR agents rose. On our cell line, there was no benefit observed after cetuximab and lovastatin association as for the cell survival.

4.1.2 A 549 cell line

For this lung cancer cell line similar results were obtained as for the previous ZMK-1 line, i. e. effect of lovastatin was dependent on dose. Its concentration that was chosen for further experiments (2.5 μM) revealed to be not high enough to induce a radiosensitization, however it decreased cell viability. Sanli et al. (Sanli et al. 2011) performed a series of assays on A 549 cells with lovastatin and found that even low concentrations of this statin (5 μM) induced an important decrease of cell survival. Moreover, they tested the expression of p-Akt and p-ERK using Western blot in the same way as we did. Both of these proteins were overexpressed in our conditions after lovastatin treatment and irradiation and even enhanced after association of two of these. This was in contrast to what could be seen in the study of Sanli at al. since expression of p-Akt as well as of p-ERK was abolished after 5 or 10 μ M lovastatin treatment. The explication is not clear but we cannot exclude that low concentrated lovastatin acts in association with irradiation rather as a cell protector than sensitizer in this cell line. Unfortunately we did not test higher concentrations of lovastatin except of very first experiments of cell survival. Indeed, we noticed the first significant cell survival decrease at the dose of 10 μM and not below.

Neither cetuximab nor bevacizumab sensitized lung tumour cells to irradiation. In contrary, we observed a protection and improved cell growth after their treatment. But the addition of lovastatin decreased slightly although not significantly the cell survival.

As mentioned above, in our study p-ERK expression undesirably raised after lovastatin treatment. A recently published *in vitro* study carried out on the same cell line and using simvastatin demonstrated an inhibitory potential of simvastatin on MAPK/ERK signalling pathway (Liu et al. 2013).

4.1.3 MO59K and MO59J cell line

In the DNA-PKs proficient MO59K cell line, lovastatin did not exert a radiosensitization at 2.5 μM but did at 5 μM. However, cell viability was lowered even with lower concentrated

lovastatin. Bevacizumab alone had no radiosensitizing effect and induced neither a cell viability decrease, yet it was diminished significantly after combination of lovastatin and bevacizumab.

Contrary to our results, an *in vitro* study showed that even low concentrations of lovastatin could already induce a decreased growth of other glioblastoma cell line such as A 172, U 251, U 373, and U 87 (Prasanna et al. 1996). Though, it is difficult to compare various sorts of gliobastoma cells.

Unlike the DNA-PKs proficient MO59K cell line, MO59J cells were highly sensitized to irradiation after pre-treatment by lovastatin.

4.1.4 HUVEC cell line

Initial experiments with our conventional dose of lovastatin, i. e. 2.5 μM exerted high cellular toxicity within this cell line that was characterised by already a very slow cell growth. But interestingly the cell viability was not significantly affected by this concentration. In contrast to other normal tissue cell lines and their association with targeted therapy, bevacizumab in HUVECs, instead of toxic impact, protected cells against irradiation. But as soon as lovastatin was added, the cell growth was interrupted immediately. Cell death was probably due to apoptosis since activation of caspases could be noticed 48 hours after incubation with lovastatin.

Nübel and colleagues postulated in their *in vitro* study differing conclusions (Nübel et al. 2006). In fact a low dose of lovastatin (1 μM) protected HUVECs from radiation damages and only doses above 10 μM had pro-apoptotic potential. Hence, discordance with our outcomes is met.

4.1.5 HaCaT cell line

Similar to HUVEC cells, also this cell line was highly sensitive to all treatments performed, i. e. we observed a strong radiosensitizing effect with SER of 1.53. On the other hand, 2.5 μM lovastatin did not decrease significantly cell's viability. Cetuximab treatment turned out to be very cell toxic even at very low concentrations presumably because of high expression of EGF receptor within and on the surface of these cells.

Within molecular biology experiments we found out that various signalling cancer pathways were activated by 2.5 μM lovastatin including NF-κB. This pathway was otherwise described by Qi et al. to be inhibited by fluvastatin decreasing simultaneously cell viability (Qi et al. 2009). At the same time we could conclude that several pathways, e. g. Notch and TGF-β, were highly

affected by lovastatin, however the results are difficult to interpret because of important cell toxicity.

4.1.6 Fibroblasts

This very sensitive primary cell culture exerted an extremely slow cell growth potential thus, only initial cell survival and cell viability assays were performed as mentioned above. Even these were difficult to perform because after lovastatin treatment no colonies were observed. Hence we can conclude that fibroblasts are highly affected even by a low dose of lovastatin. Radioprotection was seen neither in our condition neither in other published studies (Nübel et al. 2005).

To summarize, it is noteworthy that according to our results and amongst tumour cells, lovastatin at physiologically and clinically relevant doses, i. e. 2.5 μM, has a radiosensitizing effect only in the highly radiosensitive MO59J cell line whereas all other tumour cell lines remained unaffected or were even protected against radiation effects. Confirmation was obtained by the results of Western blot where various proteins connected with radioresistance and cell survival were overexpressed. We are in accord with the already published *in vitro* studies that dealt equally with statin treatment that as for tumour cell lines, effects of statins are dose-dependent. Indeed, 25 μM lovastatin was clearly toxic in our tumour cells and decreased cell survival and viability.

Disappointing results were obtained with targeted therapies. No radiosensitization was seen after monoclonal antibodies administration in tumour cells. Only improvement in the term of diminished survival was gained with the addition of lovastatin. On the other hand, the same concentrations of these were harmful for normal tissue cells.

5 Conclusions

Pleiotropic potential of HMG-CoA reductase inhibitors, statins, has already been described by several authors and within various domains of medicine (cardiology, neurology, oncology, etc.). One of such potentials is an anti-cancer effect which is still not clearly explained in detail, up today. Many theories have been postulated according to *in vitro* and *in vivo* experiments but need to be validated in clinical practice.

Nowadays, statins are frequently administered to patients undergoing a treatment by radiotherapy because of their lipid-lowering purpose. Thus, the potential interactions of lovastatin, one of the members of the statin family, with irradiation could be of high interest. In order to remain clinically relevant, low dose lovastatin was used within our work.

Our *in vitro* study demonstrated that 2.5 μM lovastatin tends to protect tumour cells against irradiation, and at the same time destroys normal tissue cells. Thus we are in discordance with several already published *in vitro* studies. More methods of molecular biology might be applied in order to find an explanation of this difference. Interestingly, association of lovastatin and monoclonal antibodies enhanced the cytotoxic effect of these antibodies alone both in tumour and normal tissue cells.

It may be also of great interest to compare the effects of various lovastatin concentrations and mechanisms of action of each of them as well as the effect of other sorts of statins. Still, we assume that to remain clinically relevant only effects of low doses of statins should be taken into consideration.

Confirmation of significant and relevant anti-cancer potential of statins in men is still far from being done even if we should bear in mind that these drugs are often administered in oncological patients and thus co-administration of chemotherapeutics or targeted therapies together with irradiation may induce yet unknown positive or negative interactions.

Further experiments are required to study the exact potential of statins in tumour cells.

6 Appendix

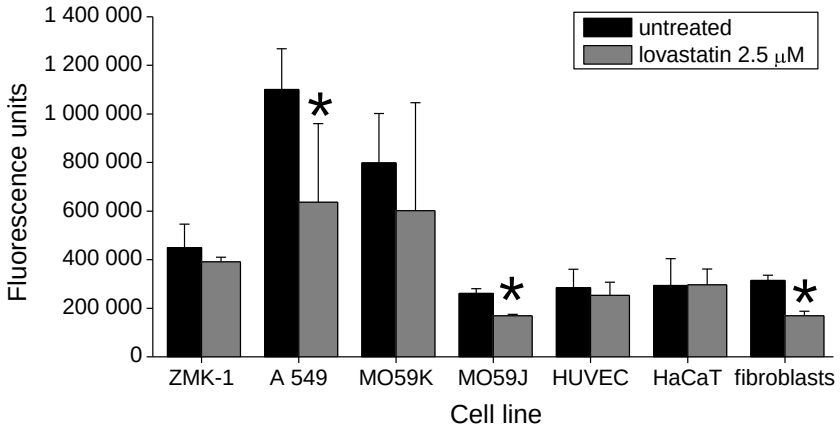


Fig. 6.1: Cell viability expressed in fluorescence units per cell line without treatment and with lovastatin 2.5 μM pre-treatment. Incubation time was 72 hours. Each experiment has been performed at least once, always in triplicate.

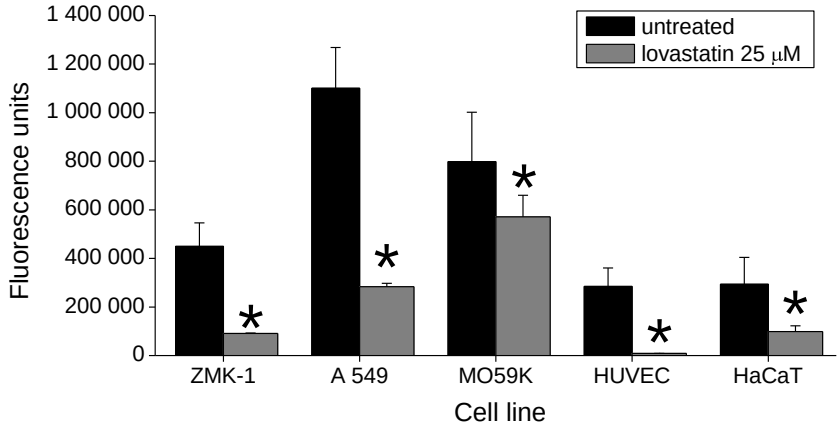


Fig. 6.2: Cell viability expressed in fluorescence units per cell line without treatment and with lovastatin 25 μM pre-treatment. Incubation time was 72 hours. Each experiment has been performed at least once, always in triplicate.

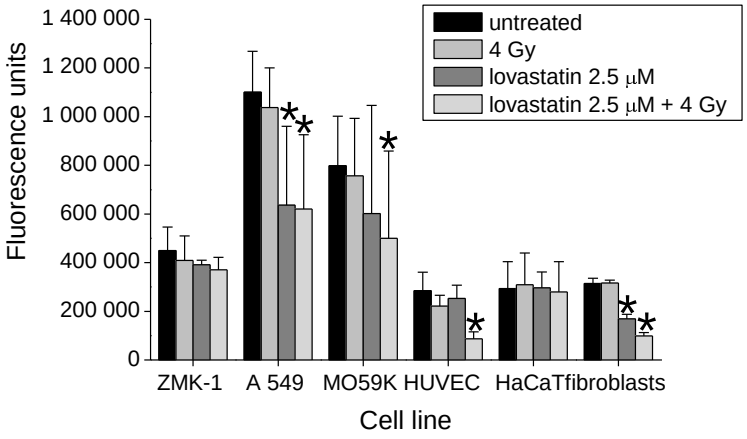


Fig. 6.3: Mean values of cell viability expressed in fluorescence units per cell line without any treatment, with irradiation alone (4 Gy), with lovastatin 2.5 μM pre-treatment alone or combination of lovastatin 2.5 μM pre-treatment 4 hours prior to irradiation (4 Gy). Incubation time was 72 hours. Each experiment has been done at least once, always in triplicate.

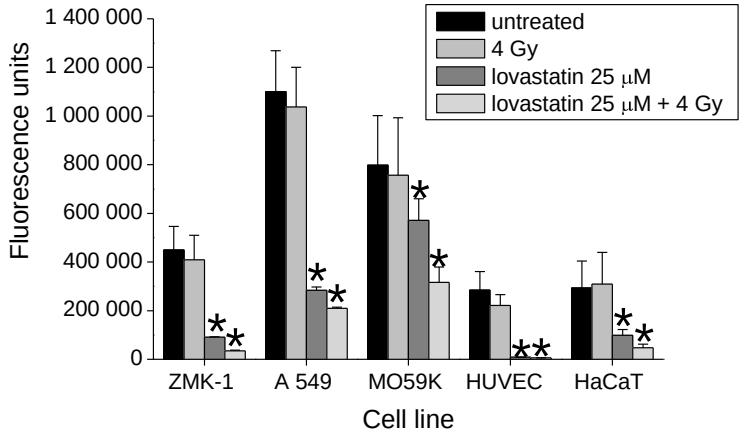


Fig. 6.4: Mean values of cell viability expressed in fluorescence units per cell line without any treatment, with irradiation alone (4 Gy), with lovastatin 25 μM pre-treatment alone or combination of lovastatin 25 μM pre-treatment 4 hours prior to irradiation (4 Gy). Incubation time was 72 hours. Each experiment has been done once, always in triplicate.

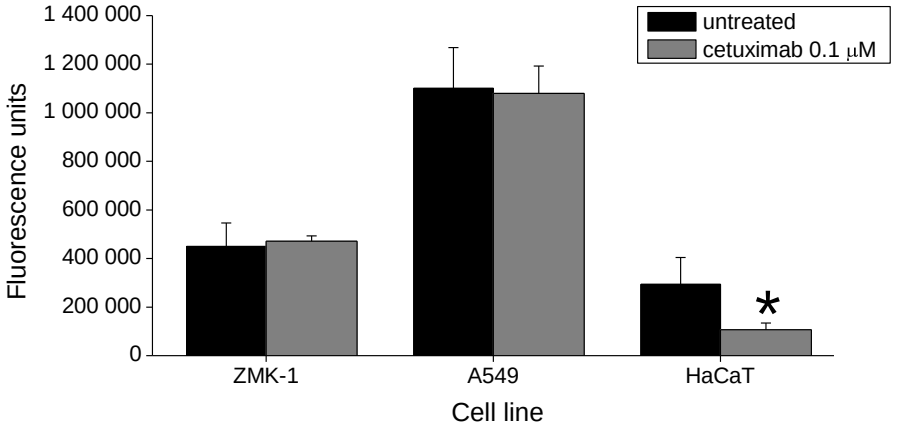


Fig. 6.5: Cell viability expressed in fluorescence units per cell line without treatment and with 0.1 μM cetuximab pre-treatment. Incubation time was 72 hours. Each experiment has been performed twice, always in triplicate.

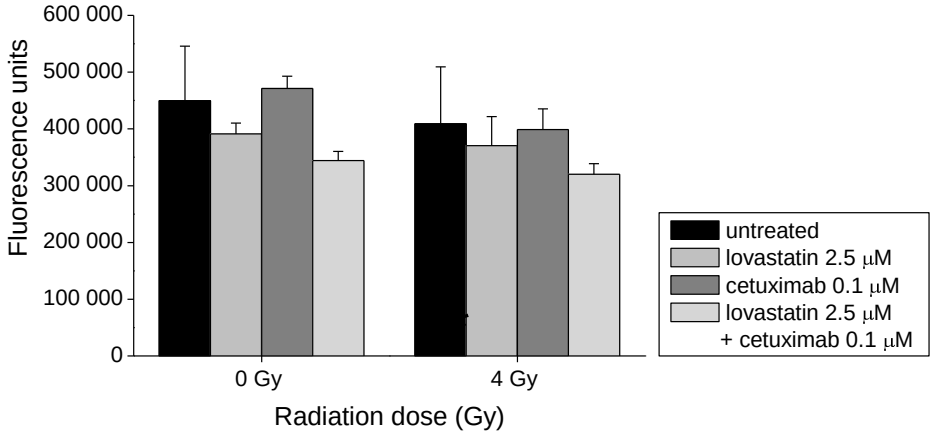


Fig. 6.6: Cell viability expressed in fluorescence units in ZMK-1 pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed at least once, always in triplicate.

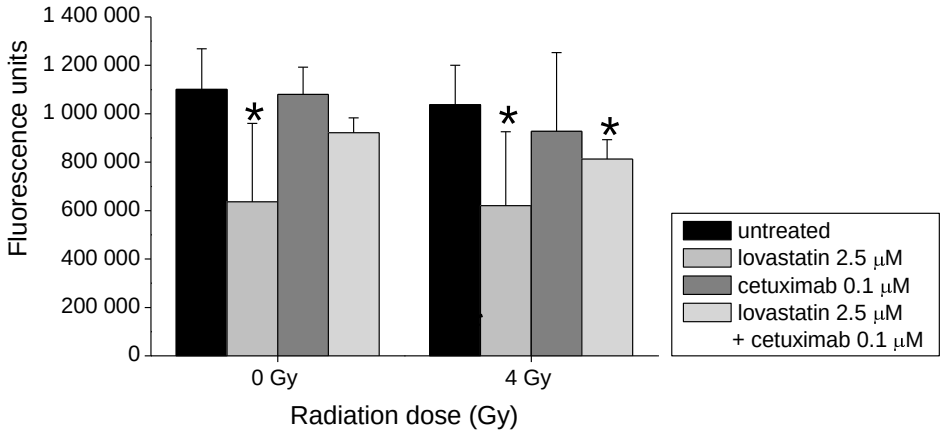


Fig. 6.7: Cell viability expressed in fluorescence units in A 549 pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed at least once, always in triplicate.

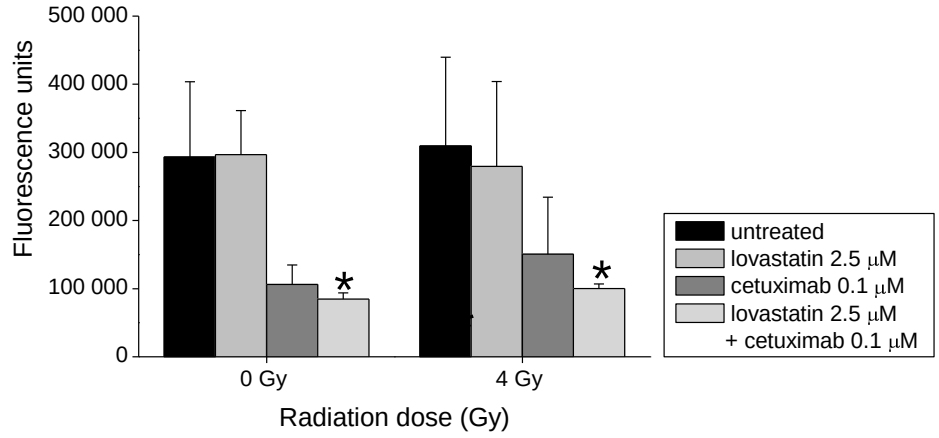


Fig. 6.8: Cell viability expressed in fluorescence units in HaCaT pre-treated with 2.5 μM lovastatin and 0.1 μM cetuximab 4 and 1 hour(s) before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed at least once, always in triplicate.

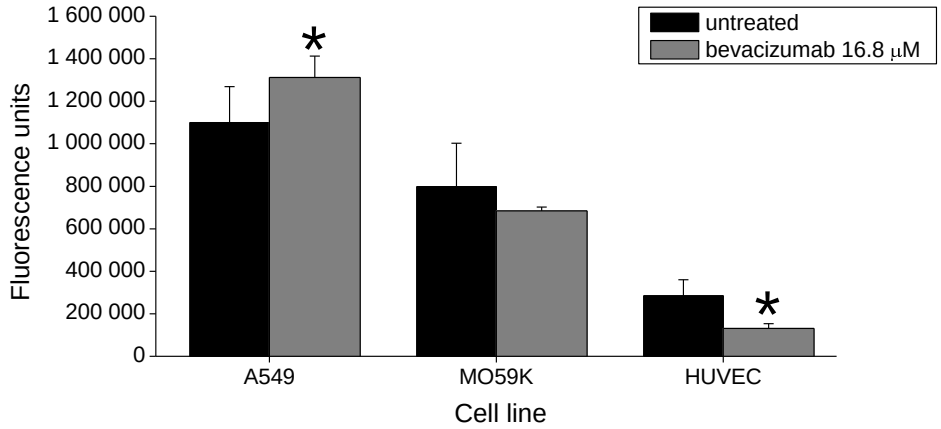


Fig. 6.9: Cell viability expressed in fluorescence units per cell line without treatment and with bevacizumab 16.8 μM pre-treatment. Incubation time was 72 hours. Each experiment has been performed twice, always in triplicate.

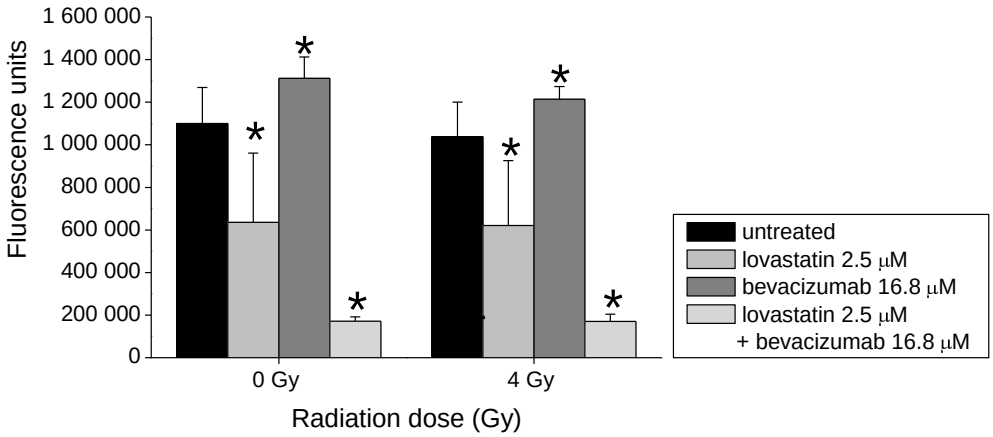


Fig. 6.10: Cell viability expressed in fluorescence units in A 549 pre-treated with 16.8 μM bevacizumab and 2.5 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed at least once, always in triplicate.

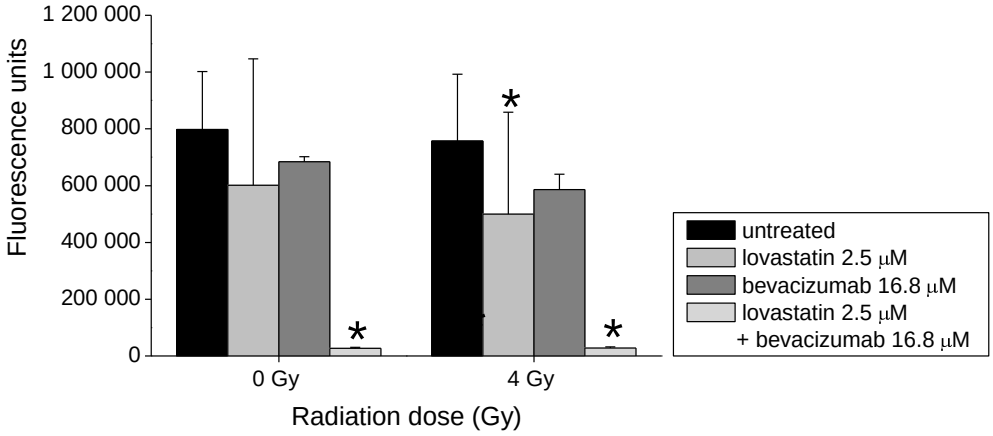


Fig. 6.11: Cell viability expressed in fluorescence units in MO59K pre-treated with 16.8 μM bevacizumab and 2.5 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed at least once, always in triplicate.

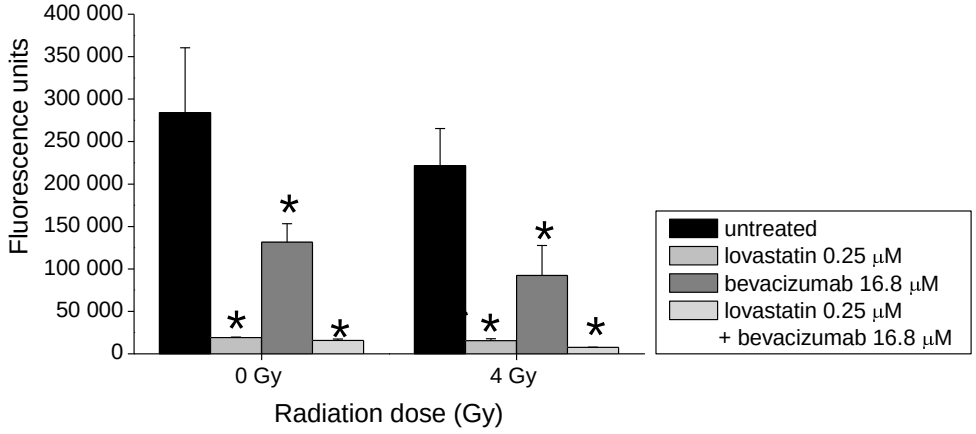


Fig. 6.12: Cell viability expressed in fluorescence units in HUVEC pre-treated with 16.8 μM bevacizumab and 0.25 μM lovastatin 24 and 4 hours before irradiation (4 Gy) respectively and the same treatment without irradiation (0 Gy). Incubation time was 72 hours. Experiment has been performed once and in triplicate.

Tab. 6.1: List of chemicals and pharmaceuticals used during the work.

Name of product	**Name of the company (city and country of origin)**
Mynox®Gold	Minerva Biolabs (Berlin, Germany)
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich (Steinheim, Germany)
Dulbecco's Minimum Essential Medium (DMEM)	Biowest (Nuaillé, France)
RPMI 1640	
Fetal calf serum (FCS) standardized	Biochrom AG (Berlin, Germany)
Penicillin/Streptomycin (10 000 U/ml / 10 000 µg/ml)	
Phophate Buffered Saline (PBS)	
0.5 % Trypsin, 0.2 % ethylenediaminetetraacetic acid in PBS, 10x	
Ampicillin 0.5 g/10 ml	Ratiopharm (Ulm, Germany)
Endothelial Cell Growth Medieum (ECGM) + supplement mix	PromoCell (Heidelberg, Germany)
Titriplex III, 100 mg diluted in 100 ml PBS	Merck (Darmstadt, Germany)
Crystal violet solution	
$CaCl_2$	
2-mercaptoethanol	
NaCl	
Erbitux®	
Avastin®	Roche (Basel, Switzerland)
Ethanol	Chemie-Vertrieb (Hannover, Germany)
Anti-EGFR antibody	Invitrogen (San Diego, USA)
Vitro-Clud	R. Langenbrinck (Emmendingen, Germany)
Opti-MEM® serum-free culture medium, 1x	Gibco (Grand Island, USA)
Non-essential amino acids (NEAA), 100x	
Attractene	Qiagen (Hilden, Germany)
Tris HCl	Roth (Karlsruhe, Germany)
$MgCl_2$	Sigma (Saint Louis, USA)
NP-40	
Sodium dodecyl sulphate (SDS)	
Anti-β-actin antibody	
Glycerol	Serva (Heidelberg, Germany)
Bromphenol blue Na-salt	
Migration buffer for Western blot	
Ponceau S diazo dye	
Mini, EDTA-free pills	Roche (Mannheim, Germany)
Protein marker Precision Plus Protein WesternC™	Bio-Rad (Hercules, USA)
Anti-ERK antibody	BD Transduction Laboratories (San Jose, USA)
Anti-caspase 3 antibody	Chemikon (Darmstadt, Germany)
Anti-p-ERK 1/2 antibody	Cell signalling (Danvers, USA)
Anti-p-Akt antibody	

Tab. 6.2: List of devices used during the work.

Name of product	Name of the company (city and country of origin)
Clean bench	Heraeus (Hanau, Germany)
Incubator	
Tube centrifuge	
Deep freezer (- 80 °C)	Sanyo (Osaka, Japan)
Refrigerator (4 – 8 °C)	Liebherr (Biberach, Germany)
Freezer (-20 °C)	
X-ray tube	Gulmay (Surrey, England, UK)
Light microscope	Carl Zeiss (Jena, Germany)
Wallec1420 VICTOR™ plate reader	PerkinElmer (Turku, Finland)
Cytospin 4 cytocentrifuge	Thermo Scientific (Rockford, USA)
Automated slide stainer	Ventana BenchMark (Tucson, USA)
Vortex shaker	Heidolph (Schwabach, Germany)
Luminometer	Tecan (Crailsheim, Germany)
Plate shaker MS1 Minishaker	IKA (Taquara, Brazil)
Ice-machine	Ziega (Isernhagen, Germany)
Ultrasonic homogenizer	Bandelin (Berlin, Germany)
Small cup centrifuge	Eppendorf AG (Hamburg, Germany)
Thermomixer comfort	
Unsterile clean bench	Norddeutsche Laborbau (Kaltenkirchen, Germany)
Western blot migration set Mini-PROTEAN Tetra System	Bio-Rad (Hercules, USA)
Electrophoresis power supply Power Pac 300	
Electroblotting transfer system Trans-Blot Turbo™	
Rotary shaker	Zeipel (Bovenden-Lenglern, Germany)

Tab. 6.3: List of experimental and detection kits used during the work.

Name of product	Name of the company (city and country of origin)
MycoAlert™ mycoplasma detection kit	Lonza (Rockland, USA)
CellTiter-Blue® Cell Viability Assay	Promega (Madison, USA)
Dual-Glo®Luciferase Assay System	
Caspase-Glo®3/7 Assay	
OptiView DAB IHC Detection Kit	Ventana BenchMark (Tucson, USA)
Cancer Cignal Finder 10-Pathway Reporter Array kit	Qiagen Science (Maryland, USA)
Bradford colorimetric protein assay	Bio-Rad (Hercules, USA)
Electophoresis transfere pack Trans-Blot Turbo	
Western blot immunodetection WesternBreez®Chromogenic kit	Invitrogen (San Diego, USA)

Tab. 6.4: List of software tools used during the work.

Name of software	**Name of the company (city and country of origin)**
Kaleidagraph® version 4.1	Synergy Software (Reading, USA)
OriginPro® software version 7.5	OriginLab Corporation (Northampton, USA)
Microsoft Office Excel	Microsoft (Albuquerque, USA)

Tab. 6.5: List of accessories used during the work.

Name of product	**Name of the company (city and country of origin)**
Freezing resistant plastic vials	Greiner Bio-One (Frickenhausen, Germany)
Plastic sterile 15 and 50 ml tubes	
Sterile/unsterile 1, 2, 10, and 25 ml pipettes	
Sterile 50 and 200 ml flasks	
Sterile 50 and 200 ml flasks	Cyto One (Tokyo, Japan)
Sterile 6-well plates	
Neubauer chamber	Paul Marienfeld (Lauda-Königshofen, Germany)
Pipette	Eppendorf AG (Hamburg, Germany)
Sterile pipette tips	
Unsterile plastic 1.5 ml cups	
Sterile 96-well black plates with clear bottom	Costar (New York, USA)
Stainless clip Shandon Cytoclip™	Thermo Scientific (Rockford, USA)
Filter card	
Re-usable sample chamber	
Glass microscopic slide	Thermo Scientific (Braunschweig, Germany)
Sterile white flat bottom 96-well plates	Nunc (Roskilde, Denmark)
Sterile cell scraper	Sarstedt (Newton, USA)
Sterile insulin injections	Braun (Bad Arolsen, Germany)
Precast 10-well polyacrylamide gels Mini-PROTEAN®TGX™, any kD™	Bio-Rad (Hercules, USA)
5 ml polystyrene test tubes	BD Falcon (San Jose, USA)

7 References

Agarwal B, Bhendwal S, Halmos B, Moss SF, Ramey WG, Holt PR (1999): Lovastatin Augments Apoptosis Induced by Chemotherapeutic Agents in Colon Cancer Cells. Clin Cancer Res 5, 2223-2229

Ahern TP, Pedersen L, Tarp M, Cronin-Fenton DP, Garne JP, Silliman RA, Sørensen HT, Lash TL (2011): Statin Prescriptions and Breast Cancer Recurrence Risk: A Danish Nationwide Prospective Cohort Study. J Natl Cancer Inst 103, 1461-1468

Alberts AW (1988): Discovery, biochemistry and biology of lovastatin. Am J Cardiol 62(15), 10J-15J

Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E,et al. (1980): Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Porc Natl Acad Sci 77(7), 3957-3961

Allalunis-Turner MJ, Barron GM, Day RS 3rd, Dobler KD, Mirzayans (1993): Isolation of two cell lines from a human malignant glioma speciment differing in sensitivity to radiation and chemotherapeutic drugs. Radiat Res 134 (3), 349-354

Barnea I, Haif S, Keshet R, Karaush V, Lev-Ari S, Khafif A, Shtabsky A, Yarden Y, Vexler A, Ben Yosef R (2013): Targeting ErbB-1 and ErbB-4 in irradiated head and neck cancer: Results of in vitro and in vivo studies. Head Neck 35(3), 399-407

Boukamp P, Petressevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988): Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cel line. J Cell Biol 106 (3), 761-771

Bradford MM (1976): A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254

Broughton T, Stington J, Beales IL (2012): Statine use is associated with a reduced incidence of colorectal cancer: a colonoscopy-controlled case-control study. BMG Gastroenterol 12:36

Chan KKW, Oza AM, Siu LL (2003): The Statins as Anticancer Agents. Clin Cancer Res 9, 10-19

Chen WS, Lazar CS, Lund KA, Welsh JB, Chang CP, Walton GM, Der CJ, Wiley HS, Gill GN, Rosenfeld MG (1989): Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. Cell 59(1), 33-43

Chen YR, Wang X, Templeton D, Davis RJ, Tan TH (1996): The Role of c-Jun N-terminal Kinase (JNK) in Apoptosis Induced by Ultraviolet C and γ Radiation: Duration of JNK activation may determine cell death and proliferation. J Biol Chem 271(50), 31929-31936

Clendening JW, Penn LZ (2012): Targeting tumor cell metabolism with statins. Oncogene 31, 4967-4978

Clendening JW, Pandyra A, Boutros PC, El Ghamrasni S, Khosravi F, Trentin GA, Martirosyan A, Hakem A, Hakem R, Jurisica I et al. (2010): Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci USA 107(34), 15051-15056

Cohen S, Ushiro H, Stoscheck C, Chinkers M (1982): A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicules. J Biol Chem 257(3), 1523-1531

Cortez D, Guntuku S, Qin J, Elledge SJ (2001): ATR and ATRIP: Partners in Checkpoint Signaling. Science 294, 1713-1716

Fritz G, Brachetti C, Kaina B (2003): Lovastatin causes sensitization of HeLa cells to ionizing radiation-induced apoptosis by the abrogation of G2blockage. Int J Radiat Biol 79(8), 601-610

Fritz G, Henninger C, Huelsenbeck J (2011): Potential use of HMG-CoA reductase inhibitors (statins) as radioprotective agents. Br Med Bull 97, 17-26

Gabryś D, Dörfler A, Yaromina A, Hessel F, Krause M, Oertel R, Baumann M (2008): Effects of lovastatin alone or combined with irradiation on tumor cells in vitro and in vivo. Strahlenther Onkol 184(1), 48-53

Ghosh-Choudhury N, Mandal CC, Ghosh-Choudhury N, Ghosh-Choudhury G (2010): Simvastatin induces derepression of PTEN expression via NFkappaB to inhibit breast cancer cell growth. Cell Signal 22(5), 749-758

Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP (1973): In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 51 (5), 1417-1423

Goldstein JL, Brown MS (1990): Regulation of the mevalonate pathway. Nature 343, 425-430

Gorski DH, Beckett MA, Jaskowiak NT, Calvin DP, Mauceri HJ, Salloum S, Seetharam S, Koons A, Hari DM, Kufe DW et al. (1999): Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. Cancer Res 59(14), 3374-3378

Grundy SM (1988): HMG-CoA reductase inhibitors for treatment of hypercholesterolemia. N Eng J Med 319, 24-33

Gutt R, Tonlaar N, Kunnavakkam R, Karrison T, Weichselbaum RR, Liauw SL (2010): Statin Use and Risk of Prostate Cancer Recurrence in Men Treated With Radiation Therapy. J Clin Oncol 28, 2653-2659

Hallahan D, Kuchibhotla J, Wyble C (1996): Cell Adhesion Molecules Mediate Radiation-induced Leukocyte Adhesion to the Vascular Endothelium. Cancer Res 56, 5150-5155

Hamilton RJ, Banez LL, Aronson WJ, Terris MK, Platz EA, Kane CJ, Presti JC, Amling CL, Freedland SJ (2010): Statin Medication Use and the Risk of Biochemical Recurrence After Radical Prostatectomy: Results From the Shared Equal Access Regional Cancer Hospital (SEARCH) Database. Cancer 116, 3389-3398

Haydont V, Mathé D, Bourgier C, Abdelali J, Aigueperse J, Bourhis J, Vozenin-Brotons MC (2005): Induction of CTGF by TGF-beta1 in normal and radiation enteritis human smooth muscle cells: Smad/Rho balance and therapeutic perspectives. Radiother Oncol 76(2), 219-225

Iliakis G, Wang Y, Guan J, Wang H (2003): DNA damge checkpoint control in cells exposed to ionizing radiation. Oncogene 22, 5834-5847

Istvan ES, Deisenhofer J (2001): Structural Mechanism for Statin Inhibition of HMG-CoA Reductase. Science 292, 1160-1164

Joiner M, van der Kogel: Basic Clinical Radiobiology. 4. Edition; Hodder Arnold, London, Great Britain 2009

Keyomarsi K, Sandoval L, Band V, Pardee AB (1991): Synchronisation of Tumor and Normal Cells from G_1 to Multiple Cell Cycles by Lovastatin. Cancer Res 51, 3602-3609

Khurana V, Bejjanki HR, Caldito G, Owens MW (2007): Statins Reduce the Risk of Lung Cancer in Humans: A Large Case-Control Study of US Vetreans. Chest 131, 1282-1288

Kil WJ, Tofilon PJ, Camphausen K (2012): Post-radiation increase in VEGF enhances glioma cell motility *in vitro*. Radiat Oncol, 7;25

Koch S, Tugues S, Li X, Gualandi L, Claesson-Welsh L (2011): Signal transduction by vascular endothelial growth factor receptors. Biochem J 437, 169-183

Kollmeier MA, Katz MS, Mak K, Yamada Y, Feder DJ, Zhang Z, Jia X, Shi W, Zelefsky MJ (2011): Improved Biochemical Outcomes with Statin Use in Patients with High-Risk Localized Prostate Cancer Treated with Radiotherapy. Int J Radiation Ocology Biol Phys 79(3), 713-718

Kornbrust DJ, MacDonald JS, Peter CP, Duchai DM, Stubbs RJ, Germershausen JI, Alberts AW (1989): Toxicity of HMG-Coenzyme A Reductase Inhibitor, Lovastatin, to Rabbits. J Pharmacol Exp Ther 248(2), 498-505

Lee J, Lee I, Han B, Park JO, Jang J, Park C, Kang WK (2011): Effect of Simvastatin on Cetuximab Resistance in Human Colorectal Cancer With KRAS Mutations. J Natl Cancer Inst 103, 674-688

Lehman DM, Lorenzo C, Hermandez J, Wang CP (2012): Statin use as a moderator of metformin effect on risk for prostate cancer among type 2 diabetic patients. Diabetes Care 35, 1002-1007

Le Tourneau C, Siu LL (2008) : Molecular-targeted therapies in the treatment of squamous cell carcinomas of the head and neck. Curr Opin Oncol 20(3), 256-263

Li X, Liu L, Tupper JC, Bannerman DD, Winn RK, Sebti SM, Hamilton AD, Harlan JM (2002): Inhibition of Protein Geranylgeranylation and RhoA/RhoA Kinase Pathway Induces Apoptosis in Human Endothelial Cells. J Biol Chem 277(18), 15309-15316

Lin CL, Cheng H, Tung CW, Huang WJ, Chang PJ, Yang JT, Wang JY (2008): Simvastatin reverse high glucose-induced apoptosis in mesangial cells via modultation of Wnt signaling pathway. Am J Nephrol 28(2), 290-297

Liu H, Wang Z, Li Y, Li W, Chen Y (2013): Simvastatin prevents proliferation and bone metastases of lung adenocarcinoma in vitro and in vivo. Neoplasma 60(3), 240-246

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG et al. (2004): Activating Mutation in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non-Small-Cell Lung Cancer to Gefitinib. N Engl J Med 350, 2129-2139

Mangoni M, Vozenin MC, Biti G, Deutsch E (2012): Normal tissue toxicities triggered by combined anti-angiogenic and radiation therapies: hurdles might be ahead. Brit J Cancer 107, 308-314

Mantha AJ, McFee KE, Niknejad N, Goss G, Lorimer IA, Dimitroulakos J (2003): Epidermal growth factor receptor-targeted therapy potentiates lovastatin-induced apoptosis in head and neck squamous cell carcinoma cells. J Cancer Res Clin Oncol 129, 631-641

Mantha AJ, Hanson JEL, Goss G, Lagarde AE, Lorimer IA, Dimitroulakos J (2005): Targeting the Mevalonate Pathway Inhibits the Function of the Epidermal Growth Factor Receptor. Clin Cancer Res 11, 2398-2407

McKenna WG, Muschel RJ, Gupta AK, Hahn SM, Bernhard EJ (2002): Farnesyltransferase inhibitors as radiation sensitizers. Semin Radiat Oncol 12(3 Suppl 2), 27-32

Meyer-Ter-Vehn T, Katzenberger B, Han H, Grehn F, Schlunck G (2008): Lovastatin inhibits TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. Invest Ophtalmol Vis Sci 49(9), 3955-3960

Nakamura JL, Karlsson A, Arvold ND, Gottschalk AR, Pieper RO, Stokoe D, Haas-Kogan DA (2005): PKB/Akt mediates radiosensitization by the signaling inhibitor LY294002 in human malignant gliomas. J Neurooncol 71(3), 215-222

Niyazi M, Maihoefer C, Krause M, Rödel C, Budach W, Belka C (2011): Radiotherapy and "new"drugs-new side effects? Radiat Oncol 6, 177

Nübel T, Damrot J, Roos WP, Kaina B, Fritz G (2006): Lovastatin Protects Human Endothelial Cells from Killing by Ionizing Radiation without Impairing Induction and Repair of DNA Double-Strand Breaks. Clin Cancer Res 12(3), 933-939

O'Reilly MS (2006): Radiation Combined With Antiangiogenic and Antivascular Agents. Semin Radiat Oncol 16, 45-50

Ostrau C, Hülsenbeck J, Herzog M, Schad A, Torzewski M, Lackner KJ, Fritz G (2009): Lovastatin attenuates ionizing radiation-induced normal tissue damage in vivo. Radiother Oncol 92(3), 492-499

Paez JG, Jähne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ et al. (2004): *EGFR* Mutations in Lung Cancer: Correlation with Clinical Response to Gefitinib Therapy. Science 304, 1497-1500

Park CM, Park MJ, Kwak HJ, Lee HC, Kim MS, Lee SH, Park IC, Rhee CH, Hong SI (2006): Ionizing Radiation Enhances Matrix Metalloproteinase-2 Secretion and Invasion of Glioma Cells through Src/Epidermal Growth Factor Receptor-Mediated 38/Akt and Phosphatidylinositol 3-Kinase/Akt Signaling Pathways. Cancer Res 66(17), 8511-8519

Park IH, Park SJ, Cho JS, Moon YM, Moon JH, Kim TH, Lee SH, Lee HM (2012): Effect of simvastatin on transforming growth factor beta-1-induced myofibroblasts differentiation and collagen production in nasal polyp-derived fibroblsts. Am J Rhinol Allergy 26(1), 7-11

Platz EA, Leitzman MF, Visvanathan K, Rimm EB, Stampfer MJ, Willett WC, Giovannucci E (2006): Statin Drug and Risk of Advanced Prostate Cancer. J Natl Cancer Inst 98, 1819-1825

Poyntner JN, Gruber SB, Higgins PD, Almog R, Bonner JD, Rennert HS, Low M, Greenson JK, Rennert G (2005): Statins and the risk of colorectal cancer. N Eng J Med 352, 2184-2192

Prasanna P, Thibault A, Liu L, Samid D (1996): Lipid Metabolism as a Target for Brain Cancer Therapy: Synergistic activity of Lovastatin and Sodium Phenylacetate Against Human Glioma Cells. J Neurochem 66, 710-716

Promega, USA, Caspase-Glo®3/7 Assay, Technical Bulletin, revised 12/12

Promega, USA, CellTiter-Blue® Cell viability Assay, Technical Bulletin, revised 6/09

Promega, USA, Dual-Glo®Luciferase Assay System, Technical Manual, revised 9/11

Qi XF, Kim DH, Yoon YS, Li JH, Jin D, Teng YC, Kim SK, Lee KJ (2009): Fluvastatin inhibits expression of the chemokine MDC/CCL22 induced by interferon-gamma in HaCaT cells, a human keratinocyte cell line. Br J Pharmacol 157(8), 1441-1450

Qiagen, Sample and Assay Technologies, USA, Cignal Finder Reporter Array Tube, Format Handbook, revised 01/11

Rave-Frank M, Virsik-Peuckert P, Bertram K, Schmidberger M (1996): Chromosome analysis in X-irradiated primary cells of a human squamous cell carcinoma by fluorescence in situ hybridization. Int J Radiat Biol 69 (6), 695-700

Rodrigues-Díez R, Rodrigues-Díez R, Lavoz C, Rayego-Mateos S, Civantos E, Rodrígez-Vita J, Mezzano S, Ortiz A, Egido J, Ruis-Ortega M (2010): Statins inhibit angiotensin II/Smad pathway and related vascular fibrosis, by a TGF-β-independent process. PLoS One 5(11), e14145

Saki M, Toulany M, Sihver W, Zenker M, Heldt JM, Mosch B, Pietzsch HJ, Baumann M, Steinbach J, Rodemann HP (2012): Cellular and molecular properties of ^{90}Y-labeled cetuximab in combination with radiotherapy on human tumor cells in vitro. Strahlenther Onkol 188, 823-832

Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, Linn S (2004): Molecular Mechanisms of Mammanlian DNA Repair and the DNA Damage Checkpoints. Annu Rev Biochem 73, 39-85

Sanli T, Liu C, Rashid A, Hopmans SN, Tsiani E, Schultz C, Farrell T, Singh G, Wright J, Tsakiridis T (2011): Lovastatin Sensitizes Lung Cancer Cells to Ionozing Radiation: Modulation of Molecular Pathways and of Radioresistance and Tumor Suppression. J Thorac Oncol 6, 439-450

Sassano A, Platanias LC (2007): Statins in tumor suppression. Cancer Letters 260, 11-19

Schaafsma D, McNeill KD, Mutawe MM, Ghavami S, Unruh H, Jacques E, Laviolette M, Chakir J, Halayko AJ (2011): Simvastatin inhibits TGF β1-induced fibronectin in human airway fibroblasts. Respir Res 12, 113

Takeda I, Maruya S, Shirasaki T, Mizukami H, Takahata T, Myers JN, Kakehata S, Yagihashi S, Shinkawa H (2007): Simvastatin inactivates β1-integrin and extracellular signal-related kinase signalling and inhibits cell proliferation in head and neck squamous cell carcinoma cells. Cancer Sci 98, 890-899

Taylor RC, Cullen SP, Martin SJ (2008): Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9(3), 231-241

Thermo Scientific, UK, Thermo Scientific Shandon Cytoclip Slide Clip, TPX Sample Chambers and Filter Cards, Instructions for use, revised 6/12

Thibault A, Samid D, Tompkins AC, Cooper MR, Hohl RJ, Trepel J, Liang B, Patronas N, Venzon DJ, Reed E et al. (1996): Phase I Study of Lovastatin, an Inhibitor of the Mevalonate Pathway, in Patients with Cancer. Clin Cancer Res 2, 483-491

Tobwin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci 76(9), 4350-4354

Urakami C, Kurosaka D, Tamada K, Kishimoto S, Tezuka Y, Nishigori H (2012): Lovastatin alters TGF-β-induced epithelial-mesenchymal transition in porcine lens epithelial cells. Curr Eye Res 37(6), 479-485

Van Aelst L, D'Souza-Schorey C (1997): Rho GTPases and signaling networks. Genes Dev 11, 2295-2322

Walker K, Olson MF (2005): Targeting Ras and Rho GTPases as opportunities for cancer therapeutics. Curr Opin Genet Dev 15(1), 62-68

Wang ChY, Liu PY, Liao JK (2008): Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. Trends Mol Med 14(1), 37-44

Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ (2001): Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia 15(9), 1398-1407

Xu J, Liu X, Chen J, Zacharek A, Cui X, Savant-Bhonsale S, Liu Z, Choop M (2009): Simvastatin enhances bone marrow stromal cell differentiation into endothelial cells via notch signaling pathway. Am J Physiol Cell Physiol 296(3), 535-543

Yang J, Yu Y, Hamrick HE, Duerksen-Hughes PJ (2003): ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. Carcinogenesis 24(10), 1571-1580

Zacharek A, Chen J, Cui X, Yang Y, Chopp M (2009): Simvastatin increases notch signaling activity and promotes arteriogenesis after stroke. Stroke 40(1), 254-260

Zhou Q, Liao JK (2010): Pleiotropic Effects of Statins-Basic Research and Clinical Perspectives. Circ J 74, 818-826

Zimmermann M, Zouhair A, Azria D, Ozsahin M (2006): The epidermal growth factor receptor (EGFR) in head and neck cancer: ist role and treatment implications. Radiat Oncol 1, 11

List of abbreviations

AP-1	activator protein-1
ATM/ATR	ataxia telangiectasia mutated/ATM- and Rad3-related
ATP	adenosine triphosphate
CFU	colony formation unit
CTB	CellTiter-Blue®
CTGF	connective tissue growth factor
DMEM	Dulbecco's minimum essential medium
DMSO	dimethyl sulphoxide
DNA-PKs	DNA dependent protein kinase
ECGM	endothelial cell growth medium
EDTA	ethylenediaminetetraacetic acid
EGRF	epidermal growth factor receptor
ERK	extracellular signal-regulated kinases
FCS	fetal calf serum
FPP	farnesylpyrophosphate
GFP	green fluorescent protein
GGPP	geranylgeranylpyrophosphate
GTP-ase	guanosine triphosphatase
HMG-CoA	3 - hydroxyl - 3methylglutaryl-coenzyme A
IMRT	intensity modulated radiation therapy
IL	interleukin
IR	ionizing radiation
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinases
mTOR	mammalian target of rapamycin
NCID	Notch intracellular domain

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B
NSCLS	non–small cell lung carcinoma
PBS	phosphate buffered saline
PE	plating efficiency
PI3 - kinase	phosphatidylinositol 3-phosphate kinase
PlGF	placental growth factor
PVDF	polyvinylidene difluoride
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
Rho/ROCK	Rho/Rho-associated protein kinase
RLU	relative luminescence units
RRR	relative response ratios
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
SER	sensitizer enhancement ratio
SF	surviving fraction
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
TRE	transcriptional response element
VEGF	vascular endothelial growth factor

Acknowledgements

I would like to warmly thank to my supervisor, PD. Dr. med. H. A. Wolff, for accepting me for this dissertation and for giving me the opportunity to perform my work under his auspice.

Special thank is dedicated to Mrs. Rave-Fränk for her highly professional, methodological, and personal support and disposition whenever needed during the work.

I owe a thank to laboratory collaborators, Mrs. Kasten-Krapp, Ms. Bitter, Mrs. Hoffmeister, and Mrs. Jünemann for their everyday essential help in the laboratory.

I should not omit to express my sincere thanks to Prof. Dr. med. Dr. rer. nat. C.-F. Hess who gave me the chance to work on this study within the Department of Radiotherapy and Radiation Oncology.

At last but not at least I wish to thank Prof. Dr. med Olivier Pradier for his continuous help and support during my studies and work experiences.

Curriculum vitae

I was born on November 18, 1984 in Bratislava, in Slovakia, as a first child of Jana and Marcel Miglierini.

I was attending the primary school at Cádrova Street in Bratislava for 4 years and afterwards the gymnasium at Hubeného Street in Bratislava for 8 years.

After my baccalaureate in 2003, I have chosen the studies of human medicine and entered the Faculty of Medicine at the Comienus University in Bratislava on September 2003.

In my 5th school year of medicine (2008) I applied for an Erasmus exchange program and was accepted for a one-semester stay at the Faculty of Medicine of the University of Western Brittany in Brest, in France. After this stay I returned back to Slovakia in order to finish my studies of medicine and I have finally graduated on May 26, 2009, in Bratislava.

My desire was to go on with my studies and specialize myself in the field of radiation oncology. In August 2009, I have started my medical practice as a junior resident at the Department of Radiotherapy at the Institute of Cancerology and Haematology (ICH) of the Regional University Hospital in Brest, France.

From November 2011 to Mai 2012, I performed a 6-month stage at the Department of Oncology at ICH in Brest and subsequently returned back to the Department of Radiotherapy.

From October 2012 till the end of April 2013, I executed a stay at the Department of Radiotherapy and Radiation Oncology at Universitätsmedizin Göttingen where I have been accepted for my promotion.

Since Mai 2013, I continue my specialist training as a resident at the Department of Radiotherapy at ICH in Brest.

On July 25, 2013, I have obtained an approval to work as a physician in Lower Saxony, Germany.