Modulation of radio- and chemotherapy-induced normal tissue toxicity by the HMG-CoA reductase inhibitor lovastatin

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
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aus Schwäbisch Hall

Düsseldorf, August 2017

aus dem Institut für Toxikologie
der Heinrich-Heine Universität und des Universitätsklinikums Düsseldorf

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

Berichterstatter:
1. Prof. Dr. Gerhard Fritz
2. Prof. Dr. Matthias Kassack

Tag der mündlichen Prüfung: 22.12.2017

Die Endlosigkeit des wissenschaftlichen Ringens sorgt unablässig dafür,
daß dem forschenden Menschengeist seine beiden edelsten Antriebe erhalten bleiben
und immer wieder von neuem angefacht werden: Die Begeisterung und die Ehrfurcht.
Max Planck (1858-1947)

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Abbreviations

53BP1	p53-binding protein 1
5-FU	5-fluorouracil
8-oxoG	8-oxo-7,8-dihydroguanine
ACE	angiotensin-converting enzyme
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
Bcl-2	B-cell lymphoma 2
BED	biologically effective dose
BRCA	breast cancer
bw	body weight
C	carboxy-
Cdc	cell division cycle
Chk	checkpoint kinase
CK	creatine kinase
CD	cluster of differentiation
CoA	coenzyme A
CRISPR	clustered regularly interspaced short palindromic repeats
cs	catalytic subunit
CTGF	connective tissue growth factor
CtIP	C-terminal binding protein-interacting protein
CYP	cytochrome P450
d	day
DDR	DNA damage response
dHJ	double Holliday junction
D-loop	displacement loop
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	double-strand break
EGF	epidermal growth factor
EMA	European Medicines Agency
EMT	epithelial-mesenchymal transition

EndoMT	endothelial-mesenchymal transition
ERK	extracellular-signal regulated kinase
FPP	farnesyl pyrophosphate
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine-5'-diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine-5'-triphosphate
Gy	gray
HaCaT	human adult low calcium high temperature cells
Hdm-2	human double minute-2
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
hMSC	human mesenchymal stem cells
HMVEC-L	human microvascular endothelial cells of the lung
HNSCC	head and neck squamous cell carcinoma
HO-1	haem oxygenase-1
HPF	human pulmonary fibroblasts
HR	homologous recombination
HSAEpC	human small airway epithelial cells
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IL	interleukin
IR	ionising radiation
i.v.	intravenous
JNK	c-jun amino-terminal kinase
Kap1	KRAB-associated protein-1
KGF-1	keratinocyte growth factor-1
KRAB	krüppel associated box
Ku	Ku70/Ku80 heterodimer
LDL	low-density lipoprotein
LET	linear energy transfer
LPS	lipopolysaccharid
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1

Mdm-2	mouse double minute-2
Mre11	meiotic recombination 11
MRN	Mre11-Rad50-Nbs complex
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
Nbs	nibrin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NHEJ	non-homologous end joining
NOS	nitric oxide synthase
NOX	NADPH oxidase
Nrf2	nuclear factor erythroid 2-related factor 2
NSCLC	non-small-cell lung carcinoma
OM	oral mucositis
PAK	p21 activated kinase
PARP	poly (ADP-ribose) polymerase
pH3	phosphorylated histone 3
PI3K	phosphatidylinositol-3-kinase
PP	pyrophosphate
PP2A	protein phosphatase 2A
qPCR	real-time quantitative polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	rat sarcome
RBE	relative biological effectiveness
RCT	randomized controlled trial
Rho	Ras homologous
RILI	radiation-induced lung injury
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RPA	replication protein A
SDSA	synthesis-dependent strand annealing
Ser	serine
SI	International System of Units
siRNA	small interfering RNA

SOD	superoxide dismutase
SSB	single-strand break
tBHP	tert-butyl hydroperoxide
TGF-β	transforming growth factor-beta
Thr	threonine
TNF-α	tumour necrosis factor-alpha
TopBP1	DNA topoisomerase II-binding protein 1
topo II	type II topoisomerase
US-FDA	United States Food and Drug Administration
UV	ultraviolet
VSMC	vascular smooth muscle cells
WTI	whole thorax irradiation
XIAP	X-linked inhibitor of apoptosis protein
XRCC	X-ray repair cross-complementing
γH2AX	Ser139-phosphorylated histone 2AX

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1 Introduction

1.1 Cancer therapy

Cancer is a leading cause of death in the Western world and tumours of the lung, colon, breast and prostate are the most frequent causes of cancer-related death (Ferlay *et al.*, 2013; Siegel *et al.*, 2015). Therapeutic options include classical interventions such as surgical removal, radio- and chemotherapy. Chemotherapeutic agents, for example platinum compounds, alkylating agents, topoisomerase inhibitors, nucleotide analogs and mitotic inhibitors, mainly interfere with the tumour cell-specific rapid proliferation and cell division of these cells. However, chemotherapeutics also act on normal cells and often provoke damage to normal tissue leading to serious side effects during anticancer therapy. In recent years, numerous new tumour cell-specific, oncogenic factors, have been identified and have been successfully targeted. Kinase inhibitors (e.g. sorafenib, imatinib, gefitinib, vemurafenib), the PARP inhibitor olaparib or the proteasome inhibitor bortezomib have been approved for the treatment of various tumour entities. Although these targets are more characteristic for tumour cells and make targeted antitumour therapies promising treatment strategies, chemo- and radiotherapy are still frequently used and remain first-line therapies for multiple tumour entities (Fennell *et al.*, 2016; Ow *et al.*, 2015; Sachdev and Jahanzeb, 2016; Sunakawa *et al.*, 2016).

1.1.1 Radiotherapy

50 % of all cancer patients receive radiotherapy during their antitumour treatment. Most often high-energy X-rays generated by linear accelerators are applied. Apart from the classical X-ray irradiation techniques, radiotherapy using charged particles is becoming increasingly employed. Protons and heavy ions (e.g. carbon ions) differ from electrons/photons in their penetration depth and deliver energy to a more defined area. While protons show equal relative biological effectiveness (RBE) compared to X-rays, carbon ions have higher RBEs (Moding *et al.*, 2013). Independent of the type and source of radiation all these methods share the ability of dislodging electrons from atoms or molecules and hence the term “ionising radiation” (IR). The SI unit to quantify the amount of radiation is the absorbed dose measured in grays (Gy). One gray corresponds to one joule of energy absorbed by one kilogram of matter (Jennings, 2007).

Conventional radiotherapy, e.g. for lung cancer, usually employs fractionated irradiation schemes. Typical treatment schemes comprise 25-35 fractions of ~2 Gy daily (5 doses per week) over a period of 5-7 weeks. This results in cumulative doses of ~50-70 Gy (Ahmed *et al.*, 2014; Haslett *et al.*, 2014). Apart from the classical fractionation scheme other types of fractionation are applied. Hyperfractionation delivers smaller doses (~1.2 Gy) more than once a day and in accelerated hyperfractionation schemes the radiation doses are given during a shorter period of time. Hypofractionation reduces the number of fractions while each dose consists of more than 2 Gy (Ahmed *et al.*, 2014) up to 4-6 Gy (Bogart, 2010). Depending on type, stage, location and origin of tumour, different irradiation schemes are applied.

Using fractionated irradiation, the four R's of fractionated radiotherapy, originally described by Withers, are applied. This maximises the efficacy of antitumour treatment whilst sparing normal tissue (Withers, 1975). Generally, cells in late S-phase of the cell cycle are more radioresistant than G2-phase cells (Sinclair and Morton, 1965). Thus, fractionated irradiation allows tumour cells in insensitive phases of the cell cycle to progress into more vulnerable phases (**R**edistribution). Moreover, tumours usually contain hypoxic cells which are less sensitive to radiation compared to their oxygenated counterparts (Kallman and Dorie, 1986). Irradiation of tumours depletes mainly oxygenated cells and leaves hypoxic cells, some of which become reoxygenated, especially in the tumour periphery. This renders them sensitive to radiation (**R**eoxygenation). In addition, fractionation has positive effects on normal cells. The period of time between two low-dose fractions is favourable for non-tumour cells to undergo **R**epair of sublethal damage (Hall and Giaccia, 2006). Furthermore, radiation increases proliferation of normal cells which contributes to a certain **R**epopulation and therefore regeneration of the damaged tissue. However, repopulation due to increased proliferation also occurs in tumour cells often leading to treatment failure which can partly be overcome by applying accelerated fractionation schemes (Hall and Giaccia, 2006). Finally, intrinsic **R**adiosensitivity of cells is often described as a fifth R (Steel *et al.*, 1989).

1.1.1.1 Molecular effects of radiation exposure

Ionising radiation can on the one hand directly provoke cellular damage by targeting intracellular macromolecules or on the other hand by acting through reactive oxygen species (ROS) via the radiolysis of water (Azzam *et al.*, 2012). The radiation effect mainly depends on the type or source of radiation: whereas high linear energy transfer (high-LET) radiation, such as neutrons or α-particles, triggers cellular damage mainly by directly interacting with

nucleic acids, lipids or proteins, low-LET radiation (e.g. γ-rays, X-rays) provokes damage predominantly through the generation of ROS (Panganiban *et al.*, 2013). The primary species that result from water radiolysis include e^-_{aq}, H•, OH•, OH^-, H_2O_2 and H_2, of which some can be quickly converted into reactive superoxide radicals (O_2^-•) (Le Caër, 2011). In addition, the latter are able to form highly reactive peroxynitrate anions ($ONOO^-$) following reaction with nitric oxide (NO•) arising from radiation-stimulated nitric oxide synthase (NOS) (Azzam *et al.*, 2012).

Although radiation affects all cellular components, the nuclear DNA is accepted as the most critical radiation target (Iliakis, 1991; Iyer and Lehnert, 2000). Irradiation leads to a plethora of DNA lesions including pyrimidine and purine base modifications e.g. 8-oxo-7,8-dihydroguanine (8-oxoG) and 5,6-dihydroxy-5,6-dihydrothymine, DNA single-strand breaks (SSBs) and DNA double-strand breaks (DSBs) (Roos and Kaina, 2013). An absorbed dose of 1 gray (Gy) is able to produce ~1000 SSBs and 20-40 DSBs (Lomax *et al.*, 2013). The latter are the most toxic DNA lesions following exposure to ionising radiation (Rothkamm *et al.*, 2003). 30 % of irradiation-induced DNA damage is provoked by direct effects of radiation on DNA. More importantly, ~70 % of total DNA damage is mediated by IR-induced ROS (Santivasi and Xia, 2014).

1.1.1.2 IR-induced DNA damage response and DNA damage repair

Upon DNA damage the so-called DNA damage response (DDR) is triggered. This can promote cell death or cell survival via cell cycle arrest followed by DNA repair (see Figure 1).

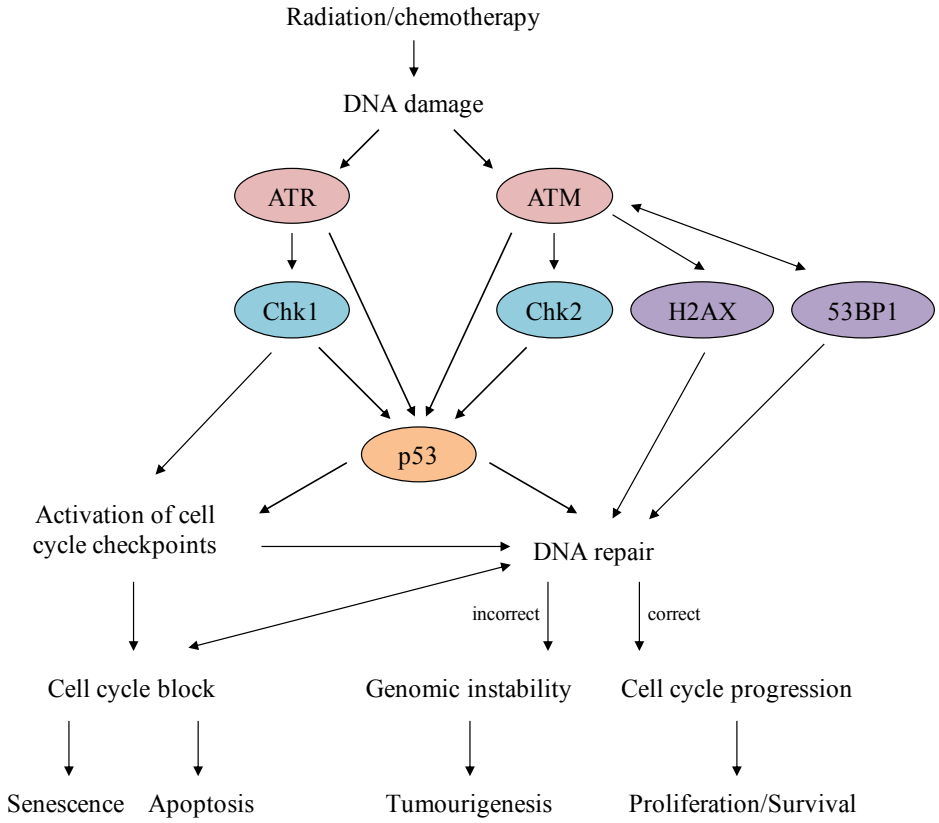

Figure 1: Recognition of DNA damage and cellular response to DNA damage. Following DNA damage recognition by ATM/ATR, downstream effectors are activated leading to checkpoint activation and cell cycle arrest in order to facilitate DNA repair. Persistent cell cycle blockage due to unrepaired DNA damage can lead to senescence or apoptosis induction. Incorrectly repaired DNA damage can be the cause for genomic instability and can promote tumourigenesis. 53BP1, p53-binding protein 1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk, checkpoint kinase; H2AX, histone 2AX (modified according to Christmann *et al.*, 2003; Houtgraaf *et al.*, 2006; Roos *et al.*, 2015).

There are three main sensor proteins which recognise DNA damage within minutes of occuring. These kinases, belonging to the family of PI3K-related kinases, are ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Houtgraaf *et al.*, 2006). After formation of DSBs ATM is recruited to the site of damage, is activated and phosphorylates various downstream signalling proteins of the DDR. ATR is mainly activated upon replication blockage and is recruited to stalled replication forks (Nam and Cortez, 2011). Moreover, ATR is activated following diverse DNA lesions including DSBs and crosslinks (Cimprich and Cortez, 2008). Downstream of ATR and ATM the transducer proteins checkpoint kinase 1 (Chk1) and 2 (Chk2) are activated by ATM/ATR-mediated phosphorylation. Chk1 is believed to be mainly activated by ATR whereas Chk2 is the main substrate of ATM (Manic *et al.*, 2015).

Phosphorylation of key residues of checkpoint kinases is indicative of the activation of the proteins. Following exposure to IR, phosphorylation of Thr68 of Chk2 by ATM is an important signalling step in the DDR (Matsuoka *et al.*, 2000). The critical residues required for Chk1 activation by ATR are Ser317 and Ser345 (Zhao and Piwnica-Worms, 2001). Upon DNA damage Chk1 and Chk2 participate in cell cycle regulation. Both checkpoint kinases share the downstream target Cdc25, a family of phosphatases that acts as a positive regulator of the cell cycle and is inactivated by Chk-mediated phosphorylation (Furnari *et al.*, 1999; Karlsson-Rosenthal and Millar, 2006; Reinhardt and Yaffe, 2009). This way, checkpoint kinases contribute to G1/S, intra-S and G2/M checkpoint activation. Apart from cell cycle regulation, the functions of Chk1 and Chk2 can be deduced from the spectrum of their substrates which are involved in DNA repair, apoptosis and p53 signalling (Zannini *et al.*, 2014), for example, BRCA1 and BRCA2 are phosphorylated by Chk2 (Bahassi *et al.*, 2008; Lee *et al.*, 2000) promoting DSB repair by homologous recombination (HR). The tumour suppressor p53, which is mutated in more than 50 % of all cancers, plays a pivotal role in the DDR and is placed at the interface between survival and cell death (Yoshida and Miki, 2010). Following genotoxic stress, p53 is stabilized by post-translational modification e.g. phosphorylation at Ser15 by ATM or ATR and Ser20 by Chk2 (Canman *et al.*, 1998; Chehab *et al.*, 2000; Hirao *et al.*, 2000; Tibbetts *et al.*, 1999). Consequently, p53 proteasomal degradation is prevented by blocking p53-Mdm-2 interaction (Yoshida and Miki, 2010). Activation of p53 leads to its accumulation in the nucleus and modulates transcription of DDR target genes (Helton and Chen, 2007). Whereas p53 phosphorylation at Ser15 is mainly pro-survival, Ser46 phosphorylation of p53 is more linked to pro-apoptotic functions of p53 (Roos *et al.*, 2015).

An early event following DNA double-strand break induction is the Ser139 phosphorylation of histone H2AX by ATM, ATR and DNA-PK. The so-called γH2AX can be detected at chromatin regions in the range of megabases up- and downstream of a single DSB (Rogakou *et al.*, 1998, 1999). 53BP1 is another important player in the DDR. The functions of 53BP1 include the promotion of checkpoint signalling by ATM activity amplification and repair pathway choice in favour of non-homologous end joining (NHEJ) (Panier and Boulton, 2013). 53BP1 is recruited to the site of DSBs with similar kinetics as γH2AX (Firsanov *et al.*, 2011; Schultz *et al.*, 2000). Therefore, formation of γH2AX and/or 53BP1 foci can be regarded as surrogate markers for DSBs.

Mammalian cells dispose of two main pathways for the repair of DNA double-strand breaks: homologous recombination (HR) and non-homologous end joining (NHEJ) (see Figure 2).

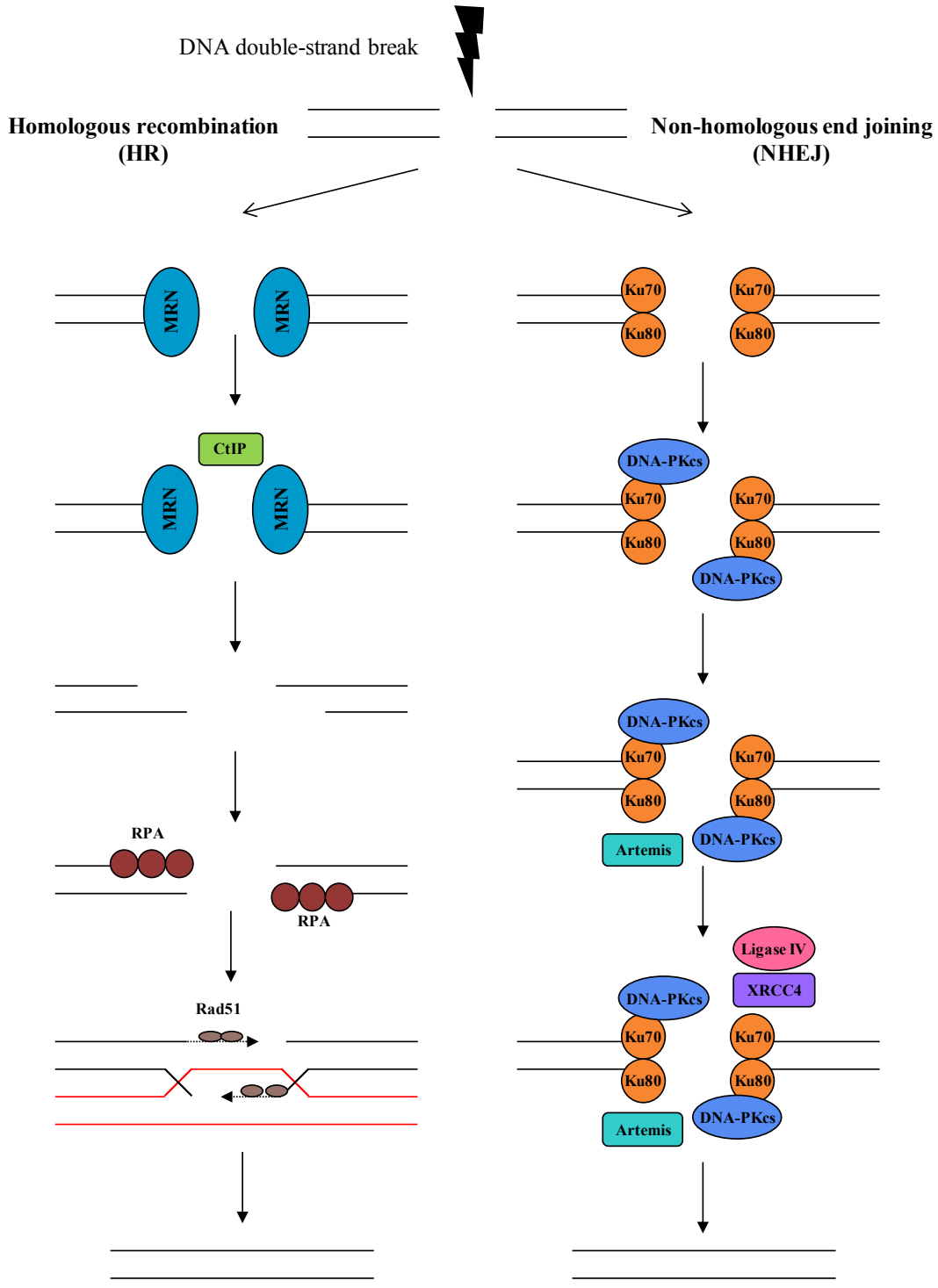

Figure 2: Mechanisms of homologous recombination (HR) and non-homologous end joining (NHEJ). See text for details. CtIP, C-terminal binding protein-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; MRN, Mre11-Rad50-Nbs complex; RPA, replication protein A; XRCC4, X-ray repair cross-complementing 4 (modified according to Christmann *et al.*, 2003).

HR is considered as an error-free repair pathway and occurs mainly during S and G2 cell cycle phases of dividing cells (Iyama and Wilson, 2013). It requires a sister chromatid which serves as a template. HR is initiated following recognition of DSBs by the MRN complex, consisting of meiotic recombination 11 (Mre11), Rad50 and nibrin (Nbs) (Lee, 2005; Uziel, 2003), which associate with C-terminal binding protein-interacting protein (CtIP) promoting 5'-3' end resection (Sartori *et al.*, 2007). The generated single stranded DNA overhangs are stabilized by binding of replication protein A (RPA) (Iyama and Wilson, 2013). With the help of Rad52, RPA is replaced by Rad51 leading to the formation of nucleoprotein filaments (Benson *et al.*, 1998; McIlwraith *et al.*, 2000). This process is BRCA2-dependent making BRCA2 a key player of HR (Jensen *et al.*, 2010). Subsequently, Rad51-mediated invasion of the homologous duplex leads to a displacement loop (D-loop) which is extended by DNA synthesis (Daley *et al.*, 2013). Following this, DNA repair can be accomplished either by synthesis-dependent strand annealing (SDSA) or via the classical pathway that includes the formation of a double Holliday junction (dHJ) (Cejka, 2015; Daley *et al.*, 2013).

Non-homologous end joining is the second pathway for the repair of DNA DSBs. Although, in contrast to HR, NHEJ is an error-prone DNA repair mechanism, it is thought to be more important for the repair of IR-induced DSBs in higher eukaryotes (Rothkamm *et al.*, 2003). One of the reasons is the presence of NHEJ in all cell cycle phases especially in the G0/G1 phase when no homologous template is available (Takata *et al.*, 1998). NHEJ is initiated by binding of the Ku70/Ku80 heterodimer (Ku) to the DNA ends (Ramsden and Gellert, 1998; Walker *et al.*, 2001) followed by the recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The resulting complex initiates DNA-PK's kinase activity which has a central role in NHEJ e.g. by controlling end-processing via Artemis nuclease (Gu *et al.*, 2010). DNA synthesis during NHEJ is fulfilled by DNA polymerases μ or λ (Kavanagh *et al.*, 2013). A complex composed of X-ray repair cross-complementing 4 (XRCC4) and ligase IV accomplishes the ligation of the processed ends which represents the last step of NHEJ. In addition, alternative end joining pathways have been described taking over DSB repair when parts of the classical NHEJ pathway are lacking (Iyama and Wilson, 2013; Wang *et al.*, 2006).

The cellular fate upon DNA damage is determined by the ratio of pro-survival and pro-death factors (see Figure 1). Persistent, unrepaired DNA damage, particularly DNA DSBs, are highly toxic and often lethal for cells (Joubert *et al.*, 2008) which subsequently undergo death

receptor- or mitochondria-dependent apoptosis or regulated necrosis (ferroptosis, necroptosis) (Roos *et al.*, 2015). This represents a tumour suppressing strategy by which cells counteract oncogenic transformation and carcinogenesis due to gene mutations and chromosomal aberrations (see Figure 1). Survival strategies include the induction of senescence, autophagy, inhibition of apoptosis by upregulation of B-cell lymphoma 2 (Bcl-2) and X-linked inhibitor of apoptosis protein (XIAP), activation of Akt or pro-survival transcription factors such as NF-κB (Roos *et al.*, 2015).

1.1.2 Chemotherapy with doxorubicin

Doxorubicin (also known as adriamycin) belongs to the group of anthracyclines and is an antitumour antibiotic used to treat a wide range of cancers including leukaemia, lymphoma as well as breast and lung carcinomas (Bonadonna *et al.*, 1969; Cimo *et al.*, 1974; Cole *et al.*, 1974; Kenis *et al.*, 1972; Wang *et al.*, 1971). Doxorubicin is administrated via *i.v.* bolus injection or continuous infusion and initial plasma concentrations are in the range of 1-2 μM (Gewirtz, 1999; Robert, 1987). Although anthracyclines represent a cornerstone in the treatment of various solid tumours and haematological malignancies the clinical use of doxorubicin is hampered by its side-effects. Doxorubicin causes nephro- (Ayla *et al.*, 2011; Injac *et al.*, 2008) and hepatotoxicity (Nagai *et al.*, 2015) and, most significantly, irreversible cardiotoxicity (Ghigo *et al.*, 2016). Moreover, patients treated with doxorubicin often suffer from myelosuppression, gastrointestinal disturbances as well as oral mucositis (Köstler *et al.*, 2001; Peterson *et al.*, 2007; Scully *et al.*, 2004; Simůnek *et al.*, 2009).

Several modes of action have been proposed for doxorubicin's biological effects on both tumour and normal cells. A main mechanism of doxorubicin is believed to be due to poisoning of type II topoisomerases (topo II). Topo II are ATP-dependent, DNA-binding enzymes that regulate DNA topology and exist in two different isoforms (α and β) (Yang *et al.*, 2014). They prevent torsional stress by introducing transient DNA DSBs and resolve DNA entanglements during replication, transcription, DNA repair as well as chromatin remodelling (Chen *et al.*, 2013). Topo II inhibitors such as doxorubicin stabilise and trap DNA-topo II complexes resulting in DNA strand breaks that are not resealed by topo II (Gruber *et al.*, 2005). This covalent topo II-DNA intermediate is designated as 'cleavable complex' and persists until it is degraded by the ubiquitin/26 S proteasome pathway (Mao *et al.*, 2001). Interaction of doxorubicin with topo II has been shown to provoke doxorubicin-induced DNA damage (Tewey *et al.*, 1984) and, in addition, doxorubicin-resistant cells

display reduced topo II levels or activity together with lower levels of doxorubicin-induced DNA strand breaks (Capranico *et al.*, 1987; Deffie *et al.*, 1989). However, the contribution of topo II poisoning and DNA damage induction to doxorubicin (cyto-) toxicity and cell death in tumour and normal cells is still a controversially discussed topic (Gewirtz, 1999; Swift *et al.*, 2006).

The generation of oxidative stress has also been proposed as playing a role in doxorubicin-mediated cell death and toxicity. Doxorubicin contains an anthraquinone structure that can be converted to a semiquinone by different enzymes including cytochrome P450 reductases and nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) dehydrogenases (Doroshow, 1983; Goodman and Hochstein, 1977). Following this, semiquinones can be re-oxidised to the quinone form thereby transferring an electron to molecular oxygen to produce superoxide radicals. Besides, superoxide radicals are further converted to hydrogen peroxide - either spontaneously or via superoxide dismutase (SOD). Both superoxide radicals and hydrogen peroxide are able to generate hydroxyl radicals that are very reactive and toxic (Simůnek *et al.*, 2009). However, the involvement of ROS in doxorubicin toxicity caused to normal tissue remains elusive since e.g. ROS scavengers failed to attenuate doxorubicin-induced cardiotoxicity *in vivo* (Ghigo *et al.*, 2016).

Additional hypotheses of doxorubicin's toxic mechanisms rest upon its ability to intercalate into nuclear and mitochondrial DNA, thereby inhibiting DNA and RNA polymerase (Ashley and Poulton, 2009; Tacar *et al.*, 2013), to form DNA adducts (e.g. with the exocyclic amino group of guanine residues) and interstrand crosslinks (Cutts and Phillips, 1995; Cutts *et al.*, 2005; Swift *et al.*, 2006; Taatjes *et al.*, 1997), to chelate iron (Muindi *et al.*, 1984) or to induce ceramide generation (Andrieu-Abadie *et al.*, 1999) or lipid peroxidation (Goodman and Hochstein, 1977).

1.1.3 Side effects of anticancer therapy

Apart from the desired effects of anticancer treatments on tumour cells, radio- and chemotherapy often provoke serious side effects on normal tissue. Following antitumour therapy unspecific effects on rapidly proliferating cells/tissues can be observed resulting for example in gastrointestinal symptoms such as nausea, vomiting and diarrhoea (Aprile *et al.*, 2015) as well as hair loss. Certain drugs exhibit specific toxicities, for example, cardio-, nephro- and neurotoxicity following chemotherapy with anthracyclines, cisplatin and

oxaliplatin, respectively (Lenneman and Sawyer, 2016; dos Santos *et al.*, 2012; Zedan *et al.*, 2014). Myelotoxicity, clinically manifested as fatigue, bleeding, infections and sepsis, greatly contributes to morbidity following anticancer therapy (Barreto *et al.*, 2014). The skin is a major target organ of anticancer therapy-induced side effects due to its rapid turnover and high metabolic rate (Payne *et al.*, 2006). Oedemas, cutaneous lesions and rash are frequently observed after treatment with various anticancer therapeutics including epidermal growth factor (EGF) receptor-targeting therapeutics such as antibodies or tyrosine kinase inhibitors (Payne *et al.*, 2006).

1.1.3.1 Oral mucositis

Oral mucositis is described as erythematous and ulcerative lesions of the mucous membranes of the oral cavity and is regarded as a major non-haematological complication following radio- or chemotherapy. Symptoms of oral mucositis comprise severe pain, oral dysfunction, bleeding, inability of swallowing and speaking, weight loss and fever amongst others (Lalla *et al.*, 2008; Scully *et al.*, 2004). Mucositis is observed following chemotherapy with doxorubicin, 5-fluorouracil (5-FU), cisplatin, cyclophosphamide, etoposide, methotrexate, vinblastine or radiotherapy (Harris, 2006; Scully *et al.*, 2004). Patients often have to undergo parenteral nutrition, take analgesics, and require extended hospitalisation periods thereby increasing the financial burden on the health system. Lesions of the mucosa can be the basis for microbial infections and sepsis. The patient's quality of life is severely affected and a dose reduction is often required limiting the efficacy of antitumour treatment (Al-Dasooqi *et al.*, 2013). There are several risk factors for the development of oral mucositis including treatment variables like type, dose and schedule of chemotherapy or radiation treatment as well as patient-specific parameters such as age, gender, genetic polymorphisms, bad medical condition or poor oral hygiene (Al-Ansari *et al.*, 2015). Radiation mucositis develops mainly in the radiation field whereas chemotherapy-induced mucositis usually involves the non-keratinized mucosa of the soft palate, tongue and cheeks (Scully *et al.*, 2004). In contrast to cancer patients undergoing chemotherapy, where mucositis typically develops shortly after the first treatment and resolves after two weeks, mucositis following fractionated irradiation starts at a cumulative dose of 15-30 Gy and takes up to four weeks after the end of the treatment period (Sonis, 2009).

The pathogenesis of oral mucositis involves a complex series of events, but can be divided in five major phases (see Figure 3).

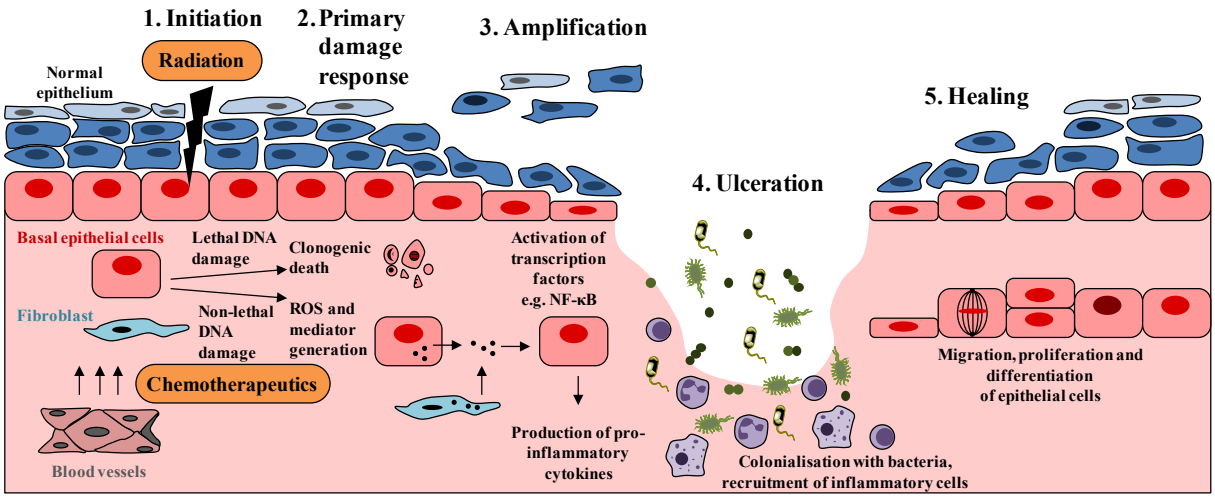

Figure 3: Pathogenesis of oral mucositis. Molecular and cellular processes during radio- and chemotherapy-induced oral mucositis. See text for details. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; ROS, reactive oxygen species (modified according to Sonis, 2011).

In the initiation phase ionising radiation or chemotherapeutics cause direct DNA damage of rapidly-dividing basal epithelial cells of the oral mucosa. In addition, ROS are produced that contribute to further damage. Following this, several biological pathways are triggered which result in the activation of different transcription factors including NF-κB. This is summarised as primary damage response phase. NF-κB can be activated by chemotherapeutics, radiation or ROS and leads to the production of cytokines, cell adhesion and to the induction of apoptosis. In the signal amplification phase local tissue responses are modulated via positive and negative feedback loops by molecules upregulated in the previous phase. Clinically, the most relevant phase is the ulceration phase. If a threshold of damaged and dead epithelial mucosa cells is reached, ulcers develop followed by bacterial colonialisation which worsens the mucositis process. Oral mucositis usually resolves spontaneously. Epithelial cells start to proliferate, migrate, differentiate and restore the surface of the damaged epithelium (Sonis, 2004, 2009, 2011).

Numerous interventions including oral hygiene, antibiotics, antifungal drugs, coating agents, laser therapy and cryotherapy have been investigated for the prevention or treatment of oral mucositis. Often they show only little or varying efficacy which is limited to specific mucositis-inducing agents, doses, irradiation schemes, sub-populations or tumour sites (Lalla

et al., 2014). Several substances which have turned out to be protective for mucositis in *in vitro* or *in vivo* preclinical models often act via their anti-inflammatory, anti-apoptotic or anti-oxidative properties (Baek *et al.*, 2014; Chang *et al.*, 2014; Charbaji *et al.*, 2013; Nakajima *et al.*, 2015; Shin *et al.*, 2013; Talwar *et al.*, 2014; Wu *et al.*, 2012). The only specific antimucositis agent approved by the US Food and Drug Administration (US-FDA) and the European Medicines Agency (EMA) is palifermin, a recombinant protein representing a truncated form of the human keratinocyte growth factor-1 (KGF-1) (Lalla *et al.*, 2014; McDonnell and Lenz, 2007). Upon binding to its receptor, KGF-1 leads to cell proliferation, differentiation, migration, and maturation of epithelial cells. Palifermin has been shown to diminish the duration and/or severity of oral mucositis following radiotherapy as well as doxorubicin- or 5-FU-based chemotherapy in different cancer entities including sarcomas, colorectal and haematological cancers (Meropol *et al.*, 2003; Rosen *et al.*, 2006; Spielberger *et al.*, 2004; Vadhan-Raj *et al.*, 2010). Amifostine (active metabolite: WR-1065) is a well-studied organic thiophosphate and is often discussed as a radio- and chemoprotective agent. It is thought to act mainly via its ROS scavenging properties thereby protecting cellular targets such as DNA and lipids (Kouvaris *et al.*, 2007; Nicolatou-Galitis *et al.*, 2013). There are studies and guidelines suggesting that the application of amifostine is clearly indicated in head and neck cancer patients receiving radio(chemo)therapy who suffer from acute and late xerostomia (Büntzel *et al.*, 1998; Hensley *et al.*, 2009; Veerasarn *et al.*, 2006; Wasserman *et al.*, 2005). However, with regard to mucositis, there is conflicting evidence whether amifostine has protective effects (Antonadou *et al.*, 2002; Brizel *et al.*, 2000; Buentzel *et al.*, 2006; Gu *et al.*, 2014; Koukourakis *et al.*, 2000; Lalla *et al.*, 2014; Stokman *et al.*, 2004). Taken together, effective measures for treatment of oral mucositis are to a large extent absent and prophylactical measures in particular are urgently needed.

1.1.3.2 Radiation-induced lung injury

Radiotherapy of the lung, a very radiosensitive organ (Giridhar *et al.*, 2015), leads to radiation-induced lung injury (RILI) that usually occurs in two phases – an early phase of ‘radiation pneumonitis’ and a late ‘radiation fibrosis’ phase. The incidence of radiation injury upon thoracic radiotherapy is up to 30 % (Kong *et al.*, 2005) and therefore represents a major dose-limiting toxicity. Radiation pneumonitis has an onset between months one and six after radiotherapy (Marks *et al.*, 2003). Patients suffering from radiation pneumonitis often have fever, coughing or suffer from dyspnoea or respiratory insufficiency (Ding *et al.*, 2013; Tsoutsou and Koukourakis, 2006). The onset of radiation fibrosis takes a few months to years

following lung radiotherapy (Ding *et al.*, 2013). Typical symptoms of radiation fibrosis are reduced gas exchange, severe respiratory failure and pulmonary heart disease (Ding *et al.*, 2013; Tsoutsou and Koukourakis, 2006).

The pathogenesis of RILI remains unclear in many areas. It is a sequential process and involves a complex interplay and an integrated response of different lung cell types, cytokines and other (growth) factors (Ding *et al.*, 2013). Lung irradiation leads to cellular and DNA damage – either directly or via the generation of ROS (see section 1.1.1.1) which provokes apoptosis in alveolar epithelial cells (Ding *et al.*, 2013). Cell death of IR-vulnerable type I pneumocytes covering 90 % of the lung surface leads to a loss of the alveolar barrier and initiates proliferation of type II cells in an attempt to replace the damaged epithelium (Graves *et al.*, 2010; Trott *et al.*, 2004). However, since radiation also targets type II epithelial cells the re-epithelialisation process is impaired meaning that the alveolar epithelium is often not fully restored (Trott *et al.*, 2004). Lung endothelial cell injury contributes to a reduced barrier function by impairing vessel integrity (Graves *et al.*, 2010). Following this, oedemas are formed and lung perfusion is reduced leading to hypoxia (Graves *et al.*, 2010). Moreover, inflammatory immune cells are recruited into the lung parenchyma and secrete factors that activate surrounding cells to participate in damage repair and wound healing (Graves *et al.*, 2010). Activated macrophages, most often derived from circulating monocytes, release cytokines and ROS. This contributes further to tissue hypoxia due to an elevated consumption of molecular oxygen by macrophages during the formation of ROS (Marks *et al.*, 2003).

Most cellular events are triggered or accompanied by the induction of diverse cytokines which begins after several hours and is maintained up to a time period of months after irradiation (Rube *et al.*, 2000; Rübe *et al.*, 2005). Transforming growth factor-beta 1 (TGF-β1), interleukins (e.g. IL-1 and IL-6) and tumour necrosis factor-alpha (TNF-α) play central roles in the pathogenesis of RILI (Kong *et al.*, 2005). TGF-β1 is one of the major mediators contributing to normal tissue damage following irradiation (Ao *et al.*, 2009). TGF-β1 is responsible for attracting monocytes/macrophages and promotes the recruitment of fibroblasts and their conversion into myofibroblasts (Graves *et al.*, 2010; Rube *et al.*, 2000). (Myo-) fibroblasts also arise from epithelial or endothelial cells through a process called epithelial/endothelial-mesenchymal transition (EMT/EndoMT) (Kim *et al.*, 2014). In the course of fibrosis formation, lung (myo-) fibroblasts proliferate and produce massive amounts

of extracellular matrix proteins which lead to an obliteration and a collapse of the alveoli and breaks down the normal lung structure (Tsoutsou and Koukourakis, 2006).

Therapeutic measures for the treatment of radiation toxicities of the lung are to a large extent absent or show only little efficacy. Corticosteroid intake (e.g. 1 mg/kg prednisolone for two weeks) is in clinical use for the treatment of radiation pneumonitis (Kim *et al.*, 2014). However, steroid therapy shows poor efficacy and can also have severe complications (e.g. Cushing syndrome) due to the narrow therapeutic window (Bista and Beck, 2014; Sekine *et al.*, 2006). Other therapeutic possibilities include the application of angiotensin-converting enzyme (ACE) inhibitors (e.g. enalapril) or pentoxifylline but there is limited data regarding their effectiveness (Giridhar *et al.*, 2015). The situation is even worse for the treatment of radiation-induced lung fibrosis as no established and effective treatment protocols exist. The treatment is based mainly on supportive measures, mobilisation of airway secretion and anti-inflammatory therapy using corticosteroids and macrolide antibiotics (Giridhar *et al.*, 2015). Amifostine (see section 1.1.3.1) is often discussed as a radioprotective agent for radiation-induced lung toxicities but there is inconclusive data regarding its efficacy (Mehta, 2005). Although Andonadou and colleagues showed that amifostine attenuates acute and late pulmonary toxicity following radiochemotherapy of non-small-cell lung carcinoma (NSCLC) patients, other studies failed to prove the efficacy of amifostine in the alleviation of RILI (Antonadou *et al.*, 2001, 2003; Werner-Wasik *et al.*, 2003). Several new preclinical strategies focus on critical signalling steps in the pathogenesis of RILI. Inhibition of TGF-β receptor reduces the extent of RILI in rodents (Anscher *et al.*, 2008; Flechsig *et al.*, 2012). Furthermore, overexpression of superoxide dismutase (SOD) or subcutaneous or intraperitoneal administration of the purified SOD, which is an important enzyme in the regulation of intracellular ROS and reactive nitrogen species (RNS) homeostasis, has favourable effects on RILI in various preclinical models (Antonic *et al.*, 2015; Breuer *et al.*, 1992; Epperly *et al.*, 1998; Kang *et al.*, 2003).

1.2 Statins and Ras-homologous GTPases

1.2.1 Statins (HMG-CoA reductase inhibitors)

Statins are first-line measures in the treatment of hyperlipidaemia. They interfere with the so-called mevalonate pathway which is required for cholesterol biosynthesis. Statins inhibit the rate limiting step of the mevalonate pathway which is the conversion of

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate by HMG-CoA reductase (Sirtori, 2014) (see Figure 4).

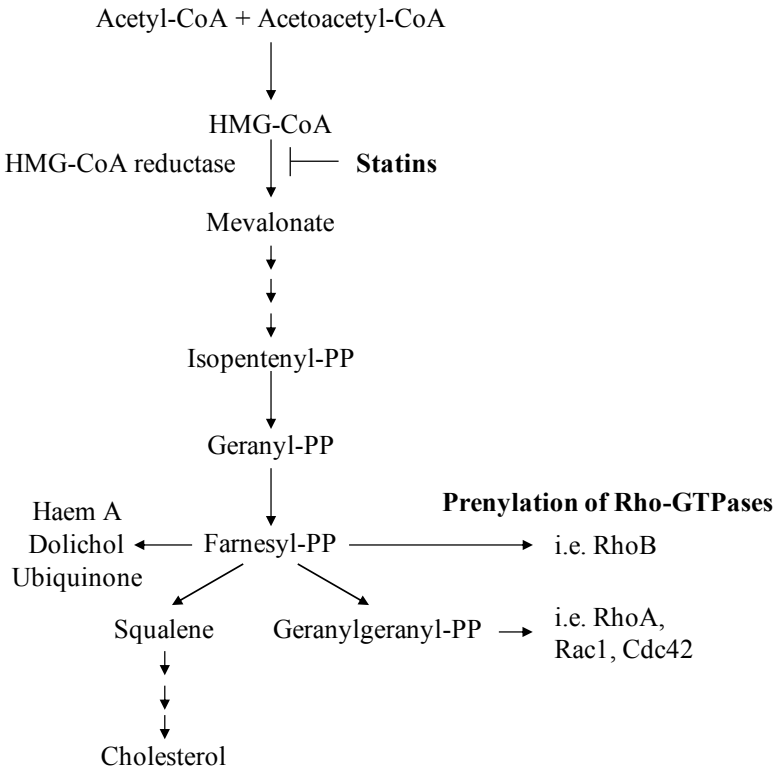

Figure 4: Inhibition of the mevalonate pathway by statins. HMG-CoA reductase inhibition by statins influences various intermediates of the mevalonate pathway including Haem A, dolichol, ubiquinone, cholesterol and isoprenoid precursors such as farnesyl- and geranylgeranylpyrophosphate which are required for isoprenylation of Rho-GTPases. Cdc 42, cell division cycle 42; CoA, Coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PP, pyrophosphate; Rac1, Ras-related C3 botulinum toxin substrate 1; Rho, Ras-homologous (modified according to Baron *et al.*, 2000; Cai *et al.*, 2015; Sirtori, 2014).

As a consequence, endogenous cholesterol biosynthesis is decreased leading to reduced intracellular cholesterol levels (Ewang-Emukowhate and Wierzbicki, 2013). Subsequently, an upregulation of low-density lipoprotein (LDL) receptors augments the clearance of LDL cholesterol from the circulation (Ewang-Emukowhate and Wierzbicki, 2013; Sirtori, 2014). By doing so, statins reduce the risk for atherosclerosis and cardiovascular complications such as ischaemic heart disease and thromboembolic stroke (Law *et al.*, 2003). Currently, there are seven statins commercially available that differ in their pharmacodynamic and –kinetic properties: lova-, simva-, prava-, atorva, fluva-, rosuva- and pitavastatin (Ewang-Emukowhate and Wierzbicki, 2013). Statins show different intestinal absorptions ranging from 30 % to

85 % (Sirtori, 2014). Due to a first pass metabolism in the liver the systemic bioavailability of statins is only 5-30 % (Sirtori, 2014). Lipophilic statins are taken up by cells via diffusion whereas more hydrophilic statins enter cells via carrier proteins (Sirtori, 2014). Most statins are metabolized by cytochrome P450 (CYP) enzymes 3A4 or 2C9 (Ewang-Emukowhate and Wierzbicki, 2013). In the case of lova- and simvastatin the presence of a closed-ring lactone renders them inactive pro-drugs which require metabolic activation, e.g. in the liver (Sirtori, 2014). Although they all inhibit the same enzyme, they have different efficacies with atorvastatin and rosuvastatin demonstrating the highest efficacy in reducing LDL cholesterol in hypercholesterolaemic patients (Jones *et al.*, 1998, 2003). Although statins are generally considered as safe and well-tolerated (Corsini *et al.*, 1995), the most important side-effect of statin intake is myopathy which occurs as myalgia, myositis or rhabdomyolysis depending on the increase of creatine kinase (CK) levels (Katz *et al.*, 2014). Additionally, hepato- and nephrotoxicity have been reported in combination with statin use but large randomised controlled trials (RCTs) do not support these concerns (Katz *et al.*, 2014). Lovastatin was the first commercially available statin approved by the US-FDA in 1987 (Tobert, 2003). Compared to other statins lovastatin, which has been used in the present studies, has a relatively low oral absorption (30 %) and a low bioavailability of 5 % (Sirtori, 2014). Lovastatin is given as daily doses of 20-80 mg/kg bw (Walker and Tobert, 1987). In humans, it reaches plasma peak levels 2-4 h post ingestion and strongly binds to plasma proteins (Frishman *et al.*, 1989).

Apart from their lipid-lowering properties statins have various pleiotropic effects which are not mediated by inhibition of cholesterol synthesis. In short, statins have been shown to possess anti-inflammatory, anti-oxidative, anti-proliferative and immunomodulatory properties (Cai *et al.*, 2015; Veillard *et al.*, 2006). Moreover, they have several positive effects on the cardiovascular system: they improve endothelial dysfunction by regulating eNOS activity and have anti-thrombotic features (Bedi *et al.*, 2016). Many of these pleiotropic, cholesterol-independent functions are based on an inhibition of members of the Rho-GTPase family (Cai *et al.*, 2015) (see section 1.2.2). By interference with HMG-CoA reductase, statins deplete not only cholesterol but also reduce the generation of isoprenoid precursors such as farnesylpyrophosphate and geranylgeranylpyrophosphate (see Figure 4) which serve as lipid attachments for Rho-GTPases (Liao and Laufs, 2005). As a consequence, the subcellular localisation of the Rho-GTPases is impeded leading to an accumulation of inactive Rho-GTPases in the cytoplasm (Yeganeh *et al.*, 2014).

1.2.2 Ras-homologous GTPases

Ras-homologous GTPases (Rho-GTPases) belong to the Ras superfamily of small GTP-binding proteins and function as molecular switches in various signalling pathways of mammalian cells. They rotate between an inactive, guanosine-5'-diphosphate (GDP)-bound form and an active, guanosine 5'-triphosphate (GTP)-bound form (Bustelo *et al.*, 2007). The latter is able to interact with different effector proteins until GTP is hydrolysed by the intrinsic GTPase activity and with the help of GTPase activating proteins (GAPs) (Bar-Sagi and Hall, 2000). Rho-GAPs increase the hydolysis rate of the GTPase up to 10^5 (Rittinger *et al.*, 1997). Moreover, the activity of Rho-GTPases is regulated by two other protein families: guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs) (Hall, 2012). Rho-GEFs interact with the GDP-GTPase complex leading to its destabilisation so that the nucleotide-free GTPase is stabilised (Schaefer *et al.*, 2014). Due to a high intracellular GTP concentration, GTP is bound to the GTPase resulting in its activation (Garcia-Mata *et al.*, 2011). Rho-GDIs hold the GTPase in its inactive state by preventing the dissociation of GDP and shield the isoprenyl group (see below) thereby preventing Rho-GTPase localisation at the membrane (Harding and Theodorescu, 2010). Apart from this, Rho-GTPases are also regulated on transcriptional level. For example, RhoB is regulated by external stimuli such as UV radiation, genotoxins as well as various growth factors and cytokines (Fritz and Kaina, 1997, 2001; Fritz *et al.*, 1995; Jähner and Hunter, 1991).

On the basis of their amino acid sequence, Rho-GTPases can be divided into six different sub-families: Rho, Rac, Cdc42, Rnd, RhoBTB and RhoT/Miro (Bustelo *et al.*, 2007). To obtain their biological activity Rho-GTPases require a specific posttranslational modification. This modification process starts with the isoprenylation of the C-terminal CAAX motive (C=cysteine, A=aliphatic amino acid, X=any amino acid) (Wennerberg *et al.*, 2005). With the help of type I transferases a geranylgeranyl tail or, in rare cases, a farnesyl tail is attached to the cysteine residue of the CAAX motive (Roberts *et al.*, 2008). Upon isoprenylation Rho-GTPases are translocated to the endoplasmatic reticulum where the AAX tripeptide is cleaved by the protease Rce I. The free carboxyl group of the cysteine is methylated to determine the subcellular localisation of the Rho-GTPase (Xu *et al.*, 2015).

Upon activation Rho-GTPases are able to interfere with several signalling cascades by interacting with effector molecules, e.g. serine/threonine kinases and scaffold/adaptor-like proteins (Bishop and Hall, 2000; Hall, 2012; Hanna and El-Sibai, 2013). Rho-GTPases fulfil

many functions in a broad range of cellular processes: aggregation of contractile actin- and myosin filaments (stress fibres) (Schaefer *et al.*, 2014), formation of lamellipodia and filopodia (Murali and Rajalingam, 2014), cell migration and adhesion as well as phago- and pinocytosis (Hall, 2012). Besides their function in the organisation of the cytoskeleton Rho-GTPases, for example Rac1, are also involved in different signalling pathways including c-jun N-terminal kinases (JNKs) as well as p38 mitogen-activated kinase and the regulation of transcription factors (e.g. NF-κB) (Hall, 2012). Moreover, Rac1 is part of the NADPH oxidase (NOX) complex (Hall, 2012). As a consequence, Rho-GTPases play important roles in physiological and pathological processes such as cell cycle progression, cell death, angiogenesis, tumour formation, metastasis and inflammation (Orgaz *et al.*, 2014). This renders them promising targets in the modulation of normal as well as tumour cell response following exposure to diverse noxae.

1.3 Objectives

To improve the efficacy of antitumour treatment, more specific anticancer drugs as well as more sophisticated radiation techniques are being continuously developed. However, the surrounding non-tumourigenic, normal tissue is often affected by chemo- or radiotherapy, thereby limiting a dose escalation and an effective tumour treatment. Therefore, preventive and therapeutic measures are urgently required which protect normal cells and tissues from the detrimental effects of anticancer therapy and also help to improve the healing process of injured cells.

Statins have been shown to exert cytoprotective properties against radiation (Nübel *et al.*, 2006; Ran *et al.*, 2010) and chemotherapeutics such as doxorubin (Damrot *et al.*, 2006) *in vitro*. *In vivo*, statins alleviate doxorubicin-induced cardiotoxicity (Feleszko *et al.*, 2000; Henninger *et al.*, 2015; Huelsenbeck *et al.*, 2011; Ramanjaneyulu *et al.*, 2013; Riad *et al.*, 2009; Yoshida *et al.*, 2009) as well as enteropathy following intestinal irradiation (Haydont *et al.*, 2007a, 2007b). However, the influence of statins on two clinically relevant normal tissue toxicities, namely oral mucositis and radiation-induced lung injury, is largely unknown since only a very limited number of preclinical studies exists (Mathew *et al.*, 2011; Medeiros *et al.*, 2011; Monceau *et al.*, 2010). Therefore, the aim of this study was to investigate potential cyto-, geno- and organoprotective effects of lovastatin following treatment with either the anthracycline doxorubicin or irradiation. In a human keratinocyte-based *in vitro* model for oral mucositis using HaCaT cells the effect of lovastatin pretreatment was investigated on

cellular and molecular level following treatment with either the anthracycline doxorubicin or irradiation. The second aim of this study was to investigate the influence of lovastatin on radiation response in primary human lung cells and mouse lung tissue. A fractionated irradiation scheme was developed and the influence of lovastatin on irradiation-induced DNA damage and apoptosis was analysed in primary lung endothelial cells, lung fibroblasts as well as lung epithelial cells. To show the transferability of these results the effect of lovastatin was also characterized in an *in vivo* mouse model using BALB/c mice. For this reason, an irradiation device was developed which allows a selective irradiation of the right lung. This device was used to investigate the effect of lovastatin on lung injury following fractionated lung irradiation.

2 Overview of manuscripts

2.1 Manuscript 1: Lovastatin protects keratinocytes from DNA damage-related proapoptotic stress responses stimulated by anticancer therapeutics.

2.1.1 Contribution statement of the first manuscript

Journal: Biochimica et Biophysica Acta - Molecular Cell Research 1863 (2016), 1082–1092.
Impact factor (2015/16): 5.13

Type of authorship: first author (shared first authorship)

Contribution:
- Design of experiments: 60 %
- Performance of experiments: 85 %
- Analysis of data: 90 %
- Contribution to discussion: 40 %
- Writing of the manuscript: 50 %
- Revision of the manuscript: 50 %
- **Total contribution to the publication: 63 %**

2.1.2 Theses of the first manuscript

Lovastatin protects keratinocytes from DNA damage-related proapoptotic stress responses stimulated by anticancer therapeutics (Ziegler *et al.*, 2016).

Thesis 1: Lovastatin prevents cell death of human keratinocytes following doxorubicin treatment and irradiation.

Pretreatment of human keratinocytes (HaCaT) with lovastatin (30 µM, 24 h) decreases both IR (10 Gy)- and doxorubicin (1 µM, 2 h)-mediated cytotoxicity by approximately 20 %. Lovastatin prevents IR- and doxorubicin-induced apoptosis as reflected on the level of subG1 induction. Analysis of cell death via Annexin V/PI staining confirms a protection from both early and late apoptosis by the statin. Cleavage of pro-caspases 3 and 7 as well as cleavage of PARP-1 were decreased by treatment with lovastatin.

Thesis 2: Lovastatin has favourable effects on DNA damage formation and/or repair following doxorubicin treatment and irradiation.

Doxorubicin treatment of HaCaT cells leads to the induction of DNA single- and double-strand breaks, which is reduced by lovastatin co-treatment independently of a statin-mediated altered drug import or export. In addition, lovastatin leads to a moderate acceleration of DNA DSBs repair as represented on the level of residual γH2AX foci. In contrast to doxorubicin treatment, lovastatin does not lead to a reduction of IR-induced initial DNA damage but improves DSB repair in HaCaT cells.

Thesis 3: Protective effects of lovastatin require cell cycle-dependent and -independent mechanisms.

Lovastatin shows inhibitory effects on IR- and doxorubicin-induced activation of ATR/Chk1-related DDR mechanisms of HaCaT cells. It preserves viability and ensures normal cell cycle progression by attenuating the IR- and doxorubicin-mediated decrease in the percentage of S-phase cells and by mitigating replication blockage caused by exposure to both noxae. The cytoprotective effects of the statin are not restricted to interference with cell cycle progression: lovastatin also protects non-proliferating HaCaT cells from apoptosis following IR or doxorubicin treatment, and, moreover, does not confer resistance following treatment of the latter with other S-phase-dependent anticancer drugs (e.g. cisplatin).

2.2 Manuscript 2: Rho inhibition by lovastatin affects apoptosis and DSB repair of primary human lung cells in vitro and lung tissue in vivo following fractionated irradiation.

2.1.1 Contribution statement of the second manuscript

Journal: Cell death & Disease 8 (2017), e2978.

Impact factor (2015): 5.97

Type of authorship: first author

Contribution: Design of experiments: 60 %

Performance of experiments: 90 %

Analysis of data: 100 %

Contribution to discussion: 50 %

Writing of the manuscript: 65 %

Revision of the manuscript: 60 %

Total contribution to the publication: 71 %

2.2.2 Theses of the second manuscript

Rho inhibition by lovastatin affects apoptosis and DSB repair of primary human lung cells in vitro and lung tissue in vivo following fractionated irradiation (Ziegler et al., 2017).

Thesis 1: Lovastatin protects primary human lung endothelial cells from apoptosis and improves DNA damage repair in different lung cells following fractionated irradiation *in vitro*.

Fractionated irradiation (4 x 4 Gy) leads to apoptosis of non-proliferating, primary human lung endothelial cells (HMVEC-L) which is prevented by low dose treatment with lovastatin. By contrast, primary human lung fibroblast cells (HPF) and primary human small airway epithelial cells (HSAEpC) do not undergo apoptosis upon fractionated irradiation. Lovastatin leads to an accelerated repair of residual DNA DSB (measured as nuclear γH2AX foci) in all three cell types which is most pronounced in fibroblasts and epithelial cells. The genoprotective effect is independent of a statin-mediated interference with the initial level of DNA damage formation.

Thesis 2: Lovastatin treatment has beneficial effects on subchronic lung tissue damage following fractionated irradiation *in vivo*.

Fractionated irradiation (4 x 4 Gy) of the right lung tissue of mice leads to a 20 % increase in the breathing frequency, which is attenuated by lovastatin co-treatment suggesting an improvement of IR-mediated reduction of lung function. Lovastatin reduces the percentage of γH2AX-positive lung cells indicating that statin treatment alleviates residual DNA damage resulting from fractionated irradiation. Moreover, it prevents apoptosis in lung tissue four weeks after irradiation. Tissue remodelling represented as mitotic index is increased by about 10-fold following exposure to radiation without histopathological detectable signs of concomitant acute inflammatory or fibrotic processes and is largely mitigated by the statin.

Thesis 3: Protective effects of lovastatin on lung tissue following fractionated irradiation are mediated by inhibition of Rac1.

Lovastatin-mediated protective effects on lung function, residual DNA damage, apoptosis, and regenerative proliferation *in vivo* are mimicked by treatment with the direct Rac1-specific small molecule inhibitor EHT1864. This suggests that inhibition of small GTPase Rac1 signalling provides beneficial effects on lung tissue upon fractionated irradiation.

3 Discussion and Conclusion

3.1 Statins show cytoprotective effects *in vitro* and *in vivo*

3.1.1 Statins protect human keratinocytes against doxorubicin- and IR-mediated cell death

In the *in vitro* mucositis model using human keratinocytes (HaCaT) lovastatin prevented cell death following exposure to ionising radiation or doxorubicin as verified by subG1 analysis and Annexin V/PI staining. In addition, statin pretreatment decreased caspase activation and cleavage of PARP-1 following exposure to both noxae. Similar cytoprotective effects of lovastatin have already been shown using primary endothelial cells (HUVEC) (Damrot *et al.*, 2006; Nübel *et al.*, 2006) or rat cardiomyocytes (H9c2) (Huelsenbeck *et al.*, 2011). In line with the findings in this thesis lovastatin-mediated protection following doxorubicin treatment turned out to be independent of cellular drug transport (Damrot *et al.*, 2006; Huelsenbeck *et al.*, 2011). Moreover, it has been demonstrated that lovastatin does not interfere with detoxification of doxrubicin-induced ROS (Damrot *et al.*, 2006). Prevention of doxorubicin injury is most likely linked to an interference of lovastatin with topoisomerase II function which reduces initial DNA damage formation (see section 3.2.1). However, statin-mediated cytoprotective effects following exposure to IR comprise different mechanisms since lovastatin does not protect from IR-induced initial DNA damage formation (see section 3.2.2). For both genotoxic noxae lovastatin enhanced repair of DSBs. Therefore, the molecular basis for the observed cyto- and genoprotective effects of the statin seems to be multifactorial.

Following induced damage to DNA, diverse stress responses are provoked which entail arrest of the cell cycle, DNA repair and subsequently cell survival or apoptosis. These are subsumed under the term DNA damage response (DDR) (see section 1.1.1.2). Both doxorubicin and irradiation triggered early phosphorylation and activation of key players of the DDR including ATM, Chk2, p53, RPA32 and Kap1, which remained unaffected by statin pretreatment. An activation of ATR by Ser428 phosphorylation was not observable under these conditions. However, IR and doxorubicin induced the phosphorylation of the ATR target kinase Chk1 which was clearly reduced by lovastatin. This suggests that in this case Chk1 might be activated by a different kinase other than ATR. Although Chk1 is known to be the main substrate of ATR, other kinases such as ATM and DNA-PK are reported to contribute to Chk1 phosphorylation (Buisson *et al.*, 2015; Gatei *et al.*, 2003; Jazayeri *et al.*, 2006). Since

additional mediator proteins such as TopBP1 and Claspin (Dai and Grant, 2010; González Besteiro and Gottifredi, 2015) are required for an ATR-dependent phosphorylation of Chk1, a putative interaction of lovastatin with those proteins might be responsible for the observed effects. Interestingly, at later time points (i.e. 24 h) ATR was phosphorylated at Ser428 following doxorubicin and IR exposure which is largely antagonised by lovastatin. This was not accompanied by a statin-mediated reduction of the level of phosphorylated Chk1. Using MCF-7 breast cancer cells, Yan and colleagues have shown that inhibition of the small GTPase Rac1 reduces ATR and Chk1 activity (Yan *et al.*, 2012). However, this corresponds with a sensitization of these cells to IR. Taken together, these data indicate that the modulation of ATR/Chk1 signalling by lovastatin is highly complex and extremely time-dependent.

Not only phosphorylation of Chk1 might be modulated by the statin, but also its dephosphorylation. Chk1 has been reported to be dephosphorylated by the protein phosphatase 2A (PP2A) among others (Leung-Pineda *et al.*, 2006). Bearing in mind that simvastatin has recently been shown to increase PP2A levels *in vivo* (Zhu *et al.*, 2012), PP2A expression following lovastatin treatment has been investigated. However, PP2A protein levels remained unchanged upon lovastatin, doxorubicin or IR exposure indicating that PP2A most likely does not contribute to the inhibitory effect of lovastatin on Chk1 activation in HaCaT cells.

In the light of an impaired Chk1 phosphorylation, it is feasible that cytoprotective effects of the statin are based on an interference with cell cycle progression of keratinocytes. Indeed, statin pretreatment temporarily reduced DNA synthesis and this was accompanied by an increase in the G1 fraction. 24 h later, however, the percentage of S-phase cells reached control level again. Exposure of HaCaT cells to doxorubicin or IR largely reduced DNA synthesis 24 h post treatment and induced a concomitant G2/M arrest. This was reflected by an increased expression of the G2/M-phase cyclins A and B1. At this time point lovastatin attenuated a reduction of S-phase cells and mitigated G2/M arrest, thereby ensuring regular cell cycle progression. According to these findings, it could be argued that the cytoprotective effects of lovastatin result from a reduction of replicative activity during genotoxin treatment. This might impact the negative consequences of doxorubicin or IR treatment by attenuating initial DNA damage formation following doxorubicin treatment or by facilitating pre-replicative repair following IR. However, as concluded from the fact that lovastatin did

not confer resistance to the S-phase dependent anticancer drug cisplatin, and, even more importantly, also protected non-proliferating HaCaT cells from destruction by doxorubicin and IR, cell cycle-independent effects most likely contribute also to the lovastatin-mediated cytoprotection. This hypothesis is supported by findings using non-proliferating lung endothelial cells which are also protected against IR-induced apoptosis by lovastatin (see section 3.1.2). Moreover, statins also protect heart tissue, which has a very low proliferation rate under physiological conditions, from doxorubicin-induced damage (Huelsenbeck *et al.*, 2011; Riad *et al.*, 2009; Yoshida *et al.*, 2009).

3.1.2 Statins protect primary human lung endothelial cells and mouse lung tissue from destruction induced by fractionated irradiation

In the clinic lung cancer patients are treated with fractionated irradiation schemes as already described above (see section 1.1.1). To investigate the effects of lovastatin on irradiated lung cells fractionated irradiation has been applied to different primary human lung cells *in vitro*. Following 4 x 4 Gy irradiation, primary microvascular endothelial cells of the lung (HMVEC-L) underwent apoptosis. This concurs with data pointing to the induction of apoptosis in other endothelial cell types. For instance, primary human umbilical vein endothelial cells (HUVEC) have been shown to undergo apoptosis upon single dose irradiation with 10 Gy (Nübel *et al.*, 2006) or 25 Gy (Ran *et al.*, 2010). The frequency of apoptotic cells ranged between 10 and 15 % (Nübel *et al.*, 2006; Ran *et al.*, 2010). In these studies cellular radiosensitivity of endothelial cells has usually been investigated using proliferating cells that were exposed to single dose irradiation. However, endothelial cells have a very slow turnover *in vivo* (Hobson and Denekamp, 1984) and, additionally, cell cycle distribution and progression determine the outcome of radiation treatment markedly (Dai *et al.*, 2015). Thus, in this thesis non-proliferating HMVEC-L, which reflect better the *in vivo* situation, were subjected to fractionated irradiation. Lacking proliferation might be the reason for a relatively low (~10 %), but significant induction of apoptosis upon fractionated irradiation with 4 x 4 Gy.

In this thesis low dose lovastatin treatment (1-5 μM) of HMVEC-L reduced the number of IR-induced apoptotic cells. Employing HUVECs, protection from IR-induced apoptotic cell death has been observed for both lova- and atorvastatin (Nübel *et al.*, 2006; Ran *et al.*, 2010). Moreover, pravastatin has been demonstrated to prevent an IR-mediated increase of the protein production of interleukins (IL-6, IL-8), monocyte chemotactic protein-1 (MCP-1) and

intercellular adhesion molecule (ICAM) in confluent HMVEC-L (Gaugler *et al.*, 2005). Since addition of mevalonate can reverse these effects, the data indicate that anti-inflammatory effects of statins are due to the inhibition of isoprenoid synthesis (Gaugler *et al.*, 2005). Accordingly, this points to a prevention of IR-mediated lung endothelial dysfunction by statins, which may hamper the onset and progression of radiation injury (Gaugler *et al.*, 2005). This is very important considering the fact that endothelial cells have been shown to be a major contributor to radiation-induced normal tissue damage including injury to the lung (Corre *et al.*, 2013).

Furthermore, lung epithelial (HSAEpC) and lung fibroblast (HPF) cells have been analysed following exposure to fractionated IR. In contrast to endothelial cells, non-proliferating HSAEpCs and HPFs did not undergo apoptosis upon irradiation. This concurs with other reports showing that lung epithelial and fibroblast cells become senescent upon radiation exposure (Citrin *et al.*, 2013; Wang *et al.*, 2011).

Fractionated irradiation of the right mouse lung (4 x 4 Gy) led to an increase in apoptotic cells (~5-fold) four weeks after the last irradiation. This tallies with an earlier report showing an induction of apoptosis in lung tissue six weeks post whole thorax irradiation (WTI) with 15 Gy, which occurred in both endo- and epithelial cells (Zhang *et al.*, 2012). Moreover, irradiation elevated the number of cells that stain positive for pH3, a G2/M-phase marker, representing cell proliferation (mitotic index). Under physiological conditions the basal proliferation rate of lung tissue was only low (~0.1 %), which is in agreement with data from literature (Parent, 2015). However, upon irradiation the mitotic index was increased 8-fold. In light of the increased apoptosis rate this is presumably due to adaptive type II pneumocyte proliferation that aims to replace the damaged tissue. In the present study co-treatment with lovastatin reduced the number of apoptotic cells four weeks after the last irradiation and, moreover, prevented an IR-mediated increase in the number of pH3-positive cells. This points to radioprotective effects of lovastatin and accords with earlier reports providing evidence that atorvastatin prevents apoptosis of intestinal cells early after single dose irradiation with 7.5 Gy (Mahmoudi *et al.*, 2008). Moreover, simvastatin prevented apoptotic cell death in bone marrow and intestine at seven days post 4 Gy or 8 Gy irradiation (Zhao *et al.*, 2014).

In the experimental setting of this thesis, fractionated lung irradiation (4 x 4 Gy) did not lead to an acute inflammation or an induction of fibrosis when histopathologically analysed four

weeks post IR. The analysis of lung function revealed only a slight increase in breathing frequency at this time point. Interestingly, two weeks earlier the breathing frequency was elevated by ~20 % which was prevented by lovastatin treatment. This transient increase in breathing frequency might be due to a temporary inflammation two weeks after the last irradiation. This assumption is supported by an IR-mediated increase in CD68-positive alveolar macrophages four weeks post IR, which might result from an earlier inflammation. Moreover, a transient early inflammation (~two weeks post IR) has been reported for several mouse strains which was accompanied by histopathological changes and cytokine induction (Jackson *et al.*, 2011). However, the contribution of these early, acute changes to long-term, chronic radiation injury remains unknown and requires further investigation.

3.2 Statins interfere with DNA damage formation and repair

3.2.1 Lovastatin prevents doxorubicin-induced DNA damage formation

Lovastatin pretreatment prevented initial DNA damage formation following doxorubicin treatment in HaCaTs. This effect has been demonstrated by directly monitoring DNA damage using the comet assay as well as on the level of nuclear γH2AX/53BP1 foci formation, which is a widely used surrogate marker for DNA DSBs. Depending on the assay lovastatin pretreated cells exhibited a ≥ 50 % reduction of DNA damage when compared to non-statin treated cells. The genoprotective effect was maintained for up to 6 h and 24 h after the end of treatment and was independent of doxorubicin import or export. Similarly, statins are known to lower DNA damage in HUVECs following exposure to doxorubicin (Damrot *et al.*, 2006) but do not influence irradiation-induced DNA damage in this cell type (Nübel *et al.*, 2006).

The genotoxic effects of doxorubicin are multifactorial and related to topoisomerase II inhibition and ROS formation amongst others (see section 1.1.2). It appears a reasonable assumption that lovastatin has anti-oxidative properties thereby reducing doxorubicin-induced DNA damage. Indeed, different statins have been shown to fortify the cellular ROS defense via nuclear factor erythroid 2-related factor 2 (Nrf2) activation (Chartoumpekis *et al.*, 2010) or upregulation of haem oxygenase-1 (HO-1) (Grosser *et al.*, 2004) and catalase (Wassmann *et al.*, 2002). This is in agreement with previous studies showing an induction of oxidative stress and DNA damage response by doxorubicin in cardiac myocytes which is attenuated by pitavastatin (Yoshida *et al.*, 2009). Moreover, it has been shown that a protective effect of atorvastatin on doxorubicin toxicity in the heart is accompanied by reduced oxidative stress and decreased DNA damage (Ramanjaneyulu *et al.*, 2013). However, there are several

arguments against substantial antioxidative properties of lovastatin in the prevention of doxorubicin-induced DNA damage. In HaCaT cells lovastatin does not prevent IR-induced DNA damage although 70 % of this damage is mediated by ROS. Moreover, it has been demonstrated that lovastatin-mediated protection from doxorubicin-induced DNA damage in HUVECs and rat cardiomyocytes (H9c2) is not accompanied by reduced ROS levels (Damrot *et al.*, 2006). Additionally, classical ROS scavengers do not protect from doxorubicin-induced toxicity (Ghigo *et al.*, 2016). Furthermore, noticeable levels of ROS have only been detected using supraclinical levels of doxorubicin (Gewirtz, 1999). In light of these findings it is likely that genoprotection by lovastatin might be based on an interference with topoisomerase II (topo II). This is supported by the fact that lovastatin also confers genoprotection to etoposide (Damrot *et al.*, 2006), a specific topo II poison which induces DNA breakage predominantly by inhibiting the ability of topo II to religate (Burden *et al.*, 1996; Montecucco and Biamonti, 2007). Moreover, topo II interacts with nuclear Rac1 as shown by co-immunoprecipitation (Sandrock *et al.*, 2010). Since lovastatin prevents isoprenylation of Rac1 and therefore inhibits its activity, it is very likely that Rac1 inhibition is at least in part responsible for statin-mediated DNA damage prevention and cytoprotection following exposure to doxorubicin. Indeed, the Rac1-specific inhibitor EHT1864 prevents doxorubicin-mediated DNA damage and cytotoxicity in cells of different origin (Huelsenbeck *et al.*, 2012; Wartlick *et al.*, 2013), which points to a contribution of Rac1 in lovastatin-mediated protective effects following doxorubicin treatment.

3.2.2 Lovastatin improves DNA repair following doxorubicin treatment and irradiation

In the *in vitro* mucositis model using HaCaT cells lovastatin attenuated the number of residual IR- and doxorubicin-induced γH2AX foci 24 h post treatment. Comparing foci numbers in lovastatin-treated cells with that of non-treated cells at different time points post treatment allows an estimate of DNA repair. Following low dose doxorubicin treatment, the percentage of foci in lovastatin-treated cells in comparison to non-treated cells was 43 % immediately after treatment. 24 h later, the statin-treated cells exhibited only 23 % of foci in comparison to untreated cells indicating an accelerated or improved DNA repair. Regarding low dose irradiation, lovastatin had no influence on initial DNA damage formation in HaCaT cells, but lowered residual DNA damage 24 h after irradiation. To substantiate the hypothesis and the relevance of lovastatin's interference with DNA repair, residual DNA damage was additionally analysed following high, apoptosis-inducing doses. Likewise, lovastatin also diminished γH2AX fluorescence intensity 24 h post treatment confirming statin-mediated

stimulation of DNA DSB repair in HaCaTs. Similarly, lovastatin also led to a slight acceleration of early DNA repair in human hepatoma cells (HepG2) following irradiation with 2.5 Gy (Ostrau *et al.*, 2009). In contrast, lovastatin did not influence IR-induced DNA repair in cervix carcinoma cells (Fritz *et al.*, 2003) as well as primary endothelial cells (Nübel *et al.*, 2006). However, in the latter study lovastatin pretreatment was performed with a lower dose followed by single dose irradiation with 20 Gy. It is therefore tempting to speculate that the dose range at which lovastatin has favourable effects on DNA repair is critical. Moreover, a certain cell-type specificity cannot be excluded. In fact, this cell-type specificity is partly verified when taking into consideration the analysis of γH2AX foci formation and resolution in different primary human lung cells following fractionated irradiation (4 x 4 Gy). Although low-dose treatment of primary human lung cells with lovastatin did not influence initial DNA damage formation, it did lower the number of residual γH2AX foci in both lung epithelial and endothelial cells as well as in lung fibroblasts, though to a different degree. The effect was most pronounced in fibroblasts and epithelial cells. A reduction of residual γH2AX foci is of particular relevance in view of recent findings showing an association between persistent DNA damage and senescence (Zhang *et al.*, 2016) and that IR-induced senescence correlates with lung injury (Citrin *et al.*, 2013).

Additionally, lovastatin treatment of BALB/c mice decreased the level of residual DNA damage four weeks after fractionated irradiation with 4 x 4 Gy which might also be a consequence of an improved DNA damage repair. This concurs with an earlier report showing an accelerated DNA damage repair of 7.5 Gy-induced DSBs by atorvastatin in intestinal tissue at very early time points (2-4 h) post irradiation (Mahmoudi *et al.*, 2008).

Regarding the influence of lovastatin on DNA repair, it is conceivable that this is a result of an altered DDR following irradiation. Consequently, the effect of lovastatin on IR-induced activation of key players of the DDR was analysed *in vitro* and *in vivo*. In primary human lung cells lovastatin had no major influence on IR-mediated DDR activation. In mouse lung tissue ATM, Chk2, p53 and Kap1 were still activated four weeks after the last irradiation as evidenced by an enhanced phosphorylation state. Lovastatin, however, did not have any modulating effect on the phosphorylation of these proteins. It can therefore be assumed that an improved DNA damage repair by lovastatin is presumably independent of interference with the DDR.

The mechanism of statin-mediated interference with DNA repair remains rather speculative since only a small number of studies report a modulation of DNA repair by statins. Mahmoudi and colleagues showed that low dose incubation of human atherosclerotic plaque vascular smooth muscle cells (VSMC) with atorvastatin activates DNA repair following treatment with the oxidative agent tert-butyl hydroperoxide (tBHP) (Mahmoudi *et al.*, 2008). This was not accompanied by a reduction of oxidative stress or initial DNA damage. In fact, statin treatment led to an accelerated phosphorylation of ATM and an increased nuclear appearance of Nbs following tBHP treatment which was mediated by an inhibition of the human Mdm-2 homolog Hdm-2 by the statin (Mahmoudi *et al.*, 2008). Additionally, treatment of quiescent Huntington's disease skin fibroblasts with a combination of pravastatin and the bisphosphonate zoledronate improved DSB recognition following IR by facilitating the nucleo-shuttling of ATM (Ferlazzo *et al.*, 2014). This entails a better DNA repair, presumably through NHEJ, which is the dominant DSB repair pathway in non-proliferating cells. However, it is not clear whether pravastatin or zoledronate would also be capable of improving IR-induced DSB repair on their own. Surprisingly, in human mesenchymal stem cells (hMSCs), zoledronate alone was able to enhance DNA repair 4 h to 48 h following irradiation without a significant difference in DNA damage levels between zoledronate and non-treated cells directly after irradiation (Misra *et al.*, 2016). These results are interesting from a mechanistic point of view because zoledronate has been shown to interfere with the mevalonate pathway by inhibiting farnesyl pyrophosphate (FPP) synthase and the effects can be reversed by supplementation with farnesol and geranylgeraniol. Interestingly, two repair-related factors, namely ligase 4 and X-ray repair cross-complementing 3 (XRCC3), who play roles in NHEJ and HR respectively, have been upregulated in a qPCR screening array following a three-week treatment of mice with lovastatin (Fritz *et al.*, 2011). Together with the results presented in this thesis, it appears feasible that inhibition of the mevalonate pathway and a subsequent inhibition of Rho-GTPases might contribute to an improved DNA repair by statins. In light of the fact that a small number of DNA DSB is sufficient for cell death and that the number of unrepaired DSBs correlates with clonogenic cell survival (Joubert *et al.*, 2008) even small favourable effects on DNA repair might significantly modulate the cellular sensitivity to genotoxins.

3.3 Rac1 inhibition contributes to the protective effects of statins on normal tissue toxicity following irradiation

The *in vivo* data of this thesis point to a contribution of Rac1-GTPase in pulmonary radiation injury. Treatment of mice with the direct small molecule inhibitor EHT1864 reduced IR-induced breathing frequency, apoptosis, tissue remodelling and residual DNA damage in the same range as the protection provided by lovastatin treatment. This suggests that inhibition of Rac1 – either directly by EHT1864 or via a statin-mediated prevention of Rac1 isoprenylation – protects from IR-induced lung injury. Interestingly, Rac1 activity is increased in various cell types upon exposure to IR (Espinha *et al.*, 2015; Hein *et al.*, 2016). In the literature, a direct link between radiation-induced lung injury and Rac1 inhibition has not yet been described. However, a few studies revealed that inhibition of Rac1 has beneficial effects on lung injury following exposure to noxae other than IR. Rac1 inhibition has been shown to attenuate pancreatitis-associated lung injury (Binker *et al.*, 2008) and, moreover, reduces lung inflammation following abdominal sepsis or LPS instillation by inhibition of neutrophil recruitment and reduction of vascular permeability (Hwaiz *et al.*, 2013; Yao *et al.*, 2011). Not only pneumonitis but also fibrotic processes in the lung as stimulated by agents other than IR (e.g. bleomycin) are alleviated by Rac1 inhibition (Liang *et al.*, 2016; Lin *et al.*, 2014; Osborn-Heaford *et al.*, 2015; Shen *et al.*, 2014). In light of the aforementioned data, it is easily conceivable that IR-induced lung injury is alleviated by Rac1 inhibition and is supported by data presented in this thesis. This is supported by the finding that lovastatin treatment of human endothelial cells led to a large reduction of GTP-bound active Rac1.

The signalling pathways by which Rac1 inhibition via EHT1864 contributes to protective effects is still unclear. EHT1864 has been shown to bind strongly to Rac1 arresting the latter in an inactive state and preventing its interaction with downstream effectors (Onesto *et al.*, 2008). It is therefore tempting to speculate that signalling downstream of Rac1 contributes to the observed effects. p21 activated kinase (PAK) is a prominent downstream effector of Rac1 (Manser *et al.*, 1994). Binding of PAK to GTP-bound Rac1 is known to activate downstream mitogen-activated protein kinases (MAPK) such as c-jun amino-terminal kinase (JNK) (Brown *et al.*, 1996; Coso *et al.*, 1995) and p38 kinase (Zhang *et al.*, 1995). MAPKs regulate various cellular processes such as proliferation, differentiation, apoptosis and inflammation (Krishna and Narang, 2008). MAPK activation has been shown to induce apoptosis following IR exposure (Chen *et al.*, 1996; Verheij *et al.*, 1998) and to contribute to the pathogenesis of

radiation-induced lung injury (Ryu *et al.*, 2011). This suggests that Rac1 inhibition might interfere with IR-induced MAPK activation thereby preventing cell death in the lung.

Moreover, Rac1 is part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) complex and is involved in the regulation of its catalytic activity (Diekmann *et al.*, 1994). NADPH oxidase has been shown to produce ROS, e.g. superoxide anions, in many cells including lung endothelial cells, epithelial cells and fibroblasts (Carnesecchi *et al.*, 2012). Additionally, irradiation leads to an upregulation or persistent activation of different NOX isoforms (Ameziane-El-Hassani *et al.*, 2015; Wang *et al.*, 2010; Zhang *et al.*, 2013). Consequently, inhibition of Rac1 might result in a reduction of NOX-mediated ROS generation and, therefore, might contribute to a prevention of ROS-mediated cellular damage and cell death. Indeed, most of the protective effects of Rac1 inhibition in the cardiovascular system or the kidneys have been related to a reduction of ROS generation via NOX inhibition (Shan *et al.*, 2010; Shen *et al.*, 2009; Thamilselvan *et al.*, 2012; Vecchione *et al.*, 2006). More importantly, it has been recently demonstrated that NOX1 knockdown in human pulmonary artery endothelial cells prevents fibrotic changes following irradiation (Choi *et al.*, 2016). Accordingly, NOX1 inhibition *in vivo* protects mice from radiation-induced fibrotic changes in lung endothelial cells following exposure to a single dose of 25 Gy (Choi *et al.*, 2016). Additionally, NOX inhibition prevents IR-induced senescence of type II alveolar epithelial cells and radiation fibrosis following fractionated irradiation (Citrin *et al.*, 2013). Based on these data a contribution of a reduced Rac1/NOX signalling to the statin-mediated protective effects following lung irradiation is quite possible. However, four weeks after fractionated irradiation no clear signs of oxidative stress have been observed, but the contribution of Rac1 inhibition to early protective effects following irradiation will be elucidated in forthcoming studies.

Data concerning Rac1 and its effects on DDR or DNA repair are mainly lacking. However, the fact that Rac1-GTPase has been detected inside the nucleus (Sandrock *et al.*, 2010) suggests a role of Rac1 in nuclear processes such as DNA repair. In fact, the above described reduction of residual DNA damage would support this hypothesis. Interestingly, 8-oxo-7,8-dihydroguanine (8-oxoG), an oxidative DNA lesion that is caused by IR-mediated ROS amongst others, has been demonstrated to act as a GEF for nuclear Rac1 (Hajas *et al.*, 2013). Thus, it is feasible that IR induces 8-oxoG which in turn activates nuclear Rac1 contributing to cellular damage. Inhibition of Rac1 (e.g. by EHT1864) might counteract this.

3.4 Statins do not have cyto- and genoprotective effects on tumour cells

Radioprotective effects of statins render them promising as therapeutic options in normal tissue protection during antitumour therapy. However, statins would not be clinically usable in terms of normal tissue protection if they also showed protective effects on tumour cells. For this reason, the effect of statin treatment on two tumour cell lines which are relevant for thoracic irradiation (see Figure S1 and S2 of the appendix) were investigated. Lung adenocarcinoma cells (A549) and breast adenocarcinoma cells (MCF-7) were subjected to fractionated irradiation (4 x 4 Gy) in accordance with the treatment scheme that was used for the analysis of radiation effects in normal lung cells.

In low, clinically-relevant concentrations (e.g. 1 μM) lovastatin alone had no major effect on A549 and MCF-7 cell death. Fractionated irradiation (4 x 4 Gy) did not induce cell death in A549 cells whereas MCF-7 cells underwent apoptosis upon irradiation. Concomitant lovastatin treatment did not prevent MCF-7 cells from undergoing apoptosis (see Figure S1 of the appendix). DNA damage formation and repair had also been analysed in A549 and MCF-7 cells upon irradiation showing neither modulating effects of lovastatin on initial numbers of γH2AX foci nor on residual foci 24 h after the last irradiation (see Figure S2 of the appendix). This indicates that, in contrast to primary lung cells, lovastatin has no influence on DNA damage formation and DSB repair of tumour cells.

Following single dose irradiation Sanli and colleagues reported a sensitizing effect of lovastatin on lung cancer cells (Sanli *et al.*, 2011). Using proliferation and clonogenic assays, higher concentrations of lovastatin (≥ 10 μM) have been suggested to sensitize A549 cells to IR (Sanli *et al.*, 2011). However, at these concentrations lovastatin alone already exhibited a large reduction of clonogenic survival of A549 cells. In line with the experiments in this thesis (see Figure S1 of the appendix), irradiation of A549 cells with 8 Gy does not lead to apoptosis as analysed on the level of subG1 induction (Sanli *et al.*, 2011). Moreover, irradiation accumulates cells in G2/M cell cycle phase which is diminished by lovastatin. The authors state that the abrogation of G2/M arrest prevents DNA repair and contributes to a statin-mediated radiosensitisation (Sanli *et al.*, 2011). This is consistent with earlier studies showing an abrogation of G2/M arrest by lovastatin in HeLa cells which concurs with lovastatin-induced apoptosis (Fritz *et al.*, 2003). In addition, statins have been shown to radiosensitize other tumour cells including prostate cancer cells (He *et al.*, 2012), breast cancer cells (Lacerda *et al.*, 2014) and glioma cells (Tsuboi *et al.*, 2009). A proposed

mechanism is based on the induction of autophagic cell death by IR which is amplified by statins (He *et al.*, 2012; Tsuboi *et al.*, 2009). Moreover, statins have been shown to induce apoptosis on their own by a reduction of anti-apoptotic Bcl-2 protein (Wood *et al.*, 2013).

Regarding doxorubicin, different *in vitro* and *in vivo* reports show sensitizing effects of statins in combination with the anthracycline. Statins sensitize various cancer cell lines including osteosarcoma cells, rhabdomyosarcoma cells and ovarian cancer cells to doxorubicin *in vitro* (Fromigué *et al.*, 2008; Martirosyan *et al.*, 2010; Werner *et al.*, 2004). Furthermore, lovastatin potentiates the cytotoxic effects of doxorubicin on melanoma cell lines by induction of apoptosis and, in line with this, a combination of doxorubicin and lovastatin leads to a retardation of melanoma tumour growth *in vivo* (Feleszko *et al.*, 2002). Lovastatin also potentiates the antitumour efficacy of doxorubicin in lymphomas and mammary adenocarcinomas which is paralleled by an increased apoptosis rate (Rozados *et al.*, 2008). In a xenograft mouse model, lovastatin has been shown to augment doxorubicin's antitumour effects on human fibrosarcoma cells (Huelsenbeck *et al.*, 2011).

In vivo studies in this work show that Rac1 inhibition provides a beneficial effect on normal lung tissue following irradiation, suggesting Rac1 targeting as a protective strategy for RILI. Regarding the influence of Rac1 inhibition on the radiosensitivity of lung carcinoma cells, no data exist so far. However, Rac1 inhibition has been shown to sensitize other tumour cells such as breast carcinoma cells (Hein *et al.*, 2016) as well as head and neck squamous cell carcinoma (HNSCC) cells (Skvortsov *et al.*, 2014) to ionising radiation. Moreover, inhibition of Rac1 sensitises pancreatic cancer cells to radiation treatment by abrogation of G2/M checkpoint activation and induction of apoptosis without exhibiting any radiosensitising effect on normal pancreas cells (Yan *et al.*, 2014).

In summary, the data presented in this thesis together with findings from previous studies point to sensitising effects of statins and Rac1 inhibitors to radiation and doxorubicin treatment of tumour cells while protecting normal tissue.

3.5 Statins for normal cell and tissue protection

3.5.1 Statins as anti-mucositis agents

In this thesis human keratinocytes (HaCaT) have been used as an *in vitro* model to investigate the effects of lovastatin in doxorubicin- and IR-induced oral mucositis. Certainly, HaCaT

cells have a number of limitations to serve as a model for such a complex series of events as it occurs during oral mucositis. Using keratinocytes one does not take into account the presence of other cells (i.e. fibroblasts, endothelial cells) and oral microorganisms which modulate the onset and progression of oral mucositis (De Ryck *et al.*, 2014; Sonis *et al.*, 2000). Although HaCaT cells are non-tumourigenic, primary-like keratinocytes (Boukamp *et al.*, 1988), they are spontaneously immortalised and exhibit two p53 point mutations (Henseleit *et al.*, 1997), which might modulate response and cell death induction following genotoxin treatment. However, HaCaT cells retain many of the functions of normal keratinocytes (Boukamp *et al.*, 1988). Moreover, HaCaT cells are frequently used for the identification of anti-mucositis drugs (Baek *et al.*, 2014; Chang *et al.*, 2014; Chen *et al.*, 2011; Shin *et al.*, 2013) and provide a simple and easily accessible model for oral mucositis research. Indeed, for several substances an *in vitro* (using HaCaT cells)/*in vivo* correlation regarding anti-mucositis properties has been demonstrated (Chang *et al.*, 2014; Shin *et al.*, 2013). Human keratinocyte growth factor-1 (palifermin) (see section 1.1.3.1) is the only approved drug for the treatment of mucositis (Lalla *et al.*, 2014) which emphasizes the importance of keratinocytes in the pathogenesis of mucositis and the meaningfulness of studying the effects of putative anti-mucositis therapeutics in this cell type. Palifermin, as with many other potential anti-mucositis agents currently under investigation, e.g. pentoxifylline (Gruber *et al.*, 2015), improves the healing process by accelerating the re-epithelisation of the damaged mucosa. In contrast, statin treatment which protects keratinocytes from the deleterious effects of anticancer treatment by antagonising DNA damage and counteracting pro-apoptotic responses, represents a preventive rather than a curative approach. However, its *in vivo* efficacy has still to be verified since only a very limited number of studies has been performed so far. In accordance with the findings in this thesis atorvastatin, for example, alleviates inflammation and damage of the oral mucosa of hamsters following administration of 5-fluorouracil (5-FU) (Medeiros *et al.*, 2011), a typical mucositis agent (Mahood *et al.*, 1991). Apart from epithelial cells, endothelial cells have been shown to play a pivotal role in oral mucositis (Sonis *et al.*, 2000). The findings of this thesis together with earlier reports showing protective effects of statins also on primary endothelial cells (HUVEC) following doxorubicin (Damrot *et al.*, 2006) or IR (Nübel *et al.*, 2006) treatment provide a holistic approach conferred by multiple beneficial effects of statins.

3.5.2 Statins as radioprotectors of the lung

In this thesis protective effects of statins on irradiation-induced DNA damage and apoptosis in lung cells and tissue have been demonstrated, which are at least partially based on inhibition of the small GTPase Rac1. The experimental setting involved a fractionated irradiation which mimics the clinical treatment scheme of lung cancer radiotherapy where fractionated radiation doses are applied. However, in contrast to the clinical setting, where ~30 doses of ~2 Gy (BED 100 Gy) are applied during a period of six weeks, a (hypo)-fractionated irradiation scheme 4 x 4 Gy (BED 37.3 Gy) over a period of two weeks has been used. Very probably, this leads to different effects, particularly when considering different BEDs. The reason for the modified approach used in our preclinical setting is the impossibility of irradiating animals on 30 consecutive days since anaesthesia is quite stressful or even lethal for mice and requires certain recovery phases. To be able to compare the results obtained with data from *in vitro* experiments, 4 x 4 Gy irradiation has also been employed on different primary human lung cells as well as on breast and lung tumour cells. This has several limitations, but is considerably closer to the clinical situation than commonly-used single high-dose irradiation protocols (Chiang *et al.*, 2005; Christofidou-Solomidou *et al.*, 2011; Eldh *et al.*, 2012; Heinzelmann *et al.*, 2006; Jackson *et al.*, 2011; Mathew *et al.*, 2011; Yang *et al.*, 2011) for investigating IR-induced lung injury in mice. Moreover, in other preclinical studies, whole thorax irradiation is frequently applied which includes the irradiation of other organs. Undesired radiation effects e.g. on the heart, might interfere with specific effects of radiation on the lung. In the experimental setting of this thesis these concerns have been overcome by developing an irradiation device with which a selective irradiation of the right lung is possible. Therefore, it can be mostly excluded that the effects on breathing frequency result from a reduced cardiac function due to irradiation of the heart.

Treatment of primary human lung cells and mice with lovastatin led to beneficial effects on DNA repair and cell death following exposure to ionising radiation. This shows an *in vitro* to *in vivo* transferability and provides the basis for further preclinical and subsequent epidemiological and intervention studies. On the basis of these data, it can be hypothesised that prevention of early radiation-induced lung injury by statins might provide beneficial effects on long-term radiation pneumonitis and lung fibrosis. Regarding lung irradiation there are only very few data available related to the influence of statins on RILI. Simvastatin has been shown to reduce inflammatory cytokine expression, inflammatory cell infiltrates and vascular leakage in mouse lung six weeks post 25 Gy whole thorax irradiation (WTI)

(Mathew *et al.*, 2011). This alleviated IR-induced oedema formation and lung pneumonitis. Monceau and colleagues showed that pravastatin prevents lung fibrosis following WTI with 19 Gy (Monceau *et al.*, 2010). However, both studies were performed with clinically less relevant single high-dose irradiation and the whole thorax has been exposed to IR. DNA damage as well as early apoptosis have not been investigated in these studies. In some cases, studies that examined potential lung radioprotectors have been performed with the radiomimetic drug bleomycin. In a bleomycin fibrosis model, atorva- and pravastatin mitigated lung fibrosis by inhibiting connective tissue growth factor (CTGF) and the extracellular-signal regulated kinase (ERK) pathway (Kim *et al.*, 2010; Zhu *et al.*, 2013).

The molecular mechanism by which statins might alleviate RILI is still unknown. The data in this thesis point to an involvement of Rac1 inhibition in the statin-mediated protective effects. In light of recent findings that suggest, for example, that NOX and MAPK kinase signalling contribute to RILI (see section 3.3), an inhibition of these signalling pathways by Rac1 inhibition is quite feasible. In previous studies a protection from IR-induced normal tissue damage, especially fibrosis, by statins has been mainly associated with a statin-mediated inhibition of the Rho/ROCK pathway (Haydont *et al.*, 2007c; Monceau *et al.*, 2010). Thus, it appears possible that an inhibition of Rac1 signalling by statins is involved in early protective effects of IR-induced lung injury whereas a statin-mediated inhibition of the Rho/ROCK pathway reduces late fibrotic events following irradiation (see Figure 5).

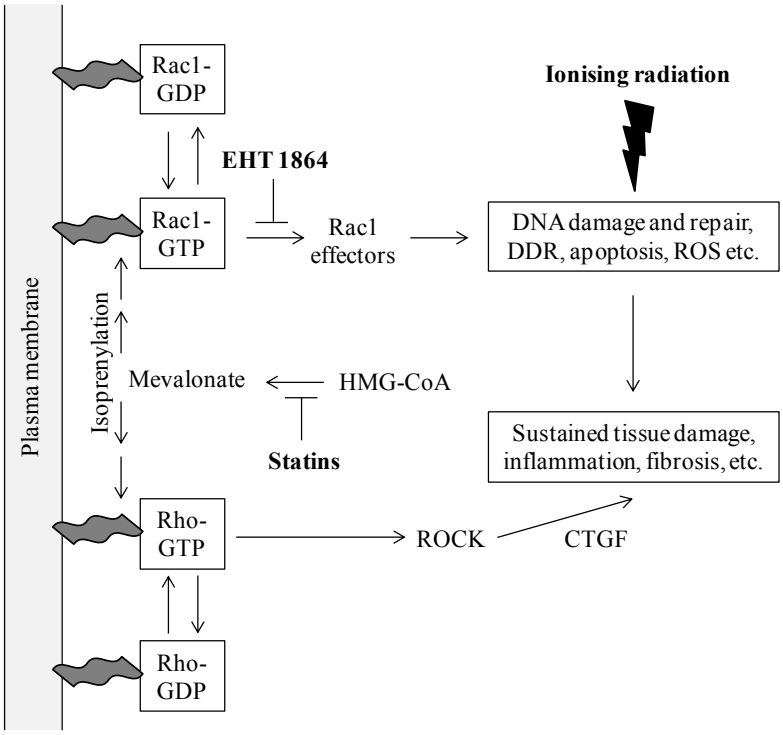

Figure 5: Proposed model of the protective effects of statins and EHT1864 on radiation response following fractionated irradiation of the lung. Pharmacological targeting of Rac1-GTPase by statins and EHT1864 lowers early adverse radiation responses following fractionated irradiation of the lung. A statin-mediated attenuation of late injury is associated with an inhibition of Rho/ROCK signalling by statins. CTGF, connective tissue growth factor; DDR, DNA damage response; GDP, guanosine-5'-diphosphate; GTP, guanosine-5'-triphosphate; Rac, Ras-related C3 botulinum toxin substrate 1; Rho, Ras-homologous; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species.

3.5.3 General conclusion

Although the identification of radioprotective substances in *in vitro* and preclinical studies is reported occasionally, they do not reach the stage of clinical trials in humans due to, amongst other things, high costs. Since statins have been in clinical use for many years it would be relatively easy and cheap to extend the indication of statins for normal tissue protection during radiotherapy. It would be preferable if statin doses for lipid-lowering purposes and normal tissue protection are in the same range. A comparison between the latter indicates that this is indeed the case. Oral daily doses in humans are in the range of 10-80 mg for most of the statins (Armitage, 2007) which corresponds to 1-8 mg/kg bw/week. This is slightly less compared to the experiments in this thesis where BALB/c mice were given three times weekly 10 mg/kg bw corresponding to 30 mg/kg bw/week.

Plasma levels of lovastatin treated patients are in the range of 0.05-0.3 µM (Bellosta *et al.*, 2004; Thibault *et al.*, 1996) which is slightly less than the concentration (1 µM) used for the *in vitro* experiments with primary human lung cells. To investigate the influence of lovastatin on human keratinocytes, a pretreatment scheme using 30 µM lovastatin has been applied which is in the range of concentrations that have been used for radio- and chemosensitising effects *in vitro* (Fromigué *et al.*, 2008; Sanli *et al.*, 2011). This is considerably higher than plasma peak levels observed in statin-treated patients. Although higher doses than 10 mg/kg/d are usually not required for a sufficient cholesterol reduction, even higher doses up to 25 mg/kg/d are considered safe since no side-effects are recognised even under long-term treatment (Thibault *et al.*, 1996). Another study showed that plasma peak levels of up to 15 µM do not exert any adverse effects in humans (Holstein *et al.*, 2006). Therefore, it seems feasible that a short-term, high dose treatment with lovastatin might be applied shortly before or during radiotherapy to protect normal tissue, including oral mucosa.

All the experiments in this thesis have been performed using lovastatin. Noteworthy is the fact that the protective effects of statins on normal tissue toxicities in different model systems have been shown for various statins including lovastatin, simvastatin, atorvastatin and pravastatin (Gaugler *et al.*, 2005; Mathew *et al.*, 2011; Medeiros *et al.*, 2011; Ostrau *et al.*, 2009). This indicates that the beneficial effects of statins are not restricted to a certain statin e.g. not via off-target effects but rather by an interference with HMG-CoA reductase as the primary target. This hypothesis is supported by experiments showing that a concurrent supplementation of mevalonate rescues the statin-provoked phenotype (Gaugler *et al.*, 2005; Oka *et al.*, 2013). Since statins are frequently prescribed drugs, epidemiological studies could easily scrutinize putative beneficial effects of statins following radio- and chemotherapy in humans. Indeed, there are first hints that statins might counteract normal tissue damage in humans following radiotherapy. It has been demonstrated that statin intake lowers gastrointestinal toxicity upon radiotherapy in the pelvic region (Wedlake *et al.*, 2012). Moreover, in two recent clinical studies statin intake was associated with a smaller decline of the left ventricular ejection fraction and a lower incidence of heart failure following anthracycline-based chemotherapy in breast cancer patients (Chotenimitkhun *et al.*, 2015; Seicean *et al.*, 2012).

It is essential for a radio- and chemoprotective drug to shield normal cells and tissues from the devastating consequences of anticancer treatment, but not to provide protection of tumour

cells since this would reduce the efficacy of anticancer treatment. Indeed, nonspecific properties of potential radiation modulators are a major reason for a failure to implement them in the clinic (Moding *et al.*, 2013). Fortunately, most of the research that has been undertaken in investigating the influence of statins on tumour cells indicates that to a certain degree statins exhibit radiosensitising properties in tumour cells. This leads to a win-win situation since the therapeutic window would be largely widened by statins (see Figure 6).

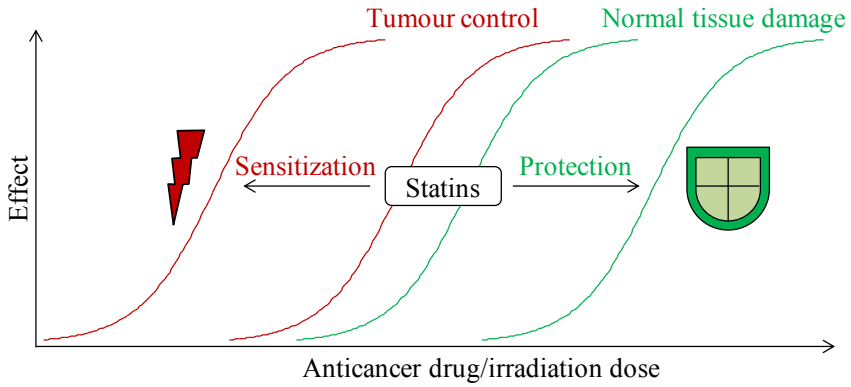

Figure 6: Model of statin-mediated enlargement of the therapeutic window of antitumour treatment. Tumour control and normal tissue damage in relation to the dose of antitumour treatment are outlined as idealised sigmoidal curves. Statins are suggested to widen the therapeutic window by a sensitisation of tumour cells (red) and a concurrent protection of normal tissue (green).

3.6 Perspective

In forthcoming studies the influence of lovastatin on IR- and doxorubicin-induced oral mucositis has to be confirmed *in vivo*. A suitable preclinical oral mucositis model has been described for instance by studies of Dörr and colleagues applying fractionated irradiation to the nose and the mouth or locally to the tongue (Albert *et al.*, 2012; Dörr *et al.*, 2005). Moreover, the molecular mechanisms by which lovastatin provides beneficial effects of keratinocytes have to be studied, especially the involvement of Rac1 signalling remains to be clarified for the oral mucositis model. Additionally, it would be interesting to know how statins exactly interfere with DNA damage repair and DDR (i.e. ATR-Chk1 signalling). For this reason, a siRNA- or CRISPR-based approach down-regulating/knocking out single DNA repair or DDR factors might help to understand the underlying molecular mechanisms.

Furthermore, the studies in this thesis provide evidence of a statin-mediated protection of early cellular and DNA damage in primary human lung cells and mouse lung tissue *in vivo*. The hypothesis that prevention of early damage might result in a prevention of long-term IR

effects needs to be verified. For instance, using the herein developed irradiation device, further studies are required to assess the effects of lovastatin on late/chronic radiation pneumonitis and fibrosis several months after exposure to fractionated irradiation. Organ specific and/or conditional Rac1 knockout mice are suitable *in vivo* models to verify the protective effects of Rac1 inhibition on lung damage following fractionated irradiation. Moreover, epidemiological studies that investigate the correlation between statin intake and lung or oral mucosa toxicity following radiotherapy in lung and breast or head and neck cancer patients would provide helpful information regarding the clinical relevance of the *in vitro* and preclinical *in vivo* data described in this thesis.

Summary

Oral mucositis (OM) and radiation-induced lung injury (RILI) are two dose-limiting side effects of chemo- and radiotherapy and often impair effective antitumour treatment. Preventive and curative measures for OM and RILI are largely missing.

In this study, the influence of the HMG-CoA reductase inhibitor lovastatin was investigated in an *in vitro* model of oral mucositis using human keratinocytes. Lovastatin pretreatment preserved cell viability and prevented apoptotic cell death following irradiation and doxorubicin treatment. The statin accelerated DNA repair upon treatment with both noxae and, in addition to that, prevented doxorubicin-induced initial DNA damage formation, which was independent of altered drug import or export. Moreover, lovastatin attenuated the doxorubicin- and irradiation-induced replication blockage by interference with cell cycle progression. Since lovastatin has been shown to protect both proliferating and non-proliferating keratinocytes and does not lead to a protection against S-phase dependent anticancer drugs such as cisplatin, the cyto- and genoprotective effect of lovastatin is likely not to be solely due to a cell cycle interference.

Furthermore, the effect of lovastatin was analysed on different confluent primary human lung cells following fractionated irradiation (4 x 4 Gy) *in vitro*. Irradiation led to apoptosis in lung endothelial cells which was prevented by the statin. Lovastatin evoked an accelerated repair of DNA DSB in lung endothelial cells, lung fibroblast cells as well as in small airway epithelial cells and this was independent of a statin-mediated interference with initial DNA damage formation. Moreover, lovastatin improved lung function and reduced residual DNA damage in mouse lung tissue following fractionated irradiation. Apoptosis and regenerative proliferation stimulated by irradiation were also attenuated by the statin. Treatment of mice with the Rac1 small molecule inhibitor EHT1864 mimicked the lovastatin-mediated protective effects suggesting that Rac1 contributes to the pathogenesis of RILI.

In summary, the data point to a radio- and chemoprotective effect of lovastatin on normal cells and tissues - which is at least partially mediated by Rac1 inhibition - without a concurrent protection of tumour cells. Since statins have been clinically-used for lipid lowering purposes for many years and since statin treatment is known to have only very few side-effects, an off-label use of statins for normal tissue protection in the context of anticancer

therapy is considered as a promising approach and encourages further *in vivo* and epidemiological studies.

Zusammenfassung

Orale Mukositis und strahleninduzierte Lungenschäden sind zwei dosislimitierende Nebenwirkungen von Chemo- und Radiotherapie und verhindern oftmals eine effektive Antitumortherapie. Präventive und kurative Ansätze für orale Mukositis und strahleninduzierte Lungenschäden fehlen hierbei größtenteils.

In dieser Arbeit wurde der Einfluss des HMG-CoA-Reduktasehemmers Lovastatin auf orale Mukositis in einem Keratinozyten-basierten *in vitro* Modell untersucht. Eine Vorbehandlung der Zellen mit Lovastatin führte zu einer erhöhten Zellviabilität und verhinderte Apoptose nach Bestrahlung oder Behandlung mit Doxorubicin. Das Statin beschleunigte die DNA-Reparatur nach Behandlung mit beiden Noxen und verhinderte außerdem die Doxorubicin-induzierte initiale DNA-Schadensbildung, die unabhängig von einem veränderten Import oder Export des Doxorubicins war. Des Weiteren verminderte Lovastatin die durch Doxorubicin und Strahlung induzierte Replikationsblockade durch eine Interferenz mit dem Zellzyklus. Da Lovastatin sowohl proliferierende als auch nicht-proliferierende Keratinozyten schützte und darüber hinaus nicht zu einer Protektion gegenüber dem S-Phase abhängigen Antikrebsmedikament Cisplatin führte, kann geschlussfolgert werden, dass die zyto- und genoprotektiven Effekte von Lovastatin wahrscheinlich nicht ausschließlich auf einer Interferenz mit dem Zellzyklus beruhen.

Weiterhin wurde der Effekt von Lovastatin auf verschiedene primäre humane Lungenzellen *in vitro* nach einer fraktionierten Bestrahlung (4 x 4 Gy) untersucht. Die Bestrahlung führte zu Apoptose in Lungenendothelzellen, die durch das Statin verhindert wurde. Die Behandlung mit Lovastatin resultierte in einer beschleunigten Reparatur von DNA-Doppelstrangbrüchen in Lungenendothelzellen, in Lungenfibroblasten sowie in Lungenepithelzellen, die unabhängig von einer Modulation der initialen DNA-Schadensbildung durch das Statin war. *In vivo* verbesserte Lovastatin die durch Strahlung beeinträchtigte Lungenfunktion von Mäusen und verringerte die Anzahl residueller DNA-Schäden. Apoptose und regenerative Proliferation wurden ebenfalls durch das Statin vermindert. Die Behandlung der Mäuse mit dem Rac1-spezifischen, niedermolekularen Inhibitor EHT1864 zeigte ähnliche zytoprotektive

Effekte, woraus geschlossen werden kann, dass Rac1 an der Pathogenese strahleninduzierter Lungenschäden beteiligt ist.

Zusammenfassend lässt sich sagen, dass die vorliegenden Daten auf radio- und chemoprotektive Effekte von Lovastatin gegenüber Normalzellen und -gewebe hindeuten. Diese Protektion beruht zumindest teilweise auf einer Rac1-Inhibition. Lovastatin hat dabei keine schützenden Effekte auf Tumorzellen. Da Statine aufgrund ihrer lipidsenkenden Eigenschaften seit vielen Jahren in klinischem Einsatz sind und nur geringe Nebenwirkungen aufweisen, ist der zulassungsüberschreitende Einsatz von Statinen für die Normalgewebsprotektion ein vielversprechender Ansatz und verlangt nach weiteren präklinischen sowie epidemiologischen Studien.

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Appendix

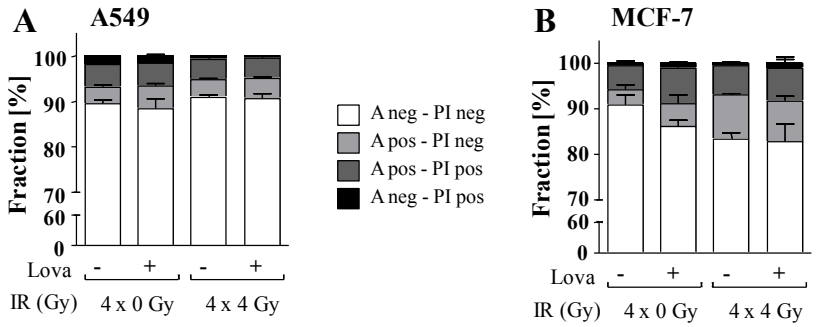

Figure S1: Effect of lovastatin on fractionated irradiation-induced apoptosis in A549 and MCF-7 cells. Human lung adenocarcinoma cells (A549, A) and human breast cancer cells (MCF-7, B) were seeded 24 h before pretreatment with 5 µM lovastatin (Lova). 24 h later the lovastatin concentration was reduced to 1 µM and cells were irradiated on four consecutive days with 4 Gy. Control cells were vehicle-treated and subjected to sham-irradiation. 24 h after the last irradiation apoptosis was measured by Annexin V/PI staining. Results show the mean ± SD from n = 3 independent experiments.

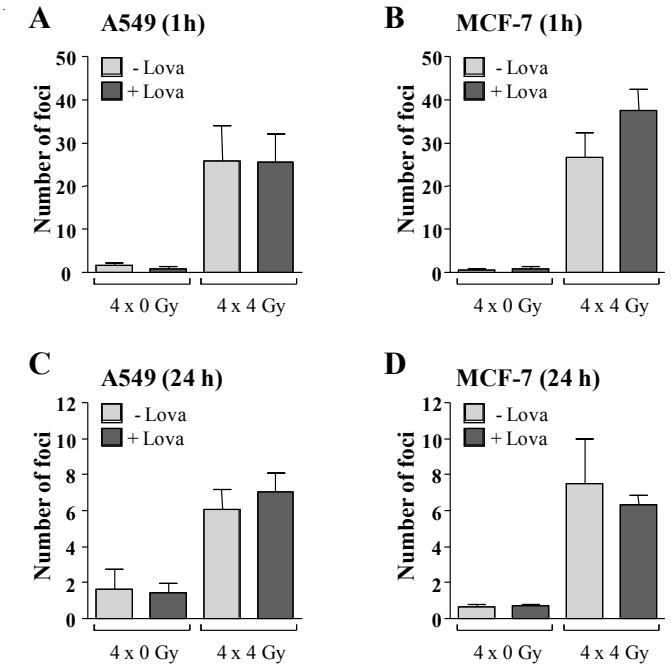

Figure S2: Effect of lovastatin on fractionated irradiation-induced DNA damage formation and repair in A549 and MCF-7 cells. Human lung adenocarcinoma cells (A549, A, C) and human breast cancer cells (MCF-7, B, D) were seeded 24 h before pretreatment with 5 µM lovastatin (Lova). 24 h later the lovastatin concentration was reduced to 1 µM and cells were irradiated on four consecutive days with 4 Gy. Control cells were vehicle-treated and subjected to sham-irradiation. 1 h (A, B) and 24 h (C, D) after the last irradiation γH2AX foci formation was analysed indicative for DNA double-strand breaks. Results show the mean ± SD from n = 3 independent experiments.

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Acknowledgements

Acknowledgements are omitted in this version of the thesis.

Publications and congress contributions

Publications

Krüger K., **Ziegler, V**., Hartmann, C., Henninger, C., Thomale, J., Schupp, N., Fritz, G. Lovastatin prevents cisplatin-induced activation of pro-apoptotic DNA damage response (DDR) of renal tubular epithelial cells. Toxicol. Appl. Pharmacol. 292 (2016), 103-14.

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Talks

Ziegler, V., Albers, A., Henninger, C., Fritz, G. Lovastatin protects human keratinocytes from ionising radiation- and doxorubicin-induced cell death in an in vitro model of oral mucositis. Young Scientists Workshop of the German Society for Environmental Mutation Research, Düsseldorf, July 2014.

Henninger, C., Wartlick, F., Bopp, A., Albers, A., **Ziegler, V.**, Fritz, G. Ras-homologous GTPases in DNA repair and DNA damage response. 13th biennial Conference of the German Society for Research on DNA Repair (DGDR), Mainz, September 2014.

Ziegler, V., Henninger, C., Fritz, G. *In vitro* and *in vivo* effects of statins on normal tissue damage induced by fractionated irradiation. Young Scientists Workshop of the German Society for Environmental Mutation Research, Potsdam, July 2015.

Ziegler, V., Henninger, C., Fritz, G. Protective effects of statins on lung toxicity after fractionated irradiation. 82nd Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology, Berlin, March 2016.

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Ziegler, V., Albers, A., Henninger, C., Fritz, G. DNA damage and related stress responses stimulated by ionizing radiation (IR): prevention of IR-induced normal tissue damage by statins. 19th Annual Meeting of the German Society for Radiobiology, Erlangen, September 2016.

Ziegler, V. Advanced training in toxicological research. Symposium of the Master's Programme in Toxicology, Düsseldorf, October 2016

Posters

Ziegler, V., Henninger, C., Fritz, G. Influence of low oxygen pressure and Rho inhibitors on radiation-induced stress response of human keratinocytes (HaCaT). 16th Annual Meeting of the German Society for Radiobiology, Darmstadt, September 2013.

Ziegler, V., Henninger, C., Fritz, G. Effect of oxygen pressure and statins on ionizing radiation-induced stress response of human keratinocytes. 80th Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology, Hannover, April 2014.

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Ziegler, V., Albers, A., Henninger, C., Fritz, G. Statins for the prevention of anticancer therapy-related normal tissue toxicity. Symposium DNA damage response, genetic instability and cancer, German Society of Toxicology, Mainz, November 2016.

Ziegler, V., Albers, A., Henninger, C., Fritz, G. Antitumor therapy-related normal tissue toxicities: prevention of oral mucositis and radiation-induced lung damage by statins. 83rd Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology, Heidelberg, March 2017.

Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass diese Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Ich versichere ferner, dass die Dissertation - weder in dieser noch in ähnlicher Form - an keiner anderen Fakultät vorgelegt wurde. Ich habe keine vorherigen erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 30.08.2017

Verena Ziegler