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Overexpression of Heat-Shock Protein 27 (HSP27) Increases Gemcitabine Sensitivity in Pancreatic Cancer Cells through S-Phase Arrest and Apoptosis

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The secret of getting ahead is getting started. - - Mark Twain

DECLARATION

I hereby declare that the thesis is my original work and I have not received outside assistance. All the work and results presented in the thesis were performed independently. Anything from the literature was cited and listed in the reference. Part of the results has been published in a previous publication (*Schaefer C, Seeliger H, Bader DC, Assmann G, Buchner D, Guo Y, et al. Heat shock protein 27 as a prognostic and predictive biomarker in pancreatic ductal adenocarcinoma. Journal of cellular and molecular medicine. 2012*). No unauthorized data was included.

All the data presented in the thesis will not be used in any other thesis for scientific degree application.

The work for the thesis began from Oct. 2010 with the supervision from PD. Dr. med. Eike Gallmeier in Medizinischen Klinik II und Poliklinik Großhadern, Ludwig-Maximilians University Munich, Germany.

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CONTENTS

1. ABSTRACT

Pancreatic cancer is a highly aggressive cancer with gemcitabine as its standard therapeutic basis. We previously established a role for HSP27 as a prognostic and predictive marker in pancreatic cancer: Tissue-microarray studies showed increased HSP27 expression in pancreatic cancer specimens to be associated with better patient survival, while HSP27 overexpression unexpectedly increased susceptibility specifically towards gemcitabine in a pancreatic cancer cell line model. Vice versa, HSP27 protein depletion in HSP27 high-expressing AsPC-1 cells caused increased gemcitabine resistance. Here, we investigate the underlying mechanisms of *HSP27*-mediated gemcitabine sensitivity in pancreatic cancer cells. Utilizing a pancreatic cancer cell model with stable HSP27 overexpression, cell cycle arrest and apoptosis induction were analyzed by flow cytometry, nuclear staining, immunoblotting and mitochondrial staining. Drug sensitivity studies were performed by proliferation assays. Hyperthermia was stimulated using mild heat-shock at 41.8 °C. Upon gemcitabine treatment, HSP27-overexpressing cells displayed an early S-phase arrest subsequently followed by a strongly increased sub-G1 fraction. Apoptosis was characterized by PARP-, CASPASE 3-, CASPASE 8-, CASPASE 9- and BIM- activation along with a mitochondrial membrane potential loss. It was reversible through chemical caspase inhibition. Importantly, heat shock-mediated HSP27 induction similarly increased gemcitabine sensitivity in a panel of pancreatic cancer cell lines. Finally, HSP27-overexpressing pancreatic cancer cells displayed an increased sensitivity also towards death receptor-targeting agents, suggesting additional pro-apoptotic functions of HSP27 along the extrinsic apoptosis pathway. Taken together, in contrast to the well-established anti-apoptotic properties of HSP27 in cancer, our study reveals novel pro-apoptotic functions of HSP27- mediated through both the intrinsic and the extrinsic apoptotic pathways- at least in pancreatic cancer cells. HSP27 could represent a predictive marker of therapeutic response towards specific drug classes in pancreatic cancer and provides a novel molecular rationale for current clinical trials applying the combination of gemcitabine with regional hyperthermia in pancreatic cancer patients.

2. INTRODUCTION

2.1 Pancreatic cancer

2.1.1 Status Quo

Since forty years ago the US President Nixon "declared" War on Cancer [1], there have been obvious triumphs, for instance, it has been recognized that cancer harbors six significant hallmarks, sustaining proliferative signaling, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, evading growth suppressors [2,3]. During the past two decades, remarkable improvements have been made in treating malignant tumors. However, the war towards pancreatic cancer seems to be one of the most difficult tasks with dismal success in decreasing the mortality of the patients. In 2012, a statistical report has illustrated that pancreatic cancer remains the fourth leading cause of cancer modality among both men and women in the United States and little improvements have been shown in survival over the past 30 years [4]. The clinical treatment failure is often attributed to the early metastatic growth, a high drug-resistance to standard therapy options and high rates of local recurrence [5,6]. Thus the development of new and efficacious treatments is urgently demanded.

2.1.2 General principles for treating pancreatic cancer

The current management of pancreatic cancer is mainly guided by tumor stage, comorbidities and performance status of the patients. Surgical resection and a 6-month-course of adjuvant gemcitabine-based chemotherapy is the standard treatment for pancreatic cancer patients at an early stage [7]. In contrast, systemic palliative chemotherapy is applied for the patients with metastatic disease, while optimal treatment, with chemotherapy alone and/or chemoradiation, is still debated for patients with locally advanced disease but without evidence of metastasis [8].

2.1.2.1 Surgery

At present, surgical resection with negative margins (R0) is the only opportunity for cure [9]. Curative resection approaches contain pancreaticoduodenectomy, which is suitable for the majority of pancreatic cancer patients because of uncinate infiltration with the head and/or neck, and distal pancreatectomy for tumors which infiltrated the body and tail part [10]. However, as most patients have locally advanced or metastatic pancreatic cancer at the time of diagnosis, surgery is applicable in less than 20% [4].

2.1.2.2 Systemic chemotherapy: gemcitabine as the first-line regimen

Single-agent gemcitabine (GEM) has been approved as the first line chemotherapy for locally advanced or metastatic nonresectable pancreatic cancer by the National Comprehensive Cancer Network (NCCN) practice guidelines since 1997 [11]. Although gemcitabine could improve the survival rate only a few months [12,13], it remains the standard therapeutic basis in most clinical settings for pancreatic cancer patients [14].

Until now, numerous chemotherapeutic agents including 5-FU, cisplatin, oxaliplatin, and capecitabine have been evaluated alone or in combination in clinical trials and some promising findings have been reported [8]. A recent combinational regimen using oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX) significantly improved overall survival compared with gemcitabine and it was suggested that FOLFIRINOX might be an option for the patients with metastatic pancreatic cancer and good performance status [15]. Moreover, in patients with metastatic pancreatic cancer, nab-paclitaxel plus gemcitabine significantly improved overall survival, progression-free survival, and response rate, but rates of peripheral neuropathy and myelosuppression were increased [16].

Additionally, with the great breakthroughs on molecularly targeted molecules in cancer and understandings on the biological properties of pancreatic cancer, novel targeted agents have been evaluated in pancreatic cancer [17,18]. Since earlier and

later studies on anti-estrogen receptor therapies such as tamoxifen failed to demonstrate any survival benefit for the treatment [19-21], recent studies are mainly focused on the genes overexpressed or mutated in pancreatic cancer such as epidermal growth factor receptor (*EGFR*), human epidermal growth factor receptor type 2 (*HER2*) and vascular endothelial growth factor (*VEGF*), etc [22]. Molecular drugs including anti-HER1/EGFR inhibitor erlotinib, anti-VEGF antibody bevazicumab, anti-HER2 antibody trastuzumab, or multikinase inhibitors with antiangiogenic activity such as axitinib, sunitinib and sorafenib [23-28] have been tested in the clinical settings. Nevertheless, only erlotinib in combination with gemcitabine showed a minimal increase in median overall survival of about two weeks compared with gemcitabine plus placebo. In particular, a small subgroup of patients with more skin rash as a side effect of erlotinib treatment showed a significantly prolonged median survival of approximately 10 months [28].

To date, only a few clinical trials have evaluated the use of second-line regimens after the failure of first-line gemcitabine therapy [7]. One randomized trial tested the combination of oxaliplatin and 5-FU versus best supportive care after failure of gemcitabine treatment and indicated a potentially benefit from the 5-FU and platinum-based combination regimen after gemcitabine-based monotherapy, with an increased survival of 4.82 months versus 2.3 months [7,29]. But definitive results from large randomized clinical trials about second-line chemotherapy are still needed [30].

2.1.3 Gemcitabine resistance

Although gemcitabine remains the cornerstone of neoadjuvant and adjuvant chemotherapy in pancreatic cancer, the response rate is only 5.4% [11] and a progression-free survival interval imparts a range from 0.9 to 4.2 months [31]. Moreover, increasing reports have illustrated the dismal prognosis of pancreatic cancer is partly ascribable to the inherent or secondarily acquired resistance towards gemcitabine treatment [32,33].

2.1.3.1 Metabolism of gemcitabine

Gemcitabine (2, 2-difluorodeoxycytidine; dFdC) is a nucleoside prodrug with a complex metabolism (**Fig. 1**). After entering across the plasma membrane through nucleoside transporters, gemcitabine is phosphorylated into gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK) in a rate-limiting manner. Subsequently, dFdCMP is converted through nucleoside kinases to its active metabolites, the triphosphate- (dFdCTP) and diphosphate- (dFdCDP) nucleoside analogues [34]. dFdCTP confers its subsequent cytotoxicity through inhibition of DNA synthesis, while dFdCDP inhibits ribonucleotide reductase (RR), converting ribonucleosides to deoxyribonucleoside triphosphates (dNTPs), which is essential for DNA polymerisation and repair [35,36]. More than 90% of transported gemcitabine is inactivated by cytidine deaminase (CDA) into 2'-deoxy-2', 2'-difluorouridine (dFdU). Additionally, phosphorylated gemcitabine is converted by cellular 5'-nucleotidase (5'-NT), and dFdCMP can also be inactivated by deoxycytidine monophosphate deaminase (DCTD) into 2'-deoxy-2', 2'-difluorouridine monophosphate (dFdUMP) [37-39].

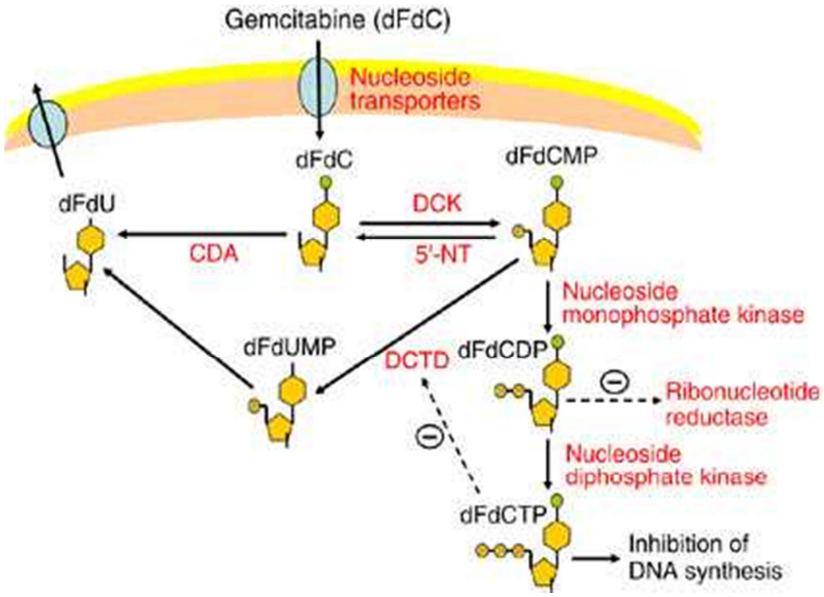


Figure 1. Metabolism of gemcitabine. *(copied from Ueno et al 2007 [39])*

2.1.3.2 Strategies to improve gemcitabine resistance

In the past decades, drug delivery, transport and metabolism of gemcitabine have been extensively studied [40] and various approaches have been developed to improve the sensitivity towards gemcitabine [41,42].

Innovative drug delivery

One of the most promising approaches for improving gemcitabine resistance is the use of innovative drug delivery devices [32,43]. Encouraging results have been achieved through the use of modified gemcitabine by liposomal encapsulation over the past 5 years [44,45]. In addition, novel supramolecular vesicular aggregates (SVAs) [46], polymeric nanoparticles [47,48] and squalenoylation [49-51] are also interesting to be used for the administration of the nucleoside analog [52].

Effective drug transport

Another intriguing approach is to increase the expressions of effective nucleoside transporters [53-55]. Plasma nucleoside transporter proteins majorly comprise two functionally distinct groups, the sodium-independent hENTs (human equilibrative nucleoside transporters) and the sodium-dependent hCNTs (human concentrative nucleoside transporters) [56]. The former group encompasses two transproters, hENT1 and hENT2, whereas the latter includes three isoforms, hCNT1, hCNT2, hCNT3 [53]. Of interest, hCNT1, hCNT3 and hENT1 are found to be the major transporters responsible for gemcitabine uptake into cells [57-59]. Recent clinical studies have reported overexpression of the three transporters is correlated with a significantly improved outcome for pancreatic cancer patients treated with gemcitabine [58,60-62].

Improved metabolism

Activities of some metabolic enzymes during the activation of gemcitabine have been reported to represent one major mechanism for gemcitabine resistance [63].

Modulation or genetic changes of these enzymes could improve therapeutic responses for pancreatic cancer. For example, mutation or deficiency of dCK has been considered to be one important mechanism for gemcitabine resistance [64,65]. In contrast, high expressions of the catabolic enzymes 5'-NT and CDA are also associated with cellular resistance to gemcitabine [66,67]. Moreover, gemcitabine resistance has been correlated with the overexpression of two RR subunits, RRM1 [68,69] and RRM2 [70,71].

2.1.4 Biomarkers for gemcitabine sensitivity in pancreatic cancer

In the past ten years, several potential predictive biomarkers alone or in combination have been mentioned for gemcitabine-based treatments in pancreatic cancer. It was reported that CA19-9 might serve as an indicator of response to gemcitabine-based chemotherapy in advanced pancreatic cancer [72,73]. Also, the expression levels of hENT1 were suggested for stratification of patients to chemotherapy including gemcitabine [60]. Moreover, dCK [74], RR subunits (RRM1 [75] and RRM2 [63]), Notch3 [76], Hu protein antigen R (HuR) [77], microRNAs [78] were found to be useful in predicting gemcitabine sensitivity of patients with pancreatic cancer. However, none of these markers has yet proven sufficiently robust to achieve clinical implementation in the gemcitabine-based treatment of pancreatic cancer.

2.1.5 Small summary

Despite much effort in the past decades, pancreatic cancer remains to be one of the most difficult-to-treat diseases. Except for surgery and systemic chemotherapy using gemcitabine, few therapeutic options have yet led to a measurable improvement in outcome. Therefore, it will be crucial to identify promising predictive markers for individualized treatment approaches based on molecular tumor characteristics and/or prognostic factors to improve the response towards traditional treatments. Particularly, new biomarkers predicting response towards gemcitabine treatment are urgently needed [79].

2.2 Heat shock protein 27

2.2.1 Heat shock proteins

Heat shock proteins (HSPs) are a set of evolutionarily conserved proteins first discovered in 1962 by Ritossa [80]. In human, HSPs are classified into two groups according to their sizes: large molecular weight HSPs and small molecular weight HSPs. The former group includes HSP110, HSP90 (HSPC), HSP70 (HSPA) and HSP60 (HSPD), and the latter are small HSPs (15 to 30 kDa) including HSP27 [81-83].

Normally, HSPs function as molecular chaperones for a large panel of 'client' proteins and have strong cytoprotective properties through the maintenance of cellular homeostasis under various physiological and stress conditions, including heat [84] and oxidative stress [85,86]. As has been convincingly shown in the last decades, HSPs are implicated in the pathogenesis of many diseases, such as neurodegeneration, myopathies, cardiomyopathies, cataracts, inflammatory diseases, and cancer [87]. Recently, various HSPs such as HSP90, HSP70, HSP27 were shown to be constitutively overexpressed in a wide range of tumor malignancies and are associated with prognosis, carcinogenesis, tumor differentiation and drug resistance [88]. Many reports reported overexpression of HSPs in various tumor entities to be connected to the suppression of apoptosis, exerted likely by their cytoprotective origin as chaperones [82,89]. However, HSPs appear to also exhibit pro-apoptotic functions and conflicting data exist [90], which is particularly true for HSP27 [90].

2.2.2 Roles of HSP27 in cancer

As one of the small HSPs, HSP27 possesses chaperone-like activity with preventing aggregation of misfolded proteins and has been implicated in proteasome-mediated protein degradation as well as in the modulation of cell death pathways [83,91-93]. Interestingly, HSP27 is found to be constitutively highly expressed in various tumor entities such as lung [94], gastric [95], prostate [96] and pancreatic cancers [97]. In

some tumor entities, this HSP27 overexpression appears to be associated with prognosis [98], tumor progression [90,99] or response to treatment [88,100-102]. Thus HSP27 has been suggested as a diagnostic, predictive and prognostic marker, implicating it as a potential therapeutic target molecule [88,103]. However, there are debates about the functional roles of HSP27 in cancer because conflicting findings have been reported [90]. Regarding HSP27 as prognostic marker, HSP27 overexpression was associated with poor prognosis in osteosarcomas [104], gastric [105,106], liver [107], prostate [108,109] and rectal cancer [110], while it was associated with favorable prognosis in endometrial adenocarcinomas [111], esophageal cancer [112], neuroblastomas [113] and malignant fibrous histiocytoma [114]. Inconclusive or conflicting findings were reported in breast [115-117], ovarian [118,119], oral [120,121] and pancreatic cancer [122]. Regarding HSP27 as a predictive marker, its upregulation was associated with radioresistance [123] in nasopharyngeal carcinoma cells [102], while data were inconsistent in head and neck cancer [124,125]. In regard to chemosensitivity, increased HSP27 expression has been reported to be associated with chemoresistance in a variety of cancers [88]. However, high HSP27 expression was strongly associated with tumor response to neoadjuvant chemotherapy in esophageal adenocarcinomas [126].

2.2.2.1 HSP27-regulated apoptosis in cancer

Previously, numerous reports indicated that HSP27 exerts predominantly anti-apoptotic effects in a variety of cancers [83], which is supported by its interaction with a plethora of apoptotic mediators. For example, HSP27 inhibits apoptosis through a caspase-dependent (intrinsic) pathway by binding to CASPASE 3 and CYTOCHROME C, preventing its interaction with APAF-1 and pro-CASPASE 9, and by inactivating BAX [127-130]. Furthermore, it has been reported that low expression of HSP27 induced apoptosis through a caspase-dependent pathway [128]. However, there are also conflicting findings about the roles of HSP27 in regard to apoptosis [131]. For instance, reports indicate that HSP27-dependent apoptosis was

caspase-independent [132,133].

2.2.2.2 Paradoxical roles of HSP27 in pancreatic cancer

As described above, the specific functional roles of HSP27 on the molecular level remain insufficiently understood and seem variable depending on tumor type or entity [88,134]. This applies particularly to the role of HSP27 as a prognostic marker in pancreatic cancer, for which on the one hand, HSP27 overexpression was associated with poor prognosis [135,136] and chemoresistance towards gemcitabine in some studies [137-140], while on the other hand, it was associated with good prognosis and increased sensitivity towards gemcitabine in others [122].

Similarly, the role of HSP27 as a predictive marker for gemcitabine sensitivity remains controversial [90,122,138]: In pancreatic cancer, HSP27 overexpression was shown to correlate with tumor aggressiveness and chemoresistance, indicating it as a potentially negative marker for gemcitabine response [97,135,136,138]. In contrast to those results, we have recently shown that high expression of HSP27 could serve as a positive prognostic marker in pancreatic cancer and that exogenous overexpression of HSP27 in pancreatic cancer cells conferred increased sensitivity to gemcitabine, indicating HSP27 as a potentially positive predictive marker for therapeutic response [122].

2.2.2.3 Roles of posttranslational modifications of HSP27 in cancer

HSP27 is regulated through posttranslational modifications such as phosphorylation [141]. Phosphorylation of HSP27 is mediated by the p38 MAPK stress kinase pathway through various kinases, including MAPK-activated protein kinase-2 (MK2), MK3, MK5, protein kinase A (PKA), AKT/protein kinase B (PKB), Protein kinase C (PKC), Protein kinase D (PKD), cGMP-dependent protein kinase, Ribosomal protein S6 kinase II ($p70^{RSK}$) and apoptosis signal-regulating kinase (ASK1) [142]. HSP27 is phosphorylated at three serine residues (Ser^{15}, Ser^{78} and Ser^{82}) [143]. This phosphorylation is a reversible process that regulates another posttranslational

modification of HSP27, the oligomerization of HSP27 [128]. This modification switches the formation of large oligomers to monomers or dimers. However, phosphorylation of HSP27 appears not to represent the only mechanism for regulating the HSP27 oligomerization including the formation of larger oligomers [128,144].

As compared to the less-studied oligomerization of HSP27, HSP27 phosphorylation has been more frequently examined in various malignancies. Of interest, increased HSP27 phosphorylation was found in different types of tumors [145,146] and might be associated with drug resistance [147,148]. For example, elevated phosphorylation of HSP27 might inhibit apoptosis [132] and was therefore proposed as a potential therapeutic target [149]. In pancreatic cancer, phosphorylated HSP27 might be a predictive biomarker for the sensitivity towards gemcitabine [150].

2.2.2.4 Other related signaling pathways

In addition to its implication in apoptotic pathways, overexpressed HSP27 interacts with many other signaling pathways in cancer, such as ASK1/p38/JNK pathway [151]. However, debated reports exist [152,153].

2.2.3 Small summary of HSP27 in cancer

HSP27 is constitutively overexpressed in various tumor malignancies and its overexpression has been associated with prognosis, diagnosis and therapeutic response in some tumor entities. Thus HSP27 could serve as a biomarker in various tumor types. However, the specific molecular functions of HSP27 in cancer are insufficiently understood. In particular, the role of HSP27 as a predictive marker for gemcitabine response in pancreatic cancer remains controversial.

2.3 Preceding own studies and deducted hypothesis

Previously, in order to identify novel biomarkers for pancreatic cancer, our group used Tissue-microarray (TMA) studies to analyze 86 specimens from pancreatic cancer patients after surgery in the Klinikum Grosshadern of Ludwig-Maximilians

University in Munich in Germany. We found that HSP27 could serve as a prognostic biomarker in pancreatic cancer. To test its potential role as a predictive marker for therapeutic response, a cellular HSP27 overexpression model was generated in PL5 pancreatic cancer cells. In this model, we were able to show that stably overexpressing HSP27 cell clones displayed drastically increased sensitivity specifically towards gemcitabine but not towards other agents commonly used for pancreatic cancer treatment [122].

2.4 Aim of the work

Pancreatic cancer is a highly aggressive cancer. Despite encouraging recent advances, gemcitabine remains the standard therapeutic basis in most clinical settings. Due to the common primary or acquired resistance of pancreatic cancer cells towards gemcitabine, predictive markers for chemotherapeutic response are urgently needed.

In our previous publication, we demonstrated that HSP27 could serve as a predictive marker for gemcitabine response since HSP27 overexpression drastically increased gemcitabine sensitivity. Thus the aims of this thesis can be summarized as follows:

1. To create a complementary cellular model system using RNA-interference technology to validate the results observed in the HSP27 overexpression model.

2. To explore the mechanistic basis of the observed effects, particularly in regard to:
    a. Cell cycle modulation;
    b. Apoptosis;
    c. Others (the metabolism of gemcitabine; other related signaling pathways; postmodifications of HSP27; etc.)

3. To analyze potential synergistic interactions between gemcitabine treatment and hyperthermia-induced HSP27 overexpression.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 General consumables

Consumables	Manufacturers
Non-pyrogenic serological pipet (2, 5, 10, 25 ml)	Sigma-Aldrich Chemie GmbH, Germany
PCR soft tubes	Fisher Scientific-Germany GmbH, Schwerte, Germany
Polyallomer tubes for ultracentrifugation (5 mm×10 mm) (#342630)	Beckman Coulter GmbH, Krefeld, Germany
Coverslip	Thermo Scientific Gerhard Menzel, Braunschweig, Germany
Cell Scraper 16cm	Sarstedt, Germany
Cuvettes	Sarstedt
Cover glass	Fisher Scientific- Germany GmbH
10cm cell culture dishes	BD Biosciences, Heidelberg, Germany
96-/12-/6-well plates	BD Biosciences
Cubic tubes (15, 50 ml)	BD Biosciences
Cryotubes Cryo.S^{TM}	Greiner Bio-one GmbH, Germany
Micro tubes (1.5 ml)	Sarstedt
Micro tubes (2 ml)	Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany
General pipettes (1 ml, 200 µl, 20 µl, 10 µl)	Eppendorf Vertrieb Deutschland GmbH
Glas Pasteur Pipettes	BRAND GmbH, Wertheim, Germany
Ranin pipettes/tips	Mettler-Toledo, LLC, Columbus, USA
Pipet tips	VWR International GmbH, Darmstadt, Germany
Gel Transfer filter paper	Germany
PVDF Membrane	Zefa-Laborservice GmbH, Harthausen, Germany
X-ray film for immunoblotting	Fuji Film Europe GmbH, Düsseldorf, Germany
PARAFILM® M	Sigma-Aldrich Chemie GmbH

3.1.2 Reagents

Reagents	Manufacturers
G418 (neomycin)	Carl Roth GmbH, Karlsruhe, Germany
Oligofectamine™ Reagent	Invitrogen, Life Technologies GmbH, Darmstadt, Germany
Lipofectamine™ 2000 Reagent	Invitrogen
Lipofectamine™ Reagent	Invitrogen
Polyethylenimine (PEI)	a gift from Benjamin Hirschi (benjamin.hirschi@helmholtz-muenchen.de)
Gemcitabine (Gemza)	Lilly Deutschland GmbH, Bad Homburg, Germany
SYBR Green I Nucleic Acid Gel Stain	Molecular Probes, Invitrogen, Darmstadt, Germany
Z-VAD-FMK	Promega GmbH, Mannheim, Germany
LBY135	Novartis, Basel, Switzerland
Tigatuzumab (CS-1008)	Daiichi Sankyo, Edison, NJ, USA
DMSO	Carl Roth GmbH
TEMED	Bio-Rad Laboratories GmbH, Munich, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH
DN/RNase-Free H_2O	Qiagen GmbH, Hilden, Germany
2-Mercaptoethanol	Sigma-Aldrich Chemi GmbH
Tween® 20	Sigma-Aldrich Chemi GmbH
propidium iodide	Sigma-Aldrich Chemi GmbH
Non-fat dry milk	Bio-Rad Laboratories GmbH
Other common chemicals (Ethidium Bromide (EB), Agarose, Tris, sodium dodecyl sulphate (SDS), methanol, sodium citrate, Triton X-100, etc)	Carl Roth GmbH

3.1.3 Media, solutions for cell culture

Product information	Manufacturers
DMEM high Glucose (4.5 g/l)	GE Healthcare, PAA Laboratories GmbH, Pasching, Austria
Dulbecco's PBS	Sigma-Aldrich Chemi GmbH
RPMI medium	GE Healthcare

Opti-MEM® I Reduced Serum Medium	Gibco, Life Technologies GmbH, Darmstadt, Germany
FBS Superior	Biochrom AG, Berlin, Germany
Penicilin/Streptomycin (100 ×)	PAA Laboratories GmbH
Trypsin	PAA Laboratories GmbH
Poly-L-lysine	Trevigen, R&D systems GmbH, Wiesbaden-Nordenstadt, Germany

3.1.4 Vector

Vector pcDNA3.1 with neomycin resistance (**Fig. 2**) was purchased from Invitrogen. Transfected vectors contained the complete coding sequence of either of three different HSP27 constructs under the control of the human HSP27 promoter were provided by Prof. Dr. Med. Claus Schaefer (Department of Medicine II, Klinikum Neumarkt, Neumarkt i. d. OPf., Germany). The HSP27 constructs consisted either of wild-type human HSP27 (hu) or mutants with the serines 15, 78 and 82 substituted to alanines (3A) or aspartic acids (3D). The mutant 3A represents a non-phosphorylatable kinase-dead form of HSP27 while 3D imitates a permanently phosphorylated form through insertion of the three negatively charged residues [154,155]. In short, the transfected plasmids/cells were labeled with ‘hu’, ‘3A’, ‘3D’, respectively.

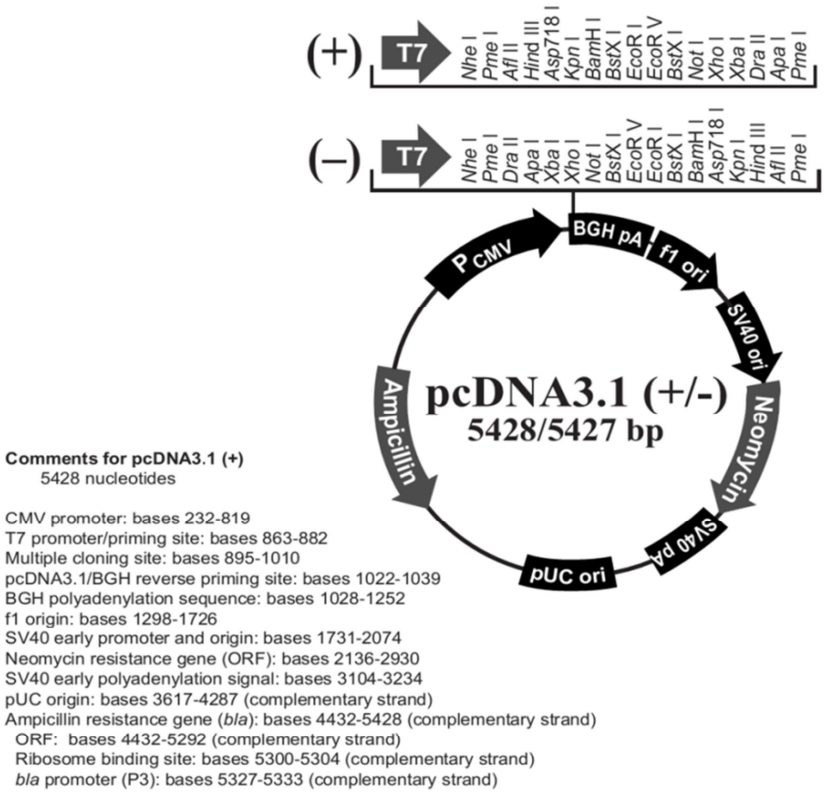


Figure 2. Map of pcDNA 3.1 *(Invitrogen™ Life Technologies)*

3.1.5 Preparation of buffers, solutions, and gels

1 mg/ml BSA (-20 °C)

Amount	Ingredients
10 mg	BSA
10 ml	ddH_2O

Hoechst 33342 (Sigma-Aldrich Chemie GmbH)

A stock of 10 mg/ml was prepared in ddH_2O and stored at room temperature (RT), avoiding light.

Resolving gel solution (4 °C)

Amount	Ingredients
400 ml	ddH_2O
250 ml	1.5 M Tris pH 8.8
10 ml	10% SDS

Stacking gel solution (4 °C)

Amount	Ingredients
340 ml	ddH_2O
62.5 ml	1.0 M Tris pH 6.8
5 ml	10% SDS

0.2 mol/l PMSF solution (-20 °C, fresh for use in max. 6 months)

Amount	Ingredients
34.8 g	PMSF
1 ml	2-propanol

5 g/l Aprotinin (-20 °C)

Amount	Ingredients
5 mg	Aprotinin
1 ml	ddH_2O

10 mmol/l Leupeptin hemisulfate (-20 °C)

Amount	Ingredients
4.8 mg	Leupeptin hemisulfate
1 ml	ddH_2O

Homogenization buffer (pH 7.4, 4 °C)

Amount	Ingredients
8.55 g	Sucrose
242.2 mg	Tris Base
76 mg	EGTA
Up to 100 ml	ddH_2O

H buffer: 1ml homogenization buffer+5 µl 5 g/l aprotinin+ 5 µl 10 mmol/l leupeptin hemisulfate+ 5 µl 0.2 mol/l PMSF + 0.7 µl 2-Mercaptoethanol

5 × sample loading buffer (Laemmli buffer) (-20 °C)

Amount	Ingredients
2.13 ml	0.5 M Tris HCl pH 6.8+ 0.4% SDS
1 g	SDS
5 ml	Glycerol
2.8 ml	ddH_2O
traces	bromophenol blue
2.56 ml	ß-Mercaptoethanol

RIPA (Radio Immuno Precipitation Assay) Buffer (4 °C)

Amount	Ingredients
5 ml	1 M Tris-Cl pH 7.4
3 ml	5 M NaCl
5 ml	20% NP-40
5 ml	10% sodium deoxycholate
0.5 ml	20% SDS
81.5 ml	ddH_2O

Additionally, ready-to-use cocktails of inhibitors containing 1 mM PMSF, 10mM NaF, 1 mM Natrium Orthovanadate (Na_3VO_4), 1 x Protein Inhibitors (complete from Roche) were added before using.

1 M Tris-Cl 1 L (pH 7.4 to pH 8.0)

Amount	Ingredients
121.1 g	Tris Base
700 ml	ddH_2O
70 ml	Concentrated HCl (37.2% - 12.1 M)

Directions:

1) Mix 121.1 g of Tris Base with 700 ml of ddH_2O by stirring.

2) Add HCl in the following volumes to reach the desired pH:

pH 7.4 = 70 ml of HCl; pH 7.6 = 60 ml of HCl; pH 8.0 = 42 ml of HCl

3) Fine adjust to the desired pH (7.4 - 8.0) with concentrated HCl.

4) Add ddH_2O until final volume is 1 L.

5) Autoclave to sterilize.

5 M NaCl (sodium chloride) 500 ml (RT)

Amount	Ingredients
146.1 g	NaCl
450 ml	ddH_2O

20% NP-40 (Nonidet P-40) 100 ml (4 °C)

Amount	Ingredients
20 ml	NP-40
80 ml	ddH_2O

10% sodium deoxycholate (Deoxycholic acid) 100 ml (RT)

Amount	Ingredients
10 g	sodium deoxycholate
80 ml	ddH_2O

20% SDS (sodium dodecyl sulfate) 100 ml (RT)

Amount	Ingredients
20 g	SDS
80 ml	ddH_2O

SDS-PAGE Electrophoresis Running Buffer (10x) 10L (RT)

Amount	Ingredients
303 g	Tris Base
1440 g	glycine
100 g	SDS
10 L	ddH_2O

Direction: For 1x Running Buffer: 100 ml from 10x buffer plus 900 ml ddH_2O.

Transfer Buffer without SDS (10x) 10 L (RT)

Amount	Ingredients
303 g	Tris Base
1440 g	glycine

10 L	ddH_2O

1x Transfer Buffer 500 ml (RT)

Amount	Ingredients
50 ml	10x Transfer buffer without SDS
100 ml	Methanol (final 20% methanol)
350 ml	ddH_2O

10 × TBS (concentrated TBS) 1 L (RT)

Amount	Ingredients
24.1 g	Tris Base
80 g	NaCl
800 ml	ddH_2O

Directions: Adjust pH through pure HCl and add ddH_2O up to 1 L

1x TBST 1 L (RT)

Amount	Ingredients
100 ml	TBS 10 ×
800 ml	ddH_2O
1 ml	Tween20

Directions: Adjust pH to 7.6 and add ddH_2O to 1 L.

HBSS solution 1 L (4 °C)

Amount	Ingredients
8.00 g	NaCl
0.40 g	KCl
2.38 g	HEPES
0.35 g	$NaHCO_3$
0.06 g	KH_2PO_4
0.06 g	Na_2HPO_4
0.1 g	$MgSO_4$•7 H_2O
0.1 g	$MgCl_2$•10 H_2O
0.185 g	$CaCl_2$•2 H_2O
1 g	Glucose

Directions:

1) Add ddH_2O to near 1 L, and adjust PH to 7.4 and the end volume to 1 L.

2) Use 0.2 μm filter to sterilize the solution.

Agarose gel 2% (4 °C)

Amount	Ingredients
4 g	Agarose
200 ml	ddH_2O

Directions:

1) Use the oven to heat and mix, add 10 μl 10mg/ml EB before coagulation;
2) Prepare gels with combs.

PCR loading dye: 10× OG loading dye

Amount	Ingredients
0.05 g	Orange G
1.5 g	Ficol (type 400)
1 ml	0.5 M EDTA (pH 8)
9 ml	ddH_2O (sterile)

Directions:

1) Add Ficol to a 10~15 ml tube first then add all other ingredients inside;

2) Mix well by vortex;

3) Load 2~3 μl of OG for 10 μl of PCR solution;

4) Store at RT.

Preparing SDS-PAGE gels 10%

Directions:

1) Clean the plates and combs;
2) Set up the plates on the rack;
3) Prepare the separating gel: Pipette solutions below in order. Swirl the solution gently to mix thoroughly after addition of each component.

Components	Volume
ddH_2O	2.50 mL
40% acrylamide/bis stock	1.25 mL
1.5 M Tris, pH 8.8	1.25 mL
10% ammonium persulfate	50 µL
TEMED	5 µL
Total volume	~5 mL

4) Prepare the stacking gel: Prepare around 1.2 mL mixture for each gel. Pipette the solutions carefully and swirl to mix after addition of each component. Pipette the gel mix between the plates up to just below the edge of the short plate quickly. Carefully place in the comb.

Components	Volume
ddH_2O	3.13 mL
40% acrylamide/bis stock	0.62 mL
1.5 M Tris, pH 8.8	1.25 mL
10% ammonium persulfate	50 µL
TEMED	5 µL
Total volume	~5 mL

5) Gel storage. The gel can be stored horizontally at 4 °C for up to 7 days.

Gemcitabine

Gemcitabine was dissolved at a stock concentration of 10 mM in DN/RNase-free H_2O and stored at RT.

LBY135

A stock solution was made at a concentration of 50mg/ml and stored at 4 °C, protected from light.

Tigatuzumab

A stock solution was prepared at a concentration of 10 mg/ml and stored at 4 °C, protected from light.

The broad-caspase inhibitor, Z-VAD-FMK

The solution was prepared at a stock concentration of 20 mM in DMSO and stored at -20 °C prior to incubation.

3.1.6 Standard markers

Products	Manufacturers
Precision Plus Protein^{TM} Standards	Bio-Rad Laboratories GmbH
O'GeneRuler^{TM} 1 kb Plus DNA Ladder	Fermentas Life Sciences, Fisher Scientific - Germany GmbH, Schwerte , Germany
Low Molecular Weight DNA Ladder	New England Biolabs GmbH, Frankfurt am Main, Germany

3.1.7 Instruments

Equipements	Manufacturers
Cell counting chamber (0.0025 mm^2/ 0.1 mm)	Carl Roth GmbH
Inverted Microscope CK2	Olympus Optical Co. (Europa) GmbH
Vortex Mixer VM-300	NeoLab Migge, Heidelberg, Germany
MiniSpin	Eppendorf Vertrieb Deutschland GmbH
Airfuge® Air-Driven Ultracentrifuge	Beckman Coulter GmbH
Sonifier	Branson, Germany
PCR gel electrophoresis chamber; power supply	Bio-Rad Laboratories GmbH
UV/Visable Spectrophotometer Ultrospec 3100 pro	Amersham Biosciences, Germany
PH meter	InoLab® - WTW inoLab Laborgeräte, Germany
Weighing machine	SCALTEL, Germany
Mixer RCT basic	KIKA® Werke, Germany
Water bath for cell culture	Medingen, Germany
Electronic Thermometer	Amarell GmbH, Kreuzwertheim, Germany
Water bath for heat shock	GFL®, Germany
Tri-Carb® 2100TR Liquid Scintillation Counter	PerkinElmer Life Science, Rodgau, Germany
Ranin pipettes/tips for PCR/Cell culture	Mettler-Toledo GmbH, Ockerweg, Gießen, Germany
Cooling centrifuge	Eppendorf Vertrieb Deutschland GmbH
Centrifuge	Hettich Rotantab, Germany

PCR cycler	Eppendorf Vertrieb Deutschland GmbH
HERA cell culture Incubator	Fisher Scientific- Germany GmbH
Laminar Hood for cell culture	Fisher Scientific- Germany GmbH
MultiDoc-It Digital Imaging System (UV Transilluminator) + FujiTsu Siemens Computer	UVP, LLC, USA
Milli-Q water	Merck Chemicals GmbH, Schwalbach, Germany
Heater (for protein denaturation)	Kobe, Germany
Western Blot shaking machine	Rocky® 3D
Western Blot Gel making unit (chamber, glasses, combs, etc)	Bio-Rad Laboratories GmbH
Electrophoresis chamber for immunoblotting + power supply	Bio-Rad Laboratories GmbH
Electrophoresis transfer unit	PeQLab Biotechnologies GmbH
Refrigerators (+4 and -20 °C)	LIEBHERR, Germany
CytoFluor 4000 plate reader	Per-Septive Biosystems, Framingham, MA, USA
Zeiss Axiovert 135 TV fluorescence microscope	Carl Zeiss, Jena, Germany
fluorescence-activated cell sorter (FACS)	Accuri C6 Flow Cytometer®, BD Biosciences, Heidelberg, Germany

3.1.8 Kits

BioRad Protein assay kit (Catalog No.500-0001, Bio-Rad Laboratories)

Kapa Taq kit for PCR (Kapa Biosystems)

Code	Description	Kit Contents
KK1015	KAPA Taq DNA Polymerase (500 U)	500 units, 5 U/µL. Wild-type Taq with 10× KAPA Taq Buffer A, 10× KAPA Taq Buffer B (both with Mg^{2+} at a concentration of 1.5 mM), and extra $MgCl^2$ (25 mM)

Mitochondrial membrane potential staining kit (Sigma Aldrich)

Catalog Number	Description	Components
CS0390	Mitochondria Staining Kit For mitochondrial potential changes detection	1 mg JC-1, 1 ml DMSO, 0.1 ml Valinomycin Ready Made Solution (1 mg/ml)

Western Blot detection kits

Three different detection kits for immunoblotting were purchased from Thermo Scientific.

Product name and Number	Description
Pierce ECL Western Blotting Substrate (Product # 32106)	An entry-level ECL substrate
SuperSignal West Pico Chemiluminescent Substrate (Product # 34077)	Twice the signal duration and sensitivity of entry-level ECL substrates
SuperSignal West Dura Chemiluminescent Substrate (Product # 34075)	Long signal duration and high signal intensity

3.2 Methods

3.2.1 Cell culture

Pancreatic cancer cell lines PL5 and PL11 were kindly provided by S.E. Kern (Johns Hopkins University, Baltimore, MD, USA). PL5 cells were also named Panc 04.03 (ATCC®CRL-2555™). All other pancreatic cancer cell lines, Su86.86, MIA PaCa-2, AsPc1, CFPAC1, Panc1, BxPc3, Capan1, Capan2, PL3, PL45-12, PL8, were purchased from the European Collection of Cell Cultures (Sigma-Aldrich, Munich, Germany) or the American Type Culture Collection (LGC Standards, Wesel, Germany), respectively. Early-passage primary human pancreatic cancer cell lines 518-665703 (short for 518), 202-587027 (202), 311-368623 (311), 520-051403 (520), 455-903753 (455), PPC0039 were derived and propagated in our laboratory from surgical specimens of pancreatic adenocarcinoma from patients (Klinikum Grosshadern, Ludwig Maxilians University, Munich, Germany). Cells were grown routinely in DMEM media (PL5, PL11, Su86.86, MIA PaCa-2, CFPAC1, Panc1,

Capan1, Capan2, PL3, PL45-12, PL8 cells) or in RPMI media (AsPc1, BxPc3 cells), supplemented with 10% FCS, L-glutamine and 1% P/S and incubated in a 5% CO_2 humidified atmosphere. Every 2~3 days, cells were digested with trypsin.

3.2.2 Detection of Mycoplasma contamination

Many methods are available to detect mycoplasma, including isolation on selective microbiological growth media, direct or indirect fluorescent staining, the enzyme-linked immunosorbent assay (ELISA), autoradiography, immunostaining and direct or nested PCR [156]. Among all methods, PCR appears to be a fast, sensitive method in the laboratories, although it might give false positives or false negatives [157].

In order to obtain reliable results with a relatively high sensitivity and specificity, our lab has optimized this method through regular examinations under the inverted or fluorescence microscope and by PCR, usage of fresh cells less than two months and treating the contaminated cells with drugs or discarding contaminated cells immediately. We used one standard primer pair (Forward Primer: 5' GGG AGC AAA CAG GAT TAG ATA CCC T 3'; Reverse Primer: 5' TGC ACC ATC TGT CAC TCC GTT AAC CTC 3'; Product size: 280 bp) [158] purchased from Metabion (Metabion international AG, Munich, Germany) and prepared the PCR reaction solution according to the following recipe. 1 µl of cell culture media was regarded as the template, and GAPDH was used as a loading control, while 1 µl sterilized DN/RNase-Free H_2O as the negative control and two of 1 µl formerly contaminated samples as the positive controls.

Ingredients	1× Master Mix
10× Kapa Buffer B	7.36 µl
dNTP (10 mM)	0.2 µl
100% DMSO	0.2 µl
Primer Mixture (10 mM)	0.2 µl
Kapa Taq polymerase (1 U/µl)	0.04 µl
Template	1 µl
add DN/RNase-Free H_2O H_2O to	10 µl

The PCR reaction procedures were as follows,

95°C	2 min	
95°C	30 sec	35 cycles
62°C	30 sec	
72°C	40 sec	
72°C	2 min	
4°C	forever	

3.2.3 Generation of cell clones stably overexpressing different HSP27 protein variant

3.2.3.1 G418 selection test

Cells were seeded with at 300000 cells/well in 6-well plates to make sure the confluence reached ~50% the next day. Cells were washed once with fresh DMEM medium (containing 10% FCS, 1% P/S) and cultured with G418 at concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.6 mg/ml or 0, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml. Cells were examined, growth patterns recorded after 5, 10, and 15 days, and optimized cell concentrations (i.e. 100% cell death) used for consecutive drug selection.

3.2.3.2 Stable transfection by PEI, Lipofectamine™ or Lipofectamine™ 2000

Cells were cultured in 10 cm dishes to reach a confluence of 60~ 80%. On the next day, 2 µg transfected vector and a related volume of transfection reagent were incubated separately in 250 µl OptiMEM medium for 5 min. Two solutions were

mixed and incubated for 20 min. Cultured cells were washed once with DMEM medium and the above mixture was added drop-wise to the cells. 4~ 6 h later, 10 ml fresh DMEM medium was added to stop the transfection. 24 h later, culture medium was changed with fresh DMEM medium containing previously optimized concentrations of G418. Four weeks later, surviving clones were transferred into 6-well plates.

Three transfection reagents including PEI, Lipofectamine™ or Lipofectamine™ 2000 were tested. For the generation of a control cell line, cells were transfected with unaltered vector pcDNA3.1 (empty vector, EV).

3.2.3.3 Single cell dilution

The above surviving polyclonal cells (harboring different expression levels of HSP27 in each clone) were single-cell selected and single clones displaying high overexpression of HSP27 picked. Single-cell dilution was performed by cell seeding at concentrations of 0.2, 1, 5 cell(s)/well in 96-well plates. Two weeks later, single clones were labeled. Four weeks later, cells were transferred into 6-well plates for propagation and consecutive screening by immunoblotting.

3.2.3.4 Screening the clones with the overexpression of HSP27

When cells reached a confluence of around 85%, around 80% cells were harvested for immunoblotting to compare the expression of HSP27 among parental, EV-transfected, HSP27 construct-transfected clones. The clones with a high expression of HSP27 were cultured in 10 cm dishes and frozen for further experiments.

3.2.4 Gemcitabine metabolism

3.2.4.1 Uptake of [^{3}H] Gemcitabine

Cells were cultured in 6-well plates to reach a confluence of around 60~70%. The next day, cells were washed three times with sodium-containing (+Na) and

sodium-free (-Na) buffer, respectively. Cells were then cultured in Incubation Buffer (above buffers containing 1 µM [^{3}H] Gemcitabine) for different time points (10, 20, 40, or 80 min and 0, 1, 2, 4, or 8 h). Afterwards cells were washed three times with 1 ml Stop Buffer (above buffers containing 10 µM [^{3}H] Gemcitabine). Consequently, buffer was discarded and 0.5 ml Trito-X-100 (1%) was added. 0.4 ml of the samples was collected for radioactivity detection through a Radiation Counter and 0.1 ml for protein measurement (Bradford method [159]).

3.2.4.2 Immunoblotting of membrane transporters, HSP27 phosphorylation, p38 and JNK pathways

To detect membrane transporters, cells were either treated to dissect cytosol and membrane lysates or whole lysates were used. All protocols are explained in the part 'Immunoblotting' (see below).

3.2.5 Flow cytometry

Apoptosis and cell cycle were analyzed by assessing sub-G1 events or cell cycle distribution using a fluorescence-activated cell sorter (FACS) (Accuri C6 Flow Cytometer®, BD Biosciences, Heidelberg, Germany) and CFlow Plus software (BD Biosciences) according to the method by Nicoletti [160]. Cells were seeded in 6-well plates or 12-well plates to reach a confluence of 50~60% the next day. Gemcitabine was then added at the end concentrations of 0, 6.25, 15 or 25 nM. After 24 and 48 h, the cells were split by trypsin, collected and washed with sterilized, ice-cold PBS once, then incubated in staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/mL propidium iodide (PI). Sub-G1 events and cell cycle distribution were measured using CFlow Plus software. Specific rate of apoptosis was calculated as $(rate_{sample} - rate_{control}) / (1 - rate_{control})$.

3.2.6 Hoechst staining

Cells were cultured in 6-well plates with or without sterilized slides inside. The next

day, cells were treated with gemcitabine at 0, 6.25, 15, 25 nM for 24, 36, or 48 h. Afterwards, cells were fixed with 4% paraformaldehyde in PBS for 20 min followed by washing with PBS twice. Fixed cells were then stained using Hoechst 33342 at 2.5 µg/mL in PBS for 20~30 min and subsequently examined using a Zeiss Axiovert 135 TV fluorescence microscope for morphological changes.

3.2.7 Immunoblotting

3.2.7.1 Lysate preparation

Whole cell lysates

Cells were scraped off the culture dishes/plates and centrifuged at a low speed (i.e. 800 rpm) for 10 sec. The supernatant was discarded and cells were washed with PBS twice. PBS was then carefully completely removed. Cell pellets were lysed in RIPA buffer for 20~30 min on ice. The cell lysate was finally centrifuged at high speed (i.e. 1000 rpm) at 4 °C for 10 min. The supernatant was stored at -80 °C as 'whole cell lysate'.

Preparation of crude membrane fraction by ultracentrifugation

Cells were cultured in 6-well plates until a confluence of around 50~60% was reached and treated with a therapeutic agent(s) according to respective experiments. Cells were then washed with cold PBS twice and 150 µl H buffer was added. Afterwards, cells were collected, centrifuged at 4 °C for 2 min and the supernatant was discarded. 150 µl H buffer was added and sonificated for 5 sec. Mixture was centrifuged for 2 min to precipitate the nucleus. The supernatant was transferred to a microtube and centrifuged again using Airfuge at the highest speed. The supernatant was collect as the cytosolic fraction. An appropriate volume (20~30 µl) of H buffer was added and the membrane pellet was rinsed with a tip, resuspended and transferred as the membrane suspension.

3.2.7.2 Protein quantification

The Bradford assay was used to measure protein concentrations of lysates [159]. First, the Bradford reagent was diluted fivefold in ddH_2O (1 part Bradford: 4 parts ddH_2O). Second, a serial diluted BSA solution (0.2, 0.4, 0.6, 0.8 mg/ml) was used to create a standard curve. Third, 1~5 µl of the protein extract was mixed with 1 ml the diluted reagent in a clean and dry plastic cuvette. The plate was incubated at RT for 10 min before the absorbance was measured at 595 nm with a spectrophotometer. The concentration of each sample was calculated according to the established standard curve.

3.2.7.3 Immunoblotting

Gel running

The cell extracts were mixed with 5 × Laemmli loading buffer, ddH_2O and adjusted with water to reach identical end concentrations. Lysates were then cooked at 95 °C for 10 min, spun down and loaded on precast SDS-PAGE gels (10%) using identical amounts (20~60 µg). Gels were run at 80 V for 30 min and then at 120 V for 30~60 min. One lane was used for the molecular weight standard, Precision Plus Protein™ All Blue Standards (6µl).

Gel transferring

After separation, proteins were transferred to a PVDF membrane in a transfer chamber. Before the transfer, the membranes are activated by isopropanol incubation for 10 s. After activation, the membrane was briefly washed with deionized water and rinsed in transfer buffer to be equilibrated. For the transfer, the PeQLab semi-dry transfer system was used. The transfer took place at a constant flow of 125 mA for 45~ 60 min.

Blocking, incubation and detection

Membranes were blocked for 1 h in TBS solution containing 0.1% Tween 20 (TBST) and 5% milk powder. Membranes were then incubated with primary antibodies in TBST containing 5% milk powder or 5% BSA, respectively, at 4 °C overnight, followed by the appropriate secondary antibody for 1 h at RT. Immunoblotting were developed using the ECL system according to the manufacturer's instructions. The following first antibodies were used: anti- HSP27 (SPA-803, Stressgen/Enzo, Lörrach, Germany); anti- phospho-HSP27 (ser^{15}, ser^{78}, ser^{82}) (Stressgen/Enzo) ; anti- hCNT1, anti- hCNT3, anti- hENT1 (kindly provided by Ralf Wimmer); anti- p38α, anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (Santa Cruz Biotechnology, Dallas Texas USA); anti- SAPK/JNK, anti- phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), anti- CASPASE 3, anti- CASPASE 8, anti- CASPASE 9, anti- PARP, anti- BCL-2, anti- BCL-xL, anti-BID, anti- BAD, anti- BAX, anti- MCL-1 (Cell Signaling Technology, Boston MA USA); anti- BAK, anti- FADD, anti- FLIP (Santa Cruz Biotechnology); anti- BIM (BD Biosciences). Anti-ß-ACTIN antibody (Sigma) served as loading control. The membranes were washed and stained with either anti-mouse or anti-rabbit HRP-conjugated antibody (GE Healthcare). Enhanced chemo-luminescence was elicited using ECL Western Blotting Substrate.

3.2.8 RNA interference

Cells were plated to reach a confluence of 40~ 60%. On the next day, cells were transfected using Oligofectamine (Invitrogen) and siRNA directed against HSP27 (sense: GGACGAGCAUG-GCUACAUCTT, antisense: GAUGUAGCCAUGCUCGUCCTT; Qiagen) at a final concentration of 100 nM. The transfection proceeded for 4 h before adding serum-containing medium. HSP27 protein depletion was quantified by immunoblotting at 24, 48, 72, 96, 120, 144, 168, or 192 h after transfection. Gemcitabine was added to the cells at previously determined time point of maximal HSP27 down-regulation, which was separately confirmed for each experiment.

3.2.9 Cell proliferation assay

The assays were performed over a broad range of concentrations covering 100 to 0% cell survival. 600 to 2500 cells/well were seeded in 96-well plates to reach confluence on day 6. After settling, the cells were incubated with various drugs at the indicated concentrations. For caspase inhibition experiments, cells were pre-treated using the Z-VAD-FMK pan-caspase inhibitor [161] at 20 μM for 3 h and then treated with gemcitabine. Following incubation for 6 d, the cells were washed, lysed in 100 μl H2O, and 0.2% SybrGreen (Invitrogen) was added. Fluorescence was measured using a CytoFluor 4000 plate reader (Applied Biosystems, Darmstadt, Germany) and growth inhibition calculated as compared to the untreated control samples. At least three independent experiments were performed per agent, with each data point reflecting triplicate wells.

3.2.10 Evaluation of mitochondrial membrane potential by JC-1 staining

Cells were cultured in poly-L-Lysine-coated 6-well or 96-well plates for 24 h and then treated with gemcitabine at 0, 25, or 50 nM for 6 or 24 h, respectively. Next, cells were stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbo-cyaniniodide (JC-1) solution at a final concentration of 25 μg/ml for 30 min. Fluo-rescence was measured employing a CytoFluor 4000 plate reader at 485 nm (excitation) and at 530 and 580 nm (emission). The ratio of green and red fluorescence signals served as surrogate readout for the mitochondrial membrane potential ($\Delta\psi_m$) independent of the mitochondrial mass; For fluorescence imaging, cells were grown directly on slides, treated as described above and pictures taken using the Zeiss Axiovert 135 TV fluorescence microscope; For FACS analysis, cells were harvested and analyzed using the Accuri C6 Flow Cytometer®. Qualitative and quantitative data were recorded through CFlow Plus software. Valinomycin or carbonyl cyanide 3-chlorophenylhydrazone served as negative controls. All experiments were performed at least in triplicate.

3.2.11 Experimental heat shock in combination with gemcitabine treatment

After individual determination of the half maximal inhibitory concentration (IC_{50}) of gemcitabine for each cell line, the heat-shock conditions were individually optimized for each cell line in regard to maximal HSP27 induction, as determined by immunoblotting, along with minimal toxicity, as determined by proliferation assays. After cell seeding and attachment, culture dishes at 60 to 70% confluence were wrapped with parafilm and immersed in a water bath at 41.8 or 43 °C for 15, 30, 60, 90, 120, or 150 min, respectively. Cells subjected to 37 °C served as controls. Constant temperature was verified through continuous monitoring. Afterwards, the cells were recovered at 37 °C for 24 h and harvested for immunoblotting. In another set of experiments, heat-shock was performed at 41.8 °C for 0, 60, or 90 min, respectively, depending on the respective cell line. Afterwards, cells were incubated with gemcitabine at 0, 25, 50, 100, or 200 nM for 5 d. Cell survival rates were measured using a CytoFluor 4000 plate reader. Experiments were repeated at least in triplicate.

3.2.12 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL, USA). Error bars represent standard error of the mean (SEM) from three experiments. Sensitivity studies were statistically interpreted using a paired Student's *t*-test. P-values <0.05 were considered statistically significant.

4. RESULTS

4.1 Exclusion of mycoplasma contamination

To detect mycoplasma contamination, polymerase chain reaction (PCR) was applied. In a representative PCR result, our three cell lines were mycoplasma negative as compared to the two positive controls and one negative control (**Fig. 3**).

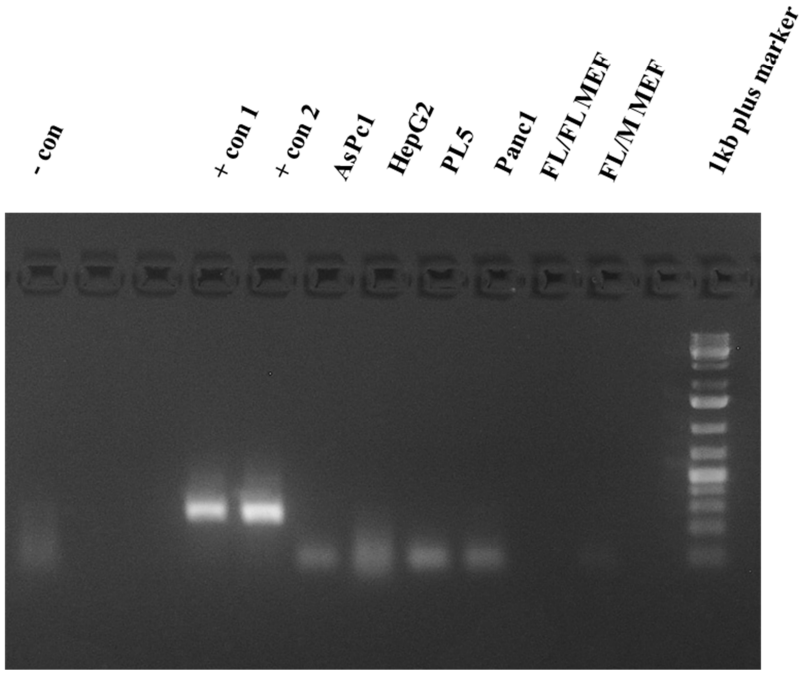


Figure 3. Detection of mycoplasma contamination. *A representative PCR result using two positive controls (+ con 1, + con 2), one negative control (- con), three pancreatic cancer cell lines (AsPc1, PL5, Panc1) from our group and three other cell lines (HepG2, FL/FL MEF, FL/M MEF) from other groups. Positive band: 280 bp; Marker: 1 kb plus.*

4.2 Negative findings in gemcitabine metabolism

To assess whether nucleoside transport might be involved in HSP27 overexpression-mediated gemcitabine sensitivity, we first tested the uptake of [^{3}H] gemcitabine among parental PL5 cells, empty-vector PL5/EV cells and the respective HSP27 overexpression clones (PL5/hu16, PL5/3A6). No significant differences were detected (data not shown).

Next, we quantified the expression levels of the three major nucleoside membrane transporters involved in gemcitabine metabolism, namely hCNT1, hCNT3 and hENT1, by immunoblotting. Likewise, no significant differences were detected among parental PL5 cells, empty-vector PL5/EV cells and the respective HSP27 overexpression clones (data not shown).

4.3 Independence of p38 and JNK in HSP27-dependent gemcitabine sensitivity

Since HSP27 was reported to be regulated through p38/JNK signaling pathway [151], we examined whether JNK and p38 were involved in HSP27-dependent gemcitabine sensitivity. We used immunoblotting to detect the expression levels of p38 and phospho- p38 as well as JNK and phospho-JNK. As compared to parental cells, HSP27-overexpressing PL5/hu18 cells displayed no detectable differences in regard to the expression levels of JNK and p38 upon gemcitabine treatment at 25 nM for 0, 6, 12, 24 h (**Fig. 4**). In addition, no constitutive phospho-p38 or phospho-JNK expression was detected in any of the cell clones tested (data not shown).

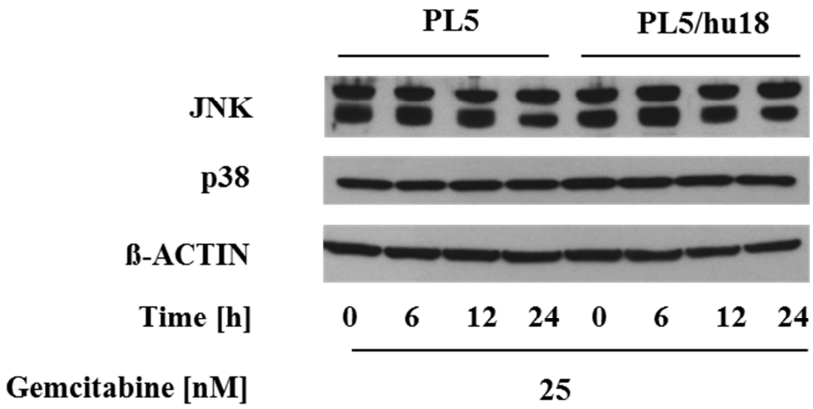


Figure 4. Detection of JNK and p38 during HSP27-dependent gemcitabine-induced apoptosis. *Immunoblotting displaying time-dependent changes of JNK and p38 expressions in PL5/hu18 cells treated with gemcitabine at 25 nM. ß-ACTIN served as the loading control. Experiments were performed at least in duplicate and representative results are shown.*

4.4 Influence of HSP27 depletion on chemosensitivity in pancreatic cancer cells

We additionally used RNA-interference in a pancreatic cancer cell line exhibiting

constitutively high HSP27 expression to validate the experiments applying HSP27 overexpression and to exclude cell line-specific or methodology-dependent artefacts. Gemcitabine sensitivity was assessed upon siRNA-mediated HSP27 down-regulation in AsPC-1 cells. HSP27 protein depletion efficiency was validated by immunoblotting 48–192 h after siRNA transfection and the time points of maximal HSP27 down-regulation were used for gemcitabine treatment. A significantly decreased sensitivity of HSP27 siRNA-treated AsPC-1 cells, expressing virtually no detectable HSP27 protein at 120–144 h after siRNA transfection as compared to HSP27-expressing untreated or control-transfected cells, was observed upon treatment with gemcitabine at 120–144 h (**Fig. 5**).

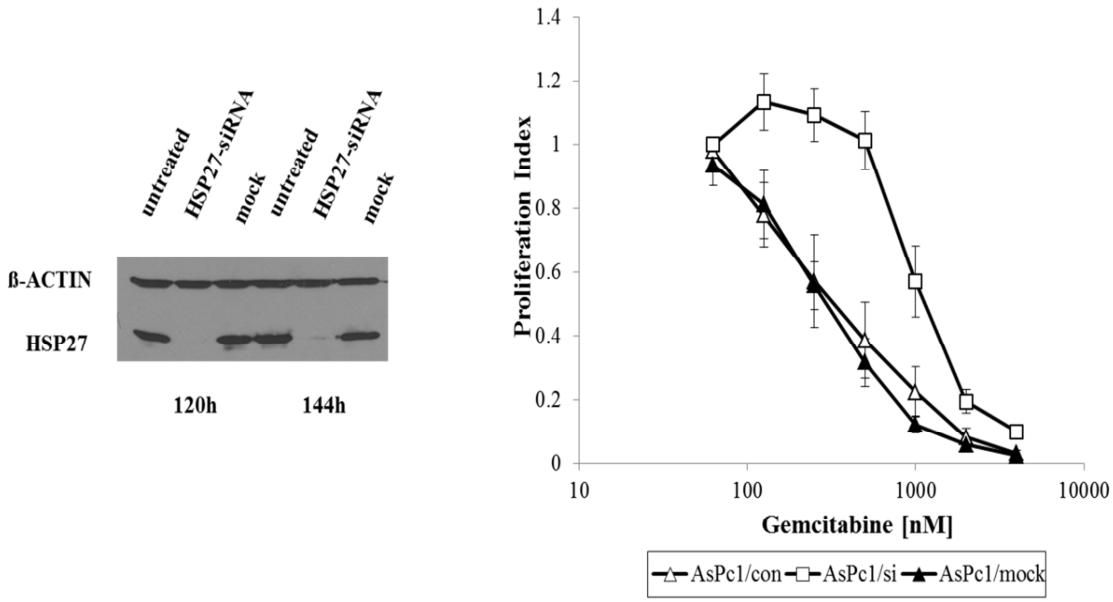


Figure 5. HSP27 knockdown- mediated gemcitabine sensitivity in AsPc1 cells. *Left: Immunoblotting displaying the expression of HSP27 upon siRNA treatment from one representative experiment in AsPc1 cells. Right: Cell proliferation assays comparing gemcitabine sensitivity of untreated, control-transfected and HSP27-siRNA-transfected AsPC-1 cells. Error bars represent SEM of four independent experiments.*

4.5 Mediation of HSP27-dependent gemcitabine sensitivity through early S-phase arrest and consequent apoptosis

To investigate the mechanism underlying HSP27-mediated gemcitabine sensitivity, we applied flow cytometry to analyze cell cycle distribution and quantify the sub-G1 cell fraction as surrogate marker for apoptosis upon treatment of cells with gemcitabine, comparing parental PL5 cells to their corresponding HSP27-overexpressing counterparts (labeled PL5/hu18). As compared to parental cells, PL5/hu18 cells displayed a strongly increased S-phase fraction (50%) 24 h after gemcitabine treatment at 15 or 25 nM (**Fig. 6 A+B**). While only a slightly increased sub-G1 fraction (~15%) was detectable at the time of S-phase arrest (**Fig. 6 B**), a strong and dose-dependent increase (~90%) was observed at 48 h in PL5/hu18 cells (**Fig. 7 A+B**).

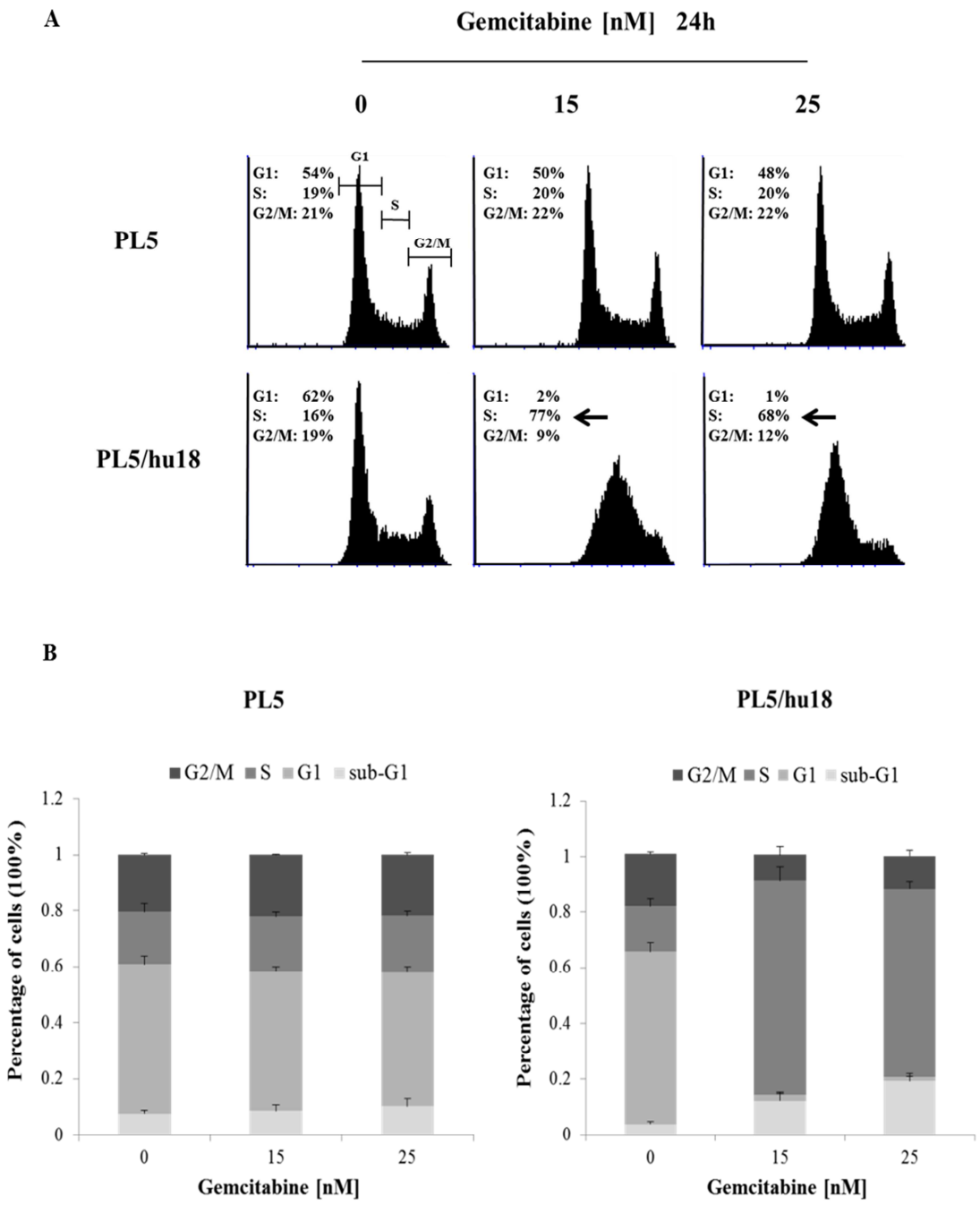


Figure 6. HSP27-overexpression-mediated gemcitabine sensitivity through early S-phase arrest. *Flow cytometry comparing cell cycle distribution upon gemcitabine treatment at 0, 15, or 25 nM for 24 h between parental PL5 cells and their HSP27-overexpressing counterparts PL5/hu18: Histograms of cell cycle distributions* ***(A)*** *and statistical analyses from at least three independent experiments* ***(B)****(Left: PL5; Right: PL5/hu18). Error bars represent SEM.*

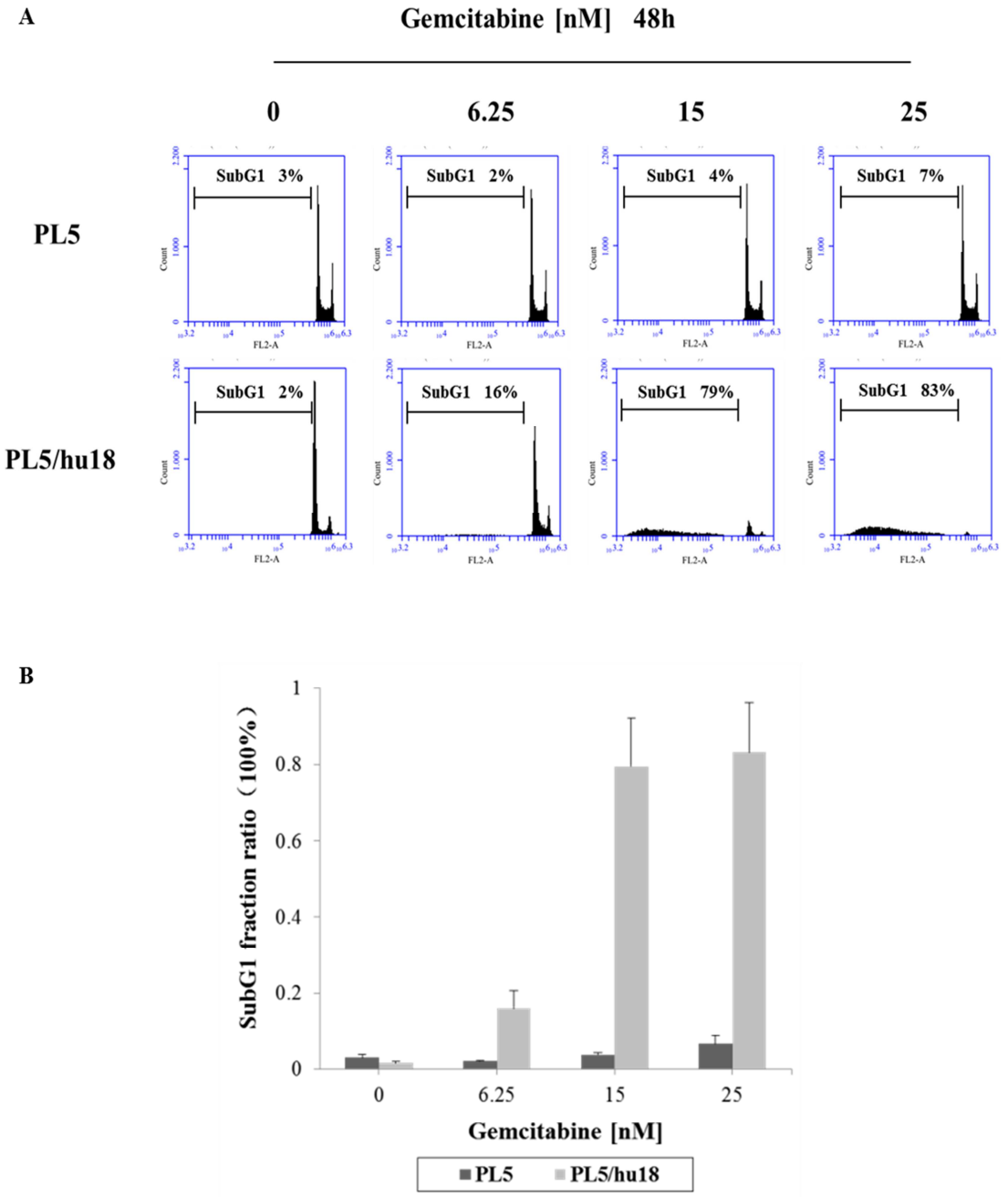


Figure 7. HSP27-overexpression-mediated gemcitabine sensitivity through consequent apoptosis. *Flow cytometry comparing sub-G1 cell fractions upon gemcitabine treatment at 0, 6.25, 15, or 25 nM for 48 h between parental PL5 cells and their HSP27-overexpressing counterparts PL5/hu18: Histograms of cell cycle distributions* ***(A)*** *and statistical analyses from at least three independent experiments* ***(B)****. Error bars represent SEM.*

4.6 Validation of apoptosis by Hoechst staining

Next, Apoptosis indicated by the sub-G1 cell fraction was morphologically validated

using nuclear staining. Consistently, increased chromatin condensation and nuclear fragmentation were observed in HSP27-overexpressing PL5/hu18 cells upon gemcitabine treatment but not in parental PL5 cells (**Fig. 8 A**). And the morphological changes were in a dose- (**Fig. 8 A**) and time- (**Fig. 8 B**) dependent manner.

Our data thus demonstrated that HSP27-dependent gemcitabine sensitivity was mediated first through S-phase arrest and consequently through apoptosis.

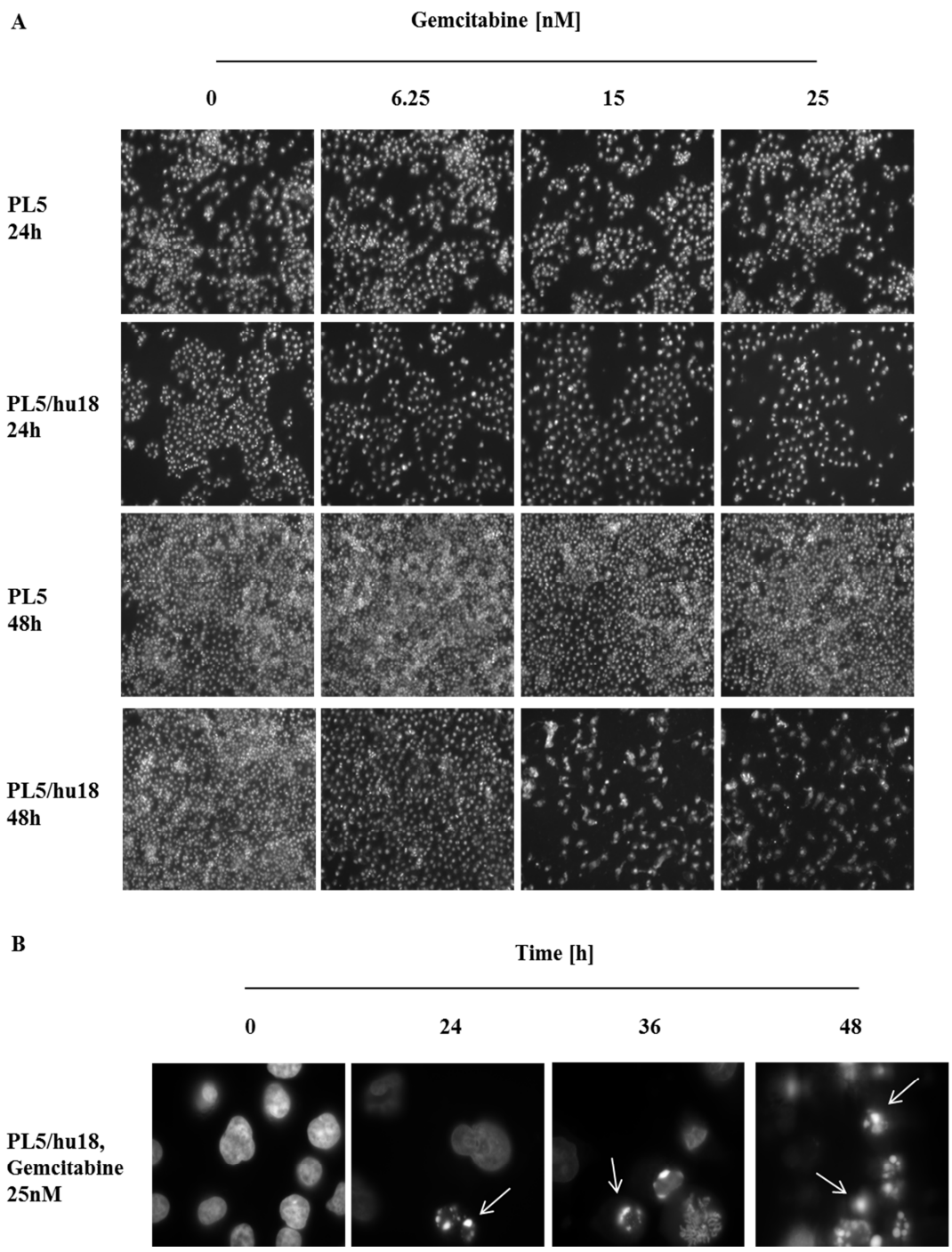


Figure 8. HSP27-dependent gemcitabine sensitivity through apoptosis. *Hoechst staining comparing morphological changes upon gemcitabine treatment at 0, 6.25, 15, or 25 nM for 0, 24, 36 or 48 h between parental PL5 cells and their HSP27-overexpressing counterparts PL5/hu18:* ***(A)*** *Representative microscopic pictures (magnification, ×10) displaying morphological differences upon gemcitabine at the indicated concentrations for 24 or 48 h in PL5 and PL5/hu18 cells.* ***(B)*** *Higher magnification (×100) showing typical morphological features of chromatin condensation and nuclear fragmentation upon gemcitabine at 0, 25 nM for the indicated time points in PL5/hu18 cells (arrows).*

4.7 HSP27-dependent cleavage of PARP, CASPASE 3, CASPASE 8, CASPASE 9

To confirm and validate HSP27-dependent induction of apoptosis upon gemcitabine treatment, we assessed Poly (ADP-ribose) polymerase (PARP) cleavage as a general apoptosis marker, followed by assessment of cleavage of initiator caspases CASPASE 8 and CASPASE 9 and of the central effector CASPASE 3. As compared to parental control cells, cleavage of PARP and all three caspases upon treatment was observed virtually exclusively in the HSP27-overexpressing PL5/hu18 cells, but not in parental PL5 cells. Moreover, all four proteins were cleaved in a dose-dependent (**Fig. 9 left**) and time-dependent (**Fig. 9 right**) manner.

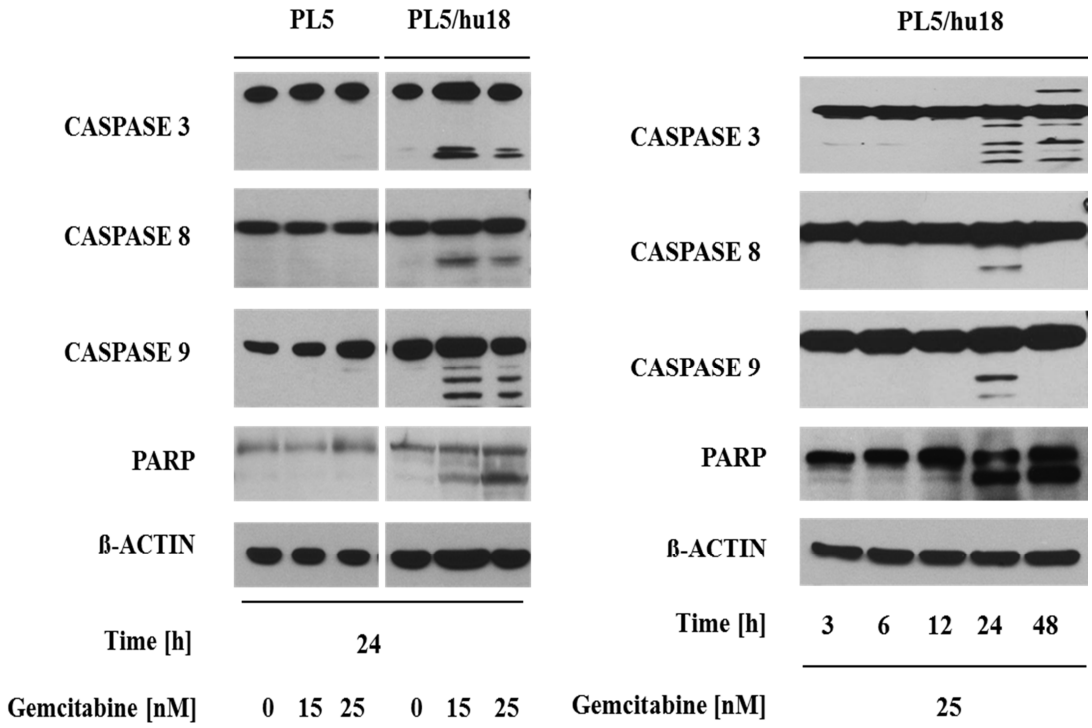


Figure 9. Activation of PARP and caspases during HSP27-dependent gemcitabine-induced apoptosis. *Immunoblotting showing cleavage of PARP, CASPASE 3, CASPASE 8 and CASPASE 9 upon gemcitabine treatment in HSP27-overexpressing PL5/hu18 but not parental control cells in a dose-dependent* ***(left)*** *and time-dependent* ***(right)*** *manner. Experiments were performed at least in duplicate and representative results are shown.*

4.8 Caspase inhibitor-induced abrogation of HSP27-dependent gemcitabine sensitivity

To confirm caspase-dependency of HSP27-mediated gemcitabine-induced apoptosis, the caspase inhibitor Z-VAD-FMK [161] was utilized. Consistent with the observed cleavage of CASPASE 3, CASPASE 8, and CASPASE 9 upon gemcitabine treatment, Z-VAD-FMK at 20 µM for 3 h virtually completely reversed gemcitabine sensitivity specifically in HSP27-overexpressing PL5/hu18 cells at 1 or 2 nM of gemcitabine. At higher concentrations (4 nM), caspase-independent gemcitabine toxicity was additionally observed (**Fig. 10**).

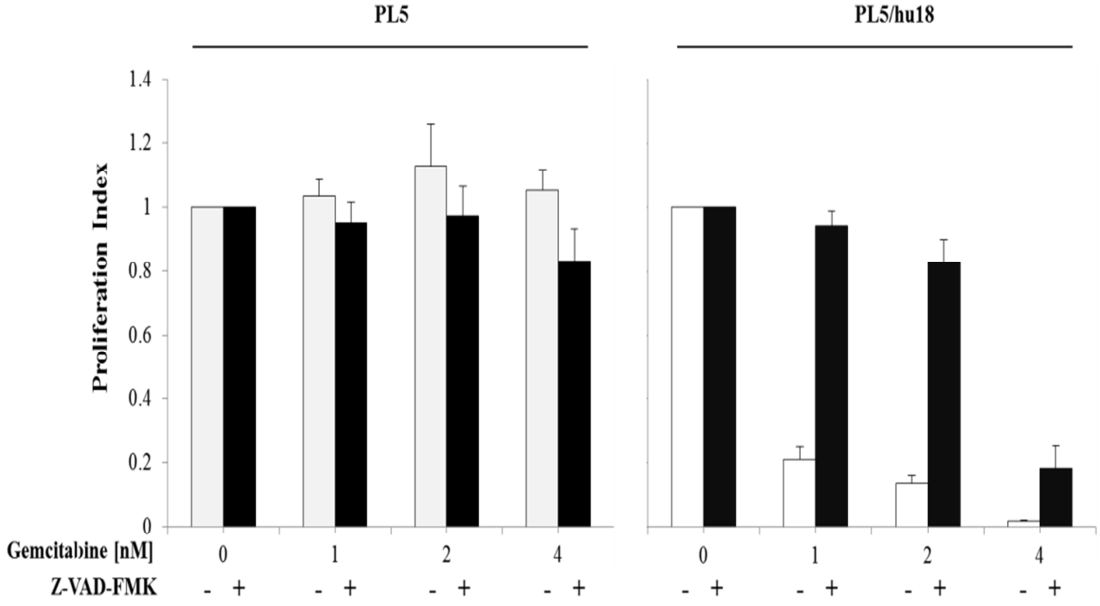


Figure 10. Reversal of HSP27 overexpression-mediated gemcitabine sensitivity through caspase inhibition. *Proliferation assays displaying abrogation of HSP27-dependent gemcitabine sensitivity through pre-incubation with the pan-caspase inhibitor Z-VAD-FMK at 20 µM for 3 h. Error bars represent SEM of at least three independent experiments.*

4.9 Identification of specific apoptotic pathways involved in HSP27-dependent gemcitabine sensitivity

4.9.1 HSP27-dependent activation of BIM upon gemcitabine

To evaluate which apoptotic molecules participated in caspase-dependent apoptosis, we analyzed the cellular expression of major pro- and anti-apoptotic mediators along the intrinsic pathway upon gemcitabine treatment at 25 nM. All three isoforms of pro-apoptotic BIM (BIM_{EL}, BIM_L and BIM_S) [162] were significantly upregulated in a time-dependent manner specifically in HSP27-overexpressing PL5/hu18 but not in parental control cells, while no discernible expression changes were detected in any other pro-apoptotic (BID, BAX and BAK) or anti-apoptotic protein (BCL-xL, BCL-2, MCL-1) (**Fig. 11 A+B**) tested.

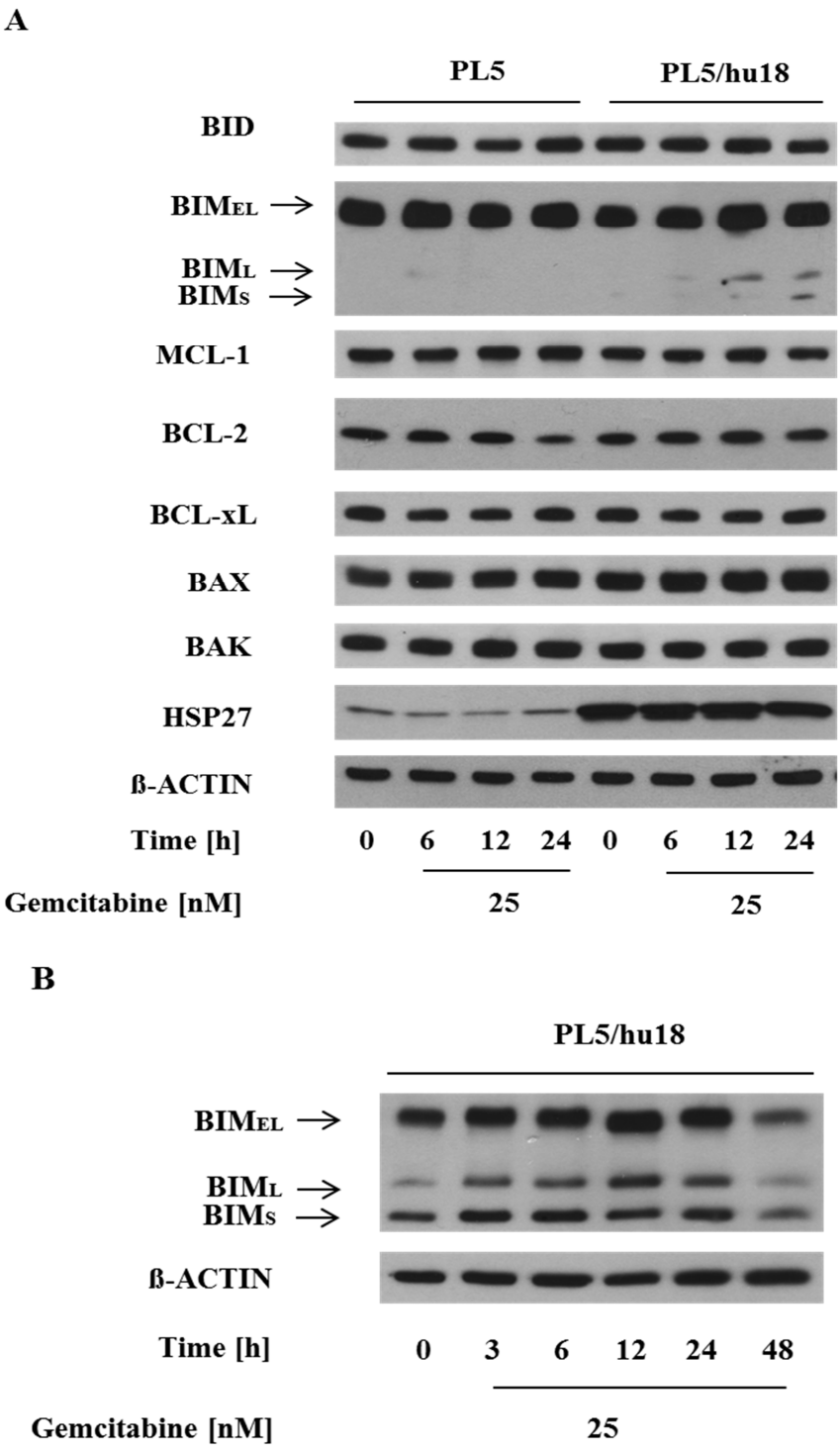


Figure 11. Modifications of apoptosis mediators in HSP27-dependent gemcitabine sensitivity. ***(A)*** *Immunoblotting assessing expression changes of the indicated pro- or anti-apoptotic molecules in parental PL5 and PL5/hu18 cells upon treatment with gemcitabine at 25 nM at the indicated time points. ß-ACTIN served as the loading control.* ***(B)*** *Immunoblotting displaying time-dependent BIM expression changes in PL5/hu18 cells treated with gemcitabine at 25 nM. ß-ACTIN served as the loading control. Experiments were performed at least in duplicate and representative results are shown.*

4.9.2 HSP27-dependent mitochondrial membrane potential loss upon gemcitabine

As BIM is commonly regarded as a central player in the control of mitochondrion-mediated apoptotic processes [163], we next applied three assays using JC-1 staining [164] to analyze mitochondrial membrane depolarization upon HSP27-dependent gemcitabine-induced apoptosis. While under normal conditions, the JC-1 dye concentrates due to the electrochemical potential gradient in the mitochondrial matrix and forms red fluorescent aggregates (JC-1 aggregates), any event that dissipates the mitochondrial membrane potential leads to the prevention of JC-1 accumulation in the mitochondria and JC-1-dispersion throughout the entire cell leading to a shift from red to green fluorescence (JC-1 monomers) [165]. Upon gemcitabine at 25 or 50 nM for 24 h, $\Delta\psi_m$ was quantitatively decreased in HSP27-overexpressing PL5/hu18 but not in parental control cells, as determined by JC-1 fluorescence (**Fig. 12**). Consistently, fluorescence imaging displayed a strong decrease of JC-1 aggregates in PL5/hu18 cells treated with gemcitabine at 25 nM for 24 h (**Fig. 13**). Similar data were obtained using FACS analysis, in which a clear and dose-dependent shift from JC-1 aggregates to JC-1 monomers was detected in PL5/hu18 cells 24 h after treatment with gemcitabine at 15 or 25 nM (**Fig. 14+15**). This effect was completely blocked by valinomycin (data not shown) or CCCP (Fig. 3C), both serving as negative controls.

Taken together, these data demonstrated mitochondrion-mediated apoptosis specifically in PL5/hu18 cells upon treatment with gemcitabine, pointing at involvement of the intrinsic apoptosis pathway in HSP27-dependent gemcitabine sensitivity in our model.

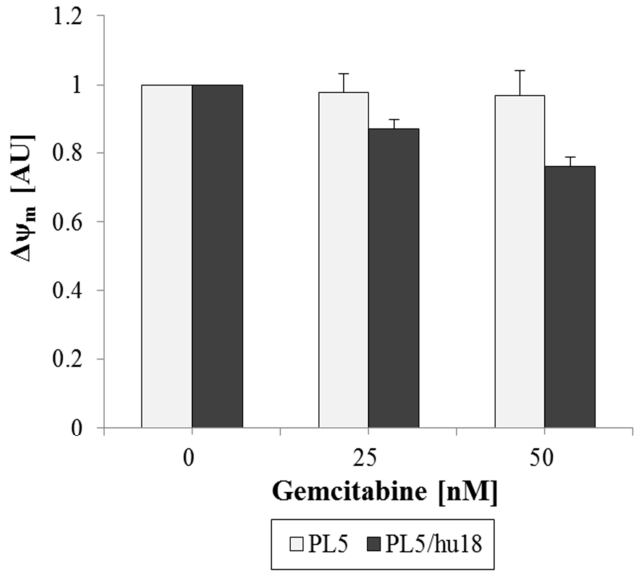


Figure 12. Influence of HSP27 overexpression on mitochondrial potential upon gemcitabine treatment using fluorimetric measurements. *Quantitative assessment of dose-dependent mitochondrial membrane potential ($\Delta\psi_m$) in PL5/hu18 as compared to parental PL5 control cells 24 h after gemcitabine treatment by determination of fluorescence upon JC-1 staining. Error bars represent SEM of three independent experiments.*

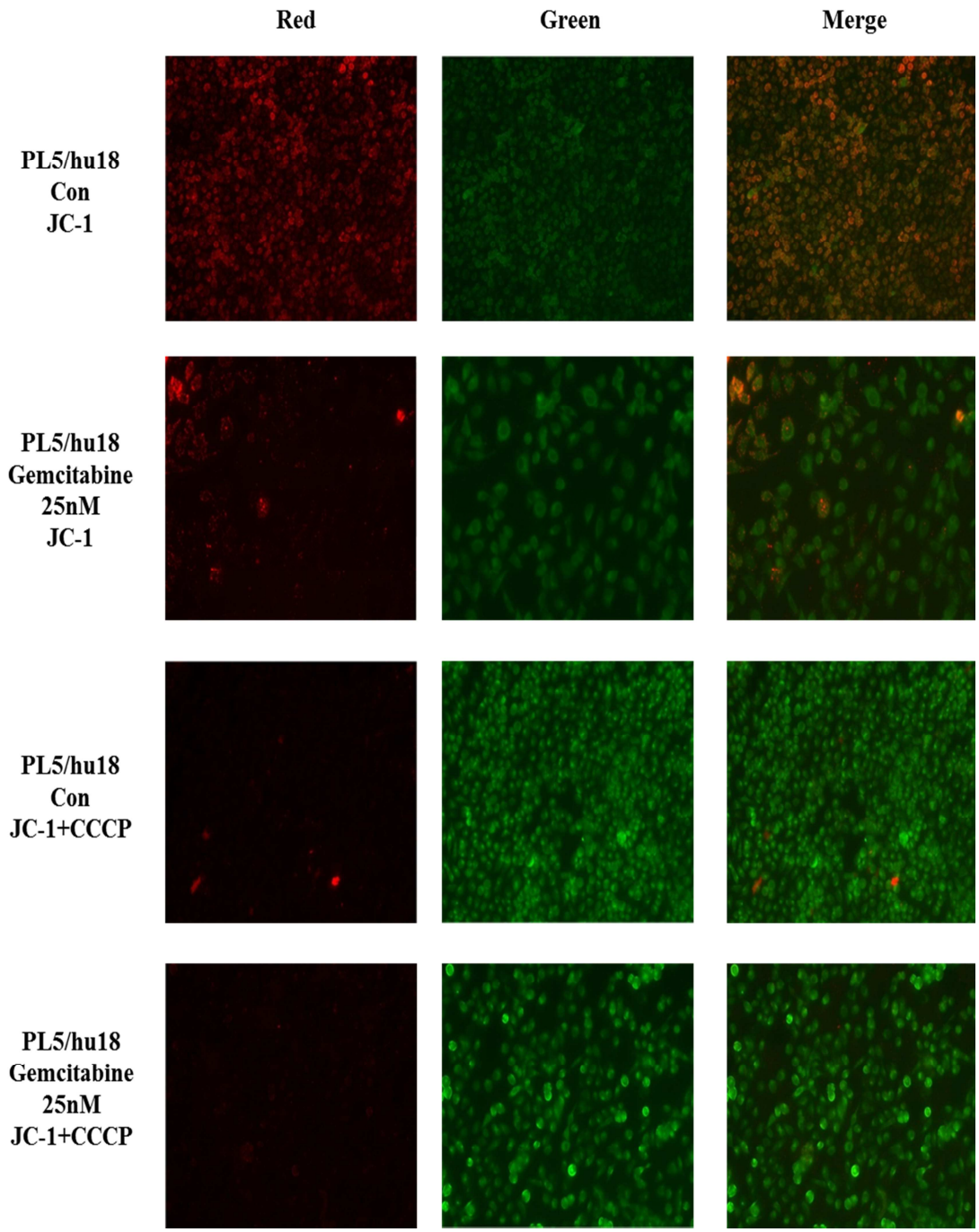


Figure 13. Influence of HSP27 overexpression on mitochondrial potential upon gemcitabine treatment using fluorescence imaging. *Representative pictures from fluorescent imaging (magnification, ×10) showing JC-1 aggregates (red) and JC-1 monomers (green) in PL5/hu18 cells 24 h after treatment with gemcitabine at 0, 25 nM. Control experiments were blocked by CCCP. Experiments were performed in triplicate and representative results are shown.*

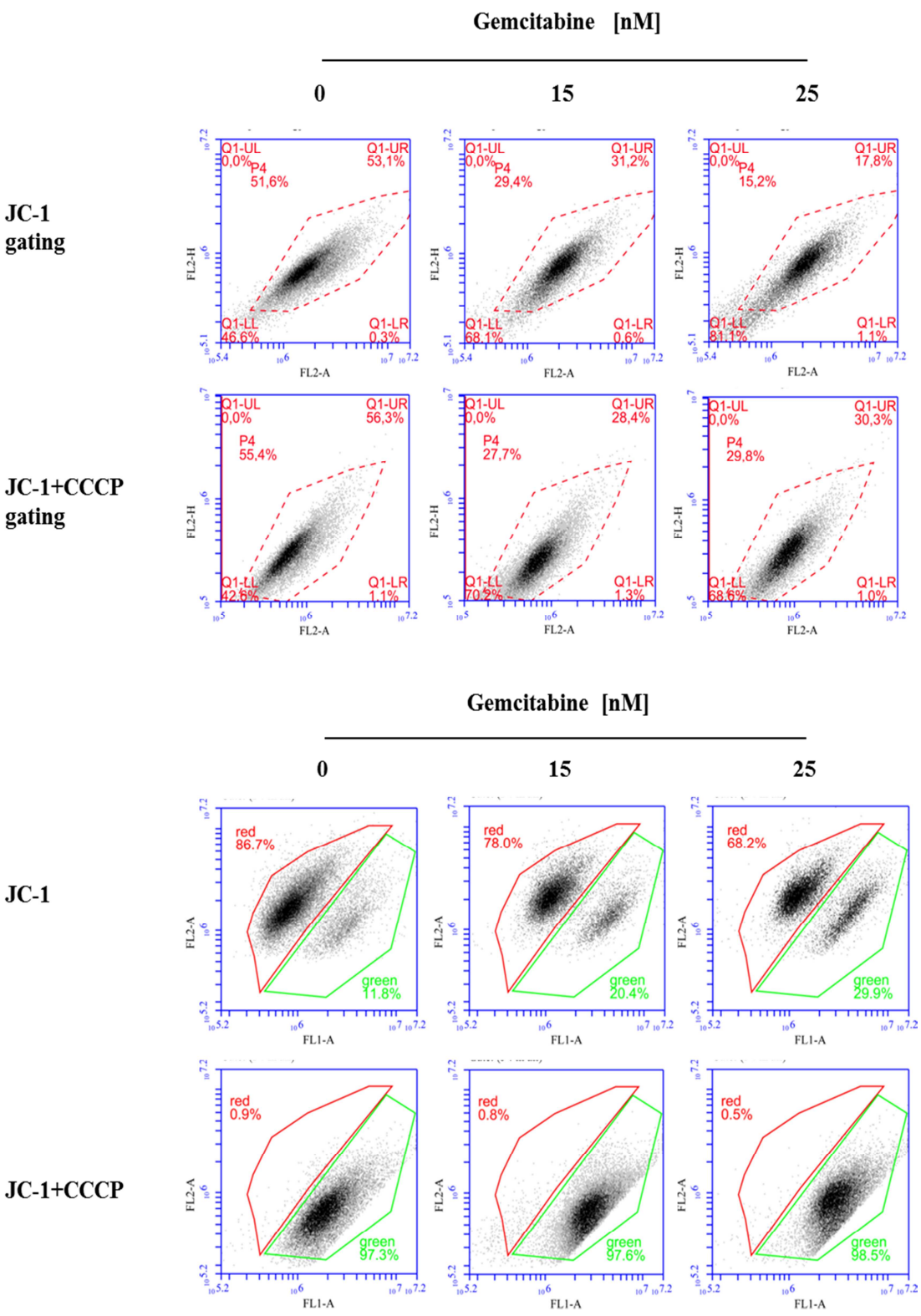


Figure 14. Influence of HSP27 overexpression on mitochondrial potential upon gemcitabine treatment using FACS. *FACS analyses displaying representative dot blots of dose-dependent $\Delta\psi_m$ loss in PL5/hu18 cells treated with gemcitabine at 0, 15, 25 nM for 24 h (the third panel) and control experiments using CCCP block (the fourth panel). All experiments were repeated at least three times.*

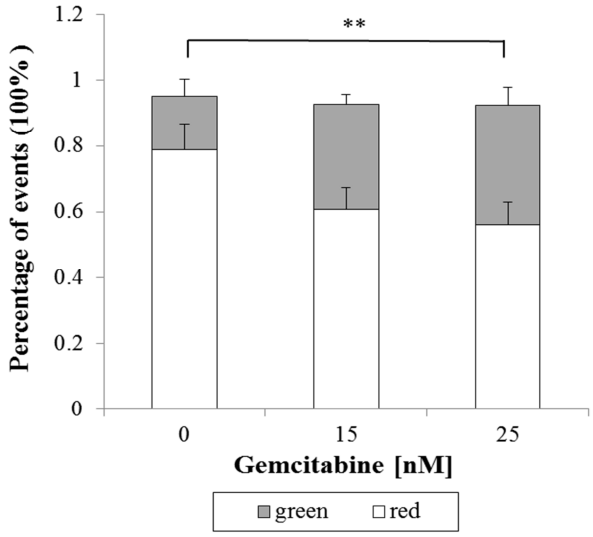


Figure 15. Statistical analysis of the impact of HSP27 overexpression on mitochondrial potential upon gemcitabine treatment using FACS. *Statistical analyses of three independent experiments using the Student's t-test (**p <0.01) with error bars representing SEM.*

4.10 Heat shock-inducible sensitization of pancreatic cancer cell lines towards gemcitabine

4.10.1 Heat shock treatment

Heat-shock induces upregulation of HSP27 expression in a wide variety of cells including pancreatic cancer cells [122,166-169]. We therefore asked, whether heat-shock mediated HSP27 induction would be capable of increasing gemcitabine sensitivity in a similar manner as did engineered HSP27 overexpression in our model, which would support potential clinical implications of our study in regard to the combination of hyperthermia with chemotherapy for pancreatic cancer patients [170-173]. To test this hypothesis, a panel of established pancreatic cancer cell lines (Capan1, MIA PaCa-2, Panc1, PL5, PL11, Su86.86) as well as short-term propagated, early-passage primary human pancreatic cancer cell lines previously established by us (202, 311, 455, 518, 520, PPC-0039) was screened for heat-shock-inducible HSP27 upregulation along with minimal heat-shock-induced toxicity. After identification of suitable cell lines, optimized heat-shock conditions in regard to temperature and duration of heat shock were defined. At conditions causing only minimal heat-induced toxicity (41.8 °C for

30 to 120 min, depending on the respective cell line), eight of twelve lines were HSP27-inducible (PL5, PL11, Su86.86, 202, 311, 455, 518, PPC-0039). Of these, five lines were picked for subsequent experiments according to their favorable growth characteristics in cell culture (PL5, PL11, Su86.86, 202, 518).

Of note, due to the optimized heat shock-conditions used in our experiments, we were able to induce HSP27 without significant concomitant increase of HSP70 or HSP90 expression levels in most cell lines (**Fig. 16**), excluding HSP70 or HSP90 as a predominant mechanism for the observed results.

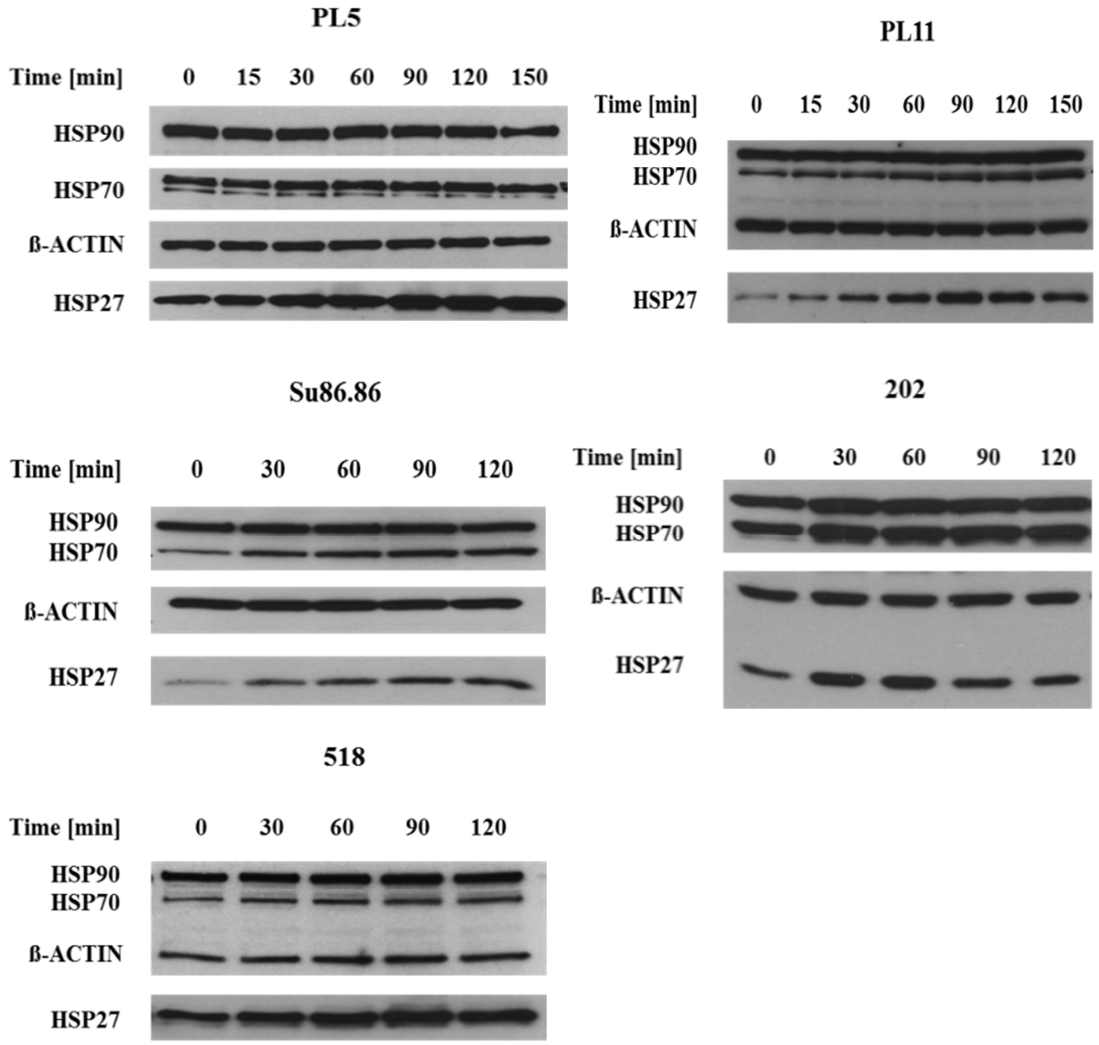


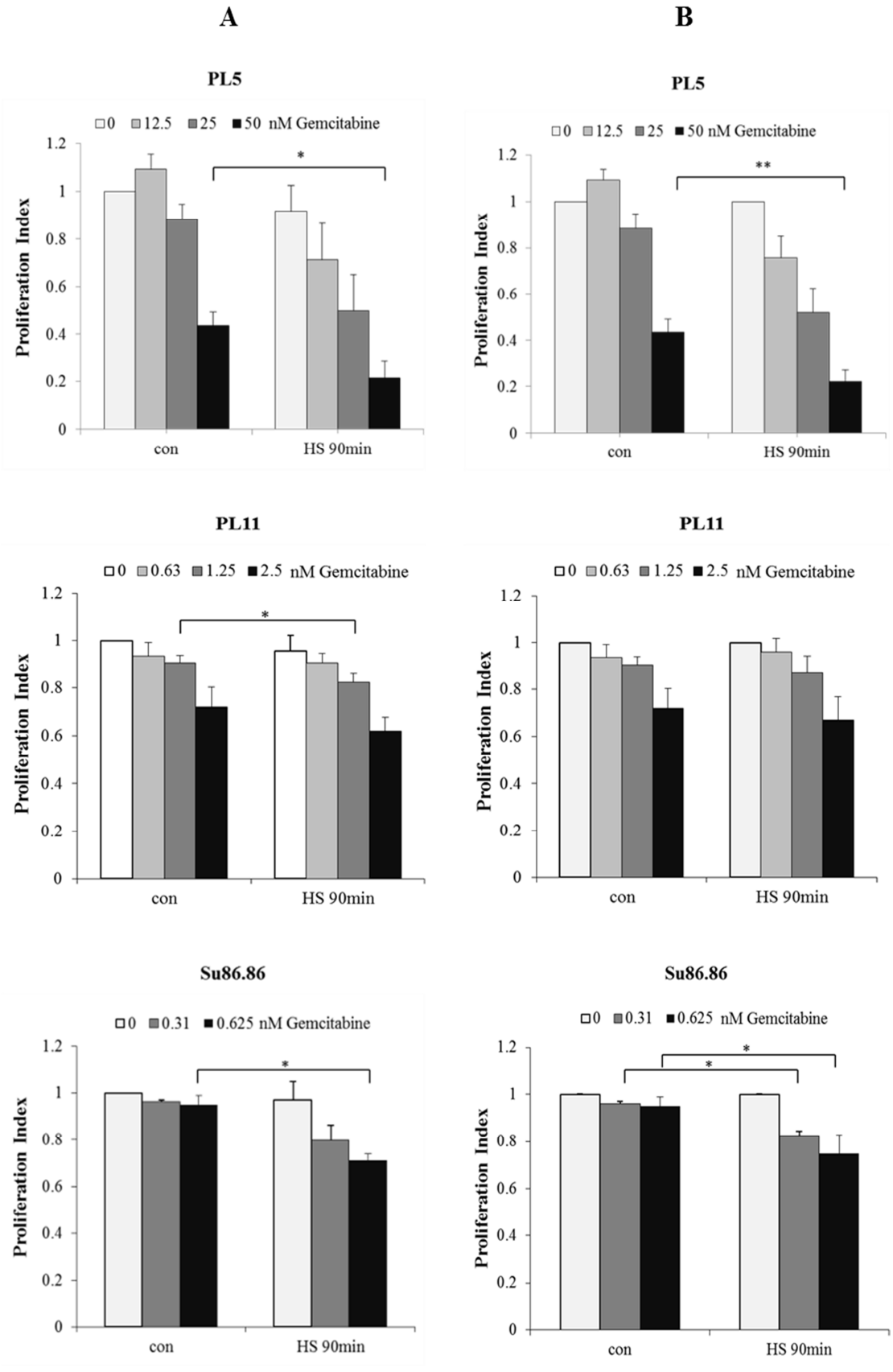
Figure 16. Mild heat shock-mediated HSP27 induction. *Immunoblotting displaying the expression levels of HSP27, HSP70, and HSP90 in the indicated established and short-term propagated primary pancreatic cancer cell lines upon heat-shock at 41.8 °C for 0, 15, 30, 60, 90, 120, or 150 min, respectively. ß-ACTIN served as loading control.*

4.10.2 Effects of gemcitabine treatment in combination with heat-shock

At the previously determined individual time points of maximal heat shock-mediated HSP27 induction (**Tbl. 1**), all cell lines displayed increased sensitivity towards gemcitabine (**Fig. 17 A**). These results were statistically significant for four out of the five cell lines tested (PL5, PL11, Su86.86, 202). Similar results were obtained when excluding the slight detrimental effects of mild heat-shock alone (i.e. when defining the surviving fraction of cells treated with heat shock but without gemcitabine as 100%) (**Fig. 17 B**). Taken together, mild heat shock enhanced the sensitivity of multiple pancreatic cancer cell lines towards gemcitabine, independent of directly heat-induced detrimental effects.

Cell type	Suitable heat shock time points
PL5	60, 90 min
PL11	60, 90 min
Su86.86	90, 120 min
202	30, 60 min
518	60, 90 min
311	30, 60 min

Table 1. Heat shock time points of maximal HSP27 induction.


A
B
PL5
0 12.5 25 50 nM Gemcitabine
Proliferation Index
1.2
1
0.8
0.6
0.4
0.2
0
*
**
con
HS 90min
PL11
0 0.63 1.25 2.5 nM Gemcitabine
Su86.86
0 0.31 0.625 nM Gemcitabine

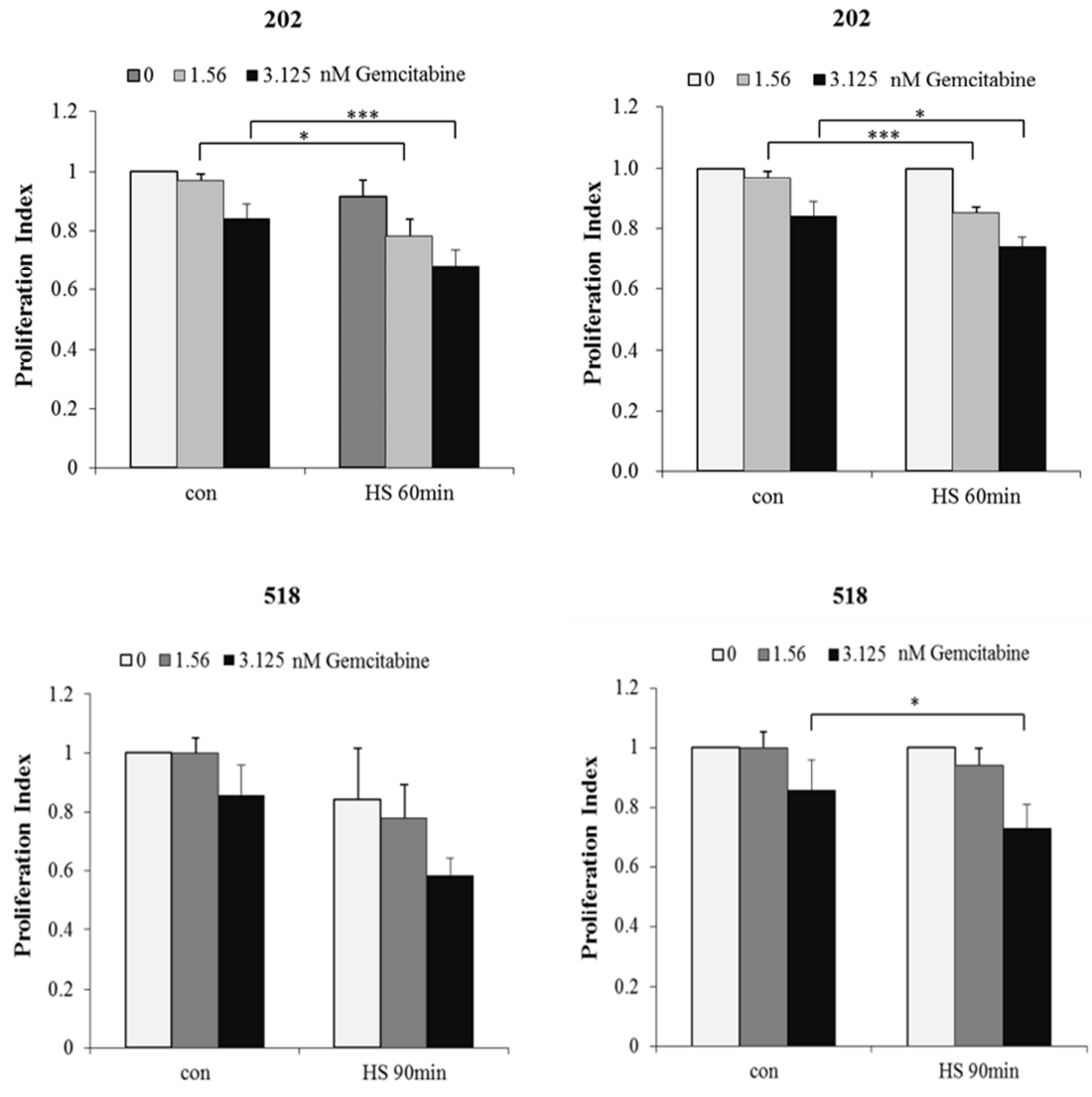


Figure 17. Mild heat shock-mediated sensitization of pancreatic cancer cell lines towards gemcitabine. *Proliferation assays comparing the sensitivity towards gemcitabine, with or without prior heat shock at 41.8 °C for the indicated time points of maximal HSP27 induction, including* ***(A)*** *or excluding* ***(B)*** *the detrimental effects of heat-shock alone. Error bars represent SEM of at least three independent experiments. Asterisks mark statistical significance between two samples using the Student's t-test (*p <0.05 **p <0.01 ***p <0.005).*

4.11 Impact of HSP27-overexpression on death receptor-targeting agents

As we unexpectedly found HSP27-dependent gemcitabine sensitivity to be partly mediated through CASPASE 8, indicating involvement of the extrinsic apoptosis pathway (**Fig. 18 A+B**), we asked whether HSP27 overexpression would accordingly directly influence the cellular sensitivity towards drugs targeting the extrinsic death

receptor (DR)-pathway. To test this, parental PL5, empty-vector transfected PL5/EV and two HSP27-overexpressing cell clones (PL5/hu16, PL5/hu18) were treated with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor-targeting agents currently tested in clinical trials, specifically the anti-death receptor 5 (DR5) agonistic antibodies tigatuzumab and LBY135. As determined by the IC50 ratios, two independently derived HSP27-overexpressing cell clones (PL5/hu16, PL5/hu18) were approximately three to four-fold more sensitive towards these drugs than parental or empty-vector transfected (PL5/EV) control cells, supporting our hypothesis that overexpression of HSP27 not only modulated the cellular sensitivity towards gemcitabine, but also towards agents directly targeting the extrinsic DR pathway (**Fig. 5A+B**).

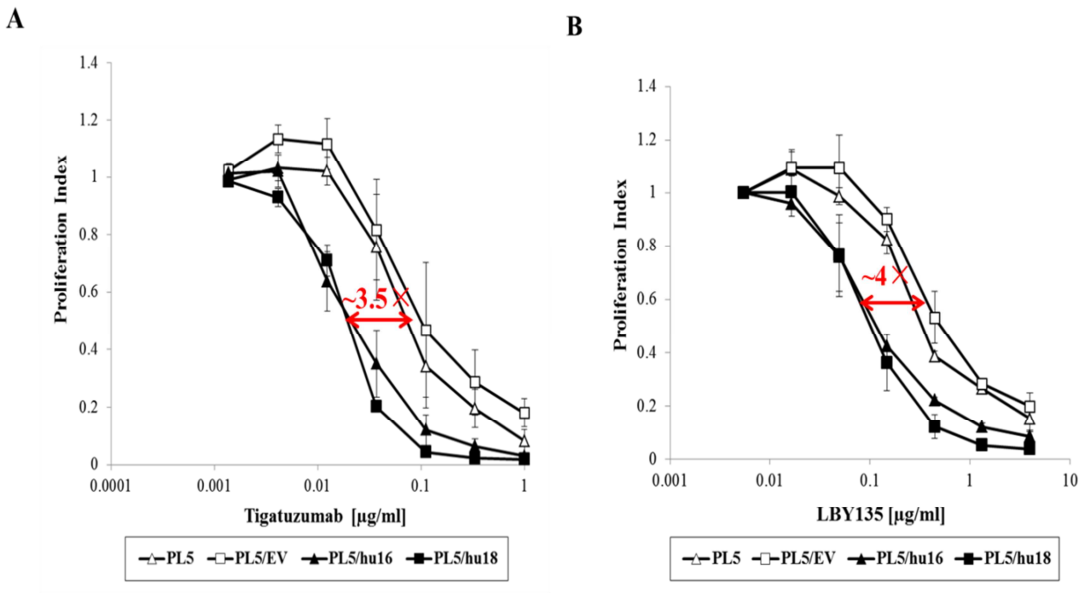


Figure 18. Influence of HSP27 overexpression on the cellular sensitivity towards DR-targeting agents. *Proliferation assays comparing the sensitivity of PL5, PL5/EV cells versus two independently derived HSP27-overexpressing cell clones (PL5/hu16, PL5/hu18) towards DR5-agonistic antibodies tigatuzumab **(A)**, LBY135 **(B)**, respectively at the indicated concentrations. Error bars represent SEM of at least three independent experiments.*

5. DISCUSSION

5.1 Summary of the present study

Previously, we have shown that HSP27 expression significantly correlated with better patient survival in pancreatic cancer in our TMA studies, suggesting that HSP27 could serve as a prognostic marker in this tumor type. Furthermore, exogenous overexpression of HSP27 in HSP27 low-expressing PL5 pancreatic cancer cells conferred increased sensitivity to gemcitabine. Vice versa, HSP27 high-expressing AsPC-1 pancreatic cancer cells exhibited a significantly increased resistance towards gemcitabine upon siRNA-mediated HSP27 protein depletion, additionally substantiating our results from the PL5 overexpression model and further excluding cell line-specific phenomena or methodological artefacts due to clonal variability [122].

On the basis of our previous study, the present study served to depict the underlying mechanism of HSP27-mediated gemcitabine sensitivity in pancreatic cancer cells. Here we demonstrate that gemcitabine treatment caused an early S-phase arrest followed by BIM-, mitochondrion- and caspase-mediated apoptosis specifically in HSP27-overexpressing pancreatic cancer cells. Furthermore, we were able to extend and generalize our data by showing that mild heat shock-mediated HSP27 induction increased the gemcitabine sensitivity in a panel of pancreatic cancer cell lines in a similar manner as did genetically engineered HSP27 overexpression. Finally, our study unexpectedly revealed that HSP27 overexpression sensitized pancreatic cancer cells not only towards gemcitabine, but also towards the DR5-targeting agonistic antibodies tigatuzumab [174] and LBY135 [175].

5.1.1 HSP27-mediated gemcitabine-induced S-phase arrest and apoptosis

The predominant cell cycle effect observed in our experiments, i.e. the early S-phase arrest in HSP27-overexpressing pancreatic cancer cells treated with gemcitabine, is in concordance with the known pharmacological mechanism of action of gemcitabine: The nucleoside analogue belongs to the chemotherapeutic group of antimetabolites,

and, after cell entry and phosphorylation, becomes incorporated into DNA. This DNA incorporation causes stalled replication forks during replication and consequently leads to an S-phase checkpoint activation followed by an S-phase arrest [176]. In our experiments, this S-phase arrest was subsequently followed by caspase-mediated apoptosis, as illustrated by the activation of the initiator caspases CASPASE 8 and CASPASE 9 and the executioner CASPASE 3 [177] and confirmed by the virtually complete abrogation of these effects by a broad caspase inhibitor [161] (**Fig. 19**). Thus, the observed gemcitabine-induced pro-apoptotic effects of HSP27 overexpression in pancreatic cancer cells were strictly caspase-dependent in our experiments, whereas the anti-apoptotic properties of HSP27 in other cancers have been reported to be mediated partly by caspase-dependent and partly by caspase-independent mechanisms [89].

Mechanistically, HSP27-dependent gemcitabine-induced apoptosis was accompanied by activation of BIM (**Fig. 19**), a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members [178], which has just recently been linked to gemcitabine sensitivity in pancreatic cancer [179]. The fact that BIM is tightly involved in mitochondrion-mediated apoptosis [163] is mechanistically substantiated by our data on gemcitabine-induced mitochondrial membrane potential loss in HSP27-overexpressing pancreatic cancer cells. Cytoskeleton remodeling represents one of the major functions of HSP27 besides the regulation of apoptosis [92,93]. Thus, it is tempting to speculate that HSP27-induced cytoskeleton dissociation of BIM and its consecutive mitochondrial translocation - the previously described process through which BIM exerts its pro-apoptotic effects [163,180]-constitutes the initiating event of HSP27-dependent gemcitabine-induced apoptosis. These pro-apoptotic effects of HSP27 overexpression probably counteract and potentially outweigh any anti-apoptotic properties of HSP27 under certain circumstances or in certain subsets of tumors. This supports our previously established hypothesis of an important pro-apoptotic role of HSP27 in pancreatic cancer [122], which appears mechanistically clearly distinguishable from the well-established anti-apoptotic properties of

HSP27 in a variety of other cancers [83,90].

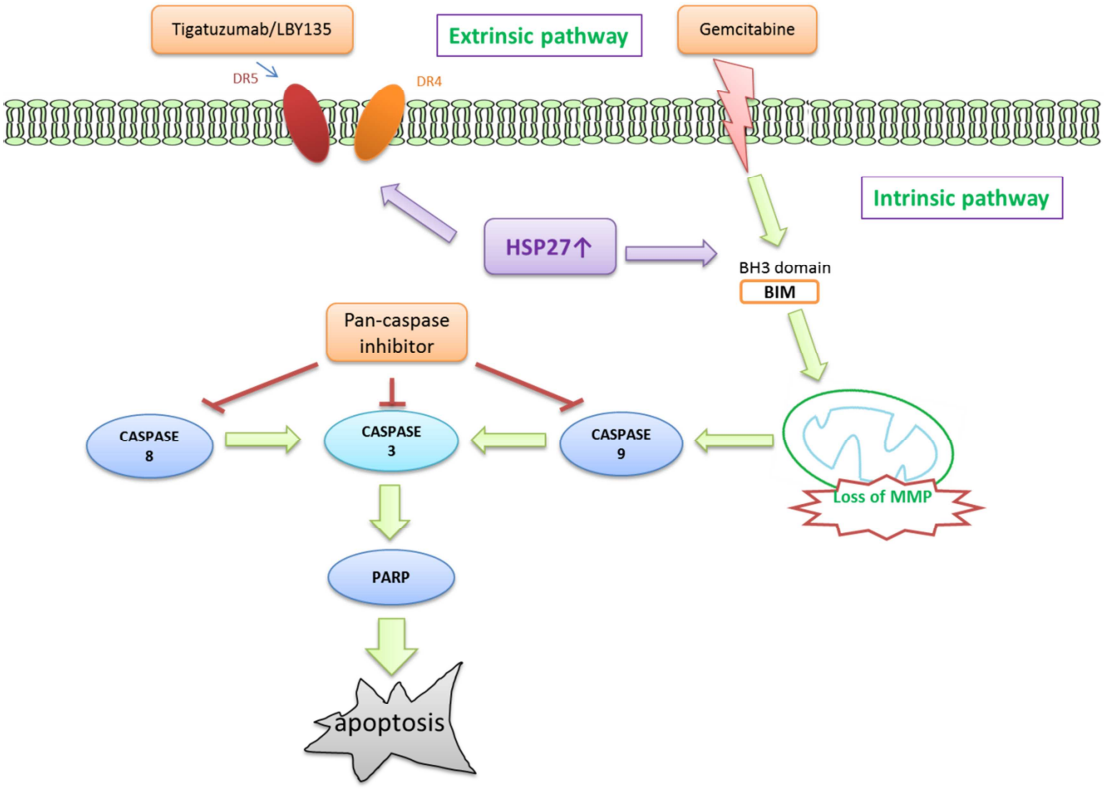


Figure 19. A pro-apoptotic role of HSP27 in pancreatic cancer.

5.1.2 Heat shock-enhanced gemcitabine sensitivity

Experimental studies indicate that heat-shock induces HSP27 expression in a wide variety of cell types including pancreatic cancer [122,166-169]. Therefore, it appeared mandatory to test whether heat-shock mediated HSP27 induction would be capable of increasing gemcitabine sensitivity in a similar manner as did engineered HSP27 overexpression in pancreatic cancer cells. In fact, we were able to demonstrate that mild experimental heat shock enhanced the sensitivity towards gemcitabine in the majority of cell lines from a panel of established as well as short-term propagated primary pancreatic cancer cell lines. Importantly, individual pre-optimization of each cell line facilitated the definition of particularly mild heat-shock conditions causing only minimal cellular toxicity, which helped to discriminate the heat shock-induced increase in cell death upon gemcitabine from potential directly heat-induced detrimental effects.

5.2 Clinical prospects about our study

5.2.1 Hyperthermia in cancer

5.2.1.1 Hyperthermia as cancer therapy

Since 1968, heating was reported to be used in cancer treatment [181]. Since then, clinical application of hyperthermia for cancer treatment was rapidly developed. At present, heat can be introduced through electromagnetic field technique, ultrasound, or perfusion methods [182]. Clinical hyperthermia is divided into three separate domains: whole body hyperthermia (WBH), regional hyperthermia (RHT), and local hyperthermia (including superficial local and interstitial local hyperthermia) (LHT) [182]. Various modalities of hyperthermia are applied mainly as an addition of chemotherapy alone [183] or with radiation [182,184]. Of Interest, clinical trials have shown benefit to add hyperthermia as an effective strategy in various tumor entities such as soft-tissue sarcoma [185], breast cancer [186], non-small cell lung cancer [187], pancreatic cancer [170,172,173].

Although hyperthermia has proven effective to enhance sensitivity towards certain drugs or radiation in various cancers [183,188,189], several obstacles need to be overcome. First, the optimum temperature and duration of hyperthermia for clinical purposes are not sufficiently known [190]. Second, the molecular mechanism, through which hyperthermia increases the cellular sensitivity towards chemotherapy or radiation has not yet been sufficiently clarified [183,191-193]. Third, thermotolerance [194] has been an obstacle for adapting hyperthermia as a standard regimen [195], and the mechanism remains unclear.

5.2.1.2 Effects of hyperthermia in combination with gemcitabine in our experiments

Our data showing heat shock-inducible sensitization of pancreatic cancer cell lines towards gemcitabine, further underscore the concept of clinical trials applying treatment protocols including regional hyperthermia in combination with gemcitabine

for pancreatic cancer patients and provide a novel molecular mechanism for the clinically established value of this combination [170-173], i.e. hyperthermia-mediated HSP27 induction and consecutive increased cellular sensitivity towards gemcitabine. Of note, while most studies concentrate on the role of other HSPs, particularly of HSP70, as critical mediators of the molecular effects observed upon hyperthermia [183], the effects described in our study were elicited in a HSP70- and HSP90-independent manner, as can be derived from the optimized heat shock conditions, allowing HSP27 induction without significant concomitant increases of HSP70 or HSP90 expression in our experiments.

5.2.2 HSP27 as a predictive marker for gemcitabine treatment

Pancreatic cancer is a highly aggressive cancer and is clinically characterized by early metastatic growth, extensive drug-resistance, and high rates of recurrence [5,6]. During the past ten years, numerous chemotherapeutic and molecularly targeted agents have been evaluated alone or in combination in clinical trials. Despite encouraging recent advances [15,16], gemcitabine - introduced more than 15 years ago - remains the standard therapeutic basis in most clinical settings [14]. Due to the common primary or acquired resistance of pancreatic cancer cells towards gemcitabine [32], predictive markers for chemotherapeutic response are urgently needed [196,197]. Various potentially predictive biomarkers have previously been reported, including carbohydrate antigen 19-9 (CA19-9) [72,73], human equilibrative nucleoside transporter-1 (hENT1) [60], deoxycytidine kinase (dCK) [74], ribonucleotide reductase subunits (RRM1 [75] and RRM2 [63], Notch3 [76], Hu protein antigen R (HuR) [77], microRNA expression [78] or heat shock protein 27 (HSP27). Still, none of these markers has yet proven sufficiently robust to achieve clinical implementation in the gemcitabine-based treatment of pancreatic cancer. Particularly, the role of HSP27 as a predictive marker for gemcitabine sensitivity remained controversial [90,122,138]. Our study now substantiates a role for HSP27 as a predictive marker for gemcitabine sensitivity in pancreatic cancer.

5.3 Novel finding: the pro-apoptotic role of HSP27 in pancreatic cancer

Unexpectedly, we found that gemcitabine-induced HSP27-dependent apoptosis was mediated partly through CASPASE 8, representing the initiator caspase upon extrinsic DR stimulation [198]. This indicated a potential cross-talk between the extrinsic and intrinsic apoptosis pathways. A highly similar extrinsic/intrinsic crosstalk, functionally linking BIM- and mitochondrion-dependent intrinsic apoptotic signaling (both observed in our experiments) to the extrinsic DR cascade, has previously been reported in a different experimental setting [199]. Consequently we asked, whether HSP27 overexpression influenced sensitivity not only towards gemcitabine but additionally towards drugs directly targeting the extrinsic DR pathway. In fact, we found that HSP27-overexpressing cells displayed an increased sensitivity towards the DR5-agonistic antibodies tigatuzumab and LBY135, thus potentially identifying a new predictive marker of therapeutic response towards this drug class in pancreatic cancer. In this context, it will be important to test in future studies, whether heat-shock-mediated HSP27 induction increased sensitivity not only towards gemcitabine, as shown here, but similarly also towards DR-targeting drugs in pancreatic cancer. Recent studies already support this hypothesis, reporting hyperthermia to enhance apoptosis induced by TRAIL or the DR4-targeting mapatumumab in colon cancer cells *in vitro* and *in vivo* [200,201].

5.4 Conclusion and future prospects

In summary, we identified and mechanistically characterized a novel link between HSP27 expression and gemcitabine sensitivity in pancreatic cancer cells. In contrast to the well-established anti-apoptotic roles of HSP27, our study revealed clearly distinguishable pro-apoptotic functions of HSP27 in certain subsets of cancer cells. This could have direct clinical implications: First, HSP27 might serve as a predictive marker of therapeutic response towards gemcitabine or DR-targeting drugs in stratifying pancreatic cancer patients for tailored drug administration. Second, our data further substantiate the molecular basis for clinical trials applying combinations

of gemcitabine and regional hyperthermia for the treatment of pancreatic cancer [170-173]. Likewise, the combination of hyperthermia with TRAIL-receptor targeted therapies appears to represent a promising avenue to be further developed in future studies [200,202,203].

6. TABLES AND FIGURES

7. ABBREVIATIONS

Tissue-microarray, TMA;

Carbohydrate antigen 19-9, CA19-9;

Epidermal growth factor receptor, EGFR;

Human epidermal growth factor receptor type 2, HER2;

Vascular endothelial growth factor, VEGF;

Deoxycytidine kinase, dCK;

Ribonucleotide reductase, RR;

Deoxyribonucleoside triphosphate, dNTP;

Cytidine deaminase, CDA;

5'-nucleotidase, 5'-NT;

Deoxycytidine monophosphate deaminase, DCTD;

Deoxycytidine triphosphate, dCTP;

Human equilibrative nucleoside transporter, hENT;

Human concentrative nucleoside transporter, hCNT;

Multidrug resistance protein, MRP;

Focal adhesion kinase, FAK;

Hu protein antigen R, HuR;

Heat shock protein, HSP;

Small HSPs, sHSPs;

Heat shock transcription factors, HSFs;

MAPK-activated protein kinase-2, MK2;

Protein kinase C, PKC;

Protein kinase D, PKD;

Apoptosis signal-regulating kinase, ASK1;

Supramolecular vesicular aggregates, SVAs;

Tissue microarray analyses, TMA;

Fetal calf/bovine serum, FCS/FBS;

Penicillin/streptomycin, P/S;

Dulbecco's Modified Eagle Medium, DMEM;

Roswell Park Memorial Institute medium, RPMI;

Radio Immuno Precipitation Assay, RIPA;

Phosphate Buffered Saline, PBS;

Bovine serum albumin, BSA;

Sodium dodecylsulfate, SDS;

Hydroxyethyl piperazineethanesulfonic acid, HEPES;

Ethidium Bromide, EB;

Dimethyl sulfoxide, DMSO;

Mitochondrial membrane potential, $\Delta\psi_m$;

5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-benzimidazolocarbocyanine iodide, JC-1;

Carbonyl cyanide 3-chlorophenylhydrazone, CCCP;

Tetramethylethylenediamine, TEMED;

Polyvinylidene Difluoride, PVDF;

Phenylmethanesulfonyl fluoride, PMSF;

Ethylene glycol tetraacetic acid, EGTA;

Ethylenediaminetetraacetic acid, EDTA;

Fluorescence-activated cell sorting, FACS;

Polymerase chain reaction, PCR;

Room temperature, RT;

Propidium iodide, PI;

Round per minute, RPM;

Enzyme-linked immunosorbent assay, ELISA;

Polyethylenimine, PEI;

Poly (ADP-ribose) polymerase, PARP;

Tumor necrosis factor (TNF)-related apoptosis inducing ligand, TRAIL;

The half maximal inhibitory concentration, IC_{50};

The maximal inhibitory concentration, IC_{100};

Fas-Associated protein with Death Domain, FADD;

FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein, FLIP;

Poly-L-lysine, PLL;

Standard error of the mean, SEM;

C-Jun N-terminal kinases, JNK;

Death receptor, DR.

8. ZUSAMMENFASSUNG

Das Pankreaskarzinom ist eine hochaggressive maligne Erkrankung mit limitierten therapeutischen Optionen, insbesondere im metastasierten Stadium. Gemcitabin stellt hier den therapeutischen Standard dar, allerdings existieren bislang keine belastbaren prädiktiven Marker, die ein Therapieansprechen vorhersagen. In Vorarbeiten konnten wir zeigen, daß das kleine Hitzeschockprotein 27 (HSP27) einen prognostischen und prädiktiven Marker im Pankreaskarzinom darstellt: So zeigte einerseits eine Tissue-Microarray-Analyse eine Korrelation zwischen HSP27-Expression und verbesserter Überlebensrate bei Pankreaskarzinompatienten. Andererseits führte die stabile HSP27-Überexpression *in-vitro* in Pankreaskarzinomzellen zu einer erhöhten Chemosensitivität spezifisch gegenüber Gemcitabin, nicht aber gegenüber anderen im Pankreaskarzinom gebräuchlichen Chemotherapeutika.

Ziel dieser Arbeit war zum Einen die Etablierung eines RNA-Interferenz Modellsystems zur Validierung der im Überexpressionsmodell generierten Daten, zum Anderen weiterführende mechanistische Untersuchungen zur Charakterisierung der HSP27-abhängigen Chemosensitivierung gegenüber Gemcitabin in Pankreaskarzinomzellen.

In RNA-Interferenz Modell konnte oben genannte Hypothese untermauert werden, indem mittels siRNA-vermittelten HSP27-Knockdowns belegt wurde, daß die Herabregulation von HSP27 zu einer erhöhten Gemcitabin-Resistenz in Pankreaskarzinomzellen führt.

Mechanistische Untersuchungen zu Zellzyklusarrest und Apoptoseinduktion wurden in stabil HSP27-überexpremierenden Pankreaskarzinomzellen mittels Durchflußzytometrie, nukleärer Färbung, Immunoblotting, Mitochondrienfärbung und Proliferationsassays durchgeführt. Desweiteren wurde Hyperthermie-bedingte HSP27-Induktion durch zellulären Hitzeschock bei 41.8°C im Wasserbad simuliert. Hierdurch konnte gezeigt werden, daß Gemcitabine in stabil

HSP27-überexprimierenden Zellen im Vergleich zu parentalen isogenen Kontrollzellen einen S-Phase-Arrest, gefolgt von Apoptose, induziert. Letztere wurde über die sub-G1-Fraktion primär quantifiziert und durch Aktivierung von PARP, CASPASE 3, CASPASE 8, und CASPASE 9 charakterisiert. Die beobachteten Effekte waren reversibel durch chemische Caspase-Inhibition. Zusätzlich konnte in Gemcitabin-behandelten, stabil HSP27-überexprimierenden Zellen eine Hochregulation von BIM, begleitet durch einen Verlust des mitochondrialen Membranpotentials, beobachtet werden.

Interessanterweise, da potentiell auch klinisch relevant, zeigten Pankreaskarzinomlinien, in denen HSP27 mittels Hitzeschock induziert wurde, ebenfalls eine Gemcitabin-Sensitivierung. Schliesslich konnte nachgewiesen werden, daß HSP27-Überexpression eine Sensitivierung nicht nur gegenüber Gemcitabin, sondern auch gegenüber direkt apoptose-induzierenden Agenzien via *Death Receptor 5 (DR5)* bewirkt. Dies belegt, daß HSP27-abhängige pro-apoptotische Funktionen nicht nur über den intrinsischen, sondern auch über den extrinsischen Apoptosesignalweg vermitteln werden können.

Zusammengefasst illustriert diese Arbeit komplementär zu vielen gut untersuchten anti-apoptotischen Eigenschaften von HSP27 eine neue, bislang unbekannte pro-apoptotische Rolle von HSP27 in Tumoren, speziell im Pankreaskarzinom, die sowohl über den intrinsischen als auch den extrinsischen Apoptosesignalweg vermittelt wird. Dies legt nahe, daß HSP27 einen prädiktiven Marker für das Therapieansprechen auf Gemcitabin oder direkt apoptose-induzierende Agenzien beim Pankreaskarzinom darstellen könnte. Desweiteren definieren diese Daten eine neue molekulare Grundlage für klinische Studien zur Kombination von Hyperthermie mit Gemcitabine für Patienten mit dieser Erkrankung.

9. REFERENCES

1. **Sporn MB.** The war on cancer. *Lancet*. 1996; 347: 1377-81.
2. **Hanahan D, Weinberg RA.** The hallmarks of cancer. *Cell*. 2000; 100: 57-70.
3. **Hanahan D, Weinberg RA.** Hallmarks of cancer: the next generation. *Cell*. 2011; 144: 646-74.
4. **Siegel R, Naishadham D, Jemal A.** Cancer statistics, 2012. *CA Cancer J Clin*. 2012; 62: 10-29.
5. **Gungor C, Hofmann BT, Wolters-Eisfeld G, Bockhorn M.** Pancreatic cancer. *Br J Pharmacol*. 2014; 171: 849-58.
6. **Strobel O, Hartwig W, Hackert T, Hinz U, Berens V, Grenacher L, Bergmann F, Debus J, Jager D, Buchler M, Werner J.** Re-resection for isolated local recurrence of pancreatic cancer is feasible, safe, and associated with encouraging survival. *Ann Surg Oncol*. 2013; 20: 964-72.
7. **Stathis A, Moore MJ.** Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol*. 2010; 7: 163-72.
8. **Michl P, Gress TM.** Current concepts and novel targets in advanced pancreatic cancer. *Gut*. 2013; 62: 317-26.
9. **Heinrich S, Pestalozzi BC, Schafer M, Weber A, Bauerfeind P, Knuth A, Clavien PA.** Prospective phase II trial of neoadjuvant chemotherapy with gemcitabine and cisplatin for resectable adenocarcinoma of the pancreatic head. *J Clin Oncol*. 2008; 26: 2526-31.
10. **Bond-Smith G, Banga N, Hammond TM, Imber CJ.** Pancreatic adenocarcinoma. *BMJ*. 2012; 344: e2476.
11. **Burris HA, 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD.** Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997; 15: 2403-13.
12. **Chauffert B, Mornex F, Bonnetain F, Rougier P, Mariette C, Bouche O, Bosset JF, Aparicio T, Mineur L, Azzedine A, Hammel P, Butel J, Stremsdoerfer N, Maingon P, Bedenne L.** Phase III trial comparing intensive induction chemoradiotherapy (60 Gy, infusional 5-FU and intermittent cisplatin) followed by maintenance gemcitabine with gemcitabine alone for locally advanced unresectable pancreatic cancer. Definitive results of the 2000-01 FFCD/SFRO study. *Ann Oncol*. 2008; 19: 1592-9.
13. **Loehrer PJ, Sr., Feng Y, Cardenes H, Wagner L, Brell JM, Cella D, Flynn P, Ramanathan RK, Crane CH, Alberts SR, Benson AB, 3rd.** Gemcitabine alone versus gemcitabine plus radiotherapy in patients with locally advanced pancreatic cancer: an Eastern Cooperative Oncology Group trial. *J Clin Oncol*. 2011; 29: 4105-12.
14. **Berlin J, Benson AB, 3rd.** Chemotherapy: Gemcitabine remains the standard of care for pancreatic cancer. *Nat Rev Clin Oncol*. 2010; 7: 135-7.
15. **Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y,**

Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M, Groupe Tumeurs Digestives of U, Intergroup P. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011; 364: 1817-25.

16. **Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF.** Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med*. 2013; 369: 1691-703.
17. **Van Laethem JL, Verslype C, Iovanna JL, Michl P, Conroy T, Louvet C, Hammel P, Mitry E, Ducreux M, Maraculla T, Uhl W, Van Tienhoven G, Bachet JB, Marechal R, Hendlisz A, Bali M, Demetter P, Ulrich F, Aust D, Luttges J, Peeters M, Mauer M, Roth A, Neoptolemos JP, Lutz M, European Society for Medical O.** New strategies and designs in pancreatic cancer research: consensus guidelines report from a European expert panel. *Ann Oncol*. 2012; 23: 570-6.
18. **Paulson AS, Tran Cao HS, Tempero MA, Lowy AM.** Therapeutic advances in pancreatic cancer. *Gastroenterology*. 2013; 144: 1316-26.
19. **Keating JJ, Johnson PJ, Cochrane AM, Gazzard BG, Krasner N, Smith PM, Trewby PN, Wheeler P, Wilkinson SP, Williams R.** A prospective randomised controlled trial of tamoxifen and cyproterone acetate in pancreatic carcinoma. *Br J Cancer*. 1989; 60: 789-92.
20. **Fazeny B, Baur M, Prohaska M, Hudec M, Kremnitzer M, Meryn S, Huber H, Grunt T, Tuchmann A, Dittrich C.** Octreotide combined with goserelin in the therapy of advanced pancreatic cancer--results of a pilot study and review of the literature. *J Cancer Res Clin Oncol*. 1997; 123: 45-52.
21. **Tomao S, Romiti A, Massidda B, Ionta MT, Farris A, Zullo A, Brescia A, Santuari L, Frati L.** A phase II study of gemcitabine and tamoxifen in advanced pancreatic cancer. *Anticancer Res*. 2002; 22: 2361-4.
22. **Herreros-Villanueva M, Hijona E, Cosme A, Bujanda L.** Adjuvant and neoadjuvant treatment in pancreatic cancer. *World J Gastroenterol*. 2012; 18: 1565-72.
23. **Kindler HL, Niedzwiecki D, Hollis D, Sutherland S, Schrag D, Hurwitz H, Innocenti F, Mulcahy MF, O'Reilly E, Wozniak TF, Picus J, Bhargava P, Mayer RJ, Schilsky RL, Goldberg RM.** Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). *J Clin Oncol*. 2010; 28: 3617-22.
24. **Kindler HL, Wroblewski K, Wallace JA, Hall MJ, Locker G, Nattam S, Agamah E, Stadler WM, Vokes EE.** Gemcitabine plus sorafenib in patients with advanced pancreatic cancer: a phase II trial of the University of Chicago Phase II Consortium. *Invest New Drugs*. 2012; 30: 382-6.

25. **O'Reilly EM, Niedzwiecki D, Hall M, Hollis D, Bekaii-Saab T, Pluard T, Douglas K, Abou-Alfa GK, Kindler HL, Schilsky RL, Goldberg RM, Cancer, Leukemia Group B.** A Cancer and Leukemia Group B phase II study of sunitinib malate in patients with previously treated metastatic pancreatic adenocarcinoma (CALGB 80603). *Oncologist*. 2010; 15: 1310-9.
26. **Kindler HL, Ioka T, Richel DJ, Bennouna J, Letourneau R, Okusaka T, Funakoshi A, Furuse J, Park YS, Ohkawa S, Springett GM, Wasan HS, Trask PC, Bycott P, Ricart AD, Kim S, Van Cutsem E.** Axitinib plus gemcitabine versus placebo plus gemcitabine in patients with advanced pancreatic adenocarcinoma: a double-blind randomised phase 3 study. *Lancet Oncol*. 2011; 12: 256-62.
27. **Safran H, Iannitti D, Ramanathan R, Schwartz JD, Steinhoff M, Nauman C, Hesketh P, Rathore R, Wolff R, Tantravahi U, Hughes TM, Maia C, Pasquariello T, Goldstein L, King T, Tsai JY, Kennedy T.** Herceptin and gemcitabine for metastatic pancreatic cancers that overexpress HER-2/neu. *Cancer Invest*. 2004; 22: 706-12.
28. **Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W, National Cancer Institute of Canada Clinical Trials G.** Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007; 25: 1960-6.
29. **Pelzer U, Schwaner I, Stieler J, Adler M, Seraphin J, Dorken B, Riess H, Oettle H.** Best supportive care (BSC) versus oxaliplatin, folinic acid and 5-fluorouracil (OFF) plus BSC in patients for second-line advanced pancreatic cancer: a phase III-study from the German CONKO-study group. *Eur J Cancer*. 2011; 47: 1676-81.
30. **Custodio A, Puente J, Sastre J, Diaz-Rubio E.** Second-line therapy for advanced pancreatic cancer: a review of the literature and future directions. *Cancer Treat Rev*. 2009; 35: 676-84.
31. **Moore MJ, Hamm J, Dancey J, Eisenberg PD, Dagenais M, Fields A, Hagan K, Greenberg B, Colwell B, Zee B, Tu D, Ottaway J, Humphrey R, Seymour L, National Cancer Institute of Canada Clinical Trials G.** Comparison of gemcitabine versus the matrix metalloproteinase inhibitor BAY 12-9566 in patients with advanced or metastatic adenocarcinoma of the pancreas: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2003; 21: 3296-302.
32. **Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C, Yao Q, Li M.** Overcoming drug resistance in pancreatic cancer. *Expert Opin Ther Targets*. 2011; 15: 817-28.
33. **Wang Z, Li Y, Ahmad A, Banerjee S, Azmi AS, Kong D, Sarkar FH.** Pancreatic cancer: understanding and overcoming chemoresistance. *Nat Rev Gastroenterol Hepatol*. 2011; 8: 27-33.

34. **Ruiz van Haperen VW, Veerman G, Vermorken JB, Peters GJ.** 2',2'-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. *Biochem Pharmacol*. 1993; 46: 762-6.
35. **Wright JA, Chan AK, Choy BK, Hurta RA, McClarty GA, Tagger AY.** Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase, and the significance to DNA synthesis. *Biochem Cell Biol*. 1990; 68: 1364-71.
36. **Hurta RA, Wright JA.** Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. *J Biol Chem*. 1992; 267: 7066-71.
37. **Fukunaga AK, Marsh S, Murry DJ, Hurley TD, McLeod HL.** Identification and analysis of single-nucleotide polymorphisms in the gemcitabine pharmacologic pathway. *Pharmacogenomics J*. 2004; 4: 307-14.
38. **Mini E, Nobili S, Caciagli B, Landini I, Mazzei T.** Cellular pharmacology of gemcitabine. *Ann Oncol*. 2006; 17 Suppl 5: v7-12.
39. **Ueno H, Kiyosawa K, Kaniwa N.** Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? *Br J Cancer*. 2007; 97: 145-51.
40. **El Maalouf G, Le Tourneau C, Batty GN, Faivre S, Raymond E.** Markers involved in resistance to cytotoxics and targeted therapeutics in pancreatic cancer. *Cancer Treat Rev*. 2009; 35: 167-74.
41. **Galmarini CM, Warren G, Senanayake MT, Vinogradov SV.** Efficient overcoming of drug resistance to anticancer nucleoside analogs by nanodelivery of active phosphorylated drugs. *Int J Pharm*. 2010; 395: 281-9.
42. **Galmarini CM, Popowycz F, Joseph B.** Cytotoxic nucleoside analogues: different strategies to improve their clinical efficacy. *Curr Med Chem*. 2008; 15: 1072-82.
43. **Zhang Y, Satoh K, Li M.** Novel therapeutic modalities and drug delivery in pancreatic cancer - an ongoing search for improved efficacy. *Drugs Context*. 2012; 2012: 212244.
44. **Gentile E, Cilurzo F, Di Marzio L, Carafa M, Ventura CA, Wolfram J, Paolino D, Celia C.** Liposomal chemotherapeutics. *Future Oncol*. 2013; 9: 1849-59.
45. **Yang F, Jin C, Jiang Y, Li J, Di Y, Ni Q, Fu D.** Liposome based delivery systems in pancreatic cancer treatment: from bench to bedside. *Cancer Treat Rev*. 2011; 37: 633-42.
46. **Paolino D, Licciardi M, Celia C, Giammona G, Fresta M, Cavallaro G.** Folate-targeted supramolecular vesicular aggregates as a new frontier for effective anticancer treatment in in vivo model. *Eur J Pharm Biopharm*. 2012; 82: 94-102.
47. **Sloat BR, Sandoval MA, Li D, Chung WG, Lansakara PD, Proteau PJ, Kiguchi K, DiGiovanni J, Cui Z.** In vitro and in vivo anti-tumor activities of a gemcitabine derivative carried by nanoparticles. *Int J Pharm*. 2011; 409: 278-88.
48. **Papa AL, Basu S, Sengupta P, Banerjee D, Sengupta S, Harfouche R.**

Mechanistic studies of Gemcitabine-loaded nanoplatforms in resistant pancreatic cancer cells. *BMC Cancer*. 2012; 12: 419.

49. **Reddy LH, Khoury H, Paci A, Deroussent A, Ferreira H, Dubernet C, Decleves X, Besnard M, Chacun H, Lepetre-Mouelhi S, Desmaele D, Rousseau B, Laugier C, Cintrat JC, Vassal G, Couvreur P.** Squalenoylation favorably modifies the in vivo pharmacokinetics and biodistribution of gemcitabine in mice. *Drug Metab Dispos*. 2008; 36: 1570-7.
50. **Desmaele D, Gref R, Couvreur P.** Squalenoylation: a generic platform for nanoparticular drug delivery. *J Control Release*. 2012; 161: 609-18.
51. **Bildstein L, Dubernet C, Marsaud V, Chacun H, Nicolas V, Gueutin C, Sarasin A, Benech H, Lepetre-Mouelhi S, Desmaele D, Couvreur P.** Transmembrane diffusion of gemcitabine by a nanoparticulate squalenoyl prodrug: an original drug delivery pathway. *J Control Release*. 2010; 147: 163-70.
52. **Celia C, Cosco D, Paolino D, Fresta M.** Gemcitabine-loaded innovative nanocarriers vs GEMZAR: biodistribution, pharmacokinetic features and in vivo antitumor activity. *Expert Opin Drug Deliv*. 2011; 8: 1609-29.
53. **Garcia-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M.** Nucleoside transporter profiles in human pancreatic cancer cells: role of hCNT1 in 2',2'-difluorodeoxycytidine- induced cytotoxicity. *Clin Cancer Res*. 2003; 9: 5000-8.
54. **Mackey JR, Yao SY, Smith KM, Karpinski E, Baldwin SA, Cass CE, Young JD.** Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. *J Natl Cancer Inst*. 1999; 91: 1876-81.
55. **Paproski RJ, Yao SY, Favis N, Evans D, Young JD, Cass CE, Zemp RJ.** Human concentrative nucleoside transporter 3 transfection with ultrasound and microbubbles in nucleoside transport deficient HEK293 cells greatly increases gemcitabine uptake. *PLoS One*. 2013; 8: e56423.
56. **Zhang J, Visser F, King KM, Baldwin SA, Young JD, Cass CE.** The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs. *Cancer Metastasis Rev*. 2007; 26: 85-110.
57. **Damaraju VL, Damaraju S, Young JD, Baldwin SA, Mackey J, Sawyer MB, Cass CE.** Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. *Oncogene*. 2003; 22: 7524-36.
58. **Marechal R, Mackey JR, Lai R, Demetter P, Peeters M, Polus M, Cass CE, Young J, Salmon I, Deviere J, Van Laethem JL.** Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma. *Clin Cancer Res*. 2009; 15: 2913-9.
59. **Ko AH, Tempero MA.** Personalized medicine for pancreatic cancer: a step in the right direction. *Gastroenterology*. 2009; 136: 43-5.
60. **Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, Iannopollo M, Bevilacqua G, Mosca**

F, Danesi R. Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res*. 2006; 66: 3928-35.

61. **Farrell JJ, Elsaleh H, Garcia M, Lai R, Ammar A, Regine WF, Abrams R, Benson AB, Macdonald J, Cass CE, Dicker AP, Mackey JR.** Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer. *Gastroenterology*. 2009; 136: 187-95.
62. **Spratlin J, Sangha R, Glubrecht D, Dabbagh L, Young JD, Dumontet C, Cass C, Lai R, Mackey JR.** The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. *Clin Cancer Res*. 2004; 10: 6956-61.
63. **Nakano Y, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, Izawa T, Mizukami Y, Okumura T, Kohgo Y.** Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. *Br J Cancer*. 2007; 96: 457-63.
64. **Blackstock AW, Lightfoot H, Case LD, Tepper JE, Mukherji SK, Mitchell BS, Swarts SG, Hess SM.** Tumor uptake and elimination of 2',2'-difluoro-2'-deoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with in vivo tumor response. *Clin Cancer Res*. 2001; 7: 3263-8.
65. **Marechal R, Mackey JR, Lai R, Demetter P, Peeters M, Polus M, Cass CE, Salmon I, Deviere J, Van Laethem JL.** Deoxycitidine kinase is associated with prolonged survival after adjuvant gemcitabine for resected pancreatic adenocarcinoma. *Cancer*. 2010; 116: 5200-6.
66. **Lotfi K, Mansson E, Chandra J, Wang Y, Xu D, Knaust E, Spasokoukotskaja T, Liliemark E, Eriksson S, Albertioni F.** Pharmacological basis for cladribine resistance in a human acute T lymphoblastic leukaemia cell line selected for resistance to etoposide. *Br J Haematol*. 2001; 113: 339-46.
67. **Eda H, Ura M, K FO, Tanaka Y, Miwa M, Ishitsuka H.** The antiproliferative activity of DMDC is modulated by inhibition of cytidine deaminase. *Cancer Res*. 1998; 58: 1165-9.
68. **Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA.** An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. *Cancer Res*. 2004; 64: 3761-6.
69. **Bergman AM, Eijk PP, Ruiz van Haperen VW, Smid K, Veerman G, Hubeek I, van den Ijssel P, Ylstra B, Peters GJ.** In vivo induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. *Cancer Res*. 2005; 65: 9510-6.
70. **Dumontet C, Bauchu EC, Fabianowska K, Lepoivre M, Wyczechowska D, Bodin F, Rolland MO.** Common resistance mechanisms to nucleoside analogues in variants of the human erythroleukemic line K562. *Adv Exp Med Biol*. 1999; 457: 571-7.

71. **Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE.** RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene*. 2004; 23: 1539-48.
72. **Heinemann V, Schermuly MM, Stieber P, Schulz L, Jungst D, Wilkowski R, Schalhorn A.** CA19-9: a pedictor of response in pancreatic cancer treated with gemcitabine and cisplatin. *Anticancer Res*. 1999; 19: 2433-5.
73. **Bauer TM, El-Rayes BF, Li X, Hammad N, Philip PA, Shields AF, Zalupski MM, Bekaii-Saab T.** Carbohydrate antigen 19-9 is a prognostic and predictive biomarker in patients with advanced pancreatic cancer who receive gemcitabine-containing chemotherapy: a pooled analysis of 6 prospective trials. *Cancer*. 2013; 119: 285-92.
74. **Fujita H, Ohuchida K, Mizumoto K, Itaba S, Ito T, Nakata K, Yu J, Kayashima T, Souzaki R, Tajiri T, Manabe T, Ohtsuka T, Tanaka M.** Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy. *Neoplasia*. 2010; 12: 807-17.
75. **Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S, Yamasaki M, Marubashi S, Takemasa I, Miyamoto A, Takeda Y, Nagano H, Dono K, Umeshita K, Sakon M, Monden M.** Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *Int J Cancer*. 2007; 120: 1355-63.
76. **Eto K, Kawakami H, Kuwatani M, Kudo T, Abe Y, Kawahata S, Takasawa A, Fukuoka M, Matsuno Y, Asaka M, Sakamoto N.** Human equilibrative nucleoside transporter 1 and Notch3 can predict gemcitabine effects in patients with unresectable pancreatic cancer. *Br J Cancer*. 2013; 108: 1488-94.
77. **Richards NG, Rittenhouse DW, Freydin B, Cozzitorto JA, Grenda D, Rui H, Gonye G, Kennedy EP, Yeo CJ, Brody JR, Witkiewicz AK.** HuR status is a powerful marker for prognosis and response to gemcitabine-based chemotherapy for resected pancreatic ductal adenocarcinoma patients. *Ann Surg*. 2010; 252: 499-505; discussion -6.
78. **Ohuchida K, Mizumoto K, Kayashima T, Fujita H, Moriyama T, Ohtsuka T, Ueda J, Nagai E, Hashizume M, Tanaka M.** MicroRNA expression as a predictive marker for gemcitabine response after surgical resection of pancreatic cancer. *Ann Surg Oncol*. 2011; 18: 2381-7.
79. **Hidalgo M.** Pancreatic cancer. *N Engl J Med*. 2010; 362: 1605-17.
80. **Ritossa P.** [Problems of prophylactic vaccinations of infants]. *Riv Ist Sieroter Ital*. 1962; 37: 79-108.
81. **Khalil AA, Kabapy NF, Deraz SF, Smith C.** Heat shock proteins in oncology: diagnostic biomarkers or therapeutic targets? *Biochim Biophys Acta*. 2011; 1816: 89-104.
82. **Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR.** Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci*. 2006; 31: 164-72.
83. **Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G.** Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties.

Cell Cycle. 2006; 5: 2592-601.

84. **Landry J, Chretien P, Lambert H, Hickey E, Weber LA.** Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J Cell Biol*. 1989; 109: 7-15.
85. **Mehlen P, Briolay J, Smith L, Diaz-latoud C, Fabre N, Pauli D, Arrigo AP.** Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the Drosophila 27-kDa heat-shock protein. *Eur J Biochem*. 1993; 215: 277-84.
86. **Huot J, Roy G, Lambert H, Chretien P, Landry J.** Increased survival after treatments with anticancer agents of Chinese hamster cells expressing the human Mr 27,000 heat shock protein. *Cancer Res*. 1991; 51: 5245-52.
87. **A.-P.Arrigo.** Pathology-Dependent Effects Linked to Small Heat Shock Proteins Expression: An Update. *Scientifica*. 2012; 2012: 19.
88. **Ciocca DR, Calderwood SK.** Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones*. 2005; 10: 86-103.
89. **Sreedhar AS, Csermely P.** Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. *Pharmacol Ther*. 2004; 101: 227-57.
90. **Ciocca DR, Arrigo AP, Calderwood SK.** Heat shock proteins and heat shock factor 1 in carcinogenesis and tumor development: an update. *Arch Toxicol*. 2013; 87: 19-48.
91. **Ciocca DR, Oesterreich S, Chamness GC, McGuire WL, Fuqua SA.** Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst*. 1993; 85: 1558-70.
92. **Acunzo J, Katsogiannou M, Rocchi P.** Small heat shock proteins HSP27 (HspB1), alphaB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death. *Int J Biochem Cell Biol*. 2012; 44: 1622-31.
93. **Wettstein G, Bellaye PS, Micheau O, Bonniaud P.** Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *Int J Biochem Cell Biol*. 2012; 44: 1680-6.
94. **Malusecka E, Zborek A, Krzyzowska-Gruca S, Krawczyk Z.** Expression of heat shock proteins HSP70 and HSP27 in primary non-small cell lung carcinomas. An immunohistochemical study. *Anticancer Res*. 2001; 21: 1015-21.
95. **Huang Q, Ye J, Huang Q, Chen W, Wang L, Lin W, Lin J, Lin X.** Heat shock protein 27 is over-expressed in tumor tissues and increased in sera of patients with gastric adenocarcinoma. *Clin Chem Lab Med*. 2010; 48: 263-9.
96. **Rocchi P, So A, Kojima S, Signaevsky M, Beraldi E, Fazli L, Hurtado-Coll A, Yamanaka K, Gleave M.** Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. *Cancer Res*. 2004; 64: 6595-602.
97. **Melle C, Ernst G, Escher N, Hartmann D, Schimmel B, Bleul A, Thieme H, Kaufmann R, Felix K, Friess HM, Settmacher U, Hommann M, Richter**

KK, Daffner W, Taubig H, Manger T, Claussen U, von Eggeling F. Protein profiling of microdissected pancreas carcinoma and identification of HSP27 as a potential serum marker. *Clin Chem*. 2007; 53: 629-35.

98. **Zoubeidi A, Gleave M.** Small heat shock proteins in cancer therapy and prognosis. *Int J Biochem Cell Biol*. 2012; 44: 1646-56.
99. **Sherman M, Multhoff G.** Heat shock proteins in cancer. *Ann N Y Acad Sci*. 2007; 1113: 192-201.
100. **Kamada M, So A, Muramaki M, Rocchi P, Beraldi E, Gleave M.** Hsp27 knockdown using nucleotide-based therapies inhibit tumor growth and enhance chemotherapy in human bladder cancer cells. *Mol Cancer Ther*. 2007; 6: 299-308.
101. **Hsu HS, Lin JH, Huang WC, Hsu TW, Su K, Chiou SH, Tsai YT, Hung SC.** Chemoresistance of lung cancer stemlike cells depends on activation of Hsp27. *Cancer*. 2011; 117: 1516-28.
102. **Zhang B, Qu JQ, Xiao L, Yi H, Zhang PF, Li MY, Hu R, Wan XX, He QY, Li JH, Ye X, Xiao ZQ, Feng XP.** Identification of heat shock protein 27 as a radioresistance-related protein in nasopharyngeal carcinoma cells. *J Cancer Res Clin Oncol*. 2012; 138: 2117-25.
103. **Bunger S, Laubert T, Roblick UJ, Habermann JK.** Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview. *J Cancer Res Clin Oncol*. 2011; 137: 375-89.
104. **Uozaki H, Ishida T, Kakiuchi C, Horiuchi H, Gotoh T, Iijima T, Imamura T, Machinami R.** Expression of heat shock proteins in osteosarcoma and its relationship to prognosis. *Pathol Res Pract*. 2000; 196: 665-73.
105. **Harrison JD, Jones JA, Ellis IO, Morris DL.** Oestrogen receptor D5 antibody is an independent negative prognostic factor in gastric cancer. *Br J Surg*. 1991; 78: 334-6.
106. **Takeno S, Noguchi T, Kikuchi R, Sato T, Uchida Y, Yokoyama S.** Analysis of the survival period in resectable stage IV gastric cancer. *Ann Surg Oncol*. 2001; 8: 215-21.
107. **King KL, Li AF, Chau GY, Chi CW, Wu CW, Huang CL, Lui WY.** Prognostic significance of heat shock protein-27 expression in hepatocellular carcinoma and its relation to histologic grading and survival. *Cancer*. 2000; 88: 2464-70.
108. **Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke Y, Foster CS.** Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Res*. 2000; 60: 7099-105.
109. **Bostwick DG.** Immunohistochemical changes in prostate cancer after androgen deprivation therapy. *Mol Urol*. 2000; 4: 101-6;discussion 7.
110. **Tweedle EM, Khattak I, Ang CW, Nedjadi T, Jenkins R, Park BK, Kalirai H, Dodson A, Azadeh B, Terlizzo M, Grabsch H, Mueller W, Myint S, Clark P, Wong H, Greenhalf W, Neoptolemos JP, Rooney PS, Costello E.** Low molecular weight heat shock protein HSP27 is a prognostic indicator in rectal

cancer but not colon cancer. *Gut*. 2010; 59: 1501-10.

111. **Geisler JP, Geisler HE, Tammela J, Miller GA, Wiemann MC, Zhou Z.** A study of heat shock protein 27 in endometrial carcinoma. *Gynecol Oncol*. 1999; 72: 347-50.
112. **Nakajima M, Kuwano H, Miyazaki T, Masuda N, Kato H.** Significant correlation between expression of heat shock proteins 27, 70 and lymphocyte infiltration in esophageal squamous cell carcinoma. *Cancer Lett*. 2002; 178: 99-106.
113. **Ungar DR, Hailat N, Strahler JR, Kuick RD, Brodeur GM, Seeger RC, Reynolds CP, Hanash SM.** Hsp27 expression in neuroblastoma: correlation with disease stage. *J Natl Cancer Inst*. 1994; 86: 780-4.
114. **Tetu B, Lacasse B, Bouchard HL, Lagace R, Huot J, Landry J.** Prognostic influence of HSP-27 expression in malignant fibrous histiocytoma: a clinicopathological and immunohistochemical study. *Cancer Res*. 1992; 52: 2325-8.
115. **Love S, King RJ.** A 27 kDa heat shock protein that has anomalous prognostic powers in early and advanced breast cancer. *Br J Cancer*. 1994; 69: 743-8.
116. **Conroy SE, Sasieni PD, Amin V, Wang DY, Smith P, Fentiman IS, Latchman DS.** Antibodies to heat-shock protein 27 are associated with improved survival in patients with breast cancer. *Br J Cancer*. 1998; 77: 1875-9.
117. **Thanner F, Sutterlin MW, Kapp M, Rieger L, Morr AK, Kristen P, Dietl J, Gassel AM, Muller T.** Heat shock protein 27 is associated with decreased survival in node-negative breast cancer patients. *Anticancer Res*. 2005; 25: 1649-53.
118. **Geisler JP, Geisler HE, Tammela J, Wiemann MC, Zhou Z, Miller GA, Crabtree W.** Heat shock protein 27: an independent prognostic indicator of survival in patients with epithelial ovarian carcinoma. *Gynecol Oncol*. 1998; 69: 14-6.
119. **Langdon SP, Rabiasz GJ, Hirst GL, King RJ, Hawkins RA, Smyth JF, Miller WR.** Expression of the heat shock protein HSP27 in human ovarian cancer. *Clin Cancer Res*. 1995; 1: 1603-9.
120. **Mese H, Sasaki A, Nakayama S, Yoshioka N, Yoshihama Y, Kishimoto K, Matsumura T.** Prognostic significance of heat shock protein 27 (HSP27) in patients with oral squamous cell carcinoma. *Oncol Rep*. 2002; 9: 341-4.
121. **Wang A, Liu X, Sheng S, Ye H, Peng T, Shi F, Crowe DL, Zhou X.** Dysregulation of heat shock protein 27 expression in oral tongue squamous cell carcinoma. *BMC Cancer*. 2009; 9: 167.
122. **Schafer C, Seeliger H, Bader DC, Assmann G, Buchner D, Guo Y, Ziesch A, Palagyi A, Ochs S, Laubender RP, Jung A, De Toni EN, Kirchner T, Goke B, Bruns C, Gallmeier E.** Heat shock protein 27 as a prognostic and predictive biomarker in pancreatic ductal adenocarcinoma. *J Cell Mol Med*. 2012; 16: 1776-91.
123. **Kabakov AE, Malyutina YV, Latchman DS.** Hsf1-mediated stress response can transiently enhance cellular radioresistance. *Radiat Res*. 2006; 165: 410-23.

124. **Fortin A, Raybaud-Diogene H, Tetu B, Deschenes R, Huot J, Landry J.** Overexpression of the 27 KDa heat shock protein is associated with thermoresistance and chemoresistance but not with radioresistance. *Int J Radiat Oncol Biol Phys*. 2000; 46: 1259-66.
125. **Guttmann DM, Hart L, Du K, Seletsky A, Koumenis C.** Inhibition of Hsp27 radiosensitizes head-and-neck cancer by modulating deoxyribonucleic acid repair. *Int J Radiat Oncol Biol Phys*. 2013; 87: 168-75.
126. **Langer R, Ott K, Specht K, Becker K, Lordick F, Burian M, Herrmann K, Schrattenholz A, Cahill MA, Schwaiger M, Hofler H, Wester HJ.** Protein expression profiling in esophageal adenocarcinoma patients indicates association of heat-shock protein 27 expression and chemotherapy response. *Clin Cancer Res*. 2008; 14: 8279-87.
127. **Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E.** HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *FASEB J*. 1999; 13: 2061-70.
128. **Bruey JM, Paul C, Fromentin A, Hilpert S, Arrigo AP, Solary E, Garrido C.** Differential regulation of HSP27 oligomerization in tumor cells grown in vitro and in vivo. *Oncogene*. 2000; 19: 4855-63.
129. **Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, Doseff AI.** Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J Biol Chem*. 2007; 282: 25088-99.
130. **Arrigo AP, Simon S, Gibert B, Kretz-Remy C, Nivon M, Czekalla A, Guillet D, Moulin M, Diaz-Latoud C, Vicart P.** Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. *FEBS Lett*. 2007; 581: 3665-74.
131. **Mosser DD, Morimoto RI.** Molecular chaperones and the stress of oncogenesis. *Oncogene*. 2004; 23: 2907-18.
132. **Charette SJ, Lavoie JN, Lambert H, Landry J.** Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol*. 2000; 20: 7602-12.
133. **Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB.** Heat shock protein 27 controls apoptosis by regulating Akt activation. *J Biol Chem*. 2003; 278: 27828-35.
134. **Parcellier A, Schmitt E, Brunet M, Hammann A, Solary E, Garrido C.** Small heat shock proteins HSP27 and alphaB-crystallin: cytoprotective and oncogenic functions. *Antioxid Redox Signal*. 2005; 7: 404-13.
135. **Tsiaousidou A, Lambropoulou M, Chatzitheoklitos E, Tripsianis G, Tsompanidou C, Simopoulos C, Tsaroucha AK.** B7H4, HSP27 and DJ-1 molecular markers as prognostic factors in pancreatic cancer. *Pancreatology*. 2013; 13: 564-9.
136. **Baylot V, Andrieu C, Katsogiannou M, Taieb D, Garcia S, Giusiano S, Acunzo J, Iovanna J, Gleave M, Garrido C, Rocchi P.** OGX-427 inhibits tumor progression and enhances gemcitabine chemotherapy in pancreatic cancer. *Cell Death Dis*. 2011; 2: e221.
137. **Kuramitsu Y, Wang Y, Taba K, Suenaga S, Ryozawa S, Kaino S, Sakaida I,**

Nakamura K. Heat-shock protein 27 plays the key role in gemcitabine-resistance of pancreatic cancer cells. *Anticancer Res*. 2012; 32: 2295-9.

138. **Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Mikuria K, Fujimoto M, Maehara S, Maehara Y, Okita K, Nakamura K, Sakaida I.** Proteomics finding heat shock protein 27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine. *Int J Oncol*. 2007; 31: 1345-50.
139. **Mori-Iwamoto S, Kuramitsu Y, Ryozawa S, Taba K, Fujimoto M, Okita K, Nakamura K, Sakaida I.** A proteomic profiling of gemcitabine resistance in pancreatic cancer cell lines. *Mol Med Rep*. 2008; 1: 429-34.
140. **Liu QH, Zhao CY, Zhang J, Chen Y, Gao L, Ni CY, Zhu MH.** Role of heat shock protein 27 in gemcitabine-resistant human pancreatic cancer: comparative proteomic analyses. *Mol Med Rep*. 2012; 6: 767-73.
141. **Benjamin IJ, McMillan DR.** Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res*. 1998; 83: 117-32.
142. **Kostenko S, Moens U.** Heat shock protein 27 phosphorylation: kinases, phosphatases, functions and pathology. *Cell Mol Life Sci*. 2009; 66: 3289-307.
143. **Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J.** Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol*. 1995; 15: 505-16.
144. **Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M.** Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. *J Biol Chem*. 1999; 274: 18947-56.
145. **Madsen PS, Hokland P, Clausen N, Ellegaard J, Hokland M.** Differential expression levels of the heat shock protein 27 isoforms in pediatric normal, nonleukemic and common acute lymphoblastic leukemia B-cell precursors. *Blood*. 1995; 85: 510-21.
146. **Guo K, Kang NX, Li Y, Sun L, Gan L, Cui FJ, Gao MD, Liu KY.** Regulation of HSP27 on NF-kappaB pathway activation may be involved in metastatic hepatocellular carcinoma cells apoptosis. *BMC Cancer*. 2009; 9: 100.
147. **Nakashima M, Adachi S, Yasuda I, Yamauchi T, Kawaguchi J, Itani M, Yoshioka T, Matsushima-Nishiwaki R, Hirose Y, Kozawa O, Moriwaki H.** Phosphorylation status of heat shock protein 27 plays a key role in gemcitabine-induced apoptosis of pancreatic cancer cells. *Cancer Lett*. 2011; 313: 218-25.
148. **Sakai A, Otani M, Miyamoto A, Yoshida H, Furuya E, Tanigawa N.** Identification of phosphorylated serine-15 and -82 residues of HSPB1 in 5-fluorouracil-resistant colorectal cancer cells by proteomics. *J Proteomics*. 2012; 75: 806-18.
149. **Chandrika BB, Maney SK, Lekshmi SU, Retnabhai ST.** Endoplasmic reticulum targeted Bcl2 confers long term cell survival through phosphorylation

of heat shock protein 27. *Int J Biochem Cell Biol*. 2010; 42: 1984-92.

150. **Taba K, Kuramitsu Y, Ryozawa S, Yoshida K, Tanaka T, Maehara S, Maehara Y, Sakaida I, Nakamura K.** Heat-shock protein 27 is phosphorylated in gemcitabine-resistant pancreatic cancer cells. *Anticancer Res*. 2010; 30: 2539-43.
151. **Schepers H, Geugien M, van der Toorn M, Bryantsev AL, Kampinga HH, Eggen BJ, Vellenga E.** HSP27 protects AML cells against VP-16-induced apoptosis through modulation of p38 and c-Jun. *Exp Hematol*. 2005; 33: 660-70.
152. **Maizels ET, Peters CA, Kline M, Cutler RE, Jr., Shanmugam M, Hunzicker-Dunn M.** Heat-shock protein-25/27 phosphorylation by the delta isoform of protein kinase C. *Biochem J*. 1998; 332 (Pt 3): 703-12.
153. **Casado P, Zuazua-Villar P, Prado MA, Valle ED, Iglesias JM, Martinez-Campa C, Lazo PS, Ramos S.** Characterization of HSP27 phosphorylation induced by microtubule interfering agents: implication of p38 signalling pathway. *Arch Biochem Biophys*. 2007; 461: 123-9.
154. **Schafer C, Clapp P, Welsh MJ, Benndorf R, Williams JA.** HSP27 expression regulates CCK-induced changes of the actin cytoskeleton in CHO-CCK-A cells. *Am J Physiol*. 1999; 277: C1032-43.
155. **Mehlen P, Hickey E, Weber LA, Arrigo AP.** Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem Biophys Res Commun*. 1997; 241: 187-92.
156. **Young L, Sung J, Stacey G, Masters JR.** Detection of Mycoplasma in cell cultures. *Nat Protoc*. 2010; 5: 929-34.
157. **Hopert A, Uphoff CC, Wirth M, Hauser H, Drexler HG.** Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. *J Immunol Methods*. 1993; 164: 91-100.
158. **Choppa PC, Vojdani A, Tagle C, Andrin R, Magtoto L.** Multiplex PCR for the detection of Mycoplasma fermentans, M. hominis and M. penetrans in cell cultures and blood samples of patients with chronic fatigue syndrome. *Mol Cell Probes*. 1998; 12: 301-8.
159. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72: 248-54.
160. **Zhao M, Shen F, Yin YX, Yang YY, Xiang DJ, Chen Q.** Increased expression of heat shock protein 27 correlates with peritoneal metastasis in epithelial ovarian cancer. *Reprod Sci*. 2012; 19: 748-53.
161. **Zhu H, Fearnhead HO, Cohen GM.** An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett*. 1995; 374: 303-8.
162. **Li R, Moudgil T, Ross HJ, Hu HM.** Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein

Bim. *Cell Death Differ*. 2005; 12: 292-303.

163. **Tong T, Ji J, Jin S, Li X, Fan W, Song Y, Wang M, Liu Z, Wu M, Zhan Q.** Gadd45a expression induces Bim dissociation from the cytoskeleton and translocation to mitochondria. *Mol Cell Biol*. 2005; 25: 4488-500.
164. **Feeney CJ, Pennefather PS, Gyulkhandanyan AV.** A cuvette-based fluorometric analysis of mitochondrial membrane potential measured in cultured astrocyte monolayers. *J Neurosci Methods*. 2003; 125: 13-25.
165. **Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB.** Mitochondrial membrane potential monitored by JC-1 dye. *Methods Enzymol*. 1995; 260: 406-17.
166. **Coss RA, Storck CW, Daskalakis C, Berd D, Wahl ML.** Intracellular acidification abrogates the heat shock response and compromises survival of human melanoma cells. *Mol Cancer Ther*. 2003; 2: 383-8.
167. **Tabuchi Y, Takasaki I, Wada S, Zhao QL, Hori T, Nomura T, Ohtsuka K, Kondo T.** Genes and genetic networks responsive to mild hyperthermia in human lymphoma U937 cells. *Int J Hyperthermia*. 2008; 24: 613-22.
168. **Rashmi R, Santhosh Kumar TR, Karunagaran D.** Human colon cancer cells differ in their sensitivity to curcumin-induced apoptosis and heat shock protects them by inhibiting the release of apoptosis-inducing factor and caspases. *FEBS Lett*. 2003; 538: 19-24.
169. **Kato K, Ito H, Kamei K, Iwamoto I.** Stimulation of the stress-induced expression of stress proteins by curcumin in cultured cells and in rat tissues in vivo. *Cell Stress Chaperones*. 1998; 3: 152-60.
170. **Tschoep-Lechner KE, Milani V, Berger F, Dieterle N, Abdel-Rahman S, Salat C, Issels RD.** Gemcitabine and cisplatin combined with regional hyperthermia as second-line treatment in patients with gemcitabine-refractory advanced pancreatic cancer. *Int J Hyperthermia*. 2013; 29: 8-16.
171. **Ohguri T, Imada H, Yahara K, Narisada H, Morioka T, Nakano K, Korogi Y.** Concurrent chemoradiotherapy with gemcitabine plus regional hyperthermia for locally advanced pancreatic carcinoma: initial experience. *Radiat Med*. 2008; 26: 587-96.
172. **Maluta S, Schaffer M, Pioli F, Dall'oglio S, Pasetto S, Schaffer PM, Weber B, Giri MG.** Regional hyperthermia combined with chemoradiotherapy in primary or recurrent locally advanced pancreatic cancer : an open-label comparative cohort trial. *Strahlenther Onkol*. 2011; 187: 619-25.
173. **Ishikawa T, Kokura S, Sakamoto N, Ando T, Imamoto E, Hattori T, Oyamada H, Yoshinami N, Sakamoto M, Kitagawa K, Okumura Y, Yoshida N, Kamada K, Katada K, Uchiyama K, Handa O, Takagi T, Yasuda H, Sakagami J, Konishi H, Yagi N, Naito Y, Yoshikawa T.** Phase II trial of combined regional hyperthermia and gemcitabine for locally advanced or metastatic pancreatic cancer. *Int J Hyperthermia*. 2012; 28: 597-604.
174. **Yada A, Yazawa M, Ishida S, Yoshida H, Ichikawa K, Kurakata S, Fujiwara K.** A novel humanized anti-human death receptor 5 antibody CS-1008 induces apoptosis in tumor cells without toxicity in hepatocytes. *Ann Oncol*. 2008; 19:

1060-7.

175. **Li J, Knee DA, Wang YZ, Zhang QX, Johnson JA, Cheng J, He H, Miller C, Li ZF, Kowal C, Eckman J, Tang B, Yuan J, Chen L, Deveraux Q, Nasoff MS, Stover D.** LBY135, a novel anti-DR5 agonistic antibody induces tumor cell-specific cytotoxic activity in human colon tumor cell lines and xenografts. *Drug Development Research*. 2008; 69: 69-82.
176. **Ewald B, Sampath D, Plunkett W.** H2AX phosphorylation marks gemcitabine-induced stalled replication forks and their collapse upon S-phase checkpoint abrogation. *Mol Cancer Ther*. 2007; 6: 1239-48.
177. **Chen M, Wang J.** Initiator caspases in apoptosis signaling pathways. *Apoptosis*. 2002; 7: 313-9.
178. **O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC.** Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J*. 1998; 17: 384-95.
179. **Yan HJ, Liu WS, Sun WH, Wu J, Ji M, Wang Q, Zheng X, Jiang JT, Wu CP.** miR-17-5p inhibitor enhances chemosensitivity to gemcitabine via upregulating Bim expression in pancreatic cancer cells. *Dig Dis Sci*. 2012; 57: 3160-7.
180. **Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A.** The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell*. 1999; 3: 287-96.
181. **Shingleton WW, Parker RT.** Abdominal Perfusion for Cancer Chemotherapy Using Hypothermia and Hyperthermia. *Acta Unio Int Contra Cancrum*. 1964; 20: 465-8.
182. **Falk MH, Issels RD.** Hyperthermia in oncology. *Int J Hyperthermia*. 2001; 17: 1-18.
183. **Issels RD.** Hyperthermia adds to chemotherapy. *Eur J Cancer*. 2008; 44: 2546-54.
184. **Rao W, Deng ZS, Liu J.** A review of hyperthermia combined with radiotherapy/chemotherapy on malignant tumors. *Crit Rev Biomed Eng*. 2010; 38: 101-16.
185. **Issels RD, Lindner LH, Verweij J, Wust P, Reichardt P, Schem BC, Abdel-Rahman S, Daugaard S, Salat C, Wendtner CM, Vujaskovic Z, Wessalowski R, Jauch KW, Durr HR, Ploner F, Baur-Melnyk A, Mansmann U, Hiddemann W, Blay JY, Hohenberger P, European Organisation for R, Treatment of Cancer Soft T, Bone Sarcoma G, European Society for Hyperthermic O.** Neo-adjuvant chemotherapy alone or with regional hyperthermia for localised high-risk soft-tissue sarcoma: a randomised phase 3 multicentre study. *Lancet Oncol*. 2010; 11: 561-70.
186. **Varma S, Myerson R, Moros E, Taylor M, Straube W, Zoberi I.** Simultaneous radiotherapy and superficial hyperthermia for high-risk breast carcinoma: a randomised comparison of treatment sequelae in heated versus non-heated sectors of the chest wall hyperthermia. *Int J Hyperthermia*. 2012; 28: 583-90.

187. **Ohguri T, Imada H, Yahara K, Moon SD, Yamaguchi S, Yatera K, Mukae H, Hanagiri T, Tanaka F, Korogi Y.** Re-irradiation plus regional hyperthermia for recurrent non-small cell lung cancer: a potential modality for inducing long-term survival in selected patients. *Lung Cancer*. 2012; 77: 140-5.
188. **Triantopoulou S, Efstathopoulos E, Platoni K, Uzunoglou N, Kelekis N, Kouloulias V.** Radiotherapy in conjunction with superficial and intracavitary hyperthermia for the treatment of solid tumors: survival and thermal parameters. *Clin Transl Oncol*. 2013; 15: 95-105.
189. **Stewart JR, Gibbs FA, Jr.** Hyperthermia in the treatment of cancer. Perspectives on its promise and its problems. *Cancer*. 1984; 54: 2823-30.
190. **Pennacchioli E, Fiore M, Gronchi A.** Hyperthermia as an adjunctive treatment for soft-tissue sarcoma. *Expert Rev Anticancer Ther*. 2009; 9: 199-210.
191. **Wissing D, Jaattela M.** HSP27 and HSP70 increase the survival of WEHI-S cells exposed to hyperthermia. *Int J Hyperthermia*. 1996; 12: 125-38.
192. **Jolesch A, Elmer K, Bendz H, Issels RD, Noessner E.** Hsp70, a messenger from hyperthermia for the immune system. *Eur J Cell Biol*. 2012; 91: 48-52.
193. **Vertrees RA, Das GC, Popov VL, Coscio AM, Goodwin TJ, Logrono R, Zwischenberger JB, Boor PJ.** Synergistic interaction of hyperthermia and Gemcitabine in lung cancer. *Cancer Biol Ther*. 2005; 4: 1144-53.
194. **Urano M.** Kinetics of thermotolerance in normal and tumor tissues: a review. *Cancer Res*. 1986; 46: 474-82.
195. **Douglas WG, Wang Y, Gibbs JF, Tracy E, Kuvshinoff B, Huntoon K, Baumann H.** Proinflammatory cytokines increase hepatocellular carcinoma cells thermotolerance: evidence of how local inflammation may negatively impact radiofrequency ablation local control rates. *J Surg Res*. 2008; 150: 118-24.
196. **Dhayat S, Mardin WA, Mees ST, Haier J.** Epigenetic markers for chemosensitivity and chemoresistance in pancreatic cancer--a review. *Int J Cancer*. 2011; 129: 1031-41.
197. **Soo RA, Yong WP, Innocenti F.** Systemic therapies for pancreatic cancer--the role of pharmacogenetics. *Curr Drug Targets*. 2012; 13: 811-28.
198. **Fulda S, Debatin KM.** Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006; 25: 4798-811.
199. **Han J, Goldstein LA, Gastman BR, Rabinowich H.** Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. *J Biol Chem*. 2006; 281: 10153-63.
200. **Song X, Kim HC, Kim SY, Basse P, Park BH, Lee BC, Lee YJ.** Hyperthermia-enhanced TRAIL- and mapatumumab-induced apoptotic death is mediated through mitochondria in human colon cancer cells. *J Cell Biochem*. 2012; 113: 1547-58.
201. **Alcala MA, Jr., Park K, Yoo J, Lee DH, Park BH, Lee BC, Bartlett DL, Lee YJ.** Effect of hyperthermia in combination with TRAIL on the JNK-Bim signal transduction pathway and growth of xenograft tumors. *J Cell Biochem*. 2010; 110: 1073-81.

202. **Yoo J, Kim HR, Lee YJ.** Hyperthermia enhances tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human cancer cells. *Int J Hyperthermia*. 2006; 22: 713-28.
203. **Yoo J, Lee YJ.** Effect of hyperthermia and chemotherapeutic agents on TRAIL-induced cell death in human colon cancer cells. *J Cell Biochem*. 2008; 103: 98-109.

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Publications

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Guo Y, Ziesch A, Hocke S, Kampmann E, Ochs S, De Toni EN, Göke B, Gallmeier E. Overexpression of Heat-Shock Protein 27 (HSP27) increases gemcitabine sensitivity in pancreatic cancer cells through S-phase arrest and apoptosis. J Cell Mol Med. 2014 (In revision)

Abstracts

1. **Guo Y**, Ziesch A, De Toni EN, Gallmeier E. Overexpression of heat-shock protein 27 (HSP27) increases gemcitabine sensitivity in human pancreatic cancer cells through cell cycle arrest and apoptosis. Z Gastroenterol. 51(9): 942, 2013.
   Oral presentation, 68. Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten mit Sektion Endoskopie (DGVS), Nürnberg, Sept 11th-14th, 2013.
2. Harnicek D, Eric Kampmann E, Tanović A, Cardoso Martins AS, **Guo Y**, Gallmeier E, Kanaar R, Lauber K, Knösel T, Lindner L, Issels RD. Hyperthermia combined with Trabectedin prolongs G2 cell cycle arrest and reduces cellular survival in human tumor cells.
   Poster, Deutscher Krebskongress (DKK), Berlin, Feb 19th- 22th, 2014.
3. Kampmann E, Harnicek D, Cardoso Martins AS, Knösel T, Tanović A, **Guo Y**, Gallmeier E, Lindner L, Kanaar R, Issels RD. Thermo-sensitization of Trabectedin in human soft-tissue sarcoma (STS) cells: Hyperthermia-mediated BRCA2-degradation is involved in enhanced cytotoxicity.
   Poster, Connective Tissue Oncology Society Annual Meeting (CTOS), New York, Oct 30th-Nov 2nd, 2013.
4. **Guo Y**. A paradoxical role of Hsp27 in pancreatic cancer: HSP27-overexpression increases gemcitabine sensitivity via s-phase arrest and apoptosis.
   Oral presentation, 16. Retreat of Med 2 in Klinikum Grosshadern of Ludwig-Maximilians Universitaet Muenchen (LMU), Munich, Dec 12th, 2012;
5. **Guo Y**. Heat shock protein 27 as a prognostic and predictive biomarker in pancreatic ductal adenocarcinoma; LKB1 gene knock out on a pancreatic cancer

cell line (PL5 cells).

Oral presentation, Auditing Seminar of CSC-LMU PhD program, Ludwig-Maximilians Universitaet Muenchen (LMU), May 30th, 2012.

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