Impact on angiogenesis and ER stress and its effect on chemoresistance through PIGF inhibition in a mouse model of hepatocellular carcinoma

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2015

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Artwork cover:

Painting of a tumour-bearing liver of Dr. A. Macphail (left) and a transmission electron microscopy image of a murine hepatocellular carcinoma cell showing the expanded endoplasmic reticulum (right).
What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what’s going on.

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<th>Acronym</th>
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<tr>
<td>4-PBA</td>
<td>4-phenylbutyric acid</td>
</tr>
<tr>
<td>AASLD</td>
<td>American association for the study of liver diseases</td>
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<tr>
<td>AGREE</td>
<td>appraisal of guidelines research and evaluation</td>
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<tr>
<td>aPlGF</td>
<td>anti-PlGF antibody</td>
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<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase</td>
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<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2-associated X protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BCLC</td>
<td>Barcelona-clinic liver cancer</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>BH</td>
<td>BCL-2 homology</td>
</tr>
<tr>
<td>BI-1</td>
<td>BAX inhibitor 1</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>Bim</td>
<td>BCL-2 interacting mediator of cell death</td>
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<tr>
<td>BiP</td>
<td>binding immunoglobulin Protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BWGE</td>
<td>Belgian week of gastroenterology</td>
</tr>
<tr>
<td>CALR</td>
<td>calreticulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-dependent kinase II</td>
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<tr>
<td>CANX</td>
<td>calnexin</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CMA</td>
<td>chaperone-mediated autophagy</td>
</tr>
<tr>
<td>DCE</td>
<td>dynamic contrast-enhanced</td>
</tr>
<tr>
<td>DEB-TACE</td>
<td>drug-eluting beads transarterial chemoembolization</td>
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</tbody>
</table>
DEN diethylnitrosamine
DESPARTH dose-escalation study evaluating the safety and pharmacokinetics of oral artesunate in patients with advanced hepatocellular carcinoma
DHA dihydroartemisinin
DUBs deubiquitinases
EASL European association for the study of the liver
EC endothelial cells
ECCO European cancer organization
EDEM1 degradation-enhancing α-mannosidase-like protein
eIF2α α subunit of eukaryotic translation initiation factor 2
ER endoplasmic reticulum
ERAD ER-associated protein degradation
ERDJ4 endoplasmic reticulum DnaJ homolog 4
ERO1L endoplasmic oxidoreductin-1-like protein
ERO1α endoplasmic reticulum oxidase 1α
{18}F-FDG {18}F-fluoro-deoxyglucose
FGF fibroblast growth factor
FRG Fah<sup>−/−</sup>/Rag2<sup>−/−</sup>/Il2rg<sup>−/−</sup>
GADD34 growth arrest and DNA damage-inducible 34
GCLC glutamate-cysteine ligase, catalytic subunit
GPX3 glutathione peroxidase 3
GRP78 glucose-regulated protein, 78 kDa
GRP94 glucose-regulated protein, 94 kDa
Gsta1 glutathione-S-transferase A1
Gsta2 glutathione-S-transferase A2
HBV hepatitis B
HCC hepatocellular carcinoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HCV</td>
<td>hepatitis C</td>
</tr>
<tr>
<td>HERPUD1</td>
<td>homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>IG</td>
<td>intragastric</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP3R1</td>
<td>inositol-1,4,5-trisphosphate receptor 1</td>
</tr>
<tr>
<td>IRE1</td>
<td>inositol-requiring enzyme-1</td>
</tr>
<tr>
<td>ISRIB</td>
<td>integrated stress response inhibitor</td>
</tr>
<tr>
<td>JIK</td>
<td>c-Jun NH2-terminal inhibitory kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MCL-1</td>
<td>myeloid cell leukaemia 1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum tolerated dose</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>Nfe2l2</td>
<td>nuclear factor erythroid-derived 2, like 2</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-B</td>
</tr>
<tr>
<td>NRF2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>OZ</td>
<td>oprozomib</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDIA4</td>
<td>protein disulfide-isomerase A4</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PERKi</td>
<td>PERK inhibitor</td>
</tr>
<tr>
<td>PET/CT</td>
<td>positron emission tomography/computed tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PI GF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>PI GF KO</td>
<td>PI GF knockout</td>
</tr>
<tr>
<td>PP1C</td>
<td>protein phosphatase 1C</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RIDD</td>
<td>regulated IRE1-dependent decay of mRNA</td>
</tr>
<tr>
<td>RIP</td>
<td>regulated intramembrane proteolysis</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>serine protease site-1</td>
</tr>
<tr>
<td>S2P</td>
<td>site-2 protease</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>sterol regulatory element binding protein-1</td>
</tr>
<tr>
<td>TACE</td>
<td>transarterial chemoembolization</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>TUDCA</td>
<td>tauroursodeoxycholic acid</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>UEG</td>
<td>united European gastroenterology</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>W</td>
<td>week</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box-binding protein 1</td>
</tr>
<tr>
<td>XBP1s</td>
<td>spliced X-box-binding protein 1</td>
</tr>
<tr>
<td>XBP1u</td>
<td>unspliced X-box-binding protein 1</td>
</tr>
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Summary

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide. Because current treatment options for HCC are capable of providing good survival rates to only a small subset of patients, innovative therapeutic strategies are urgently required. The endoplasmic reticulum (ER) is responsible for protein translocation, folding mediated by a machinery of molecular chaperones, and post-translational modifications that allow further transport of proteins to the Golgi apparatus. Protein folding and modification in the ER is highly sensitive to disturbances of homeostasis. Accumulation of unfolded proteins in the ER lumen, termed ER stress, activates intracellular signalling pathways to resolve the protein-folding defects. This unfolded protein response (UPR) increases the protein-folding capacity, reduces global protein synthesis and stimulates the ER-associated protein degradation. Three major ER stress transducers regulating the UPR have been identified: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6). Paradoxically, if ER stress is too severe or persistent, numerous apoptotic signalling pathways are activated. The UPR has been implicated in a variety of diseases including metabolic diseases, neurodegenerative diseases, inflammatory diseases and cancer. Recently, UPR activation was shown in human HCC samples. Given the dichotomy in outcomes of UPR activation, it remains unclear whether a window exists in which manipulation of the UPR can be harnessed therapeutically. Thus, it is crucial to develop a precise understanding of the signal transduction in the UPR.

The present work focused on the exact role and therapeutic potential of ER stress and the UPR in hepatocarcinogenesis, which starts from the initial genotoxic insult, through the clonal expansion from a premalignant to a tumoural lesion (promotion) and finally to tumour progression.

First, we sequentially monitored the UPR over time in a commonly used orthotopic mouse model for HCC induced by 30 weeks of the carcinogen diethylnitrosamine and explored the effects of UPR modulation on cell viability and proliferation in vitro and in the mouse model. We observed that the expression of ER-resident chaperones peaked during tumour initiation and increased further during tumour progression, predominantly within the nodules. A peak in Ire1 signalling was observed early during tumour initiation. The Perk pathway was activated during tumour progression. The Atf6 pathway was modestly activated only after tumour initiation. Consistent with the UPR activation, electron microscopy demonstrated ER expansion and reorganization in isolated HCC cells in vivo.
Strikingly, under ER stress or hypoxia, the Perk inhibitor and not the Ire1 inhibitor reduced cell viability via escalating proteotoxic stress. Importantly, the Perk inhibitor significantly decreased tumour burden in the mouse model. Thus, we provided the first evaluation of the UPR dynamics in a long-term cancer model and identified a small molecule Perk inhibitor as a new strategy for HCC therapy. However, the antitumour effect of this inhibitor was modest and we found induction of hyperglycaemia in the mice treated with the Perk inhibitor, confirming the essential role of Perk in insulin biosynthesis.

Secondly, we assessed the impact of ER stress in tumour initiation and progression by applying tauroursodeoxycholic acid (TUDCA), a bile acid with chaperone properties reducing ER stress, in the diethylnitrosamine-induced HCC model in preventive and therapeutic settings. Administration of TUDCA in the preventive setting reduced carcinogen-induced elevation of liver enzyme levels, apoptosis of hepatocytes and tumour burden. TUDCA also suppressed carcinogen-induced pro-apoptotic UPR. Furthermore, TUDCA administration after tumour development did not alter orthotopic tumour or HepG2 xenograft growth. Thus, these results identified the UPR as a key pathway in hepatocarcinogenesis and showed that TUDCA attenuates hepatocarcinogenesis by suppressing carcinogen-induced ER stress-mediated cell death without stimulating tumour progression. Therefore, this chemical chaperone could represent a novel chemopreventive agent.

Thirdly, we assessed the effect of established or potential HCC treatments possibly modulating the UPR, including sorafenib, artesunate, PlGF inhibition and proteasome inhibition, on the tumour growth and UPR signature in HCC. Sorafenib, a multi-kinase inhibitor, is currently the only drug available for the treatment of advanced HCC, but effects are limited. Given the importance of the UPR and UPR-induced autophagy in HCC, we questioned whether the use of rationally designed combinations of sorafenib with clinically applicable modulators of the UPR and autophagy would be more effective than sorafenib monotherapy. We found that sorafenib activated the UPR and autophagy in HCC cells. Furthermore, sorafenib-mediated reduction in tumour cell proliferation was dependent on proteotoxicity and IRE1 RNase activity. Targeting of the UPR or autophagy separately did not enhance the antitumour efficacy of sorafenib. However, the combination with an ER stress inducer and an inhibitor of adaptive protein refolding or autophagy potentiated the efficacy. Thus, triple therapies of sorafenib with inducers of ER stress and inhibitors of autophagy seem to enhance the antitumour potential of sorafenib.
Artemisinins are antimalarial drugs that exert anticancer activity, possibly by inducing ER stress. We evaluated the effects of artesunate, a semisynthetic derivative of artemisinin, on tumour growth, angiogenesis and the UPR in HCC. We found that artesunate dose dependently reduced cell viability in different HCC cells. These effects were enhanced by hypoxia. In mice, artesunate decreased vessel density and tumour burden. These in-vivo effects were enhanced by combination with sorafenib. Furthermore, artesunate modulated the UPR in vitro and in vivo, increasing proapoptotic signalling. Thus, clinical trials with artesunate as monotherapy or in combination with current hypoxia-inducing approaches are necessary. Therefore, we initiated a phase I dose-escalation study evaluating the safety and pharmacokinetics of oral artesunate in patients with advanced HCC (DESPARTH trial).

We previously showed that the inhibition of placental growth factor (PlGF) exerts antitumour effects and induces vessel normalisation, possibly reducing hypoxia. However, the exact mechanism underlying these effects remains unclear. Because hypoxia and ER stress have been implicated in HCC progression, we assessed the interactions between PlGF and these microenvironmental stresses. Both the genetic and pharmacological inhibitions of PlGF reduced the chaperone levels and the activation of the PERK pathway in experimental HCC. We identified that tumour hypoxia was attenuated. Furthermore, hypoxic exposure activated the PERK pathway in vitro, suggesting PlGF inhibition may diminish PERK activation by improving oxygen delivery via vessel normalisation.

The first-in-class proteasome inhibitor bortezomib has been approved in clinical use against hematologic malignancies and has shown modest activity in a variety of solid tumours. However, a considerable proportion of subjects fail to respond to bortezomib and have adverse events. Recently, the next-generation orally bioavailable proteasome inhibitor oprozomib was developed. Oprozomib dose dependently reduced viability and proliferation of human HCC cells. The effect of oprozomib on the UPR pattern was dual: oprozomib inhibited the cytoprotective transcriptional program of ATF6 but increased the pro-apoptotic UPR-mediated protein levels by prolonging protein half-life. Oral oprozomib displayed antitumour effects in the HCC models. Importantly, combination of oprozomib with PERK activators stimulating CHOP induction improved the antitumour efficacy compared to oprozomib monotherapy without cumulative toxicity.

In conclusion, the UPR seems to be involved in the different steps of hepatocarcinogenesis and HCC survival under tumour microenvironmental stress. Consequently, chemopreventive and therapeutic targeting of the UPR holds significant potential in HCC.
Samenvatting

Hepatocellulair carcinoom (HCC) is wereldwijd de 2de belangrijkste oorzaak van kankergerelateerde mortaliteit. Aangezien de huidige behandelingsopties slechts voor een klein deel van de patiënten goede overlevingskansen bieden is er een dringende nood aan innovatieve behandelingen.

Het endoplasmatisch reticulum (ER) is verantwoordelijk voor eiwittranslocatie, opvouwing en post-translatiele modificaties. Vervolgens worden de mature eiwitten na een kwaliteitscontrole verder getransporteerd naar het Golgi apparaat. Eiwitopvouwing is gevoelig aan stress in de cellulaire micro-omgeving en accumulatie van ongevouwen eiwitten in het ER lumen, een status die ER stress wordt genoemd, activeert intracellulaire signaalwegen die trachten de eiwithomeostase in het ER te herstellen. Deze “unfolded protein response” (UPR) verhoogt de eiwitopvouwingscapaciteit, vermindert de globale eiwitsynthese en stimuleert de ER-geassocieerde eiwitafbraak. Binnen de UPR zijn er drie voornaam ER stress transducers geïdentificeerd: inositol-requiring enzyme-1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) en activating transcription factor 6 (ATF6). Echter, als de ER stress te ernstig of persistent is, wordt apoptose geïnitieerd. De UPR is betrokken bij diverse pathologieën, waaronder metabole, neurodegeneratieve en inflammatoire pathologieën, maar ook bij kanker. Zo werd recent UPR activatie in humane HCC stalen aangetoond. Aangezien de UPR kan leiden tot paradoxale effecten op celoverleving is het onduidelijk of er een therapeutisch venster bestaat waarbij UPR manipulatie kan worden aangewend in kankerbehandeling of -preventie. Hiervoor is een adequaat inzicht van de UPR signaaltransductie tijdens de verschillende stappen van de carcinogenese van essentieel belang.

Hepatocarcinogenese begint met de eerste genotoxische schade, gevolgd door klonale expansie van een premaligne naar een maligne laesie (tumorpromotie) en finaal tot tumorprogressie. Deze studie focust op de rol en het therapeutisch potentieel van ER stress en de UPR in de hepatocarcinogenese.

Vooropstelling hebben we de UPR dynamiek gemonitord in de tijd in een orthotoop muismodel geïnduceerd door toediening van het carcinogeen diethylnitrosamine. Vervolgens hebben we de effecten van UPR-modulatie op de viabiliteit en proliferatie van HCC cellen in vitro en in het desbetreffende muismodel onderzocht. Hierbij stelden we vast dat de expressie van ER-residente chaperones piekte tijdens tumorinitiatie en verder toenam tijdens tumorprogressie. Tijdens tumorinitiatie werd eveneens een piek in het Ire1 signaal waargenomen.
De Perk pathway werd geactiveerd tijdens tumorprogressie. De Atf6 pathway werd mild geactiveerd na tumorinitiatie. Elektronenmicroscopie identificeerde ER expansie en reorganisatie, ultrastructurele kenmerken van ER stress, in HCC cellen in vivo. Verder werd er vastgesteld dat bij ER stress of hypoxie, de Perk inhibitor en niet de Ire1 inhibitor de celviabiliteit via escalerende proteotoxiciteit verminderde. Toediening van de Perk inhibitor reduceerde de tumorgroei. Kortom, deze studie leidde tot de eerste evaluatie van de UPR dynamiek in een kankermodel geïnduceerd door chronische carcinogeneexpositie en identificeerde de Perk inhibitor als een potentiële therapeutische strategie. Echter, het antitumoraal effect van deze inhibitor was beperkt en we detecteerden inductie van diabetes door de Perk inhibitor.

Ten tweede hebben we de impact van ER stress op tumorinitiatie en -progressie geanalyseerd door het toepassen van tauroursodeoxycholic acid of kortweg TUDCA, een galzuur met chaperone eigenschappen die ER stress antagoneert, in het HCC model in de preventieve en therapeutische setting. TUDCA toediening in de preventieve setting reduceerde de carcinogeengeïnduceerde leverenzymen, incidentie van apoptotische hepatocyten, pro-apoptotische UPR signalisatie en tumorlast. TUDCA toediening na tumorontwikkeling daarentegen had geen effect op de tumorgroei. Deze resultaten identificeerden de UPR als een belangrijke as in hepatocarcinogenese en toonden aan dat TUDCA hepatocarcinogenese reduceert door onderdrukking van carcinogeengeïnduceerd UPR-gemiedeerde apoptose zonder stimulatie van tumorprogressie. Hierdoor kan dit chemisch chaperone als een nieuw chemopreventief agens worden beschouwd.

Ten derde onderzochten we het effect van erkende of potentiële HCC behandelingen die mogelijk de UPR moduleren. Hierbij includeerden we sorafenib, artesunaat, PlGF inhibitie en proteasoom inhibitie en onderzochten we het effect van deze op de tumorgroei en het UPR patroon in HCC. Sorafenib, een multi-kinase inhibitor, is momenteel het enige geneesmiddel beschikbaar voor gevorderd HCC. Echter is het overlevingsvoordeel door sorafenib beperkt tot enkele maanden. Gezien het belang van de UPR en autofagie, een katabool proces waarbij subcellulaire componenten via lysosomale enzymen worden afgebroken, vroegen we ons af of het gebruik van rationeel gedesigneerde combinaties van sorafenib met klinisch toepasbare modulators van de UPR en/of autofagie effectiever zou zijn dan sorafenib monotherapie. We stelden vast dat sorafenib de UPR en UPR-gemiedeerde autofagie activeert in HCC cellen en dat de sorafenib-gemiedeerde reductie in cellulaire proliferatie afhankelijk is van proteotoxiciteit en IRE1. Inhibitie van de UPR of autofagie afzonderlijk verbeterde het antitumor effect van sorafenib echter niet.
Maar combinatie van een ER stress inductor samen met een remmer van adaptieve heropvouwing of autofagie versterkte het effect van sorafenib. Artemisinines zijn antimalariamiddelen die een antitumoraal effect blijken te hebben in verschillende kankertypes, eventueel door inductie van ER stress. We beoordeelden de effecten van artesunaat, een semi-synthetisch derivaat van artemisinine, op tumorgroei, angiogenese en de UPR in HCC. We vonden dat artesunaat dosisafhankelijk de viabiliteit van HCC cellen verminderde en dat deze effecten bovendien werden versterkt door hypoxie. Artesunaat verminderde tevens de bloedvatdichtheid en tumorlast in vivo. Hetgeen werd versterkt in combinatie met sorafenib. Verder moduleerde artesunaat de UPR waardoor pro-apoptotische UPR signalering optrad. Bijgevolg lijken klinische studies met artesunaat als monotherapie of in combinatie met huidige hypoxie-inducerende behandelingen aangewezen. Daarom zijn we gestart met een fase I dosisescalatie studie ter evaluatie van de veiligheid en farmacokinetiek van oraal artesunaat bij HCC patiënten (DESPARTH trial).

We toonden eerder aan dat inhibitie van de placentale groeifactor (PlGF) bloedvatnormalisatie en antitumurale effecten induceert. Het onderliggende mechanisme is echter onduidelijk. Omdat hypoxie en ER stress betrokken blijken bij HCC, onderzochten we de interacties tussen PlGF en deze stressoren. Zowel genetische als farmacologische PlGF inhibitie onderdrukte de chaperones en Perk pathway in HCC. We identificeerden dat PlGF inhibitie tumorhypoxie tempert en dat hypoxie de PERK pathway activeert, wat erop wijst dat PlGF inhibitie de Perk activatie reduceert door verbeterde zuurstofvoorziening via bloedvatnormalisatie.

De first-in-class proteasoominhibitor bortezomib is in klinisch gebruik bij hematologische maligniteiten en heeft een bescheiden activiteit aangetoond in verschillende solide tumoren. Echter, een aanzienlijk deel van patiënten reageren niet op bortezomib en hebben majeure bijwerkingen. Recent werd de next-generation proteasoominhibitor oprozomib ontwikkeld. Oprozomib verminderde de proliferatie van HCC cellen op een dosisafhankelijke manier. Het effect van oprozomib op het UPR patroon was tweeledig: oprozomib remde het cytoprotectief transcriptioneel programma van ATF6, maar verhoogde de pro-apoptotische UPR-gemedieerde eiwitniveaus door verlengde halfwaardetijd. Oraal oprozomib reduceerde de tumorgroei in vivo. Combinatie van oprozomib met PERK activatoren verbeterde de werkzaamheid in vergelijking met oprozomib monotherapie zonder cumulatieve toxiciteit.

We besluiten dat de UPR betrokken is bij verschillende stappen van hepatocarcinogenese en de overleving van HCC cellen in de stressvolle tumor micro-omgeving. Bijgevolg heeft het chemopreventief en therapeutisch manipuleren van de UPR een belangrijk potentieel in HCC.
Chapter 1

General introduction
1. Hepatocellular carcinoma

1.1. Introduction

Tumours of the liver are classified as being either primary (originating from the liver) or metastatic (spread from another organ to the liver). Primary liver tumours may be further divided into those that are benign, which are not cancerous and remain in the liver, or malignant, which progress with local expansion, intrahepatic spread and distant metastases [1]. The most common primary malignant tumour of the liver is a hepatocellular carcinoma (HCC). HCC occurs predominantly in patients with underlying chronic liver disease and cirrhosis [1], [2].

Figure 1. Large hepatocellular carcinoma. Note the heterogeneity within the nodule. Image courtesy of Arief Suriawinata, Department of Pathology, Dartmouth Medical School, USA.

1.2. Pathophysiology of hepatocellular carcinoma

The ratio of cell growth and death is precisely balanced through developmental and homeostatic processes in multi-cellular organisms [3]. Sensu stricto, cancer is a genetic disease and the genetic alterations that lead to most cancers arise in the DNA of a somatic cell [4]. Because of these genetic changes, cancer cells proliferate irrepressibly, producing malignant cells that invade surrounding healthy tissue and tend to metastase, that is, to spawn cells that break away from the parent mass, enter the lymphatic or vascular circulation, and spread to distant sites in the body where they establish metastases that are no longer amenable to surgical resection [2].
The **hallmarks** of cancer comprise biological capabilities acquired during multistep carcinogenesis [3]. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming of energy metabolism, evading immune destruction and activating invasion and metastasis. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmarks. In addition to cancer cells, tumours exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that create the tumour microenvironment. Recent evidence suggests that pathophysiologic conditions unique to the tumour microenvironment initiate tumour cell stress signals that converge upon the endoplasmic reticulum (ER), resulting in a condition termed “ER stress” (see section 2.2) [5].

Also hepatocarcinogenesis is a **multistep** process: the presence of specific risk factors (see section 1.4) promotes genotoxicity leading to a cascade of molecular and cellular deregulations that ultimately result in malignant transformation of hepatocytes and generally develops within liver cirrhosis related to various aetiologies [1], [2]. In cirrhosis, precancerous dysplastic macronodules transform into early HCCs that evolve into small and progressed HCCs, and finally lead to advanced HCC [6], [7]. In rare cases, HCC develops in a normal liver, with some of these tumours potentially resulting from malignant transformation of hepatocellular adenomas [8].

High-resolution analyses have depicted the **molecular heterogeneity** of HCC [9]. A broad variety of **pathways** activated in HCC have been reported including the Wnt/β-catenin, Ras/MAPK, insulin-like growth factor, PI3K/Akt/mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), hepatocyte growth factor/cMET signalling among many others [4]. Depending on the specific alterations, distinct molecular subclasses have been defined (see section 1.6) [10].
Furthermore, several **structural alterations** have been characterized. Recent studies identified an average of 30-40 mutations per tumour, among which 6–8 are considered oncogenic drivers [11]. Mutations in TERT (60%, telomere stability), TP53 (30%, genome integrity guardian), CTNNB1 (25%, Wnt signalling), NFE2L2 (6-10%, oxidative stress) and ARID1A (10-16%, chromatin remodelling) and high-level amplifications in 11q13 (7%) and 6p21 (5%, contains VEGF) are the most relevant ones [4], [10]. Another study identified 161 putative driver genes associated with 11 recurrently altered pathways [9]. Associations of mutations defined 3 groups of genes related to aetiology. However, no oncogenic addiction loop for any driver has been defined in HCC. Intriguingly, well-known genes commonly mutated in other solid tumours such as EGFR, PIK3CA or KRAS are rarely mutated in HCC [11].

Next to the structural alterations, several **prognostic mRNA-based molecular signatures** from tumour tissue have been reported [12], [13]. Signatures identifying progenitor cell-like and/or cholangiocyte profile (EPCAM signature3, CK19 signature) show worse prognosis [13]. Likewise, a 5-gene score signature (TAF9, RAN, RAMP3, KRT19, HN1) predicted overall survival in four independent cohorts [14]. However, there is significant **intratumour heterogeneity** (Figure 1) leading to an underestimation of the tumour genomics landscape portrayed from single tumour biopsy [15].

In conclusion, the interindividual and intratumour heterogeneity, biological redundancies and presence of several growth factors and cytokines potentially involved in HCC progression make it extremely difficult to select the best genetic target for therapy and stimulated us to target other hallmarks of cancer, such as angiogenesis or effect of peritumoural microenvironmental stress.

### 1.3. Epidemiology of hepatocellular carcinoma

HCC is the fifth most common cancer in men (554,000 cases, 7.5% of total cancer incidence) and the ninth in women (228,000 cases, 3.4%) [16]. HCC has a strong male preponderance with a male to female ratio estimated to be 2.4, which is partially explained by the protective role of oestrogens and HCC-promoting effects of androgens [17]. It is mainly a problem of the less developed regions where 83% of the estimated 782,000 new cases worldwide occurred in 2012. In men, high incidence regions are Eastern and South-Eastern Asia (age-standardised rates: 31.9 and 22.2 resp.). Intermediate rates occur in Southern Europe (9.5) and Northern America (9.3) and the lowest rates are in Northern Europe (4.6).
HCC is the second leading cause of cancer-related mortality worldwide, estimated to be responsible for nearly **746,000 deaths in 2012** (9.1% of total cancer-related mortality) [16]. The prognosis is very poor (overall ratio of mortality to incidence of 0.95). The incidence increases progressively with advancing age in all populations, reaching a peak at 70 years. Unfortunately, HCC is one of the cancers with a still increasing incidence rate [18], [19].

**Figure 2. Estimated liver cancer incidence worldwide in 2012: men.** Estimated age-standardised rates per 100,000 [16].
1.4. Aetiology of hepatocellular carcinoma

Hepatitis viral infection (hepatitis B (HBV) and C (HCV)), chronic alcohol abuse and non-alcoholic steatohepatitis (NASH) are the major risk factors of liver cirrhosis and cancer [1]. Other risk factors include fungal aflatoxin B1 exposure, hemochromatosis, α-1-antitrypsin deficiency and tyrosinaemia [2]. Worldwide, 54% of HCC cases can be attributed to HBV infection (which affects 400 million people globally), while 31% can be attributed to HCV infection (which affects 170 million people), leaving 15% to other aetiologies. In Africa and East Asia, the largest attributable fraction is due to HBV (60%) whereas in the Western world, only 20% of cases can be attributed to HBV, while chronic HCV and alcohol consumption appear to be the major risk factors (Table 1) [20]. Recently, great advances in HCV therapies were made. For more information, please see our review concerning ‘New therapies for hepatitis C’ (YP. Vandewynckel et al., Tijdschr. voor Geneeskunde, 2014). Long-term follow-up studies have demonstrated that 1-8% of cirrhotic patients per year develop HCC [21]. Ultimately, one-third of cirrhotic patients will develop HCC during their lifetime [22]. In general, liver disease severity, age and male gender correlate with HCC development among cirrhotic patients [23]. Since risk factors are well known, prevention by risk factor reduction is an achievable objective. Control of HBV and HCV, as well as reduction in alcohol consumption is recommended [24]. While health plans are implemented to achieve this goal, the epidemic of overweight and metabolic syndrome leading to NASH-induced HCC has emerged as a significant risk factor [25]. Alternatively, development of chemopreventive agents could drastically reduce HCC mortality [26]. However, randomized controlled chemoprevention trials are logistically and ethically challenging.

Table 1. Geographical distribution of main risk factors for hepatocellular carcinoma worldwide.

<table>
<thead>
<tr>
<th>Geographic area</th>
<th>AAIR</th>
<th>Risk factors</th>
<th>Alcohol</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M/F</td>
<td>HBV (%)</td>
<td>HCV (%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Europe</td>
<td>6.7/2.3</td>
<td>60-70</td>
<td>15-20</td>
<td>10</td>
</tr>
<tr>
<td>Southern</td>
<td>10.5/3.3</td>
<td>60-70</td>
<td>15-20</td>
<td>10</td>
</tr>
<tr>
<td>Northern</td>
<td>4.1/1.8</td>
<td>50-60</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>North America</td>
<td>6.8/2.3</td>
<td>60-70</td>
<td>15-20</td>
<td>10</td>
</tr>
<tr>
<td>Asia and Africa</td>
<td>20</td>
<td>70</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Asia</td>
<td>21.6/6.2</td>
<td>(NASH)</td>
<td>(Allatoxin)</td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>23/6.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>20/5.7/8</td>
<td>70</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Africa</td>
<td>1.6/5.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WORLD</td>
<td>16/6</td>
<td>31</td>
<td>54</td>
<td>15</td>
</tr>
</tbody>
</table>

AAIR, age-adjusted incidence rate. Adapted from [1].
1.5. Diagnosis of hepatocellular carcinoma

Diagnosis of HCC is based on the diagnostic algorithm in Figure 4 [1]. In cirrhotic patients, nodules less than 1 cm in diameter detected by ultrasound should be followed every 4 months the first year and every 6 months thereafter. In cirrhotic patients, diagnosis of HCC for nodules of 1-2 cm should be based on non-invasive criteria or biopsy-proven pathological confirmation. In cirrhotic patients, nodules more than 2 cm can be diagnosed for HCC based on typical features on one imaging technique. In case of uncertainty or atypical radiological findings, diagnosis should be confirmed by biopsy.

Pathological diagnosis is based on the recommendations of the International Consensus Panel [6]. Non-invasive criteria can only be applied to cirrhotic patients and are based on imaging techniques obtained by 4-phase multidetector CT or dynamic contrast-enhanced MRI [1]. Diagnosis is based on the typical hallmark of HCC, i.e. hypervascular in the arterial phase with washout in the portal venous or late phases (see section 4.1).

Figure 4. Diagnostic algorithm for hepatocellular carcinoma. **HCC radiological hallmark: arterial hypervascularity and venous/late phase wash-out. Adapted from [1].
1.6. Treatment of hepatocellular carcinoma

The **Barcelona-Clinic Liver Cancer (BCLC) staging** system is recommended for prognostic prediction and treatment allocation [1]. The BCLC classification divides HCC patients in 5 stages (0, A, B, C and D) according to pre-established prognostic variables and allocates therapies according to treatment-related status (Fig. 5). **Prognosis prediction** is defined by variables related to tumour status (size, number, vascular invasion, N1, M1), liver function (Child–Pugh’s) and performance status of the patient (ECOG). **Treatment allocation** incorporates treatment dependant variables, which have been shown to influence therapeutic outcome, such as bilirubin, portal hypertension or presence of symptoms.

**Figure 5. BCLC staging system and treatment strategy.** Adapted from [1].
Resection is the treatment option for patients with solitary tumours and well-preserved liver function, as normal bilirubin with either hepatic venous pressure gradient ≤10 mmHg or platelet count ≥100,000. Large tumours are not well served by ablation because long lasting complete response in HCC >3 cm is less frequent and recurrence rate is high [27]. However, resection may be successful and this is why tumour size does not constitute a contraindication by itself [1]. Additional indications for patients with multifocal tumours meeting Milan criteria (≤3 nodules ≤3 cm) or with mild portal hypertension not suitable for liver transplantation require prospective comparisons with loco-regional treatments. Peri-operative mortality of resection in cirrhotic patients is expected to be 2-3%. Tumour recurrence represents the major complication of resection [28]. In case of recurrence, the patient will be re-assessed by BCLC staging and re-treated accordingly [1].

If patients present hepatic decompensation, the expected outcome offered by liver transplantation, if the Milan criteria are not exceeded, is superior to resection and ablation [18]. Peri-operative mortality and one-year mortality are expected to be approximately 3% and ≤10%, respectively [1]. Modest expansion of the Milan criteria applying the “up-to-seven” in patients without microvascular invasion achieves competitive outcomes.

Local ablation with radiofrequency or percutaneous ethanol injection is considered the standard of care for patients with BCLC 0-A tumours not suitable for surgery [1]. Chemoembolization is recommended for patients with BCLC B, multinodular asymptomatic tumours without vascular invasion or extra-hepatic spread. However, although severe events are infrequent, chemoembolization is associated with transient post-embolization syndrome in most cases and there is still no standardized protocol for chemoembolization in terms of treatment schedule, type and dosage of anti-cancer drug [18].

Multiple guidelines have been developed to assist clinicians in HCC management. Though, we reported that the methodological quality of guidelines on chemoembolization in HCC management is poor [29]. This results in important discrepancies between guideline recommendations, creating confusion in clinical practice. Incorporation of the Appraisal of Guidelines Research and Evaluation II (AGREE II) instrument in guideline development may improve quality of future guidelines by increasing focus on methodological aspects [30].
Conventional chemotherapy, either alone or in combination, administered intravenously or intra-arterially, never reached positive results [2]. Based on the positive phase III SHARP trial demonstrating a median overall survival of 10.7 months for sorafenib-treated vs. 7.9 months for placebo-treated HCC patients [31], sorafenib is the standard systemic therapy for advanced HCC. In terms of toxicity, there were more cases of diarrhoea, weight loss, hand-foot skin reaction and hypophosphatemia among the patients receiving sorafenib [31]. In addition, we reported (YP. Vandewynckel et al. Cerebellar stroke in a low cardiovascular risk patient associated with sorafenib treatment for fibrolamellar hepatocellular carcinoma. Clin Case Rep. 2014,2,4-6) a case of a 26-year-old man with fibrolamellar HCC, who had a cerebrovascular accident while being treated with sorafenib, illustrating a probable relationship between the use of sorafenib and vascular adverse effects in low cardiovascular risk patients.

Sorafenib acts by inhibiting the serine threonine kinases Raf-1 and B-Raf and the receptor tyrosine kinase activity of VEGF and platelet-derived growth factor (PDGF) receptors, among others [32] and is indicated for patients with well-preserved liver function (Child-Pugh A) and with advanced tumours (BCLC C) or those progressing upon loco-regional therapies [1]. To date, there are no biomarkers available to identify the best responders and no second-line treatment for patients with intolerance or failure to sorafenib.

Molecular classification should aid in understanding the biological subclasses and oncogenic drivers and optimize benefits from targeted therapies and enrich trial populations [1], [32]. Different molecular classes have been characterized including a Wnt class (25% of cases; enriched with CTNNB1 mutations and HCV aetiology), a proliferation/progenitor class (50% of cases; worse prognosis; with two subclasses: S1-TGF-β and S2-EpCAM positive) and an inflammation/interferon class [10], [12]. Nonetheless, no molecular subclass has been reported to respond to a specific targeted therapy.

Several therapies targeting signalling cascades involved in hepatocarcinogenesis have been explored in phase III trials [32]. However, none of the drugs tested have shown positive results in the first-line (tyrosine kinase inhibitors (TKI): brivanib, sunitinib, erlotinib and linifanib) or second-line (TKI: brivanib and mTOR inhibitor: everolimus) setting after sorafenib progression (Table 2). Regorafenib, lenvatinib, cabozantinib and tivantinib are currently being evaluated in phase III trials [18].
Reasons for **failure** are diverse and include lack of understanding of critical drivers of tumour progression, liver toxicity or marginal antitumour potency [32]. If targeted therapy is aimed to act on specific targets it would make sense to select patients according to the recognition of the pathway to be modulated. This enrichment policy is sound but the challenge is how to properly profile the biomarker status. As indicated earlier, HCCs present a marked heterogeneity within the same nodule (Figure 1) and across nodules making a single biopsy unlikely to provide an accurate profiling of the tumour.

### Table 2. Randomized phase III clinical trials completed in HCC in first and second line (2007–2014).
(Modified from [18])

<table>
<thead>
<tr>
<th>Year</th>
<th>Randomized drugs</th>
<th>n</th>
<th>TTP (mo)</th>
<th>p value</th>
<th>OS (mo)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Sorafenib vs. placebo</td>
<td>299/303</td>
<td>5.5 vs. 2.8</td>
<td>&lt;0.001</td>
<td>10.7 vs. 7.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2009</td>
<td>Sorafenib</td>
<td>150/76</td>
<td>2.8 vs. 1.4</td>
<td>&lt;0.001</td>
<td>6.5 vs. 4.2</td>
<td>0.01</td>
</tr>
<tr>
<td>2012</td>
<td>Sorafenib + erlotinib vs. sorafenib</td>
<td>362/358</td>
<td>3.2 vs. 4</td>
<td>n.s.</td>
<td>9.5 vs. 8.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>2012</td>
<td>Linifanib vs. sorafenib</td>
<td>514/521</td>
<td>5.4 vs. 4</td>
<td>0.001</td>
<td>9.1 vs. 9.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>2013</td>
<td>Sunitinib vs. sorafenib</td>
<td>530/544</td>
<td>3.6 vs. 3.6</td>
<td>n.s.</td>
<td>7.9 vs. 10.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>2013</td>
<td>Brivanib vs. sorafenib</td>
<td>577/578</td>
<td>4.2 vs. 4.1</td>
<td>n.s.</td>
<td>9.5 vs. 9.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>2013</td>
<td>FOLFOX-4 vs. doxorubicin</td>
<td>184/187</td>
<td>2.9 vs. 1.8</td>
<td>n.s.</td>
<td>6.4 vs. 4.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>2013</td>
<td>Brivanib vs. placebo</td>
<td>263/132</td>
<td>4.2 vs. 2.7</td>
<td>0.001</td>
<td>9.4 vs. 8.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>2014</td>
<td>Everolimus vs. placebo</td>
<td>362/184</td>
<td>2.9 vs. 2.6</td>
<td>n.s.</td>
<td>7.6 vs. 7.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>2014</td>
<td>Ramicirumab vs. placebo</td>
<td>283/282</td>
<td>3.5 vs. 2.6</td>
<td>&lt;0.0001</td>
<td>9.2 vs. 7.6</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., not significant; TTP, time to progression; OS, overall survival.
2. Endoplasmic reticulum stress and the unfolded protein response

2.1. The endoplasmic reticulum: a biosynthetic factory

The endoplasmic reticulum (ER; Figure 6) is the central organelle in the secretory pathway and provides a specialized environment for protein translocation, protein folding by a machinery of molecular chaperones and post-translational modifications that allow further transport of proteins to the Golgi and ultimately to vesicles for secretion or display on the membrane [33], [34]. The rates of protein synthesis, folding and trafficking are precisely coordinated by an efficient system termed quality control to ensure that only properly folded proteins exit the ER. Misfolded proteins are either retained within the ER or subject to degradation by the proteasome-dependent ER-associated protein degradation (ERAD) pathway or by autophagy (see section 3).

Furthermore, the ER is the major site for the synthesis of sterols and phospholipids that constitute the bulk of the lipid components of all membranes. The ER, therefore, plays an essential role in regulating the lipid composition of membranes, which, in turn, determines the biophysical properties and functions of cell membranes [35]. ER membrane expansion generally reflects the increased secretory capacity or the accumulation of unfolded proteins in the ER [35], [36].

Finally, the ER is the main site for storage of intracellular Ca\(^{2+}\). The concentration of Ca\(^{2+}\) in the ER lumen can reach ~5 mM [37]. The majority of ER-luminal Ca\(^{2+}\) is bound to molecular chaperones and is essential for their function.

Figure 6. Transmission electron microscopy showing the normal morphology of the endoplasmic reticulum of a hepatocyte (left) versus the expanded morphology of a hepatocellular carcinoma cell (right).
2.2. Endoplasmic reticulum stress

The ER is a highly dynamic organelle and responds to perturbations in its function, a process named **ER stress**, through a series of signalling cascades known as the **unfolded protein response** (UPR) [38]. The UPR is a tightly orchestrated collection of intracellular signal transduction reactions designed to cope with misfolded proteins and restore ER proteostasis [33], [34]. However, chronic or irremediable ER stress results in cell apoptosis [39].

The UPR regulates the size, the shape and the components of the ER machinery to accommodate the fluctuating demands on protein folding, as well as other ER functions in coordination with different physiological and pathological conditions [40]. Recent studies on the integration of the UPR with metabolic stress, oxidative stress and inflammatory signalling highlight new insights into the diverse cellular processes regulated by the UPR [41], [42]. For example, UPR signalling was shown to intersect at many levels with the innate and adaptive immune responses [42].

Three canonical UPR branches have been identified: inositol-requiring protein-1α (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Next to the canonical UPR, the OASIS family represents a group of additional ER stress transducers and UPR regulators [43]. These ER membrane-bound sensors operate in parallel and use unique mechanisms of signal transduction. The IRE1 branch is the most conserved and sole branch of the UPR in lower eukaryotes [44]. Evolution later added the PERK and ATF6 branches to metazoan cells.

Under physiological conditions, the luminal domains of PERK and ATF6 are bound to the ER-resident chaperone Binding immunoglobulin Protein or glucose-regulated protein, 78 kDa (BiP or GRP78), which keeps them inactive [34]. When unfolded proteins accumulate in the ER, GRP78 binds with high affinity to the exposed hydrophobic regions of the unfolded proteins and is thereby released from these complexes to assist with the folding of accumulated proteins. Upon activation, PERK, IRE1 and ATF6 induce signal transduction that alleviate the accumulation of misfolded proteins by increasing expression of ER chaperones, inhibiting protein entry into the ER by arresting global mRNA translation and stimulating retrograde transport of misfolded proteins from the ER into the cytosol for ubiquitination and destruction by ERAD [45]. Under conditions when ER stress is chronically prolonged and the protein load on the ER greatly exceeds its folding capacity, cellular dysfunction and UPR-mediated apoptosis occur [33].
Among the UPR pathways IRE1 is a key molecule that functions as a rheostat regulating cell fate upon ER stress [38]. The alternative outputs from IRE1-mediated downstream signalling dictate opposing cell fates (survival versus death) during ER stress, which are critically influenced by the intensity and longevity of ER stress [34]. IRE1 is a transmembrane protein that consists of an N-terminal luminal sensor domain, a transmembrane domain and a C-terminal cytosolic effector that is responsible for both protein kinase and endoribonuclease activities [38]. Accumulation of unfolded proteins in the ER triggers oligomerization and autophosphorylation. The RNase mediates splicing of an intron from the X-box-binding protein-1 (XBP1) mRNA to allow production of the spliced XBP1 (XBP1s) protein, which is a stronger transcription factor compared to unspliced XBP1 (XBP1u) protein. Notably, XBP1u is not the only mRNA targeted by the IRE1 RNase [46]. For example, IRE1 also controls its own expression by cleaving its own mRNA. The XBP1s protein binds to promoters of several genes involved in the UPR and ERAD to restore proteostasis [47]. In addition to its cytoprotective function, IRE1 also stimulates activation of the apoptotic-signalling kinase-1 (ASK1), which causes activation downstream of stress kinases Jun-N-terminal kinase (JNK) and p38 MAPK that promote apoptosis. Among the apoptosis-inducing substrates of JNK are Bcl-2 and Bim, which are inhibited and activated, respectively, by JNK [33]. Also, p38 MAPK phosphorylates and activates the transcription factor C/EBP homologous protein (CHOP), which leads to major changes in gene expression that favour cell apoptosis, including increasing expression of Bim and DR5, while decreasing expression of Bcl-2 [48]. Recently, regulated IRE1-dependent decay of mRNA (RIDD) was shown to reduce ER localized mRNAs [46]. RIDD selectively targets and degrades mRNAs encoding proteins involved in protein folding. Prolonged RIDD can promote cell death, but its exact role is currently not well understood.

ATF6 is a transcriptional factor that upon ER stress translocates to the Golgi where it is cleaved by the action of two proteases: the serine protease site-1 (S1P) cleaves ATF6 in the luminal domain, while the N-terminal portion is subsequently cleaved by the site-2 protease (S2P) [34]. The cleaved N-terminal cytosolic domain of ATF6 subsequently translocates into the nucleus where it binds to ATF/cAMP-response elements and ER stress-response elements to activate target genes, such as chaperones GRP78, PDIA4, GRP94 but also CHOP [49].
When activated upon sensing ER stress, PERK oligomerizes and phosphorylates itself and the α subunit of eukaryotic translation initiation factor 2 (eIF2α), inactivating eIF2α and inhibiting global mRNA translation. Phosphorylation of eIF2α inhibits the recycling of eIF2α to its active GTP-bound form, which is required for the initiation of polypeptide chain synthesis [33]. Thereby, PERK prevents influx of de novo synthesized proteins into the stressed ER providing a time frame to restore ER function [50]. However, some mRNAs containing short open reading frames in their 5′-untranslated regions are preferentially translated when eIF2α is limiting. One of these encodes the activating transcription factor 4 (ATF4), whose translation is thus induced upon eIF2α phosphorylation. Two important target genes driven by ATF4 are the proapoptotic CHOP and growth arrest and DNA damage–inducible 34 (GADD34) [51]. Thus, the PERK pathway is strongly protective at modest levels of signalling but, paradoxically, can contribute signals to apoptosis [33]. This dualism is likely played out at the level of eIF2α. GADD34 encodes a regulatory subunit of protein phosphatase 1C that dephosphorylates eIF2α. Selective inhibition of the GADD34-PP1c complex, either by salubrinal or GADD34 deletion, protects cells against ER stress by prolonging low-level eIF2α phosphorylation. Besides eIF2α, PERK can phosphorylate nuclear erythroid 2-related factor 2 (NRF2), which contributes to dissociation of the NRF2-Keap1 complex and thereby promotes expression of genes containing antioxidant response elements, preventing oxidative stress [52].

2.3. Endoplasmic reticulum stress in disease

The UPR has been implicated in a variety of diseases including metabolic such as diabetes and atherosclerosis, neurodegenerative such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, as well as rare prion disorders, and inflammatory diseases, and cancer [53],[54]. Signalling components of the UPR are rapidly emerging as targets for treatment of human diseases [41]. Many extracellular stimuli and fluctuations in intracellular homeostasis disrupt protein folding. Consequently, the cell uses its ER protein-folding status as an exquisite sensor to monitor intracellular homeostasis. Pharmacological insults were used to elucidate how cells cope with immediate and severe challenges to the protein-folding quality control. It is now evident that intracellular signalling, such as insulin anabolic responses, as well as pathophysiological conditions including hypoxia, oxidative stress, high or low glucose levels, acidosis, hyperlipidaemia, hyperhomocysteinaemia and inflammatory cytokines all disrupt protein folding and activate the UPR [33]. In solid tumours, the UPR mediates adaptation to various micro-environmental stressors [55].
2.4. Endoplasmic reticulum stress in cancer

**Review: The Paradox of the Unfolded Protein Response in Cancer**

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### 2.4.1. Abstract

The endoplasmic reticulum (ER) is an elaborate organelle that is essential for cellular function and survival. Conditions that interfere with ER functioning can lead to the accumulation of unfolded proteins, which are detected by transmembrane sensors that then initiate the unfolded protein response (UPR) to restore ER proteostasis. If the adaptive response fails, apoptotic cell death ensues. Many studies have focused on how this failure initiates apoptosis, particularly because ER stress-induced apoptosis is implicated in the pathophysiology of several diseases, including cancer. Whether the UPR inhibits tumour growth or protects tumour cells by facilitating their adaptation to stressful conditions within the tumour microenvironment is unknown, and dissection of the UPR network will likely provide answers to this question. In this review, we aim to elucidate the paradoxical role of the UPR in apoptosis and cancer.
2.4.2. Introduction

The endoplasmic reticulum (ER) consists of a membranous network that extends throughout the cytosol; here, proteins are synthesized, post-translationally modified and folded into correct conformations. Unlike the cytosol, the ER luminal environment is sufficiently oxidised to permit for cysteine oxidation and subsequent formation of the disulfide bonds that are critical to the correct conformations of many mature proteins (1). The ER contains stringent quality control systems that selectively export correctly-folded proteins and extract terminally-misfolded proteins for ubiquitin-dependent proteolytic degradation, a process known as ER-associated protein degradation (2) (Figure 1). However, if degradation is insufficient, misfolded proteins can accumulate. This phenomenon is called ER stress, and it activates the unfolded protein response (UPR). The UPR is generally considered to be the transcriptional induction of molecular chaperones in response to ER stress (3). However, gene expression profiling has demonstrated that, parallel to the chaperones, the UPR regulates genes involved in protein entry into the ER, calcium and redox homeostasis, ER quality control, autophagy, lipid biogenesis and vesicular trafficking. Additionally, ER stress attenuates global protein synthesis, a process that subsequently reduces the protein load to help re-establish equilibrium and is associated with cell-cycle arrest and tumour dormancy. Three ER stress transducers have been identified: protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor-6 (ATF6; Figure 2) (4, 5). Most targets are co-regulated by IRE1, PERK and ATF6 to ensure the redundancy and robustness of this adaptive response (6).

Following initiation of malignancy, rapid tumour growth and inadequate vascularization result in microenvironmental stress. This condition activates a range of stress response pathways, including the UPR, which meticulously coordinate adaptive and apoptotic responses to ER stress. During tumourigenesis, the UPR enhances the ER protein-folding capacity and maintains ER protein homeostasis (or proteostasis), thereby counteracting apoptosis. The UPR, when coupled with induced tumour dormancy, dually protects neoplastic cells from apoptosis and permits recurrence once favourable growth conditions have been restored (7, 8). However, if ER stress is prolonged and the UPR fails to restore ER proteostasis, tumour cell apoptosis ensues. This review addresses this paradoxical role in cancer.
Figure 1. Cellular stress as the cause of protein misfolding. Molecular chaperones stabilise and (un)fold newly-synthesised proteins into their proper conformations. During tumour formation, continuous endoplasmic reticulum (ER) stress eventually causes damage that the chaperones cannot correct. These proteins might then be recognised and degraded by the ubiquitin-proteasome system (UPS). However, if this process is insufficient to counter the accumulation of misfolded proteins, the unfolded protein response (UPR) is activated to induce chaperones, protein quality control and degradation.

2.4.3. Extrinsic and Intrinsic Stressors that Activate the UPR During Tumourigenesis

Although tumours secrete angiogenic factors to promote angiogenesis, this is often insufficient to meet the elevated tumour metabolic requirements. Therefore, in addition to hypoxia (9), cells in developing tumours are subject to glucose deprivation, lactic acidosis, oxidative stress and decreased amino acid supplies (Figure 1). In addition to these extrinsic stressors, tumour-intrinsic stressors, such as errors in glycoprotein and lipid biosynthesis that result from an increased mutation rate, might also contribute to the induction of ER stress (10).
Hypoxia-mediated UPR activation is essential for tumour cell survival. The major UPR-inducing pathway in tumours is mediated by hypoxia. Human fibrosarcoma and lung carcinoma cells up-regulate 78-kDa glucose-regulated protein (GRP78) and X-box-binding protein 1 (XBP1) splicing under hypoxic conditions in vitro, whereas in human colon cancer cells, hypoxia induces the PERK-dependent phosphorylation of eukaryotic initiation factor-2α (eIF2α) and the translation of activating transcription factor-4 (ATF4; Figure 2) (8). A strong positive correlation was demonstrated between spliced XBP1 (XBP1s)-induced bioluminescence and tumour hypoxia in transgenic mice that developed spontaneous mammary carcinomas and exhibited luciferase reporter coupled XBP1 splicing (11). Additionally, the exposure of transformed mouse embryonic fibroblasts (MEFs) to hypoxia led to increased GRP78 and XBP1 expression, as well as increased ATF4 and C/EBP homologous protein transcription factor (CHOP) expression. A potential UPR trigger in hypoxic conditions is ER oxidase 1α (ERO1α), an oxidoreductase that catalyses disulfide bond formation in nascent proteins in an oxygen-dependent manner. Although hypoxia transcriptionally induces ERO1α, reduced oxygen tension impairs ERO1α activity and subsequent protein folding. Another UPR-inducing mechanism is the upregulation of glycogen synthase kinase 3B, which activates the PERK branch (12).

The UPR is required for tumour cell growth under hypoxic conditions (13). Cells are sensitised to hypoxia in vitro by antisense-mediated GRP78 inhibition (14). PERK inactivation due to the generation of mutations in its kinase domain impairs cell survival under extreme hypoxia (15). PERK promotes cancer cell proliferation by limiting oxidative DNA damage through ATF4 (16).

Additionally, XBP1-deficient tumour cell survival was reduced during severe hypoxia in vitro, and these cells were unable to grow as tumours in vivo. Spliced XBP1 expression restored tumour growth, suggesting that the IRE1 branch is also required for tumour cell survival during hypoxia (17).

Thus, tumour formation with aberrant microcirculation leads to hypoxia, which induces the UPR. In turn, the UPR increases cellular survival and proliferation, which further enlarges the tumour and thereby increases hypoxia in the tumour core (Figure 3).
Activation of the UPR by glucose deprivation and subsequent acidosis. Tumour cells adapt to low glucose levels by switching to a high rate of aerobic glycolysis, which is known as the Warburg effect (18). The resulting lactic acid production reduces the pH, leading to aggravated local distress. Acidosis is a prominent feature of the tumour microenvironment that surprisingly promotes tumour survival and progression by regulating several B-cell leukaemia/lymphoma-2 (BCL-2) family members and CHOP (see below) (19). The glucose-regulated protein family, which includes the master UPR regulator GRP78, was originally discovered due to the up-regulation of its members in response to glucose deprivation (20). In the XBP1s reporter mouse model, which develops spontaneous mammary tumours, XBP1 splicing was found to increase upon exposure to a non-metabolizable glucose analog that simulates glucose deprivation (11). CHOP deletion in a mouse model of Kirsten rat sarcoma viral oncogene homolog-induced lung cancer increases tumour incidence and thus supports the notion that ER stress serves as a barrier to malignancy. UPR activation and the subsequent p58IPK expression control the fates of malignant cells that face glucose deprivation. Overcoming this barrier requires for selective attenuation of the PERK-CHOP branch by p58IPK. Furthermore, this p58IPK-mediated fine-tuning enables cells to benefit from the protective features of chronic UPR (21).
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Figure 2. **Endoplasmic reticulum (ER) stress induces the unfolded protein response (UPR) through a triple transcription factor system.** Misfolded proteins sequestre 78-kDa glucose-regulated protein (GRP78), thus allowing the activation of three ER membrane-associated proteins. Activating transcription factor-6 (ATF6) translocates to the Golgi for cleavage, and the cleaved fragment subsequently regulates UPR gene expression. Inositol requiring enzyme 1 (IRE1) cleaves X-box-binding protein 1 (XBP1) mRNA to a spliced form (XBP1s) that is translated to a strong transcription factor. Along with selective XBP1 mRNA splicing, other mRNAs are degraded by the IRE1 RNase activity (RIDD). IRE1 promotes c-Jun N-terminal kinase (JNK) and p38 phosphorylation through direct interactions. Caspase-12 (murine) or -4 (human) activation is ER stress-dependent. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic initiation factor 2α (eIF2α) to attenuate global translation. Phosphorylated eIF2α favours activating transcription factor 4 (ATF4) translation. The latter induces growth arrest and DNA damage-inducible protein (GADD34), which dephosphorylates eIF2α. PERK also phosphorylates nuclear factor erythroid 2-related factor 2 (NRF2), which induces an anti-oxidative response. ASK1: apoptosis signal-regulating kinase; CHOP: C/EBP homologous protein transcription factor; ERAD: ER-associated protein degradation; ERO1α: ER oxidase 1α; JIK: jun kinase-inhibitory kinase; ROS: reactive oxygen species; TRAF2: tumour necrosis factor receptor-associated factor-2.

2.4.4. **Dual Role of GRP78 in and on Surface of Tumour and Endothelial Cells**

GRP78 is a key player in tumourigenesis and is involved in the three major hallmarks of cancer, namely the enhancement of cell proliferation, protection against apoptosis and promotion of tumour angiogenesis (22). The phosphoinositide-3 kinase (PI3K)/phosphatase and tensin homolog (PTEN)/protein kinase B (PKB) pathways play central roles in these hallmark processes. In mice, PKB activation in PTEN-null prostate epithelium was potently suppressed in a GRP78-knockout model, and a similar suppression of PKB activation was observed in human prostate cancer cells that had been transfected with small interfering RNA (siRNA) targeted against GRP78 (23, 24). As PTEN mutations and PKB activation are major drivers of tumourigenesis, GRP78 inactivation might represent a novel approach to reducing tumourigenesis that results from loss of PTEN tumour suppression or oncogenic PKB activation (1). Apart from its abundant expression in the ER, GRP78 can localise at the cell surface, within the cytoplasm, in the mitochondria and in the nucleus, as well as in secretions from tumour and endothelial cells, and this protein is implicated in processes beyond protein folding.
Figure 3. The paradoxical role of the unfolded protein response (UPR) in cancer. During tumourigenesis, specific stressors activate the UPR in both cancer and endothelial cells (EC). In cancer cells, both apoptosis and survival can be induced by UPR components. Furthermore, cell-cycle progression or arrest (e.g. by reduced cyclin D1 translation) can occur in response to protein kinase RNA-like endoplasmic reticulum kinase (PERK) activation. This arrest can be temporary during stressful conditions such as chemotherapy. After the induced dormancy, tumour re-growth can occur upon the restoration of more favourable conditions. A positive feedback loop increases ER stress via cellular adaptation during tumour formation. Due to its effects on endothelial and cancer cell survival and function, the UPR also modulates metastasis and angiogenesis, which, if functional, reduces ER stress. ROS: Reactive oxygen species.

GRP78 in tumour cells. The first causal correlation between GRP78 and in vivo carcinogenesis was reported in fibrosarcoma cells. GRP78 silencing in these cells inhibited their ability to form tumours upon xenografting into mice (25). The essential role of GRP78 was confirmed in a transgenic mouse mammary tumour model. Mice that lacked one GRP78 allele exhibited decreased breast adenocarcinoma growth and angiogenesis as well and showed survival compared to wild-type mice (26). Likewise, in glioma cells, high GRP78 levels were found to correlate with increased proliferation, and siRNA-mediated GRP78 suppression reduced the cell proliferation rate (27).
GRP78 levels are known to be increased in various solid tumour types, including prostate, head and neck, melanoma, breast, lung, brain, gastric, colon, and hepatocellular carcinoma (HCC) (14, 28). Furthermore, elevated GRP78 levels correlate with gastric, breast, and liver cancer metastasis (7). In contrast, a recent report suggested that GRP78 is downregulated in mouse prostate cancer models (29). Thus, although GRP78 and malignancy appear to be positively correlated, exceptions might occur. However, these unexpected results might be due to time-dependent alterations. Additionally, GRP78 plays a dual role in tumour cells. GRP78 controls early tumour development through tumour suppressive mechanisms such as the induction of dormancy (30). On the other hand, at more advanced stages of progression, during which tumours are exposed to more severe stress, GRP78 has been shown to promote tumour progression through its pro-survival (26) and pro-metastatic functions (7).

**GRP78 on the tumour cell surface.** Severe ER stress promotes GRP78 cell surface localization in various types of neoplastic and endothelial cells (14). The cell surface form of GRP78 affects cell membrane signalling pathways that regulate proliferation, apoptosis and tumour immunity (31). A growing number of cell surface GRP78-binding partners have been identified (1, 14). In prostate cancer cells, cell surface GRP78 binds the activated form of the proteinase inhibitor α2-macroglobulin. This interaction promotes cell proliferation by activating p38 and PI3K (32). In addition to α2-macroglobulin, cell surface GRP78 can interact with Cripto, a small tumour cell surface protein that regulates tumour progression by blocking the growth-inhibitory transforming growth factor β and activating c-SRC and PKB. Interestingly, antibody-mediated blockade of this interaction with cell surface GRP78 is sufficient to inhibit its oncogenic signalling (14, 33). Finally, neovascularization, together with the formation of cell surface GRP78/T-cadherin complexes, was accelerated by ER stress (34), whereas cell surface GRP78 might also serve as a receptor for the angiogenesis inhibitor Kringle 5; the binding of Kringle 5 to GRP78 is required to exert its anti-angiogenic and pro-apoptotic activities in stressed tumour and endothelial cells (14). Thus, the dual effects of cell surface GRP78 signalling depend on the availability of binding partners in the tumour microenvironment.
**GRP78 in endothelial cells.** The importance of GRP78 in tumour angiogenesis is reflected by its constitutively high expression within the glioblastoma vasculature, which is suggestive of the sustained stress experienced by tumour-associated endothelial cells (31). In a mammary tumour model, conditional heterozygous *GRP78* knockout in endothelial cells led to a dramatic reduction in tumour angiogenesis and metastatic growth, with minimal effects on normal tissue microvascular densities. *GRP78* knockdown in immortalised human endothelial cells revealed that *GRP78* regulated endothelial cell proliferation, survival and migration (7). Vascular endothelial growth factor (VEGF) is a major driver of endothelial proliferation, survival and migration (35). However, the downstream target *GRP78* also plays an active role in VEGF regulation. *GRP78*-knockdown suppresses VEGF receptor-2, as well as VEGF-induced endothelial cell proliferation (14).

### 2.4.5. Three Proximal UPR Sensors in Cancer: An Integrated View

After the sequestration of GRP78 by unfolded proteins, ATF6, IRE1, and PERK are activated to transduce the ER stress signal to the cytosol and nucleus (Figure 2).  

**ATF6: Fine-tuning of the UPR.** Although the ATF6 branch in cancer is the least investigated, its potential as an effector of clinical outcomes should not be underestimated. Activated ATF6 translocates to the Golgi, where proteases cleave it and release a fragment into the cytosol. Indeed, enhanced nuclear translocation of the ATF6 fragment is observed in various types of cancer, including HCC (28) and Hodgkin’s lymphoma (36), and its expression has been linked to metastasis and relapse (37). Additionally, whereas XBP1s is required for organismal development, the functional roles of ATF6 in ER proteostasis remodelling are adaptive and can adjust the ER capacity to match demand. Therefore, ATF6 modulation might sensitively tune proteostasis without globally influencing proteome folding, trafficking, or degradation (38).

In contrast to PERK and IRE1, ATF6 activation has no obvious paradoxical outcomes. The latter primarily induces cytoprotective responses, such as ER biogenesis, chaperone up-regulation and protein degradation (38, 39). Moreover, ATF6 induces transcription of *XBP1* mRNA, the major splicing target of the IRE1 endonuclease.
Recently, ATF6 was identified as a survival factor for quiescent, but not proliferative, squamous carcinoma cells and as essential for the adaptation of dormant tumour cells to chemotherapy, a process that is mediated by Ras homolog enriched in brain (RHEB) and mammalian target of rapamycin (mTOR) activation (37). ATF6 or RHEB down-regulation was able to reverse dormant cell resistance \textit{in vivo}. Therefore, targeting survival signalling in dormant tumour cells after chemotherapy by abrogating the adaptive ATF6-RHEB-mTOR pathway might reduce the metastatic cancer relapse rate.

\textit{IRE1: The conserved core branch.} After oligomerisation, IRE1 has at least three established outputs: \textit{XBP1} mRNA splicing, regulation of IRE1-dependent decay (RIDD) of other mRNAs and direct interactions with downstream mediators (40) (Figure 2). Increased \textit{XBP1} splicing has been demonstrated in numerous haematological and solid types of cancer and has been associated with more malignant phenotypes and poor survival (41-43). IRE1 has been shown to promote cell proliferation by regulating cyclin A1 expression through \textit{XBP1} splicing in prostate cancer cell lines (44). Notably, \textit{XBP1}s enhances catalase expression, and the loss of \textit{XBP1}s sensitizes cells to oxidative stress-induced apoptosis. Indeed, \textit{XBP1}-deficient cells produce less catalase, which is associated with reactive oxygen species (ROS) generation and p38 activation (45). Moreover, \textit{XBP1} splicing itself might directly lead to tumourigenesis, as was evidenced by the observation that the maintenance of elevated \textit{XBP1}s levels in B and plasma cells could drive multiple myeloma pathogenesis and promote hallmark myeloma characteristics, including bone lytic lesions and sub-endothelial immunoglobulin deposition (46). Moreover, a putative inhibitor of IRE1 RNase exhibited anti-myeloma activity in xenograft mice, suggesting that the IRE1-\textit{XBP1} pathway is an appealing target for anticancer therapies (47).

Xenograft glioma cells that expressed dominant-negative \textit{IRE1} exhibited reduced proliferation. In this model, wildtype gliomas were characterised by an angiogenic/massive phenotype, whereas tumours that expressed dominant-negative \textit{IRE1} exhibited an avascular/diffuse phenotype, suggesting that IRE1 is required for angiogenesis and functions as a switch between angiogenesis and invasion (48). The requirement for IRE1 in tumour angiogenesis during stress conditions \textit{in vitro} could be attributed to its role in VEGF expression regulation (49). Additionally, the loss of \textit{XBP1} was shown to inhibit both tumour growth and blood vessel formation. However, these effects appeared to be VEGF-independent, indicating that the IRE1-\textit{XBP1}s-VEGF axis only partially regulates the angiogenic functions of IRE1 (50). On the other hand, VEGF induces internalization of the VEGF receptor, which subsequently interacts with IRE1 to enhance \textit{XBP1} splicing (51).
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The role of RIDD and the interactions of IRE1 with several downstream mediators during tumour growth and angiogenesis are not currently understood. Prolonged RIDD activation has been reported to increase apoptosis (40). Activated IRE1 recruits the adaptor protein tumour necrosis factor associated factor-2 (TRAF2) to the ER membrane, which has been reported to further activate c-Jun N-terminal kinase (JNK) (see below), resulting in caspase-12 activation and apoptosis in a mouse model (52). The JNK pathway is a member of the mitogen-activated protein kinase superfamily, which also includes p38 (53), and this activated pathway is involved in ER stress-mediated apoptotic cascades.

XBP1s overexpression in breast cancer cells increased BCL-2 levels after antiestrogen stimulation, thereby suppressing apoptosis (54); however, JNK phosphorylates and paradoxically inhibits BCL-2. Thus, the effects of IRE1 on the BCL-2 family vary according to the output, which is anti-apoptotic when mediated by XBP1 splicing versus proapoptotic when mediated by JNK.

*PERK and protein translation in cancer.* PERK phosphorylates eIF2α, leading to a translation blockade and cap-independent ATF4 translation, as well as nuclear factor erythroid 2-related factor-2 (NRF2), leading to the upregulation of antioxidative enzymes (6) (Figure 2). PERK has been implicated in tumour progression and angiogenesis. PERK inactivation in mouse fibroblasts and human colon cancer cells, using targeted mutagenesis or a dominant-negative PERK, resulted in smaller tumours that demonstrated impaired angiogenic abilities upon grafting into immunodeficient mice (13, 55). PERK deletion in a mammary tumour mouse model was found to modestly increase tumour latency while profoundly inhibiting metastatic spread (16).

Similar observations were made in a colorectal carcinoma xenograft model that expressed a dominant-negative PERK. PERK-knockdown in human esophageal and breast carcinomas resulted in cell-cycle arrest at the G2/M phase (16). This G2/M arrest could likely be attributed to reduced NRF2 activity in these PERK-deficient cells, resulting in ROS accumulation that causes oxidative DNA damage and subsequently triggers cell-cycle arrest via the DNA double strand-break checkpoint (31). Similar to IRE1 deficiency, PERK-deficient tumours exhibited reduced viability and impaired angiogenic ability during hypoxia; these effects were attributed to the losses of phosphorylated eIF2α and ATF4. The requirement for PERK in tumour angiogenesis was further confirmed with a mouse PERK−/− insulinoma model in which PERK−/− tumours exhibited reduced vascularity (56). Thus, both downstream transcription factors of PERK, namely ATF4 and NRF2, contribute to cellular adaptation and tumour promotion.
In contrast to the previous results, p38-induced dormancy in squamous cell carcinoma cells was associated with increased PERK activation. Accordingly, pharmacologically activated PERK was found to induce growth arrest in vitro and to suppress tumour growth in vivo, indicating an additional role for PERK in tumour growth suppression (57). Indeed, eIF2α phosphorylation-induced translational arrest down-regulates cell-cycle regulators such as cyclin D1, resulting in cell-cycle arrest in the G1 phase. Accordingly, a non-phosphorylatable eIF2α mutant was sufficient to drive the malignant transformation of human kidney cells or fibroblasts, and conditional PERK deletion was found to deregulate mammary acinar morphogenesis and to cause hyperplastic growth in vivo (58, 59).

Taken together, activation of the PERK axis induces tumour suppression (by G1/S arrest) and dormancy, whereas inactivation appears to induce paradoxical effects on specific hallmarks of carcinogenesis (22), such as tumourigenesis, angiogenesis and metastasis.

Recently, a context-dependent impact of PERK on cell fate has been indicated. Downstream of PERK, CHOP directly transactivates the growth arrest and DNA damage inducible protein (GADD34). The latter promotes eIF2α dephosphorylation, thereby creating a negative feedback loop that leads to translational recovery (60). Additionally, both ATF4 and CHOP induce protein synthesis (61). This finding could explain the time-dependent balance in protein synthesis. After acute ER stress, protein synthesis is inhibited by eIF2α phosphorylation. However, downstream induction of ATF4, CHOP, and GADD34 leads to protein synthesis recovery. If acute ER stress is addressed, survival is promoted by the restoration of translation. Conversely, if chronic ER stress continues or the acute ER stress was too severe to be addressed during a transient reduction of translation, protein synthesis leads to ROS formation and ultimately triggers apoptosis. Accordingly, salubrinal, an eIF2α dephosphorylation inhibitor, protects cells from ER stress-associated apoptosis (62).

2.4.6. The UPR and Apoptosis: Adaptation or Suicide – A Double-edged Sword

During ER stress, cells either survive by inducing adaptation mechanisms or commit suicide by apoptosis. The intrinsic apoptosis pathway is closely related to factors anchored on the mitochondria. The membrane insertion of pro-apoptotic proteins changes mitochondrial membrane permeability, resulting in cytochrome c release and caspase activation (53, 63).
CHOP: A key mediator of ER stress-induced apoptosis. Notably, CHOP induction strongly correlates with the onset of ER stress-associated apoptosis, and CHOP silencing protects cells (53). However, mouse embryonic fibroblasts (MEFs) derived from CHOP-knockout mice exhibit only partial resistance to ER stress-driven apoptosis, indicating that CHOP is not the only death pathway in this context (64). Precisely how CHOP mediates ER stress-induced apoptosis remains controversial because CHOP regulates numerous genes, the majority of which are involved in hallmarks of cancer, such as cell migration, proliferation, and survival (22, 65).

The down-regulation of anti-apoptotic BCL-2 and the induction of the proapoptotic BCL-2 interacting mediator of cell death (BIM), p53 up-regulated modulator of apoptosis (PUMA) and BCL-2-associated X protein (BAX) are believed to contribute to CHOP-mediated apoptosis (63). In vivo data from breast carcinoma-derived cells corroborate these findings (66).

CHOP transcriptionally induces ERO1α (see above), which promotes disulfide bond formation but also generates hydrogen peroxide leakage into the cytoplasm (60). In vivo, partial ERO1α silencing was shown to protect against ER stress-induced death, and CHOP deficiency suppressed pancreatic β-cell apoptosis, which was associated with decreased ERO1α expression and oxidative stress markers. ERO1α activates the ER calcium channel inositol-1,4,5trisphosphate receptor 1 (IP3R1) (67). Upon release from the ER, calcium triggers apoptosis by activating calcium/calmodulin-dependent kinase II (CaMKII), which subsequently induces four apoptotic pathways. Firstly, CaMKII triggers JNK-mediated Fas antigen induction. Secondly, CaMKII promotes mitochondrial calcium uptake, thereby activating intrinsic apoptosis. Thirdly, CaMKII activates signal transducer and activator of transcription-1 (STAT1), a pro-apoptotic signal transducer (68). Finally, the CHOP-ERO1α-IP3R1-CaMKII axis induces NADPH oxidase subunit 2 and generates ROS to possibly amplify CaMKII activation as part of a positive feedback loop because ROS induces CHOP expression. Surprisingly, NADPH oxidase-induced ROS are also part of a second positive feedback loop that activates dsRNA-dependent protein kinase to subsequently phosphorylate eIF2α, thereby amplifying CHOP expression. For example, CHOP-induced hepatocyte death in a mouse protein-misfolding model was associated with oxidative stress and was relieved by an antioxidant. Because CHOP-induced apoptosis can be blocked by buffering cytosolic calcium, the ERO1α-IP3R1 pathway appears to comprise its main signalling axis (69).
Furthermore, CHOP activity is increased in response to phosphorylation by p38. p38 is a substrate of apoptosis signal-regulating kinase (ASK1, see below), which is recruited to the IRE1-TRAF2 complex upon ER stress. Thus, during prolonged stress, the PERK and IRE1 pathways might converge on CHOP, with IRE1-mediated ASK1 activation potentiating CHOP activity (4).

The JNK pathway in ER stress-mediated apoptosis. Several studies indicate a pivotal role for JNK in the mediation of ER stress-induced apoptosis (70). JNK recruitment by IRE1 is regulated by c-Jun NH2-terminal inhibitory kinase (JIK), which has been reported to interact with both IRE1 and TRAF2. The IRE1-TRAF2 complex then recruits ASK1, causing ASK1 activation and regulating the JNK pathway that leads to cell death. In a mouse HCC model, ASK1 deficiency promoted HCC, whereas the reintroduction of ASK1 suppressed tumour development (71). Furthermore, cells from ASK1-knockout mice were found to be resistant to ER stress-associated apoptosis and exhibited reduced JNK and p38 activity (72). JIK overexpression was shown to promote the interaction between IRE1 and TRAF2 and JNK activation in response to ER stress, whereas the overexpression of an inactive JIK mutant inhibited JNK activation (52). Thus, the IRE1-TRAF2-JIK-ASK1-JNK pathway exerts an opposite effect on cell survival than that of the cytoprotective IRE1-XBP1s pathway. The regulation of these paradoxical IRE1 outputs requires further investigation. As described previously, JNK is a downstream effector of the CHOP-CaMKII pathway. Therefore, in conditions where both proapoptotic IRE1 activation and CHOP expression are prolonged, additive JNK activation might play a crucial role in apoptosis regulation.

Downstream apoptosis-related targets of JNK include antiapoptotic BCL-2, B-cell lymphoma-extra-large (BCLXL) and myeloid cell leukaemia sequence 1 (MCL-1), all of which are inhibited by JNK, and proapoptotic BID and BIM, which are activated by JNK-mediated phosphorylation (63, 73). During non-stress conditions, BIM is sequestered by dynein motor complexes. ER stress increases BIM levels by reducing BIM degradation and by CHOP-mediated gene induction. Phosphorylation by JNK releases BIM from its inhibitory association with the motor complexes, thus permitting its translocation to the mitochondrial outer membrane where it promotes cytochrome c release and caspase activation. Interestingly, a positive feedback loop exists between BIM and caspase-3. Phosphorylated BIM is a caspase-3 target and, once cleaved, becomes a more potent inducer of cytochrome c release (74, 75).
The mitochondria and the BCL-2 family. The proapoptotic BCL-2 family members trigger mitochondrial dysfunction and are sub-divided into the multi-BCL-2 homology (BH) domain proteins, such as BAX and BAK, and the BH3-only proteins, such as BAD. Of the 11 BH3-only protein subfamily members, PUMA, NOXA, BID, HARAKIRI and BIM have been reported to mediate ER stress-induced apoptosis (74, 75).

Additionally, the BCL-2 family also regulates ER stress through physical interactions with certain UPR components. For example, BAX and BAK directly interact with the IRE1 cytosolic domain upon ER stress; this interaction is essential for IRE1 activation (76). In cells that exclusively express ER-localised BAX, BIM and PUMA selectively activate the TRAF2-JNK arm of IRE1 in the absence of XBP1 splicing (77). In BAX/BAK double-knockout mice, ER stress failed to induce XBP1s, IRE1 or JNK. Moreover, BAX/BAK double-knockout MEFs are resistant to apoptosis mediated by various ER stressors, and the reconstitution of BAK expression in these MEFs restored JNK phosphorylation, suggesting a direct connection between the UPR and the apoptotic machinery (76). Thus, BAX and BAK are required for IRE1 signalling, although both are also involved in ER stress-induced apoptosis. This response could represent a switch toward pro-apoptotic signalling by IRE1. The association of IRE1 with BAX and BAK is influenced by BAX inhibitor-1 (BI-1), an ER transmembrane protein. BI-1 directly interacts with the IRE1 cytosolic domain to inhibit its endoribonuclease activity. BI-1-deficient cells were found to exhibit enhanced IRE1 activity and sustained XBP1 splicing, whereas BI-1 overexpression disrupted the interaction between IRE1 and BAX or BAK (78). Similar to ER-localised BAX/BAK oligomers (see below), BI-1 also modulates ER calcium homeostasis by forming a calcium-permeable channel pore (79).

BCL-2, BAX and BAK associate with both mitochondrial and ER membranes. During ER stress, ER-targeted BAX and BAK undergo conformational changes and oligomerisation, which leads to calcium release from the ER to the cytosol to activate m-calpain and, subsequently, procaspase-12 (see below) (81, 82). In contrast, mitochondria-targeted BAK enhances caspase-7 cleavage to create parallel pathways of caspase activation by BAX and BAK (80).
Each branch acts on different levels to tightly modulate the BCL-2 family. During hypoxia, ATF4 induces the BH3only proteins HARAKIRI, PUMA and NOXA following PERK activation (75). Additionally, CHOP transactivates BIM and down-regulates BCL-2 and MCL-1. The less studied ATF6 has been linked to ER stress-induced apoptosis in a myoblast cell line through the indirect inhibition of MCL-1 expression (83). The IRE1 branch can affect BH3only proteins such as PUMA or BID. Functional integration likely occurs because BAX/BAK acts through mitochondrial permeabilisation, a key pro-apoptotic effect of the CHOPERO1α-CaMKII pathway.

**Caspases.** The processing of caspases-2 to -9 and caspase-12 has been observed in various models of ER stress-induced apoptosis (84, 85). In mouse models, caspase-12 was proposed as a key mediator of ER stress-induced apoptosis. Caspase-12 knockout MEFs exhibited partial resistance, specifically against ER stressors. However, another study that used different caspase-12 knockout MEFs did not show any resistance to apoptosis (86). Procaspase-12 is localised on the cytosolic ER surface and is activated by ER stress via IRE1-TRAF2 dependent mechanisms. TRAF2 promotes procaspase-12 clustering at the ER membrane (52). The interaction between TRAF2 and procaspase-12 is inhibited by ER stress, and IRE1 overexpression. Therefore, caspase-12 activation might require for the dissociation of procaspase-12 from TRAF2, which is subsequently recruited to IRE1. Calpains, a family of calcium-dependent proteases, also play a role in caspase-12 activation, and calpain-deficient MEFs exhibit reduced ER stress-mediated caspase-12 activation and apoptosis (85). Therefore, it is plausible that a CHOP-ERO1α-IP3R-calcium-calpain pathway contributes to caspase-12 activation. Surprisingly, human caspase-12 has no similar function because its gene has been disrupted by a frame shift. Instead, human caspase-4 is specifically cleaved under ER stress, suggesting that it might be a functional mouse caspase-12 ortholog. Transmembrane protein 214 (TMEM214) mediates stress-induced apoptosis by acting as an anchor for the ER recruitment and subsequent activation of procaspase-4 (87).

Caspase-7, which also translocates from the cytosol to the cytosolic ER surface in response to ER stress, cleaves procaspase-12. A dominant-negative catalytic caspase-7 mutant was shown to inhibit caspase-12 activation. Caspase-7 is also a downstream executioner of caspase-12, a fact that suggests an amplification loop in the ER stress-induced apoptotic cascade (53).
2.4.7. Future Perspectives and Remaining Conundrums

Recently, ER stress research has received unprecedented attention. A basic PubMed search revealed that more than 830 ER stress investigational studies have so far been published in 2013. However, to integrate these links with hypoxia-inducible factor 1 (HIF1)-VEGF or inflammatory pathways in a comprehensive network, the focus should be placed on elucidating the downstream mediators and crosstalk for all three UPR pathways. The observed paradox of the UPR in cancer (Figure 3) is likely due to functional redundancy and time-dependent outcomes of the UPR, although there are also some methodological issues.

ER stress-induced apoptosis is not completely suppressed when a single UPR effector is experimentally silenced. The fact that CHOP is a transcriptional target of both PERK and IRE, and even ATF6 provides an obvious link among all three branches. One caveat is that the IRE1 and ATF6 branches have weaker activities, compared to the PERK-CHOP branch, during prolonged ER stress (63). Most targets can be regulated separately by each pathway; moreover, each pathway possesses its own transcriptional activity for a certain target that determines its effect on cell fate. Some targets even require the concomitant activation of two pathways, for example, p58IPK requires ATF6/IRE1 cooperation (38). Additionally, a single downstream effector can exhibit different mechanisms of action. For example, the anti-apoptotic mechanisms of GRP78 include the prevention of UPR sensor activation, and the preservation of ER calcium homeostasis and its chaperone activity by limiting misfolded protein aggregation (1, 26).

The majority of studies measured the expression of only two or three ER stress markers such as GRP78 or CHOP; only a minority included target genes from each branch. Future therapeutic targeting of the UPR will likely affect one branch. The pleiotropic effects and acute toxicities of global UPR inducers, including the most commonly used thapsigargin (an ER calcium pump inhibitor) and tunicamycin (an N-linked glycosylation inhibitor), complicate studies that focus on an understanding of how the UPR remodels ER proteostasis in the absence of acute ER stress or how partitioning between ER client protein folding and trafficking versus degradation can be influenced by arm-selective UPR modulation. Moreover, UPR research that includes a variety of acute ER stress inducers introduces difficulties when comparing data from different studies. The recent development of targeted inhibition [e.g. PERK inhibitors (88)] or activation [e.g. PERK activators (89)] approaches, as opposed to the concomitant activation of all three branches, will lead to a new era in UPR research.
The duration and severity of ER stress determine the survival/apoptosis switch. The three branches provide opposing signals, and the timing of their induction shifts the balance between cytoprotection and apoptosis in response to unmitigated ER stress. For example, IRE1 signalling is an early event that is attenuated upon prolonged ER stress (90), and likewise, PERK induces its own de-activation via the upregulation of GADD34 (Figure 2). Both pathways thus contain intrinsic ‘timers’ that likely contribute to cellular life-or-death decisions. Because CHOP mRNA and protein half-lives were found to be short, compared to those of prosurvival UPR outputs such as GRP78, sustained PERK activity (which is primarily responsible for CHOP upregulation) might therefore be necessary to accumulate CHOP levels sufficiently to stimulate the pro-apoptotic BCL-2 family proteins. Additionally, despite ATF4, CHOP, and GADD34 being able to restore protein synthesis, sustained PERK activity results in a protracted translational block that is incompatible with cell survival (38, 61). Similarly, sustained IRE1-mediated mRNA degradation might deplete ER cargo and protein-folding activities (40). Currently, it is unclear how tumour cells adapt to chronic ER stress in vivo. Although the UPR components are clearly activated in several types of tumours, the long-term evolution of this activity is unknown. Therefore, the use of experimental models with which to monitor temporal dynamics is required. For example, under hypoxia, there is a bi-phasic response to eIF2α phosphorylation. Phosphorylation is increased after 8 h but reduced after 24 h (possibly by PERK-ATF4-CHOPGADD34) and is again enhanced after 48 h. Thus, following the initial attenuation in protein translation, there might be a transient period in which additional protein synthesis is permitted before a more permanent reduction occurs (15). Consequently, the effects of future drug interventions might be time-dependent, and whether cancer incidence might be reduced through the enhancement of protein-folding capacities during carcinogen exposure remains unknown. For example, the development of molecules that protect the liver by reducing alcohol-induced ER stress might dramatically reduce the incidence of HCC because chronic alcohol use increases HCC risk (91).

Deciphering this paradox could permit for the development of novel therapeutic modalities. In cancer, the UPR could be targeted to promote apoptosis by inhibiting UPR components and thus abrogating cellular adaptation (e.g. the use of versipelostatin, a repressor of GRP78 expression) or overloading the UPR (e.g. the use of proteasomal inhibitors). Overall, an ideal approach would integrate both targets without any toxicity.
In principle, UPR inhibitors should specifically target the tumour tissue. However, certain normal cell types place high demands on ER function, such as antibody-producing B-cells or insulin-secreting β-cells, and the potential toxicities against these cell types would require close monitoring during future drug discovery efforts (92). Notably, tissue-specific UPR patterns might help to differentiate target tissues. However, current molecular insights into the adaptation/apoptosis switch during ER stress are insufficient, and UPR drugs might block ER stress-mediated apoptosis and might unintentionally promote tumour progression. In general, protein kinases such as PERK represent favourable targets for the development of small-molecule inhibitors. However, as described above, PERK exhibits both pro- and anti-tumour properties. PERK-targeted therapies might facilitate the proliferation of dormant tumour cells or might drive cancer cells into dormancy, thereby protecting them from chemotherapy. Additionally, the inhibition of one branch might result in altered signalling through the other branches. Indeed, HEK293 cells that overexpressed a kinase-dead PERK mutant were found to exhibit increased XBP1 splicing and ATF6 activation in response to ER stress. Despite delayed dynamics, these cells still induced CHOP expression, which partially accounts for the increased susceptibility of these cells to ER stress-induced apoptosis (93).

In conclusion, the ability of the UPR to regulate cell fate has been highlighted as a primary pathophysiology research focus and represents a potential cancer therapeutic axis. However, its paradoxical effects on survival and proliferation of neoplastic and endothelial cells complicate the clinical applications of UPR modulators. This paradox is primarily due to our incomplete understanding of redundancy, the opposing effects of the separate outputs of each pathway, the interplay between the UPR and other pathways and the temporal UPR dynamics in cancer, as well as other confounding factors, including the absence of a standardized definition of ER stress, and a lack of branch-specific research.
2.4.8. References


Chapter 1


Chapter 1


2.5. Endoplasmic reticulum stress in hepatocellular carcinoma

Rapid tumour growth creates hypoxia, glucose deprivation and oxidative stress, activating the UPR in various solid tumour types [33]. In human HCC, Shuda et al. (2003) showed that elevated expression of GRP78 and ATF6 and splicing of XBP1 mRNA occurred in HCC tissues with increased histological grading [56]. Higher accumulation of the GRP78 product in the cytoplasm, concomitantly with marked nuclear localization of the activated ATF6 fragment, was observed in moderately to poorly differentiated HCC tissues. In addition, Al-Rawashdeh et al. (2010) showed that 100% of the 86 investigated human HCC samples exhibited increased expression of ER stress marker GRP78 [57]. Proteomic profiling of 146 HCC samples revealed that in response to the stressful microenvironment, tumour cells strived to increase the expression of chaperone proteins including GRP78 for cytoprotective function [58]. Moreover, upregulation of GRP78 was significantly associated with tumour venous infiltration. Also CHOP expression was shown to be upregulated in human HCC [59]. In contrast, unaffected liver tissue from HCC patients showed significantly less CHOP staining and control liver samples showed less still. In summary, the UPR was shown to be activated in human HCC [56]–[58]. However, the dynamics and the role of these pathways in hepatocarcinogenesis is currently unknown.
3. **Autophagy**

3.1. **Autophagy: a lysosomal degradation pathway**

Autophagy or "self-eating" digests proteins and organelles to reuse. Autophagy is an evolutionary highly conserved mechanism involved in cellular homeostasis under basal conditions and is upregulated during cellular stress [60]. During autophagy, cytoplasmic content is delivered to lysosomes for degradation to macronutrients and energy. As shown in Figure 7, there are three isoforms: 1) micro-autophagy, i.e. direct engulfment of cytoplasmic content by a lysosome, 2) chaperone-mediated autophagy (CMA), i.e. delivery of cytoplasmic proteins by specific chaperones to the lysosome and 3) macro-autophagy, i.e. isolation of cytoplasmic material in a double membrane structure, which will subsequently fuse with the lysosome [61], [62]. **Macro-autophagy** is thought to play the most significant role under pathophysiological conditions and we refer to this isoform through this thesis [62]. Autophagy is a context-dependent tumour-suppressing mechanism that can also promote tumour cell survival upon stress and treatment resistance [63]. Because of this ambiguity, autophagy is considered as a double-edged sword, making therapeutic approaches highly challenging.

![Figure 7. The three forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy.](image)

Macroautophagy starts with the de novo formation of a cup-shaped double membrane that engulfs a portion of cytoplasm. Microautophagy involves the engulfment of cytoplasm instantly at the lysosomal membrane by invagination, protrusion and separation. Chaperone-mediated autophagy is a process of direct transport of unfolded proteins via the lysosomal chaperonin hsc70 and LAMP-2A. All forms of autophagy subsequently lead to the degradation of intra-autophagosomal components by lysosomal hydrolases. PE, phosphatidylethanolamine. [64]
3.2. Autophagy and endoplasmic reticulum stress: integration of two double-edged swords

ER stress induces autophagy directly through upregulation of GRP78 and through mechanisms downstream of the three UPR signal transducers [65]. A critical role for GRP78 in autophagy was demonstrated with GRP78 knockdown in normal and cancer cells, which prevented autophagosome formation in response to starvation or in response to tunicamycin, an inhibitor of N-linked glycosylation required for proper protein folding. The massively dilated and disrupted ER and deficient autophagosome formation induced by GRP78 knockdown were both alleviated by simultaneous knockdown of the IRE1-regulated XBP1 [66], suggesting that intact ER is maintained by and required for autophagy.

The link between the IRE1 branch and autophagy is mediated by IRE1-activated JNK. A study conducted in neuroblastoma cells using siRNA knockdown of IRE1, PERK or ATF6 or using a JNK inhibitor, demonstrated that ER stress upregulated autophagy through a mechanism dependent on IRE1, but independently of PERK and ATF6 [67]. ER stress was induced with ER stress inducers tunicamycin or thapsigargin, an agent that blocks ER Ca$^{2+}$ uptake by inhibiting ER Ca$^{2+}$-ATPase. In this model, autophagy protected against cell death as demonstrated by the increased cell death when autophagy was inhibited by chemical (3-methyladenine) or genetic manipulation (ATG7 siRNA) and suppressed cell death when autophagy was induced by chemical stimulation by the mTOR inhibitor rapamycin. The mechanism of autophagy induction downstream of JNK is a result of JNK phosphorylation of Bcl-2, which releases Bcl-2 repression of the autophagy factor Beclin1 [68].

The link between the PERK arm and autophagy is mediated by ATF4-driven upregulation of the ATG genes [69]. Treatment of embryonic carcinoma cells with misfolded polyglutamine repeats caused accumulation of polyubiquitinated protein aggregates and induced LC3 conversion through a PERK-dependent mechanism and resulting in autophagic elimination of the aggregates [70]. When proteasomal inhibitor bortezomib was used in pancreatic cancer cells, phospho-eIF2α led to ATF4-driven transcription of ATG5 and ATG7 [71].

All three of the UPR arms were involved in the induction of autophagy in breast cancer cells caused by accumulation of sphingosine-1-phosphate [72]. The resulting autophagy was prevented by silencing of PERK, IRE1 or ATF6. In conclusion, multiple levels of integration between autophagy and the UPR can maintain cellular homeostasis or default into apoptosis. Upon stress, the ultimate consequence of cell survival or apoptosis depends on the balance of events linked at multiple network connections between the UPR and autophagy.
Chapter 1

4. Angiogenesis and endoplasmic reticulum stress

4.1. Angiogenesis

Angiogenesis refers to the sprouting, migration and remodelling of existing blood vessels and plays a crucial role in several physiological processes as well as in a number of diseases including liver diseases and cancer [73], [74]. Endothelial cells exhibit the ability to divide rapidly in response to physiological stimuli, such as hypoxia, inflammation and shear stress. However, when these stimuli become too pronounced, angiogenesis becomes a key pathophysiological process, for example, in tumour growth. HCC cells exhibit rapid growth and consequently require high oxygen and nutrient supply [75]. Hence, these tumour cells induce the formation of new blood vessels to counteract hypoxia. As mentioned previously, HCC lesions are characterised by arterial hypervascularity to provide the tumour with oxygenated blood [1]. However, these neo-vessels are marked by a disorganised vasculature, consisting of leaky and tortuous vessels, resulting in a chaotic and dysfunctional blood flow [75].

Angiogenesis is tightly regulated by pro- and anti-angiogenic factors that are produced in steady-state. Pro-angiogenic factors such as VEGF, fibroblast growth factor (FGF), PDGF and interleukin-8 (IL-8), bind to their receptors on endothelial cells causing proliferation, release of matrix metalloproteinases and migration towards the angiogenic stimuli, and finally inducing the formation of new blood vessels [76]. The best characterized pathway regulating VEGF is the hypoxia inducible factor (HIF) pathway activated by hypoxia [77]. HIF1 and HIF2 are heterodimeric transcription factors composed of an α and β subunit. The β subunit is constitutively expressed but the α subunit is labile in an oxygen rich setting. When hypoxia occurs the α subunit is stabilized, thereby activating the HIF-complex, which induces transcription of pro-angiogenic factors such as VEGF and leads to angiogenesis restoring the oxygen levels [77], [78].
4.2. Angiogenesis and endoplasmic reticulum stress

It is well established that hypoxia and glucose deprivation induce angiogenesis in tumours, triggering the growth of new capillaries from pre-existing vessels [79]. Importantly, ER stress was shown to stimulate angiogenesis in tumour cells, where the UPR shifts the balance from anti-angiogenic to pro-angiogenic events through modulating the expression of different factors including VEGF, FGF, interleukin (IL)-1β, IL-6 and IL-8 [79], [80]. In fact, hypoxia causes upregulation of VEGF through the HIF pathway as well as via the UPR [73]. Glucose or amino acid deprivation, as well as chemical ER stress inducers thapsigargin and tunicamycin also induce VEGF upregulation, but only via the UPR [81]. The PERK target ATF4 directly binds to the VEGF promoter [73]. The IRE1/XBP1 axis also increases angiogenesis in cancer models, including VEGF and IL-6 expression [73]. Moreover, GRP78 expression accelerates cancer progression also through induction of tumour angiogenesis [82]. Recently, Karali et al. (2014) revealed that VEGF signalling engages UPR sensors in an unconventional manner independent of ER stress, mediated by mTOR signalling, to promote endothelial cell survival and angiogenesis [81]. This finding extends the role of the UPR sensors beyond adaptation to ER stress.

4.3. The placental growth factor

Another member of the VEGF family which contributes to pathological angiogenesis is the placental growth factor (PlGF) [83], [84]. The PlGF gene is highly expressed in the placenta at all stages of human gestation. Unlike VEGF, the role of PlGF during embryogenesis and physiological angiogenesis is redundant. PlGF-deficient mice are viable and fertile and do not have any visible abnormalities [85]. Recently, HIF-1α and chromatin remodelling were shown to be involved in the hypoxia-mediated upregulation of PlGF [86]. Studies in transgenic mice revealed that the angiogenic activity of PlGF is restricted to pathological conditions. Loss of PlGF impairs angiogenesis in wounded skin, ischemic retina, limb or heart and in cancer, whereas administration of recombinant PlGF promotes collateral vessel growth in models of limb and myocardial ischemia [85], [87].
The effect of PlGF is mediated by binding to VEGFR-1 and neuropilin-1 [88]. VEGFR1 is minimally expressed in adult quiescent vessels but membranous localization is upregulated during pathological conditions, stimulating PlGF-dependent angiogenesis. Different direct and indirect effects after PlGF-receptor interaction can lead to increased angiogenesis [89]. PlGF has been proposed to stimulate angiogenesis by displacing VEGF from VEGFR-1, thereby increasing the fraction of VEGF available to activate its main receptor, VEGFR-2. Activation of VEGFR-1 can also induce a crosstalk with VEGFR-2 resulting in transphosphorylation of VEGFR-2, which becomes more active in signalling VEGF-driven angiogenesis. Alternatively, PlGF might stimulate angiogenesis by direct signalling via VEGFR-1 or by forming heterodimers with VEGF [89].

As the role of PlGF is mainly restricted to pathological conditions, blocking PlGF signalling may be an attractive target to avoid potential side-effects related to VEGF inhibition such as thrombosis, hypertension and proteinuria [90]. In contrast to VEGF inhibitors, a monoclonal anti-PlGF antibody (aPlGF) has been shown to reduce pathological angiogenesis in various spontaneous cancer and other disease models without affecting healthy blood vessels, resulting in no major side effects [91]–[93]. We previously showed that PlGF inhibition exerts antitumour effects and induces vessel normalization in experimental HCC (Fig. 8) [91], [93]. However, the exact role of PlGF in tumoural UPR activation remains elusive.
Figure 8. PlGF inhibition induces vessel normalization and antitumour effects in experimental HCC. (A) Mean tumour burden is reduced in placental growth factor knockout (PlGFKO) compared to wild type mice following 20, 25 and 30 weeks of diethylnitrosamine (DEN). *p<0.05, **p<0.01, ***p<0.001. (B) Scanning electron microscope images showing the microvasculature of IgG (chaotic) and anti-PlGF (αPlGF) (normalised) treated peritumoural vessels. Adapted from [91].
Chapter 1

5. Artemisinins, cancer and endoplasmic reticulum stress

5.1. Artemisinins

Artemisinins are a family of sesquiterpene trioxane lactone anti-malarial agents originally derived from sweet wormwood Artemisia annua L. [94]. With its established safety record in millions of malarial patients, Artemisinins are also being investigated in cancer [95]. The active moiety is dihydroartemisinin (DHA), which is the reduced form of Artemisinin. Since DHA has unfavourable thermal stability, it is more convenient for pharmaceutical applications to use DHA prodrugs, including the stable succinate-ester derivative, Artesunate [96]. The latter confers substantial water-solubility and high oral bioavailability, resulting in a more favourable pharmacological profile. Artesunate is recommended by the World Health Organization in preference to quinidine for the treatment of malaria and has been used worldwide for many years. In 2007, the Food and Drug Administration made intravenous Artesunate available for treatment of malaria in the United States.

5.2. Artesunate and cancer: old drug, new tricks?

Artesunate has demonstrated remarkable cytotoxicity against a wide range of human tumour cell lines and showed anticancer activity in different animal models, including HCC [97], [98]. The multi-faceted mechanism of action of Artesunate involves the formation of free radicals via cleavage of the endoperoxide bond in its structure, protein alkylation, induction of apoptosis, angiogenesis inhibition, cell cycle regulation and abrogating cancer invasion and metastasis [99]. Importantly, the anti-cancer activity of DHA is also associated with induction of iron-dependent ER stress [100]. These preclinical results led to the development of clinical trials with Artesunate in cervical [101] and lung [102] cancer, which showed promising results.
6. Experimental mouse models for hepatocellular carcinoma

6.1. Carcinogen-induced mouse model

When selecting a model for HCC research, one must first understand the limitations and advantages that the specific model possesses. The advantage of chemically-induced models is the similarity with the injury-fibrosis-malignancy cycle observed in humans [103]. Diethylnitrosamine (DEN) is often used as a carcinogen and the target organ in which DEN induces tumours is species-dependent. Mice mostly develop liver tumours [104]. The carcinogenic capacity of DEN is situated in its capability of alkylating DNA-structures. In the first step DEN is hydroxylated to hydroxynitrosamine. This bioactivation step is oxygen- and NADPH-dependent and mediated by cytochrome P450, an enzyme that has its highest activity in the centrilobular hepatocytes [105]. After cleavage of acetaldehyde, an electrophilic ethylidiazonium ion is formed and causes DNA-damage by reacting with nucleophiles such as DNA-bases. Furthermore, oxidative stress caused by DEN can contribute to the hepatocarcinogenesis [106]. Reactive oxygen species (ROS) generated by cytochrome P450 induce oxidative stress due to the formation of hydrogen peroxide and superoxide anions [106]. Production of ROS is known to cause DNA, protein and lipid damage; consequently oxidative stress has been known to play a significant role in DEN-elicited carcinogenesis.

Chronic DEN exposure for 25 weeks, in contrast to single DEN injection, induces small cell dysplasia, ductular hyperplasia and readily distinguishable HCC nodules occurring in a background of liver inflammation and mild fibrosis [104]. Additionally, we reported (YP. Vandewynckel, R. De Rycke, E. Bogaerts, H. Van Vlierberghe. Intestinal metaplasia in an orthotopic mouse model for hepatocellular carcinoma. Dig Liver Dis. 2014;46,e17) the rare occurrence of intestinal metaplastic cells in the liver parenchyma of DEN-treated mice. Intestinal metaplasia is the transformation of epithelium, usually of the stomach or the oesophagus but here of the hepatocytes, to an intestinal type. The existence of this phenotype is evidence of the multipotency of hepatic stem/progenitor cells involved in the hepatic healing response to DEN.

Limitations of this model include the high variability in tumour burden and high mortality (i.e. mean survival of 70% in our hands). In conclusion, DEN has been shown to induce tumours which are histologically similar to human HCC with poor prognosis.
6.2. Xenograft mouse model

In xenograft models, injecting human cancer cells from a cell culture in immune-deficient mice creates the tumours. Athymic (nude) or severe combined immune deficient mice are often used as hosts [103]. In the ectopic xenograft model, human tumour cells are subcutaneously injected in these mice and tumour volume is followed over time. The advantages of xenograft mouse models is the short time span needed for tumour development, low mortality and the low variability in tumour burden. However, the histological resemblance between xenograft and human tumours is rather poor [107].
7. Reference list


Chapter 1


Chapter 2

Aims of the work
1. Aims of the work

A role for ER stress signalling in cancer was initially proposed in 1996, introducing the concept that it could either be beneficial for tumour growth or play a guardian role to prevent cell transformation [108], [109]. Accumulating evidence in the course of the last decade indicated that all branches of the UPR contribute to the tumourigenesis, affecting diverse aspects of the disease including angiogenesis, cell differentiation, cell migration, tumour growth and the inflammatory microenvironment [41]. Also in human HCC, the UPR was shown to be activated [56].

- First, we aimed to determine the presence of ER stress and the temporal dynamics of UPR activation in the well-known diethylnitrosamine-induced mouse model for HCC. Additionally, we aimed to validate innovative imaging techniques for the functional assessment of experimental HCC burden (in collaboration with Infinity Lab, Ghent University).

- Secondly, we aimed to explore the effects of rational UPR modulation on HCC cell viability and proliferation in vitro and in the mouse model.

These objectives were assessed in the following manuscript:

- Thirdly, we assessed the impact of ER stress in tumour initiation and progression by applying tauroursodeoxycholic acid (TUDCA), a bile acid with chaperone properties reducing ER stress, in the diethylnitrosamine-induced HCC model in preventive and therapeutic settings.
This objective was assessed in the following manuscript:

- Finally, we assessed the effect of established or potential HCC treatments possibly modulating the UPR, including sorafenib, artemesunate, PlGF inhibition and proteasome inhibition, on the tumour growth and UPR signature in HCC and subsequently aimed to enhance the antitumour efficacy of these treatments by UPR modulation subverting ER stress towards apoptosis of the tumour cells.

These objectives were assessed in the following manuscripts:


Chapter 3

Results
1. Identification and modulation of the ER stress phenotype in experimental hepatocellular carcinoma

1.1. Modulation of the unfolded protein response impedes tumor cell adaptation to proteotoxic stress: a PERK for hepatocellular carcinoma therapy

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Modulation of the unfolded protein response impedes tumor cell adaptation to proteotoxic stress: a PERK for hepatocellular carcinoma therapy

1.1.1. Abstract

Background

Functional disturbances of the endoplasmic reticulum (ER) lead to activation of the unfolded protein response (UPR), which is involved in the consecutive steps of carcinogenesis. In human hepatocellular carcinoma (HCC), the UPR is shown to be activated; however, little is known about the UPR kinetics and effects of UPR modulation in HCC.

Methods

We sequentially monitored the UPR over time in an orthotopic mouse model for HCC and explored the effects of UPR modulation on cell viability and proliferation in vitro and in the mouse model.

Results

The expression of ER-resident chaperones peaked during tumor initiation and increased further during tumor progression, predominantly within the nodules. A peak in Ire1 signaling was observed during tumor initiation. The Perk pathway was activated during tumor progression, and the proapoptotic target Chop was upregulated from week 5 and continued to rise, especially in the tumors. The Atf6 pathway was modestly activated only after tumor initiation. Consistent with the UPR activation, electron microscopy demonstrated ER expansion and reorganization in HCC cells in vivo. Strikingly, under ER stress or hypoxia, the Perk inhibitor and not the Ire1 inhibitor reduced cell viability and proliferation via escalating proteotoxic stress in vitro. Notably, the Perk inhibitor significantly decreased tumor burden in the mouse model.

Conclusion

We provide the first evaluation of the UPR dynamics in a long-term cancer model and identified a small molecule inhibitor of Perk as a promising strategy for HCC therapy.
### 1.1.2. Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GRP78</td>
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</tr>
<tr>
<td>GRP94</td>
<td>Glucose-regulated protein, 94 kDa</td>
</tr>
<tr>
<td>PDIA4</td>
<td>Protein disulfide-isomerase A4</td>
</tr>
<tr>
<td>CANX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated protein degradation</td>
</tr>
<tr>
<td>EDEM1</td>
<td>Degradation-enhancing a-mannosidase-like protein</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA-dependent protein kinase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
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<td>Inositol requiring enzyme 1</td>
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<td>Activating transcription factor 6</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>W</td>
<td>Week</td>
</tr>
<tr>
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</tr>
<tr>
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1.1.3. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide [1]. Conventional chemotherapy is ineffective, and targeted therapy for advanced HCC with sorafenib shows only a limited survival benefit [2]. The endoplasmic reticulum (ER) consists of a membranous network in which proteins are synthesized, posttranslationally modified and folded. Therefore, the ER lumen houses a large array of chaperones, including glucose-regulated protein-78 (GRP78) and -94 (GRP94), and protein disulfide-isomerase A4 (PDIA4), and contains stringent quality-control systems that selectively extract terminally misfolded proteins for degradation, a process known as ER-associated protein degradation (ERAD), which is accelerated by ER degradation-enhancing amannosidase-like 1 (EDEM1) [3]. Several perturbations in protein folding lead to the accumulation of unfolded proteins that trigger the unfolded protein response (UPR). The UPR engages a transcriptional response involved in protein quality control, ERAD, redox homeostasis and ER expansion. Paradoxically, the UPR also coordinates proapoptotic responses to ER stress [3, 4].

Three major ER stress sensors have been identified: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) [3]. Upon irremediable ER stress, the effect of ATF6 on cell fate is primarily cytoprotective, whereas the effect of IRE1 and PERK is presumed to be both proadaptive and proapoptotic [4, 5].

Following the release of GRP78, PERK phosphorylates eukaryotic initiation factor 2a (eIF2a), leading to the attenuation of global translation. However, the translation of certain transcripts, such as activating transcription factor 4 (ATF4), is favored. ATF4 induces genes involved in protein quality control, amino acid biosynthesis and the induction of apoptosis via CCAAT/enhancer-binding protein homologous protein (CHOP) [3]. PERK-regulated nuclear factor-(erythroid-derived-2)-like-2 (NRF2) promotes cell survival via antioxidant enzymes, such as glutamate-cysteine ligase, catalytic (GCLC) and glutathione peroxidase-3 (GPX3) [6]. IRE1 activation results in XBP1 mRNA splicing to generate a more active spliced XBP1 (XBP1s), which induces genes involved in protein folding, such as ERDJ4 and CANX, ERAD and redox homeostasis [7].
ATF6 is mobilized to the Golgi where it is cleaved, releasing a transcriptionally active fragment, which in turn induces the expression of chaperones, such as PDIA4 and endoplasmic oxidoreductin-1-like protein (ERO1L), unspliced XBP1 (XBP1u) and ERAD components, such as homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 (HERPUD1) [3, 7]. Although each UPR pathway targets a specific set of genes, certain targets require the concomitant activation of two pathways, e.g., P58IPK and EDEM1 transcription require the cooperation of ATF6 and XBP1s [7].

Rapid tumor growth creates hypoxia, glucose deprivation and oxidative stress, activating the UPR in various solid tumor types [3]. In human HCC, elevated GRP78, ATF6 and IRE1 activation was observed [8–10]. The UPR demonstrates a specific time-dependent pattern of activation that determines its cytoprotective versus proapoptotic outcome [11]. However, these alterations have only been investigated in vitro during acute ER stress in a maximum time period of 48 h [3, 11], and it is unknown how tumor cells adapt to chronic ER stress in vivo. In the present study, we monitored the kinetics of the UPR, a potential tumoral Achilles’ heel, in an orthotopic mouse model for hepatocarcinogenesis [12, 13]. Our data showed IRE1 signaling during tumor initiation and robust PERK activation once the tumors had been established. Furthermore, we explored the therapeutic potential of UPR modulation in vitro and in vivo. We observed that in contrast to the dogma stating that PERK induces apoptosis via CHOP accumulation, a small molecule inhibitor of PERK actually reduces HCC burden via proteotoxicity.
1.1.4. Materials and methods

Cell culture
HepG2, BWTG3 and Hepa1-6 (ATCC, Manassas, VA, USA) cells were cultured with DMEM supplemented with 10% fetal bovine serum (Life Technologies, Ghent, Belgium). Cells were incubated for 48 h with tunicamycin (1.25 µg/ml), a PERK inhibitor (0.3 µM; GSK2656157, NoVi Biotechnology, Shandong, China), an IRE1 inhibitor (8 µM; 4µ8C, Calbiochem, Cambridge, MA, USA), salubrinal (50 µM; Tocris, Bristol, UK), tauroursodeoxycholic acid (1 µM), cycloheximide (5 µM), ascorbic acid (50 µM) or pretreated with N-acetylcysteine (2 h; 5 µM), SP600125 and SB203580 (30 min; 10 µM), and compared to equal volumes of solvent in normoxic and hypoxic (1% oxygen; AnaeroGen, Oxoid, Hampshire, UK) conditions. All reagents were from Sigma (Diegem, Belgium) unless stated otherwise. Each experiment was performed in quadruplicate.

Animals
Wild-type 129S2/SvPasCrl mice (Charles River, Brussels, Belgium) were maintained as previously described [12]. Five-week-old males received weekly intraperitoneal saline or diethylnitrosamine (DEN; 35 mg/kg). Every 5 weeks (W) until W30 (12 groups with n = 12), a group of mice was euthanized. The mice were killed 1 week after the last DEN injection. By macroscopic and microscopic assessments, three liver tissue areas, non-HCC (no nodules in the close proximity), surrounding (perinodular ring of altered hepatic tissue) and HCC (nodules), were isolated by microdissection (Carl Zeiss, Bernreid, Germany) from W25 because at this time, nodules could be clearly distinguished from the surrounding tissue. The number of tumors with a minimum diameter of 2 mm was counted. In addition, 30 µg of tunicamycin was injected intraperitoneally in four untreated 30-week-old male mice 72 h before sacrifice. After randomizing mice treated for 25 W with DEN or saline, GSK2656157 (100 mg/kg bid) was administered intraperitoneally for 4 W and compared with the vehicle (4 groups with n = 12).
Blood was collected from the ophthalmic artery. All organs were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin or snap frozen in liquid nitrogen. Hematoxylin & eosin and reticulin stainings were performed to assess the tumor burden as shown by the mean total tumor surface of six slides per liver and blindly evaluated by two independent observers. Interobserver reliability was substantial (intraclass correlation coefficient = 0.74). Mean intercapillary distance was measured on CD105-stained slides as previously described [12]. Sirius Red staining enabled fibrosis assessment by Metavir scoring. Serum alanine aminotransferase and glycemia were measured at Ghent University Hospital. All protocols were approved by the Ethics Committee of experimental animals, Ghent University (ECD 11/52).

Detailed information regarding RNA extraction, quantitative real-time PCR, Western blot analysis, immunohistochemistry, TUNEL immunofluorescence, WST-1 and caspase-3 activity assays, electron microscopy and positron emission tomography is provided in the Supplementary Materials and Methods.

Statistics
Statistical analysis was performed using SPSS 21 (SPSS, Chicago, IL, USA). Data are presented as the mean ± SD or as the fold change relative to the expression in the controls. Normally distributed data were subjected to unpaired Student’s t test. Multiple groups were compared by one-way ANOVA with Bonferroni correction. Non-normally distributed data were tested using the Mann-Whitney U test. The Friedman test with Bonferroni correction was applied to compare non-HCC, surrounding and tumor tissues of the same liver. The chi-squared test was used to compare mortality. Reported p values were two sided and considered significant when lower than 0.05.
1.1.5. Results

Kinetics of chaperone expression in the HCC model

Every 5 weeks (W) after DEN administration, mice were killed for the analysis of tumor progression and the expression of the (co-)chaperones Grp78, Grp94 and P58\textsuperscript{IPK} (Fig. 1a). At W25, tumor nodules were observed in a background of fibrosis (Fig. S1). The expression of Grp78 was upregulated at W10 (mRNA: p<0.05; protein: Fig. 1b) but reduced again at W15 (mRNA: p<0.05), and once tumors were established, Grp78 mRNA was elevated in the nodules compared to the surrounding (p<0.05) and non-HCC tissue (p<0.01). By immunohistochemistry, we demonstrated an inhomogeneous pattern of Grp78-positive HCC cells within the nodules, but only a few Grp78-positive cells in the surrounding tissue (Fig. 1c). Grp94 mRNA followed a similar temporal pattern, i.e., increased from W25 only in the surrounding and tumor tissue (p<0.05, Fig. 1a). Accordingly, in addition to a tendency to increase at W10, co-chaperone P58\textsuperscript{IPK} exhibited upregulation in the surrounding tissue and nodules from W25 (Fig. 1a).

The Ire1 pathway is activated before tumor promotion

At W10, phospho-Ire1 levels (Fig. 1b), detected by the use of a phosphate-binding tag, and Xbp1 splicing (p<0.05, Fig. 1d) were increased. Two targets of Xbp1s (7), Canx mRNA and Erdj4 mRNA showed a similar evolution until W20, but in contrast to the Xbp1 splicing, they continued to rise in the tumor tissue (p<0.05, Fig. 1d). Additionally, Edem1 mRNA peaked at W5 (p<0.05) but not during tumor growth. Although splicing activity was not significantly altered in the DEN- compared with saline-treated mice at W30 (Fig. 1d), phospho-Ire1 levels were increased in the tumors (Fig. 1b), suggesting additional regulation of the Ire1 endoribonuclease, possibly by reduced oligomerization [3, 14].

The Perk pathway is robustly activated in HCC

At W25, during tumor progression, Atf4 mRNA upregulation was observed and limited to the nodules (Fig. 2a). At W30, expression expanded to the surrounding tissues (Fig. 2a, b).
Phosphorylation of eIf2a was increased in both surrounding and tumor tissue at W30 (Fig. 2b). Immunostaining for phospho-eIf2a showed a diffuse distribution in the surrounding tissue, intensifying toward the core of the nodules at W30. The expression of Chop, a presumed proapoptotic target of Perk [4], was increased from the beginning, then stable between W5 and W20 and from W25 continued to increase. Although expression was higher in HCC compared to non-HCC tissue (p<0.05), both Chop mRNA (Fig. 2a) and protein (Fig. 1b, 2b) levels were increased in all three isolated areas. Furthermore, caspase-3 activity, an indicator of apoptosis, was increased at W30 in the surrounding tissue and nodules (p<0.01, Fig. 2c). Growth arrest and DNA damage-inducible protein (Gadd34) demonstrated a similar pattern as Atf4, with a marked increase from W25, especially in the nodules (p<0.05; Fig. 2a, b), suggesting Perk activation in HCC. Concerning the Perk/Nrf2 axis, we observed the upregulation of Gpx3 mRNA at W5, W10 and W20, and Gclc mRNA showed a tendency to upregulation at these time points (Fig. S2). Surprisingly, in the tumors both these transcripts showed a tendency to downregulation compared to the non-tumor liver.

**The Atf6 pathway is modestly activated after tumor initiation**

To examine the Atf6 pathway, Pdia4, Herpud1 and Ero1L expressions were monitored [7]. The expression of Pdia4 mRNA (p<0.05; Fig. 2d) and protein (Fig. 1b) was modestly increased from W30 in the tumors compared with the surrounding tissue. In accordance, Ero1L and Herpud1 mRNA was upregulated in the nodules from W30 compared with saline-treated livers (p<0.05; Fig. 2d).

**Murine HCC cells exhibit ultrastructural hallmarks of ER stress**

Electron microscopy of the tumors in the mouse model at W30 revealed an extensive expanded ribosome-bound ER in a lamellar pattern (Fig. 3a, middle panel) in the HCC cells compared with the ER in the hepatocytes of saline-treated mice (Fig. 3a, left panel). The liver of mice treated with the ER stress inducer tunicamycin showed extremely dilated ER in the hepatocytes (Fig. 3a, right panel).
Interestingly, some HCC cells demonstrated a remarkable ER reorganization with disruption of ribosome-bound ER and extension of smooth ER (Fig. 3b). These observations provide structural evidence for the hypothesis that DEN-induced hepatocarcinogenesis leads to ER stress. Consistent with histological Metavir scoring of fibrosis (Fig. S1C), we observed the increased presence of collagen fibers in the extracellular matrix (data not shown).

**PERK inhibition but not IRE1 inhibition diminishes the viability and proliferation of HCC cells under stressed conditions**

To address whether interfering with the UPR affects tumor growth, we first validated the effect of the PERK inhibitor (PERKi) and IRE1 inhibitor on HepG2 cells under hypoxia and in the presence of tunicamycin-induced ER stress. The expression of ER stress markers was induced by tunicamycin and hypoxia (Fig. 4b).

PERKi induced no alterations in basal XBP1 splicing or on the basal expression of PERK targets (Fig. 4a, b). However, under hypoxia or ER stress, PERKi diminished the expression of chaperones GRP78 and PDIA4, the adaptive factor ATF4 and the proapoptotic factor CHOP and elevated IRE1 splicing activity (both p<0.05) compared with vehicle-treated cells (Fig. 4a, b). Under these conditions, PERKi decreased eIF2a phosphorylation, suggesting the unblocking of protein synthesis. Indeed, PERKi restored the tunicamycin-attenuated protein synthesis rate (Fig. S3A). The IRE1 inhibitor abolished basal and induced XBP1 splicing (p<0.001; Fig. 4a) and attenuated the induction of PDIA4 and GRP78, although to a lesser extent than in the presence of PERKi. ATF4 and CHOP were unchanged at the mRNA level but slightly increased at the protein level. We next defined the effect of these small molecules on the cell viability and proliferation rate in different HCC cell lines. Here, we also tested salubrinal, which is known to prevent eIF2a dephosphorylation, thus prolonging PERK activation.

Neither hypoxia nor tunicamycin affected cell viability (Fig. 4c), and under normal conditions, UPR modulation did not alter cell viability in HepG2 cells. However, following ER stress induction, PERKi, but not the IRE1 inhibitor, strikingly reduced cell viability (p<0.001), while salubrinal increased cell viability (p<0.05). PERKi also impaired cell viability under hypoxia (p<0.01). Accordingly, PERKi elevated caspase-3 activity under these stressed conditions (p<0.001, Fig. 4d).
To further confirm the potential of PERKi-induced cell death, cell proliferation was assessed by the incorporation of the thymidine analog bromodeoxyuridine into DNA. Under ER stress, PERKi (p<0.01) and the IRE1 inhibitor (p<0.05) reduced the cell proliferation rate. Hypoxia increased the proliferation rate (p<0.001; Fig. S3B), and only PERKi was able to temper the hypoxia-stimulated proliferation (p<0.001). Viability experiments were repeated in BWTG3 and Hepa1-6 cells with comparable results (Fig. S3C). These data suggest that PERKi is able to suppress HCC cell growth under conditions comparable to the tumor microenvironment.

**Antitumor effect of the PERK inhibitor is mediated by proteotoxic stress and not oxidative stress**

Because the PERK/NRF2 pathway is known to upregulate antioxidant enzymes [6], which were increased in our mouse model (Fig. S2), we examined whether the killing of HepG2 cells by PERKi depends on oxidative stress. Under ER stress, the co-incubation of PERKi with N-acetylcysteine (NAC) or ascorbic acid, which attenuate oxidative stress by directly scavenging ROS, was unable to counteract the PERKi-mediated reduction of cell viability (Fig. S3D) or the IRE1 hyperactivation (data not shown).

To examine whether the compensatory hyperactivation of the proapoptotic IRE1/JNK/p38 pathway [4] is involved in the antitumor effect of PERKi, cells were pretreated with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 (validation not shown). Both compounds did not affect the PERKi-mediated reduction of cell viability (Fig. S3D).

Additionally, the combination of PERKi with the IRE1 inhibitor did not modify cell viability under ER stress compared with PERKi alone. These findings suggest that neither oxidative stress nor the observed IRE1 hyperactivation contribute to PERKi-induced cell death. In addition, we tested whether the PERKi-induced cell death was due to autophagy modulation by using inhibitors (3-methyladenine and chloroquine) or activators (rapamycin); however, despite validation by LC3 and P62 blotting, the effect of PERKi on viability was unaltered (data not shown).

PERK is known to inhibit protein synthesis in response to accumulation of misfolded proteins [3]. To check whether the cellular stress caused by PERKi was due to proteotoxicity, cycloheximide was used to inhibit protein synthesis.
Additionally, the chemical chaperone tauroursodeoxycholic acid (TUDCA) was applied to reduce the load of misfolded proteins. Both cycloheximide and TUDCA decreased PERKi-induced cell death under ER stress (p<0.001; Fig. S3D). These findings highlight a key role of proteotoxic stress in PERKi-induced cell death.

**The PERK inhibitor disrupts the UPR and reduces tumor growth in vivo**

Based on the in vitro data, we investigated the effects of PERKi in an orthotopic model of HCC. While the administration of PERKi in saline- or DEN-treated mice did not significantly alter weight or survival (Table 1), a tendency to increase the survival of the PERKi-treated HCC-bearing mice was observed (83 vs. 66 % in the vehicle-treated group). No other clinical signs of toxicity were observed in any of the PERKi-treated groups. Furthermore, PERKi induced no inherent hepatotoxicity, as the serum alanine aminotransferase levels were similar to those in the vehicle-treated control mice (35.6 ± 11 vs. 41.3 ± 14 U/l).

PERKi reduced PERK autophosphorylation in DEN-induced HCC, validating the in vivo activity of the small molecule used. Atf4 expression and elf2a phosphorylation were only slightly reduced (Fig. 5a). The quantification analysis of HCC burden, characterized by the loss of normal reticulin staining, revealed that PERKi reduced the mean tumor number (10.5 ± 2.4 vs. 7.8 ± 3.4; p<0.05) and burden (p<0.001; Fig. 5b) of the DEN-treated mice and rendered the tumors more spongiform with increased intercellular spaces. Choline positron emission tomography, used to visualize cellular membrane biosynthesis, demonstrated a decreased number of loci with high mean standardized uptake values after PERKi compared with vehicle administration (p<0.05; Fig. 5c). In addition, TUNEL immunofluorescence showed that DEN administration significantly increased the hepatic apoptosis rate compared with saline-treated livers (p<0.01) and that the PERK inhibitor induced a tendency to further elevate the apoptosis rate (Fig. S4).

No abnormalities were found during the pathologic examinations of the heart, spleen and kidney. Because PERK is known to play an essential role in insulin biosynthesis [15], we examined the fasting blood sugar levels. Hyperglycemia was observed in the PERKi-treated mice (174 ± 43 vs. 118 ± 31 mg/dl, p<0.01). Taken together, PERKi decreased the tumor burden without inducing significant toxicity.
1.1.6. Discussion

HCC is the second leading cause of cancer-related mortality [1]. Hepatocarcinogenesis starts from the initial genotoxic insult, through the clonal expansion from a premalignant to a tumoral lesion (promotion) and finally to tumor progression [16]. In the present study, we monitored the UPR during hepatocarcinogenesis. We observed a differential induction of the UPR pathways with a strong activation of the PERK pathway in HCC. Initially, PERK induces eIF2a phosphorylation to globally attenuate translation, thus reducing the load of unfolded proteins entering the ER. Under irremediable ER stress, PERK induces CHOP accumulation and subsequently apoptosis. Interestingly, we found that a small molecule inhibitor of PERK leads to ER-stress-driven cell death and HCC regression via proteotoxicity.

The chaperones, which assist protein folding, were elevated early during tumor initiation and even more so later on in the tumors. In contrast, the Atf6 pathway, a cytoprotective fine-tuner of the UPR [7], was not activated until W30, suggesting that Atf6-mediated adaptation is not a prerequisite for HCC initiation. Interestingly, the increased expression of Grp78 and Pdia4 mRNA in the tumor nodules compared with the perinodular ring of hepatic tissue suggests that these targets are additionally upregulated by tumoral UPR fine-tuning.

Ire1 signaling peaked before tumor development at W5-15, indicating a possible modulatory role for the Ire1 pathway during tumor initiation. The rise in Xbp1s could be an adaptation to the environment created by DEN-mediated inflammation [4, 12]. Surprisingly, Edem1 and P58IPK, both requiring the co-activation of Atf6 and Ire1, followed a different pattern. P58IPK was upregulated in the tumors, while Edem1, an ERAD accelerator [17], showed an early peak that faded thereafter.

In C57Bl/6J models of HCC induced by a single DEN injection, Chop expression was only found in the tumors and, in contrast to our model with repeated DEN injections, not in the surrounding tissue [18, 19]. Because Chop expression was increased from the first time point in the liver parenchyma without canonical UPR activation, Chop may be upregulated by an integrated stress response induced by DEN-mediated oxidative stress [20, 21].
To inhibit PERK, a recently developed small molecule (PERKi) was used and had no effect under normal conditions but potently diminished the viability of HCC cells under ER stress or hypoxia. In contrast, salubrinal treatment improved viability, suggesting a cytoprotective role of persistent eIF2a phosphorylation for attenuating protein synthesis [22]. In the mouse model, PERKi reduced the tumor burden. Collectively, these findings highlight the importance of the proadaptive outputs of PERK in HCC biology. Recently, the dogma of the proapoptotic PERK/CHOP pathway was challenged by the observation that PERK-mediated eIF2a-phosphorylation-attenuated protein synthesis is crucial for cell survival [5] and that CHOP promotes inflammation-mediated hepatocarcinogenesis [18, 19]. Accordingly, in the present study, PERKi augmented UPR-mediated cell death despite reduced expression of CHOP. PERKi decreased the expression of UPR-induced chaperones and the phospho-eIF2a/ATF4 pathway, which protect tumor cells from UPR-induced apoptosis [3, 23]. Furthermore, PERKi elevated global protein synthesis during ER stress, possibly escalating the unfolded protein load and ROS formation. Moreover, a protein synthesis inhibitor and a chemical chaperone were each able to hamper the cytotoxic effect of PERKi. Intriguingly, eIf2a phosphorylation was only modestly reduced in vivo, suggesting that other eIf2a kinases are able to bypass PERK inhibition [3]. Importantly, antioxidants were unable to limit PERKi-mediated cell death, suggesting that oxidative stress is not required for its cytotoxicity and that the inhibition of the PERK/NRF2 pathway, which promotes the antioxidant defense, is not the dominant antitumor action. Notably, PERKi enhanced IRE1 activation under ER stress, indicating a compensatory adaptation, consistent with the hyperactivity of the IRE1/XBP1 system in embryonic stem cells with defective PERK signaling [24]. However, because the inhibition of the IRE1/p38/JNK pathway did not affect cell death by PERKi, this pathway seems to be redundant for its antitumor effect. These findings reveal a pivotal survival role for the PERK pathway under stressed conditions as present in the tumor microenvironment.

PERK deficiency during development leads to Wolcott-Rallison syndrome [25]. However, in the therapeutic study with PERKi in adult mice, we observed no increased mortality or apparent toxicity with the exception of hyperglycemia, possibly due to reduced pancreatic b-cell mass [15]. In conclusion, our study sheds light on the UPR fine-tuning during hepatocarcinogenesis. Furthermore, our in vitro and in vivo results identified PERK inhibition as a novel approach to modulate the UPR in order to selectively kill ER-stressed HCC cells.
1.1.7. References


1.1.8. Figures

Fig. 1 Temporal dynamics of chaperones and the Ire1 pathway in the HCC model.

a) Real-time PCR of Grp78, Grp94 and P58IPK during hepatocarcinogenesis. Orange line compares DEN-treated groups at different time points. From W25, different tissue compartments were isolated: green, non-HCC; blue, surrounding; red, tumors. Dashed lines compare these compartments at W30. Relative fold change was calculated using the DDCT method.

b) Immunoblotting for UPR-mediated proteins. Results are representative of two independent experiments. Densitometric analysis of the p-Ire1:Ire1 ratio is indicated.

c) Immunostaining for Grp78 in livers treated for 30 W. Arrows indicate tumor. Positive control received a single injection with tunicamycin. Scale bar 100 lm.

d) Real-time PCR of Ire1-mediated splicing activity, Erdj4, Canx and Edem1. The Ire1-mediated splicing of Xbp1 mRNA is calculated as the relative ratio of spliced Xbp1 mRNA over total Xbp1 mRNA. Horizontal axis in (a) and (d) indicates the number of weeks of DEN treatment. *p<0.05, **p<0.01.
Fig. 2 Temporal dynamics of genes regulated by Perk or Atf6 in the HCC model. a Real-time PCR analysis of Perk-regulated genes. b Immunoblotting and immunostaining for Perk-regulated genes in livers treated for 30 W with DEN or saline. Positive control received tunicamycin for 72 h. Arrows indicate tumors. Scale bar 100 lm. c Caspase-3 activity of liver lysates of the indicated tissue after 30 weeks of saline or DEN administration. d Real-time PCR analysis of Atf6 target genes. Horizontal axis in (a) and (d) indicates the number of weeks of DEN treatment. Data are presented as the mean ± SD. One-way ANOVA was applied for statistical analysis. *p<0.05, **p<0.01.
Fig. 3 Transmission electron microscopy of saline- and tunicamycin-treated livers and DEN-induced tumors of the mouse model. a Expansion of the endoplasmic reticulum (ER; arrows) in HCC cells (middle panel) compared with saline-treated livers (left panel). Dilated ER in the liver of mice that received tunicamycin for 72 h (right panel). CF, collagen fibers. Tunicamycin-induced lipid droplets are indicated by arrowheads. b Reorganization of the endoplasmic reticulum after 30 weeks of DEN administration. DEN-induced HCC: hepatocellular carcinoma nodules isolated from the diethylnitrosamine-treated mouse liver.
Fig. 4 Effect of salubrinal, PERK or IRE1 inhibitor on the UPR and cell viability in HepG2 cells. Cells were subjected to hypoxia or tunicamycin as indicated for 48 h. a Effect on UPR marker mRNA expression by a PERK inhibitor and by an IRE1 inhibitor compared with solvent-treated cells under the same condition. b Immunoblotting for UPR markers. c Cell viability of HepG2 cells was assessed by a WST-1 assay. d Caspase-3 activity of HepG2 cells treated with the indicated compounds. These experiments were repeated six times with similar results. *p<0.05, ***p<0.001.
Fig. 5 A PERK inhibitor reduces HCC burden in the orthotopic mouse model. a Immunoblotting for phospho-Perk, Atf4 and phospho-eIf2α in the lysates of isolated tumors after 25 weeks of DEN followed by treatment with a PERK inhibitor or vehicle. Experiments were repeated twice with similar results. b Reticulin staining to quantify the tumor burden as shown by mean total tumor surface. Arrows indicate tumors. c 18F-Choline positron emission tomography visualizes cell membrane synthesis after the indicated treatments. Arrows indicate the left kidney for reference density. Standardized uptake values of the mouse livers are presented as the mean ± SD. One-way ANOVA was applied for statistical analysis. *p<0.05, **p<0.01
1.1.9. Tables

Table 1 Mouse body weights (g) (mean ± SD) and survival rates (%)

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NS not significant compared with vehicle
*** p<0.001: 25 weeks DEN versus saline
1.1.10. **Supplementary Figures**

**Fig. S1 Confirmation of the mouse model** (A) Number of macroscopic tumors. (B) Mean tumor burden. (C) Fibrosis as shown by mean Metavir-score. Data are presented as the mean±SD of n=12. *p<0.05, **p<0.01, ***p<0.001. (D) Representative images of a 25 week saline- or DEN-treated liver. Arrows indicate tumors.

**Fig. S2 Temporal dynamics of Nrf2-mediated genes** (A) Real-time PCR analysis of *Gpx3* and (B) *Gclc*. Data are presented as the mean ± SD of n=12. *p<0.05, **p<0.01.
Fig. S3 Effect of UPR modulation on cell viability, proliferation and protein synthesis in HCC cells
(A) Protein synthesis rate as assessed by puromycin incorporation in HepG2 cells. (B) Proliferation rate was assessed by measurement of BrdU incorporation in HepG2 cells. (C) Cell viability of BWTG3 and Hepa1-6 cells, as assessed by a WST-1 assay. For cytotoxic control, 1% Triton X-100 was applied. (D) Effect of a PERK inhibitor on cell viability of HepG2 cells under ER stress in combination with the indicated compounds for 48 hours. Experiments were repeated twice with similar results. Data are presented as the mean ± SD. *p<0.05, **p<0.01, ***p<0.001.
Fig. S4 The PERK inhibitor induces a tendency to increased hepatic apoptosis rate (A) Representative TUNEL immunofluorescence and (B) quantification of the TUNEL-positive index. *p<0.05, **p<0.01.
1.2. Tauroursodeoxycholic acid dampens oncogenic apoptosis induced by endoplasmic reticulum stress during hepatocarcinogen exposure

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Tauroursodeoxycholic acid dampens oncogenic apoptosis induced by endoplasmic reticulum stress during hepatocarcinogen exposure

1.2.1. Abstract
Hepatocellular carcinoma (HCC) is characterized by the accumulation of unfolded proteins in the endoplasmic reticulum (ER), which activates the unfolded protein response (UPR). However, the role of ER stress in tumor initiation and progression is controversial. To determine the impact of ER stress, we applied tauroursodeoxycholic acid (TUDCA), a bile acid with chaperone properties. The effects of TUDCA were assessed using a diethylnitrosamine-induced mouse HCC model in preventive and therapeutic settings. Cell metabolic activity, proliferation and invasion were investigated in vitro. Tumor progression was assessed in the HepG2 xenograft model. Administration of TUDCA in the preventive setting reduced carcinogen-induced elevation of alanine and aspartate aminotransferase levels, apoptosis of hepatocytes and tumor burden. TUDCA also reduced eukaryotic initiation factor 2α (eIf2α) phosphorylation, C/EBP homologous protein expression and caspase-12 processing. Thus, TUDCA suppresses carcinogen-induced pro-apoptotic UPR. TUDCA alleviated hepatic inflammation by increasing NF-κB inhibitor IκBα. Furthermore, TUDCA altered the invasive phenotype and enhanced metabolic activity but not proliferation in HCC cells. TUDCA administration after tumor development did not alter orthotopic tumor or xenograft growth. Taken together, TUDCA attenuates hepatocarcinogenesis by suppressing carcinogen-induced ER stress-mediated cell death and inflammation without stimulating tumor progression. Therefore, this chemical chaperone could represent a novel chemopreventive agent.

1.2.2. List of abbreviations
HCC, hepatocellular carcinoma; TUDCA, tauroursodeoxycholic acid; NFκB, nuclear factor kappa-B; ER, endoplasmic reticulum; UPR, unfolded protein response; Grp78, glucose-regulated protein, 78 kDa; eIf2α, eukaryotic initiation factor 2α; Chop, C/EBP homologous protein; DEN, diethylnitrosamine; LDH, lactate dehydrogenase; BrdU, bromodeoxyuridine; Gsta1, glutathione-S-transferase A1; Gsta2, glutathione-S-transferase A2; Nfe2l2, nuclear factor erythroid-derived 2, like 2; Gclc, glutamate-cysteine ligase
1.2.3. Introduction

Hepatocellular carcinoma (HCC) ranks as the second leading cause of cancer-related mortality worldwide and is frequently associated with liver cirrhosis [1,2]. Resection and transplantation are the only potentially curative treatments available following detection of a small HCC [1]. For the majority of patients with locally advanced disease, however, the multi-kinase inhibitor sorafenib and transarterial embolization are the only approved treatments. Unfortunately, both of these treatments provide a limited survival benefit [1]. Given that risk factors for HCC, such as liver cirrhosis, are fairly well established, chemopreventive strategies may help combat the disease. Ideally, these drugs should be safe for long-term use in the at-risk population. However, no chemopreventive drugs are currently available for HCC [3].

Tauroursodeoxycholic acid (TUDCA) is a hydrophilic bile acid that is produced endogenously in humans at very low levels [4]. TUDCA is synthesized in the conjugation pathway of ursodeoxycholic acid, which is effectively used for treating cholestatic liver diseases, including primary biliary cirrhosis, without major adverse reactions [4]. These hydrophilic bile acids act as bile secretagogues and immunomodulators that can prevent apoptosis induced by several agents, such as hydrophobic bile acids, alcohol, transforming growth factor β1, and Fas ligand, in hepatic and non-hepatic cells [5,6]. In addition, TUDCA protects rat livers during long-term ethanol feeding [7] and human livers from ischemia-reperfusion injury during harvesting and cold storage [8]. The mechanisms involved in the antiapoptotic properties of TUDCA include targeting mitochondrial function and integrity and interactions with the nuclear factor kappa-B (NFκB) signaling pathways [9].

The endoplasmic reticulum (ER) is an important organelle required for cell survival and is highly sensitive to homeostatic alterations. Disruption of ER homeostasis leads to the accumulation of unfolded proteins, which disturb ER function and result in a state known as ER stress [10]. In resting cells, all ER stress receptors are maintained in an inactive state through association with the ER chaperone glucose-regulated protein, 78 kDa (Grp78). Upon ER stress, Grp78 dissociates and triggers the unfolded protein response (UPR), which orchestrates cellular adaptation to stress by inducing transcriptional programs and by repressing global translation by eukaryotic initiation factor 2α (eIf2α) phosphorylation [11].
However, if the stress is too severe, UPR signaling switches from a pro-survival to a pro-apoptotic state, leading to increased pro-apoptotic transcription factor C/EBP homologous protein (Chop) expression and the cleavage of procaspase-12 to its active caspase-12 form [11]. Interestingly, the UPR is activated in several liver diseases, including fatty liver disease, viral hepatitis, alcohol-induced liver injury and HCC [12,13].

Importantly, TUDCA has been shown to act as a chemical chaperone that decreases UPR signaling and protects hepatocytes against cytotoxicity caused by the ER stress inducer thapsigargin [14,15]. TUDCA has also been shown to abolish ER stress-induced caspase-12 processing and to subsequently inhibit effector caspases-3/7 activation and apoptosis [15].

Recent studies have demonstrated that hepatocyte apoptosis is a pathogenic event in several liver diseases [13,16]. Chronically increased hepatocyte apoptosis in genetic mouse models is carcinogenic and leads to compensatory liver regeneration, oxidative stress and DNA hypermethylation [16,17]. In this study, we investigated the preventive and therapeutic potential of TUDCA in HCC. Our data demonstrate that TUDCA reduces carcinogen-induced liver dysfunction and HCC incidence, likely through the prevention of ER stress-induced apoptosis and inflammation. Importantly, once HCC nodules are established, TUDCA does not modulate tumor growth.
1.2.4. Results

Preventive administration of TUDCA reduces HCC burden in an orthotopic mouse model

The ER stress-inducing hepatocarcinogen diethylnitrosamine (DEN) promotes multifocal HCC after 25 weeks of administration [18,19]. To explore the effects of TUDCA on hepatocarcinogenesis, we supplemented the animals with low or high dose TUDCA in their drinking water. Administration of DEN resulted in reduced body weights and 25% increased mortality (p<0.001; Table 1) compared to saline administration. TUDCA treatment did not affect the weight loss or mortality of DEN-treated mice, and no clinical signs of toxicity were observed in any of the TUDCA-treated groups.

DEN-treated mice that received low- or high-dose TUDCA developed fewer nodules per liver (all sizes: 18.9 ± 5.3 after vehicle versus 11.5 ± 3.9 after low-dose TUDCA [p<0.01] and 12.3 ± 4.1 after high-dose TUDCA [p<0.05]). HCC burden, quantified by the loss of reticulin staining, was reduced in DEN-treated mice following the co-administration of low- or high-dose TUDCA (p<0.01; Fig. 1A-C). Choline positron emission tomography, which visualizes cellular membrane biosynthesis, demonstrated that administration of low-dose TUDCA in DEN-treated mice resulted in fewer hepatic loci with high mean standardized uptake values compared with animals receiving vehicle alone (p<0.05; Fig. 1D-E). Repeated DEN administration induces HCC in a background of liver fibrosis [18]. However, no difference in grade of fibrosis, as determined by Sirius Red staining, was found between TUDCA- and vehicle-treated livers (data not shown).

DEN administration increased the levels of serum ALT and AST compared to saline administration (p<0.001; Fig. 2A). Importantly, serum ALT and AST levels were reduced in DEN-treated mice receiving TUDCA-supplemented drinking water compared to those receiving regular drinking water (p<0.05; Fig. 2A). This suggests that both low- and high-dose TUDCA protect the liver from DEN-induced hepatotoxicity. Collectively, these data indicate that TUDCA decreases the susceptibility of mice to DEN-induced hepatocarcinogenesis in a preventive setting.

TUDCA attenuates UPR-induced apoptosis in DEN-treated mice

Repeated DEN administration results in significant apoptosis and ER stress in hepatocytes [18,19]. We measured the activity of effector caspase-3/7 ex vivo and, as expected, observed increased levels in DEN-treated mouse livers (p<0.001, Fig. 2B).
Interestingly, the hepatic caspase-3/7 activity was lower in DEN/TUDCA-treated mice compared to DEN/vehicle-treated mice (Fig. 2B). In agreement with these data, TUNEL immunofluorescence demonstrated a significant reduction of TUNEL-positive hepatocytes following TUDCA supplementation (p<0.05, Fig. 2C-D), thus confirming reduced hepatocyte apoptosis upon DEN challenge.

Grp78 and Chop expression and elf2α phosphorylation were increased in the mice treated with DEN, reflecting robust UPR activation (Fig. 3A-B). As a positive control, tunicamycin, a nucleoside antibiotic that inhibits protein glycosylation and thereby elicits acute ER stress [11], was administered to naive mice. These control mice showed significantly increased expression of Grp78 and Chop and elf2α phosphorylation (Fig. 3B). Administration of TUDCA during the 25 weeks of carcinogen exposure consistently reduced the expression of Grp78 and Chop and inhibited elf2α phosphorylation, thereby possibly restoring global translation (Fig. 3A-B). Caspase-12, the central player in ER stress-induced apoptosis [11], was markedly activated by both tunicamycin and DEN administration, whereas TUDCA reduced the DEN-induced cleavage of procaspase-12 (p<0.05; Fig. 3C). Overall, these data strongly suggest that TUDCA decreases carcinogen-induced ER stress and thereby attenuates caspase-12-mediated hepatocyte apoptosis.

**TUDCA increases cellular metabolic activity independent of ER stress**

To further explore the effect of TUDCA on cell viability and proliferation, HepG2, BWTG3 and Hepa1-6 cells were incubated with a dilution series of TUDCA. As shown by lactate dehydrogenase (LDH) activity in the cell supernatant, TUDCA at 0.1-1 mM did not induce cytotoxicity in HepG2 cells (Fig. S1A). At 10 mM, however, TUDCA increased the LDH activity (p<0.001). Next, we examined the effect of TUDCA on the MTT metabolic activity of cells under basal conditions. The reduction of tetrazolium salts such as MTT depends on both cellular metabolic activity and proliferation rate. Intriguingly, 0.1-1 mM TUDCA increased the MTT metabolic activity of HepG2 cells in a dose-dependent manner to supranormal levels of up to 150% (p<0.001; Fig. 3D).
Incorporation of the thymidine analogue bromodeoxyuridine (BrdU) into the DNA of HepG2 cells under basal conditions showed that TUDCA modestly decreased the cell proliferation rate at 1 mM (p<0.05, Fig. S1B). Furthermore, we directly counted HepG2 cells after 48 h of incubation with 0.1-1 mM TUDCA and found no difference in cell number compared to controls (data not shown). The MTT metabolic activity and BrdU incorporation experiments were repeated in BWTG3 and Hepa1-6 cells with similar results (Fig. S1C-F). These data suggest that, under basal conditions, TUDCA dose-dependently enhances cellular metabolic activity without increasing the absolute number of cells.

Next, we examined whether TUDCA would have the same effects in the presence of ER stress [11]. Tunicamycin (0.5 μg/ml) did not alter MTT metabolic activity or LDH release in HepG2 cells, suggesting no inherent cytotoxicity at the concentration used (Fig. 3D and S1A, respectively). However, tunicamycin did impair BrdU incorporation (p<0.01; Fig. S1B), confirming the well-known ER stress-mediated induction of cell cycle arrest [11]. Finally, TUDCA was able to enhance cellular metabolic activity even in the presence of acute ER stress (Fig. 3D and S1C-D); however, this had no effect on the antiproliferative effect of ER stress (Fig. S1B and S1E-F).

**TUDCA does not reduce oxidative stress-induced cell death or DEN-induced oxidative stress**

TUDCA has been shown to exert cytoprotective effects in different models by reducing oxidative stress [20], and ER stress is closely connected to the oxidative stress response [10]. Thus, we evaluated the effect of TUDCA on the cytotoxicity of H$_2$O$_2$-induced oxidative stress in HepG2 cells. Cell viability declined following the addition of 1-5 mM of H$_2$O$_2$ in a dose-dependent manner (Fig. S2A). In contrast to the established antioxidant properties of N-acetylcysteine, co-incubation with 0.1 or 1 mM TUDCA did not protect HepG2 cells against H$_2$O$_2$-induced cell death (p<0.01; Fig. S2A).

In the orthotopic HCC model, TUDCA supplementation did not alter the expression levels of DEN-induced antioxidant response genes such as glutathione-S-transferase A1 (*Gsta1*), glutathione-S-transferase A2 (*Gsta2*), nuclear factor erythroid-derived 2 like 2 (*Nfe2l2*) and glutamate-cysteine ligase (*Gclc*; Fig. S2B). Malondialdehyde (MDA) is a toxic product of reactions between reactive oxygen species (ROS) and polyunsaturated lipids that is commonly used as a biomarker to quantify oxidative stress.
DEN administration resulted in substantial accumulation of MDA protein adducts ($p<0.001$), and this was found to be unaltered by TUDCA supplementation (Fig. S2C). Thus, the chemopreventive action of TUDCA seems to be independent of oxidative stress.

**TUDCA does not affect DEN-induced autophagic flux**

ER stress is able to induce autophagy [21], which has been shown to protect against hepatocarcinogenesis [22]. We therefore tested whether TUDCA modulates autophagy in vivo (Fig. S3). DEN-treated liver tissue exhibited enhanced expression of Beclin-1 and increased conversion of LC3B-I to LC3B-II, indicating the activation of autophagic signals. The cellular content of p62, a receptor and substrate of selective autophagy, is a critical indicator of autophagic flux [23]. Immunoblotting for p62 showed that DEN slightly decreased the hepatic p62 content (Fig. S3A). TUDCA administration did not alter the stably elevated LC3 conversion and reduced p62 content. Thus, TUDCA was unable to alter the enhanced autophagic flux.

**TUDCA attenuates DEN-induced hepatic inflammation**

Human HCC usually develops in the background of chronic hepatic inflammation [1]. Repeated DEN-induced murine tumors share similar pathogenesis [18], where pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (Tnf-α), promote tumor development [24]. Indeed, accumulating evidence suggests extensive cross-talk between UPR and the inflammatory response [25]. Chop-deficient livers have been shown to exhibit reduced inflammation and hepatocarcinogenesis [26]. Because TUDCA abolished the DEN-induced Chop expression in our study, we hypothesized that TUDCA might alter DEN-induced hepatic inflammation.

Immunohistochemical staining demonstrated robust accumulation of F4/80+ macrophages in the livers of animals treated with DEN for 25 weeks (Fig. 4A-B). However, TUDCA significantly decreased the number of liver-infiltrating macrophages ($p<0.05$). Using multiplex microbead immunoassays, we observed that hepatic expression of inflammatory cytokines, including interleukin-1 beta (IL-1β), IL-6, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1 (Mcp-1), and Tnf-α, was augmented following DEN administration. Importantly, IL-6 ($p<0.05$), KC ($p<0.05$) and Tnf-α ($p<0.01$) levels were reduced following TUDCA supplementation (Fig. 4C).
Elevated eIf2α phosphorylation leads to translational repression of the NF-κB inhibitor IκBα and thereby promotes NF-κB signaling [27], which exerts a pro-carcinogenic role in inflammation-related hepatocarcinogenesis [28]. Using salubrinal, which inhibits eIf2α dephosphorylation, we confirmed that sustained eIf2α phosphorylation leads to translational repression of IκBα in HepG2 cells (data not shown). Because DEN-induced eIf2α phosphorylation was abolished by TUDCA supplementation (Fig. 3B), we assessed the effect of TUDCA on hepatic IκBα expression and NF-κB activation to investigate this potential mechanism of action. Interestingly, TUDCA supplementation restored IκBα expression and slightly decreased phospho-NFκB p65 levels, the active form of NF-κB, in the DEN-treated livers (Fig. 4D-E). These data indicate that TUDCA suppresses the immune response to DEN-induced liver injury by reducing phospho-eIf2α-mediated repression of IκBα translation.

**TUDCA alters invasiveness in vitro**

Because ER stress was previously linked to cell invasion [29,30], we questioned whether TUDCA could modify HCC cell invasion in vitro. Therefore, we examined the effect of TUDCA in a hepatocyte growth factor-induced invasion assay in which spheroids of the Hepa1-6 HCC cell line embedded in collagen matrix were observed over time (Fig. 5A-C). TUDCA decreased the sphere area after 60 h of incubation (p<0.05; Fig. 5A), whereas the perimeter of the TUDCA-treated spheres increased compared with controls after 24 (p<0.001) and 48 h (p<0.05) of incubation (Fig. 5B). Although TUDCA may have decreased sphere area by affecting cell proliferation in this context (e.g., as observed for HepG2 in Fig. 3B), the larger perimeter combined with a smaller area suggests an altered invasive phenotype. This result was also supported by the more irregular sphere shape observed upon invasion in the presence of TUDCA (Fig. 5C). However, in a Boyden chamber assay, addition of 1-2 mM of TUDCA induced no significant alterations in the hepatocyte growth factor-stimulated invasion of the HCC cells over a 48-hour period (Fig. 5D).

Degradation of the extracellular matrix is one pivotal step and occurs due to the actions of matrix metalloproteinases (MMP). MMP-2, MMP-9 and MMP-14 enzymes play important roles in the degradation of the extracellular matrix and exist extensively in HCC tissues. MMP profiling showed that addition of TUDCA did not alter the protein levels of MMP-2, -9 and -14 (Fig. 5E). Accordingly, we observed no changes in the extracellular MMP-2 and -9 activity in the concentrated conditioned medium (Fig. 5F). In conclusion, although TUDCA alters the invasive phenotype, TUDCA does not induce increased HCC cell invasion.
**TUDCA did not affect tumor progression**

Given our findings that TUDCA attenuated UPR-induced apoptosis and increased cellular metabolic activity, we evaluated whether TUDCA could stimulate tumor progression via its cytoprotective effect. To assess the effect of TUDCA on established tumors, we used orthotopic and xenograft mouse models of HCC. Following orthotopic HCC induction with 25 weeks of DEN, 5 weeks of low- or high-dose TUDCA supplementation had no significant effect on mortality (Table 1) or tumor number (all sizes: 17.4 ± 4.9 after vehicle versus 19.7 ± 4.5 after low-dose TUDCA and 18.8 ± 5.3 after high-dose TUDCA). Accordingly, microscopic quantification confirmed that TUDCA produced no significant effects on HCC burden (Fig. 6A-C). Next, we investigated the effects of 5 weeks of TUDCA supplementation following 25 weeks of DEN-induced tumor development on the UPR (Fig. 6D-E). TUDCA did not alter the protein levels of Chop or phospho-eIF2α in HCC-bearing livers (Fig. 6E). Moreover, no difference in the apoptosis rate was observed (data not shown). These results suggest that TUDCA was unable to restore ER function after prolonged hepatocarcinogen exposure.

In the HepG2 xenograft model, TUDCA supplementation for 5 weeks did not significantly modify tumor growth compared to control treatment (Fig. 6F-G), and no mortality occurred in any of these groups. Finally, no metastases were detected in any group of the preventive or therapeutic settings of the orthotopic or of the HepG2 xenograft model.
1.2.5. Discussion

There is an urgent need for innovative preventive and therapeutic options for HCC [3]. Previous studies have demonstrated that the chemical chaperone TUDCA serves as a cytoprotective agent by reducing ER stress and apoptosis [5,7,8,14]. In this study, we evaluated the preventive and therapeutic potential of TUDCA and its effect on carcinogen-induced ER stress in HCC. Our results reveal that TUDCA supplementation during carcinogen exposure reduces the carcinogen-induced apoptosis of hepatocytes and HCC incidence (Fig. 7). Furthermore, it does not stimulate the progression and invasion of established tumors.

A recent double-blind randomized trial demonstrated that daily TUDCA therapy (750 mg) for 6 months is safe and appears to be an effective liver cirrhosis treatment; in particular, it improved several biochemical parameters [31]. In addition, TUDCA therapy in cirrhotic patients awaiting liver transplantation supported their functional stability during the wait time [32]. Our results suggest that TUDCA therapy in patients with a high HCC risk, such as cirrhotic patients with continuous exposure to carcinogens, not only improved liver function but could also prevent HCC incidence.

We selected a low dosage (60 mg/kg/day), which corresponds to dosages administrable and tolerable for humans, and a high dosage (300 mg/kg/day) to assess dose-dependent effects and potential side effects. Both low- and high-dose TUDCA administration decreased tumor burden in our mouse models. However, the trend of reduced weight and survival was only observed in the high-dose TUDCA group. This reduction in body weight was also observed in a mouse model of retinitis pigmentosa, where animals were treated with high-dose TUDCA (500 mg/kg/day) [33]. Additionally, TUDCA has been shown to increase energy expenditure by promoting intracellular thyroid hormone activation [34]. Therefore, a low dose of TUDCA is advisable for chemoprevention.

Chemically improving the ER folding capacity by TUDCA administration was previously shown to protect from UPR signaling and ER stress-induced apoptosis, which are primarily regulated by the phospho-eIf2α/Chop cascade [10,15]. Although Chop plays critical pro-apoptotic roles, it was recently shown to promote carcinogenesis in a DEN-induced model of HCC [26]. Accordingly, TUDCA efficiently diminished Chop expression and tumorigenesis during carcinogen exposure in our study.

In hepatocytes, DEN is metabolized by cytochrome P450 2E1 through a ROS-generating reaction that induces liver injury and DNA damage [35,36].
Subsequently, danger signals released from injured hepatocytes induce liver inflammation. Accordingly, certain antioxidants, such as N-acetylcysteine [35] or lycopene [37], have been shown to attenuate DEN-induced hepatocarcinogenesis. Of note, N-acetylcysteine was recently shown to accelerate lung cancer progression in mice [38]. Because TUDCA did not affect oxidative stress-induced cytotoxicity in vitro or hepatic oxidative stress in vivo, we presume that the chemopreventive effect of TUDCA was not mediated by directly antagonizing oxidative stress but rather by modulating ER stress-induced apoptosis. Therefore, the combination of TUDCA with antioxidants could represent a dual-targeting chemopreventive strategy for HCC.

HCC has been characterized as a chronic inflammation-driven cancer, and chemically induced models have revealed the crucial roles of inflammatory signaling in disease onset and severity [35]. Therefore, we assessed whether TUDCA affected tumor-immune system crosstalk. TUDCA administration resulted in a decrease in hepatic macrophage infiltration and IL-6, KC and Tnf-α levels. These results suggest that TUDCA attenuates inflammation in response to DEN-induced liver injury and thus interrupts positive feedback from inflammation-induced ER stress and UPR-induced inflammation [13]. Interestingly, a similar effect of TUDCA on macrophage infiltration and Tnf-α expression was also recently observed in a model of ER stress-mediated steatohepatitis-induced HCC [39]. However, additional studies are needed to uncover the precise mechanism of the interaction between ER stress and inflammation and the effects of TUDCA on these signaling pathways.

Uncontrolled regeneration of hepatocytes, which occurs after repeated cycles of cell death and compensatory proliferation in chronic hepatitis, appears to be an important factor in hepatocarcinogenesis [16,40]. Apparently, increased hepatocyte apoptosis contributed to the development of HCC in our DEN-induced mouse model. TUDCA interrupts the positive feedback from UPR-mediated apoptosis-induced hepatocarcinogenesis, leading to UPR activation by tumor microenvironmental stresses [11].

Finally, an oral treatment option for preventing HCC would be highly beneficial for cirrhotic patients with high HCC risk. In this study, we showed that TUDCA could represent a clinically applicable chemopreventive agent for HCC. Whether hepatocyte susceptibility to other carcinogens, such as viral replication or alcohol, can be limited by chemical chaperones will be of great interest. In conclusion, our study demonstrates that supplementation with TUDCA diminishes carcinogen-induced hepatotoxicity and prevents tumor induction by, at least partially, alleviating positive feedback from ER stress, inflammation and apoptosis in carcinogen-injured liver tissue.
1.2.6. Methods

Animal studies

Ethics statement. The investigation was conducted in accordance with the ethical standards and according to the Declaration of Helsinki as well as the national and international guidelines. The investigation was also approved by the institutional review board of Ghent University (ECD 11/52).

Orthotopic model. Wild-type 129S2/SvPasCrl mice were purchased from Charles River (Belgium) and maintained as previously described [18]. In the preventive arm of the study, 5-week-old male mice were randomly divided into 4 groups (n=12 in each group). Three groups received weekly intraperitoneal DEN (35 mg/kg, in saline) injections for 25 weeks with either regular drinking water or drinking water supplemented with varying amounts of TUDCA (low dose of 60 mg/kg/day or high dose of 300 mg/kg/day). Mice in the control group received 25 weeks of saline injections and regular drinking water. In the therapeutic arm of the study, mice received DEN for 25 weeks before being treated with saline- or TUDCA-supplemented (low or high dose) drinking water for 5 weeks (n=12 in each group). In addition, 30 μg of tunicamycin was intraperitoneally injected in four naive 30-week-old male 129S2/SvPasCrl mice 72 h before sacrifice. Blood was collected from the retro-orbital sinus under isoflurane anesthesia. After macroscopic evaluation and quantification of hepatic tumor number, all organs were fixed in 4% phosphate-buffered formaldehyde (Klinipath) and embedded in paraffin or snap frozen in liquid nitrogen. Hematoxylin/eosin and reticulin staining were performed to assess tumor burden, and the results were evaluated by two independent observers. Sirius Red staining enabled fibrosis assessment according to Metavir scoring. Serum alanine and aspartate aminotransferase (ALT and AST, respectively) levels were measured at the Lab of Clinical Biology, Ghent University Hospital.

Xenograft model. HepG2 cells (6x10^6) were re-suspended in 100 μl of serum-free media and mixed with 100 μl of Matrigel (BD Biosciences, Bedford, MA, USA). The cell preparation was injected subcutaneously into the right flank of 8-week-old male athymic nude mice. Tumor volumes were calculated using the following formula: volume (mm³) = ab^2/2; where b is the smaller dimension.
When the mean tumor volume reached 150 mm³, animals were randomized into three groups (n=6 in each group) as follows: regular drinking water, low-dose (60 mg/kg/day) TUDCA-supplemented drinking water and high-dose (300 mg/kg/day) TUDCA-supplemented drinking water. Tumor dimensions were recorded two times per week with a digital caliper starting on the first day of treatment. Tumor weights were recorded at the time of sacrifice.

**Cell culture**

HepG2, BWTG3 and Hepa1-6 (ATCC, Virginia, USA) cells were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Ghent, Belgium). Cells were incubated for 48 h with tunicamycin (0.5 μg/ml), TUDCA (0.1–10 mM, Calbiochem, MA, USA), salubrinal (50 μM, Tocris, Bristol, UK), N-acetylcysteine (5 mM), H₂O₂ (1-5 mM) or equal volumes of solvent. For direct cell counting, cells were trypsinised and counted in trypan blue. All reagents were obtained from Sigma (Diegem, Belgium) unless stated otherwise. Experiments were performed in quadruplicate and independently repeated three times.

Detailed information regarding choline positron emission tomography, caspase-3/7 activity, TUNEL apoptosis, RNA extraction, quantitative real-time PCR, western blot analysis, LDH, MTT, BrdU incorporation, lipid peroxidation, immunohistochemistry, multiplex microbead and spheroid invasion assays is provided in the Supporting Information.

**Statistics**

Statistical analyses were performed using SPSS version 21 (SPSS, Chicago, USA). Data are presented as the mean ± SD or fold change relative to expression in controls. Normally distributed data were subjected to unpaired student’s t-tests. Multiple groups were compared by one-way analysis of variance (ANOVA) with Bonferroni correction. Non-normally distributed data were tested using the Mann-Whitney U test. The chi-squared test was used to compare mortality. Student’s paired t-test was used to compare area or perimeter fold change. Reported p-values were two-sided and considered significant when less than 0.05.
1.2.7. References

Chapter 3


Fig. 1. TUDCA prevents the development of HCC during carcinogen exposure. (A) Representative images of livers treated for 25 weeks with the indicated treatments. (B) Quantitative analysis of the tumor burden as assessed by (C) Reticulin staining. Scale bar: 100 µm. (D) \(^{18}\)F-Choline positron emission tomography was performed to visualize cell membrane synthesis after the indicated treatments (blue: low, red: high activity). (E) Quantification of \(^{18}\)F-Choline positron emission tomography. Standardized uptake values of the mouse livers are presented as the mean ± SD. One-way ANOVA was applied for statistical analysis. *p<0.05, **p<0.01.
Fig. 2. TUDCA reduces DEN-induced apoptosis of hepatocytes. (A) Liver damage was assessed by measuring ALT and AST levels in the serum of mice after the indicated treatments. (B) Caspase-3/7 activity \textit{ex vivo} (n=8). (C) TUNEL immunofluorescence and (D) quantification of the TUNEL-positive index. *p<0.05, **p<0.01, ***p<0.001.
Fig. 3. Effect of TUDCA on the hepatic UPR pattern in the DEN-induced mouse model of HCC. (A) Real-time PCR analysis of the UPR targets Grp78 and Chop. (B) Expression of Grp78, elf2α, phospho-elf2α, Chop, procaspase-12 and cleaved caspase-12 was detected using Western blotting. Results are representative of 2 independent experiments. (C) Cleaved caspase-12/procaspase-12 ratio obtained using densitometric analysis of the Western blot shown in B. (D) Effect of TUDCA treatment on the MTT metabolic activity of HepG2 cells (treated with tunicamycin (Tunica) or not). *p<0.05, **p<0.01, ***p<0.001. Results are representative of 3 independent experiments.
Fig. 4. Effect of TUDCA on DEN-induced hepatic inflammation. (A) Immunostaining for F4/80 and (B) quantification of F4/80-positive macrophages in the liver after the indicated treatments (n=5). (C) Determination of the indicated hepatic cytokine levels by multiplex microbead immunoassay (n=8). Values represent the mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (D) Expression of IκBα, phospho-NFκB p65 and NFκB p65 was detected using Western blotting. Results are representative of 2 independent experiments. (E) Phospho-NFκB p65/ NFκB p65 ratio obtained using densitometric analysis of the Western blot shown in D.
Fig. 5. Effects of TUDCA on HCC cell invasion. (A) The area and (B) length of the outside boundary (perimeter) of collagen-embedded Hepa1-6 spheroids (n=9) was measured at 0, 24, 48 and 60 h of incubation with 2 mM TUDCA or control medium. Results are representative of 2 independent experiments. (C) Representative images of invasive capacity of cells present in a collagen-embedded multicellular spheroid are shown at different time points. The multicellular spheroids are approx. 150 µm in diameter at start. At 60 h, the spheroid perimeter is marked. Scale bar is 50 µm. (D) Boyden chamber invasion assay with Hepa1-6 cells following 48 h of incubation with control medium, control medium with chemoattractant hepatocyte growth factor (HGF) without or with 1 or 2 mM TUDCA. Samples were run in quadruplicate. (E) Western blot analysis of MMP-2, -9 and -14 levels in Hepa1-6 cell lysates of control cells and cells treated with 1 or 2 mM TUDCA (48h), in the presence or absence of 50 ng/ml HGF stimulation. Arrows indicate MMP-2 (72 and 63-66 kDa), MMP-9 (78-82 kDa) and MMP-14 (66-57 kDa). The β-actin signal is used as loading control. (F) Gelatin zymography of concentrated culture medium of Hepa1-6 control cells or cells treated with 1 or 2 mM TUDCA (48 h), in the presence or absence of 50 ng/ml HGF stimulation. The white bands indicate MMP-activity; arrowheads: signal for MMP-9 (glycosylated, 92 kDa) and MMP-2 (58/62 kDa, active). Values represent the mean ± SD. *p<0.05, **p<0.001.
Fig. 6. Effect of TUDCA on the orthotopic and xenograft mouse models of HCC progression. (A) Representative images of murine livers treated for 25 weeks with DEN injections followed by 5 weeks of TUDCA-supplemented or control drinking water. (B) Reticulin staining of the DEN-treated livers for (C) quantification of tumor burden. Scale bar: 100 µm. (D) Real-time PCR analysis of the UPR targets Grp78 and Chop. (E) Expression of Grp78, eIf2α, phospho-eIf2α, Chop, procaspase-12 and cleaved caspase-12 was detected using Western blotting. Results are representative of 2 independent experiments. (F) Effect of indicated treatments on growth of HepG2 xenografts in athymic nude mice (n=6). The volume of each tumor was measured for 33 days. Values represent the mean ± SD. (G) At the end of the treatment period, animals were sacrificed and tumor weights were recorded. *p<0.05, **p<0.01.
Fig. 7. Schematic overview outlining the mechanisms of the chemopreventive effects of TUDCA.
1.2.9. Supplementary Figures

**Fig. S1. Effect of TUDCA on viability and proliferation of HCC cells.** Control cells or cells treated with 0.5 microgram/ml tunicamycin were treated with increasing concentrations of TUDCA as indicated. (A) LDH release in HepG2 cells. As positive control, TritonX 1% was applied. (B) Proliferation rate as assessed by BrdU incorporation in HepG2 cells. (C) MTT metabolic activity and (D) proliferation rate in BWTG3 cells. (E) MTT metabolic activity and (F) proliferation rate in Hepa1-6 cells. Data are presented as the mean ± SD of n=4. Statistical significance was determined by one-way analysis of variance (ANOVA) with Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001.
Fig. S2. Effect of TUDCA on oxidative stress-induced cytotoxicity in HepG2 cells. (A) MTT assay of HepG2 cells incubated for 48 h. Concentrations are indicated in mM. NAC, N-acetylcysteine. (B) Real-time PCR analysis of the indicated antioxidant genes after indicated cell treatments. (C) Malondialdehyde (MDA) levels were quantified as a measure of lipid peroxidation in the mouse livers after the indicated treatments. All the values are expressed as the mean ± SD, n=5. *p<0.05, **p<0.01, ***p<0.001.
Fig. S3. Effect of TUDCA on DEN-induced autophagy. (A) The expression level of autophagy-associated proteins in the livers was analyzed by western blotting. β-actin: loading control. (B) Densitometric analysis of LC3 II/actin ratio.
### Table 1: Mouse body weight (g) (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Average body weight 25 weeks (g)</th>
<th>Average body weight 30 weeks (g)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preventive study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>31.22 ± 1.39</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>DEN + control</td>
<td>26.75 ± 1.96***</td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>DEN + low dose TUDCA</td>
<td>27.39 ± 1.45#</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td>DEN + high dose TUDCA</td>
<td>24.64 ± 2.17#</td>
<td></td>
<td>67</td>
</tr>
<tr>
<td><strong>Therapeutic study</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN =&gt; control</td>
<td>25.96 ± 3.65</td>
<td>22.92 ± 3.19</td>
<td>67</td>
</tr>
<tr>
<td>DEN =&gt; low dose TUDCA</td>
<td>23.86 ± 2.70</td>
<td>22.13 ± 2.57¥</td>
<td>75</td>
</tr>
<tr>
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<td>24.53 ± 2.13</td>
<td>23.43 ± 2.61¥</td>
<td>67</td>
</tr>
</tbody>
</table>

***p<0.001: 25 weeks DEN vs. saline. 
#not significant compared to DEN + control. ¥not significant compared to DEN => control. 
DEN, diethylnitrosamine.
2. Sorafenib and the unfolded protein response in hepatocellular carcinoma

2.1. Antitumor efficacy of sorafenib is potentiated by modulation of the interplay between the unfolded protein response and autophagy in hepatocellular carcinoma

Manuscript in preparation, patent application filed

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Chapter 3


Abstract of the patent

The present invention relates to compositions which can be used for the treatment of hepatocellular carcinoma. More in particular, the present invention discloses that the addition of both an endoplasmic reticulum stress inducer and an inhibitor of autophagy significantly improve the antitumoral efficacy of the kinase inhibitor sorafenib in a synergistic manner. Hence, the present invention relates to compositions comprising the kinase inhibitor sorafenib, an endoplasmic reticulum stress inducer such as nelfinavir and an inhibitor of autophagy such as clarithromycin. The present invention also discloses the combination of an endoplasmic reticulum stress inducer and an inhibitor of autophagy—without sorafenib—for the treatment of hepatocellular carcinoma.

Claims of the patent

1. A composition comprising the kinase inhibitor sorafenib, an endoplasmic reticulum stress inducer and an inhibitor of autophagy.
2. A composition according to claim 1 for use as a medicament.
3. A composition according to claim 1-2 for use to treat hepatocellular carcinoma.
4. A composition comprising an endoplasmic reticulum stress inducer and an inhibitor of autophagy for use to treat hepatocellular carcinoma.
5. A composition according to claims 3-4 wherein said hepatocellular carcinoma is advanced hepatocellular carcinoma.
6. A composition according to claims 1-5 wherein said endoplasmic reticulum stress inducer is a) the HIV protease inhibitor nelfinavir, saquinavir, fosamprenavir, atazanavir, darunavir, tipranavir, indinavir or liponavir, or b) the proteasome inhibitor bortezomib, carfilzomib or oprozomib, or c) quercetin, or any combination of said endoplasmic reticulum stress inducers.
7. A composition according to claims 1-6 wherein said inhibitor of autophagy is a) the neomacrolide clarithromycin, azithromycin or roxithromycin, or b) chloroquine or hydroxychloroquine, or, any combination of said inhibitors of autophagy.
8. A composition according to claims 1-2 and 4-7 comprising the kinase inhibitor sorafenib, the endoplasmic reticulum stress inducer nelfinavir and the inhibitor of autophagy clarithromycin.
9. A composition according to claims 1- and 4-7 comprising the kinase inhibitor sorafenib, the endoplasmic reticulum stress inducer nelfinavir and the inhibitor of autophagy chloroquine.
10. A composition according to claims 1-7 wherein said endoplasmic reticulum stress inducer is nelfinavir and wherein said inhibitor of autophagy is clarithromycin.

11. A pharmaceutical composition comprising a composition according to claims 1-10.

12. A process to treat hepatocellular carcinoma comprising administering a therapeutically effective amount of a pharmaceutical composition according to claim 11 to a person in need thereof.

13. A composition comprising the inhibitor of autophagy clarithromycin and the endoplasmic reticulum stress inducer nelfinavir for use to treat hepatocellular carcinoma wherein said nelfinavir is given at a dose of maximally 89.3 mg/kg.

Full patent application is provided in the Supplemental information.
Antitumor efficacy of sorafenib is potentiated by modulation of the interplay between the unfolded protein response and autophagy in hepatocellular carcinoma

2.1.2. Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality and is resistant to cytotoxic and targeted therapies. Although sorafenib, a multi-kinase inhibitor, is the standard therapy, the antitumor effect is limited and average survival benefit is less than 3 months. Given the emerging importance of the unfolded protein response (UPR) and autophagy in HCC, we questioned whether the use of rationally designed combinations of sorafenib with clinically applicable modulators of the UPR and autophagy would be more effective than current sorafenib monotherapy. The UPR and autophagy and their relevance in cell viability and proliferation in vitro and in orthotopic and xenograft mouse models were assessed. Sorafenib activated the UPR and autophagy in HCC cells. Importantly, sorafenib induced inositol-requiring enzyme 1 (IRE1) RNase-mediated mRNA cleavage, a pro-apoptotic process called regulated IRE1-dependent decay. Moreover, sorafenib-mediated reduction in tumor cell proliferation was dependent on proteotoxicity and IRE1 RNase activity. Also autophagy induction by sorafenib was in part mediated by IRE1 activation. Targeting of the UPR or autophagy separately did not synergistically enhance the antitumor efficacy of sorafenib. However, combination with an UPR inducer and an inhibitor of adaptive protein refolding or autophagy potentiated the efficacy synergistically in vitro and in the used mouse models without significant toxicity.

Conclusion: Clinically applicable triple therapies of sorafenib with modulators of the UPR and autophagy seem to suppress intrinsic sorafenib resistance and open new avenues for a multi-target approach that may produce durable responses against HCC.
2.1.3. List of abbreviations

HCC, hepatocellular carcinoma; ER, endoplasmic reticulum; GRP78, glucose-regulated protein, 78 kDa; PDI, protein disulfide isomerases; ERAD, ER-associated protein degradation; UPR, unfolded protein response; PERK, PKR-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor 6; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding homologous protein; XBP1u, unspliced X-box-binding protein 1; XBP1s, spliced X-box-binding protein 1; RIDD, regulated IRE1-dependent decay; IP, intraperitoneal; IG, intragastric; ERDJ4, endoplasmic reticulum DnaJ homolog 4; DEN, diethylnitrosamine; GADD34, growth arrest and DNA damage inducible 34; HERPUD1, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1.

2.1.4. Introduction

Hepatocellular carcinoma (HCC) represents a major health problem that causes over 700,000 deaths annually worldwide [1]. Conventional chemotherapy is ineffective and targeted therapy for advanced HCC with sorafenib provides a limited and variable survival benefit [2]. Sorafenib was identified as a multi-kinase inhibitor, targeting Raf (serine/threonine-specific protein kinase), Fms-like tyrosine kinase 3,4 c-Kit, p38, stem cell growth factor receptor 1, vascular endothelial growth factor receptors, and platelet-derived growth factor receptor-β to induce growth arrest in a variety of tumors, including HCC [3]. Resistance may be either pre-existent (intrinsic resistance), or drug-induced (acquired resistance). So far, no clinical strategy has been found to overcome sorafenib resistance. Long-term exposure to sorafenib of HCC cells induces acquired resistance with epithelial-to-mesenchymal transition. The sensitivity for sorafenib after resistance development can partially be restored by inhibition of phosphatidylinositol 3-kinase/Akt, BCRP/ABCG2 or NF-κB-upregulated CD47 [4]–[6]. In addition, Rudalska et al. (2014) identified by in vivo shRNA screening the p38/ATF2 pathway as a potential mechanism of acquired sorafenib resistance in HCC [7]. However, the molecular mechanisms by which sorafenib exerts its effect or how intrinsic resistance to sorafenib is facilitated remain unclear [2].

Certain chemotherapeutic drugs provoke apoptosis through the mitochondrial pathway, whereas others, most notably the proteasome inhibitors, induce cell death via endoplasmic reticulum (ER) stress. The ER consists of a membranous network in which proteins are synthesized, post-translationally modified and folded.
Therefore, the ER lumen houses a large array of chaperones, including glucose-regulated protein-78 (GRP78) and protein disulfide isomerase A4 (PDIA4) and contains quality-control systems that extract terminally misfolded proteins for degradation, a process known as ER-associated protein degradation (ERAD) [8]. Disturbances in ER homeostasis activate the unfolded protein response (UPR), which initially compensates for damage, but will activate apoptosis during intense or persistent ER stress [9]. Three major ER stress transducers have been identified: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) [8]. Following release of GRP78, PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) leading to attenuation of global translation. Accompanying this translational control, phosphorylated eIF2α selectively enhances translation of activating transcription factor 4 (ATF4), a transcriptional activator of genes involved in protein quality control, amino acid biosynthesis as well as induction of apoptosis such as CCAAT/enhancer-binding homologous protein (CHOP) [8]. Upon ER stress, ATF6 is mobilized to the Golgi where it is cleaved, which releases the transcriptionally active fragment and induces chaperones such as PDIA4, unspliced X-box-binding protein-1 (XBP1u) and ERAD components such as homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1 (HERPUD1) [10]. Activation of IRE1 results in splicing of XBP1u mRNA to generate a more active spliced XBP1 (XBP1s), which induces genes involved in protein folding such as ERDJ4 [10], [11]. Next to XBP1 mRNA, also other mRNAs can be cleaved by IRE1, a process known as regulated IRE1-dependent decay (RIDD) [8], [12]. The effect of sorafenib on the UPR in HCC is controversial; both UPR inhibition [13] as activation [14]–[16] have been reported.

Besides canonical UPR activation, sustained ER stress can elicit autophagy, which allows cells to sequester cytoplasmic material by forming double-membrane vesicles and to target the material for degradation [17]. Interestingly, sorafenib was previously shown to generate cytoprotective autophagy in different types of tumor cells including HCC [16], [18].

We further investigated the effect of rationally designed combinations of clinically applicable UPR- and autophagy-modulating agents and human equivalent dose of sorafenib on HCC in vitro and validated these results in an orthotopic and xenograft mouse model. Our data indicate that the effect of sorafenib is in part mediated by proteotoxic stress, which activates compensatory mechanisms, such as adaptive protein refolding and autophagy. Inhibition of these survival mechanisms under amplified ER stress synergistically enhanced the antitumor efficacy.
2.1.5. Materials and Methods

**Cell culture**

Human HepG2 cells (HB-8065; ATCC, Virginia, USA) were cultured with Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Ghent, Belgium). Cells were incubated for 24 hours with sorafenib (5-20 μM; Bay43-9006, Nexavar®), tunicamycin (1 μg/ml), thapsigargin (50 ng/ml), PERK inhibitor (0.5 μM; GSK2656157, Chengdu novi biotechnology, Shandong, China), IRE1 RNase inhibitor (8 μM; 4μ8C, Calbiochem, Massachusetts, USA), quercetin (25-100 μM), bacitracin (3 mM), nelfinavir (10 μM), chloroquine (10 μM), clarithromycin (50 μg/ml), bortezomib (50 nM; Santa Cruz, Heidelberg, Germany), tauroursodeoxycholic acid (1 mM), cycloheximide (5 μM) or MEK inhibitor PD98059 (30 minutes; 10 μM; Cell signaling) or equal volumes of solvent as control. All reagents were obtained from Sigma (Diegem, Belgium) unless stated otherwise. Each experiment was performed in quadruplicate.

**Animals**

Wild-type 129S2/SvPasCrl mice and athymic nude mice were purchased respectively from Charles River (L'Arbresle Cedex, France) and Harlan (Horst, The Netherlands) and maintained as previously described [19]. All protocols were approved by the Ethical Committee of experimental animals at the Faculty of Health Sciences, Ghent University, Belgium (ECD 11/52 and 13/39).

**Orthotopic model.** Five-week-old males received weekly intraperitoneal (IP) saline or diethylnitrosamine (DEN) (35 mg/kg, in saline). After 25 weeks, saline-treated mice were randomly divided into 2 groups: control (intragastric (IG) 100 μl of vehicle once daily) or sorafenib (IG 30 mg/kg once daily, as indicated by human equivalent plasma levels [20]); and DEN-treated into 5 groups (each n=12): control, sorafenib, sorafenib + clarithromycin (IG 50 mg/kg bid), sorafenib + nelfinavir (IP 50 mg/kg bid), sorafenib + nelfinavir + clarithromycin (idem) and nelfinavir (IP 250 mg/kg bid) + clarithromycin (idem). After 30 weeks, blood was collected from the retro-orbital sinus under isoflurane anaesthesia, and the mice were euthanized. All organs were fixed in 4% phosphate-buffered formaldehyde (Klinipath) and embedded in paraffin or snap frozen in liquid nitrogen. Hematoxylin/eosin and reticulin staining were performed and the results were blindly evaluated by two independent observers. Mean intercapillary distance was measured on CD105-stained slides as previously described [19].
**Xenograft model.** HepG2 cells (6x10⁶) were mixed with 40% Matrigel (BD Biosciences, Bedford, MA, USA). The cell preparation was injected subcutaneously into the right flank of 8-week-old male athymic nude mice housed in filter-topped cages. Tumor dimensions were recorded three times per week with a digital caliper starting with the first day of treatment. Tumor volumes were calculated using the following formula: volume (mm³) = \( \frac{ab^2}{2} \), where b was the smaller dimension. When mean tumor volume reached 150 mm³, animals were randomized into eight groups (n=6): sorafenib (IG 30 mg/kg once daily), sorafenib + clarithromycin (IG 50 mg/kg bid), sorafenib + nelfinavir (IP 50 mg/kg bid), sorafenib + nelfinavir + clarithromycin (idem), nelfinavir (IP 50 mg/kg bid) + clarithromycin (idem), nelfinavir (IP 250 mg/kg bid) + clarithromycin (idem), nelfinavir (IP 250 mg/kg bid) and control group. Final tumor weights were recorded at the time of sacrifice.

Detailed information of total RNA extraction, quantitative real-time PCR, Western blotting, immunohistochemistry, MTT, bromodeoxyuridine (BrdU) incorporation and caspase-3 activity assays and positron emission tomography is provided in the Supplementary Materials and Methods.

**Statistics**

Statistical analyses were performed using SPSS 21 (SPSS, Chicago, USA). Data are presented as the mean ± SD or percentage relative to expression in controls. Variables were tested for normality using the Shapiro-Wilk test. Normally distributed data were subjected to the unpaired student’s t-tests. Data involving more than two groups were assessed by one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test. Non-normally distributed data were tested using the Mann-Whitney-U test. The chi-squared test was used to compare mortality. Continuous data (xenograft tumor volume changes) were evaluated using linear mixed models. CompuSyn program (ComboSyn, NJ, USA) was used to compute a combination index for drug combinations studied with cell proliferation assays. Reported p-values were two-sided and considered significant when less than 0.05.
2.1.6. Results

*Sorafenib activates the IRE1/XBP1/RIDD and PERK/eIF2α/ATF4/GADD34 pathways in vitro*

The effect of sorafenib on the UPR was assessed in HepG2 cells at concentrations achieved in clinical practice (between 1 and 10 μM [21], [22]). Sorafenib dose dependently increased the mRNA levels of chaperones GRP78 (5 μM: p<0.05; 10 μM: p<0.001) and PDIA4 (from 5 μM: p<0.001) compared to control (Fig. 1A). GRP78 protein levels were decreased by sorafenib, which is consistent with observations in sorafenib-treated human leukemia cells [14]. In contrast, sorafenib increased PDIA4 protein levels (Fig. 1A-B). In addition, sorafenib increased the mRNA levels of HERPUD1 (10 μM: p<0.01; Fig. 1A).

Expression of total IRE1 mRNA (p<0.001, Fig. 1A) and protein (Fig. 1B) was markedly increased by sorafenib. Through the use of Phos-tag (a reagent that selectively binds to phosphorylated amino acids), we readily detected the active phosphorylated form of IRE1 in sorafenib-treated cells (Fig. 1B) [23]. Accordingly, the IRE1-mediated splicing of XBP1 and expression of a target of XBP1s, ERDJ4 [10], were increased by sorafenib (p<0.001, Fig. 1A). 5 μM sorafenib decreased XBP1u mRNA levels (p<0.05). However, 10 μM sorafenib increased XBP1u mRNA (p<0.001; Fig. 1A). The IRE1 inhibitor attenuated induction of XBP1s and ERDJ4 mRNA and restored XBP1u levels (p<0.001; Fig. 1A). Surprisingly, the IRE1 inhibitor increased IRE1 mRNA (p<0.001), suggesting negative regulation of the sorafenib-induced IRE1 mRNA expression by the IRE1 RNase, possibly by RIDD [12].

Interestingly, liver-expressed transcripts which are known targets of RIDD were all downregulated by sorafenib (Fig. 1D) [24]. Addition of the IRE1 inhibitor abolished the sorafenib-mediated downregulation of the tested RIDD targets. These results confirm that sorafenib induced IRE1-mediated RIDD. Importantly, sorafenib-induced RIDD downregulated Glypican-3 (GPC3), which encodes a cell-surface heparan-sulfate proteoglycan expressed in HCC [25]. Chemical ER stress inducers, thapsigargin and tunicamycin [8], downregulated all tested RIDD targets, with exception of CYP2E1, which was upregulated by tunicamycin, although thapsigargin downregulated this transcript (Fig. 1D).
Next, we examined whether sorafenib also activates the PERK pathway. Sorafenib promoted phosphorylation of eIF2α and expression of PERK targets ATF4, CHOP and growth arrest and DNA damage inducible 34 (GADD34) (mRNA: Fig. 1A; protein: Fig. 1B). The PERK inhibitor decreased sorafenib-induced phosphorylation of eIF2α and expression of ATF4 and GADD34 (Fig. 1B). However, addition of the PERK inhibitor to sorafenib further upregulated GRP78, PDIA4 and HERPUD1 transcripts (Fig. 1A), which are primary regulated by ATF6 [10], suggesting enhanced cytoprotective ATF6 signaling upon pharmacological blockade of the PERK pathway. Interestingly, IRE1 inhibition modestly decreased sorafenib-induced expression of CHOP (Fig. 1B).

Since sorafenib is developed as an inhibitor of the RAF/MEK/ERK pathway, we examined the effect of the MEK inhibitor PD98059 on the UPR. Next to a slight decline in GRP78, PD98059 was unable to alter the expression of the UPR-mediated proteins (Fig. 1B), suggesting sorafenib induces ER stress in HCC cells independent of MEK inhibition.

Since sorafenib induces ER stress, we questioned whether pre-existent ER stress, as present in human HCC in vivo [26], modulates the effect of sorafenib on the UPR. Therefore, we examined the effect of sorafenib on the UPR in the presence of tunicamycin in HepG2 cells (Fig. 1C). Despite the presence of tunicamycin-induced ER stress, sorafenib was able to further enhance the expression of GRP78 (p<0.001), XBP1s (p<0.05), ERDJ4 (p<0.001), CHOP (p<0.05) and GADD34 (p<0.001) mRNA. At the protein level, addition of sorafenib to tunicamycin potentiated the PERK-mediated phosphorylation of eIF2α and expression of GADD34 and CHOP.

Sorafenib-mediated reduction in tumor cell proliferation is dependent on proteotoxic stress and IRE1 RNase activity

Sorafenib at 5-20 μM dose dependently reduced HepG2 cell viability and proliferation and triggered caspase-3 activation (Fig. S1A-C). Although tunicamycin alone did not affect cell viability, we observed slight enhancement of the sorafenib-mediated reduction in proliferation and induction of caspase-3 by addition of tunicamycin (p<0.05, Fig. S1A-C). In the next experiments, sorafenib was applied at 10 μM, since this was the lowest concentration significantly reducing cell viability in the range of the human intratumor concentration.
To demonstrate that proteotoxic stress caused by sorafenib originates during the synthesis of new proteins, cycloheximide (CX) was used to block protein synthesis. In addition, chemical chaperone tauroursodeoxycholic acid (TUDCA) was applied to reduce the load of misfolded proteins. Cell viability was reduced by CX and increased by TUDCA, confirming their effect on metabolic activity (p<0.01; Fig. 2A). Interestingly, CX increased the proliferation and TUDCA reduced the caspase-3 activity of sorafenib-challenged cells (p<0.01; Fig. 2B-C).

Next, we assessed the effect of inhibition of the sorafenib-activated IRE1 or PERK pathway on cell viability. The IRE1 inhibitor counteracted sorafenib-mediated suppression of viability (p<0.05, Fig. 2A), proliferation (p<0.01, Fig. 2B) and induction of caspase-3 activity (p<0.001, Fig. 2C), possibly by downregulating CHOP and inhibition of pro-apoptotic RIDD (Fig. 1). Inhibition of IRE1-activated JNK [8] by SP600125 did not alter the effect of sorafenib (data not shown). The PERK inhibitor did not alter sorafenib-induced cell death (Fig. 2A-C). Thus, these data demonstrate that IRE1 is partly responsible for the cytotoxicity of sorafenib, likely through induction of CHOP and RIDD.

**Sorafenib induces autophagy in part via induction of IRE1**

Sorafenib at 10 μM induced autophagy-related genes ATG5, ATG7 and BECLIN1 (Fig. S3A), LC3 conversion and P62 degradation (Fig. S3B) in HepG2 cells. Addition of the IRE1 inhibitor attenuated the induction of ATG5 and -7 (p<0.01) and LC3 conversion in sorafenib-challenged cells. The PERK inhibitor did not affect sorafenib-induced LC3 II (Fig. S3B). Chloroquine inhibits autophagy by increasing lysosomal pH [17]. Sorafenib-induced conversion of LC3 and P62 degradation in HepG2 cells was inhibited by chloroquine (Fig. S3B), suggesting sorafenib promotes autophagic flux. Additionally, we confirmed that neomacrolide antibiotic clarithromycin [27] was able to inhibit sorafenib-induced autophagic flux (Fig. S3B).

Further, we evaluated the effect of sorafenib in combination with autophagy inhibition. Surprisingly, chloroquine was unable to potentiate the effect of sorafenib (Fig. 3A-C). Addition of clarithromycin to sorafenib further reduced the proliferation rate (p<0.01; Fig. 3B) and enhanced the caspase-3 activity (p<0.01; Fig. 3C) compared to sorafenib alone. Again, this rather seems to be an additive effect because clarithromycin monotherapy was able to induce both effects compared to control (Table S1). Thus, combination of sorafenib with autophagy inhibitors did not induce synergistic effects to HCC cells.
Autophagy inhibition together with IRE1 hyperactivation enhanced the efficacy of sorafenib

Because the activation of the IRE1 pathway was critical for the sorafenib-induced antiproliferative effect, we questioned whether IRE1 hyperactivity could enhance its efficacy. Therefore, we used the clinically applicable flavonoid quercetin which is known to directly bind and activate IRE1 [28]. Indeed, in HepG2 cells, quercetin dose dependently downregulated the RIDD targets (Fig. 1D) and induced XBP1 splicing without affecting ATF6 targets such as HERPUD1 (Fig. S2A). Surprisingly, quercetin decreased sorafenib-induced CHOP expression (Fig. S2A and 1B) and caspase-3 activity (p<0.001; Fig. S2B-D).

In order to find a clinically applicable efficacy-enhancing combination with sorafenib, we evaluated the combination of sorafenib with general ER stress inducers. Therefore, we selected the proteasome inhibitor bortezomib [8], [29] and the HIV protease inhibitor nelfinavir [30]. Surprisingly, combination of sorafenib with bortezomib did not show additive effects, although both demonstrated in monotherapy significant antitumor effects (p<0.001; Fig. 3A-C). Addition of nelfinavir at a noncytotoxic but ER stress-inducing concentration of 10 μM (data not shown) to sorafenib reduced the proliferation rate (p<0.05; Fig. 3B) and increased the caspase-3 activity (p<0.001; Fig. 3C) compared to sorafenib alone, however, this did not represent a synergistic effect (Table S1).

Since intensification of ER stress was insufficient to increase the efficacy of sorafenib, we questioned whether the observed induction of resistance mechanisms, such as autophagy and adaptive protein refolding by upregulating chaperones, such as PDIA4, which help in relieving ER stress, enables tumor cell survival under ER stress. To modulate protein refolding, we applied bacitracin, which is known to affect PDI activity [31]. Although the efficacy of sorafenib was unaltered by addition of bacitracin, the efficacy of sorafenib/bortezomib was enhanced by triple therapy with bacitracin (Fig. 3A-C), indicating that adaptive protein refolding is an essential response to proteotoxic stress induced by sorafenib/bortezomib. Similar results on cell viability were obtained by triple therapy with sorafenib/nelfinavir and bacitracin compared to sorafenib/nelfinavir (p<0.05; Fig. 3A).
The efficacy of sorafenib with autophagy inhibition was synergistically enhanced by addition of ER stress inducers nelfinavir or bortezomib (p<0.001; Fig. 3A-C and Table S1), confirming the need for a triple therapy approach tackling cellular adaptation. In contrast to the autophagy inhibitors, addition of bacitracin to sorafenib/nelfinavir was unable to change the antitumor effect, suggesting autophagy is a more important survival mechanism instigated by sorafenib/nelfinavir than adaptive refolding. In addition, triple therapy with sorafenib, quercetin, and autophagy inhibition synergistically decreased tumor cell proliferation (p<0.001; Fig. S2E and Table S1), suggesting a pivotal role for IRE1 in this strategy.

In addition, testing of this combination in HepG2 cells with acquired sorafenib resistance obtained by slowly increasing sorafenib concentrations, as described in [4], is ongoing. Interestingly, UPR-regulated CHOP mRNA was significantly upregulated in the sorafenib-resistant compared to sorafenib-sensitive HepG2 cells (p<0.001).

The antitumor efficacy of sorafenib is potentiated by combination with nelfinavir and clarithromycin in mouse models of HCC

Since triple therapy with sorafenib, nelfinavir and clarithromycin demonstrated the strongest synergistic effects in vitro (combination index: 0.63, Table S1), we tested this triple therapy in an orthotopic and a xenograft mouse model. In saline-treated and DEN-treated mice, sorafenib administration did not alter body weight or mortality (Table S2). In the DEN-treated mice, sorafenib reduced the hepatic tumor burden (p<0.01, Fig. 4A-B and S4A-B). Addition of nelfinavir, and not clarithromycin, to sorafenib, augmented the sorafenib-mediated antitumor effect (p<0.01). Moreover, triple therapy with sorafenib/nelfinavir/clarithromycin synergistically reduced tumor burden (p<0.001) and showed a tendency to reduce mortality (p=0.17, Table S2). Visualization of the cellular membrane biosynthesis by $^{18}$F-choline positron emission tomography demonstrated a decreased number of loci with high mean standardized uptake values after triple therapy compared to vehicle-treated mice (Fig. S4D).
Hematoxylin/eosin staining demonstrated intense cytoplasmic vacuolization, which is related to ER stress [32], by sorafenib and, even more, by triple therapy (Fig. S4A). Sorafenib decreased tumor angiogenesis (p<0.05), however, addition of nelfinavir or clarithromycin did not alter vessel density (data not shown). Triple therapy increased caspase-3 activity, measured in lysates of HCC-bearing livers compared to vehicle-treated mice (p<0.05; Fig. S4C). Analysis of HepG2 xenograft tumors from the control- and sorafenib-treated mice showed that 30 mg/kg/day sorafenib did not significantly reduce HCC growth in vivo (Fig. 4C-D). However, triple therapy demonstrated strong antitumor efficacy (p<0.001, Fig. 4C-D). Taken together, tackling the interplay between sorafenib-induced adaptation mechanisms strikingly suppressed HCC growth (Fig. 4E).

Finally, we tested the possibility to omit sorafenib, which can induce serious adverse events in clinical use [3], from the triple therapy of sorafenib, nelfinavir and clarithromycin. At the dose of 50 mg/kg bid of nelfinavir, omitting sorafenib dramatically reduced the antitumor effect in the HepG2 xenograft model, however, increasing the dose of nelfinavir to 250 mg/kg bid resulted in a comparable antitumor action compared to the triple therapy, while 250 mg/kg bid nelfinavir without clarithromycin did not suppress tumor growth (Fig. 4C-D). Based on these results, we evaluated the combination of 250 mg/kg bid nelfinavir and clarithromycin in the orthotopic model. Importantly, this combination was superior in inhibiting tumor progression compared to treatment with sorafenib monotherapy (p<0.001).

These data indicate that the threshold for ER stress-induced cell death during autophagy inhibition in HCC cells is elevated by omission of sorafenib from the triple therapy, which can be overcome by increasing the dose of nelfinavir.
2.1.7. Discussion

While sorafenib heralded a major breakthrough in HCC therapy, the clinical benefit is at best modest and transient [3]. HCC is a complex disease which needs multi-targeting approaches for effective therapy. ER stress and autophagy are two basic cell survival mechanisms often occurring in concert. In this study, we assessed the effect of sorafenib on the UPR and autophagy and on HCC growth in combination with modulation of these cellular adaptation processes.

Sorafenib activates the IRE1/XBP1/RIDD and PERK/eIF2α/ATF4/GADD34 pathways, but reduced GRP78 protein expression. Since IRE1 knockdown increased induction of apoptosis by 20 μM sorafenib in MHCC97-L and PLC/PRF/5 HCC cells [16], we hypothesized that IRE1 inhibition could boost the effect of sorafenib. Surprisingly, a small molecule inhibitor binding the IRE1 RNase domain [33] attenuated the antiproliferative effect of sorafenib in HepG2 cells.

In this experiments, IRE1 is still present, upregulated and able to mediate the well-known protein-protein interactions, such as the IRE1-TRAF2 interaction [8]. Another difference is the concentration used, we applied 1-10 μM sorafenib, which is in the range of mean intratumor concentration of 8 μM in case of the recommended oral dose of 400 mg bid [21], [22]. For example, XBP1u mRNA levels decreased from 1 to 5 μM sorafenib, probably by depletion via IRE1-mediated splicing. However, 10 μM sorafenib increased XBP1u mRNA, which might suggest that XBP1s-mediated induction of XBP1u mRNA overruled the IRE1 splicing capacity [9]. Next to the known cytoprotective effects of IRE1 signaling [8], sustained XBP1 splicing is able to induce CHOP expression, cell dysfunction and apoptosis [9], [34]. In addition, the IRE1 RNase performs RIDD on specific target transcripts, a pro-apoptotic process activated by sorafenib [24].

Inhibition of protein synthesis increased cell survival of sorafenib-treated cells, while tunicamycin slightly increased the antiproliferative effect of sorafenib, suggesting ER stress plays a significant role in the effect of sorafenib. Next, we selected two clinically applicable ER stress inducers: the proteasome inhibitor bortezomib and the HIV protease inhibitor nelfinavir. Interestingly, only nelfinavir induces an additive effect in combination with sorafenib in HCC. This effect was also observed in leukemia cells [35]. Because sorafenib was shown to induce apoptosis through induction of growth arrest and DNA damage 45β (GADD45β) and mitochondrial fragmentation [36], which both can be induced by ER stress [37], [38], it would be interesting to investigate whether sorafenib-induced mitochondrial fragmentation and GADD45β expression is dependent on UPR activation.
Sorafenib promotes adaptive autophagy in HCC cells, as shown previously [39]. To inhibit autophagy, we used antimalarial chloroquine and neomacrolide antibiotic clarithromycin [27]. However, combination of sorafenib with these autophagy inhibitors again induces only additive, but no synergistic, effects.

Since sorafenib activates the UPR and thereby induces adaptive protein refolding and autophagy, we evaluated whether triple therapies could improve the antitumor efficacy of sorafenib. Combination of sorafenib with an ER stress inducer and an inhibitor of the observed compensatory mechanisms, adaptive protein refolding or autophagy, synergistically potentiated the antitumor efficacy of sorafenib in vitro. Of these multiple combinations, we tested the combination with nelfinavir and clarithromycin in different HCC models, which showed the strongest antiproliferative effects in vitro. Nelfinavir and clarithromycin synergized with sorafenib to reduce tumor growth in vivo without toxicity.

The p38/ATF2 pathway was recently identified as a mechanism of sorafenib resistance in HCC [7]. Investigating the effect of the proposed triple therapy modulating the induced UPR/autophagy on this sorafenib-resistance pathway may uncover downstream interconnections. For example, oligomerized IRE1 binds TNF receptor-associated factor 2 (TRAF2), activating apoptosis signal-regulating kinase 1 (ASK1) and downstream kinases that activate p38 [8], [40]. Therefore, an altered IRE1 pathway activation pattern may modulate the p38/ATF2 pathway and thereby sorafenib resistance.

A recent phase I trial of the HIV protease inhibitor nelfinavir in adults with solid tumors showed an ER stress-inducing maximum tolerated dose of 6250 mg/day or, for a person of 70 kg, 89 mg/kg/day, which is 2.5 fold over the doses typically used in HIV patients [41]. This human dose correspond to a dose of 1094 mg/kg/day in mice [42], which highly exceeds the dose used in this study. The threshold for ER stress-induced cell death during autophagy inhibition in HCC cells is elevated by omission of sorafenib, however, this can be counterbalanced by increasing the dose of nelfinavir without increased toxicity given its high maximum tolerated dose. Further investigation needs to determine whether daily administration of this dual therapy is feasible and sufficient for sustained antitumor response, as evidenced for sorafenib [3].

Clarithromycin and nelfinavir are inhibitors of cytochrome P450 CYP3A4, which is involved in the metabolism of sorafenib [20], and could thereby increase the tissue concentration of sorafenib in vivo. However, combination of clarithromycin with another CYP3A4 substrate and multi-kinase inhibitor sunitinib showed no effect on the pharmacokinetics of sunitinib [43].
Combined therapy with multiple drugs is a common practice in cancer therapy and can achieve better therapeutic effects than a single drug. Furthermore, the utility of combining approved drugs by rational drug repositioning may be rapidly implemented in HCC patients. Therefore, clinical evaluation of the investigated combinations subverting ER stress towards apoptosis is indicated.
2.1.8. References


2.1.9. Figures

Fig. 1. Sorafenib activates the UPR in HepG2 cells irrespective of pre-existent ER stress. (A) Real-time PCR analysis of chaperones, IRE1 targets and PERK targets. (B) Cells receiving the indicated treatments were harvested for Western blot analysis, and probed for indicated UPR-mediated proteins. Quercetin concentration: 100 μM. (C) Real-time PCR analysis of UPR-mediated genes in the presence of tunicamycin. (D) Real-time PCR analysis of RIDD targets. One way ANOVA test was applied for statistical analysis. Data are presented as mean ± SD of n=4. All experiments were repeated three times with similar results. Concentrations are indicated in μM. Sora= sorafenib. *p<0.05, **p<0.01, ***p<0.001 compared to control. #p<0.05, ##p<0.01, ###p<0.001 compared to 5 μM sorafenib.
Fig. 2. Sorafenib-mediated reduction in tumor cell viability and proliferation depends on proteotoxic stress and the IRE1 RNase. (A) Cell viability as shown by MTT assay. (B) Cell proliferation was assessed by BrdU incorporation. (C) Caspase-3 activity of HepG2 cells treated with the indicated compounds. Data are presented as mean ± SD. These experiments were repeated three times with similar results. One way ANOVA test was applied for statistical analysis. CX= cycloheximide. *p<0.05, **p<0.01, ***p<0.001.
Fig. 3. Combination of sorafenib with an ER stress inducer and an inhibitor of adaptive protein refolding or autophagy potentiates the antitumor activity in vitro. (A) Cell viability, (B) proliferation rate and (C) caspase-3 activity of HepG2 cells. *p<0.05, **p<0.01, ***p<0.001 compared to control; *p<0.05, **p<0.01, ***p<0.001 compared to 10 μM sorafenib alone; §p<0.05, §§p<0.01, §§§p<0.001 compared to the indicated group. Sora=sorafenib, BORT=bortezomib, NEL=nelfinavir, BAC=bacitracin, CQ=chloroquine, CLAR=clarithromycin.
Fig. 4. Combination of sorafenib with an ER stress inducer and an inhibitor of autophagy potentiates the antitumor activity in vivo. (A) Representative photographs of the DEN-treated livers. (B) Tumor burden as assessed by reticulin staining in randomly selected high-power fields. *p<0.05, **p<0.01, ***p<0.001. (C) Effect of indicated treatments on growth of HepG2 xenografts in athymic nude mice (n=6). The tumor volume was measured three times per week for 4 weeks. Values represent the mean ± SD. (D) At the end of the treatment period, animals were sacrificed and xenograft tumor weights were recorded. (E) Schematic model outlining the modulation of sorafenib-induced resistance mechanisms. Sora= sorafenib, NEL 50= nelfinavir 50 mg/kg bid, NEL 250= nelfinavir 250 mg/kg bid, CLAR= clarithromycin.
2.1.1. Supplementary figures

Fig. S1. Sorafenib induces apoptosis and reduces proliferation in human and murine HCC cells irrespective of pre-existent ER stress. (A) Cell viability, (B) caspase-3 activity and (C) proliferation assessment in HepG2, BWTG3 and Hepa1-6 cells. Data are expressed as the percentage of control and presented as the mean ± SD of three separate experiments, each of which was performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 compared to 0 μM sorafenib in control conditions. #p<0.05, ##p<0.01, ###p<0.001 compared to indicated sorafenib concentration.
Fig. S2. IRE1 activator quercetin induces no synergistic antitumor effects in sorafenib-treated HepG2 cells. (A) Effect of 25-100 μM quercetin on the mRNA expression of UPR-mediated genes with and without sorafenib. (B) Proliferation rate. (C) Caspase-3 activity. (D) Cell viability. (E) Proliferation rate. Concentrations are indicated in μM. *p<0.05, **p<0.01, ***p<0.001 compared to sorafenib. Sora 5=sorafenib 5 μM. CQ=chloroquine.
Fig. S3. Sorafenib dose dependently induces autophagy in part via ER stress modulation. (A) mRNA expression of autophagy-related genes and (B) Western blotting for LC3 and P62 in HepG2 cells. Blots are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to saline.
Fig. S4. Antitumor efficacy of sorafenib is potentiated by combination with nelfinavir and clarithromycin in xenograft and orthotopic models for HCC. (A) Hematoxylin/eosin and (B) Reticulin staining of HCC-bearing livers. Scale bar: 100 µm. Arrows indicate cytoplasmic vacuolization. (C) Caspase-3 activity in lysates of HCC-bearing livers, *p<0.05. (D) $^{18}$F-Choline positron emission tomography visualizes cell membrane synthesis.
3. Antimalarial Artesunate and the unfolded protein response in hepatocellular carcinoma

3.1. Therapeutic effects of artesunate in hepatocellular carcinoma: repurposing an ancient antimalarial agent

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Therapeutic effects of artesunate in hepatocellular carcinoma: repurposing an ancient antimalarial agent

3.1.1. Abstract

Objectives

Artemisinins are antimalarial drugs that exert potent anticancer activity. We evaluated the effects of artesunate, a semisynthetic derivative of artemisinin, on tumor growth, angiogenesis, the unfolded protein response, and chemoresistance in hepatocellular carcinoma.

Materials and methods

The effect of artesunate was examined in HepG2 and BWTG3 cells under normoxic and hypoxic conditions and in a diethylnitrosamine-induced mouse model. Histology was performed with hematoxylin/ eosin and reticulin staining. The expression of chemoresistance-related transporters and angiogenic and unfolded protein response factors was determined. Cytotoxicity was assessed by alanine and aspartate transaminase, lactate dehydrogenase, water-soluble tetrazolium salt, and caspase-3 activity assays. Small animal imaging was performed using dynamic contrastenhanced MRI and choline PET to assess tumor progression.

Results

Artesunate dose dependently reduced cell viability (from 50 μmol/l; P< 0.05) and increased caspase-3 activity (P< 0.05) in HepG2 and BWTG3 cells. These effects were enhanced by hypoxia (from 12.5 μmol/l; P< 0.01). Moreover, artesunate downregulated vascular endothelial growth factor and placental growth factor expression in vitro (both P< 0.05) and in vivo (both P< 0.01). In mice, artesunate decreased vessel density and tumor burden (both P< 0.05). These in-vivo effects were enhanced by combination with sorafenib (P< 0.05 and P= 0.07, respectively), without apparent hepatotoxicity. Furthermore, artesunate modulated the unfolded protein response in vitro and in vivo, increasing proapoptotic signaling, and did not induce doxorubicin chemoresistance.

Conclusion

These findings indicate that artesunate could offer a new approach to the therapy of hepatocellular carcinoma. Clinical trials with artesunate as monotherapy or in combination with current hypoxia-inducing approaches are necessary.
3.1.2. Introduction

Hepatocellular carcinoma (HCC) remains a major health problem and is the third leading cause of cancer-related mortality worldwide. HCC is frequently associated with liver cirrhosis and dysfunction, complicating therapeutic strategies [1]. Resection and transplantation are the only curative treatments available. However, only ~20% of patients with HCC are eligible for surgery, and recurrence rates are high [2,3]. Classical chemotherapy is ineffective because HCC cells are chemoresistant, partially because of the increased cellular efflux of cytostatic compounds, including doxorubicin, by transmembrane ATP-binding cassette (ABC) transporters [4–7]. To date, the antiangiogenic multikinase inhibitor sorafenib, which targets Raf and platelet-derived and vascular endothelial growth factor (VEGF) receptor tyrosine kinase signaling, and transarterial embolization are the only approved treatments for advanced disease. Unfortunately, both treatments provide only a limited survival advantage [3], necessitating the development of innovative compounds. A phase III trial of sorafenib showed prolonged survival but only minimal tumor shrinkage, thus increasing the use of functional imaging (e.g. PET) [8].

Artemisinins, compounds extracted from the herb Artemisia annua, are safe and effective antimalarial drugs. Recent studies have suggested that artemisinins also exert antiangiogenic and cytotoxic effects on cancer cells [9]. Dihydroartemisinin, the main active metabolite, exerts antitumor effects against various human cancers, including liver, lung, ovarian, and pancreatic cancer [9,10]. Artesunate (ART) is a water-soluble semisynthetic artemisinin with improved pharmacokinetic properties and is approved by the Food and Drug Administration for the treatment of malaria [11]. The mechanism through which artemisinins inhibit cancer growth is not fully understood, but ferrous iron is considered to induce cytotoxic radicals by reductive scission of the endoperoxide bridge of artemisinins [9]. Blocking the transferrin receptor by specific monoclonal antibodies abrogates the antitumoral activity of ART [9]. Because the liver serves as an iron storehouse, we expect ART to be active in hepatocytes. Moreover, continuous HCC cell growth requires high iron metabolism, and cancer cells show increased transferrin receptor expression. As transferrin receptors are responsible for the uptake and regulation of intracellular iron [12], ART may have greater efficacy in cancer cells.
In addition to oxidative stress, ART inhibits angiogenesis and causes calcium dysregulation [9,13]. These effects may induce the unfolded protein response (UPR), which stimulates cellular adaptation to stress or, if the stress is too severe, apoptosis. In addition, the UPR regulates chemoresistance and angiogenesis and is activated in HCC [14–16]. Three UPR transducers have been identified: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) [17]. In the absence of misfolded proteins, these stress sensors exist in an inactive state in association with binding immunoglobulin protein (BiP). During endoplasmic reticulum stress, misfolded proteins sequester BiP, allowing sensor activation. PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to transcription factor 4 (ATF4) and C/EBP homologous protein transcription factor (CHOP) activation, growth arrest, and DNA damage-inducible protein (GADD34) expression [17]. Furthermore, dihydroartemisinin induces CHOP in HepG2 cells [9]. The effects of ART on HCC growth, angiogenesis, UPR induction, and chemoresistance in vitro and in vivo are unknown.

In this study, we tested the hypothesis that ART has antitumor activity in HCC and elucidated potential modes of action, such as the induction of tumor hypoxia and endoplasmic reticulum stress. We then evaluated dual therapy with sorafenib (ART/Sora) and the effect of ART on chemoresistance.
3.1.3. Materials and methods

Cell culture

The human HepG2 (HB-8065; ATCC, Manassas, Virginia, USA) and mouse BWTG3 [18] cell lines were cultured in DMEM supplemented with 10% FBS (Life Technologies, Ghent, Belgium) and incubated at 37°C in a water-saturated atmosphere of 5% CO₂/95% air. Cells were seeded in six-well plates at 2.5×10⁶ cells/well and incubated for 1 or 4 days with different ART concentrations (provided by Dafra Pharma Research, Turnhout, Belgium); equal volumes of PBS and ethanol were included as controls. Cell density was assessed daily. Selective PERK (Toronto Research Chemicals, Toronto, Canada) and IRE1 (4μ8C; Calbiochem, Billerica, Massachusetts, USA) inhibitors were applied at concentrations of 0.1 and 7 μmol/l, respectively. Each experiment was conducted in quadruplicate under normoxic and hypoxic conditions (1% oxygen in a hypoxia chamber, AnaeroGen; Oxoid, Basingstoke, UK). Subsequently, the cells were lysed for RNA extraction.

Animals

Wild-type 129S2/SvPasCrl mice were purchased from Charles River (Brussels, Belgium) and maintained as described previously [19]. Five-week-old males received weekly intraperitoneal saline or diethylnitrosamine (DEN) (35 mg/kg, in saline) injections [20]. After 25 weeks, the mice were divided randomly into six groups (each n=14) including two control groups [intraperitoneal injections with 100μl of saline once daily with or without ART (30mg/kg/day)] and four DEN-treated groups [saline, ART, intragastric sorafenib (Bayer, Diegem, Belgium) once daily (10 mg/kg/day) or ART/Sora]. After 30 weeks, blood was collected from the ophthalmic artery under isoflurane anesthesia and the mice were euthanized. Pimonidazole HCl (Hypoxyprobe-1; Natural Pharmacia International Inc., Burlington, Massachusetts) was administered intraperitoneally to four random mice per group at a single dose of 60 mg/kg 1 h before they were killed. After macroscopic and microscopic assessment, all organs were fixed in 4% phosphate-buffered formaldehyde (Klinipath, Olen, Belgium) and embedded in paraffin or snap frozen in liquid nitrogen. Hematoxylin/eosin and reticulin staining was performed to assess tumor burden (size×number) and evaluated by two independent observers. All animals received humane care, and all protocols were approved by the Ethical Committee of Experimental Animals at the Faculty of Health Sciences, Ghent University, Belgium (ECD 11/52).
WST-1 and LDH cytotoxicity assays

HepG2 cells were seeded in 96-well plates at 10^4 cells/well in DMEM supplemented with 10% FBS and stimulated with an ART dilution series or 1% Triton X-100. After 24 h, 100 µl of supernatant was collected for lactate dehydrogenase (LDH) measurement according to the manufacturer’s protocol (Biovision, Milpita, California, USA). Cell viability was determined using water-soluble tetrazolium salt 1 (WST-1) (Roche Diagnostics, Mannheim, Delaware, USA). After supernatant collection, the reagent was added and cells were incubated for 4 h at 37°C. The absorbance of the bioreduced WST-1 (formazan) was measured at 450 nm against a background control at 655 nm (Multiskan Ascent, Leuven, Belgium). Experiments were conducted in quadruplicate.

Detailed information on the caspase-3 activity assay, total RNA extraction, quantitative real-time PCR, western blotting, immunohistochemistry, enzyme-linked immunosorbent assay and dynamic contrast-enhanced MRI (DCE-MR), and choline PET imaging is provided in the Supplementary Materials and methods section.

Statistics

Statistical analysis was carried out using SPSS 21 (SPSS Inc., Chicago, Illinois, USA). Values are presented as the mean ± SEM or log 2[fold change (FC)] relative to the average expression in controls. Variables were tested for normality using the Shapiro–Wilk test. Normally distributed data were subjected to the unpaired Student’s t-test. Non-normally distributed data were tested using the Mann-Whitney U-test. Two-tailed probabilities were calculated; a P-value less than 0.05 was considered statistically significant. Survival was analyzed using the log-rank test.
Chapter 3

3.1.4. Results

Dose-dependent and time-dependent inhibitory effects of ART on tumor cell viability

To assess the effects of ART on HCC cells, cell viability, cytotoxicity, and caspase-3 activity were assessed in HepG2 and BWTG3 cells treated with different ART concentrations. The WST-1 assay indicated that ART dose dependently decreased HepG2 cellular metabolic activity and, thus, cell viability (Fig. 1a, P<0.05 at 50 μmol/l). Because liver tumors rapidly outgrow their vascular supply and become hypoxic, all experiments were conducted under normoxic and hypoxic conditions [21]. In HepG2 and BWTG3 cells, hypoxia enhanced the reduction in cell viability [Fig. 1b (P<0.05 at 12.5 μmol/l) and Fig. 1Sb (P<0.001 at 12.5 μmol/l), respectively].

Dose-dependent cellular cytotoxicity was measured by LDH released into the supernatant and observed at 25 and 50 μmol/l ART in HepG2 cells (Fig. 1c; P<0.01 and P<0.001, respectively) and 50 μmol/l ART in BWTG3 cells (Fig. 1Sc, P<0.05).

The activity of caspase-3, a downstream effector of the intrinsic and extrinsic apoptotic pathways, time dependently increased after 24 and 48 h of incubation with 50 μmol/l ART in HepG2 cells, indicating induction of apoptosis (Fig. 1e, P<0.05 after 24 h). All of these effects led to a significant reduction in the number of HepG2 cells by 25 and 50 μmol/l ART after 4 days of incubation (P<0.01, Fig. 1f).

ART monotherapy and ART/Sora show antitumor efficacy in a mouse model of HCC

To confirm the antitumor activity of ART observed in our in-vitro studies, we evaluated its antitumor activity in an HCC mouse model. Twenty-five weeks of DEN administration reduced the body weight of mice when compared with controls (P<0.001). Subsequent treatment with ART or ART/Sora for 5 weeks did not significantly alter the average body weight when compared with vehicle (Table 1). ART did not increase mortality in saline-injected mice; in DEN-injected mice with HCC, survival rates were slightly, but not significantly, increased by ART or ART/Sora therapy (P>0.05, 71.43, 85.71, and 82.62%; Fig. 2a). Furthermore, neither ART nor sorafenib induced apparent hepatotoxicity as the serum alanine aminotransferase and aspartate aminotransferase levels were equally increased compared with vehicle-treated mice after administration of DEN (Fig. 2b). Macroscopic evaluation (Fig. 3a) and tumor burden quantification (size×number), as determined by reticulin staining (Fig. 2c), showed reduced tumor number (Fig. 2d) and burden (Fig. 2e) in ART-treated mice (both P<0.05).
In addition, although sorafenib did not enhance the ART-induced reduction in HCC nodules (P=0.76, Fig. 2c), a borderline significant additive effect on tumor burden was observed microscopically (P=0.07, Fig. 2e). Interestingly, 25 weeks of DEN administration significantly increased transferrin receptor mRNA levels (1.93 FC compared with 25 weeks of saline, P<0.01) and protein levels (Fig. 2Sg).

Caspase-3 activity levels were increased by administration of DEN (Fig. 2f, P<0.05) and significantly increased in HCC livers after ART monotherapy compared with controls (P<0.01). Administration of sorafenib also increased caspase-3 activity (P<0.05) and an additive effect was observed after ART/Sora (P<0.001).

Functional choline PET and DCE-MRI studies were used to visualize cellular membrane biosynthesis [22] and time-dependent contrast uptake, respectively. Choline PET showed a reduced number of loci with high mean standardized uptake values according to the PERCIST criteria [23] after the administration of ART and ART/Sora compared with saline (Fig. 3b). DCE-MRI showed smaller and reduced numbers of nodules with low vascularization following the administration of ART and ART/Sora (Fig. 3c).

ART decreases tumor angiogenesis in HCC

In addition to its direct effect on tumor cell viability, the antitumor efficacy of ART has been associated with antiangiogenic activity [9,13]. Therefore, we assessed the effects of ART on the expression of key angiogenic factors VEGF and placental growth factor (PlGF), intratumoral hypoxia, and vessel density in this HCC model. After 25 weeks of DEN administration, Vegf mRNA and protein levels were upregulated (P<0.001 and P<0.01, respectively; Fig. 2S and Fig. 4a). An increase in vessel density after the administration of DEN further led to neovascularization, as shown by CD105 immunohistochemistry (1.49 FC, P<0.001, Fig. 4b–d). However, pimonidazole binding showed reduced oxygen levels within the tumor nodules, suggesting the formation of dysfunctional vessels.

Remarkably, Plgf and Vegf mRNA (P<0.01 and P<0.05, respectively; Fig. 2S) and protein levels (P<0.05 and P<0.01, respectively; Fig. 4a) were reduced after 5 weeks of ART treatment compared with controls. Moreover, ART decreased the vessel density in the tumor (Fig. 4b) and surrounding tissue (Fig. 4c) compared with vehicle (both P<0.05). ART/Sora reduced angiogenesis more than ART monotherapy, but the difference was significant only in the tumor tissue (P<0.05).
Reduced vascularization was associated with increased nodular hypoxia in ART-treated and ART/Sora-treated mice compared with vehicle-treated mice (Fig. 4e). Finally, the addition of 25 μmol/l ART for 24 h to HepG2 cells under hypoxia reduced VEGF and PlGF secretion (both P<0.05) (Fig. 2Sb), suggesting that these effects are cell intrinsic.

ART modulates the UPR and ABC transporter induction in vitro and in vivo

Because the induction of endoplasmic reticulum stress is a proposed ART mechanism of action [9], we evaluated the expression of key markers of the three UPR pathways in vitro and in vivo. In HepG2 cells, ART upregulated ATF4 and CHOP mRNA (both P<0.05, Fig. 3Sa) and protein (Fig. 2Sf) under normoxia. In addition, induction of these PERK targets was observed in BWTG3 cells (Fig. 4Sa). In mice, ART increased the levels of Atf4 mRNA (P<0.01, Fig. 3Sa) and phospho-eIf2α (P<0.01, Fig. 3Sb), Atf4 (P<0.01), Chop (P<0.05), and Gadd34 (P=0.12) protein expression (Fig. 3Sc). No changes were observed in IRE1-mediated X-box-binding protein 1 (XBP1) splicing activity in vitro or in vivo. Remarkably, in HepG2 cells, 25 μmol/l ART reduced the mRNA levels of BiP and other ATF6 pathway targets, protein disulfide isomerase family A, member 4 (PDIA4), and unspliced XBP1. Western blot analysis showed reduction of BiP by ART therapy in vitro (Fig. 2Sf) and in vivo (Fig. 3Sc). Importantly, VEGF and PlGF expression and the reduced cell viability induced by ART were not altered by an IRE1 or a PERK inhibitor in HepG2 cells despite their effective inhibition of XBP1 splicing and CHOP expression (Fig. 2Sb–f). Therefore, ART attenuates the expression of chaperones, including BiP and PDIA4, and the cytoprotective ATF6 pathway, and increases certain PERK targets (e.g. the proapoptotic protein CHOP).

ABC transporters determine cell viability following chemotherapy, and doxorubicin is the most commonly used chemotherapeutic agent for HCC [6,24,25]. Because hypoxia is a well-known ABC transporter inducer and because reduced oxygen levels were observed after ART therapy, we examined the effects of ART on the chemoresistance profile in HCC, which is primarily determined by the ABCB1 and ABCG2 levels [24,26]. Furthermore, we examined the effects of the ART-induced expression pattern by exposing HepG2 cells to ART for 4 h before incubation with doxorubicin for 24 h. In HepG2 cells, ABCB1 and ABCG2 were decreased by ART under normoxia, but not hypoxia (P<0.01, Fig. 3Sd). In BWTG3 cells, only Abcg2 was decreased (P<0.05, Fig. 4Sb).

In addition, the expression of other ABC transporters implicated in chemoresistance, for example ABCC10, was altered [6]. ART pretreatment did not alter doxorubicin cytotoxicity (Fig. 2Sc), excluding direct resistance induction.
In mice, ART decreased Abcg2 and did not alter Abcb1 at the mRNA (Figs 3Sd and 4Sb) and protein levels [ABCG2: P<0.05; ABCB1 (Fig. 3Se): NS]. Only Abcc6 mRNA was upregulated (P<0.05).
3.1.5. Discussion

To date, only sorafenib has provided limited benefits for advanced HCC. ART is an approved treatment option for malaria and is well tolerated by patients at therapeutic doses of $\sim 5$ mg/kg/day [27,28]. The antitumor activity of ART has been described [9,29,30], but its biological activity has not been completely elucidated. In this study, ART (30 mg/kg/day) exerted potent cytotoxic effects on HCC in vitro and in vivo without inducing body weight reduction, hepatotoxicity, or increased mortality in an orthotopic mouse model of HCC.

The effects of ART on angiogenesis and VEGF downregulation have been reported in other cell types [31,32]. We showed that ART reduced the angiogenic factors VEGF and PlGF in vitro and in vivo and decreased tumor angiogenesis in a mouse model [19]. Because the UPR is crucial for angiogenesis regulation and hypoxia is a well-known endoplasmic reticulum stress inducer, we evaluated the effects of ART on the UPR and, consequently, angiogenic factors [33]. ART fortified the PERK pathway in HCC. However, the addition of a selective PERK inhibitor did not counteract the ART-mediated reduction of VEGF or PlGF. In addition, ART did not affect the IRE1 pathway, and in-vitro IRE1 inhibition did not alter its effects on angiogenic factors or cell viability. These data suggest that ART-induced reductions in tumor angiogenesis could be dependent on the UPR, but upstream selective pathway inhibition was insufficient, confirming the known functional redundancy of the UPR. Validation in knockout models of downstream UPR factors is required.

Remarkably, the antitumor activity of ART was increased under hypoxia. In addition, because hypoxic conditions are more distinctly present in intratumoral regions than in healthy livers [34], this finding also allows for dual therapy with ART and conventional hypoxia-inducing therapeutic strategies, for example transarterial embolization [34] and sorafenib therapy [35]. Because we previously showed the antitumor efficacy of sorafenib in DEN-induced HCC [36], we investigated combined ART/Sora and showed that it exerted an additive effect on the microscopic tumor burden and further reduced tumor neovascularization compared with monotherapy.

Notably, macroscopic tumor counting in the ART/Sora group could have been biased by difficulty in identifying smaller nodules in contrast to the quantification of tumor burden determined by reticulin staining. In addition, no significant alterations were observed in the vascularization of the nontumoral liver tissue by ART/Sora compared with ART monotherapy. Apparently, the different modes of action of ART and sorafenib allow them to act in parallel. Further investigation to determine downstream interactions is needed.
In the mouse model, ART decreased tumor neovascularization. Moreover, enhanced antiproliferative activity was observed under hypoxia in HCC cell lines. Consequently, we hypothesize that a positive feedback loop exists: repeated ART administration reduces angiogenesis, increasing intratumoral hypoxia and thus enhancing ART efficacy (Fig. 4f).

Given the modest effects of ART on ABC transporters in the mouse model and the unaltered doxorubicin cytotoxicity after pretreatment with ART in vitro, there is no indication for combining ART with chemotherapeutics such as doxorubicin for HCC therapy or to presume enhanced chemoresistance after ART therapy. Moreover, although doxorubicin is a substrate, the relevance of the only in-vivo upregulated ABC transporter, Abcc6, to chemoresistance is questionable [4]. Finally, in line with findings in non-small-cell lung cancer [37], Abcg2 was significantly reduced by ART. In this study, the effect of ART on ABC transporter expression was examined. Future studies should also investigate efflux pump activity.

3.1.6. Conclusion

ART, as a monotherapy or in combination with sorafenib, exerted antitumor effects in experimental HCC models, and ART/Sora therapy was more effective than sorafenib alone and did not show cumulative toxicity. Experience in the use of ART and sorafenib in clinical practice is a considerable advantage. Furthermore, as a safe and low cost drug, ART might be of particular interest for developing countries with a high incidence of HCC [3, 38]. These results collectively suggest that ART could be a promising candidate drug for HCC treatment.

3.1.7. Acknowledgements

The authors thank the Department of Biostatistics, Ghent University, for support with statistical analysis.

3.1.8. Funding

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3.1.9. References


Fig. 1 Artesunate (ART) reduces proliferation and increases cytotoxicity in HepG2 cells. Cells were incubated for 24 h with 6.25–50 µmol/l ART or PBS or ethanol as a control. (a) Assessment of cell viability by optical density (OD) under normoxia and (b) hypoxia in HepG2 cells. (c) Assessment of cytotoxicity by LDH release in HepG2 under normoxia and (d) hypoxia. 1% Triton X-100 = positive control. (e) Assessment of caspase-3 activity over time in HepG2 cells treated with 50 µmol/l ART. (f) Incubation of HepG2 cells with ART for 4 days with a daily assessment of cell density. *P< 0.05, **P < 0.01, ***P <0.001.
Fig. 2 Artesunate (ART) shows antitumor activity in an hepatocellular carcinoma (HCC) mouse model. (a) Kaplan–Meier survival curve. (b) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. (c) Tumor number and (d) tumor burden. (e) Caspase-3 activity in saline (S)-treated mouse liver tissue and tumor nodules from diethylnitrosamine (DEN)-treated mice. (f) Representative reticulin-stained slides showing the absence of reticulin in HCC. Following ART, smaller nodules with less defined tumor borders were observed. Scale bars =100 µm. *P < 0.05, **P <0.01, ***P <0.001. Sora, sorafenib.
Fig. 3 Structural and functional imaging of the hepatocellular carcinoma model. (a) Representative liver images. (b) Representative images of choline PET and (c) DCE-MRI. ART, artesunate; DEN, diethylnitrosamine; Sora, sorafenib.
Fig. 4 Artesunate (ART) shows antiangiogenic properties in the hepatocellular carcinoma (HCC) model. (a) Protein concentrations of Vegf and Plgf (pg/ml) in liver lysates. (b) Assessment of vessel density by CD105 staining in tumor nodules and (c) surrounding tissue. (d) Representative CD105 staining of liver tissue after different treatments. (e) Pimonidazole staining. Scale bars =100 µm; S, saline; *P < 0.05, **P <0.01 and ***P <0.001. (f) Schematic representation of the modes of action of ART related to modulation of the unfolded protein response and hypoxia. DEN, diethylnitrosamine; Sora, sorafenib.
### Table 1  Mouse body weights (g) (mean ± SEM)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average body weight 25 weeks (g)</th>
<th>Average body weight 30 weeks (g)</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>27.22 ± 1.32</td>
<td>26.03 ± 1.61</td>
</tr>
<tr>
<td>Saline + ART</td>
<td>26.31 ± 2.28</td>
<td>25.62 ± 2.03</td>
</tr>
<tr>
<td>DEN + saline</td>
<td>19.75 ± 1.76***</td>
<td>18.30 ± 1.92</td>
</tr>
<tr>
<td>DEN + ART</td>
<td>20.86 ± 1.45</td>
<td>10.17 ± 2.10 (NS)</td>
</tr>
<tr>
<td>DEN + sorafenib</td>
<td>22.96 ± 3.65</td>
<td>17.92 ± 3.19 (NS)</td>
</tr>
<tr>
<td>DEN + ART/sorafenib</td>
<td>18.53 ± 2.13</td>
<td>17.43 ± 3.61 (NS)</td>
</tr>
</tbody>
</table>

ART, artesunate; DEN, diethylnitrosamine; NS, not significant compared with DEN + saline.

***$P < 0.001$: 25 weeks DEN versus saline.
3.1.12. Supplementary figures

Fig. 1S: ART dose dependently reduces proliferation and increases cytotoxicity in BWTG3 cells. Cells were incubated for 24 hours with 6.25-50 μM of ART or PBS or ethanol as a control. (A) Assessment of cell viability under normoxia and (B) hypoxia. (C) Assessment of LDH release under normoxia and (D) hypoxia. 1% Triton-X100= positive control. *p<0.05, ***p<0.001.
Fig. 2S: The effect of ART on angiogenic factors and cell viability is independent of PERK and IRE1 signaling. (A) Relative VEGF and PlGF mRNA expression in the mouse model. (B) Protein concentrations of VEGF and PlGF in supernatant from HepG2 cells incubated with ART and a PERK or IRE1 inhibitor. (C) Assessment of cell viability. Relative mRNA levels of (D) XBP1s and (E) CHOP in HepG2 cells. (F) Western blotting of lysates from HepG2 cells with anti-BiP and anti-CHOP antibodies. ART=Artesunate; S=Saline; *p<0.05, **p<0.01 and ***p<0.001.
Fig. 3S: The mRNA and protein levels of UPR- and chemoresistance-related proteins. (A) The mRNA log2(fold change) of UPR-related genes in HepG2 cells following 25 μM ART compared with saline treatment, and the mRNA log2(fold change) of UPR-related genes in the mouse HCC model following 5 weeks of 30 mg/kg per day ART compared with saline administration. (B) Representative immunohistochemistry images of phospho-eIf2α, which was increased (p<0.01) by ART treatment. Positive controls received only intraperitoneal tunicamycin for 72 hours. (C) Western blotting of lysates from liver tissue after saline administration or tumors after DEN administration, as described in the Materials and Methods. The outer right lane in the blot for BiP represents a positive control. The mean densitometric values are shown below each group. (D) mRNA log2(fold change) of chemoresistance-related genes compared with those following saline administration. (E) Abcb1 expression was unaltered, whereas Abcg2 expression was decreased (p<0.05) by ART. Scale bars: 100 μm. (F) Western blot of Abcc1. Experiments were repeated twice with similar results. *p<0.05 and **p<0.01.
Fig. 4S: The mRNA and protein levels of UPR- and chemoresistance-related proteins in BWTG3 cells. (A) The mRNA log2(fold change) of UPR- and (B) chemoresistance-related genes following 25 μM ART compared with saline treatment. *p<0.05, **p<0.01 and ***p<0.001.
4. **PlGF inhibition and the UPR in hepatocellular carcinoma.**

4.1. Placental growth factor inhibition modulates the interplay between hypoxia and the unfolded protein response in hepatocellular carcinoma.

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Placental growth factor inhibition modulates the interplay between hypoxia and unfolded protein response in hepatocellular carcinoma

4.1.1. Abstract

Background. Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality. We previously showed that the inhibition of placental growth factor (PIGF) exerts antitumour effects and induces vessel normalisation, possibly reducing hypoxia. However, the exact mechanism underlying these effects remains unclear. Because hypoxia and endoplasmic reticulum stress, which activates the unfolded protein response (UPR), have been implicated in HCC progression, we assessed the interactions between PIGF and these microenvironmental stresses.

Methods. PIGF knockout mice and validated monoclonal anti-PIGF antibodies were used in a diethylnitrosamine-induced mouse model for HCC. We examined the interactions among hypoxia, UPR activation and PIGF induction in HCC cells.

Results. Both the genetic and pharmacological inhibitions of PIGF reduced the chaperone levels and the activation of the PKR-like endoplasmic reticulum kinase (PERK) pathway of the UPR in diethylnitrosamine-induced HCC. Furthermore, we identified that tumour hypoxia was attenuated, as shown by reduced pimonidazole binding. Interestingly, hypoxic exposure markedly activated the PERK pathway in HCC cells in vitro, suggesting that PIGF inhibition may diminish PERK activation by improving oxygen delivery. We also found that PIGF expression is upregulated by different chemical UPR inducers via activation of the inositol-requiring enzyme 1 pathway in HCC cells.

Conclusions. PIGF inhibition attenuates PERK activation, likely by tempering hypoxia in HCC via vessel normalisation. The UPR, in turn, is able to regulate PIGF expression, suggesting the existence of a feedback mechanism for hypoxia-mediated UPR that promotes the expression of the angiogenic factor PIGF. These findings have important implications for our understanding of the effect of therapies normalising tumour vasculature.
4.1.2. Background

Hepatocellular carcinoma (HCC) ranks as the second leading cause of cancer-related mortality worldwide [1]. Conventional chemotherapy is ineffective, and targeted therapy for advanced HCC with sorafenib, which targets Raf and platelet-derived and vascular endothelial growth factor (VEGF) receptor tyrosine kinase signalling, shows only a limited survival benefit [2].

The VEGF signalling pathways play central roles in angiogenesis [3]. VEGF-A binds to two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Most of the biological effects of VEGF-A are mediated by VEGFR-2 [3]. The placental growth factor (PlGF, four isoforms: PlGF-1-4) binds to VEGFR-1 and induces responses in endothelial, malignant, and immune cells [4]. VEGFR-1 has weak tyrosine kinase activity but a substantially higher binding affinity for VEGF-A than VEGFR-2. Although VEGFR-1 may act as a trap for VEGF-A, it also transmits signals in response to PlGF via its tyrosine kinase domains [4], [5]. A role for VEGFR-1 during tumour angiogenesis has been suggested [5], [6]. VEGFR-1 expression in HCC tissues is higher than that in peritumoural tissues and correlates with worse survival after resection [7], [8]. Importantly, genetic or pharmacological inhibition of PlGF reduces tumour growth and induces vessel normalisation in different preclinical models, including the diethylnitrosamine-induced HCC model [5], [9], [10]. Although anti-PlGF antibodies are controversial [11], evidence for the dose and specificity of the anti-PlGF-2 antibody clone 5D11D4 was previously provided [5]. Furthermore, disease stabilization for 12 months has been observed with anti-human PlGF monoclonal antibody TB403 in 2 out of 23 patients with advanced solid tumours refractory to standard therapy, confirming the need for a better understanding of the effect of PlGF inhibition on tumour biology [12].

The endoplasmic reticulum (ER) consists of a membranous network in which proteins are synthesised, post-translationally modified and folded. Therefore, the lumen houses chaperones, including protein disulfide isomerase A4 (PDIA4), calnexin (CANX), glucose-regulated protein-78 (GRP78) and -94 (GRP94) [12–14]. Several perturbations in the protein folding, such as hypoxia, glucose deprivation and oxidative stress, lead to the accumulation of unfolded proteins in the ER, a phenomenon called ER stress. ER stress triggers the unfolded protein response (UPR), which leads to an adaptive transcriptional response involved in protein quality control, redox homeostasis and angiogenesis. Paradoxically, the UPR also coordinates pro-apoptotic responses to ER stress [13], [14]. Interestingly, ER stress is present in human and experimental HCC, and modulating the UPR could hold important therapeutic potential [16], [17].
Three major ER stress sensors have been identified, as follows: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) [13]. The effect of ATF6 on cell fate is primarily cytoprotective, whereas the effect of IRE1 and PERK is presumed to be both pro-adaptive and pro-apoptotic [13], [14], [18]. However, inhibition of the PERK pathway induces antitumour effects in experimental HCC [14]. Following the release of GRP78, PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α), leading to the attenuation of global translation. However, the translation of certain transcripts, such as activating transcription factor 4 (ATF4), is favoured. ATF4 induces genes involved in protein quality control, amino acid biosynthesis and the induction of apoptosis via C/EBP homologous protein (CHOP) [13]. IRE1 activation results in X-box-binding protein 1 (XBP1) mRNA splicing to generate a more active spliced XBP1 (XBP1s), which induces the genes involved in protein folding, such as endoplasmic reticulum DnaJ homolog 4 (ERDJ4) and CANX [19]. ATF6 is mobilised to the Golgi, where it is cleaved, releasing a transcriptionally active fragment, which in turn induces the expression of homocysteine-responsive ER-resident ubiquitin-like domain member 1 (HERPUD1), unspliced XBP1 (XBP1u) and chaperones including PDIA4 [12, 18].

In this study, we investigated whether vessel normalisation induced by PlGF blockade modulates the activation of the UPR or oxygen levels in experimental HCC and whether PlGF expression is regulated by ER stress. Collectively, we revealed that PlGF inhibition reduced hypoxia and the activation of the PERK pathway of the UPR in the tumour nodules of the carcinogen-induced mouse model. Furthermore, PlGF expression was upregulated by divergent ER stress stimuli in vitro. These results provide important insight into the reciprocal interactions between PlGF and the tumour microenvironment.
4.1.3. Materials and Methods

**Animals**

Wild type 129S2/SvPasCrl mice were purchased from Charles River (Belgium), and PI GF/- knockout (PIGFKO) 129S2/SvPasCrl mice were obtained from the laboratory of Angiogenesis & Neurovascular link (Leuven, Belgium). Both were maintained as previously described [5]. All mice were genotyped by PCR before the start of the experiments. PI GF-deficient mice are born at normal Mendelian ratios and do not show any obvious vascular anomalies [20]. Five-week-old males received weekly intraperitoneal saline or diethylnitrosamine (DEN) (35 mg/kg, in saline) injections [21]. A murine anti-PI GF monoclinal antibody (validated clone 5D11D4 [5]; referred to as aPI GF) was obtained from Thrombogenics (Leuven, Belgium). Wild type mice that received DEN for 25 weeks were subsequently treated for 5 weeks with aPI GF (intraperitoneally, 25 mg/kg; 2x/week) or IgG (same regimen, n=10 in each group). Wild type mice that received saline for 25 weeks were subsequently treated for 5 weeks with aPI GF (same regimen) or IgG (same regimen, n=10 in each group). Pimonidazole HCl (Hypoxyprobe-1 Inc., Burlington, MA, USA) was intraperitoneally administered to 4 random mice per group in a single dose of 60 mg/kg one hour before sacrifice. Male PIGFKO mice and their wild type littermates received DEN for 30 weeks (n=12 in each group). After 30 weeks, blood was collected from the retro-orbital sinus under isoflurane anaesthesia. After macroscopic evaluation and the quantification of the number of hepatic tumours with a minimum diameter of 2 mm, the livers were fixed in 4% phosphate-buffered formaldehyde (Klinipath) and embedded in paraffin or snap frozen in liquid nitrogen. Tumour nodules were isolated by microdissection (Carl Zeiss, Bernreid, Germany) for expression analysis. Haematoxylin/eosin and reticulin staining were performed to assess the tumour burden, and the results were assessed by 2 independent observers. All protocols were approved by the Ethical Committee of experimental animals at the Faculty of Health Sciences, Ghent University, Belgium (ECD 11/52).

**Cell culture**

HepG2 cells (HB-8065; ATCC, Virginia, USA) were cultured in DMEM supplemented with 10% foetal bovine serum (Life Technologies, Ghent, Belgium).
Cells were incubated for 24 h or 48 h with a PERK inhibitor (0.3 μM; GSK2656157, NoVi Biotechnology, Shandong, China), an IRE1 inhibitor (8 μM; 4μ8C, Calbiochem, Massachusetts, USA), tauroursodeoxycholic acid (TUDCA, 1 mM), tunicamycin (1 μM), thapsigargin (150 nM) or quercetin (100-300 μM) and compared to equal volumes of solvent. All reagents were obtained from Sigma (Diegem, Belgium) unless stated otherwise. Hypoxic atmosphere (1% oxygen) was established in a hypoxic chamber (AnaeroGen; Oxoid, Basingstoke, UK). Experiments were carried out in quadruplicate and independently repeated three times.

Detailed information regarding total RNA extraction, quantitative real-time PCR, Western blotting, and immunohistochemistry is provided in the Supplementary Materials and Methods.

**Statistics**

Statistical analyses were performed using SPSS 21 (SPSS, Chicago, USA). Values are presented as the means ± SD or fold change relative to the mean expression in controls. Kolmogorov-Smirnov test was used to test for normality. Normally distributed data were subjected to the unpaired Student’s t-tests. Multiple groups were compared by one-way analysis of variance (ANOVA) with Bonferroni correction. Non-normally distributed data were tested using the Mann-Whitney U-test. Two-tailed probabilities were calculated; a p-value less than 0.05 was considered statistically significant.
4.1.4. Results

**PIGF inhibition induces antitumour effects and vessel normalisation in experimental HCC**

First, we validated the previously reported antitumour effects and vessel normalisation induced by PIGF blockage [5, 9]. When wild type mice with established HCC were treated with aPIGF (n=10) or IgG (n=10) from 25 weeks onward for 5 weeks, 20% of mice receiving control IgG died, whereas only 10% died in the aPIGF group. Additionally, aPIGF-treated mice developed fewer nodules per liver (all sizes: 17.6 ± 4.9 after IgG versus 12.7 ± 3.2 after aPIGF; p<0.05). After 30 weeks of DEN administration to wild type (n=12) or PlGFKO (n=12) mice, 25% of wild type mice compared to 16% of PlGFKO mice succumbed, and fewer tumour nodules per liver were observed in PlGFKO mice (22.4 ± 4.8 in wild type versus 15.8 ± 6.2 in PlGFKO; p<0.05). Furthermore, several capillaries in control HCC nodules had an abnormal shape and size (Fig. S1A). In PIGF-blocked tumours, fewer capillaries, as shown by endoglin staining, were tortuous (aPIGF: p<0.05 and PlGFKO: p<0.01; Fig. S1B). These results confirm that PIGF blockage induces antitumour effects and partially normalises the abnormal tumour vessel structure.

**PIGF inhibition reduced chaperone expression and activation of the Perk pathway in experimental HCC**

We among others previously described the UPR pattern in DEN-induced HCC [16]. Here, we evaluated the effect of PIGF inhibition on this pattern in isolated tumours. The administration of aPIGF downregulated the mRNA expression of the ER stress-induced chaperones Grp78 and Grp94 in the tumours, compared to the IgG group (p<0.05; Fig. 1A). Additionally, the PlGFKO mice that received DEN for 30 weeks showed reduced levels of Grp78 (p<0.05) and Grp94 (p<0.05) in the tumours compared to their wild type littermates. Western blotting demonstrated reduced protein expression of Grp78 in the tumours of the aPIGF-treated and PlGFKO mice compared to those of the IgG-treated and wild type control group, respectively (Fig. 1B). The Ire1-mediated splicing of Xbp1 was unaltered by PIGF inhibition (Fig. 1C). Accordingly, the targets of Xbp1s, Canx and Erdj4, showed a similar expression level compared to the corresponding controls.
Western blot analysis showed that the Perk-mediated phosphorylation of eIf2α was reduced in the HCC tissues of aPIGF-treated and PIGFKO mice compared to IgG-treated and wild type controls resp. (Fig. 1B and S2). Atf4 mRNA (aPIGF: p<0.05 and PIGFKO: p<0.01; Fig. 1D) and protein (Fig. 1B) expression in the nodules were decreased by PIGF inhibition. Further, Chop mRNA (p<0.05; Fig. 1D) and protein (Fig. 1B) levels were decreased. Next, we assessed the expression of Growth arrest and DNA damage-inducible protein (Gadd34), which initiates eIf2α dephosphorylation leading to a negative feedback loop of the Perk pathway [13]. Gadd34 levels were unaltered (Fig. 1B and 1D), indicating that PIGF inhibition did not enhance this negative feedback loop. Also, the mRNA and protein levels of the UPR sensor Perk itself were unaltered, excluding a direct effect of PIGF on Perk expression (Fig. S3A-B). Overall, these data indicate that PIGF inhibition indirectly diminished Perk signalling in HCC.

To examine the Atf6 pathway, Pdia4 and Herpud1 mRNA expression was monitored (Fig. 1A). Only Pdia4 mRNA was downregulated in the tumours of PIGFKO mice compared to their wild type littermates (p<0.05).

Importantly, wild type mice that received saline for 25 weeks and were subsequently treated with aPIGF for 5 weeks demonstrated no significant differences in the hepatic mRNA expression of the selected UPR targets compared to those receiving control IgG treatment (data not shown). Thus, these results demonstrate that PIGF inhibition reduces the intratumour expression of chaperones, such as Grp78, Grp94 and Pdia4, as well as the activation of the Perk pathway.

**PIGF inhibition reduces intratumour hypoxia**

We previously showed that PIGF inhibition induces vessel normalisation (Fig. S1; [5]). To investigate whether these vascular changes were functionally relevant or, in other words, whether PIGF inhibition effectively increased the oxygen levels in the hepatic tumours of the used mouse model, we applied pimonidazole, a molecule that binds only hypoxic areas *in vivo* and can be detected after sacrifice by immunohistochemistry (Fig. 2A). Indeed, administration of aPIGF significantly reduced tumoural pimonidazole binding (p<0.05; Fig. 2A-B). To improve the quantification method of the binding of pimonidazole, Western blotting for detection of pimonidazole adducts in isolated DEN-induced tumours was performed (Fig. 2C). Densitometry analysis confirmed that the liver tumours were characterized by increased pimonidazole binding and that administration of aPIGF reduced pimonidazole binding in the tumours (p<0.05; Fig. 2C-D).
Finally, aPlGF downregulated the expression of hypoxia-inducible genes *Glut1* (p<0.05) and *Pfk* (p=0.07) in the DEN-induced HCC (Fig. 2E). Thus, aPlGF effectively tempered the induction of tumour hypoxia.

**Hypoxia activates the PERK pathway**

Because PIGF inhibition reduced tumour hypoxia and PERK activation in vivo, we questioned whether hypoxia mediates PERK activation in HCC cells. Therefore, we examined the effect of hypoxia (<1% O<sub>2</sub> or 7.6 mmHg [24]) for 24 h or 48 h on the expression of PERK targets in HepG2 cells (Fig. 3A-B). Hypoxic exposure upregulated the mRNA expression of GRP78 (p<0.001), ATF4 (p<0.05), CHOP (p<0.001) and GADD34 (p<0.001). Furthermore, hypoxic exposure also increased the phosphorylation of eIF2α (24 h: p<0.05 and 48 h: p<0.01; Fig. 3B-C) and protein expression of ATF4 and CHOP (Fig. 3B). These data indicate that hypoxic exposure causes potent activation of the PERK pathway in HCC cells.

**Activation of the IRE1 pathway promotes PIGF expression**

Because the UPR is activated in HCC and PIGF inhibition is able to reduce activation of at least the Perk branch of the UPR, we next analysed the effect of ER stress on PIGF expression in vitro. Therefore, we used two different ER stress inducers: tunicamycin, an inhibitor of protein glycosylation, and thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup> ATPases [13], [25]. Both significantly increased the mRNA levels of PIGF (Fig. 4A). As shown in Fig. 4B, an increase in the expression of faster-migrating unglycosylated PIGF was detected in HepG2 cells treated with tunicamycin.

The addition of the chemical chaperone TUDCA to tunicamycin-treated cells attenuated the ER stress-mediated induction of PIGF mRNA (p<0.01), whereas the addition of TUDCA to untreated cells had no effect on the PIGF mRNA levels (Fig. 4A). Quercetin, an IRE1 activator [26] (Fig. S4A), induced PIGF expression (300 μM: p<0.01; Fig. 4A-B). Accordingly, the addition of a small-molecule inhibitor of the IRE1 pathway reduced the tunicamycin-mediated upregulation of PIGF mRNA (p>0.001, Fig. 4A) and protein (Fig. 4B) levels. In contrast, the addition of a small-molecule inhibitor of the PERK pathway did not affect PIGF expression. These data show that the ER stress-mediated upregulation of PIGF is primary regulated by the IRE1 pathway of the UPR.
4.1.5. Discussion

Growing tumours are often subjected to deficiencies in vital nutrients and oxygen. These inadequate extracellular conditions can adversely affect the environment of the ER and impinge on the maturation of nascent proteins. We recently reported that PlGF inhibition induces vessel normalisation, potentially supporting the delivery of nutrients and oxygen to tumour cells [5], [27].

In this study, we found that PlGF inhibition reduced intratumour hypoxia and ER stress levels (Fig. 5). In fact, PlGF inhibition attenuates the carcinogen-induced upregulation of chaperones, such as Grp78 and Grp94, and the activation of the Perk pathway without affecting Ire1 activation. These chaperones and Perk activation are pro-survival and pro-proliferative modulators in tumour cells [13]–[15]. Probably, the aPlGF-mediated reduction of these UPR factors tempers the aggressive growth of HCC cells.

Recently, hypoxia-inducible factor-1α (HIF-1α), a key transcription factor in the cellular response to hypoxia, was shown to be an important driver of HCC growth [28]. In this study, we showed that PlGF inhibition reduced tumour hypoxia and PERK activation in vivo and that hypoxia activates the PERK/phospho-IF2α/ATF4 cascade in HCC cells, suggesting that tumour hypoxia mediates the observed PERK activation in HCC. Possibly, tumour hypoxia is also involved in the pronounced activation of PERK in other tumour types, such as glioma [29]. Finally, because PERK is, next to hypoxia, able to stimulate tumour growth [13], [30], the normalisation of tumoural oxygen levels by PlGF inhibition is able to dually target pro-survival signalling via reduced activation of the HIF-1α and PERK pathway.

Because PlGF inhibition was previously reported to reduce experimental liver fibrosis [31], the contribution of hypoxia and ER stress modulation, which both have been implicated in fibrogenesis [32], [33], to this outcome requires further investigation.

Whereas the UPR has previously been shown to upregulate several angiogenic factors, including VEGF [34], this is, to our knowledge, the first report to demonstrate the induction of PlGF by ER stress in tumour cells. Because studies on transgenic mice have revealed that PlGF expression is restricted to pathological conditions [35], the further investigation of the role of ER stress in the selectivity of PlGF expression to pathological conditions, potentially characterised by ER stress, is indicated. Finally, the role of the UPR in vessel abnormalisation induced by excessive production of angiogenic factors requires further investigation [35].
Vice versa, the effect of therapies modulating tumour angiogenesis on the UPR activation pattern, which affects tumour growth, is currently unknown. To our knowledge, this is the first study to provide evidence that vessel normalisation regulates the UPR in cancer cells. We speculate that anti-VEGF therapies may exert their therapeutic effect in part by UPR modulation.

The promising preclinical findings of anti-PIGF in HCC but also in other tumour types such as medulloblastoma [36], together with the acceptable safety profile of anti-PIGF administration in Phase I clinical trials, have attracted attention to PIGF as a potential target for therapy. However, improved understanding of the effect on tumour biology is required. This study indicates that anti-PIGF modulates the tumour microenvironment and cell adaptation mechanisms, which have been linked to tumour behavior [13], [37].

4.1.6. Conclusions

In summary, we have shown that inhibition of PIGF tempers UPR activation in HCC, most likely by improved oxygen delivery via the induced normalisation of tumour vessels. Moreover, we revealed that the UPR, in turn, regulates the expression of PIGF in HCC cells. Thus, our study sheds light on the reciprocal interactions between PIGF, hypoxia and the UPR and suggests that the antitumour effects of angiogenesis-modulating therapy could be mediated by modifying the tumour microenvironmental stresses in HCC.
4.1.7. List of abbreviations

HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; ER, endoplasmic reticulum; PDIA4, protein disulfide isomerase family A, member 4; CANX, calnexin; GRP78, glucose-regulated protein 78; GRP94, glucose-regulated protein 94; UPR, unfolded protein response; PERK, PKR-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor 6; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; XBP1, X-box-binding protein 1; XBP1s, spliced XBP1; ERDJ4, endoplasmic reticulum DnaJ homolog 4; XBP1u, unspliced XBP1; HERPUD1, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein; PlGFKO, PlGF knockout; DEN, diethylnitrosamine; aPlGF, anti-PlGF monoclonal antibody; TUDCA, tauroursodeoxycholic acid; GADD34, growth arrest and DNA damage-inducible protein; HIF-1α, hypoxia-inducible factor-1α.

4.1.8. Competing interests:

Bart Jonckx is an employee of Thrombogenics NV. The other authors have no conflicts of interest to declare.

4.1.9. Acknowledgements

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4.1.10. References


Fig. 1. PIGF inhibition tempers the activation of the UPR in an orthotopic mouse model of HCC.
(A) Quantitative real-time PCR analysis of the ER chaperones *Grp78*, *Grp94* and *Pdia4* and *Herpud1* in aPlGF-treated and PIGFKO mice. Relative fold changes were calculated using the ΔΔCT method.
(B) Immunoblotting for UPR-mediated proteins. (C) Quantitative real-time PCR analysis of ER chaperones of Ire1-mediated splicing of *Xbp1* and Ire1 targets *Canx* and *Erdj4*. (D) Perk-related genes *Atf4*, *Chop* and *Gadd34*. *p*<0.05, **p*<0.01, ***p*<0.001. IgG= 25w DEN + 5w IgG, aPlGF= 25w DEN + 5w aPlGF, WT= 30w DEN in wild type (WT) mice, PIGFKO= 30w DEN in PIGF⁻/⁻ knockout mice.
Fig. 2. PIGF inhibition reduces intratumour hypoxia in experimental HCC. (A) Immunostaining for pimonidazole in mouse livers following the indicated treatment. Arrows indicate tumours. Scale bars: 100 µm. (B) Quantification of the immunostaining for pimonidazole. (C) Lysates of control liver tissue or isolated DEN-induced tumours were subjected to Western blotting for detection of pimonidazole adducts (Pimo). Blotting of β-actin is shown as a loading control. (D) Densitometry analysis of the pimonidazole blot in (C). (E) Real-time PCR analysis of Glut1 and Pfk mRNA levels in tumour tissues. IgG= 25w DEN + 5w IgG, aPlGF= 25w DEN + 5w aPlGF. Data are presented as the means ± SD. *p<0.05.
Fig. 3. Hypoxia activates the PERK pathway in HCC cells. (A) HepG2 cells were cultured in normoxia or hypoxia for 24 h or 48 h. The PERK targets, GRP78, ATF4, CHOP and GADD34 mRNA were detected by Real-time PCR analysis. (B) Expressions of phospho-eIF2α, eIF2α, ATF4, and CHOP protein were detected using Western blotting. All experiments were repeated three times with similar results. (C) Densitometry analysis of the ratio of phosphorylated eIF2α to total eIF2α bands normalised to tubulin and relative to the corresponding control. Quantitative results of the phosphorylation of eIF2α are presented as the mean ± SD. *p<0.05, **p<0.01, ***p<0.001.
Fig. 4. ER stress induces PlGF expression in HepG2 cells. (A) Relative PlGF mRNA levels in HepG2 cells treated for 48 hours with the indicated treatments. *p<0.05, **p<0.01, ***p<0.001 compared to control. *p<0.05, **p<0.01, ***p<0.001 compared to the indicated group. TUDCA: tauroursodeoxycholic acid. (B) Immunoblotting of cell lysates was performed to detect PlGF protein levels. All experiments were repeated three times with similar results.
Fig. 5. Schematic model outlining the interactions among PIGF, ER stress and hypoxia and their effects on HCC growth.
4.1.12. Supplementary Figures

Fig. S1. PIGF blockage induces vessel normalization. (A) Immunostaining for the endothelial marker endoglin (CD105). In HCC nodules, the capillary network is chaotically organized with tortuous vessels (indicated by red arrows) laying at large distances from each other. However, the capillaries in HCC after aPIGF treatment or in PIGFKO mice have a more normal appearance with regular pattern, size, and shape (indicated by green arrows). Black arrows and dashed lines indicate tumours. (B) Quantification of tortuous vessels per mm²; n= 5; *p<0.05, **p<0.01.
Fig. S2. Densitometry analysis of the phosphorylation of eIf2α in isolated HCC. (A) Densitometry analysis of the ratio of phosphorylated eIf2α to total eIf2α bands normalized to tubulin and relative to the corresponding control. Quantitative results of phosphorylation of eIf2α are presented as the mean ± SD. *p<0.05.

Fig. S3. Effect of PlGF inhibition on the expression of the UPR sensor Perk. (A) Quantitative real-time PCR analysis of Perk. Relative fold changes were calculated using the ΔΔCT method. IgG= 25w DEN + 5w IgG, aPlGF= 25w DEN + 5w aPlGF, WT= 30w DEN in wild type (WT) mice, PlGFKO= 30w DEN in PlGF^−/−_ knockout mice. (B) Immunoblotting for Perk protein.
Fig. S4. Validation of quercetin as activator of the IRE1 RNase. (A) Quantitative real-time PCR analysis of unspliced and spliced XBP1 mRNA to evaluate the effect of 100-300 μM quercetin for 24 hours on HepG2 cells. Results are representative of 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001.
5. **Proteasome inhibition and the UPR in hepatocellular carcinoma**

5.1. Next-generation proteasome inhibitor oprozomib synergizes with modulators of endoplasmic reticulum stress in hepatocellular carcinoma

*Manuscript in preparation, patent application filed.*

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Next-generation proteasome inhibitor oprozomib synergizes with modulators of endoplasmic reticulum stress in hepatocellular carcinoma

5.1.1. Abstract

**Background & Aims:**
Advanced hepatocellular carcinoma (HCC) responds poorly to conventional systemic therapies. The first-in-class proteasome inhibitor bortezomib has been approved in clinical use for hematologic malignancies and has shown modest activity in a variety of solid tumours including HCC. However, a considerable proportion of subjects fail to respond and experience adverse events. Recently, the next-generation orally bioavailable proteasome inhibitor oprozomib was developed. In this study, we assessed the efficacy of oprozomib and its effects on the unfolded protein response (UPR) in HCC.

**Methods:**
Oprozomib was evaluated in vitro and in diethylnitrosamine-induced and xenograft mouse models for HCC. Also, the role of the UPR in HCC cells treated with oprozomib was determined.

**Results:**
Oprozomib dose-dependently reduced the viability and proliferation of human HCC cells. Unexpectedly, oprozomib-treated cells displayed reduced cytoprotective ATF6-mediated transcription, whereas PERK and IRE1 pathway activation was not observed. However, oprozomib increased pro-apoptotic UPR-mediated protein levels, including CHOP, by prolonging their half-life. Supplementary boosting UPR activity improved the sensitivity of HCC cells to oprozomib, an effect that was blocked by inhibition of PERK. Oral oprozomib monotherapy displayed significant antitumour effects in the experimental HCC models, and importantly, the combination of oprozomib with different UPR activators further improved the antitumour efficacy in vitro and in vivo by stimulating UPR-induced apoptosis without cumulative toxicity.

**Conclusion:**
Next-generation proteasome inhibition by oprozomib significantly dysregulates UPR activation in HCC. This finding can be exploited to enhance the antitumour efficacy by combining oprozomib with UPR modulators for the treatment of advanced HCC.
5.1.2. List of nonstandard abbreviations

HCC, hepatocellular carcinoma; ER, endoplasmic reticulum; GRP78, glucose-regulated protein, 78 kDa; OZ, oprozomib; UPR, unfolded protein response; PERK, PKR-like endoplasmic reticulum kinase; IRE1, inositolrequiring enzyme-1; ATF6, activating transcription factor 6; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; CHOP, c/EBP-homologous protein; XBP1u, unspliced X-box-binding protein 1; XBP1s, spliced X-box-binding protein 1; ERDJ4, endoplasmic reticulum DnaJ homolog 4; RIP, intramembrane proteolysis; S1P, site-1 protease; S2P, site-2 protease; GRP94, glucose-regulated protein, 94 kDa; PDIA4, protein disulfide isomerase A4; CALR, calreticulin; ERO1L, endoplasmic oxidoreductin-1-like protein; HERPUD1, homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1; DEN, diethylnitrosamine; NAC, N-acetyl-L-cysteine; GADD34, growth arrest and DNA damage inducible 34; SREBP-1, sterol regulatory element binding protein-1.
5.1.3. **Introduction**

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide [1]. Resection and transplantation are the only potentially curative treatments available following detection of a small HCC [2]. For the majority of patients with locally advanced disease, however, the multi-kinase inhibitor sorafenib and transarterial embolization are the only approved treatments. Unfortunately, both provide a limited survival benefit [2].

The ubiquitin-proteasome pathway is responsible for the degradation of misfolded proteins as well as short-lived mediators of signalling cascades regulating cell proliferation and survival pathways [3]. Proteasome inhibition leads to accumulation of these substrates, resulting in concomitant activation of pro- and anti-proliferative signals, disruption of cell-cycle regulation, and, ultimately, apoptosis. Bortezomib is a first-in-class proteasome inhibitor clinically used for the treatment of multiple myeloma. Bortezomib-induced cell death is related with induction of endoplasmic reticulum (ER) stress, inhibition of nuclear factor kappa B, activation of caspase-8 and generation of oxidative stress [3], [4]. Multiple clinical trials have demonstrated that this small-molecule possesses antitumour activity in a variety of human cancers [5], [6]. Despite promising preclinical results [5], a multicentre, single-arm, phase II trial assessing the activity of bortezomib in HCC has demonstrated modest antitumour effects, indicating intrinsic or acquired resistance [4], [7]. In addition, the majority of the patients developed adverse events including peripheral neuropathy [7]. However, the good clinical outcome of bortezomib in myeloma led to the development of next-generation proteasome inhibitors, such as carfilzomib, that selectively and irreversibly bind to the proteasome aspiring to enhance inhibition, improve antitumour activity and decrease toxicity by reducing off-target effects, such as peripheral neuropathy [8], [9]. A phase III trial showed that intravenously administered carfilzomib improved progression-free survival in myeloma with a favourable risk-benefit profile [10]. Recently, an orally bioavailable analogue of carfilzomib, called oprozomib (OZ), was developed [9].
Proteasome inhibition is thought to trigger accumulation of misfolded proteins in the ER, which activates the unfolded protein response (UPR) [4]. Three major ER stress transducers have been identified: PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6) [1]. Following release of chaperone glucose-regulated protein, 78 kDa (GRP78), PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) leading to attenuation of global translation. However, the phosphorylated form of this factor favours selective translation of activating transcription factor 4 (ATF4), which regulates genes involved in protein quality control, amino acid biosynthesis as well as apoptosis regulators such as c/EBP-homologous protein (CHOP) [11]. Activation of IRE1 results in splicing of unspliced X-box-binding protein 1 (XBP1u) mRNA to generate a more active spliced XBP1 (XBP1s), which induces genes involved in protein folding such as endoplasmic reticulum DnaJ homolog 4 (ERDJ4), protein degradation and redox homeostasis [11]. ATF6 is mobilized to the Golgi where it is cleaved by regulated intramembrane proteolysis (RIP), which involves the site-1 (S1P) and site-2 (S2P) proteases, releasing a transcriptionally active fragment, which in turn induces the expression of chaperones, such as GRP78, glucose-regulated protein, 94 kDa (GRP94), protein disulfide isomerase A4 (PDIA4), calreticulin (CALR) and endoplasmic oxidoreductin-1-like protein (ERO1L), XBP1u and of proteins stimulating protein degradation, such as homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1 (HERPUD1) [12].

In this study, we provide a molecular clue to the how OZ might work and identified the therapeutic potential of OZ in monotherapy or in combination with modulators of the induced interplay with the UPR in vitro and in mouse models for HCC.
5.1.4. Materials and Methods

Cell culture

HepG2 (ATCC, Virginia, USA) and Huh7 cells (JCRB, Japan) were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% foetal bovine serum (Life Technologies, Ghent, Belgium). Cells were incubated for 48 hours with oprozomib (100-400 nM; ApexBio, USA), tunicamycin (1 μg/ml; Sigma, Diegem, Belgium), PERK inhibitor (14 μM; GSK2656157, Chengdu novi, Shandong, China), salubrinal (50 μM; Sigma), IRE1 inhibitor (25 μM; 4μ8C, Calbiochem, Massachusetts, USA), cycloheximide (5 μM; Sigma), 1,10-phenanthroline (5-50 μM; Sigma), ascorbic acid (50 μM; Sigma), nelfinavir (10 μM; Sigma) or pre-treated with N-acetyl-L-cysteine (2 hours; 5 μM; Sigma) and compared to equal volumes of solvent as control. Each condition was performed in quadruplicate.

Animals

Wild-type 129S2/SvPasCrl mice were purchased from Charles River, Belgium, and were housed as previously described [13]. The animals had free access to water and to a commercial chow (mice maintenance chow, Carfil Labofood, Pavan Service, Belgium). Five-week-old male mice received weekly intraperitoneal injections with saline or diethylnitrosamine (DEN) (35 mg/kg, Sigma) for 25 weeks. Then, 4 DEN-treated groups (n=12) were treated for 4 weeks with oprozomib (intragastric 30 mg/kg/day for 3 consecutive days per week) alone or in combination with salubrinal (intraperitoneally 1 mg/kg/day) or nelfinavir (intraperitoneally 250 mg/kg/day) and compared to a similar volume of vehicle. Blood was collected from the retro-orbital sinus under isoflurane anaesthesia. After macroscopic evaluation and quantification of the number of hepatic tumours, all organs were fixed in 4% phosphate-buffered formaldehyde (Klinipath, Belgium) and embedded in paraffin or snap frozen in liquid nitrogen. Haematoxylin/eosin and reticulin stainings were performed to assess tumour burden and the results were blindly evaluated by two independent observers (YV and CC).
For the xenograft model, HepG2 cells (6x10^6) were resuspended in 100 µl serum-free media and mixed with 100 µl Matrigel (BD Biosciences, Bedford, MA, USA). Cell/Matrigel mixture was injected subcutaneously into the right flank of 8-week-old athymic nude (Foxn1\textsuperscript{nu}/Foxn1\textsuperscript{nu}) mice housed in filter-topped cages. Tumour dimensions were recorded three times per week with a digital calliper starting with the first day of treatment. Tumour volumes were calculated using the following formula: volume (mm³) = ab²/2, where b was the smaller dimension. When tumours reached 150 mm³, animals were randomized into four groups (n=6) with the same treatment regime as the DEN-treated mice plus monotherapy with salubrinal (intraperitoneally 1 mg/kg/day) or nelfinavir (intraperitoneally 250 mg/kg/day). The ethical committee of experimental animals at Ghent University, Belgium, approved the protocols (ECD 13/39).

Detailed information of MTT, TUNEL, caspase-3/7 activity, bromodeoxyuridine (BrdU) incorporation assays, total RNA extraction, quantitative real-time PCR and Western blotting is provided in the Supplementary Materials and Methods.

**Statistics**

Statistical analyses were performed using SPSS 21 (SPSS, Chicago, USA). Data are presented as the mean ± SD or percentage relative to controls. Variables were tested for normality using the Shapiro-Wilk test. Normally distributed data were subjected to the unpaired student’s t-tests. Data involving more than two groups were assessed by one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test. Non-normally distributed data were tested using the Mann-Whitney-U test. The chi-squared test was used to compare mortality. The IC50 values were obtained using the Bliss method. Interpretation of combination index (CI) values, as calculated by the method of Chou and Talalay [14] using CompuSyn software (ComboSyn Inc., Paramus, NJ), is defined such that CI= 1 indicates an additive effect, and CI<1 and a CI>1 indicate synergism and antagonism, respectively. Reported p-values were two-sided and considered significant when less than 0.05.
5.1.5. Results

Supplementary ER stress improves the sensitivity of HCC cells to proteasome inhibition

In HepG2 cells, 48 hours of incubation with 100-400 nM OZ dose-dependently reduced cell viability, as shown by a tetrazolium MTT spectrophotometric assay (p<0.001; Fig. 1A and Table S1). Combination with the chemical ER stress inducer tunicamycin or with the recently developed small-molecules selectively inhibiting the IRE1 or PERK pathway or salubrinal, which inhibits eIF2α dephosphorylation, was evaluated. Addition of tunicamycin or salubrinal synergistically decreased cell viability (p<0.05, CI=0.71 and 0.60 respectively). As shown by BrdU incorporation, OZ decreased the proliferation rate (p<0.001; Fig. 1B), whereas the addition of tunicamycin or salubrinal further impeded cell proliferation (p<0.05). As revealed by activation of executioner caspase-3/7, OZ dose-dependently induced apoptosis in HepG2 cells (p<0.001; Fig. 1C). Again, addition of tunicamycin or salubrinal further increased caspase-3/7 activity (p<0.001). Although the IRE1 and PERK inhibitors were previously validated [15], these compounds did not affect the sensitivity of HCC cells to OZ. Since tunicamycin increased the sensitivity, but is not clinically applicable because of its toxicity, the HIV protease inhibitor nelfinavir, which represents one of the few clinically applicable ER stress-inducing agents [16], was tested. Interestingly, also addition of nelfinavir synergistically increased the sensitivity to OZ (CI=0.68). The MTT viability and BrdU incorporation experiments were repeated in Huh7 cells with similar results (Fig. S1A-B and Table S1).

Next, we questioned whether the efficacy of other proteasome inhibitors, such as the first-in-class bortezomib, could also be enhanced by combination with UPR inducers. Interestingly, a similar synergistic increase in antiproliferative efficacy was observed with 25 nM bortezomib in combination with tunicamycin, nelfinavir or salubrinal in HepG2 cells (Fig. S2A-B). Finally, we assessed whether OZ or bortezomib altered the chemosensitivity of HepG2 cells to 25-100 µM doxorubicin and observed that proteasome inhibition did not alter the chemosensitivity (data not shown). Together, these results indicate that the sensitivity of human HCC cells to proteasome inhibition is enhanced by ER stress.

Antiproliferative effect of oprozomib depends on the build-up of proteotoxic stress
After 24 hours of incubation, no significant alteration in proliferation rate, caspase-3 activity or cell viability is induced by 400 nM OZ (data not shown). However, after 48 hours, as stated above, OZ displayed robust growth-inhibitory effects, suggesting sufficient time is required to build-up proteotoxicity induced by proteasome inhibition in HCC cells. To demonstrate that proteotoxic stress caused by OZ originates during the synthesis of new proteins, cycloheximide was used to inhibit protein synthesis. Interestingly, treatment with cycloheximide readily increased proliferation (p<0.001; Fig. S3A) and cell viability (p<0.01; Fig. S3B) and reduced caspase-3 induction (p<0.001; Fig. S3C) of OZ-challenged HepG2 cells.

Since bortezomib generates oxidative stress, which is crucial for its antitumour activity [17] and is reported to induce ER stress [11], we investigated whether the antiproliferative effect of OZ is also dependent on oxidative stress. Therefore, we measured cell viability and proliferation after treatment with OZ only or in combination with the antioxidants N-acetyl-L-cysteine (NAC) or ascorbic acid in HepG2 cells. Surprisingly, addition of these antioxidants to OZ did not alter its antitumour action (Fig. S3A-C). Thus, these findings suggest that the build-up of proteotoxic, and not oxidative, stress is indispensable for the effects of OZ.

**Oprozomib upregulates UPR-mediated proteins without induction of the transcriptional UPR program**

First, we examined the induction of UPR targets at the mRNA level by 200 or 400 nM OZ in HepG2 cells. Surprisingly, incubation with 400 nM OZ downregulated the ATF6-mediated GRP78 and PDIA4 mRNA levels (p<0.05; Fig. 2A) [18]. In addition, incubation with 400 nM OZ repressed CHOP and ATF4 transcription (p<0.05) but did not alter the mRNA levels of IRE1-generated XBP1u and its target ERDJ4. Even in presence of ER stress induced by tunicamycin, OZ reduced the transcription of GRP78, PDIA4, XBP1u and CHOP compared to cells treated with tunicamycin alone. Intriguingly, the levels of growth arrest and DNA damage inducible 34 (GADD34) mRNA, a downstream target of ATF4 and CHOP protein, were increased by incubation with OZ and tunicamycin compared to tunicamycin alone (p<0.001). Collectively, these data indicate that OZ impeded the transcriptional induction of target genes of the ATF6 and PERK pathway without altering the IRE1 RNase activity.
In contrast to the mRNA levels, 400 nM OZ triggered increased expression of GRP78, PDIA4, ATF4 and CHOP protein (Fig. 2B). In line with its transcriptional activation, also GADD34 protein levels were elevated. Based on these findings, we determined the protein half-life of the transcription factor CHOP in HepG2 cells by performing a time-course in the presence of cycloheximide blocking protein synthesis (Fig. 2C). The half-life of CHOP protein increased from 5.75 hours in vehicle-treated to 12.82 hours in OZ-treated cells (fold change: 2.23, p<0.001; Fig. 2D). Thus, OZ increased the UPR protein levels by inhibition of their proteasomal degradation and not by enhanced \textit{de novo} synthesis following induction of the transcriptional UPR program.

\textbf{OZ inhibits cytoprotective ATF6 signalling by direct RIP inhibition}

Because OZ increased the levels of full ATF6 protein without any change in the levels of the transcriptionally active cleaved ATF6 fragment, consistent with inhibition of ATF6 activation (Fig. 2B), the effect of OZ on tunicamycin-induced ATF6 activation was evaluated (Fig. S4). First, we assessed the effect of OZ on the tunicamycin-mediated transcriptional induction of additional ATF6-regulated UPR targets, such as \textit{GRP94}, \textit{ERO1L}, \textit{CALR} and \textit{HERPUD1} mRNA (Fig. S4A) [12], [18]. As expected, upregulation of all ATF6 targets by tunicamycin was evident. Interestingly, addition of OZ to tunicamycin downregulated these selected ATF6 targets. To validate this hypothesis, the processing of another target of RIP, sterol regulatory element binding protein-1 (SREBP-1), was examined. Western blotting confirmed accumulation of precursor SREBP-1 in OZ-treated HepG2 cells (Fig. S4B). These results indicate that OZ inhibits ATF6 signalling by RIP inhibition.

RIP inhibition could be either directly by inhibition of the S1P or S2P expression or activity or indirectly by upregulation of a repressor of protease-mediated ATF6 activation. OZ does not alter the \textit{S1P} or \textit{S2P} mRNA levels (Fig. S4C), suggesting OZ functions through post-translational RIP inhibition without affecting \textit{S1P} or \textit{S2P} expression. Nucleobindin 1 is a reported ATF6 repressor [19]. While tunicamycin increased nucleobindin 1 expression, the expression in OZ-treated cells was indistinguishable from vehicle-treated cells (Fig. S4B).
Treatment of HepG2 cells with 25 μM 1,10-phenanthroline, a metalloprotease-specific S2P inhibitor [20], leads to accumulation of precursor SREBP-1 and to the absence of processed SREBP-1 detection and did not alter the nucleobindin 1 expression (Fig. S4B). In addition, 5 to 50 μM of 1,10-phenanthroline dose-dependently reduced the HepG2 cell viability (Fig. S4D), phenocopying the effects of OZ (Fig. 1 and S4B, respectively). Thus, OZ at an effective dose of 400 nM inhibits cytoprotective ATF6 signalling by RIP inhibition, probably, via off-target protease inhibition.

The PERK pathway regulates the nelfinavir-mediated increase in sensitivity to OZ

OZ slightly increased the eIF2α phosphorylation, which could not be inhibited by the PERK inhibitor (Fig. 2B), suggesting other eIF2α kinases such as heme-regulated inhibitor are involved, as previously reported for eIF2α phosphorylation induced by proteasome inhibitor MG-132 [21]. Importantly, addition of salubrinal or nelfinavir profoundly increased the OZ-induced eIF2α phosphorylation and pro-apoptotic CHOP protein levels (Fig. 2B), which may contribute to the increased sensitivity to OZ (Fig. 1). Indeed, addition of the PERK inhibitor increased the proliferation rate of HepG2 cells treated with the combination of OZ and nelfinavir (p<0.05; Fig. 2E), validating the role of the PERK pathway in the mechanism of this combination. Furthermore, addition of nelfinavir to OZ abolished the protein levels of the ATF6-dependent chaperones GRP78 and PDIA4 (Fig. 2B), possibly exacerbating the generated proteotoxicity. Interestingly, nelfinavir was previously reported to induce apoptosis in liposarcoma cells by direct S2P inhibition [22]. These data suggest that dysregulation of the transcriptional UPR program and decreased proteasomal degradation of short-lived pro-apoptotic UPR proteins are involved in OZ-induced HCC cell death and the observed synergy with PERK inducers.

OZ reduced tumour burden in orthotopic and xenograft mouse models for HCC

Prior to evaluating the antitumour activity of OZ in the DEN-induced mouse model characterized by severe liver dysfunction [23], several dosing regimen for 2 weeks were tested in 25 weeks saline-treated and DEN-treated mice (n=3, Table 1). We observed 100% mortality in the mice with liver dysfunction treated with 50 mg/kg/day for 5 consecutive days per week. At 30 mg/kg/day for 3 consecutive days per week, no mortality occurred. Therefore, this dosing regimen was applied in the following experiments.
OZ for 4 weeks did not affect mortality in saline- or DEN-injected mice (n=12, Table 2). Average body weight of mice was decreased following 25 weeks of DEN compared to saline administration (p<0.001, Table 2). Subsequent treatment with OZ did not alter the average body weight compared to vehicle. Although serum ALT and AST levels were elevated by DEN administration (p<0.001), treatment with OZ did not alter these levels in the surviving mice (Fig. S5A).

DEN-treated mice that received OZ developed fewer macroscopic nodules per liver (all sizes: 16.2 ± 4.5 after vehicle versus 11.1 ± 3.9 after OZ; p<0.05). HCC burden, microscopically quantified by the loss of reticulin staining, was reduced in OZ-treated compared to vehicle-treated mice (p<0.01, Fig. 3A-C). Hepatic caspase-3/7 activity levels ex vivo were elevated by DEN compared to saline administration (p<0.001, Fig. 3D). OZ monotherapy further increased these levels compared to vehicle-treated HCC-bearing mice (p<0.05), consistent with in vivo apoptosis induction. We previously reported the UPR pattern in the DEN-induced mouse model [15]. Here, OZ administration reduced the levels of Grp78 mRNA (p<0.05) and induced a tendency to reduce Pdia4 and Chop in the isolated tumours (Fig. 4A). However, immunoblotting of lysates of isolated tumours revealed that OZ promoted CHOP protein expression in vivo (Fig. 4B). Yet, UPR-regulated caspase-12 cleavage was only slightly increased by OZ. These observations provide evidence that the efficacy of OZ in inhibiting the growth of DEN-induced HCC is through similar mechanisms as those observed in vitro.

Secondly, the effect of OZ was assessed in a HepG2 xenograft model. No significant differences in body weight or appearance between control and OZ-treated animals were observed during the course of the xenograft study (data not shown). OZ administration suppressed the growth rate of the HepG2-derived tumours (p<0.05, Fig. 4E). Accordingly, TUNEL immunofluorescence demonstrated a significant increase of TUNEL-positive apoptotic HepG2 cells following OZ administration (p<0.01, Fig. 4C-D).

**Nelfinavir and salubrinal potentiate the therapeutic efficacy of OZ in experimental HCC**

Administration of nelfinavir or salubrinal did not alter the mean body weight compared to vehicle (Table 1). Although hepatic caspase-3/7 activity was not significantly increased (Fig. 3D), combining OZ with nelfinavir was more efficacious compared to OZ monotherapy in the orthotopic model (number of macroscopic nodules per liver: 11.1 ± 3.9 after OZ versus 7.2 ± 5.2 after OZ and nelfinavir; microscopic tumour burden: p<0.05; Fig. 3A-C).
Addition of salubrinal at 1 mg/kg/24 hrs similarly improved the effect of OZ (number of macroscopic nodules per liver: 6.7 ± 4.8 after OZ and salubrinal; microscopic tumour burden: p<0.05; Fig. 3A-C). Interestingly, addition of nelfinavir or salubrinal to OZ promoted elf2α phosphorylation and downstream Chop mRNA and protein levels (Fig. 4A-B), suggesting intensified pro-apoptotic UPR signalling. Caspase-12 cleavage was indeed markedly increased by addition of nelfinavir or salubrinal to OZ treatment (Fig. 4B). In contrast, salubrinal at 1 mg/kg/72 hrs induced no detectable effects on the antitumour efficacy of OZ, elf2α phosphorylation or Chop expression (data not shown).

Accordingly, in the HepG2 xenograft model, dual therapy with OZ and nelfinavir or OZ and salubrinal strikingly inhibited tumour growth compared to vehicle-treated (both p<0.001) and to OZ-treated mice (both p<0.01), whereas nelfinavir or salubrinal monotherapy did not alter the xenograft growth (Fig. S5B-C). Furthermore, addition of nelfinavir or salubrinal augmented the number of TUNEL-positive HepG2 cells in the xenograft tumours (p<0.05 and p<0.01 respectively, Fig. 4C-D), suggesting robust induction of apoptosis when UPR modulators are added. Thus, we identified that nelfinavir and salubrinal potentiate the therapeutic efficacy of OZ in different models for HCC, likely by increased UPR-mediated apoptosis via induction of Chop synthesis while OZ diminishes its proteasomal degradation.
5.1.6. Discussion

Proteasome inhibition could represent a novel therapeutic strategy for advanced HCC [5], [7]. The next-generation orally bio-available irreversible proteasome inhibitor oprozomib (OZ) is assumed to evoke less adverse events and improved antitumour activity compared to the first-in-class bortezomib [3]. Recently, OZ was shown to exert antitumour activity on myeloma and head and neck cancer xenograft models [3]. In this study, OZ exerted potent anti-tumour effects in vitro and in different in vivo models for HCC, supporting the potential value of irreversibly targeting the proteasome in the treatment of HCC. Moreover, we revealed a strategy to further enhance the efficacy of OZ by modulation of the UPR.

When unfolded proteins accumulate in the ER, the UPR is initiated to allow the cells to restore homeostasis by proteasomal degradation of unfolded proteins, translational arrest and increasing protein folding capacity [11]. The association between UPR activity and therapeutic efficacy of proteasome inhibition was first illustrated in myeloma, in which patient serum levels of XBP1 correlated with the clinical response towards bortezomib [24]. Theoretically, decreased protein degradation by proteasome inhibition could lead to oxidative stress and UPR activation. However, the effect on the UPR is not well understood. We showed that the cytotoxic effect of OZ depends on the build-up of proteotoxic stress without an important contribution of oxidative stress. Although OZ did not induce the transcriptional UPR program and even inhibited the protease-dependent activation of the cytoprotective ATF6 pathway, OZ increased the protein levels of different UPR markers. Notably, OZ increased the protein stability of the pro-apoptotic transcription factor CHOP and did induce CHOP-mediated transcription of GADD34. Thus, OZ increased the levels of the UPR-regulated proteins by abrogated proteasomal degradation of these rather than a general activation of the UPR program by increased unfolded protein load. Apparently, rapid proteasomal degradation of UPR proteins is a pivotal negative feedback mechanism following recovery of the ER proteostasis.

Consistent with the effect of OZ on the UPR, PERK or IRE1 inhibition did not alter its effect, whereas ER stress inducers, such as tunicamycin or nelfinavir, or an inhibitor of eIF2α dephosphorylation enhanced the growth-inhibitory effects of OZ in HCC cells. Interestingly, a similar synergy was observed with bortezomib, suggesting this concept can also be applied to other proteasome inhibitors. Since the maximum serum levels attained in patients are much higher (bortezomib: 0.16 μM (1.3 mg/m² intravenous); oprozomib: 3.8 μM (30 mg per os) [25]), the concentrations used in vitro are clinically relevant.
Based on these results, we evaluated the in vivo effects of the combination of nelfinavir or salubrinal and OZ in HCC. Both nelfinavir and salubrinal enhanced the growth-inhibitory effect without cumulative toxicity, suggesting this rational combination presents a safe strategy to potentiate the antitumour effects of proteasome inhibition in HCC. Experience in the use of proteasome inhibitors and nelfinavir in clinical practice and the oral bio-availability are considerable advantages for implementation. Bortezomib resistance in myeloma cells was shown to be induced by attenuated eIF2α phosphorylation [26]. Salubrinal typically provides for enhanced resistance to stress conditions, such as those triggered by oxidizing or UPR-inducing agents [27], [28]. However, here we provide evidence that salubrinal renders HCC cells more susceptible to proteasome inhibition by activating the PERK/phospho-eIF2α/CHOP pathway. Of note, human HCC, in contrast to unaffected adjacent liver tissue, is characterized by increased CHOP staining [29]. Although a recent phase I trial with OZ demonstrated that OZ has an acceptable safety profile when given daily for 5 consecutive days every 2 weeks in patients with solid tumours [30], significant toxicity occurred when OZ was administrated for several consecutive days in mice with DEN-induced liver dysfunction. However, identification of sensitivity enhancers by UPR modulation could allow for dose and, possibly, toxicity reduction. Because ER stress potentiates the antitumour efficacy of OZ, we assume a stronger effect on hypoxic ER-stressed tumour cells compared to the normal liver tissue [15]. Consequently, OZ could be more efficacious in combination with antiangiogenic treatments increasing tumour hypoxia-induced UPR [11], [31]. In conclusion, dysregulation of the transcriptional UPR program and reduced proteasomal degradation of pro-apoptotic UPR-mediated proteins are involved in OZ-induced cell death. Furthermore, modulation of the interplay between OZ and the UPR enhances its antitumour efficacy without cumulative toxicity. Therefore, OZ monotherapy or in combination with UPR modulators may present a novel and clinically applicable therapeutic strategy for HCC.

5.1.7. Acknowledgements
The authors thank P. Vanwassenhove at the Department of Gastroenterology and Dr. M. Bol at the Department of Basic Medical Sciences, Ghent University (Ghent, Belgium) for their expert technical assistance.
5.1.8. References


5.1.9. Tables

Table 1. Mortality at different dosing regimens (n=3 in each group).

<table>
<thead>
<tr>
<th>Consecutive-day dosing in weekly cycle</th>
<th>Mortality in saline-treated mice</th>
<th>Mortality in DEN-treated mice</th>
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<tr>
<td>50 mg/kg/day for 5 days</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>30 mg/kg/day for 5 days</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>30 mg/kg/day for 4 days</td>
<td>0/3</td>
<td>1/3</td>
</tr>
<tr>
<td>30 mg/kg/day for 3 days</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

Table 2. Average body weight (g) ± SD and survival of mice (n=12 in each group).

<table>
<thead>
<tr>
<th>Group</th>
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<th>Average body weight 29 weeks (g)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline =&gt; vehicle</td>
<td>27.22 ± 1.32</td>
<td>26.03 ± 1.61</td>
<td>100</td>
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<tr>
<td>DEN =&gt; vehicle</td>
<td>19.75 ± 1.76***</td>
<td>18.30 ± 2.92</td>
<td>58.33</td>
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<tr>
<td>DEN =&gt; OZ</td>
<td>20.86 ± 1.45</td>
<td>19.17 ± 2.10 NS</td>
<td>58.33</td>
</tr>
<tr>
<td>DEN =&gt; OZ + nelfinavir</td>
<td>22.96 ± 3.65</td>
<td>17.92 ± 3.19 NS</td>
<td>66.67</td>
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<tr>
<td>DEN =&gt; OZ + salubrinal</td>
<td>21.23 ± 5.89</td>
<td>18.09 ± 6.23 NS</td>
<td>66.67</td>
</tr>
</tbody>
</table>

***p<0.001: 25 weeks DEN vs. saline, NS= not significant compared to vehicle.
5.1.10. Figures

Fig. 1. Antiproliferative and pro-apoptotic effects of oprozomib in monotherapy or in combination with modulators of ER stress in human hepatoma HepG2 cells. (A) MTT assay (B) BrdU incorporation (C) Caspase-3/7 activity. OZ: oprozomib. *p<0.05, **p<0.01, ***p<0.001 compared to oprozomib 0 nM; #p<0.05, ##p<0.01, ###p<0.001 compared to respective concentration of oprozomib alone.
Fig. 2. Oprozomib modulates the UPR pattern in HepG2 cells. (A) Real-time PCR analysis of relative mRNA levels of UPR-mediated genes after 48 hours of incubation with the indicated treatments. (B) Immunoblotting of UPR-mediated proteins. (C) To measure the half-life of CHOP protein in HepG2 cells, cells were pre-treated for 1 hour with 1 μg/ml tunicamycin and a time-course in the presence of 50 μg/ml cycloheximide, which blocks protein synthesis, was performed. Band intensities were quantified using ImageJ software. (D) Half-life of CHOP protein was determined by plotting optical density (arbitrary unit) calculated from densitometric analysis of bands in panel C versus hours of treatment. Data represent the mean ± SD of three independent experiments. (E) BrdU incorporation of HepG2 cells incubated with indicated treatments for 48 hours. OZ: 400 nM oprozomib. *p<0.05, **p<0.01, ***p<0.001.
Chapter 3

Fig. 3. Impact of oprozomib in monotherapy or in combination with ER stress modulators on an orthotopic and a xenograft model for HCC. (A) Photographs of representative livers after different treatments. (B) Reticulin staining. Scale bar: 100 µm. (C) Assessment of tumour burden in the carcinogen-induced mouse model in randomly selected high-power fields. (D) Hepatic caspase-3/7 activity *ex vivo*. (E) Evolution of tumour volume in mice bearing HepG2-derived xenograft tumours. (F) Final tumour weights. Values represent the mean ± SD. *p<0.05, **p<0.01, ***p<0.001.
Fig. 4. Oprozomib and UPR modulation in experimental HCC. (A) Real-time PCR analysis and (B) Immunoblotting of UPR targets in isolated DEN-induced tumours following the indicated treatments. (C) TUNEL immunofluorescence in HepG2 xenografts and (D) quantification of TUNEL-positive index (n=6). OZ: oprozomib. *p<0.05, **p<0.01, ***p<0.001.
Fig. 5. Schematic model outlining the mechanisms of oprozomib with indication of the point of action of the applied products. Persistent ER stress activates the tripartite UPR-mediated transcriptional program followed by translation of these UPR proteins, which leads to proteotoxicity-mediated tumour cell death. Although oprozomib did not induce the UPR and even inhibited ATF6-mediated transcription, it increased the UPR-mediated protein levels by prolonging their half-life. This UPR dysregulation allows for enhanced proteotoxicity by supplementary boosting PERK activity by tunicamycin, nelfinavir (also inhibits ATF6) or salubrinal.
5.1.11. Supplementary Figures

Fig. S1. Growth-inhibitory effects of oprozomib in Huh7 cells. (A) Assessment of cell viability by MTT assay. (B) Cell proliferation rate as assessed by BrdU incorporation in Huh7 cells. One-way ANOVA was applied for statistical analysis. *p<0.05, **p<0.01, ***p<0.001 compared to oprozomib 0 nM; *p<0.05, **p<0.01, ***p<0.001 compared to respective concentration of oprozomib alone. OZ: oprozomib. Results are representative of 2 independent experiments.
Fig. S2. Bortezomib and ER stress modulation in HepG2 cells. (A) Cell viability as assessed by MTT assay. (B) Cell proliferation rate as assessed by BrdU incorporation. *p<0.05, **p<0.01, ***p<0.001. BZ: bortezomib 25 nM. Results are representative of 3 independent experiments.
Fig. S3. Modulation of proteotoxic, not oxidative, stress affects antitumour activity of oprozomib in HepG2 cells. (A) Cell proliferation rate. (B) Cell viability. (C) Caspase-3/7 activity. Values represent the mean ± SD. *p<0.05, **p<0.01, ***p<0.001 compared to oprozomib alone. OZ: oprozomib 400 nM, CX: cycloheximide. NAC: N-acetyl-L-cysteine. Results are representative of 2 independent experiments.
Fig. S4. OZ inhibits cytoprotective ATF6 activation in HepG2 cells. (A) Quantitative real-time PCR analysis of the ATF6 targets GRP94, ERO1L, CALR and HERPUD1. (B) Western blotting for RIP-dependent SREBP-1 cleavage and the ATF6 repressor Nucleobindin 1. RIP inhibitor 1,10-phenanthroline was applied at a dose of 25 μM. (C) Quantitative real-time PCR analysis of S1P and S2P. OZ: oprozomib 400 nM. (D) Effect of 5 to 50 μM 1,10-phenanthroline on the MTT cell viability of HepG2 cells. Results are representative of 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001.
Fig. S5. Effect of nelfinavir and salubrinal on the serum levels of liver enzymes in the DEN-induced model and on HepG2 xenograft growth. (A) Liver damage was assessed by measuring ALT and AST levels in the serum of surviving mice after the indicated treatments. Values represent the mean ± SD (n=7). ***p<0.001. (B) Evolution of tumour volume in mice bearing HepG2-derived xenograft tumours. (C) Final tumour weights. Values represent the mean ± SD of the tumour volumes of each group.
Chapter 4

Conclusions and future perspectives
1. Conclusions and future perspectives

1.1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide with a very poor prognosis [1]. Few therapeutic strategies have been proven efficient, particularly for those patients not indicated for curative resection or transplantation [2]. To date, sorafenib remains the only approved, targeted molecule for the treatment of advanced HCC. Although a small survival benefit was demonstrated, it remains only true in the population of patients with Child-Pugh A liver disease [3]. Moreover, sorafenib has distinct side effects that require close monitoring [2]. Recently, newer but similar tyrosine kinase inhibitors (TKIs) and angiogenesis inhibitors have been evaluated in clinical trials with disappointing results [4]. Therefore, innovative therapeutic strategies are urgently required.

Cancer cells survive the self-created hostile microenvironmental conditions, such as hypoglycaemia, hypoxia, acidosis and nutrient scarcity by activation of adaptive processes [5]. These processes render cancer cells resistant to stress-induced cell death allowing for continuous proliferation. However, elucidation of these adaptive processes makes it possible to exploit this characteristic feature to selectively target the cancer cells. The aim of the present thesis is to explore this hypothesis in the setting of HCC.

The unfolded protein response (UPR) and autophagy are two fundamental adaptive mechanisms [6]. As a consequence of the already engaged ER stress system, fewer contingencies may be left to accommodate additional intensities of ER stress in cancer cells. In comparison, normal cells without chronic ER stress may harbour greater reserves to withstand intervention with agents aimed at aggravating the pro-apoptotic UPR signalling. Thus, manipulation of the UPR could provide a novel strategy to treat HCC.
1.2. The unfolded protein response is dynamically fine-tuned during murine multistep hepatocarcinogenesis

Animal models can provide essential knowledge about the pathogenesis of cancer, particularly when they mimic the natural environment in which human tumours develop. Diethylnitrosamine (DEN) has been shown to induce hepatic tumours, which are similar to human HCC with poor prognosis [7]. In our study, weekly intraperitoneal injections with 35 mg/kg DEN gave rise to HCC formation in a background of inflammation and fibrosis, thus mimicking the human setting. By monitoring the UPR in this model, we provided the, to our knowledge, first evaluation of the UPR dynamics in a long-term cancer model [8]. Surprisingly, we observed that the UPR is not an all-or-nothing system. Activation of the UPR pathways is dynamically fine-tuned during the different phases of hepatocarcinogenesis. In fact, each phase of hepatocarcinogenesis is characterized by a specific pattern of UPR signalling. For example, a peak in Ire1 signalling was observed during tumour initiation, while the Perk pathway was activated during tumour progression. Possibly, the dynamic regulation of the UPR is mediated by variations in tissue oxygen levels, which is influenced by the interactions between the UPR and angiogenesis [9].

How the shift from pro-survival to pro-apoptotic UPR signalling is regulated remains unclear with both IRE1 and PERK signalling associated with pro-survival as well as pro-apoptotic signalling [10], [11]. A recent study indicated that the relative dynamics of IRE1/XBP1 and PERK/ATF4 signalling rather than a switch between branches determine cell survival [11]. Moreover, the exact role of cell apoptosis [12] and, more specifically, UPR-induced cell apoptosis in the different steps of hepatocarcinogenesis is also unclear. Counterintuitively, recent studies have demonstrated that hepatocyte apoptosis is a pathogenic event in several liver diseases [13], [14]. Chronically increased hepatocyte apoptosis in genetic mouse models is carcinogenic and leads to compensatory liver regeneration, oxidative stress and DNA hypermethylation [13], [15]. However, once tumours are established and develop resistance against cell death signaling pathways, pharmacological or genetic induction of tumour cell apoptosis slows down the tumour growth [12]. Again, the timing of the event determines its outcome.
It is important to note that the hepatic expression of major ER stress markers is age-dependent. For example, we observed a decrease in Grp78 mRNA levels and an increase in Chop mRNA levels in the control mice of 5 weeks of age compared to those of 35 weeks of age (fold change: 0.57 and 1.43 respectively). Apparently, there is a shift in the balance between the adaptive response of the UPR and pro-apoptotic signalling during aging; where the protective arm is reduced and the apoptotic arm is more robust [16]. Hence, we decided to use age-matched controls during the monitoring of the UPR dynamics in hepatocarcinogenesis.

Carrara et al. (2015) revealed that transient conversion from a dimeric to a previously unknown and more active tetrameric state of PERK is an important regulatory mechanism in PERK signalling [17]. Therefore, analysis of the state and effect of tetramerization of PERK in HCC may offer important insights in the downstream effects of this UPR pathway.

We strengthened the manuscript by performing transmission electron microscopy of the tumours in the mouse model at week 30 revealing an extensive expanded ribosome-bound ER in a lamellar pattern and the presence of remarkable ER reorganization in HCC cells [18]. Additional investigation of the temporal pattern of these changes in size, preferably measured quantitatively, and organization of the ER in experimental and human HCC is indicated.

Also, exploration of the UPR dynamics in the multistep process of human hepatocarcinogenesis from low-grade to high-grade dysplastic nodules, dysplastic nodules with HCC foci to HCCs and comparison to the UPR dynamics in experimental HCC is indicated.

During the progress of our work on the UPR in the DEN model, we encountered the need for functional assessment of the tumour burden in this model. Therefore, we validated innovative small animal imaging techniques for the functional assessment of the experimental HCC burden, including $^{18}$F-Fluoro-deoxyglucose (FDG) positron emission tomography–computed tomography (PET/CT), $^{18}$F-Choline PET/CT and (dynamic contrast-enhanced) magnetic resonance imaging (DCE-MRI), and compared these different imaging techniques evaluating the tumour burden in the DEN-induced mouse model (in collaboration with Infinity Lab, Ghent University). We concluded that $^{18}$F-Choline PET/CT, which visualizes the increased cell membrane synthesis requiring choline uptake in tumour cells, was the optimal imaging technique providing high spatial resolution and 3D image reconstructions to assess tumour burden in this model. Furthermore, we validated the quantification of this technique by using the left kidney as reference density and calculating the standardized uptake values of the voxels within the mouse liver [19]. This technique is and will be of uttermost importance in the setting of testing new pharmacological or genetic tools minimizing the number of animals needed, creating smaller standard deviations and eliminating possible confounders between animals.
1.3. Role of the unfolded protein response in the pathogenesis of hepatocellular carcinoma

1.3.1. Modulation of the unfolded protein response impedes tumour cell adaptation to proteotoxic stress: a PERK for hepatocellular carcinoma therapy

In an effort to better understand the underlying mechanisms of HCC cell survival under ER stress, we demonstrated that under ER stress, Perk inhibition and not Ire1 inhibition reduced HCC cell viability and proliferation via escalating proteotoxic stress, and that the small-molecule inhibitor of PERK leads to HCC regression in the orthotopic mouse model [8]. However, important drawbacks to clinical application of Perk modulation were identified and need to be discussed.

First, we observed only a modest antitumour effect in vivo compared to the dramatic effects on cell viability under chemically induced ER stress in vitro. Our preliminary validation studies of the Perk inhibitor used (GSK2656157 [20] at 100 mg/kg bid) showed that the tunicamycin-mediated activation of the Perk pathway in hepatocytes was inhibited for only 6 hours following administration (data not shown), explaining the use of twice a day dosing. Consequently, tumoural Perk signalling was probably not completely and continuously inhibited during the treatment. Development of sustained release formulations containing GSK2656157 could solve this issue.

Secondly, the Perk inhibitor-treated mice exhibited hyperglycaemia compared to the vehicle-treated mice. Induction of diabetes in cancer patients could be an important limitation of therapeutic Perk inhibition. Possibly, the development of a targeted drug delivery system could reduce the toxicity of this compound. A recent study showed that nanoparticles conjugated with an antibody against GRP78 promote drug delivery of 5-fluorouracil into human HCC cells with high expression of surface-exposed GRP78 [21]. Nanoparticles increase the stability of chemical agents by encapsulation, protecting these agents from the extracellular environment and regulating the drug release in a prolonged and controllable manner. The technical feasibility of Perk inhibitor-loaded nanoparticles conjugated with anti-Grp78 and the effect of Perk inhibition on the surface Grp78 expression of HCC cells require further investigation. Taken together, although our results are promising, pharmacological efforts to establish a drug formulation with sustained release and tumour-targeted drug delivery are required.
Recently, the small-molecule \( \text{N,N'}-\text{trans-}(\text{cyclohexane-1,4-diyl})-\text{bis-}(2-(4\text{-chlorophenoxy}) \text{acetamide} \) (integrated stress response inhibitor, **ISRIB**), which prevents translational inhibition downstream of the Perk/phospho-\text{eIF}2\alpha pathway, was developed [22]. Interestingly, this small-molecule partially restores global translation without adverse effects on the pancreas, such as induction of diabetes. In prion disease, restoration of global protein synthesis using the Perk inhibitor was profoundly neuroprotective [23]. However, in line with our results, this also occurred at the cost of toxicity to the pancreas. Recently, the effect of ISRIB was tested in this model and conferred similar neuroprotection in prion-diseased mice but without the pancreatic toxicity [24]. Whether the ISRIB-mediated modulation of translational control could mimic the antitumour effects of the Perk inhibitor in the DEN-induced mouse model without the observed deleterious effects is currently unknown.

**1.3.2. Tauroursodeoxycholic acid dampens oncogenic apoptosis induced by endoplasmic reticulum stress during hepatocarcinogen exposure**

To assess the role of ER stress in the biology of tumour initiation and progression, we applied the chemical chaperone TUDCA in preventive and therapeutic settings of experimental HCC [25], [26]. We discovered that TUDCA attenuates hepatocarcinogenesis by suppressing carcinogen-induced ER stress-mediated cell death and inflammation without stimulating tumour progression [25]. Therefore, this chemical chaperone could represent a novel chemopreventive agent. An important limitation of these interpretations is that the effect of TUDCA was assessed in a model induced by the carcinogen DEN. Although this is a commonly used and well-known carcinogen for experimental HCC, it is noteworthy that assessment of the effects of TUDCA on hepatocarcinogenesis induced by **human-relevant carcinogens**, such as chronic HCV replication or alcohol exposure, could further support the clinical application of this putative chemopreventive strategy. Regarding HCV, preliminary experiments with HCV-infected humanized livers in Fah\(^{-}\)/Rag2\(^{+/}\)/Il2rg\(^{-}\) (FRG) mice [27] and with transgenic mice expressing the full HCV protein repertoire at levels corresponding to the natural human infection [28] were initiated in collaboration with Prof. Ph. Meuleman, Ghent university, Ghent, Belgium and Prof. H. Lerat, INSERM U955, Créteil, France. Assessment of the tumour-promoting effects of chronic HCV infection and effects of targeted UPR modulation in these models is planned.
The mechanism of action of chemical chaperones is complex and their application is hindered by limited knowledge of their molecular activities [29]. It is thought that chemical chaperones are typically poor solvents for a protein backbone and hence facilitate native structure formation [30]. However, it is unknown whether different chemical chaperones can act differently to modulate folding energy landscapes. Recently, a classification of chemical chaperones based on their thermodynamic effect on protein folding landscapes was proposed [29]. The canonical chemical chaperones, trimethylamine-N-oxide and trehalose, accelerate refolding by decreasing the flexibility of the refolding intermediate, thereby decreasing the entropic barrier of refolding (i.e. reduced disorder of the refolding intermediate), while other small-molecules stabilize the transition state enthalpically (i.e. reduced sum of the internal energy of the refolding intermediate). Additional studies on the exact chaperone mechanism of TUDCA are required.

Next to TUDCA, assessment of the effects of other established and safe chemical chaperones, such as 4-phenylbutyric acid (4-PBA) or the novel 4-PBA derivatives with a greater chemical chaperone activity than 4-PBA [31], on hepatocarcinogenesis, is indicated to better understand the exact role of chemically improved protein folding capacity in carcinogenesis during carcinogen exposure.

TUDCA has next to its chaperone properties other direct or indirect downstream effects [32]. For example, the reported mechanisms involved in the antiapoptotic properties of TUDCA include targeting of mitochondrial function and integrity and interactions with survival signals in the cAMP, Akt, NF-κB, mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling pathways [33]. Next to the reduced pro-apoptotic UPR signalling, these cytoprotective effects could attribute to the observed chemopreventive action of TUDCA.

The cellular and molecular pathways involved in metastasis have been studied in many models; however, the link between ER stress signaling and invasion/metastasis is poorly understood [34]. Because ER stress was previously linked to cell invasion [29,30], we assessed the effect of TUDCA on cell invasion in a hepatocyte growth factor-induced invasion assay in which spheroids of Hepa1-6 HCC cells embedded in collagen matrix were observed over time. Additionally, we assessed its effect in a Boyden chamber assay.
In the spheroid assay, the perimeter of the TUDCA-treated spheres increased compared to controls after 24 and 48 hours of incubation, suggesting an altered invasive phenotype. However, in the Boyden chamber assay, addition of TUDCA induced no significant alterations in the hepatocyte growth factor-stimulated invasion of these HCC cells over a 48 hour period. These differential results could possibly be explained by the different experimental set-up to assess cell invasion. In the spheroid assay, cells at the border of the spheroid directly invade the surrounding collagen without a chemoattractant force. In contrast, in the Boyden chamber assay, plated HCC cells migrate in the direction of the source of chemoattractants (i.e. added serum in the lower chamber) through the microporous filter membrane and subsequently invade into the attached basement membrane extract. Indeed, adequate *in vivo* evaluation of invasion/metastasis could solve this issue. Despite recent advances in this field [34], systematic studies on the role of the UPR in cancer invasion and metastasis and its effects on the degradation of the extracellular matrix by matrix metalloproteinases are required.

In conclusion, improved cellular coping with carcinogen-induced stress by assisting protein folding holds great potential to prevent tumourigenesis. However, validation in other models and identification of the best chemical chaperone in this context is required prior to clinical evaluation.
1.4. Sorafenib interacts reciprocally with the unfolded protein response in hepatocellular carcinoma

While the multi-kinase inhibitor sorafenib heralded a major breakthrough in HCC therapy, the clinical benefit is at best modest and transient [3]. Biomarkers determined at diagnosis to identify the patients who will have the best tumour response and/or survival benefit are currently lacking and efforts to attenuate the sorafenib resistance or toxicity were hitherto disappointing [37]. Numerous clinical trials have been conducted to evaluate a large number of molecularly targeted drugs for treating HCC, but most drugs exhibited less efficacy and/or higher toxicity compared to sorafenib. Therefore, considering that this ‘specific’ inhibitor could target unexpected molecules depending on the biological context [38], defining the mechanisms underlying sorafenib resistance of HCC cells is critical for treating HCC by maximizing its treatment efficacy, while minimizing adverse effects [39].

We found that sorafenib dose-dependently activates the IRE1/XBP1/RIDD and PERK/eIF2α/ATF4/CHOP/GADD34 pathways leading to adaptive protein refolding, but reduced the GRP78 protein expression. Since IRE1 knockdown increased induction of apoptosis by sorafenib in MHCC97-L and PLC/PRF/5 HCC cells [40], we hypothesized that IRE1 inhibition could boost the effect of sorafenib. Unexpectedly, a small-molecule inhibitor binding the IRE1 RNase domain [41] attenuated the antiproliferative effect of sorafenib in HepG2 cells. In our experiments, IRE1 is still present, upregulated and able to mediate the well-known protein-protein interactions, such as the IRE1-TRAF2 interaction [10]. In addition, the IRE1 RNase performs RIDD on specific target transcripts, a pro-apoptotic process unexpectedly but significantly activated by the multi-TKI sorafenib [42]. Mechanistically, further characterisation of the mRNA targets of sorafenib-activated RIDD in HCC cells could offer important insights into its mechanism of action.

Recently, So et al. (2015) showed that the IRE1/RIDD pathway could mediate the inhibitory effects of ER stress on global protein translation by decay of the mRNA of CREP/Ppp1r15b, a regulatory subunit of eIF2α phosphatase, which is similar to the PERK-mediated GADD34/Ppp1r15a. Therefore, analysis of the effects of sorafenib on this IRE1/RIDD/CREP/eIF2α/translation-axis could provide additional information on the mechanism of action of sorafenib.
Interestingly, **GRP78 protein** expression, a chaperone protein classically induced during the UPR [10], was decreased in sorafenib-treated cells, whereas no major change was observed in the protein levels of PDIA4, another chaperone protein. In view of evidence that GRP78 overexpression confers resistance to sorafenib in HCC cells [43] and that GRP78 plays a cytoprotective role in the setting of ER stress [44], these findings raise the possibility that the decrease in GRP78 levels contribute to the pro-apoptotic effects of sorafenib and that additional blockade of GRP78 induction may enhance the sorafenib-mediated lethality. To elucidate the underlying mechanism of the selective decrease in GRP78 protein expression by sorafenib, analysis of the protein half-life of GRP78 in the presence of sorafenib is indicated to differentiate between decreased production and increased degradation.

Next, we selected two **clinically applicable ER stress inducers**: the proteasome inhibitor bortezomib and the HIV protease inhibitor nelfinavir [45]. Interestingly, only nelfinavir induces an additive effect in combination with sorafenib in HCC, suggesting that the effect of UPR activation depends on the used ER stress inducer. By analysis of the effect of proteasome inhibition in HCC, we found that the proteasome inhibitor oprozomib did not activate the canonical UPR signalling pathway but rather increased the protein levels of UPR proteins by reduced proteasomal degradation. This observation could possibly explain the differential impact of the used ER stress inducers. Finally, further elucidation of clinical applicable pharmacological ER stress inducers is ongoing and a recent study confirmed that several commonly used drugs modulate the UPR pathways [46]. Evaluation of the effects of combination with these newly identified ER stress inducers is optional.

Sorafenib also promotes **autophagic flux** in HCC cells, as shown previously [47]. However, combination of sorafenib with these autophagy inhibitors again induces only additive, but no synergistic, effects in HCC cells. The role of autophagy in sorafenib resistance was previously investigated in other HCC cell lines [48], [49]. These studies indicate that the autophagic responsiveness to sorafenib is distinct between Hep3B and Huh7 cells and that resistance of Hep3B cells to sorafenib may be associated with altered autophagy signalling pathways. Accordingly, in our study we used HepG2 cells, but further research in other HCC cell lines could improve our understanding of the exact role of autophagy in sorafenib resistance.
Since sorafenib activates the UPR and thereby induces adaptive protein refolding and autophagy, we assessed whether triple therapies could improve the antitumour efficacy of sorafenib. It was striking that combination of sorafenib with an ER stress inducer and an inhibitor of the observed compensatory mechanisms, i.e. adaptive protein refolding or autophagy, synergistically potentiated the antitumour efficacy of sorafenib *in vitro*. Of these multiple combinations, we assessed the combination with nelfinavir and clarithromycin in different models for HCC, which showed the strongest antiproliferative effects *in vitro*. Of note, nelfinavir and clarithromycin synergized with sorafenib to reduce tumour growth *in vivo* without significant toxicity.

In the last section of our study, we assessed whether it is possible to omit sorafenib in this strategy. Interestingly, the threshold for ER stress-induced cell apoptosis during autophagy inhibition in HCC cells is elevated by omission of sorafenib, however, this can be counterbalanced by increasing the dose of nelfinavir without increased toxicity given its relatively high maximum tolerated dose (MTD) [50]. Further investigations need to determine whether daily or cyclic administration of this dual therapy is feasible and sufficient for sustained antitumour response, as evidenced for sorafenib [3]. In line with these results, a patent application involving the use of ER stress inducers together with autophagy inhibitors in HCC with or without sorafenib was filed (P2014/060, Ghent University, in collaboration with Bimetra). Prior to clinical evaluation of these combinations, further investigations including the determination of the optimal dose and ratio of the involved compounds and the efficacy in sorafenib-resistant HepG2 cells [51] is required.

Rudalska et al. (2014) elegantly identified by *in vivo* shRNA screening the p38/ATF2 pathway as a mechanism of sorafenib resistance in HCC [52]. Investigating the effect of the proposed triple therapy modulating the induced UPR/autophagy on this presumed sorafenib-resistance pathway may uncover downstream interconnections. For example, oligomerized IRE1 binds TNF receptor-associated factor 2 (TRAF2), activating apoptosis signal-regulating kinase 1 (ASK1) and downstream kinases that activate p38 [10], [53]. Therefore, we speculate that an altered IRE1 pathway activation pattern modulates the p38/ATF2 pathway and thereby sorafenib resistance.
The complex, highly heterogeneous nature of HCC makes it unlikely that targeting only one pathway will achieve optimal disease control. Moreover, the efficacy of multi-TKIs including sorafenib and the recently tested compounds, such as sunitinib, also appears to be insufficient to meaningfully prolong the overall survival of the patients. However, the strategy of combining targeted disruption of the cellular protein homeostasis with inhibition of the cellular “waste disposal” via autophagy (and/or proteasomal degradation) alone or added to the multi-TKI could offer new perspectives in HCC therapy. Combined therapy with multiple drugs is a common practice in cancer therapy and can achieve better therapeutic effects than a single drug. Furthermore, the utility of combining approved drugs by rational drug repositioning may be rapidly clinically implemented in patients. Therefore, clinical evaluation of the investigated combinations subverting ER stress in HCC cells towards apoptosis is indicated.

1.5. Therapeutic effects of Artesunate in hepatocellular carcinoma: repurposing an ancient antimalarial agent

The antimalarial Artesunate exerts potent cytotoxic effects on HCC in vitro and in vivo without inducing body weight reduction, hepatotoxicity or increased mortality in experimental HCC [54]. Furthermore, we found that Artesunate fortified the PERK pathway but did not affect the IRE1 pathway. However, the relevance of this effect on the UPR in the cytotoxic effects of Artesunate requires further investigation. Of note, the antitumour activity of Artesunate was increased under hypoxia and dual therapy with Artesunate and sorafenib exerted an additive effect on the microscopic tumour burden and further reduced tumour neovascularization compared to sorafenib monotherapy, the current standard-of-care [2], without cumulative toxicity. Again, whether the altered UPR signalling is involved in the efficacy of this dual therapy remains to be elucidated.
Experience in the use of both Artesunate and sorafenib in clinical practice is a considerable advantage. However, in contrast to sorafenib, the safety of Artesunate in HCC patients with mild impaired liver function is currently unknown. Therefore, we recently initiated the DESPARTH trial (NCT02304289; in collaboration with Prof. Dr. L. Van Bortel, clinical pharmacology, Ghent University, Belgium and the “Anticancer Fund”), a phase I dose-escalation trial, to assess the safety and determine the MTD of oral Artesunate in HCC patients with mild impaired liver function. This trial is approved and now recruiting. In parallel, the effect of DEN-induced liver dysfunction on Artesunate metabolism and pharmacokinetics following intragastric administration will be investigated (in collaboration with Prof. Dr. B. De Spiegeleer. Lab of Drug Quality & Registration, Ghent University, Belgium). We consider the present studies as an important step forward in moving our understanding of Artesunate mechanisms into the clinical stage.

1.6. PlGF inhibition modulates hypoxia and the unfolded protein response in hepatocellular carcinoma

Our group previously showed that the inhibition of PlGF exerts antitumour effects and induces vessel normalisation [55]. Our most recent study indicates that PlGF inhibition significantly suppresses UPR activation, potentially by tempering hypoxia in HCC and that the UPR, in turn, is able to induce PlGF, suggesting the existence of a feedback mechanism of hypoxia-mediated UPR that stimulates PlGF-mediated angiogenesis. Thus, anti-angiogenic therapies, including the blockade of the PlGF pathway that ‘normalise’ the abnormal blood vessels in tumours, seem to effectively temper tumour hypoxia and hypoxia-induced adaptation processes, such as the UPR, in experimental HCC. However, whether the diminished activation of these adaptation processes accounts for the observed reduction in tumour growth remains elusive.

Our present work in HCC models confirmed the excellent safety profile of monoclonal antibodies against PlGF. Because previous studies on transgenic mice have revealed that PlGF expression is mainly restricted to pathological conditions [56], characterisation of the role of ER stress in the selectivity of PlGF expression to pathological angiogenesis, potentially characterised by ER stress, is indicated. Finally, the role of the UPR in vessel abnormalisation induced by excessive production of angiogenic factors [57] remains to be elucidated. The UPR induces angiogenic factors [9], possibly resulting in an excessive production and hence vessel abnormalisation.
Because we observed that the chemotherapeutic doxorubicin promotes PIGF production and that PIGF inhibition leads to hypoxia-reducing vessel normalisation, potentially decreasing hypoxia-mediated chemoresistance and improving tumoural drug delivery, we questioned whether the inhibition of this angiogenic factor was able to increase the antitumour effect of doxorubicin in vivo. However, addition of anti-PIGF to doxorubicin did not alter the weight, survival or tumour burden compared to mice treated with doxorubicin and IgG (data not shown). Thus, we were not able to detect any improvement, suggesting the inhibition of this specific angiogenic factor was insufficient to improve the efficacy of doxorubicin. Possibly, the lack of PIGF can be bypassed by increased production of other angiogenic factors or the tumoural drug delivery may be still impaired despite the observed vessel normalisation. Further investigation is needed to support these hypotheses.

1.7. Proteasome inhibition and the unfolded protein response in hepatocellular carcinoma: an unexpected pathway dysregulation provides an opportunity to boost its efficacy

At its most simple level the ubiquitin-proteasome system (UPS) is composed of an E3 ligases-mediated tagging factor in the form of ubiquitin which marks unwanted or damaged proteins for degradation, and the proteasome, a large molecular shredder that degrades proteins into smaller peptides for use in other anabolic processes [58]. The multifaceted role of the UPS includes the degradation of misfolded and damaged proteins, cell cycle regulators, oncogene and tumour suppressor proteins, as well as the regulation of antigen processing and control of transcription factor activity [59].

The next-generation orally bio-available irreversible proteasome inhibitor oprozomib (OZ) is assumed to have less adverse events and improved antitumour activity compared to the first-in-class proteasome inhibitor bortezomib [60]. Recently, OZ was shown to exert antitumour activity on myeloma and head and neck cancer xenograft models [60]. Our study showed for the first time that OZ exerted potent anti-tumour effects in vitro and in different in vivo models for HCC, supporting the therapeutic potential of irreversibly targeting the proteasome.
Theoretically, decreased protein degradation by proteasome inhibition could lead to oxidative stress and UPR activation. However, the mechanism of action and links with the UPR pathways are not well understood. We showed that the cytotoxic effect of OZ depends on the build-up of proteotoxic stress without an important contribution of oxidative stress. Although OZ did not induce the transcriptional UPR program and even inhibited the protease-dependent activation of the cytoprotective ATF6 pathway, OZ elevated the protein levels of different UPR markers. Notably, OZ increased the protein stability of the pro-apoptotic transcription factor CHOP. Thus, decreased proteasomal degradation of pro-apoptotic UPR proteins leads to elevated levels of these proteins rather than a general UPR activation by increased unfolded protein load in the ER. Apparently, rapid proteasomal degradation of UPR proteins is a pivotal negative feedback mechanism following recovery of the ER protein homeostasis.

Identification of the specific components of the UPS regulating degradation of these UPR proteins is indicated. Therefore, we initiated a research project investigating the role of deubiquitinases (DUBs) in the regulation of the UPR and autophagic flux in HCC. DUBs are components of the UPS that catalyse the removal of ubiquitin moieties from target proteins or polyubiquitin chains, resulting in altered signalling or changes in protein stability. Analysis of the human genome shows the presence of ~80 functional DUBs [61]. A number of DUBs regulate processes associated with cell proliferation and apoptosis, and as such represent candidate targets for cancer therapeutics [62]. Interestingly, the majority of DUBs are cysteine proteases and are more "druggable" compared to the E3 ligases.

Intriguingly, addition of ER stress inducers nelfinavir or salubrinal enhanced the growth-inhibitory effects of OZ in both models without cumulative toxicity, suggesting that this rational combination could represent a safe strategy to potentiate the antitumour effect of proteasome inhibition in HCC. Finally, assessment of the role of autophagy in OZ effect and of the effect and toxicity of a triple strategy consisting of oprozomib, an autophagy inhibitor (i.e. clarithromycin) and an ER stress inducer (i.e. nelfinavir), a concept that may retain the unfolded proteins in the ER by blocking the major degradation pathways while inducing additional ER stress, provides great opportunities for future research.
Of note, human HCC, in contrast to the unaffected adjacent liver tissue, is characterized by extensive CHOP staining and, assuming the cellular threshold for CHOP-induced apoptosis is similar in hepatocytes and HCC cells, CHOP-promoting therapy could selectively drive the HCC cells toward apoptosis [63]. Because ER stress potentiates the antitumour efficacy of OZ, we assume a stronger effect on the hypoxic ER-stressed tumour cells compared to normal liver tissue. Consequently, we speculate that OZ could be more efficacious in combination with antiangiogenic treatments, presumably increasing tumour hypoxia-induced UPR [9], [10], [25].

In conclusion, modulation of the interplay between OZ and the UPR enhances the antitumour efficacy of OZ in vitro and in experimental HCC models without cumulative toxicity. Therefore, OZ monotherapy or in combination with UPR modulators may represent a novel and clinically applicable therapeutic strategy for advanced HCC.
2. General conclusions

Although extensive HCC research is in progress, to this date, the results are unsatisfactory with limited long-term survival. In the fight against this lethal disease, there is still a long way to go. New therapeutic tools need to be not only therapeutically effective but also tolerable and economically within the reach of the neediest people. The potential impact of the UPR in many human diseases makes UPR signalling a promising target for therapeutic intervention. In this thesis, the role and translational potential of the UPR in HCC biology were explored. The UPR protects cells against defects in protein folding in the ER. Because many mechanisms come into play, the cell carefully balances various means at its disposal to protect against proteotoxicity while also providing adequate protein synthesis to sustain cellular fitness. Tumour cells continuously divide and grow under oncogenic stress caused by hypoxia, nutrient deprivation, metabolic stress and oxidative stress, leading to UPR activation as a central cellular adaptation strategy. The role of the UPR in malignancy has inspired great interest in exploring the therapeutic potential of targeting UPR components. Because most normal cells are not subjected to stress and the UPR pathways remain inactive in these cells, this difference between tumour and normal cells might offer the advantage of achieving specificity in cancer therapy by UPR targeting. If tumour cells are exposed to another form of ER stress in addition to the pre-existent ER stress, the intensity of ER stress might exceed a threshold, thereby inducing cell death specifically in tumour cells. Therefore, it is of considerable importance to understand the impact and dynamics of the UPR in HCC.

As a general conclusion, this thesis provides evidence that the UPR plays a pivotal role in the multistep process of hepatocarcinogenesis. Furthermore, this work indicates that modulation of the UPR could potentially be applied as an innovative strategy against HCC initiation and progression and as a method to enhance the efficacy of certain drugs acting on these adaptive signalling pathways. Based on the novel concepts discussed here, the future challenge is to integrate our understanding of how the UPR regulates distinct aspects of cancer biology as a global network integrating the different cellular stress responses. Although the obtained results hold great potential, several questions remain and require further fundamental and clinical research in the near future.
3. References


Chapter 5

Curriculum Vitae
1. Curriculum Vitae

1.1.1. Personal information

- Address: Warandemolen 20, 9890 Gavere, Belgium
- Tel: +32 495/342794
- E-mail: yvespaul.vandewynckel@ugent.be
- Nationality: Belgian
- Date of birth: 06/07/1987

1.1.2. Education

- 30/06/2012: Resident Internal Medicine, Ghent University Hospital
- 2008-2012: Master of Medicine, Ghent University
  *Achieved with “magna cum laude”*
- 2005-2008: Bachelor of Medicine, Ghent University
  *Achieved with “magna cum laude”*

1.1.3. Courses

- 24/04/2015: Abdominal ultrasound hands-on course: “Hepatology diagnostics by US” during ILC of EASL 2015 at Vienna, Austria
- 16-17/04/2015: Advanced endoscopy hands-on course in SkillsLab of Erasmus MC, Rotterdam, The Netherlands.
- 14/06/2014: Young VVGE course: “Pre-malign conditions of the GI tract”, Belgium
- 06/2014: "Medical English" with proof of competence (UGent)
- 09/2013: Doctoral schools: clinical trial design course (UGent)
- 07/2013: Interuniversity Special Education Antibiotic Policy course (4 days) + certificate
- 06/2013: “Advanced academic conference skills” doctoral schools course (UGent)
- 13/06/2013: Course and certificate of clinical trial investigator skills (UGent)
- 16/03/13: Course and certificate of clinical trial investigator skills (UGent)
- 29/09/2012: Course and certificate Abdominal ultrasound at "Flemish Association for Ultrasound"
- 09/2012: Doctor in "Child and Family" after training course
- 08/2012: Doctoral schools: “English presentation techniques” course (UGent)
- 06/2012: "Medical French" with proof of competence (UGent)
- 20/01/2012: Laboratory animal science course (FELASA approved)
- 2011-12: Introductory Statistics: principles of statistical interference, SPSS and ANOVA, Institute for Continuing Formation in Sciences (UGent)
- 2011: Radiation and radioprotection (UGent)
- 2009: Postacademic formation in electrocardiography (UGent)
1.1.4. Teaching experience

- Start in 03/2015: Clinical education: tutor 2nd Bachelor, Medicine: Skills Lab, UGent: "Clinical examination of the abdomen".
- 09/2014-06/2016: Thesis supervisor of M.Sc project Margot Vandenbossche, Biomedical sciences, Ghent University: “The interconnection between chaperone-mediated autophagy and endoplasmic reticulum stress in HCC”.
- 09/2014-06/2016: Thesis co-supervisor of M.Sc project Laure De Maere, Biomedical sciences, Ghent University: “The role of endoplasmic reticulum stress in the neuronal cell death in a rat model for hepatic encephalopathy”.
- 09/2013-06/2015: Thesis supervisor of M.Sc project Céline Coucke, Biomedical sciences, Ghent University: “Therapeutic potential of next-generation proteasome inhibitor Oprozomib in HCC”.
- 09/2012-06/2014: Thesis supervisor of M.Sc project Ine De Saegher, Biomedical sciences, Ghent University: “Modulation of Sorafenib resistance in HCC”.

1.1.5. Management skills

- 18/08/2014: Member of the steering committee UGent for Internal Medicine lectures
- 17/06/2014: Elected president of “Young BASL” + active member of BASL as Young BASL representative
- 08/2012: Member of program committee UGent " ManaMa Specialist Medicine" as Resident representative for Internal Medicine

1.1.6. Conference presentations

- Yves-Paul Vandewynckel. Journal Watch in Internal Medicine: What’s new?. Internal Medicine lecture (Ghent University), 11/06/2015, Ghent, Belgium. Oral presentation


- Yves-Paul Vandewynckel, Xavier Verhelst, Klara Rombauts, Bart De Spiegeleer, Luc Van Bortel, Anja Geerts, Hans Van Vlierbergh. Phase I dose-escalation study evaluating the safety and pharmacokinetics of oral Artesunate in patients with advanced hepatocellular carcinoma (DESPARTH trial). International Liver Congress (EASL), 24/04/2015, Vienna, Austria. Poster presentation

- Yves-Paul Vandewynckel, Debby Laukens, Lindsey Devisscher, Annelies Paridaens, Eliene Bogaerts, Anja Van den Bussche, Sarah Raevens, Xavier Verhelst, Christophe Van Steenkiste, Bart Jonckx, Louis Libbrecht, Anja Geerts, Peter Carmeliet, Hans Van Vlierbergh. Placental growth factor inhibition modulates the interplay between hypoxia and
the unfolded protein response in hepatocellular carcinoma. *International Liver Congress (EASL)*, 24/04/2015, Vienna, Austria. Poster presentation


- **Yves-Paul Vandewynckel**. Introduction of the Young BASL. *BASL winter meeting*, 05/12/2014, La Hulpe, Belgium. Oral presentation


• Eliene Bogaerts, Annelies Paridaens, **Yves-Paul Vandewynckel**, Anja Geerts, Femke Heindryckx, Hans Van Vlierberghe. Time-dependent effects of hypoxia on liver progenitor cell activation in HCC. *26th Belgian Week of Gastroenterology*, 14/02/2014, La Hulpe, Belgium. Oral presentation

• Annelies Paridaens, Eliene Bogaerts, **Yves-Paul Vandewynckel**, Lien Thoen, Hans Van Vlierberghe, Leo Van Grunsven, Anja Geerts, Isabelle Colle. Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress in cholestatic liver disease. *26th Belgian Week of Gastroenterology*, 14/02/2014, La Hulpe, Belgium. Oral presentation

• Eliene Bogaerts, Aurelie Comhaire, Annelies Paridaens, **Yves-Paul Vandewynckel**, Peter Carmeliet, Anja Geerts, Femke Heindryckx, Hans Van Vlierberghe. The role of hypoxia on liver progenitor cell activation in HCC. *26th Belgian Week of Gastroenterology*, 14/02/2014, La Hulpe, Belgium. Oral presentation


• **Yves-Paul Vandewynckel**, Debby Laukens, Eliene Bogaerts, Annelies Paridaens, Anja Van den Bussche, Xavier Verhelst, Christophe Van Steenkiste, Benedicte Descamps, Chris Vanhove, Louis Libbrecht, Riet De Rycke, Bart N. Lambrecht, Anja Geerts, Sophie


Oral presentation

1.1.7. Awards and patents

- 22/04/2015: EASL Vienna, Austria: “Top 10% abstract” award for “TUDCA dampens oncogenic apoptosis induced by endoplasmic reticulum stress during hepatocarcinogen exposure”.
- 10/03/2015: Patent application: invention of a novel treatment option for HCC.
- 27/02/2015: “Belgian Week of Gastroenterology (BWGE) Research Grant” based on evaluation by external referees of previous work, CV and research project proposal (TUDCA in HCC).
- 14/10/2013: “National Award” for best Belgian UEG abstract 2013 of the “United European Gastroenterology” (UEG) week in Berlin, Germany.
- 30/05/2005: Finalist international “Flemish Biology Olympiad”.

1.1.8. Research skills

- qRT-PCR
- Western blotting, IHC and ELISA
- Cell culture techniques
- Cell viability and proliferation assays and colony formation assays
- Mouse models for HCC and cirrhosis
- Clinical trial design

1.1.9. Reviewer for journals

Reviewed manuscripts for the following international scientific journals:

- 20/05/2015: *Journal of Hepatology* (IF: 10.4)
- 13/04/2015: *Apoptosis* (IF: 3.6)
- 16/03/2015: *World Journal of Gastroenterology* (IF: 2.4)
- 02/05/2013: *Carcinogenesis* (IF: 5.7)
- 01/02/2014: *European Journal of Gastroenterology and Hepatology* (IF: 2.2)

1.1.10. Participation in popular media

- 10/11/2014: Interview regarding the foundation of Young BASL in “Artsenkrant”

1.1.11. Active memberships

- EASL: European association for the study of the liver
- AASLD: American association for the study of liver diseases
- ECCO: European cancer organisation
- VVGE: Vlaamse vereniging van Gastro-enterologie
- Jong-VVGE: Jong Vlaamse vereniging van Gastro-enterologie
- BASL: Belgian association for the study of liver diseases
- Young BASL: Young Belgian association for the study of liver diseases (President: 2014-up till now)
1.1.12. Bibliography


=> Case report showing the increased risk of stroke by sorafenib in low cardiovascular risk patient.


=> Review of the literature concerning the unfolded protein response in cancer.


=> In this study, we showed the therapeutic potential of the antimalarial Artesunate in HCC. This study led to the start of a phase I trial of Artesunate in advanced HCC patients (NCT02304289).


=> Review concerning the unfolded protein response and angiogenesis.


=> Review concerning the key pathways regulating liver progenitor cells in HCC.


=> Review concerning the new treatment options for hepatitis C in a Belgian context.


=> In this paper, we observed the presence of intestinal metaplasia following carcinogen-induced liver damage and regeneration.

Cytoprotective effect of the chemical chaperone TUDCA reduces experimental colitis.


In this study, we showed that UPR activation is fine-tuned during the different phases of tumorigenesis and that the PERK pathway is essential for tumour cell survival upon ER stress.


In this study, we demonstrated that the effect of hypoxia depends on the phase of tumorigenesis.


In this study, we demonstrated the chemopreventive potential of TUDCA and the pivotal role of the pro-apoptotic UPR in the initiation of hepatocarcinogenesis.
Chapter 6

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Chapter 7

Supplemental information
1. Supplementary files

Supplementary files can be downloaded using the following URL:

https://docs.google.com/document/d/15PN0_GCWz3y_i0Z7XMNgvrJCNgqqfMUr6aLMYbV4/edit?usp=sharing

Or by scanning the following QR-code: