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Effects of Lactatedehydrogenase inhibition via GNE-140 on the expression of heat shock proteins

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1 Abbreviations

17-AAG ..... 17-(allylamino)-17-demethoxy-geldanamycin
17-DMAG 17-N,N-dimethylaminoethylaminogeldanamycin
AMP ..... adenosine-monophosphate
Apaf-1 ..... apoptotic protease-activating factor 1
APC ..... Adenomatous polyposis coli
APS ..... Ammonium persulfate
ATP ..... adenosine triphosphate
Bax ..... Bcl-2-associated X protein
BCA ..... bicinchoninic acid
Bcl2 ..... B-cell lymphoma 2
Bim ..... Bcl-2-like protein 11
BRAF ..... B- rapidly accelerated fibrosarcoma
CA ..... California
Cas ..... CRISPR-associated
CFA ..... Colony formation assays
CRC ..... colorectal cancer
CRISPR ..... Clustered Regularly Interspaced Short Palindromic Repeats
CT ..... computed tomography, computed tomography
ctrl ..... control group
D ..... dose
DCC ..... Deleted in Colorectal Carcinoma
DMEM ..... Dulbecco's Modified Eagle's Medium
DMSO ..... Dimethyl sulfoxid
DNA ..... deoxyribonucleic acid
EDTA ..... ethylene- diamine-tetra-acetic acid
EGFR ..... epidermal growth factor receptor
FACS ..... fluorescence-activated cell sorting
FBS ..... Fetal Bovine Serum
FDA ..... Food and Drug Administration
FITC ..... fluorescein isothiocyanate
FOLFOX ..... folinic acid, 5-fluorouracil, oxaliplatin
Gb3 ..... glycosphingolipid globotriaosylceramide
GLUT1 ..... glucose transporter 1
GLUT4 ..... glucose transporter 4
HIF1 ..... hypoxia- inducible factor 1
HSF1 ..... heat-shock factor 1
HSP ..... heat shock proteins
Hsp70.1 ..... key representative heat shock protein from the HSP70 family
HSPs ..... heat shock proteins
IC50 ..... half maximal inhibitory concentration
IgG ..... immunoglobulin G
IgG1 ..... immunoglobulin subclass G1

KRAS ..... Kirsten rat sarcoma virus
LDH ..... lactate dehydrogenase
LDH-1 ..... lactatedehydrogenase consisting of four H subunits
LDH-5 ..... lactatedehydrogenase consisting of four M subunits
LDHA ..... subunit M of lactate dehydrogenase
LDHB ..... subunit H of lactatedehydrogenase
LQ ..... linear-quadratic
MA ..... Massachusetts
MAPK ..... mitogen-activated protein kinase
mHsp70 ..... membrane Hsp70
MLH1 ..... human mutL homolog 1
MRI ..... magnetic resonance imaging
MSH2 ..... human mutS homolog 2
mTOR ..... mechanistic Target of Rapamycin
NaCl ..... Sodium chloride
NAD ..... nicotinamide adenine dinucleotide
NAD+ ..... oxidated form of nicotinamide adenine dinucleotide
NADH ..... reduced form of nicotinamide adenine dinucleotide
NK ..... natural killer
OXPHOS ..... oxidative phosphorylation
PBS ..... Phosphate buffered saline
PDPK1 ..... 3-phosphoinositide-dependent protein kinase 1
PE ..... Plating efficiency
PI ..... Propidium- iodid
PI3/AKT ..... phosphatidylinositol-3-kinase/ protein-kinase- B
PS ..... phosphatidylserine, Penicillin/Streptomycin
PUMA ..... p53 upregulated modulator of apoptosis
RAF ..... Rapidly Accelerated Fibrosarcoma
RAS ..... Rat sarcoma
RIPA ..... radioimmunoprecipitation assay
RNA ..... ribonucleic acid
ROS ..... reactive oxygen species
SDS ..... Sodium dodecyl sulfate
SDS-PAGE ..... SDS polyacrylamid gel electrophoresis
SER ..... sensitizer enhancement ratio
SF ..... surviving fraction
TBS ..... tris-buffered saline
TBST ..... TBS with Tween20
TEMED ..... Tetramethylethylenediamine
TGF- β ..... transforming growth factor beta
TNF- alpha ..... tumor necrosis factor alpha
TNM ..... tumor, nodes, metastasis
TP53 ..... Tumor protein 53
TRIS ..... Tris(hydroxymethyl)aminomethane

USA ..... United States of America
VEGF ..... anti-epidermal growth factor receptor
WNT ..... wingless-type MMTV integration site
XELOX ..... Capecitabin, Oxaliplatin

2 Graphical abstract

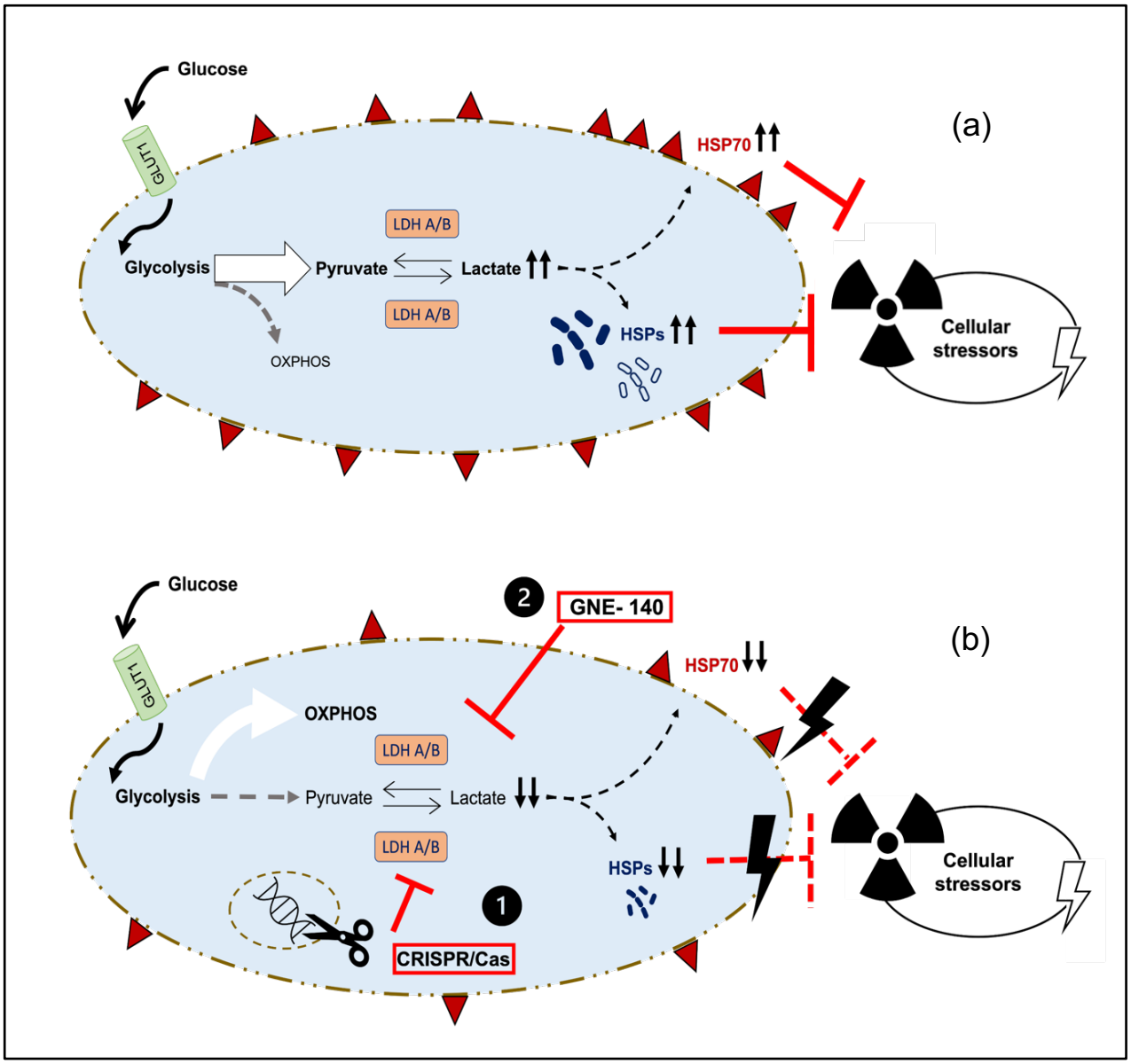


Figure 1. Effects of LDH inhibition via GNE-140 treatment and double genetic disruption of *LDH A* and *B*. The metabolism of tumor cells is schematically illustrated in (a). The glycolytic phenotype leads to an overexpression of heat shock proteins which increases the capacity to counteract cellular stressors, e.g., radiation. Impaired lactate metabolism, either via genetic disruption (1) or pharmacological inhibition (2) of LDH, consequently leads to impaired heat shock response which hinders effective cellular defense as shown in (b).

3 Introduction

3.1 Colorectal carcinoma

3.1.1 Disease characterization, epidemiology and etiology

Colorectal cancer (CRC) ranks as the third most common cancer globally. Over 1.9 million cases are diagnosed and lead to more than 900,000 deaths annually. This makes it the second leading cause of cancer related death, exceeded only by lung cancer. The gender distribution is similar accounting for up to 9.4% of cancer incidence in women and 10.6% in men. (Sung et al., 2021) By the year 2040 incidence rates are estimated to rise by over three million causing over 1.5 million deaths in a 12-month period (Ferlay et al., 2020). Colorectal cancer is highly associated with economic transition hence, the highest incidence rates of approximately 55% are found in Western countries. Nonetheless, developing countries are catching up. Regions with increasing human development indices face a corresponding rise in incidence and mortality which underlines the role of colorectal cancer as an indicator for socioeconomic development. (Brody, 2015; Holmes, 2015) Changes in lifestyle and diet towards increased consumption of highly processed food and meat combined with decreased physical activity and excess body weight seem to be independently associated with the risk of developing colorectal cancer although the underlying mechanisms are not fully understood yet (Clinton et al., 2020). Excessive alcohol consumption and cigarette smoking display additional risk factors (Islami et al., 2018; Secretan et al., 2009). The main risk factor, however, is age and therefore non-modifiable. Here, the risk significantly increases after the fifth decade. (Levin et al., 2008) Besides age, there are certain genetic conditions such as familial hereditary nonpolyposis colon cancer or adenomatous polyposis which significantly increase the risk of disease when inherited (Lynch & De la Chapelle, 2003). Regarding the predictions and ongoing socioeconomic

and lifestyle changes within the scope of globalization, CRC is conceivably becoming an even greater global burden in the near future.

The pathogenesis of sporadic CRC derives from certain genetic and epigenetic events. Most colorectal tumors arise from prelesions developing according to a morphological adenoma-carcinoma sequence. This transition derives from a characteristic cluster of genetic alterations including protooncogenes and tumor suppressor genes, both promoting malignancy. Usually, the transformation of a normal colonic stem cell into a neoplastic one is propelled by the accumulation of two to eight driver gene alterations. (Carethers & Jung, 2015)

Although each patient's CRC is genetically unique, the malignant progression appears to follow distinct patterns, which can be classified into three major molecular pathways at present: chromosomal instability, microsatellite instability and serrated neoplasia (Müller et al., 2016). The former displays the most common (Fearon & Vogelstein, 1990), the latter, on the other hand, is less frequent (Bettington et al., 2013). Moreover, new pathways continue to be discovered as a result of integrated data on genotype and expression (Lee et al., 2015b)

Hypermethylation of specifically arranged deoxyribonucleic acid (DNA) regions, so-called CpG islands, is typically found in the serrated pathway and causes silencing of tumor suppressor genes often associated with B-rapidly accelerated fibrosarcoma (*BRAF*) mutations (Tadros & Anderson, 2013). Failed DNA mismatch repair, on the other hand, leads to microsatellite instability (Ionov et al., 1993). Whereas the other pathways are found in both hereditary as well as sporadic cancer forms, microsatellite instability is mostly associated with hereditary cancer (Lee et al., 2015a).

Adenomatous polyposis coli (*APC*) is usually the first tumor suppressor gene to lose its function which triggers the formation of polyps.

Subsequent genetic alterations depend on the specific pathway engaged. (Nguyen et al., 2020) Following the chromosomal instability pathway, a Kirsten rat sarcoma virus (*KRAS*) mutation is likely to occur afterwards, followed by Deleted in Colorectal Carcinoma (*DCC*) gene and a conclusive loss of the Tumor protein 53 (*TP53*) (Fearon & Vogelstein, 1990).

However, pathophysiological changes cannot only be observed at the genetic level. Due to their reliance on oncogenic signals, as described above, CRC cells are particularly prone to proteotoxic stresses. Therefore, heat shock proteins (HSP), a specific group of chaperones, have a decisive influence on the pathogenesis of colorectal cancer. (Abi Zamer et al., 2021)

Furthermore, key dysregulated signaling pathways in colorectal cancer, such as phosphatidylinositol-3-kinase/ protein-kinase-B (PI3/AKT), mitogen-activated protein kinase (MAPK) and the wingless-type MMTV integration site (WNT) pathway, were discovered to be modulated by several HSPs, providing them with even greater significance in tumorigenicity (Lee et al., 2015a).

Given these properties, overexpression of HSP has a positive influence on the oncogenic progression in CRC. A detailed explanation of HSPs, including their functions and effects, especially with regard to tumorigenicity, will be given in the theoretical part.

3.1.2 Diagnosis and staging

Patients with symptoms characteristic for colorectal cancer should undergo an endoscopic examination. Clinical manifestations range from changes in bowel habits, rectal bleeding to anemia or weight loss. Nonetheless, symptoms usually do not occur until late stages. (Thanikachalam & Khan, 2019) To face this challenge, a variety of screening methods have been implemented, the gold standard of which is colonoscopy. During this intervention, polyps can be removed and

biopsies can be taken for histopathological examination of suspicious lesions. In case of confirmed suspicion, proper staging must be performed, including a computed tomography (CT) scan of at least the abdomen, a chest X-ray, plus a pelvic magnetic resonance imaging (MRI) scan if the rectum is affected. (Brenner et al., 2014; Lee et al., 2015a; Letlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2019))

The differentiation between these two entities has diagnostic as well as therapeutic consequences. By definition, colon carcinomas are localized at least 16 cm above the anocutaneous line, whereas malignant neoformations below this threshold are referred to as rectum carcinomas. (Letlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2019))

The pathological and radiological findings can be classified according to the tumor, node, metastasis system (TNM) and the corresponding staging which is decisive for treatment strategies and prognosis (Sobin et al., 2011)

AJCC stage	TNM categories	Description
0	Tis; N0; M0	This stage is also referred to as carcinoma in situ (Tis), which implies that the tumor has not spread past the colon's or rectum's mucosa.
I	T1 or T2; N0; M0	The tumor has progressed and is now invading the submucosa (T1) or muscularis propria (T2). Lymph nodes (N0) or distant sites (M0) are not involved.
II A-C	T3-T4b; N0; M0	The subserosa, the pericolic/-rectal fat tissue (T3) is affected or even the wall is perforated (T4a). The tumor is possibly attached to or has penetrated surrounding tissues (T4b).
III A-C	Any T; N1-N2b, M0	At this stage surrounding lymph nodes are involved. The affection of less than four lymph nodes is described as N1, N2a includes up to six lymph nodes and N2b covers everything above this level. This stage is independent of the primary's tumor infiltration.
IV A-C	Any T; any N, M1	Metastases are present (M1). The cancer has spread to distant organs or lymph nodes.

Table 1. Staging classification of colorectal cancer according to the American Joint Committee on Cancer (AJCC). Adapted from (Vogel et al., 2017) and (American Cancer Society, 2024)

3.1.3 Treatment

As indicated above, the therapeutic algorithm depends on the progression of the disease, the patient's general condition, and, with regard to novel therapeutic approaches, also on the molecular biological features of the tumor. Surgery represents the major pillar in fighting CRC and is usually combined with chemo- and targeted therapy as part of a multimodal treatment. Whereas colon carcinoma is only irradiated in a palliative setting, irradiation of rectum carcinoma is broadly implemented in therapeutic regimes. Stage I of CRC requires only surgical therapy or local excision, depending on the infiltration depth. Radical surgery involves a systematic lymph node extirpation and removal of the mesocolon/-rectum. In the case of colon carcinoma, the surgical approach generally comprises

a hemicolectomy. Adjuvant therapy in colon carcinoma is usually not performed below stage III. Stage II high-risk patients, however, should also be considered for this regime. Chemotherapeutic agents are used to prevent recurrency and treat micrometastases. The following regimes are commonly applied: FOLFOX (folinic acid, 5-fluorouracil, oxaliplatin) and XELOX (Capecitabin, Oxaliplatin). (Letlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2019))

Patients with rectum carcinoma, on the other hand, already receive multimodal therapy at stage II. Tumors of the rectum's upper third are usually treated with adjuvant (radio)chemotherapy. For those localized in the middle or lower third, a neoadjuvant radio(chemo)therapeutic approach including capecitabin/5-fluorouracil and different irradiation schemes (short-term vs. fractionized) have been established. (Letlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2019))

Metastatic disease, i.e., stage IV, requires an interdisciplinary assessment regarding the resectability of metastases and whether the patient is fit enough for intensive treatment. A molecular biological analysis should be conducted first to evaluate the efficacy and potential benefits of targeted therapy. Therefore, at least mutations of the Rat sarcoma (RAS) genes (KRAS and NRAS), the BRAF gene and microsatellite instability should be investigated. Molecularly targeted therapy comprises Bevacizumab, an anti-vascular endothelial growth factor (VEGF) receptor monoclonal antibody, and anti-epidermal growth factor receptor (EGFR) antibodies like Cetuximab or Panitumumab. These should always be administered in combination with chemotherapy doublets/triplets. (Letlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2019)

The approximate 5-year survival rate for CRC is 65%. That being said, the prognosis strongly relies on the progression of the disease. In localized stages the rate reaches 90%, declining to 12% in case of distant tumor

spread (Miller et al., 2019). Furthermore, over 20% of patients newly diagnosed with CRC present with distant metastases, that are usually found in the liver (Leufkens et al., 2011).

Given the variety of signaling pathways involved in the carcinogenesis of CRC, single targeted therapies, unfortunately, only show limited success. The approximate overall survival in advanced colorectal cancer does not climb above 30 months despite intensive treatment. This further stresses the need for innovative and improved strategies to support the established therapeutic cornerstones. Targeting a component that affects multiple signaling pathways towards less proliferative and more apoptotic stimuli would therefore provide great therapeutic potential with regard to the complexity and diversity of CRC. (Scudellari, 2015)

3.2 Cancer cell biology

Cancer is a disease rooted in a multistep development that cells undergo during their transformation towards malignancy. Dynamic changes in the phenotype result from genetic alterations as cells gradually evolve. Those events of spontaneous mutation and consecutive selection of well-adapted cancer cells foster the neoplastic disease by the acquisition of specific tumorigenic traits. (Hanahan & Weinberg, 2000)

Hanahan and Weinberg (2000) suggested that this circuit is based on the following six major modifications in cell biology: “self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.” These so-called hall marks represent a logical framework to depict the complexity and diversity of cancer evolution. Moreover, these biological features are expressed in a broad variety not only between different entities but also within one macroscopic population, making tumors heterotypic tissues. (Hanahan & Weinberg, 2011)

One of the most fundamental characteristics of cancer cells is their capacity for autonomous (hyper-)proliferation. Contrary to normal cells, where growth signals are strictly regulated in their release and activation to maintain the homeostasis in number and architecture, cancer can induce and sustain proliferative signaling in multiple ways. Apart from growth factor overproduction and the upregulation of corresponding receptors, further mechanisms such as constitutive activation of agents in mitogenic signaling pathways (BRAF, Pi3-kinase) and dysfunctional feedback loops lead to proliferative stimulation. (Davies & Samuels, 2010; Hanahan & Weinberg, 2011; Jiang & Liu, 2009)

To sustain hyperproliferation over the long-term, cancer cells have managed to achieve replicative immortality. Usually, telomers, sequences

of multiple tandem repeats, protect the DNA sequence during replication. Nonetheless, frequent cycles of cell division cause a shortening of telomers and ultimately a loss that inevitably triggers cell death. (Hanahan & Weinberg, 2000) Cancer cells, however, have managed to compensate this shortening by the upregulation of telomerase, the corresponding enzyme to extend telomers. (Blasco, 2005; Shay & Wright, 2000)

The loss of tumor suppressors, e.g., p53 and downregulation of proapoptotic factors, display further strategies of cell death evasion making cancer even more insensitive towards apoptosis-inducing signals. Especially members of the B-cell lymphoma 2 (Bcl2) protein family such as Bcl-2-associated X protein (Bax), Bcl-2-like protein 11 (Bim) or p53 upregulated modulator of apoptosis (PUMA) are of great importance here. (Hanahan & Weinberg, 2011)

Cancer cells are not only able to evade apoptosis but also manage to compensate sublethal damage through autophagy. Its cytoprotective property resides in the ability to generate low-molecular-weight metabolites, which can be used for biosynthesis and energy metabolism during a state of reversible dormancy. (Hanahan & Weinberg, 2011) Apparently, this survival response enables eventual regrowth. However, due to its conflicting effects, it remains unclear whether the tumorigenic traits outweigh the apoptotic ones. (Apel et al., 2009)

Angiogenesis and metastasis display two further hallmarks, which are based on cooperative interaction with the tumor's microenvironment. Contrary to the former belief that tumors are simply homogenous masses of cancer cells, research of the past decade has shown that tumors consist of multiple geno- and phenotypes. (Hanahan & Weinberg, 2011) These different subpopulations do not only interact with each other but also with the surrounding endothelial, pericytes, and immune cells, thus creating favorable environmental conditions for angiogenesis and invasion (Coffelt

et al., 2010; Egeblad et al., 2010; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). Neoangiogenesis and metastasis enable effective nutrient and oxygen supply as well as access to the lymphatic and blood systems. After entering the circulation, tumor cells can spread over the body to colonize and establish new permissive microenvironments (Fidler, 2003).

These six hallmarks of cancer facilitate cancer cell survival, proliferation and spreading. Driving mechanism for this multi-step acquisition are genome instability and inflammation (Colotta et al., 2009; Grivennikov et al., 2010; Negrini et al., 2010). Yet, the core characteristics above seemed to lack distinct attributes regarding tumorigenesis. Hanahan and Weinberg (2011) revised their six hallmarks and proposed two emerging ones: immunoevasion and reprogramming of energy metabolism.

The first one implies the ability to escape immunosurveillance and disruption. This can be achieved by the release of immunosuppressive factors such as transforming growth factor beta (TGF-β) which inhibits cytotoxic T-cells and natural killer (NK) cells, for example (Yang et al., 2010). The second hallmark proposed comprises the reorganization of the energy supply. Chronic hyperproliferation is not only based on cell cycle dysregulation and insensitivity to growth inhibition but requires corresponding adaptions in the metabolic system to sustain the reproductive capacity (Hanahan & Weinberg, 2011).

Normally, cells process glucose in a multistep reaction: pyruvate is produced through the process of glycolysis and then converted to carbon dioxide with the help of oxidative phosphorylation (OXPHOS). This conversion, however, requires oxygen supply, meaning that cells are forced to switch towards glycolysis under anerobic conditions. Contrary to normal cells, cancer can reprogram its energy metabolism towards so-called aerobic glycolysis. In this case, energy production is mainly limited

to glycolysis regardless of environmental conditions such as oxygen supply (Warburg, 1956). This widespread metabolic switch has been intensively studied and substantiated in the last two decades (DeBerardinis et al., 2008; Jones & Thompson, 2009; Zdralevic et al., 2018). Its role in tumorigenicity will be discussed in the following sections. To understand the complex biology of cancer, these hallmarks should be seen as a dynamic theory requiring constant evaluation and reassessment. Hanahan and Weinberg (2011) have established the conceptual framework for many studies including this one.

3.2.1 Stress response in cancer: heat shock proteins

Heat shock proteins (HSPs) represent a heterogenous group of proteins which is part of the molecular chaperone family (Ellis, 2006). The latter displays a diverse protein assortment that assists the (un)folding and (dis)assembly of non-functional macromolecules due to incorrect surface interactions (Ellis et al., 2005). This can occur in newly synthesized, native ,and already folded proteins (Ellis & Hemmingsen, 1989) and ribonucleic acid (RNA) which is also a suitable substrate for chaperones (Ellis, 2006). All organisms ubiquitously express HSPs in different subcellular departments (Chatterjee & Burns, 2017). While chaperones are essential for maintaining homeostasis under normal metabolic conditions, their stress-inducible HSP subset copes with increased amounts of damaged polypeptides to prevent cell death (Craig, 1985).

The so-called heat shock response is a cellular mechanism to deal with a range of physical, chemical, and metabolic stressors, mostly mediated by heat-shock factor 1 (HSF1). Although the molecular trigger for HSF1 activation remains unclear, it is known that the factor undergoes posttranslational modifications and assembles as a homotrimer before entering the nucleus. By binding to the 5’ promoter of *HSP* genes, it serves

as a transcriptional factor orchestrating the synthesis of these chaperones. (Calderwood et al., 2010)

HSF1 recruitment is normally suppressed by heat shock protein 90. However, this formation can be reversed if cellular stress arises. (Zou et al., 1998)

HSPs are subdivided into numerous genetically encoded families: HSP70, small HSPs, HSP90, HSP60, and large HSPs – all classifying similarly structured polypeptides (Kampinga et al., 2009). Small HSPs act independently of adenosine triphosphate (ATP) whereas large ones require the nucleotide for their function (Kumar et al., 2016).

HSPs fulfill a variety of tasks in cellular processes. Their primary role, however, is to serve as molecular chaperones, which comprises the maintenance of innate structures and functions of client proteins (Chatterjee & Burns, 2017; Macario & Conway de Macario, 2007). Besides protein folding, assembly, and proteasome-mediated protein degradation, HSPs fulfill tasks such as cytoskeletal organization, small-molecule transport, and energy generation (Hahn et al., 2004). These characteristic cytoprotective properties of HSPs are inevitable for normal cells when subjected to homeostatic challenges (Lindquist & Craig, 1988). Nonetheless, imbalance in the HSF1-HSP axis, e.g., dysregulated expression, can lead to malignant transformation. Higher metabolic needs, expression of oncoproteins, and inappropriate activation of signaling pathways demand increased chaperonage function (Chatterjee & Burns, 2017). To meet these needs, cancer cells exhibit increased HSP concentrations. This mechanism is triggered by an overactivation of HSF1 (Mendillo et al., 2012). Furthermore, this set of evolutionary conserved proteins is capable of opposing cell death through caspase-dependent as well as senescence pathways (Garrido et al., 2006). Particularly, the latter stresses the importance of heat shock proteins and their crucial role in the

pathophysiological changes of aging and apoptosis. Specific interactions and functions of concrete chaperones are discussed in the following sections.

3.2.1.1 Hsp70

13 members constitute the HSP70 family, all of which play crucial roles in cell survival and growth (Rosenzweig et al., 2019). These members are subdivided into four major groups with a molecular weight of 70 kDa: constitutively expressed Hsp70 (Hsp73), mortalin, Grp78, and stress-inducible Hsp70 (Moradi-Marjaneh et al., 2019). Hsp70.1 (Hsp72, Hsp70, HSPA1A), the major stress-inducible member of the HSP70 family, is described as the key representative of the latter group. HSP70 is widely spread over the nucleus and cytosol, plus two members also reside in mitochondria and the endoplasmic reticulum, where they can bind a variety of substrates from primary protein stretches to complex tertiary structures due to their flexible interaction mechanism (Ciocca et al., 2013). Structurally, HSP70 incorporates an N-terminal nucleotide-binding region for ATPase function and is connected to a C-terminal substrate-binding domain via a J-domain (Kumar et al., 2016). Co-substrates regulate the chaperonage function either by silencing or enhancing ATPase activity by binding to different regions (Moradi-Marjaneh et al., 2019). Besides maintaining a normal cell's homeostasis by (re)assembling polypeptides, preventing their aggregation, and assisting cross-membrane transport (Hartl, 1996), Hsp70 is also highly active in cancer cells with regard to survival and tumorigenicity (Aghdassi et al., 2007; Rohde et al., 2005). Herein, a major aspect apart from its metastatic potential (Calderwood et al., 2006) and the disturbance of lysosomal permeabilization (Nylandsted et al., 2004) is the ability to inhibit apoptosis. Specific regulators, such as apoptotic protease-activating factor 1 (Apaf-1) (Beere et al., 2000) and apoptosis-inducing factor (Ravagnan et al., 2001) are bound by Hsp70 on

many levels of pre-and post-caspase recruitment. Further targets are the tumor suppressor p53 (Fourie et al., 1997) and apoptosis signal-regulating kinase 1 (Park et al., 2002) which are either inhibited or degraded after binding. However, the role of the HSP70 family is multidimensional and therefore not limited to promoting cell death resistance.

Several observations have been made confirming Hsp70's contribution to pro-apoptotic mechanisms, such as the sensitization to certain stimuli like tumor necrosis factor alpha (TNF- alpha) (Ran et al., 2004). Moreover, members of the HSP70 group are involved in signaling pathways that especially promote proliferation and survival in colorectal carcinoma cells. Besides stimulating the extracellular signal-regulated kinase (Zhou et al., 2016), Hsp70 urges downstream activation of PI3/AKT and WNT/β-Catenin via autocrine signaling (Fu et al., 2014) both of which are crucial in CRC pathogenesis. Hsp70 has also been reported to modulate epithelial-mesenchymal transition of colorectal cancer cells, which enables dissemination and metastatic spread (Chang et al., 2015).

However, Hsp70 is not only expressed intracellularly, but also on the plasma membrane. This property was only found in tumor cells, which further distinguishes them from normal tissue cells (Multhoff, 2007). Under physiological conditions, membrane Hsp70 (mHsp70) colocalizes with the lipid raft glycosphingolipid globotriaosylceramide (Gb3), whereas increased stress levels lead to an association with phosphatidylserine (PS), probably as a result of the switch to the outer membrane (Gehrmann et al., 2008).

Cellular stress factors such as heat, hypoxia, irradiation, and drugs induce increased levels of cytosolic and membrane Hsp70, indicating their major role in recovery. Moreover, membrane-bound and extracellular Hsp70 have immunomodulatory effects. After being recognized by pre-activated NK cells, Hsp70 can stimulate the innate immune system (Multhoff et al.,

2001). It can further initiate a response of the adaptive immune system when loaded with immunogenic tumor-deprived peptides in terms of antigen cross-presentation (Binder, 2008). In contrast to normal cells, tumors can actively release Hsp70 in lipid microvesicles with biophysical properties of exosomes via an alternative pathway that does not include the endoplasmic reticulum or the Golgi apparatus (Gastpar et al., 2005). These amounts of secreted Hsp70 positively correlate with the viable tumor volume and pro-/regression (Bayer et al., 2014). Hence, the properties and functions of Hsp70 far exceed the maintenance of homeostasis, playing a crucial role in the pathophysiology of malignant transformation.

3.2.1.2 Hsp90

The constitutively expressed Hsp90β and stress-inducible Hsp90α isotypes belong to the HSP90 family, which comprise four members in total. Both, Hsp90α and β, form homo- or heterodimers and can perform ATP-dependent chaperone activity making up to 2% of a cell's total protein amount. The ratio between both isotypes varies and shifts in favor of Hsp90α under stressful conditions (Ciocca et al., 2013). Besides its assembly and folding duties, Hsp90 assists the maturation as well as the membrane-translocation of signaling molecules usually in association with co-chaperones (Buchner, 1999).

Hsp90 is further involved in many crucial mechanisms of carcinogenesis, including the stabilization of mutant proteins (e.g., growth factor receptors, survival-signaling kinases), metastasis, and environmental-independent growth signaling (Ciocca et al., 2013; Taipale et al., 2010). Many of these oncoproteins, such as TGF-β, Rapidly Accelerated Fibrosarcoma (RAF) proteins, MAP-kinase, 3-phosphoinositide-dependent protein kinase 1 (PDPK1), mechanistic Target of Rapamycin (mTOR), and glycogen synthase kinase-3, serve as Hsp90 clients due to their dependence on

chaperones. Consequently, they can only sustain accelerated growth in the presence of Hsp90 for stabilization. These signaling pathways and their corresponding oncogenic proteins are of great importance in the development and progression of CRC (Kryeziu et al., 2019; Lee et al., 2015a).

Moreover, Hsp90 achieves inhibition of apoptosis via cytosolic pathways by antagonizing Apaf-1 or activating cytochrome c (Pandey et al., 2000). According to Fulda et al. (2010) anti-apoptotic activity also results from high Hsp90 levels found within the mitochondria of cancer cells. Targeting Hsp90 is therefore likely to affect many precancerous signaling pathways, which can potentially have a lethal effect on cancer cells.

3.2.1.3 Hsp27

Hsp27 (HspB1) represents one of the 10 structurally similar small heat shock proteins with a molecular weight ranging from 20-40 kDa. Some of these, including Hsp27, are stress-inducible and feature ATP-independent chaperone activity. (Ciocca et al., 2013) This way, misfolded proteins can be retained to prevent aggregation under stress conditions before they are refolded or degraded (Parcellier et al., 2006). This is usually performed in cooperation with the Hsp70-Hsp90 complex (Parcellier et al., 2006). Hsp27 further participates in promoting cellular resistance to apoptosis by interacting with crucial agents like procaspase-3 (Gibert et al., 2012). Moreover, it stimulates tumor progression, resistance to several anti-cancer drugs (Arrigo, 2000; Kang et al., 2008) and metastasis (Katoh et al., 2000). The latter could be explained, at least in part, by its ability to influence the cytoskeleton and, thus indirectly, the extracellular matrix (Dalle-Donne et al., 2001). This broad variety of functions results from the structural plasticity allowing rapid adaptation to environmental changes so that appropriate client proteins can be recognized and bound effectively (Paul et al., 2010). Hsp27-client-interaction leads to activity or half-life

modulation of the targeted protein (Ciocca et al., 2013). Targeting the specific binding domain for pathological clients might be a promising therapeutic approach, which has already been investigated in (pre)clinical studies (Gibert et al., 2011; J. C. Heinrich et al., 2011).

3.2.1.4 HSP: Role in carcinogenesis and tumor development

Contrary to their role in a normal cell's metabolism and cycle, much less is known about HSPs and their contribution to carcinogenesis. Cancers from a wide range of histological entities such as breast, prostate, lung, ovarian, gastric, esophageal, and colorectal cancer have been reported to express elevated HSP levels (Ciocca, Oesterreich, et al., 1993; Fuller et al., 1994; Love & King, 1994; Saini & Sharma, 2018; Tang et al., 2005; Yun et al., 2019) as well as activated HSF1 (Mendillo et al., 2012; Santagata et al., 2011; Tang et al., 2005). The malignant phenotype emerges from 'hallmarks' comprising several biological capabilities (Hanahan & Weinberg, 2011). These are acquired through the multistage progression of a tumor involving HSF1 and HSPs. A popular hypothesis suggests that HSP levels increase through the proliferation of mutated proteins requiring stabilization to sustain cancer growth and to counteract proteotoxic stressors (Ciocca et al., 2013). Inhibiting this mechanism, e.g., targeting Hsp90, leads to the destruction of associated oncoproteins. (Trepel et al., 2010)

As indicated before, HSF1 and HSP were observed to promote resistance to cell death by impairing the caspase-dependent apoptosis pathway (Gabai et al., 2005). Likewise, replicative senescence was affected possibly due to the antagonization of wildtype p53 (Sherman et al., 2011). Thus, elevated HSP levels have been described to positively affect tumor growth and progression due to their ability to modulate key dysregulated signaling pathways involving PI3/AKT, WNT/ β-Catenin, MAP-kinase, and TGF-β (Lee et al., 2015a). HSPs are further involved or at least associated

with increasing metastatic potential, tumor invasion and metabolic reprogramming towards aerobic glycolysis (Ciocca et al., 2013).

3.2.1.5 Clinical implication and therapy relevance

The HSF1-HSP axis is a concept of great complexity with a relevance expanding far beyond the molecular context. HSPs are of diagnostic, prognostic, as well as therapeutic value. In patients with hepatocellular carcinoma Hsp70 serum levels were found to be significantly higher compared to those with chronic inflammation or the control group (Gehrmann et al., 2014). This confirms the results of mouse studies, which demonstrated a positive correlation between the serum levels and tumor volume/progression (Bayer et al., 2014). Studies in lung cancer patients displayed similar results (Ren et al., 2017). Those findings imply that HSP serum levels could serve as a potential non-invasive biomarker to monitor and diagnose cancer in the future. Albakova et al. (2021) summarized the correlations between HSP levels and cancer diseases that are currently under investigation.

It was repeatedly discovered that these chaperones have a prognostic relevance: high Hsp27 levels in over seven cancer types were found to be associated with poor clinical outcome (Ciocca et al., 2013). Furthermore, high levels are linked to shorter survival rates (Klimczak et al., 2019; Yu et al., 2010). Similar associations in terms of prognostic significance have been discovered for Hsp70 (Ciocca, Clark, et al., 1993; Nanbu et al., 1998; Thanner et al., 2003; Thomas et al., 2005).

In CRC, Hsp70, as well as Hsp27, were found to be correlated with worse clinical outcome, poor prognosis, and survival (Bauer et al., 2012; Ge et al., 2018; Hrudka et al., 2021).

Another aspect of clinical importance is the ability of HSP to mediate therapy resistance. Besides the cytoprotective property of protein refolding, degradation, or the disruption of apoptosis induction (Arrigo et

al., 2002), HSPs can also positively interact with DNA repair proteins, e.g., mismatch repair genes human mutL homolog 1 (MLH1) and human mutS homolog 2 (MSH2), and therefore contribute to DNA repair (Nadin et al., 2007; Sottile & Nadin, 2018). Even the susceptibility of hormone therapy, a crucial pillar in fighting breast or prostate cancer, is inflicted by HSPs due to their interaction with specific receptors (Ciocca et al., 2010; Kang et al., 2008). The success of chemotherapy is further limited by the acquisition of multi-drug resistance. The underlying mechanism here involves the cellular efflux of substances via ATP-binding cassette transporters, the expression of which is linked to HSF1. (Tchénio et al., 2006) Thus, HSPs assist in the formation of a mutated p53-oncoprotein complex known to diminish chemosensitivity (Wawrzynow et al., 2018).

It has been repeatedly demonstrated that the efficacy of radiation is also impaired (Hadchity et al., 2009; Hunt et al., 2004; Teimourian et al., 2006). The ability of Hsp70 to interact with and stabilize a tumor cell's telomerase (Agarwal et al., 2008) or the involvement of Hsp27 in decreasing anti-oxidative glutathione levels (Aloy et al., 2008) places an additional burden on radiosensitivity. Damage caused by ionizing irradiation can therefore be counteracted by HSPs. Membrane-bound Hsp70 appears to be of great importance here (Ciocca et al., 2013; Multhoff et al., 2015; Murakami et al., 2015).

The investigation of HSPs, however, does not only focus on mediated effects in established therapeutic procedures. Due to their enormous relevance in carcinogenesis, HSPs are considered to have great potential as therapeutic targets. Besides newer immunotherapeutic approaches, e.g., vaccinations (Binder, 2008) and NK-cell infusion (Krause et al., 2004), inhibition of HSPs became an excessively studied strategy with promising results.

The proof-of-concept for targeting HSPs has been accomplished by the excessive evaluation of HSP90 inhibitors. Whitesell et al. (1994) discovered the first specific inhibitor, Geldanamycin, which targets the chaperone's N-terminal ATP-binding domain. Since subsequent studies have proven its therapeutic potency, novel derivates such as 17-(allylamino)-17-demethoxy-geldanamycin (17-AAG) or 17-desmethoxy-17-*N,N*-dimethylaminoethylaminogeldanamycin (17-DMAG)) have been developed to overcome the hepatotoxicity and poor water solubility (Shevtsov et al., 2019). Although the thoroughly investigated 17-AAG presented even higher *in vitro* efficacy compared to its precursor, subsequent clinical studies failed to demonstrate its therapeutic success (Gartner et al., 2012; Pacey et al., 2012; Pedersen et al., 2015).

Despite their promising results in cancer cell killing, more than 18 Hsp90 inhibitors, which have been investigated in clinical studies, didn't get the Food and Drug Administration's (FDA) approval. Most of the drugs proposed even failed to pass Phase I. Limitations comprise dose-dependent toxicities and unfavorable pharmacokinetic properties. (Sanchez et al., 2020) Moreover, the inhibition of HSP90 leads to a compensatory upregulation of HSP70 counteracting the cellular stress caused by the drug application which further decreases the efficacy (Goloudina et al., 2012).

Combinatorial regimes of HSP90 inhibitors with conventional treatment modalities (chemo-/radiotherapy) (He et al., 2014), targeted therapy (kinase inhibitors) (Vaishampayan et al., 2010) or even immunotherapy (check-point-inhibitors) (Proia & Kaufmann, 2015) seem to bear higher therapeutic potential compared to monotherapy.

Combined therapy was also tested for HSF1 inhibitors. For instance, the benzylidene lactam compound represents one of the HSF1 inhibitors tested in clinical studies, where higher therapeutic efficacy could be

reached when combined with bortezomib (Bustany et al., 2015). Likewise, strong anticancer activity was found for the HSF1 inhibiting compound KRIBB11 (N(2)-(1H-indazole-5-yl)-N(6)-methyl-3-nitropyridine-2,6-diamine) when combined with danusertib (Shen et al., 2021).

Contrary to the presumption that HSF1 impacts tumor biology only indirectly through initiating HSP expression, an alternate concept has been proposed. HSF1 was identified to independently promote invasion and metastasis by regulating a distinct transcriptional program. These activated cellular processes are not only limited to the heat shock response but also comprise cell cycle signaling and energy metabolism. (Mendillo et al., 2012)

RP101 (Brivudine), a nucleoside that directly inhibits Hsp27, did not only display chemo-sensitizing properties *in vitro* but was also successful in clinical trials. An increase of the overall-survival rate in patients with pancreatic cancer could be accomplished when Brivudine was applied together with gemcitabine. Clinical application, however, is limited due to its toxic side effects. (J.-C. Heinrich et al., 2011)

The antisense oligonucleotide, apatorsen, which reduces the expression of Hsp27 by targeting its messenger ribonucleic acid, has also demonstrated promising *in vivo* results. Mouse-models showed that this drug led to a decrease in tumor size when combined with chemotherapeutic agents (Lelj-Garolla et al., 2015). Clinical studies reported that apatorsen combined chemotherapy achieved a prolonged progression-free and overall-survival in patients with metastatic pancreatic cancer. Unfortunately, this effect could only be detected in a small proportion of patients. (Ko et al., 2017)

The search for Hsp70 inhibitors led to the discovery of a variety of pharmacologically different agents (Albakova et al., 2021; Chatterjee & Burns, 2017). Blocking of the chaperone's ATP-binding site is the

underlying mechanism that unites these inhibitors. (Kumar et al., 2016) After many attempts, however, only few have proven to be successful (Chatterjee & Burns, 2017). 15-deoxyspergualin, a dihydropyrimidine-derived inhibitor, is one of the few substances tested in clinical trials. Yet, due to the frequent occurrence of neuromuscular side effects, it could not pass Phase II. (Eguchi et al., 2018) Other inhibitors with dihydropyrimidine compounds have not displayed convincing *in vivo* results so far (Moradi-Marjaneh et al., 2019). The rhodacyanine-derived agent MKT-077 targets the nucleotide-binding site, resulting in altered chaperoning function (Chatterjee & Burns, 2017). Although clinical studies have demonstrated significant anti-proliferative activity, its application as an anti-cancer agent is limited due to rapid metabolization and nephrotoxicity (Britten et al., 2000).

So far, most of the research on HSP inhibitors has been conducted *in vitro* since *in vivo* application is often limited due to unfavorable bioavailability or severe side effects (Chatterjee & Burns, 2017). Thus, targeting distinct HSP members such as Hsp90 can be counteracted by HSF1 release and subsequent activation, which in turn leads to increased Hsp70 synthesis (Prince et al., 2018; Shevtsov et al., 2019). Presumably, the combination of HSP inhibitors with conventional anti-cancer treatment as well as the simultaneous application of different HSP inhibitors might improve the anti-tumor potency and clinical outcome. (Du et al., 2022; Kumar et al., 2016; Nagaraju et al., 2014; Shevtsov et al., 2019).

3.2.2 Cancer metabolism

3.2.2.1 Lactate dehydrogenase

The lactate dehydrogenase (LDH) is the key glycolytic enzyme, which catalyzes the reversible alteration of pyruvate to lactate linked to the oxidation or reduction of nicotinamide adenine dinucleotide (NAD): NAD+

represents the oxidated form whereas NADH represents the reduced form (Figure 2).

In its active form, the enzyme appears as a tetramer, consisting of the subunits M (LDHA) and H (LDHB), which are coded separately in the genome. There are five isoenzymes, LDH1-5, which vary in their amounts of M and H units and are associated with specific tissue distribution. (Markert & Møller, 1959; Zdralevic et al., 2018)

The homotetramer LDH-1, consisting of four H subunits, and LDH-5 the counterpart composed of M components, proved to be the most metabolically active (Read et al., 2001).

Contrary to the common assumption that only one unit, LDHA, catalyzes the reduction of pyruvate, while LDHB enables the reverse reaction, recent studies (Zdralevic et al., 2018) have shown that both metabolites are suitable substrates for LDHA and B.

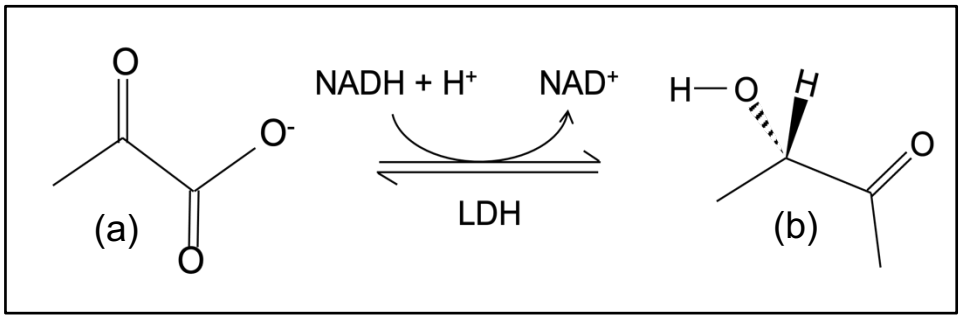


Figure 2. Conversion of pyruvate (a) to lactate (b) catalyzed by LDH.

3.2.2.2 Role of metabolism in carcinogenesis

A major characteristic of tumor cells, distinguishing them from normally differentiated ones, is increased glycolysis and therefore glucose consumption, followed by lactate formation (Warburg, 1925). This phenomenon known as the ‘Warburg effect’ originates from high metabolic demands and fast growth rates and can even be observed in aerobic environments (Liberti & Locasale, 2016; Vaupel & Multhoff, 2020). In

contrast, normal cells would primarily rely on oxidative phosphorylation under these conditions.

At first, this metabolic switch seems counterintuitive, as cells must compensate the 18-fold lower efficiency, since glycolysis only generates two ATPs, whereas 38 could potentially be gained via oxidative phosphorylation. To face this discrepancy, cancer cells expand their capacities to perform glycolysis on a larger and faster scale. Upregulation of glucose transporters via GLUT1, enhances the cytoplasmic influx of glucose. There, it can be further processed. (Hanahan & Weinberg, 2011; Hsu & Sabatini, 2008; Wieman et al., 2007)

The herein-formed lactate can be recycled to serve as an energy fuel. Hypoxic cells secrete lactate, which can then be incorporated into oxygenated cells. After import via the monocarboxylate transporter 1, lactate can be reconverted in the citric acid cycle and used for oxidative phosphorylation. Considering the varying environmental conditions within a population, this symbiosis meets the metabolic requirements. Therefore, cancer cells can generate energy independent of oxygen supply, which is a great advantage regarding the hypoxic conditions many tumors are confronted with. (Feron, 2009; Hardee et al., 2009)

In addition to ATP synthesis, increased amounts of glucose can also be used for anabolic reactions (Kroemer & Pouyssegur, 2008). Intermediates and metabolites generated during this process can be utilized as substrates for biosynthetic pathways. Products such as nucleotides and amino acids can, in turn, be used for the synthesis of macromolecules required for active cell proliferation (Vander Heiden et al., 2009).

Aerobic glycolysis not only offers numerous survival benefits, but also promotes the progression and spread of malignancies (Vaupel et al., 2019). High lactate levels lead to acidification, thus creating an immunosuppressive environment that interferes with the functions of

cytotoxic T lymphocytes and NK cells (Brand et al., 2016; Fischer et al., 2007). Lactate further contributes to immune escape by impairing cytokine release and inhibiting the differentiation of monocytes. (Hirschhaeuser et al., 2011) These circumstances condition the tumor's stroma to form a functional and co-dependent unit, which in turn promotes the processes mentioned above (Swietach et al., 2007).

This metabolic switch occurs at an early stage and is driven by several tumorigenic mechanisms. Activation of the c-myc oncogene, for instance was linked to the induction of LDHA, the expression of which increases lactate production required for c-myc-mediated transformation (Shim et al., 1997). Dysregulation of signaling pathways results in constitutive activation of growth signals, which results in metabolic reprogramming. Self-sufficient activation of the PI3K/Akt system enhances glucose and amino acid flux due to Akt stimulated GLUT1 expression, glucose transporter 4 translocation (GLUT4) and phosphorylation of 6-phosphofructo-2-kinase which directly stimulates glycolysis. In addition, PIK3 inhibits β-oxidation trough transcriptional downregulation of membrane transporters needed for fatty acids, thus promoting the so-called glucose addiction of cancer cells. (Kroemer & Pouyssegur, 2008)

Besides, tumorigenic mutations can result in the loss of the tumor suppressor p53, which directly induces the Warburg phenomenon. Since p53 stimulates the expression of OXPHOS related proteins such as cytochrome c oxidase 2 (Matoba et al., 2006), while being responsible for downregulation of the glycolytic enzyme phosphoglycerate mutase (Kondoh et al., 2005), its inactivation inevitably increases lactate production.

In addition to tumor suppressors and oncogenes, one of the most important mechanisms responsible for this change, is the activation of hypoxia-inducible factor (HIF1). Inflammation, hypoxia, metabolic and

oxidative stress all trigger HIF1 activation which facilitates the transcription of genes associated with glycolysis (Kroemer & Pouyssegur, 2008). Furthermore, HIF-1 concomitantly induces anabolic pathways such as the synthesis of lipids or glycogen favoring the glucose storage for later use (Krishnan et al., 2009; Pescador et al., 2010). A balance between catabolism and anabolism is achieved by the adenosine-monophosphate-(AMP) activated protein kinase, which reacts to the AMP/ATP ratio (Brahimi-Horn et al., 2011).

Moreover, HIF-1 initiates epithelial-to-mesenchymal transition, a mechanism demonstrated to promote cell survival by allowing further oxygen-independent invasion of the tissue (Brahimi-Horn et al., 2011).

LDH represents the key player of the Warburg effect, which is responsible for the glycolytic phenotype (Warburg, 1925). Its subunit, LDHA, is frequently overexpressed in cancer cells and known to be HIF-1 regulated (Hirschhaeuser et al., 2011). LDHA and its role in tumor progression have been examined in lots of studies. The homotetramer LDH-5, consisting of four M subunits, was found to be frequently expressed in many tumors, particularly in highly aggressive ones (Yao et al., 2014). Recent studies, however, showed that its corresponding subunit B should not be underestimated either regarding its contribution to tumorigenesis (McCleland et al., 2013).

The glycolytic phenotype is therefore closely linked to the oncogenic transformation, maintenance, and progression (DeBerardinis et al., 2008; Le et al., 2010). Thus, the metabolic reprogramming is not only a cancer associated characteristic that occurs in 70-80% of human cancers (Vaupel & Multhoff, 2020) and is a distinctive feature, but also provides favorable conditions for the development of the other six hallmarks. Hence, aerobic glycolysis should be considered a promising therapeutic target regarding

its role in tumorigenesis and the codependence between other hallmarks. (Hanahan & Weinberg, 2011)

3.2.2.3 Clinical implication

High glucose-to-pyruvate turnover and increased lactate production correlate with aggressive tumor phenotypes. Tumors with increased lactate levels display a significantly higher incident of distant metastases (Walenta et al., 2000). Lactate accumulation due to increased LDH activity, especially LDH-5, inversely correlates with patient survival in primary cervical cancer, head and neck squamous cell and colorectal carcinoma whereas low lactate levels are associated with longer overall and disease-free survival (Walenta & Mueller-Klieser, 2004; Walenta et al., 2000). Similar results were found in patients with glioblastoma multiforme, where high lactate levels correlated with poor survival (Saraswathy et al., 2009).

To assess its role in tumorigenicity, several studies investigated the effects of either LDHA inhibition or knockout which resulted in anti-proliferative effects (Augoff et al., 2015), tumor growth suppression (Sheng et al., 2012) and even regression (Xie et al., 2014), *in vitro* and *in vivo*. Even though less is known about the role of LDHB, further findings also indicate a contribution to tumor progression in several entities (McCleland et al., 2012).

Moreover, increased LDH activity correlates with impaired responses to radio-, chemo- and immunotherapy (Koukourakis et al., 2006; Koukourakis et al., 2011; Sattler, Meyer, et al., 2010). This can partly be explained by the antioxidant properties of lactate, which can compensate the overproduction of reactive oxygen species (ROS) as a result of cell stress caused by anticancer therapy (Sattler, Hirschhaeuser, et al., 2010).

Bearing those clinical findings in mind, LDH displays a promising target in cancer therapy, as it enables a decisive impairment of the tumor's metabolic system.

3.2.2.4 GNE-140

Much effort has been spent developing suitable agents to target LDH. The first inhibitor described was the natural analog of pyruvate – Oxamate (Novoa et al., 1959). Since the importance of LDH has been demonstrated repeatedly, the search for suitable inhibitors continues and has led to the discovery of several more, mostly targeting LDH-5. Currently, the inhibitors, which consist of either natural or synthetic molecules, can be categorized mainly according to the following criteria: pyruvate-competitive, NADH-competitive, free enzyme-binding or other mechanisms (Cheng et al., 2021).

Inhibitors such as 3-((3-carbamoyl-7-(3,5-dimethylisoxazole-4-yl)−6-methoxyquinolin-4-yl) amino) benzoic acid (Billiard et al., 2013), FX11 [3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid]) (Le et al., 2010) or N-hydroxyindole-based agents (Granchi et al., 2011) mimic the substrates or cofactors interaction with the enzyme whereas others like Galloflavin, for example, are non-competitive (Farabegoli et al., 2012). Only few of these inhibitors, however, are suitable for experimental use as they are not selective or lack cellular activity, leading to non-specific toxicity.

Besides, *in vivo* practice requires not only efficacy at the cellular level but also suitable pharmacokinetic properties such as good oral bioavailability and adequate clearance. Hence, most of the inhibitors above are limited to *in vitro* practice. (Billiard et al., 2013; Dragovich et al., 2013; Fauber et al., 2013; Kim et al., 2019).

GNE-140 was first described and developed by Dragovich et al. (2013). Its chemical structure is depicted in Figure 3. This novel small-molecule

LDH inhibitor manages to target LDHA and LDHB with a half maximal inhibitory concentration (IC50) of 0.003 and 0.005 μM without affecting structurally similar dehydrogenase enzymes, thus indicating a high substrate specificity (Boudreau et al., 2016). The ability to inhibit both subunits increases efficiency in terms of the fact that LDHA and LDHB can compensate for the deficiency of the other subunit under metabolic pressure (Zdralevic et al., 2018). According to 2.2-Å X-ray structure, GNE-140 binds to the active site of the enzyme via a hydrogen bridge bond, adjacent to the cofactor NADH, which means that it acts as a non-competitive inhibitor (Boudreau et al., 2016). Regarding its high potency, specificity, and favorable pharmacokinetic properties, GNE-140 can be utilized for *in vitro* as well as for *in vivo* studies (Boudreau et al., 2016).

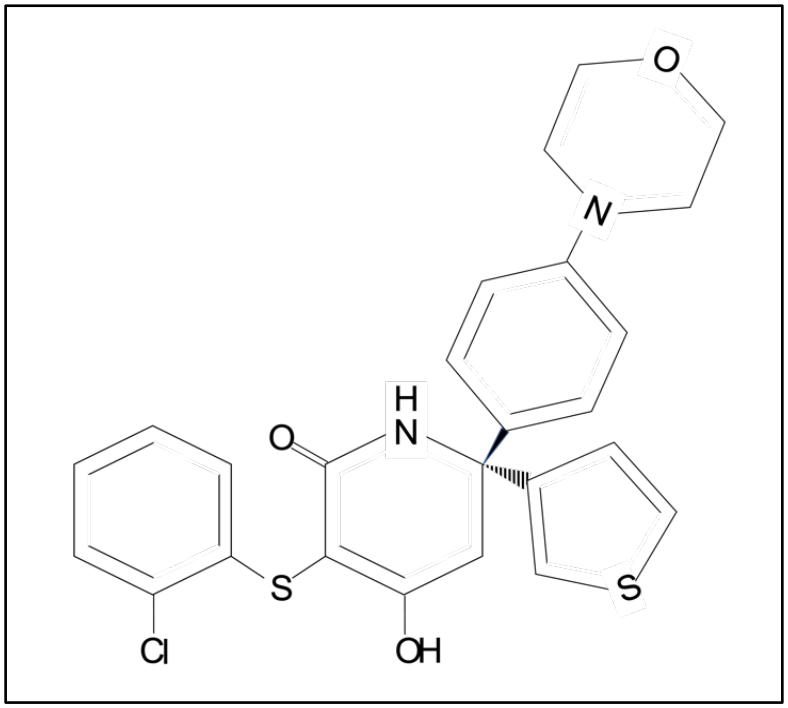


Figure 3. Chemical structure of GNE-140.

4 Aim of the study

Colorectal carcinoma represents one of the most common malignancies worldwide. Despite the variety of therapeutic regimes including surgery, combined chemotherapeutic regimes, molecular targeting, and irradiation it remains a major cause of cancer-related deaths. Rising incidence and high mortality rates further stress the need for novel therapy approaches. (Ferlay et al., 2020; Sung et al., 2021)

One major characteristic uniting CRC with a variety of other tumor cell lines is the overexpression of heat shock proteins (Chen et al., 2011; Ciocca & Calderwood, 2005; Gunaldi et al., 2015; Kocsis et al., 2010). This subset of chaperones is known to immensely support tumor cell survival and resistance to apoptosis (Soo et al., 2008). Knowledge regarding the interaction of the stress response with the lactate metabolism, however, is currently limited.

The glycolytic phenotype of tumors, which results from increased glucose uptake due to high metabolic demands as it was described by Warburg (1925), not only maintains malignancy, but also promotes aggressiveness via immunosuppression (Brand et al., 2016; Fischer et al., 2007) and increases the metastatic potential (Swietach et al., 2007).

Both mechanisms – the stress response system and the aerobic glycolysis – have repeatedly been demonstrated to play a decisive role in tumorigenesis. In order to assess whether there is a dependence between these two major pillars of cancer, LDH, the key enzyme of lactate metabolism, was specifically targeted using the inhibitor GNE-140.

The intervention's effects on the HSP-HSF1 axis were determined by analyzing protein expression levels both intracellularly and on the membrane.

Thus, this work aimed to study, whether these metabolic changes would affect the phenotype, since increased heat shock protein expression is

known to mediate radioresistance. In this respect, radiosensitivity was determined in LDH-suppressed cells compared to untreated control cells. Despite auspicious discoveries from *in vitro* studies, the direct targeting of HSPs has not been proven to be a successful strategy so far. Regarding its profound contribution to tumorigenicity, impairing the HSP-HSF1 axis is nevertheless a promising approach. Moreover, pharmacological targeting of LDH might inflict the stress response system in a way that sensitizes colorectal cancer cells to break therapy resistance.

The overall aim of this study was therefore to investigate the relationship between lactate metabolism and HSP expression in the human colorectal adenocarcinoma cell line, LS174T, as a basis to develop more efficient therapy approaches for CRC.

5 Material and methods

5.1 Material

5.1.1 Chemicals and reagents

Product	Manufacturing company
Acetic acid	Roth, Karlsruhe, Germany
Acrylamide/Bis Rotiphorese 40%	Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Roth, Karlsruhe, Germany
Ampicillin	Roth, Karlsruhe, Germany
β-Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
Bromophenol blue	Serva, Heidelberg
Crystal violet 0.1%	Sigma-Aldrich, Taufkirchen, Germany
Dimethyl sulfoxid (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
Ethanol	Brüggemann, Heilbronn, Germany
GNE-140 (LDH A/B/C IC50 = 3/5/5 nM)	Sigma-Aldrich, Taufkirchen, Germany
Methanol	Sigma-Aldrich, Taufkirchen, Germany
Propanol	Otto Fischar, Saarbrücken
Propidium-iodid (PI)	Thermo Fisher Scientific, Waltham, Massachusetts (MA), United States of America (USA)
Ponceau S	Sigma-Aldrich, Taufkirchen, Germany
Skim milk powder	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe, Germany
Sodium deoxycholate	Roth, Karlsruhe, Germany
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe, Germany
Tris(hydroxymethyl)aminomethane (TRIS)	Roth, Karlsruhe, Germany
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany
Tween20	Sigma-Aldrich, Taufkirchen, Germany

Table 2. List of chemicals and reagents.

5.1.2 Cell culture media and supplements, tissue culture flasks

Product	Manufacturing company
12-well, 96-well plates	Sigma-Aldrich, Taufkirchen, Germany

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, Taufkirchen, Germany
Eppendorf tubes (1,5 ml, 2 ml)	Sarstedt AG, Nümbrecht, Germany
Falcon® Round Bottom Polystyrene Tubes (5 ml)	Corning, Corning, New York, USA
Fetal Bovine Serum (FBS)	Sigma-Aldrich, Taufkirchen, Germany
L-glutamine	Sigma-Aldrich, Taufkirchen, Germany
Penicillin/Streptomycin (10,000 IU/ml; 10 mg/ml) (PS)	Sigma-Aldrich, Taufkirchen, Germany
Phosphate buffered saline (PBS)	Sigma-Aldrich, Taufkirchen, Germany
Sodium pyruvate	Sigma-Aldrich, Taufkirchen, Germany
T25, T75 CELLSTAR® cell culture flasks	Greiner Bio-One International GmbH, Frickenhausen, Germany
tips (1 ml, 250 µl, 20 µl)	Gilson, Middleton, USA
Trypsin-ethylene-diamine-tetra-acetic acid (EDTA) solution	Sigma-Aldrich, Taufkirchen, Germany

Table 3. List of cell culture media, supplements and tissue culture flasks.

5.1.3 Buffers

Buffer	Composition	Application
Fluorescence-activated cell scanning (FACS) buffer	– PBS – 10% FBS	FACS
Loading buffer, 4× concentrated	– 8% SDS – 40% glycerol – 0.008 % bromophenol blue – 250 mM TRIS – 20% β-mercaptoethanol	Gel electrophoresis
Ponceau dilution	– 0,1 % Ponceau S – 5 % acetic acid	Western Blot
Radioimmunoprecipitation assay (RIPA) buffer	– 150 mM NaCl – 1 mM EDTA – 1% Triton X-100 – 50 mM Tris, pH 8.0	Sample preparation for Western Blot

	– 0.5% sodium deoxycholate – 0.1% SDS – Protease inhibitor cocktail (Roche, Basel, Switzerland)	
Running buffer, 5× concentrated	– 125 mM TRIS – 1,25 M glycine – 0.5% SDS	Gel electrophoresis
Transfer buffer	– 38 mM glycine – 48 mM TRIS – 2.88 M methanol – 0.04% SDS	Western Blot
Tris-buffered saline (TBS), 5× concentrated	– 100 mM TRIS – 680 mM NaCl	Western Blot
TBS with Tween20 (TBST)	– ×1 TBS – 0.05% Tween20	Western Blot

Table 4. List of buffers.

5.1.4 Molecular biology kits

Product	Manufacturing company
Lactate Dehydrogenase Activity Kit	Sigma-Aldrich, Taufkirchen, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
Pierce™ ECL Western Kit	Thermo Fisher Scientific, Waltham, MA, USA

Table 5. List of molecular biology kits.

5.1.5 Antibodies

Antibody	Code	Donor	Features	Dilution	Manufacturing company
Anti-ß-Actin	A2228	mouse	primary	1:10,000	Sigma-Aldrich, Taufkirchen, Germany
Anti-Hsp27	NBP2-32972	mouse	primary	1:1,000	Novus Biologicals, Centennial, Colorado, USA
Anti-Hsp70 IgG1	cmHsp70.1	mouse	primary	1:500	multimmune, Munich,Germany
Anti-membrane-Hsp70 IgG1	mAb	mouse	primary, fluorescein isothiocyanate (FITC)-labeled	1:15	multimmune, Munich,Germany
Anti-Hsp90	4874	rabbit	primary	1:1,000	Cell Signaling Technology, Danvers, MA, USA
Anti-mouse immunoglobulin G (IgG)	P0260	rabbit	secondary	1:2,000	Dako-Agilent, Santa Clara,

					California (CA), USA
Anti-rabbit IgG	P0217	swine	secondary	1:1,000	Dako-Agilent, Santa Clara, CA, USA
Isotype matched control immunoglobulin subclass G1 (IgG1)	345815	mouse	primary, FITC-labeled	-	BD Biosciences, Heidelberg, Germany

Table 6. List of antibodies.

5.1.6 Cell lines

Cell line	Dilution	Type	Medium	Supplier
LS174T wildtype	0.06×10^6 cells/ml	Human colorectal adenocarcinoma cell line	DMEM + 10% FBS + 1% P/S + 2 mM L-glutamine + 1 mM sodium pyruvate	ATCC, Manassas, USA
LS174T *LDHA/B* double knockout	0.12×10^6 cells/ml	Human colorectal adenocarcinoma cell line	DMEM + 10% FBS + 1% P/S + 2 mM L-glutamine + 1 mM sodium pyruvate	Kindly provided by Marina Kreutz and Jacques Pouyssegur

Table 7. List of cell lines.

5.1.7 Instruments and devices

Product	Manufacturing company
Amersham™ Hybond™ ECL Nitrocellulose Membrane	GE Healthcare UK LIMITED, Amersham Place, United Kingdom

Bioreader® 3000	Bio-Sys GmbH, Karben, Germany
Cell Culture CO_2-Incubator Hera cell 240i	Thermo Fisher Scientific, Waltham, MA, USA
ChemiDoc™ Touch Imaging System	Bio-Rad, Hercules, CA, USA
CellRad compact benchtop X-ray irradiator	Precision X-Ray, North Branford, CT, USA
CELLSTAR® serological pipette 2/5/0/5/50 ml	Greiner Bio-One International GmbH, Frickenhausen, Germany
FACSCalibur™ flow cytometer	BD Biosciences, Heidelberg, Germany
Hoefer™ Mighty Small™ II electrophoresis system	Hoefer Scientific instruments, San Francisco, CA, USA
Hoefer™ Mighty Small™ II multiple gel caster SE 200 series	Hoefer Scientific instruments, San Francisco, CA, USA
Intelli-Mixer™ rm-2l Rotating wheel	LTF- Labortechnik GmbH, Wasserburg, Germany
Magnet Thermo Stirrer RCT basic	IKA, Staufen, Germany
Microscope Primovert	Carl Zeiss AG, Oberkochen, Germany
Neubauer hemocytometer	Marienfeld, Lauda- Königshofen, Germany
Safety hood ENVAIR eco Safe Comfort	Envair Deutschland GmbH, Emmendingen, Germany
Stripettor™ Ultra Pipet Controller	Corning, Corning, New York, USA
Thermo Scientific™ Heraeus™ Fresco 17 centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Thermo Scientific™ Heraeus™ Fresco 21 centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Thermo Scientific™ Heraeus™ Megafuge 16R centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
ThermoStat plus heating block	Eppendorf, Hamburg, Germany
Trans-Blot® Turbo™ Transfer System	Bio-Rad, Hercules, CA, USA
Electrophoresis Power Supply EPS 301	Amersham pharmacia biotech
ph- meter ph720 InoLab	WTW, Weilheim, Germany
Pioneer™ Precision Weighing station	Ohaus Europe GmbH, Nänikon, Switzerland
Pipettes Pipetman Classic P2, P10, P20, P100, P200, P1000	Gilson, Middleton, USA
VICTOR™ X4 Multilabel Reader 2030	PerkinElmer, Waltham, MA, USA

Vortex	Starlab International GmbH, Hamburg, Germany

Table 8. List of instruments and devices.

5.1.8 Software

Software	Supplier
BD CellQuest™ Pro	BD Biosciences, Heidelberg, Germany
ImageJ	https://imagej.nih.gov/ij/download/, accessed on 12 October 2022 (Schneider et al., 2012)
PerkinElmer 2030 Manager	PerkinElmer, Waltham, MA, USA
Microsoft® Excel	Microsoft Corporation, Redmond, WA USA

Table 9. List of software.

5.2 Methods

5.2.1 Cell culturing

Wildtype, *LDHA* and *LDHB* double knockout cells of the human colorectal adenocarcinoma cell line LS174T were used for this study. All cells were incubated at a temperature of 37°C in a 95%-humidified atmosphere with 5% CO_2. T75 cell culture flasks were used to grow the cells in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Media were exchanged along with the cell splitting at least twice a week, depending on the color of the medium, which indicates more acidic conditions when changing to orange. The growth density was aimed at being in the range of 75-90%. It is crucial to maintain standardized culturing conditions to exclude these as a cause of fluctuations in HSP levels.

Adherent tumor cells were first briefly washed with 6 ml of phosphate buffered saline after removing the medium. Two milliliters of trypsin-EDTA-solution were added followed by a 2-3 min incubation at 37°C to remove the cells from the surface. To inactivate trypsin, the threefold volume, 6 ml, of fresh medium was applied. After proper resuspension and counting, the cells were seeded in new flasks filled with 12 ml of medium. Due to lower proliferation rates of the knockout cells, a larger quantity had to be seeded to establish growth conditions similar to those of the wildtype (Zdralevic et al., 2018). For a splitting rhythm of four days 0.8×10^6 wildtype cells and 1.6×10^6 knockout cells were seeded. A span of three days required an increased number of 1.2×10^6 and 2.4×10^6 cells.

To harvest the cells, they were detached according to the splitting protocol above. If cells were seeded in T25 flasks used for FACS analysis and LDH activity assays, only 1 ml of Trypsin was used and inhibited with 3 ml of medium. A medium volume of 5 ml was added to smaller flasks, where

cells were harvested 48 h later. Cells were routinely checked for mycoplasma contamination.

5.2.2 Cell counting

The cell number was estimated with a Neubauer hemocytometer according to the Trypan Blue exclusion method. After proper resuspension, 20 µl of Trypan Blue were added to the same volume of cell-containing medium which again was thoroughly mixed to fill up one side of the hemocytometer below the cover slip. Trypan blue is a non-permeable dye that cannot pass intact membranes, e.g., the ones of viable cells, meaning that dye-uptake only takes place in dead cells. Therefore, it can serve as a method to visualize viability. To assess the proliferation, cells were counted in two of four 4×4 large corner squares excluding the colored, non-viable ones. The total cell number was calculated according to the following formula: $\frac{cells}{ml} = number\ of\ cells\ counted\ \times 10^4$.

A more accurate approach was pursued when seeding cells for colony forming assays (CFA) by counting all four large squares and dividing them in half afterwards.

5.2.3 Treatment procedure

GNE-140 was dissolved in DMSO, creating a 4mM stock solution. Cells were incubated with a sublethal dose of 10 µM of the GNE-140 solution for 24 hours before experiments were conducted. Control groups were treated with the same concentration of DMSO to exclude any effects the dilutant might have on HSP expression.

5.2.4 LDH activity assay

The assay was performed to measure the enzyme's activity in the LS174T cell lines: the wildtype and the *LDHA*- and *B* double knockout. Moreover, control and treated groups were established to assess the GNE-140 inhibitor in a qualitative and quantitative manner.

The Lactate Dehydrogenase Activity Assay Kit from Sigma-Aldrich® was used for this purpose. Based on the enzyme's ability to reduce NAD^+ during the conversion of lactate to pyruvate, this method is able to detect increased NADH colorimetrically at a wavelength of 450 nm, which indicates high LDH activity. (Sigma- Aldrich, 2018)

Before conducting the assay, cells were seeded two days in advance: 0.5 Mio for the wildtype and 1 Mio for the knockout. Doublets were prepared for each cell line. The treatment-group was incubated with 10 µMol of GNE-140 for 24 hours, whereas the control group only received the solvent DMSO.

The following steps were carried out according to the manufacturer's protocol. After harvesting and counting, 1×10^6 cells were rapidly homogenized on ice with 500 µl of cold LDH assay buffer and then centrifuged at 10,000× g and 4°C for 15 min. The soluble fraction was used for the assay, the insoluble material was removed. If not directly assayed, samples could be stored at -20°C. Wildtype samples were diluted in PBS creating a 1:25 ratio solution before being measured and filled up to a final volume of 50 µl with the assay buffer. All NADH standards and samples were run in duplicates. To start the reaction, 50 µl of the Master reaction mix were put into each well. The initial measurement was taken after 2-3 min [(A_{450nm})initial], then every 10 min until the least active sample reached the lowest value of the standard [(A_{450nm})final]. To correct the final measurements for background signal, the NADH blank standard value had to be subtracted. After establishing the linear standard curve derived from the absorbance and known NADH concentrations, the amount of NADH produced between (A_{450nm})initial and (A_{450nm})final in each sample could be determined. The LDH activity was calculated according to following formula:

$$\frac{amount\ of\ NADH\ (nmole)}{reaction\ time\ (min) \times sample\ volume\ (ml)} \times sample\ dilution\ factor = LDH - activity\ (\frac{mU}{ml}).$$

(Sigma- Aldrich, 2018)

5.2.5 Flow cytometry analysis

Flow cytometry analysis, also known as FACS-analysis, allows the characterization of single-cell suspensions with regard to surface markers, granularity, size and also intracellular localized features. When passing through the capillary of the flow cytometer, cells absorb the incident light, usually coming from a laser beam and emit photons at certain wavelengths. Photodetectors collect a fraction of this emitted light and generate electrical signals as a response to the optical impulses. Each cell is analyzed by its light scattering in at least two directions. The forward scatter indicates the cell size, whereas the sideward scatter at 90° represents a cell's granularity. Besides light scattering, further analysis can be achieved using fluorescence-labeled substrates, e.g., antibodies against surface proteins. Laser light can excite fluorophores, which leads to the emission of photons at certain wavelengths. The fluorescence activity can be detected and measured independently of the light scattering. Therefore, the flow cytometer machine is able to measure multiple emissions at specific wavelengths and repeat this process for each passing cell. After amplification and accumulation, these electrical signals can be presented in multiparameter dot plots, which are used for characterization. (Tanke & van der Keur, 1993)

In this study flowcytometry was used to measure the Hsp70 membrane expression on the LS174T cells after incubation with GNE-140 compared to the untreated wildtype and the *LDHA*- and *B* double knockout cells. Cells, 0.5×10^6 of the wildtype and 1.0×10^6 of the knockout line, were seeded two days in advance. A control group (ctrl.) only treated with the

solvent DMSO was established for each cell line. 24 hours after incubation with GNE-140, cells were harvested and counted. 0.2 $\times 10^6$ cells were transferred into Eppendorf tubes creating doublets for each sample. The cell pellets were resuspended in 1 ml of PBS before being centrifuged again at 500 *g* and 4°C for 4 min. After taking off the supernatant, the cell pellets were dried and each sample doublet was incubated on ice for 30 min with either 20 µl of FITC-labeled mHsp70 antibody (cmHsp70.1, IgG1; multimmune GmbH, Munich, Germany) or 5 µl of a FITC-conjugated control antibody (Mouse IgG1 FITC, 345815, BD Biosciences). The isotype-matched control antibody was used to detect the background staining due to unspecific binding and to exclude it from further analysis. One more washing step with PBS was repeated after the incubation, before adding 200 µl of FACS buffer to the cell pellet. Shortly before measuring, 2 µl of propidium iodide were added and transferred into tubes. PI is membrane impermeant dye which is used to distinguish between dead and viable cells. To analyze the amount of membrane-Hsp70 positive cells only propidium negative and therefore viable cells were gated. The membrane-Hsp70-positivity was determined as the percentage of antibody-bound cells among viable ones from which the staining of the control group was subtracted. All data was generated by the FACSCalibur™ flow cytometer and analyzed with BD CellQuest™ Pro (BD Biosciences, Heidelberg, Germany).

5.2.6 Protein analysis

5.2.6.1 Sample preparation (cell lysis & BCA assay)

Cells were seeded three days before harvesting: 0.8 $\times 10^6$ cells for the LS174T wildtype and 1.6 $\times 10^6$ of the *LDHA*- and *B* double knockout cells. For each cell line there was a control group incubated with DMSO and a counterpart which was treated with GNE-140.

After harvesting, counting, and washing three times with PBS, 2×10^6 cells were incubated on ice with 80-100 µl of RIPA buffer for 30 min, followed by centrifugation for 20 min at 12,000 *g* and 4°C to pellet the non-soluble material. The supernatant with cytosolic proteins was collected afterwards and stored at -20°C.

Protein concentrations were determined colorimetrically with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The underlying mechanism here is the combination of a biuretic reaction and the selective detection of cuprous cations using bicinchoninic acid (BCA). BCA reacts with the reduced Cu^{1+} formed during chelation with protein in an alkaline environment. This reaction leads to the formation of an intensely violet-colored product with a linear absorbance at a wavelength of 562 nm, which is proportional to the protein concentration. (Thermo Fisher Scientific, n.d.)

The working reagent was mixed with 50 parts of agent A and one part of agent B. Then, 200 µl of the solution were added to 25 µl of the samples to start the reaction. 30 minutes after the plate was incubated at a temperature of 37°C, the absorbance was measured at a wavelength of 562 nm using a photometer. For a loading amount of 20 µg and a desired sample volume of 20 µl, protein solutions were mixed with 5µl of loading buffer and filled up to 20 µl with distilled water. SDS-containing loading buffer was added before the samples were heated at 95°C for 10 min to denature the proteins. If not used immediately, samples could be stored at -20°C.

5.2.6.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

To analyze and quantify the cytoplasmic amounts of Hsp90, Hsp70 and Hsp27, an SDS-PAGE was performed. This analytical method established by Laemmli (Laemmli, 1970) allows the separation of proteins based solely on their molecular weight.

For this, sodium-dodecyl-sulfate-containing buffer was used to denature the proteins and to coat them with a negative charge. After applying an electric field, the proteins were pulled towards the positively charged anode and separated according to their molecular mass. As a result, similarly-sized proteins formed characteristic bands.

Gels were prepared in advance consisting of acrylamide, Tris buffer with a pH of 8.6 for the upper and pH 6.8 for the lower separating component, 10% ammonium persulfate, 10% SDS, and TEMED. Various acrylamide concentrations of 34-40% were established to prepare the gels. High percentages allowed proper separation in the range of small-sized proteins, e.g., Hsp27.

To identify the bands after the run, a colored standard protein marker was used as a reference. The power supply was set at 80 V and 20 mA per gel and applied for 2 h 30 min.

5.2.6.3 Western Blotting

After SDS-PAGE, the size-separated protein samples were transferred to nitrocellulose membranes by semi-dry Western Blotting based on the protocol of Goldman et al. (2015).

Gels, membranes, and filter paper were soaked in transfer buffer for 10 minutes before being placed in the blotting machine, the setup of which is illustrated schematically in Figure 4.

The transfer was performed at 60 V and 45mA per gel for 1h 30 min. The negatively charged proteins were pulled towards the anode and onto the membrane, where immunological detection was then possible. After blotting, Ponceau S solution was used to stain the membranes and make the bands visible to cut them into strips.

Membranes were blocked with 5% milk in TBST for one hour at room temperature and then incubated with the primary antibody-solution overnight at 4°C. This was followed by three washing steps with TBST for 15 min each. Matching secondary antibodies were used to incubate the membranes afterwards for one hour before repeating the washing steps. After developing the membranes with the Pierce™ ECL Western Kit (Thermo Fisher Scientific, Waltham, MA, USA), immune complexes were visualized via chemiluminescence using the ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). The intensities of the resulting bands were quantified with the ImageJ-program. All analyzed heat shock protein bands were normalized to β-actin and compared to the untreated wild type cells as a reference.

Figure 4. Schematic assembly of a semi-dry Western Blot. 1: Filter paper; 2: Polyacrylamide gel; 3: Nitrocellulose membrane. Flow direction for proteins from anode (+) to cathode (-).

5.2.7 Colony formation assays

Colony formation assays were performed to analyze cell survival based on the ability of single cells to establish colonies *in vitro* (Franken et al., 2006). It is the method of choice to assess reproductive capacities and viability both of which can be impaired by cytotoxic agents and radiation

(Franken et al., 2006). In this study, CFAs were carried out to analyze the effects of LDH inhibition on radiosensitivity.

Cells, 800/1,000 for the wildtype and 1,600/2,000 for the knockout line, were seeded in 12-well plates two days before irradiation after a 24-hour-incubation with GNE-140. Irradiation was performed with the CellRad compact benchtop X-ray irradiator (Precision X-Ray, North Branford, CT, USA). Single doses of 0 Gy, 2 Gy, 4 Gy, and 6 Gy were applied at a dose rate of 1 Gy/min (5 mA, 130 kV).

After approximately seven days, the plates were stained. This required a washing step with PBS and a 5 min fixation with ice-cold methanol, followed by a 3 min stain with 0.1% crystal violet. Colonies reaching an appropriate size of more than 50 cells were counted manually. Plating efficiency (PE) and surviving fraction (SF) were calculated for each group to assess the effects of the intervention.

5.2.8 Statistical analysis

Experiments were conducted at least three times independently of each other to establish biological replicates. For the comparison of two groups, statistical analysis was performed using the Student's t-test. Significant results are displayed with the following marks: $* \, p \leq 0.05$; $** \, p \leq 0.01$;$*** \, p \leq 0.001$. Most graphs show standard deviation of the data.

6 Results

This study focuses on the biological aspects of impaired lactate metabolism.

For this purpose, LS174T colorectal adenocarcinoma wildtype cells were treated with the LDH inhibitor GNE-140 and compared with *LDHA* and *B* double knockout cells regarding heat shock protein expression and its effects on the phenotypic level, i.e., radiosensitivity.

Within the framework of a greater study, several additional experiments were performed to analyze the effects of the metabolic switch, including proteomic and ROS analysis, cell proliferation assays, and immunohistochemistry. (Schwab et al., 2021) These results will be addressed later in the discussion section.

6.1 LDH activity

LDH activity was measured colorimetrically using an assay to demonstrate the successful knockout of *LDHA* and *B* and to examine the efficacy of the inhibitor. It therefore served as a quality control.

Cell lines treated with GNE-140 in fact showed a significantly decreased LDH activity compared to the control group (Figure 5). The activity levels of the knockout cell lines were barely measurable which proved that the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR-associated (Cas) application was successful. *LDHA/B* double knockout cells treated with GNE-140 had very low and almost identical activity levels compared to the control group, confirming the hypothesis that the LDH inhibitor has no impact if the corresponding enzyme is not expressed.

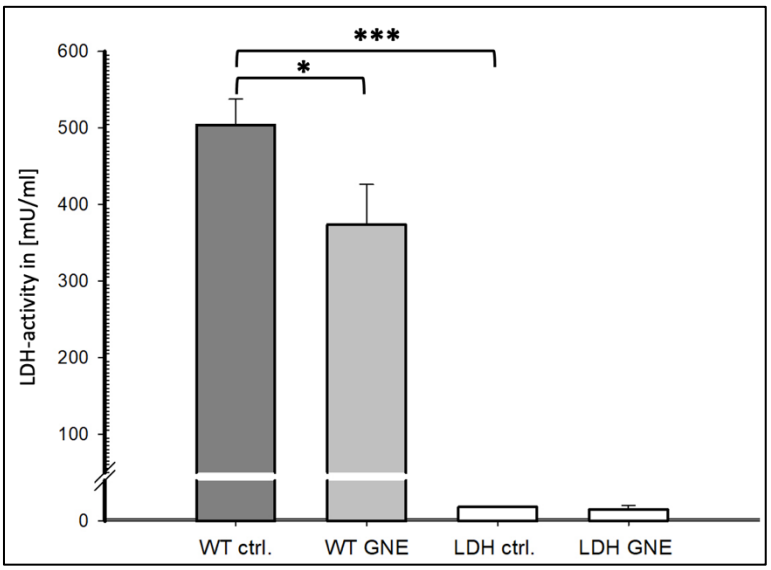


Figure 5. Lactate dehydrogenase activity in LS174T cells. Effects of GNE-140 (10 μM, 24h) on wildtype (WT) and *LDHA/B* double knockout cells (LDH) compared to control groups (ctrl.) (*: p ≤0.05 and ***: p ≤ 0.001)

6.2 Effects on the expression of Hsp90, Hsp70 and Hsp27

Western Blots were conducted to analyze whether the inhibition of LDH affects the expression of heat shock proteins. The measured intracellular levels of Hsp90, Hsp70 and Hsp27 were normalized to β-actin, which serves as a housekeeping protein. Accordingly, the values were compared with the wildtype control group, which was determined to be 100%. The results are depicted in Figure 6.

The greatest reduction in the amount of protein was observed for Hsp27 levels, which fell by more than 19% in LS174T wildtype cells after treatment with GNE-140 and by approximately 40% in *LDH*-knockout cells. The amount of Hsp90 decreased significantly by 6% after treatment with GNE-140. In *LDH*A/B double knockout cells, the Hsp90 level dropped by 10%. Similar to the other proteins, the amount of intracellular Hsp70 was reduced by 14% in the inhibited wildtype and by up to 26% in the genetically disrupted cell line. In summary, the protein levels of all HSPs investigated were concomitantly downregulated upon LDH inhibition. HSP suppression reached its maximum in knockout cells confirming that this

effect is strictly mediated by LDH inhibition. No remarkable difference was observed in the treatment of *LDH*A/B double knockout cells with GNE-140 compared to the control group. This is in line with the results of the LDH activity measurements.

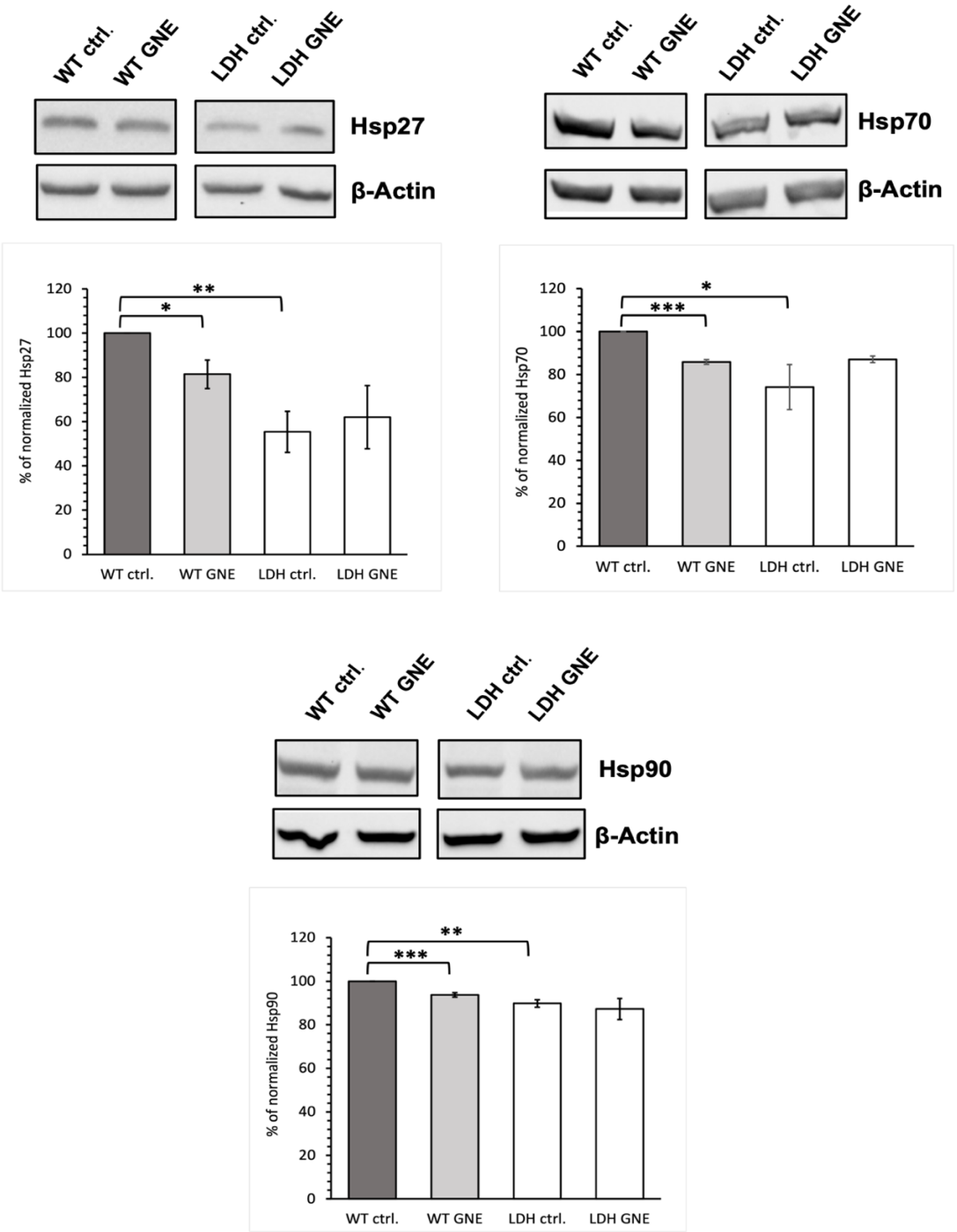
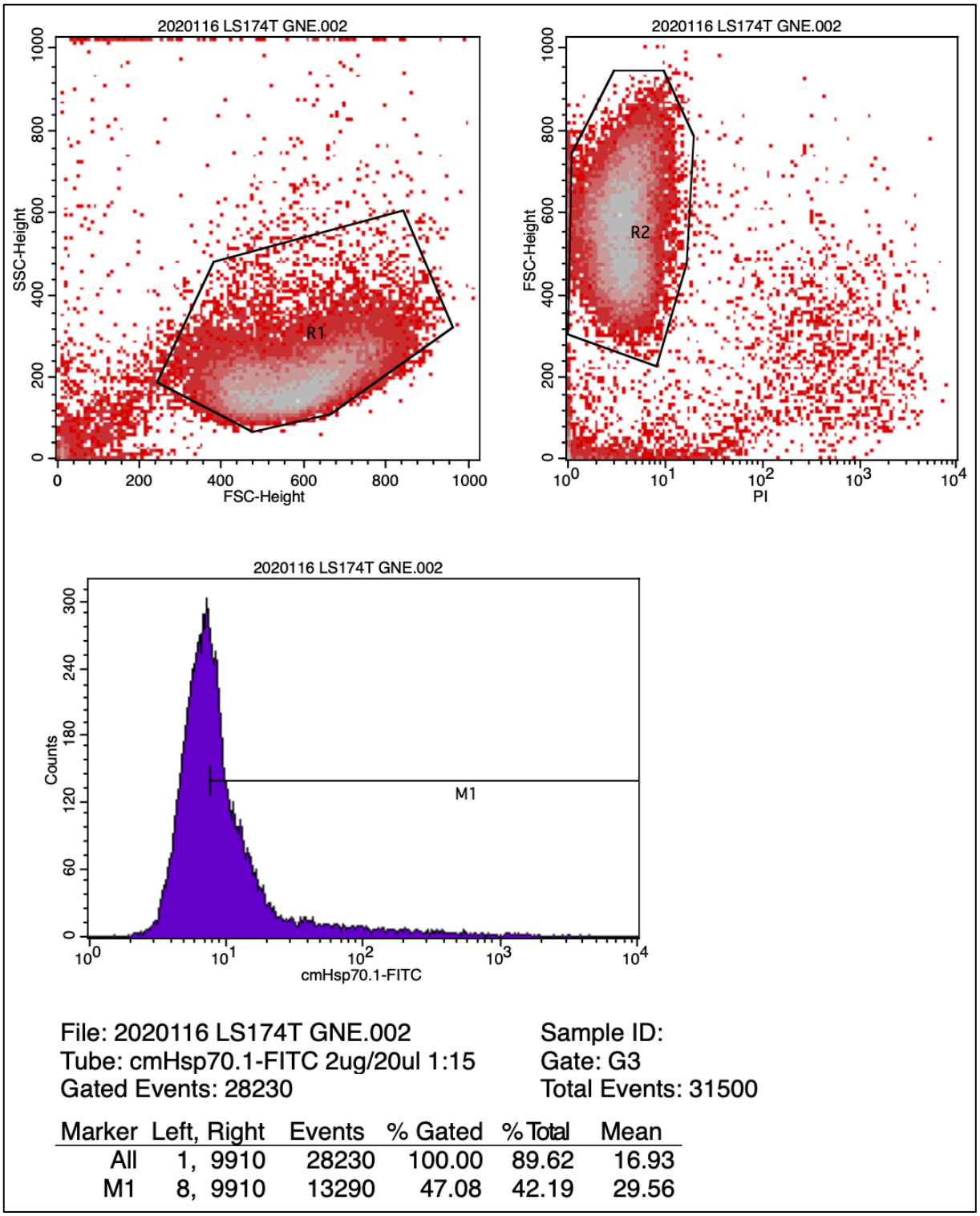


Figure 6. Representative immunoblot displaying intracellular levels of Hsp90, Hsp70 and Hsp27 in LS174T wildtype and *LDHA/B*-double-knockout cells. Control groups and GNE-140 treated groups were compared. β-Actin was used as a loading control. HSP-levels were relatively quantified according to the wildtype control group. Error bars show the standard deviation (*: p ≤0.05, **: p ≤ 0.01 and ***: p ≤ 0.001).

FACS analysis was performed to analyze membrane Hsp70 levels. Figure 7 depicts representative data of untreated wildtype populations. The population of interest was gated after staining with an antibody against membrane-bound Hsp70. Only viable, i.e., PI-negative populations, were analyzed. The corresponding Hsp70 membrane expression of the gated population is illustrated in histograms (Figure 7). Unspecific binding was detected using an isotype-matched IgG control antibody (Figure 8b). The percentage of non-specific staining was deducted from the one of Hsp70-positively stained cells (Figure 8a).

In addition to the reduction of intracellular chaperone levels, FACS analysis revealed that Hsp70 membrane expression was similarly affected (Figure 9, 10). The histograms in Figure 9 depict the specific mHsp70 positivity for each group investigated. Whereas over 50% of the untreated wildtype cells presented Hsp70 on the plasma membrane, populations treated with GNE-140 only reached 27% (Figure 10). *LDHA/B* double knockout cells in turn showed an even greater decline, with values around 23% (Figure 10).



Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	28230	100.00	89.62	16.93
M1	8, 9910	13290	47.08	42.19	29.56

Figure 7. Representative FACS analysis of the untreated wildtype population. The upper two Dotplots show the cell population of interest analyzed after staining with Hsp70 membrane antibodies. The histogram at the bottom depicts the corresponding percentage of mHsp70 expression in the gated population.

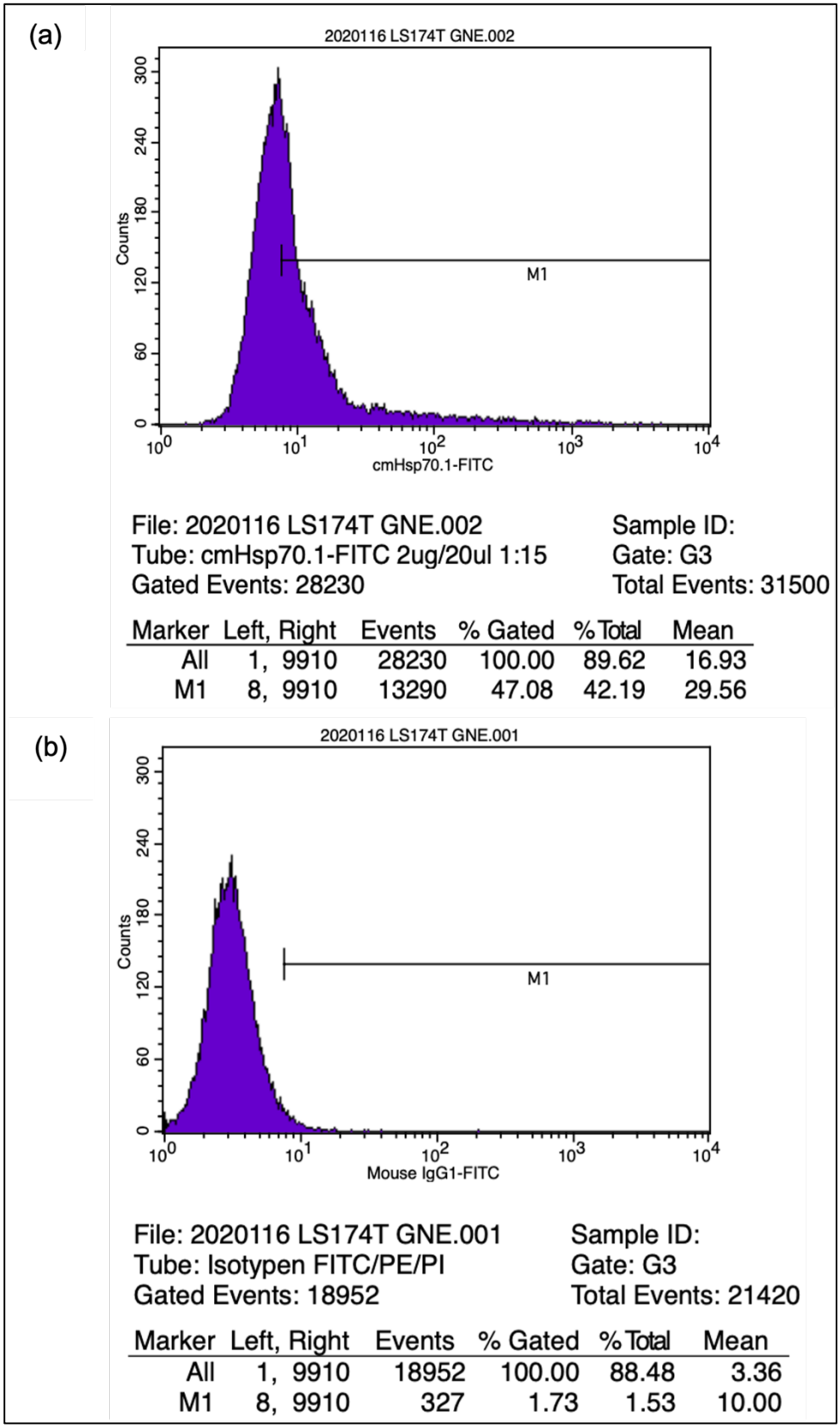


Figure 8. Representative histograms of the untreated wildtype population. Each group (WT ctrl/GNE; LDH ctrl/GNE) was stained with a membrane Hsp70 antibody and an isotype IgG to detect unspecific binding. (a): WT ctrl. Hsp70 IgG-stained; (b): WT ctrl. IgG isotype-stained.

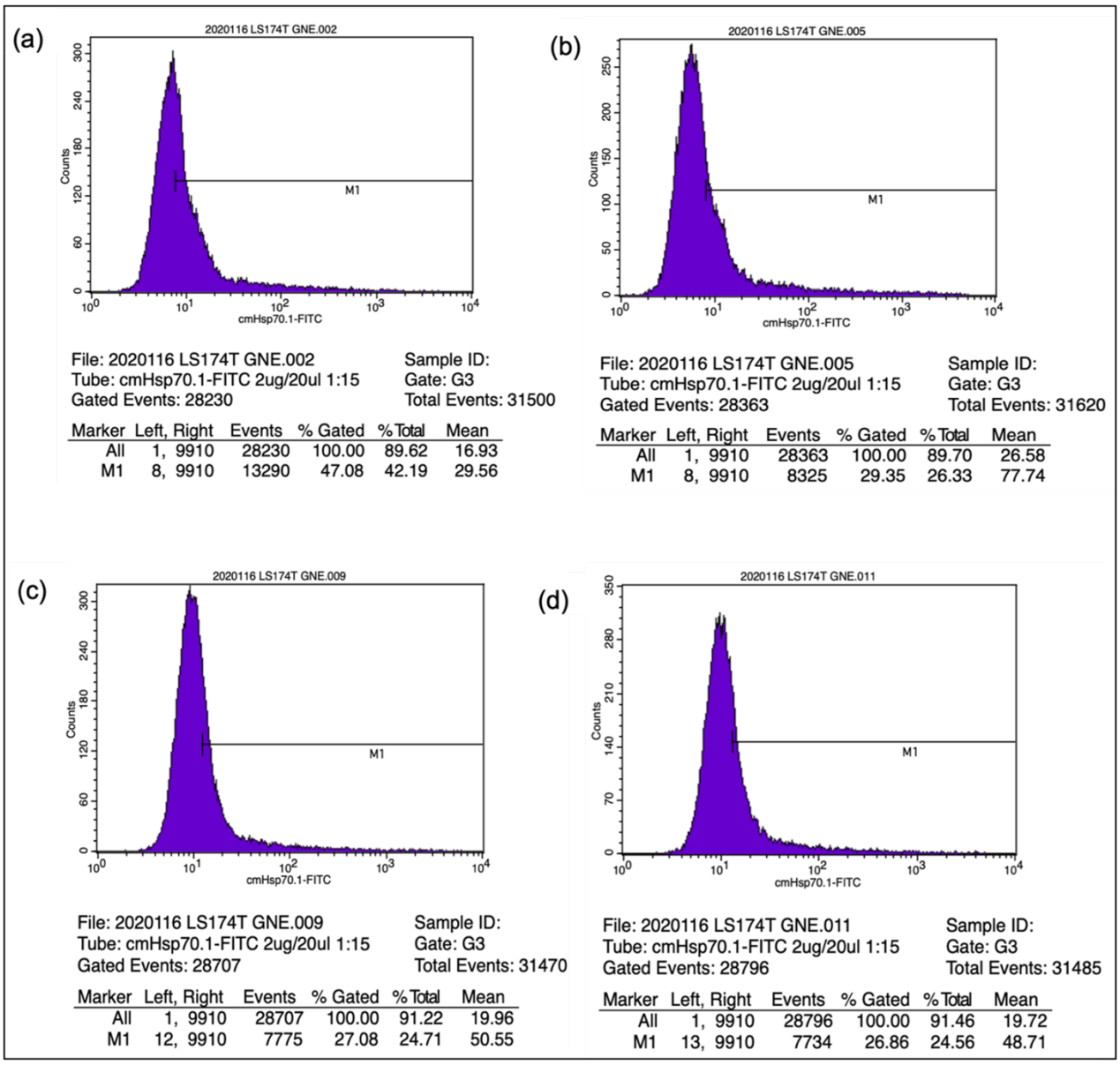


Figure 9. Representative histograms comparing the mHsp70 expression of the wildtype and knockout cell groups (% gated). (a): WT ctrl. (b): WT GNE (c) LDH ctrl. (d) LDH GNE.

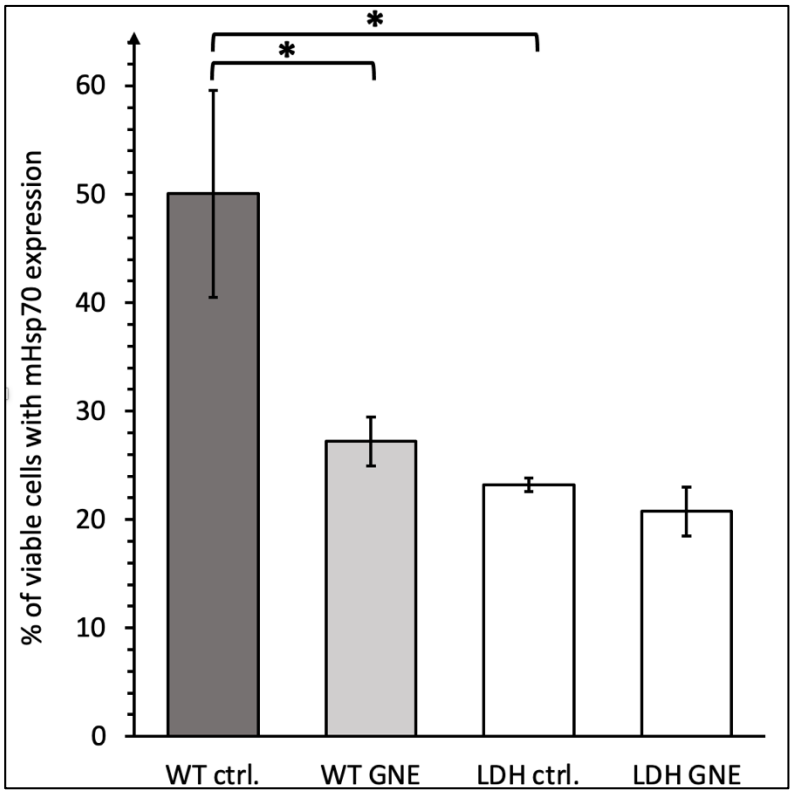


Figure 10. Hsp70 membrane expression in different cell populations.

6.3 Consequences of LDH suppression on radiosensitivity

As mHsp70 overexpression is known to mediate radioresistance, CFAs were conducted to evaluate whether these effects could be counteracted by LDH inhibition. The results are demonstrated in cell survival curves which describe the relationship between the SF of cells and the radiation exposure dose. Following equation was used to calculate the SF:

$$SF(\%) = \frac{number\ of\ colonies\ counted}{number\ of\ colonies\ seeded \times PE} \times 100$$

Accordingly, the PE was determined:

$$PE\ (\%) = \frac{number\ of\ colonies\ counted}{number\ of\ colonies\ seeded} \times 100$$

Clonogenicity is defined as the capacity of a single cell to survive and proliferate after a certain treatment. Loss of reproductive integrity therefore determines reproductive death, as it happens to take place in non-static cells such as those of a tumor. This may result from a biological disorder due to irradiation-impaired DNA or other structures. X-rays, which were used for irradiation, are considered to be beams with low linear energy transfer. In this case, two thirds of the damage is caused by indirect effects via the formation of free radicals leading to changes in chemical bonds that can be lethal. (Radiation Oncology Physics, 2005)

The cell survival curves represent a function in which the SF on the ordinate is logarithmically plotted against the dose on the abscissa. Effects of radiation on tumor cells, i.e., quantification of radiosensitivity, were described using the linear-quadratic (LQ) model.

$$SF = e^{-\alpha D - \beta D^2}$$

It derives from the hypothesis that, in addition to the applied dose (D) tissue specific parameters α and β further affect the SF. Alpha serves as the linear component of the function. Hence, it determines the initial slope. The term beta, on the other hand, represents the quadratic portion, which defines the curvature in the range of higher doses. (Lütter, 2012)

Specific LQ-models were established for each cell line (Table 10).

	WT ctrl.	WT GNE	LDH ctrl.	LDH GNE
α	0.3607	0.5000	0.8078	0.6796
β	-0.0055	-0.0249	-0.0369	-0.0110

Table 10. Alpha and beta values for wildtype and knockout cell lines according to the linear quadratic model.

To evaluate how quickly cells can cope and respond to irradiation, the α/β ratio can be used as a reference. In this case, however, β values were negative so that $\beta \approx 0$ was approximated (Table 10). Consequently, the

equation developed into a linear dependency, whereby the quadratic component, which was represented by β, was omitted.

This relationship could be deduced from the shape of the curve (Figure 11b). As expected, high irradiation led to a significant decrease in the SF, resulting in a gradient between the wildtype and the knockout cell lines, the latter of which appear to be more radiosensitive (Figure 11). Although the gradient between the control group and the one treated with GNE-140 diminished with higher irradiation doses, a tendency towards increased radiosensitivity was observed for the intervention group.

Likewise, knockout cell lines demonstrated even greater radiosensitivity when higher doses were administered, so that the SF was even lower compared to the other two groups (Figure 11). Statistically significant results, however, could only be reached for an irradiation dose of 2 Gy. D_{50} values, i.e., the half maximal inhibitory dose required to reduce the SF down to 50%, confirmed the indicated relative radiosensitivity above (Table 11).

	WT ctrl	WT GNE	LDH ctrl	LDH GNE
D_{50}	1.40	1.20	0.95	1.02

Table 11. D_{50} values for wildtype and knockout cell lines.

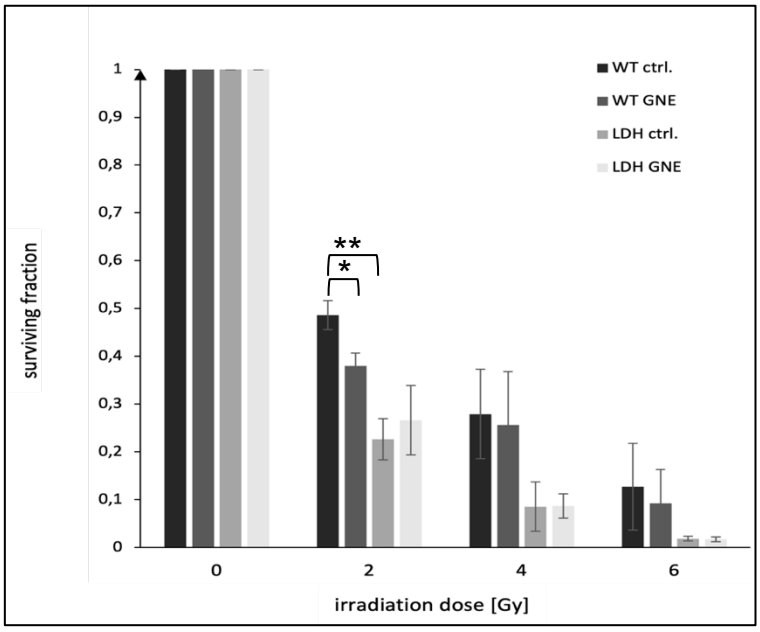
The highest of approximately 1.4 Gy was determined for the wildtype control group followed by the GNE-140-treated group with a decrease of 0.2 Gy. Knockout cell lines achieved the lowest D_{50} values. The sensitizer enhancement ratio (SER) can used to evaluate the properties of the LDH inhibitor to make cells more susceptible to irradiation.

$$SER = \frac{D_{50}(control\ group)}{D_{50}(intervention\ group)}.$$

GNE-140-treated groups reached a respective SER of 1.16, whereas the knockout cells achieved one of 1.47 compared to the wildtype control

group. In conclusion, radiosensitivity was more pronounced in knockout cell lines and in the intervention group.

(a)



(b)

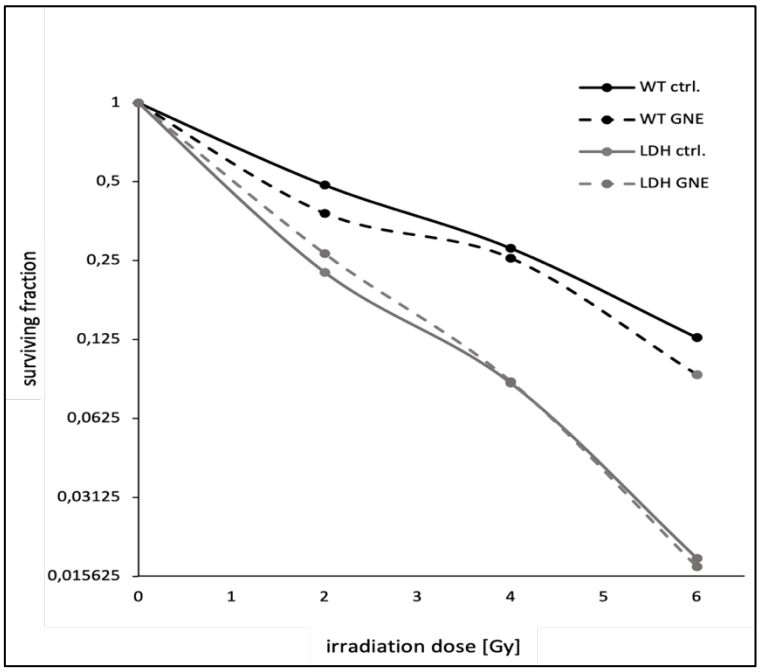


Figure 11. Survival fractions of the subpopulation as a function of the radiation dose, illustrated in bar charts (a) and the corresponding survival curves (b). Error bars in (a) show the standard deviation (*: $p \leq 0.05$, **: $p \leq 0.01$).

7 Discussion

This study contributes to the investigation of cancer metabolism and its interaction with the stress response system within the framework of a comprehensive project (Schwab et al., 2021). The glycolytic phenotype of many cancer entities, including CRC, is characterized by increased energetic demands. This leads to higher LDH activity – a phenomenon also known as the Warburg effect (Walenta et al., 2000; Warburg et al., 1927). Moreover, not only metabolic changes have a positive effect on tumorigenicity but also the upscaling of the heat shock response, which is reflected in elevated levels of HSPs (Ciocca et al., 2013; Lee et al., 2015a). This unique group of proteins is of further interest due to its interaction with multiple clients many of which are involved in carcinogenesis and metastasis (Hanahan & Weinberg, 2011).

A co-dependent relationship between these pro-tumorigenic mechanisms was already suggested by Guan and colleagues. They have demonstrated that heat-induced overexpression of Hsp70 was associated with increased LDHA activity and concomitantly enhanced lactate production in immature Sertoli cells of the boar (Guan et al., 2018). Similar effects were observed in human cervical cancer cells: high cytosolic Hsp70 levels correlated with enhanced glycolytic activity, thus promoting the Warburg effect (Wang et al., 2012).

Nevertheless, not only lactate production seems to affect the HSP-axis. Elevated glycolytic flux leads to accumulation of methylglyoxal (MGO), a well-known and highly reactive by-product (Richard, 1993), which binds Hsp27 to form an even more stable and active agent (Oya-Ito et al., 2011). MGO-Hsp27 favors cancer cell escape from apoptosis by suppressing caspase activation (Sakamoto et al., 2002) and stabilizing AKT (Mearow et al., 2002). This triggers a series of survival-promoting and anti-apoptotic signaling (Bellier et al., 2020). Besides, AKT-activation itself affects

glucose consumption by triggering the uptake, creating a self-sustaining vicious cycle between the glycolytic phenotype and apoptotic evasion mediated by HSPs (Elstrom et al., 2004).

The concrete link between the mechanisms, the glycolytic phenotype and the upregulation of the stress response system, however, remains largely unexplored. To elucidate a possible interdependence of these two cancer-promoting mechanisms, this study focused on the effects that inhibition of the crucial glycolytic enzyme LDH might have on HSP expression.

The main consequence of reduced lactate levels due to LDH interference was reflected in the decrease of Hsp27, 70 and 90 levels, resulting in an impaired stress response system. Decreased intracellular amounts could be detected in GNE-140-treated groups and the *LDHA*/*B* double knockout cell line, which displayed even lower levels (Figure 7). Similar effects of HSP expression in genetically modified and inhibitor-treated cells largely exclude off-target effects. In addition, LDH inhibition via Oxamate showed comparable results in terms of reducing HSP (Manerba et al., 2017). Considering these results, it seems unlikely that the inhibitor directly interacts with HSP or undefined agents.

One possible mediator between the cellular metabolism and the stress response could be the transcription factor HSF1. Besides regulating the expression of many HSPs, it plays a crucial role in the glucose metabolism. (Dai et al., 2007) HSF1 was found to display a binding site for the *LDHA* promoter, leading to a subsequent reduction in its levels when the transcription factor was downregulated (Zhao et al., 2009). Consistent with these findings, Schwab et al. (2021) detected a reduction of HSF1 in *LDHA/B* double knockout cells, where HSP levels were lower compared to the WT.

The correlation between HSF1 and LDH activity might also explain the differences in the HSP expression levels: Hsp27 and Hsp70, both of which

are known to be regulated by HSF1 (Calderwood et al., 2010), were decreased the most when LDH was inhibited (Figure 7). Hsp90 on the other hand has two isoforms: the constitutively expressed Hsp90β and the stress-inducible from Hsp90α. The transcription of the latter is known to be mediated by HSF1. (Zuehlke et al., 2015) According to these assumptions, a decrease of HSF1 levels due to impaired LDH activity as demonstrated by Schwab et al. (2021) should have less impact on the total amounts of Hsp90 compared to the other HSPs investigated (Figure 7). This study confirmed that Hsp90 levels were least reduced in case of LDH inhibition or genetic depletion. Therefore, these findings suggest that HSF1 might function as a crucial mediator between the heat shock response and the metabolic reprogramming. However, further research is needed to better understand the underlying mechanism.

Another possible link between metabolic activity and HSP expression is indicated by the change in ROS, which accumulate during cell stress. ROS, in turn, leads to an upregulation of HSP as an attempt to compensate stress damage. (Driedonks et al., 2015) Enhanced aerobic glycolysis in WT cells hence results in ROS overproduction, which again correlates with high HSP levels (Li et al., 2016; Schwab et al., 2021). *LDHA/B* double knockout cells with less metabolic activity and lower HSP levels, on the other hand, displayed lower ROS production (Schwab et al., 2021).

The correlation between low metabolic activity, ROS levels and therefore less HSP synthesis could be explained by the metabolic shift from aerobic glycolysis back to OXPHOS, which takes place in *LDHA/B* double knockout cells and can be phenocopied by GNE-140 application. This change in the metabolic status was shown to be accompanied by a restricted *in vitro* growth. However, it does not lead to a complete

suppression, contradicting the common assumption that the Warburg effect is indispensable. (Zdralevic et al., 2018)

Similar results were obtained by Schwab et al. (2021), who demonstrated that LDH depletion leads to reduced proliferative activity. *In vivo* growth could not be halted but was reduced in its rate. This suggests that although the glycolytic phenotype provides a definite growth advantage, the metabolic shift is sufficient to sustain growth. In case of additional OXPHOS inhibition via phenformin, a mitochondrial complex I inhibitor, complete eradication could be achieved. (Zdralevic et al., 2018) Dual metabolic targeting should therefore be considered regarding future therapeutic approaches.

As opposed to normal cells, Hsp70 is not only found in the cytosol but also on the plasma membrane of cancer cells (Multhoff et al., 1995). In this study, flow cytometry analysis revealed that the membrane expression of Hsp70 was similarly affected by LDH inhibition (Figure 3). *LDHA/B* double knockout cells carried the lowest Hsp70 membrane density followed by the ones treated with GNE-140. Similar results were observed using Oxamate as an LDH inhibitor (Schwab et al., 2021). Moreover, these effects on HSP expression could be reproduced in cells cultured in low-glucose medium, which impairs the glycolytic activity and therefore mimics LDH inhibition. Higher glucose intake hence leads to increased mHsp70. (Li et al., 2016; Schwab et al., 2021)

As mentioned before, mHsp70 is known to colocalize with Gb3, functioning as an anchor to the plasma membrane (Gehrmann et al., 2008). A decrease in mHsp70 was found to be associated with less Gb3. This could be explained by the major differences found in the cellular proteomes between WT and *LDHA/B* double knockout cells, especially with regard to proteins that participate in carbohydrate and lipid metabolism. This might explain the differences in Gb3 expression and thus the lower capacity to

anchor mHsp70. Once again, this indicates a connection between the metabolic changes and HSP expression. (Schwab et al., 2021)

An important mechanism by which cells compensate for and react to potentially lethal damage caused by physical and chemical stress factors is the heat shock response system. HSPs participate in antiapoptotic pathways (Garrido et al., 2006) and exhibit cytoprotective properties, e.g., protein-refolding (Hahn et al., 2004) both of which promote resistance to cell death. Whereas in normal cells this is part of the damage-control system, in cancer it serves as one of the causes for therapy resistance (Sottile & Nadin, 2018). Tumor cells with high expression of mHsp70, for instance, were demonstrated to be less radiosensitive compared to sublines with lower membrane density (Murakami et al., 2015).

This study has demonstrated that cells with impaired lactate-metabolism became more radiosensitive either by GNE-140 application or genetic disruption (Figure 11a,b). Irradiation with a low dose of 2 Gy showed a significant reduction in the reproductive capacities, reflected by a decrease in the surviving fractions compared to the wildtype which was even more pronounced in the *LDHA/B* double knockout cell line. Higher irradiation doses of 4 or 6 Gy only showed a trend towards increased radiosensitivity in cells with impaired lactate metabolism. This could indicate that doses above the 2 Gy threshold might override the effects of LDH inhibition due to their lethal properties. To elucidate this phenomenon, however, further investigations of radiation response in the lower dose range are required. The SER and decrease of D50 values were concomitant with previous studies where Oxamate-treated LS174T and B16F10 cells and *LDHA* and *B* double knockout lines were investigated (Schwab et al., 2021).

Following these results, LDH-mediated downregulation of mHsp70 has been proven to break radioresistance by impairing the lactate metabolism.

The latter should be borne in mind when evaluating future therapeutic approaches.

In summary, this study's findings support the hypothesis of a codependent relationship and suggest ways the Warburg effect could be linked to the heat shock response of cancer cells. Targeting both mechanisms bears great potential as a new approach complementing existing therapeutic regimes especially in CRC where HSPs have a decisive role in the development, sustainment, aggressiveness, and therapeutic burdens of the tumor (Lee et al., 2015a).

Besides dose-dependent toxicities and unfavorable pharmacokinetic properties (J.-C. Heinrich et al., 2011; Sanchez et al., 2020), therapeutic targeting of the HSP-axis, i.e., the inhibition of specific members, has not proven to be successful. This lack of efficiency due to compensatory upregulation of other HSP members (Goloudina et al., 2012) illustrates the need for a universal inhibitor or mediator, such as LDH inhibitors, to cover the broad spectrum and escape the redundant system.

In this study, GNE-140 was used as a pharmacologic agent to achieve LDH inhibition in CRC cells. While newer inhibitors have been reported, only few of them have been found to have cellular activity. Thus, LDHB usually remains unaffected since many inhibitors only modulate LDHA activity. (Boudreau et al., 2016) Given the fact that both subunits contribute to carcinogenesis, this is not favorable (McCleland et al., 2013). According to Boudreau et al. (2016), GNE-140 seemed to be a promising candidate targeting both, LDHA and B. Its efficacy has been demonstrated repeatedly not only in CRC cell lines (Zdralevic et al., 2018) but also in in pancreatic (Boudreau et al., 2016) and breast cancer (Mazzio et al., 2021). Contrary to the pyruvate analog Oxamate, which was the first inhibitor described, GNE-140 causes less side effects due to its potency and high specificity. Hence, lower concentrations are needed to sufficiently inhibit

the enzyme. The pharmacological efficacy of GNE-140 displays an enormous benefit compared to its predecessor Oxamate which has led to further evaluation of this substance. *In vivo* data, however, was less convincing. LDH inhibition could not be maintained for longer than one hour as the substance is rapidly cleared and modulates LDH activity only temporarily. (Boudreau et al., 2016) Zdralevic et al. (2018) confirmed that it cannot suppress growth *in vivo*. GNE-140 is therefore not suitable for *in vivo* use which severely limits its field of application. Clinical implementation demands safe and effective *in vivo* practice. Consequently, the research on agents with higher efficacy and more favorable biodistributive characteristics must be pursued.

A promising strategy appears to be the disruption of the enzyme's tetramerization. Novel peptide inhibitors are being designed to interfere with the protein-protein interaction of the subunits. Compared to the commonly used small molecules, which act as (non-)competitive inhibitors, the disruption of the assembly process represents a completely new approach. (Jafary et al., 2019; Nadal-Bufi et al., 2021) The use of peptides as anti-cancer drugs has several advantages, such as ease of synthesis, better tissue penetrability, lower toxicity (Craik et al., 2013) and higher specificity due to their larger site of action (Cunningham et al., 2017). Higher affinity on the other hand allows the use of lower dosages to achieve the same effects. A previously designed peptide, cGmC9, which impairs the LDH-5 association, showed high potency and even lower IC_{50} values compared to GNE-140 (Nadal-Bufi et al., 2021). Nonetheless, this new group of inhibitors requires extensive *in vivo* studies before clinical implementation. The investigation of more potential inhibitors continues (Christov et al., 2021; Laganá et al., 2019; Nadal-Bufi et al., 2021; Rai et al., 2017).

8 Conclusion

The glycolytic phenotype of tumors such as CRC is accompanied by changes in the stress response system. Besides the accumulation of immunosuppressive lactate (Brand et al., 2016; Fischer et al., 2007), the metabolic change also leads to an upregulation of HSPs. Both mechanisms – the adaptive metabolism and the heat shock response – promote tumorigenicity via direct or indirect cellular mechanisms (Gabai et al., 2005; Sherman et al., 2011) and therefore burden conventional therapeutic regimes (Nadin et al., 2007; Sattler, Meyer, et al., 2010). Hsp90, 70 and 27 are correlated with increased therapy resistance, higher invasiveness, and consequently poor survival in CRC (Kocsis et al., 2011; Milicevic et al., 2008; Tweedle et al., 2010). The available preclinical data demonstrates the role of HSP in anti-apoptotic signaling cascades in CRC, promoting tumorigenicity and mediating chemo- and radioresistance (Abi Zamer et al., 2021; Lee et al., 2015a; Lopez-Camarillo & Arechaga-Ocampo, 2015). Given the scientific evidence, the potential of HSP as a therapeutic target now demands proper investigation in clinical trials.

This study demonstrated that LDH inhibition via GNE-140 leads to decreased HSP expression which in turn increases radiosensitivity in colorectal adenocarcinoma cells. Pharmacological inhibition of LDH represents a promising therapeutic approach, since it inflicts the metabolism and the stress response system. Thus, it provides the opportunity to affect the HSP axis on a large scale by suppressing the synthesis of numerous members. Since targeting single HSPs has not proven to be successful, this might be an approach to circumvent compensatory upregulation (Goloudina et al., 2012).

Particularly entities such as CRC with rising epidemiological significance (Ferlay et al., 2020) and exhausted therapeutic regimes (Abi Zamer et al., 2021; Manfredi et al., 2006) would profit from such a targeted strategy.

Once again, this stresses the need to drive forward research into LDH inhibitors suitable for clinical application to set a milestone in the fight against cancer.

9 List of figures and tables

9.1 List of figures

9.2 List of tables

10 Literature

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