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Targeting cancer metabolism enhances radiosensitivity by impairing the heat shock response

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Contents

Own research publications and poster presentation

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List of abbreviations

5-FU.....5-fluorouracil
ADP.....Adenosine diphosphate
AIF.....Apoptosis-inducing factor
Apaf-1.....Apoptosis protease-activating factor 1
APC.....Allophycocyanin
Ask-1.....Apoptosis signal-regulated kinase 1
ATP.....Adenosine triphosphate
ATPase.....Adenosine triphosphatase
BAG.....Bcl-2-associated athanogene
Bax.....Bcl-associated X protein
BCA.....Bicinchoninic acid
BMP.....Bis(monoacylglycero)phosphate
BSA.....Bovine serum albumin
CAR.....Chimeric antigen receptor
CCK-8.....Cell Counting Kit-8
CHIP.....C-terminal Hsp70-interacting protein
COX.....Cyclooxygenase
C-terminal.....Carboxy terminal
CTP.....Cytosine triphosphate
DAMP.....Damage-associated molecular pattern
DCFDA.....Dichlorodihydrofluorescein diacetate
DMEM.....Dulbecco's modified eagle's medium
DMSO.....Dimethyl sulfoxide
DNA.....Deoxyribonucleic acid
ECL.....Enhanced chemiluminescence
EDTA.....Ethylenediamine tetraacetic acid
ELISA.....Enzyme-linked immunosorbent assay
ER.....Endoplasmic reticulum
ERK.....Extracellular signal-regulated kinase
FACS.....Fluorescence-activated cell sorting
FBS.....Fetal bovine serum

FITC..... Fluorescein isothiocyanate
Gb3.....Globotriaosylceramide
GBM ..... Glioblastoma
Glut.....Glucose transporter
GLUT1.....Glucose transporter 1
GLUT3.....Glucose transporter 3
GrB ..... Granzyme B
GTP ..... Guanosine triphosphate
HCL .....Hydrochloric acid
HIF-1..... Hypoxia-inducible factor 1
HIP .....Hsp70 interacting protein
HRP.....Horseradish peroxidase
HSBP1..... Heat shock factor binding protein 1
Hsc70 ..... Cognitive heat shock protein 70
HSE .....Heat shock elements
HSF .....Heat shock factor
HSP .....Heat shock protein
HSP70 .....HSP70 protein family
Hsp70 (old nomenclature Hsp70i/Hsp72).....Stress-inducible heat shock protein 70
HSPBP1 .....Hsp70 binding protein 1
HSR.....Heat shock response
IFN-γ..... Interferon-γ
IgG..... Immunoglobulin G
IL .....Interleukin
IL-2 .....Interleukin-2
JDPs.....J-domain proteins
JNK.....C-Jun N-terminal kinase
LAMP2A .....Lysosomal-associated membrane protein 2A
LDH .....Lactate dehydrogenase
LDH$^{-/-}$..... *Lactate dehydrogenase A* and *B* double knockout
LDHA.....Lactate dehydrogenase A
LDHB.....Lactate dehydrogenase B
mAb .....Monoclonal antibody
MCT.....Monocarboxylate transporter

MCT1 ..... Monocarboxylate transporter 1
MCT4 ..... Monocarboxylate transporter 4
miRNA ..... Micro-RNA
mRNA ..... Messenger RNA
mTORC1 ..... Mammalian target of rapamycin complex 1
NaCl ..... Sodium chloride
NAD^+ ..... Nicotinamide adenine dinucleotide
NADH ..... Nicotinamide adenine dinucleotide hydride
NaLac ..... Sodium lactate
NBD ..... Nucleotide binding domain
NEFs ..... Nucleotide exchange factors
NK cell ..... Natural killer cell
NSAID ..... Nonsteroidal anti-inflammatory drug
NSCLC ..... Non-small cell lung cancer
N-terminal/NH_2-terminal ..... Amino terminal
OXPHOS ..... Oxidative phosphorylation
P ..... Phosphate
p38 MAPK ..... p38 mitogen-activated protein kinase
PARP1 ..... Poly(ADP-ribose) polymerase 1
PBMCs ..... Peripheral blood mononuclear cells
PBS ..... Phosphate-buffered saline
PE ..... Phycoerythrin
Phgdh ..... Phosphoglycerate dehydrogenase
Pkm1/2 ..... Pyruvate kinase muscle isoforms 1 and 2
RIPA ..... Radioimmunoprecipitation assay
RNA ..... Ribonucleic acid
ROS ..... Reactive oxygen species
RPMI ..... Roswell Park Memorial Institute
SBD ..... Substrate binding domain
SD ..... Standard deviation
SDS ..... Sodium dodecyl sulfate
SDS-PAGE ..... Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA ..... Short interfering RNA
STI1 ..... Stress-inducible protein 1

TCA .....Tricarboxylic acid cycle
TKD .....14-mer Hsp70 peptide
TLR.....Toll-like receptor
TNF.....Tumor necrosis factor
TNF-α .....Tumor necrosis factor-α
TNFR1.....Tumor necrosis factor receptor 1
T_{reg} cells .....Regulatory T cells
TRIS ..... Tris(hydroxymethyl)aminomethane
TTP.....Thymine triphosphate
UTP .....Uracil triphosphate
WT .....Wild type

List of figures

Summary

Increased lactate dehydrogenase (LDH) activity, elevated levels of the oncometabolite lactate in the tumor microenvironment and overexpression of anti-apoptotic heat shock proteins (HSPs) can mediate therapy resistance (i.e. radioresistance) of cancer cells. In this study, I aimed to investigate the relationship of LDH and the cellular stress response with respect to radiation resistance. My results showed that the lactate metabolism and heat shock response are co-regulated in two different tumor cell systems (murine B16F10 melanoma and human LS174T colorectal adenocarcinoma). A CRISPR/Cas9-induced double knockout of *LDHA/B* ($LDH^{-/-}$) and pharmacological inhibition of LDH by the pyruvate analog oxamate reduced tumor growth, reactive oxygen species (ROS) production and synthesis of several HSPs, including Hsp90, Hsp70 and Hsp27. In addition, *LDHA/B* knockout cells exhibited an altered lipid metabolism including a decreased expression of the tumor-specific glycosphingolipid globotriaosylceramide (Gb3) which enables membrane anchorage of Hsp70 to the plasma membrane of tumor cells. Therefore, the Hsp70 membrane positivity of cancer cells with a diminished LDH activity was also significantly reduced. As a functional consequence of the impaired lactate metabolism and heat shock response the radiosensitivity of cancer cells was significantly enhanced. In order to study the effects of an Hsp70-inducing Hsp90 inhibitor on cells with impaired lactate metabolism wild type (WT) and $LDH^{-/-}$ cells were treated with non-lethal concentrations of the Hsp90 inhibitor NVP-AUY922. As expected, NVP-AUY922 increased the cytosolic expression of Hsp70 and Hsp27 in the cytosol of both cell types, but did not alter the Hsp70 membrane status. Despite increased cytosolic levels of Hsp70 and Hsp27, NVP-AUY922 exhibited a radiosensitizing effect which was even more pronounced in $LDH^{-/-}$ cells with a lower membrane Hsp70 positivity. These data suggest that the membrane Hsp70 status and not the cytosolic amount of HSPs determines the radiosensitizing potential of NVP-AUY922. In conclusion, targeting lactate metabolism of tumor cells might provide a promising strategy to improve the success of radiation therapy in cancer patients by impairing the stress response.

Zusammenfassung

Therapieresistenz (wie zum Beispiel Strahlenresistenz) von Krebszellen kann durch eine erhöhte Laktatdehydrogenase (LDH)-Aktivität, erhöhte Konzentrationen des Onkometaboliten Laktat und eine Überexpression von anti-apoptotisch wirkenden Hitzeschockproteinen verursacht werden. In meiner Promotion habe ich mir die Frage gestellt, inwieweit Therapieresistenz (z.B. Strahlenresistenz) durch einen Eingriff in den Laktatmetabolismus der Krebszellen überwunden werden kann. Meine Daten zeigten, dass der Laktatstoffwechsel und die Hitzeschockantwort in zwei verschiedenen Tumorzellsystemen (murines B16F10-Melanom und humanes LS174T-Kolorektal-Adenokarzinom) co-reguliert sind. Ein CRISPR/Cas9-induzierter Doppel-Knockout von *LDHA/B* ($LDH^{-/-}$) sowie eine pharmakologische Hemmung von LDH durch das Pyruvat Analogon Oxamat reduzierte das Tumorzellwachstum, die Produktion von reaktiven Sauerstoffspezies und die Synthese verschiedener Hitzeschockproteinen, einschließlich Hsp90, Hsp70 und Hsp27. Darüber hinaus wiesen die *LDHA/B* knockout Zellen einen veränderten Lipidstoffwechsel auf, was zu einer verminderten Expression des tumorspezifischen Glykosphingolipids Globotriaosylceramid (Gb3) führte, das für die Verankerung von Hsp70 in der Plasmamembran von Tumorzellen verantwortlich ist. Als Folge einer reduzierten Gb3 Expression war auch die Hsp70-Membranpositivität von Tumorzellen nach Hemmung der LDH-Aktivität signifikant vermindert. Funktional führte eine LDH Inhibition und die damit verbundene Beeinträchtigung der Hitzeschockantwort zu einer erhöhten Strahlenempfindlichkeit der Tumorzellen. Um den Einfluss des Hsp70-induzierenden Hsp90 Inhibitors NVP-AUY922 auf den Laktatmetabolismus zu untersuchen, wurden Wildtyp (WT) und $LDH^{-/-}$ Zellen mit einer subletalen Konzentration von NVP-AUY922 behandelt. Wie erwartet führte die Behandlung zu einer erhöhten zytosolischen Expression von Hsp70 und Hsp27, die Hsp70 Membran-Expression blieb jedoch unverändert. Trotz erhöhter zytosolischer Hsp70 und Hsp27 Konzentrationen zeigte NVP-AUY922 eine radiosensitivierende Wirkung in beiden Zelltypen, die in $LDH^{-/-}$ Zellen noch signifikant stärker ausgeprägt war. Diese Daten weisen darauf hin, dass das radiosensibilisierende Potenzial von NVP-AUY922 von der Hsp70-Membranpositivität und nicht von der zytosolischen Expression von Hitzeschockproteinen bestimmt wird. Zusammenfassend lässt sich sagen, dass eine gezielte Beeinflussung des Laktatstoffwechsels von Tumorzellen über eine

Beeinträchtigung der Stressantwort (insbesondere der Hsp70 Membranexpression) die Strahlensensitivität von Tumorzellen positiv beeinflussen kann.

1 Introduction

1.1 Cancer Metabolism

Cancer is a heterogenous group of diseases that includes more than 100 different types (Hanahan & Weinberg, 2000). In 2020, nearly 20 million new cancer cases were diagnosed with almost 10 million cancer deaths worldwide. The global cancer burden is expected to continue to rise in the coming years due to an increased life expectancy (Sung et al., 2021). Considerable research efforts have been undertaken to determine the underlying mechanisms of transformation in cancer development. Six hallmarks of cancer have been proposed (Figure 1) providing a solid basis for the understanding of the biology of cancer development. These hallmarks comprise a sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (Hanahan & Weinberg, 2000). A decade after publication of the initial hallmarks of cancers, progress in technology and cancer research led Hanahan and Weinberg to add reprogramming of energy metabolism and evading immune destruction as two additional emerging hallmarks (Hanahan & Weinberg, 2011).

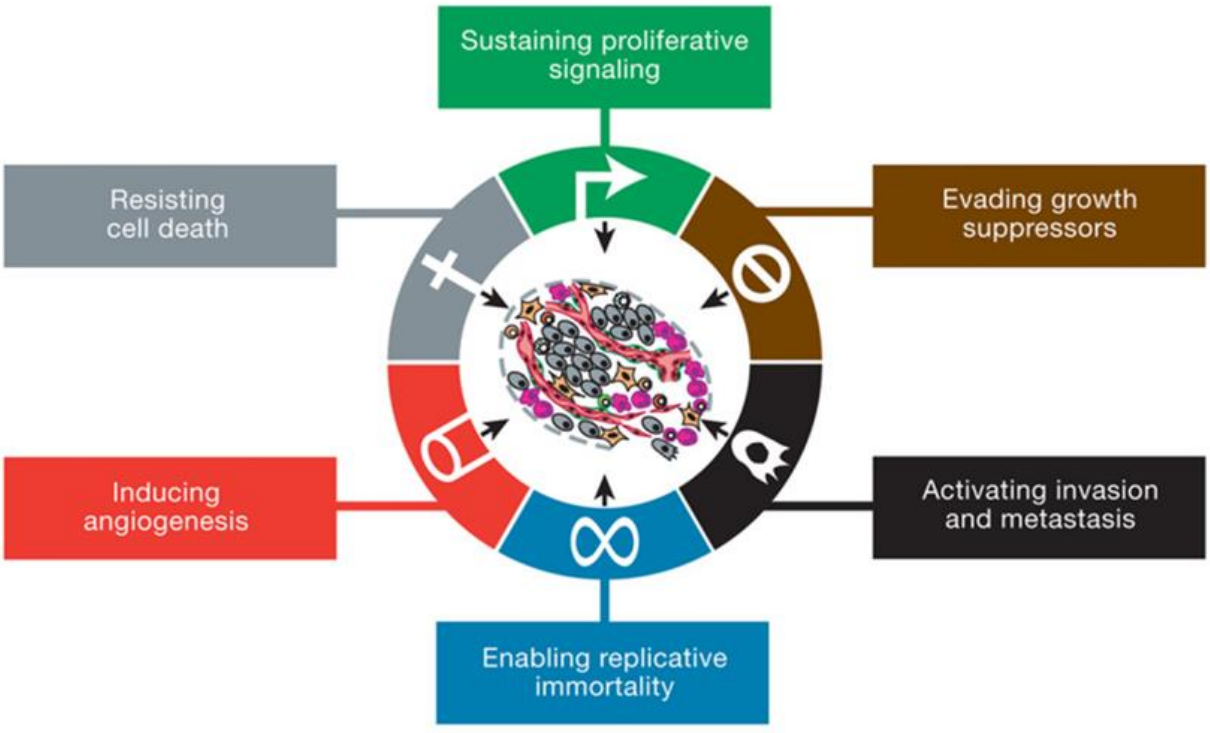

Figure 1: The six hallmarks of cancer (Hanahan & Weinberg, 2011).

Already a century ago, the German biochemist Otto Warburg and his colleagues discovered that cancer cells in contrast to normal cells use glycolysis for adenosine triphosphate (ATP) production even in the presence of oxygen (Figure 2). This elevated glycolytic activity, which is associated with an increased lactate production and release, is known as the Warburg effect or aerobic glycolysis (Pavlova & Thompson, 2016; Warburg, 1956a; Warburg et al., 1927). Warburg originally suggested that the aerobic glycolysis in cancer cells is due to a defect in the mitochondrial respiration (Warburg, 1956b). However, later investigations demonstrated that the function of mitochondrial oxidative phosphorylation (OXPHOS) is not impaired in most cancer types (Vaupel & Multhoff, 2021). Therefore, it remains unclear why cancer cells favor the much less energy efficient process of aerobic glycolysis over OXPHOS (Figure 2).

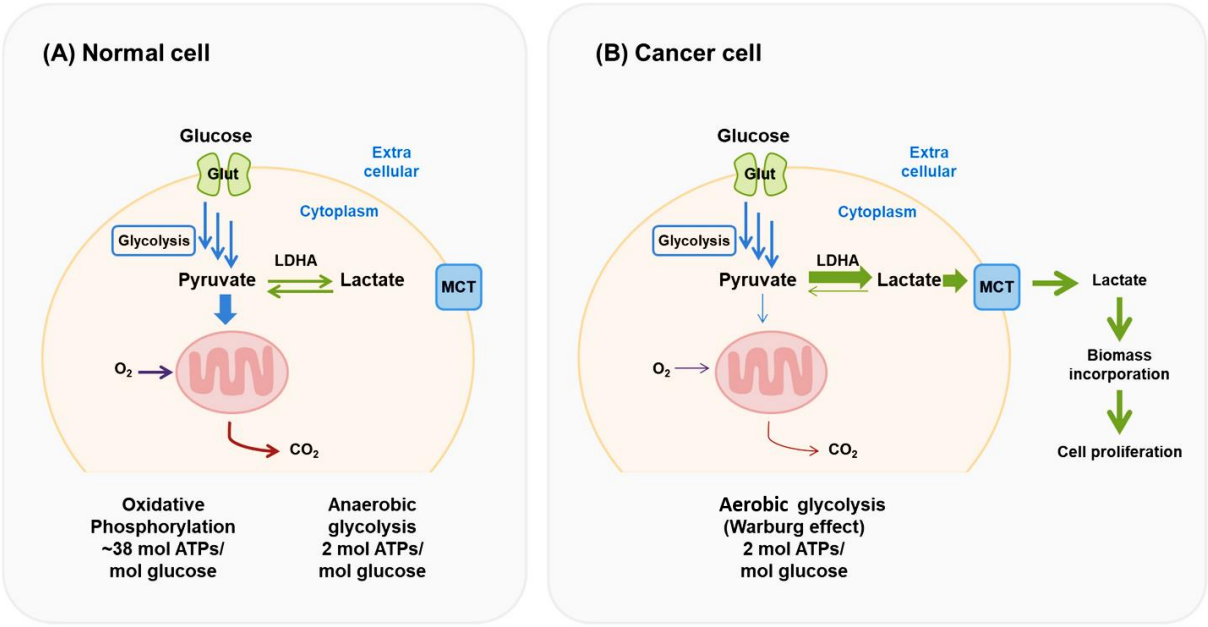

Figure 2: Energy generation in normal and cancer cells. (**A**) In the presence of O_2 (oxidative phosphorylation), normal cells produce up to 38 mol adenosine triphosphate (ATP) per mol glucose through glycolysis, tricarboxylic acid cycle (TCA), and electron transport chain while only 2 mol ATP are formed under anaerobic conditions. (**B**) Cancer cells exclusively use the glycolysis pathway independent of the level of oxygen (adapted from (Kim & Baek, 2021)). Glut, glucose transporter ; LDHA, Lactate dehydrogenase A ; MCT, monocarboxylate transporter.

Only 2 ATPs instead of up to 38 ATPs are produced from one glucose molecule during aerobic glycolysis (Kim & Baek, 2021). However, the speed of generating ATP by glycolysis is approximately 100 times faster than by OXPHOS. Due to their highly increased ATP demand, cancer cells may switch to aerobic glycolysis because ATP production can only be moderately increased by mitochondrial respiration (Vaupel & Multhoff, 2021). Another explanation might be that intermediates of the glycolytic pathway are required for the biosynthesis of macromolecules like nucleotides, amino acids and lipids which are essential for accelerated cell division (Figure 3, (Lunt & Vander Heiden, 2011)).

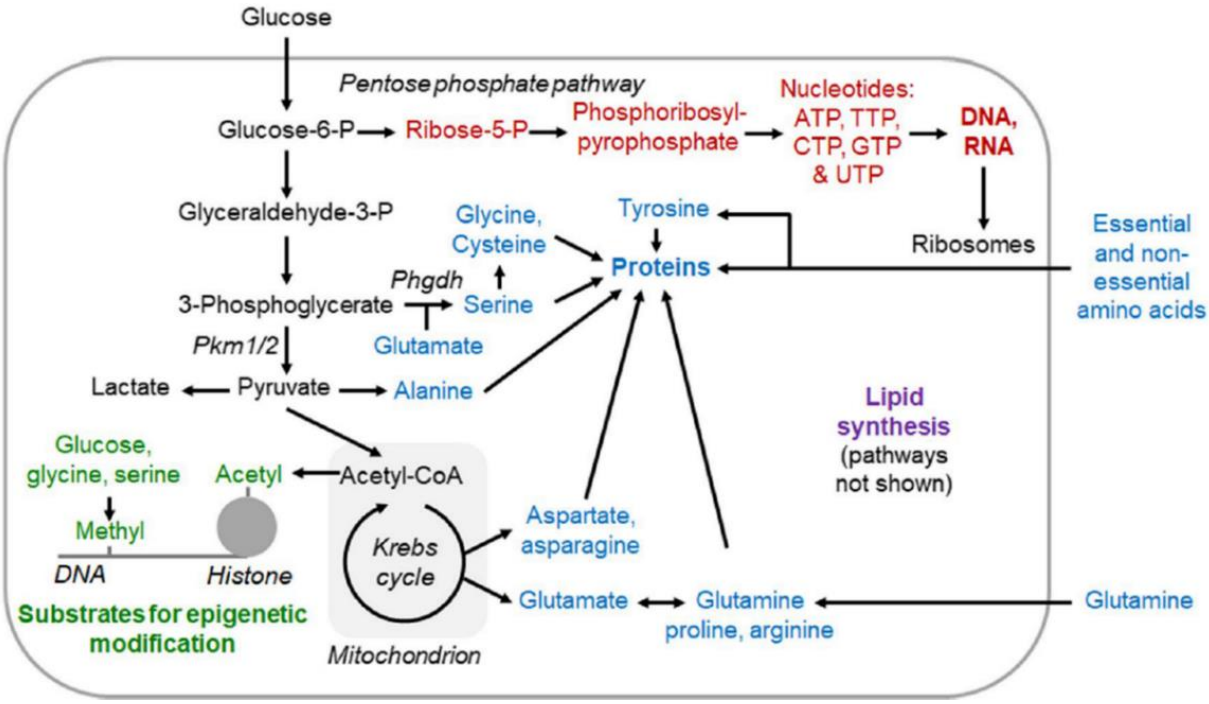

Figure 3: Relationships between energy metabolism and anabolism of macromolecules. In proliferating cells, glycolysis is typically enhanced, generating glycolytic intermediates and other energy metabolites required for the biosynthesis of macromolecules such as nucleotides (red), amino acids (blue), lipids (purple) and small groups used for epigenetic modification (green). In addition to glucose, glutamine is also increasingly taken up by proliferating cells (Wackerhage et al., 2022). ATP, adenosine triphosphate; CTP, cytosine triphosphate; DNA, deoxyribonucleic acid; GTP, guanosine triphosphate; P, phosphate; *Phgdh,* phosphoglycerate dehydrogenase; *Pkm1/2*, pyruvate kinase muscle isoforms 1 and 2; RNA, ribonucleic acid, TTP, thymine triphosphate; UTP, uracil triphosphate.

In addition, the generation of reactive oxygen species (ROS) is reduced when cells shift their metabolism from OXPHOS to aerobic glycolysis, promoting cell survival (Vaupel & Multhoff, 2021). Furthermore, secretion of high lactate levels acidifies the tumor microenvironment which in turn supports tumor growth (Hirschhaeuser et al., 2011), survival and the invasive capacity of tumor cells and suppresses the effector cell function of T and natural killer (NK) cells (Bohn et al., 2018; Brand et al., 2016; Lunt & Vander Heiden, 2011; Siska et al., 2020). Additionally, immunosuppressive cells like regulatory T (T_{reg}) cells show a high metabolic flexibility and can use lactic acid as an alternative fuel. Therefore, high lactate levels in the tumor microenvironment can support the immunosuppressive function of T_{reg} cells (Angelin et al., 2017; Watson et al., 2021).

The key metabolic enzymes of the aerobic glycolysis are the lactate dehydrogenases (LDHs), which catalyze the reversible conversion of pyruvate and nicotinamide adenine dinucleotide hydride (NADH) to lactate and nicotinamide adenine dinucleotide (NAD^+) and *vice versa*. There are five LDH isomeric forms (LDH1-5), assembled by two different types of subunits, named muscle and heart, depending on their original location and encoded by the *LDHA* and *LDHB* genes, respectively (Figure 4).

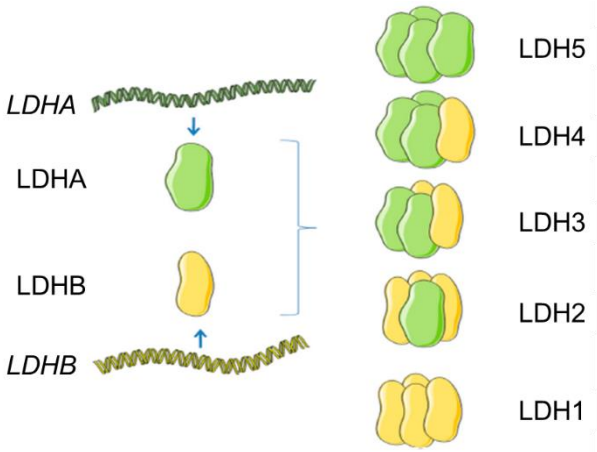

Figure 4: Lactate dehydrogenase (LDH) genes, subunits and enzyme oligomers. Two LDH genes (*LDHA* and *B*) provide the subunits for five LDH enzyme variants (LDH1-5) (adapted from (Urbanska & Orzechowski, 2019)).

LDH1-5 differ in their tissue distribution (Farhana & Lappin, 2022; Markert & Moller, 1959; Perez-Tomas & Perez-Guillen, 2020). In the cancer metabolism, LDH5 and LDH1 also termed as LDHA and LDHB, respectively, are of particular interest due to their overexpression in many tumor cells. LDHA composed of four LDHA subunits preferentially supports the pyruvate reduction to lactate during glycolysis (Figure 4, (Perez-Tomas & Perez-Guillen, 2020; Zdralevic et al., 2017)).

Hypoxia-inducible factor 1 (HIF-1) (Semenza et al., 1996), c-Myc (Shim et al., 1997) and micro-RNA (miRNA) miR-34a (Kaller et al., 2011) are known regulators of LDHA expression. Already in 1964 it could be demonstrated that many human cancers have higher LDHA levels than normal tissues (Goldman et al., 1964). High lactate levels in human cervical patients have been shown to correlate with an increased risk of developing metastases, tumor recurrence, and poorer disease-free and overall survival (Walenta et al., 2000). In addition, patients with non-small cell lung cancer (NSCLC) bearing overexpression of LDHA have a poor prognosis (Koukourakis et al., 2003). Downregulation of LDHA by small interfering RNA (siRNA) or by the LDH inhibitor FX11 inhibited tumor progression in preclinical tumor models (Le et al., 2010). Knockdown of LDHA in esophageal squamous cell carcinoma and pancreatic cancer cells reduced cell growth and cell migration *in vitro* as well as tumorigenesis *in vivo* (Rong et al., 2013; Yao et al., 2013). In addition to LDHA, LDHB also plays an important role in tumor progression. LDHB oxidizes lactate to pyruvate, coupled with NADH formation (Zdralevic et al., 2017). Depletion of LDHB can inhibit cell proliferation in lung cancer cell lines (McCleland et al., 2013), triple-negative breast cancer cells (McCleland et al., 2012) and in osteosarcoma cells (Li et al., 2016). Furthermore, high LDHB levels in patients are linked to poorer prognosis and decreased overall survival (Li et al., 2016; McCleland et al., 2013; McCleland et al., 2012; Sun et al., 2015). Elevated serum LDH is negatively associated with clinical efficacy of anticancer therapies including radiotherapy (de la Cruz-Lopez et al., 2019; Dimopoulos et al., 1991; Forkasiewicz et al., 2020; Hirschhaeuser et al., 2011). Therefore, there is great interest in developing drugs, which reduce either the level of LDH or other molecules involved in lactate metabolism to improve anti-cancer therapy and patient survival. Although promising results were obtained with the LDH inhibitors oxamate (Li et al., 2013; Manerba et al., 2017; Yang et al., 2014), FX11 (Le et al., 2010; Xian et al., 2015), and GNE-140 (Boudreau et al., 2016; Zdralevic et al., 2018) in preclinical models, the

currently available substances are not suitable for clinical use due to unfavorable off-targets effects and a poor bioavailability (Feichtinger & Lang, 2019).

An association between the glucose/lactate metabolism and the cellular stress response has been previously described for the human HeLa cervical cancer cell line. Cells overexpressing heat shock protein 70 (Hsp70) altered their energy metabolism by increasing aerobic glycolysis (Wang et al., 2012). Furthermore, immature boar Sertoli cells showed an upregulation of Hsp70, glucose transporter 3 (GLUT3), and lactate production upon mild heat stress, thereby improving spermatogenesis and male fertility in boars (Guan et al., 2018). In addition, reduction of LDHA activity by the LDH inhibitors galloflavin and oxamate resulted in decreased expression of Hsp90, Hsp70, and Hsp27 in hepatocellular carcinoma cells (Manerba et al., 2017). *Vice versa*, overexpression of HSPA12A, a distant member of the HSP70 protein family, led to an enhanced lactate export and glycolytic activity in hepatocellular carcinoma cells by promoting membrane localization of the monocarboxylate transporter 4 (MCT4) (Min et al., 2022). These are first indications for a potential link between the lactate metabolism and the stress response.

1.2 Heat shock response

The heat shock response (HSR) is a mechanism which includes an increased synthesis of molecular chaperones like heat shock proteins (HSPs) to prevent toxic effects on proteins caused by physical or biological stressors such as increased temperatures, heavy metals, toxins, oxidative stress and infections by bacteria or viruses (Morimoto, 1993). HSP genes were firstly described in the early 1960's by Ferruccio Ritossa as a set of evolutionary highly conserved genes in salivary gland cells of *Drosophila melanogaster,* which are activated upon exposure to elevated temperatures (Rittosa, 1962). HSPs are highly conserved and ubiquitously expressed in various organisms from bacteria over plants to animals (Lindquist & Craig, 1988). Based on their molecular weight, ranging from 27 to 110 kDa, and their functions HSPs are divided into five major groups (HSP27, HSP60, HSP70, HSP90 and HSP110) (Moseley, 1997). Under physiological conditions they are involved in maintaining protein homeostasis by supporting the transport, folding/unfolding and degradation of proteins (Hartl, 1996). Further, it has been shown that HSPs exhibit regulatory functions in apoptosis (Garrido et al., 2001). One of the best-studied classes of HSPs

is the HSP70 family, therefore this family will be considered in more detail in the next sections with respect to its structure, regulation, function and role in cancer and immunity.

1.2.1 Structure of Hsp70

The highly conserved Hsp70 molecules consist of a 44 kDa amino (N)-terminal nucleotide binding domain (NBD) containing the adenosine triphosphatase (ATPase) domain, which binds and hydrolyzes ATP, a hydrophobic linker region, and a 28 kDa carboxy (C)-terminal substrate binding domain (SBD) (Figure 5, (Bertelsen et al., 2009; Flaherty et al., 1990; Radons, 2016)). Eukaryotic cytosolic but not endoplasmic reticulum (ER)-located or mitochondrial Hsp70 possess a C-terminal EEVD domain mediating binding of co-chaperones and other HSPs (Hartl, 1996; Radons, 2016).

Figure 5: Schematic representation of the common domain structures of the human Hsp70 chaperone (Radons, 2016).

Hsp70 is constitutively expressed and found within nearly all major subcellular compartments (Tavaria, Gabriele et al. 1996) like the nucleus, the cytosol, the mitochondria, the ER and lysosomes (Daugaard, Rohde et al. 2007, Radons 2016). Furthermore, they are present on the plasma membrane of virally infected cells (Di Cesare, Poccia et al. 1992) and tumor cells (Multhoff, Botzler et al. 1995) as well as in the extracellular milieu (Pockley, Henderson et al. 2014).

The human HSP70 family consists of 13 gene products that differ in terms of their expression level, subcellular localization, and amino acid composition (Radons 2016). They are encoded by a multigene family consisting of 47 hsp70 sequences, 17 genes and 30 pseudogenes (Brocchieri, Conway de Macario et al. 2008). There are two main representatives of the HSP70 family: the major stress-inducible Hsp70 and the cognitive heat shock protein 70 (Hsc70). They are found in all nucleated eukaryotic cells and show a high sequence homology of 86% (Daugaard, Rohde et al. 2007). The

major stress-inducible Hsp70s, Hsp70-1 (HSPA1A) and Hsp70-2 (HSPA1B), collectively named Hsp70 or Hsp70-1, differ by only two amino acids. The basal messenger RNA (mRNA) expression of HSPA1A/B is variable in most tissues and shows the highest expression levels of all Hsp70 isoforms in humans (Daugaard, Rohde et al. 2007, Radons 2016). Hsp70 has been found to be upregulated following a series of stress stimuli, and is thought to play an important role in protecting cells from lethal damage and maintaining cell survival (Daugaard, Rohde et al. 2007, Murphy 2013). In contrast, Hsc70 is constitutively expressed in all cells at a low level and is not stress inducible. Therefore, it is responsible for the housekeeping functions of the HSP70 family and plays an important role in the chaperone-mediated autophagy (Murphy 2013).

1.2.2 Regulation of Hsp70

The HSR is regulated, in addition to altered mRNA stability, mainly at the transcriptional level by different heat shock factors (HSFs) (Shamovsky & Nudler, 2008). Six heat shock transcription factors are known in the human genome: HSF1, HSF2, HSF4, HSF5, HSFX and HSFY (Gomez-Pastor et al., 2018). HSF1 is ubiquitously expressed and considered as the major transcription factor for the stress-induced expression of major HSPs (Jolly & Morimoto, 2000). HSF1 is activated in response to a variety of physiological and environmental stressors, whereas HSF2 activity appears to be more selective and is induced upon inhibition of the ubiquitin-proteasome, during differentiation and early development. HSF4 is important for growth and differentiation during the development of the eye lens (Gomez-Pastor et al., 2018; Jolly & Morimoto, 2000). HSF5 has only been validated in humans at the transcriptional level. HSFX, located on the X chromosome, has not yet been studied in detail with respect to its function. HSFY located on the Y chromosome is mainly expressed in the testis and contributes to male fertility (Gomez-Pastor et al., 2018).
Under physiological, non-stressed conditions, HSF1 is located in the cytoplasm predominantly as an inactive monomer complexed with regulatory proteins including Hsp90, Hsp70 and Hsp40 (Gomez-Pastor et al., 2018; Murphy, 2013). After stress stimuli, inhibitory proteins are separated from the complex and HSF1 forms a homotrimer with the ability to translocate into the nucleus. Its DNA binding capacity and transcriptional activity can be modulated by posttranscriptional modifications such as stress-dependent phosphorylation mediated by kinases including the mammalian

target of rapamycin complex 1 (mTORC1) kinase (Gomez-Pastor et al., 2018; Morimoto, 1993; Murphy, 2013). In the nucleus HSF1 binds to the heat shock element (HSE) upstream of the HSP promotor and thereby facilitates HSP transcription by promoting RNA polymerase II escape from the paused pre-initiation complex and starting elongation (Figure 6, (Murphy, 2013; Radons, 2016)). When a sufficient amount of HSPs is synthesized and binding to all non-native proteins is saturated after cell stress, excess HSPs bind to the HSF trimers. This results in a dissociation and return of HSF1 to its inactive, monomeric form and thereby further synthesis of HSPs is stopped (Shamovsky & Nudler, 2008). The heat shock factor binding protein 1 (HSBP1) can bind to both, trimeric state of HSF1 and Hsp70, resulting in a negative regulation of the heat shock response (Morimoto, 1998). The Hsp70 expression levels are also regulated by miRNAs at the posttranscriptional level which is essential in transformation, differentiation and proliferation (Radons, 2016).

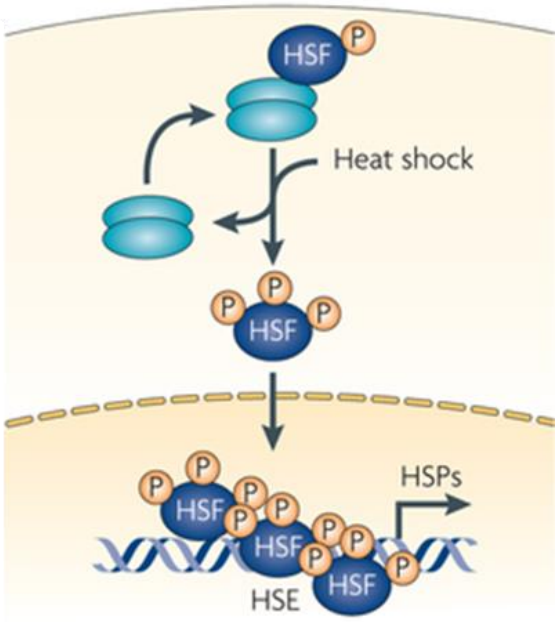

Figure 6: Heat shock factor 1 (HSF1) activation cycle. Under non-stressed conditions, HSF1 is located in its monomeric, inactive form in the cytoplasm and remains repressed by regulatory proteins including Hsp90, Hsp70 and Hsp40. After stress e.g. heat shock, HSF1 is activated, trimerize, translocate to the nucleus and its DNA binding capacity is mediated by phosphorylation (P). HSF1 binds to the heat shock element (HSE) and thereby enabling the transcription of heat shock proteins (HSPs) (Trepel et al., 2010).

1.2.3 Functions of Hsp70

Originally, molecular chaperones were described as a group of proteins mediating the correct assembly of other proteins without being part of the final functional assemblies. More recently, chaperones have been defined as proteins that bind and stabilize other proteins. Through selective binding and release, they enable the proteins to fold correctly, be transported to a specific subcellular compartment inside the cell, or be removed by degradation (Hartl, 1996).

The best known functions of Hsp70 is to support the folding of nascent polypeptides exiting the ribosome or stress-denatured proteins (Ambrose & Chapman, 2021), prevention of protein aggregation, solubilization and refolding of aggregated proteins (Mayer & Bukau, 2005), disassembly of protein complexes and the regulation of protein activity (Rosenzweig et al., 2019). Chaperones recognize hydrophobic residues and/or unstructured backbones of stable misfolded or aggregated proteins and ensure their unfolding and correct refolding. The folding process is mediated by chaperones through repeated cycles of substrate binding and release, which are controlled by their ATPase activity and by cofactors (Hartl & Hayer-Hartl, 2002; Radons, 2016). Unfolded or non-native polypeptides are recognized by Hsp40, a member of the J-domain proteins (JDPs), and transferred to Hsp70 to prevent aggregation. The hydrolysis of ATP to adenosine diphosphate (ADP) and the associated conformational change that closes the substrate binding groove is stimulated by the interaction of Hsp40 with the N-terminal ATPase domain of Hsp70. In the ADP-bound state, Hsp70 exhibit high substrate affinity and low substrate exchange rate. The Hsp70 interacting protein (HIP) stabilizes this conformation by binding to the NBD of Hsp70 and thereby prevents ADP dissociation. The stress-inducible protein 1 (STI1 or Hop) binds to the C-terminal domain of Hsp70 and Hsp90 thus passing client proteins to Hsp90. STI1 is responsible for the association of other chaperones. Interaction of nucleotide exchange factors (NEFs) like Bcl-2-associated athanogene (BAG) domain proteins and Hsp70 binding protein 1 (HSPBP1) with the Hsp70 ATPase domain results in the release of ADP and a conformational change leading to a low substrate affinity and finally to the release of the substrate. The released polypeptide can either be folded into its native state or bound back to Hsp70 (Fernandez-Fernandez et al., 2017; Radons, 2016). The activity of Hsp70 is connected to proteolysis via the C-terminal Hsp70-interacting protein (CHIP). CHIP inhibits the ATPase activity of cytosolic Hsp70 by inhibiting HSP70-mediated protein folding, and through its function as E3-ubiquitin ligase, it can

ubiquitinate proteins, leading to their degradation by the proteasome (Mayer & Bukau, 2005; Radons, 2016).

The transport of proteins to their target organelles is another important function of Hsp70 in eukaryotes. For the mitochondrial and the ER transport, proteins are tagged with N-terminal leader peptide sequences and the proteins must be kept in an unfolded state until they reach their appropriate compartment. Once the unfolded polypeptides have started to cross the membrane of their target organelle, Hsp70 binds to the entering polypeptides to support translocation of the peptides into the organelle by an entropic force (Ambrose & Chapman, 2021).

In addition to its role in maintaining protein homeostasis, Hsp70 is also involved in the cellular processes of senescence and autophagy (Radons, 2016). Cellular senescence is a stable arrest of the cell cycle that is initiated in response to various stress factors like oxidative stress, mitochondrial dysfunction, irradiation or chemotherapeutic drugs (Herranz & Gil, 2018). Downregulation of Hsp70 by siRNA in cancer cells caused senescence, activated and stabilized tumor protein p53 by destabilizing the p53 inhibitor Hdm2. Furthermore, the cell cycle kinase Cdc2 is phosphorylated and thereby inhibited (Yaglom et al., 2007). Autophagy is an intracellular pathway for degradation of proteins and is important for the cellular energy supply during development and nutrient stress. Furthermore, it is involved in the removal of misfolded or aggregated proteins, damaged organelles or intracellular pathogens in autophagic structures (Glick et al., 2010). One of the three main types of autophagy is chaperone-dependent. Cytosolic proteins with a specific sequence motif are recognized by chaperones and following binding to lysosomal-associated membrane protein 2A (LAMP2A) the proteins are unfolded and degraded in the lysosomes (Dokladny et al., 2015).

Moreover, Hsp70 is an important anti-apoptotic protein, which interferes with apoptotic pathways at different levels. These pathways can be activated either intrinsically with a mitochondrial involvement and the formation of the apoptosome, or extrinsically with involvement of tumor necrosis factor (TNF)-family receptors. Hsp70 can inhibit the formation of the apoptosome by interacting with apoptosis protease-activating factor 1 (Apaf-1) (Takayama et al., 2003). In addition, Hsp70 prevents mitochondrial membrane permeabilization and the secretion of pro-apoptotic factors by inhibiting the mitochondrial translocation and activation of Bcl-associated X protein (Bax). Other components of apoptotic pathways that are affected by Hsp70 are caspase-3, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK),

extracellular signal-regulated kinase (ERK), and the mitochondrial intermembrane flavoprotein apoptosis-inducing factor (AIF). Hsp70 together with the E3 ubiquitin ligase CHIP support the proteasomal degradation of the apoptosis signal-regulated kinase 1 (Ask-1) and a complex consisting of these three proteins prevents apoptosis. In the nucleus, Hsp70 can bind to poly(ADP-ribose) polymerase 1 (PARP1) and thereby promote the single-strand DNA break repair machinery (Radons, 2016).

1.2.4 The role of Hsp70 in cancer and immunology

In many different tumor entities, including melanoma and colorectal carcinoma, HSPs are overexpressed which results in poorer prognosis and therapy resistance (Ciocca & Calderwood, 2005). Hsp70 is involved in several hallmarks of cancers (Albakova et al., 2020), which were described in Figure 1.

High levels of Hsp70 impairs apoptosis (Mosser et al., 1997) and leads to cancer progression (Ciocca & Calderwood, 2005). *Vice versa,* cancer cell death is induced by a downregulation of Hsp70 in human breast cancer cell lines (Nylandsted et al., 2000). Cell death can also be escaped by Hsp70-mediated stabilization of lysosomal membranes to prevent permeabilization (Nylandsted et al., 2004). Permeabilization of lysosomal membranes by the Hsp70 inhibitor apoptozole causes apoptosis in different human cancer cell lines (Park et al., 2018). Furthermore, Hsp70 protects cancer cells from necrosis by JNK inhibition (Yaglom et al., 2003). Depending on its localization (intra/extracellular) Hsp70 can fulfill different functions. On the one hand, necrotic cells can release Hsp70 as a free molecule, which can act as a damage-associated molecular pattern (DAMP) triggering antitumoral immune responses. On the other hand, cytosolic Hsp70 prevents necrotic cell death in cancer cells and thereby supports tumor progression (Albakova et al., 2020). By regulating p53 and cell cycle kinase Cdc2, Hsp70 can downregulate also cancer cell senescence (Yaglom et al., 2007). Besides its roles in preventing cancer cell death, Hsp70 is also involved in angiogenesis (Albakova et al., 2020; Park et al., 2017), invasiveness (Teng et al., 2012), and metastasis of cancer cells (Kluger et al., 2005).

In contrast to normal cells, a large number of cancer cells not only overexpress Hsp70 in the cytosol (Ciocca & Calderwood, 2005), but also present it on their plasma membrane (Multhoff et al., 1995) and actively release it in exosomes (Gastpar et al., 2005). This tumor specific membrane localization of Hsp70 is enabled by a spontaneous interaction of Hsp70 with negatively charged glycosphingolipids including

sulfogalactosyl ceramide (Mamelak et al., 2001) and globotriaosylceramide (Gb3) (Gehrmann et al., 2008) which are compounds of cholesterol-rich microdomains also termed as lipid rafts (Horvath et al., 2008). It is assumed that Hsp70 trafficking to the cell membrane and its exosomal export is mediated via a non-classical vesicular pathway, since inhibitors of the ER/Golgi pathway such as brefeldin A or monensin do not affect the membrane transport and release of Hsp70 (Bausero et al., 2005). Under stress conditions like irradiation (Fellinger et al., 2020; Gehrmann et al., 2005; Murakami et al., 2015) or treatment with tubulin-interacting chemotherapeutics like vincristine and paclitaxel (Gehrmann et al., 2002) the density of Hsp70 on the plasma membrane of cancer cells but not on normal cells can be upregulated.

As mentioned before, Hsp70 is not only located in the cytosol but can also be found in the extracellular milieu (Pockley et al., 2014) or on the cell membrane of tumor cells (Multhoff et al., 1995) where it mediates a number of immunomodulatory effects on both, the innate and adaptive immune system (Albakova et al., 2020). Two types of Hsp70 are found in the circulation of cancer patients. On the one hand, viable cancer cells release Hsp70 in extracellular vesicles with biophysical properties of exosomes, and on the other hand, dying cells can release Hsp70 as a free molecule, which can serve as a DAMP (Albakova et al., 2020).

Hsp70 released from necrotic cells can trigger a strong T cell response (Albakova et al., 2020; Breloer et al., 1999), whereas immune tolerance is induced when immune cells are exposed to high amounts of free Hsp70 for a long period of time (i.e. after radiation) and thereby promotes tumor progression (Rothammer et al., 2019). In addition, low doses of an Hsp70 peptide have been shown to induce antitumor immunity (Blachere et al., 1997). Therefore, it can be assumed that the endogenously released Hsp70 from cancer cells initially has a tumor suppressive effect, but in larger amounts may contribute to tumor progression (Albakova et al., 2020).

Not only extracellular Hsp70 modulates immunomodulatory effects, plasma membrane bound Hsp70 can serve as a target structure for pre-stimulated NK cells. An incubation of NK cells with low dose interleukin-2 (IL-2) and Hsp70 protein or a 14-mer Hsp70 peptide (TKD) derived from the oligomerization domain of Hsp70 for 3-5 days stimulates their proliferative as well as their cytolytic activity towards tumor cells which express Hsp70 on their plasma membrane (Multhoff et al., 2001). Only $CD3^-/CD56^+$ NK cells, but not $CD3^+$ T cells, are activated by a stimulation with TKD/IL-2. Stimulation of NK cells is associated with increased cell surface density of the cell surface

receptors CD94, CD56 and CD69 (Gastpar et al., 2005; Gross, Schmidt-Wolf, et al., 2003). Tumor cell killing occurs via a perforin-independent granzyme B (GrB)-mediated apoptosis pathway (Gross, Koelch, et al., 2003). A phase I clinical trial in patients with colorectal or lung cancer (Krause et al., 2004) as well as a phase II clinical trial with NSCLC patients (Multhoff et al., 2020) showed safety and therapeutic potential of *ex vivo* Hsp70-activated, autologous NK cells in human patients. Another mechanism how extracellular Hsp70 interact with the innate immune system is the formation of a complex with Tag7. Permeabilization of lysosomal membranes and consecutively cell death is mediated by the interaction of the Tag7-Hsp70 complex with tumor necrosis factor receptor 1 (TNFR1) (Albakova et al., 2020; Yashin et al., 2016). In addition, Hsp70 can stimulate the expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNF-α) after binding to the toll-like receptors (TLR)-2 and -4 via the MyD88/NF-κB transduction pathway (Asea et al., 2000; Asea et al., 2002).

1.2.5 Heat shock proteins and cancer treatment

Cancer cells often overexpress HSPs in the cytosol which is associated with treatment resistance and poorer prognosis (Ciocca & Calderwood, 2005). Therefore, intensive research is being conducted on therapies that target the HSR in cancer cells either by HSPs-targeting immunotherapies or by modulating the expression and/or activity of HSPs. Besides the already mentioned autologous NK cells therapy which showed positive clinical response in a phase II clinical trial with NSCLC patient after radiochemotherapy (Multhoff et al., 2020), different vaccines have been developed on the basis of HSPs (Yang et al., 2021). Various autologous cancer vaccines consisting of HSP peptide complex-96 have been tested in clinical trials for glioblastoma (Bloch et al., 2014), melanoma (Testori et al., 2008) and renal cell carcinoma patients (Wood et al., 2008).

In recent years, several inhibitors have been developed for various HSPs including Hsp90, Hsp70 and Hsp27. However, despite promising preclinical data, none of the inhibitors has yet been approved in clinical practice (Yang et al., 2021). One promising Hsp90 inhibitor is NVP-AUY922, which is a resorcinylic isoazole amide. It showed negative effects on tumor cell proliferation and viability *in vitro* and *in vivo* (Brough et al., 2008; Eccles et al., 2008). Therefore, NVP-AUY922 was used in a phase I/II clinical trial for NSCLC, breast, colorectal and advanced gastric cancer (Soga et al., 2013).

However, its adverse side effects including hepatotoxicity, poor water solubility, and a compensatory upregulated expression of anti-apoptotic HSPs like Hsp70 limits its broader clinical application. Therefore, combination therapies using inhibitors targeting different HSPs or combining HSPs inhibitors at lower concentrations with conventional therapies like chemo- or radiotherapy are recently under investigation, although clinical data are still missing (Jego et al., 2013; Kijima et al., 2018; Kinzel et al., 2016; Sanchez et al., 2020; Soga et al., 2013; Yang et al., 2021). Therefore, there remains a great medical need for further research into inhibitors of the heat shock response in cancer cells to break resistance to therapy.

1.3 Aim of the study

More than 50% of all solid tumors are treated with radiation therapy, although its therapeutical success is limited by normal tissue toxicity and radioresistance of the cancer cells. Radioresistance of cancer cells can be caused by an increased LDH activity, associated with elevated levels of the oncometabolite lactate, and overexpression of anti-apoptotic HSPs. A potential mechanistic link between the pro-tumorigenic lactate metabolism and the anti-apoptotic stress response in tumor cells is not well understood.

Therefore, in this study I investigated the co-regulation of LDH and the heat shock response with respect to radiation resistance in two different cancer cell systems (murine melanoma B16F10 and human colorectal carcinoma LS174T). LDH inhibition was performed either genetically by an *LDHA/B* double knockout or pharmacologically by the treatment with the pyruvate analog oxamate. Furthermore, I analyzed the mechanism by which the radiosensitizing potential of the Hsp90 inhibitor NVP-AUY922 is mediated in cancer cells with an impaired lactate metabolism. The overall aim was to radiosensitize cancer cells by targeting different pathways of the cancer metabolism.

2 Materials and methods

Cells and cell culture

The human mouse B16F10 wild type (WT) melanoma cell line (ATCC® CRL-6475) and *LDHA/B* double knockout cell line ($LDH^{-/-}$) - kindly provided by Marina Kreutz and Jacques Pouyssegur (Zdralevic et al., 2018) - were cultured in Roswell Park Memorial Institute (RPMI)-1640 Medium (Sigma-Aldrich, St. Louis, MO, USA). The human LS174T colorectal adenocarcinoma cell line (ATCC® CL-188™) WT and $LDH^{-/-}$ - kindly provided by Marina Kreutz and Jacques Pouyssegur (Zdralevic et al., 2018) - were cultured in high glucose Dulbecco`s Eagle`s Minimum Essential Medium (DMEM) (Sigma-Aldrich). All media were supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (10,000 IU/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). Cells were routinely checked and confirmed negative for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Rockland, ME, USA).

Reagents and treatment

The pyruvate analog sodium oxamate (Santa Cruz, Dallas, TX, USA), was dissolved in the relevant cell culture medium in which the cells were grown and tumor cells were incubated with a sublethal concentration of oxamate (60 mM) for 48 h. Sodium lactate (NaLac) (Sigma-Aldrich) was dissolved in H_2O and 100 mM sodium pyruvate solution was purchased from Sigma-Aldrich. Cells were incubated with 15 mM NaLac or 15 mM pyruvate for 6 h. A stock solution (10 mM) of the Hsp90 inhibitor NVP-AUY922 (Santa Cruz Biotechnology, Dallas, TX, USA) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and further diluted in phosphate buffered saline (PBS). Cells were treated with the indicated doses of NVP-AUY922 for 24 h. Control cells were incubated with the respective amounts of diluents, media or DMSO, as appropriate.

Lactate dehydrogenase (LDH) activity measurement

LDH activity was determined using the Lactate Dehydrogenase Activity kit (Sigma-Aldrich) following the manufacturer’s protocol.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1% v/v Triton-X-100, 0.1% w/v sodium dodecyl sulphate (SDS), 0.5% w/v sodium deoxycholate, protease inhibitor cocktail (Roche, Basel, Switzerland). The protein content in the cell lysates was determined using the BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes and detected by immunoblotting using the following primary and secondary antibodies: anti-HSF1 (ADI-SPA-901-D, Enzo Life Sciences, Farmingdale, NY, USA), anti-HSF1 phospho S326 (ab76076, Abcam, Cambridge, MA, USA), anti-Hsp27 (NBP2-32972, Novus Biologicals, Centennial, CO, USA), anti-Hsp70 (cmHsp70.1, immunoglobulin G (IgG)1, multimmune GmbH, Munich, Germany), anti-Hsp90 (4874, Cell Signaling Technology, Danvers, MA, USA), anti-AKT (9272S, Cell Signaling Technology), anti-LDHA (NBP1-48336, Novus Biologicals, Centennial, CO, USA), anti-LDHB (NBP2-53421, Novus Biologicals, Centennial, CO, USA), anti-ß-Actin (A2228, Sigma-Aldrich), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins (P0260, Dako-Agilent, Santa Clara, CA, USA) and HRP-conjugated swine anti-rabbit immunoglobulins (P0217, Dako-Agilent). Immune complexes were detected using the Pierce™ enhanced chemiluminescence (ECL) Western Kit (Thermo Fisher Scientific) and imaged digitally (ChemiDoc™ Touch Imaging System, Bio-Rad, Hercules, CA, USA). Fiji software (Schindelin et al., 2012) was used for quantification of the Western blot signals.

Flow cytometry

Single cell suspensions of the tumor cells were collected. Viable cells (0.2 x 10^6 cells) were washed once with flow cytometry buffer (10% FBS in PBS) and incubated either with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody (mAb) specific for membrane bound Hsp70 (cmHsp70.1, IgG1, multimmune GmbH, Munich, Germany) or an isotype matched FITC-labeled control antibody (IgG1, FITC, 345815, BD Biosciences, Heidelberg, Germany) on ice in the dark for 30 min. Dead cells were stained with propidium iodide (0.2 µg/mL, Sigma-Aldrich) and only viable cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences). Fluorescence data were plotted by using the CellQuest software (Becton Dickinson, Heidelberg, Germany).

For analyzing the expression of monocarboxylate transporter 1 (MCT1) by flow cytometry LS174T cells were permeabilized with 70% v/v methanol and stained using the allophycocyanin (APC)-conjugated human MCT1/SLC16A1 monoclonal antibody (mAb, IgG2A, R&D systems, Minneapolis, MN, USA) or an isotype matched APC-labeled control antibody (mouse IgG2A, Beckman Coulter, Brea, CA, USA).

Dichlorodihydrofluorescein diacetate (DCFDA) assay for measuring reactive oxygen species (ROS)

The DCFDA Cellular ROS Detection Assay Kit (Abcam) was used to determine intracellular levels of reactive oxygen species (ROS). Fluorescence signals were measured on a VICTOR Multilabel plate reader (PerkinElmer, Waltham, MA, USA) with 485/535 nm excitation/emission filters. The average relative fluorescence signal of WT cells was set to 1 and that of $LDH^{-/-}$ cells calculated proportionally.

Cell counting Kit-8

Cell viability and cell proliferation was measured using a Sigma-Aldrich Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol.

Immunocytochemistry

LS174T cells were grown on poly-L-lysine-coated glass slides. After blocking with 5% w/v bovine serum albumin (BSA) in PBS, cells were co-stained with FITC-labeled cmHsp70.1 mAb (multimmune GmbH) and phycoerythrin (PE)-labeled CD77 (Gb3) mAb (563631, BD Biosciences) on ice for 20 min. After a washing step with ice-cold PBS, cells were fixed in 0.5% w/v paraformaldehyde in PBS. Nuclei were counter-stained with Hoechst 33342 (H3570, Invitrogen, Carlsbad, CA, USA). A Leica TCS SP8 confocal microscope were used to take fluorescence images.

Measurement of extracellular Hsp70 levels

Extracellular Hsp70 levels in the supernatant of LS174T cells were determined by enzyme-linked immunosorbent assay (ELISA) (R&D systems) following the manufacturer's recommendations. The measured values of extracellular Hsp70 were normalized to 1×10^6 viable tumor cells.

Irradiation

Tumor cells were irradiated with the indicated doses using the CellRad compact benchtop X-ray irradiator (Precision X-Ray, North Branford, CT, USA) at a dose rate of 1 Gy/min (5 mA, 130 kV) or using the Gulmay RS225A device (Gulmay Medical Ltd., Camberley, UK) at a dose rate of 1.1 Gy/min (15 mA, 200 kV).

Analysis of cell survival using the clonogenic assay

Tumor cells were seeded into 12-well plates, treated with 5 nM NVP-AUY922 (24 h) or 60 mM oxamate (48 h) and irradiated with the indicated doses. After irradiation, cells were washed and incubated in drug-free medium. After 5-10 days, plates were washed with PBS, fixed with ice-cold methanol and colonies were stained with 0.1% w/v crystal violet. The number of colonies (≥ 50 cells) was measured automatically in a Bioreader® 3000 (Bio-Sys GmbH, Karben, Germany). Survival curves were fitted to the linear quadratic model using SigmaPlot (Systat Software Inc, San Jose, CA, USA).

Cell cycle analysis

For cell cycle analysis, cells were fixed in 70% v/v methanol overnight at 4°C, incubated with RNAse for 15 min at 37°C, stained with propidium iodide (50 µg/mL) and analyzed using a FACSCalibur™ flow cytometer (BD Biosciences).

Statistics

Each experiment was independently performed at least 3 times (biological replicates). The Student t-test was used to evaluate significant differences between two groups. When comparing multiple groups, the Tukey test was applied (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). Data are presented as mean values with standard deviation (SD).

3 Summary of the included publications

3.1 Targeting Cancer Metabolism Breaks Radioresistance by Impairing the Stress Response

Cancers 2021, 13, 3762. doi:10.3390/cancers13153762

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Background: Due to their enhanced energetic demand, cancer cells shift their metabolism towards aerobic glycolysis with an increased LDH activity and elevated levels of the corresponding oncometabolite lactate. Furthermore, many cancer cell types exhibit an overexpression of HSPs and thereby promote therapy resistance. Herein, I investigated the co-regulation of the lactate metabolism and the cellular stress response with respect to radiation resistance in two cancer cells systems (B16F10 murine melanoma and LS174T human colorectal adenocarcinoma).

Principal findings: The pharmacologically inhibition of LDH activity by oxamate and GNE-140, glucose deprivation and the genetically disruption of *LDHA/B* by a CRISPR/Cas9 mediated *LDHA/B* double knockout in mouse B16F10 and human LS174T cells significantly decreased cancer cell proliferation, ROS production and the synthesis of different HSPs, including Hsp90, Hsp70 and Hsp27, and HSF1. A *LDHA/B* double knockout resulted in an altered lipid metabolism and a downregulation of the Hsp70-anchoring, tumor-specific glycosphingolipid Gb3 on the cell surface of tumor cells. Reduced levels of Gb3, in turn, leads to a decreased membrane Hsp70 positivity and elevated extracellular Hsp70 levels. Conversely, the membrane Hsp70 expression in WT cancer cells was upregulated by treatment with increased extracellular lactate/pyruvate concentrations. Functionally, pharmacological or genetic inhibition of LDH reduces both cytosolic and membrane-bound HSPs in cancer cells and results in a significant reduction in radioresistance associated with G_2/M arrest.

Conclusion: In summary, I have shown that targeting lactate/pyruvate metabolism significantly reduces the radioresistance of cancer cells by impairing the heat shock response. Consequently, a combination therapy of pharmacological inhibition of LDH and ionizing radiation should be used to reduce radiation doses for tumor cell killing, thus sparing normal tissue.

Own contribution to the publication:

- Conception and performance of all experiments in which LS174T WT cells were treated with oxamate, including data analysis, processing and statistical analysis. Supervision of Katharina Thunborg who performed all experiments with GNE-140.
- Conception and performance of all experiments in which *LDHA/B* double knockout cells were used, with the exception of proteomics analysis, ELISA measurement and fluorescence microscopy, including data analysis, processing and statistical analysis.
- Writing the first draft of the manuscript and making adjustments for the revision of the manuscript.

3.2 A Low Membrane Hsp70 Expression in Tumor Cells With Impaired Lactate Metabolism Mediates Radiosensitization by NVP-AUY922

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Background: Radioresistance of cancer cells is mediated by high lactate concentrations in the tumor microenvironment, and overexpression and membrane localization of heat shock proteins like the major stress-inducible Hsp70. A previous study from our laboratory showed that *LDHA/B* double knockout significantly diminished both cytosolic and membrane HSP levels, leading to radiosensitization. Herein, I analyzed the mechanism underlying the radiosensitizing effect of the Hsp90 inhibitor NVP-AUY922 in mouse B16F10 and human LS174T *LDHA/B* double knockout cancer cells.

Principal findings: *LDHA/B* double knockout cells exhibit reduced cytosolic as well as membrane HSP levels. The Hsp90 inhibitor NVP-AUY922 elevates the cytosolic expression of Hsp27 and Hsp70, while leaving membrane Hsp70 expression unaltered. Although NVP-AUY922 induces the cytosolic levels of HSPs, radioresistance was significantly reduced in WT cells and even more pronounced in $LDH^{-/-}$ cells.

Conclusion: In summary, I demonstrated that a low membrane Hsp70 expression but not cytosolic Hsp70 levels, are responsible for the radiosensitizing potential of the Hsp90 inhibitor NVP-AUY922 in cancer cells with an impaired lactate metabolism.

Own contribution to the publication:

- Conception and performance of all experiments in this publication, including data analysis, processing and statistical analysis.
- Writing the original draft.

4 Discussion

The expression of HSPs such as Hsp90, Hsp70 and Hsp27 is increased in many tumor cell types including colorectal carcinoma and melanoma, and promotes tumor progression and therapy resistance by protecting tumor cells from apoptosis (Ciocca & Calderwood, 2005). Furthermore, due to their fast proliferation rates (Vaupel & Multhoff, 2021), many solid tumor cells have an increased uptake of glucose and an elevated glycolytic capacity (Warburg effect) compared to normal cells (Warburg et al., 1927). The resulting high concentrations of the oncometabolite lactate, caused by an enhanced LDH activity, lead to an acidification of the tumor microenvironment promoting tumor growth (Hirschhaeuser et al., 2011), aggressiveness, metastatic spread and tumor recurrence (Walenta et al., 2000). Additionally, high lactate levels suppress effector T and NK cell functions (Bohn et al., 2018; Brand et al., 2016; Siska et al., 2020) and promote the proliferation and activity of immunosuppressive cells including regulatory T_{reg} cells (Angelin et al., 2017). Recently, it has been shown that due to their metabolic flexibility T_{reg} cells can use lactic acid as an alternative fuel to maintain their immunosuppressive identity (Watson et al., 2021) and first reports indicated that the glucose/lactate metabolism might be linked to the HSR in immature boar Sertoli cells (Guan et al., 2018). The transcription HSF1, which is the master regulator of the stress response in eukaryotes, also regulates glucose metabolism (Dai et al., 2007), binds to the LDHA promotor and thereby increases the LDHA expression in breast cancer cells (Zhao et al., 2009). Furthermore, overexpression of Hsp70 in human HeLa cervical cancer cells leads to increased aerobic glycolysis (Wang et al., 2012). In hepatocellular carcinoma cells, enhanced lactate export and glycolytic activity are observed in cells overexpressing HSPA12A (Min et al., 2022). Despite these studies, only little is known about the relationship between oncometabolite lactate and the antiapoptotic stress response, particularly with respect to the functional consequences. Therefore, this study aimed to investigate the co-regulation of the lactate metabolism and the HSR with respect to radiation resistance in two different cancer cell systems. LDH inhibition was performed either genetically by an CRISPR/Cas9 mediated *LDHA/B* double knockout or pharmacologically by a treatment with the pyruvate analog oxamate.

4.1 Heat shock response of cancer cells can be significantly reduced by targeting the lactate/pyruvate metabolism

The inhibition of the LDH activity in the human colorectal carcinoma cell line LS174T with sublethal doses of oxamate (60 mM, 48 h) resulted in a significantly down regulation of the cytosolic stress proteins Hsp90, Hsp70 and Hsp27 (Figure 7, (Schwab et al., 2022; Schwab et al., 2021)). These data are consistent with earlier findings demonstrating a reduction in HSPs by oxamate in hepatocellular carcinoma cells (Manerba et al., 2017). To confirm that the effects of oxamate on the synthesis of HSPs are mediated solely by LDH inhibition and not by direct impairment of HSPs expression or stability, cancer cells (LS174T and B16F10) with CRISPR/Cas9 generated *LDHA/B* double knockout were investigated with respect to their HSPs expression. Similar to the LDH inhibitor, depletion of *LDHA/B* significantly decreased the cytosolic HSP levels (Figure 7). Additionally, $LDH^{-/-}$ cancer cells showed reduced metabolic and proliferative capacity which was associated with decreased ROS production (Schwab et al., 2022; Schwab et al., 2021). The genetic depletion of *LDHA/B* results in a metabolic shift in the tumor cells from glycolysis towards oxidative phosphorylation (OXPHOS) (Zdralevic et al., 2018). A reduced oxidative metabolism is often related to a decreased ROS production, however, our results, which are consistent with those of an earlier study (Li et al., 2017), show that $LDH^{-/-}$ cells with an inhibited glycolysis exhibit lower ROS levels than the corresponding WT cells (Schwab et al., 2022; Schwab et al., 2021). Potential explanations are that ROS production is promoted by lactate and pyruvate by triggering their mitochondrial activity (Tauffenberger et al., 2019) or that an excess of NADH, which is produced during the conversion of lactate to pyruvate, could provoke electron leakage and ROS production by the respiratory complex I (Vinogradov & Grivennikova, 2016).

The HSR is a key survival mechanism to protect cells from toxic effects caused by physical or biological stressors such as heat, heavy metals, toxins, oxidative stress, irradiation and infections by bacteria or viruses. This molecular response to cellular stress includes an increased synthesis of molecular chaperones like HSPs (Jolly & Morimoto, 2000; Morimoto, 1993). Tumor cells often show a constitutively upregulated HSP expression in the cytosol to prevent them from lethal damage induced by environmental stress and to enhance cell proliferation. The overexpression also promotes therapeutic resistance by affecting apoptotic pathways (Ciocca & Calderwood, 2005) and by stabilizing proteins which are involved in DNA repair

mechanism (Sottile & Nadin, 2018). Radioresistance of tumor cells is mediated not only by an upregulation of cytosolic, but also of plasma membrane bound Hsp70 levels (Murakami et al., 2015). This tumor specific membrane localization and anchorage of Hsp70 is enabled by a tumor-specific lipid composition. On the membrane, Hsp70 can bind to negatively charged glycosphingolipids including sulfogalactosyl ceramide (Mamelak et al., 2001) and globotriaosylceramide (Gb3) (Gehrmann et al., 2008) which are located in lipid rafts of tumor cells (Horvath et al., 2008). Furthermore, Hsp70 can interact with phosphatidylserine, which translocate to the outer plasma membrane leaflet upon environmental stress (De Maio & Hightower, 2021; Schilling et al., 2009). Pharmacological inhibition of LDH by oxamate or genetic inhibition by an *LDHA/B* double knockout significantly reduced Hsp70 membrane positivity in cancer cells (Figure 7). Confocal microscopy analysis revealed that the diminished Hsp70 membrane positivity was associated with a dramatic reduction in the Gb3 expression on the plasma membrane of the LS174T LDH$^{-/-}$ cells compared to the corresponding WT cells. Comparative analysis of the cellular proteomes of LS174T WT and LDH$^{-/-}$ showed substantial differences in major pathways related to cholesterol biosynthesis, mitochondrial dysfunction and lipid-, carbohydrate-, nucleic acid- and protein metabolism which might explain the reduced Gb3 expression in LDH$^{-/-}$ cells (Schwab et al., 2022; Schwab et al., 2021).

The expression of Hsp70 on tumor cell membranes correlates with the localization of Hsp70 in lysosomal membranes. Membrane-bound Hsp70 mediates resistance to chemically and physically-induced membrane permeabilization by anticancer drugs and irradiation, respectively and prevents apoptosis by stabilizing plasma as well as lysosomal membranes (Kirkegaard et al., 2010; Multhoff et al., 2015; Nylandsted et al., 2004). Hsp70 contributes to membrane stability through its resistance to lysosomal hydrolases. This is mediated by an effective anchorage of Hsp70 to the lysosomal membranes via its high-affinity binding to bis(monoacylglycero)phosphate (BMP) which increases the lipid catabolism in lysosomes. The high-affinity association of Hsp70 and BMP facilitates the BMP binding of sphingolipid-degrading enzymes, thereby increasing their activity and inhibiting their degradation. Furthermore, Hsp70 can protect the membranes from oxidative stress and thereby stabilizes lysosomal membranes (Balogi et al., 2019). Due to the fact that both, elevated cytosolic and membrane bound HSPs, can lead to therapy resistance (Ciocca & Calderwood, 2005; Murakami et al., 2015), inhibition of the cellular stress response appears to be a

promising strategy to increase the efficacy of cancer therapies such as ionizing radiation.

4.2 Heat shock proteins as a target in cancer therapy

In recent years inhibitors of the major heat shock proteins Hsp27, Hsp70 and Hsp90 have been developed and tested in preclinical and clinical studies (Jego et al., 2013). One promising candidate is the potent synthetic small-molecule Hsp90 inhibitor NVP-AUY922 which binds to the NH_2-terminal nucleotide binding site of Hsp90. It inhibits tumor cell proliferation and viability (Brough et al., 2008; Eccles et al., 2008) while exhibiting radiosensitizing potential *in vitro* and *in vivo* (Schilling et al., 2012; Zaidi et al., 2012). This radiosensitization could be potentiated when NVP-AUY922 was combined with a HSF1 knockdown in lung cancer cells by impairing DNA double-strand break repair (Kuhnel et al., 2019). Inhibition of the LDH activity, genetically or pharmacologically, decreased the cytosolic expression of HSF1, Hsp90, Hsp70 and Hsp27 as well as membrane bound Hsp70 and thereby increased the radiosensitivity of cancer cells (Figure 7). Therefore, LDH inhibition might be an effective strategy to improve the clinical outcome of radiotherapy (Schwab et al., 2022; Schwab et al., 2021). Despite an increase in cytosolic Hsp70 and Hsp27 expression, leading to comparable cytosolic levels in WT and $LDH^{-/-}$ cells, treatment with the Hsp90 inhibitor NVP-AUY922 resulted in radiosensitization of WT cells, which was even more pronounced in $LDH^{-/-}$ cells (Figure 7). In contrast, the membrane Hsp70 expression remained unaltered in WT and $LDH^{-/-}$ cells suggesting that the lower membrane Hsp70 positivity is responsible for the higher radiosensitizing effect in $LDH^{-/-}$ cells (Schwab & Multhoff, 2022). Although a high Hsp70 expression, also on the plasma membrane, can mediate radioresistance (Murakami et al., 2015), membrane Hsp70 can serve as a target for activatory C-type lectin receptors on NK cells (Gross, Schmidt-Wolf, et al., 2003; Multhoff et al., 2015). A higher density of plasma membrane bound Hsp70 enhances their visibility towards NK cells, activated *ex vivo* with Hsp70 peptide TKD plus low dose IL-2, thereby increasing the killing of these tumor cells (Multhoff et al., 2001). The cytolytic function of NK cells is reflected by an increased release of apoptosis-inducing serine proteases such as GrB and pro-inflammatory cytokines including interferon-γ (IFN-γ). LS174T WT cells, which exhibit a higher membrane Hsp70 positivity than the $LDH^{-/-}$ cells (Schwab et al., 2022; Schwab et al., 2021),

induced a higher GrB and IFN-γ release after co-incubation with TKD/IL-2 activated NK cells compared to $LDH^{-/-}$ cells. Furthermore, transduced peripheral blood mononuclear cells (PBMCs)-derived T cells with a novel anti-Hsp70 chimeric antigen receptor (CAR), capable of recognizing membrane Hsp70 on cancer cells, showed comparable results to TKD/IL-2 stimulated NK cells in terms of their cytolytic activity against membrane Hsp70 positive tumor cells (Bashiri Dezfouli et al., 2022). Ionizing radiation can further increase the density of Hsp70 on cancer cell membranes (Fellinger et al., 2020; Schwab et al., 2021), therefore a combination therapy consisting of radiation followed by NK/CAR-T cell therapy might improve clinical outcome of patients with membrane Hsp70 positive tumor cells. In a randomized phase II clinical trial patients with advance unresectable, membrane Hsp70 positive NSCLC were treated after radiochemotherapy with *ex vivo* Hsp70-activated, autologous NK cells. The NK cell therapy was well tolerated and delivered positive clinical responses in the patients receiving activated NK cells (Multhoff et al., 2020). High levels of Hsp70 may be associated with increased tumor aggressiveness (Ciocca & Calderwood, 2005). A study with glioma patients showed that Hsp70 protein levels are higher in glioblastoma (GBM) patients than in low-grade gliomas (Thorsteinsdottir et al., 2017) and GBM patients with low Hsp70 levels might favor a beneficial clinical outcome (Lobinger et al., 2021). Therefore, free and exosomal Hsp70 in blood of patients may represent a tumor-specific biomarker, which can be quantified by the Hsp70 compELISA (Werner et al., 2021).

4.3 Overcoming the resistance mechanisms of cancer cells

One of the major challenges in cancer therapy remains the resistance mechanisms of cancer cells. To overcome drug resistance, combination therapies using inhibitors targeting different HSPs or combining HSP inhibitors with conventional therapies such as chemo- or radiotherapy have recently been investigated, although clinical data are still missing (Jego et al., 2013; Yang et al., 2021). High lactate levels (Hirschhaeuser et al., 2011) as well as enhanced HSP expression increase the radioresistance of cancer cells (Ciocca & Calderwood, 2005; Murakami et al., 2015). LDH inhibition, genetically or pharmacologically, impairs both the lactate (Zdralevic et al., 2018) and HSP response (Schwab et al., 2022; Schwab et al., 2021) and thereby increases the radiosensitizing capacity of the HSP90 inhibitor NVP-AUY922 (Schwab & Multhoff,

2022). Therefore, LDH inhibition might provide a promising strategy to combat radioresistance and improve the clinical outcome of cancer patients. Various LDH inhibitors like FX11 (Le et al., 2010; Xian et al., 2015) and oxamate (Li et al., 2013; Manerba et al., 2017; Yang et al., 2014) showed promising results in inhibiting tumor growth and progression in preclinical models. Furthermore, the pyruvate analog oxamate reduced the expression levels of HSPs in a human hepatocellular carcinoma cell line (Manerba et al., 2017) and in a human colorectal adenocarcinoma cell line (Schwab et al., 2022; Schwab et al., 2021). Due to limited cell membrane permeability the effective dose of oxamate is too high for clinical use (Feng et al., 2018). Therefore, there is a need for developing more potent and less toxic LDH inhibitors that are able to interfere with the HSR. One promising candidate is GNE-140 (Boudreau et al., 2016) which showed similar results to oxamate in terms of inhibiting LDH and reducing HSPs (Schwab et al., 2022; Schwab et al., 2021). Nevertheless, GNE-140 is presently not able to maintain inhibition of LDH activity *in vivo* longer than 1 h due to its rapid *in vivo* clearance (Boudreau et al., 2016). Because gene knockout is difficult to translate into clinical practice and the currently available LDH inhibitors have poor stability and are highly toxic *in vivo*, their clinical use is limited. One possibility would be to search for compounds that phenocopy the effects of a genetic depletion of *LDHA/B*. The easiest and fast-forward way would be to screen already approved drugs for their potential impact on the lactate metabolism. This would avoid costly and time-consuming toxicity studies of newly designed drug candidates. The clinical approved nonsteroidal anti-inflammatory drug (NSAID) diclofenac might provide a promising candidate. Diclofenac reduced tumor growth *in vitro* and *in vivo* by its inhibitory effect on the cyclooxygenases COX1 and COX2 (Johnsen et al., 2004). For human colon cancer cell lines, it has been known for almost three decades that diclofenac exert antiproliferative effects (Hixson et al., 1994). Previous studies showed also COX-independent antitumoral effects of diclofenac. It can inhibit glycolytic genes such as the *glucose transporter 1* (*GLUT1*), *LDHA* and *MCT1* (Gottfried et al., 2013) and diminish MYC expression resulting in reduced uptake of glucose and lactate production (Chirasani et al., 2013; Gottfried et al., 2013; Leidgens et al., 2015). Furthermore, it was shown that diclofenac could act as a radiosensitizer *in vitro* and *in vivo* in COX2 overexpressing prostate cancer cells (Inoue et al., 2013). My unpublished data showed that diclofenac reduces LDH activity, decreases MYC expression, diminishes cytosolic HSF1, Hsp70 and Hsp27 levels and membrane Hsp70 positivity in colorectal LS174T and LoVo cancer cells, but not in lung

A549 carcinoma cells, MDA-MB-231 breast cancer cells and COLO-357 pancreatic adenocarcinoma cells. The impaired lactate metabolism and stress response was associated with a significantly increased sensitivity towards radiation and 5-fluorouracil (5-FU) in LS174T and LoVo cells. Therefore, monitoring of the LDH activity and heat shock response upon diclofenac treatment might be predictive for the radiation and/or chemo-sensitizing capacity of the drug. It remains to be investigated why diclofenac shows different effects in different cell lines. Nevertheless, diclofenac is a promising candidate, which could potentially be repurposed as a LDH inhibitor although further studies are needed in preclinical animal models.

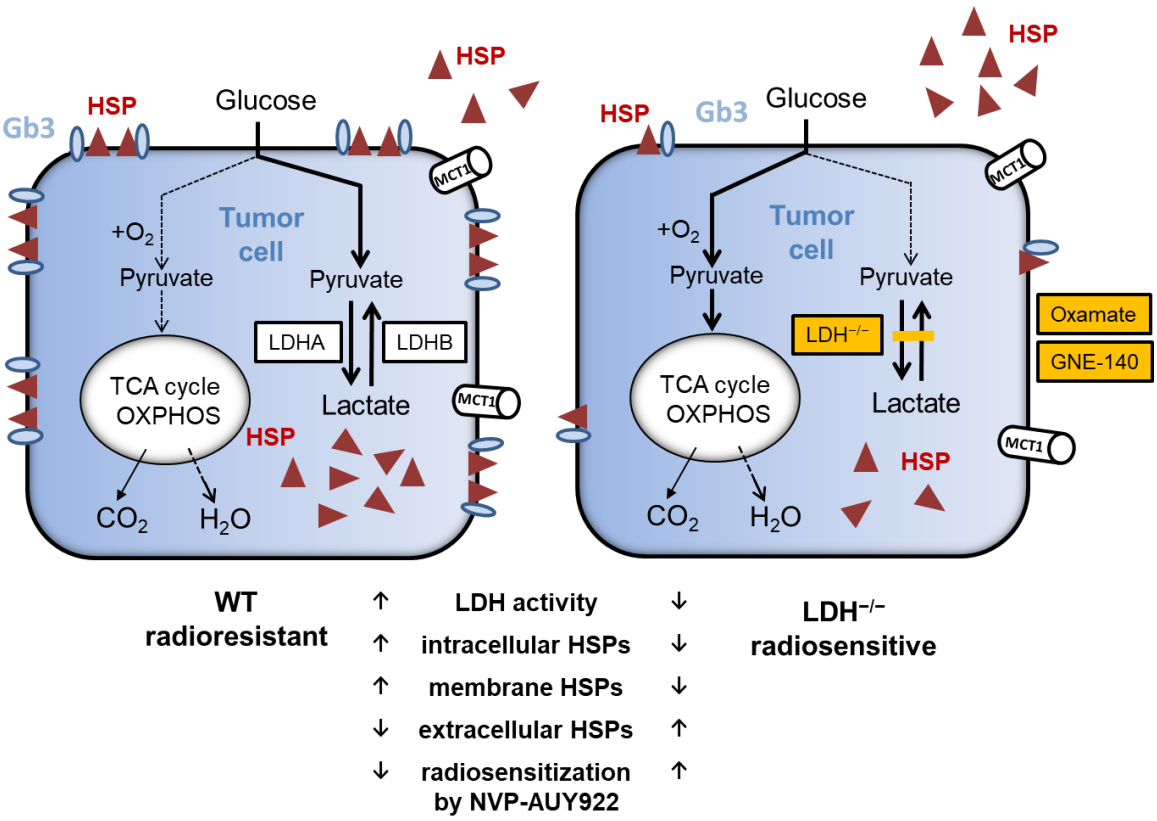

Figure 7: Schematic illustration of the main findings. LDH inhibition by LDH inhibitors oxamate and GNE-140 or by *LDHA/B* double knockout ($LDH^{-/-}$) reduces the radioresistance of cancer cells by a downregulation of cytosolic, anti-apoptotic heat shock proteins (HSPs), a decrease in plasma membrane bound Hsp70 and an impaired lactate dehydrogenase (LDH) activity. Whereas the lactate transporter monocarboxylate transporter 1 (MCT1) remains unchanged. Elevated extracellular Hsp70 levels in cancer cells with an impaired lactate metabolism are associated with a diminished membrane expression of the Hsp70-anchoring glycosphingolipid globotriaosylceramide (Gb3). Treatment with the Hsp90 inhibitor NVP-AUY922 resulted in radiosensitization of wild type (WT) cells, which was even more pronounced in $LDH^{-/-}$ cells (adapted from (Schwab et al., 2021)).

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6 Appendix

6.1 Eidesstattliche Erklärung

Ich, Melissa Elisabeth Schwab, erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung School of Life Sciences der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

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