ROLE OF BCL-2 PROTEIN FAMILY MEMBERS AND ASSOCIATED MITOCHONDRIAL FACTORS IN HYPOXIA-MEDIATED RESISTANCE OF TUMOR CELLS TO APOPTOSIS AND RADIOTHERAPY

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“Science is simply the word we use to describe a method of organizing our curiosity”

Tim Minchin
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1 INTRODUCTION

1.1 CANCER INCIDENCE AND MORTALITY

Cancer constitutes one of the leading causes of morbidity and mortality. According to the GLOBOCAN database, an estimated 14.1 million people worldwide were diagnosed with cancer in 2012 and the number of new cases is expected to further rise over the next two decades [1-3]. Breast, colorectal, and lung malignancies were the most frequently diagnosed cancer among women, while cancers of the lung, prostate, and colon were the most commonly diagnosed malignancies in men (Figure 1.1, excluding non-melanoma skin cancer). Overall, lung and colorectal cancer accounted for nearly a quarter of all new cases. Despite the increasing number of cancer treatment options developed during the last decades, 8.2 million people died from cancer in 2012, most of which from lung cancer [3].

![Figure 1.1: Worldwide cancer incidence and mortality in men and women in 2012 [1]. Estimated numbers of ten leading cancer types are enlisted (excluding non-melanoma skin cancer).](image-url)
1.2 RADIOTHERAPY

1.2.1 IMPORTANCE OF RADIOTHERAPY FOR CANCER TREATMENT

Owing to lifestyle behaviors, the cumulated cancer incidence rate in more developed countries is almost twice that of less developed countries [3]. Strikingly, cancer mortality is only 8-15% higher in more developed regions. This discrepancy is, among other factors, associated with disparities in treatment availability, such as access to radiotherapy (Figure 1.2). This highlights the importance of comprehensive medical care for optimal cancer treatment.

Figure 1.2: Estimated rate of cancer patients with access to radiotherapy in 2013 [3]. The majority of cancer patients in less developed regions has only limited access to radiotherapy as compared to greater radiotherapy supply reliability in Northern America, parts of Europe, and several other countries.

Delaney et al. estimated the proportion of cancer cases that should have been treated with radiotherapy as opposed to actual radiotherapy utilization rates in the years 1990-2001 [4]. Of the four most common cancers that together account for almost half of all cancer-related deaths [1, 3], the optimal radiotherapy utilization rate for lung cancer would be 76%, for breast cancer 83%, for colon cancer 14%, and for prostate cancer 60%. In total, an estimated 52% of patients with a registered cancer would benefit from radiotherapy, whereas actual utilization rates vary regionally from 20-55% - even in more developed countries [5-9]. As growth and aging of the population is expected to result in further increasing cancer incidence rates [2, 3], structural improvement of radiotherapy availability is one of the
requirements for the reduction of the growing cancer burden. However, despite good health infrastructure and optimal therapy supply in several regions, cancer is still one of the leading causes of death in more as well as less developed parts of the world [3]. Alongside the demand for improved cancer prevention strategies and diagnostic procedures, this points at the fact that much remains to be explored concerning mechanisms of malignant transformation, tumor progression, and especially therapy resistance in order to improve treatment options and ameliorate cancer related suffering and premature death.

1.2.2 CELL DEATH SIGNALING IN RESPONSE TO IONIZING RADIATION

Despite the recent shift in therapeutic approaches and cancer drug design towards molecularly targeted therapies, radiation therapy still is an essential mode of treating various types and stages of solid tumors [10]. Radiation is administered either alone or combined with surgery and chemotherapy in curative, palliative, adjuvant, and neoadjuvant approaches. The most frequently applied form of radiotherapy is ionizing radiation with low-linear-energy-transfer photon beams from cesium-137, cobalt-60, iridium-192, and low-linear-energy-transfer electron beams produced by linear accelerators [11]. The absorption of radiation results in the production of fast recoil electrons [12]. These can directly damage DNA by breaking chemical bonds, thereby creating single-strand and double-strand breaks. However, most damage resulting from ionizing radiation is attributed to the indirect effect, which is constituted by ions and ion pairs that emerge as high-energy particles move through the biological material and free electrons from atoms and molecules. Having an unpaired valence electron, these radicals are highly reactive and can severely damage cellular macromolecules, such as DNA, proteins, and lipids, as well as activate several intracellular signaling pathways [13-15].

The ultimate goal of radiotherapy is to induce an amount of damage that overcomes repair capacities of the tumor cell. This supposedly stops the unleashed replication and proliferation of cancer cells, triggers accelerated senescence, or directs them to cell death via apoptosis, necrosis, or autophagy [16-18]. As shown in Figure 1.3, several of these cellular responses are orchestrated by p53, which is transcriptionally upregulated and post-translationally modified in response to radiation-induced DNA damage [19]. p53 can initiate cell cycle arrest via induction of p21 and p16 [20, 21], induce intrinsic apoptosis via interaction with and transcriptional upregulation of Bcl-2 protein family members [22], or extrinsic apoptosis via
induction of Fas [23]. Furthermore, irradiation can directly activate sphingomyelinases (SMases) and thereby increase protein levels of ceramide, which can act as a second messenger in initiating apoptotic responses [24]. Radiation-induced endoplasmic reticulum (ER) stress and autophagy are activated as a result of accumulating radical-damaged proteins and organelles and can also shift the cells’ fate towards apoptosis [25]. The exact biological effect of ionizing radiation varies depending on diverse factors, such as received dose, cell type, pre-existing genetic alterations, and other aspects of the cellular context, which together determine treatment sensitivity [15].

![Figure 1.3: Cellular responses to radiation-induced damage](image)

Over the past decades, radiation research focused mainly on technique refinement, in order to enable the precise deposition of an effective dose to the tumor while sparing normal tissue [26, 27]. However, despite valuable technical improvements and the substantial therapeutic benefit that can be achieved in individual tumors, many patients experience tumor recurrence after radiotherapy [10]. The exploration of the molecular profiles of tumor cells has therefore moved more into focus of research in radiation biology, bearing the potential for refined
targeting and selective sensitization to radiotherapy [28]. Among the identified factors that affect radiosensitivity are cell intrinsic features, such as aberrantly activated signal transduction pathways, defective DNA damage response, and improved DNA repair [29] as well as survival benefits conferred by microenvironmental parameters [30, 31]. Of the latter ones, tumor hypoxia is one of the main obstacles to successful radiotherapy [32].

1.3 TUMOR HYPOXIA

1.3.1 TUMOR HYPOXIA PHYSIOLOGY

The tumor microenvironment is created by non-transformed cells and their interactions with malignant cells, but also by distinctly altered physiological features, such as pH, nutrient, and oxygen levels [33]. The complexity of cellular, physical, and chemical properties of the micromilieu is highly heterogeneous between different tumor entities. A common feature found within virtually all locally advanced solid tumors is the presence of fluctuating and chronically low oxygenation levels – known as tumor hypoxia [34]. It arises, amongst others, due to accelerated cancer cell proliferation and the consequential abnormal tumor vascularization [33]. There are two forms of tumor hypoxia, resulting from different mechanisms: perfusion limited or acute hypoxia and diffusion-limited or chronic hypoxia (Figure 1.4). Dilated, immature vessels and chaotic architecture of the vascular network are typical abnormalities in solid tumors that lead to an inadequate blood flow and consequently poor oxygen and nutrient supply within parts of the tumor [35, 36]. Regions of acute hypoxia occur as a result of fluctuations in blood flow, caused by transient closing or blockage of particular parts of the malformed vasculature [37, 38]. As perfusion in these areas changes dynamically over time, tumor cells close to blood vessels are exposed to transient periods of acute hypoxia and reoxygenation [39-41]. Chronic hypoxia is the consequence of tumor cells spreading beyond the diffusion distance of oxygen through respiring tissue, which is about 70 µm at the arterial and less at the venous end of a capillary [34]. Chronically hypoxic cells at greater distances to a blood vessel can remain viable for hours to days [42-44]. However, as reoxygenation is less likely to occur in chronically hypoxic than in acutely hypoxic tumor regions, these tumor cells eventually die - unless they are able to adapt to these unfavorable oxygen conditions.
Figure 1.4: Schematic representation of tumor cells under acute or chronic hypoxia in dependence of vascular oxygen supply [45]. Oxygen from a functional blood vessel is used up by respiring cells, resulting in an oxygen gradient (left). Outer-most viable cells are exposed to chronic hypoxia. Transient closure of blood vessels results in acute hypoxia (right). Tumor cells are consequently temporarily deprived of oxygen.

The imbalance between oxygen delivery and high oxygen consumption of tumor cells triggers a complex compensatory cellular response that allows continued survival [30, 46]. Thus, tumor hypoxia is considered a major determinant of malignant behavior and poor treatment outcome [32, 47]. Hypoxia-mediated malignant progression and resistance to radiotherapy is considered to be driven by three key factors: i) reduced availability of molecular oxygen hampers manifestation of radiation-induced DNA damage, ii) acute hypoxia activates survival signaling pathways, and iii) cycling hypoxia allows the clonal selection and evolution of death resistant cancer cells.

1.3.2 Permanent DNA damage induced by radiation requires molecular oxygen

The extent of radiation-induced DNA damage is critically influenced by the oxygen level in the cell [12]. If molecular oxygen is present, DNA radicals can react to form peroxy radicals. These represent the “fixed” form of DNA damage that leads to cell death, if the rate of DNA damage exceeds repair capacities of the cell (Figure 1.5). In absence of oxygen, DNA radicals can be restored through chemical reduction and the DNA recovers to its normal function, attenuating the effect of radiotherapy. Cells irradiated under hypoxic conditions are therefore 2-3 times more resistant to cell death induction by ionizing radiation than oxygenated cells, which is referred to as the “oxygen effect” [12, 48].
The majority of biological damage produced by ionizing radiation is mediated through free radicals (indirect effect). Formation of organic peroxides \((\text{RO}_2\cdot)\) in presence of molecular oxygen lead to permanent, “fixed” damage [12].

1.3.3 Exposure to acute hypoxia triggers increased survival signaling

In addition to hampered manifestation of DNA damage, exposure to acute hypoxia activates several oxygen-sensitive survival pathways [50] (Figure 1.6). Of these, hypoxia inducible transcription factors (HIFs 1-3) play a key role in cellular response to hypoxic stress [51]. Both HIF subunits, \(\alpha\) and \(\beta\), are constitutively expressed. Under well oxygenated conditions, HIF-\(\alpha\) subunits are constantly degraded by the ubiquitin-proteasome pathway after hydroxylation by the von-Hippel Lindau protein. This process is initiated by oxygen-dependent prolyl-hydroxylases. Consequently, hypoxia stabilizes HIF-\(\alpha\) subunits, which then form heterodimers with HIF-\(\beta\) subunits and activate expression of hypoxia-inducible genes by binding to hypoxia responsive elements (HRE) within target gene promoters. Among others, HIF enhances cancer cell survival by inducing gene expression of the vascular endothelial growth factor VEGF [52], which increases oxygen availability due to formation of new blood vessels. Moreover, HIF mediates metabolic responses to hypoxia by enhancing transcription of genes that promote glycolysis, such as glucose transporter GLUT1, to compensate for decreased energy production through oxygen-dependent oxidative phosphorylation (OXPHOS) [53]. Hypoxia furthermore triggers the unfolded protein response (UPR) through the activation of several ER stress sensors that can induce selective gene expression and inhibit translation [54, 55]. Resulting alterations of processes including protein production, maturation, and degradation can re-establish the ER homeostasis following hypoxic stress.
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[56]. Furthermore, inhibition of global mRNA translation can be essential for cell survival by contributing to preservation of energy homeostasis. The mammalian target of rapamycin (mTOR) kinase, an important transmitter of metabolic signals to the translational machinery, is another factor that influences downstream pathways in an oxygen-sensitive manner [57, 58]. Inhibition of the mTOR kinase under hypoxic conditions leads to specific changes in mRNA translation, resulting in a differential protein expression pattern in favor of cell survival [50, 59].

![Diagram of hypoxia tolerance mechanisms](image)

**Figure 1.6: Response of oxygen-sensitive signaling pathways to hypoxia [50].** Hypoxia tolerance is promoted by three main oxygen-sensitive pathways that regulate transcription and translation. i) Hypoxia inducible transcription factors (HIFs): HIF-α is stabilized under hypoxic conditions, which allows heterodimerization with HIF-β subunits and thereby activates target gene transcription. ii) Unfolded protein response (UPR): ER stress sensors inositol-requiring protein 1 (IRE1) and PKR-like ER kinase (PERK) are activated in response to accumulation of misfolded proteins, which can result from exposure to hypoxia. IRE1 activity induces transcription of chaperone proteins that facilitate protein folding. Activated PERK inhibits mRNA translation and thereby prevents further accumulation of misfolded proteins. iii) Hypoxia-mediated inhibition of mTOR (mammalian target of rapamycin) kinase results in differential mRNA translation, which contributes to altered protein expression. Collectively, hypoxic activation of HIFs, the UPR, and inhibition of mTOR kinase enhance hypoxia tolerance by regulating metabolism, angiogenesis, autophagy, and ER homeostasis.
Furthermore, gene expression of Bcl-2 protein family members, which are central regulators of cell death induction via intrinsic apoptosis [60], was reported to be differentially regulated under hypoxic conditions [61-64]. The direct association between tumor hypoxia, reduced apoptosis, and poor survival of cancer patients was demonstrated by Höckel and colleagues already in 1999 [65]. However, while some details of hypoxia-mediated evasion of apoptosis were shown in vitro and in vivo so far [66-70], the mechanisms especially regarding the involvement of the Bcl-2 protein family require further investigation.

Taken together, hypoxia signaling contributes to tumor aggressiveness and therapy resistance by increasing the cell death threshold via the mechanisms described above. Thus, tumor hypoxia is critically associated with poor prognosis. Due to the complexity of hypoxia biology, approaches to reduce hypoxia-mediated survival signaling have been lacking clinical success to date [47]. However, as hypoxia is rather unusual in the surrounding normal tissue, it is an attractive tumor-specific target for new therapeutic approaches and currently under investigation [71, 72].

1.3.4 CYCLING HYPOXIA RESULTS IN HYPOXIA ADAPTATION AND INCREASED THERAPY RESISTANCE

As the tumor microenvironment is highly dynamic, oxygen levels in solid tumors are commonly found to fluctuate in temporal and spatial manners [41, 73, 74]. Particularly exposure to repeating cycles of hypoxia and reoxygenation is linked to phenotypic and genotypic adaptation of tumor cells to this adverse situation. Reynolds et al. demonstrated the general potential of cycling hypoxia as a physiologic condition that selects for certain mutations in tumor cells [75]. The study revealed that the mutation frequency increased with each additional cycle of hypoxia. Moreover, it was shown that cycling hypoxia selected for apoptosis-resistant cells, in particular for cell populations carrying nonfunctional alleles encoding for p53 [76]. In line with this finding, Weinmann et al. reported that lung cancer cells exposed to multiple rounds of severe hypoxia and reoxygenation resulted in the selection of cells with an increased threshold specifically toward induction of intrinsic apoptosis [77]. Cycling hypoxia was furthermore associated with an aggressive tumor phenotype in vivo, based on the finding that whole-body exposure of tumor-bearing mice to cycling hypoxia resulted in an increased incidence of lung and lymph node metastases [78, 79]. Collectively, these studies have shown that repeated exposure to hypoxia and reoxygenation induces mutagenesis and selects for resistance to apoptosis. Consequently, cycling hypoxia gives rise
to expansion of hypoxia-adapted tumor cells that are able to survive and proliferate in a wide range of stress conditions, thereby contributing to tumor progression and therapy resistance. Further exploration of the mechanisms of adaptation to cycling hypoxia might therefore provide valuable insights and targets for improved therapeutic eradication of hypoxic and hypoxia-adapted tumor cell fractions.

1.4 ROLE OF THE BCL-2 PROTEIN FAMILY IN TUMORIGENESIS AND CANCER THERAPY

1.4.1 REGULATION OF INTRINSIC APOPTOSIS BY BCL-2 FAMILY PROTEINS

Apoptosis is a key cell death mechanism, serving the purpose of eliminating normal or dysfunctional cells and maintaining tissue homeostasis [80]. This mode of programmed cell death is characterized by cellular shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and disintegration into apoptotic bodies [80, 81]. Apoptotic cells are engulfed and degraded by macrophages or neighboring cells [82, 83]. The morphological changes are caused by caspases [84]. These are special proteases that are expressed as inactive proenzymes, so-calledzymogenes, and become activated upon intrinsic or extrinsic apoptotic triggers. While the extrinsic (or death receptor) apoptosis pathway is initiated by ligands binding to cell surface death receptors, the intrinsic (or mitochondrial) pathway is activated in response to internal cell death stimuli such as excessive DNA damage [85, 86]. The two pathways engage discrete initiator caspases but converge with activation of common effector caspases (caspase-3, 7, and 6) [84]. Activation of these proteolytic caspase cascades leads to degradation of key cellular components, such as structural proteins and DNA, resulting in irreversible cellular dismantling and clearance [87, 88].

In the intrinsic apoptosis pathway, the caspase cascade is activated by cytochrome c release from the mitochondrial inter-membrane space [89, 90]. This step is cardinaly regulated by the members of the B-cell leukemia (Bcl)-2 protein family [60, 91, 92]. As displayed in Figure 1.7, this protein family is defined by structural homology of conserved Bcl-2 homology (BH) domains 1-4 and can be divided according to their function in anti-apoptotic and pro-apoptotic members [93]. The anti-apoptotic family subgroup, including Bcl-2, Bcl-xl, Myeloid cell leukemia (Mcl)-1, and Bcl-w, contain up to 4 BH domains and a C-terminal transmembrane domain (TM) for anchoring to organelles such as mitochondria or the ER [94-97]. Pro-apoptotic Bax and Bak contain a C-terminal membrane-anchoring domain, BH domains 1-3 [94-97] as well as BH domain 4, considering structural and sequence homology of BH4
present in a wide range of Bcl-2 family members [98]. Members of the third Bcl-2 subfamily share only a single BH3 domain and are referred to as BH3-only proteins.

![Figure 1.7: Bcl-2 family proteins are defined by structural homology (adapted from [98]).](image)

Figure 1.7: Bcl-2 family proteins are defined by structural homology (adapted from [98]). Schematic representation of Bcl-2 homology (BH1-4) and transmembrane (TM) domains of Bcl-2 protein family members, categorized by their function. BH4 domain refers to a structural motif with sequence homology found in anti-apoptotic family members and in Bax, Bak, and possibly Bok. Exemplary sequences of human Bcl-2 and Bax are shown.

The Bcl-2 family proteins regulate apoptosis by activating and neutralizing each other’s function in a complex network of more or less selective interactions [99] (Figure 1.8 A). Anti-apoptotic members preserve the integrity of the mitochondrial outer membrane (MOM) by sequestering pro-apoptotic family members, which inhibits MOM permeabilization by Bax and Bak [100-102]. Bax and Bak activation enables their oligomerization and pore formation at the MOM, resulting in cytochrome c release from the mitochondrial inter-membrane space [103].

Three models exist addressing the interactions of Bcl-2 family subgroups that control cell fate [99] (Figure 1.8 B). The direct activation model suggests that an “activator” subgroup of BH3-only proteins (truncated form of Bid (tBid), Bim, and possibly Puma) directly bind to Bax and Bak, triggering oligomerization and subsequent pore formation by induction of structural changes [104, 105]. Other BH3-only proteins act as “sensitizers” by binding to anti-apoptotic members, thereby releasing activator BH3-only proteins [106, 107]. The displacement (or indirect activation) model proposes that in addition to BH3-only proteins, anti-apoptotic members also sequester activated Bax and Bak [108, 109]. For release of Bax and Bak, all anti-apoptotic Bcl-2 proteins need to be neutralized by BH3-only proteins [110, 111]. In a combining, unified model, anti-apoptotic members sequester BH3-only proteins as well as Bax and Bak [112-114]. Which mode dominates, is proposed to depend on the biological context.
Figure 1.8: Schematic representation of proposed binding specificities and models of functional interactions between pro- and anti-apoptotic Bcl-2 protein family subgroups (adapted from [99]). A) While some BH3-only family members can bind to all anti-apoptotic Bcl-2 proteins, others (Bad and Noxa) interact with a subset. Bax can be inhibited by all anti-apoptotic members, whereas Bak is inhibited primarily by Bcl-xl, Mcl-1 and A1. B) Overview of the three models of apoptosis regulation by Bcl-2 protein family subgroups, represented by individual members. “Activator” BH3-only proteins (e.g. Bim) can directly interact with Bax. Anti-apoptotic members (e.g. Bcl-2) prevent Bax activation by direct interaction and/or by sequestering BH3-only activator proteins. “Sensitizer” BH3-only proteins (e.g. Bad) are not able to directly activate Bax, but binding to anti-apoptotic members releases Bax and activator BH3-only proteins.

In healthy, non-transformed cells, BH3-only proteins are usually present in relatively small quantities or engaged in non-apoptotic cellular functions [113]. In response to various stress signals, including genotoxic stresses as induced by ionizing radiation or DNA-damaging chemotherapeutics, induction of intrinsic apoptosis is actively promoted by tumor suppressor protein p53 [19]. p53 is stabilized by a series of post-translational modifications and contributes to apoptosis by several mechanisms. p53 can directly interact with Bcl-2 family members in the cytosol and at the mitochondria. It interacts with Bcl-xl and Bcl-2, thereby antagonizing their anti-apoptotic effects, and furthermore can induce Bax and Bak oligomerization at the MOM [22]. The pivotal role of p53 in promoting apoptosis, however,
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relies on its function as transcription factor, as it activates expression of pro-apoptotic Bax and BH3-only proteins Noxa and Puma [115]. Furthermore, various post-translational mechanisms induce translocation, stabilization, and pro-apoptotic activity of BH3-only proteins. These include proteolytic cleavage of Bid to tBid, translocation of Bim from microtubules to mitochondria, and various modifications as phosphorylation and ubiquitylation [116]. Finally, if the mitochondrial enrichment of pro-apoptotic Bcl-2 family members exceeds a certain threshold, effector pore formation and subsequent cytochrome c release irreversibly induce apoptosis [93, 99].

1.4.2 ROLE OF BCL-2 FAMILY PROTEINS IN MITOCHONDRIAL METABOLISM AND DYNAMICS

In addition to regulation of intrinsic apoptosis, Bcl-2 family proteins are involved in a range of non-apoptotic processes. A considerable part of these roles relate to mitochondrial physiology, including metabolism and morphology [117] (Figure 1.9).

Mitochondria are central organelles of energy production, as they host key components of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) [118]. Cytosolic metabolites such as pyruvate and fatty acids can pass the MOM through the voltage dependent anion channel (VDAC) [119]. Following transport into the mitochondrial matrix, they are broken down to acetyl CoA. The molecule is further metabolized by enzymes of the TCA cycle [118]. The TCA cycle generates reducing equivalents NADH and FADH$_2$, which fuel the process of OXPHOS. At the IMM, the electron transport chain (ETC), comprised of complexes I-IV, shuttles electrons from the reducing equivalents to molecular oxygen. The electron transfer through the ETC is coupled to the transport of protons into the intermembrane space, creating an electrochemical proton gradient. The resulting flow of protons back into the matrix through the oligomeric ATP synthase drives the production of ATP, the primary cellular energy source. Maintenance of an adequate balance between nutritional supply and energy demand is tightly linked to mitochondrial morphology, which is controlled by complementary fusion and fission events [120].

The main mediators of mitochondrial fusion are Opa1 (Optic atrophy 1) and mitofusin 1 and 2 (Mfn1/2) [121]. Mfn1 and Mfn2 are embedded in the MOM and engage in oligomeric complexes to mediate fusion of juxtaposed mitochondria. Opa1 is integrated in and controls fusion of the inner mitochondrial membrane (IMM). Fission, the process balancing fusion, requires the translocation of cytosolic protein Drp1 to the MOM. Drp1 is recruited to
mitochondrial fission sites by interaction with its mitochondrial receptors, including Mff and Fis1. Drp1 assembles ring-like oligomers around the organelle and constricts the membranes, which results in smaller fragmented mitochondria.

The dynamic balance between fusion and fission is required to maintain mitochondrial function and furthermore is regulated in response to physiological stresses such as hypoxia and nutrient deprivation [120]. Stress-induced “hyperfusion” is linked to increased metabolic productivity. Although the mechanisms are not fully resolved, evidence has been provided that Opa1-dependent cristae stabilization promotes ATP synthase assembly and organization of the ETC into so-called supercomplexes, thus increasing OXPHOS efficiency [122]. Furthermore, several anti-apoptotic Bcl-2 proteins were implicated in the regulation of mitochondrial energy production. Bcl-xL, for instance, was found to localize to the IMM and interact with the ATP synthase, thereby decreasing ion leak and enhancing ATP production [123]. Moreover, a truncated form of Mcl-1 was reported to reside inside the mitochondria and contribute to efficient ATP production [124]. In particular, cleaved Mcl-1 was required for the assembly of respiratory supercomplexes and ATP synthase oligomers. This Mcl-1 isoform furthermore facilitated mitochondrial fusion, but was not able to prevent apoptosis. Nevertheless, as cristae contain a large part of the cytochrome c, enhanced fusion and cristae stabilization is found to protect from apoptosis [125]. In contrast to mitochondrial fusion, fission is an integral part of apoptosis induction and execution [126]. Consequently, Bcl-2 protein family members are also implicated in the regulation of mitochondrial dynamics [125]. The suggested roles of pro-and anti-apoptotic Bcl-2 family members in fusion and function are, however, controversial. Bcl-xL, which resides at the MOM, has been shown to interact with Drp1 and promote fission. Conversely, Bax and Bak seem to promote mitochondrial fusion by interacting with Mfn1 and Mfn2 [127]. However on the contrary, it was also shown that the fission-promoting association of Drp1 at the MOM is stabilized by sumoylation through activated Bax or Bak [128]. Moreover, inhibition of Drp1 and resulting blockage of fission was found to reduce cytochrome c release and protect from apoptosis [129, 130]. The exact mechanisms and functional consequences of the relationship between Bcl-2 family members and mitochondrial dynamics thus remain to be elucidated. It is moreover not yet clear, how this interrelation is linked to metabolic stress responses and whether the proposed additional roles of Bcl-2 family proteins are distinct from their function in apoptosis. However, mitochondrial dysfunction, metabolic alterations, and evasion of
apoptosis are characteristic features of cancer cells [131, 132]. Understanding re-programming of mitochondrial functions therefore might contribute to the development of therapeutic strategies that target mitochondrial alterations for cancer treatment.

Figure 1.9: Proposed additional functions of Bcl-2 protein family members at the mitochondria (adapted from [117]). Several Bcl-2 family proteins are proposed to additionally contribute to regulation of mitochondrial metabolism as well as fusion and fission.

1.4.3 DEFECTIVE APOPTOSIS SIGNALING IN CANCER

The delicately balanced activity of pro- and anti-apoptotic Bcl-2 family members, the so-called Bcl-2 rheostat, is essential for tissue homeostasis. Pathological deregulation of their expression and function can enable evasion of programmed cell death and is considered one of the hallmarks of cancer [132]. Indeed, genetic and biochemical studies have revealed a deregulated Bcl-2 rheostat in a wide variety of human tumors [133, 134]. Anti-apoptotic Bcl-2 family proteins are broadly found overexpressed, while several pro-apoptotic members exhibit reduced expression due to loss of heterozygosity or promoter methylation as well as functional inactivation as a result of mutations [135-137].

Besides in various hematopoietic malignancies, overexpression of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL was reported in several solid tumors, including small cell lung cancer (SCLC), glioblastoma, colon carcinoma, head and neck squamous cell carcinoma, and others [134, 138-140]. Of importance, such deregulations of Bcl-2 and Bcl-xL often correlated with therapy resistance and poor clinical prognosis. Mcl-1 overexpression is likewise frequently detected in leukemic and lymphatic malignancies as well as in solid tumors and is associated
with therapy resistance and poor outcome [141-145]. Mcl-1 is furthermore considered a short-lived protein whose stability is tightly regulated by post-translational mechanisms, which can be altered in cancer cells [146]. Radioresistance of glioblastoma cells, for instance, was associated with Mcl-1 stabilization by deubiquitinase USP9x, an enzyme that interferes with degradation [143, 147]. While the chromosomal translocation t(14;18) accounts for Bcl-2 overexpression in various hematopoietic malignancies [148, 149], gene amplification was reported to play a role in overexpression of Bcl-2, Bcl-xL, and also Mcl-1 in other cancer entities [150-152]. Several mechanisms accounting for underrepresentation of functional pro-apoptotic Bcl-2 family members have also been described in human cancers. BAX frameshift and inactivating mutations were found in hematopoietic and colorectal tumors [153-155], BAK mutations in gastric and colon cancers [156], and NOXA mutations in diffuse large B-cell lymphoma [157]. Loss of heterozygosity was reported for BIM in mantle cell lymphoma and for BIK in renal cell carcinoma [158, 159]. Furthermore, hypermethylation of BIM, BIK, HRK, and PUMA gene promoters were proposed to reduce expression of pro-apoptotic proteins in different lymphatic and solid malignancies [157, 159-162].

These findings corroborate the critical role of deregulations in the Bcl-2 rheostat, which is frequently found in cancer cells escaping from apoptosis. Consequently, apoptosis resistance is an almost universal feature that contributes to disease progression by allowing tumor cells to overcome intrinsic and therapy-induced cell death signals [132, 136]. Alterations of the Bcl-2 rheostat in favor of the anti-apoptotic function, such as overexpression of Bcl-2 and Bcl-xL, are therefore often correlated with therapy resistance and poor clinical prognosis [134, 140]. The elevated apoptotic threshold furthermore enables cancer cells to survive various stressful environmental conditions such as hypoxia [163-165]. Interfering with the altered Bcl-2 rheostat therefore might specifically sensitize apoptosis resistant cancer cells to anti-neoplastic therapy.
1.4.4 SMALL MOLECULE INHIBITORS OF ANTI-APOPTOTIC BCL-2 FAMILY MEMBERS

Elucidation of the mechanisms involved in defective apoptosis signaling in malignancy and treatment resistance have stimulated the exploration of apoptosis-directed therapeutic strategies [136, 166]. These strategies aim to restore cancer cell sensitivity to cell death-inducing therapy by either correcting the defect or by activating the apoptosis pathway downstream of the defect. Of the possible targets, most effort has been directed to the inhibition of anti-apoptotic Bcl-2 protein family members [167]. Numerous inhibitors were identified or designed using approaches such as high-throughput screenings and structure-based drug design. Of these, ABT-737 and ABT-263 (Figure 1.10) were shown to be particularly promising candidates.

Figure 1.10: Chemical structures of ABT-737 and ABT-263 [168]. The three indicated key sites in ABT-737 were identified to affect charge balance, metabolism, and oral absorption. Chemical optimization resulted in identification of ABT-263.

ABT-737 was discovered in a nuclear magnetic resonance-based screening aimed at rationally designing molecules that target the BH3-binding groove of Bcl-xL [169]. The small molecule binds to and inhibits Bcl-xL, Bcl-2, and Bcl-w with high affinity (Ki ≤ 1 nM) but with comparatively low affinity to Mcl-1 and other anti-apoptotic Bcl-2 family members (Ki ≥ 0.46 μM). It was further found to compete with binding of a Bad BH3 peptide to Bcl-xL and is therefore categorized as a Bad-like BH3-mimetic.

In leukemic and lymphatic cancer cells, ABT-737 was capable of inducing apoptosis by disrupting Bcl-2/Bax complexes and release Bim from Bcl-2 sequestration [170, 171]. Affirmatively, ABT-737 showed single-agent cytotoxic activity against various hematological cell lines with low Mcl-1 levels [172-174]. Except for SCLC cells, however, solid tumor cell lines were largely insensitive [169, 175, 176]. Furthermore, solubility of ABT-737 was insufficient
for intravenous delivery and consequently ABT-263 (navitoclax), an orally bioavailable derivate, was developed by chemical optimization efforts and further structure-based design [168]. ABT-263 was demonstrated to have similar binding affinities and therefore similar potency in inducing intrinsic apoptosis. Like ABT-737, ABT-263 has no significant affinity to Mcl-1. Correspondingly, increased presence of Mcl-1 protein is a commonly reported mechanism of resistance to inhibitor-induced cell death in various cancer types [170, 175, 177]. Due to some promising results from in vitro and in vivo studies, ABT-263 was nevertheless rapidly advanced to clinical testing and valuable progress has been made in the clinical evaluation [167]. The results obtained so far suggest that this BH3-mimetic may be particularly useful when combined with conventional chemotherapeutic or molecularly targeted agents [178-181]. Several preclinical studies further reported synergistic effects of BH3-mimetics that improved cancer cell response to radiation in vitro and in vivo [182-184].
1.5 **Aims**

Hypoxia is a common feature of most human solid tumors and is considered a major factor that drives malignant progression and limits the success of chemo- and especially radiotherapy. The present study was based on the hypothesis that exposure to acute and cycling hypoxia alters the balance between pro- and anti-apoptotic Bcl-2 family members in favor of a pro-survival function and that the resulting elevated apoptotic threshold might critically contribute to increased radioresistance of hypoxic tumor cell fractions. Consequently, targeting anti-apoptotic Bcl-2 family members might enhance therapy success by lowering the apoptotic threshold of hypoxic cancer cells.

HCT116 colon carcinoma and NCI-H460 lung adenocarcinoma cells were used as experimental models, extended by previously described cells obtained by exposing NCI-H460 cells to 25 cycles of severe hypoxia and reoxygenation (hypoxia/reoxygenation-stress tolerant NCI-H460 cells [185], in this study referred to as *hypoxia-selected* NCI-H460 cells). This cell model enables exploration and identification of mechanism of adaptation to adverse hypoxic conditions that might be relevant for improved survival and therapy resistance of hypoxic tumor cells.

The first objective of this study was to validate the suggested association of apoptosis resistance and decreased sensitivity to radiotherapy of cancer cells exposed to acute and cycling severe hypoxia in an experimental setup. Furthermore it was intended to compare gene expression and protein levels of Bcl-2 family proteins in normoxia and after exposure to acute and cycling hypoxia to enlighten their regulation and determine how an altered Bcl-2 rheostat contributes to therapy resistance.

The second aim was the definition of therapeutic approaches to overcome radioresistance of hypoxic and hypoxia-selected cancer cells. To this end, it was intended to employ ABT-263, a clinically relevant inhibitor of Bcl-2 and Bcl-xL, in combination with radiotherapy. Flow cytometry based cell analyses as well as short- and long-term survival assays should indicate whether targeting anti-apoptotic Bcl-2 family members improves radiotherapy success in normoxic and particularly hypoxic cancer cells.
The third objective was to evaluate possible alterations of other mitochondrial functions that might contribute to improved survival of hypoxia-selected cancer cells. For this purpose, hypoxia-selected and non-selected NCI-H460 cells should be analyzed regarding expression of genes involved in mitochondrial dynamics and respiration as well as their metabolic potential. This might identify novel and specific targetable factors and in the long term allow the design of rational strategies for overcoming radioresistance acquired due to adaptation to cycling hypoxia.
2 MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 TECHNICAL EQUIPMENT

<table>
<thead>
<tr>
<th>Device</th>
<th>Manufacturer</th>
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<tr>
<td>ABI 7900HT Real-Time PCR System</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Analytic scale ABT 120-4</td>
<td>Kern</td>
</tr>
<tr>
<td>Biological irradiator X-RAD 320</td>
<td>Precision X-Ray</td>
</tr>
<tr>
<td>Blotting chamber Trans-Blot®</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Cell counter CASY® Model TT</td>
<td>OMNI Life Science</td>
</tr>
<tr>
<td>Centrifuge 5417 R</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>Centrifuge Biofuge® pico</td>
<td>Heraeus Instruments</td>
</tr>
<tr>
<td>Centrifuge Megafuge™ 1.0R</td>
<td>Heraeus Instruments</td>
</tr>
<tr>
<td>Chemiluminescence-imager Fusion Solo</td>
<td>Vilber Lourmat</td>
</tr>
<tr>
<td>Extracellular flux analyzer Seahorse XFe96</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Flow cytometer BD Accuri™ C6</td>
<td>Becton Dickinson Bioscience</td>
</tr>
<tr>
<td>GelCount™ colony counter</td>
<td>Ophord Optronix</td>
</tr>
<tr>
<td>Incubator C200</td>
<td>Labotect</td>
</tr>
<tr>
<td>Inverted light microscope Diavert</td>
<td>Ernst Leitz</td>
</tr>
<tr>
<td>Invivo2 400 hypoxia chamber</td>
<td>Ruskinn Technology</td>
</tr>
<tr>
<td>Laminar-flow bench</td>
<td>BDK</td>
</tr>
<tr>
<td>Magnetic separation rack MagnaRack™</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Magnetic stirrer RH basic 2</td>
<td>IKA-Werke</td>
</tr>
<tr>
<td>Microplate reader BioTec® Synergy™ 2</td>
<td>Bio-Tek Instruments</td>
</tr>
<tr>
<td>Microplate reader Spark®</td>
<td>Tecan Trading</td>
</tr>
<tr>
<td>Orbital shaker MTS 2/4</td>
<td>IKA-Werke</td>
</tr>
<tr>
<td>pH electrode edge®</td>
<td>Hanna Instruments</td>
</tr>
<tr>
<td>Photometer Nanodrop™ ND-1000</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Pipette controller ErgoOne® FAST</td>
<td>STARLAB</td>
</tr>
<tr>
<td>Pipette PIPETMAN® Classic</td>
<td>Gilson</td>
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<tr>
<td>Power supply PowerPac HC</td>
<td>Bio-Rad</td>
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<tr>
<td>Power supply VWR 250V</td>
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<td>Protein electrophoresis system</td>
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<td>RS 320 cabinet irradiator</td>
<td>XStrahl Limited</td>
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<td>ThermoMixer® comfort</td>
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<td>Vortexer Reax 2000</td>
<td>Heidolph</td>
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<td>Water bath</td>
<td>GFL</td>
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## MATERIAL AND METHODS

### 2.1.2 CONSUMABLES

Table 2.2: Consumable material

<table>
<thead>
<tr>
<th>Item</th>
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<td>0.45 µm filter</td>
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<td>6-well plates</td>
<td>Eppendorf</td>
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<td>384-well qPCR plates</td>
<td>4titude</td>
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<td>96-well plates</td>
<td>TPP</td>
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<tr>
<td>CASYcups</td>
<td>OMNI Life Science</td>
</tr>
<tr>
<td>Cell culture dishes</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Cell culture flasks</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Centrifuge tubes</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Combitips advanced®</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>Cryo tubes</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Flow cytometry tubes</td>
<td>BD Falcon</td>
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<tr>
<td>GasPak™ Anaerobe Pouch System</td>
<td>Becton Dickinson Diagnostics</td>
</tr>
<tr>
<td>Injection needles</td>
<td>Becton Dickinson Diagnostics</td>
</tr>
<tr>
<td>Pasteur pipettes</td>
<td>Brand</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>Starlab</td>
</tr>
<tr>
<td>Plastic syringes</td>
<td>Becton Dickinson Diagnostics</td>
</tr>
<tr>
<td>PVDF membrane</td>
<td>Roth</td>
</tr>
<tr>
<td>qPCR seal</td>
<td>4titude</td>
</tr>
<tr>
<td>Reaction tubes</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Seahorse XF96 cell culture microplates</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Seahorse XF96 sensor cartridges</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Serological pipettes</td>
<td>Sarstedt</td>
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<tr>
<td>Whatman® paper</td>
<td>Schleicher &amp; Schuell</td>
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### 2.1.3 KITS

Table 2.3: Utilized commercial kits

<table>
<thead>
<tr>
<th>Kit</th>
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<tbody>
<tr>
<td>Pierce™ BCA Protein Assay Kit</td>
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<tr>
<td>qPCR MasterMix for SYBR® Green I</td>
<td>Eurogentec</td>
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<tr>
<td>QuantiTect® Reverse Transcription Kit</td>
<td>Qiagen</td>
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<tr>
<td>RNeasy® Mini Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Seahorse XF Cell Phenotype Test Kit</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>Seahorse XF Glycolysis Stress Test Kit</td>
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<tr>
<td>Seahorse XF Mito Stress Test Kit</td>
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# MATERIAL AND METHODS

## 2.1.4 GENERAL CHEMICALS

Table 2.4: Utilized chemicals

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<tr>
<th>Chemical</th>
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<tr>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)</td>
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<tr>
<td>Acetic acid</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ammonium persulfate (APS)</td>
<td>Carl Roth</td>
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<tr>
<td>Bovine serum albumin (BSA)</td>
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<tr>
<td>Bromphenol blue</td>
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<tr>
<td>CHAPS</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Coomassie brilliant blue</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Crystal violet</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ethanol</td>
<td>Carl Roth</td>
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<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Formaldehyde</td>
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<td>Glutar aldehyde</td>
<td>Carl Roth</td>
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<td>Glycerol</td>
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<td>Glycine</td>
<td>Merck Millipore</td>
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<td>Isoflurane</td>
<td>Sigma-Aldrich</td>
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<td>Isopropanol</td>
<td>Sigma-Aldrich</td>
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<td>Methanol</td>
<td>Carl Roth</td>
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<td>Non-fat dry milk</td>
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<td>Paraformaldehyde</td>
<td>Sigma-Aldrich</td>
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<td>Phenylmethanesulfonyl fluoride</td>
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<td>Propidium iodide (PI)</td>
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<td>Sodium chloride (NaCl)</td>
<td>Merck Millipore</td>
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<td>Sodium citrate</td>
<td>Carl Roth</td>
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<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
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<tr>
<td>Sodium fluoride</td>
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<td>Sodium orthovanadate</td>
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<td>Sodium pyrophosphate</td>
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<td>Tetramethylethylenediamine (TEMED)</td>
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<tr>
<td>Tetramethylrhodamine ethyl ester (TMRE)</td>
<td>Thermo Fisher Scientific</td>
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<td>Tris(hydroxymethyl)aminomethane (Tris)</td>
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<td>Triton X-100</td>
<td>Sigma-Aldrich</td>
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<td>Tween-20</td>
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<tr>
<td>β-mercaptoethanol</td>
<td>Merck Millipore</td>
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### MATERIAL AND METHODS

#### 2.1.5 MEDIA, REAGENTS, AND COMMERCIAL BUFFERS

**Table 2.5: Utilized media, reagents, and commercial buffers**

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<thead>
<tr>
<th>Medium/Reagent/Buffer</th>
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<tr>
<td>ABT-263 (navitoclax)</td>
<td>Selleckchem</td>
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<td>Acrylamide solution (Rotiphorese® Gel 30)</td>
<td>Carl Roth</td>
</tr>
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<td>Aprotinin</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>CASYton</td>
<td>OMNI Life Science</td>
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<td>D-glucose</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Dynabeads® M-280 Sheep Anti-Rabbit IgG</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>ECL™ Prime Reagent</td>
<td>GE Healthcare Life Sciences</td>
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<td>ECL™ Select Reagent</td>
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<tr>
<td>Fetal Calf Serum (FCS)</td>
<td>Gibco</td>
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<td>Hoechst 33342</td>
<td>Thermo Fisher Scientific</td>
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<td>Leupeptin</td>
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<td>L-glutamine</td>
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<tr>
<td>Matrigel® Matrix</td>
<td>Corning</td>
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<td>MCL1-directed siRNA (ON-TARGET SMARTpool)</td>
<td>Dharmacon</td>
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<td>Non-targeting siRNA (ON-TARGET Non-targeting Control Pool)</td>
<td>Dharmacon</td>
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<td>OPA1-directed siRNA (ON-TARGET SMARTpool)</td>
<td>Dharmacon</td>
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<tr>
<td>Opti-MEM™</td>
<td>Gibco Thermo Fisher</td>
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<td>Page Ruler™ Pre-Stained Protein Ledder</td>
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<td>Pepstatin</td>
<td>Carl Roth</td>
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<td>Phosphate Buffered Saline (PBS)</td>
<td>Gibco</td>
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<td>RPMI 1640 (with L-glutamine)</td>
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<td>Seahorse XF Calibrant</td>
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<td>Sodium pyruvate</td>
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<td>Trypsin-EDTA (0.05 %)</td>
<td>Biochrom</td>
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2.1.6 Buffers and Solutions

Table 2.6: Composition of staining solutions for flow cytometric analyses

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<th>Staining solution</th>
<th>Component</th>
<th>Amount/Concentration</th>
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<tr>
<td>Nicoletti</td>
<td>Sodium citrate</td>
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<td></td>
<td>Triton X-100</td>
<td>0.1 % (v/v)</td>
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<tr>
<td></td>
<td>Propidium iodide in PBS</td>
<td>50 µg/mL</td>
</tr>
<tr>
<td>PI exclusion</td>
<td>Propidium iodide in complete medium</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>TMRE</td>
<td>tetramethylrhodamine ethyl ester in complete medium</td>
<td>25 nM</td>
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Table 2.7: Composition of staining solutions for cell survival assays

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<th>Buffer/Solution</th>
<th>Components</th>
<th>Amount/Concentration</th>
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<td>Coomassie brilliant blue staining solution</td>
<td>Coomassie brilliant blue</td>
<td>0.05 % (w/v)</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>20 % (v/v)</td>
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<td></td>
<td>Acetic acid</td>
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<tr>
<td>Crystal violet staining solution</td>
<td>Crystal violet in PBS</td>
<td>0.1 % (w/v)</td>
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Table 2.8: Composition of assay media for Seahorse XF cellular bioenergetics tests

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</tr>
</thead>
<tbody>
<tr>
<td>Cell phenotype test</td>
<td>Seahorse XF Base Medium</td>
<td></td>
</tr>
<tr>
<td>Mito stress test</td>
<td>Pyruvate</td>
<td>1 mM</td>
</tr>
<tr>
<td></td>
<td>L-glutamine</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>D-glucose</td>
<td>10 mM</td>
</tr>
<tr>
<td></td>
<td>adjusted to pH 7.4</td>
<td></td>
</tr>
<tr>
<td>Glycolysis stress test</td>
<td>Seahorse XF Base Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-glutamine</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>adjusted to pH 7.4</td>
<td></td>
</tr>
</tbody>
</table>
## Table 2.9: Composition of buffers and solutions for protein analysis

<table>
<thead>
<tr>
<th>Buffer/Solution</th>
<th>Components</th>
<th>Amount/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis buffer</strong></td>
<td>HEPES (pH 7.5)</td>
<td>50 mM</td>
</tr>
<tr>
<td></td>
<td>sodium chloride</td>
<td>150 mM</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>1 % (v/v)</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td></td>
<td>sodium pyrophosphate</td>
<td>10 mM</td>
</tr>
<tr>
<td></td>
<td>sodium fluoride</td>
<td>10 mM</td>
</tr>
<tr>
<td></td>
<td>sodium orthovanadate</td>
<td>2 mM</td>
</tr>
<tr>
<td></td>
<td>phenylmethanesulfonyl fluoride</td>
<td>100 mM</td>
</tr>
<tr>
<td></td>
<td>Aprotinin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td></td>
<td>Leupeptin</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td></td>
<td>Pepstatin A</td>
<td>3 µg/mL</td>
</tr>
<tr>
<td></td>
<td>in H2O</td>
<td></td>
</tr>
<tr>
<td><strong>4x SDS sample buffer</strong></td>
<td>Glycerol</td>
<td>40 % (v/v)</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl (pH 6.8)</td>
<td>240 mM</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>8 % (w/v)</td>
</tr>
<tr>
<td></td>
<td>Bromphenol blue</td>
<td>0.04 % (w/v)</td>
</tr>
<tr>
<td></td>
<td>β-mercaptoethanol</td>
<td>10 % (v/v)</td>
</tr>
<tr>
<td></td>
<td>in H2O</td>
<td></td>
</tr>
<tr>
<td><strong>15 % SDS PAGE resolving gel (for 1 gel)</strong></td>
<td>H2O</td>
<td>3.4 mL</td>
</tr>
<tr>
<td></td>
<td>Acrylamid (30 %)</td>
<td>7.5 mL</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl (1.5 M, pH 8.8)</td>
<td>3.8 mL</td>
</tr>
<tr>
<td></td>
<td>SDS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>APS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.006 mL</td>
</tr>
<tr>
<td><strong>12 % SDS PAGE resolving gel (for 1 gel)</strong></td>
<td>H2O</td>
<td>4.9 mL</td>
</tr>
<tr>
<td></td>
<td>Acrylamid (30 %)</td>
<td>6 mL</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl (1.5 M, pH 8.8)</td>
<td>3.8 mL</td>
</tr>
<tr>
<td></td>
<td>SDS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>APS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.006 mL</td>
</tr>
<tr>
<td><strong>10 % SDS PAGE resolving gel (for 1 gel)</strong></td>
<td>H2O</td>
<td>5.9 mL</td>
</tr>
<tr>
<td></td>
<td>Acrylamid (30 %)</td>
<td>5 mL</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl (1.5 M, pH 8.8)</td>
<td>3.8 mL</td>
</tr>
<tr>
<td></td>
<td>SDS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>APS (10 %)</td>
<td>0.150 mL</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.006 mL</td>
</tr>
<tr>
<td><strong>5 % SDS PAGE stacking gel (for 1 gel)</strong></td>
<td>H2O</td>
<td>3.4 mL</td>
</tr>
<tr>
<td></td>
<td>Acrylamid (30%)</td>
<td>0.83 mL</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl (1 M, pH 6.8)</td>
<td>0.63 mL</td>
</tr>
<tr>
<td></td>
<td>SDS (10%)</td>
<td>0.05 mL</td>
</tr>
<tr>
<td></td>
<td>APS (10%)</td>
<td>0.05 mL</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>0.005 mL</td>
</tr>
</tbody>
</table>
10x SDS running buffer

- **Tris-HCl**
- **Glycine**
- **SDS**

*in H₂O; adjusted to pH 8.3*

- **250 mM**
- **192 mM**
- **1% (w/v)**

10x Transfer buffer

- **Tris-HCl**
- **Glycine**

*in H₂O; adjusted to pH 8.3*

- **250 mM**
- **192 mM**

10x TBS-T

- **Tris**
- **NaCl**
- **Tween-20**

*Adjusted to pH 7.4*

- **0.5 M**
- **1.5 M**
- **1% (v/v)**

Blocking solution

- **Non-fat dry milk**

*in 1x TBS-T*

- **5% (w/v)**

### 2.1.7 Antibodies

#### Table 2.10: Antibodies applied for immunoprecipitation and Western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Bax (N-terminus)</td>
<td>rabbit</td>
<td>1 µg/250 µg total protein</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Anti-mouse (HRP-conjugated)</td>
<td>goat</td>
<td>1:2000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Anti-rabbit (HRP-conjugated)</td>
<td>goat</td>
<td>1:2000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>rabbit</td>
<td>1 µg/250 µg total protein</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Bak</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Bax (D2E11)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>mouse</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Bim (C34C5)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Bnip3</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Cleaved caspase-3 (Asp175)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Mcl-1 (D35A5)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Noxa (114C307)</td>
<td>mouse</td>
<td>1:1000</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Opa1 (1E8-1D9)</td>
<td>mouse</td>
<td>1:1000</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>PARP (46D11)</td>
<td>rabbit</td>
<td>1:2000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>Puma</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technologies</td>
</tr>
<tr>
<td>β-actin</td>
<td>mouse</td>
<td>1:20000</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
2.1.8 qRT-PCR PRIMERS

All primers were produced by Metabion.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M forwards</td>
<td>TGCTGTCTCCATGTTTGATGTATCT</td>
</tr>
<tr>
<td>reverse</td>
<td>TCTCTGCTCCCCACCTCTAAGT</td>
</tr>
<tr>
<td>DNM1L forwards</td>
<td>AGAAAATGGGGTGGAAGCAGA</td>
</tr>
<tr>
<td>reverse</td>
<td>CACCTTGGTCACTTCGAC</td>
</tr>
<tr>
<td>FIS1 forwards</td>
<td>GGAACACCGGGATTACGTCT</td>
</tr>
<tr>
<td>reverse</td>
<td>TGCCCAAGAGTCCATTTTC</td>
</tr>
<tr>
<td>MCL1 forwards</td>
<td>TCTCATTTCTTTTGGTGCCT</td>
</tr>
<tr>
<td>reverse</td>
<td>GATATGCCAAACCAGCTCCTAC</td>
</tr>
<tr>
<td>MFN1 forwards</td>
<td>TAGTTGGAGGGAGACCTTAGC</td>
</tr>
<tr>
<td>reverse</td>
<td>TCTACCAGATCATCTTCAAGTGC</td>
</tr>
<tr>
<td>MFN2 forwards</td>
<td>CCCCTTGTCTTTTATGCGATGT</td>
</tr>
<tr>
<td>reverse</td>
<td>TTTGGGAGAGGTGTTGATTTTC</td>
</tr>
<tr>
<td>OPA1 forwards</td>
<td>TGCCGTACATTGATGGGAAAA</td>
</tr>
<tr>
<td>reverse</td>
<td>TTCCGGAGAACCCTGAGGTAAAA</td>
</tr>
<tr>
<td>VDAC1 forwards</td>
<td>AAGTGAACAACTCCAGCTGA</td>
</tr>
<tr>
<td>reverse</td>
<td>CACCAGGAGTCCCTCGTCT</td>
</tr>
<tr>
<td>VDAC2 forwards</td>
<td>CATTTCGAAAGTCAACAAGTC</td>
</tr>
<tr>
<td>reverse</td>
<td>TCCCATTACCAGACAGAG</td>
</tr>
<tr>
<td>VDAC3 forwards</td>
<td>AGCCTGATTGGACCTGGCT</td>
</tr>
<tr>
<td>reverse</td>
<td>CTGTGACCTCCTGCACTGA</td>
</tr>
</tbody>
</table>

2.1.9 EUKARYOTIC CELL LINES

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>human colon carcinoma</td>
<td>ATCC</td>
</tr>
<tr>
<td>Non-selected NCI-H460</td>
<td>human non-small cell lung cancer</td>
<td>ATCC</td>
</tr>
<tr>
<td>Hypoxia-selected NCI-H460</td>
<td>human non-small cell lung cancer</td>
<td>previous work Jendrossek lab [185]</td>
</tr>
</tbody>
</table>
### 2.1.10 SOFTWARE AND TOOLS

**Table 2.13: Applied software and tools**

<table>
<thead>
<tr>
<th>Software</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD Accuri™ C6</td>
<td>Becton Dickinson Biosciences</td>
</tr>
<tr>
<td>BLAST 2.2</td>
<td>National Center for Biotechnology Information (NCBI)</td>
</tr>
<tr>
<td>Fusion</td>
<td>Vilber Lourmat</td>
</tr>
<tr>
<td>GelCount™ 1.4</td>
<td>Oxford Optronix</td>
</tr>
<tr>
<td>GraphPad Prism 6</td>
<td>GraphPad Software</td>
</tr>
<tr>
<td>KC4™</td>
<td>BioTek Instruments</td>
</tr>
<tr>
<td>Primer-BLAST</td>
<td>National Center for Biotechnology Information (NCBI)</td>
</tr>
<tr>
<td>SDS 2.2</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>SparkControl™</td>
<td>Tecan Trading</td>
</tr>
<tr>
<td>Wave Desktop 2.3</td>
<td>Agilent Technologies</td>
</tr>
</tbody>
</table>
2.2 CELL LINES AND CULTIVATION

2.2.1 HYPOXIC SELECTION OF NCI-H460 CANCER CELLS

In order to analyze mechanisms and effects of adaptation to cycling hypoxia, a sub-cell line of NCI-H460 cells was generated within the group of Prof. Jendrossek (not part of this work). The cell line was established by exposing NCI-H460 cells to 25 cycles of severe hypoxia (48 h 0.1 % O₂) and reoxygenation (120 h 20 % O₂), which resulted in the selection of hypoxia/reoxygenation stress tolerant cells as described recently by Matschke et al. [185]. In the present study, this cell line is referred to as hypoxia-selected NCI-H460 as opposed to non-selected NCI-H460 cells.

2.2.2 EUKARYOTIC CELL CULTURE

HCT116, non-selected and hypoxia-selected NCI-H460 cells were cultivated in RPMI medium containing L-glutamine and supplemented with 10 % heat-inactivated FCS (complete medium). For standard cultivation under normoxic conditions, cells were kept in a 20 % O₂ and 5 % CO₂ humidified atmosphere at 37 °C.

Cells were split as they reached a confluence of 80-90 %. For passaging, they were washed with PBS, detached by incubation with Trypsin-EDTA at 37 °C, and seeded in 75 cm² cell culture flasks at appropriate dilutions in fresh complete medium. Cells used for experiments did not exceed 20 passages. When seeding of specific cell numbers was required, the cell suspension was diluted with CASYton and the number of viable cells was assessed using CASY® cell counter. All cell lines were routinely checked for mycoplasma.

For conservation, cells were spun down by centrifugation for 5 minutes at 1400 rpm and resuspended in 1.5 mL RPMI medium supplemented with 20 % FCS and 10 % DMSO. The cell suspension was transferred to cryo tubes and frozen at -80 °C. After a few days, the cells were transferred to liquid nitrogen. For reactivation, cells were thawed in a water bath at 37 °C, washed with complete medium to remove DMSO, and further cultivated at standard cell culture conditions.
2.3 CELL TREATMENT AND EXPERIMENTAL CONDITIONS

2.3.1 TREATMENT CONDITIONS

Cells were seeded in complete medium at appropriate numbers the day before treatment and incubated overnight under normoxic conditions. All experiments were simultaneously performed under normoxic and severely hypoxic conditions. For latter, cells were treated in a humidified hypoxia workstation (Invivo2 400) at 37 °C, 0.2 % O₂, and 5 % CO₂ (referred to as severe hypoxia or severely hypoxic conditions). Prior to any treatment, cells, media, and solutions were equilibrated to hypoxic conditions in the hypoxia work station for 2 hours. Following treatment under hypoxic conditions, cells were further incubated in hypoxia for indicated periods before proceeding with the experiment or analyses.

2.3.2 ABT-263 TREATMENT

For treatment, the medium of cells seeded the day before was removed and replaced by complete medium containing ABT-263 (stock solution dissolved in DMSO) at concentration between 0.1 and 10 µM. Control medium contained DMSO instead of ABT-263, at the same concentration as in the sample with the highest ABT-263 concentration.

2.3.3 IRRADIATION OF CELLS

Irradiation of cells was performed with an X-ray machine (X-RAD 320) operated at 320 kV, 10 mA, with a 1.65 mm aluminum filter. Irradiation was carried out at room temperature at a distance of 75 cm. The dose rate was ~2.76 Gy/min, with effective photon energy of ~90 kV. For irradiation under hypoxic conditions, plates or dishes containing cells were transferred to GasPak™ airtight pouches and returned to the hypoxia chamber immediately after irradiation. For combined treatment with ABT-263, the inhibitor was added directly after irradiation (unless stated otherwise).

2.3.4 siRNA-MEDIATED GENE SILENCING

Downregulation of Mcl-1 and Opa1 were performed by siRNA-mediated gene silencing. Transfection mixtures containing siRNA targeting MCL1, OPA1, or non-targeting siRNA were prepared as shown in Table 2.14.
Table 2.14: Composition of siRNA transfection mixtures

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per mL complete medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM™</td>
<td>100 µL</td>
</tr>
<tr>
<td>TransIT-siQUEST® Reagent</td>
<td>3 µL</td>
</tr>
<tr>
<td>siRNA (10 µM)</td>
<td>2.8 µL</td>
</tr>
</tbody>
</table>

After 25 minutes incubation at room temperature, transfection mixtures were added drop-wise to different areas of the well and distributed by gentle shaking. 24 hours after transfection, medium was exchanged and cells were further treated as described in 2.3.2 and 2.3.3 or 2.6.2. Knockdown efficiency was assessed at mRNA level by qPR-PCR 24 hours after transfection (see 2.10) and at protein level by Western blot analysis at 24, 48, and 72 hours after transfection (see 2.8).

2.4 FLOW CYTOMETRY-BASED CELL ANALYSES

2.4.1 FLOW CYTOMETRY PRINCIPLES AND ASSAYS

Flow cytometry is a laser based method that allows analysis of multiple characteristics of individual cells [186]. As cells flow in single file in a stream of fluid, they pass a beam of monochromatic light. The light is scattered at characteristic angles, determined by cell size (forward scattering, FSC) and granularity (sideward scattering, SSC). Furthermore, specific fluorescent dyes and fluorescently labelled antibodies are used to qualitatively and quantitatively analyze physiological and cellular properties, such as membrane potential, DNA content, and membrane-bound or intracellular proteins. As this technique allows detailed analysis of complex cell population in a short time, flow cytometry has many applications in research and clinical practice [187].

2.4.1.1 MEASUREMENT OF APOPTOSIS INDUCTION VIA NICOLETTI STAINING

The “Nicoletti” staining allows flow cytometric assessment of the cell cycle distribution in a cell population [188]. For this, fluorescent agent propidium iodide (PI) is used to stain the DNA content in permeabilized cells. As PI intercalates between nucleotide base pairs, fluorescence emission is proportional to the DNA content of a cell, which is characteristic for the cell cycle phase. In apoptotic cells, DNA is fragmented and low molecular DNA fragments are generated,
represented in the “Sub G1” population. This enables quantification of apoptotic cells within a cell population (Figure 2.1).

![Figure 2.1: Representative cell cycle distribution histogram generated by flow cytometry. Staining of cellular DNA with propidium iodide allows the quantification of cells in G1, S and G2 cell cycle phase. Apoptotic cells are represented in the Sub G1 fraction.](image1)

2.4.1.2 **Propidium Iodide Exclusion Staining**

PI can furthermore be used as an indicator for general cell viability. This is based on the fact that PI is a membrane impermeable dye that is excluded from viable cells. In dead cells, when the cellular membranes become permeable, PI can diffuse into the cell nucleus where it intercalates with DNA, resulting in fluorescent signal. As shown in Figure 2.2, PI exclusion staining can be used to quantify viable and dead cells in a cell suspension by flow cytometric analysis of fluorescence intensity.

![Figure 2.2: Representative density plot of flow cytometric viability analysis based on PI exclusion staining. Membranes of viable cells are impermeable for PI. Cells with intact membranes therefore have low fluorescence intensity. Non-viable or dead cells are characterized by high fluorescence intensity, as PI can pass damaged membranes and bind to DNA.](image2)
2.4.1.3 Mitochondrial membrane potential dissipation measurement via TMRE staining

Tetramethylrhodamine ethyl ester (TMRE) is a positively charged, lipophilic fluorescence dye. In healthy cells with intact mitochondria, TMRE accumulates at the inner mitochondrial membrane, directed by the proton gradient that constitutes the mitochondrial membrane potential (MMP, ΔΨm) [189, 190]. As apoptotic and dying cells are characterized by dissipation of the MMP, TMRE staining can be used to assess the amount of viable cells with intact MMP (high ΔΨm) as opposed to dead cells with dissipated MMP (low ΔΨm) via flow cytometry (Figure 2.3).

Figure 2.3: Representative flow cytometric analysis of the mitochondrial membrane potential of TMRE-stained cells. The density plot represents a cell population stained with ΔΨm-specific fluorescent dye TMRE. Cells with intact mitochondria are characterized by high ΔΨm and therefore have higher fluorescence intensity. Apoptotic and dead cells have weaker fluorescence intensities, as ΔΨm is dissipated.

2.4.2 Experimental procedure for flow cytometric analyses

In this study, flow cytometry-based analyses were employed to assess apoptosis induction, cell death, and MMP dissipation. For this, cell culture medium and supernatants from washing steps were collected and cells were harvested by Trypsin-EDTA incubation. Cell suspensions were split into 3 tubes and spun down by centrifugation for 5 minutes at 1400 rpm. Cell pellets were resuspended in 200 µL Nicoletti, PI exclusion, or TMRE staining solution and incubated in the dark at 37 °C (PI exclusion, TMRE) or at room temperature (Nicoletti) for 30 minutes. Flow cytometric measurements were performed with a BD Accuri™ C6 flow cytometer, employing fluorescence channel 2 (FL-2, 597 nm), and analyzed with respective BD Accuri™ C6 software.
2.5 **SHORT- AND LONG-TERM CELL SURVIVAL ASSAYS**

2.5.1 **CRYSTAL VIOLET SHORT-TERM SURVIVAL ASSAY**

The crystal violet assay enables determination of short-term cell survival and differences in proliferation upon treatment with death-inducing agents [191]. The dye binds to protein and DNA of viable, attached cells. In contrast, dying cells detach and are consequently removed during the washing step. Furthermore, treatment can influence or halt proliferation. Both characteristics reduce the amount of crystal violet staining. In this study, the assay was used for a relative quantification of cell viability of HCT116 and NCI-H460 cells upon treatment with ionizing radiation and ABT-263 in normoxia and severe hypoxia.

Cells were seeded at different numbers (5000, 10000, 15000 cells per well) in 96-well plates. Each plate has been set up with 4 replicates per cell line, group, and condition. Cells were irradiated with 10 Gy, treated with 0.4-4 µM ABT-263, or both. To avoid detaching and loss of cells during medium exchange required for treatment, fresh complete medium containing ABT-263 for appropriate final concentrations was added directly to the wells, without removing the medium the cells were seeded in.

Following 48 hours treatment in normoxia or severe hypoxia, medium containing detached, non-viable cells was discarded. Remaining viable cells were fixed with glutaraldehyde (1 % in PBS) for 15 minutes and stained with crystal violet (0.1 % in PBS) for 25 minutes. Following removal of the staining solution, plates were rinsed with water and left to dry. For cell lysis and release of the dye, cells were incubated with Triton X-100 (0.2 % in PBS) for at least 25 minutes on an orbital shaker at room temperature. Absorption was measured spectrophotometrically at a wavelength of 540 nm. As optical density of the dye is directly proportional to the number of attached, viable cells, changes in cell viability upon treatment were calculated by normalizing the values to respective controls.

2.5.2 **COLONY FORMATION ASSAY**

The colony formation assay determines reproductive cell death after treatment with cytotoxic agents [192]. This assay is based on the ability of single cells to grow into colonies, detecting those which have retained the capacity to undergo unlimited division despite treatment with cytotoxic agents. To analyze clonogenic survival after treatment with ionizing radiation and ABT-263 treatment in normoxia and severe hypoxia, cells were seeded in triplicates at various
concentrations (HCT116: 100-6400, NCI-H460: 50-3200 cells per well of a 6-well plate). The following day, cells were irradiated with doses of 0-5 Gy under normoxic or hypoxic conditions, with or without subsequent addition of ABT-263. For treatment under hypoxic conditions, cells were pre-incubated for 2 hours before irradiation and remained in severe hypoxia for 48 hours, before all cells were further incubated under normoxic conditions for 8-10 days to allow growth of single cell colonies.

As soon as sufficiently large colonies (≥50 cells) had formed in untreated controls, medium was removed and cells were fixed by 10 minutes incubation with 3.7 % paraformaldehyde (PFA) and permeabilized with 70 % ethanol for further 10 minutes. After removing ethanol, Coomassie brilliant blue staining solution was added for 1 hour. Following removal of the staining solution, plates were rinsed with water and left to dry.

Colonies were counted using the GelCount™ colony counter and respective software. The plating efficiency (PE) was calculated according to numbers of counted colonies and seeded cells in untreated normoxia and hypoxia controls:

\[
PE \% = \frac{\text{counted colonies}_{\text{control}}}{\text{seeded cell number}_{\text{control}}} \times 100
\]

Surviving fractions (SF) were then calculated as ratio of counted colonies to seeded cells for each treatment condition and dose and normalized to respective untreated controls:

\[
SF = \frac{\text{counted colonies}_{\text{treatment}}}{\text{seeded cell number}_{\text{treatment}} \times PE}
\]

For better graphical representation, survival curves were generated by plotting radiation doses (linear) versus surviving fractions (logarithmic).

### 2.6 Analysis of Cellular Bioenergetics

#### 2.6.1 Seahorse XF Extracellular Flux Analyzer - Principle

The Seahorse XF Extracellular Flux technology was developed to determine the bioenergetic phenotypes and metabolic potential of cells [193]. The technique allows analysis and comparison of cellular engagement of glycolysis and mitochondrial respiration under base and energetic stress conditions. Key parameters of the two main energy-generating pathways are assessed by simultaneous fiber-optic-based measurement of the extracellular acidification.
rate (ECAR; indicator of glycolysis) and the oxygen consumption rate (OCR; indicator of OXPHOS). In metabolic assays, inhibitors, stimulators, or substrates interfering with glycolysis and oxidative phosphorylation are sequentially injected through built-in ports to evaluate immediate effects on the cellular bioenergetic state. Assessing the capability of cells to respond to induced energetic demands enables estimation of their metabolic potential, which can be a measure for cellular fitness or flexibility.

### 2.6.2 Assessment of metabolic phenotypes, glycolytic function, and mitochondrial respiration

In this study, a Seahorse XFe96 Extracellular Flux Analyzer was used to assess bioenergetic characteristics of hypoxia-selected and non-selected NCI-H460 cells. For an optimal cell density of 60-80% at the time point of measurement, a range between 5000 and 12500 cells in 80 µL complete medium were seeded per well of a Seahorse XF96 cell culture microplate. Each plate has been set up with 4 replicates for each cell line and condition. 48 hours after seeding or 24 hours after siRNA transfection (see 2.3.4), cell culture medium was removed and cells were washed with assay medium. After adding a total volume of 180 µL assay medium per well, cells were incubated in a non-CO₂ incubator for 1 hour prior to the assay. Metabolic profiles were assessed using Seahorse XF cell energy phenotype, glycolysis stress, and mito stress test kits following the manufacturer’s protocols as described below.

As measured ECAR and OCR sensitively depend on the cell amount in each well, values were normalized to Hoechst nuclear staining. For this, supernatants were removed directly after completion of assays and cells were fixed by incubation in 4% PFA for 10 minutes. Fixed cells were incubated with 10 µg/mL Hoechst33342 in PBS for further 20 minutes. Following removal of the staining solution and a PBS washing step, 100 µL PBS were added per well. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

Metabolic data were analyzed using Wave Desktop 2.3 software.
2.6.2.1 Seahorse XF Mito Stress Test

The Seahorse XF mito stress test is designed to evaluate mitochondrial function [193]. Modulators of respiration, targeting distinct components of the ETC, are injected serially (Figure 2.4 A). Measurement of the OCR and changes in response to compound injection reveal key parameters of mitochondrial function (Figure 2.4 B).

Oligomycin, used at a final concentration of 1 µM, is an ATP synthase (complex V) inhibitor. The decrease in OCR after injection indicates the amount of oxygen consumed for mitochondrial ATP generation, which is calculated as follows:

\[ \text{ATP production} = \text{last OCR before oligom.} - \text{minimum OCR after oligom.} \]

Fluoro-carbonyl cyanide phenylhydrazide (FCCP) depolarizes the mitochondrial membrane. FCCP was used a final concentration of 2 µM. Injection results in an uninhibited electron flow through the ETC and drives maximal oxygen consumption by complex IV. FCCP-driven oxygen consumption reveals how close to their bioenergetic limit the cells utilize mitochondrial respiration. The difference is indicative of the cellular capacity to respond to an elevated energy demand via mitochondrial respiration, defined as spare respiratory capacity:

\[ \text{spare respiratory capacity} = \text{maximal respiration} - \text{basal respiration} \]

Rotenone, an inhibitor of complex I, and antimycin A, an inhibitor of complex III (used at final concentration of 0.5 µM each), completely shut down the ETC and hence oxygen consumption when added together. Consequently, the remaining oxygen consumption is non-mitochondrial. This measurement can be used to calculate the proton leak:

\[ \text{proton leak} = \text{minimum OCR after oligom.} - \text{no-n-mitochondrial respiration} \]
Figure 2.4: Seahorse XF mito stress test modulators and their effect on the cellular profile of key parameters of mitochondrial respiration [193]. A) Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, and rotenone and antimycin A inhibit complexes I and III, respectively. B) Key parameters of mitochondrial function, in particular ATP production, maximal respiration, and non-mitochondrial respiration, are assessed by measuring changes of the OCR in response to compound injection. These parameters furthermore reveal proton leak and spare respiratory capacity.

2.6.2.2 Seahorse XF Glycolysis Stress Test

The Seahorse XF glycolysis stress test assesses key parameters of the glycolytic flux: glycolysis, glycolytic capacity, and glycolytic reserve [193] (Figure 2.5). In this test, cells are incubated in assay medium without glucose or pyruvate. Glucose is injected at a saturating concentration (10 mM). As the cells catabolize glucose to pyruvate, ATP, NADH, water, and protons are produced. The release of protons is measured as increase of the ECAR, considered as glycolysis rate at base conditions. ATP synthase inhibitor oligomycin (1 µM) is injected to shut down mitochondrial ATP production. Consequently, cellular energy production is shifted to glycolysis, revealing the maximal ECAR (glycolytic capacity). The parameters can further be used to calculate the glycolytic reserve:

\[ \text{glycolytic reserve} = \text{glycolytic capacity} - \text{glycolysis} \]

Injection of 2-deoxy-glucose (2-DG, used at a final concentration of 50 mM), an inhibitor of glucose hexokinase, results in a decreased ECAR. It is used as a control to confirm that the protons produced and ECAR measured in the experiment was due to glycolysis.
Figure 2.5: Seahorse XF glycolysis stress test modulators and their effect on the cellular profile of key parameters of glycolysis [193]. A) Glycolysis is fueled by glucose and triggered in response to ATP synthase inhibitor oligomycin. 2-DG is a glucose analog and inhibits glycolysis by binding to glucose hexokinase. B) Measuring changes the ECAR in response to compound injection reveals key parameters of glycolytic flux: glycolysis, glycolytic capacity, and glycolytic reserve.

2.6.2.3 Seahorse XF Cell Energy Phenotype Test

In the Seahorse XF Cell Energy Phenotype Test kit, OCR and ECAR are measured under baseline and stressed conditions to assess the baseline phenotype, stressed phenotype, and the metabolic potential, which are indicative for the relative utilization of glycolysis and mitochondrial respiration [193] (Figure 2.6). To induce an energetic demand, oligomycin (1 µM) and FCCP (2 µM) are injected simultaneously. As oligomycin inhibits mitochondrial ATP production, cells react by a compensatory increase of glycolysis to produce energy. At the same time, the mitochondrial membrane is depolarized by FCCP, which allows proton flux into the matrix independent of ATP synthase. The attempt to restore the mitochondrial membrane potential requires the use of the ETC and therefore drives oxygen consumption rates higher. The metabolic potential is considered as the cells’ ability to respond to an energy demand via respiration and glycolysis. Both are calculated as increased rates over baseline rates:

\[
\text{stressed OCR} \, (\%) = \frac{\text{stressed OCR}}{\text{baseline OCR}} \times 100
\]

\[
\text{stressed ECAR} \, (\%) = \frac{\text{stressed ECAR}}{\text{baseline ECAR}} \times 100
\]
2.6 Exemplary Seahorse XF cell energy phenotype profile [193]. Baseline phenotype and stressed phenotype of a cell population are determined by the relative utilization of glycolysis and mitochondrial respiration, revealing their metabolic potential.

2.7 Mouse xenograft tumor generation and in vivo treatment

Animal experiments were conducted according to German animal welfare regulations and approved by local authorities (registration number AZ 84-02.04.2013). Male and female immunodeficient nude mice (NMRI-nu/nu) were purchased from University Hospital Essen. Animals were housed in individually ventilated cages and fed with sterile high calorie laboratory food. Food and water were provided ad libitum.

In order to analyze sensitivity of non-selected and hypoxia-selected NCI-H460 cells to treatment with ionizing radiation and ABT-263 under physiological conditions, tumor xenograft experiments were performed in NMRI nude mice. For this, tumors of both NCI-H460 cell lines were generated by subcutaneous injection of 5x10^5 cells in 50 μL RPMI 1640 containing 50 % growth factor reduced Matrigel® Matrix into the right hind leg. As soon as tumor volume reached ~50 mm³, mice were randomly allocated to treatment groups of 6-8 animals each.

Radiotherapy was delivered by an RS 320 cabinet irradiator. The irradiator was operated at 300 kV and 10 mA, with an output of 1.53 Gy/min. For radiation therapy, mice were anesthetized with 2 % isoflurane and the tumor bearing leg exposed to a single dose of 5 Gy in 5 mm tissue depth, via a collimated beam with a field size of 25x13 mm at a focus distance.
of 60 cm and Cu filtration. ABT-263 was given at a dose of 75 mg/kg via intraperitoneal injection in a total volume of 50 μL every second day starting at day 0 and ending at day 10 after treatment start (6 injections total). DMSO was used as a solvent control. For combined treatment, ABT-263 was initially injected 2 hours after irradiation. Tumor dimensions were assessed by caliper measurement and volume calculated by the modified ellipsoid formula:

$$\text{volume (mm}^3\text{)} = \frac{\pi}{6} \cdot a \cdot b \cdot \frac{a + b}{2}$$

### 2.8 Protein Analysis

#### 2.8.1 Preparation of Whole Cell Protein Lysates

After treatment and incubation in normoxia or hypoxia for the indicated time, total protein of HCT116 and NCI-H460 cells was extracted to evaluate changes in the protein levels of Bcl-2 protein family members. Furthermore, activation of caspase-3 was analyzed as an indicator of apoptosis. This is represented by the evidence of subunits that are generated following activating cleavage. Activity of caspase-3 moreover results in inactivating cleavage of DNA repair enzyme PARP, a direct substrate of caspase-3, which leads to the generation of an 89 kDa fragment [194, 195]. Furthermore, protein was extracted 24, 48, and 72 hours after transfection with MCL1- and OPA1-targeting siRNA in order to assess knockdown efficiency.

After removing medium, cells were harvested and washed with PBS. If necessary, cell pellets were frozen in liquid nitrogen and stored at -80 °C until further processing. To prevent protein degradation, the following steps were carried out on ice. For whole cell protein extraction, cell pellets were resuspended in lysis buffer. Following incubation for 30 minutes, lysates were centrifuged at 14000 rpm for 10 minutes at 4 °C. The supernatants containing solubilized protein were transferred to new Eppendorf tubes. Protein concentration was determined using Pierce™ BCA Protein Assay kit following the manufacturer’s protocol. Concentrations were calculated on the basis of a bovine serum albumin (BSA) calibration curve. Where necessary, samples were diluted with lysis buffer to obtain same protein concentrations within sample sets. Protein samples were complemented with an appropriate volume of 4x SDS sample buffer and denatured by incubation at 95 °C for 5 minutes. Samples were stored at -20 °C or directly separated by SDS PAGE followed by Western blot analysis.
2.8.2 IMMUNOPRECIPITATION

In healthy cells, most Bax protein resides in the cytosol in an inactive form [98]. In response to an apoptotic stimulus, Bax becomes activated. The activation process is accompanied by a conformational change that involves exposure of the N-terminus [196]. Therefore, antibodies that specifically bind to the N-terminal sequence of Bax can be used to detect apoptotic Bax activation. In this study, an antibody recognizing the N-terminus of Bax was used for immunoprecipitation of active Bax in HCT116 and NCI-H460 cells in response to treatment with ionizing radiation (10 Gy), ABT-263 (HCT116: 0.4 µM, NCI-H460: 1 µM), or both under normoxic and hypoxic conditions. For this, whole cell lysates were prepared as described above, using less denaturing detergent CHAPS (1%) instead of Triton X-100. Protein concentrations were assessed using Pierce™ BCA Protein Assay kit in order to employ equal protein amounts in all samples. A total amount of 250 µg protein per sample was mixed with 1 µg N-terminus-specific Bax antibody, or with 1 µg unspecific anti-rabbit IgG antibody as control, and incubated rotating for 2 hours at 4 °C. After addition of magnetic beads conjugated with secondary anti-rabbit antibody, the mixture was incubated similarly for further 2 hours. Using a magnetic separation rack, the bead-protein-complexes were washed twice with lysis buffer containing 0.4 % CHAPS. For protein denaturation and separation from the beads, samples were heated at 95 °C for 5 minutes in 1x SDS sample buffer. Precipitates were separated by SDS PAGE and protein detected by Western blot analysis.

2.8.3 SDS PAGE AND WESTERN BLOT ANALYSIS

Proteins were separated according to size by reducing SDS PAGE on SDS polyacrylamide gels (10, 12, or 15 %). A pre-stained protein standard (PageRuler™) was additionally loaded onto each gel to visualize protein separation and to indicate protein size for detection. After loading with protein samples and the protein standard, SDS PAGE was run in 1x SDS running buffer at 40 V overnight, until sufficiently separated. Proteins were then transferred from polyacrylamide gels onto methanol-activated PVDF membranes using a tank transfer system. Electrophoretic transfer was performed in 1x transfer buffer at 1.5 A for 1.5 hours. In order to block unoccupied binding sites, membranes were incubated in blocking solution containing 5 % non-fat milk for 1 hour at room temperature. Incubation with appropriate dilutions of primary antibodies in 1x TBS-T containing 5 % BSA was carried out at 4 °C overnight. Following 3 washing steps with 1x TBS-T for 10 minutes, membranes were incubated with 1:2000
dilutions of appropriate horseradish peroxidase-conjugated secondary antibody in blocking solution for 1 hour at room temperature. After 3 further washing steps with 1x TBS-T, membranes were incubated with ECL™ Prime or Select detection reagent. Antibody binding was detected using chemiluminescence imager Fusion Solo and Fusion software.

2.9 MICROARRAY GENE EXPRESSION PROFILING

Gene expression measurements of non-selected and hypoxia-selected NCI-H460 cells in normoxic conditions (20 % O₂) as well as upon exposure to severe hypoxia (6 h, 0.2 % O₂) were performed by Dr. Helena Riffkin prior to the current study. cDNA microarray analysis was conducted using Affymetrix® GeneChip® Whole-Transcript (Human Gene 1.0 ST) Array under supervision of PD. Dr. Ludger Klein-Hitpass (BioChip Lab, University Hospital Essen). Data was processed using Affymetrix® Expression Console™ software to produce normalized and background corrected expression values [197]. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Benjamini-Hochberg multiple testing correction for controlling the false discovery rate. Differentially expressed genes were combined to gene sets or assigned to pathways using GeneTrail analysis tool [198]. Expression profiles presented in this work are shown as heat maps, with gene expression illustrated by a red-to-green color gradient representing high-to-low levels in comparison to the mean expression level across all samples.

2.10 QUANTITATIVE REAL TIME PCR

2.10.1 PRINCIPLES AND UTILIZATION OF qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) is based on the detection of the amount of PCR product at every cycle of the PCR using fluorescent probes or dyes. The approach used in this study is based on the specific binding of fluorescent dye SYBR® Green I to double stranded DNA [199]. Consequently, fluorescence signal increases with every PCR cycle as a function of the target gene’s initial amount. The number of PCR cycles necessary to reach a given level of fluorescence is designated as cycle threshold (Ct) value and is used for relative quantification. This reflects relative target gene concentration compared to an internal reference, which is a non-regulated housekeeping gene. As qRT-PCR is a highly sensitive method that allows transcript quantification, it is a valuable tool for the analysis of changes in gene expression. In this study, qRT-PCR was employed to evaluate gene expression
of proteins involved in mitochondrial function and dynamics of non-selected and hypoxia-selected NCI-H460 cells in normoxic conditions (20 % O₂) and after exposure to severe hypoxia (6 h, 0.2 % O₂). Furthermore, the efficiency of siRNA-mediated silencing of MCL1 and OPA1 gene expression in HCT116 and NCI-H460 cells was analyzed via qRT-PCR 24 hours post transfection (see 2.3.4).

2.10.2 RNA ISOLATION AND CDNA SYNTHESIS

Following 6 hours incubation in normoxia or severe hypoxia, or 24 hours after siRNA transfection, respectively, medium was removed and the cells were harvested and washed with PBS. To avoid RNA degradation, the following steps were carried out on ice. RNA was isolated using RNeasy® Mini Kit according to the manufacturer’s protocol. RNA concentration in the eluates was assessed using a Nanodrop™ photometer. If necessary, samples were stored at -80 °C. cDNA was synthesized from 1 µg purified RNA using QuantiTect® Reverse Transcription Kit following the manufacturer’s protocol, whereby synthesis time was extended to 25 minutes. cDNA samples were stored at -20 °C.

2.10.3 PRIMER DESIGN

Specific primers were designed based on NCBI nucleotide sequences, using NCBI’s online tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ [200]). Primer pairs were designed to span introns and cross-reactivity was excluded by Blast 2.2 database comparison. Primers were 20-25 and PCR products 100-250 base pairs in size, with a GC content between 50-60 % and annealing temperatures of 57-63 °C. B2M gene, encoding β2-microglobulin, was used as a non-hypoxia-regulated housekeeping gene [201]. All primers were synthesized by Metabion.
2.10.4 qRT-PCR

qRT-PCR reaction mixtures were prepared with qPCR MasterMix for SYBR® Green I according to the manufacturer’s protocol (Table 2.15).

Table 2.15: Composition of qRT-PCR reaction mixtures

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x reaction buffer</td>
<td>5 µL</td>
</tr>
<tr>
<td>Primer forward (10 µM)</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>Primer reverse (10 µM)</td>
<td>0.2 µL</td>
</tr>
<tr>
<td>SYBR® Green I</td>
<td>0.3 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>2.3 µL</td>
</tr>
</tbody>
</table>

8 µL reaction mixture was added per well of 384-well qPCR plates and complemented with 2 µL (=2 ng) cDNA per well. qRT-PCR was carried out using the ABI 7900HT Real-Time PCR System. Cycling conditions are shown in Table 2.16.

Table 2.16: qRT-PCR cycling conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uracil-N-Glycosilase (UNG) activation</td>
<td>50 °C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>DNA polymerase activation</td>
<td>95 °C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>UNG inactivation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 s</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Melting curve analysis</td>
<td>60-95 °C</td>
<td>+ 0.5 °C every 3 s</td>
<td></td>
</tr>
</tbody>
</table>

Reactions containing cDNA were run in triplicates, controls (either without cDNA or reverse transcriptase) were run in duplicates. Normalized target gene expression was calculated with the 2^ΔΔCt method [202].

2.11 Statistical analysis

Experiments were usually performed at least 3 times. All numerical data show means ± standard deviation (SD), unless stated otherwise. The results were subjected to statistical analysis using GraphPad Prism 6 software. Groups of data were analyzed for statistical significance using t-test or, for multiple comparison, two-way ANOVA followed by Bonferroni correction. A p value of ≤ 0.05 was considered statistically significant. Significance levels are indicated with asterisks as follows: * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001.
3 RESULTS

3.1 EXPOSURE TO ACUTE AND ADAPTATION TO CYCLING HYPOXIA REDUCES RADIATION-INDUCED APOPTOSIS AND ENHANCES CLONOGENIC CELL SURVIVAL

The impact of tumor hypoxia on apoptosis resistance and sensitivity to radiotherapy was analyzed in an experimental *in vitro* setting using the colon carcinoma cell line HCT116 and the lung adenocarcinoma cell line NCI-H460. The cells were exposed to ionizing radiation either under normoxic (20 % O₂) or under severely hypoxic conditions (0.2 % O₂) and analyzed in regard to apoptosis induction and clonogenic survival. The analyses also included a hypoxia-selected NCI-H460 sub-cell line that was obtained by exposing NCI-H460 cells to repeated cycles of severe hypoxia and reoxygenation (see 2.2.1). This model allows the investigation of the possible contribution of adaptive changes to resistance to radiotherapy acquired in consequence of adaptation to cycling hypoxia. In the following, this sub-cell line is referred to as hypoxia-selected NCI-H460, as opposed to non-selected NCI-H460 cells.
48 and 72 hours after irradiation of HCT116 and non-selected NCI-H460 cells with a single dose of 10 Gy under normoxic or severely hypoxic conditions, DNA fragmentation was measured as a means of apoptosis induction by flow cytometry (Figure 3.1). While irradiation in normoxia induced apoptotic DNA fragmentation in a time-dependent manner in both cell lines, apoptosis was induced upon irradiation in severe hypoxia only after 72 hours and the levels were significantly lower than in normoxia.

Figure 3.1: Irradiation under hypoxic conditions resulted in reduced apoptosis induction in HCT116 and non-selected NCI-H460 cells. A) HCT116 and B) non-selected NCI-H460 cells were irradiated with a single dose of 10 Gy under normoxic (Nx; 20 % O2) or severely hypoxic conditions (Hx; 0.2 % O2). 48 and 72 hours after irradiation, apoptosis levels were assessed by flow cytometric analysis of DNA fragmentation (Sub G1 fraction). Data represent mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). Under normoxic conditions, irradiation induced DNA fragmentation in a time-dependent manner in HCT116 (A) and non-selected NCI-H460 cells (B). In both cell lines, levels of apoptotic DNA fragmentation assessed 48 hours after irradiation remained at similar levels as non-irradiated controls. Apoptosis induction increased 72 hours after irradiation in hypoxia, but DNA fragmentation levels were significantly lower than after irradiation in normoxia.
To further examine the observed effect of acute hypoxia on sensitivity to radiotherapy, clonogenic survival of HCT116 and non-selected NCI-H460 cells was analyzed after irradiation under normoxic and hypoxic conditions employing colony formation assays (Figure 3.2). Surviving fractions of both cell lines were reduced in a dose-dependent manner after irradiation in normoxia. Compared to normoxia, surviving fractions of both cell lines remained at significantly higher levels when the cells were irradiated with respective doses under hypoxic conditions. The cytotoxic effect of ionizing radiation was generally smaller in non-selected NCI-H460 cells, as irradiation with a dose of 1 Gy in normoxia reduced the surviving fraction by 35 % compared to 50 % in HCT116 cells. Moreover, 3 Gy were sufficient to eradicate 90 % of clonogenic HCT116 cells, while a radiation dose of 4 Gy was required to achieve the same effect in non-selected NCI-H460 cells.

**Figure 3.2:** Clonogenic survival of HCT116 and non-selected NCI-H460 cells was enhanced after irradiation under hypoxic compared to normoxic conditions. HCT116 (A) and non-selected NCI-H460 cells (B) were irradiated with doses of 0-5 Gy under normoxic (Nx; 20 % O2) or severely hypoxic conditions (Hx; 0.2 % O2). Following irradiation under hypoxic conditions, cells remained in severe hypoxia for 48 hours, before all cells were further incubated under normoxic conditions for 8-10 days to allow growth of single cell colonies. Surviving fractions were calculated as ratio of seeded cells to counted colonies and normalized to respective untreated controls. Data show mean values of at least 3 independent experiments ±SD and the resulting survival curves, with indicated statistical significance between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). Clonogenic survival of HCT116 (A) and non-selected NCI-H460 cells (B) decreased with increasing doses of ionizing radiation in normoxia and in severe hypoxia. Compared to normoxia, irradiation with the respective doses under hypoxic conditions resulted in significantly higher surviving fractions in both cell lines.
Irradiation of hypoxia-selected NCI-H460 cells in normoxia induced apoptotic DNA fragmentation in a time-dependent manner (Figure 3.3 A). In contrast, apoptosis induction was detected only after 72 hours and at significantly lower levels, when the cells were irradiated in severe hypoxia. Compared to non-selected NCI-H460 cells, radiation-induced apoptosis was significantly reduced in hypoxia-selected cells under normoxic and hypoxic conditions (Figure 3.3 B). Moreover, background apoptosis in non-irradiated cells exposed to severe hypoxia for 48 h was lower in the hypoxia-selected NCI-H460 cells, pointing at increased hypoxia tolerance of this sub-cell line.

Figure 3.3: Hypoxia- and radiation-induced apoptosis were reduced in hypoxia-selected compared to non-selected NCI-H460 cells under normoxic and hypoxic conditions. Hypoxia-selected and non-selected NCI-H460 cells were irradiated with 10 Gy under normoxic (Nx; 20 % O₂) or severely hypoxic conditions (Hx; 0.2 % O₂). Apoptotic DNA-fragmentation (Sub G1) was analyzed after 48 and 72 hours. Data are presented as mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (A) or cell lines (B) (n.s. p > 0.05 ; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). A) Apoptosis induction in hypoxia-selected NCI-H460 cells increased 48 and 72 hours after irradiation under normoxic conditions. Apoptosis levels slightly increased 72 hours after irradiation in hypoxia, but significantly less than in normoxia. B) Compared to non-selected NCI-H460 cells, apoptosis levels in hypoxia-selected cells were significantly lower after irradiation in normoxia and in hypoxia.
Similar to non-selected NCI-H460 cells, surviving fractions of hypoxia-selected NCI-H460 cells irradiated in severe hypoxia were higher than after irradiation in normoxia (Figure 3.4 A). Compared to non-selected cells, hypoxia-selected NCI-H460 cells showed a tendency towards increased clonogenic survival after irradiation in normoxia, whereas surviving fractions of both cell lines were similar, when the cells were irradiated under hypoxic conditions (Figure 3.4 B).

**Figure 3.4:** Clonogenic survival of hypoxia-selected NCI-H460 cells irradiated in normoxia was less reduced than in non-selected cells. Hypoxia-selected and non-selected NCI-H460 cells were irradiated at indicated doses and incubated for further 48 hours in normoxia (Nx; 20 % O₂) or severe hypoxia (Hx; 0.2 % O₂). Following further 8-10 days incubation in normoxia, formed colonies were counted and used to calculate surviving fractions. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance between treatment groups (A) or cell lines (B) (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001). A) Clonogenic survival of hypoxia-selected NCI-H460 cells decreased with increasing irradiation doses. Irradiation under hypoxic conditions resulted in significantly higher surviving fractions than in normoxia. B) Compared to non-selected cells, surviving fractions of hypoxia-selected NCI-H460 cells were slightly but at doses of 1-3 Gy significantly higher after irradiation in normoxia, while survival curves of both cell lines overlapped after irradiation in hypoxia.

In summary, the results show that exposure to acute hypoxia reduces apoptosis induction in response to ionizing radiation. Along with this, clonogenic survival is improved when
irradiation is applied under hypoxic conditions, suggesting a direct relation between attenuated apoptosis induction and radioresistance caused by exposure to acute hypoxia. Furthermore, adaptation to cycling hypoxia results in increased resistance to radiation-induced apoptosis not only in hypoxia, but also under normoxic conditions. However, this only resulted in improved clonogenic survival of irradiated hypoxia-selected NCI-H460 cancer cells in normoxia but not in hypoxia.

3.2 Exposure to acute and adaptation to cycling hypoxia alters the balance of pro- and anti-apoptotic Bcl-2 protein family members

Bcl-2 family proteins are important regulators of intrinsic apoptosis. In order to explore mechanisms of hypoxia-mediated resistance to radiation-induced apoptosis and enhanced clonogenic survival, expression of Bcl-2 family members was analyzed in HCT116 cells and in non-selected and hypoxia-selected NCI-H460 cell lines. Differences in transcriptional regulation of Bcl-2 protein family members resulting from adaptation to cycling hypoxia were determined by cDNA microarray-based analysis of non-selected and hypoxia-selected NCI-H460 cells (Figure 3.5). Gene expression profiles of both cell lines differed more prominently under normoxic (panel 1) than under hypoxic conditions (panel 2). Furthermore, exposure to acute hypoxia for 6 hours affected Bcl-2 family gene expression more severely in hypoxia-selected (panel 3) than in non-selected NCI-H460 cells (panel 4). Most noticeable changes in hypoxia-selected compared to non-selected cells in normoxia were enhanced expression of BCL2L1 (encoding for Bcl-xL and the shorter isoform Bcl-xS) and PMAIP1 (Noxa) and reduced expression of BCL2L11 (Bim). In response to acute hypoxia, hypoxia-selected cells markedly downregulated transcription of genes encoding Bax, Bcl-xL (and Bcl-xS, respectively), and Noxa. This suggests that transcriptional regulation of the Bcl-2 rheostat might predominantly be a feature of hypoxia-selected cancer cells.
Figure 3.5: Hypoxia-selected NCI-H460 cells showed altered gene expression of several Bcl-2 family members compared to non-selected cells in normoxia and in response to acute hypoxia. Non-selected and hypoxia-selected NCI-H460 cells were incubated in normoxia (Nx; 20 % O₂) or severe hypoxia (Hx; 0.2 % O₂) for 6 hours. Following mRNA isolation and cDNA transcription, expression profiles were generated using microarray technology. Expression of Bcl-2 family protein-encoding genes is illustrated by a red-to-green color gradient representing high-to-low levels in comparison to the mean expression level across all samples (black). While expression of several Bcl-2 family protein-encoding genes differed between both NCI-H460 cell lines in normoxia (panel 1), expression patterns were more similar after exposure to hypoxia (panel 2). Changes in gene expression in response to acute hypoxia were more prominent in hypoxia-selected (panel 3) than in non-selected cells (panel 4).

Due to differential translation efficiency as well as post-transcriptional and -translational modifications, Bcl-2 family members can be distinctly regulated at the protein level, which is pivotal for apoptosis regulation at the mitochondria. Therefore, changes in the expression of pro- and anti-apoptotic family members in both NCI-H460 cell lines and in HCT116 cells were additionally determined at protein levels by Western blot analysis (Figure 3.6). Exposure to hypoxia for 24 hours affected levels of several Bcl-2 family proteins. Protein levels of pro-apoptotic BH3-only regulators Noxa, Puma, and Bim and of apoptosis effector Bax were reduced in all three cell lines. In contrast, levels of anti-apoptotic proteins broadly increased in response to acute hypoxia. Of these, HCT116 cells showed pronounced hypoxia-mediated increase of Bcl-2 and Bcl-xL levels. Both NCI-H460 cell lines were rather characterized by elevated Mcl-1 protein levels in hypoxia. To enable direct comparison, protein levels of both hypoxia-selected and non-selected NCI-H460 cells were analyzed on the same membranes.
Marked differences were detected for Bcl-xL protein levels, which were generally increased, whereas levels of pro-apoptotic members Puma and Bak were reduced in hypoxia-selected cells.

**Figure 3.6:** Exposure to acute and adaptation to cycling hypoxia altered protein levels of several Bcl-2 family members in HCT116 and NCI-H460 cells. HCT116 and non-selected as well as hypoxia-selected NCI-H460 cells were incubated under normoxic (Nx; 20 % O2) or severely hypoxic conditions (Hx; 0.2 % O2) for 24 hours. Whole cell protein lysates were prepared, separated by SDS PAGE, and protein levels of main Bcl-2 family members assessed by Western blot analysis. To allow direct comparison, protein levels of non-selected and hypoxia-selected NCI-H460 cells (and where possible of HCT116 cells) were analyzed on the same membranes or at similar detection conditions. β-actin was used as loading control. Figures show representative results. After exposure to severer hypoxia, all three cell lines broadly showed decreased protein levels of pro-apoptotic BH3-only regulators and apoptosis effectors, whereas protein levels of anti-apoptotic members increased. Compared to non-selected cells, Bcl-xL protein levels were generally higher and of Bak and Puma lower in hypoxia-selected NCI-H460 cells.
Taken together, exposure to acute hypoxia shifts the Bcl-2 protein family balance towards the anti-apoptotic members, possibly contributing to increased apoptosis resistance observed in HCT116 and NCI-H460 cells exposed to severe hypoxia. Furthermore, the Bcl-2 rheostat is generally altered in hypoxia-selected NCI-H460 cells, with a tendency towards increased weighting of anti-apoptotic family members. These alterations might promote adaptation to cycling hypoxia and contribute to their increased apoptosis resistance.

3.3 **Hypoxia-mediated resistance to ionizing radiation can be overcome by BH3-mimetic ABT-263**

The results obtained so far indicated that a deregulated balance between pro- and anti-apoptotic Bcl-2 family members might reduce cancer cell sensitivity to radiation-induced cell death in hypoxia. Accordingly, interfering with the Bcl-2 rheostat by shifting the balance towards pro-apoptotic proteins might lower the cell death threshold and restore radiosensitivity. Therefore, BH3-mimetic ABT-263, an inhibitor of Bcl-2, Bcl-xL, and Bcl-w, was used to assess whether targeting anti-apoptotic Bcl-2 family proteins improves radiation-induced cytotoxicity in acute hypoxia and hypoxia-adapted cancer cells. In the following, the therapeutic potential of ABT-263 was assessed as single-agent or in combination with ionizing radiation in normoxia and severe hypoxia. Following treatment, HCT116 cells and both NCI-H460 cell lines were analyzed with regard to apoptosis induction as well as short- and long-term survival.

3.3.1 **ABT-263 induces apoptosis in HCT116 colon carcinoma cells and improves the cytotoxic effect of ionizing radiation in hypoxia**

In HCT116 cells, single-agent treatment with ABT-263 induced apoptotic DNA fragmentation, which is indicative for apoptosis execution, in a concentration dependent manner (Figure 3.7). Effectivity in normoxia started at a concentration of 0.4 µM, while 0.1 µM ABT-263 were sufficient to significantly induce apoptosis under hypoxic conditions. Apoptosis levels were significantly higher upon ABT-263 treatment in severe hypoxia than in normoxia and further increased after additional irradiation under both conditions. Although apoptosis induction in response to irradiation alone was reduced in severe hypoxia compared to irradiation in normoxia, as shown before, combined treatment with ABT-263 and ionizing radiation raised apoptosis to similar levels as detected in normoxia.
RESULTS

Figure 3.7: ABT-263 induced apoptosis in HCT116 cells under normoxic and pronouncedly under hypoxic conditions and further enhanced apoptosis when combined with ionizing radiation. HCT116 cells were treated with indicated concentrations of ABT-263, 10 Gy ionizing radiation (IR), or both in normoxia (Nx; 20% O₂) or in severe hypoxia (Hx; 0.2% O₂). After 48 hours, apoptosis levels were determined by flow cytometric analysis of DNA fragmentation (Sub G1 fraction). Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). Apoptosis levels of HCT116 cells rose with increasing concentrations of ABT-263, with significantly higher levels under hypoxic than under normoxic conditions. Apoptosis induction was further enhanced by additional irradiation. While radiation-induced apoptosis was reduced in hypoxia, combined treatment resulted in comparably high levels under normoxic and severely hypoxic conditions.
Apoptosis induction upon treatment was corroborated by Western blot analysis (Figure 3.8 A). Active Bax, which was precipitated with an activation-specific antibody, as well as apoptotic cleavage of caspase-3 and its substrate PARP were detected in HCT116 cells treated with ABT-263 alone or combined with irradiation. Moreover, flow cytometric analysis of cell death, quantified by propidium iodide exclusion assay and by dissipation of the mitochondrial membrane potential (Figure 3.8 B), showed similar levels as apoptosis levels detected by DNA fragmentation. This indicated that cell death following treatment mainly occurred via apoptosis.

Figure 3.8: ABT-263-, irradiation-, and co-treatment-induced cell death in HCT116 cells occurred via apoptosis induction. HCT116 cells were treated with ABT-263, ionizing radiation, or both as indicated in normoxia (Nx; 20 % O2) or severe hypoxia (Hx; 0.2 % O2). A) 48 hours after treatment, active Bax was precipitated from whole cell lysates using a conformation-specific antibody. Precipitation with unspecific isotype-matched IgG was performed as a negative control. Precipitates and whole cell protein lysates were separated by SDS PAGE and protein levels of cleaved caspase-3, PARP, cleaved PARP, total Bax, and active Bax were assessed by Western blot analysis. β-actin was used as loading control for whole cell lysates. Figures show representative results. Cleavage of caspase-3, PARP, as well as active Bax were detected in lysates of HCT116 cells after treatment with ABT-263 alone more prominently in hypoxia and in combination with irradiation similarly in normoxia and in hypoxia. B) Cell death induction was assessed by PI exclusion staining (PI positive cells) and mitochondrial membrane potential (MMP) dissipation (ΔΨm low) by TMRE staining and subsequent flow cytometric analysis. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; *** p ≤ 0.001; **** p ≤ 0.0001). Cell death induction and MMP dissipation following treatment increased to a similar extent as DNA fragmentation displayed in Figure 3.7.
Short-term cell survival in response to treatment was further analyzed employing crystal violet assay (Figure 3.9). While higher ABT-263 concentrations were required to reduce cell viability in normoxia (1; 4 µM), 0.4 µM already significantly reduced cell viability in hypoxia and to a higher extent than in normoxia. Cell viability was further reduced after combined treatment with ABT-263 and ionizing radiation in normoxia. As cell viability levels in severe hypoxia were already relatively low upon single-agent ABT-263 treatment, further reduction by additional irradiation was only observed at the lowest applied concentration of 0.4 µM.

Figure 3.9: ABT-263 reduced short-term survival of HCT116 cells under normoxic and pronouncedly under hypoxic conditions and additional irradiation further decreased cell viability. HCT116 cells were treated as indicated in normoxia (Nx; 20 % O₂) or severe hypoxia (Hx; 0.2 % O₂). After 48 hours, attached cells were fixed and stained with crystal violet solution. Cell lysis and spectrophotometrical measurement of released dye was used to calculate the amount of viable cells by normalizing the values to untreated controls or irradiation-only treated cells. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001).

A) Cell viability of HCT116 cells decreased with increasing concentrations of ABT-263, starting at a dose of 1 µM under normoxic and 0.4 µM under hypoxic conditions. Reduction of cell viability upon ABT-263 treatment was significantly stronger in severe hypoxia than in normoxia. B) After combined treatment with ABT-263 and ionizing radiation in normoxia, cell viability was further significantly reduced. In hypoxia, irradiation in addition to treatment with 0.4 µM ABT-263 further reduced short-term survival, whereas cell viability levels upon co-treatment with higher ABT-263 doses were similar to those without irradiation.
To further assess the therapeutic potential of ABT-263 as single- and radiosensitizing agent, clonogenic survival of HCT116 cells was analyzed using the colony formation assay. Compared to the decrease of surviving fractions after irradiation alone in normoxia, combined treatment showed a largely insignificant tendency to further reduce clonogenic survival (Figure 3.10 A). In contrast to normoxia, treatment with ABT-263 alone in hypoxia already resulted in decreased clonogenic survival (Figure 3.10 B). Compared to radiotherapy alone, combined treatment applied in severe hypoxia significantly reduced surviving fractions to levels observed after irradiation in normoxia.

Figure 3.10: ABT-263 and ionizing radiation pronouncedly reduced clonogenic survival of HCT116 cells when applied together in hypoxia. HCT116 cells were irradiated with doses of 0-5 Gy without or with subsequent addition of 1 µM ABT-263. Following treatment under hypoxic conditions (Hx; 0.2 % O₂), the cells were kept in severe hypoxia for 48 hours, before all cells were further incubated in normoxia (Nx; 20 % O₂) for 8-10 days to allow growth of single cell colonies. Formed colonies were counted and used to calculate surviving fractions. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance between treatment groups (* p ≤ 0.05; ** p ≤ 0.01). A) Surviving fractions were reduced in a dose-dependent manner after irradiation in normoxia. After co-treatment with ABT-263 and ionizing radiation, clonogenic survival was slightly but, except at an irradiation dose of 1 Gy, insignificantly decreased. B) ABT-263 reduced surviving fractions in severe hypoxia as single-agent treatment. Compared to the reduction of surviving fractions after irradiation-only treatment in hypoxia, co-treatment with ABT-263 significantly reduced clonogenic survival.

Taken together, the results show that treatment with ABT-263 improves radiosensitivity in normoxia and overcomes hypoxia-mediated radioresistance of HCT116 colon cancer cells.
3.3.2 ABT-263 IMPROVES SENSITIVITY OF NON-SELECTED AND HYPOXIA-SELECTED NCI-H460 LUNG CANCER CELLS TO THE CYTOTOXIC ACTION OF IONIZING RADIATION IN NORMOXIA AND HYPOXIA

3.3.2.1 ABT-263 ENHANCES THE CYTOTOXIC EFFECT OF IONIZING RADIATION IN NON-SELECTED NCI-H460 CELLS IN NORMOXIA AND HYPOXIA

In contrast to HCT116 cells that clearly induced apoptosis after treatment with ABT-263, the inhibitor alone had no effect on non-selected NCI-H460 cells, as flow cytometric analysis of apoptotic DNA fragmentation shows in Figure 3.11. However, apoptosis induction was clearly enhanced after combined treatment with ionizing radiation and ABT-263. Although DNA fragmentation levels upon irradiation in severe hypoxia were lower than in normoxia, combined treatment enhanced apoptosis induction to similar levels as achieved in normoxia.

![Figure 3.11: ABT-263 treatment enhanced radiation-induced apoptosis in non-selected NCI-H460 cells in normoxia and hypoxia. NCI-H460 cells were treated with ABT-263, ionizing radiation (IR), or both as indicated in normoxia (Nx; 20 % O₂) or hypoxia (Hx; 0.2 % O₂). Apoptosis levels were analyzed by flow cytometric assessment of Sub G1 fractions 48 hours after treatment. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; **** p ≤ 0.0001). ABT-263 treatment did not affect apoptosis levels in non-selected NCI-H460 cells, whereas apoptosis induction upon combined treatment was significantly enhanced in normoxia and hypoxia. While apoptosis induction in response to irradiation alone was reduced in severe hypoxia compared to normoxia, DNA fragmentation levels were similarly high after combined treatment.](image-url)
Conform to apoptosis induction assessed by flow cytometry, apoptotic cleavage of caspase-3, its substrate PARP, and activation of Bax were detected in non-selected NCI-H460 cells after combined treatment with ABT-263 and ionizing radiation in normoxia as well as in hypoxia (Figure 3.12 A). In addition, these protein signals were evident in cells treated with ABT-263 alone under hypoxic conditions. This suggests that ABT-263 might slightly induce apoptosis in hypoxia, but higher concentrations are necessary to reveal this effect on levels of DNA fragmentation. PI exclusion and mitochondrial membrane potential dissipation levels (Figure 3.12 B) matched those of DNA fragmentation displayed in Figure 3.11, confirming that treatment-induced cell death was largely accounted for by apoptosis.

Figure 3.12: Apoptosis accounted for treatment-induced cell death of non-selected NCI-H460 cells. Non-selected NCI-H460 cells were treated as indicated in normoxia (Nx; 20 % O2) or severe hypoxia (Hx; 0.2 % O2). A) Following treatment, active Bax was precipitated from whole cell lysates using a conformation-specific antibody. Precipitation with unspecific isotype-matched IgG was performed as a negative control. Precipitates and whole cell protein lysates were separated by SDS PAGE. Protein levels of cleaved caspase-3, PARP, cleaved PARP, total Bax, and active Bax were analyzed by Western blot. In whole cell lysates, β-actin was used as loading control. Figures show representative results. Caspase-3 fragments, cleavage of PARP, and active Bax were evident in lysates of non-selected NCI-H460 cells after treatment with ABT-263 alone in hypoxia and in combination with irradiation in normoxia and hypoxia. B) Cell death induction following treatment was analyzed by measurement of PI exclusion (PI positive cells) and dissipation of the mitochondrial membrane potential (MMP; ΔΨm low) by TMRE staining. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). Levels of cell death induction and MMP dissipation increased similarly to treatment-induced DNA fragmentation shown in Figure 3.11.
Short-term survival analyzed by crystal violet assay confirmed that treatment of non-selected NCI-H460 cells with ABT-263 alone had no significant effect on cell viability (Figure 3.13). However, combined treatment with ABT-263 and ionizing radiation resulted in significantly reduced short-term survival in normoxia and to an even greater extent under severely hypoxic conditions.

Figure 3.13: Combined treatment with ABT-263 and ionizing radiation reduced short-term survival of non-selected NCI-H460 cells under normoxic and pronouncedly under hypoxic conditions. 48 hours after indicated treatment in normoxia (Nx; 20% O₂) or severe hypoxia (Hx; 0.2% O₂), viable cells were fixed and stained with crystal violet solution. Following subsequent lysis, spectrophotometrical measurement of released dye was used to calculate the amount of viable cells by normalizing the values to untreated controls or irradiation-only treated cells. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). A) Cell viability of non-selected NCI-H460 cells was not significantly affected by single-agent treatment with ABT-263. B) Combined treatment with ABT-263 and irradiation in normoxia reduced cell viability clearly stronger than irradiation alone. Under hypoxic conditions, combined treatment reduced cell viability more efficiently than either treatment alone.
As Bax activation and apoptotic cleavage of caspase-3 and PARP in response to ABT-263 treatment in hypoxia indicated that the therapeutic potential might not be fully exhausted by the concentrations applied thus far, clonogenic survival of non-selected NCI-H460 cells was analyzed at a slightly increased ABT-263 concentration of 10 µM. At this, treatment with ABT-263 alone reduced surviving fractions in normoxia and in hypoxia (Figure 3.14). Compared to the effect of irradiation alone, combined treatment further decreased clonogenic survival with increasing radiation doses under normoxic and hypoxic conditions.

Figure 3.14: Clonogenic survival of non-selected NCI-H460 cells was decreased by treatment with ABT-263 alone and in combination with ionizing radiation in normoxia and hypoxia. Non-selected NCI-H460 cells were irradiated with 0-5 Gy without or with subsequent addition of 10 µM ABT-263. After treatment in hypoxia, the cells were kept under hypoxic conditions for 48 hours, followed by further incubation in normoxia for 8-10 days. Surviving fractions were calculated from counted colonies and normalized to respective untreated controls. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance between treatment groups (*p ≤ 0.05; **p ≤ 0.01). A) ABT-263 reduced surviving fractions as single-agent treatment in normoxia. Compared to the reduction of surviving fractions upon irradiation, combined treatment with ABT-263 and irradiation further decreased clonogenic survival. B) Treatment with ABT-263 alone decreased clonogenic survival under hypoxic conditions. Combined treatment with ABT-263 and ionizing radiation reduced surviving fractions more potently than irradiation alone also in hypoxia.
3.3.2.2 **ABT-263 INCREASES THE CYTOTOXIC EFFECT OF IONIZING RADIATION IN HYPOXIA-SELECTED NCI-H460 CELLS IN NORMOXIA AND PRONOUNCEDLY IN HYPOXIA**

While non-selected NCI-H460 cells showed no significant apoptosis induction in response to 0.1-4 \( \mu \text{M} \) ABT-263, flow cytometry-based analysis of hypoxia-selected NCI-H460 cells revealed slight sensitivity towards ABT-263-induced apoptosis, but only when the inhibitor was applied under hypoxic conditions (Figure 3.15). Similarly to non-selected cells, apoptotic DNA fragmentation was markedly increased after combined treatment with ABT-263 and ionizing radiation in a concentration dependent manner in the hypoxia-selected sub-cell line. When irradiation was combined with higher concentrations of ABT-263, apoptosis levels increased more pronounced in hypoxia than in normoxia.

![Graphs showing apoptosis levels in normoxia and hypoxia](image)

**Figure 3.15:** Hypoxia-selected NCI-H460 cells showed slight sensitivity to ABT-263-induced apoptosis and pronounced sensitivity to combined treatment with ionizing radiation in hypoxia. Hypoxia-selected NCI-H460 cells were treated as indicated in normoxia (N\(_x\); 20 % O\(_2\)) or hypoxia (H\(_x\); 0.2 % O\(_2\)). Apoptosis levels were assessed by measuring DNA fragmentation (Sub G1 fraction) 48 hours after treatment. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* \( p \leq 0.05 \); ** \( p \leq 0.01 \); **** \( p \leq 0.0001 \)). ABT-263 treatment at higher concentrations in hypoxia slightly but significantly induced apoptosis in hypoxia-selected NCI-H460 cells. Combined treatment with ABT-263 and irradiation strongly increased apoptosis levels in normoxia and hypoxia. At higher ABT-263 concentrations, co-treatment induced apoptosis more effectively in hypoxia than in normoxia.
Apoptosis induction following treatment with ABT-263 alone in hypoxia and combined treatment in normoxia and hypoxia could be corroborated by apoptotic cleavage of caspase-3 and PARP as well as activation of Bax (Figure 3.16 A). Similar to non-selected cells, the comparable extent of cell death measured by DNA fragmentation and PI exclusion or dissipation of the mitochondrial membrane potential, respectively, confirmed that apoptosis was the major mechanism responsible for treatment-induced cell death also in hypoxia-selected NCI-H460 cells (Figure 3.16 B).

Figure 3.16: ABT-263- and co-treatment-induced cell death of hypoxia-selected NCI-H460 cells occurred via apoptosis. Hypoxia-selected NCI-H460 cells were treated with ABT-263, ionizing radiation, or both in normoxia (Nx; 20 % O2) or severe hypoxia (Hx; 0.2 % O2). A) After 48 hours of indicated treatment, active Bax was precipitated using a conformation-specific antibody. Precipitation with unspecific isotype-matched IgG was performed as a negative control. Precipitates and whole cell protein lysates were separated by SDS PAGE. Protein levels of cleaved caspase-3, PARP, cleaved PARP, total Bax, and active Bax were assessed by Western blot analysis. β-actin was used as loading control for whole cell lysates. Figures show representative results. Active Bax as well as caspase-3 and PARP cleavage were detected after ABT-263 treatment in hypoxia, combined treatment with ABT-263 and irradiation in normoxia, and pronouncedly after co-treatment under hypoxic conditions. B) Flow cytometric measurement of cell death induction (PI exclusion staining; PI positive cells) and of mitochondrial membrane potential (MMP) dissipation (ΔΨm low) following treatment showed similar levels as DNA fragmentation (Figure 3.15). Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (**p ≤ 0.001; ****p ≤ 0.0001).
In accordance to increased apoptosis induction upon treatment with ABT-263 alone in hypoxia (Figure 3.15, upper left panel), the inhibitor also reduced short-term survival of hypoxia-selected NCI-H460 cells at higher concentrations in hypoxia as determined by crystal violet assay (Figure 3.17 A). ABT-263 treatment in combination with ionizing radiation furthermore significantly reduced cell viability levels under normoxic and hypoxic conditions (Figure 3.17 B).

**Figure 3.17:** Short-term survival of hypoxia-selected NCI-H460 was reduced by single-agent ABT-263 treatment in hypoxia. Combined treatment with ABT-263 and ionizing radiation reduced cell viability under normoxic and hypoxic conditions. Following 48 hours treatment with indicated concentrations of ABT-263, irradiation with 10 Gy (IR), or both under normoxic (Nx; 20 % O2) or in severely hypoxic conditions (Hx; 0.2 % O2), viable cells were fixed and stained with crystal violet solution. Subsequent lysis and spectrophotometrical measurement of released dye was used to calculate the amount of viable cells. Values were normalized to untreated controls or irradiation-only treated cells. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance to respective controls and between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). A) Viability of hypoxia-selected NCI-H460 cells was significantly reduced after treatment with 4 µM ABT-263 in hypoxia. B) ABT-263 treatment in combination with ionizing radiation significantly reduced short-term survival under normoxic and hypoxic conditions, resulting in slightly but insignificantly lower cell viability levels in hypoxia than in normoxia.
Similar to non-selected NCI-H460 cells, surviving fractions decreased slightly after treatment with ABT-263 alone in normoxia as well as in hypoxia (Figure 3.18). Combined treatment with ABT-263 and ionizing radiation reduced surviving fractions more potently than irradiation alone in normoxia as well as in hypoxia also in hypoxia-selected NCI-H460 cells.

Figure 3.18: Clonogenic survival of hypoxia-selected NCI-H460 cells was reduced after ABT-263 treatment in combination with ionizing radiation under normoxic and hypoxic conditions. Hypoxia-selected NCI-H460 cells were irradiated with 0-5 Gy without or with addition of 10 µM ABT-263. After treatment in hypoxia, the cells were kept in hypoxia for further 48 hours, before all cells were further incubated in normoxia for 8-10 days. Surviving fractions were calculated from formed colonies and normalized to respective untreated controls. Data show mean values of 3 independent experiments ±SD, with indicated statistical significance between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001). Compared to the reduction of surviving fractions upon irradiation alone, combined treatment with ABT-263 significantly reduced clonogenic survival under normoxic (A) and hypoxic conditions (B).

Summarized, the results show that combined treatment with ionizing radiation and Bcl-2/Bcl-xL inhibitor ABT-263 potently increased apoptosis induction and reduced short- and long-term survival of both NCI-H460 cell lines in normoxia and in hypoxia. Thus, hypoxia-mediated resistance to radiation-induced apoptosis can be overcome and the cytotoxic action of ionizing radiation enhanced by ABT-263 treatment.
3.4 **Anti-apoptotic Bcl-2 family member Mcl-1 determines sensitivity to ABT-263- and radiation-induced apoptosis**

3.4.1 **ABT-263 treatment, ionizing radiation, and combined treatment have differential effects on protein levels of anti-apoptotic Bcl-2 family proteins**

Treatment with ABT-263 generally improved sensitivity of HCT116 cells and both NCI-H460 cell lines to the cytotoxic effects of radiotherapy. The individual responses of the cells to the respective treatments, however, differed. The therapeutic effect of ABT-263 is based on neutralization of anti-apoptotic Bcl-2 and Bcl-xL [168]. Both proteins were upregulated in HCT116 cells exposed to hypoxia, while Bcl-xL levels were higher in hypoxia-tolerant NCI-H460 cells than in non-selected control cells. In addition, high protein levels of anti-apoptotic family member Mcl-1 observed in both NCI-H460 cell lines could confer resistance to ABT-263 and ionizing radiation. To explore the relevance of deregulated expression of Bcl-2, Bcl-xL, and Mcl-1 to the therapy response, changes in their protein levels following treatment with ABT-263, ionizing radiation, and combined treatment were assessed by Western blot analysis.

While protein levels of Bcl-2 and Bcl-xL in HCT116 colon cancer cells were generally higher under hypoxic conditions, treatment with ABT-263, irradiation, or combined treatment had no prominent effect on their levels (**Figure 3.19 A**). In contrast, protein levels of Mcl-1, which is not targeted by ABT-263, greatly increased in response to inhibition of Bcl-2 and Bcl-xL in normoxia and to a lesser extent in hypoxia. This effect of ABT-263 persisted when the HCT116 cells were irradiated in addition to ABT-263 treatment.

In both NCI-H460 cell lines, Mcl-1 protein levels strongly decreased upon irradiation alone as well as in combination with ABT-263 treatment under normoxic conditions (**Figure 3.19 B and C**). However, while a similar reduction of Mcl-1 was detected in non-selected cells irradiated under hypoxic conditions, Mcl-1 protein levels remained unchanged in hypoxia-selected NCI-H460 cells irradiated in hypoxia.
Figure 3.19: ABT-263, irradiation, and combined treatment differentially altered Mcl-1 protein levels in HCT116 as well as non-selected and hypoxia-selected NCI-H460 cells. 48 hours after treatment with ABT-263 at indicated doses, irradiation with 10 Gy, or both in normoxia (Nx; 20 % O2) or severe hypoxia (Hx; 0.2 % O2), whole cell protein lysates were prepared and separated by SDS PAGE. Protein levels of Mcl-1, Bcl-2, and Bcl-xL were assessed by Western blot analysis. β-actin was used as loading control. Figures show representative results. A) In HCT116 cells, Mcl-1 protein levels strongly increased after ABT-263 single-agent and combined treatment with ABT-263 and irradiation in normoxia and to a lesser extent in hypoxia. Bcl-2 and Bcl-xL protein levels were generally increased in severe hypoxia but not altered by any treatment. B) Irradiation alone and in combination with ABT-263 resulted in a strong decrease of Mcl-1 protein levels under normoxic and hypoxic conditions in non-selected NCI-H460 cells. C) In hypoxia-selected NCI-H460 cells, Mcl-1 protein levels decreased after irradiation and combined treatment with ABT-263 in normoxia only.
The protein analyses implicated that particularly Mcl-1 might have a critical impact on sensitivity to ABT-263, ionizing radiation, and combined treatment. In HCT116 cells, a compensatory increase of Mcl-1 protein levels in response to Bcl-2/Bcl-xL inhibition might attenuate the effect of ABT-263 and combinatory treatment with ionizing radiation. While radiation-induced downregulation of Mcl-1 potentially determined sensitivity of non-selected NCI-H460 cells to combined treatment with ABT-263, hypoxia-selected cells preserved Mcl-1 at higher levels in hypoxia. This might be a particular resistance mechanism of hypoxia-selected NCI-H460 cells that may account for their increased resistance to radiation-induced apoptosis under hypoxic conditions. The impact of Mcl-1 protein on treatment outcome was therefore further analyzed in knockdown experiments.

3.4.2 **Silencing of MCL1 Increases Sensitivity of HCT116 Cells to Apoptosis Induction by ABT-263 Combinatory Treatment with Ionizing Radiation**

To address the potentially compensatory function of Mcl-1 upon inhibition of Bcl-2 and Bcl-xL by ABT-263 and the influence on treatment outcome in HCT116 colon cancer cells, siRNA-mediated silencing of MCL1 gene expression was performed in combination with ABT-263 treatment and irradiation. Knockdown efficiency was confirmed by qRT-PCR and Western blot analysis and apoptosis levels were determined by flow cytometric analysis of DNA fragmentation (Figure 3.20). Silencing of MCL1 expression significantly improved apoptosis induction in response to treatment with ABT-263 under normoxic and hypoxic conditions. Similar as in controls transfected with non-targeting siRNA, ABT-263 induced apoptosis more efficiently in hypoxia than in normoxia also when Mcl-1 was knocked down. Moreover, downregulation of MCL1 significantly sensitized HCT116 cells to apoptosis induction by combined treatment with ABT-263 and ionizing radiation under normoxic and under hypoxic conditions.
Figure 3.20: Silencing of MCL1 increased sensitivity of HCT116 cells to apoptosis induced by ABT-263 alone and combinatorial treatment with ionizing radiation. HCT116 cells were transfected with MCL1-targeting (siMCL1) or non-targeting control siRNA (siNT). A) Knockdown efficiency was assessed by qRT-PCR and Western blot analysis at indicated time points after transfection. siRNA-mediated silencing of MCL1 reduced the cellular amount of Mcl-1-encoding transcripts to 24%. Mcl-1 protein was undetectable between 24 and 72 hours post transfection. B) 24 hours after transfection, the cells were treated with ABT-263 in normoxia (Nx; 20% O<sub>2</sub>) or severe hypoxia (Hx; 0.2% O<sub>2</sub>). C) In addition, the cells were irradiated with 10 Gy. Apoptosis levels (Sub G1 fraction) were determined 48 hours later. Data show mean values of 3 independent experiments ±SD. Statistical significance is for clarity only indicated to oppose treatment outcome in siNT versus siMCL1-transfected cells and treatment outcome in normoxia versus hypoxia in siMCL1-transfected cells (* p ≤ 0.05; **** p ≤ 0.0001). Apoptosis levels significantly increased in response to ABT-263 treatment in normoxia and severe hypoxia in cells transfected with MCL1-targeting siRNA compared to non-targeting controls (B). Analogous to NT-controls, ABT-263-mediated apoptosis induction was significantly stronger under hypoxic than under normoxic conditions in siMCL1-transfected HCT116 cells. Silencing of MCL1 showed no effect on radiation-induced apoptosis (C). Compared to NT-controls, apoptotic DNA fragmentation upon combined treatment with ABT-263 and ionizing radiation was enhanced in siMCL1-transfected cells in normoxia and hypoxia.
3.4.3 **Silencing of MCL1 sensitizes NCI-H460 lung adenocarcinoma cells to the cytotoxic effects of ABT-263 and improves radiation-induced apoptosis**

While protein levels of anti-apoptotic Mcl-1 were markedly reduced upon irradiation in normoxic as well as hypoxic conditions in non-selected NCI-H460 cells, ionizing radiation decreased Mcl-1 levels in hypoxia-selected NCI-H460 cells only in normoxia but not in hypoxia. To exploit the influence of the differential Mcl-1 regulation on their sensitivity to ABT-263- and radiation-induced apoptosis, treatment outcome was analyzed in NCI-H460 cells with downregulated Mcl-1 expression.

qRT-PCR and Western blot analysis confirmed the successful knockdown of *MCL1* in non-selected NCI-H460 cells (**Figure 3.21 A**). Downregulation of Mcl-1 rendered non-selected NCI-H460 cells sensitive to ABT-263-induced apoptosis in normoxia and significantly stronger in severe hypoxia (**Figure 3.21 B**). Apoptosis levels in response to combined treatment with ABT-263 and irradiation further increased in normoxia and in hypoxia, when *MCL1* gene expression was silenced (**Figure 3.21 C**). In addition, knockdown of Mcl-1 increased sensitivity to radiation-induced apoptosis of non-selected NCI-H460 cells under hypoxic conditions, elevating apoptosis inductions to the levels detected in cells irradiated in normoxia.
Figure 3.21: Silencing of MCL1 sensitized non-selected NCI-H460 cells to ABT-263 treatment as single-agent and in combination with ionizing radiation and enhanced apoptosis induction upon irradiation in hypoxia. Non-selected NCI-H460 cells were transfected with siRNA targeting MCL1 (siMCL1) or non-targeting control siRNA (siNT). A) qRT-PCR and Western blot analysis were performed at indicated time points after transfection to assess knockdown efficiency. Transfection with MCL1-targeting siRNA reduced the cellular amount of mRNA encoding Mcl-1 to 21%. Mcl-1 protein levels were markedly reduced between 24 and 72 hours after transfection. B) 24 hours after transfection, the cells were treated with ABT-263 under normoxic (Nx; 20% O<sub>2</sub>) or severely hypoxic conditions (Hx; 0.2% O<sub>2</sub>). C) Additionally, the cells were irradiated with 10 Gy. After 48 hours, apoptosis levels were determined by measuring DNA fragmentation (Sub G1 fraction). Data are presented as mean values of 3 independent experiments ±SD. Statistical significance is for clarity only indicated to oppose treatment outcome in siNT- versus siMCL1-transfected cells and treatment outcome in normoxia versus severe hypoxia in siMCL1-transfected cells (** p ≤ 0.001; **** p ≤ 0.0001). Apoptosis levels increased in response to ABT-263 treatment in cells transfected with MCL1-targeting siRNA under normoxic and significantly stronger under hypoxic conditions (B). After combinatory treatment with ABT-263 and ionizing radiation, apoptotic DNA fragmentation was significantly elevated in cells with downregulated Mcl-1 compared to NT-controls, and to similar levels in normoxia and severe hypoxia (C). MCL1 gene expression silencing furthermore significantly increased apoptosis levels in non-selected NCI-H460 cells after irradiation under hypoxic conditions.
Downregulation of Mcl-1 in hypoxia-selected NCI-H460 cells following siRNA transfection was verified by qRT-PCR and Western blot analysis (Figure 3.22 A). Similarly to non-selected cells, downregulation of Mcl-1 led to increased apoptosis levels in response to treatment with ABT-263 also in hypoxia-selected NCI-H460 cells (Figure 3.22 B). Likewise, Mcl-1 knockdown-mediated sensitization to ABT-263-induced cell death was significantly stronger under hypoxic conditions, resulting in even higher apoptosis levels of 28 % compared to 19 % in non-selected NCI-H460 cells. Furthermore, Mcl-1 downregulation slightly but significantly increased apoptosis levels after sole incubation in hypoxia for 48 hours without any further treatment in hypoxia-selected NCI-H460 cells. Similarly to non-selected NCI-H460 cells, apoptosis induction in response to combined treatment with ABT-263 and irradiation was greatly enhanced in hypoxia-selected cells, when MCL1 gene expression was silenced (Figure 3.22 C). Moreover, MCL1 silencing in hypoxia-selected cells not only increased sensitivity to radiation-induced apoptosis in hypoxia, as observed in non-selected NCI-H460 cells, but also under normoxic conditions.
Figure 3.22: Silencing of MCL1 sensitized hypoxia-selected NCI-H460 cells to ABT-263, radiation-, and co-treatment-induced apoptosis. A) Following transfection of hypoxia-selected NCIH460 cells with siRNA against MCL1 (siMCL1) or non-targeting control siRNA (siNT), qRT-PCR and Western blot analysis were performed to determine knockdown efficiency. Transfection with siRNA targeting MCL1 reduced the cellular amount of Mcl-1-encoding transcripts to 13%. Mcl-1 protein levels were strongly reduced between 24 and 72 hours after transfection. B) 24 hours after transfection, the cells were treated with ABT-263 under normoxic (Nx; 20% O2) or severely hypoxic conditions (Hx; 0.2% O2). C) In addition, the cells were irradiated with 10 Gy. Apoptosis levels were analyzed by flow cytometric analysis of DNA fragmentation (Sub G1 fraction) 48 hours after treatment. Data are presented as mean values of 3 independent experiments ±SD. Statistical significance is for clarity only indicated to oppose treatment outcome in siNT- versus siMCL1-transfected cells and treatment outcome in normoxia versus hypoxia in siMCL1-transfected cells (*** p ≤ 0.01; **** p ≤ 0.001; ***** p ≤ 0.0001). Silencing of MCL1 in hypoxia-selected NCI-H460 cells led to slightly but significantly increased apoptosis levels after 48 hours incubation in severe hypoxia (B). In response to ABT-263 treatment, apoptosis levels increased in cells with downregulated Mcl-1 as compared to NT-controls, and the levels were significantly higher in hypoxia than in normoxia. Furthermore, MCL1 silencing resulted in increased apoptosis levels upon irradiation with 10 Gy in normoxia and in severe hypoxia (C). Apoptotic DNA fragmentation in response to combined treatment with ABT-263 and ionizing radiation further increased in cells transfected with MCL1-targeting siRNA and to a similar extent under normoxic and hypoxic conditions.
Collectively, downregulation of Mcl-1 improved sensitivity to apoptosis induction by ABT-263 alone and in combination with irradiation in all examined cell lines. In NCI-H460 cells, silencing of MCL1 moreover enhanced radiation-induced apoptosis. Thus, compensatory upregulation of Mcl-1 upon ABT-263 treatment as shown in HCT116 cells, or the failure to downregulate Mcl-1 levels as observed in hypoxia-selected NCI-H460 cells upon irradiation in hypoxia, may promote resistance to the cytotoxic effects of anti-cancer therapies.

3.5 Combined treatment with ABT-263 and ionizing radiation decelerates NCI-H460 xenograft tumor growth in vivo

To investigate whether the beneficial effect of ABT-263 on the cytotoxic action of ionizing radiation observed for non-selected and hypoxia-selected NCI-H460 cells in vitro translates to tumor growth deceleration in vivo, the effects of ABT-263, irradiation, and combined treatment (see treatment schedule in Figure 3.23 A) were assessed in xenograft tumors of non-selected and hypoxia-selected NCI-H460 cells in NMRI nude mice.

As shown in Figure 3.23 B, treatment with ABT-263 alone had no significant effect on growth of tumors derived from non-selected NCI-H460 cells. Tumor growth was significantly decreased by irradiation, as 6-fold increase of tumor volume was reached 5 days later than in the control group. As observed in vitro, ABT-263 improved the radiotherapy response also in vivo. Compared to irradiation alone, combined treatment with ABT-263 and ionizing radiation resulted in a more pronounced tumor growth delay. Significantly smaller tumor volumes were apparent from day 15 after irradiation, with a prolonged period of 2 days until the tumor volume reached 6-fold size.

Unlike tumors derived from non-selected cells, xenograft tumors of hypoxia-selected NCI-H460 cells showed slight but significant sensitivity to treatment with ABT-263 alone, as 6-fold tumor volume was reached significantly later than in control animals (Figure 3.23 C). Irradiation of these tumors resulted in a comparatively reduced growth delay, as 6-fold tumor volume was reached only 3 days later than in untreated controls. However, compared to the irradiation-only treated group, combined treatment with ABT-263 and ionizing radiation prolonged the period until 6-fold tumor volume was reached by 4 days. Significant differences between tumor volumes of groups treated with irradiation only and combined treatment with ABT-263 were observed from day 13 after irradiation.
Figure 3.23: Combined treatment with ABT-263 and ionizing radiation decelerated growth of non-selected and hypoxia-selected NCI-H460 xenograft tumors. A) Treatment schedule: Xenograft tumors of non-selected and hypoxia-selected NCI-H460 cells were generated by subcutaneous injections into the hind leg of NMRI nude mice. As soon as tumor volume reached ~50 mm³ (modified ellipsoid formula, see 2.7), tumors were irradiated with a single dose of 5 Gy. ABT-263 (75 mg/kg) was applied via intraperitoneal injection every other day (6 injections in total), starting 2 hours after tumor irradiation for combined treatment. B; C) Tumor growth is presented as mean tumor volume of 6-8 animals per group ±SEM, with indicated statistical significance between treatment groups (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001). Growth delay upon treatment is represented as median time until tumors reached 6-fold volume, with tumor volumes at day 0 defined as individual starting points. Treatment with ABT-263 alone had no significant effect on growth of tumors derived from non-selected NCI-H460 cells (B). Irradiation of these tumors resulted in a growth delay of 5 days. Combined treatment with ABT-263 and ionizing radiation further prolonged the time until the tumor volume reached 6-fold size by 2 days. In xenograft tumors of hypoxia-selected NCI-H460 cells, tumor growth was delayed upon treatment with ABT-263 by 2 days and upon irradiation by 3 days (C). Compared to only irradiated tumors, combined treatment with ABT-263 delayed growth by further 4 days.
Direct comparison of growth curves of untreated non-selected and hypoxia-selected NCI-H460-derived tumors showed that both tumor entities had comparable growth rates (Figure 3.24 A). Treatment with ABT-263 alone had similar effects on growth of tumors derived from non-selected and hypoxia-selected NCI-H460 cells (Figure 3.24 B). As expected from in vitro findings, following radiotherapy, tumors derived from hypoxia-selected cells grew faster than those derived from non-selected cells NCI-H460 (Figure 3.24 C). Significant differences between mean tumor volumes were evident from day 13 after irradiation. Nevertheless, groups treated with the combination of ABT-263 and ionizing radiation showed similar tumor volumes and growth delay (Figure 3.24 D).

Figure 3.24: Growth comparison of non-selected and hypoxia-selected NCI-H460 xenograft tumors upon treatment with ABT-263, irradiation, and combined treatment. Tumors derived from non-selected and hypoxia-selected NCI-H460 cells show similar growth in untreated (A) and in ABT-263-treated animals (B). Regrowth after irradiation (5 Gy) started earlier in tumors derived from hypoxia-selected NCI-H460 cells than in those derived from non-selected NCI-H460 cells (C). Combined treatment with ABT-263 and ionizing radiation resulted in similar growth delay and tumor volumes (D). * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001.

The results obtained from tumor xenograft experiments confirmed that treatment with ABT-263 improves effectivity of radiotherapy of non-selected and pronouncedly hypoxia-selected NCI-H460 cells also in vivo.
3.6 Adaptation to Cycling Hypoxia Alters Mitochondrial Features and Function

3.6.1 Exposure to Acute Hypoxia and Adaptation to Cycling Hypoxia Alters Expression of Genes Involved in Mitochondrial Energy Metabolism and Mitochondrial Dynamics

The data presented above clearly show that the Bcl-2 rheostat influences the sensitivity of hypoxic and hypoxia-selected cancer cells to anti-neoplastic therapies. The Bcl-2 protein family is most notable for regulation of apoptosis by controlling mitochondrial integrity [101]. However, Bcl-2 family members have also been shown to participate in the regulation of mitochondrial metabolism and morphology [117]. This led to the hypothesis that the deregulated Bcl-2 rheostat in hypoxia-selected NCI-H460 cells might be accompanied by additional alterations in mitochondrial functions that might contribute to hypoxia tolerance and increased apoptosis resistance. Moreover, a previous study associated distinct metabolic dependencies of hypoxia-selected NCI-H460 cells with a characteristic occurrence of enlarged mitochondria, when the cells were incubated under severely hypoxic conditions (Dr. Johann Matschke, unpublished data). To explore additional alterations of mitochondrial features and function, hypoxia-selected and non-selected NCI-H460 cells were analyzed for differential expression of genes involved in mitochondrial energy metabolism and dynamics by qRT-PCR and cDNA microarray analysis.

Compared to non-selected cells, hypoxia-selected NCI-H460 cells showed increased gene expression of several proteins involved in mitochondrial metabolism and mitochondrial dynamics in normoxia (Figure 3.25 A). Genes encoding for malate dehydrogenase and subunits of isocitrate dehydrogenase, which are enzymes of the TCA cycle, as well as regulators of mitochondrial fusion (Mfn1, Opa1) and fission (Drp1 (encoded by DNM1L), Fis1) were expressed at significantly higher levels in hypoxia-selected than in non-selected cells. Moreover, hypoxia-selected cells showed a slight but insignificant tendency (p=0.051) to increased expression of VDAC1. VDAC is a pore forming protein that mediates influx and efflux of ions, nucleotides, and other metabolites across the mitochondrial outer membrane [203]. After exposure to acute hypoxia for 6 hours, both cell lines upregulated VDAC1-3 (Figure 3.25 B and C). Of the proteins involved in regulation of mitochondrial dynamics, gene expression of fusion protein Opa1 was significantly upregulated in both cell lines in response to acute hypoxia.
Figure 3.25: Gene expression of proteins involved in regulation of mitochondrial metabolism and dynamics was altered in hypoxia-selected NCI-H460 cells and in both NCI-H460 cell lines in response to acute hypoxia. Following incubation of non-selected and hypoxia-selected NCI-H460 cells in normoxia (Nx; 20% O₂) or in severe hypoxia (Hx; 0.2% O₂) for 6 hours, mRNA was isolated and used for cDNA transcription. Gene expression of proteins involved in mitochondrial respiration (IDH1A, IDH1B, IDH1G, MDH2, VDAC1-3) and mitochondrial
RESULTS

dynamics (MFN1, MFN2, OPA1, DNM1L, FIS1) was analyzed by qRT-PCR and cDNA microarray. Statistically significant fold change (FC) values from microarray analysis are specified in the tables. Graphs show relative gene expression assessed by qRT-PCR, normalized as indicated to allow comparison of gene expression in both cell lines in normoxia (A) and cell-individual changes in hypoxia (B and C). Data represent mean values of 3 independent experiments ±SD (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001). Expression of several regulators of mitochondrial metabolism and dynamics was upregulated in hypoxia-selected compared to non-selected NCI-H460 cells and in both cell lines upon exposure to acute hypoxia. While hypoxia-selected NCI-H460 cells showed upregulation of enzymes of the TCA cycle in normoxia (A), both cell lines increased expression of VDAC1-3 after incubation in hypoxia (B and C). Moreover, OPA1 was significantly upregulated in hypoxia-selected compared to non-selected cells in normoxia as well as in both cell lines in response to exposure to acute hypoxia.

Taken together, the results show that expression of several genes involved in mitochondrial dynamics was altered in response to acute hypoxia. Furthermore, particularly hypoxia-selected NCI-H460 cells were characterized by increased expression of enzymes of the TCA cycle. This suggested that both, altered mitochondrial dynamics and mitochondrial metabolism could represent mechanisms of adaptation to acute and cycling hypoxia.

3.7 PARAMETERS OF MITOCHONDRIAL RESPIRATION ARE ALTERED IN HYPOXIA-SELECTED NCI-H460 CELLS

The differential expression of genes involved in mitochondrial dynamics and metabolism suggested a metabolic switch in response to acute hypoxia and adaptation to cycling hypoxia. Thus, metabolic parameters in hypoxia-selected and non-selected NCI-H460 cells were measured by extracellular flux analysis. For technical reasons, these measurements could be conducted under normoxic conditions only.

Analysis of the cell energy phenotype provided insight into the relative utilization of glycolysis and mitochondrial respiration. As shown in Figure 3.26, similar bioenergetic profiles were detected for non-selected and hypoxia-selected NCI-H460 cells. Both cell lines had similar metabolic phenotypes under basal conditions and responded to the induced energetic stress mainly by increasing glycolysis. However, compared to non-selected NCI-H460 cells, hypoxia-selected cells showed slightly but significantly enhanced response to the energy demand via mitochondrial respiration.
RESULTS

Figure 3.26: Utilization of mitochondrial respiration in response to energetic demand was slightly enhanced in hypoxia-selected compared to non-selected NCI-H460 cells. The cell energy phenotype and metabolic potential of non-selected and hypoxia-selected NCI-H460 cells was assessed by simultaneous measurement of extracellular acidification rates (ECAR; indicator of glycolysis) and oxygen consumption rates (OCR; indicator of oxidative phosphorylation) under basal and energetically stressed conditions. For latter, ATP synthase inhibitor oligomycin (1 µM) and mitochondrial uncoupler FCCP (2 µM) were injected simultaneously. Oligomycin drives a compensatory increase of glycolysis, while FCCP reveals the maximum capacity of oxidative phosphorylation. Right panel shows representative cell energy phenotype profiles of non-selected and hypoxia-selected NCI-H460 cells. Left panel shows their metabolic potential, calculated as increased OCR and ECAR over baseline rates. Data in left panel show mean values of 3 independent experiments ± SD (* p ≤ 0.05). Cell energy phenotypes of non-selected and hypoxia-selected NCI-H460 cells were similar under basal conditions. In response to energetic stress, hypoxia-selected cells showed a slightly increased utilization of mitochondrial respiration.

To elaborate the differences in the metabolic potential of non-selected and hypoxia-selected NCI-H460 cells shown in the analysis of their bioenergetic phenotypes, key parameters of glycolytic function and mitochondrial respiration were assessed in more detail. Additional extracellular flux analyses showed that glycolytic parameters were similar in both cell lines, whereas hypoxia-selected NCI-H460 cells showed a change in parameters of mitochondrial respiration (Figure 3.27). Compared to non-selected cells, they were characterized by a significantly increased spare respiratory capacity. This suggests that the elevated capability of hypoxia-selected NCI-H460 cells to increase productivity of mitochondrial respiration in response to energetic stress might be a mechanism for adapting to adverse hypoxic conditions.
Figure 3.27: Hypoxia-selected NCI-H460 cells were characterized by an increased spare respiratory capacity, while glycolytic function was similar to non-selected NCI-H460 cells. Key parameters of glycolytic function and mitochondrial respiration were assessed in non-selected and hypoxia-selected NCI-H460 cells by extracellular flux analysis. A) Right panel shows representative measurements of extracellular acidification rates (ECAR) in NCI-H460 cells with indicated time points of addition of glucose (10 mM), oligomycin (1 µM), and 2-DG (50 mM). Left panel shows key parameters of glycolysis. Basal glycolysis was determined after addition of glucose. ATP synthase inhibitor oligomycin was added to assess the maximum glycolytic capacity, which allows calculation of the glycolytic reserve. B) Right panel shows representative measurements of oxygen consumption rates (OCR) in NCI-H460 cells with addition of oligomycin (1 µM), mitochondrial uncoupler FCCP (2 µM), and electron transport chain inhibitors antimycin A and rotenone (0.5 µM) at indicated time points. Left panel shows key parameters of mitochondrial respiration. Basal respiration was determined prior to addition of oligomycin and maximal respiratory capacity after addition of FCCP. Addition of antimycin A and rotenone revealed non-mitochondrial respiration. The measurements were used to calculate proton leak, spare respiratory capacity, and ATP production. Data in left panels represent mean values of 3 independent experiments ±SD (* p ≤ 0.05). While glycolytic function was similar in both cell lines (A), mitochondrial metabolism was altered in hypoxia-selected NCI-H460 cells, showing a significant increase in spare respiratory capacity (B).
3.7.1 **FUSION PROTEIN OPA1 INFLUENCES MITOCHONDRIAL RESPIRATION OF HYPOXIA-SELECTED NCI-H460 CELLS**

In addition to its role in mitochondrial inner membrane fusion, Opa1 protein function has also been linked to increased mitochondrial energy production [122]. In the previously shown results (Figure 3.25), expression of *OPA1* was significantly upregulated in hypoxia-selected compared to non-selected NCI-H460 cells in normoxia. Moreover, expression of *OPA1* was also increased in both cell lines 6 hours after exposure to acute severe hypoxia. Thus, regulation of Opa1 and its impact on the increased spare respiratory capacity observed in hypoxia-selected NCI-H460 cells were further analyzed in knockdown experiments.

qRT-PCR data presented in 3.6.1 were elaborated for *OPA1* gene expression by direct comparison of non-selected and hypoxia-selected NCI-H460 cells. Additional western blot analyses were performed to assess regulation on protein level. Figure 3.28 A shows that upregulation of *OPA1* expression after exposure to acute hypoxia was significantly stronger in hypoxia-selected than in non-selected cells. The hypoxia-induced transcriptional upregulation of *OPA1* may contribute to the less pronounced decrease in Opa1 protein levels under hypoxic conditions in hypoxia-selected NCI-H460 cells compared to non-selected cells (Figure 3.28 B).
Figure 3.28: Expression of Opa1 was differentially regulated on transcriptional and protein level in non-selected and hypoxia-selected NCI-H460 cells. A) Gene expression of OPA1 was assessed by qRT-PCR analysis. Graphs show relative gene expression normalized as indicated to allow comparison of gene expression in both cell lines (left panel) and cell-individual changes in hypoxia (right panel). Data represent mean values of 3 independent experiments ±SD (* p ≤ 0.05; ** p ≤ 0.01). OPA1 was expressed at higher levels in hypoxia-selected than in non-selected NCI-H460 cells in normoxia (Nx; 20% O2) as well as after exposure to severe hypoxia (Hx; 0.2% O2) for 6 hours. Upregulation in response to acute hypoxia was significantly higher in hypoxia-selected than in non-selected cells. B) Protein levels of Opa1 following 48 hours incubation in normoxia (Nx; 20% O2) or severe hypoxia (Hx; 0.2% O2) were assessed by Western blot analysis. β-actin was used as loading control. Prolonged exposure to severe hypoxia resulted in decreased levels of Opa1 in non-selected cells, whereas the protein remained at higher levels in hypoxia-selected NCI-H460 cells.

The differential regulation of Opa1 in hypoxia-selected NCI-H460 cells might influence mitochondrial function, which might allow adaptation to hypoxia by contributing to increased survival throughout exposure to adverse conditions including hypoxic and energetic stress. To address the protein’s influence on mitochondrial integrity and function, siRNA-mediated silencing of OPA1 gene expression was performed. As shown in Figure 3.29, knockdown of Opa1 had no direct impact on both NCI-H460 sub-cell lines in terms of apoptosis induction and integrity of the mitochondrial membrane potential after incubation for 24 and 48 hours in severe hypoxia.
Figure 3.29: Silencing of OPA1 expression had no influence on hypoxia tolerance of non-selected and hypoxia-selected NCI-H460 cells. A) Following transfection with siRNA against OPA1 (siOPA1) or non-targeting control siRNA (siNT), qRT-PCR and Western blot analysis were performed 24 h (qRT-PCR) and, respectively, 24-72 h after transfection (Western blot) to determine knockdown efficiency. Analyses confirmed that siRNA mediated silencing of OPA1 efficiently reduced Opa1 transcription and protein levels in non-selected and hypoxia-selected NCI-H460 cells. B; C) 24 hours after siRNA transfection, the cells were incubated under normoxic (Nx; 20 % O2) or severely hypoxic conditions (Hx; 0.2 % O2). Apoptotic DNA fragmentation (Sub G1 fraction) and dissipation of the mitochondrial membrane potential (MMP; ΔΨm low) were analyzed after 24 and 48 hours. Data present mean values of 3 independent experiments ±SD. Downregulation of Opa1 did not induce apoptosis (B) or MMP dissipation (C) under normoxic or hypoxic conditions in neither NCI-H460 cell line.
Furthermore, key parameters of mitochondrial respiration were examined following siRNA mediated downregulation of Opa1. While the spare respiratory capacity was increased in hypoxia-selected cells under control conditions, as shown before (Figure 3.27), OPA1 silencing significantly decreased this property in hypoxia-selected cells (Figure 3.30). In contrast, key parameters of mitochondrial respiration such as basal respiration and ATP production were unaffected by Opa1 downregulation.

**Figure 3.30:** Spare respiratory capacity was specifically reduced in hypoxia-selected NCI-H460 cells upon OPA1 silencing. 24 hours after transfection with siRNA targeting OPA1 (siOPA1) or non-targeting siRNA (siNT), mitochondrial function of non-selected and hypoxia-selected NCI-H460 cells was evaluated by extracellular flux analysis. Modulators of respiration, targeting distinct components of the electron transport chain, were injected serially and changes in oxygen consumption rates (OCR) were used to analyze key parameters of mitochondrial respiration. Data show mean values of 3 independent experiments ±SD (* p ≤ 0.05; ** p ≤ 0.01). Downregulation of Opa1 had no influence on basal respiration, proton leak, or ATP production of non-selected or hypoxia-selected cells. While spare respiratory capacity was increased in hypoxia-selected NT-control cells, OPA1 silencing decreased the levels significantly. The resulting levels were significantly lower than in non-selected NCI-H460 cells, the spare respiratory capacity of which was not affected by OPA1 silencing.

Collectively, the results show that hypoxia-selected NCI-H460 cells are characterized by the capability to increase mitochondrial energy production in response to energetic stress. This elevated bioenergetic capability depends on fusion protein Opa1. Thus, specific alterations in mitochondrial function could provide a mechanism of adaptation to hypoxia that improves cell survival in adverse conditions.
4 DISCUSSION

4.1 EXPOSURE TO ACUTE AND ADAPTATION TO CYCLING HYPOXIA REDUCE RADIATION-INDUCED APOPTOSIS AND EFFICACY OF IONIZING RADIATION

Accelerated cancer cell proliferation and resulting abnormal tumor vascularization give rise to chronic and alternating periods of low oxygenation levels within the tumor, known as tumor hypoxia [204]. Ranging from mild (>5 % O₂) to severe (<0.5 % O₂), hypoxia is a characteristic feature of most human solid tumors and a major factor that limits success of chemotherapies and especially radiotherapy [205]. The dramatic effect of cellular hypoxia during irradiation is based on the necessity of molecular oxygen to enable permanent, irreparable DNA damage produced by reactive oxygen species (ROS) [12, 206]. In addition to hampered manifestation of DNA damage, exposure to acute hypoxia activates several oxygen-sensitive signaling cascades that increase the cell death threshold [207]. Consequently, hypoxia signaling further promotes therapy resistance by allowing survival and expansion of tumor cells that are insensitive to death signals [163].

The first experiments conducted in the present study were intended to examine the relationship between tumor hypoxia, apoptosis resistance, and failure of radiotherapy described in various studies [65, 208-212]. The results showed that in HCT116 colon carcinoma and NCI-H460 lung adenocarcinoma cells, apoptosis induced by ionizing radiation was reduced when the cells were irradiated under severely hypoxic conditions (0.2 % O₂) as compared to irradiation in normoxia (Figure 3.1). Along with increased resistance to apoptosis induction, clonogenic survival was enhanced in both cell lines upon irradiation under severely hypoxic compared to normoxic conditions (Figure 3.2). This suggests a direct relationship between hampered apoptosis induction and increased radioresistance caused by exposure to acute hypoxia. Moreover, these findings confirm that the experimental setup is suitable for a thorough examination of hypoxia-mediated apoptosis resistance and the contribution to associated radioresistance and to explore strategies for overcoming therapy resistance of hypoxic cancer cells.
To investigate changes induced by adaptation to cycling severe hypoxia, the present work extended the initial studies with NCI-H460 cells exposed to 10 cycles of severe hypoxia (48 h 0.1 % O₂) and reoxygenation (120 h 20 % O₂) conducted by Weinmann et al. [77, 213] towards a sub-cell line established by additional 15 cycles of hypoxic selection (hypoxia-selected NCI-H460) as described recently by Matschke et al. [185]. Particularly exposure to repeating cycles of severe hypoxia and reoxygenation is known to trigger adaptation processes, which promote clonal selection of hypoxia tolerant cells with characteristically reduced apoptotic potential and increased therapy resistance [76, 77, 185, 213-216]. Apoptosis resistance in these reports had mostly been associated with defects in the mitochondrial apoptosis pathway, partially resulting from adaptive changes in the regulation of pro- or anti-apoptotic Bcl-2 protein family members [76, 214, 215]. Moreover, several studies documented the importance of improved ROS defense in radiotherapy resistant hypoxic cancer cells [185, 216]. In particular, Matschke et al. recently demonstrated that increased antioxidant capacity, which included enhanced expression of ROS scavenging enzymes and increased glutathione levels, contributes to apoptosis and radiation resistance of the hypoxia-selected NCI-H460 cell line employed in the present study [185]. Weinmann et al. similarly observed upregulation of genes involved in stress resistance including ROS metabolism in NCI-H460 cells already after 10 cycles of hypoxic selection [213]. In addition, functional analyses of these cells led to the conclusion that impaired Bax activation is a critical factor of apoptosis resistance mediated by adaptation to cycling hypoxia [77].

In the present study, radiation-induced apoptosis in hypoxia-selected NCI-H460 cells was significantly reduced compared to non-selected cells in normoxia (Figure 3.3). Apoptosis induction was further attenuated when hypoxia-selected cells were irradiated under severely hypoxic conditions. Interestingly, apoptosis levels of non-irradiated controls, which were incubated in hypoxia for 48 hours, were slightly but significantly lower in hypoxia-selected than in non-selected NCI-H460 cells, pointing at increased hypoxia tolerance of the hypoxia-selected sub-cell line. Moreover, clonogenic survival after irradiation in normoxia was less reduced in hypoxia-selected than in non-selected NCI-H460 cells (Figure 3.4). In line with this finding, Verduzco et al. recently demonstrated that adaptation to cycling hypoxia involves permanent changes that persist under normoxic conditions in various cancer cell lines [217]. This suggests that hypoxic signaling not only transiently increases the cell death threshold during episodes of hypoxia, but by retaining the "pseudohypoxic" phenotype in normoxia,
hypoxia-selected cancer cells might critically promote tumor progression and therapy resistance.

The results obtained in the present study corroborate previous findings on apoptosis resistance and associated radioresistance of hypoxia-selected NCI-H460 cells of Weinmann et al. and Matschke et al. [77, 185], providing evidence that adaptation to cycling hypoxia offers a survival advantage also under normoxic conditions. In addition, the present study demonstrates that hypoxic selection of cancer cells with a diminished apoptotic potential also attenuates the cytotoxic effect of ionizing radiation in vivo (see 4.3.2). Collectively, these findings underline that the cell model employed in this study is suitable to examine alterations of cancer cells adapted to cycling hypoxia/reoxygenation stress. This model can consequently be used to identify adaptation mechanisms that can be targeted to re-sensitize hypoxia-selected cancer cells to radiotherapy.

4.2 Exposure to acute and adaptation to cycling hypoxia result in alterations in the Bcl-2 rheostat

Tumor hypoxia contributes to invasion, metastasis, and resistance to chemo- and especially radiotherapy [218, 219]. Pathways activated in response to acute hypoxia include activation of the hypoxia-inducible factor family of transcription factors (HIFs) and the unfolded protein response (UPR) as well as inhibition of the mammalian target of rapamycin (mTOR) kinase [50]. These oxygen sensitive pathways function as tolerance mechanisms in cells deprived of oxygen by regulating metabolism, angiogenesis, autophagy, and endoplasmic reticulum (ER) homeostasis. Moreover, evidence has been provided that hypoxia tolerance and resulting clonal evolution of therapy resistant cancer cells can be promoted through alterations in the mitochondrial apoptosis program, which is regulated by the Bcl-2 protein family [61-64]. Particularly the preceding study on hypoxia-selected NCI-H460 cells of Weinmann et al., who observed impaired Bax activation in irradiated cells exposed to 10 cycles of hypoxic selection [77], led to the speculation that alterations in the regulation of Bcl-2 protein family members might be an underlying mechanism of apoptosis resistance in hypoxic and hypoxia-selected cancer cells and provided the basis for the present work.
Gene and protein expression analyses employed to explore mechanisms of apoptosis resistance of HCT116 and NCI-H460 cells indeed revealed altered expression of several Bcl-2 protein family members after exposure to acute and cycling severe hypoxia (Figure 3.5 and Figure 3.6). HCT116 cells and both NCI-H460 sub-cell lines broadly showed decreased protein levels of pro-apoptotic BH3-only regulators and apoptosis effectors, whereas protein levels of anti-apoptotic family members rather increased.

Decreased protein levels of pro-apoptotic BH3-only regulators Noxa, Puma, and Bim as well as of apoptosis effector Bax after exposure to acute hypoxia were a common finding among the three examined cell lines. A similar tendency of downregulated pro-apoptotic Bcl-2 family members was previously reported for several colon cancer cell lines exposed to acute hypoxia [62]. In the study of Erler et al., hypoxia-mediated downregulation of Bid, Bad, and Bax were shown to contribute to enhanced apoptotic as well as clonogenic resistance to various chemotherapeutics. Another BH3-only protein with documented implication in hypoxic apoptosis regulation is Puma, as presence of this Bcl-2 family member was reported to be critical for apoptosis-induction in hypoxia [220]. Intriguingly, Puma protein levels in hypoxia-selected NCI-H460 cells were lower than in non-selected cells in both normoxia and hypoxia, which correlated with increased resistance to radiation-induced apoptosis in hypoxia-selected compared to non-selected cells. In accordance with the mentioned and other reports [68-70], the findings of the present study implicate that downregulation of pro-apoptotic Bcl-2 family members may be a general response of cancer cells to hypoxia and contribute to hypoxia-mediated apoptosis resistance.

On the other hand, upregulation of anti-apoptotic Bcl-2 family members was likewise associated with hypoxia-mediated evasion of apoptosis in a cell type- and context-dependent manner in several studies [61, 63, 68]. Accordingly, levels of distinct anti-apoptotic Bcl-2 family proteins increased in HCT116 and NCI-H460 cells in response to acute hypoxia in the present work. HCT116 cells showed markedly elevated Bcl-xL and Bcl-2 protein levels in hypoxia. Consistent with this finding, overexpression of Bcl-2 and Bcl-xL were reported to abrogate hypoxia-induced cell death in early studies exploring hypoxia-mediated cell death resistance [221-223]. Moreover, resistance of renal carcinoma cells to chemically induced hypoxia was reported to be conferred by a strong upregulation of Bcl-2, which depended on the presence of the von Hippel-Lindau protein (pVHL), an oxygen-sensitive regulator of HIF-1α-activity [224]. However, while HIF-1α activity in cancer cells is clearly related to increased resistance
to chemo- and radiotherapy [225], the link between hypoxia-induced HIF-1α activity and apoptosis regulation by Bcl-2 family members is controversial. It was reported that expression of Bcl-2 inversely correlated with HIF-1α and HIF-2α activity in non-small cell lung cancer [226], whereas Bos et al. provided evidence for a strongly positive correlation of increased HIF-1α and Bcl-2 expression and the pathologic state of breast carcinoma [227]. These differences, however, might be due to tissue specific regulation of hypoxia-induced apoptosis and the extent of oxygen deprivation.

Both NCI-H460 cell lines were characterized by higher Mcl-1 protein levels than observed in HCT116 cells. Mcl-1 protein levels furthermore strongly increased in the NCI-H460 cell lines after exposure to acute severe hypoxia. In contrast to this observation, Brunelle et al. demonstrated that Mcl-1 protein was depleted rapidly in complete absence of oxygen (anoxia), which in combination with inhibition of Bcl-2 and Bcl-xL potently induced cell death [228]. Exposure to mild hypoxia (1.5 % O₂), however, had no influence on Mcl-1 protein levels and did not trigger cell death in their study. The findings of Brunelle et al. emphasize the critical role of Mcl-1 for the switch from cell survival to cell death under conditions of oxygen deprivation. With regard to the results obtained in the present study, it can thus be concluded that strong increase of Mcl-1 protein levels in both NCI-H460 cell lines upon exposure to acute hypoxia might critically determine their enhanced resistance to radiation-induced apoptosis under hypoxic conditions. Similarly, Piret et al. reported that incubation in hypoxia increased Mcl-1 expression of hepatoma cells and overexpression of this Bcl-2 family member protected the cells from apoptosis [63]. Piret and co-workers furthermore identified a HIF-1 binding site within the Mcl-1 promotor sequence, which corroborates the distinct role of Mcl-1 regulation upon oxygen deprivation.

Furthermore, Bcl-xL was generally upregulated on protein and transcription levels in hypoxia-selected compared to non-selected NCI-H460 cells. Interestingly, upregulated Bcl-xL expression in cancer cells adapted to cycling hypoxia was previously observed by Dong and Wang, who used a similar model as in the present study [215]. These hypoxia-selected cells were reported to be largely insensitive to apoptosis-inducing agents and preserved mitochondrial integrity and cell viability. As selective down-regulation of Bcl-xL by antisense oligonucleotides and inhibitors restored their sensitivity, Bcl-xL was proposed to be a key molecule for hypoxia-mediated selection of apoptosis resistance. Consequently, general upregulation of Bcl-xL, as detected in hypoxia-selected NCI-H460 cells in the present study,
might be a mechanism of adaptation to hypoxia and a critical factor for increased apoptosis resistance of these cells. Although Weinmann et al. did not observe significant changes in the expression of Bcl-xL or other Bcl-2 protein family members in NCI-H460 cells after 10 cycles of hypoxic selection, it is conceivable that impaired Bax activation upon irradiation observed in the initial study [77] resulted from a slight, unobvious shift of the Bcl-2 protein family balance towards survival. As shown in the present study, exposure to 15 additional cycles of severe hypoxia and reoxygenation resulted in the selection of NCI-H460 cells with clearly increased protein levels of Bcl-xL and decreased expression of Puma and Bak, suggesting that the altered balance in favor of anti-apoptotic Bcl-2 protein family members determines the increased resistance of hypoxia-selected NCI-H460 cells to radiation-induced apoptosis. Moreover, the present work extended the initial studies by providing insight into the regulation of Bcl-2 protein family members in hypoxia-selected cells under severely hypoxic conditions. The marked increase in Mcl-1 protein levels and decrease of Bax, Puma Noxa, and Bim upon exposure to acute severe hypoxia, however, resembled the proteins’ regulation in non-selected NCI-H460 cells.

Taken together, the results obtained from protein expression analyses showed that exposure to acute and cycling hypoxia shifts the delicate balance between pro- and anti-apoptotic Bcl-2 protein family members towards survival in all three examined cell lines. Importantly, these findings correlated with increased resistance to radiation-induced apoptosis in hypoxia and pronouncedly in hypoxia-selected NCI-H460 cells. This implied that interfering with the deregulated Bcl-2 rheostat might be a useful approach to therapeutically target hypoxic and hypoxia-selected cancer cells.
4.3 Radioresistance conferred by exposure to acute and adaptation to cycling hypoxia can be overcome by Bcl-2/Bcl-xL inhibitor ABT-263

4.3.1 ABT-263 improves sensitivity of hypoxic and hypoxia-selected cancer cells to the cytotoxic action of ionizing radiation in vitro

As protein expression analyses of HCT116 colon cancer and NCI-H460 lung cancer cells have implied that an altered Bcl-2 rheostat might determine hypoxia-mediated radioresistance, BH3-mimetic ABT-263 was applied to assess whether targeting anti-apoptotic Bcl-2 family proteins improves radiation-induced cytotoxicity in severe hypoxia and in hypoxia-selected cancer cells. ABT-263 is an orally bioavailable analog to ABT-737, a molecule that was previously discovered and optimized by NMR-based screening and structure-based design. The compounds bind with high affinity to Bcl-2, Bcl-xL, and Bcl-w, thereby disrupting interactions with pro-apoptotic proteins and ultimately leading to apoptosis induction [168].

In the present study, treatment with ABT-263 greatly enhanced the cytotoxic effect of ionizing radiation in HCT116 cells and both NCI-H460 cell lines under normoxic and, importantly, also under hypoxic conditions.

In HCT116 colon carcinoma cells, treatment with the BH3-mimetic alone effectively induced apoptosis in normoxia and even stronger in severe hypoxia (Figure 3.7). In contrast, both NCI-H460 cell lines were largely insensitive towards apoptosis induction by ABT-263 (Figure 3.11 and Figure 3.15). However, despite differential sensitivity to treatment with the BH3-mimetic alone, ABT-263 clearly sensitized all three cell lines to radiation-induced apoptosis. Importantly, ABT-263 and irradiation applied together in hypoxia induced apoptosis at similar levels as in normoxia in HCT116 and non-selected NCI-H460 cells, although apoptosis induced by irradiation alone was decreased under hypoxic conditions. ABT-263 sensitized hypoxia-selected NCI-H460 cells even stronger to irradiation-induced apoptosis in hypoxia, as apoptosis levels were significantly higher than in normoxia.

Similarly pronounced sensitivity to ABT-737-induced apoptosis in hypoxia was demonstrated by others in neuroblastoma, colon, and small cell lung cancer (SCLC) cells [229, 230]. In several of these, sensitivity correlated with increased protein levels of Bcl-2 and Bcl-xL or decreased Mcl-1 levels. Moreover, the synergistic effect of combined treatment with BH3-mimetics and irradiation has been attributed to distinct regulation of certain Bcl-2 family members by irradiation or by ABT-737 [147, 182-184, 231]. Consistently, differential sensitivity of HCT116
and NCI-H460 cells observed in the study at hand was found to underlie intrinsic and treatment-induced regulation of Bcl-2 family proteins.

4.3.1.1 DEPENDENCE OF HCT116 COLON CARCINOMA CELLS ON BCL-2 AND BCL-XL DETERMINES THEIR SENSITIVITY TO ABT-263

In HCT116 cells, in which Mcl-1 protein was present at rather low levels, inhibition of Bcl-2 and Bcl-xL by ABT-263 was able to overcome the apoptotic threshold and induce cell death. Release of sequestered apoptosis effectors Bax and Bak and possibly BH3-only proteins upon ABT-263 binding to Bcl-2 and Bcl-xL apparently is sufficient to allow Bax and Bak pore formation at the MOM, which leads to cytochrome c release and finally induces apoptosis. This implies that HCT116 colon cancer cells depend on Bcl-2 and Bcl-xL to counteract apoptosis signals. “Bcl-2-like dependence” describes a state in which individual or subsets of anti-apoptotic Bcl-2 family proteins are essential for sustained survival of cancer cells [136]. Upregulation of anti-apoptotic proteins in response to environmental cues is thought to reinforce dependency, concomitantly increasing vulnerability to their inhibition. According to this concept, the strong upregulation of Bcl-2 and Bcl-xL in HCT116 cells in hypoxia is suggestive of increased dependence on these anti-apoptotic Bcl-2 family members. Consequently, their inhibition by ABT-263 resulted in even stronger apoptosis induction in hypoxia, despite downregulation of pro-apoptotic family members.

The fact that combinatory treatment with ABT-263 and ionizing radiation further increased apoptosis levels in normoxia and in hypoxia suggests that irradiation additionally interferes with the Bcl-2 rheostat. Protein analyses showed that irradiation did not affect protein levels of anti-apoptotic family members Bcl-2, Bcl-xL, and Mcl-1 (Figure 3.19 A). However, irradiation-induced DNA damage is known to trigger transcriptional and post-translational activation of p53 [19]. This protein can contribute to apoptosis induction by direct interaction with Bcl-2 family members [22]. More importantly, acting as a transcription factor, p53 activates expression of apoptosis effector Bax and pro-apoptotic BH3-only proteins Noxa and Puma [85]. While Bax levels were not affected by any treatment (Figure 3.8 A), radiation-induced upregulation of Puma and Noxa might add to ABT-263-induced apoptosis. Puma is able to neutralize all anti-apoptotic Bcl-2 family members, thus might enhance release of sequestered pro-apoptotic BH3-only and apoptosis effector proteins in HCT116 cells. Moreover, Puma was proposed to directly activate Bax and Bak pore formation, thus
facilitating apoptosis induction. Noxa, however, selectively binds to Mcl-1 [93]. Despite relatively low Mcl-1 protein levels in HCT116 cells, upregulation of Puma and Noxa and enhanced release of other BH3-only proteins as Bim or tBid upon irradiation might further contribute to enhanced apoptosis induction by inhibiting Mcl-1.

In the present study, inhibition of Bcl-xL and Bcl-2 in HCT116 cells by treatment with ABT-263 revealed a prominent increase in Mcl-1 protein in normoxia and, although less prominently, also under hypoxic conditions (Figure 3.19 A). In line with this finding, several studies reported of increased Mcl-1 protein levels upon treatment with ABT-737 [183, 184]. Wu et al., for instance, showed that ABT-737 upregulated protein levels of Mcl-1, which was blunted again by additional irradiation, resulting in a strong synergistic induction of apoptosis in breast cancer cells [183]. Unlike this observation, irradiation did not prevent ABT-263-induced increase of Mcl-1 protein levels in HCT116 cells in the present study. However, despite markedly elevated Mcl-1 protein levels, HCT116 cells reacted very sensitive to treatment with ABT-263 alone and in combination with irradiation. Thus, release of pro-apoptotic Bcl-2 family members from sequestration by Bcl-2 and Bcl-xL upon ABT-263 treatment might be sufficient to overcome anti-apoptotic function of upregulated Mcl-1. Moreover, ABT-263 treatment might concomitantly upregulate or activate pro-apoptotic Bcl-2 family members. In head and neck squamous cell carcinoma, for instance, treatment with ABT-737 was found to result in a parallel increase of Mcl-1 and Noxa [184]. A similar upregulation of Noxa by ABT-263 might inactivate Mcl-1 in HCT116 cells and thereby sustain their observed sensitivity to inhibition of Bcl-2 and Bcl-xL. Another Bcl-2 family member that was reported to be upregulated upon ABT-737 treatment is BH3-only protein Bim [182]. Bim is able to neutralize all anti-apoptotic Bcl-2 family members and can directly activate apoptosis effectors Bax and Bak [93]. Simultaneous induction of Bim by ABT-263 might therefore add to outweighing increased Mcl-1 protein levels and promote effective apoptosis induction in HCT116 cells as observed in the present study.

Figure 4.1 shows a schematic overview of assessed and putative Bcl-2 family protein regulation in HCT116 cells and observed sensitivity to the respective treatments under normoxic and severely hypoxic conditions.
Figure 4.1: Schematic representation of assessed and putative Bcl-2 protein family member regulation in HCT116 colon cancer cells upon treatment with ABT-263, ionizing radiation (IR), and combinatorial administration in normoxia and severe hypoxia. Vertical position represents the ability of the respective treatment to overcome the apoptotic threshold.
Although HCT116 cells seemingly depend on Bcl-2 and Bcl-xL for sustained survival, silencing of Mcl-1 gene expression yet increased their sensitivity to apoptosis induction by ABT-263 alone and in combination with irradiation (Figure 3.20). This indicates that, although HCT116 cells are relatively sensitive to ABT-263-induced apoptosis despite increased Mcl-1 levels, Mcl-1 attenuates the therapeutic efficacy. The importance of treatment-induced regulation of Bcl-2 family proteins was also emphasized in a study by Tahir et al. Here, initial sensitivity of SCLC cells to ABT-737 was associated with relatively high expression levels of Bcl-2, Bcl-xL, Bim, and Noxa and lower Mcl-1 levels. However, chronic exposure to ABT-737 resulted in resistance, accompanied by decreased Bcl-2 and increased Mcl-1 protein levels [175]. Thus, regulation of Bcl-2 family members, in particular stabilization of Mcl-1 after ABT-263 treatment, provides a mechanism that might interfere with a successful therapy based on BH3-mimetics.

4.3.1.2 Mcl-1 Determines Resistance to ABT-263 and Ionizing Radiation in NCI-H460 Cells

In contrast to HCT116 cells, ABT-263 treatment did not affect Mcl-1 protein levels in non-selected NCI-H460 cells (Figure 3.19 B). However, levels of Mcl-1 markedly decreased after irradiation in normoxia and in hypoxia. This suggests that irradiation-induced decrease of Mcl-1 protein levels “primes” non-selected NCI-H460 cells to apoptosis induction by ABT-263. The conceptual state in which cells are “primed for death” was introduced by Certo et al. to characterize cellular addiction to certain anti-apoptotic Bcl-2 protein family members in an approach to model selective cancer cell sensitivity towards BH3-mimetics [106]. Corroborating the assumption in non-selected NCI-H460 cells, knocking down Mcl-1 rendered these cells sensitive to ABT-263-induced apoptosis (Figure 3.21). The fact that knockdown of Mcl-1 or inhibition of Bcl-2 and Bcl-xL alone had only marginal effects on survival leads to the conclusion that the efficiency of combined treatment in non-selected NCI-H460 cells underlies the synergistic effect of radiation-induced reduction of Mcl-1 protein levels and inhibition of Bcl-2 and Bcl-xL by ABT-263. Although similar Mcl-1 depletion following irradiation was observed in several other studies, for example in human leukemic cells [232] and mouse peritoneal resident macrophages [233], the underlying mechanisms are not clear at present.
While Mcl-1 protein levels likewise decreased after irradiation in hypoxia-selected NCI-H460 cells in normoxia, Mcl-1 levels did not change after irradiation in severe hypoxia (Figure 3.19 C). The failure to downregulate Mcl-1 after irradiation in hypoxia might be a result of adaptation to hypoxia and underlie the increased resistance of hypoxia-selected cells to radiation-induced apoptosis. Moreover, the abrogation of radiation-induced Mcl-1 depletion in hypoxia might attenuate the combinatorial effect of ionizing radiation and ABT-263. As Mcl-1 degradation is mainly regulated by the ubiquitin proteasome system [146], specific alterations in this program might lead to the stabilization of Mcl-1 in hypoxia-adapted cancer cells. Decreased ubiquitin ligase activity could result in reduced ubiquitylation, thus increase Mcl-1 stability following irradiation in hypoxia. Alternatively, increased deubiquitylation of Mcl-1 could prevent the protein’s degradation and increase its half-life time. Deubiquitinase USP9x, an enzyme that interferes with Mcl-1 degradation, for instance, was demonstrated to stabilize Mcl-1 in lymphoma and glioblastoma cells, thereby increasing resistance to radiation-induced cell death [143, 147]. It is therefore tempting to speculate that a similar mechanism might be responsible for stabilization of Mcl-1 in hypoxia-selected NCI-H460 cells in severe hypoxia. Nevertheless, these cells showed high sensitivity to apoptosis induction by combined treatment with ABT-263 and ionizing radiation in hypoxia. This suggests that other processes as upregulation or activation of pro-apoptotic BH3-only proteins, as described above for HCT116 cells, might outweigh increased Mcl-1 protein levels and potently induce apoptosis. Nevertheless, similar as in non-selected NCI-H460 cells, knocking down Mcl-1 sensitized also hypoxia-selected NCI-H460 cells to ABT-263-induced apoptosis (Figure 3.22).

In both NCI-H460 cell lines, downregulation of Mcl-1 resulted in significantly stronger sensitivity to ABT-263-induced apoptosis in hypoxia than in normoxia. Moreover, knocking down Mcl-1 significantly sensitized both NCI-H460 cell lines to radiation-induced apoptosis - non-selected cells in hypoxia and hypoxia-selected cells under both normoxic and hypoxic conditions. This strongly suggests that particularly the upregulation of Mcl-1 seems to control the elevated apoptotic threshold in cancer cells exposed to acute or cycling severe hypoxia, thereby promoting radioresistance. Hypoxia-selected NCI-H460 cells might additionally depend on the anti-apoptotic function of Bcl-xL, which is generally upregulated in these cells. The increased dependence of hypoxia-selected NCI-H460 cells on the deregulation of the Bcl-2 rheostat might furthermore determine their enhanced sensitivity to treatment with ABT-263 alone and combined with ionizing radiation in hypoxia.
The assessed and putative regulation of Bcl-2 protein family members in non-selected and hypoxia-selected NCI-H460 cancer cells and the ability of the respective treatments to overcome the apoptotic threshold in these cell lines are summarized in Figure 4.2 and Figure 4.3.

Figure 4.2: Schematic representation of assessed and putative Bcl-2 protein family member regulation in non-selected NCI-H460 lung cancer cells upon treatment with ABT-263, ionizing radiation (IR), and combinatorial administration in normoxia and severe hypoxia. Vertical position represents the ability of the respective treatment to overcome the apoptotic threshold.
Figure 4.3: Schematic representation of assessed and putative Bcl-2 protein family member regulation in hypoxia-selected NCI-H460 lung cancer cells upon treatment with ABT-263, ionizing radiation (IR), and combinatorial administration in normoxia and severe hypoxia. Vertical position represents the ability of the respective treatment to overcome the apoptotic threshold.
Similarly as in HCT116 cells, the highest levels of apoptosis induction were achieved by combining knockdown of Mcl-1, inhibition of Bcl-xL and Bcl-2 by ABT-263, and ionizing radiation also in both NCI-H460 cell lines. As Mcl-1 has an apparently distinguished role in regulation of apoptosis in severe hypoxia, combinatory treatment with agents that target Mcl-1 represents a favorable strategy to treat hypoxia-prone solid tumors. In the past, attempts to design specific and tolerable inhibitors of Mcl-1 failed. Approaches to target this Bcl-2 protein family member mainly focused on inhibitors of cyclin-dependent kinases, which downregulate transcription of several short-lived proteins including Mcl-1 and thus potentially inherit undesired toxicity and side effects [234-236]. However recently, Kotschy et al. described S63845, a novel small molecule that specifically binds to the BH3-binding groove of Mcl-1 [237]. It is proposed to meet important criteria for an advantageous Mcl-1 inhibitor, as the compound binds Mcl-1 with high affinity, induces Bax-/Bak-dependent apoptosis in Mcl-1-dependent cancer cells, and shows sufficient activity and tolerability in vivo. The benefit of this novel Mcl-1 inhibitor to potentiate cell death induction by ABT-263 and ionizing radiation remains to be examined. Moreover, this inhibitor might provide further insight to the mechanistic involvement of Mcl-1 in radioresistance mediated by exposure to acute and adaptation to cycling severe hypoxia.

Importantly, enhancement of radiation-induced apoptosis by ABT-263 correlated with reduced short- and long-term survival of HCT116 cells and both NCI-H460 cell lines. Of greater importance regarding the therapeutic benefit, clonogenic survival of HCT116 was reduced more efficiently by combined treatment with ABT-263 and ionizing radiation than by irradiation alone and to an even greater extent under hypoxic conditions (Figure 3.10). Combinatory treatment with ABT-263 and ionizing radiation also reduced clonogenic survival of both NCI-H460 cell lines better than either treatment alone and with similar effectivity in normoxia as in hypoxia, despite pronounced resistance to the cytotoxic action of ionizing radiation under hypoxic conditions (Figure 3.14 and Figure 3.18). As a consequence of the apparently critical importance of the deregulated Bcl-2 rheostat for increased survival of hypoxic and hypoxia-selected cancer cells, the combination of targeting the Bcl-2 rheostat by ABT-263 and ionizing radiation is a particularly promising approach for overcoming resistance to radiation-induced cell death mediated by exposure to acute or adaptation to cycling hypoxia.
4.3.2 ABT-263 TREATMENT ENHANCES THE RESPONSE OF XENOGRAFT TUMORS OF NON-SELECTED AND HYPOXIA-SELECTED NCI-H460 CELLS TO RADIOTHERAPY

The promising results obtained in vitro provided a rationale to investigate the therapeutic potential of ABT-263 in combination with ionizing radiation in vivo. Tumor xenograft experiments performed to this end confirmed the beneficial effect of combined treatment also in vivo.

Tumor growth was significantly delayed in animals exposed to combined treatment as compared to the groups receiving radiotherapy or the BH3-mimetic only (Figure 3.23). Regrowth of xenograft tumors derived from hypoxia-selected NCI-H460 cells after radiotherapy was faster than of the tumors derived from non-selected NCI-H460 cells (Figure 3.24). These observations are in accordance with the results of the in vitro studies, corroborating the suitability of hypoxia-selected NCI-H460 cells as a model for radioresistance due to adaptation to cycling hypoxia. Despite growth dissimilarities after irradiation, tumors of both NCI-H460 sub-cell lines showed similar volumes and growth rates after combined treatment with ABT-263 and ionizing radiation. This demonstrates that inhibition of Bcl-2 and Bcl-xL is particularly able to overcome radioresistance acquired in response to cycling hypoxia. Furthermore, only tumors derived from hypoxia-selected NCI-H460 cells additionally showed slight but significant sensitivity to treatment with the BH3-mimetic alone, matching the in vitro findings. This further emphasizes the distinguished importance of the altered Bcl-2 rheostat found in the hypoxia-selected lung cancer cell line, which, suggested by the findings of the study at hand, contributes to increased radioresistance in vitro as well as in vivo.

Results from clinical trials evaluating the therapeutic benefit of Bcl-2/Bcl-xL-targeting BH3-mimetics so far indicate that these inhibitors may be particularly useful in combination with conventional chemotherapeutic or molecularly targeted agents [178-181]. Combinatory treatment with ionizing radiation has not yet been considered for clinical testing. However, similar to the findings of the present study, several other preclinical investigations also demonstrated synergistic effects of BH3-mimetics that improved cancer cell response to radiation in vitro and in vivo [182-184]. Interestingly, combinatory treatment with ABT-737 and its derivatives and cytotoxic drugs proved to be particularly effective in hypoxia in several cancer cell lines including neuroblastoma, SCLC, and colon carcinoma cells [229, 230]. However, although hypoxia is recognized as a major obstacle to successful cancer management [32], the therapeutic potential of combining radiotherapy and BH3-mimetics in
hypoxia is hardly examined to date. The results shown in the present study demonstrate that targeting the Bcl-2 rheostat by ABT-263 not only improves the cytotoxic effect of radiotherapy on cancer cells in normoxic conditions, but particularly overcomes radioresistance of cancer cells exposed to acute or cycling hypoxia \textit{in vitro} and \textit{in vivo}. This strongly suggests that the combinatory treatment with BH3-mimetics and radiotherapy is a promising approach that should be evaluated thoroughly, as this therapy concept could be of great benefit for patients with hypoxic tumors refractory to radiotherapy.

4.4 Mitochondrial features and function is altered in hypoxia-adapted NCI-H460 lung cancer cells

Mitochondria are primary sites for Bcl-2 regulation of intrinsic apoptosis, but also play a central role in metabolic functions that are considered to participate in the regulation of cell survival and death [120]. For example, they host the enzymes of the tricarboxylic acid (TCA) cycle in the mitochondrial matrix and components of the electron transport chain (ETC), which are found in the inner mitochondrial membrane and produce ATP through oxidative phosphorylation (OXPHOS) [118]. Both functions, mitochondrial apoptosis and energy metabolism, are linked to the organelle’s morphology [120]. In particular, complementary fission and fusion events are regulated in response to metabolic demands and furthermore are integral components of apoptosis [126]. Various studies have demonstrated that control of mitochondrial dynamics and bioenergetics involves Bcl-2 family proteins [117, 238]. Some of these interact with resident mitochondrial proteins, exerting functions distinct from apoptosis. Thus, it was speculated that the deregulated Bcl-2 rheostat in hypoxia-selected NCI-H460 cells described in the previous sections might be accompanied by alterations of other mitochondrial functions. These could be critical mechanisms of adaptation to hypoxia that promote cell survival and therefore contribute to therapy resistance. Indeed, transcriptional and metabolic analyses revealed that hypoxia-selected NCI-H460 cells were characterized by an elevated capability to enhance mitochondrial respiration, which was associated with a distinguished role of fusion protein Opa1.
In more detail, compared to non-selected NCI-H460 cells, hypoxia-selected cells showed upregulation of genes encoding for malate dehydrogenase and subunits of isocitrate dehydrogenase, which are enzymes of the TCA cycle (Figure 3.25 A). Correspondingly, hypoxia-selected NCI-H460 cells had an increased ability to enhance the productivity of mitochondrial respiration under energetically demanding conditions (Figure 3.26 and Figure 3.27 B). Moreover, voltage dependent anion channel (VDAC1-3) was upregulated after exposure to acute hypoxia in both hypoxia-selected and non-selected cells (Figure 3.25). In addition, VDAC1 expression was slightly higher in hypoxia-selected than in non-selected cells in normoxia. VDAC is known to affect cancer cell metabolism by tightly interacting with usually cytosolic hexokinase, which catalyzes the first step of glycolysis [239]. This interaction is thought to increase productivity of both OXPHOS and glycolysis, by enhancing ATP release from mitochondria for glucose phosphorylation and by facilitating influx of ADP into mitochondria for OXPHOS. Thus, upregulation of VDAC1 might contribute to the enhanced spare respiratory capacity of hypoxia-selected NCI-H460 cells, which remains to be explored.

VDAC is furthermore involved in the regulation of mitochondrial apoptosis by channeling the release of cytochrome c [240]. Binding of hexokinase as well as Bcl-xL and potentially Bcl-2 protects cells from apoptosis. Conversely, interaction of VDAC with Bax or Bak facilitates the release of cytochrome c into the cytosol, resulting in induction of apoptosis. Pronounced sensitivity to Bcl-2/Bcl-xL inhibitor ABT-263 when applied in combination with irradiation under hypoxic conditions might therefore be a result of enhanced capacity to form cytochrome c-releasing pores due to increased abundance of VDAC.

Moreover, specific occurrence of a truncated form of VDAC1 (VDAC1-ΔC) has been described in mitochondria of cancer cells exposed to hypoxia [241]. Possession of VDAC1-ΔC was linked to protection from apoptosis and increased metabolic capacity. These mitochondria were furthermore characterized by an abnormal, enlarged morphology, which was attributed to enhanced fusion. Intriguingly, the appearance of enlarged mitochondria after exposure to acute hypoxia was also observed specifically in hypoxia-selected NCI-H460 cells in a previous study (Dr. Johann Matschke, unpublished data). With respect to this mitochondrial phenotype, prominent regulation of fusion protein Opa1 was found in hypoxia-adapted NCI-H460 cells in the present study. In particular, Opa1 was stronger expressed in hypoxia-selected than in non-selected cells under normoxic and especially hypoxic conditions (Figure 3.28). Opa1 is responsible for fusion of the inner mitochondrial membrane and
maintenance of cristae structure \cite{242}. Thus, abnormal fusion due to a distinguished regulation of Opa1 might contribute to a similar mitochondrial phenotype in the hypoxia-selected NCI-H460 cells as described for VDAC1-ΔC-possessing mitochondria. Accordingly, siRNA mediated knockdown of Opa1 specifically reduced the spare respiratory capacity in hypoxia-selected NCI-H460 cells, decreasing stress-induced mitochondrial respiration below the levels of non-selected NCI-H460 cells (Figure 3.30). The data suggest that the enhanced spare respiratory capacity of hypoxia-selected NCI-H460 cells could be ascribed to elevated Opa1 expression. Intriguingly, Opa1-dependent cristae stabilization was reported to determine assembly and efficiency of respiratory chain supercomplexes \cite{122}, directly linking Opa1 to increased mitochondrial energy production, similar to the observation in the hypoxia-selected NCI-H460 sub-cell line.

Together these findings suggest that the capability to increase mitochondrial respiration in response to energetic stress might be a mechanism of adaptation to cycling severe hypoxia. Especially energetically demanding conditions such as oxidative stress following hypoxia and reoxygenation or irradiation might render cancer cells particularly dependent on the provision of sufficient energy, building blocks, or both to repair damaged DNA, proteins, and lipids. Thus, metabolic adaptation might offer a survival advantage to hypoxia-selected cancer cells.

Silencing expression of Opa1 per se had no influence on apoptosis induction or mitochondrial integrity of non-selected or hypoxia-selected NCI-H460 cells (Figure 3.29). However, stabilization of Opa1 has been associated with impaired completion of apoptosis by others \cite{243, 244}. In particular, Opa1 is thought to tighten cristae junctions, which counteracts mitochondrial structural remodeling, permeabilization, and cytochrome c release in response to apoptotic insults \cite{245}. Thus, in addition to contributing to enhanced respiratory capacity of hypoxia-selected NCI-H460 cells, Opa1 might counteract the structural remodeling of mitochondria that is necessary for apoptosis induction. However, the mechanisms leading to Opa1 upregulation in normoxia and stabilization in hypoxia and the associated metabolic benefit in the hypoxia-selected sub-cell line require further experimental evaluation. Furthermore, it remains to be examined whether Opa1 function influences mitochondrial morphology and correlates with increased levels of VDAC or VDAC-ΔC, and thereby also protects from apoptosis.

Regulation of mitochondrial metabolism and morphology has also been proposed to involve several Bcl-2 family members \cite{117}. While the contribution of Bcl-2 family proteins in fusion
and fission is controversial, particularly Bcl-xL and Mcl-1 are implicated in the regulation of mitochondrial respiration. Both were proposed to enhance ATP production under energetically demanding conditions by increasing efficiency of OXPHOS components [123, 124]. Intriguingly, both proteins showed a prominent regulation in hypoxia-selected NCI-H460 cells. Bcl-xL was generally upregulated in hypoxia-selected compared to non-selected cells, whereas Mcl-1 showed markedly increased levels after incubation in severe hypoxia. This implicates that in addition to upregulated Opa1, upregulated anti-apoptotic Bcl-2 family members in hypoxia-selected NCI-H460 cells might contribute to their characteristically enhanced spare respiratory capacity. Consequently, inhibition of Bcl-xL by ABT-263 might also interfere with the enhanced ability to increase mitochondrial respiration, thus contributing to the pronounced sensitivity of hypoxia-adapted cells to ABT-263 and combined treatment with irradiation in hypoxia. This remains a further point to be examined.

Collectively, these findings demonstrate that an elevated capability to enhance mitochondrial respiration might be a critical factor of tolerance to adverse hypoxia/reoxygenation cycling. This characteristic of hypoxia-selected NCI-H460 cancer cells seems to be associated with increased levels of mitochondrial fusion protein Opa1 and putatively involves other mitochondrial proteins, such as VDAC1. Importantly, features of mitochondrial metabolism and dynamics are linked to regulation of intrinsic apoptosis by Bcl-2 family proteins and thus might influence therapy resistance. The identification of factors involved in re-programmed mitochondrial function of hypoxia-selected cancer cells might therefore open novel possibilities to specifically target their ability to evade apoptosis and should be further explored in future investigations.
5 SUMMARY

Hypoxia, a characteristic of most human solid tumors, is a major obstacle to successful radiotherapy. Aim of this PhD study was to define the role of the Bcl-2 protein family in radioresistance conferred by exposure to acute and adaptation to cycling hypoxia and to evaluate whether pharmacologic targeting of the Bcl-2 rheostat improves sensitivity of hypoxic and hypoxia-adapted cancer cells to the cytotoxic action of radiotherapy.

The results demonstrated that exposure to acute and adaptation to cycling hypoxia (represented by hypoxia-selected NCI-H460 cell model) alters the balance of Bcl-2 family proteins in favor of anti-apoptotic family members, thereby elevating the apoptotic threshold and attenuating the success of radiotherapy. It was further shown that blocking anti-apoptotic Bcl-2 family members by the clinically relevant BH3-mimetic ABT-263 enhanced the sensitivity of HCT116 colon cancer and NCI-H460 lung cancer cells to the cytotoxic action of ionizing radiation. Importantly, this effect was not only observed in normoxia, but to a similar or even higher extent in severe hypoxia. ABT-263 furthermore enhanced the response of xenograft tumors of non-selected and hypoxia-selected NCI-H460 cells to radiotherapy, thereby confirming the beneficial effect of combined treatment in vivo. Targeting the Bcl-2 rheostat with ABT-263 therefore is a particularly promising approach to overcome radioresistance of hypoxic and hypoxia-adapted cancer cells. Moreover, intrinsic as well as ABT-263- and irradiation-induced regulation of Bcl-2 family members was found to determine therapy sensitivity. In this context, Mcl-1 was identified as a resistance factor that interfered with apoptosis induction by ABT-263, ionizing radiation, and combinatorial administration.

Finally, hypoxia-selected NCI-H460 cells were characterized by an elevated capability to enhance mitochondrial respiration, which depended on fusion protein Opa1. This represents an additional mechanism of adaptation to adverse hypoxic conditions that might be relevant for improved survival and therapy resistance of cancer cells adapted to cycling hypoxia.

Collectively, the findings of the present PhD thesis provide novel insights into the molecular determinants of hypoxia-mediated resistance to apoptosis and radiotherapy and a rationale for future therapies of hypoxic and hypoxia-adapted tumor cell fractions.
6 REFERENCES


### 7 Appendix

#### 7.1 Abbreviation

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<td>2-DG</td>
<td>2-deoxy-glucose</td>
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<td>A</td>
<td>Ampere</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>APS</td>
<td>Ammonium persulfate</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl</td>
<td>B-cell leukemia</td>
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<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>cDNA</td>
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<td>ECAR</td>
<td>Extracellular acidification rate</td>
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<td>EDTA</td>
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<td>Endoplasmic reticulum</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FCCP</td>
<td>Fluoro-carbonyl cyanide phenylhydrazon</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scattering</td>
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<td>g</td>
<td>Gram</td>
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h
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF  Hypoxia inducible transcription factor
HRE  Hypoxia responsive elements
HRP  Horseradish peroxidase
Hx  Hypoxia
Hx-sel.  Hypoxia-selected
IgG  Immune globulin G
IMM  Inner mitochondrial membrane
IR  Ionizing radiation
IRE1  Inositol-requiring protein 1
kDa  Kilodalton
kg  Kilogram
kV  Kilovolt
mA  Milliampere
Mcl  Myeloid cell leukemia
Mfn  Mitofusin
mg  Milligram
min  Minutes
mL  Milliliter
mM  Millimolar
mm  Millimeter
mm²  Square millimeter
MMP  Mitochondrial membrane potential
MOM  Mitochondrial outer membrane
mRNA  Messenger ribonucleic acid
mTOR  Mammalian target of rapamycin
NaCl  Sodium chloride
nm  Nanomolar
Non-sel.  Non-selected
NT  Non targeting
Nx  Normoxia
O₂  Oxygen
OCR  Oxygen consumption rate
Opa1  Optic atrophy 1
OXPHOS  Oxidative phosphorylation
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PERK  PKR-like ER kinase
PFA  Paraformaldehyde
PI  Propidium iodide
pmol  Picomolar
<table>
<thead>
<tr>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
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<td>Small interfering ribonucleic acid</td>
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</tr>
<tr>
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<td>Transmembrane</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
<tr>
<td>XF</td>
<td>Extracellular flux</td>
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7.5 **CURRICULUM VITAE**

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The curriculum vitae is not included in the online version for reasons of data protection.
7.6 DECLARATIONS

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur
Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Role of Bcl-2 protein
family members and associated mitochondrial factors in hypoxia-mediated resistance of tumor
cells to apoptosis and radiotherapy“ zuzuordnen ist, in Forschung und Lehre vertrete und den
Antrag von Violetta Ritter befürworte.

Essen, den ____________________________

Verena Jendrossek

Erklärung:
Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie
zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und
mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder
inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den ____________________________

Violetta Ritter

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abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den ____________________________

Violetta Ritter
“It’s easier, at a dinner party, to say "science" than to say "the incremental acquisition of understanding through observation, humbled by an acute awareness of our tendency towards bias."”

Tim Minchin