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Radiotherapy plus Heat Shock Protein 90 Inhibition: Sensitizing Cancer Cells to Irradiation-Induced Cell Death and Enhancing their Immunogenicity

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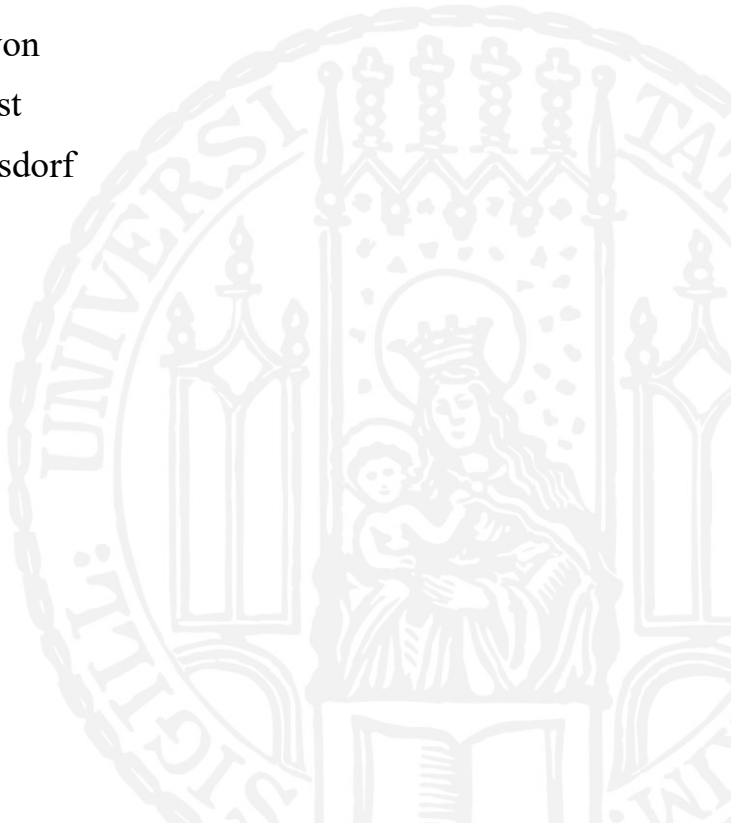

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Abbreviations

µ	Micro (10^{-6})
ADP	Adenosine diphosphate
AKT	Protein kinase B
APC	Adenomatosis polyposis coli or antigen-presenting cell
ATM	Serine-protein kinase ATM; alternative name: Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Serine/threonine-protein kinase ATR; alternative name: Ataxia telangiectasia and Rad3-related protein
BAX	Apoptosis regulator BAX
BCL-2	Apoptosis regulator Bcl-2
BRAF	B-Raf proto-oncogene, serine/threonine kinase; alternative name: v-raf murine sarcoma viral oncogene homolog B
CD	Cluster of differentiation
CDK	Cyclin-dependent kinases
CHK	Serine/threonine-protein kinase Chk; alternative name: checkpoint kinase
CRC	Colorectal cancer
CX3CR1	CX3C chemokine receptor 1
Da	Dalton
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DFSP	Dermatofibrosarcoma protuberans
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DDR	DNA damage response
DSB	DNA double-strand break
e.g.	For example
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPHA2	Ephrin type-A receptor 2
g	Gram
GIST	Gastrointestinal stromal tumor
H	Hour

γH2AX	Phosphorylates histone H2AX
HDAC	Histone deacetylase
hTERT	Human telomerase reverse transcriptase
Gy	Gray
HER2/NEU	Human epidermal growth factor receptor 2; alternative name: receptor tyrosine-protein kinase erbB-2 (ERBB2)
hi	High
HIF-1α	Hypoxia-inducible factor 1-alpha
HMGB1	High mobility group protein B1
HR	Homologous recombination
HSP	Heat shock protein
IFN	Interferon
i.e.	That is
IL	Interleukin
int	Intermediate
IR	Ionizing radiation
IRTG	Integrated research training group
k	Kilo (10^3)
KIT	KIT proto-oncogene receptor tyrosine kinase Kit
KRAS	KRAS proto-oncogene, GTPase; alternative name: kirsten rat sarcoma viral oncogene homolog
KRAS	GTPase KRas
l	Liter
lo	Low
Ly	Lymphocyte antigen
m	Milli (10^{-3})
M	Molar (mol/l)
mDCs	Myeloid DCs
MDSC	Myeloid-derived suppressor cell
min	Minute
MET	Tyrosine-protein kinase Met
MHC	Major histocompatibility complex
MOMP	Mitochondrial outer membrane permeabilization
MRN complex	MRE11-RAD50-NBS1 complex
mTOR	Serine/threonine-protein kinase mTOR
MW	Molecular weight

MYC	Myc proto-oncogene protein
m	Micro (10^{-6})
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
NKC	Natural killer cell
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
NW457	*epi*-pochoxime F
P	Phosphate
p53	Cellular tumor antigen p53
RAD51	DNA repair protein RAD51 homolog 1
RAF1	RAF proto-oncogene serine/threonine-protein kinase
PARP	Poly(ADP-ribose)polymerase
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PRA	Replication protein A
PUMA	Bcl-2-binding component 3
qRT-PCR	Quantitative real-time RT-PCR
RIP kinase	Receptor-interacting serine/threonine-protein kinase
RT	Radiotherapy
SCFR	Mast/stem cell growth factor receptor Kit; alternative name: proto-oncogene c-Kit
s.d.	Standard deviation
SDF-1α	Stromal cell-derived factor 1
s.e.m.	Standard error of the mean
STS	Soft tissue sarcoma
TCGA	The Cancer Genome Atlas
TP53	Tumor protein p53
Treg	Regulatory T cell
U	Unit
VEGF-A	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
zVAD-fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Zusammenfassung

Die Strahlentherapie ist ein essentieller Bestandteil der multimodalen Behandlung von Krebserkrankungen. Ein Versagen dieser Therapieform wird mit intrinsischer Strahlenresistenz in Verbindung gebracht. Deshalb ist der Fokus der vorliegenden Arbeit die Identifizierung von geeigneten Biomarkern für intrinsische Strahlenresistenz und deren pharmakologische Nutzung, um die therapeutische Breite der Strahlentherapie zu verbessern.

Die Effizienz der Behandlung von Weichteilsarkomen mittels Strahlentherapie ist aufgrund von Unterschieden in der Strahlensensibilität verschiedener Sarkoma-Subtypen leider eingeschränkt. In der vorliegenden Arbeit wurden systematische Analysen der intrinsischen Strahlenresistenz verschiedener Weichteilsarkom-Zelllinien durchgeführt, und anhand einer Hauptkomponentenanalyse von Klonogenitätsdaten wurden Radioresistenz-Scores extrahiert. In Korrelationsanalysen dieser Radioresistenz-Scores mit Transkriptomprofilen von DNA-Schadensreparatur-Regulatoren zeigten starke positive Korrelationen für die mRNA-Expressionslevels des molekularen Chaperons Hitzeschockprotein 90 (HSP90) und seiner Klientenproteine ATM, ATR und NBS1. Dadurch wurden diese Kandidaten als potentielle Biomarker für strahlenresistente, aggressive Weichteilsarkome identifiziert. Untersuchungen des Datensatzes der Weichteilsarkomkohorte des *Cancer Genome Atlas* (TCGA) bestätigten eine gesteigerte Expression dieser Kandidaten-Biomarker in Tumorproben von Patienten mit schlechterem klinischem Verlauf und besonders kurzem Gesamtüberleben. Die Inhibition von HSP90 und die damit einhergehende Beeinträchtigung seiner Klientenproteine (u.a. ATM, ATR und NBS1) könnte daher ein vielversprechender Ansatz zur zielgerichteten Modulation der intrinsischen Strahlenresistenz sein. In der vorliegenden Arbeit wurde der neue Pochoxim-basierte Wirkstoff NW457 für die Inhibition von HSP90 verwendet. Tatsächlich reichten niedrige nanomolare Konzentrationen von NW457 aus, um strahlenresistente Weichteilsarkom-Zelllinien gegenüber ionisierender Strahlung *in vitro* zu sensibilisieren. NW457 erhöhte den strahleninduzierten klonogenen Zelltod begleitet von einer Hemmung bzw. Verzögerung der DNA-Schadensreparatur. Während strahlungsvermittelte Apoptose und Nekrose in diesem Konzentrationsbereich unbeeinflusst waren, scheint die zelluläre Seneszenz in der NW457-vermittelten Strahlensensibilisierung eine Rolle zu spielen. Hier sind weitere mechanistische Untersuchungen erforderlich und geplant.

Die Suche nach strahlensensibilisierenden Wirkstoffen ist auch für die Behandlung des kolorektalen Karzinoms von großem Interesse, da die Anwendung ionisierender Strahlung aufgrund der hohen Darmmobilität und der relativen Strahlensensitivität des umliegenden Normalgewebes Schwierigkeiten in dieser Entität mit sich bringt. Daher wurde das strahlensensibilisierende Potential des neuen HSP90-Inhibitors NW457 auch in dieser

Krebsentität analysiert. Tatsächlich sensibilisierte NW457 kolorektale Karzinom-Zelllinien wirksam gegenüber ionisierender Strahlung *in vitro* und *in vivo*. Die durch eine Inhibition von HSP90 vermittelte Beeinträchtigung der DNA-Schadensreparatur resultierte in einer signifikanten Verminderung des klonogenen Überlebens nach Bestrahlung – im Wesentlichen unabhängig vom p53- und BAX-Status. Hohe Konzentrationen von NW457 in Kombination mit Bestrahlung induzierten Apoptose und förderten den Übergang zur sekundären Nekrose durch BAX-abhängige und -unabhängige Mechanismen während p53 in dem Szenario vernachlässigbar zu sein scheint. In einem murinen syngenen, heterotopen kolorektalen Tumormodel verbesserte NW457 die durch Bestrahlung erreichte Tumorkontrolle signifikant und zeigte eine reduzierte Hepatotoxizität im Vergleich zu HSP90-Inhibitoren der ersten und zweiten Generation. Neben seiner strahlensensibilisierenden Wirkung beeinflusste NW457 die Art des strahleninduzierten kolorektalen Tumorzelltods und führte zum verstärkten bzw. beschleunigten Auftreten von Sekundärnekrose. Die damit verbundene Freisetzung von immunogenen DAMPs (damage-associated molecular patterns) und die Rekrutierung von monozytären Zellen *in vitro* und *in vivo* weisen auf eine gesteigerte Tumorimmunogenität hin, und es bleibt zu klären, inwiefern diese Mechanismen einen Beitrag zur beobachteten verbesserten Tumorkontrolle leisten.

Zusammenfassend lässt sich festhalten, dass der neue Pochoxim-basierte HSP90 Inhibitor NW457 die therapeutische Breite der Strahlentherapie steigern kann und gleichzeitig die Tumorimmunogenität erhöht. Demzufolge erscheint dieser Ansatz als eine vielversprechende Kombinationsstrategie für die Strahlentherapie resistenter, aggressiver Weichteilsarkome und kolorektaler Karzinome.

Abstract

Radiotherapy is an essential part of multimodal anti-cancer treatment. Failure of this therapy is considered to be associated with intrinsic radioresistance. Therefore, this study focuses on the identification of potential biomarkers of intrinsic radioresistance and their pharmacological exploitation in order to improve the therapeutic width of radiotherapy.

For soft tissue sarcomas, the efficacy of radiotherapy is limited due to strong differences in radiosensitivity of distinct sarcoma subtypes. In the present study, systematic analyses of the intrinsic radioresistance of a panel of soft tissue sarcoma cell lines were performed, and quantitative scores of radioresistance were extracted by principal component analysis. Correlation analyses of these radioresistance scores with transcriptomic profiling data of DNA damage response regulators revealed strong positive correlations for the mRNA expression levels of the molecular chaperone heat shock protein 90 (HSP90) and its client proteins ATM, ATR, and NBS1, thereby identifying these candidates as potential biomarkers for radioresistant, aggressive soft tissue sarcomas. Query of the soft tissue sarcoma cohort of the Cancer Genome Atlas (TCGA) database displayed upregulated expression of these candidate biomarkers in tumor samples of patients with dismal clinical outcome and particularly poor overall survival. Thus, HSP90 inhibition and concomitant client protein deprivation could represent a promising approach to target intrinsic radioresistance. In the present study, the novel pochoxime-based compound NW457 was utilized for HSP90 inhibition. Indeed, low nanomolar concentrations of NW457 potently sensitized radioresistant soft tissue sarcoma cell lines towards ionizing radiation *in vitro*. NW457 increased irradiation-induced clonogenic cell death accompanied by impairment of the DNA damage response. Whereas irradiation-induced apoptosis and necrosis virtually remained unaffected, cellular senescence appears to be involved in NW457-mediated radiosensitization. Further studies elucidating the underlying mechanisms in greater depth are necessary and planned.

Finding radiosensitizing agents is also of high interest for the treatment of colorectal cancers, since application of ionizing radiation can be difficult in this entity due to the high degree of mobility of the large intestine and considerable radiosensitivity of the surrounding normal tissue. Therefore, the radiosensitizing potential of the novel HSP90 inhibitor NW457 was also analyzed in models of this cancer entity. Indeed, NW457 potently sensitized colorectal cancer cells towards ionizing radiation *in vitro* and *in vivo*. HSP90 inhibitor-mediated impairment of the DNA damage response resulted in significantly decreased clonogenic survival upon irradiation – virtually irrespective of the p53 and BAX status. Higher concentrations of NW457 in combination with irradiation induced apoptosis and accelerated the transition into secondary necrosis by BAX-dependent and -independent mechanisms, whereas p53 appears to be

dispensable in this scenario. In a murine syngeneic, heterotopic colorectal cancer model, NW457 potently improved irradiation-mediated tumor control and displayed reduced hepatotoxicity compared to HSP90 inhibitors of the first and second generation. Apart from its radiosensitizing effects, NW457 affected the mode of irradiation-induced colorectal cancer cell death and favored the onset of secondary necrosis. The concomitant release of immunogenic DAMPs (damage-associated molecular patterns) and the attraction of monocytes *in vitro* and *in vivo* are indicative for enhanced tumor immunogenicity, and it remains to be elucidated how far these mechanisms contribute to the observed improvement in tumor control.

In summary, the novel pochoxime-based HSP90 inhibitor NW457 improves the therapeutic width of radiotherapy and enforces tumor immunogenicity. Hence, this approach appears as a promising combined modality strategy for radiotherapy of resistant, aggressive soft tissue sarcomas and colorectal cancers.

1 Introduction

Cancer is the second leading cause of death worldwide. In 2012, 14.1 million new cases were diagnosed, with the most common entities being cancers of the lung, breast, and colorectum. In terms of mortality, cancers of the lung, liver, stomach, and colorectum account for the majority of all cases (i.e. 8.2 million deaths in 2012) (Ferlay et al., 2015). For the future, increasing incidences are predicted due to the aging world population and the adoption of cancer-causing westernized lifestyles within economically developing countries (i.e. unhealthy diet and less physical activity) (Bray et al., 2012; Jemal et al., 2011). Although there are genetic predispositions, inherited factors account for the minority of cancers (approximately 5%-10%) (Anand et al., 2008).

Cancer is a disease of multicellular organisms that can arise from almost every tissue in the body, and more than 100 different forms are currently known (Hanahan and Weinberg, 2000). The well-acknowledged multi-step model of cancerogenesis describes the transformation of a normal cell into a malignant cell by stepwise accumulation of somatic mutations in proto-oncogenes and/or tumor suppressor genes and subsequent clonal selection, analogous to Darwinian evolution (Kinzler and Vogelstein, 1996; Nowell, 1976; Vogelstein et al., 1988; Vogelstein and Kinzler, 1993). During this process, whose duration and molecular details are distinct for different cancer entities, cancer cells acquire a set of crucial capabilities, the so-called hallmarks of cancer. The first six hallmarks of cancer that were originally defined by Hanahan and Weinberg in 2000 comprise features allowing tumor growth, survival, and formation of metastasis: ‘sustaining proliferative signaling’, ‘evading growth suppressors’, ‘resisting cell death’, ‘enabling replicative immortality’, ‘inducing angiogenesis’, and ‘activating invasion and metastasis’ (Hanahan and Weinberg, 2011). In 2011, two further hallmarks and two enabling characteristics crucial to the acquisition of the hallmarks were defined: The enabling characteristic ‘genome instability and mutation’ favors the accumulation of mutations and the emergence of tumor cell variants with acquired hallmarks and corresponding survival benefits, and ‘tumor-promoting inflammation’ refers to an immune-mediated, inflammatory, tumor-promoting microenvironment that is enriched in growth, survival, and proangiogenic factors. The two new hallmarks encompassed ‘deregulating cellular energetics’ necessary to fulfil the energetic and anabolic requirements of the growing tumor and ‘avoiding immune destruction’ describing the observation that most clinically manifest tumors are poorly immunogenic and have managed to escape the immune system (Hanahan and Weinberg, 2011). This poorly immunogenic status is established in a process known as cancer-immunoediting consisting of three steps: elimination, equilibrium, and escape (Dunn et al., 2006; Schreiber et al., 2011). During the elimination phase, the immune system initially attacks and kills cells of the growing tumor.

However, individual tumor cells can survive the elimination phase because they have acquired non-immunogenic features and thus have growth advantages (equilibrium phase). Finally, the tumor cell variants that have been selected in the equilibrium phase successfully evade the immune attack and give rise to a poorly immunogenic tumor with a high degree of resistance against immune detection (escape phase).

1.1 Soft Tissue Sarcoma

Soft tissue sarcomas (STSs) are rare, heterogeneous tumors accounting for about 1% of all adult and 8% of all pediatric cancers in children <15 years of age (Linch et al., 2014; Sangkhathat, 2015). These malignancies are of mesenchymal, non-epithelial origin and are the most frequent among all sarcomas. The majority of STSs occur in the extremities and the abdomen (Clark et al., 2005; Demetri et al., 2010). More than 50 histological subtypes are defined, including rhabdomyosarcoma, leiomyosarcoma, liposarcoma, fibrosarcoma, synovial sarcoma, and gastrointestinal stromal tumors (GISTs) arising from skeletal muscle, smooth muscle, fat, fibrous tissue, tissue around the joints, or the gastrointestinal tract, respectively (Clark et al., 2005; Fletcher, 2014). Rhabdomyosarcoma is the most common STS in children (Sangkhathat, 2015). The majority of STSs are sporadic. Risk factors for STS development are environmental influences (e.g. exposure to vinyl chloride, ionizing radiation (IR)), viral infections (e.g. kaposi´s sarcoma, leiomyosarcoma), and genetic predisposition syndromes, such as the Li-Fraumeni syndrome in which a germline mutation of the tumor suppressor tumor protein p53 (*TP53*) is associated with occurrence of rhabdomyosarcoma (Clark et al., 2005; Thomas and Ballinger, 2015). From a genetic point of view, STSs can be categorized into two groups with different pathogenesis-driving genetic aberrations. The first comprises malignancies with simple karyotypes and distinct translocations generating fusion genes or specific oncogenic mutations, respectively. The second group contains tumors with complex karyotypes characterized by numerous chromosomal aberrations due to a high degree of genome instability (Bridge, 2014; Taylor et al., 2011).

The standard treatment for non-metastatic, localized STSs comprises surgery in combination with adjuvant (post-operative) radiotherapy (RT) to improve the local control rate. In cases in which the feasibility of surgery is questionable, neoadjuvant (pre-operative) RT is implemented in order to reduce the tumor burden prior to resection. For unresectable disease, RT can be administrated as a primary treatment. Multimodal treatment with chemotherapy (e.g. doxorubicin, ifosfamide), RT, and surgery shows efficacy in patients with advanced disease (Demetri et al., 2010; Group, 2014; von Mehren et al., 2014). The five-year overall survival rates of STSs vary between tumor stages, declining from approximately 90% for initial stages to 10%-20% for advanced stages. Local recurrence and

metastasis, typically to the lungs, are the major underlying reasons (Clark et al., 2005; Kneisl et al., 2014). Since pathogenesis of STSs is driven by specific genetic aberrations, molecular targeting of protein kinases (e.g. serine/threonine-protein kinase mTOR (mTOR), vascular endothelial growth factor receptor (VEGFR)) or histone deacetylases (HDACs) has been explored to improve treatment outcome. So far, the most effective targeted drug is the tyrosine kinase inhibitor imatinib that has been approved for the treatment of GISTs and dermatofibrosarcoma protuberans (DFSP). 80% of GIST patients respond to imatinib – at least partially – when carrying activating mutations in the KIT proto-oncogene receptor tyrosine kinase (*KIT*). In DFSP, imatinib inhibits platelet derived growth factor receptor (PDGFR), which is overexpressed due to gene fusion with the collagen type I alpha 1 chain (*COL1A1*) promotor (Demicco et al., 2012; Taylor et al., 2011). Since treatment options especially for advanced, recurrent, and metastatic STSs are limited, novel and effective molecularly targeted therapies as well as immunotherapies are of high clinical interest. In this regard, the heterogeneity of STS subtypes represents a crucial and challenging factor demanding for rational and adequate patient stratification (Tseng et al., 2014).

1.2 Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer worldwide accounting for more than one million new diagnoses per year (about 10% of all new cancer cases). In terms of mortality, CRC is the fourth most common cause of cancer-related deaths with approximately 600.000 cases per year (Ferlay et al., 2015). CRCs originate from epithelial cells of the colon or rectum, usually beginning as benign adenomatous polyps before transforming into invasive, malignant cancers (Markowitz and Bertagnolli, 2009). The development of CRCs follows the classical multi-step model of carcinogenesis involving the gradual accumulation of genetic and epigenetic alterations over years and decades. In fact, the multi-step model was established at this cancer entity (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996; Vogelstein et al., 1988; Vogelstein and Kinzler, 1993). The majority of CRCs are sporadic (70%-80%) and not inherited. Risk factors include increasing age, previous polyps, male sex, and environmental factors comprising high-fat diet, red meat consumption, obesity, elevated consumption of alcohol, smoking, and a sedentary lifestyle (Binefa et al., 2014). Inflammatory bowel diseases, such as Crohn´s disease and ulcerative colitis, strongly increase the risk of colitis-associated cancer and thus are considered as precancerosis (Garg and Loftus, 2016). Rare inherited forms of CRCs are familial adenomatous polyposis, *MYH*-associated polyposis, and Lynch syndrome (hereditary non-polyposis colorectal cancer) (Binefa et al., 2014; Cunningham et al., 2010). CRCs are classified into three phenotypes with different potentially overlapping genetic instabilities. The most common phenotype has a chromosomal instability in which amplifications or

translocations of chromosomes contribute to aneuploidy. Chromosomal instability is associated with accumulation of mutations in proto-oncogenes (e.g. KRAS proto-oncogene, GTPase (*KRAS*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)) and tumor suppressor genes (e.g. adenomatosis polyposis coli (*APC*), *TP53, SMAD4*). The microsatellite instability phenotype accumulates errors during DNA replication in short tandem repeat sequences (microsatellites) due to mutations in replication mediating genes (e.g. *MLH1*, *MSH2*, *MSH6*). The CpG island methylator phenotype exhibits hypermethylation of CpG islands, which is a mechanism of gene silencing (Aghagolzadeh and Radpour, 2016; Bogaert and Prenen, 2014; Markowitz and Bertagnolli, 2009; Walther et al., 2009).

Surgery is the main treatment option for CRC, mostly with curative intention in case of initial stages. For more advanced stages, surgery is complemented with adjuvant or neoadjuvant chemotherapy, RT, or radiochemotherapy, respectively. Different regimes of 5-fluorouracil, its pro-drug capecitabine, oxaliplatin, folinic acid, and irinotecan are used for systemic chemotherapy (Binefa et al., 2014; Cunningham et al., 2010). Whereas RT is commonly employed as treatment modality for rectal cancer, its application in colon cancer is largely restricted to high-risk cases due to the relevant degree of mobility within the colon and the resulting radiation toxicity to surrounding organs (Glimelius, 2002; van de Velde et al., 2014; Willett et al., 1999). Disseminated CRCs, which are characterized by typical metastasis formation in liver and lungs, require multimodal treatment with surgery, chemotherapy, RT, and targeted therapies against VEGFR and/or epidermal growth factor receptor (EGFR) (Ahn et al., 2016; Binefa et al., 2014). Mutations in the proto-oncogenes *KRAS* (in up to 50% of all CRCs) and *BRAF* (in approximately 10% of CRCs) encoding for downstream mediators in the EGFR signaling pathway represent contraindications for EGFR-directed therapies. These mutations lead to constitutive activation of the GTPase KRas (KRAS) and the serine/threonine-protein kinase B-raf, thereby inducing tumor-promoting signaling of mitogen-activated protein kinase (MAPK)- and phosphatidylinositol 3-kinase (PI3K) pathway irrespective of EGFR blockade (Bazan et al., 2005; Binefa et al., 2014; Cunningham et al., 2010). In addition, inactivating mutations in the tumor suppressor *TP53* (in up to 50% of all CRCs) encoding for the central cell cycle regulator and transcription factor cellular tumor antigen p53 (p53) have been reported to associate with poorer prognosis (Bazan et al., 2005; Iacopetta et al., 2006; Russo et al., 2005).

Despite a decline in CRC deaths over the last decades as a result of better prevention, early detection, and more elaborate treatment, the five-year survival rates still range from 75%-90% for initial stages to less than 15% for advanced stages (Binefa et al., 2014). Advances in classifying CRC phenotypes and the associated cell populations and/or mechanisms mediating therapy resistance in greater depth as well as the development of

novel therapy strategies will hopefully contribute to further improve CRC treatment in the future.

1.3 Radiotherapy: A Central Cornerstone of Cancer Treatment

RT is one of the cornerstones of cancer treatment besides surgery and systemic chemotherapy. The medical application of IR improves tumor control and prolongs disease-free and overall patient survival. More than 60% of all cancer patients receive RT at one point during their treatment, and various settings ranging from definitive to multimodal strategies, including neoadjuvant and adjuvant as well as palliative settings, are in clinical use (Orth et al., 2014). Implementation of RT varies according to tumor type, size, and stage. Conventionally fractionated RT comprises five daily fractions of 1.8-2 Gy per week up to a total dose of 45-70 Gy. For certain cancer entities, hypofractionation regimes with fewer and larger fractions are employed (Lauber et al., 2015). For instance, neoadjuvant RT with a daily dose of 5 Gy in 5 fractions is used in localized rectal cancer (Chen et al., 2015; van de Velde et al., 2014). In breast cancer, ongoing clinical trials will clarify if higher doses up to 5 Gy per fraction in comparison to the commonly applied 2.66 Gy will define new alternatives. In case of ablative RT, such as stereotactic body RT, intraoperative RT, or brachytherapy, reduction of the fraction number is escalated to 1-5 high doses of more than 10 Gy (Lauber et al., 2015).

The aim of RT is to eradicate the tumor, while sparing the normal surrounding tissue. State-of-the-art RT comprises photon irradiation with intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT). These modern treatment technologies facilitate advanced precision treatment planning and image-guided administration of target volumes in order to achieve improved accuracy and to spare organs at risk. With the advent of new irradiation qualities, including protons and heavy ions, this preciseness has been brought to an even higher level (Baumann et al., 2016). However, due to high investment costs, the clinical availability of proton and heavy ion therapy remains largely limited.

1.3.1 Molecular Responses to Ionizing Radiation

The cytotoxic effects of IR derive from direct or indirect deoxyribonucleic acid (DNA) damage, with DNA double-strand breaks (DSBs) being the most lethal lesions. In order to guarantee genomic integrity, a plethora of sophisticated DNA repair mechanisms – termed DNA damage response (DDR) – have emerged. Upon DNA damage, the DDR orchestrates activation of cell cycle checkpoints, recruitment as well as activation of DNA repair proteins, and finally the repair of the damage. The success of DNA repair decides over cell survival or death, respectively (Begg et al., 2011; Roos et al., 2016). In principle, RT takes advantage

of the higher DNA repair capacity of normal cells in comparison to malignant cells, thereby aiming at abrogation of clonogenic survival and induction of cell death in the latter.

1.3.1.1 DNA Damage Response

DNA double-strand breaks, the most severe type of damage caused by IR, are repaired via two different pathways: Non-homologous end joining (NHEJ) or homologous recombination (HR) repair, respectively. Although NHEJ is highly error-prone due to ligation of the ends of two nearby broken DNA sequences, this mechanism can rapidly restore the majority of IR-induced DSBs during all phases of the cell cycle. In contrast, HR

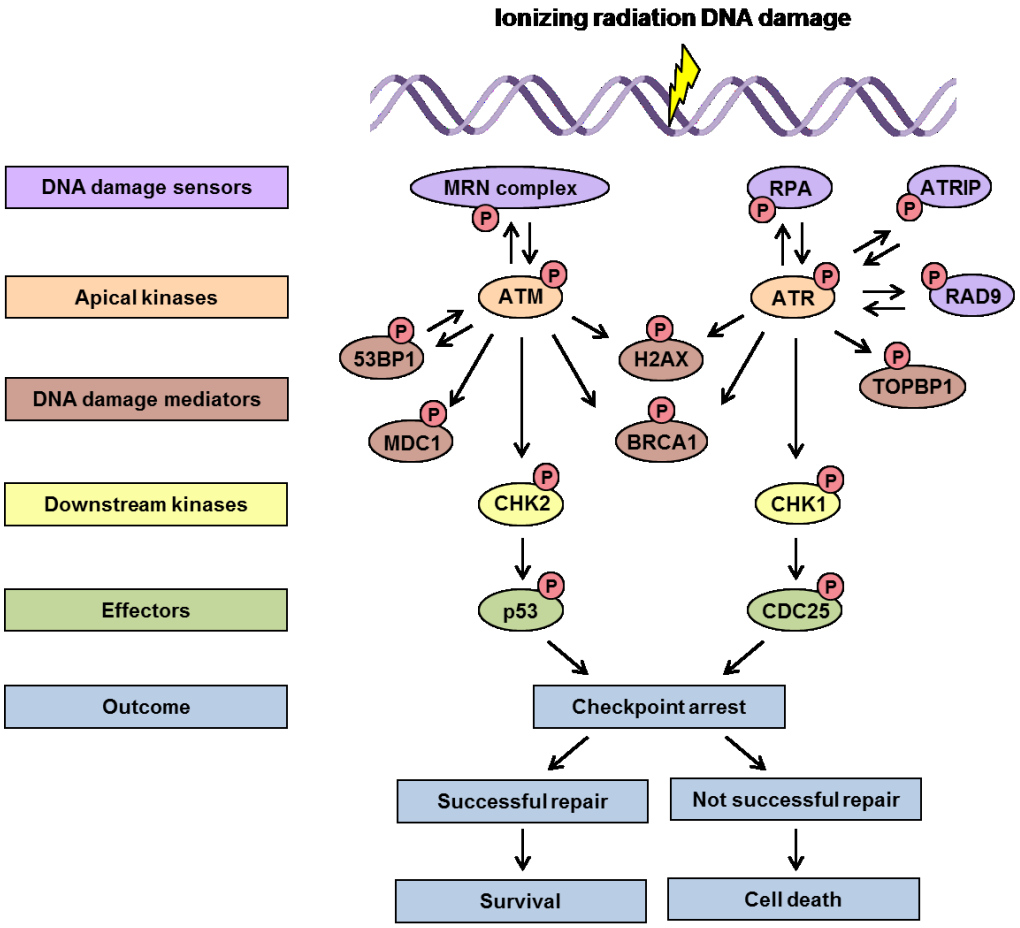

Figure 1 The DNA damage response.
IR causes DNA damage that can be repaired via the DDR. A plethora of regulators detect, mediate, and eliminate DNA damage. The success of DNA repair determines cell proliferation, cellular senescence, and/or cell death. MRN complex, MRE11-RAD50-NBS1 complex; RPA, replication protein A; ATM, serine-protein kinase ATM; ATR, serine/threonine-protein kinase ATR; ATRIP, ATR-interacting protein; RAD9, RAD9-RAD1-HUS1 complex; H2AX, histone H2AX; BRCA1, breast cancer type 1 susceptibility protein; 53BP1, tumor suppressor p53-binding protein 1; MDC1, mediator of DNA damage checkpoint protein 1; TOPBP1, DNA topoisomerase 2-binding protein 1; p53, cellular tumor antigen p53; CDC25, cell division cycle 25. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Sulli et al., 2012), copyright (2012).

is a highly accurate DNA repair mechanism as it uses the sister chromatid as template. HR repairs the minority of DSBs and is restricted to S- and G2-phase of the cell cycle, when sister chromatids are available (Begg et al., 2011; Curtin, 2012; Maier et al., 2015). DSBs are recognized by the MRE11-RAD50-NBS1 (MRN) complex that recruits and activates the central protein kinase serine-protein kinase ATM (ATM) (Figure 1). Subsequently, ATM phosphorylates histone H2AX (γH2AX) that, in turn, recruits multiple DNA repair factors. Single-stranded DNA resulting from DSB strand resection is recognized by replication protein A (RPA) that recruits and activates the next protein kinase within the cascade, serine/threonine-protein kinase ATR (ATR). ATM and ATR, together with their downstream protein kinases, serine/threonine-protein kinase Chk1 and Chk2 (CHK1 and CHK2), phosphorylate and activate various effector proteins, such as the central tumor-suppressor protein p53 (Sengupta and Harris, 2005; Sulli et al., 2012). Phosphorylation of p53 leads to disruption of its interaction with the E3 ubiquitin-protein ligase Mdm2 (MDM2), which under normal conditions mediates p53's continuous proteasomal degradation. Stabilized p53 and other effector proteins trigger transient or permanent cell cycle arrest as well as cell death (Ciccia and Elledge, 2010; Maier et al., 2015; Sengupta and Harris, 2005).

1.3.1.2 Irradiation-induced Forms of Cell Death and Cellular Senescence

In response to excessive, unrepaired DNA damage, different forms of cell death as well as cellular senescence can be induced – depending on the cellular origin, the genetic background, and the functionality of cell cycle checkpoints (Figure 2) (Lauber et al., 2012; Roos et al., 2016). Proliferating cells of the hematopoietic system, including lymphoma and leukemia, predominantly undergo apoptotic cell death (Eriksson and Stigbrand, 2010). Characteristic features of apoptosis include cellular shrinkage, membrane blebbing, exposure of the phospholipid phosphatidylserine, condensation of chromatin, and nuclear fragmentation (Taylor et al., 2008). Importantly, the plasma membrane stays intact during apoptosis. However, if apoptotic cells are not timely engulfed by surrounding cells or professional phagocytes, including macrophages and dendritic cells (DCs), they transit into post-apoptotic, secondary necrosis, and the plasma membrane disintegrates (Silva, 2010).

IR stimulates apoptosis preferentially via the intrinsic death pathway (Rudner et al., 2001). Prolonged activation of p53 due to massive DNA damage results in activation of pro-apoptotic apoptosis regulator Bcl-2 (BCL-2) family members (e.g. apoptosis regulator BAX (BAX), Bcl-2-binding component 3 (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA)), and subsequent mitochondrial outer membrane permeabilization (MOMP) together with release of mitochondrial cytochrome c into the cytosol activate the caspase cascade. However, IR can also stimulate the upregulation of death receptor family

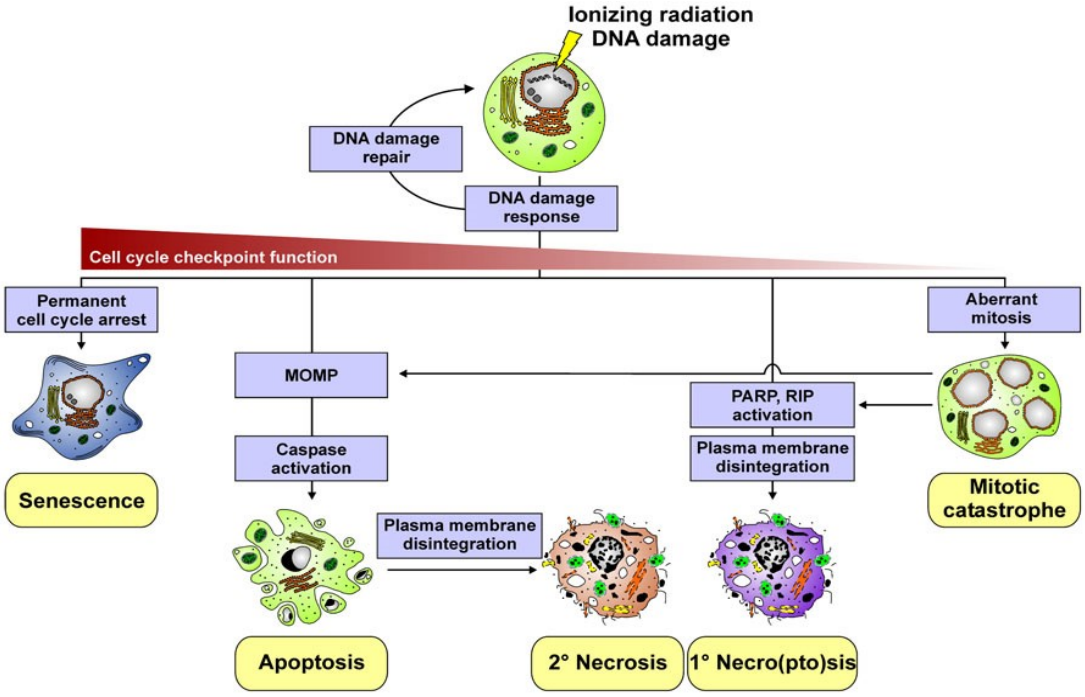

Figure 2 Ionizing radiation induces different forms of cell deaths and cellular senescence.
IR causes DNA damage. When the DNA damage cannot be repaired via DDR, cancer cells undergo different forms of cell death or permanent cell arrest termed cellular senescence. Crucial determinants in this regard are the cellular origin, the genetic background, and the functionality of cell cycle checkpoints. MOMP, mitochondrial outer membrane permeabilization; PARP, poly(ADP-ribose)polymerase; RIP kinase, receptor-interacting serine/threonine-protein kinase. Figure taken from (Lauber et al., 2012).

members that are required for the extrinsic apoptosis pathway. Although p53 is a central player in apoptosis induction, malignant cells with inactivating mutations in p53 can undergo IR-induced apoptosis via other mechanisms (e.g. p63/p73-dependent expression of pro-apoptotic proteins) (Lauber et al., 2012).

Apoptosis is less common in cells of epithelial origin (i.e. in most solid tumors). These cells display different forms of cell death upon irradiation (Deloch et al., 2016; Eriksson and Stigbrand, 2010). Limitations and deficiencies in apoptosis regulators can stimulate programmed necroptosis, which comprises hyperactivation of poly(ADP-ribose)polymerase (PARP), activation of receptor-interacting serine/threonine-protein (RIP) kinase, and depletion of intracellular adenosine triphosphate (ATP) levels. Characteristics of necroptosis are reactive oxygen species production, lipid peroxidation, organelle swelling, and disintegration of the cell membrane (Pasparakis and Vandenabeele, 2015; Vandenabeele et al., 2010). In case of very high irradiation doses, uncontrolled rupture of the plasma membrane and massive release of intracellular contents – termed primary necrosis – can occur (Lauber et al., 2012). Functionality of cell cycle checkpoints allows cells to leave the

cell cycle irreversibly upon IR. They do not further proliferate but stay metabolically active. This permanent cell cycle arrest is termed cellular senescence and exhibits features of flattened and enlarged morphology, upregulation of cyclin-dependent kinase inhibitors (e.g. p16, p21, p27,), senescence-associated β-galactosidase activation, and involvement of p53 (Nardella et al., 2011; Roos et al., 2016). Defects in cell cycle checkpoints force cells to undergo aberrant mitosis upon IR resulting in mitotic catastrophe as characterized by formation of giant cells with multiple nuclei and centrosome hyperamplification. Eventually, these cells can undergo cellular senescence, delayed apoptosis and/or necro(pto)sis, respectively (Eriksson and Stigbrand, 2010; Lauber et al., 2012).

1.3.1.3 Immunological Potential of Irradiation-induced Cell Death Modalities

Not only the induction of cancer cell death and the abrogation of clonogenic survival, but also the immunological potential of the different cell death modalities appears to be important for the treatment outcome of RT. Enhancing dying cancer cell immunogenicity by RT may favor the induction of anti-tumor immune mechanisms that can contribute to local as well as systemic tumor control. Along these lines, regression of distant, out-of-field metastases upon local RT (so-called abscopal effects) has been occasionally reported in the clinic (Demaria et al., 2004; Formenti and Demaria, 2009; Mole, 1953; Postow et al., 2012).

Apoptotic tumor cells with intact plasma membrane are removed by antigen-presenting cells (APCs), including macrophages and DCs, in an immunologically silent or tolerogenic manner (Poon et al., 2014). Under certain conditions, this can even promote tumor progression (Ford et al., 2015; Huang et al., 2011). In contrast, necrotic forms of cell death are rather immunogenic and can trigger pro-inflammatory immune responses. Due to the loss of plasma membrane integrity, intracellular contents comprising tumor-antigens and damage-associated molecular patterns (DAMPs) are released and/or exposed from necrotic cells (Derer et al., 2015; Lauber et al., 2012). DAMPs include heat shock proteins (HSPs), high mobility group protein B1 (HMGB1), nucleotides, S100 proteins, and others (Garg et al., 2015; Kroemer et al., 2013; Krysko et al., 2012). They can instigate the recruitment of monocytes and DCs, the uptake of tumor antigens by DCs, their maturation, lymph node homing, and activation of naïve $CD4^+$ and $CD8^+$ T cells via (cross-) presentation of tumor-antigens by DCs (Kroemer et al., 2013; Lauber et al., 2015). The resulting $CD8^+$ T cell responses can contribute to the eradication of tumor cells – within the irradiation field, but in principle also systemically (Deloch et al., 2016; Formenti and Demaria, 2009). For senescent cells, only limited data regarding their immunogenic potential are available. They are known to produce various cytokines and chemokines (collectively termed 'senescence-associated secretory phenotype') that has been reported contradictorily to initiate tumor

regression as well as tumor progression (Coppe et al., 2010; Perez-Mancera et al., 2014; Ruhland et al., 2016).

For ablative RT regimes with high single doses, accumulating evidence suggests that several steps within the cancer-immunity cycle can be enforced (Figure 3) (Deloch et al., 2016; Gaipl et al., 2014; Lauber et al., 2015). As such, the release of DAMPs derived from irradiated, necrotic cancer cells has been reported to recruit monocytes, which can function as precursor cells of APCs (Hennel et al., 2014). *In vivo* evidence has been provided for the intratumoral accumulation of APCs, originating from peripheral blood monocytes, upon ablative irradiation (Burnette et al., 2011). These intratumoral APCs can produce type I interferons (IFNs), thus supporting APC maturation, lymph node homing, and DC-mediated cross-priming of $CD8^+$ T cells in the tumor draining lymph nodes (Burnette et al., 2011; Lugade et al., 2005). In turn, type II IFN-producing $CD8^+$ T cells have been described to kill tumor cells via their cytolytic capacity (Gerber et al., 2013; Gupta et al., 2012). Finally, IR-mediated upregulation of stress-induced ligands, adhesion molecules, death receptors, and major histocompatibility complex (MHC) class I molecules can enhance tumor immunogenicity, thereby increasing anti-tumor $CD8^+$ T cell responses. For non-ablative RT

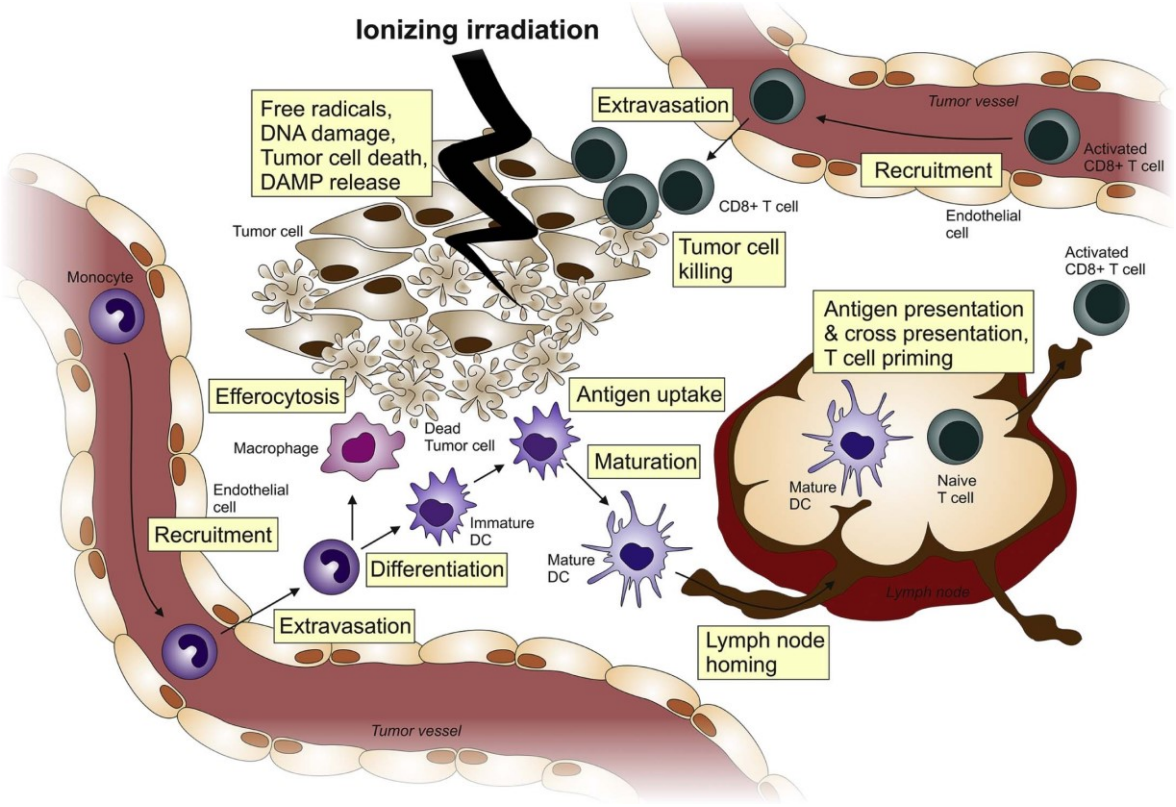

Figure 3 Ablative radiotherapy and the cancer immunity cycle.
Ablative RT with doses of more than 10 Gy can enforce various steps involved in the induction of anti-tumor immune responses finally resulting in tumor-specific killing by $CD8^+$ T cells. DAMP, damage-associated molecular pattern. Figure taken from (Lauber et al., 2015).

regimes, including conventionally fractionated and hypofractionated RT, the involvement of effective anti-tumor immune responses is only poorly understood and currently under discussion. In these cases, combination with immunotherapeutic approaches appears promising in order to overcome the immunosuppressive tumor microenvironment and stimulate systemic anti-tumor immune mechanisms (Deloch et al., 2016; Frey et al., 2014; Lauber et al., 2015).

1.3.2 Limitations of Radiotherapy

Despite its essential role in cancer treatment, RT still faces several limitations regarding side effects and therapeutic failure. Side effects are caused by normal tissue damage, inflammation, and fibrosis. Their occurrence depends on various factors, including the RT scheme and total dose applied, the location of the tumor, radiosensitivity of the surrounding normal tissue, and patient-related, individual factors (Begg et al., 2011; Orth et al., 2014). Therapeutic failure, in contrast, can derive from intrinsic tumor cell radioresistance, increased repopulation, poor cell cycle redistribution of surviving tumor cells, and impaired reoxygenation between RT fractions (Baumann et al., 2016). Of special interest is intrinsic radioresistance that can be caused by the upregulation of various DDR mediators (Begg et al., 2011). In addition, the tumor microenvironment can contribute to impaired responses towards IR caused by hypoxia, fibrosis, and immunosuppressive effects (Barker et al., 2015).

Consequently, a major focus of modern radiation oncology is to improve the therapeutic outcome by preferential sensitization of the tumor to IR and concurrent avoidance of side effects. In this regard, combined modality approaches of RT with molecularly targeted agents that interfere with essential drivers of malignancy, including regulators of the DDR, the EGFR pathway, angiogenesis, and other pathways, appear particularly promising (Begg et al., 2011; Maier et al., 2015; Morris and Harari, 2014).

1.4 Molecularly Targeted Therapy

Conventional chemotherapy is a systemic and cytotoxic anti-cancer treatment damaging all rapidly proliferating cells in the body. Over the last decades, cancer research focused on more selective and personalized approaches, such as molecularly targeted therapies. These molecularly designed agents address only a limited set of proteins – sometimes only one individual protein – which are essential for maintaining the malignant phenotype. Therefore, molecularly targeted therapies are assumed to have less frequent and less severe side effects as well as a higher efficacy than chemotherapeutics. These small molecule inhibitors or monoclonal antibodies specifically target individual signaling cascades, such as the EGFR pathway, the VEGFR pathway, DDR regulators, and others.

Several molecularly targeted therapies have already been integrated in current multimodal treatment approaches of certain cancers. The tyrosine kinase inhibitor imatinib initially received approval for Philadelphia chromosome-positive chronic myeloid leukemia inhibiting the BCR-ABL1 fusion protein (Druker, 2008). With identification of other imatinib targets, such as mast/stem cell growth factor receptor Kit (SCFR) and PDGFR, approval was extended to GISTs harboring mutations in *KIT* and to DFSP with a gene fusion leading to overexpression of *PDGFR* (Demicco et al., 2012; Taylor et al., 2011). Meanwhile, treatment of metastatic CRC comprises the application of the molecularly designed agents bevacizumab targeting vascular endothelial growth factor A (VEGF-A), as well as cetuximab and panitumumab both interfering with EGFR signaling (Ahn et al., 2016; Cunningham et al., 2010). Interestingly, the observation that EGFR overexpression correlates with radioresistance was the initial step for the approval of cetuximab in combination with RT for squamous cell carcinoma of the head and neck. Several preclinical studies provide evidence for molecularly targeted sensitization to RT by different substances (Giaccia, 2014; Morris and Harari, 2014). Nevertheless, the efficacy of these approaches will clearly depend on appropriate patient stratification with versatile predictive biomarkers and the absence and/or the control of severe toxicities.

1.5 Heat Shock Protein 90 as Promising Target for Anti-Cancer Therapy

Since multiple and – to a certain degree – redundant signaling pathways contribute to the maintenance of malignant phenotype, pharmacological interference with one single protein and/or pathway might be compensated by others and can lead to inefficient response or therapy resistance, respectively. Therefore, it appears promising to target multiple oncogenic signaling pathways simultaneously. A protein involved in diverse signaling cascades orchestrating the hallmarks of cancer is the chaperone HSP90.

1.5.1 The Molecular Chaperone Heat Shock Protein 90

HSP90 is a member of the heat shock protein family with a molecular weight (MW) of 90 kDa. The ATP-dependent chaperone prevents protein aggregation by mediating maturation, activation, and stabilization of its substrates, referred to as client proteins (Saibil, 2013; Taipale et al., 2010). These client proteins are involved in several signaling pathways and control various cellular features, including cellular growth, proliferation, differentiation, and death. HSP90 and other HSPs are upregulated and activated under different stress conditions, such as heat, irradiation, hypoxia, and acidosis (Whitesell and Lindquist, 2005). Evolutionarily, HSP90 is highly conserved and can be found in all organisms apart from archaea. In humans, the HSP90 family comprises different gene products. HSP90-α_1 and

HSP90-α_2 are encoded by *HSP90AA1* and *HSP90AA2*, and HSP90-β by *HSP90AB1*. Under non-stress conditions, gene products of these paralogs account for 1%-2% of the total protein content in the cytoplasm, but they can also be found in the nucleus. Further HSP90 family members are localized in other cellular compartments: 94 kDa glycose-related protein 94 (GRP94) in the endoplasmic reticulum and tumor necrosis factor type 1 receptor-associated protein (TRAP1) in the mitochondria (Chen et al., 2006; Johnson, 2012; Taipale et al., 2010).

HSP90 clients are folded via the HSP90 chaperone cycle with the help of several co-chaperones, such as stress-induced-phosphoprotein 1 (STI1), HSP70, HSP40, and activator of 90 kDa heat shock protein ATPase homolog 1 (AHA1) (Figure 4A). The flexible HSP90 dimer consists of three different domains exerting specific functions: the N-terminal domain binds ATP, the middle domain interacts with the client protein, and the C-terminal domain is responsible for dimerization during the chaperone cycle. In the course of this highly dynamic process, HSP90 switches between an open conformation via an intermediate state to the closed and twisted conformation. Eventually, under hydrolysis of ATP, the client protein is correctly folded and dissociates from the chaperone complex (Trepel et al., 2010). Meanwhile, several asymmetric processes, such as interactions with co-chaperones and posttranslational modifications (e.g. phosphorylation, acetylation), are known to fine-tune the chaperone cycle (Mayer and Le Breton 2015). All small molecule inhibitors of HSP90 that have been clinically evaluated so far bind competitively to the unique N-terminal ATP-

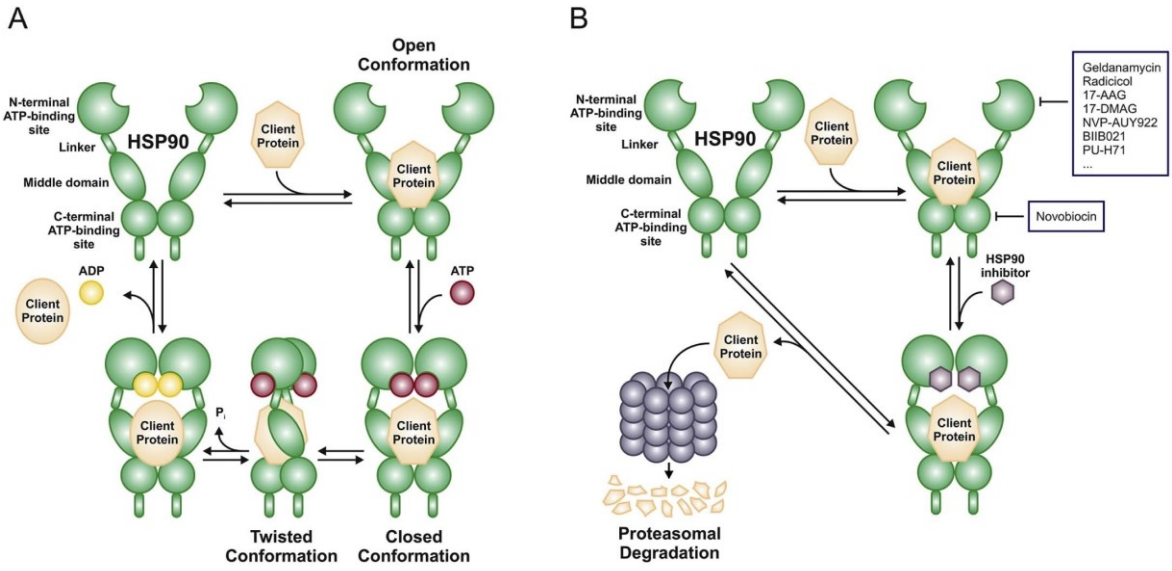

Figure 4 Simplified scheme of the dynamic HSP90 chaperone cycle.
(A) HSP90 and various co-chaperones (not shown here) mediate the folding of client proteins under several conformational changes and ATP hydrolysis. (B) The vast majority of HSP90 inhibitors competitively interfere with N-terminal ATP binding and prevent the chaperoning activity of HSP90. This hampers the maturation of the client protein that will be released and subjected to proteasomal degradation. ATP, adenosine triphosphate; ADP, adenosine diphosphate; HSP90, heat shock protein 90; P, phosphate. Figure taken from (Lauber et al., 2015).

binding domain with a high degree of affinity. This leads to the sequence of events: prevention of ATP binding and hydrolysis, release of misfolded client proteins, and finally their proteasomal degradation (Figure 4B). Depletion of several hundred client proteins, including protein kinases and transcription factors (for an up-to-date list see http://www.picard.ch/downloads/HSP90interactors.pdf), can interfere with basically all hallmarks of cancer (Lauber et al., 2015; Neckers and Workman, 2012).

1.5.2 Heat Shock Protein 90 Inhibition Interferes with Multiple Hallmarks of Cancer

Cancer cells exhibit a stronger dependency on HSP90 folding assistance than normal cells. Overexpressed HSP90 in cancer cells compensates for stress associated with the malignant phenotype, such as aneuploidy, proteotoxic stress, nutrient starvation, and hypoxia. In addition, HSP90 supports the stability and function of mutated, translocated, and/or overexpressed oncoproteins, which are particularly labile. Thus, HSP90 has emerged as an interesting target for anti-cancer therapies. Pharmacological inhibition of HSP90 represents a multi-target approach, since many of the hundreds of HSP90 client proteins contribute to the establishment and the maintenance of the hallmarks of cancer (Figure 5) (Calderwood and Gong, 2016; Neckers and Workman, 2012). 'Sustaining proliferative signaling' in cancer cells is facilitated by ligand-mediated oncogenic growth factor receptor signaling, mainly due to activating mutations or overexpression of cell surface tyrosine kinases. These tyrosine kinases, including receptor tyrosine-protein kinase erbB-2 (ERBB2) or EGFR, as well as their downstream signaling mediators in the PI3K/protein kinase B (AKT) and the mitogen-activated (MAP) kinases pathway are HSP90 client proteins and, thus, sensitive to its inhibition (Lauber et al., 2015). For 'evading growth suppression', the chaperone is involved in the stabilization of clients regulating the cell cycle, the cyclin-dependent kinases (CDKs), and their ligands. Hence, cell cycle progression can be disrupted by HSP90 inhibition (Xu and Neckers, 2007). 'Resisting cell death' is sustained by HSP90 through stabilization of anti-apoptotic AKT signaling associated with inactivation of pro-apoptotic BCL-2 family member Bcl2-associated agonist of cell death (BAD), prevention of cytochrome C release via overexpression of anti-apoptotic BCL-2, and activation of pro-survival factors (Lauber et al., 2015). Malignant cells have the feature to overcome the life-span limiting shortening of telomeres by overexpression of the enzyme telomerase. HSP90 is required for the enzymatic activity of the human telomerase reverse transcriptase (hTERT). Correspondingly, inhibition of HSP90 prevents elongation of telomeres and therefore interferes with the hallmark 'enabling replicative immortality'. The chaperone also contributes to 'inducing angiogenesis'. HSP90 stabilizes the oxygen level-sensing transcription factor hypoxia-inducible factor 1-alpha (HIF-1α) that controls the transcription

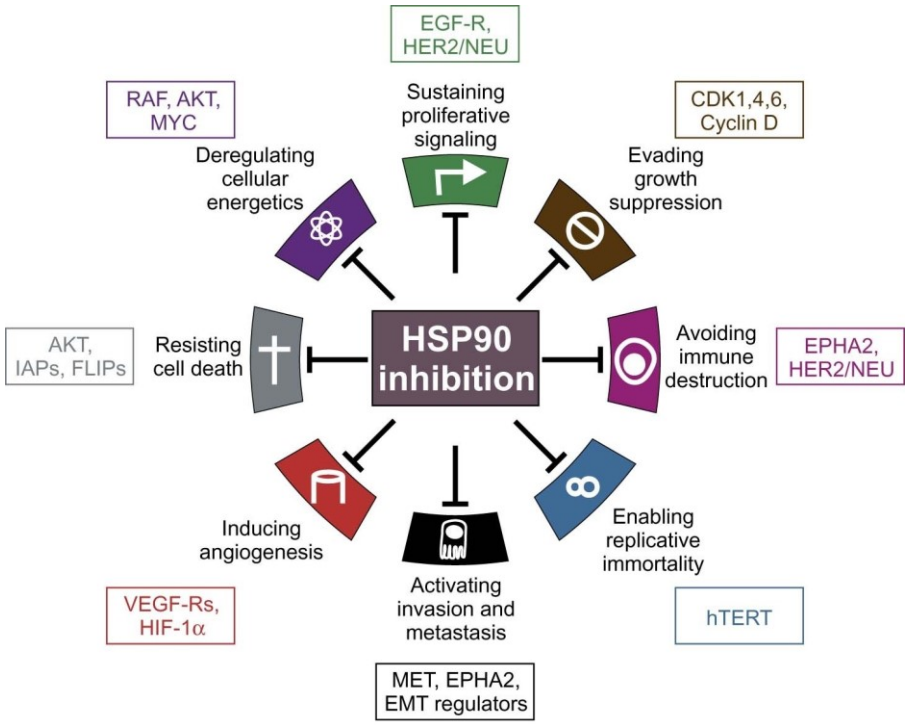

Figure 5 Overview of HSP90 inhibitors interfering with multiple hallmarks of cancer simultaneously.
Exemplary selected HSP90 client proteins are displayed that contribute to the establishment and the maintenance of different hallmarks of cancers, thereby promoting the transition of a normal cell into a malignant cell. AKT, protein kinase B; CDK, cyclin-dependent kinase; EGF-R, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EPHA2, ephrin type-A receptor 2; FLIP, FLICE-inhibitory protein; HER2/NEU, human epidermal growth factor receptor 2; HIF-1α, hypoxia-inducible factor 1-alpha; hTERT, human telomerase reverse transcriptase; IAP, inhibitor of apoptosis protein; MET, tyrosine-protein kinase Met; MYC, Myc proto-oncogene protein; RAF, RAF proto-oncogene serine/threonine-protein kinase; VEGF-R, vascular endothelial growth factor receptor. Figure taken from (Lauber et al., 2015).

of VEGF. Binding of VEGF to its receptor VEGFR that is also a known HSP90 client protein induces – amongst many other aspects of angiogenesis – endothelial cell proliferation (Xu and Neckers, 2007). Furthermore, HSP90 inhibition interferes with ‘activating invasion and metastasis’, since several of its clients are involved in this hallmark. In this context focal adhesion kinase should be mentioned exemplarily as well as ephrin receptors, the tyrosine-protein kinase Met (MET), matrix-metalloproteases, and mediators of epithelial-mesenchymal transition. A direct effect on tumor cell migration has been reported for ectopic, membrane-bound HSP90 that is only expressed in malignant cells. Several HSP90 clients (e.g. RAF proto-oncogene serine/threonine-protein kinase, AKT, Myc proto-oncogene protein (MYC)) support ‘deregulating cellular energetics’ that e.g. includes anaerobic glycolysis for generation of glycolytic intermediates needed for assembling new

cells. Nevertheless, the influence of HSP90 inhibition on malignant cell metabolism is only poorly understood (Lauber et al., 2015).

Finally, HSP90 inhibition also counteracts ‘avoiding immune destruction’ in various ways. First, stress-inducible proteins, such as HSP70 and the natural killer group 2D (NKG2D) ligands MHC class I polypeptide-related sequence A and B (MIC-A and -B), are upregulated on the tumor cell surface upon HSP90 inhibition. These proteins stimulate activation, migration, and cytolytic activity of natural killer cells (NKCs) (Lauber et al., 2015). Second, HSP90 inhibition can restore the recognition of tumor cells by overcoming the loss of tumor antigen presentation. HSP90 inhibitors promote the accelerated degradation of highly expressed oncogenic HSP90 clients (e.g. HER2/NEU, ephrin type-A receptor 2 (EPHA2)) and the presentation of peptides derived thereof on MHC class I molecules on the tumor cell surface. Thus, tumor cells are sensitized towards $CD8^+$ T cell-mediated lysis (Castilleja et al., 2001; Kawabe et al., 2009). Additionally, increased expression of tumor-specific differentiation antigens and MHC class I molecules that are no HSP90 client proteins contribute to enhanced tumor cell recognition by $CD8^+$ T cells (Haggerty et al., 2014). Third, HSP90 inhibition renders the tumor microenvironment favorable to the infiltration of $CD4^+$ and $CD8^+$ T cells and reduces the intratumoral levels of immunosuppressive myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) (Rao et al., 2012). In contrast, even low concentrations of HSP90 inhibitors are considered to suppress DC and T cell functions (Bae et al., 2007; Schnaider et al., 1998). Nevertheless, a dominance of immunostimulatory effects is assumable, since HSP90 inhibitors specifically accumulate in cancer cells (see 1.5.3). Further studies will elucidate whether HSP90 inhibition might also interfere with the two enabling characteristics ‘genome instability and mutation’ and ‘tumor-promoting inflammation’.

1.5.3 Heat Shock Protein 90 Inhibitors Exhibit a High Degree of Cancer Cell Selectivity

The strong dependency of cancer cells on HSP90 chaperoning assistance and the association of elevated HSP90 expression in cancer with poor overall survival make HSP90 an attractive target for anti-cancer therapy (Cheng et al., 2012; Neckers and Workman, 2012; Wang et al., 2013; Whitesell and Lindquist, 2005). Nevertheless, a prerequisite for clinical translation of HSP90 inhibitors is a relevant degree of selectivity for HSP90 in cancer cells as compared to HSP90 in normal cells particularly when some normal tissues express HSP90 at higher levels (brain, spleen, bladder) than e.g. breast cancer cells (Barrott and Haystead, 2013). First of all, the majority of HSP90 inhibitors bind to the N-terminal ATP-binding domain that is defined as the Bergerat fold. This Bergerat fold is structurally unique to the small GHKL (Gyrase, HSP90, histidine kinase, MutL) subgroup within the ATPase

superfamily, thereby exhibiting high specificity for HSP90 and not for other ATPases or kinases (Neckers and Workman, 2012; Pearl and Prodromou, 2006). Second, HSP90 inhibitors were indeed found to accumulate specifically in tumors and are rapidly cleared from circulation (Porter et al., 2010). Although the molecular basis of this observation remains to be fully elucidated, some suggestions were already made ranging from existence of high multi-chaperone complexes in malignant cells with high affinity for HSP90 inhibitors, preferred binding of small molecule drugs to HSP90 found in complex with oncoproteins, to posttranslational modifications of the chaperone influencing the affinity for HSP90 inhibitors (Kamal et al., 2003; Mollapour et al., 2014; Moulick et al., 2011). In addition, HSP90 inhibitor binding to ectopic, membrane-localized HSP90 that is only expressed in malignant cells might contribute to accumulation of small molecule HSP90 inhibitors in tumor cells via internalization of the formed complexes (Barrott et al., 2013). All this contributes to preferential anti-cancer activity of HSP90 inhibitors at doses that are in principal tolerated by the normal tissue.

1.5.4 Clinical Development of the Heat Shock Protein 90 Inhibitors

The first HSP90 inhibitors were the natural compounds geldanamycin (a benzoquinone ansamycin antibiotic) and radicicol (an antifungal, antibiotic macrocyclic resorcinol). This first generation of HSP90 activity blocking compounds exhibited unfavorable pharmacological properties, including toxicities, poor solubility, and insufficient bioavailability, thus prohibiting their translation into the clinic (Table 1). The second generation of HSP90 inhibitors, which are derivatives of the natural compounds, displayed improved properties (Alarcon et al., 2012; Garcia-Carbonero et al., 2013). However, geldanamycin and its derivatives 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) still exhibited hepatotoxicity that derives from their quinone-structure. In the hepatic metabolism of these drugs, NADPH-cytochrome P450 reductase and NADH cytochrome b5 reductase generate unstable semi-quinones and toxic xenobiotic epoxides (Egorin et al., 1998; Guo et al., 2008). Reaction of the drugs with thiol groups of hepatic glutathione forms further toxic conjugates (Cysyk et al., 2006). This led to the development of a third generation of fully synthetic HSP90 inhibitors that are structurally unrelated to the precursor drugs, including purine scaffold compounds, resorcinylic pyrazole amides, and isoxazole amides. These compounds exhibit improved potency, bioavailability, and application (Lauber et al., 2015). Currently, 17 different HSP90 inhibitors are undergoing clinical evaluation, but so far none of them received approval for clinical use (Neckers and Trepel, 2014). Among the most advanced agents is the resorcinol-based STA-9090 (ganetespib, Synta Pharmaceuticals) that is

Table 1 Overview of HSP90 inhibitors under clinical evaluation.
Table taken from (Lauber et al., 2015).

Classes of HSP90 inhibitors.

Inhibitor class	Compound	Binding site	Inhibitor generation	Clinical studies
Benzoquinone ansamycins	Geldanamycin	N-terminal ATP binding pocket	1st	None
	17-AAG (Tanespimycin, KOS-935, CNF1010)	N-terminal ATP binding pocket	2nd	(advanced) solid tumors, multiple myeloma, kidney tumors in von-Hippel–Lindau disease, chronic lymphatic leukemia (ZAP70 positive), other types of leukemia
	17-DMAG (Alvespimycin, KOS-1022)	N-terminal ATP binding pocket	2nd	(Metastatic) solid tumors, lymphoma, intestinal cancer, B-cell leukemia, breast cancer (HER2/NEU positive)
	IPI-504 (Retaspimycin)	N-terminal ATP binding pocket	2nd	Metastatic melanoma, non-small cell lung cancer, breast cancer, gastrointestinal stromal tumors
	IPI-493	N-terminal ATP binding pocket	2nd	Hematologic malignancies, advanced solid tumors
Macrolide resorcinols	Radicicol	N-terminal ATP binding pocket	1st	None
	KW-2478	N-terminal ATP binding pocket	2nd	Multiple myeloma
	AT13387	N-terminal ATP binding pocket	2nd	(metastatic) solid tumors, breast cancer, melanoma, non-small cell lung cancer, prostate cancer
	STA-9090 (Ganetespib)	N-terminal ATP binding pocket	2nd	Solid tumors, small cell lung cancer, prostate cancer, myeloid leukemia, melanoma, non-small cell lung cancer, breast cancer, esophagogastric cancer, gastrointestinal carcinomas, pancreas cancer, acute myeloid leukemia, chronic myeloid leukemia, myeloproliferative disorders, ovarian cancer, peritoneal cavity cancer, sarcoma, hepatocellular cancer, colon cancer, rectal cancer
	NVP-HSP990	N-terminal ATP binding pocket	2nd	Advanced solid tumors
	NVP-AUY922	N-terminal ATP binding pocket	2nd	(advanced) solid tumors, multiple myeloma, non-small cell lung cancer, myelofibrosis, breast cancer, gastric cancer, gastrointestinal stromal tumors, colorectal cancer, pancreatic cancer
Purine scaffold compounds	BIIB021 (CNF-2024)	N-terminal ATP binding pocket	3rd	Advanced solid tumors, B-cell chronic lymphatic leukemia
	BIIB028	N-terminal ATP binding pocket	3rd	Advanced solid tumors
	PU-H71	N-terminal ATP binding pocket	3rd	(metastatic) solid tumors, lymphoma
	MPC-3100	N-terminal ATP binding pocket	3rd	Cancer patients who failed other treatments
Pyrazole compounds	SNX-5422	N-terminal ATP binding pocket	3rd	Solid tumors, hematopoietic malignancies, lung adenocarcinoma, neuroendocrine tumors, HER2/NEU positive cancers
Other small molecule inhibitors, structurally unrelated	XL888	N-terminal ATP binding pocket	3rd	Solid tumors, melanoma
	Debio-0932	N-terminal ATP binding pocket	3rd	Advanced solid tumors, lymphoma
	DS-2248	N-terminal ATP binding pocket	3rd	Solid tumors, non-small cell lung cancer
Noviosylcoumarin crosslinker	Novobiocin	C-terminal domain	1st	None

currently undergoing phase III evaluation in combination with docetaxel in patients with advanced non-small-cell lung cancer (NCT01798485).

In comparison to geldanamycin and its derivatives, the natural, none-quinone structured radicicol avoids hepatotoxicity, but has weak bioactivity *in vivo* presumably due to lack of stability (Agatsuma et al., 2002). Hence, diverse efforts were undertaken in order to develop derivatives of radicicol with improved stability and pharmacokinetics. Prof. Nicolas Winssinger and his lab generated a library of pochoximes, which are based on the radicicol pharmacophore, identifying novel, highly potent HSP90 inhibitors with improved efficacy at nanomolar concentrations. Pochoxime-derived HSP90 inhibitors have been reported to induce apoptosis and reduce tumor growth in breast cancer xenografts and in a genetically engineered mouse model of glioblastoma multiforme (Barluenga et al., 2009; Barluenga et al., 2008; Wang et al., 2009; Zhu et al., 2010). For the present study, *epi*-pochoxime F (NW457) was used. It is a highly potent HSP90 inhibitor of the pochoxime series (Nexgenix Pharmaceuticals) with an HSP90 binding K_D (dissociation constant) of approximately 14 nM (Karthikeyan et al., 2012).

In general, the best therapeutic success of HSP90 inhibitors is expected in tumors that rely heavily on driver oncogenes requiring HSP90 assistance (e.g. ALK tyrosine kinase receptor (ALK), HER2/NEU, EGFR) and tumors in which HSP90 buffers high levels of proteotoxic stress (e.g. multiple myeloma) (Neckers and Workman, 2012). So far, anti-tumor

efficacy of HSP90 inhibition was moderate when applied as monotherapy. Interestingly, accumulating evidence suggests a synergistic mode of action for HSP90 inhibitors in combination with other anti-cancer therapies, including RT (Butler et al., 2015; Whitesell and Lin, 2012).

1.6 Radiotherapy plus Heat Shock Protein 90 Inhibition

HSP90 inhibition provides a multi-target approach of cancer cell-selective radiosensitization by overcoming intrinsic radioresistance – a complex phenomenon that is coordinated by several regulators involved in pleotropic signaling pathways (e.g. ATM, CHK1, focal adhesion kinase 1 (FAK), EGFR). Some of these molecular drivers are known HSP90 client proteins. Consequently, pharmacological blockade of HSP90 leads to simultaneous depletion of proteins that are essential for the response to IR, particularly DDR regulators and protein kinases. Degradation of DDR regulators results in hampered repair of irradiation-induced DNA damage, which can be measured by delayed or impaired clearance of irradiation-induced γH2AX foci (Camphausen and Tofilon, 2007; Lauber et al., 2015). On the molecular level, HSP90 inhibition compromises the two major repair mechanisms for irradiation-induced DSBs, NHEJ and HR. NHEJ is affected by impaired activation of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), and HR is hampered by proteasomal degradation of the HSP90 client proteins BRCA1, BRCA2, and DNA repair protein RAD51 homolog 1 (RAD51). In addition, the function of central upstream regulators of the DDR is disrupted. Upon HSP90 inhibition, the HSP90 client protein CHK1 lacks folding assistance, and activation of ATM is impaired due to reduced MRN complex-dependent phosphorylation, since MRN's ability to form IR-mediated nuclear foci is compromised (Pennisi et al., 2015). Furthermore, ATR has been reported as a *bona fide* HSP90 client protein, and therefore HSP90 inhibition reduces ATR levels (Ha et al., 2011).

Preclinical studies in different cancer entities revealed an association of HSP90 inhibitor-mediated radiosensitization with impairment of the DDR, disruption of cell cycle arrest, reduction in cellular proliferation, increase in cell death, abrogation of clonogenic survival, and attenuation of tumor growth (Camphausen and Tofilon, 2007; Lauber et al., 2015). However, although the radiosensitizing potential of HSP90 inhibitors has been known for more than a decade, it took several years for the first HSP90 inhibitor (the resorcinol-based AT13387) to enter clinical testing in combination with RT and cisplatin in patients with locoregionally advanced squamous cell carcinoma of the head and neck (NCT02381535).

Interestingly, combination of RT with HSP90 inhibition appears to have even more advantages. HSP90 inhibitors specifically radiosensitize cancer cells, thereby improving the therapeutic width (Kabakov et al., 2010). Furthermore, the underlying synergistic mode of action between RT and HSP90 inhibition seems to provide a better therapeutic outcome with

the same irradiation dose, or a reduced risk of irradiation-induced side effects by allowing the use of lower irradiation doses, respectively. It might also be speculated that RT plus HSP90 inhibition has the potential to enhance tumor cell immunogenicity, since some RT regimes and HSP90 inhibition alone display immunostimulatory potential. However, the impact of the immune system on the efficacy of HSP90-mediated radiosensitization so far is poorly understood (Lauber et al., 2015).

Hence, HSP90 inhibition appears to be a promising partner for RT with regard to reduction of RT-related side effects by overcoming intrinsic radioresistance, improving the therapeutic width, and possibly enhancing tumor cell immunogenicity.

2 Objective

Intrinsic radioresistance is a major cause of radiotherapeutic failure. To overcome this limitation, the aim of the present study was the identification of biomarkers associated with intrinsic radioresistance and their potential pharmacological exploitation in order to improve the therapeutic width. Based on a set of newly identified biomarkers, the efficacy of HSP90 inhibitor-mediated sensitization of radioresistant STSs towards IR was analyzed *in vitro*. Moreover, in models of CRC, HSP90 inhibition was examined with special regard to its radiosensitizing potential, its tolerability, and its capacity to enhance tumor cell immunogenicity in combination with RT *in vitro* and *in vivo*.

3 List of Publications

Publications Included in the Thesis

Ernst, A.*, Anders, H.*, Kapfhammer, H.*, Orth, M., Hennel, R., Seidl, K., Winssinger, N., Belka, C., Unkel, S., and Lauber, K. (2015). HSP90 inhibition as a means of radiosensitizing resistant, aggressive soft tissue sarcomas. Cancer letters 365, 211-222.

Kinzel, L.*, Ernst, A.*, Orth, M.*, Albrecht, V., Hennel, R., Brix, N., Frey, B., Gaipl, U. S., Zuchtriegel, G., *et al.* (2016). A novel HSP90 inhibitor with reduced hepatotoxicity synergizes with radiotherapy to induce apoptosis, abrogate clonogenic survival, and improve tumor control in models of colorectal cancer. Oncotarget 7, 43199-43219.

Additional Publications

Lauber, K., Brix, N., Ernst, A., Hennel, R., Krombach, J., Anders, H., and Belka, C. (2015). Targeting the heat shock response in combination with radiotherapy: Sensitizing cancer cells to irradiation-induced cell death and heating up their immunogenicity. Cancer letters 368, 209-229.

Wunderlich, R., Ernst, A., Rodel, F., Fietkau, R., Ott, O., Lauber, K., Frey, B., and Gaipl, U. S. (2015). Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. Clinical and experimental immunology *179*, 50-61.

Orth, M., Lauber, K., Niyazi, M., Friedl, A. A., Li, M., Maihofer, C., Schuttrumpf, L., Ernst, A., Niemoller, O. M., and Belka, C. (2014). Current concepts in clinical radiation oncology. Radiation and environmental biophysics *53*, 1-29.

Hennel, R., Brix, N., Seidl, K., Ernst, A., Scheithauer, H., Belka, C., and Lauber, K. (2014). Release of monocyte migration signals by breast cancer cell lines after ablative and fractionated gamma-irradiation. Radiation oncology *9*, 85.

Lauber, K., Ernst, A., Orth, M., Herrmann, M., and Belka, C. (2012). Dying cell clearance and its impact on the outcome of tumor radiotherapy. Frontiers in oncology *2*, 116.

Rosenwald, M.*, Koppe, U.*, Keppeler, H., Sauer, G., Hennel, R., Ernst, A., Blume, K. E., Peter, C., Herrmann, M., Belka, C., *et al.* (2012). Serum-derived plasminogen is activated by apoptotic cells and promotes their phagocytic clearance. Journal of immunology *189*, 5722-5728.

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4 Publications

4.1 HSP90 Inhibition as a Means of Radiosensitizing Resistant, Aggressive Soft Tissue Sarcomas

Ernst, A.*, Anders, H.*, Kapfhammer, H.*, Orth, M., Hennel, R., Seidl, K., Winssinger, N., Belka, C., Unkel, S., and Lauber, K. (2015). HSP90 inhibition as a means of radiosensitizing resistant, aggressive soft tissue sarcomas. Cancer letters 365, 211-222.

DOI: 10.1016/j.canlet.2015.05.024

4.2 A Novel HSP90 Inhibitor with Reduced Hepatotoxicity Synergizes with Radiotherapy to Induce Apoptosis, Abrogate Clonogenic Survival, and Improve Tumor Control in Models of Colorectal Cancer

Kinzel, L.*, Ernst, A.*, Orth, M.*, Albrecht, V., Hennel, R., Brix, N., Frey, B., Gaipl, U. S., Zuchtriegel, G., *et al.* (2016). A novel HSP90 inhibitor with reduced hepatotoxicity synergizes with radiotherapy to induce apoptosis, abrogate clonogenic survival, and improve tumor control in models of colorectal cancer. Oncotarget 7, 43199-43219.

DOI: 10.18632/oncotarget.9774

5 Summary and Discussion of Publications

5.1 HSP90 Inhibition as a Means of Radiosensitizing Resistant, Aggressive Soft Tissue Sarcomas

RT is an integral part of the multimodal treatment of STSs (Demetri et al., 2010; Group, 2014; von Mehren et al., 2014). Nonetheless, radiotherapeutic failure commonly occurs as a result of intrinsic tumor radioresistance (Barker et al., 2015; Begg et al., 2011). Since the sensitivity towards irradiation differs considerably among STSs (Rhomberg, 2006), identification of appropriate biomarkers of radioresistance is of high interest – not only in terms of risk assessment and individualization of treatment plans, but also for improvement of RT by combination with radiosensitizing agents that can target those biomarkers (Wong et al., 2014).

The motivation of this study was to identify biomarkers of intrinsic radioresistance that can be used for STS patient stratification and to provide new potential approaches for molecularly targeted radiosensitization. To the best of our knowledge, this is the first systematic study on intrinsic radioresistance in this cancer entity. We analyzed several human STS cell lines regarding their intrinsic radioresistance. Colony formation assays revealed strong differences in clonogenic survival upon IR. The survival data were subjected to linear-quadratic fitting to obtain α/β values as well as to principal component analysis (PCA) to extract scores of radioresistance, both parameters describing radioresistance in a quantitative manner (Franken et al., 2013; Unkel et al., 2016). These analyses separated two clusters of STS cell lines, one with lower and one with higher resistance towards irradiation. Since improved and/or accelerated DNA damage repair is commonly considered to contribute to intrinsic radioresistance (Begg et al., 2011; Bouwman and Jonkers, 2012), we performed transcriptomic profiling by quantitative real-time RT-PCR (qRT-PCR) analyses of DDR regulators in order to find of candidates whose expression levels correlate with radioresistance. The three DDR regulators whose mRNA expression levels revealed the strongest positive correlations with the PCA-derived radioresistance scores were ATM, ATR, and Nijmegen breakage syndrome (NBS1, a member of the MRN complex). The MRN complex is crucial for the recognition of irradiation-induced DSBs and subsequent activation of the protein kinase ATM as well as for the regulation of ATR-mediated processes within the DDR, thereby activating downstream DNA repair mediators (Ciccia and Elledge, 2010). Intriguingly, ATM, ATR, and NBS1 have been reported to be dependent on the folding assistance of the molecular chaperone HSP90, either via direct protein stabilization (in the case of ATR and the MRN complex) or via mechanisms involved in protein activation (in the case of ATM) (Dote et al., 2006; Ha et al., 2011; Pennisi et al., 2015). In line with this, the expression levels of the HSP90 isoform

HSP90AB1 also strongly correlated with the scores of radioresistance. Query of the STS cohort of the Cancer Genome Atlas (TCGA) database revealed that upregulated expression levels of HSP90 (particularly isoform *HSP90AB1*), ATM, ATR, and/or NBS1 are associated with impaired clinical outcome. This underlines the clinical relevance of these candidate biomarkers as well as the potential of their molecular targeting in the context of multimodal RT of STSs.

The results described above encouraged us to examine whether HSP90 inhibition can sensitize STS cell lines to IR, since regulators of the DDR have been reported to be preferentially sensitive to HSP90 inhibition (Sharma et al., 2012). Hence, HSP90 inhibition has emerged as a promising means of radiosensitization (Camphausen and Tofilon, 2007; Kabakov et al., 2010). For our study, we used the novel HSP90-inhibiting pochoxime-derivative NW457 (Barluenga et al., 2009; Barluenga et al., 2008; Karthikeyan et al., 2012; Wang et al., 2009). Pretreatment with low nanomolar concentrations of NW457 for 24 h was sufficient to sensitize radioresistant STS cell lines towards irradiation as revealed by a significant reduction in clonogenic survival. Mechanistically, NW457-mediated radiosensitization was accompanied by a delayed clearance of γH2AX DNA damage repair foci and by a time-dependent downregulation of the crucial DNA damage repair mediators ATM, ATR, CHK1, and RAD51 on the protein level. Abrogation of clonogenic survival has been reported to be mediated by cell cycle arrest and induction of cell death upon HSP90 inhibitor-mediated radiosensitization (Chettiar et al., 2016; Kinzel et al., 2016; Spiegelberg et al., 2015). Indeed, pretreatment with NW457 led to an enhancement of basal and irradiation-induced cellular senescence in radioresistant cell lines. This suggests an involvement of cellular senescence in this context, whereas apoptosis and necrosis were only observed upon treatment with higher concentrations of NW457.

So far, different efforts have been undertaken in order to improve RT of STSs. Very promising strategies appear to be the use of hyperthermia integrated into multimodal therapy concepts and irradiation with carbon ions instead of photons (DeLaney et al., 2005; Jingu et al., 2012; Lindner and Issels, 2011). The efficacy of radiochemotherapy for STSs is controversially discussed, and the risk of subsequently occurring side effects should not be neglected (Curtis et al., 2011; Look Hong et al., 2013). With increasing knowledge of the mechanistic characteristics of STS radioresistance, molecularly targeted agents have been developed. These molecular drugs, which specifically target protein kinases (e.g. VEGFR, mTOR) or HDACs, respectively, have shown potent radiosensitization in preclinical studies, and thus are currently being evaluated in clinical trials (Blattmann et al., 2010; Canter et al., 2014; Murphy et al., 2009; Rao et al., 2014; Wong et al., 2014). As shown here, HSP90 inhibition is a specifically attractive approach for targeted radiosensitization. HSP90 inhibitors have the potential to target multiple HSP90 client proteins that contribute to intrinsic radioresistance at once, and they can simultaneously interfere with the function of other client proteins that are involved in various

oncogenic signaling pathways, including growth factor receptors, downstream signaling mediators, and others (Camphausen and Tofilon, 2007; Kabakov et al., 2010; Lauber et al., 2015).

This first systemic study on intrinsic radioresistance in STSs describes HSP90 inhibition as a promising approach for targeted radiosensitization. Our *in vitro* studies identified ATM, ATR, NBS1, and HSP90 as potential biomarkers for intrinsic radioresistance due to strong positive correlations of their mRNA expression levels with intrinsic STS cell radioresistance. The clinical relevance of our findings is underlined by poor overall survival of patients with aggressive STSs displaying elevated expression levels of HSP90 itself and/or its client proteins ATM, ATR, and/or NBS1. Pharmacological targeting of HSP90 and its client proteins with the novel HSP90 inhibitor NW457 led to preferential radiosensitization of particularly resistant STS cell lines. Mechanistically, induction of cellular senescence but not apoptosis or necrosis appears to be involved.

With respect to the TCGA data, a specific subgroup of STS patients with particularly high expression levels of the identified biomarkers might benefit most from HSP90 inhibitor-mediated radiosensitization. So far, preclinical studies with HSP90 inhibition as monotherapy have reported its efficacy in different histological subtypes of STSs as measured by a reduction in cell proliferation, induction of apoptosis, and improvement of tumor control in xenograft models (Lesko et al., 2007; Smyth et al., 2012; Steinmann et al., 2015). A phase I study with HSP90 inhibitor single treatment has shown a stable disease in 60%-70% of STS patients and a good tolerability (Wagner et al., 2013). However, accumulating evidence suggests that HSP90 inhibition is considerably more powerful when combined with other anti-cancer therapies, including RT, as compared to monotherapy concepts (Butler et al., 2015). This is in accordance with our results showing that HSP90 inhibition has much stronger effects in combination with RT than as mono-agent. In addition, it is feasible to assume that HSP90 inhibitors perform better in combination with RT than specific inhibitors of ATM, ATR, or the MRN complex (if available), respectively, since HSP90 inhibitors can target multiple client proteins involved in the DDR simultaneously. Nevertheless, further studies have to evaluate this assumption in greater depth.

Besides enhancing irradiation-induced cell death and enforcing the reduction in clonogenic survival, HSP90 inhibition has the potential to favor anti-tumor immune mechanisms (Castilleja et al., 2001; Fionda et al., 2009; Haggerty et al., 2014; Kawabe et al., 2009). This is of special interest for the establishment of local and particularly systemic tumor control. Upon HSP90 inhibition, the immunogenicity of cancer cells can be enhanced by increased production and release of DAMPs (Lauber et al., 2015). Furthermore, studies in an *in vivo* sarcoma model provided evidence for HSP90 inhibitor-mediated reprogramming of the tumor microenvironment. This comprised an increase in intratumoral $CD8^+$ T cells and a decrease in

tumor infiltrating immunosuppressive cell subsets, such as MDSCs and Tregs (Rao et al., 2012). Since RT has been reported to stimulate upregulation of immune effectors markers and downregulation of immune suppressor markers in sarcoma samples (Sharma et al., 2013), additional HSP90 inhibition may even be able to boost these immunological effects. Further investigations are needed in order to dissect the immunostimulatory potential of RT plus HSP90 inhibition in general and to elucidate the specific relevance of different modes of cell death in this scenario, particularly that of cellular senescence.

In summary, our study identifies overexpression of HSP90 (particularly isoform *HSP90AB1*) and its client proteins ATM, ATR, and NBS1 as potential biomarkers of radioresistance in aggressive STSs. This special subgroup of STS patients may particularly benefit from molecularly targeted therapy with HSP90 inhibitors in combination with RT.

My contribution to this study:

As shared first author of this publication, I made major contributions to the initiation and design of this study. I planned, designed, and performed experiments. I analyzed and discussed experimental results leading to the next steps in the study. In addition, I discussed and commented on the manuscript. As part of this study, I methodologically supervised Heidi Kapfhammer´s medical doctoral thesis.

In detail, I made the following experimental contributions:

- Experiments on the induction of apoptosis and necrosis upon treatment with different concentrations of NW457 (Fig. 5A,B)
- Colony formation assays for analyses of clonogenic survival of STS cell lines in response to irradiation and additional HSP90 inhibitor pretreatment (Fig. 4A)
- qRT-PCR analyses for transcriptional profiling of DDR regulators (Fig. 2A)

5.2 A Novel HSP90 Inhibitor with Reduced Hepatotoxicity Synergizes with Radiotherapy to Induce Apoptosis, Abrogate Clonogenic Survival, and Improve Tumor Control in Models of Colorectal Cancer

For rectal cancer, RT is commonly applied in neoadjuvant or adjuvant settings within multimodal therapy concepts. In case of colon cancer, it is only occasionally used due to a high degree of colon mobility, considerable radiosensitivity of the surrounding normal tissue, and the corresponding difficulties in designing and delivering proper RT treatment plans (Glimelius, 2002; van de Velde et al., 2014; Willett et al., 1999). Therefore, it is of great interest to find substances that can sensitize CRC cells to IR and thereby can increase the therapeutic width. HSP90 inhibitors have emerged as attractive partners for RT, since HSP90 client

proteins, such as DDR regulators, play essential roles in the cellular response to IR and mediate intrinsic radioresistance (Begg et al., 2011; Maier et al., 2015; Sharma et al., 2012). Whereas preclinical studies have shown a strong synergizing potential of HSP90 inhibitors towards irradiation in several cancer entities, *in vivo* studies are rare and are mainly confined to xenograft models in immunocompromised mice (Kabakov et al., 2010). Moreover, clinical translation of first and second generation of HSP90 inhibitors has been hampered due to suboptimal pharmacological properties and severe hepatotoxicity (Garcia-Carbonero et al., 2013).

The study was designed to examine whether the novel pochoxime-based HSP90 inhibitor NW457, which was developed with the special focus on improved pharmacological properties and tolerability (Barluenga et al., 2009; Barluenga et al., 2008; Karthikeyan et al., 2012; Wang et al., 2009), has the potential to effectively sensitize CRC towards IR *in vitro* and *in vivo*. Our tolerability analyses provided encouraging results as they showed virtually no impairment in primary murine hepatocyte viability *in vitro*. Cell death assays disclosed that pretreatment with NW457 for 24 h synergistically sensitizes human HCT116 CRC cells to irradiation-induced apoptosis over a wide range of irradiation doses and inhibitor concentrations as determined by isobologram analysis and calculation of combination indices (Chou and Talalay, 1984; Niyazi and Belka, 2006). Apoptosis induction was accompanied by activation of caspases 9 and 3, cleavage of caspase substrates, chromatin condensation, and fragmentation of DNA, thus suggesting an involvement of the intrinsic apoptosis signaling pathway in this scenario. Later on, apoptotic cells underwent subsequent secondary necrosis as measured by time course flow cytometric analyses in the presence or absence of the poly-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk). In contrast, addition of the RIP-1 kinase inhibitor necrostatin-1s excluded a relevant contribution of necroptosis in this context, which is in line with other studies reporting interference of HSP90 inhibition with necroptosis induction (Jacobsen et al., 2016; Zhao et al., 2016).

For mechanistic studies on irradiation-induced intrinsic apoptosis with or without additional HSP90 inhibition, two subclones of HCT116 wildtype cells with genetic deficiencies in *TP53* or *BAX* were employed. Although intrinsic apoptosis is considered to be predominately regulated by the tumor suppressor p53 (Belka et al., 2004; Eriksson and Stigbrand, 2010), the *TP53*$^{-/-}$ subclone showed apoptosis levels comparable to those of the HCT116 wildtype cells upon NW457 treatment and irradiation. However, the number of synergistic combinations was reduced. A more detailed examination of pro- and anti-apoptotic proteins suggested a contribution of the pro-apoptotic cell cycle regulator p14^{ARF} in p53-deficient cells. *BAX*$^{-/-}$ cells lacking the pro-apoptotic BCL-2 family member BAX displayed a strong reduction in apoptosis levels with almost no synergistic mode of action at all, thus indicating the dominance of BAX-dependent apoptosis mechanisms upon NW457 treatment in combination with irradiation and

only a minor contribution of BAX-independent mechanisms. Transition into secondary necrosis was reduced in p53-deficient cells as compared to the wildtype controls, and in the BAX-deficient cells secondary necrosis was hardly detectable, which is in accordance with the low levels of apoptosis.

Long-term colony formation assays showed a significant reduction in clonogenic survival of HCT116 cells upon HSP90 inhibition in combination with irradiation, irrespective of the p53 or BAX status. This was accompanied by the degradation of two essential upstream kinases of the DDR, ATM and CHK1, and a delay in DNA damage repair as indicated by impaired clearance of γH2AX foci.

In vivo anti-tumor efficacy of NW457 treatment in combination with RT was analyzed in a syngeneic, heterotopic tumor model. Colorectal CT26 tumor-bearing Balb/c mice were subjected to repeated intraperitoneal administration of 100 mg/kg NW457 or the vehicle control and/or local irradiation with two fractions of 5 Gy. In comparison to the exponentially growing tumors in the vehicle control animals, NW457 delayed tumor expansion in the beginning, but exponential growth was rapidly restored. Irradiation alone revealed a stronger attenuation of tumor growth than NW457 treatment. However, the best tumor control was observed in mice that were subjected to the combined treatment, and Kaplan-Meier analyses disclosed the best tumor-specific survival in animals with the combination treatment. No evidence of toxic alterations upon NW457 treatment was observed in histologic examinations of mouse livers, and serum levels of liver enzymes were only marginally elevated suggesting that NW457 treatment was well-tolerated.

Different approaches have been developed to find radiosensitizing agents to improve the therapeutic width of RT for the treatment of CRCs. Even though neoadjuvant radiochemotherapy with 5-fluorouracil or its oral prodrug capecitabine has become a standard treatment for advanced rectal cancer, the treatment benefits appear to be limited to local tumor control, whereas survival times remain virtually unaffected (Binefa et al., 2014; Sauer et al., 2012). The majority of clinical evaluations of chemotherapeutics (e.g. oxaliplatin) and molecularly targeted drugs (e.g cetuximab, bevacizumab) failed to show benefits in comparison to the standard treatment, while to some extent severe side effects were observed (Dipetrillo et al., 2012; Eisterer et al., 2014; Ricardi et al., 2013). Very rarely, partial or even complete responses were described (Garcia et al., 2015). Since targeted inhibition of HSP90 interferes simultaneously with the function of different client proteins involved in maintaining the malignant phenotype and the repair of irradiation-induced DNA damage (Lauber et al., 2015; Pennisi et al., 2015), it is of high interest to analyze HSP90 inhibitor-mediated sensitization of CRCs towards IR. In this regard, a first phase I study has recently been completed. Here, multimodal treatment of rectal cancer with the HSP90 inhibitor ganetespib and capecitabine in combination with RT (NCT01554969) was examined, but so far no results are available.

In the present study, the novel pochoxime-based HSP90 inhibitor NW457 was shown to potently sensitize CRC cells towards IR. NW457 treatment led to rapid degradation of the two upstream kinases of the DDR, ATM and CHK1, which translated into delayed repair of irradiation-induced DNA damage and reduced clonogenic survival of CRC cells. This is in accordance with other studies in the same as well as in other cancer entities (Chettiar et al., 2016; Dote et al., 2006; Ernst et al., 2015; Moran et al., 2008; Spiegelberg et al., 2015; Stingl et al., 2010). Interestingly, NW457 treatment interfered with DNA damage repair and clonogenic survival independently of the p53 and BAX status. The dispensability of p53 is in line with data from another report in colorectal cancer cell lines in which HSP90 inhibitor-mediated abrogation of irradiation-induced G2 arrest, premature mitosis, and subsequent mitotic catastrophe have been suggested as underlying mechanisms (Moran et al., 2008). However, p53's role in this scenario is still controversially discussed, and further analyses are needed (Fujii et al., 2010; Moran et al., 2008; Shintani et al., 2006).

In terms of apoptosis induction, NW457 treatment in combination with IR revealed a largely synergistic mode of action. Predominantly the intrinsic apoptosis pathway was involved as indicated by caspase 9 cleavage and strongly reduced apoptosis levels in BAX-deficient cells. This is in accordance with other reports in this cancer entity (Azoitei et al., 2012; He et al., 2013; He et al., 2014). Induction of apoptosis upon NW457 treatment in combination with irradiation only partially required p53, since apoptotic cell death was clearly observed in p53-deficient cells. This is of high clinical relevance, since *TP53* is mutated or lost in approximately 50% of all CRCs indicating the potential benefit of NW457 treatment in CRCs lacking functional p53 (Naccarati et al., 2012). Several attempts have been made to explain p53-independent induction of apoptosis in response to DNA damage on the molecular level. On the one hand, $p14^{ARF}$ that was constitutively upregulated in the p53-deficient cells in the present study can stimulate intrinsic apoptosis in a Bcl-2 homologous antagonist/killer (BAK)-dependent manner and in the absence of p53 and BAX (Hemmati et al., 2006). On the other hand, p63 and p73, two close relatives of p53, have been reported to induce intrinsic apoptosis by transactivation of promotors of pro-apoptotic genes encoding for BAX, NOXA, and PUMA (Cai et al., 2012; Flinterman et al., 2005). Whereas apoptosis induction upon treatment with higher concentrations of NW457 was largely BAX-dependent, reduction in clonogenic survival by low nanomolar concentrations of NW457 was virtually independent of BAX. Hence, different concentrations of NW457 appear to trigger different mechanisms of cell death.

The *in vivo* efficacy of HSP90 inhibitor monotherapy has been proven in models of CRC (He et al., 2014; Powers et al., 2013). However, studies of the therapeutic efficacy of HSP90 inhibition in combination with IR so far are limited to xenograft models and other cancer entities (Bisht et al., 2003; Bull et al., 2004; Chettiar et al., 2016; Yin et al., 2010; Zaidi et al., 2012). In these models, the combination treatment has been reported to be superior to the

monotherapies as measured by tumor growth control and survival. Our study analyzes for the first time HSP90 inhibitor-mediated radiosensitization in a syngeneic murine CRC model. HSP90 inhibition with NW457 in combination with irradiation showed significantly improved therapeutic efficacy as measured by tumor growth and tumor-specific survival in comparison to the monotherapies. It is worth mentioning that the murine CT26 CRC cell line utilized in this model carries an activating mutation in the proto-oncogene *KRAS* as reported for up to 50% of all CRCs, thereby strengthening the clinical relevance of this treatment approach (Bazan et al., 2005). Moreover, NW457, which was developed with special focus on improved water solubility, bioavailability, and reduced liver toxicity, showed no evidence of toxic alterations in liver histology and only slight increases in serum levels of liver enzymes.

In summary, this study characterizes HSP90 inhibition by the novel pochoxime-derivative NW457 as a promising combined modality approach with RT for the treatment of CRC on the basis of its good *in vivo* efficacy and tolerability.

My contribution to this study:

My shared first authorship results from the major contributions I made to the design of this study and its experiments. Besides performing a large proportion of the experiments, I evaluated, interpreted, and discussed the results. Furthermore, I corrected, discussed, and gave scientific input to the manuscript.

Experimentally, I contributed the following:

- Measurements of apoptotic and necrotic cell death in different cell lines by flow cytometry (Fig. 2E-G, Fig. 4D-I, Suppl. Fig. 4A,B, Suppl. Fig. 5A,C)
- Western blot analyses of caspase and caspase substrate cleavage and enzymatic assays for determination of caspase activity (Fig. 3A,B,D, Fig. 4A)
- Measurements of liver enzyme serum levels and preparation of mouse livers for histopathologic analyses (Fig. 6D,E)
- Cell cycle analyses (Suppl. Fig., 1G,H)

5.3 Additional Unpublished Data

Accumulating evidence suggests that the efficacy of RT relies, apart from the induction of DNA damage resulting in cell cycle arrest and cell tumor death, on the induction of specific adaptive anti-tumor immune responses that contribute to tumor control (Frey et al., 2014; Lauber et al., 2015). This contribution of the immune system appears to be particularly relevant in the context of irradiation regimes with high, ablative doses. However, for several cancer entities, including CRC, ablative doses cannot be used due to safety concerns. Instead, RT is performed using conventionally fractionated (1.8-2 Gy per fraction) or hypofractionated (2.66-5.0 Gy per fraction) RT strategies (Binefa et al., 2014; Glimelius, 2002). Immunostimulatory

effects of these RT regimes are currently under investigation and are controversially discussed (Filatenkov et al., 2015; Kulzer et al., 2014). Since HSP90 inhibition has been considered to favor anti-tumor immune mechanisms (Alarcon et al., 2012; Lauber et al., 2015), it is of high interest whether HSP90 inhibition can enforce anti-tumor immune responses of non-ablative, hypofractionated irradiation doses in CRC. Although HSP90 inhibitors have been shown to be potent radiosensitizers in CRC models (He et al., 2014; Spiegelberg et al., 2015), the immunological potential of HSP90 inhibition-mediated radiosensitization has not been studied so far.

Therefore, this study focused on elucidating the immunogenicity of irradiation-induced colorectal cancer cell death with or without additional HSP90 inhibition in the sense of an *in situ* vaccination strategy. The initial steps required for stimulating systemic anti-tumor immune responses in this context are characterized by the release of immunostimulatory DAMPs and tumor antigens via induction of immunogenic forms of tumor cell death (Chen and Mellman, 2013; Kroemer et al., 2013). *In vitro*, high concentrations of NW457 resulted in increased and accelerated irradiation-induced apoptosis and subsequent secondary necrosis of human HCT116 CRC cells (Figure 6A) (Kinzel et al., 2016). This was accompanied by an augmented release of DAMPs, including HMGB1, HSP70, and ATP into the culture supernatants as measured by enzyme-linked immunosorbent assay (ELISA) and luciferase assays (Figure 6B). Hence, HSP90 inhibition apparently can shift irradiation-induced cell death towards more immunogenic forms with increased DAMP release.

Extracellular DAMPs can stimulate the attraction of monocytes which then can differentiate into DCs – the central players in the induction of adaptive anti-tumor immune responses (Lauber et al., 2012). *In vitro*, cell-free supernatants derived from NW457-treated and irradiated HCT116 cells potently attracted monocytic THP-1 cells in transwell migration assays (Figure 6A). To investigate which form of cell death was crucial for the release of monocyte attraction signals, different genetic subclones of HCT116 cells were utilized. These subclones showed clear differences in the mode of cell death induced by HSP90 inhibition in combination with irradiation. Whereas the parental cells ($BAX^{+/+}$, $KRAS^{+/-}$) first underwent apoptosis and then transited into secondary necrosis, the BAX-deficient subclone displayed limited apoptosis induction and very little secondary necrosis (Figure 6C) (Zhang et al., 2000). Interestingly, *KRAS* wildtype cells underwent apoptosis to a comparable extent as the parental cells (Shirasawa et al., 1993). However, they did not transit into secondary necrosis. In turn, only cell-free supernatants of secondary necrotic HCT116 wildtype cells attracted monocytic cells upon combination treatment. No relevant monocyte migration was observed in response to supernatants of the other subclones, suggesting that secondary necrosis is essential for the release of monocyte attracting DAMPs. This was further confirmed by analyses in the presence

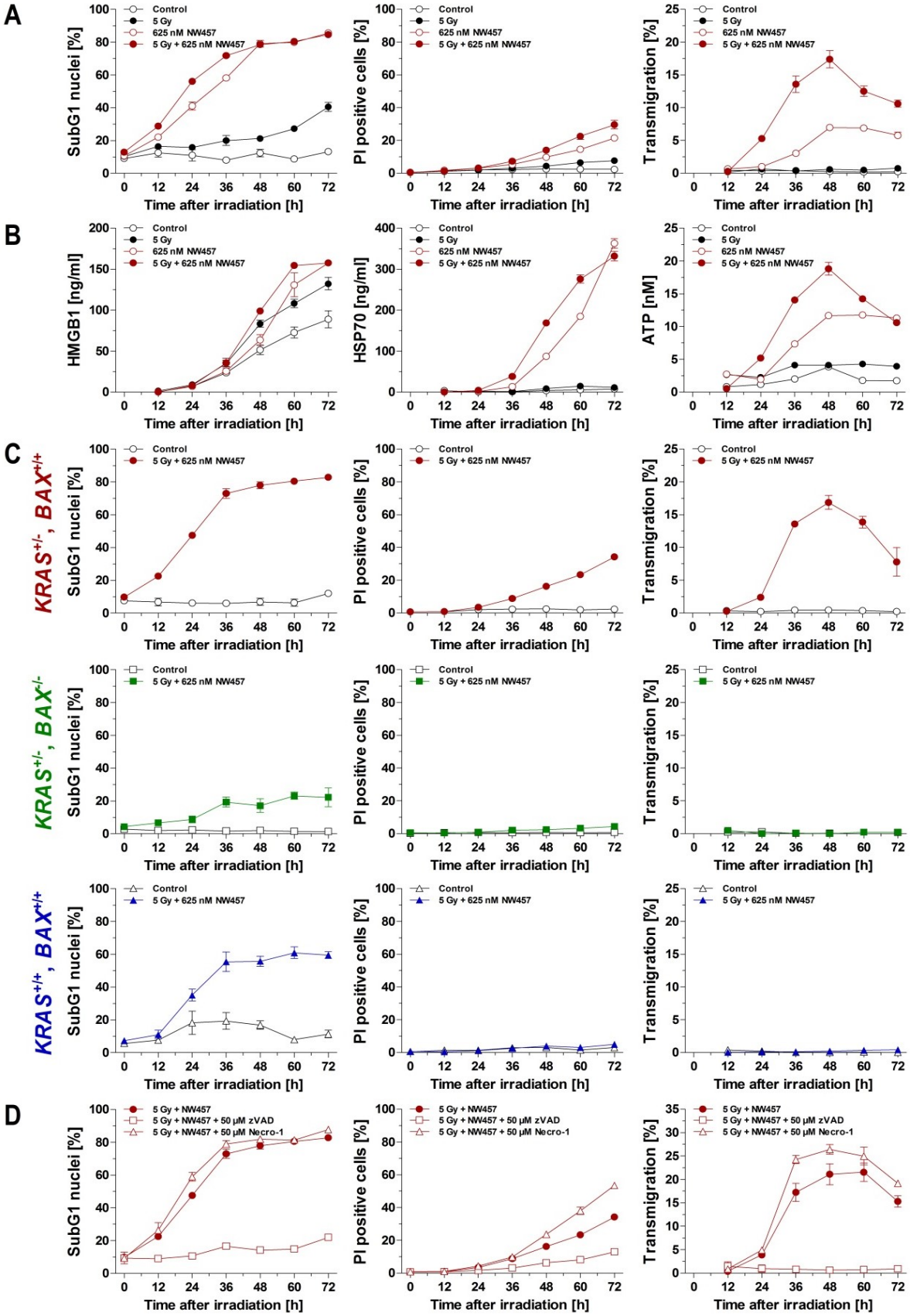
A
B
C
D
KRAS+/-, BAX+/+
KRAS+/-, BAX-/-
KRAS+/+, BAX+/+
Control
5 Gy
625 nM NW457
5 Gy + 625 nM NW457
5 Gy + NW457
5 Gy + NW457 + 50 µM zVAD
5 Gy + NW457 + 50 µM Necro-1
SubG1 nuclei [%]
PI positive cells [%]
Transmigration [%]
HMGB1 [ng/ml]
HSP70 [ng/ml]
ATP [nM]
Time after irradiation [h]

Figure 6 HSP90 inhibition shifts ionizing radiation-induced colorectal cancer cell death towards more immunogenic forms with increased DAMP release.
(A) Induction of apoptosis and necrosis, and transwell migration of THP-1 cells towards cell-free culture supernatants of irradiated and NW457-treated HCT116 cells. Cells were irradiated at 0-5 Gy and concomitantly treated with 0-625 nM NW457. At the indicated time points, induction of apoptosis and necrosis was assessed by flow cytometric measurements of subG1 nuclei or propidium iodide exclusion staining, respectively. Harvested cell-free supernatants were subjected to transwell migration assays with monocytic THP-1 cells. Means ± s.d. of quadruplicates are shown. (B) Time-dependent release of HMGB1, HSP70, and ATP from irradiated and NW457-treated HCT116 cells. Cells were treated as in (A). In cell-free supernatants, HMGB1 and HSP70 were quantified by ELISA, and ATP was measured by luciferase assay. Means ± s.d. of triplicates are depicted. (C) Induction of apoptosis, necrosis, and transwell migration of THP-1 cells towards cell-free culture supernatants of irradiated and NW457-treated HCT116 wildtype cells (heterozygous *KRAS* mutation) and the *BAX*$^{-/-}$ subclone or the wildtype *KRAS* subclone. Cells were left untreated or irradiated at 5 Gy and treated with 625 nM NW457. At the indicated time points, apoptosis and necrosis induction as well as transwell migration of THP-1 cells towards cell-free supernatants were analyzed as in (A). Means ± s.d. of quadruplicates are shown. (D) Relevance of secondary necrosis or necroptosis for transwell migration of THP-1 cells. Induction of apoptosis and necrosis, and transwell migration of THP-1 cells were measured as in (A) in the presence or absence of the poly-caspase inhibitor zVAD-fmk and the necroptosis inhibitor necrostatin-1. Means ± s.d. of quadruplicates are displayed.

or absence of the poly-caspase inhibitor zVAD-fmk and the necroptosis inhibitor necrostatin-1. Upon addition of zVAD-fmk, induction of apoptosis was virtually abrogated resulting in very low levels of subsequent secondary necrosis and no detectable transwell migration of THP-1 cells (Figure 6D). In contrast, addition of necrostatin-1 affected the induction of secondary necrosis or THP-1 cell migration only marginally. Accordingly, secondary necrosis appears to be the decisive mode of cell death that is required for the release of monocyte attraction signals in this scenario.

A characterization of the monocyte attraction signals derived from secondary necrotic cells upon HSP90 inhibition in combination with irradiation was performed with cell-free supernatants of HCT116 cells harvested 48 h after treatment (which revealed the highest migratory potential). After subjection of the supernatants to heat-treatment, filtration (MW cut-off 10 kDA), and enzymatic digestion with apyrase (ATP diphosphohydrolase) or proteinase K (serine protease), respectively, the remaining migratory potential was analyzed. ATP (MW = 507 Da, apyrase-sensitive) and stromal cell-derived factor 1-alpha (SDF-1α) (MW = 11 kDa, apyrase-insensitive) served as controls. Our data indicate that the majority of attraction signals are heat-stable, smaller than 10 kDa, and sensitive to apyrase treatment. Hence, the factors are apparently nucleotides (Figure 7A). Time-lapse video microscopy with 2D chemotaxis assay slides were performed to characterize the nature of the migratory response: chemotaxis (directional movement of cells towards a stimulus) or chemokinesis (non-directional movement in response towards a stimulus). As shown in Figure 7B, supernatants of HSP90 inhibitor-

treated HCT116 cells induced chemokinesis rather than chemotaxis. This was observed already with NW457 monotherapy, and it was even more enhanced when cells were additionally irradiated. For comparison, purified ATP was used as a chemokinetic stimulus, and WKYMVm (an agonist of formyl-peptide receptors 1, 2, and 3) served as prototypical chemotactic stimulus. Digestion of the supernatants with apyrase again confirmed the importance of nucleotides in the migratory response (Figure 7C). Therefore, it can be concluded that NW457 treatment in combination with irradiation induces the release of apyrase-sensitive nucleotides from secondary necrotic CRC cells, which are able to stimulate monocyte migration *in vitro*.

The question that arises at this point is whether the non-directional migratory response of monocytes observed *in vitro* can be converted into directional recruitment of monocytes *in vivo*. In a first approach to address this question, the recruitment of leukocyte subsets by supernatants of untreated or combinedly treated HCT116 cells was examined in two *in vivo* surrogate models. In the peritonitis model, intraperitoneal injection of conditioned supernatants from combinedly treated HCT116 cells stimulated infiltration of the following myeloid leukocyte subsets to a significantly stronger extent than conditioned supernatants from untreated control cells: Inflammatory monocytes (Gr-1^{hi}F4/80^{hi} & Gr-1^{hi}F4/80^{int}), macrophage-like cells (Gr-1^{low}F4/80^{hi}), and neutrophils (Gr-1^{hi}F4/80^{low}) (Figure 7D). In analogy, significantly increased adhesion and extravasation of leukocytes and CX3C chemokine receptor 1 (CX3CR1)-expressing monocytic cells from postcapillary venules were observed in the cremaster model upon intrascrotal injection of conditioned supernatants from combinedly treated HCT116 cells (Figure 7E). Inflammatory monocytes express low levels and resident monocytes high levels of CX3CR1 (Soehnlein et al., 2009). CX3CR1-expressing DCs were excluded from the analyses on the basis of their characteristic stellate morphology.

Encouraged by these positive *in vivo* results, intratumoral recruitment of different leukocyte subsets was examined in a heterotopic, syngeneic colorectal tumor model upon irradiation and/or HSP90 inhibitor treatment. Murine CT26 CRC cells were subcutaneously inoculated into the right flanks of Balb/c mice. Tumors were allowed to grow for 7 days and tumor-bearing mice were randomly distributed into four treatment groups: Vehicle (dimethyl sulfoxide (DMSO)) only, 2x 5 Gy + vehicle, NW457 only, and 2x 5 Gy + NW457. 100 mg/kg of NW457 or DMSO were administered intraperitoneally on days 7 and 10 after tumor implantation. Local irradiation was performed on days 8 and 11. Mice were sacrificed for analysis of intratumoral leukocyte infiltration on day 14. Tumor volume measurements revealed exponential tumor growth in the vehicle controls, whereas NW457 slightly and irradiation strongly delayed tumor growth (Figure 7F). Importantly, the best tumor growth control was observed in mice receiving the combination treatment, thus confirming our previous observations (Kinzel et al., 2016). Our flow cytometry measurements show that irradiation stimulated a strong intratumoral accumulation of $CD45^{+}$ leukocytes that was even increased by additional HSP90 inhibition.

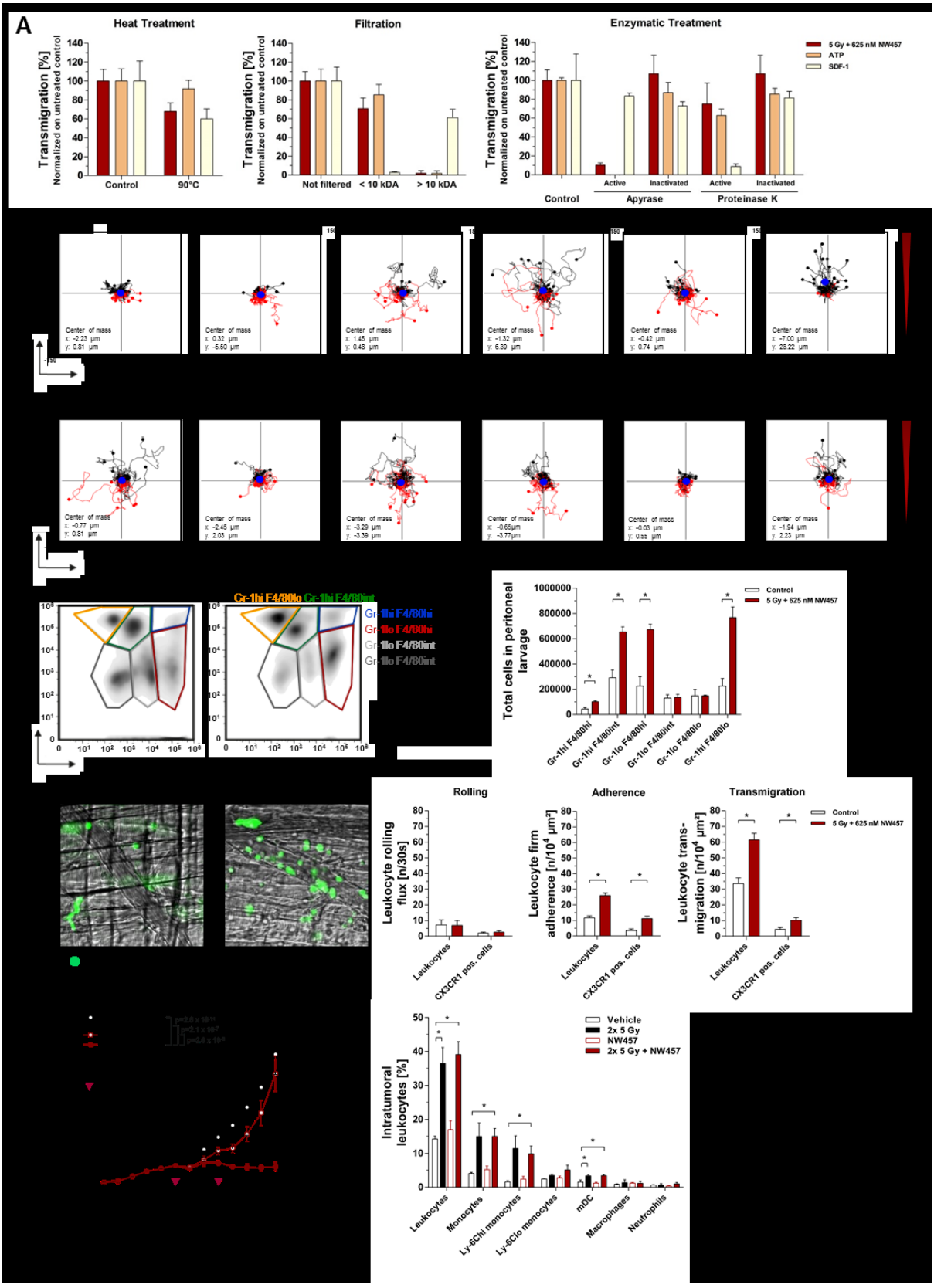
A
Heat Treatment
Filtration
Enzymatic Treatment
Transmigration [%]
Normalized on untreated control
Control
90°C
Not filtered
< 10 kDA
> 10 kDA
Active
Inactivated
Apyrase
Proteinase K
5 Gy + 625 nM NW457
ATP
SDF-1
Center of mass
Gr-1hi F4/80lo
Gr-1hi F4/80hi
Gr-1lo F4/80hi
Gr-1lo F4/80int
Gr-1lo F4/80lo
Total cells in peritoneal larvage
Control
5 Gy + 625 nM NW457
Rolling
Adherence
Transmigration
Leukocyte rolling flux [n/30s]
Leukocyte firm adherence [n/10⁴ µm²]
Leukocyte trans-migration [n/10⁴ µm²]
Leukocytes
CX3CR1 pos. cells
Intratumoral leukocytes [%]
Vehicle
2x 5 Gy
NW457
2x 5 Gy + NW457
Monocytes
Ly-6Chi monocytes
Ly-6Clo monocytes
mDC
Macrophages
Neutrophils

Figure 7 Secondary necrotic colorectal cancer cells release DAMPs that stimulate chemokinesis of monocytes *in vitro* and induce recruitment of monocytes and DCs *in vivo*. (A) Characterization of monocyte attraction signals. HCT116 cells were treated with 5 Gy and 625 nM NW457 and incubated for 48 h. Migratory capacity of ultracentrifugated (42000 G, 143 min, 4°C) cell- and apoptotic microbleb-free supernatants was determined after heat-treatment, ultrafiltration (MW cut-off 10 kDA), or enzymatic digestion for 30 min at 30°C with active and heat-inactivated proteinase K (3 U/ml) or apyrase (50 mU/ml), respectively, in transwell migration assays with THP-1 cells. Culture media supplemented with ATP (200 nM) and SDF-1α (200 ng/ml) severed as controls. The percentage of transmigrated THP-1 cells was normalized on the untreated samples or control stimuli. Means + s.d. from quadruplicates are shown. (B) Induction of chemotaxis and chemokinesis in primary human monocytes. Conditioned supernatants of HCT116 cells were generated as in (A), and stimulation of chemotaxis and chemokinesis in primary human monocytes was analyzed by live cell tracking in 2D-chemotaxis chambers for 3 h (analysis window 10 min - 160 min). ATP (1 µM) and WKYMVm (1 µg/ml) served for comparison. Trajectory paths of 40 randomly picked single cells and resulting centers of mass (filled blue circles) are displayed. (C) Characterization of chemokinesis inducing factors. Conditioned supernatants of HCT116 cells were harvested as in (B) and were digested for 30 min at 30°C with active or heat-inactivated apyrase (50 mU/ml). Chemokinesis was analyzed as in (B) with purified ATP (1 µM) as a control. (D) Recruitment of myeloid leukocyte subsets in the peritonitis model. Conditioned supernatants harvested as in (B) were intraperitoneally injected into C57BL/6 mice. After 6 h of stimulation, myeloid leukocytes ($CD45^+$ $CD11b^+$ cells) recruited into the peritoneum were characterized by flow cytometry. Representative plots of two mice are shown (*left panel*). Quantification of recruited leukocytes: Inflammatory monocytes ($Gr\text{-}1^{hi}F4/80^{hi}$, $Gr\text{-}1^{hi}F4/80^{int}$), macrophage-like cells ($Gr\text{-}1^{low}F4/80^{hi}$, $Gr\text{-}1^{low}F4/80^{int}$, $Gr\text{-}1^{low}F4/80^{low}$), and neutrophils ($Gr\text{-}1^{hi}F4/80^{low}$) (*right panel*). Means + s.e.m. are shown (n=4 per group). Asterisks indicate $p<0.05$ as calculated by unpaired Student's *t*-test to supernatants from untreated control cells. (E) Extravasation of leukocytes in the cremaster model. Conditioned supernatants harvested as in (B) were intrascrotally injected into $CX3CR1^{GFP/+}$ mice. After 6 h of stimulation, microscopy images of postcapillary venules are shown for two representative mice (*left panel*), and quantification of intravascular rolling, firm adherence, and transmigration of leukocytes and CX3CR1-positive monocytic cells is displayed (*right panel*). Means + s.e.m. are depicted (n=6 per group). Asterisks indicate $p<0.05$ as determined by unpaired Student's *t*-test to supernatants from untreated control cells. (F) Analysis of intratumoral recruitment of leukocytes in a syngeneic, heterotopic mouse model. $1.2x\ 10^6$ murine CT26 CRC cells were subcutaneously inoculated into the right flanks of Balb/c mice and allowed to grow for 7 days. Mice were randomly distributed into four treatment groups (n=5 per each treatment group): Vehicle (DMSO) only, 2x 5 Gy + vehicle, NW457 only, and 2x 5 Gy + NW457. 100 mg/kg of NW457 or DMSO were administered intraperitoneally on days 7 and 10 (red arrowheads), and local irradiation was performed on days 8 and 11 (black arrowheads). On day 14, mice were sacrificed and tumors were explanted for flow cytometric analysis of leukocyte infiltration. Tumor volumes are displayed as percentage of the respective starting volume on day 7 (*left panel*). Means ± s.e.m are depicted and two-way ANOVA was used for overall comparison of the curves. Flow cytometric data of intratumoral leukocyte subsets are shown as the percentage of all cells following doublet exclusion (*right panel*). Single cell suspensions of the tumors were stained against surface markers of leukocytes for quantification of the following subsets: Leukocytes ($CD45^+$), all monocytes, inflammatory monocytes ($Ly\text{-}6C^{hi}$), resident/maturing monocytes ($Ly\text{-}6C^{low}$), myeloid DCs ($F4/80^+$MHC II $(I\text{-}A/I\text{-}E)^{hi}$), macrophages ($Ly\text{-}6G^-F4/80^+$), and neutrophils ($Ly\text{-}6G^+F4/80^{low}$). Means + s.e.m. are depicted, and asterisks indicate $p<0.05$ as calculated by unpaired Student's *t*-test to the vehicle controls.

Among the $CD45^+CD11b^+$ myeloid leukocytes, inflammatory monocytes (lymphocyte antigen (Ly)-$6C^{hi}$) were the dominant population followed by Ly-$6C^{lo}$ monocytes, which either can be resident monocytes or recruited monocytes in the process of differentiation. Importantly, enhanced intratumoral infiltration of Ly-$6C^{hi}$ monocytes was already observed upon irradiation alone, but only after additional HSP90 inhibition statistical significance was reached. Interestingly, intratumoral numbers of Ly-$6C^{lo}$ monocytes and myeloid DCs (mDCs) expressing MHC II (I-A/I-E) showed basically the same trend although the absolute numbers were much lower. Hence, HSP90 inhibition obviously can improve irradiation-mediated tumor control accompanied by enhanced intratumoral infiltration of leukocytes – in particular Ly-$6C^{hi}$ and Ly-$6C^{lo}$ monocytes as well as mDCs.

Tumor infiltrating immune cells are known to affect the clinical outcome of CRC. In this regard, T cell infiltration into tumors has been reported as a biomarker for the treatment response, which led to the development of a predictive 'immunoscore' (Perez-Ruiz and Berraondo, 2016). Therefore, therapeutic strategies with the aim of enhancing the immunological potential of RT in CRC are of special interest (Derer et al., 2015). One mechanism how anti-tumor therapies can elicit specific adaptive anti-tumor immune responses is the induction of immunogenic forms of cell death with the concomitant release of DAMPs (Bezu et al., 2015; Galluzzi et al., 2015). The present study demonstrates for the first time that the novel HSP90 inhibitor NW457 can shift the form of irradiation-induced cell death towards more immunogenic secondary necrosis with concurrent release of DAMPs leading to attraction of monocytes *in vitro* and *in vivo*. Furthermore, control of tumor growth upon NW457 treatment in combination with irradiation was accompanied by increased intratumoral levels of leukocytes, in particular monocytes and mDCs.

Tumor cells undergoing therapy-induced apoptosis may be insufficiently cleared resulting in increased secondary necrosis. This happens especially when the load of dying cells is too high and overwhelms the phagocytic compartment. In contrast to apoptosis, post-apoptotic, secondary necrosis is considered as an immunogenic form of cell death. Due to the rupture of the plasma membrane, several immunogenic DAMPs are released and can contribute to the stimulation of anti-tumor immune responses (Garg et al., 2013; Silva, 2010). In the present study, several DAMPs were observed to be liberated upon HSP90 inhibition in combination with irradiation. For instance, HMGB1, HSP70, and ATP were released from dying colorectal cancer cells upon the combined treatment in a time-dependent manner and in parallel to the onset of secondary necrosis (data not shown). Extracellular DAMPs may promote tumor immunogenicity potentially resulting in the stimulation of systemic anti-tumor immune responses (Deloch et al., 2016; Garg et al., 2015). For instance, the DNA chaperoning protein HMGB1 binds – among others – to TLR-4 on DCs and promotes processing and cross-presentation of tumor-associated antigens to T cells, thereby mediating T cell activation

(Apetoh et al., 2007; Tesniere et al., 2008). HSP70 has a dual role in cancer biology. The intracellular chaperoning function of HSP70 promotes tumor progression by its anti-apoptotic properties. Membrane-bound and extracellular HSP70, in contrast, link innate and adaptive immune responses by carrying immunogenic tumor-derived peptides and by directly activating immune cells as a natural immunogen, thereby promoting maturation of DCs (Joly et al., 2010; Shevtsov and Multhoff, 2016). ATP released upon anti-cancer therapy binds to purinergic P2X7 receptors on DCs and mediates DC activation via the inflammasome. This promotes the secretion of the pro-inflammatory interleukin (IL)-1β that is required for the efficient priming of tumor-specific IFN-γ producing $CD8^+$ T cells (Aymeric et al., 2010; Ghiringhelli et al., 2009).

IR can stimulate immunogenic forms of cell death and consequently induce the release of DAMPs, but this clearly depends on the RT regime applied and intrinsic characteristics of the tumor cells (Demaria and Formenti, 2012; Formenti and Demaria, 2013). Therefore, different approaches have been utilized to boost the immunogenic potential of irradiation-induced cell death upon treatment with moderate doses of 5 Gy in other colorectal cancer models. Addition of chemotherapeutics has been observed to shift irradiation-induced colorectal cancer cell death towards higher levels of apoptosis, necrosis, and possibly also necroptosis accompanied by the release of HMGB1 and HSP70 and, in turn, maturation of DCs *in vitro* (Frey et al., 2012). Hyperthermia in combination with RT can enforce the induction of necrotic cell death and the release of HMGB1 and HSP70 (Mantel et al., 2010; Schildkopf et al., 2010; Schildkopf et al., 2011). In this context, HSP70 was identified as key driver of DC maturation and expression of the homing receptor CCR7. Interestingly, hyperthermia shares a common feature with HSP90 inhibition: The induction of the heat shock response (Lauber et al., 2015). Thus, it is tempting to speculate that HSP90 inhibition may induce similar immunomodulatory effects that enhance the immunogenicity of irradiation-induced CRC cell death, especially in regard to the observed release of HMGB1 and HSP70 from secondary necrotic tumor cells upon combined treatment.

Circulating leukocytes have to infiltrate the tumor in order to contribute to tumor control. It has been reported that the intratumoral recruitment of APCs originating from inflammatory monocytes of the peripheral blood ($CD45^+CD11b^+Gr\text{-}1^{hi}$) and their production of type I IFNs is of vital importance for the treatment efficacy of RT with ablative doses (Burnette et al., 2011; Lim et al., 2014). In our study, addition of NW457 enforced the capacity of CRC cells that were irradiated with non-ablative doses of 5 Gy to attract monocytes *in vitro*. In this scenario, secondary necrosis, which was only observed in HCT116 wildtype cells carrying a heterozygous *KRAS* mutation, was of essential importance. Mutant KRAS appears to be involved in the induction of this form of cell death and/or the associated release of DAMPs. Hence, secondary necrosis may not be as accidental as it currently still is considered to be.

Central regulators of monocyte attraction were identified as apyrase-sensitive nucleotides derived from secondary necrotic colorectal cancer cells upon HSP90 inhibition in combination with RT. Several studies have already demonstrated a role of dying cell-derived nucleotides in the recruitment of monocytes, macrophages, and DCs via ligation of P2Y purinoceptor 2 (P2RY2) (Elliott et al., 2009; Ghiringhelli et al., 2009; Ma et al., 2013a). Whether nucleotides act as chemoattractants for immune cells *per se* or rather function as auto- and/or paracrine amplifiers of other chemotactic stimuli is currently under discussion (Hennel et al., 2014; Isfort et al., 2011; Kronlage et al., 2010). In the present study, chemokinesis but not chemotaxis was observed *in vitro*. However, cell-free supernatants of irradiated and NW457-treated HCT116 cells were well able to stimulate recruitment of neutrophils and/or monocytic cells subsets in the peritonitis and the cremaster model *in vivo*. An interesting question is how the chemokinetic signals can be converted into directional recruitment of leukocytes *in vivo*. In models of laser injury, this conversion was reported to be facilitated by upregulation of adhesion molecules and inflammatory cytokines on endothelial cells and pericytes, and it is feasible to assume that similar mechanisms might also be functional in our model (McDonald et al., 2010; Pittman and Kubes, 2013; Stark et al., 2013). Additionally, the initial wave of recruited neutrophils that appears approximately 6 h after intraperitoneal injection of supernatants from combinedly treated colorectal cancer cells may contribute to monocyte recruitment in a second wave, since neutrophil-mediated monocyte recruitment to sites of sterile inflammation has been described (Soehnlein et al., 2009).

In our syngeneic, heterotopic colorectal tumor model, NW457 treatment in combination with irradiation led to significantly increased intratumoral numbers of inflammatory monocytes ($CD11b^{+}Ly\text{-}6C^{hi}$) and mDCs ($F4/80^{+}$MHC II $(I\text{-}A/I\text{-}E)^{hi}$) 7 days after treatment begin. Of note, this was accompanied by the strongest attenuation of tumor growth control, and further studies have to elucidate how far this is causally connected. Intratumoral inflammatory monocytes may be of special importance for improving irradiation-mediated tumor control, since inflammatory monocytes can differentiate into monocyte-derived DCs, which can activate adaptive immune responses as mDCs (Shi and Pamer, 2011). Recruitment of inflammatory monocytic cells has already been reported as a critical step in anti-tumor therapy with agents inducing immunogenic forms of cell death. Upon anthracycline treatment, nucleotides derived from dying tumor cells stimulated intratumoral recruitment of $CD11c^{+}CD11b^{+}Ly\text{-}6C^{hi}$ monocytic cells as well as their activation, differentiation, and intratumoral survival. These monocytic cells can differentiate into potent APCs, which engulf dying cancer cells and present tumor antigens to T cells (Ma et al., 2013a; Ma et al., 2013b).

Collectively, our results suggests that HSP90 inhibition can enforce the immunogenicity of irradiation-induced CRC cell death via stimulation of immunogenic forms of cell death and attraction of monocytes *in vitro* and i*n vivo*. Further studies have to elucidate if and to which

extent this enhanced tumor immunogenicity together with the recruitment of distinct immune cell subpopulations contribute to local and systemic tumor control in this scenario.

6 References

https://www.picard.ch/downloads/HSP90interactors.pdf as of 2016-08-07

Agatsuma, T., Ogawa, H., Akasaka, K., Asai, A., Yamashita, Y., Mizukami, T., Akinaga, S., and Saitoh, Y. (2002). Halohydrin and oxime derivatives of radicicol: synthesis and antitumor activities. Bioorg Med Chem *10*, 3445-3454.

Aghagolzadeh, P., and Radpour, R. (2016). New trends in molecular and cellular biomarker discovery for colorectal cancer. World J Gastroenterol *22*, 5678-5693.

Ahn, D. H., Ciombor, K. K., Mikhail, S., and Bekaii-Saab, T. (2016). Genomic diversity of colorectal cancer: Changing landscape and emerging targets. World J Gastroenterol *22*, 5668-5677.

Alarcon, S. V., Mollapour, M., Lee, M. J., Tsutsumi, S., Lee, S., Kim, Y. S., Prince, T., Apolo, A. B., Giaccone, G., Xu, W., *et al.* (2012). Tumor-intrinsic and tumor-extrinsic factors impacting hsp90- targeted therapy. Current molecular medicine *12*, 1125-1141.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung, B. Y., and Aggarwal, B. B. (2008). Cancer is a Preventable Disease that Requires Major Lifestyle Changes. Pharm Res-Dord *25*, 2097-2116.

Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., Mignot, G., Maiuri, M. C., Ullrich, E., Saulnier, P., *et al.* (2007). Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature medicine *13*, 1050-1059.

Aymeric, L., Apetoh, L., Ghiringhelli, F., Tesniere, A., Martins, I., Kroemer, G., Smyth, M. J., and Zitvogel, L. (2010). Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. Cancer research *70*, 855-858.

Azoitei, N., Hoffmann, C. M., Ellegast, J. M., Ball, C. R., Obermayer, K., Gossele, U., Koch, B., Faber, K., Genze, F., Schrader, M., *et al.* (2012). Targeting of KRAS mutant tumors by HSP90 inhibitors involves degradation of STK33. The Journal of experimental medicine *209*, 697-711.

Bae, J., Mitsiades, C., Tai, Y. T., Bertheau, R., Shammas, M., Batchu, R. B., Li, C., Catley, L., Prabhala, R., Anderson, K. C., and Munshi, N. C. (2007). Phenotypic and functional effects of heat shock protein 90 inhibition on dendritic cell. Journal of immunology *178*, 7730-7737.

Barker, H. E., Paget, J. T., Khan, A. A., and Harrington, K. J. (2015). The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nature reviews Cancer *15*, 409-425.

Barluenga, S., Fontaine, J. G., Wang, C., Aouadi, K., Chen, R., Beebe, K., Neckers, L., and Winssinger, N. (2009). Inhibition of HSP90 with pochoximes: SAR and structure-based insights. Chembiochem : a European journal of chemical biology *10*, 2753-2759.

Barluenga, S., Wang, C., Fontaine, J. G., Aouadi, K., Beebe, K., Tsutsumi, S., Neckers, L., and Winssinger, N. (2008). Divergent synthesis of a pochonin library targeting HSP90 and in vivo efficacy of an identified inhibitor. Angewandte Chemie *47*, 4432-4435.

Barrott, J. J., and Haystead, T. A. (2013). Hsp90, an unlikely ally in the war on cancer. The FEBS journal *280*, 1381-1396.

Barrott, J. J., Hughes, P. F., Osada, T., Yang, X. Y., Hartman, Z. C., Loiselle, D. R., Spector, N. L., Neckers, L., Rajaram, N., Hu, F.*, et al.* (2013). Optical and radioiodinated tethered Hsp90 inhibitors reveal selective internalization of ectopic Hsp90 in malignant breast tumor cells. Chemistry & biology *20*, 1187-1197.

Baumann, M., Krause, M., Overgaard, J., Debus, J., Bentzen, S. M., Daartz, J., Richter, C., Zips, D., and Bortfeld, T. (2016). Radiation oncology in the era of precision medicine. Nature reviews Cancer *16*, 234-249.

Bazan, V., Agnese, V., Corsale, S., Calo, V., Valerio, M. R., Latteri, M. A., Vieni, S., Grassi, N., Cicero, G., Dardanoni, G.*, et al.* (2005). Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *16 Suppl 4*, iv50-55.

Begg, A. C., Stewart, F. A., and Vens, C. (2011). Strategies to improve radiotherapy with targeted drugs. Nature reviews Cancer *11*, 239-253.

Belka, C., Jendrossek, V., Pruschy, M., Vink, S., Verheij, M., and Budach, W. (2004). Apoptosis-modulating agents in combination with radiotherapy-current status and outlook. International journal of radiation oncology, biology, physics *58*, 542-554.

Bezu, L., Gomes-de-Silva, L. C., Dewitte, H., Breckpot, K., Fucikova, J., Spisek, R., Galluzzi, L., Kepp, O., and Kroemer, G. (2015). Combinatorial strategies for the induction of immunogenic cell death. Frontiers in immunology *6*, 187.

Binefa, G., Rodriguez-Moranta, F., Teule, A., and Medina-Hayas, M. (2014). Colorectal cancer: from prevention to personalized medicine. World J Gastroenterol *20*, 6786-6808.

Bisht, K. S., Bradbury, C. M., Mattson, D., Kaushal, A., Sowers, A., Markovina, S., Ortiz, K. L., Sieck, L. K., Isaacs, J. S., Brechbiel, M. W.*, et al.* (2003). Geldanamycin and 17-allylamino-17-demethoxygeldanamycin potentiate the in vitro and in vivo radiation response of cervical tumor cells via the heat shock protein 90-mediated intracellular signaling and cytotoxicity. Cancer research *63*, 8984-8995.

Blattmann, C., Oertel, S., Ehemann, V., Thiemann, M., Huber, P. E., Bischof, M., Witt, O., Deubzer, H. E., Kulozik, A. E., Debus, J., and Weber, K. J. (2010). Enhancement of radiation response in osteosarcoma and rhabdomyosarcoma cell lines by histone deacetylase inhibition. International journal of radiation oncology, biology, physics *78*, 237-245.

Bogaert, J., and Prenen, H. (2014). Molecular genetics of colorectal cancer. Ann Gastroenterol *27*, 9-14.

Bouwman, P., and Jonkers, J. (2012). The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nature reviews Cancer *12*, 587-598.

Bray, F., Jemal, A., Grey, N., Ferlay, J., and Forman, D. (2012). Global cancer transitions according to the Human Development Index (2008-2030): a population-based study. The Lancet Oncology *13*, 790-801.

Bridge, J. A. (2014). The role of cytogenetics and molecular diagnostics in the diagnosis of soft-tissue tumors. Mod Pathol *27 Suppl 1*, S80-97.

Bull, E. E., Dote, H., Brady, K. J., Burgan, W. E., Carter, D. J., Cerra, M. A., Oswald, K. A., Hollingshead, M. G., Camphausen, K., and Tofilon, P. J. (2004). Enhanced tumor cell radiosensitivity and abrogation of G2 and S phase arrest by the Hsp90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin. Clinical cancer research : an official journal of the American Association for Cancer Research *10*, 8077-8084.

Burnette, B. C., Liang, H., Lee, Y., Chlewicki, L., Khodarev, N. N., Weichselbaum, R. R., Fu, Y. X., and Auh, S. L. (2011). The Efficacy of Radiotherapy Relies upon Induction of Type I Interferon-Dependent Innate and Adaptive Immunity. Cancer research *71*, 2488-2496.

Butler, L. M., Ferraldeschi, R., Armstrong, H. K., Centenera, M. M., and Workman, P. (2015). Maximizing the Therapeutic Potential of HSP90 Inhibitors. Mol Cancer Res *13*, 1445-1451.

Cai, Y., Qiu, S., Gao, X., Gu, S. Z., and Liu, Z. J. (2012). iASPP inhibits p53-independent apoptosis by inhibiting transcriptional activity of p63/p73 on promoters of proapoptotic genes. Apoptosis *17*, 777-783.

Calderwood, S. K., and Gong, J. (2016). Heat Shock Proteins Promote Cancer: It's a Protection Racket. Trends in biochemical sciences *41*, 311-323.

Camphausen, K., and Tofilon, P. J. (2007). Inhibition of Hsp90: a multitarget approach to radiosensitization. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 4326-4330.

Canter, R. J., Borys, D., Olusanya, A., Li, C. S., Lee, L. Y., Boutin, R. D., Christensen, S. D., Tamurian, R. M., and Monjazeb, A. M. (2014). Phase I trial of neoadjuvant conformal radiotherapy plus sorafenib for patients with locally advanced soft tissue sarcoma of the extremity. Annals of surgical oncology *21*, 1616-1623.

Castilleja, A., Ward, N. E., O'Brian, C. A., Swearingen, B., 2nd, Swan, E., Gillogly, M. A., Murray, J. L., Kudelka, A. P., Gershenson, D. M., and Ioannides, C. G. (2001). Accelerated HER-2 degradation enhances ovarian tumor recognition by CTL. Implications for tumor immunogenicity. Molecular and cellular biochemistry *217*, 21-33.

Chen, B., Zhong, D., and Monteiro, A. (2006). Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. BMC genomics *7*, 156.

Chen, C., Sun, P., Rong, J., Weng, H. W., Dai, Q. S., and Ye, S. (2015). Short Course Radiation in the Treatment of Localized Rectal Cancer: A Systematic Review and Meta-Analysis. Sci Rep *5*, 10953.

Chen, D. S., and Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. Immunity *39*, 1-10.

Cheng, Q., Chang, J. T., Geradts, J., Neckers, L. M., Haystead, T., Spector, N. L., and Lyerly, H. K. (2012). Amplification and high-level expression of heat shock protein 90 marks aggressive phenotypes of human epidermal growth factor receptor 2 negative breast cancer. Breast cancer research : BCR *14*, R62.

Chettiar, S. T., Malek, R., Annadanam, A., Nugent, K. M., Kato, Y., Wang, H., Cades, J. A., Taparra, K., Belcaid, Z., Ballew, M.*, et al.* (2016). Ganetespib radiosensitization for liver cancer therapy. Cancer biology & therapy *17*, 457-466.

Chou, T. C., and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*, 27-55.

Ciccia, A., and Elledge, S. J. (2010). The DNA damage response: making it safe to play with knives. Molecular cell *40*, 179-204.

Clark, M. A., Fisher, C., Judson, I., and Thomas, J. M. (2005). Soft-tissue sarcomas in adults. The New England journal of medicine *353*, 701-711.

Coppe, J. P., Desprez, P. Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol *5*, 99-118.

Cunningham, D., Atkin, W., Lenz, H. J., Lynch, H. T., Minsky, B., Nordlinger, B., and Starling, N. (2010). Colorectal cancer. Lancet *375*, 1030-1047.

Curtin, N. J. (2012). DNA repair dysregulation from cancer driver to therapeutic target. Nature reviews Cancer *12*, 801-817.

Curtis, K. K., Ashman, J. B., Beauchamp, C. P., Schwartz, A. J., Callister, M. D., Dueck, A. C., Gunderson, L. L., and Fitch, T. R. (2011). Neoadjuvant chemoradiation compared to neoadjuvant radiation alone and surgery alone for Stage II and III soft tissue sarcoma of the extremities. Radiation oncology *6*, 91.

Cysyk, R. L., Parker, R. J., Barchi, J. J., Jr., Steeg, P. S., Hartman, N. R., and Strong, J. M. (2006). Reaction of geldanamycin and C17-substituted analogues with glutathione: product identifications and pharmacological implications. Chemical research in toxicology *19*, 376-381.

DeLaney, T. F., Trofimov, A. V., Engelsman, M., and Suit, H. D. (2005). Advanced-technology radiation therapy in the management of bone and soft tissue sarcomas. Cancer control : journal of the Moffitt Cancer Center *12*, 27-35.

Deloch, L., Derer, A., Hartmann, J., Frey, B., Fietkau, R., and Gaipl, U. S. (2016). Modern Radiotherapy Concepts and the Impact of Radiation on Immune Activation. Frontiers in oncology *6*, 141.

Demaria, S., and Formenti, S. C. (2012). Radiation as an immunological adjuvant: current evidence on dose and fractionation. Frontiers in oncology *2*, 153.

Demaria, S., Ng, B., Devitt, M. L., Babb, J. S., Kawashima, N., Liebes, L., and Formenti, S. C. (2004). Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. International journal of radiation oncology, biology, physics *58*, 862-870.

Demetri, G. D., Antonia, S., Benjamin, R. S., Bui, M. M., Casper, E. S., Conrad, E. U., 3rd, DeLaney, T. F., Ganjoo, K. N., Heslin, M. J., Hutchinson, R. J.*, et al.* (2010). Soft tissue sarcoma. Journal of the National Comprehensive Cancer Network : JNCCN *8*, 630-674.

Demicco, E. G., Maki, R. G., Lev, D. C., and Lazar, A. J. (2012). New therapeutic targets in soft tissue sarcoma. Adv Anat Pathol *19*, 170-180.

Derer, A., Deloch, L., Rubner, Y., Fietkau, R., Frey, B., and Gaipl, U. S. (2015). Radio-Immunotherapy-Induced Immunogenic Cancer Cells as Basis for Induction of Systemic Anti-Tumor Immune Responses - Pre-Clinical Evidence and Ongoing Clinical Applications. Frontiers in immunology *6*, 505.

Dipetrillo, T., Pricolo, V., Lagares-Garcia, J., Vrees, M., Klipfel, A., Cataldo, T., Sikov, W., McNulty, B., Shipley, J., Anderson, E.*, et al.* (2012). Neoadjuvant bevacizumab, oxaliplatin,

5-fluorouracil, and radiation for rectal cancer. International journal of radiation oncology, biology, physics *82*, 124-129.

Dote, H., Burgan, W. E., Camphausen, K., and Tofilon, P. J. (2006). Inhibition of hsp90 compromises the DNA damage response to radiation. Cancer research *66*, 9211-9220.

Druker, B. J. (2008). Translation of the Philadelphia chromosome into therapy for CML. Blood *112*, 4808-4817.

Dunn, G. P., Koebel, C. M., and Schreiber, R. D. (2006). Interferons, immunity and cancer immunoediting. Nature reviews Immunology *6*, 836-848.

Egorin, M. J., Rosen, D. M., Wolff, J. H., Callery, P. S., Musser, S. M., and Eiseman, J. L. (1998). Metabolism of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. Cancer research *58*, 2385-2396.

Eisterer, W., De Vries, A., Ofner, D., Rabl, H., Koplmuller, R., Greil, R., Tschmelitsch, J., Schmid, R., Kapp, K., Lukas, P.*, et al.* (2014). Preoperative treatment with capecitabine, cetuximab and radiotherapy for primary locally advanced rectal cancer--a phase II clinical trial. Anticancer research *34*, 6767-6773.

Elliott, M. R., Chekeni, F. B., Trampont, P. C., Lazarowski, E. R., Kadl, A., Walk, S. F., Park, D., Woodson, R. I., Ostankovich, M., Sharma, P.*, et al.* (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature *461*, 282-286.

Eriksson, D., and Stigbrand, T. (2010). Radiation-induced cell death mechanisms. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine *31*, 363-372.

Ernst, A., Anders, H., Kapfhammer, H., Orth, M., Hennel, R., Seidl, K., Winssinger, N., Belka, C., Unkel, S., and Lauber, K. (2015). HSP90 inhibition as a means of radiosensitizing resistant, aggressive soft tissue sarcomas. Cancer letters *365*, 211-222.

Fearon, E. R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759-767.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer *136*, E359-386.

Filatenkov, A., Baker, J., Mueller, A. M., Kenkel, J., Ahn, G. O., Dutt, S., Zhang, N., Kohrt, H., Jensen, K., Dejbakhsh-Jones, S.*, et al.* (2015). Ablative Tumor Radiation Can Change the Tumor Immune Cell Microenvironment to Induce Durable Complete Remissions.

Clinical cancer research : an official journal of the American Association for Cancer Research *21*, 3727-3739.

Fionda, C., Soriani, A., Malgarini, G., Iannitto, M. L., Santoni, A., and Cippitelli, M. (2009). Heat shock protein-90 inhibitors increase MHC class I-related chain A and B ligand expression on multiple myeloma cells and their ability to trigger NK cell degranulation. Journal of immunology *183*, 4385-4394.

Fletcher, C. D. (2014). The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. Histopathology *64*, 2-11.

Flinterman, M., Guelen, L., Ezzati-Nik, S., Killick, R., Melino, G., Tominaga, K., Mymryk, J. S., Gaken, J., and Tavassoli, M. (2005). E1A activates transcription of p73 and Noxa to induce apoptosis. The Journal of biological chemistry *280*, 5945-5959.

Ford, C. A., Petrova, S., Pound, J. D., Voss, J. J., Melville, L., Paterson, M., Farnworth, S. L., Gallimore, A. M., Cuff, S., Wheadon, H.*, et al.* (2015). Oncogenic properties of apoptotic tumor cells in aggressive B cell lymphoma. Curr Biol *25*, 577-588.

Formenti, S. C., and Demaria, S. (2009). Systemic effects of local radiotherapy. The Lancet Oncology *10*, 718-726.

Formenti, S. C., and Demaria, S. (2013). Combining radiotherapy and cancer immunotherapy: a paradigm shift. Journal of the National Cancer Institute *105*, 256-265.

Franken, N. A., Oei, A. L., Kok, H. P., Rodermond, H. M., Sminia, P., Crezee, J., Stalpers, L. J., and Barendsen, G. W. (2013). Cell survival and radiosensitisation: modulation of the linear and quadratic parameters of the LQ model (Review). International journal of oncology *42*, 1501-1515.

Frey, B., Rubner, Y., Kulzer, L., Werthmoller, N., Weiss, E. M., Fietkau, R., and Gaipl, U. S. (2014). Antitumor immune responses induced by ionizing irradiation and further immune stimulation. Cancer immunology, immunotherapy : CII *63*, 29-36.

Frey, B., Stache, C., Rubner, Y., Werthmoller, N., Schulz, K., Sieber, R., Semrau, S., Rodel, F., Fietkau, R., and Gaipl, U. S. (2012). Combined treatment of human colorectal tumor cell lines with chemotherapeutic agents and ionizing irradiation can in vitro induce tumor cell death forms with immunogenic potential. Journal of immunotoxicology *9*, 301-313.

Fujii, Y., Kato, T., Kubota, N., Fujimori, A., Niwa, O., and Okayasu, R. (2010). p53 independent radio-sensitization of human lymphoblastoid cell lines by Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Oncology reports *23*, 199-203.

Gaipl, U. S., Multhoff, G., Scheithauer, H., Lauber, K., Hehlgans, S., Frey, B., and Rodel, F. (2014). Kill and spread the word: stimulation of antitumor immune responses in the context of radiotherapy. Immunotherapy *6*, 597-610.

Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L., and Kroemer, G. (2015). Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. Cancer Cell *28*, 690-714.

Garcia-Carbonero, R., Carnero, A., and Paz-Ares, L. (2013). Inhibition of HSP90 molecular chaperones: moving into the clinic. The Lancet Oncology *14*, e358-369.

Garcia, M., Martinez-Villacampa, M., Santos, C., Navarro, V., Teule, A., Losa, F., Pisa, A., Cambray, M., Soler, G., Lema, L.*, et al.* (2015). Phase II study of preoperative bevacizumab, capecitabine and radiotherapy for resectable locally-advanced rectal cancer. BMC cancer *15*, 59.

Garg, A. D., Dudek, A. M., and Agostinis, P. (2013). Cancer immunogenicity, danger signals, and DAMPs: what, when, and how? Biofactors *39*, 355-367.

Garg, A. D., Galluzzi, L., Apetoh, L., Baert, T., Birge, R. B., Bravo-San Pedro, J. M., Breckpot, K., Brough, D., Chaurio, R., Cirone, M.*, et al.* (2015). Molecular and Translational Classifications of DAMPs in Immunogenic Cell Death. Frontiers in immunology *6*, 588.

Garg, S. K., and Loftus, E. V., Jr. (2016). Risk of cancer in inflammatory bowel disease: going up, going down, or still the same? Curr Opin Gastroenterol *32*, 274-281.

Gerber, S. A., Sedlacek, A. L., Cron, K. R., Murphy, S. P., Frelinger, J. G., and Lord, E. M. (2013). IFN-gamma mediates the antitumor effects of radiation therapy in a murine colon tumor. Am J Pathol *182*, 2345-2354.

Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., Ortiz, C., Vermaelen, K., Panaretakis, T., Mignot, G., Ullrich, E.*, et al.* (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nature medicine *15*, 1170-1178.

Giaccia, A. J. (2014). Molecular radiobiology: the state of the art. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *32*, 2871-2878.

Glimelius, B. (2002). Radiotherapy in rectal cancer. Br Med Bull *64*, 141-157.

Group, E. S. E. S. N. W. (2014). Soft tissue and visceral sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *25 Suppl 3*, iii102-112.

Guo, W., Reigan, P., Siegel, D., and Ross, D. (2008). Enzymatic reduction and glutathione conjugation of benzoquinone ansamycin heat shock protein 90 inhibitors: relevance for toxicity and mechanism of action. Drug Metab Dispos *36*, 2050-2057.

Gupta, A., Probst, H. C., Vuong, V., Landshammer, A., Muth, S., Yagita, H., Schwendener, R., Pruschy, M., Knuth, A., and van den Broek, M. (2012). Radiotherapy promotes tumor-specific effector CD8+ T cells via dendritic cell activation. Journal of immunology *189*, 558-566.

Ha, K., Fiskus, W., Rao, R., Balusu, R., Venkannagari, S., Nalabothula, N. R., and Bhalla, K. N. (2011). Hsp90 inhibitor-mediated disruption of chaperone association of ATR with hsp90 sensitizes cancer cells to DNA damage. Molecular cancer therapeutics *10*, 1194-1206.

Haggerty, T. J., Dunn, I. S., Rose, L. B., Newton, E. E., Pandolfi, F., and Kurnick, J. T. (2014). Heat Shock Protein-90 Inhibitors Enhance Antigen Expression on Melanomas and Increase T Cell Recognition of Tumor Cells. PloS one *9*, e114506.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

He, K., Zheng, X., Zhang, L., and Yu, J. (2013). Hsp90 inhibitors promote p53-dependent apoptosis through PUMA and Bax. Molecular cancer therapeutics *12*, 2559-2568.

He, S., Smith, D. L., Sequeira, M., Sang, J., Bates, R. C., and Proia, D. A. (2014). The HSP90 inhibitor ganetespib has chemosensitizer and radiosensitizer activity in colorectal cancer. Investigational new drugs *32*, 577-586.

Hemmati, P. G., Guner, D., Gillissen, B., Wendt, J., von Haefen, C., Chinnadurai, G., Dorken, B., and Daniel, P. T. (2006). Bak functionally complements for loss of Bax during p14ARF-induced mitochondrial apoptosis in human cancer cells. Oncogene *25*, 6582-6594.

Hennel, R., Brix, N., Seidl, K., Ernst, A., Scheithauer, H., Belka, C., and Lauber, K. (2014). Release of monocyte migration signals by breast cancer cell lines after ablative and fractionated gamma-irradiation. Radiation oncology *9*, 85.

Huang, Q., Li, F., Liu, X., Li, W., Shi, W., Liu, F. F., O'Sullivan, B., He, Z., Peng, Y., Tan, A. C.*, et al.* (2011). Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. Nature medicine *17*, 860-866.

Iacopetta, B., Russo, A., Bazan, V., Dardanoni, G., Gebbia, N., Soussi, T., Kerr, D., Elsaleh, H., Soong, R., Kandioler, D.*, et al.* (2006). Functional categories of TP53 mutation in colorectal cancer: results of an International Collaborative Study. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO *17*, 842-847.

Isfort, K., Ebert, F., Bornhorst, J., Sargin, S., Kardakaris, R., Pasparakis, M., Bahler, M., Schwerdtle, T., Schwab, A., and Hanley, P. J. (2011). Real-time imaging reveals that P2Y2 and P2Y12 receptor agonists are not chemoattractants and macrophage chemotaxis to

complement C5a is phosphatidylinositol 3-kinase (PI3K)- and p38 mitogen-activated protein kinase (MAPK)-independent. The Journal of biological chemistry *286*, 44776-44787.

Jacobsen, A. V., Lowes, K. N., Tanzer, M. C., Lucet, I. S., Hildebrand, J. M., Petrie, E. J., van Delft, M. F., Liu, Z., Conos, S. A., Zhang, J. G.*, et al.* (2016). HSP90 activity is required for MLKL oligomerisation and membrane translocation and the induction of necroptotic cell death. Cell death & disease *7*, e2051.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., and Forman, D. (2011). Global cancer statistics. CA Cancer J Clin *61*, 69-90.

Jingu, K., Tsujii, H., Mizoe, J. E., Hasegawa, A., Bessho, H., Takagi, R., Morikawa, T., Tonogi, M., Tsuji, H., Kamada, T.*, et al.* (2012). Carbon ion radiation therapy improves the prognosis of unresectable adult bone and soft-tissue sarcoma of the head and neck. International journal of radiation oncology, biology, physics *82*, 2125-2131.

Johnson, J. L. (2012). Evolution and function of diverse Hsp90 homologs and cochaperone proteins. Biochimica et biophysica acta *1823*, 607-613.

Joly, A. L., Wettstein, G., Mignot, G., Ghiringhelli, F., and Garrido, C. (2010). Dual role of heat shock proteins as regulators of apoptosis and innate immunity. Journal of innate immunity *2*, 238-247.

Kabakov, A. E., Kudryavtsev, V. A., and Gabai, V. L. (2010). Hsp90 inhibitors as promising agents for radiotherapy. Journal of molecular medicine *88*, 241-247.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature *425*, 407-410.

Karthikeyan, G., Zambaldo, C., Barluenga, S., Zoete, V., Karplus, M., and Winssinger, N. (2012). Asymmetric synthesis of pochonin E and F, revision of their proposed structure, and their conversion to potent Hsp90 inhibitors. Chemistry *18*, 8978-8986.

Kawabe, M., Mandic, M., Taylor, J. L., Vasquez, C. A., Wesa, A. K., Neckers, L. M., and Storkus, W. J. (2009). Heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin enhances EphA2+ tumor cell recognition by specific CD8+ T cells. Cancer research *69*, 6995-7003.

Kinzel, L., Ernst, A., Orth, M., Albrecht, V., Hennel, R., Brix, N., Frey, B., Gaipl, U. S., Zuchtriegel, G., Reichel, C. A.*, et al.* (2016). A novel HSP90 inhibitor with reduced hepatotoxicity synergizes with radiotherapy to induce apoptosis, abrogate clonogenic survival, and improve tumor control in models of colorectal cancer. Oncotarget *7*, 43199-43219.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Kneisl, J. S., Coleman, M. M., and Raut, C. P. (2014). Outcomes in the management of adult soft tissue sarcomas. J Surg Oncol *110*, 527-538.

Kroemer, G., Galluzzi, L., Kepp, O., and Zitvogel, L. (2013). Immunogenic cell death in cancer therapy. Annual review of immunology *31*, 51-72.

Kronlage, M., Song, J., Sorokin, L., Isfort, K., Schwerdtle, T., Leipziger, J., Robaye, B., Conley, P. B., Kim, H. C., Sargin, S.*, et al.* (2010). Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. Science signaling *3*, ra55.

Krysko, D. V., Garg, A. D., Kaczmarek, A., Krysko, O., Agostinis, P., and Vandenabeele, P. (2012). Immunogenic cell death and DAMPs in cancer therapy. Nature reviews Cancer *12*, 860-875.

Kulzer, L., Rubner, Y., Deloch, L., Allgauer, A., Frey, B., Fietkau, R., Dorrie, J., Schaft, N., and Gaipl, U. S. (2014). Norm- and hypo-fractionated radiotherapy is capable of activating human dendritic cells. Journal of immunotoxicology *11*, 328-336.

Lauber, K., Brix, N., Ernst, A., Hennel, R., Krombach, J., Anders, H., and Belka, C. (2015). Targeting the heat shock response in combination with radiotherapy: Sensitizing cancer cells to irradiation-induced cell death and heating up their immunogenicity. Cancer letters *368*, 209-229.

Lauber, K., Ernst, A., Orth, M., Herrmann, M., and Belka, C. (2012). Dying cell clearance and its impact on the outcome of tumor radiotherapy. Frontiers in oncology *2*, 116.

Lesko, E., Gozdzik, J., Kijowski, J., Jenner, B., Wiecha, O., and Majka, M. (2007). HSP90 antagonist, geldanamycin, inhibits proliferation, induces apoptosis and blocks migration of rhabdomyosarcoma cells in vitro and seeding into bone marrow in vivo. Anti-cancer drugs *18*, 1173-1181.

Lim, J. Y., Gerber, S. A., Murphy, S. P., and Lord, E. M. (2014). Type I interferons induced by radiation therapy mediate recruitment and effector function of CD8(+) T cells. Cancer immunology, immunotherapy : CII *63*, 259-271.

Linch, M., Miah, A. B., Thway, K., Judson, I. R., and Benson, C. (2014). Systemic treatment of soft-tissue sarcoma-gold standard and novel therapies. Nature reviews Clinical oncology *11*, 187-202.

Lindner, L. H., and Issels, R. D. (2011). Hyperthermia in soft tissue sarcoma. Current treatment options in oncology *12*, 12-20.

Look Hong, N. J., Hornicek, F. J., Harmon, D. C., Choy, E., Chen, Y. L., Yoon, S. S., Nielsen, G. P., Szymonifka, J., Yeap, B. Y., DeLaney, T. F., and Mullen, J. T. (2013). Neoadjuvant chemoradiotherapy for patients with high-risk extremity and truncal sarcomas: a 10-year single institution retrospective study. European journal of cancer *49*, 875-883.

Lugade, A. A., Moran, J. P., Gerber, S. A., Rose, R. C., Frelinger, J. G., and Lord, E. M. (2005). Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. Journal of immunology *174*, 7516-7523.

Ma, Y., Adjemian, S., Mattarollo, S. R., Yamazaki, T., Aymeric, L., Yang, H., Portela Catani, J. P., Hannani, D., Duret, H., Steegh, K.*, et al.* (2013a). Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity *38*, 729-741.

Ma, Y., Adjemian, S., Yang, H., Catani, J. P., Hannani, D., Martins, I., Michaud, M., Kepp, O., Sukkurwala, A. Q., Vacchelli, E.*, et al.* (2013b). ATP-dependent recruitment, survival and differentiation of dendritic cell precursors in the tumor bed after anticancer chemotherapy. Oncoimmunology *2*, e24568.

Maier, P., Hartmann, L., Wenz, F., and Herskind, C. (2015). Cellular Pathways in Response to Ionizing Radiation and Their Targetability for Tumor Radiosensitization. Int J Mol Sci *17*.

Mantel, F., Frey, B., Haslinger, S., Schildkopf, P., Sieber, R., Ott, O. J., Lodermann, B., Rodel, F., Sauer, R., Fietkau, R., and Gaipl, U. S. (2010). Combination of ionising irradiation and hyperthermia activates programmed apoptotic and necrotic cell death pathways in human colorectal carcinoma cells. Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al] *186*, 587-599.

Markowitz, S. D., and Bertagnolli, M. M. (2009). Molecular origins of cancer: Molecular basis of colorectal cancer. The New England journal of medicine *361*, 2449-2460.

McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. C., Beck, P. L., Muruve, D. A., and Kubes, P. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science *330*, 362-366.

Mole, R. H. (1953). Whole body irradiation; radiobiology or medicine? Br J Radiol *26*, 234-241.

Mollapour, M., Bourboulia, D., Beebe, K., Woodford, M. R., Polier, S., Hoang, A., Chelluri, R., Li, Y., Guo, A., Lee, M. J.*, et al.* (2014). Asymmetric Hsp90 N domain SUMOylation recruits Aha1 and ATP-competitive inhibitors. Molecular cell *53*, 317-329.

Moran, D. M., Gawlak, G., Jayaprakash, M. S., Mayar, S., and Maki, C. G. (2008). Geldanamycin promotes premature mitotic entry and micronucleation in irradiated p53/p21 deficient colon carcinoma cells. Oncogene *27*, 5567-5577.

Morris, Z. S., and Harari, P. M. (2014). Interaction of radiation therapy with molecular targeted agents. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *32*, 2886-2893.

Moulick, K., Ahn, J. H., Zong, H., Rodina, A., Cerchietti, L., Gomes DaGama, E. M., Caldas-Lopes, E., Beebe, K., Perna, F., Hatzi, K.*, et al.* (2011). Affinity-based proteomics reveal cancer-specific networks coordinated by Hsp90. Nature chemical biology *7*, 818-826.

Murphy, J. D., Spalding, A. C., Somnay, Y. R., Markwart, S., Ray, M. E., and Hamstra, D. A. (2009). Inhibition of mTOR radiosensitizes soft tissue sarcoma and tumor vasculature. Clinical cancer research : an official journal of the American Association for Cancer Research *15*, 589-596.

Naccarati, A., Polakova, V., Pardini, B., Vodickova, L., Hemminki, K., Kumar, R., and Vodicka, P. (2012). Mutations and polymorphisms in TP53 gene--an overview on the role in colorectal cancer. Mutagenesis *27*, 211-218.

Nardella, C., Clohessy, J. G., Alimonti, A., and Pandolfi, P. P. (2011). Pro-senescence therapy for cancer treatment. Nature reviews Cancer *11*, 503-511.

Neckers, L., and Trepel, J. B. (2014). Stressing the development of small molecules targeting HSP90. Clinical cancer research : an official journal of the American Association for Cancer Research *20*, 275-277.

Neckers, L., and Workman, P. (2012). Hsp90 molecular chaperone inhibitors: are we there yet? Clinical cancer research : an official journal of the American Association for Cancer Research *18*, 64-76.

Niyazi, M., and Belka, C. (2006). Isobologram analysis of triple therapies. Radiation oncology *1*, 39.

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. Science *194*, 23-28.

Orth, M., Lauber, K., Niyazi, M., Friedl, A. A., Li, M., Maihofer, C., Schuttrumpf, L., Ernst, A., Niemoller, O. M., and Belka, C. (2014). Current concepts in clinical radiation oncology. Radiation and environmental biophysics *53*, 1-29.

Pasparakis, M., and Vandenabeele, P. (2015). Necroptosis and its role in inflammation. Nature *517*, 311-320.

Pearl, L. H., and Prodromou, C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem *75*, 271-294.

Pennisi, R., Ascenzi, P., and di Masi, A. (2015). Hsp90: A New Player in DNA Repair? Biomolecules *5*, 2589-2618.

Perez-Mancera, P. A., Young, A. R., and Narita, M. (2014). Inside and out: the activities of senescence in cancer. Nature reviews Cancer *14*, 547-558.

Perez-Ruiz, E., and Berraondo, P. (2016). Immunological Landscape and Clinical Management of Rectal Cancer. Frontiers in immunology *7*, 61.

Pittman, K., and Kubes, P. (2013). Damage-associated molecular patterns control neutrophil recruitment. Journal of innate immunity *5*, 315-323.

Poon, I. K., Lucas, C. D., Rossi, A. G., and Ravichandran, K. S. (2014). Apoptotic cell clearance: basic biology and therapeutic potential. Nature reviews Immunology *14*, 166-180.

Porter, J. R., Fritz, C. C., and Depew, K. M. (2010). Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. Current opinion in chemical biology *14*, 412-420.

Postow, M. A., Callahan, M. K., Barker, C. A., Yamada, Y., Yuan, J., Kitano, S., Mu, Z., Rasalan, T., Adamow, M., Ritter, E.*, et al.* (2012). Immunologic correlates of the abscopal effect in a patient with melanoma. The New England journal of medicine *366*, 925-931.

Powers, M. V., Valenti, M., Miranda, S., Maloney, A., Eccles, S. A., Thomas, G., Clarke, P. A., and Workman, P. (2013). Mode of cell death induced by the HSP90 inhibitor 17-AAG (tanespimycin) is dependent on the expression of pro-apoptotic BAX. Oncotarget *4*, 1963-1975.

Rao, A., Taylor, J. L., Chi-Sabins, N., Kawabe, M., Gooding, W. E., and Storkus, W. J. (2012). Combination therapy with HSP90 inhibitor 17-DMAG reconditions the tumor microenvironment to improve recruitment of therapeutic T cells. Cancer research *72*, 3196-3206.

Rao, S. S., Thompson, C., Cheng, J., Haimovitz-Friedman, A., Powell, S. N., Fuks, Z., and Kolesnick, R. N. (2014). Axitinib sensitization of high Single Dose Radiotherapy. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology *111*, 88-93.

Rhomberg, W. (2006). The radiation response of sarcomas by histologic subtypes: a review with special emphasis given to results achieved with razoxane. Sarcoma *2006*, 87367.

Ricardi, U., Racca, P., Franco, P., Munoz, F., Fanchini, L., Rondi, N., Dongiovanni, V., Gabriele, P., Cassoni, P., Ciuffreda, L.*, et al.* (2013). Prospective phase II trial of neoadjuvant chemo-radiotherapy with Oxaliplatin and Capecitabine in locally advanced rectal cancer (XELOXART). Med Oncol *30*, 581.

Roos, W. P., Thomas, A. D., and Kaina, B. (2016). DNA damage and the balance between survival and death in cancer biology. Nature reviews Cancer *16*, 20-33.

Rudner, J., Belka, C., Marini, P., Wagner, R. J., Faltin, H., Lepple-Wienhues, A., Bamberg, M., and Budach, W. (2001). Radiation sensitivity and apoptosis in human lymphoma cells. International journal of radiation biology *77*, 1-11.

Ruhland, M. K., Coussens, L. M., and Stewart, S. A. (2016). Senescence and cancer: An evolving inflammatory paradox. Biochimica et biophysica acta *1865*, 14-22.

Russo, A., Bazan, V., Iacopetta, B., Kerr, D., Soussi, T., Gebbia, N., and Group, T. C. C. S. (2005). The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *23*, 7518-7528.

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. Nat Rev Mol Cell Biol *14*, 630-642.

Sangkhathat, S. (2015). Current management of pediatric soft tissue sarcomas. World J Clin Pediatr *4*, 94-105.

Sauer, R., Liersch, T., Merkel, S., Fietkau, R., Hohenberger, W., Hess, C., Becker, H., Raab, H. R., Villanueva, M. T., Witzigmann, H.*, et al.* (2012). Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *30*, 1926-1933.

Schildkopf, P., Frey, B., Mantel, F., Ott, O. J., Weiss, E. M., Sieber, R., Janko, C., Sauer, R., Fietkau, R., and Gaipl, U. S. (2010). Application of hyperthermia in addition to ionizing irradiation fosters necrotic cell death and HMGB1 release of colorectal tumor cells. Biochemical and biophysical research communications *391*, 1014-1020.

Schildkopf, P., Frey, B., Ott, O. J., Rubner, Y., Multhoff, G., Sauer, R., Fietkau, R., and Gaipl, U. S. (2011). Radiation combined with hyperthermia induces HSP70-dependent maturation of dendritic cells and release of pro-inflammatory cytokines by dendritic cells and macrophages. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology *101*, 109-115.

Schnaider, T., Somogyi, J., Csermely, P., and Szamel, M. (1998). The Hsp90-specific inhibitor, geldanamycin, blocks CD28-mediated activation of human T lymphocytes. Life sciences *63*, 949-954.

Schreiber, R. D., Old, L. J., and Smyth, M. J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science *331*, 1565-1570.

Sengupta, S., and Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. Nat Rev Mol Cell Biol *6*, 44-55.

Sharma, A., Bode, B., Studer, G., Moch, H., Okoniewski, M., Knuth, A., von Boehmer, L., and van den Broek, M. (2013). Radiotherapy of human sarcoma promotes an intratumoral immune effector signature. Clinical cancer research : an official journal of the American Association for Cancer Research *19*, 4843-4853.

Sharma, K., Vabulas, R. M., Macek, B., Pinkert, S., Cox, J., Mann, M., and Hartl, F. U. (2012). Quantitative proteomics reveals that Hsp90 inhibition preferentially targets kinases and the DNA damage response. Molecular & cellular proteomics : MCP *11*, M111 014654.

Shevtsov, M., and Multhoff, G. (2016). Heat Shock Protein-Peptide and HSP-Based Immunotherapies for the Treatment of Cancer. Frontiers in immunology *7*, 171.

Shi, C., and Pamer, E. G. (2011). Monocyte recruitment during infection and inflammation. Nature reviews Immunology *11*, 762-774.

Shintani, S., Zhang, T., Aslam, A., Sebastian, K., Yoshimura, T., and Hamakawa, H. (2006). P53-dependent radiosensitizing effects of Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin on human oral squamous cell carcinoma cell lines. International journal of oncology *29*, 1111-1117.

Shirasawa, S., Furuse, M., Yokoyama, N., and Sasazuki, T. (1993). Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science *260*, 85-88.

Silva, M. T. (2010). Secondary necrosis: the natural outcome of the complete apoptotic program. FEBS letters *584*, 4491-4499.

Smyth, T., Van Looy, T., Curry, J. E., Rodriguez-Lopez, A. M., Wozniak, A., Zhu, M., Donsky, R., Morgan, J. G., Mayeda, M., Fletcher, J. A.*, et al.* (2012). The HSP90 inhibitor, AT13387, is effective against imatinib-sensitive and -resistant gastrointestinal stromal tumor models. Molecular cancer therapeutics *11*, 1799-1808.

Soehnlein, O., Lindbom, L., and Weber, C. (2009). Mechanisms underlying neutrophil-mediated monocyte recruitment. Blood *114*, 4613-4623.

Spiegelberg, D., Dascalu, A., Mortensen, A. C., Abramenkovs, A., Kuku, G., Nestor, M., and Stenerlow, B. (2015). The novel HSP90 inhibitor AT13387 potentiates radiation effects in squamous cell carcinoma and adenocarcinoma cells. Oncotarget *6*, 35652-35666.

Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M., von Bruhl, M. L., Gartner, F., Khandoga, A. G., Legate, K. R., Pless, R.*, et al.* (2013). Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. Nature immunology *14*, 41-51.

Steinmann, S., Gali-Muhtasib, H., Huebner, K., Al-Halabi, R., Abou Merhi, R., Aman, P., Agaimy, A., Haller, F., and Schneider-Stock, R. (2015). Hsp90 inhibition by AUY922 as an effective treatment strategy against myxoid liposarcoma. Cancer letters *367*, 147-156.

Stingl, L., Stuhmer, T., Chatterjee, M., Jensen, M. R., Flentje, M., and Djuzenova, C. S. (2010). Novel HSP90 inhibitors, NVP-AUY922 and NVP-BEP800, radiosensitise tumour cells through cell-cycle impairment, increased DNA damage and repair protraction. British journal of cancer *102*, 1578-1591.

Sulli, G., Di Micco, R., and di Fagagna, F. D. (2012). Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. Nature Reviews Cancer *12*.

Taipale, M., Jarosz, D. F., and Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol *11*, 515-528.

Taylor, B. S., Barretina, J., Maki, R. G., Antonescu, C. R., Singer, S., and Ladanyi, M. (2011). Advances in sarcoma genomics and new therapeutic targets. Nature reviews Cancer *11*, 541-557.

Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol *9*, 231-241.

Tesniere, A., Panaretakis, T., Kepp, O., Apetoh, L., Ghiringhelli, F., Zitvogel, L., and Kroemer, G. (2008). Molecular characteristics of immunogenic cancer cell death. Cell death and differentiation *15*, 3-12.

Thomas, D. M., and Ballinger, M. L. (2015). Etiologic, environmental and inherited risk factors in sarcomas. J Surg Oncol *111*, 490-495.

Trepel, J., Mollapour, M., Giaccone, G., and Neckers, L. (2010). Targeting the dynamic HSP90 complex in cancer. Nature reviews Cancer *10*, 537-549.

Tseng, W. W., Somaiah, N., and Engleman, E. G. (2014). Potential for immunotherapy in soft tissue sarcoma. Hum Vaccin Immunother *10*, 3117-3124.

Unkel, S., Belka, C., and Lauber, K. (2016). On the analysis of clonogenic survival data: Statistical alternatives to the linear-quadratic model. Radiation oncology *11*, 11.

van de Velde, C. J., Boelens, P. G., Borras, J. M., Coebergh, J. W., Cervantes, A., Blomqvist, L., Beets-Tan, R. G., van den Broek, C. B., Brown, G., Van Cutsem, E.*, et al.* (2014). EURECCA colorectal: multidisciplinary management: European consensus conference colon & rectum. European journal of cancer *50*, 1 e1-1 e34.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol *11*, 700-714.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. The New England journal of medicine *319*, 525-532.

Vogelstein, B., and Kinzler, K. W. (1993). The multistep nature of cancer. Trends in genetics : TIG *9*, 138-141.

von Mehren, M., Randall, R. L., Benjamin, R. S., Boles, S., Bui, M. M., Casper, E. S., Conrad, E. U., 3rd, Delaney, T. F., Ganjoo, K. N., George, S.*, et al.* (2014). Soft tissue sarcoma, version 2.2014. Journal of the National Comprehensive Cancer Network : JNCCN *12*, 473-483.

Wagner, A. J., Chugh, R., Rosen, L. S., Morgan, J. A., George, S., Gordon, M., Dunbar, J., Normant, E., Grayzel, D., and Demetri, G. D. (2013). A phase I study of the HSP90 inhibitor retaspimycin hydrochloride (IPI-504) in patients with gastrointestinal stromal tumors or soft-tissue sarcomas. Clinical cancer research : an official journal of the American Association for Cancer Research *19*, 6020-6029.

Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., and Kerr, D. (2009). Genetic prognostic and predictive markers in colorectal cancer. Nature reviews Cancer *9*, 489-499.

Wang, C., Barluenga, S., Koripelly, G. K., Fontaine, J. G., Chen, R., Yu, J. C., Shen, X., Chabala, J. C., Heck, J. V., Rubenstein, A., and Winssinger, N. (2009). Synthesis of pochoxime prodrugs as potent HSP90 inhibitors. Bioorganic & medicinal chemistry letters *19*, 3836-3840.

Wang, J., Cui, S., Zhang, X., Wu, Y., and Tang, H. (2013). High expression of heat shock protein 90 is associated with tumor aggressiveness and poor prognosis in patients with advanced gastric cancer. PloS one *8*, e62876.

Whitesell, L., and Lin, N. U. (2012). HSP90 as a platform for the assembly of more effective cancer chemotherapy. Bba-Mol Cell Res *1823*, 756-766.

Whitesell, L., and Lindquist, S. L. (2005). HSP90 and the chaperoning of cancer. Nature reviews Cancer *5*, 761-772.

Willett, C. G., Goldberg, S., Shellito, P. C., Grossbard, M., Clark, J., Fung, C., Proulx, G., Daly, M., and Kaufman, D. S. (1999). Does postoperative irradiation play a role in the adjuvant therapy of stage T4 colon cancer? Cancer J Sci Am *5*, 242-247.

Wong, P., Houghton, P., Kirsch, D. G., Finkelstein, S. E., Monjazeb, A. M., Xu-Welliver, M., Dicker, A. P., Ahmed, M., Vikram, B., Teicher, B. A.*, et al.* (2014). Combining targeted agents with modern radiotherapy in soft tissue sarcomas. Journal of the National Cancer Institute *106*.

Xu, W., and Neckers, L. (2007). Targeting the molecular chaperone heat shock protein 90 provides a multifaceted effect on diverse cell signaling pathways of cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research *13*, 1625-1629.

Yin, X., Zhang, H., Lundgren, K., Wilson, L., Burrows, F., and Shores, C. G. (2010). BIIB021, a novel Hsp90 inhibitor, sensitizes head and neck squamous cell carcinoma to radiotherapy. International journal of cancer Journal international du cancer *126*, 1216-1225.

Zaidi, S., McLaughlin, M., Bhide, S. A., Eccles, S. A., Workman, P., Nutting, C. M., Huddart, R. A., and Harrington, K. J. (2012). The HSP90 inhibitor NVP-AUY922 radiosensitizes by abrogation of homologous recombination resulting in mitotic entry with unresolved DNA damage. PloS one *7*, e35436.

Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. Science *290*, 989-992.

Zhao, X. M., Chen, Z., Zhao, J. B., Zhang, P. P., Pu, Y. F., Jiang, S. H., Hou, J. J., Cui, Y. M., Jia, X. L., and Zhang, S. Q. (2016). Hsp90 modulates the stability of MLKL and is required for TNF-induced necroptosis. Cell death & disease *7*, e2089.

Zhu, H., Woolfenden, S., Bronson, R. T., Jaffer, Z. M., Barluenga, S., Winssinger, N., Rubenstein, A. E., Chen, R., and Charest, A. (2010). The novel Hsp90 inhibitor NXD30001 induces tumor regression in a genetically engineered mouse model of glioblastoma multiforme. Molecular cancer therapeutics *9*, 2618-2626.

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,
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