Aus dem
CharitéCentrum 13 für Innere Medizin mit Gastroenterologie und Nephrologie
Medizinische Klinik mit Schwerpunkt Gastroenterologie, Infektiologie und Rheumatologie
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Habilitationsschrift

Bedeutung und mögliche therapeutische Nutzung von Komponenten der extrazellulären Matrix, KCa3.1-Ionenkanälen und *Lindera obtusiloba* bei Leberfibrose und dem hepatozellulären Karzinom

zur Erlangung der Lehrbefähigung
für das Fach Experimentelle Medizin
vorgelegt dem Fakultätsrat der Medizinischen Fakultät
Charité - Universitätsmedizin Berlin

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Eingereicht: **Juni 2015**

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Abkürzungsverzeichnis

α2(VI)	Tripelhelikale Domäne der alpha2-Kette von Kollagen VI
AKT	Protein Kinase B
ASH	Alkoholische Steatohepatitis
CBD	*Collagen binding domain* (der Matrix-Metalloproteinasen)
CD	*Cluster of differentiation*
CED	Chronisch entzündliche Darmerkrankungen
CLT	Clotrimazol
CM	*Calcification medium*
COX-2	Cyclooxygenase-2
DDR1	*Discoidin domain receptor 1*
DSS	Natriumdextransulfat
EGCG	Epigallocatechingallat
EMT	Epithel-Mesenchym-Transition
ERα	*Estrogen receptor alpha*
ERK1/2	*Extracellular-signal regulated kinase*
ES	(+)-Episesamin
EZM	Extrazelluläre Matrix
$(GPO)_{10}$	$[H\text{-}GCO\text{-}(GPO)_{10}\text{-}GCOG\text{-}NH_2]_3$; tripelhelikales Kollagenpeptid
HCC	Hepatozelluläres Karzinom
HSZ	Hepatische Sternzelle
HO-1	*Heme oxygenase*-1
IFN-γ	Interferon gamma
IGF-1	*Insulin growth factor*-1
IGF-1R	*Insulin growth factor*-1 *receptor*
IL	Interleukin
LOE	Wässriges *Lindera obtusiloba* Extrakt
LPS	Lipopolysaccharid
KCa3.1	Ca^{2+}-aktivierter K^{+}-Kanal mit mittlerer Leitfähigkeit
KVI-F/PR	Pepsin-resistentes, tripelhelikales Kollagen VI-Fragment
MAPK	*Mitogen-activated protein kinase* (MAP-Kinase-Signalweg)
MCP-3	*Monocyte chemotactic protein*-3
MMP(s)	Matrix-Metalloproteinase(n)
MT-MMPs	Membranständige Matrix-Metalloproteinasen
MUC-2	Mucin 2
NASH	Nicht-alkoholische Steatohepatitis
NF-κB	*Nuclear factor-kappa B*
NK-Zelle	Natürliche Killerzelle
NO	Stickstoffmonoxid
PDGF	*Platelet-derived growth factor*
PI3K	Phosphatidylinositol-4,5-Bisphosphat 3-Kinase
ProMMP	Pro-Matrix-Metalloproteinase (inaktive Proform)
qPCR	Quantitative Polymerase-Kettenreaktion
Raf-Kinase	*Rapidly accelerated fibrosarcoma*-Kinase
siRNA	*Small interfering RNA*
STAT3	*Signal transducer and activator of transcription* 3

TCM	Traditionelle Chinesische Medizin
TGF-β1	*Transforming growth factor beta 1*
TIMP	*Tissue inhibitor of metalloproteinases*
TLR	*Toll-like receptor*
TNBS	2,4,6-Trinitrobenzolsulfonsäure
TNF-α	*Tumor necrosis factor alpha*
TRAM-34	Spezifischer Inhibitor von Kca3.1
TRPM7	*Transient receptor potential melastain* 7
TRPV4	*Transient receptor potential vanilloid* 4
VEGF	*Vascular endothelial growth factor*
VSMC	Glatte Gefäßmuskelzelle

1 Einleitung

1.1 Fibrose

Als Fibrose wird die pathologische Akkumulation extrazellulärer Matrix (EZM) in Geweben bezeichnet, eine Reaktion des Körpers auf einen pathologischen Stimulus im Sinne einer Wundheilung. Eine fortgeschrittene Fibrose kann beträchtliche Einschränkungen bzw. den Verlust von Organfunktionen hervorrufen und tritt neben der Leber (Kap. 1.2) auch in Niere, Lunge, Haut und Darm (Morbus Crohn – Kap. 1.5.4) auf (**Abb. 1**). Neben organspezifischen molekularen Veränderungen im Rahmen der Fibrose, wie z.B. die vermehrte Transdifferenzierung von Perizyten zu Myofibroblasten in der renalen Fibrose, gibt es auch organübergreifende Gemeinsamkeiten wie eine verstärkte Expression des *Transforming growth factor beta* 1 (TGF-β1), so dass TGF-β1-spezifische Therapieansätze möglicherweise auch organübergreifend wirken [1].

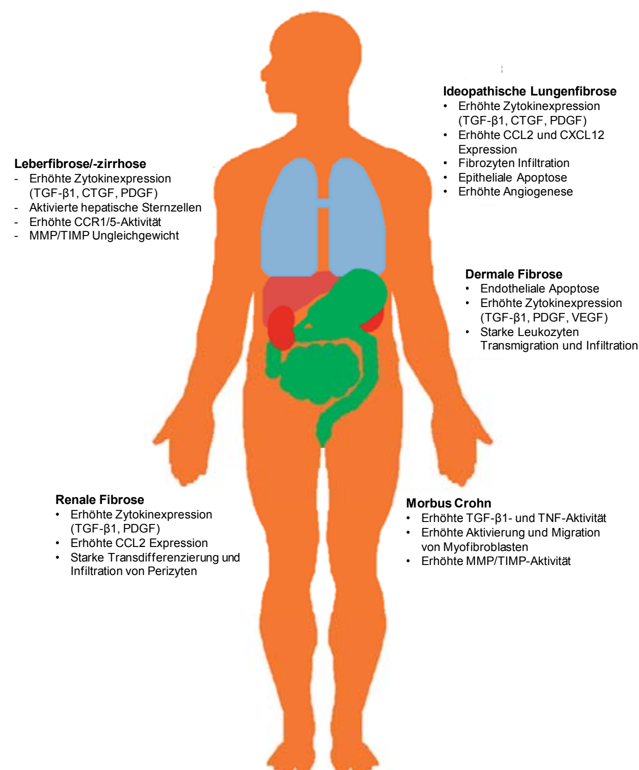

Abbildung 1: Pathologische und molekulare Merkmale von Fibrose. Der genaue molekulare Vorgang der Fibrose ist noch unverstanden, er beinhaltet spezifische pathologische bzw. molekulare Veränderungen, die in der Abbildung stichpunktartig aufgeführt sind. Ein zentrales gemeinsames Merkmal der Fibrose ist eine stark erhöhte TGF-β1-Expression. Bis heute gibt es nur wenige effektive anti-fibrotische Therapien. Viele sind gegen Fibrose-assoziierte Signalwege wie von TGF-β1 gerichtet, können also auch fibrose- bw. organübergreifend wirken. *Abkürzungen:* CCL2, CC-Ligand 2 (Synonym: MCP-1); CCR, CC Receptor; CTGF, *connective tissue growth factor*; CXCL12, CXC-Motiv-Ligand 12; PDGF, *platelet-derived growth factor*; VEGF, *vascular endothelial growth factor*; TIMP, *tissue inhibitor of metalloproteinases*; TNF, *tumor necrosis factor* (Modifiziert nach [1]).

1.2 Leberfibrose und –zirrhose

Hauptursachen der Leberfibrose sind Hepatiden wie das Hepatitis B- oder C-Virus, Autoimmun- und Gallenwegserkrankungen, die alkoholische Steatohepatitis (ASH) und mit steigender Inzidenz auch die

nicht-alkoholische Steatohepatitis (NASH) [2-6]. Bei chronischer Schädigung/Inflammation, z.B. auch durch Alkoholabusus, ist die Homöostase von Synthese und Degradation der EZM chronisch gestört. Hauptproduzenten der EZM in der Leber sind hepatische Sternzellen (HSZ), die physiologisch ein ausgeglichenes Expressionsprofil von Matrix-aufbauender EZM (u.a. Kollagen IV) und Matrix-abbauenden Matrix-Metalloproteinasen (MMPs) zeigen. Durch chronische Schädigung von Leberzellen (Hepatozyten, Endothel- und Kupfferzellen) bzw. durch von infiltrierten Makrophagen freigesetzte Zytokine wie TGF-β1, *Tumor necrosis factor* (TNF)-α, Endothelin-1, *Platelet-derived growth factor* (PDGF), Interleukin (IL)-6 oder Interferon (IFN)-γ sowie reaktive Sauerstoffradikale, kommt es zu einer Aktivierung oder auch Transdifferenzierung der quieszenten HSZ zu aktivierten HSZ, sogenannten Myofibroblasten. Diese sezernieren vermehrt fibrilläre Kollagene wie Kollagen I und III sowie die MMPs -1, -2, -3, -13 und -14 [7-10] (**Abb. 2**).

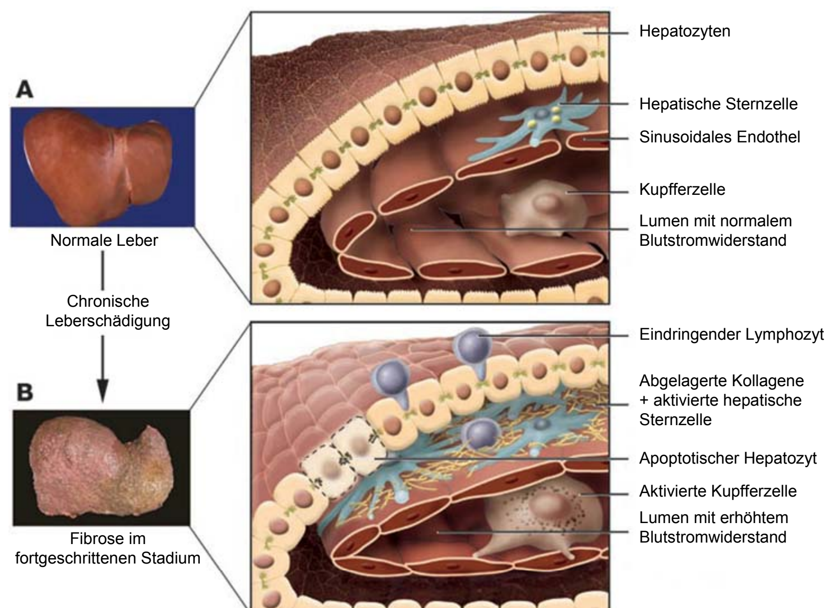

Abbildung 2: Pathologische Vorgänge in der Leber auf Zellebene. (**A**) Im normalen Zustand liegt die hepatische Sternzelle im quieszenten Zustand vor. Die Hepatozyten besitzen Mikrovilli für den Stoffaustausch. Der Blutstrom in den Sinusoiden ist ungehindert. (**B**) Bei chronischer Schädigung werden quieszente HSZ aktiviert, transdifferenzieren zu Myofibroblasten und produzieren vermehrt Bindegewebe, Wachstumsfaktoren und Chemokine. Hepatozyten verlieren ihre Mikrovilli und gehen teilweise in Apoptose. Die vermehrten Kollagen-ablagerungen und der Verlust der Endothelfenestrierung führen zu erhöhtem portalen Druck und verminderter Organfunktion [11].

Neben den MMPs erfolgt aber gleichzeitig auch die Hochregulation der natürlichen MMP-Inhibitoren *Tissue inhibitor of metalloproteinases* (TIMP)-1 und -2, wobei TIMP-1 im frühen- und TIMP-2 eher im fortgeschrittenen Stadium der Fibrose/Zirrhose exprimiert wird [8, 12]. Neben den HSZ, werden während der Fibrogenese auch portale Fibroblasten aktiviert und transdifferenzieren zu Kollagen-produzierenden Myofibroblasten [13] (**Abb. 3**).

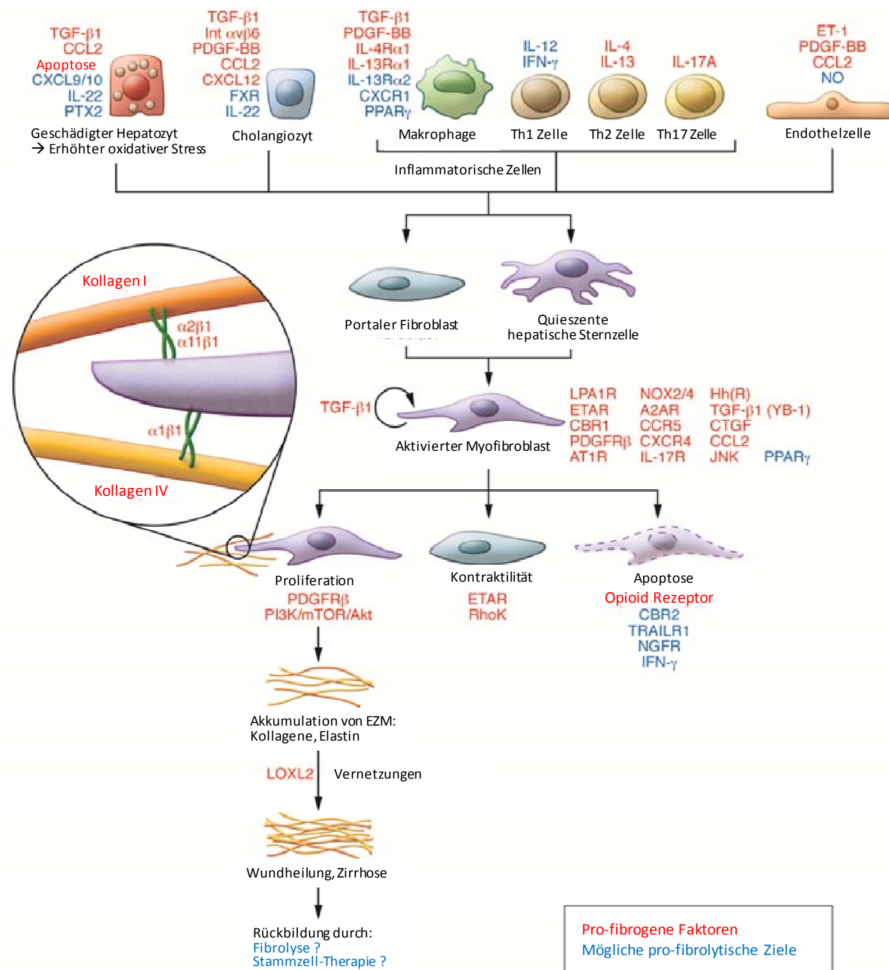

Abbildung 3: Entstehung von Myofibroblasten im Kontext der Fibrose. Dargestellt sind neben den verschiedenen Quellen von Myofibroblasten (portale Fibroblasten und quieszente HSZ) auch Ziele für anti-fibrotische Therapien. Die Matrix selbst kann als Regulator von Fibrogenese und Fibrolyse wirken. So werden Kollagenfibrillen im fortgeschrittenen Stadium der Fibrose durch *lysyl oxidase-like 2* (LOXL2) vernetzt, was eine Fibrolyse, also die Reversibilität der Fibrose erschwert. Zudem können Kollagen-bindende Matrix-Rezeptoren wie die Integrine α1β1, α2β1 und α11β1 pro-fibrogene oder pro-fibrolytische Effekte in Myofibroblasten induzieren. *Abkürzungen:* A2AR, Adenosine 2A-Rezeptor; AT1R, Angiotensin 1-Rezeptor; CBR1, Cannabinoid-Rezeptor 1; CCL2, CC-Ligand 2; CXCL, CXC-Motiv-Ligand; CXCR, CXC-Motiv-Rezeptor; ET-1, Endothelin-1; ETAR, Endothelin A-Rezeptor; FXR, Farnesoid X-Rezeptor; Hh(R), hedgehog (receptor); IFNγ, Interferon gamma; IL, Interleukin; Int, Integrin; JNK, c-Jun N-terminale Kinase; LPA1R, Lysophosphatidylsäure-Rezeptor 1; mTOR, *mechanistic target of rapamycin;* NGFR, *nerve growth factor receptor*; NO, Stickstoffmonoxid; PDGF-BB, *platelet-derived growth factor-BB*; PI3K, Phosphatidylinositol-4,5-Bisphosphat 3-Kinase, PPARγ, *peroxisome proliferator-activated receptor gamma*; PTX2, Pentraxin 2; RhoK, *G protein-coupled receptor kinase 1*; Th Zelle, T-Helfer-Zelle; TRAILR, *tumor necrosis factor-related apoptosis-inducing ligand receptor*; YB-1, *Y-box binding protein*. (Modifiziert nach [14]).

Die (pathologische) Akkumulation von EZM im Leberparenchym kann zu eingeschränkter Leberfunktion und erhöhtem intrahepatischen Blutflusswiderstand führen [11, 15]. Eine weiter anhaltende Schädigung führt zur Ausbildung der Leberzirrhose, einem knotigen Umbau des Leberparenchyms mit Ausbildung von Regeneratknoten und Bindegewebssepten. Es kommt letztlich zum Ausfall der normalen Synthese-, Exkretions-, Biotransformationsfunktion der Leber. Im Gegensatz zur Fibrose ist die Zirrhose als Endstufe irreversibel und zählt, mit zunehmender Inzidenz, zu den 20 häufigsten Todesursachen in Deutschland [16]. Als effektive therapeutische Maßnahme bleibt bisher nur eine Lebertransplantation.

1.2.1 Therapieansätze der Leberfibrose

Ein gängiger Behandlungsansatz ist die Behandlung der primären Erkrankung, mit dem Ziel, eine Perpetuierung der Fibrose zu unterdrücken. Im einfachsten Fall ist dies die Alkohol-Abstinenz bei der Alkohol-induzierten Leberfibrose, aber auch der Einsatz antiviraler Therapeutika im Fall von viraler Hepatitis oder von Chelatoren bei Morbus Wilson. Weitere Ansätze zielen auf die Suppression der hepatischen Entzündungsreaktion, um eine dadurch induzierte Aktivierung von HSZ zu unterdrücken. Hier wurden z.B. die Behandlung mit anti-inflammatorischem IL-10 oder die Inhibition von TNF-α getestet [17, 18]. Besonders in der ostasiatischen Heilkunde werden vielfach auch pflanzliche Wirkstoffe oder Extrakte mit anti-inflammatorischer bzw. anti-oxidativer Wirkung zur alternativmedizinischen, anti-fibrotischen Therapie eingesetzt [19, 20]. Ein anderes Ziel ist die Antagonisierung auto- und parakriner Prozesse durch aktivierte HSZ, z.B. durch die Inhibition mitogener und fibrogener Zytokine wie o.g. TGF-β1 oder die Inhibition von deren Rezeptoren wie z.B. den *platelet-derived growth factor receptor alpha* (PDGFR-α) [21]. Der portalen Hypertension als wichtigen Fibrosestimulus kann durch eine Manipulation des Gleichgewichts von Endothelin und Stickstoffmonoxid (NO) entgegengewirkt werden. Zudem konnte eine vermehrte Kollagensynthese durch Inhibition des Renin-Angiotensin-Systems unterbunden werden. Auch das Konzept einer gezielten Induktion der HSZ-Apoptose, mit der Idee die schädigenden (EZM- und TIMP-produzierenden) aktivierten HSZ aus dem Organismus zu entfernen, wird verfolgt. Dies kann durch Aktivierung der Caspase-Signaltransduktionswege geschehen, extrazellulär durch Stimulierung des „Todes“-Rezeptors“ Fas (Synonym: *cluster of differentiation* 95-CD95) oder intrazellulär, z.B. durch Adenoviren. Generell wird die Entwicklung anti-fibrotischer Therapien u.a. dadurch erschwert, dass Leberfibrose bzw. -zirrhose i.d.R. erst im fortgeschrittenen Stadium diagnostiziert und damit potentiell behandelt werden. Ein rationaler Ansatz bei ausgeprägter Fibrose ist eine Beeinflussung der Matrixdegradation durch gezielte Inhibition der TIMP-Expression bzw. der Induktion der MMP-Expression [11, 15, 22]. In dieser Habilitationsschrift wird mit der lokalen Freisetzung, bzw. Aktivierung EZM-assoziierter MMPs durch synthetische Kollagenmimetika ein weiterer potentieller Ansatzpunkt diskutiert.

1.3 Das hepatozelluläre Karzinom

Eine Komplikation der Leberzirrhose bzw. Leberfibrose im fortgeschrittenen Stadium stellt das hepatozelluläre Karzinom (HCC) dar, die dritthäufigste Krebs-Todesursache und die fünfthäufigste Todesart weltweit [23, 24]. Das HCC hat die am stärksten ansteigende Inzidenz für Krebserkrankungen in der westlichen Welt [25], was u.a. Folge der vermehrt auftretenden Hepatitis C-assoziierten Fibrose/Zirrhose ist. Unabhängig vom HCC ist die Leberzirrhose auch als eigene bzw. zusätzliche Krankheitsentität zu sehen, was die Behandlung mit Zytostatika weiter erschwert [26]. Kurative Ansätze wie die Resektion, die Lebertransplantation, eine lokale Ablation oder eine Chemoembolisation sind nur bedingt möglich, eingeschränkt z.B. durch die Leberfunktion, die Tumorgröße und –anzahl oder dem

Grad der Vaskularisierung [27]. Die einzige bisher zugelassene systemische Therapieoption für das HCC im fortgeschrittenen Stadium ist der Multikinaseinhibitor Sorafenib (s.u.) [28].

Die Grundlagen der Assoziationen zwischen Fibrose und dem HCC sind bisher noch wenig verstanden. Man vermutet, dass inflammatorische Zellen, Integrin-Signalwege, Wachstumsfaktor-Interaktionen mit der EZM und interzelluläre Kommunikation von HSZ mit Tumorzellen involviert sind [29]. Für eine HCC-fördernde Wirkung der Fibrose spricht die Tatsache, dass aktivierte HSZ neben Endothel- und Tumorzellen in Patienten mit HCC gefunden werden und Überstände von HSZ-Kulturen die Proliferation und Migration humaner HCC-Zellen stimulieren.

Bei Fibrose-/Zirrhose-assoziierten HCCs stellt die umgebaute EZM ein Reservoir für gebundene Wachstumsfaktoren dar, welche neben der Angiogenese auch das Überleben prä-neoplastischer Hepatozyten und aktivierter HSZ fördern. Hinzu kommt eine verminderte Aktivität natürlicher Killerzellen sowie natürlicher Killer-T-Zellen, welche Tumorzellen erkennen und abtöten können. Dies, in Zusammenspiel mit inflammatorischen Signalen, inkl. Telomerase-Reaktivierung und oxidativem Stress, begünstigt die HCC-Entstehung (**Abb. 4**) [30].

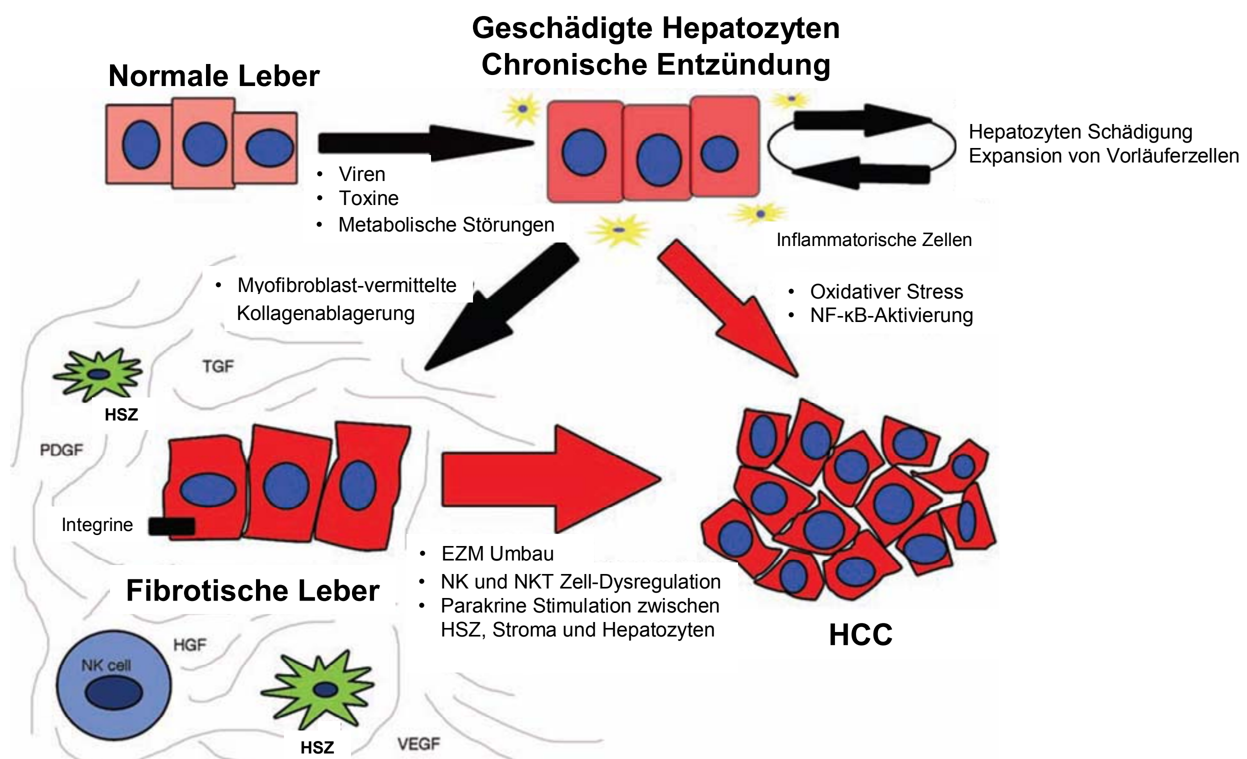

Abbildung 4: Die Progression von normalen Hepatozyten zum HCC. Chronische Schädigung durch Viren, Toxine oder metabolische Faktoren führt zu Hepatozytenschäden mit entsprechender Entzündungsreaktion. Es kommt zu vermehrtem Zellumsatz und der Expansion von Vorläuferzellen. Dies, in Kombination mit oxidativem Stress und der verstärkten Aktivierung des Transkriptionsfaktors *nuclear factor kappaB* (NF-κB), kann direkt zur Ausbildung eines HCC führen. Parallel kommt es zur Aktivierung/Transdifferenzierung von hepatischen Sternzellen zu Myofibroblasten, was zu Kollagenakkumulation bzw. verstärktem EZM-Umbau führt. Die roten Pfeile zeigen hepatokarzinogene Schritte, wobei die Pfeilgröße den relativen Anteil am Krankheitsgeschehen widerspiegelt: Das HCC entwickelt sich oft auf Basis einer fortgeschrittenen Fibrose oder Zirrhose. *Abkürzungen:* HGF, *hepatocyte growth factor*; HSZ, Hepatische Sternzelle; NK, natürliche Killerzelle; NKT, natürliche Killer-T-Zelle; PDGF, *platelet-derived growth factor;* TGF, *transforming growth factor*; VEGF, *vascular endothelial growth factor* (Modifiziert nach [30]).

Wie bei anderen Tumorentitäten sind Rezeptortyrosinkinasen und nachgeschaltete Signaltransduktionswege auch beim HCC vermehrt aktiviert und fördern so Proliferation, Angiogenese und Apoptoseresistenz [31]. Durch Multikinaseinhibitoren, die bei anderen Tumoren teilweise schon erfolgreich eingesetzt werden, ergeben sich so potentielle therapeutische Ansatzpunkte. Beim HCC hat sich bisher nur Sorafenib (hemmt u.a. die *rapidly accelerated fibrosarcoma* (Raf)-Kinase sowie die VEGF- und PDGF-Rezeptoren) als so wirksam erwiesen, dass es die bisher einzige zugelassene Substanz zur Therapie des fortgeschrittenem HCC ist [27]. Die mediane Überlebenszeit wird um ca. drei Monate verbessert [28]. Dies zeigt, dass weitere Therapiekonzepte dringend erforderlich sind.

1.4 Die extrazelluläre Matrix

Die für die Fibrose charakteristische EZM umfasst unlösliche Bestandteile und assoziierte Makromoleküle des interstitiellen Raumes [32-34]. Ursprünglich nur als eine Art Stützgerüst für Organe angesehen, erfüllt die EZM zudem eine Filter- bzw. Diffusionsbarrierefunktion und kann auch Zellfunktionen und -eigenschaften wie Proliferation, Migration, Adhäsion, Polarisation und Differenzierung beeinflussen. Zusätzlich erfüllt die EZM eine Speicherfunktion für Zytokine und Wachstumsfaktoren [35, 36]. Hauptbestandteile der EZM sind vor allem Kollagene sowie nicht-kollagene Glykoproteine und Proteoglykane [37]. Kollagene werden in fibrilläre und nicht-fibrilläre Kollagene unterschieden. Alle Kollagene weisen zumindest eine tripelhelikale Domäne auf, wobei fibrilläre Kollagene durchgehend tripelhelikal vorliegen. Die Kollagenfaser besteht aus mehreren Kollagenfibrillen welche aus mehreren Mikrofibrillen zusammengesetzt sind. Diese wiederum bestehen aus Tropokollagen-Molekülen, die aus drei Polypeptidketten aufgebaut sind, welche in Form einer rechtsgängigen Tripelhelix umeinander gewunden sind [38, 39]. Die Peptidketten fibrillärer Kollagene enthalten die charakteristische Aminosäuresequenz Gly-X-Y (Gly = Glycin, X = Prolin, Y = Hydroxyprolin). Der elektronegative Sauerstoff des Prolins und die Hydroxy-Gruppe des Hydroxyprolins begünstigen die Bildung von Wasserstoffbrückenbindungen und führen so zu hoher Stabilität und Zugfestigkeit der Kollagenfasern [38, 39].

Synthese und Degradation von EZM befinden sich unter normalen Bedingungen in einem dynamischen Gleichgewicht [40]. Ein physiologisches Verschieben dieser Homöostase tritt z.B. bei Wachstum und Embryogenese auf. Bei Fibrogenese und Tumorwachstum ist dies pathologisch. Der für den Matrixumbau wichtige Abbau von z.B. Kollagenen erfolgt durch ein Zusammenspiel mehrerer Enzyme wie Serinproteasen und vor allem den MMPs (Kap. 1.5).

1.4.1 Regulatorische Funktion der extrazellulären Matrix bei biologischen Vorgängen

Die EZM beeinflusst durch ihre verschiedenen Komponenten u.a. die Migration, Proliferation, Differenzierung, das Überleben und Apoptose von in die EZM eingebetteten Zellen [41]. EZM-Moleküle wie Kollagene, nichtkollagene Glykoproteine sowie Proteoglykane haben verschiedene, z.T.

überlappende funktionelle Domänen, die als Liganden für Matrixrezeptoren dienen können und im Zusammenspiel mit Wachstumsfaktoren, differenzierte Signaltransduktionsprozesse auslösen. Vor allem bei chronischen Entzündungen oder Tumorerkrankungen, die mit erhöhter Synthese und Degradation der EZM einhergehen, können durch Fragmentierung der EZM-Moleküle autarke Domänen freigesetzt werden, die potent Wachstum, Differenzierung oder Angiogenese induzieren können [42]. So sind chronische Leberentzündungen wie Hepatitis B oder C von verstärkter Expression z.B. der Kollagene I, III, VI und XIV begleitet, die ihrerseits die Fibroseprogression durch direkten Einfluss auf die HSZ und Myofibroblasten modulieren. Das Kollagen VI-Fragment, das in der Fibrosierung um ein Vielfaches überexprimiert ist und als Fibrosemarker dient, stimuliert das Wachstum und die Kollagensynthese dieser Zellen [41]. Ein weiteres Beispiel für den Einfluss der EZM auf Zellen ist die Epithel-Mesenchym-Transition (EMT), die u.a. beim HCC auftritt [43, 44]. Hier, wie auch bei anderen epithelialen Tumoren, spielt das Kollagen VI-Fragment eine entscheidende Rolle. Im Gegensatz zur Fibrose sind die Effekte eher parakrin [45, 46]. Endokrines und exokrines Kollagen VI-Fragment wirken auf mesenchymale und epitheliale Zellen mitogen, anti-apoptotisch und vermitteln Chemotherapeutika-Resistenz, sind also ein wesentlicher Überlebensfaktor [47, 48]. Die Adhäsion von Zellen an Kollagen VI wird nur teilweise über eine Integrin-induzierte Signaltransduktion vermittelt, was den Einfluss anderer Kollagen-Rezeptoren wie den *discoidin domain receptor* (DDR)1, das transmembranäre Proteoglykan NG2, oder auch Komplexe aus NG2, Integrinen und weiteren Rezeptoren impliziert [41, 49].

Neben Kollagen VI fördert auch das Matrixprotein Fibronektin das Überleben von Zellen, u.a. durch Aktivierung des e*xtracellular-signal regulated kinase* (ERK)- und des *c-Jun* NH_2*-terminal kinase* (JNK)-Kinase-Signaltransduktionsweges in Fibroblasten [50].

Darüber hinaus ist das ebenfalls in der Leberfibrose hochregulierte, ursprünglich Undulin benannte, Kollagen XIV biologisch aktiv. Es ist Fibrillen-assoziiert und kommt vor allem in der EZM ausdifferenzierter Gewebe aber nicht im Tumorstroma vor [51]. Als Rezeptor für Kollagen XIV wurde eine transmembranäre Chondroitin-Dermatansulfat Variante von CD44 identifiziert [52]. CD44 ist ein Zelladhäsionsmolekül, welches auf Leukozyten, epithelialen sowie mesenchymalen Zellen exprimiert wird und primär als ein Rezeptor für Hyaluronsäure identifiziert wurde [53]. Neben seiner Rolle in der Tumorgenese bzw. Metastasierung ist die Bedeutung von CD44 und seiner Varianten für entzündliche Vorgänge, z.B. für die Rekrutierung von T-Zellen bei chronisch-entzündlichen Darmerkrankungen (CED), gut belegt.

Kollagen XIV und sein N-terminales CD44-bindendes Fragment sind starke Induktoren der Differenzierung von Fibroblasten, Präadipozyten und myeloischen Zellen ([54] und eigene unveröffentliche Daten). Über die Signaltransduktionswege, u.a. in HSZ, ist bisher nichts bekannt.

1.5 Matrix-Metalloproteinasen

MMPs sind Kalzium-abhängige, proteolytische Enzyme, die in ihrer Gesamtheit alle Protein-Komponenten der EZM abbauen können. Sie werden als inaktive Proformen sezerniert (ProMMPs) und

extrazellulär durch proteolytische Spaltungen und Konformationsänderungen aktiviert. MMPs beeinflussen physiologische Prozesse wie Zellmigration [55, 56], Embryogenese [57, 58], Wundheilung [59] und den ovariellen bzw. endometrialen Zyklus [60]. MMP-assoziierte, pathologische Prozesse sind u.a. Tumorerkrankungen (Invasion, Metastasierung) [61-63], Kornea- und Magenulzera sowie Kollagenosen wie Arthritis und Fibrose [35, 64-66].

1.5.1 Struktur und Regulation von Matrix-Metalloproteinasen

Grundstrukturen aller MMPs sind das N-terminale Propeptid, die katalytische Domäne und - mit Ausnahme von MMP-7 - eine Hämopexin-ähnliche Domäne am C-terminalen Ende [67]. Das N-terminale Propeptid interagiert über einen Cysteinrest mit einem Zinkatom im katalytischen Zentrum, wodurch das Propeptid über die katalytische Domäne gefaltet wird und so das aktive Zentrum blockiert [68]. Vor Abspaltung der Prodomäne liegt das Enzym in seiner inaktiven Proform vor [68, 69]. Eine Abspaltung des Propeptids führt zur Aktivierung der MMPs [59]. Die C-terminale, Hämopexin-ähnliche Domäne ist eine „Exosite“ [70] und ist wichtig für die Substratbindung und Interaktion mit TIMPs, den endogenen Inhibitoren der MMPs, sowie mit großen Molekülen, die nicht direkt an das katalytische Zentrum gelangen können [71, 72]. MMP-7 und MMP-12 sind auch ohne diese Domäne enzymatisch aktiv [69]. Wird diese Domäne dagegen bei den Kollagenasen MMP-1 und MMP-8 entfernt, können diese keine (große) kollagene Tripelhelix degradieren [69, 73]. Die Gelatinasen MMP-2 und MMP-9 besitzen zusätzlich eine Kollagenbindungs-Domäne (*collagen binding domain* - CBD) [74], membranständige MMPs (MT-MMPs) eine entsprechende Transmembrandomäne [75].
MMPs werden in der Regel nur aufgrund (zell)spezifischer Signale synthetisiert und sezerniert. Unphysiologisch aktivierte, bzw. fehlregulierte MMPs können zu pathologischer Zerstörung von Gewebe führen. Die daher strenge MMP-Regulation erfolgt auf Ebene der Genexpression und durch Kontrolle der proteolytischen Aktivität bzw. Aktivierung. Die meisten MMPs sind durch Wachstumsfaktoren, Zytokine, chemische Reagenzien, physikalischen Stress und zelluläre Transformation bei Neoplasien hochreguliert [76-78] und können z.B. durch Glukokortikoide, Retinolsäure und Progesteron runterreguliert werden [77-79]. Nach der Sekretion als inaktive Proformen erfolgt eine regulierte Prozessierung und Aktivierung, z.B. durch Peptidasen wie Plasmin oder durch andere MMPs wie MMP-7 (aktiviert ProMMP-3 und -9) [75, 80-82] oder MMP-2 und -3 (aktivieren ProMMP-9) [83, 84]. Intrazellulär kann Furin z.B. MMP-3 und die MT-MMPs aktivieren [85, 86]. Aktivierte MMPs können umgekehrt durch o.g. TIMPs gehemmt werden. Auf Zelloberflächen dient TIMP-2 im Komplex mit MT-MMP-1 allerdings als ein natürlicher Aktivator von ProMMP-2 [59, 87-90]. Zudem können pH-Wert- und Temperaturschwankungen zur Aktivierung führen [91, 92].

1.5.2 Rolle von Matrix-Metalloproteinasen bei Fibrose und Neoplasie

Die gestörte Homöostase von EZM-Synthese und –Abbau bei der Fibrose führt zu Forschungsansätzen zur Modulierung dieses Gleichgewichtes, u.a. durch Inhibition der TIMP-Expression oder durch Induktion der MMP-Expression.

Bei Tumoren beeinflusst die Interaktion von Zellen mit benachbarter EZM und Stromazellen, der Tumormikroumgebung, das Verhalten und Eigenschaften von Tumoren [32]. Neben Effekten von EZM-Molekülen bzw. EZM-Fragmenten (Kap. 1.4.1) sind Effekte von MMPs auf die Metastasierung von Tumoren schon länger bekannt. Insbesondere die MMPs -2, -13 und -14 spielen dabei eine wichtige Rolle, was sich u.a. an erhöhter Expression der MMPs in Invasionsfronten von Tumoren zeigt [93-95]. Zudem können Hepatozyten in frühen Stadien (wenig differenziert) des HCC einen Phänotyp ausbilden, der mit erhöhter MMP-1-Produktion assoziiert ist und verstärkte Proliferation und Invasion zeigt [30]. In der Folge exprimieren differenzierte Hepatomzellen verstärkt MT-MMP-1, MMP-2 oder MMP-9, welche die Invasion durch die Stroma fördern [96]. Eine (therapeutische) Beeinflussung des MMP-TIMP-Gleichgewichtes, z.B. durch MMP-Inhibitoren, könnte so zu verminderter Progression beitragen [97, 98].

1.5.3 Chronisch entzündliche Darmerkrankungen

Ein Charakteristikum von chronisch entzündlichen Darmerkrankungen (CED) wie Morbus Crohn und Colitis ulcerosa ist eine gestörte und persistierende Aktivierung des mukosalen Immunsystems, begleitet von einer schweren Darm-Pathologie und Veränderungen in der Darm-Physiologie, u.a. des Darmepithels. Durch eine gestörte Homöostasevon Aktivierung, Wachstum bzw. Apoptose von Immunzellen in der intestinalen Mukosa kommt es zu verstärkter Infiltration von aktivierten T-Zellen, was wiederum zur Generierung eines Chemokin-/Zytokin-Milieus führt, welches weitere Immunzellen (Neutrophile, dendritische Zellen, Makrophagen, NK-Zellen) anlockt [99]. Neben Immunzellen tragen auch nicht-Immunzellen zu diesem Prozess bei. So kann die Darmflora, bzw. aus Bakterien stammende Liganden, die *toll-like receptors* (TLR)-2 und -4 in intestinalen Myofibroblasten Stimulieren, was zur Aktivierung von NF-κB und weiterer Freisetzung von Zytokinen und Chemokinen führt [100]. Diese so in ihrer Gesamtheit immer weiter verstärkte Immunantwort führt letztlich zu einem Funktionsverlust der epithelialen Barriere. Bei chronischer Schädigung kann sich im Rahmen einer dadurch induzierten Wundheilung als Komplikation eine intestinale Fibrose entwickeln [99], was u.a. zu einer Reduktion des Lumendurchmessers und intestinalen Stenosen führen kann [101]. Als Langzeitkomplikationen der CED ist die Ausbildung maligner Tumoren möglich [102].

1.5.4 Rolle von MMP-2 und -9 bei chronisch entzündlichen Darmerkrankungen

Proteolytische Enzyme wie MMPs werden als „Antwort“ auf inflammatorische Prozesse verstärkt freigesetzt und spielen damit auch eine wichtige Rolle im Rahmen der EZM-Homöostase im Darm bzw. bei CED [103, 104]. Neben der Leberfibrose trägt ein gestörtes MMP-TIMP-Gleichgewicht auch bei Morbus Crohn und Colitis ulcerosa zur Entwicklung von intestinaler Fibrose bei.

Im entzündeten intestinalen Gewebe dominiert die Expression der Gelatinase MMP-9. Die Gewebskonzentrationen von MMP-9, nicht aber die der anderen Gelatinase MMP-2, korrelieren mit der Krankheitsaktivität von Patienten mit Colitis ulcerosa und wurden mit einer Schädigung der Mukosa und Fistelbildung bei Patienten mit Morbus Crohn assoziiert. Diese Daten wurden durch Tiermodelle mit MMP-9$^{-/-}$-und MMP-2$^{-/-}$/MMP-9$^{-/-}$-Doppelknockoutmäusen bestätigt. Diese zeigen signifikant geringere klinische und histologische Manifestationen einer durch Natriumdextransulfat (DSS)-induzierten Colitis. Zudem sind MMP-2$^{-/-}$-Mäuse, denen das in der intestinalen epithelialen Barriere exprimierte MMP-2 fehlt, stark empfindlich gegenüber der Entwicklung einer Colitis [107].

Die pro-inflammatorische, eher schädigende Funktion von MMP-9 bei CED beruht neben einer möglichen Chemoattraktion von Neutrophilen [105, 106] u.a. auf einer Blockade der Differenzierung von Becherzellen, den Hauptproduzenten von Mucin-2 (MUC-2) im Darm. MUC-2 gehört zu den sekretorischen Mucinen, die Bestandteil einer schützenden Schleimschicht des intestinalen Epithels sind. Ein entsprechender MUC-2 Mangel durch erhöhte MMP-9-Aktivität führt zu Barriereschäden [108]. Darüber hinaus degradiert MMP-9 Occludine, was zu erhöhter parazellulärer Permeabilität bzw. epithelialer Dysfunktion führt [107].

Dem gegenüber stehen eher protektive Effekte der Gelatinase MMP-2, die durch eine Assoziation mit *Tight-junctions* (Claudine) zu einer Stabilisierung der Barrierefunktion beiträgt [107]. Ein weiterer protektiver Effekt von MMP-2 ist die Spaltung des Chemokins *monocyte chemotactic protein*-3 (MCP-3), wodurch es zu einer verminderten Neutrophilen-Migration bzw. -Infiltration kommt [109].

Dies zeigt, dass neben gängigen, anti-inflammatorischen Therapieansätzen auch eine Beeinflussung der EZM bzw. von MMPs ein therapeutisches Potential bei CED birgt.

1.6 Der Kalzium-aktivierte Kaliumkanal mit mittlerer Leitfähigkeit (KCa3.1): Bedeutung für Fibrogenese und Tumorwachstum

In den letzten Jahren wurden Ionenkanäle wie der Kalzium-aktivierte Kaliumkanal mit mittlerer Leitfähigkeit (KCa3.1) als mögliche therapeutische Zielstrukturen bei Entzündungen und Tumoren untersucht [110-116]. Hintergrund ist ihre wichtige regulatorische Funktion der Zellproliferation, welche einen wichtigen physiologischen Prozess z.B. bei Wundheilung und der Erneuerung von Zellen im Gewebe darstellt. Ihre Fehlregulation spielt eine Rolle z.B. bei der Pathogenese von Atherosklerose, Angiogenese und Tumorwachstum. Über beteiligte Signalwege an der Wirkung von KCa3.1 ist bisher nur wenig bekannt. In Mikrogliazellen wird eine Aktivierung der MAPK p38 durch KCa3.1 beschrieben [117]. KCa3.1 selbst werden in glatten Gefäßmuskelzellen (VSMC) durch Aktivierung der MAPK ERK1/2 reguliert [118]. ERK1/2 und p38 sind auch in der Karzinogenese des HCC involviert [119].

KCa3.1-Inhibitoren wie Clotrimazol (CLT) und CLT-Analoga wie TRAM-34 [120, 121] zeigten wachstumshemmende Effekte auf verschiedene Krebszellen *in vitro* und *in vivo* [122, 123]. Darüber hinaus hemmte CLT *in vitro,* in der humanen Kolon-Epithelzelllinie HT-29, die Aktivität des pro-inflammatorischen Transkriptionsfaktors NF-κB, was von einer verminderten TNFα-induzierten IL-8-

Freisetzung begleitet war. In der 2,4,6-Trinitrobenzolsulfonsäure (TNBS)-induzierten Colitis hemmte CLT die *signal transducer and activator of transcription* 3 (STAT3)-Phosphorylierung, zeigt also anti-inflammatorisches Potential [124]. Die systemische Anwendung von CLT, z.B. gegen Tumoren, ist jedoch durch eine stark hepatotoxische Wirkung limitiert [125]. Alternativen stellen Derivate wie TRAM-34 [120, 121, 126] dar, dessen Effekte auf die Fibrogenese und auf Tumorzellen in dieser Arbeit diskutiert werden.

1.7 Pflanzliche Wirkstoffe als Reservoir potenter Pharmaka

In der Arbeitsgruppe von Frau Prof. Ki-Young Kim (Wonkwang University, South Korea), mit der die eigene AG seit Jahren kooperiert, wurden verschiedene Pflanzenextrakte aus der Koreanischen Heilkunde auf ihre anti-fibrotischen Effekte untersucht. Da erste Ergebnisse positiv waren (persönliche Kommunikation Prof. Kim), wurden ausgewählte Extrakte systematisch in unserem Labor untersucht.
Dieser Ansatz steht in Einklang mit einem Forschungszweig, bei dem isolierte Wirkstoffe aus Pflanzen wie z.B. grünem Tee neben der präklinischen Grundlagenforschung auch vermehrt Anwendung in der klinischen Forschung finden. Grünem Tee werden präventive Wirkungen gegenüber Diabetes, Hypertonie, Neoplasien und kardiovaskulären Krankheitsbildern zugeschrieben [127], wobei die Wirkung auf anti-oxidative [128], anti-inflammatorische [129], anti-neoplastische [130] und anti-angiogenetische [131, 132] Effekte von enthaltenen Polyphenolen wie Epigallocatechingallat (EGCG) zurückgeführt wird.
Ein Vorteil der Naturstoff-basierten Forschung ist, dass viele Naturstoffe vom Menschen als Bestandteil von Lebens-/Genussmitteln bzw. Nahrungsergänzungsmitteln regulär konsumiert werden, i.d.R. also gut verträglich bzw. wenig toxisch sind, was eine Translation von Grundlagenstudien in weitere Tierversuche, bzw. perspektivisch auch in die Klinik (möglicherweise durch Ernährungsumstellung oder Nahrungsergänzung bei Patienten) begünstigt. Dies macht man sich bei komplementären Therapieansätzen, z.B. in der Onkologie, zu nutze. Einige in der Tumortherapie eingesetzte Substanzen stammen aus der Traditionellen Chinesischen Medizin (TCM) bzw. gleichwertigen alternativmedizinischen Anwendungen [133, 134]. Vor dem Hintergrund der Standardisierung der TCM-Formulate wird (in der westlichen Schulmedizin) versucht, Wirkstoffe einzelner Pflanzen zu isolieren und diese in gereinigter Form wieder in Mischungen zu applizieren [135].
Aufbauend auf den positiven Vorbefunden mit Tees, Tee-Extrakten und jeweiligen Inhaltsstoffen wie EGCG, wurde in der eigenen Arbeitsgruppe schwerpunktmäßig ein als hepatoprotektiv vorbeschriebenes wässriges Extrakt (Tee) aus der asiatischen Heilpflanze *Lindera obtusiloba* hinsichtlich anti-fibrotischer Wirkungen und Wirkmechanismen *in vitro* untersucht.

1.7.1 *Lindera obtusiloba*

Ein wässriges Extrakt der Heilpflanze *Lindera obtusiloba* (LOE), als Tee getrunken, wird in der traditionellen Koreanischen Heilkunde zur Behandlung von Entzündungen und chronischen Leberschäden sowie der Unterstützung der Blutzirkulation angewendet [136]. Eine Untersuchung dieser potentiell

hepatoprotektiven Eigenschaften des Extraktes nach westlichen Standards fehlte jedoch bisher und wird im Rahmen dieser Arbeit diskutiert.

Für LOE sind bei topischer Anwendung protektive Effekte in einem murinen Modell der atopischen Dermatitis beschrieben. LOE hemmte typische Symptome wie Ohrschwellung, erhöhte Histamin-Serumwerte, die Infiltration von Mastzellen im Ohr sowie die Sekretion pro-inflammatorischer Zytokine wie TNF-α [137]. Für ein ethanolisches Extrakt aus *Lindera obtusiloba* konnten bereits anti-oxidative Effekte *in vitro* und *in vivo* gezeigt werden, wobei das Extrakt u.a. durch künstlich induzierten oxidativen Stress hervorgerufene Leberläsionen minderte [138]. Weitere Studien zeigten protektive Effekte des Ethanolextraktes auf das vaskuläre System in diabetischen *db/db*-Mäusen (besitzen keine funktionelle Ob-Rb-Isoform des Leptinrezeptors) durch Relaxation des Endothels und reduzierten oxidativen Stress [139, 140].

Zu den bisher bekannten Wirkstoffen in *Lindera obtusiloba* gehören Lignane und Glykoside, die scheinbar für die anti-allergenen Effekte von LOE in Mastzellen (s.o.) verantwortlich sind [34, 36]. Ein bereits identifizierter anti-oxidativer Wirkstoff in LOE ist Quercitrin, welcher neben anti-oxidativen Effekten auch Melanogenese-hemmende Eigenschaften aufweist [141]. Enthaltende Butanolide und Lignane zeigten anti-neoplastische Effekte in Lungen-, Ovarial- und Colon-Tumorzellen [142, 143].

In den eigenen Arbeiten konnte mit (+)-Episesamin (ES) ein weiterer sehr potenter Wirkstoff in LOE identifiziert und isoliert werden, für den anti-adipogene, anti-fibrotische und anti-neoplastische Effekte beschrieben werden konnten, die im Rahmen der vorliegenden Arbeit diskutiert werden [144-146].

1.8 Fragestellung und Zielsetzung

Zentrale Fragestellung der hier zusammengefassten Arbeiten ist die Beeinflussung der Leberfibrose und des HCC durch Komponenten der EZM, durch Inhibition von KCa3.1-Ionenkanälen und durch LOE bzw. daraus isoliertem ES.
Die Fragestellung gliedert sich in drei Aspekte:

(1) Ziel: Ein besseres Verständnis der Interaktion von EZM mit Zellen und MMPs.
Durch die Arbeiten sollen die Funktionen von Komponenten der EZM als mögliche Werkzeuge zur Beeinflussung der Matrix-MMP-Interaktion sowie der enzymatischen Aktivität von MMPs untersucht werden. Gewonnene Erkenntnisse aus *in vitro*-Studien sollen *in vivo*, im Modell der MMP-assoziierten epithelialen Barrieredisruption bei akuter DSS-Colitis verifiziert werden.

(2) Ziel: Erkenntnisse zur potentiellen Funktion des Ionenkanals KCa3.1 bei Leberfibrose und HCC.
In *in vitro-* und *in vivo*-Modellen soll eine mögliche Rolle von KCa3.1-Ionenkanälen bzw. des KCa3.1-Inhibitors TRAM-34 auf den Prozess der hepatischen Fibrogenese untersucht werden. Parallel sollen wachstumshemmende Effekte und grundlegende Wirkmechanismen von TRAM-34 in humanen HCC-Zelllinien untersucht werden.

(3) Ziel: Etablierung von LOE bzw. dem Inhaltsstoff ES als Kandidaten für den Einsatz in weitergehenden Studien zu Leberfibrose und HCC.
Die vorbeschriebene hepatoprotektive Wirkung von LOE sowie Wirkmechanismen sollen nach westlichen Standards in *in vitro*-Modellen der Leberfibrose und des HCC verifiziert werden. Zudem sollen aktive Wirkstoffe aus LOE identifiziert, isoliert und hinsichtlich ihres anti-fibrotischen und anti-neoplastischen Potentials *in vitro* charakterisiert werden.

2 Eigene Arbeiten

2.1 Beeinflussung der Interaktion von extrazellulärer Matrix mit Matrix-Metalloproteinasen

2.1.1 Die alpha2 Kette von Kollagen VI als Modulator von Bindung und Aktivierung von Matrix-Metalloproteinasen

- (**Freise, C.**, Erben, U., Farndale, R., Muche, M., Somasundaram, R., und Ruehl, M.'The alpha 2 chain of collagen type VI sequesters latent proforms of matrix-metalloproteinases and modulates their activation and activity' *Matrix Biol*. 2009 Oct;28(8):480-9.).

Die EZM dient als Speichermedium für biologisch aktive Moleküle, die durch proteolytische Spaltungen, z.B. durch MMPs, freigesetzt werden können. Interessanterweise werden auch MMPs selbst in der Matrix gespeichert. In dieser Studie konnte gezeigt werden, dass enzymatisch inaktive Proformen der MMPs an kollagenen Septen in fibrotischem Lebergewebe assoziiert sind. Zur Identifikation von Bindungspartnern im Gewebe, wurden aus humaner Plazenta isolierte Kollagene und Kollagenketten sowie andere EZM-Moleküle isoliert und in Bindungsstudien mit MMPs eingesetzt. U.a. wurde Kollagen VI als ein MMP-Bindungspartner identifiziert. Zur Isolation der aufgespaltenen und gereinigten Einzelketten (alpha-Ketten) von Kollagen VI sowie des Pepsin-resistenten, tripelhelikalen Kollagen VI-Fragmentes (KVI-F/PR) wurde eine zweistufige Chromatographie-Methode entwickelt. In Festphasen- und Oberflächen-Plasmonen-Räsonanz-Messungen (Biacore) zeigte die tripelhelikale Domäne der alpha 2-Kette von Kollagen VI - α2(VI) - eine hohe Bindungsaffinität im starken nanomolaren Bereich zu den Kollagenasen ProMMP-1, -8, -13 und zu Stromelysin-1 (MMP-3). Zudem zeigte sich eine Beziehung zwischen Kollagenase-Aktivität und Kollagenase-Kollagen VI-Bindung: Eine α2(VI)-vermittelte, verminderte Kollagenase-Aktivität ging mit einer erhöhten Bindung an α2(VI) einher. Ähnliche Daten zeigten sich auch für die Gelatinase ProMMP-9. Im Fall der zweiten Gelatinase ProMMP-2 dagegen störte α2(VI) die Konservierung der Prodomäne des ProMMP-2-Moleküls, was zu enzymatischer Aktivität des Moleküls ohne Abspaltung der Prodomäne führte. Zudem hemmte α2(VI) die Bindung von ProMMP-2 an seinen natürlichen Liganden Kollagen I. Zusammengefasst fungiert α2(VI) als Bindungspartner von ProMMPs in der EZM und hemmt die enzymatische Aktivität von MMPs. Kollagen VI und insbesondere α2(VI) könnten deshalb in der Zukunft als „Lead"-Struktur für MMP-basierte Therapeutika dienen, mit denen MMP- und EZM-assoziierte Prozesse wie Fibrose oder Neoplasien beeinflusst werden können.

Im Rahmen der Suche nach weiteren MMP-Bindungspartnern in der Matrix, beschreibt die erste der im nachfolgenden Kapitel zusammengefassten Arbeiten zunächst die Identifikation tripelhelikaler Strukturen als Bindungspartner der Gelatinasen MMP-2 und MMP-9. Zudem wird das entsprechend tripelhelikale

Peptid $(GPO)_{10}$ als Modulator von Kollagenbindung und enzymatischer Aktivität der Gelatinasen beschrieben. In der sich anschließenden zweiten Arbeit wird darüber hinaus die *in vitro*-Testung von $(GPO)_{10}$ hinsichtlich ProMMP-2-spezifischer, mitogener Effekte in HSZ beschrieben.

http://dx.doi.org/10.1016/j.matbio.2009.08.001

2.1.2 Das hydroxyprolinhaltige Kollagenmimetikum $(GPO)_{10}$ fördert die Freisetzung und Aktivierung von kollagengebundenem ProMMP-2

- (*Ruehl, M., *Muche, M., ***Freise, C.**, Erben U, Neumann U, Schuppan D, Popov Y, Dieterich W, Zeitz M, Farndale RW, Somasundaram R.: 'Hyp-containing collagen analogs trigger the release and activation of collagen-sequestered proMMP 2 by competition with prodomain-derived peptide P33-42' *Fibrogenesis & Tissue Repair* 2011, 4:19). (*geteilte Erstautorenschaft)
- (**Freise C**., Ruehl M., Erben U., Farndale RW., Somasundaram R, Heimesaat MM.: The synthetic hydroxyproline containing collagen analogue $(Gly\text{-}Pro\text{-}Hyp)_{10}$ promotes enzymatic activity of matrixmetalloproteinase-2 *in vitro. European Journal of Microbiology and Immunology* 2012; 2(3), 186-191).

MMP-2 und -9 spielen eine zentrale Rolle im Rahmen der Leberfibrose. Aus Vorstudien war bekannt, dass die EZM-Bindung beider Gelatinasen deren Aktivität beeinflusst. Hier wurde ihre Interaktion mit Kollagen I bzw. Kollagen I-Fragmenten untersucht, den Hauptbestandteilen fibrotischer Matrix. *In situ*-Zymographien und immunhistologische Färbungen zeigten eine Assoziation der inaktiven Gelatinase Proformen ProMMP-2/-9, nicht aber ihrer aktivierten Formen, an fibrilläre Kollagenstrukturen. Parallele Festphasen-Bindungsstudien mit humanen Kollagenen und Kollagenfragmenten zeigten, dass ~45% $[^{125}I]$-markierter ProMMP-2/-9, nicht aber aktivierte MMP-2/-9 an natürliche Kollagenmoleküle bzw. synthetische Kollagenpeptide mit zehn Aminosäuresequenz-Triplets Gly-Pro-Hyp ($(GPO)_{10}$)binden. Oberflächen-Plasmonen-Räsonanz-Messungen ergaben mittlere Bindungsstärken von ProMMP-2/-9 an Kollagen I im nanomolaren Bereich. Aktivierte MMP-2/-9-Moleküle zeigten eine deutlich schwächere Kollagenbindung. Ein zehnfach molarer Überschuss des Kollagenpeptids $(GPO)_{10}$ schwächte die Bindung von ProMMP-2/MMP-2 an Kollagen I deutlich (22- bzw. 380-fach). Gelzymographien zeigten, dass $(GPO)_{10}$ bei ProMMP-2 eine Abspaltung des Propeptids bewirkt, wodurch eine erhöhte enzymatische Aktivität von MMP-2 induziert wird. Höhere Konzentrationen von $(GPO)_{10}$ dagegen (100-facher molarer Überschuss), führten zu einer geringeren enzymatischen Aktivität von MMP-2, was auf Kompetition von $(GPO)_{10}$ mit dem Gelatine-Substrat am aktiven Zentrum von MMP-2 hinweist. Um den Mechanismus der Enzymaktivierung von ProMMP-2 durch $(GPO)_{10}$ näher zu untersuchen, wurden Kompetitionsstudien mit $(GPO)_{10}$ und dem synthetischen Peptid P33-42 durchgeführt. P33-42 entstammt der Prodomäne von ProMMP-2 und bindet, wie auch Gelatine als MMP-2-Substrat, spezifisch an die Kollagen-Bindungsdomäne von ProMMP-2, was die enzymatische Inaktivität von ProMMP-2 bestimmt [147]. Erwartungsgemäß konkurrierte P33-42 mit ProMMP-2 um Bindungsstellen an Kollagen I und hemmte darüber hinaus auch die $(GPO)_{10}$–vermittelte Aktivierung von ProMMP-2. Anders als $(GPO)_{10}$ führte die Anwesenheit von P33-42 jedoch zu keiner Aktivierung von ProMMP-2, was auf einen spezifischen Effekt von $(GPO)_{10}$, bedingt durch seine Kollagen-ähnliche, tripelhelikale Struktur, schließen lässt.

In der zweiten Arbeit wurden die Auswirkungen dieser MMP-spezifischen Effekte von $(GPO)_{10}$ auf zelluläre Prozesse *in vitro* untersucht. Rekombinantes ProMMP-2 induzierte die Proliferation von HSZ,

welche als zentrale Mediatoren bei der Entstehung der Leberfibrose gelten. Dieser Effekt wurde durch Anwesenheit von $(GPO)_{10}$ verstärkt und entsprach der Proliferationsinduktion durch voll aktiviertes MMP-2. Zudem induzierte $(GPO)_{10}$ Migration in den HSZ und zwar ähnlich stark wir der Wachstumsfaktor *platelet-derived growth factor subunit-B* (PDGF-BB). In Invasionsexperimenten mit HT1080 Fibrosarkomzellen führte $(GPO)_{10}$ zu einer verstärkten MMP-2-abhängigen Invasion der Tumorzellen durch eine EZM-Barriere. Die Stimulation von Proliferation, Migration und Invasion (gleichbedeutend mit Degradation von EZM) durch $(GPO)_{10}$ zeigt, dass die in Bindungsstudien gezeigte $(GPO)_{10}$-MMP-Wechselwirkung auch im biologischen System (Zellkultur) relevant ist.

Die im nachfolgenden Kapitel zusammengefasste Arbeit beschreibt den Einsatz von $(GPO)_{10}$ auch *in vivo*, im murinen Tiermodell der akuten Natriumdextransulfat (DSS)-Colitis, bei dem die durch $(GPO)_{10}$ beeinflussten Gelatinasen ProMMP-2 und ProMMP-9 einen wesentlichen Einfluss auf die Integrität der Epithelbarriere des Darmes haben.

Ruehl et al. Fibrogenesis & Tissue Repair 2011, 4:1
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Hydroxyproline-containing collagen analogs trigger the release and activation of collagen-sequestered proMMP-2 by competition with prodomain-derived peptide P_{33-42}

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Abstract

Background: Fibrolytic and profibrotic activities of the matrix metalloproteinases (MMPs)-2 and -9 play a central role in liver fibrosis. Since binding to the extracellular matrix influences the activity of both gelatinases, here the role of fibrillar collagens as the most abundant matrix components in fibrotic tissue was investigated.

Results: *In situ* zymography and immunohistology showed association of enzymatically inactive prodomain-containing proMMP-2 and proMMP-9 but not of their activated forms to fibrillar collagen structures, which are not substrates of these gelatinases. In solid-phase binding studies with human collagens and collagen fragments, up to 45% of [^{125}I]-labeled proMMP-2 and proMMP-9 but not of active (act)MMP-2 and actMMP-9 were retained by natural collagenous molecules and by synthetic analogs containing repeated Gly-Pro-Hyp triplets (GPO). Surface plasmon resonance yielded binding constants for the interaction of collagen type I (CI) with proMMP-2 and proMMP-9 in a nanomolar range. Values for actMMP-2 and actMMP-9 were 30-40 times higher. Tenfold molar excesses of $(GPO)_{10}$ reduced the interaction of CI with pro- and actMMP-2 by 22- or 380-fold and resulted in prodomain release accompanied by high enzymatic activation and activity. Pointing to gelatine substrate displacement, higher $(GPO)_{10}$ concentrations blocked the enzymatic activity. The MMP-2 prodomain-derived collagen-binding domain peptide (P_{33-42}) binds to the collagen-binding domain of MMP-2, thereby preserving enzymatic inactivity. Synthetic P_{33-42} peptide competed with proMMP-2 binding to CI and prevented $(GPO)_{10}$-mediated proMMP-2 activation. In contrast to $(GPO)_{10}$, P_{33-42} did not activate proMMP-2, making triple helical and hydroxyproline-containing $(GPO)_{10}$ unique in modulating gelatinase availability and activity.

Conclusions: These findings suggest novel strategies using collagen analogs for the resolution of liver fibrosis via fibrotic matrix-sequestered gelatinases.

Background

Matrix metalloproteinases (MMPs) form a large family of zinc-dependent metalloendopeptidases that degrade extracellular matrix (ECM) molecules, including various collagens, gelatine, elastin, fibronectin and aggrecan [1]. The diversity of MMP-binding partners and of MMP substrates suggests a central role for MMPs in the "protease web" beyond their proteolytic activity. MMPs were described to be involved in the regulation of cellular differentiation, proliferation and migration, the regulation of growth and metastasis of tumors, and the regulation of organ fibrosis (for example, liver) [2-4]. All MMPs consist of three domains, including the catalytic domain with a zinc-binding active-site motif, the prodomain with a conserved cysteine interacting with the catalytic zinc to maintain the latency of the enzymatically inactive latent proform of MMPs (proMMPs), and the hemopexin-like domain functional in substrate binding and in the interaction with tissue inhibitors of

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metalloproteinases (TIMPs). Within their catalytic domain, the gelatinases MMP-2 and MMP-9 contain the additional fibronectin type II modules Col-1, Col-2 and Col-3 [5], forming collagen-binding domains (CBDs) that specifically interact with collagens, with other ECM molecules and with the prodomain. As for differences in gelatinases, only MMP-2 but not MMP-9 has collagenolytic activity, and a distinct MMP-2 prodomain peptide (P_{33-42}) conserves latency upon interaction with the CBD [6,7]. Here a combination of the sequence and the thermal stability of their substrate, exemplified by denatured nonhelical gelatine defines specificity [8]. MMP-2 localized at the cell surface interacts with collagen type IV (CIV), CD44, integrin receptors and the discoidin domain receptor 2 [4,9,10]. MMP-2 binds to native or denatured collagens, elastin, fatty acids and thrombospondins via its CBD exosite [11,12].

MMPs are assumed to be sequestered in the ECM [13,14]. Recently, we established the α2 chain of collagen type VI as the main binding structure for sequestration of collagenases and stromelysin-1 proforms in fibrotic tissue [15]. Gelatinase binding sites were assumed to be within the rigid triple-helical collagen structure and thus far have been described only at the oligopeptide level [7,16]. As for the α1 chain of collagen type I (α1(I)), the hydroxyproline (Hyp)-containing peptide segment P713 was identified as an exosite CBD ligand of MMP-2 [17].

The current view of progressive liver fibrosis includes neutralization of potentially matrix-degrading MMPs by an even higher expression of TIMPs. On the other hand, in the fibrosis resolution phase, MMP-2 activity in serum [18] and liver tissue [19] is high and high serum levels of MMP-9 and MMP-2 were found as early as 6 h after hepatectomy [20]. These observations pointed to a pool of ECM-stored MMPs as recently shown for collagenases [15].

The aim of this study was to characterize non-substrate-binding structures for gelatinase in the ECM and the potential of synthetic collagen-like binding competitors to modulate MMP availability or activity through exosite interaction in fibrotic diseases. Our data suggest that collagen analog-driven conformational changes of the MMP molecule are triggered by high-affinity interaction of collagen analogs with the CBD, eventually leading to MMP activation that ultimately abrogates proMMP binding to nonsubstrate collagens. We found the collagen-immanent secondary triple-helical structure and the modified amino acid Hyp to be prerequisite for gelatinase binding.

Results

Collagen fibers in cirrhotic liver tissue retain gelatinases

Thioacetamide-intoxicated rats developed liver cirrhosis with extensive deposition of scar tissue in expanding fibrotic septa showing typical extensive bridged fibrosis, in which collagen types I and III (CI and CIII) predominate (Figures 1A and 1B). In *in situ* zymography with dye quenched (DQ)-gelatine, strong gelatinolytic activity was associated with these structures, as shown by the bright fluorescence aligned with fibrillar structures (Figures 1C and 1D). In the liver, MMP-2 is mainly expressed by hepatic stellate cells, whereas Kupffer cells are the major cellular source for MMP-9. Human fibrotic tissue was stained with monoclonal antibodies specific for MMP-2 or MMP-9 and subjected to a stringent washing procedure (Figures 1E-H). Light MMP-2 labeling was detected in fibrotic septa, and more pronounced MMP-9-specific labeling was observed in the pericellular region of macrophage-like cells (Figure 1E-G). The ubiquitous fibrillar staining observed for MMP-2 and the pericellular deposition of MMP-9 suggested fibrillar collagens or associated ECM molecules to have the capacity to store MMPs. No significant binding was observed when sections were preincubated with the aminophenyl mercuric acetate (APMA)-activated form of MMP (actMMP)-2 or actMMP-9 (not shown). If sequential liver sections were preincubated with prodomain-containing proMMP-2 or proMMP-9, preferential staining of fibrotic septa was observed for both gelatinase proforms (Figures 1F-H), confirming the localization of gelatinolytic activity observed by *in situ* zymography.

ProMMP-2 and ProMMP-9, but not actMMP-2 and actMMP-9, strongly bind to immobilized native collagens, CI fragments and to Hyp-containing collagen analogs

To further elucidate the interaction of human ECM components with human pro- or actMMP-2 or pro- or actMMP-9, we studied the retention of recombinant $[^{125}I]$-labeled and enzymatically active gelatinases (Figure 2A) by highly purified and well-characterized native fibrillar collagens, CI fragments and Hyp-containing collagen analogs. Serial dilutions of potential non-substrate-binding partners dotted to a nitrocellulose membrane with high protein-binding capacity showed that natural and synthetic collagen structures sufficiently bound proMMP-2 and proMMP-9 (not shown) (Figure 2B). In this qualitative analysis, comparable signal intensities were observed for proMMP-2 binding to 0.25 to 0.5 μg/dot CI or CIII and from 2 to 4 μg/dot of the tightly packed helical Gly-Pro-Hyp $(GPO)_{10}$ (Figure 2B, left). The binding efficiencies of proMMP-2 to α1(I)-derived CB fragments declined in the following order: CB7>CB6>CB8> > CB3. Regardless of the collagenous structure immobilized, only weak binding was found for actMMP-9 (not shown) and for actMMP-2 (Figure 2B, right).

In the next step, $[^{125}I]$-labeled proMMP-2 and proMMP-9 were applied to microwell-immobilized

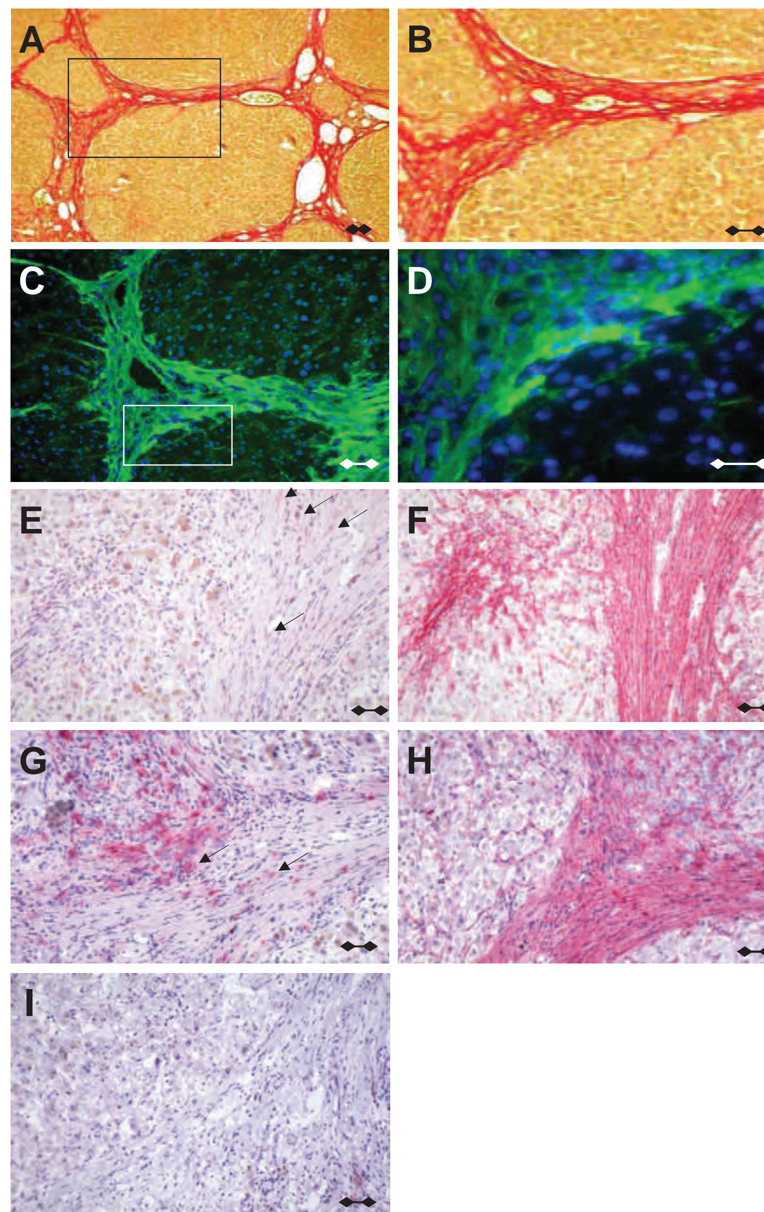
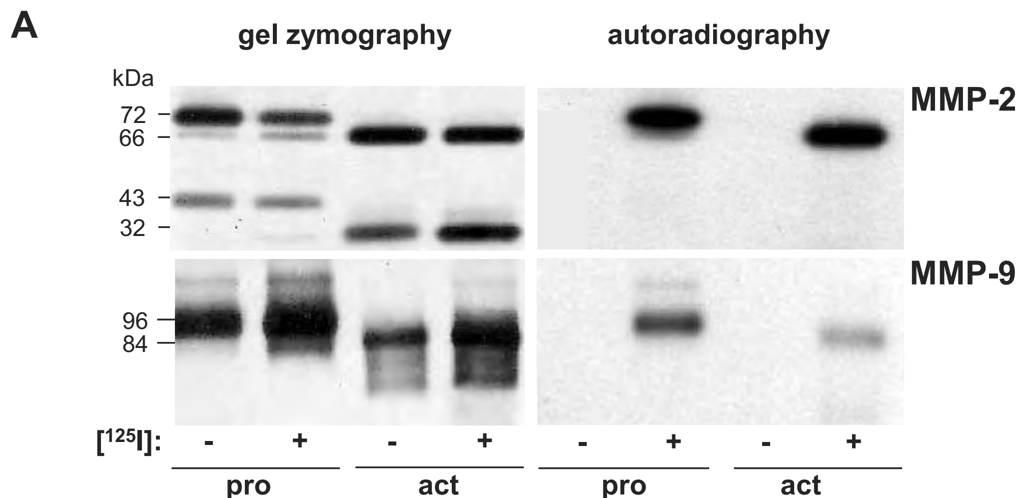


Figure 1 Localization of gelatinolytic activity in fibrotic liver tissue. **(A** and **B)** Collagenous septa in fibrotic rat liver tissue were stained with Sirius red with rectangular detail that highlights collagen fibers in **(B)**. Original magnification, ×20. **(C** and **D)** *In situ* zymography localizes strong gelatinolytic activity to fibrous structures with rectangular detail that demonstrates nonpericellular, fiber-associated gelatinase activity in **(D)**. Original magnification, ×40. **(E-H)** Cryostat sections of fibrotic human liver tissue were incubated with 25 ng of proMMP-2 **(F)** or proMMP-9 **(H)** or were left untreated **(E** and **G)**. Bound matrix metalloproteinases (MMPs) were detected using immunohistochemistry monoclonal antibodies specific for MMP-2 (arrows in **E** and **F** depict endogenous MMP expression) or MMP-9 **(G** and **H)**. An irrelevant primary antibody served as a control **(I)**. Sections shown represent three independent experiments. Scale bars, 100 μm.

collagen structures, and retained radioactivity was determined after thorough washing to estimate gelatinase binding (Table 1). Again, proMMP-2 and proMMP-9 strongly bound to CI as well as to single chains of CI and CIII (range, 22% to 45%). Confirming the results from the dot-blot analysis, a maximum binding of only 19% was found for actMMP-2 to the α1(I) chain, whereas no binding of actMMP-9 to collagenous structures was observed. The Hyp-containing $(GPO)_{10}$ peptide, which structurally resembles triple-helical collagen helices with high melting temperatures, was used to further elucidate the relevance of the GPO triplet for proMMP-2 and proMMP-9 binding. Triple-helical Gly-Pro-Pro $(GPP)_{10}$, devoid of Hyp, and the linear



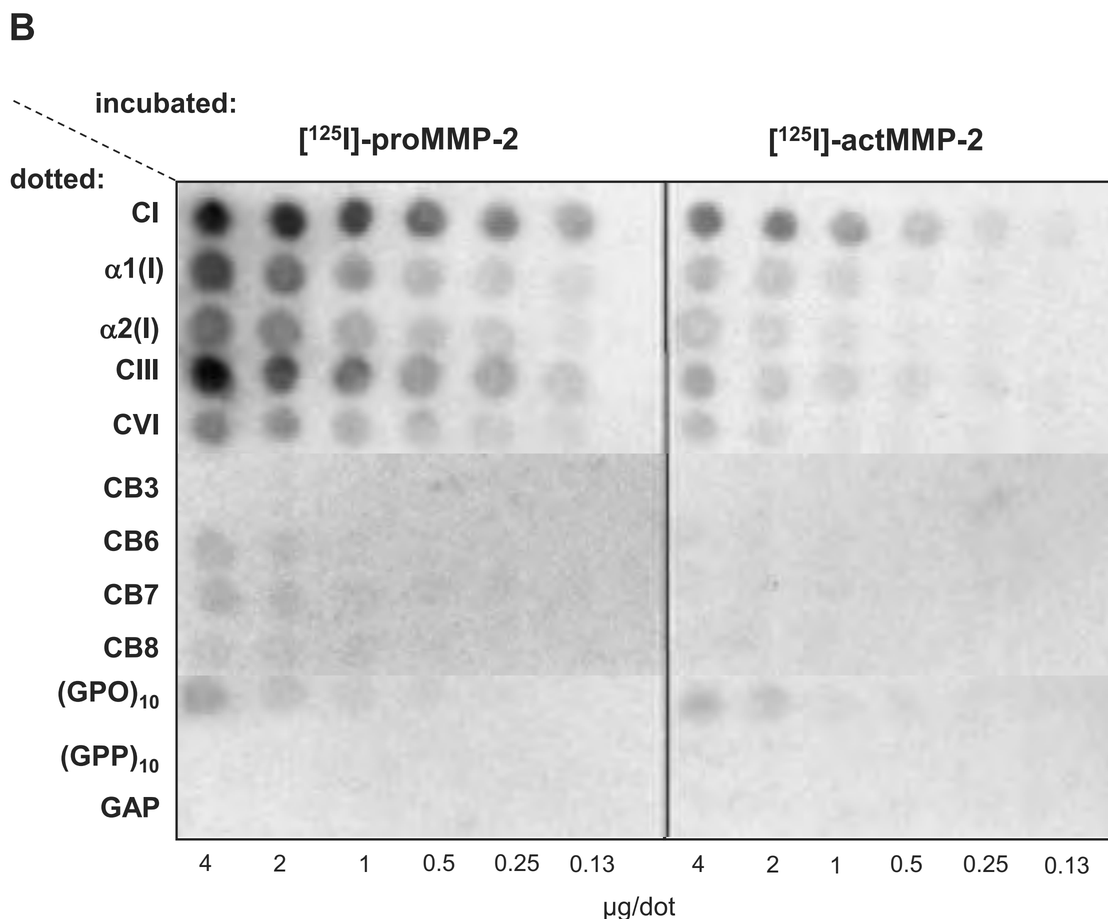


Figure 2 Quality control of [^{125}I]-(un)labeled pro/actMMP-2/-9 and dose-dependency of pro/actMMP-2-binding to nitrocellulose-immobilized collagenous molecules. **(A)** Unlabeled or [^{125}I]-labeled pro/actMMP-2/-9 (~1 ng) were quality-checked in substrate gel zymography (left) before the dried gel was subjected to autoradiography (right). Note that after [^{125}I]-labeling, MMP structure and activity remained unchanged. **(B)** Serial dilutions of collagens or collagen type I (CI) derivatives dotted to a nitrocellulose membrane were incubated with 5 ng of [^{125}I]-labeled pro/actMMP-2 before thorough washing. Bound MMP was monitored by autoradiography. Images shown represent three independent experiments.

Table 1 Binding of pro- or actMMP-2 or pro- or actMMP-9 to native collagens, single chains of CI, chain fragments and synthetic peptides[a]

	Binding of [125I]-MMPs (% of maximum control)			
	ActMMP-2	ActMMP-9	ProMMP-2	ProMMP-9
Wells coated with				
CI	11.1 ± 1.8	3.1 ± 0.5	24.0 ± 5.6	30.9 ± 3.1
CIII	14.3 ± 5.8	5.0 ± 0.4	40.0 ± 5.0	45.0 ± 2.0
CVI	4.8 ± 0.6	2.4 ± 0.1	14.1 ± 1.8	12.3 ± 0.8
α1(I)	19.0 ± 3.0	4.7 ± 0.3	36.0 ± 4.0	37.6 ± 0.9
α2(I)	10.7 ± 1.8	2.6 ± 0.2	30.2 ± 2.3	21.8 ± 1.1
α1CB3	7.4 ± 0.3	1.8 ± 0.2	13.2 ± 7.9	7.4 ± 1.2
α1CB6	4.9 ± 1.5	2.4 ± 0.2	12.8 ± 2.6	19.3 ± 0.4
α1CB7	6.8 ± 0.6	2.1 ± 0.3	18.2 ± 3.7	20.4 ± 1.3
α1CB8	4.4 ± 1.7	2.8 ± 0.4	14.0 ± 4.5	31.6 ± 0.9
$(GPO)_{10}$	6.2 ± 3.1	5.4 ± 0.6	13.0 ± 3.5	36.1 ± 1.7
$(GPP)_{10}$	2.1 ± 0.7	0.9 ± 0.4	3.6 ± 2.0	4.9 ± 0.7
GAP	1.5 ± 0.7	0.7 ± 0.2	1.7 ± 1.0	1.3 ± 0.2

[a]Proteins and peptides were immobilized to the wells of polystyrene microtiter plates before 2 ng/well [125I]-MMPs were added. Wells were washed thoroughly, and bound MMPs were determined as residual radioactivity. Binding efficiency was calculated in comparison to the amount of the respective radiolabeled MMPs initially added (100%). Results are mean values ± SD of at least five independent experiments performed in triplicate. activated form of MMP (actMMP); proMMPs, inactive latent proform of MMPs; MMP, matrix metalloproteinase; CI, collagen type I; α1(I), α1 chain of collagen type I (α1(I)); α1CB3, α1CB3, cyanogen bromide peptide 3 of the α1 chain of collagen type I;; GPO, Gly-Pro-Hyp triplets; GPP, Gly-Pro-Pro; GAP, Gly-Ala-Pro.

Gly-Ala-Pro (GAP) peptide served as controls. Compared to the respective actMMP, proMMP-2 and proMMP-9 bound two- to sevenfold more strongly to $(GPO)_{10}$, but both forms of the MMPs showed only marginal interactions with the control peptides $(GPP)_{10}$ and linear GAP (Table 1).

Repeated GPO peptides interfere with binding of proMMP-2 and proMMP-9 to CI

The kinetics of gelatinase binding to CI were determined by surface plasmon resonance (SPR) measurements. The linear control peptide GAP had no effect on the interaction of the MMPs with CI (not shown). As for the assumed binding competitor $(GPO)_{10}$, K_d values were determined using standard conditions for SPR measurements (Table 2). These nonactivating conditions without divalent metal ions are known to interfere with MMP activity and conformation. Since the Off rates for proMMP-2 and actMMP-2 binding to CI were in the same range (0.48 s^{-1}), the reduced binding strength of actMMP-2 (K_d, 170 ± 5 nM) compared to proMMP-2 (K_d, 70 ± 3 nM) was due to differences in the On rates. The effects seen with MMP-9 were more dramatic, since actMMP-9 bound to CI (K_d, 870 ± 39 nM) sevenfold less effectively than proMMP-9 (K_d, 120 ± 9 nM).

A 10-fold molar excess of $(GPO)_{10}$ only slightly impaired the binding of proMMP-2 and actMMP-2 to CI but had more pronounced effects on proMMP-9 (Table 2). Using MMP activity buffer conditions, addition of $(GPO)_{10}$ abolished binding of proMMP-9 to CI (Figure 3A), and $(GPO)_{10}$ treatment of proMMP-2 led to a reduction in resonance units below baseline levels (Figure 3B). Since under these conditions actMMP-2 effectively degraded the CI matrix with a final loss of about 400 resonance units after 60 s (not shown), the sensorgram of proMMP-2 in the presence of $(GPO)_{10}$ (Figure 3B) was most likely due to $(GPO)_{10}$-induced collagenolytic MMP-2 activity, which is absent with MMP-9 (Figure 3A).

Table 2 Effect of soluble (GPO)10 on the binding of pro- or actMMP-2 or pro- or actMMP-9 to CI[a]

		Binding to CI			
		Without $(GPO)_{10}$		10× $(GPO)_{10}$	
		Off rate (s^{-1})	K_d (μM)	Off rate (s^{-1})	K_d (μM)
MMP-2	Pro	0.48	0.07 ± 0.03	0.37	0.10 ± 0.04
	Act	0.48	0.17 ± 0.05	0.56	0.21 ± 0.15
MMP-9	Pro	0.32	0.12 ± 0.09	0.22	1.22 ± 0.64
	Act	0.76	0.87 ± 0.39	0.61	1.79 ± 1.81

[a]Matrix metalloproteinases (MMPs) (100 nM) were passed over collagen type I (CI) immobilized to a sensor chip in the presence or absence of a 10-fold molar excess of Gly-Pro-Hyp triplets $(GPO)_{10}$. MMP self-activation and activity were prevented by a buffer system consisting of phosphate-buffered saline and 0.05% (vol/vol) Tween 20. K_d values and off rates of MMP binding to CI were determined by surface plasmon resonance (SPR) analysis and are shown as mean values ± SD from at least three experiments.

Binding of MMP-2 to CI strictly depends on the gelatinase activation status

Since K_d values for binding of proMMP-2 to CI in the presence of $(GPO)_{10}$ could not be assessed under activating buffer conditions, we studied proMMP-2 and actMMP-2 binding to immobilized CI in MMP activity buffer containing Ro 28-2653 that specifically inhibits proMMP (auto)activation and MMP enzymatic activity. The effects of strong binding of latent gelatinases to CI reduced by MMP activation were observed to be highly aggravated using this optimized buffer. ProMMP-2 bound to CI within low nanomolar K_d values representing a 10-fold binding enhancement, whereas the reduction of binding upon MMP activation was elevated from 2.5- to 35-fold (Figures 3C and 3D; Tables 2 and 3). Here the addition of $(GPO)_{10}$ to proMMP-2 resulted in a 22-fold reduced affinity for CI (Figure 3C), which was in the same range observed for MMP-2 activation (Table 3).

ProMMP-2 activation and actMMP-2 activity are impaired upon CI binding and are strongly enhanced in the presence of $(GPO)_{10}$

To further investigate the activation of MMP-2 by $(GPO)_{10}$, the cleavage of a short, gelatinase-specific

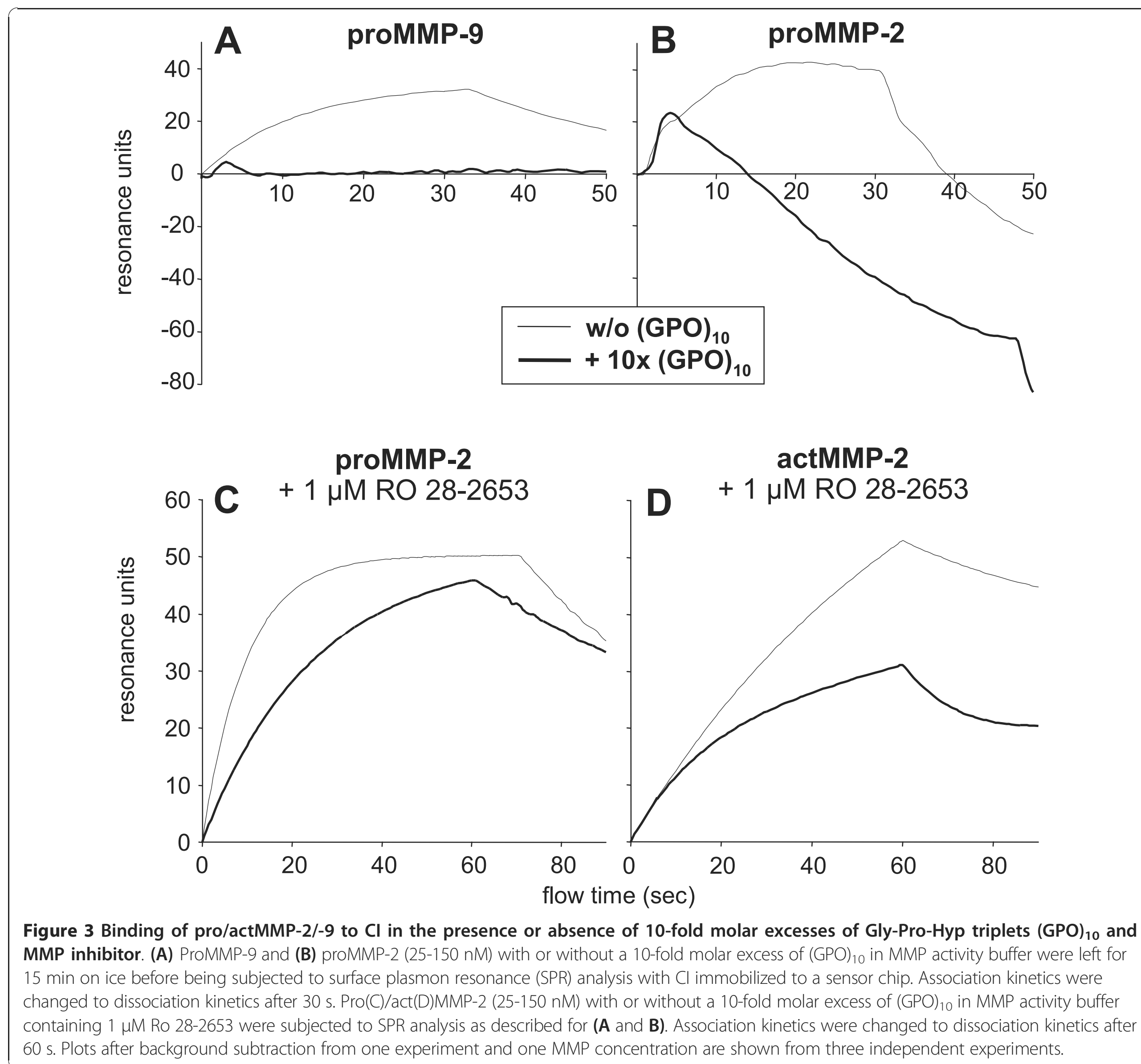


Figure 3 Binding of pro/actMMP-2/-9 to CI in the presence or absence of 10-fold molar excesses of Gly-Pro-Hyp triplets $(GPO)_{10}$ and MMP inhibitor. **(A)** ProMMP-9 and **(B)** proMMP-2 (25-150 nM) with or without a 10-fold molar excess of $(GPO)_{10}$ in MMP activity buffer were left for 15 min on ice before being subjected to surface plasmon resonance (SPR) analysis with CI immobilized to a sensor chip. Association kinetics were changed to dissociation kinetics after 30 s. Pro(C)/act(D)MMP-2 (25-150 nM) with or without a 10-fold molar excess of $(GPO)_{10}$ in MMP activity buffer containing 1 μM Ro 28-2653 were subjected to SPR analysis as described for **(A** and **B)**. Association kinetics were changed to dissociation kinetics after 60 s. Plots after background subtraction from one experiment and one MMP concentration are shown from three independent experiments.

substrate by proMMP-2 and actMMP-2 was measured with and without a 10-fold excess of $(GPO)_{10}$. In addition, some experiments used wells coated with fibrillar CI to explore its effects on activation and activity of MMP-2. Conversion of the quenched substrate peptide over time depended on the activation state of MMP-2. Activation of proMMP-2 (Figure 4A) showed sigmoid and activity of actMMP-2 (Figure 4B) immediate exponential kinetics of substrate turnover, probably due to an activation lag phase for proMMP-2. There

Table 3 Effect of thermostability and Hyp content of collagen analogs on the binding kinetics of pro- or actMMP-2 to CI[a]

	K_d for binding to CI (in nM) in the presence of 10-fold molar excess			
	-	$(GPP)_{10}$	$(POG)_5$	$(GPO)_{10}$
ProMMP-2	7.1 ± 0.1	42.1 ± 39.1	155.5 ± 40.3	155.5 ± 50.2
ActMMP-2	250.0 ± 30.0	n.d.	n.d.	2,710.0 ± 90.0

[a]Pro- or actMMP-2 (100 nM) was passed over collagen type I (CI) immobilized to a sensor chip in the presence or absence of 10-fold molar excesses of $(GPP)_{10}$, $(POG)_5$, or $(GPO)_{10}$. Reagents were dissolved in matrix metalloproteinase (MMP) activity buffer, and MMP-2 activity was specifically blocked by 1 μM Ro 28-2653. K_d values of MMP-2 binding to CI were determined by surface plasmon resonance (SPR) analysis and are shown as mean values ± SD from at least three experiments. Hyp, hydroxyproline; GPP, Gly-Pro-Pro; GPO, Gly-Pro-Hyp triplets; n.d., not determined.

was a strong reduction in activity of both proMMP-2 and actMMP-2 due to association with CI, showing that binding to fibrillar collagen results in impaired (auto)activation and activity of the enzyme (Figure 4 hatched curves). Either on bovine serum albumin (BSA) or on CI, addition of $(GPO)_{10}$ to proMMP-2 (Figure 4A, bold lines) resulted in the same plateau activity as actMMP-2 without $(GPO)_{10}$ (Figure 4B, thin line). Since substrate freshly added after 120 min did not alter the outcome and excluded substrate depletion (data not shown), an elevated substrate turnover in the presence of low molar excesses of $(GPO)_{10}$ was suggested.

The Hyp content defines the efficiency of a competitor for proMMP-2 binding to CI

Further studies on the proMMP-2 exosite ligand-binding structure focused on the role of the triple helix and the Hyp content of the repeated triplet. Midpoints of melting curves of $(POG)_{10}$ and $(PPG)_{10}$ occurred at 64°C and 43°C, respectively. The control peptides GAP and $(POG)_5$ were confirmed to be found nonhelical even at 5°C (Figure 5). Triple-helical $(GPP)_{10}$ without Hyp residues was much less efficient in competing with proMMP-2 binding to CI than the nonhelical $(POG)_5$ (Table 3). The competition potency of $(GPO)_{10}$ that was triple-helical and contained Hyp residues was comparable to that of $(POG)_5$. These results emphasize the crucial role of GPO triplets for gelatinase binding and established a 10-fold molar excess of $(POG)_5$ in relation to proMMP-2 as a minimum prerequisite for blockade of proMMP-2 binding to fibrillar CI.

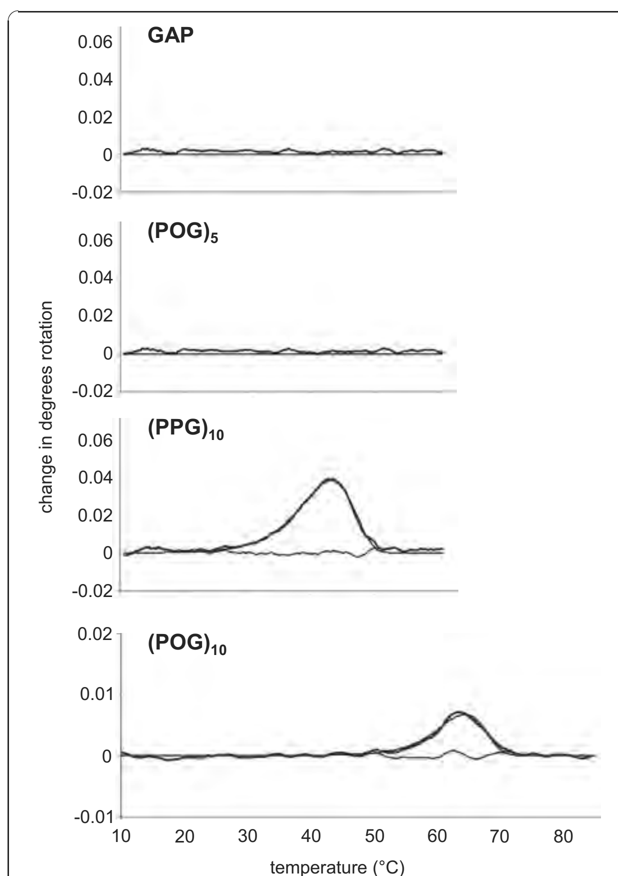


Figure 5 Polarimetric determination of melting temperatures of collagen analogs. Spontaneous triple helix assembly of collagen analogs was assessed by the midpoints of melting curves in a temperature regimen from 8°C to 60°C for Gly-Ala-Pro (**GAP**), $(POG)_5$ and $(PPG)_{10}$ and from 8°C to 90°C for $(POG)_{10}$. Graphs shown represent three independent experiments.

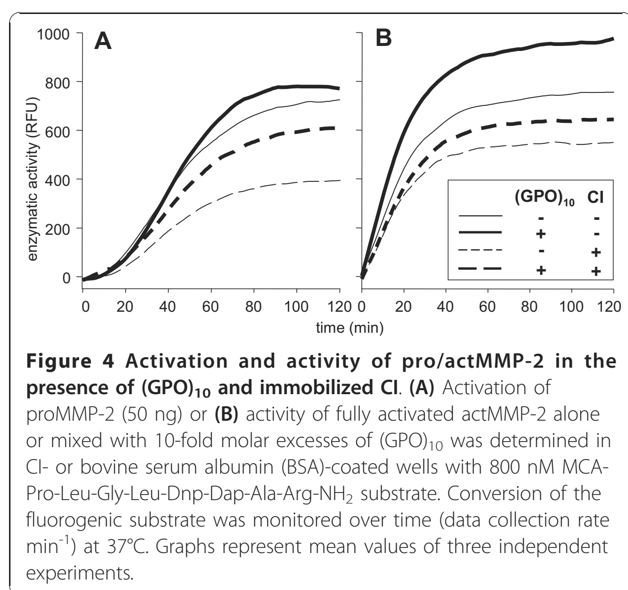


Figure 4 Activation and activity of pro/actMMP-2 in the presence of $(GPO)_{10}$ and immobilized CI. **(A)** Activation of proMMP-2 (50 ng) or **(B)** activity of fully activated actMMP-2 alone or mixed with 10-fold molar excesses of $(GPO)_{10}$ was determined in CI- or bovine serum albumin (BSA)-coated wells with 800 nM MCA-Pro-Leu-Gly-Leu-Dnp-Dap-Ala-Arg-NH_2 substrate. Conversion of the fluorogenic substrate was monitored over time (data collection rate min^{-1}) at 37°C. Graphs represent mean values of three independent experiments.

$(GPO)_{10}$ and the MMP-2 prodomain peptide P_{33-42} compete for binding to the CBD

Collagen analogs similar to $(GPO)_{10}$, as well as the proMMP-2 prodomain-derived peptide P_{33-42}, are known to bind with high affinity to the Col-modules of the CBD of MMP-2. Addressing mechanisms of $(GPO)_{10}$-induced inhibition of proMMP-2 binding to fibrillar collagen and $(GPO)_{10}$-induced enzymatic activation, we used $(GPO)_{10}$ and P_{33-42} to interfere with proMMP-2 binding to CI and to modulate CBD-dependent DQ-gelatine degradation of MMP-2.

In SPR measurements P_{33-42} was evaluated as a competitor of proMMP-2 binding to immobilized CI. High molar excesses of P_{33-42} increased the K_d values of proMMP-2 binding to CI to up to 50% (Figure 6A), which is moderate in comparison to the 22-fold inhibition in the presence of $(GPO)_{10}$ (Table 3). On the other hand, $(GPO)_{10}$ and P_{33-42} had different effects on

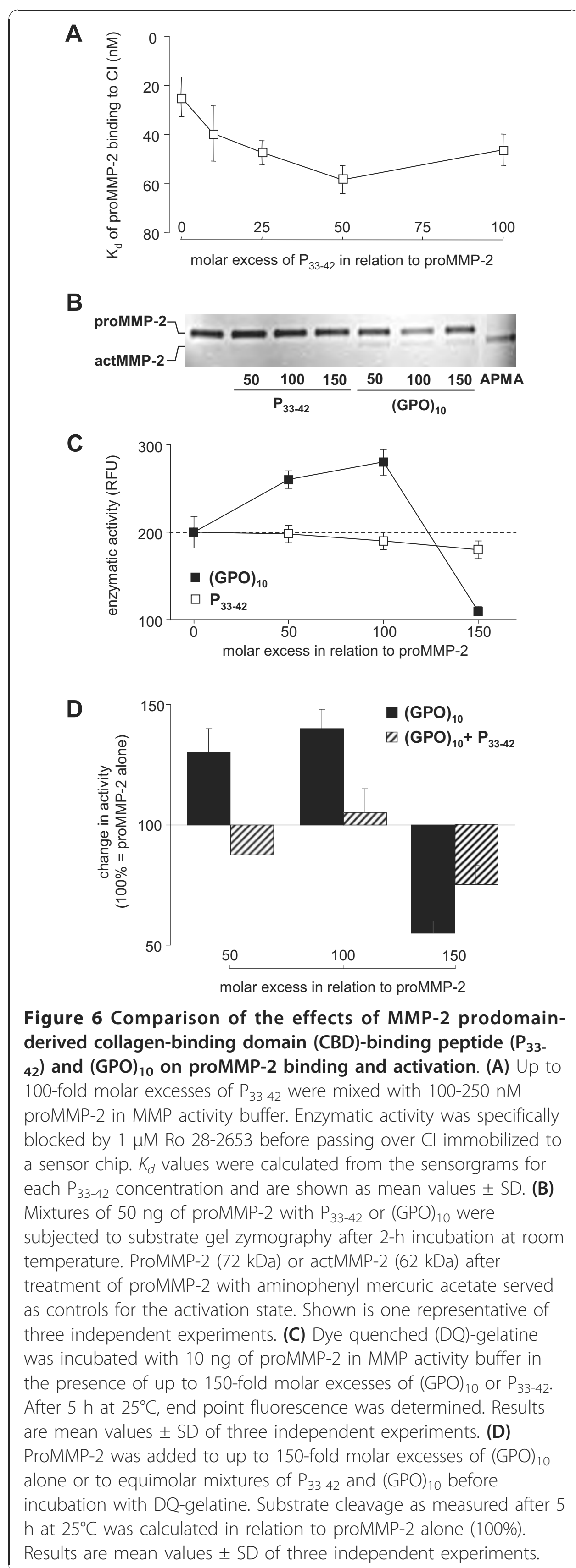


Figure 6 Comparison of the effects of MMP-2 prodomain-derived collagen-binding domain (CBD)-binding peptide (P_{33-42}) and $(GPO)_{10}$ on proMMP-2 binding and activation. **(A)** Up to 100-fold molar excesses of P_{33-42} were mixed with 100-250 nM proMMP-2 in MMP activity buffer. Enzymatic activity was specifically blocked by 1 μM Ro 28-2653 before passing over CI immobilized to a sensor chip. K_d values were calculated from the sensorgrams for each P_{33-42} concentration and are shown as mean values ± SD. **(B)** Mixtures of 50 ng of proMMP-2 with P_{33-42} or $(GPO)_{10}$ were subjected to substrate gel zymography after 2-h incubation at room temperature. ProMMP-2 (72 kDa) or actMMP-2 (62 kDa) after treatment of proMMP-2 with aminophenyl mercuric acetate served as controls for the activation state. Shown is one representative of three independent experiments. **(C)** Dye quenched (DQ)-gelatine was incubated with 10 ng of proMMP-2 in MMP activity buffer in the presence of up to 150-fold molar excesses of $(GPO)_{10}$ or P_{33-42}. After 5 h at 25°C, end point fluorescence was determined. Results are mean values ± SD of three independent experiments. **(D)** ProMMP-2 was added to up to 150-fold molar excesses of $(GPO)_{10}$ alone or to equimolar mixtures of P_{33-42} and $(GPO)_{10}$ before incubation with DQ-gelatine. Substrate cleavage as measured after 5 h at 25°C was calculated in relation to proMMP-2 alone (100%). Results are mean values ± SD of three independent experiments.

both proMMP-2 activation and activity (Figures 6B-D). Substrate zymography showed a faint band of prodomain-free actMMP-2 in the presence of $(GPO)_{10}$ which did not occur if proMMP-2 was treated with P_{33-42} (Figure 6B). In MMP activity assays with DQ-gelatine as a fluorogenic substrate, actMMP-2 used as positive control established a plateau level of substrate conversion of about 1,000 relative fluorescence units while baseline levels for proMMP-2 were at about 200 relative fluorescence units. Whereas DQ-gelatine cleavage was significantly enhanced by 50- to 100-fold molar excesses of $(GPO)_{10}$, P_{33-42} had no activating effect but a slight inhibitory effect (Figure 6C). The 150-fold excesses of P_{33-42} or $(GPO)_{10}$ in relation to proMMP-2 resulted in a more enhanced inhibitory effect of P_{33-42} and a $(GPO)_{10}$-driven shift from an active MMP-2 to an enzymatically blocked enzyme (Figure 6C). To gain insights into the mechanism of action, equimolar mixtures of $(GPO)_{10}$ and P_{33-42} were added to proMMP-2 before assaying DQ-gelatine degradation (Figure 6D). At 50- to 100-fold molar excesses compared to proMMP-2, P_{33-42} drastically diminished the activating effect of $(GPO)_{10}$ on proMMP-2. In addition, at a 150-fold molar excess of the mixture to proMMP-2, the inhibitory effect of $(GPO)_{10}$ on MMP-2 activity was diminished (Figure 6D). These findings indicated that both $(GPO)_{10}$ and P_{33-42} compete for the same proMMP-2 exosite.

$(GPO)_{10}$ prevents proMMP-2 binding and releases proMMP-2 *in situ* bound to fibrillar septa

To answer the question about the capacity of $(GPO)_{10}$ to modulate proMMP-2 binding to fibrillar structures *in situ*, cryostat sections of cirrhotic liver tissue were treated with exogenous Cy2-labeled proMMP-2 (Figure 7). Without $(GPO)_{10}$, the fluorescence pattern (Figure 7B) reflected the extent of fibrillar collagenous structures (Figure 7A), confirming that proMMP-2 can be extracellularly stored in fibrotic liver tissue. Coincubation of $(GPO)_{10}$ with Cy2-proMMP-2 (Figure 7C) or subsequent treatment of ECM-bound Cy2-proMMP-2 with $(GPO)_{10}$ (Figure 7D) either prevented gelatinase binding or promoted effective release of the sequestered enzyme.

Discussion

The ECM is known as a depot for cytokines, as described by the term *crinopexy* [21], and as a reservoir for regulative matrix fragments, for example, classified by the terms *degradomics* and *endogenous inhibitors of angiogenesis* [22,23]. Proteolytic processing of bioactive molecules is mainly performed by MMPs. In pathological processes such as organ fibrosis, this fine-tuned tissue homeostasis is lost. Because of chronic wounding, inflammation scar tissue accumulates. Its degradation is hampered by the over-expression of TIMPs and by the blockade of ECM-degrading activity via binding of latent MMPs to defined

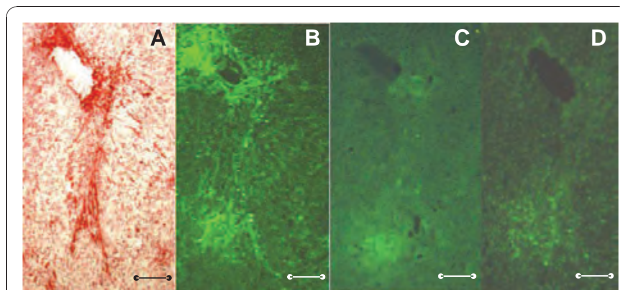


Figure 7 $(GPO)_{10}$-mediated release of proMMP-2 sequestered in cirrhotic liver tissue. **(A)** Cryostat sections of cirrhotic human liver tissue were fixed, stained with Sirius red for collagens and counterstained with Haemalaun. **(B-D)** Serial sections of the same liver tissue sample were incubated with 60 ng of Cy2-proMMP-2 in the dark for 24 h before unbound Cy2-proMMP-2 was removed by washing. **(B)** Slides were treated with Cy2-proMMP-2 alone. **(C)** Cy2-proMMP-2 was mixed with a 10-fold molar excess of $(GPO)_{10}$ before being added to the slides. **(D)** Cy2-proMMP-2 was allowed to bind before a 10-fold molar excess of $(GPO)_{10}$ was applied. Original magnification, ×40; scale bars, 200 μm.

collagen structures, identified as the α2(VI) chain for collagenases [15] and, as reported here, fibrillar collagens for gelatinases. Thus, the ECM contributes to the availability and activity of its degrading enzymes by storing the inactive collagenase and gelatinase proforms. To break this vicious cycle of enhanced scar matrix production and accompanied blockade of MMP activity, we here propose short synthetic collagen analogs to release matrix-stored collagen-degrading enzymatic activity.

In situ gelatinolytic activity of proMMP-2 and proMMP-9 collocalizes with fibrotic fibers

Apart from the well-known cell membrane localization of gelatinases [9], we demonstrated by *in situ* zymography fibril-associated gelatinolytic activity and identified it immunohistochemically as MMP-2 and MMP-9. Since neither method could discriminate inactive proforms from activated gelatinases, sections of human cirrhotic liver were preincubated with recombinant pro- or actMMP-2 or pro- or actMMP-9, demonstrating that only the inactive gelatinase proforms efficiently bound to the collagenous septa *in situ* (Figure 1). These findings are in line with previous reports describing a "distinct pool" of collagen-bound proenzyme which "appears recalcitrant to cellular activation" and a reduction of autolytic inactivation by binding to CI [13,14].

Collagen binding is strongly diminished upon proMMP-2 and proMMP-9 activation

Earlier structural analysis of proMMP-2 revealed a conformational transition from an inactive, "closed" proMMP-2 to an "open" actMMP-2 [24,25]. This activation-related conformational switch also implicated a shift in exosite binding affinities of the gelatinases. Indeed, high binding affinity of the proforms to nonsubstrate collagens at least by a factor of 35 reduced the affinity of actMMP-2 (Tables 1, 2 and 3 and Figure 2B) and supported this assumption. In addition, the "closed" conformation benefited from the high-affinity binding to nonsubstrate fibrillar CI insofar as (auto)activation of proMMP-2 and proMMP-9 is delayed (Figure 4). Importantly, this activation can be alleviated not only by gelatine binding as described earlier [26] but also by competing the proMMP exosite interactions with CI by defined low-molecular-weight, Hyp-containing, triple-helical short collagen analogs (Figure 4). Thus, autolysis of MMP-2 slowed because of binding to CI [14], and substrate turnover, especially for short substrates, is enhanced by $(GPO)_{10}$. Our data also pointed to the importance and interdependency of conformational switches and enzymatic activation, which were initially more or less excluded because of the absence of divalent cations, resulting in only marginally diminished binding of proMMP-2 to CI despite the presence of $(GPO)_{10}$ (Tables 2 and 3).

Interestingly, addition of $(GPO)_{10}$ to proMMP-2 not only affected the binding and gelatinolytic activity of proMMP-2 but also confirmed the collagenolytic potential of MMP-2 (Figure 3B) [6], whereas the loss of CI binding affinity of proMMP-9 in the presence of $(GPO)_{10}$ was not accompanied by CI degradation (Figure 3A), which is in contrast to a recent publication [27].

On the matrix site, triple helicity and Hyp are important for strong proMMP-2 and proMMP-9 exosite interaction

Generally, collagens comprise the repeated triplet sequence $(Gly\text{-}Xaa\text{-}Xaa')_n$. In stable triple helices, these triplets contain a high proportion of the imino acids Pro and Hyp at the Xaa/Xaa' positions [28]. Complementing previous reports [29], screening with highly purified liver ECM components proved fibrillar CI and CIII to be good as well as microfibrillar CVI to be inferior ligands for proMMP-2 and proMMP-9 (Table 1 and Figure 2B). Assuming that retention of proMMPs might depend on the secondary structure of the collagen, particularly on the collagen triple helix, we investigated correlations between its thermal stability and the affinity of MMP-2 and MMP-9 binding.

Starting from the soluble human placenta-isolated triple-helical portion of CI with a melting temperature of 33.8°C [30], we found high binding affinity to proMMP-2 and proMMP-9 with K_d values in a low nanomolar range (Tables 2 and 3). We tested α1(I) chains, which rapidly refold into triple-helical conformation in neutral buffers at room temperature, as well as CB-peptides of the α1(I) chain, where the length of the triple helix affects their thermal stability [31]. Here the strength of

binding of proMMP-2 and proMMP-9 to CB peptides directly correlated to the thermal stability of its triple helix that was defined by the overall size and by the GPP/O content of the peptide (Table 1). Short synthetic collagen analogs with increasing triple helicity and Hyp content confirmed the findings for the CB peptides (Figure 2B and Table 1). Keeping the development of synthetic lead structures to modulate proMMP binding to collagen in mind, we tested short soluble natural and synthetic collagen structures as competitors of proMMP-2 and proMMP-9 binding.

At low molar excesses, not only the CI-derived CB7 (not shown) but also synthetic $(GPO)_{10}$ (Table 3) were found to effectively compete proMMP-2 binding to CI. Addition of just a 10-fold molar excess of $(GPO)_{10}$ to CI-bound proMMP-2 resulted in a more than 20-fold increase in K_d values, while $(GPP)_{10}$ and GAP were less efficient. While short nonhelical $(POG)_5$ (Figure 5) increased the K_d value of binding 22-fold, triple-helical $(GPP)_{10}$ induced about a sixfold increase (Table 3). Thus, the combined presence of Hyp residues and a stable triple-helical structure might explain the efficacy of $(GPO)_{10}$ as a competitor of the gelatinase-CI binding. Subsequent experiments emphasized the Hyp content of the triple helix as the major prerequisite for strong interference with the binding of proMMP-2 and proMMP-9 to CI (Table 3).

We hypothesize that proMMP binding to GPO triplets in collagens resembles the binding of platelet glycoprotein VI or leukocyte-associated immunoglobulin-like receptor 1 to collagens, showing stronger binding of platelet glycoprotein VI to $(GPO)_{4-10}$ as compared to $(GPP)_{10}$ [32,33].

Binding constants of proMMP-CI interaction in the nanomolar range suggest binding cooperativity of CBD modules

Functionally, the CBD is critical for positioning large gelatine-like substrates, which defines MMP specificity and activity while degradation of short synthetic substrates is independent of binding to the CBD [17]. Exosites, that is, outside the catalytic center, are the crucial if not the only structures to bind collagens to MMP-2 and MMP-9 [34].

Our findings with $(GPP)_{10}$ and $(GPO)_{10}$ underline the earlier observed positive correlation between increasing rigidity of the triple helix of the collagen analogs $(PPG)_6$, $(PPG)_{12}$ and 3× $(PPG)_{12}$ and their binding to recombinant CBD modules [7]. They are also consistent with the finding that proMMP-2 preferentially binds to triple-helical collagenous analogs rather than to gelatinous analogs [16].

Affinities of single Col-modules of the CBD to synthetic collagen analogs, for example, $(PPG)_{12}$, were reported to be in the range of K_d 1.4-4.5 mM [7,35]. The strong interaction for fibrillar CI and proMMP-2 and proMMP-9 with K_d values in the low nanomolar range (Tables 2 and 3) indicate cooperativity of all three Col-modules when bound to extended collagen fibrils. Obviously, owing to interaction with $(GPO)_{10}$, major conformational changes occur in the entire proMMP-2 molecule as suggested previously [7].

Collagen analog $(GPO)_{10}$ binding at the CBD disturbs the interaction with fibrillar collagen, prodomain sequence P_{33-42} and gelatine

Finally, we asked how $(GPO)_{10}$ competes CBD-mediated binding of proMMP-2 to fibrillar collagen and thereby modulates binding as well as enzymatic activity and activation. We made use of the P_{33-42} peptide derived from the prodomain of MMP-2. The P_{33-42} is known to bind specifically to the Col-3 module of the CBD of MMP-2 with high affinity (K_d, 1.6 mM), thereby mimicking gelatine substrate [7]. $(GPO)_{10}$ weakened prodomain binding via P_{33-42} to proMMP-2, but this effect was not accompanied by strong prodomain cleavage (Figure 6B), similar to earlier reports for MMP-9 [36]. Even at high molar excesses, P_{33-42} alone only slightly inhibited proMMP-2 binding to CI (Figure 6A) and did not affect proMMP-2 activation (Figures 6B and 6C). In MMPs devoid of prodomain-CBD interaction or without CBD, such as the gelatinase MMP-9 and the collagenase MMP-13, respectively, P_{33-42} had no effects (data not shown). On the other hand, P_{33-42} blocked $(GPO)_{10}$-mediated proMMP-2 activation (Figure 6D), strongly implying that $(GPO)_{10}$ also binds to Col-3 of the CBD.

Very high molecular excesses of $(GPO)_{10}$ not only displaced the prodomain from the CBD but also competed with DQ-gelatine substrate, resulting in the reduction of MMP-2 gelatinolytic activity (Figure 6C). Thus, affinity-driven and concentration-dependent interactions of $(GPO)_{10}$, first with Col-1 and Col-2 and finally with Col-3, seem to be the trigger for proMMP-2 and proMMP-9 release and activation or for inhibition of enzymatic activity by modulating the interaction of the collagen-binding domain of gelatinases and their ligands: fibrillar collagen, the MMP-2 prodomain peptide P_{33-42}, and the gelatine substrate.

Collagen analogs have concentration-dependent differential effects on gelatinases

We here provide a hypothesis for concentration-dependent differential effects of $(GPO)_{10}$ on the binding, release and activation and activity of (pro)MMP-2. At low concentrations, interaction of Hyp-containing triple-helical collagen analogs with Col-modules compete with the binding of proMMP to collagens. Medium analog concentrations affect proMMP binding as well as its enzymatic activity. High-affinity binding of collagen analogs to Col-1 and Col-2 and low affinity to Col-3

triggers transition to the "open" conformation, resulting in release of proMMP from the collagen depot and autoactivation accompanied by replacement of P_{33-42} at Col-3. At high concentrations, Hyp-containing triple-helical collagen analogs compete with the substrate gelatine at all Col-modules, resulting in the disorientation of gelatine at the CBD and block of enzymatic activity [37].

Collagen analogs might be used in the therapy of fibrotic diseases

How does this model relate to potential therapeutic options in liver fibrosis? The failure or limited success of recent clinical trials targeting the MMP catalytic center, for example, hydroxamic acid-based inhibitors or peptide libraries [17,38], demand the reconsideration of strategies. It was assumed that competition with gelatine at the CBD might be a main mechanism for inhibition [17]. Collagen analogs were chemically linked to a hydroxamic acid derivative and spanned from the CBD to the active center. Unfortunately, this straightforward strategy did not enhance the specificity of inhibitors for MMP-2 and MMP-9 [35]. Recently, further exosite inhibitors of MMPs came into consideration [39,40]. These earlier studies did not focus on the Hyp content and triple-helical structure of small synthetic collagen analogs. As exemplified by $(GPO)_{10}$, they can release proMMP-2 and proMMP-9 from their fibrillar collagen depots, directly and sequentially interact with distinct Col-modules of the CBD, and interfere with the prodomain peptide P_{33-42}, thereby inducing conformational changes and activation of MMP-2. The first hints of the potential of collagen analogs as therapeutic tools in liver fibrosis are given by the *in situ* release of proMMP-2 sequestered by collagen in cirrhotic liver tissue by low molecular excesses of $(GPO)_{10}$ (Figure 7). Thus, our findings establish the ECM-sequestered proform as the noncellular source of high MMP-2 activity found in the fibrosis resolution phase supposed earlier [18-20] and Hyp-containing collagen analogs as tools for targeted release of proMMP-2 and proMMP-9 from their extracellular depot and concomitant activation of the enzymes.

Conclusions

In conclusion, Hyp content and rigidity of the triple helix of small collagen analogs are crucial for effective competition with the CBD-mediated proMMP-2 and proMMP-9 binding to nonsubstrate collagens, eventually leading to activation of the enzyme. Thus, for example, $(GPO)_{10}$ as a model molecule for a new class of exosite MMP modulators might mobilize the sequestered pool of gelatinolytic activity from its noncellular storage depot, inducing the degradation of excess ECM in fibrotic diseases such as liver fibrosis.

Methods

Liver tissue samples

Male Wistar rats of 200-250 g body weight were obtained from Charles River Laboratories (Wilmington, MA, USA). Liver fibrosis was induced by administering 200 mg/kg thioacetamide for 2 wk as described before [41]. If not noted otherwise, reagents were purchased from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany) and were of the highest purity available. Tissue samples were either fixed by 4% (vol/vol) formalin and embedded in paraffin or prepared for cryostat sections. Animal protocols were approved by the regional animal study committee. Specimens of cirrhotic human livers were obtained from explanted livers from patients with alcoholic cirrhosis undergoing orthotopic liver transplantation. Informed consent was obtained prior to surgery. Immediately after explantation, tissue samples were snap-frozen and stored over liquid nitrogen.

In vitro activation of MMP-2 and MMP-9

The 62-kDa actMMP-2 (Invitek, Berlin, Germany) was released from 27.8 nM 72-kDa proMMP-2 in 1 mM APMA in MMP activity buffer consisting of 50 mM Tris·HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% (vol/vol) Brij-35 for 1 h. The 86-kDa actMMP-9 (Invitek) was obtained from 217 nM 92-kDa proMMP-9 in 80-µl MMP activity buffer without Brij-35 incubated with 100 µg/ml chymotrypsin activity-blocked trypsin for 20 min. Tryptic digestion was terminated by 100 µg/ml aprotinin within 10 min. All steps were performed at 37°C. These completely activated MMPs as end products of the *in vitro* activation structurally and functionally correspond to those found *in vivo* [42,43]. All MMPs were stored in aliquots at -80°C. The (pro)MMP activation state was routinely checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) substrate zymography.

Radiolabeling of pro- and actMMP-2 and pro- and actMMP-9

Human recombinant proMMPs and *in vitro* activated MMPs were obtained from Invitek [44] and were labeled with the $[^{125}I]$-Bolton-Hunter reagent according to the manufacturer's instructions (PerkinElmer, Rodgau, Germany) before the buffer was exchanged for phosphate-buffered saline (PBS) with 0.05% (vol/vol) Tween 20 by gel filtration as described previously [45]. Specific radioactivity for $[^{125}I]$-MMPs was 3-9 × 10^4 cpm/ng. Precipitation with 10% (wt/vol) trichloroacetic acid and 200 µg of BSA recovered 96% to 100% of protein-bound radioactivity. Aliquots of labeled MMPs were frozen and stored at -80°C. The activity and integrity were checked

by substrate gel zymography using SDS-PAGE and autoradiography with overnight exposure to Biomax MS film (Kodak, Stuttgart, Germany) (Figure 2A).

Preparation of collagens, CI derivatives and structural analogs

Native human CI, CIII and CVI were purified from skin tissue and placenta. The CI single chains $\alpha 1$(I) and $\alpha 2$ (I) were obtained and modified as described previously [45]. To prepare defined fragments, 2 mg of $\alpha 1$(I) were dissolved in 1 ml of 70% (vol/vol) formic acid at room temperature, the tubes were flushed with nitrogen for 10 min, and then 2 mg of CB were added. After incubation for 4 h at 37°C, free CB was neutralized and the samples were lyophilized. The peptides CB3, CB6, CB7 and CB8 were separated from the reaction mixture by gel filtration followed by ion-exchange chromatography. The resulting peptides were characterized by amino acid analysis and SDS-PAGE [45,46]. The CB peptides had the following melting temperatures: CB3, 23.9°C; CB6, 26.7°C; CB7, 28.1°C; and CB8, 28.0°C [31].

The following collagen analogs and control peptides were synthesized as described previously [47]: $(GPO)_{10}$, H-Gly-Cys-Hyp-$(Gly\text{-}Pro\text{-}Hyp)_{10}$-Gly-Cys-Hyp-Gly-$NH_2$; $(GPP)_{10}$, H-Gly-Cys-Pro-$(Gly\text{-}Pro\text{-}Pro)_{10}$-Gly-Cys-Pro-Gly-$NH_2$; and GAP, H-Gly-Ala-Cys-$(Gly\text{-}Ala\text{-}Pro)_5$-Gly-Phe-Hyp-Gly-Glu-Arg-$(Gly\text{-}Ala\text{-}Pro)_5$-$NH_2$. Peptides $(POG)_{10}$, $(PPG)_{10}$ and $(POG)_5$ were purchased from Peptide International (Louisville, KY, USA). Spontaneous triple-helix assembly was approved by polarimetry over a 10-cm path length at 1°C/min in 10 mM phosphate buffer, pH 7.4. At 5 mg/ml, midpoints of melting curves occurred at 82.3 ± 1.4°C for $(GPO)_{10}$ and at 45.8 ± 0.8°C for $(GPP)_{10}$. Peptide GAP was determined to be nonhelical even at 5°C. Graphs were calculated from the primary data using a custom fitting program written by D. A. Slatter (Department of Biochemistry, University of Cambridge, Cambridge, UK [48]) to model different possible transitions. All collagens, CI derivatives and peptides were stored in stock solutions of 2 mg/ml in 150 mM acetic acid at -20°C.

Histological detection of connective tissue

In rat liver samples, connective tissue was visualized using Sirius red staining in thin sections of formalin-fixed, paraffin-embedded tissue samples [41]. Cryostat sections of human liver samples were fixed with 1% (vol/vol) formalin for 10 min before being stained with Sirius red. Slides were assessed using standard light microscopy (Olympus, Hamburg, Germany).

In situ zymography

As described earlier, *in situ* zymography was performed with cryostat sections (6 μm) of rat cirrhotic liver [41,49]. In brief, sections were dried, overlayed with 100 μg/ml DQ-gelatine ($\lambda_{ex/em}$, 495/515 nm; Molecular Probes, Eugene, OR, USA) and 0.5% (wt/vol) low-melt agarose in MMP activity buffer. For negative controls, 10 mM ethylenediaminetetraacetic acid or 1 mM phenanthroline was included to the reaction mixture, after which no generation of bright green fluorescence was observed, implying inhibition of gelatinase activity [50]. Samples were inserted into coverslips and incubated at 40°C for 1 h before being transferred to room temperature for an additional 2 to 16 h. Hoechst 33342 (Invitrogen, Carlsbad, California, USA) nuclear dye was used for counterstaining. Images were obtained by fluorescence microscopy using a Nikon E800 photodocumentation microscope (Nikon Imaging, Düsseldorf, Germany).

In situ binding of (pro)MMP-2 and (pro)MMP-9

Human cirrhotic liver cryostat sections (5 μm) were air-dried and fixed in ice-cold acetone for 10 min. Tissue sections were rehydrated with PBS and incubated with 25 ng/50 μl of the respective proMMP and actMMP for 30 min or were left untreated. After thorough washing with PBS, antibodies specific for human MMP-9 (clone MAB911; R&D Systems, Minneapolis, MN, USA) and human MMP-2 (clone 75-7F7; Oncogene, Cambridge, MA, USA) were applied, and primary antibody binding was detected using the alkaline phosphatase-antialkaline phosphatase detection system (Dako, Hamburg, Germany). An irrelevant primary mouse antibody served as control. Nuclei were counterstained with Hemalaun, and slides were examined by standard light microscopy.

Solid-phase binding studies

ProMMP-2 and proMMP-9 or actMMP-2 and actMMP-9 were bound to nitrocellulose and polystyrene-immobilized native collagens, CI chains, CB peptides or structural analogs. Serial dilutions of collagens or CI derivatives in 150 mM acetic acid were dotted at 3 × 3 μl to a nitrocellulose membrane with high protein-binding capacity (GE Healthcare, Munich, Germany). Air-dried membranes were blocked with PBS and 0.3% (vol/vol) Tween 20 overnight at 4°C, washed three times, and incubated with 1 ng/ml $[^{125}I]$-pro- and actMMP-2 and pro- and actMMP-9 in PBS and 0.3% (vol/vol) Tween 20 for 2 h at room temperature. Membranes were washed again and air-dried, and bound MMP was monitored by autoradiography. In parallel, polystyrene microtiter plates (Dynex, Chantilly, VA, USA) were coated with collagen proteins and peptides. Here 2 μg/well or 200 ng/well proteins and peptides or BSA as control were immobilized in 100 μl of 50 mM ammonium bicarbonate buffer, pH 9.6, by overnight incubation at 4°C. Immobilization efficacies were 20% to 45% of total proteins [45]. Wells were washed three times

with PBS, and nonspecific binding sites were blocked with PBS and 0.05% (vol/vol) Tween 20 for 1 h at room temperature. All incubation steps were performed with 2 ng of [^{125}I]-MMPs at 4°C for 2 h. Unbound reagents were removed by thorough washing with PBS and 0.05% (vol/vol) Tween 20, and residual radioactivity was determined using a gamma counter (Berthold, Bad Wildbach, Germany).

Surface plasmon resonance analysis

Sensor chip preparations and SPR measurements were performed using a BiacoreX device and the Bia-evaluation software (version 3.2; Biacore, Uppsala, Sweden). The pepsin-resistant triple-helical part of human fibrillar CI (100 μg/ml) in 10 mM acetate coupling buffer, pH 4.8, was immobilized to a dextran matrix-sensor chip at a flow rate of 5 μl/min, resulting in 5,500 resonance units from CI covalently linked via its primary amino groups. The control flow cell was prepared using the coupling buffer without CI. Surfaces were activated and blocked as described previously [51]. Immediately after thawing, pro- and actMMP-2 and pro- and actMMP-9 were diluted to 100 to 250 nM in MMP activity buffer or in PBS and 0.05% (vol/vol) Tween 20. For SPR measurements, flow rates were 10 μl/min at 25°C, and equilibrium was typically reached after 30 to 60 s. The effects of $(GPO)_{10}$, GAP, $(POG)_5$, $(GPP)_{10}$ and $P_{33\text{-}42}$ on MMP-2 and MMP-9 binding to CI and their enzymatic activity were determined by adding the binding competitors to the (pro- and act)MMP-2 and MMP-9 solution in 10- to 150-fold molar excesses. Effects independent of MMP-2 and MMP-9 activity were monitored in the presence of Ro 28-2653 (1 μM) during SPR analysis. The gelatinase inhibitor Ro 28-2653 was a generous gift from H.-W. Krell (Roche, Grenzach-Wyhlen, Germany). Sensor surfaces were regenerated with 10 mM glycine, pH 2.3, for 1 min between runs, and sensor chips were used up to 25 times. Kinetic parameters were analyzed using the 1:1 binding model with drifting baseline and subtraction of the control flow cell binding from sensorgrams obtained with immobilized CI. Binding constants (K_d) were calculated from the association (k_a) and dissociation rates (k_d) obtained from individual binding curves at different concentrations. Individual drifts of the resonance signal were fitted locally, and χ^2 values of 0.2% to 1.0% of the maximum resonance value were considered good fits.

Fluorogenic MMP activity assay

Enzymatic activities of MMPs were studied spectrofluorimetrically by cleavage of fluorogenic substrates in MMP activity buffer within 2 to 5 h. For gelatinases 800 nM MCA-Pro-Leu-Gly-Leu-Dnp-Dap-Ala-Arg-NH_2 ($\lambda_{ex/em}$ 328/393 nm; Bachem, Bubendorf, Switzerland) or 10 μg/ml DQ-gelatine were used, according to the method described by Knight *et al.* [52] for collagenases 800 nM MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 ($\lambda_{ex/em}$ 280/360 nm; Anaspec, San José, CA, USA). In some experiments, proMMP-2 was fully activated in the presence of 1 mM APMA prior to the kinetic measurements. A quantity of 50 ng pro- or actMMP-2 alone or mixed with 10- to 150-fold molar excesses of $(GPO)_{10}$, GAP, $P_{33\text{-}42}$ or mixtures of $(GPO)_{10}$ and $P_{33\text{-}42}$, were added to CI-coated, BSA-coated (1 μg/well both) or uncoated wells containing 150 μl of the respective substrate solution. The peptide $P_{33\text{-}42}$ was purchased from the Institute of Biochemistry (Humboldt-University, Berlin, Germany). The influence of CI on MMP-2 enzymatic activity against the quenched fluorescent substrate could be excluded [14]. Background subtraction (measurement without MMPs) was applied to all curves. All experiments were performed with a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) and black 96-well microtiter plates with a clear bottom (Greiner bio-one, Frickenhausen, Germany).

Gel zymography

Samples containing MMP-2 were diluted with zymogram sample buffer (Bio-Rad, Munich, Germany) and separated on homogeneous 10% SDS-PAGE gels containing 1 mg/ml (wt/vol) gelatine (Bio-Rad), washed with excess MMP activity buffer containing 2.5% (vol/vol) Triton X-100 to remove SDS, and incubated with MMP activity buffer for 24 h. Gels were stained with Coomassie Blue R-250. Gels showing proteolytic bands corresponding to proMMP-2 (72 kDa) or actMMP-2 (62 kDa) were scanned (Plustek, Norderstedt, Germany) and analyzed from inverted grayscale images.

Release of *in situ* bound proMMP-2

ProMMP-2 was labeled using the FluoroLink Cy2 Labeling Kit according to the manufacturer's instructions (Amersham Biosciences, Freiburg, Germany). Unbound fluorescent dye was removed by ultrafiltration (Nanosep, Lund, Sweden), and labeling success was monitored using a fluorescence microplate reader ($\lambda_{ex/em}$, 489/506 nm). Serial cirrhotic human liver sections were covered with 1.2 μg/ml Cy2-proMMP-2 in 50 mM Tris·HCl, pH 7.4, containing 1 mM $CaCl_2$ or with buffer alone, and were incubated in a dark humidified chamber for 24 h at 4°C. To study effects of $(GPO)_{10}$, a 10-fold molar excess in relation to proMMP-2 was added to slides prior to or after Cy2-proMMP-2 binding. Slides were washed with PBS, air-dried, and rinsed with deionized water. Bound Cy2-proMMP-2 was detected by fluorescence microscopy (Olympus, Hamburg, Germany).

Statistical Analysis

One-way analysis of variance and Tukey's tests were performed using SigmaStat for Windows version 2.03 (Sigmaplot, Erkrath, Germany), and $P < 0.05$ was considered significantly different.

Abbreviations

α1(I): α1 chain of collagen type I; actMMP: activated form of MMP; APMA: aminophenyl mercuric acetate; CB: cyanogen bromide cleavage-derived peptides of α1(I); CBD: collagen-binding domain; CI: CIII and CIV, collagen types I, III and IV; CoI: fibronectin type II module of the CBD; DQ: dye-quenched; ECM: extracellular matrix; GAP: Gly-Ala-Pro; GPO: Gly-Pro-Hyp; GPP: Gly-Pro-Pro; Hyp: hydroxyproline; MMP: matrix metalloproteinase; P_{33-42}: MMP-2 prodomain-derived CBD-binding peptide; POG: Pro-Hyp-Gly; PPG: Pro-Pro-Gly; proMMP: latent proform of MMP; SPR: surface plasmon resonance; TIMP: tissue inhibitor of metalloproteinase.

Acknowledgements

This study was supported by grants Ru 624/3-2 and Schu 646/1-10 and the Collaborative Research Centers grants SFB366 C5/C10 and SFB633 Z1 from the Deutsche Forschungsgemeinschaft, as well as by the Medical Research Council, UK.

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Authors' contributions

MR conceived of the study, participated in its design and coordination and participated in the binding studies. MM carried out the solid-phase MMP-collagen binding studies. CF carried out the surface plasmon resonance MMP-collagen-binding studies and the MMP labeling. UE performed statistical analysis and helped to draft the manuscript. UN helped to draft the manuscript. DS helped to draft the manuscript.
YP carried out *in situ* zymography and fibrillar localization of gelatinase activity. WD helped to draft the manuscript. MZ helped to draft the manuscript. RF provided collagen analogs and characterized their melting behavior. RS participated in the design of the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 7 September 2010 Accepted: 6 January 2011
Published: 6 January 2011

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doi:10.1186/1755-1536-4-1
Cite this article as: Ruehl *et al.*: Hydroxyproline-containing collagen analogs trigger the release and activation of collagen-sequestered proMMP-2 by competition with prodomain-derived peptide P_{33-42}. *Fibrogenesis & Tissue Repair* 2011 **4**:1.

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European Journal of Microbiology and Immunology 2 (2012) 3, pp. 186–191
DOI: 10.1556/EuJMI.2.2012.3.3

THE SYNTHETIC HYDROXYPROLINE-CONTAINING COLLAGEN ANALOGUE $(GLY–PRO–HYP)_{10}$ PROMOTES ENZYMATIC ACTIVITY OF MATRIXMETALLOPROTEINASE-2 *IN VITRO*

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Received: June 11, 2012; Accepted: June 17, 2012

Diseases such as liver fibrosis and intestinal inflammation are characterized by accumulated components of the extracellular matrix (ECM). Given that fibrillar collagen structures were shown to serve as storage site for inactive proforms of matrixmetalloproteinases (MMPs), modulating this MMP–collagen interaction might offer a rational interventional (therapeutic) approach to enhance degradation of accumulated ECM. The synthetic triple helical collagen analogue $(Gly–Pro–Hyp)_{10}$ – $(GPO)_{10}$ – was shown to trigger release and enzymatic activation of collagen sequestered proMMP-2. In the presented study, we, for the first time, investigated how MMP–$(GPO)_{10}$ interaction impacts cellular responses *in vitro*. We found that recombinant proMMP-2 induced proliferation of hepatic stellate cells (HSC), which was enhanced after addition of $(GPO)_{10}$ reaching comparable levels following incubation with fully activated MMP-2. In addition, $(GPO)_{10}$ induced HSC migration similar to the platelet-derived growth factor subunit-B. Further, the MMP-2-dependent invasion of HT1080 fibrosarcoma cells through an ECM membrane was enhanced after addition of $(GPO)_{10}$. Since cellular proliferation and migration concomitant with matrix degradation is stimulated, we conclude that the MMP–$(GPO)_{10}$ interaction also functions in a physiological environment. Thus, a potential therapeutic effect of $(GPO)_{10}$ should be further tested in animal models for MMP-associated diseases such as colitis or fibrosis.

Keywords: $(GPO)_{10}$, extracellular matrix, *in vitro,* matrixmetalloproteinase, proMMP-2, gelatinases, enzymatic activation, HT1080, hepatic stellate cellss

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; DQ-gelatin, dye-quenched gelatin; DSS, dextran sulfate sodium; ECM, extracellular matrix; FCS; fetal calf serum; GAP, $H–Gly–Ala–Cys–(Gly–Ala–Pro)_5–Gly–Phe–Hyp–Gly–Glu–Arg–(Gly–Ala–Pro)_5–NH_2$; $(GPO)_{10}$, $H–Gly–Cys–Hyp–(Gly–Pro–Hyp)_{10}–Gly–Cys–Hyp–Gly–NH_2$; HSC, hepatic stellate cell; HT1080, human fibrosarcoma cell line; MMP, matrixmetalloproteinase; PBS, phosphate buffered saline; PDGF-BB, platelet-derived growth factor subunit B; PI3-K, phosphoinositide 3-kinase

Introduction

Matrixmetalloproteinases (MMPs) form a large family of zinc-dependent metalloendopeptidases that degrade extracellular matrix (ECM) molecules such as collagens, gelatin, elastin, or fibronectin [1]. MMPs are assumed to be sequestered into the ECM [2, 3] and are involved in the regulation of cellular differentiation, proliferation and migration, tumour growth and metastases, and fibrosis of organs such as liver [4–6]. Among the MMPs, the gelatinases MMP-2 and MMP-9 are considered to play vital roles in tumour invasion and liver fibrosis [7–11]. Both gelatinases can degrade virtually all structural components of the ECM and are therefore considered interesting targets for potential ECM-targeted therapeutic approaches. MMP-2 and MMP-9 were shown to be involved in human inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis [12, 13], as well as experimental acute small intestinal [14] and large intestinal inflammation [15].

In previous studies, we identified the α2 chain of collagen type VI as the main binding structure for sequestration of collagenases and stromelysin-1 proforms in fibrotic tissue [16], and showed that gelatinase binding sites are located on fibrillar collagen structures and the synthetic collagen analogue $(Gly–Pro–Hyp)_{10}$ ($(GPO)_{10}$) [17]. Using $(GPO)_{10}$ as a binding competitor for the interaction of the gelatinase proMMP-2 with collagen, a dissociation of the proMMP-2 prodomain accompanied by high enzymatic activation of MMP-2 could be achieved [17].

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ISSN 2062-509X / $ 20.00 © 2012 Akadémiai Kiadó, Budapest

In the present cell culture experiments, we investigated whether $(GPO)_{10}$ modulates the enzymatic activation/activity of proMMP-2/MMP-2. We demonstrate that $(GPO)_{10}$ acts as a mitogen for hepatic stellate cells and supports the MMP-dependent invasion of HT1080 fibrosarcoma cells via *in vitro* activation of proMMP-2, implicating $(GPO)_{10}$ or structural-related peptides as therapeutic options in MMP-associated diseases.

Materials and methods

Cell culture

Hepatic stellate cells (HSC) isolated from rats and treated with carbon tetrachloride as described earlier [18] were obtained from Prof. W. Dieterich (University of Erlangen, Germany). Human fibrosarcoma cells (HT1080) were purchased from ATCC (CCL-121). All cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 U/ml penicillin–streptomycin, and incubated at 5% CO_2 and 37 °C humidified atmosphere.

Preparations of collagen peptides

The triple helical collagen analogue $(GPO)_{10}$ – H–Gly–Cys–Hyp–$(Gly–Pro–Hyp)_{10}$–Gly–Cys–Hyp–Gly–NH_2 – and the linear control peptide GAP – H–Gly–Ala–Cys–$(Gly–Ala–Pro)_5$–Gly–Phe–Hyp–Gly–Glu–Arg–$(Gly–Ala–Pro)_5$–NH_2 – were synthesized and characterized as described previously [17, 19]. The lyophilized peptides were dissolved and stored in stock solutions of 2 mg/ml in 150 mM acetic acid at –20 °C. In the cell culture experiments, the peptides were diluted in the supernatants of the cells.

In vitro *activation of MMP-2*

ProMMP-2 was activated by incubation with 4-aminophenylmercuric acetate (APMA) as described [17]. To exclude APMA-specific effects, the respective controls contained APMA alone.

Table 1. Concentrations of (pro)MMP-2, $(GPO)_{10}$, GAP, ilomastat and PDGF-BB used in the cell culture experiment

Treatment	Concentration [M]
MMP-2	1.7×10^{-9}
proMMP-2	1.7×10^{-9}
$(GPO)_{10}$	1.7×10^{-8}
GAP [17]	1.7×10^{-8}
Ilomastat	6.4×10^{-6}
PDGF-BB	2.1×10^{-6}

Determination of hepatic stellate cell proliferation

As described in a previous study [20], effects of $(GPO)_{10}$, GAP with or without proMMP-2, MMP-2, or the MMP-inhibitor ilomastat on the proliferation of HSC were tested by $[^3H]$-thymidine incorporation. All peptide analogue and chemical concentrations used in the proliferation assay and the following assays are given in *Table 1.*

Wound healing assay

For migration experiments, 5×10^4 HSC were seeded into 24-well plates in triplicate. After reaching confluence artificial wounds were created using a sterile yellow 100 μl pipette tip. After washing with PBS, the cells were treated with proMMP-2 and MMP-2 with or without $(GPO)_{10}$/GAP or platelet-derived growth factor subunit B (PDGF-BB, Biomol, Hamburg, Germany) in DMEM supplemented with 0.25% FCS. Treatment with DMEM supplemented with 5% FCS was used as positive control. After 8 h, migrated cells were photographed using a digital camera coupled to a phase contrast microscope. Five randomly chosen fields were analyzed for each well. Wound diameters were analyzed using ImageJ (version 1.41; National Institutes of Health, Bethesda, MA) software.

In vitro *invasion assay*

Fifty microliters of 3 mg/ml Matrigel™ (BD Biosciences, Heidelberg, Germany) diluted in ice cold, serum-free DMEM were used to coat the upper compartments of 24-well FluoroBlok transwell inserts (BD Biosciences; pore size 8 μm) for 16 h at 37 °C. 2×10^5 HT1080 cells diluted in 300 μl serum-free medium were seeded into the upper compartments followed by addition of ilomastat, $(GPO)_{10}$, GAP or MMP-2. The lower compartment was also filled with serum-free medium. As a positive control, an FCS gradient was created by adding DMEM supplemented with 10% FCS as stimulating agent into the lower compartment. The plates were incubated for up to 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Cells that remained in the upper compartment were gently removed with a cotton swab. The inserts were then washed with PBS and invaded cells on the lower surface were quantified after calcein AM (Life Technologies, Carlsbad, CA, USA) labeling using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Determination of MMP-activity in cellular supernatants

Enzymatic activity of cellular supernatants was studied spectrofluorometrically by cleavage of the fluorogenic MMP-substrate DQ-gelatin as described [21]. HSC and HT1080 were treated for 24 h as indicated in the respective figures. As control, MMP-activity was blocked by addition of the MMP-inhibitor ilomastat. Supernatants were collected, 40 μl was mixed with 150 μl dye-quenched

(DQ)-gelatin solution, and substrate conversion was measured for 1.5 h. Background subtraction was applied to all measurements. The experiments were performed with a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) and black 96-well microtiter plates with clear bottoms (Greiner Bio One, Frickenhausen, Germany).

Statistical analysis

One-way ANOVA/Tukey tests were performed using SigmaStat for Windows (version 2.03; Systat, San Jose, CA). $p < 0.05$ was considered significantly different.

Results

$(GPO)_{10}$ and (pro)MMP-2 enhance the proliferation of HSC

First, effects of $(GPO)_{10}$ and (pro)MMP-2 on the proliferation of HSC were tested serum-starved cells. Recombinant proMMP-2 treated HSC reached 60% of maximum proliferation obtained by treatment with 10% FCS *(Fig. 1)*. Simultaneous treatment of HSC with proMMP-2 and $(GPO)_{10}$ enhanced the stimulating effect of proMMP-2 alone, reaching 75% of maximum stimulation. This corresponded with the effects obtained by treatment of HSC with fully (pre)activated MMP-2, which induced an HSC proliferation of ~77% of the maximum level. HSC treated with $(GPO)_{10}$ showed a proliferation of ~66% of the maximum *(Fig. 1)* whereas treatment with the linear control-peptide GAP [17] showed no stimulatory effects on HSC proliferation as compared to control (not shown).

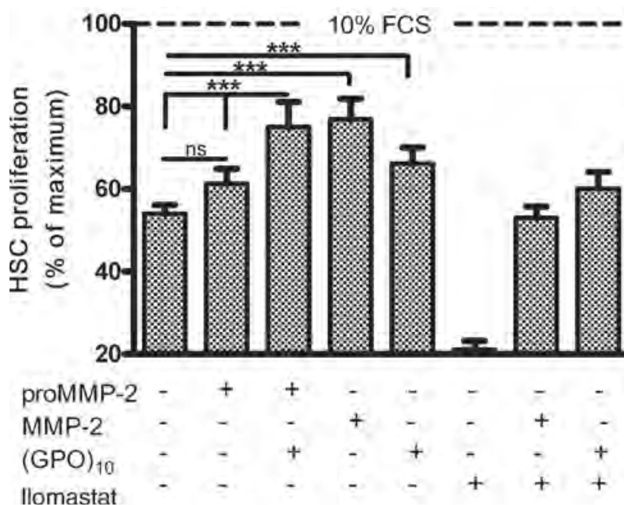

Fig. 1. Proliferation of hepatic stellate cells treated with $(GPO)_{10}$. Cell cycle-synchronized hepatic stellate cells were treated with proMMP-2 or MMP-2 with or without a tenfold molar excess of $(GPO)_{10}$ or GAP for 24 h as described in the Materials and methods section. Proliferation was determined by [^{3}H]-thymidine incorporation. The proliferation of cells treated with DMEM supplemented with 10% fetal calf serum (FCS) was set to 100%. Shown are mean values and SD of five parallel measurements. ns = non-significant, ***$p < 0.001$

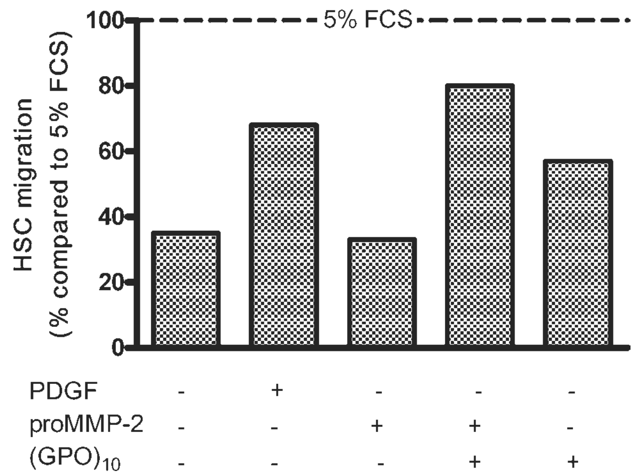

Fig. 2. Effects of $(GPO)_{10}$ on the migration of hepatic stellate cells. Confluent layers of hepatic stellate cells were scratched with a pipette tip and were treated with or without $(GPO)_{10}$, proMMP-2 or platelet-derived growth factor subunit B (PDGF-BB) and documented as described in the Materials and methods section. Indicated are the data of one representative experiment out of three individual experiments

$(GPO)_{10}$ is a mitogen for HSC

To determine $(GPO)_{10}$ effects on the motility of HSC, we next performed a wound healing migration assay. As expected, treatment of HSC in DMEM containing 0.25% FCS with the strong mitogenic homodimer of platelet-derived growth factor subunit B (PDGF-BB) provoked a migratory stimulus reaching ~70% of maximum HSC migration obtained by treatment with 5% FCS *(Fig. 2)*. In this experimental setting, proMMP-2-treated cells displayed no differences to untreated cells. The addition of $(GPO)_{10}$ to the cells, however, induced a migratory stimulus of up to 80% of maximum migration. Interestingly, $(GPO)_{10}$ treatment alone also induced a strong HSC migration reaching ~57% of the maximum *(Fig. 2)*. This on one hand demonstrates a proMMP-2-specific effect of $(GPO)_{10}$ but on the other hand also points to yet another mode of $(GPO)_{10}$ action. Of note, HSC treatment with GAP had no mitogenic activity (not shown).

$(GPO)_{10}$ enhances the invasive properties of HT1080 tumour cells

Given that HT1080 invasion through an ECM barrier depends on the proteolytic activity exerted by MMPs, we next applied $(GPO)_{10}$ to HT1080 cells in matrigel invasion assays. Addition of preactivated MMP-2 to the medium in the upper chamber induced an enhanced invasion of the tumour cells into the lower compartment reaching ~70% of maximum invasion capacity *(Fig. 3)*. By adding inactive proMMP-2 to the media, however, only ~39% of maximum invasion could be observed. Furthermore, similar as in proliferation and migration experiments, the relatively low pro-invasive effects of proMMP-2 could be increased in the presence of $(GPO)_{10}$ to the level obtained

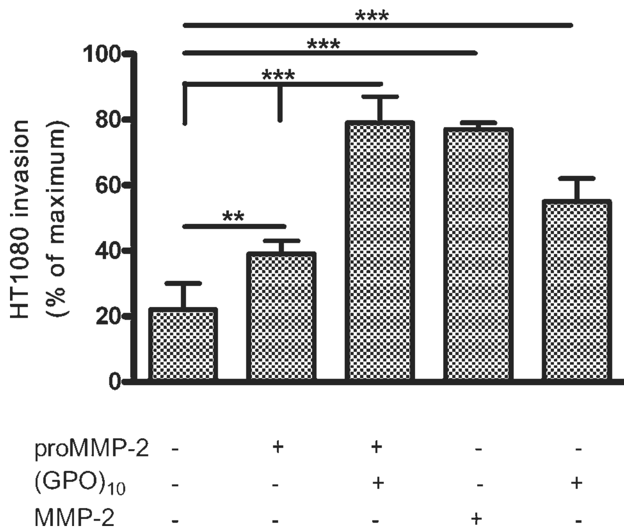

Fig. 3. Invasion of HT1080 cells following $(GPO)_{10}$ treatment. HT1080 cells were allowed to invade membranes coated with basement collagen in the absence or presence of $(GPO)_{10}$/GAP and (pro)MMP-2 as described in the Materials and methods section. After 24 h, transmigrated cells were detected by calcein AM labeling. Shown are the mean values ± SD of three independent experiments with four parallel measurements. $^{**}p<0.01$, $^{***}p<0.001$

by treatment with activated MMP-2 pointing towards an increased activation/activity of (pro)MMP-2 following $(GPO)_{10}$ treatment *(Fig. 3).*

The mitogenic effects of $(GPO)_{10}$ are due to enhanced activation of proMMP-2

The hypothesis that the stimulatory effects of $(GPO)_{10}$ on HSC proliferation and HT1080 invasion could be referred to enhanced enzymatic activity of MMP-2 in the supernatants was next investigated applying fluorescent based gelatinase-activity assays.

Supernatants from HSC and HT1080 containing exogenous (pre)activated MMP-2 showed the highest gelatinolytic activity *(Fig. 4).* In contrast, the gelatinolytic activity in supernatants containing exogenous proMMP-2 was lower, but was markedly enhanced in the presence of $(GPO)_{10}$ reaching the level of fully active MMP-2. This clearly demonstrates activating effects of $(GPO)_{10}$ on proMMP-2 *in vitro.* Taken together, we here show for the first time that the triple helical peptide $(GPO)_{10}$ modulates cellular responses *in vitro* by activation of proMMP-2 in the supernatants.

Discussion

Fibrillar collagen structures were shown to serve as storage site for inactive proforms of matrixmetalloproteinases (MMPs) and accumulate in extracellular matrices under pathological conditions such as liver fibrosis or intestinal inflammation. Thus, modulating this MMP–collagen interaction might offer a rational approach to enhance degradation of accumulated ECM. In a previous study, the potential of the synthetic collagen analogue $(GPO)_{10}$ to act as a binding partner for the gelatinases proMMP-2 and proMMP-9 could be clearly demonstrated. Furthermore, $(GPO)_{10}$ induced enzymatic activation and activity of (pro)MMP-2, but not (pro)MMP-9 [17]. However, effects of $(GPO)_{10}$ on MMPs under physiological *in vitro* conditions such as cell culture experiments have not been tested so far. As a preceding step prior to the testing of $(GPO)_{10}$ *in vivo*, the present study provides evidence that $(GPO)_{10}$ promotes the activation and activity of (pro)MMP-2 *in vitro.*

Initially, we examined the effects of $(GPO)_{10}$ on the proliferation of HSC. These cells are known to produce proMMP-2, and recent studies have suggested that MMP-2 itself acts as a mitogen for HSC [22]. The latter was confirmed by our experiments, in that the addition of pre-activated MMP-2, in contrast to enzymatically inactive proMMP-2, induced a marked increase of HSC proliferation. In the presence of $(GPO)_{10}$, treatment of HSC with proMMP-2 showed a stimulatory effect similar to activated MMP-2 alone. This points towards an activating effect of $(GPO)_{10}$ on proMMP-2 even in cell supernatants.

In another set of experiments, we investigated the potential of $(GPO)_{10}$ to act as a mitogen in a 'wound healing' model system. Given that PDGF signaling is the most effective mitogenic pathway in stellate cells [23], PDGF-BB was used as positive control. Similar to the proliferation experiments, a concomitant treatment of $(GPO)_{10}$ with proMMP-2 provoked a stronger mitogenic stimulus than proMMP-2 alone. Interestingly, even treatment with $(GPO)_{10}$ alone showed a comparable stimulatory effect like PDGF-BB. Thus, besides acting via the activation of endogenous proMMP-2, $(GPO)_{10}$ might also impact cellular processes directly, as it does via activation of immune receptors such as the platelet glycoprotein VI (GPVI) or the leukocyte-associated immunoglobulin-like receptor-1

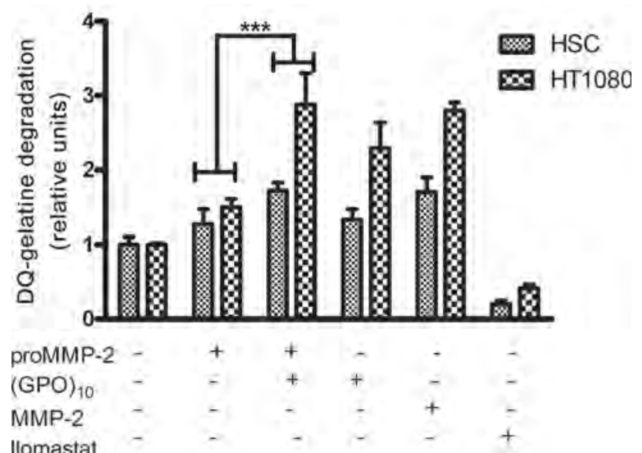

Fig. 4. Effects of $(GPO)_{10}$ on MMP activity in cellular supernatants. Supernatants from the preceding proliferation or invasion measurements were mixed with dye-quenched (DQ)-gelatin substrate, and gelatinolytic activity was measured as described in the Materials and methods section. Results are given as mean values ± SD from three independent experiments with five parallel measurements. $^{***}p<0.001$

(LAIR1) [19]. An intracellular target of (GPO)10 might be phosphatidylinositol 3-kinase (PI3-K) signaling, which is involved in PDGF-induced migration [23]. The latter needs to be addressed in ongoing studies.

In a third approach, we tested the influence of $(GPO)_{10}$ on the invasion of tumour cells through an ECM barrier which requires the release and proteolytic activity of matrix degrading enzymes such as MMPs [24]. The human fibrosarcoma cell line HT1080 is known to secrete relatively high amounts of proMMP-2 [25]. Treatment of the cells with $(GPO)_{10}$ almost doubled the invasive capacity of the tumour cells, indicating an enhanced activation of endogenous proMMP-2 by $(GPO)_{10}$ in the supernatant.

We finally confirmed our hypothesis that $(GPO)_{10}$ activates proMMP-2 *in vitro* by performing fluorescent-based MMP-activity assays with aliquots of the respective supernatants from the preceding assays. As expected, the presence of $(GPO)_{10}$ strongly increased the gelatinolytic activity in the supernatants compared to untreated cells. In line with this, adding exogenous proMMP-2 concomitant with $(GPO)_{10}$ resulted in a comparable strong increase in gelatinolytic MMP-activity as obtained by adding exogenous pre-activated MMP-2. This is in accordance with substrate gel zymography experiments in previous studies, which clearly showed the generation of activated, prodomain free MMP-2 due to the treatment with $(GPO)_{10}$ [17].

In all experiments, the usage of the linear control-peptide GAP showed no effects on investigated cellular processes (not shown in the figures). We conclude that the $(GPO)_{10}$-mediated effects result from its unique triple helical structure.

In summary, we here demonstrate that $(GPO)_{10}$ is capable to induce MMP-2 activity *in vitro* which could impact MMP-associated processes like proliferation and invasion.

In conjunction with our previous study, which focused on the mechanisms of proMMP-2 activation by $(GPO)_{10}$ in binding studies including *in situ* experiments, the present study adds a solid background of *in vitro* data, which provides a rationale for the appliance of $(GPO)_{10}$ or structure related peptides in animal models such as inflammatory bowel disease where MMPs play a central role for the inflammatory response [12, 13].

Acknowledgements

This study was supported by grants (Ru 624/3-2, Schu 646/1-10) funded by the Deutsche Forschungsgemeinschaft (DFG), and the Collaborative Research Centers (SFB366, projects C5/C10) and (SFB633, projects A7, B6, and Z1) funded by the DFG.

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2.1.3 Das Kollagenmimetikum $(GPO)_{10}$ hemmt die akute DSS-Colitis im Mausmodell

- (Heimesaat MM., Heilmann K., Ruehl M., Kuehl AA, Erben U., Fischer R., Farndale RW., Bereswill S., Göbel UB., Zeitz M., Somasundaram R and **Freise C**.: The Synthetic Hydroxyproline Containing Collagen Analogue (Gly-Pro-Hyp)10 Ameliorates Acute DSS Colitis. *European Journal of Microbiology and Immunology* 2012; 2(3), 192-200).

In experimentellen Tiermodellen sowie Patienten mit intestinaler Entzündung zeigen sich erhöhte Konzentrationen von MMP-2 und MMP-9 in entzündeten Geweben. Das synthetische Kollagenpeptid $(GPO)_{10}$ wurde in vorausgegangenen Studien als Bindungspartner von ProMMP-2/-9 und zudem als Aktivator von ProMMP-2 identifiziert. Vor dem Hintergrund einer möglichen therapeutischen Nutzung dieser MMP-$(GPO)_{10}$-Interaktion wurde $(GPO)_{10}$ erstmals *in vivo*, in einem murinen Modell der akuten DSS-Colitis, eingesetzt. Die intraperitoneale Gabe von $(GPO)_{10}$ über sieben Tage verbesserte die klinischen Symptome und histopathologischen Veränderungen des Kolons im Vergleich zu den Plazebo-Kontrolltieren mit schwerer Colitis. $(GPO)_{10}$-behandelte Tiere zeigten eine geringere Infiltration von Neutrophilen und T- bzw. B-Lymphozyten in die Kolonmukosa. Dagegen war die Zahl regulatorischer T-Zellen und regenerativer Zellen im Vergleich zu den Kontrolltieren erhöht. Die Zytokinregulation wurde in *ex vivo*-Kolonbiopsien untersucht. Biopsieüberstände $(GPO)_{10}$–behandelter Tiere zeigten einen verminderten Gehalt von pro-inflammatorischem IL-6. Interessanterweise hemmte die Behandlung mit $(GPO)_{10}$ auch die Infiltration inflammatorischer Zellen in die Lunge. Zusammengefasst zeigen diese Ergebnisse, dass $(GPO)_{10}$ eine vielversprechende therapeutische Option von intra- und extra-intestinalen Entzündungen darstellt.

Komplementär zu der Bedeutung und einer möglichen Nutzung von EZM und MMPs bei Fibrose und Neoplasie wurden auch Ionenkanäle und entsprechende Inhibitoren dahinhehend untersucht. Im nachfolgenden Kapitel werden zwei Arbeiten zusammengefasst, die zum einen erstmals die Expression und einen möglichen therapeutischen Nutzen der Inhibition von Kaliumkanälen bei Fibrose zeigen und zum anderen anti-neoplastische Effekte einer Kaliumkanalinhibition beschreiben.

European Journal of Microbiology and Immunology 2 (2012) 3, pp. 192–200
DOI: 10.1556/EuJMI.2.2012.3.4

THE SYNTHETIC HYDROXYPROLINE-CONTAINING COLLAGEN ANALOGUE (GLY–PRO–HYP)$_{10}$ AMELIORATES ACUTE DSS COLITIS

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Received: June 11, 2012; Accepted: June 17, 2012

In experimental models of and humans with intestinal inflammation, increased levels of the matrix-degrading gelatinases MMP-2 and -9 in inflamed tissues can be detected. The synthetic collagen analogue (Gly–Pro–Hyp)$_{10}$, (GPO)$_{10}$, has been identified as a relevant binding structure for proMMP-2/-9 and promotes enzymatic activity of proMMP-2. Since targeted MMP strategies might offer promising anti-inflammatory treatment options, we for the first time studied *in vivo* actions exerted by (GPO)$_{10}$ applying an acute dextrane sulfate sodium (DSS) induced colitis model. Seven-day intraperitoneal (GPO)$_{10}$ treatment ameliorated clinical symptoms and histopathological colonic changes as compared to placebo controls with severe colitis. (GPO)$_{10}$-treated mice displayed a diminished influx of neutrophils, and T- and B-lymphocytes into their colonic mucosa whereas numbers of regulatory T-cells and regenerative cells were higher as compared to placebo controls. Furthermore, IL-6 secretion was down-regulated in *ex vivo* colonic biopsies derived from (GPO)$_{10}$-treated mice whereas higher concentrations of the anti-inflammatory cytokine IL-10 in extra-intestinal compartments such as MLN and spleen could be detected. Strikingly, influx of inflammatory cells into lungs was abolished following (GPO)$_{10}$ application. We therefore propose (GPO)$_{10}$ as a promising effective and safe treatment option of intestinal and extra-intestinal inflammatory conditions in humans.

Keywords: (GPO)$_{10}$, matrix metalloproteinases, gelatinase, proMMP-2, extracellular matrix, acute DSS colitis, intestinal inflammation, pro-inflammatory cytokines, IL-10, proliferation, *in vivo*, extra-intestinal immune responses, lung, human IBD, crinopexy

Abbreviations: (GPO)$_{10}$, (Gly–Pro–Hyp)$_{10}$; PBS, phosphate buffered saline; DSS, dextrane sulfate sodium; p.i., post induction; MMP, matrix metalloproteinase; IBD, inflammatory bowel disease; i.p., intraperitoneal; p.o., peroral; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; TNF, tumor necrosis factor; IFN, interferon, Treg, regulatory T cell; Th, T helper; IL, interleukin; SPF, specific pathogen-free; HE, hematoxylin eosin; MLN, mesenteric lymphnodes

Introduction

Matrix metalloproteinases (MMP) comprise a heterogeneous family of 25 zinc- and calcium dependent endopeptidases which, with respect to their substrate specificity, can be categorized into collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), elastase (MMP-12), and membrane-type matrix metalloproteinases (MT-MMP-1 through -5) [1–4]. MMPs are tightly controlled by a balance of activators (e.g. pro-inflammatory molecules such as IL-1, IL-6, TNF-α) and inhibitors (tissue inhibitors of matrix metalloproteinases (TIMPs), anti-inflammatory cytokines such as IL-4, IL-10, TGF-β) and contribute to synthesis and degradation of extracellular matrix, cytokine activation, and ligand shedding [5, 6]. Under physiological conditions, MMPs are involved in embryonic development and differentiation, and proliferation and regeneration of tissues such as wound healing [1–4]. A disbalanced MMP expression, however, can run into pathological processes such as inflammation and tissue destruction. In experimental models of Th1-type inflammation (e.g. acute ileitis and colitis), [7–10] as well as in humans with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis [11–16], increased levels of the gelatinases MMP-2 and MMP-9 in inflamed tissue sites associated with increased mRNA levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF-α could be detected. Interestingly, MMPs are known to shed biologically active IL-1, IL-6, and TNF-α molecules from the surfaces of effector cells such

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ISSN 2062-509X / $ 20.00 © 2012 Akadémiai Kiadó, Budapest

as macrophages, which, in turn, induce MMP expression from immune, epithelial, and parenchymal cells [17, 18].

Extracellular deposition of growth factors, cytokines, and enzymatically inactive proforms of MMPs (proMMP) that makes them rapidly and locally available is a phenomenon called crinopexy [19]. We identified the rigid collagen triple helix, a secondary structure element of collagens, exemplified by the collagen analog $(Gly–Pro–Hyp)_{10}$ – $(GPO)_{10}$ –, as a relevant binding structure for proMMP-2/-9 in the extracellular matrix [20]. The tissue distribution and substrate specificity of the gelatinases MMP-2 and MMP-9 are defined by their collagen-binding domains which enhance the interaction with substrate and non-substrate molecules. We could demonstrate that collagenous peptides like the alpha 2 chain of collagen VI or the synthetic peptide $(GPO)_{10}$ act on MMP-2/-9 collagen-binding domain-exosites thus releasing and activating the collagen-sequestered proMMPs from their extracellular depot [20, 21]. In very recent work, we demonstrated that this $(GPO)_{10}$–proMMP-2 interaction also impacts cellular responses in cell culture experiments (Freise et al., this issue of EUJMI). In IBD, MMP-2 is stored within the extracellular matrix of the gut submucosa, and MMP-9 is localized pericellularly in the lamina propria [22, 23]. Thus, targeted therapies against specific MMPs should be highly promising in treating intestinal inflammatory disorders with respect to clinical efficacy and rather little adverse side effects which might be due to unselective blockage of MMPs. We were therefore interested in potential beneficial effects of $(GPO)_{10}$ in acute DSS colitis. In the study presented here, we demonstrate that in mice treated with $(GPO)_{10}$ intraperitoneally i) clinical colitis pathology was ameliorated, ii) histopathological changes in the colon were less severe which was parallel by iii) less secretion of the pro-inflammatory cytokine IL-6 in their colon whereas iv) concentrations of the anti-inflammatory cytokine IL-10 in extraintestinal compartment such as MLN and spleen were increased. Furthermore, v) influx of pro-inflammatory immune cell influx into mucosa and submucosa was reduced whereas vi) numbers of Tregs and regenerative cells in the colon were increased. Finally, vii) influx of inflammatory cells into extra-intestinal tissue sites such as lungs was abolished following $(GPO)_{10}$ i.p.. We therefore propose $(GPO)_{10}$ as a promising future option for anti-inflammatory/immunomodulatory treatment options of intestinal and extra-intestinal inflammatory conditions in humans.

Materials and methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, Germany). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice and colitis induction

Female C57BL/6j wildtype mice were bred and maintained under specific pathogen-free (SPF) conditions in the Forschungsinstitut für Experimentelle Medizin (FEM, Charité, Berlin, Germany). For colitis induction, mice 3 months of age were treated with 3.5% (wt/vol) DSS (40 kDa, MP Biomedicals, Illkirch, France) in drinking water *ad libitum* for 6 days. Prior necropsy, mice received water without DSS for 24 h. The intake of the DSS solution was controlled, and mice were weighed daily. Mice without colitis induction served as negative controls.

Determination of clinical scores of large intestinal shortening

Total clinical scores with a maximum of 12 were generated daily by combined data of weightloss, occurrence of blood in stool (as determined by the Guajak method using Haemoccult™, Beckman Coulter/PCD, Krefeld, Germany), and stool consistency, as described [24, 25]. The relative shortening of the colon was calculated by dividing the difference of the mean lengths of the large intestines from age- and sex-matched control mice minus the length of mice subjected to DSS at day 7 p.i. (post induction) and then multiplied by 100 over the mean colon length of naïve control mice (relative shortening in length = (mean d0 – d7 p.i.) × 100/mean d0). Results were expressed as % shortage.

Treatment with $(GPO)_{10}$

The triple helical collagen analogue $(GPO)_{10}$ – H–Gly–Cys–Hyp–$(Gly–Pro–Hyp)_{10}$–Gly–Cys–Hyp–Gly–NH_2 – was synthesized and characterized as described previously [20, 26]. Mice (with and without DSS induction) were treated perorally (p.o.) by gavage or intraperitoneally (i.p.) twice daily with $(GPO)_{10}$ (2 mg/kg body weight/day) dissolved in 0.3 ml PBS starting at day 0 for 7 days until necropsy. PBS treated animals (0.3 ml perorally twice daily for the respective period of time) served as placebo controls.

Sampling procedures and histologic scoring

Mice were sacrificed by isofluran treatment (Abbott, Germany) on day 7 after induction of colitis. Colon samples from each mouse were removed under sterile conditions and collected in parallel for histopathological and immunohistochemical analyses as well as for detection of cytokines. For immunohistochemical stainings, colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 μm) were stained with the respective antibodies as described below. Histopathology was investigated in paraffin-embedded hematoxylin and eosin (HE)-stained tissue sections of colon and lung. A published standardized histologic score ranging from

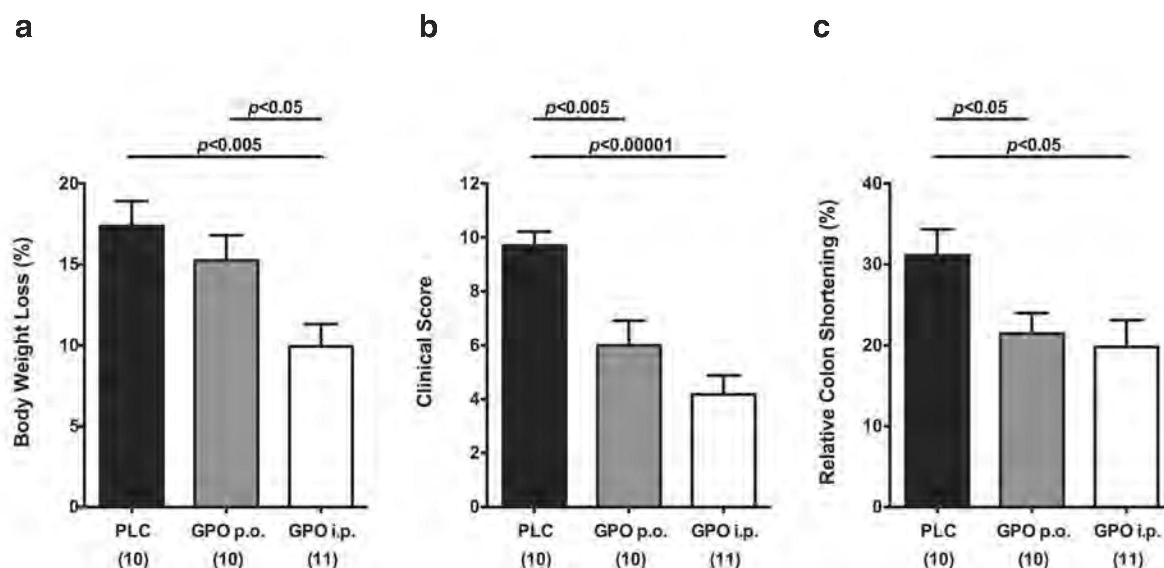

Fig. 1. Better clinical outcome of acute DSS colitis following $(GPO)_{10}$ treatment.
(a) Relative body weight loss, (b) clinical condition (as indicated by a clinical colitis score, see methods), and (c) relative colon length loss (in %) at day 7 following treatment with placebo (PLC; black bars), $(GPO)_{10}$ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 were recorded in mice after DSS treatment for 7 days. Numbers of analyzed animals are given in parentheses. Mean values, standard errors of the mean (SEM), and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

0 to 6 was used for blinded evaluation of the inflammatory processes in the colon [24, 25].

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [27, 28]. Primary antibodies against CD3 (#N1580, Dako, Denmark, dilution 1:10), B220 (eBioscience, San Diego, CA, USA, 1:200), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:10000), FOXP-3 (FJK-16s, eBioscience, 1:100), and Ki-67 (TEC3, Dako, 1:100) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, ×400 magnification) was determined by light microscopy.

Cytokine detection in colon culture supernatants

Colon biopsies were cut longitudinally, washed with PBS and strips of approximately 1 cm^2, as well as mesenteric lymphnodes (MLNs) and spleens were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 μl serum-free RPMI 1640 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml; PAA Laboratories). After 18 h at 37 °C, culture supernatants were tested for IL-6, TNF-α, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences) as described previously [25, 29, 30].

Statistical analysis

Medians, mean values, standard error of the means (SEM), and levels of significance were determined using appropriate tests as indicated (two-tailed Student's *t*-test). Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

Results

Less intestinal immunopathology following $(GPO)_{10}$ treatment of mice with acute DSS colitis

Recent studies using mice lacking genes for MMP-2 and/or MMP-9 in experimental colitis models revealed that epithelial-derived MMP-9 is an important mediator in colitis induction whereas MMP-2 exerts protective function preserving intestinal epithelial barrier integrity [31, 32]. $(GPO)_{10}$ has been identified as a relevant binding structure for proMMP-2/-9 interfering with proMMP-2/-9 binding to the extracellular matrix. In our very recent *in vitro* study (published in the same issue), we could clearly demonstrate that, in cell lines, cellular proliferation and migration concomitant with matrix degradation could be stimulated due to MMP–$(GPO)_{10}$ interaction. Given that $(GPO)_{10}$ is capable of inducing MMP-2 activity *in vitro*, we were interested whether $(GPO)_{10}$ treatment could impact acute large intestinal inflammation *in vivo*. Mice were subjected to DSS in order to induce acute colitis (day 0) and treated with synthetic $(GPO)_{10}$ for 7 days either via the intraperitoneal (i.p.) or peroral (p.o.) route. At day 7

post induction (p.i.), i.p. $(GPO)_{10}$-treated mice had lost significantly less body weight as compared to placebo controls (10.0± 1.3% versus 17.5± 1.5%, $p < 0.005$; *Fig. 1a*). In addition, applying a "cumulative" clinical colitis score assessing body weight loss, occurrence of blood and consistency of stool revealed that mice benefitted from either $(GPO)_{10}$ treatment as compared to placebo controls suffering from severe colitis at day 7 p.i. *(Fig. 1b)*. Given that colonic inflammation is accompanied by a significant shortening of the lower intestinal tract, we determined the lengths of the large intestines in treated and control animals. Mice treated with $(GPO)_{10}$ either p.o. or i.p. displayed significantly less colonic shortening (21.4± 1.5% and 20.0± 3.2%, respectively, $p < 0.05$) as compared to placebo mice (31.2± 3.1%; *Fig. 1c*). To further characterize the beneficial effects of $(GPO)_{10}$ during acute colonic inflammation, we studied the effects on colitis severity related histopathological changes in the colon. $(GPO)_{10}$ treatment via the i.p., but not p.o. route resulted in significantly less distinct colonic histopathology as compared to placebo controls at day 7 p.i. as indicated by lower histopathological scores in the i.p. $(GPO)_{10}$ group *(Fig. 2)*. In i.p. $(GPO)_{10}$-treated animals, only few epithelial lesions and a discrete influx of inflammatory cells into the colonic lamina propria, but not into the submucosal layer, could be observed (mean histopathological score of approximately 3.0; *Fig. 2*). However, severe mucosal damage with extended ulcerations affecting the entire colon length and complete loss of the epithelium as well as a thickened submucosa with distinct transmural leukocyte infiltrates were present in the large intestine of the placebo group at day 7 p.i. (mean histopathological score of nearly 5.0; *Fig. 2*). Taken together, following i.p. $(GPO)_{10}$ treatment mice exhibited less macroscopic, clinical, and histopathological signs of large intestinal inflammation as well as less distinct inflammation-induced colon shortening following induction of acute DSS colitis.

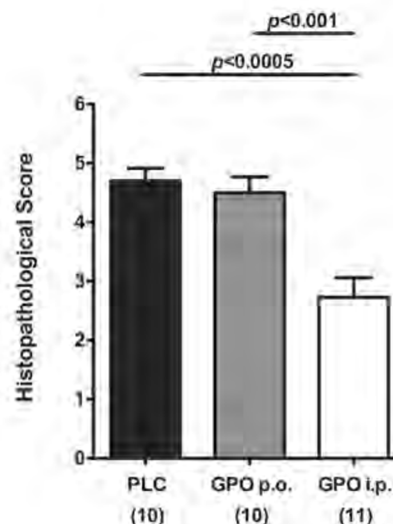

Fig. 2. Less colonic histopathology of acute DSS colitis following $(GPO)_{10}$ treatment.
Histopathology scores of the colon were determined at day 7 following treatment with placebo (PLC; black bars), $(GPO)_{10}$ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days. Numbers of analyzed animals are given in parentheses. Mean values, standard errors of the mean (SEM), and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

Less distinct immune cell responses in colonic mucosa in situ *following $(GPO)_{10}$ treatment of mice with acute DSS colitis*

Given that human colitis is accompanied by the recruitment of pro-inflammatory immune cell populations to sites of inflammation in the large intestine [25], we next quantitated immune cell responses by immunohistochemical staining of colonic paraffin sections of animals with antibodies against CD3 (T-lymphocytes), B220 (B-lymphocytes), MPO7 (neutrophils), and FOXP3 (regulatory T-cells, Treg). In addition, colonic $Ki67^+$ cells indicative for cell proliferation and regeneration were determined. At day 7, following colitis induction, placebo control mice displayed a substantial increase in T- and B-lymphocytes, neutrophils, and Tregs within their colonic mucosa as compared to naïve placebo mice *(Fig. 3)*. The influx of $CD3^+$, $B220^+$, and $MPO7^+$ cells, however, was significantly less pronounced in mice treated with $(GPO)_{10}$ by either route at day 7 p.i. *(Fig. 3a–c)* whereas following i.p. $(GPO)_{10}$ application mice exhibited significantly higher $FOXP3^+$ cell numbers ($p < 0.005$; *Fig. 3d*). Due to a relatively high standard deviation in the $(GPO)_{10}$ p.o. cohort only a trend towards higher colonic Tregs versus placebo controls at day 7 p.i. could be determined (n.s.; *Fig. 3d*). Interestingly, application of $(GPO)_{10}$ to naïve mice (i.e. without colitis induction) resulted in a 2- to 4-fold increase of neutrophils and Tregs, respectively, but not of T- and B-lymphocytes in the colonic mucosa *in situ (Fig. 3)*. In addition, irrespective of the application mode, $(GPO)_{10}$-treated mice exhibited significant higher numbers of $Ki67^+$ proliferative cells as compared to placebo mice with severe colitis *(Fig. 3e)*. Taken together, less distinct clinical and histopathological signs of colonic inflammation following $(GPO)_{10}$ treatment were accompanied by lower numbers of pro-inflammatory immune cell populations such as T- and B-lymphocytes, but higher Treg and regenerative cell counts in the colonic mucosa *in situ*.

Intestinal and extra-intestinal cytokine secretion following $(GPO)_{10}$ treatment of mice with acute DSS colitis

To further underline the beneficial (i.e. immunomodulatory, anti-inflammatory) properties of synthetic $(GPO)_{10}$ treatment in acute DSS colitis, we next determined protein levels of the pro-inflammatory cytokine IL-6 in *ex vivo* colon cultures. Whereas during colitis induction,

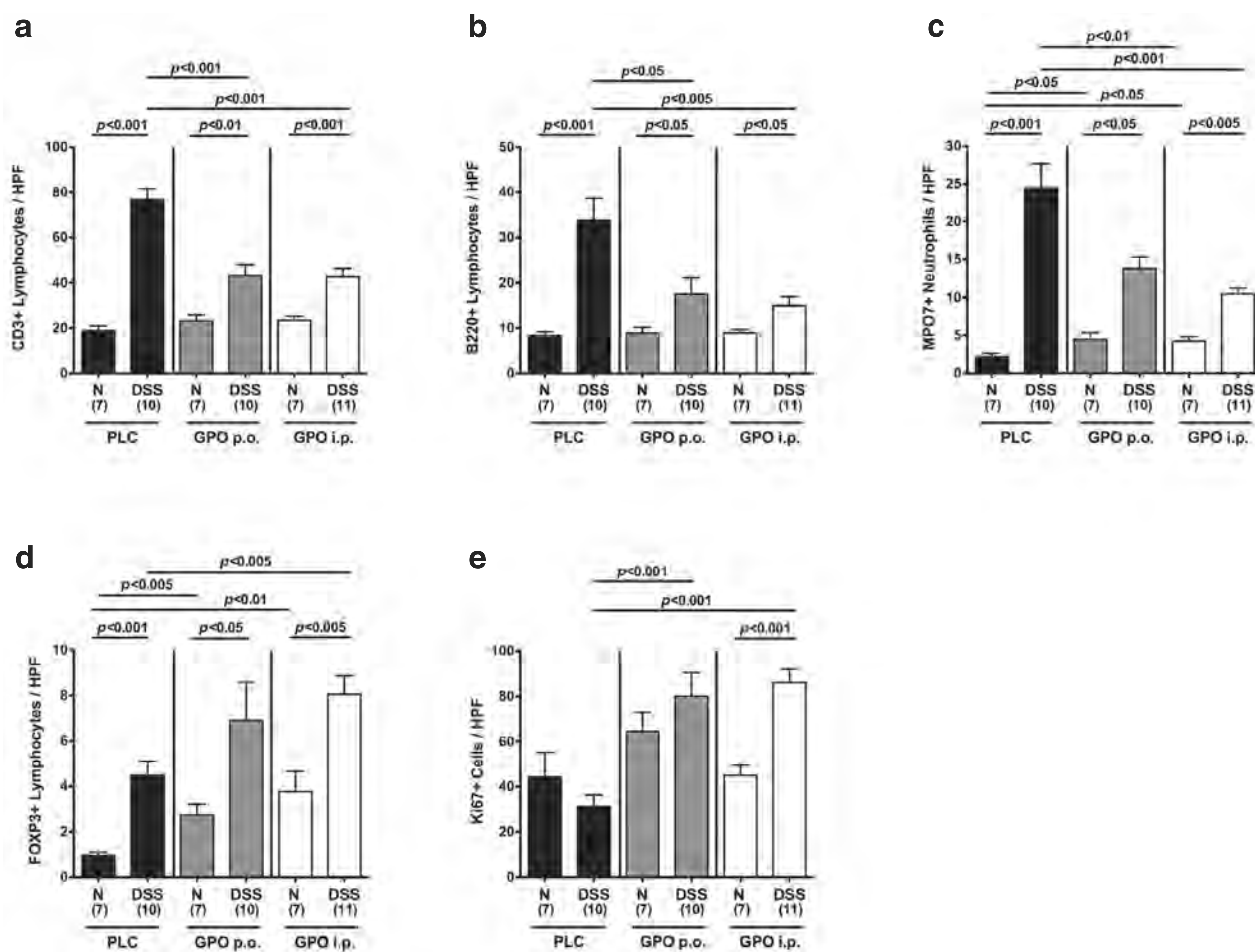

Fig. 3. Quantification of defined cell populations in the colon of mice with acute DSS colitis *in situ* following $(GPO)_{10}$ treatment. The average number of cells positive for (a) CD3 (T-Lymphocytes), (b) B220 (B-Lymphocytes), (c) MPO7 (Neutrophils), (d) FOXP3 (regulatory T cells, Treg), and (e) Ki67 (proliferating cells) from at least six high power fields (HPF, ×400 magnification) per animal was determined microscopically in immunostained colon sections isolated at day 7 following treatment with placebo (PLC; black bars), $(GPO)_{10}$ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations, and significance levels as indicated were determined by the Student's *t*-test. Data are pooled from three independent experiments

colonic IL-6 levels had increased multifold as compared to healthy mice, this increase, however, was significantly less pronounced following i.p. $(GPO)_{10}$ treatment as indicated by IL-6 concentrations reaching approximately 50% of levels in the placebo group at day 7 p.i. *(Fig. 4)*. Thus, amelioration of colitis severity following i.p. $(GPO)_{10}$ application was paralleled by less IL-6 secretion from the inflamed colonic epithelium.

We were next interested whether $(GPO)_{10}$ treatment might also impact pro- and anti-inflammatory immune responses in extra-intestinal compartments such as the colon draining MLNs and the spleens. TNF-α levels increased more than 5-fold in MLNs derived from placebo control animals with severe colitis as compared to healthy mice *(Fig. 5a)*. This effect was not as distinct following i.p. $(GPO)_{10}$ application as indicated by approximately 50% lower TNF-α levels in MLNs compared to placebo controls *(Fig. 5a)*. In addition, i.p. $(GPO)_{10}$-treated mice exhibited significantly higher IL-10 levels in *ex vivo* MLN and spleen biopsies as compared to placebo mice *(Fig. 5b, c)*. Taken together, amelioration of acute DSS colitis following i.p. GPO treatment was paralleled by less distinct pro- and more pronounced anti-inflammatory cytokine responses in extra-intestinal compartments.

Pulmonary inflammatory responses following $(GPO)_{10}$ treatment of mice with acute DSS colitis

Given that intestinal inflammation might also be associated with extra-intestinal inflammatory responses, e.g. in the lungs as shown very recently by our group [33], we next examined HE-stained pulmonary paraffin sections of $(GPO)_{10}$ versus placebo-treated mice with acute colitis. At day 7, p.i. placebo mice with acute colitis displayed significant perivascular immune cell infiltrates in their lungs whereas these infiltrates were virtually missing in naïve as well as following p.o. and i.p. $(GPO)_{10}$ treatment *(Fig. 6)*.

Thus, the presented data underline the beneficial anti-inflammatory effect exerted by $(GPO)_{10}$ treatment not only locally (i.e. at intestinal) but also at extra-intestinal tissue sites implicating therapeutic properties in acute intestinal inflammatory conditions in humans.

Discussion

Matrix metalloproteinases are essentially involved in mediating inflammatory responses in human IBD. So far, clinical studies investigating beneficial effects of synthetic compounds non-selectively blocking MMPs in cancer or inflammation were rather disappointing either due to lack of effectiveness or major negative side effects such as painful arthralgia as a consequence of collagenase blockage [34–38]. Thus, as potential pharmacological treatment options more effective and rather selective MMP blocking agents would be desirable.

The gelatinases A (MMP-2) and B (MMP-9) have been shown to be highly upregulated in intestinal inflammation in mice and men, *in vitro* and *in vivo* [11–16]. We have recently reported that selective blockage of gelatinases by the synthetic compound RO28-2653 was effectively ameliorating acute murine ileitis and colitis [39, 40]. Furthermore, studies in experimental colitis models revealed that MMP-2 exerts protective function preserving intestinal epithelial barrier integrity whereas tissue MMP-9 levels correlate with disease activity of patients with ulcerative colitis and have been associated with mucosal damage and fistulae in Crohn's disease. Further, MMP-9 is suggested to be important for neutrophil recruitment [31, 32, 41].

The synthetic collagen analogue $(Gly–Pro–Hyp)_{10}$, $(GPO)_{10}$, has been identified as a relevant binding structure for proMMP-2/-9. Given that $(GPO)_{10}$ interferes with collagen binding of proMMP-2 and proMMP-9 [20] and is capable of inducing MMP-2 activity *in vitro* (as shown by our publication in the same issue), we were hypothesizing that $(GPO)_{10}$ might be a promising compound in pathophysiological conditions such as intestinal inflammation. Therefore, we investigated potential anti-inflammatory effects of $(GPO)_{10}$ in acute DSS colitis comparing peroral with intraperitoneal application mode. Our data revealed that intraperitoneal $(GPO)_{10}$ injection was more effective as compared to peroral application. This is not surprising

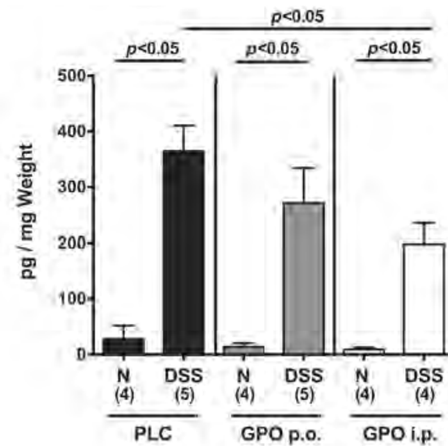

Fig. 4. Less colonic IL-6 secretion following $(GPO)_{10}$ treatment in acute DSS colitis.
IL-6 protein concentrations were determined in supernatants of colonic *ex vivo* cultures isolated at day 7 following treatment with placebo (PLC; black bars), $(GPO)_{10}$ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations, and significance levels as indicated were determined by the Student's *t*-test. Data are representative for three independent experiments

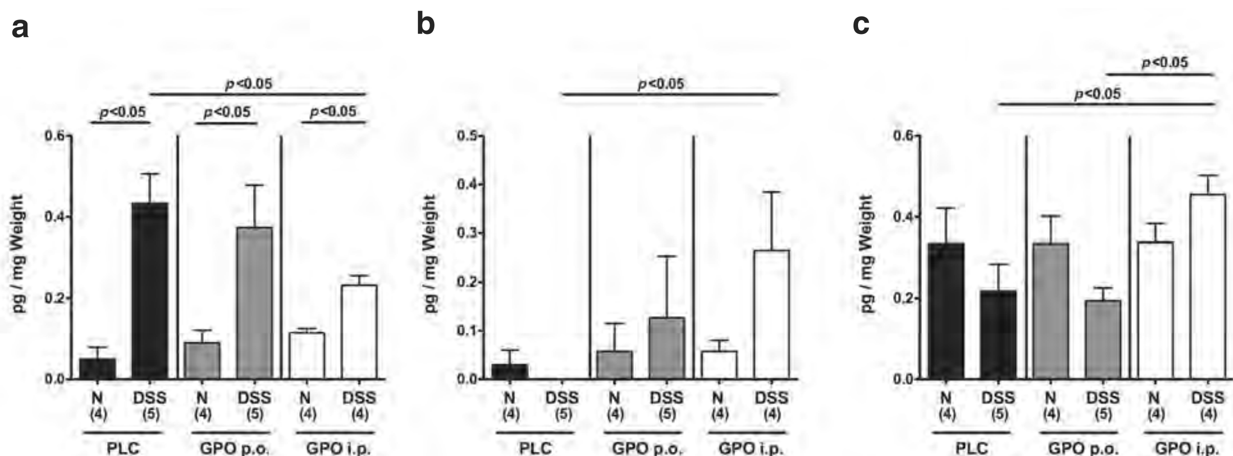

Fig. 5. Extra-intestinal cytokine secretion following $(GPO)_{10}$ treatment in acute DSS colitis.
Cytokine secretion was determined in supernatants of *ex vivo* biopsies taken from mesenteric lymphnodes (MLNs; (a) TNF-α, (b) IL-10) and (c) spleen (IL-10) isolated at day 7 following treatment with placebo (PLC; black bars), $(GPO)_{10}$ (GPO) application via the peroral (p.o.; gray bars) or intraperitoneal (i.p.; white bars) route from day 0 until day 7 in mice after DSS treatment for 7 days and compared to naïve (N) mice without colitis induction. Numbers of analyzed animals are given in parentheses. Mean values, standard deviations and significance levels as indicated were determined by the Student's *t*-test. Data are representative for three independent experiments

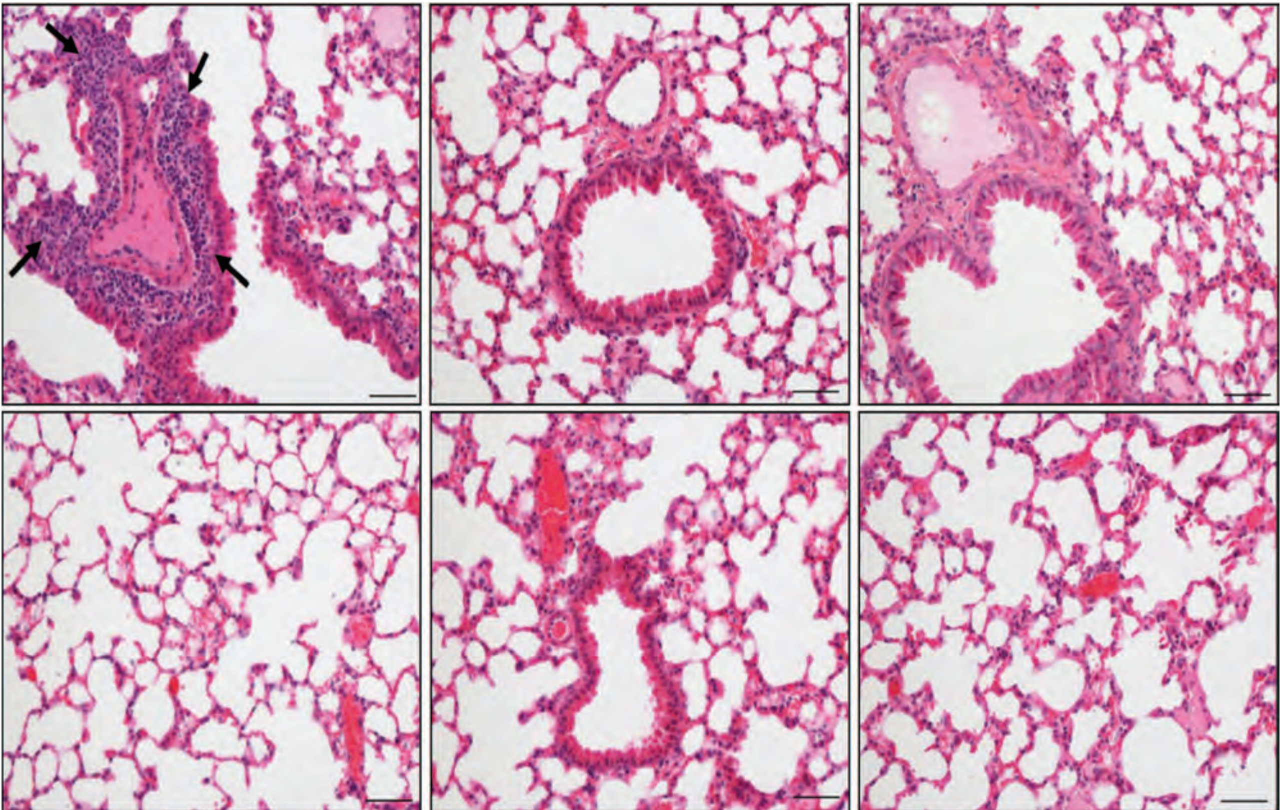

Fig. 6. Less pulmonary inflammatory responses following $(GPO)_{10}$ treatment in acute DSS colitis.
Paraffin sections of lungs taken at day 7 following treatment with placebo (left panel), $(GPO)_{10}$ (GPO) application via the peroral (middle panel) or intraperitoneal (right panel) route from day 0 until day 7 in mice after DSS treatment for 7 days (upper panel) were HE-stained and compared to naïve (N) mice without colitis induction (lower panel).
Representative photomicrographs for three independent experiments are shown (×200 magnification, scale bar 50 μm). Perivascular cuffs of infiltrating lymphocytes (arrows) are shown in lungs of placebo-treated mice with acute colitis (upper left), but missing in naïve mice or following $(GPO)_{10}$ treatment

given that $(GPO)_{10}$ is a polypeptide of 10 amino acid triplets which might have been substantially degraded during the gastroduodenal passage due to inactivating effects exerted by gastric and bile acids as well as pancreatic enzymes within the duodenal lumen.

Intraperitoneal $(GPO)_{10}$ treatment starting upon colitis induction resulted in significant better clinical and macroscopic outcomes as well as in significantly less histopathological changes of the colonic mucosa at day 7 as compared to placebo controls with severe colitis. Given that MMPs are involved in the recruitment of immune cells into the gut [18, 42] and shed biologically active IL-1, IL-6, and TNF-α molecules from the surfaces of effector cells such as neutrophils and macrophages, which, in turn, are able to induce MMP expression from immune, epithelial, and parenchymal cells [17, 18], it would be desirable to pharmacologically cut this vicious cycle all in one. Following i.p. $(GPO)_{10}$ treatment, amelioration of clinical symptoms and histopathology was paralleled by less infiltration of the colonic mucosa and lamina propria by immune and effector cells such as T- and B-lymphocytes, as well as neutrophilic granulocytes. This less distinct influx of immune cells into the large intestinal mucosa was accompanied by lower levels of pro-inflammatory cytokines such as IL-6 and TNF-α determined in the colonic mucosa of $(GPO)_{10}$-treated animals as compared to placebo mice. The decrease in neutrophil numbers following $(GPO)_{10}$ application is well in line with previous reports by us [40, 43] and others [44, 45]: in acute experimental colitis, MMP blockage resulted in a diminished influx of neutrophilic granulocytes into the colon thereby reducing oxidative stress for the colon epithelium.

Importantly, at day 7, following colitis induction, numbers of $FOXP3^+$ Tregs and $Ki67^+$ regenerative cells were higher in the colon of $(GPO)_{10}$-treated mice as compared to placebo controls. Thus, $(GPO)_{10}$ exerts its beneficial effects *in vivo* by diminishing pro- and enhancing anti-inflammatory and thus promoting regenerative effects following intraperitoneal application in acute colitis. One explanation might be the interference of $(GPO)_{10}$ with the matrix binding of anti-inflammatory proMMP-2 and even more pronounced of pro-inflammatory proMMP-9. In contrast to proMMP-2, the effects of $(GPO)_{10}$ on proMMP-9 were not accompanied by an occurrence of proteolytic activity [20]. Another possible mode of action of $(GPO)_{10}$ might therefore be the release/removal of tissue sequestered pro-inflammatory proMMP-9 and concurrently the induction of MMP-2 with its protective function in preserving intestinal epithelial barrier integrity.

Recently, comparable results could be achieved by peroral treatment of mice suffering from acute DSS colitis with the selective gelatinase blocker RO28-2653 [40].

In this study, however, amelioration of acute colitis was accompanied by decreasing (and not increasing) $FOXP3^+$ cell numbers in the colonic mucosa. Of note, in the study presented here, i.p. $(GPO)_{10}$ application resulted in increasing $FOXP3^+$ Tregs in the colons not only of diseased, but also in healthy, naïve animals further underlying its potent anti-inflammatory capacity.

Surprisingly, the beneficial action exerted by $(GPO)_{10}$ was not restricted to the intestinal tract but also effective in extra-intestinal compartments: MLNs derived from i.p. $(GPO)_{10}$-treated mice secreted significantly less TNF-α as compared to placebo controls. Additionally, expression levels of the anti-inflammatory cytokine IL-10 were higher in MLNs as well as in spleens of i.p. $(GPO)_{10}$-treated mice versus placebo controls at day 7 p.i. Furthermore, whereas placebo mice displayed significant perivascular cuffs of inflammatory cells at day 7 p.i., the influx of inflammatory cells into lungs was virtually abolished following i.p.. $(GPO)_{10}$ treatment. Thus, $(GPO)_{10}$ might be a promising pharmacological compound exerting its potent immunomodulatory (i.e. anti-inflammatory) effects locally in the intestinal tract as well as in extra-intestinal compartments.

Beside MMP-specific effects, $(GPO)_{10}$ might also impact immune-specific cellular processes directly, as it does via activation of immune receptors such as the platelet glycoprotein VI (GPVI) or the leukocyte-associated immunoglobulin-like receptor-1 (LAIR1) [26].

Given that to our knowledge $(GPO)_{10}$ is lacking nonselective MMP blocking, such as anti-MMP-1 and anti-MMP-7, properties the risk of significant unwanted side effects, such as arthralgia for instance, might be regarded as rather low [46]. Thus, from our current point of view, $(GPO)_{10}$ might be regarded a safe compound which needs to be further proven in future *in vivo* experimental and clinical studies.

Taken together, the data presented here demonstrate for the first time that the synthetic compound $(GPO)_{10}$ ameliorates acute murine DSS colitis. $(GPO)_{10}$ exerts its beneficial immuno-modulatory effect by disrupting the vicious cycle of the positive feedback loop between 'immune cell stimulation and MMP induction' and promotes anti-inflammatory and regenerative effects locally as well as systemically. Further mechanisms of $(GPO)_{10}$ action in experimental *in vivo* conditions by using MMP-2 and/or -9 deficient mice, however, need to be unraveled in ongoing studies. In conclusion, the beneficial features described here put $(GPO)_{10}$ in a promising position for future intervention strategies in human intestinal and extra-intestinal inflammatory diseases.

Acknowledgements

This work was supported by grants from the German Research Foundation (DFG) to S.B. and A.F. (SFB633, TP A7), A.A.K. and U.E. (SFB633, TP Z1), M.M.H. (SFB633, TP B6), R.S. (SFB366, TP C5 and C10; Ru624/3-2, Schu 646/1-10), and from the German Federal Ministry of Education and Research (BMBF) to S.B. ('Lab in a hanky' projects TP 1.1 and TP 8.2).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Michaela Wattrodt, Ursula Rüschendorf, Gernot Reifenberger, Uwe Lohmann, and the staff of the animal research facility for excellent technical assistance and animal breeding. We are grateful to Simone Spieckermann for immunohistochemistry staining of colon sections.

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2.2 Präventive Effekte der Inhibition von Ionenkanälen

2.2.1 Inhibition des Kalzium-aktivierten Kaliumkanals mit mittlerer Leitfähigkeit KCa3.1 als therapeutisches Ziel bei Leberfibrose und dem hepatozellulären Karzinom

- (**Freise C**., Heldwein S., Erben U., Hoyer J., Köhler R., Jöhrens K., Patsenker E., Ruehl M., Seehofer D., Stickel F., Somasundaram R.: K^+-channel inhibition reduces portal perfusion pressure in fibrotic rats and fibrosis associated characteristics of hepatic stellate cells. *Liver International* 2015 Apr;35(4):1244-52).
- (**Freise C**., Ruehl M., Seehofer D., Hoyer J., Somasundaram R.: The inhibitor of Ca2+-dependent K+ channels TRAM-34 blocks growth of hepatocellular carcinoma cells via downregulation of estrogen receptor alpha mRNA and nuclear factor-kappaB. *Invest New Drugs* 2013 Apr;31(2):452-7).

Im Rahmen der Leberfibrose produzieren und sezernieren aktivierte HSZ vermehrt Moleküle der EZM und stellen damit ein Ziel für anti-fibrotische Therapieansätze dar. KCa3.1 regulieren u.a. Proliferation, Migration und den Gefäßwiderstand und ist so ebenfalls ein rationales Ziel anti-fibrotischer Therapieansätze. Bisher lagen keine Informationen über eine mögliche KCa3.1-Expression in HSZ vor. Ziel dieser Arbeit war es daher, die Expression von KCa3.1 in HSZ in Abhängigkeit des HSZ-Aktivierungsstatus sowie potentielle anti-fibrotische Wirkungen einer pharmakologischen Inhibition von KCa3.1 *in vitro* und *in vivo* zu untersuchen. Die Expression und Funktionalität von KCa3.1 in TGF-β1-aktivierten HSZ wurde mittels quantitativer Polymerase-Kettenreaktion (qPCR), per Western-Blot und durch *patch-clamp* Analysen bestimmt. Effekte des spezifischen KCa3.1-Inhibitors TRAM-34 auf die Proliferation, den Zellzyklus und die Expression von Fibrose-assoziierten Genen wurde mittels [^{3}H]-Thymidineinbau, FACS-Analyse und qPCR untersucht. Im Tiermodell der durch Gallengangsligation induzierten Leberfibrose wurden Effekte von TRAM-34 auf den Gefäßwiderstand sowie die Gen- und Proteinexpression von KCa3.1 durch *in situ* Leberperfusion, Taqman PCR bzw. durch Immunhistochemie bestimmt. Es zeigte sich, dass fibrotisches Gewebe und TGF-β1-aktivierte HSZ eine stärkere KCa3.1-Expression aufweisen als jeweils normales Lebergewebe bzw. nicht-aktivierte HSZ. Die Inhibition von KCa3.1 mit TRAM-34 führte zu einer verminderten Proliferation von HSZ durch die Induktion eines Zellzyklusarrestes in der G_0/G_1-Phase. Zudem hemmte TRAM-34 die TGF-β1-induzierte Genexpression von Kollagen I, *alpha smooth muscle actin* sowie TGF-β selbst und verminderte die Aktivierung des TGF-β-induzierten TGF-β-Signalweges in HSZ. *In vivo* reduzierte TRAM-34 den durch einen Thromboxan-Agonisten induzierten portalen Druck. Die Inhibition von KCa3.1 durch TRAM-34 hemmt also die Genexpression fibrosespezifischer Gene und führt zu einem verminderten portalen Druck *in vivo*. KCa3.1 bzw. spezifische Modulatoren wie TRAM-34 stellen so rationale Ziele anti-fibrotischer Therapieansätze dar.

In der zweiten Arbeit wurden neben anti-fibrotischen Effekten auch potentielle anti-neoplastische Wirkungen von TRAM-34 untersucht. Das HCC ist das häufigste Lebermalignom und weist nur beschränkte Therapiemöglichkeiten auf. Für TRAM-34 wurden bereits wachstumshemmende Effekte in verschiedenen anderen Tumorzellen beschrieben. In dieser Arbeit wurde gezeigt, dass TRAM-34 das Wachstum von drei unterschiedlichen humanen HCC-Zelllinien effektiv hemmt und dass dies u.a. auf einer reduzierten Expression des *estrogen receptors alpha* (ERα) sowie einer verringerten Aktivierung des Transkriptionsfaktors NF-κB beruht. Sowohl für ERα als auch für NF-κB ist eine Beteiligung an der Entwicklung und Progression des HCC bekannt. TRAM-34 hat also neben der anti-fibrotischen Wirkung auch ein hemmendes Wirkpotential beim HCC, welches *in vivo* weiter untersucht werden soll.

Neben Leberfibrose und HCC stellen KCa3.1 auch potentielle therapeutische Zielstrukturen bei kardiovaskulären Erkrankungen dar. Im nachfolgenden Kapitel wird eine Veröffentlichung zusammengefasst, die die Inhibition der künstlich induzierten (Trans)differenzierung bzw. Verkalkung von VSMC durch TRAM-34 beschreibt.

http://dx.doi.org/10.1111/liv.12681
http://dx.doi.org/10.1007/s10637-012-9879-6

2.2.2 Der Kalzium-aktivierte Kaliumkanal mit mittlerer Leitfähigkeit KCa3.1 als therapeutisches Ziel bei Gefäßverkalkung

- (**Freise C.**, Querfeld U.: Inhibition of vascular calcification by block of intermediate conductance calcium-activated potassium channels with TRAM-34. *Pharmacological Research* 2014 (85):6-14).

Nicht nur in der Leber oder dem Darm, sondern auch bei Gefäßerkrankungen wie der medialen (Arteriosklerose) und der intimalen Verkalkung (Atherosklerose), spielt der strukturelle Gefäß- bzw. Matrixumbau, u.a. durch MMPs eine wichtige Rolle. Vaskuläre Verkalkungen generell sind Zeichen fortgeschrittener kardiovaskulärer Erkrankungen, u.a. bei Patienten mit chronischer Niereninsuffizienz. Im Zentrum dabei steht die (Trans)differenzierung von VSMC von einem kontraktilen zu einem synthetischen Phänotyp. KCa3.1 werden in diversen Geweben inklusive VSMC exprimiert. Vor dem Hintergrund möglicher neuer Therapien bei Gefäßverkalkungen wurden in dieser Studie Effekte des spezifischen KCa3.1-Inhibitors TRAM-34 auf die künstlich induzierte Verkalkung von VSMC untersucht. Verkalkungen der murinen VSMC-Zelllinie MOVAS-1 und von primären Ratten VSMC wurden durch ein *calcification medium* (CM) induziert, welches künstlich erhöhte Ca^{2+}- und PO_4^{3-}-Konzentrationen aufweist.
TRAM-34 (10 nM) hemmte die CM-induzierte Verkalkung in VSMC, induzierte die Freisetzung von NO und reduzierte gleichzeitig die Aktivierung des TGF-β-Signalweges. Zudem wurde die CM-induzierte Expression der Verkalkungsmarker Osterix und Osteocalcin gehemmt, ebenso wie die Expression der MMPs-2 und -9. Auf Signaltransduktionsebene hemmte TRAM-34 die TNF-α-induzierte Aktivierung des Transkriptionsfaktors NF-κB und reduzierte die Sekretion der MMPs-2 und -9. Neben TRAM-34 führte auch ein transienter KCa3.1-*knockdown* in den VSMC durch *small interfering RNA* (siRNA) zu einer verminderten Verkalkung der VSMC.
Zusammengefasst hemmt der KCa3.1-Inhibitor TRAM-34 die Aktivierung der im Rahmen der Gefäßverkalkung wichtigen Signalwege von NF-κB und TGF-β. Zusätzlich wird die Produktion bzw. Freisetzung der MMPs-2 und -9 gehemmt, wodurch die (Trans)differenzierung der VSMC und damit deren Verkalkung unterbunden wird. Die Ergebnisse dieser Studie stellen eine Rationale für weitere Studien hinsichtlich einer potentiellen therapeutischen Rolle von KCa3.1 bzw. TRAM-34 bei Gefäßerkrankungen in Tiermodellen dar.

Im nachfolgenden Kapitel werden, analog zu KCa3.1, zwei Arbeiten zur Bedeutung und Wirkung eines als hepatoprotektiv vorbeschriebenen pflanzlichen Extraktes aus *Lindera obtusiloba* zusammengefasst. In der ersten Arbeit werden anti-fibrotische, in der zweiten Arbeit anti-neoplastische Effekte des Extraktes beschrieben.
http://dx.doi.org/10.1016/j.phrs.2014.04.013

2.3 *Lindera obtusiloba* als Reservoir potenter Pharmaka bei Fibrose und Neoplasie

2.3.1 Ein wässriges *Lindera obtusiloba* Extrakt zeigt anti-fibrotisches und anti-neoplastisches Wirkpotential *in vitro*

- (Ruehl, M., Erben, U., Kim, K., **Freise, C.**, Dagdelen, T., Trowitzsch-Kienast, W., Zeitz, M., Jia, J., Stickel, F. and Somasundaram, R.: ‘Extracts of Lindera obtusiloba induce antifibrotic effects in hepatic stellate cells via suppression of a TGF-β mediated profibrotic gene expression pattern’ *J Nutr Biochem*. 2009 Aug;20(8):597-606)
- (**Freise C**, Ruehl M, Erben U, Neumann U, Seehofer D, Kim KY, Trowitzsch-Kienast W, Stroh T, Zeitz M, Somasundaram R.: ‘A hepatoprotective Lindera obtusiloba extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines‘ *BMC Complement Altern Med*. 2011 May 12;11(1):39.)

In der traditionellen Koreanischen Heilkunde wird ein Extrakt der Heilpflanze *Lindera obtusiloba* (LOE) zur Behandlung von Entzündungen und chronischen Leberschäden sowie der Unterstützung der Blutzirkulation eingesetzt. Experimentelle Studien zur Wirksamkeit nach westlichen Standards fehlten jedoch bisher. Die Leberfibrose geht aus verschiedenen chronischen Vorerkrankungen der Leber wie z.B. der chronischen viralen Hepatitis hervor und kann bis heute nicht hinlänglich behandelt werden. Dies erklärt den Bedarf an neuen Therapieformen. Ein Charakteristikum der Leberfibrose ist die erhöhte Expression des profibrotischen “Masterzytokins” TGF-β und des endogenen MMP-Inhibitors TIMP-1 u.a. durch HSZ, was zu einer erhöhten netto-Matrixakkumulation führt. Mögliche protektive Effekte von LOE auf die Fibrogenese wurden in einem Zellkulturmodell mit HSZ als zentralen Mediatoren der Leberfibrose untersucht. LOE (135 µg/ml) führte zu einer um bis zu 90% verminderten Proliferation von humanen und Ratten-HSZ, ohne zytotoxisch zu wirken, was auf eine Quieszenzinduktion hinweist. LOE hemmte zudem die TGF-β-induzierte Genexpression von TIMP-1 und TGF-β selbst, induzierte aber die Genexpression von fibrolytischer MMP-3. Enzymatische Aktivitätsassays zeigten eine reduzierte gelatinolytische Aktivität von pro-fibrotischer MMP-2 in Kulturüberständen LOE-behandelter HSZ. Ähnlich wie grüner Tee, zeigte LOE zudem starke anti-oxidative Effekte. Zusammengefasst hat LOE durch Modulation der Expression von TGF-β, MMPs und TIMPs sowie durch potente anti-oxidative Wirkung ein anti-fibrotisches Wirkpotential, welches im Tiermodell der Fibrose weiter untersucht werden sollte.

In der zweiten Arbeit wurden darüber hinaus auch anti-neoplastische Effekte von LOE in humanen HCC-Zelllinien untersucht. Das HCC ist weltweit einer der häufigsten und am schwierigsten zu behandelnden Tumore. Einzig zugelassenes Medikament beim (fortgeschrittenen) HCC ist Sorafenib, das über die Hemmung von Rezeptortyrosinkinasen und Signaltransduktionswegen im Median eine um ca. drei Monate verlängerte Überlebenszeit zeigt. Aufgrund der limitierten Prognoseverbesserung und teils

erheblichen Nebenwirkungen unter Therapie, wird nach neuen komplementären und vor allem wenig hepatotoxischen Therapieoptionen gesucht. In vier unterschiedlichen humanen HCC-Zelllinien hemmte LOE Wachstum und Invasion, u. a. durch Hemmung der IGF-1R-Signaltransduktion, durch verminderte Aktivierung des Transkriptionsfaktors NF-κB sowie einer enzymatischen MMP-Hemmung. Zusammen mit der von uns gezeigten und in der traditionellen Koreanischen Heilkunde beschriebenen hepatoprotektiven bzw. wenig toxischen Wirkung in „normalen" Leberzellen, stellt LOE auch einen möglichen Kandidaten für komplementäre Therapieansätze beim HCC dar.

Nachdem anti-fibrotische und anti-neoplastische Effekte von LOE *in vitro* gezeigt werden konnten, war nun die Kenntnis der pharmakologisch aktiven Inhaltsstoffe in dem Extrakt von Interesse. Die im nachfolgenden Kapitel zusammengefassten Arbeiten beschreiben die Isolierung und Identifizierung von ES und die Austestung von dessen biologischen Wirkungen *in vitro*.
http://dx.doi.org/10.1016/j.jnutbio.2008.06.003

Freise *et al. BMC Complementary and Alternative Medicine* 2011, **11**:39
http://www.biomedcentral.com/1472-6882/11/39

BMC
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A hepatoprotective *Lindera obtusiloba* extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines

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Abstract

Background: In traditional Chinese and Korean medicine, an aqueous extract derived from wood and bark of the Japanese spice bush *Lindera obtusiloba* (*L.obtusiloba*) is applied to treat inflammations and chronic liver diseases including hepatocellular carcinoma. We previously demonstrated anti-fibrotic effects of *L.obtusiloba* extract in hepatic stellate cells. Thus, we here consequently examine anti-neoplastic effects of *L.obtusiloba* extract on human hepatocellular carcinoma (HCC) cell lines and the signaling pathways involved.

Methods: Four human HCC cell lines representing diverse stages of differentiation were treated with *L.obtusiloba* extract, standardized according to its known suppressive effects on proliferation and TGF-β-expression. Beside measurement of proliferation, invasion and apoptosis, effects on signal transduction and NF-κB-activity were determined.

Results: *L.obtusiloba* extract inhibited proliferation and induced apoptosis in all HCC cell lines and provoked a reduced basal and IGF-1-induced activation of the IGF-1R signaling cascade and a reduced transcriptional NF-κB-activity, particularly in the poorly differentiated SK-Hep1 cells. Pointing to anti-angiogenic effects, *L.obtusiloba* extract attenuated the basal and IGF-1-induced expression of hypoxia inducible factor-1α, vascular endothelial growth factor, peroxisome proliferator-activated receptor-γ, cyclooxygenase-2 and inducible nitric oxide synthase.

Conclusions: The traditional application of the extract is confirmed by our experimental data. Due to its potential to inhibit critical receptor tyrosine kinases involved in HCC progression via the IGF-1 signaling pathway and NF-κB, the standardized *L.obtusiloba* extract should be further analysed for its active compounds and explored as (complementary) treatment option for HCC.

Background

Hepatocellular carcinoma (HCC) results from chronic liver disease and is the most common malignancy of the liver [1]. Chronic Hepatitis B or C leading to liver cirrhosis are major risk factors for the development of HCC [2]. Even in developing countries less than 40% of patients have a chance for cure when the tumor is diagnosed. In more advanced stages there are only reduced therapeutic options, since e.g. the use of more aggressive chemotherapeutic approaches is often limited by significant liver dysfunction/cirrhosis. Thus, the median survival in advanced HCC without therapy ranges from 4.2 to 7.9 months or even less [3,4]. Small molecules, targeting tumor angiogenesis, apoptosis or specific signal transduction pathways, have gained growing attention in cancer therapy. The multikinase inhibitor sorafenib is currently the only approved drug for the treatment of HCC, prolonging median survival of advanced HCC from 7.9 to 10.4 months [4]. But side effects and upcoming resistances reveal that monotherapies with

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the kinase inhibitors alone are not sufficient suggesting the need for combinatory and/or multitargeted therapies [5].

The receptor tyrosine kinase insulin like growth factor-1 receptor (IGF-1R) and its ligands, IGF-1 and IGF-2, are essential for cell growth and development [6] but also in the progression of various types of cancer, including HCC [7-10]. In addition, IGF-1R signaling protects cells from apoptosis mainly through the PI3K/Akt and Ras-Raf-MAPK pathways [11,12]. Activation of IGF-1R critically impacts HCC angiogenesis by induced expression of vascular endothelial growth factor (VEGF) and its transcription factor hypoxia inducible factor (HIF)-1α [13-15]. Inhibition of IGF-1R, e.g by monoclonal antibodies against IGF-1R, has been shown to block tumor growth *in vitro* and in a xenograft model of HCC and to sensitize cells for anti-tumor treatment, indicating that IGF-1R is a promising antineoplastic target [16-18]. A clinical trial targeting IGF-1R inhibition is currently ongoing in patients with advanced solid tumors. Preliminary data suggest evidence of clinical activity and good tolerance [19].

IGF-1R signaling via the PI-3K/AKT-axis also impacts the nuclear factor-kappaB (NF-κB), which is not only considered a key factor in inflammation but also regulates angiogenesis and as a major characteristic mediates inhibition of apoptosis [20]. NF-κB is spontaneously activated in HCC cells [21,22] and induces expression of cyclooxygenase-2 (COX-2) or inducible nitric oxide synthase (iNOS) which support cell survival and might contribute to the resistance against exogenously induced tumor cell apoptosis [23,24].

Traditionally, Oriental medicine makes use of compositions from or mixtures of different plants to prevent or to treat cancer and liver diseases [25,26]. Novel multitargeted therapeutics including natural compounds such as epigallocatechin-3-gallate from green tea have gained growing attention [27].

In traditional Chinese and Korean medicine preparations from *Lindera obtusiloba* (*L.obtusiloba*) comprise a good physiological compatibility and are applied to treat inflammations and to improve blood circulation [28]. Especially in Korean medicine an extract of *L.obtusiloba* is used for a long time for the treatment of chronic liver diseases which includes treatment of HCC the endstage of chronic liver disease (personal communication, Prof. Ki Young Kim, Wonkwang University, Korea).

Bioactive components from the leaves of *L.obtusiloba* described so far exert cytotoxicity against tumors as shown with human cancer cell lines from lung (A549), ovarian cancer (SK-OV-3), skin (SK-MEL-2), the central nervous system (XF498) or colon (HCT15) with half-maximum inhibitory concentration (IC_{50}) values ranging from 3-20 μg/ml of the respective compounds [29,30]. We previously found an aqueous extract from wood and bark of *L.obtusiloba* to suppress profibrotic stimuli, exerting anti-oxidative activity, reduction of the expression of pro-fibrotic marker proteins and inhibition of matrix-metalloproteinases in hepatic stellate cells [31]. In addition, this extract displayed anti-inflammatory and anti-adipogenic activity in 3T3-L1 preadipocytes [32].

However, experimental data from in vitro or in vivo studies on anti-neoplastic effects of *L.obtusiloba* extracts in human HCC as complication of chronic liver disease were not reported so far. We here used well established HCC cell lines that represent diverse stages of differentiation and different degrees of invasiveness to examine direct anti-neoplastic effects of *L.obtusiloba* extract, standardized to its antiproliferative and anti-fibrotic effects, on tumor cells and to get insights into signaling pathways involved. With a clear focus on aspects affecting angiogenesis and tumor cell invasion, we aimed to understand mechanisms of action of *L.obtusiloba* extract.

Methods

Materials and reagents

Tissue culture plates and polystyrene microtiter for ELISA as well as for fluorimetric analysis were from Nunc (Roskilde, Denmark) and Dynex (Chantilly, VA), respectively. If not stated otherwise, all reagents were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) and were of the highest purity available. Cell culture media and solutions were purchased from Invitrogen (Karlsruhe, Germany) or Biochrom (Berlin, Germany).

Preparation and standardization of *L.obtusiloba* extract

Freeze-dried extracts of *L.obtusiloba* were obtained as described previously [31]. To obtain stock solutions, 10 mg powder was redissolved in 10 ml sterile phosphate-buffered saline (PBS) at 60°C for 30 min. Aliquots were stored at -20°C. Freshly prepared working solutions of *L. obtusiloba* extract were routinely standardized according to their anti-fibrotic and anti-inflammatory activity as previously described [31,32]. Briefly, 100 μg/ml *L.obtusiloba* extract had to reduce proliferation of 3T3-L1 preadipoctyes by 45% and to suppress the autocrine stimulation of TGF-β expression of hepatic stellate cells by 50% before to be used in the assays with HCC cells.

Cell culture

The human HCC cell lines HepG2 (ATCC HV-8062), Hep3B (ATCC HV-8064), Huh-7 (JCRB 0403; Tokio, Japan) and SK-Hep1 (ATCC HTB-52) cells (Fuchs et al., 2008) were cultured in a humidified atmosphere at 37°C and 5% CO_2. Standard culture medium consisted of DMEM with 862 mg/l L-alanyl-L-glutamine, 4.5 g/l glucose, 50 μg/ml streptomycin, 50 units/ml penicillin,

50 μg/ml L-ascorbic acid, supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cell layers were detached with 0.05% trypsin/0.02% EDTA solution. Cell morphology in culture was directly examined by inverse phase contrast microscopy (Zeiss, Oberkochen, Germany).

HCC cell proliferation

HCC cells (5×10^3) were seeded into 96-well tissue culture plates in 100 μl standard culture medium. After 24 h, cells were cell cycle synchronized in 100 μl culture medium containing 0.2% FBS for additional 24 h. Cultures were treated with up to 200 μg/ml *L.obtusiloba* extract as indicated for 20 h. Proliferation was determined by adding 0.5 μCi/well [^{3}H]-thymidine (GE Healthcare, Munich, Germany) for 4 h. Cells were fixed with 10% trichloro acetic acid and the DNA was solubilized with 200 mM NaOH, neutralized with an equal volume of 800 mM HCl and transferred to glass filter pads. Radioactive decay was monitored by liquid β-scintillation counting within 1 min (LKB Wallac Turku, Finland).

Cell invasion assays

50 μl of 3 mg/ml Matrigel™ (BD Biosciences, Heidelberg, Germany) diluted in ice cold, serum free DMEM were used to coat the upper compartments of 24-well transwell inserts (BD Biosciences; pore size 8 μm) for 16 h at 37°C. 2×10^5 cells diluted in 300 μl serum free medium were seeded into the upper compartments and *L.obtusiloba* extract was added at a final concentration of 100 μg/ml. DMEM containing 10% FBS as stimulating agent was added to the lower compartment and the plates were incubated for up to 24 h at 37°C in a humidified atmosphere with 5% CO_2. Cells that remained in the upper compartment were gently removed with a cotton swab. The inserts were then washed with PBS and invaded cells on the lower surface of the insert were fixed for 20 min with 2% glutaraldehyde in PBS and stained using 0.1% crystal violet in water. The stained cells on each insert were visualized by light microscopy and manually counted in three independent spots per insert.

Apoptosis by caspase 3/7 activity

Apoptosis was quantified fluorimetrically from caspase-3/7 activity. In brief, 2×10^5 HCC cells in standard culture medium were seeded into 24-well tissue culture plates. Confluent cell layers were thoroughly washed with DMEM and subsequently incubated with culture medium containing 0.2% FBS for 24 h. Cells were then treated for another 24 h in the presence of 100 μg/ml L. obtusiloba extract or 100 nM staurosporine and 0.2% FBS. Apoptosis was determined using the SensoLyte™ Homogenous AFC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA) according to the manufactures instructions. Briefly, cells were lysed in 200 μl lysis buffer for 1 h at 4°C. The clear supernatant obtained after centrifugation at 2,500 × g for 30 min was stored at -80°C until measurement. Caspase 3/7-mediated conversion of the substrate N-acetyl-Asp-Glu-Val-Asp-7 amino-4 trifluoromethyl coumarin was monitored fluorometrically using a Spectramax Gemini EM microplate reader (λex: 380 nm, λem: 500 nm; Molecular Devices, Sunnyvale, CA).

Western-blot

HCC cells cultured in 6-well tissue culture plates with 125 ng/ml human recombinant IGF-1 (Biomol, Hamburg, Germany), 100 μg/ml *L.obtusiloba* extract and a combination of both for 48 h were rinsed with ice-cold PBS and lysed with a lysis-buffer containing 50 mM Tris-HCl pH 7.4, 2.25 M urea, 1.4% sodium dodecyl sulfate, 100 mM dithiothreitol, 2 mM $NaVO_3$, 5 mM NaF, and per 10 ml buffer one tablet of Complete Mini Protease Inhibitor cocktail (Roche, Penzberg, Germany). Aliquots of 333 μl lysate were transferred to 0.5 ml reaction tubes and frozen at 80°C. Protein content was determined using the Nano Orange Protein Assay Kit (Molecular Devices) according to the manufactures instructions. From each cell lysate, 25 μg protein per lane were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Munich, Germany) using a tank blot apparatus (Hoefer, Holliston, MA). Membranes blocked for 1 h with 5% skim milk powder in a buffer containing 10 mM Tris, 154 mM NaCl, 0.1% Tween 20 were incubated over night at 4°C with the following specific primary antibodies with the dilution given: Akt (1:1,250), COX-2 (1:1,000), Erk1/2 (1:1,000), iNOS (1:1,500), pAkt (1:1,250), pErk1/2 (1:1,250), Stat3 (1:1,250; Cell Signaling, Beverly, MA), β-Actin (1:10,000), HIF-1α (1:2,000; Novus Biologicals, Littleton, CO, USA), IGF-1R (1:1,250), pIGF-1R (1:1,250; Imgenex, San Diego, CA) and PPARγ (1:2,000), pStat3 (1:1,250), VEGF (1:800; Santa Cruz, Santa Cruz, CA). After washing, membranes were incubated for 1 h with rabbit or mouse immunoglobulin G-specific horseradish peroxidase-labeled secondary antibodies (1:2,500; Dako, Hamburg, Germany). Bands were detected by enhanced chemiluminescence (GE Healthcare) using the Luminescent Image Analyser LAS-4000 (Fujifilm, Düsseldorf, Germany). Band intensities were quantified using Image J and normalized to the β-actin loading control.

Transient transfection of HCC cells

Transfection of the cells was performed using the electroporation method and a NF-κB-luciferase reporter plasmid as described by Stroh et al. [33,34]. Detached cells (2×10^5) were resuspended in 100 μl electroporation buffer containing 90 mM phosphate buffer pH 7.2,

10 mM $MgCl_2$, and 50 mM glucose before 4 μg of the NF-κB-luciferase reporter plasmid pNF-κB-TA-Luc (Clontech, Mountain View, CA) were added. In an electroporation cuvette with a gap of 2 mm (Biozym, Hessisch Oldendorf, Germany), cells were subjected to single square pulses of 400 V for 400 μs (HepG2, Hep3B and Huh-7) or 600 V for 400 μs (SK-Hep1), allowed to rest for 1 min, and transferred into pre-warmed standard culture medium. A total of 1×10^5 transfected cells in 1 ml culture medium were seeded into a 24-well plate. Cell viability as determined by Calcein AM staining [32] was about 85% in conjunction with a cell transfection efficacy of ~75%.

Assessment of NF-κB activation by luciferase assay

Twenty hours after transfection with the NF-κB-luciferase reporter plasmid [33] cells were treated with 10 μg/ml recombinant human TNFα (Peprotech, Hamburg, Germany), 100 μg/ml *L.obtusiloba* extract, a combination of both and 15 nM of the NF-κB inhibitor 17-Dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG, InvivoGen, San Diego, CA). Cells were incubated for 24 h, washed twice with PBS, and lysed in 80 μl of reporter lysis buffer (Promega, Mannheim, Germany). Protein concentrations were determined using the Nano Orange Protein Assay Kit. Samples (20 μl) were transferred into a white 96 well plate before 60 μl of luciferase substrate were added and mixed for 5 s. Luciferase activity was measured for 0.5 s using a Mithras LB 940 luminescence reader (Berthold Technologies, Bad Wildbad, Germany). NF-κB activity was estimated as relative luminescence units (RLU) corresponding to equal protein amounts.

Statistical Analysis

One way ANOVA/Tukey Tests were performed using SigmaStat for Windows (version 2.03; Systat, San Jose, CA). $P < 0.05$ was considered significantly different.

Results

L.obtusiloba extract reduces proliferation, induces apoptosis and blocks invasion of HCC cells

Effects of *L.obtusiloba* extract on the proliferation of human HCC cells were tested in cell-cycle synchronized cell lines. To define effective dose ranges, HCC cells in culture were treated with up to 200 μg/ml *L.obtusiloba* extract (Figure 1A). The range of concentration of *L.obtusiloba* extract and the experimental protocols were adapted from preceding studies dealing with the extract [31,32].

L.obtusiloba extract reduced the proliferation of all four human HCC cell lines in a dose-dependent manner. The IC_{50} values for the inhibition of the de novo DNA synthesis were approximately 100 μg/ml *L.obtusiloba* extract for all HCC cell lines. This concentration was used in all subsequent experiments. Induction of apoptosis due to exposure of cells with *L.obtusiloba* extract was determined by the enzymatic activity of pro-apoptotic caspase-3/-7 (Figure 1B). As shown for the apoptosis inducer and kinase inhibitor staurosporine used as control, all cell lines were highly susceptible to induction of apoptosis by *L.obtusiloba* extract as shown by 2.2- to 20-fold enhanced caspase activity. In the differentiated HCC cell lines HepG2, Hep3B and Huh-7, this effect of *L.obtusiloba* extract did not exceed 60% of the effect of 100 nM staurosporine. In contrast, *L.obtusiloba* extract provoked a caspase activity that corresponded to ~80% of apoptosis induced by staurosporine in the poorly differentiated SK-Hep1 cells ($P < 0.001$). Since their migratory potential mainly defines their aggressiveness, 100 mg/ml *L.obtusiloba* extract was applied to HCC cells in matrigel invasion assays. Again, while *L.obtusiloba* extract only slightly attenuated the invasion of HepG2, Huh-7 ($P < 0.05$) and Hep3B cells through a reconstituted basement membrane, it led to a stronger reduction of invasion in SK-Hep1 cells by 55% ($P < 0.01$) (Figure 1C). As for direct effects of *L.obtusiloba* extract on tumor cells, it diminished the invasive potential of HCC cell lines and was most effective on cells displaying a highly aggressive phenotype.

L.obtusiloba extract reduces basal and IGF-1-induced protein expression of VEGF and its transcription factor HIF-1α

HCC represents a highly vascularized tumor entity and the tumor cells contribute to that process by production of proteins regulating angiogenesis. Thus, we next investigated whether *L.obtusiloba* extract impacts the expression of VEGF and HIF-1α in HCC cell lines. Linking Huh-7 to SK-Hep1 cells, stimulation with exogenous IGF-1 enhanced basal expression of VEGF by 1.4- or 3.3-fold, while in HepG2 and Hep3B no effects of IGF-1 were observed (Table 1). *L.obtusiloba* extract alone reduced VEGF expression in all four cell lines but strongest in Huh-7 cells. In combination with IGF-1, *L.obtusiloba* extract did not affect the IGF-1-induced VEGF expression in HepG2 cells, but in Hep3B, Huh-7 and SK-Hep1. The IGF-1-induced enhancement of HIF 1α expression was most prominent in differentiated HepG2 cells (3.6-fold) and intermediate in Hep3B (1.5-fold) and SK-Hep1 cells (1.3-fold). In Huh-7 cells no significant IGF-1-mediated effects on HIF 1α expression were observed. Similar to VEGF, *L.obtusiloba* extract distinctly reduced basal and IGF-1-induced HIF-1α expression in each of the HCC cell lines to comparable individual levels that were independent of the presence of IGF-1. These findings on VEGF and HIF-1α pointed to a strong anti-angiogenic potential of *L.obtusiloba* extract. Consequently, we studied the

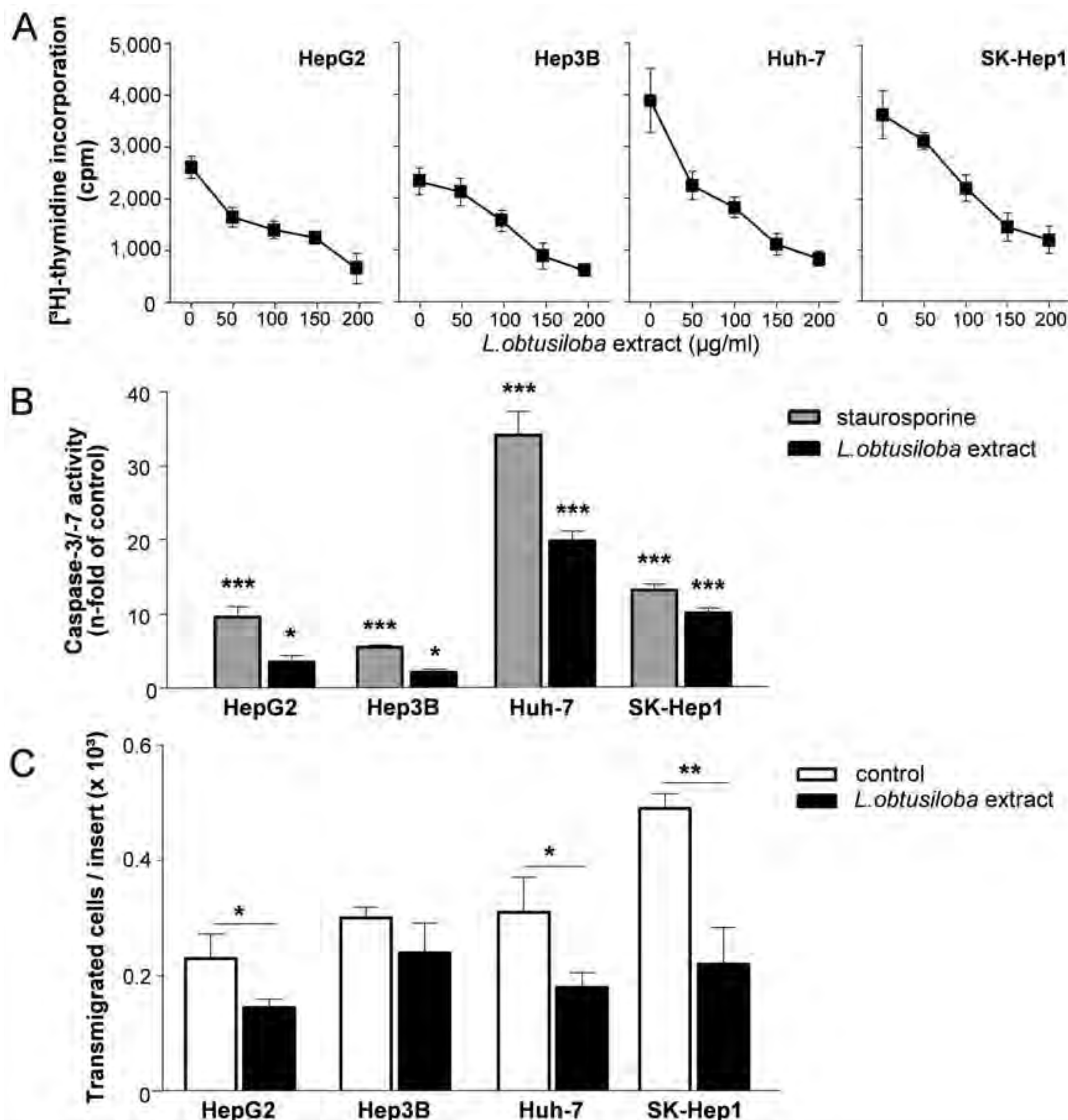

Figure 1 Proliferation, apoptosis and invasion of HCC cell lines treated with *L.obtusiloba* extract. (A) Cell cycle synchronized HepG2, Hep3B, Huh 7 and SK-Hep1 cells were treated with up to 200 µg/ml *L.obtusiloba* extract for 24 h. Cultures without *L.obtusiloba* extract served as controls. Proliferation was determined by [^{3}H]-thymidine incorporation within the last 4 h of the culture. Mean values ± SD of three parallel measurements. (B) HCC cells were incubated with 100 µg/ml *L.obtusiloba* extract for 24 h. Cultures without additives or with 100 nM staurosporine served as negative or positive controls, respectively. Enzymatic activities of caspase-3/7 were determined from cell lysates by fluorogenic substrate conversion. Shown are the mean values ± SD of four parallel measurements. (C) HCC cells were allowed to invade membranes coated with basement collagen in the absence or presence of 100 µg/ml *L.obtusiloba* extract. After 24 h, transmigrated cells were stained with crystal violet and numbers were counted. Shown are the mean values ± SD of three independent experiments with four parallel measurements.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

impact of *L.obtusiloba* extract on the expression of other proteins crucial in neo-angiogenesis.

L.obtusiloba extract decreases the protein expression of PPARγ, COX-2 and iNOS

The expression of the nuclear transcription factor PPARγ and its target genes COX-2 and iNOS are implicated in hepatocarcinogenesis and in the formation of enhanced microvessel density in HCC tissues. Effects of *L.obtusiloba* extract on the expression of PPARγ, COX-2 and iNOS were examined at protein level (Table 2). The expression of PPARγ in all four HCC cell lines was enhanced after stimulation with IGF-1. *L.obtusiloba* extract reduced both, basal and IGF-1-induced PPARγ

Table 1 Expression of VEGF and HIF-1α in human HCC cell lines

	VEGF expression			HIF-1α expression		
	IGF-1	*L.obtusiloba* extract	IGF-1 and *L.obtusiloba* extract	IGF-1	*L.obtusiloba* extract	IGF-1 and *L.obtusiloba* extract
HepG2	1.02 ± 0.03	0.75 ± 0.10*	0.93 ± 0.06	3.58 ± 0.26*	0.72 ± 0.07*	0.82 ± 0.11#
Hep3B	0.82 ± 0.18	0.67 ± 0.09*	0.54 ± 0.10*,#	1.52 ± 0.21*	0.62 ± 0.11*	0.63 ± 0.07*,#
Huh-7	1.38 ± 0.05*	0.28 ± 0.10*	0.47 ± 0.08*,#	0.89 ± 0.12	0.14 ± 0.04*	0.05 ± 0.03*,#
SK-Hep1	3.28 ± 0.24*	0.93 ± 0.10	0.83 ± 0.09#	1.28 ± 0.13*	0.67 ± 0.09*	0.68 ± 0.12*,#

Whole cell lysates from cells treated with 100 μg/ml *L.obtusiloba* extract, 125 ng/ml human IGF-1 or a combination of both for 48 h and from untreated cells as control were analyzed by western-blot specific for VEGF and HIF-1α. β-Actin was stained for equal loading control and specific band intensities were normalized to β-actin. VEGF and HIF-1α protein expression levels were calculated in relation to the respective untreated cells. Mean values ± SD from three independent experiments. *P < 0.05 compared to the untreated control, # P < 0.05 compared to IGF-1-treated cells.

expression with the same pattern as HIF-1α (Table 1). COX-2 was not detected in HepG2 and Huh-7 cells (Table 2). On the other hand, Hep3B and SK-Hep1 showed a high IGF-1-induced expression of COX-2 by 2.3- and 3.2-fold, respectively and with *L.obtusiloba* extract a reduction of both, the basal and the IGF-1-induced COX-2 expression. Hep3B and Huh 7 cells showed no expression of iNOS. In HepG2 and SK-Hep1 cells the basal expression of iNOS was enhanced by IGF-1 by 1.2- and 1.9-fold, respectively. *L.obtusiloba* extract reduced the basal and the IGF-1-induced iNOS expression of both cell lines by ~80%.

Taken together and complementing the results from the preceding experiments, these data suggest direct effects of *L.obtusiloba* extract on the angiogenic program of HCC cells via decreased expression of PPARγ and its target genes COX-2 and iNOS thus contributing to dampened growth and motility of HCC cells.

L.obtusiloba extract blocks expression of VEGF and HIF-1α via attenuated activation of IGF 1R downstream targets

The IGF-1/IGF-1R axis plays an important role in angiogenesis and therefore the development of HCC. To investigate signaling pathways involved, western-blots specific for (p)IGF-1R and the activation states of its target proteins were focused on Hep3B as one out of the three less invasive HCC cells (compare Figure 1C) and the more aggressive SK-Hep1 cells. In both cell lines 100 μg/ml exogenous IGF-1 increased the phosphorylation state of the IGF-1R (Figure 2, Table 3). This IGF-1-mediated activation of the IGF-1R was strongly reduced in the presence of *L.obtusiloba* extract; by half in the Hep3B and to about a quarter in SK-Hep1 cells. As for the downstream signaling molecules Akt, Stat3 and Erk, *L.obtusiloba* extract did not alter basal phosphorylation. IGF-1 induced phosphorylation of Akt, Stat3 and Erk were tested in both cell lines. Increased pAkt levels that were at least partially abrogated by *L.obtusiloba* extract were found to be the most prominent effect. Treatment with *L.obtusiloba* extract in combination with IGF-1 markedly decreased the levels of pAkt, pStat3 and pErk in Hep3B and SK-Hep1 cells.

These findings that *L.obtusiloba* extract decreased the basal phosphorylation of Akt, Stat3 and Erk in Hep3B cells as well as in poorly differentiated SK-Hep1 cells as a result of reduced stimulatory effects of IGF-1 on its receptor explains the inhibition of growth and motility and the induction of apoptosis in HCC cells.

L.obtusiloba extract decreases transcriptional activity of NF-κB

NF-κB is a key regulator of crucial pro-inflammatory cytokines during carcinogenesis and promotes cell survival and angiogenesis. Since *L.obtusiloba* extract induces apoptosis (Figure 1B) and displays anti-inflammatory activity [32], we assessed whether the extract decreases

Table 2 Expression of PPARγ, COX-2 and iNOS in human HCC cell lines

	PPARγ expression			COX-2 expression			iNOS expression		
	IGF-1	*L. obtusiloba* extract	IGF-1 and *L. obtusiloba* extract	IGF-1	*L.obtusiloba* extract	IGF-1 and *L. obtusiloba* extract	IGF-1	*L. obtusiloba* extract	IGF-1 and *L. obtusiloba* extract
HepG2	4.31 ± 0.51*	0.76 ± 0.14	1.15 ± 0.09#	n.d.	n.d.	n.d.	1.17 ± 0.07	0.21 ± 0.14*	0.21 ± 0.09*;#
Hep3B	1.33 ± 0.12*	0.84 ± 0.09	0.81 ± 0.05#	2.28 ± 0.19*	0.77 ± 0.08*	1.09 ± 0.04*;#	n.d.	n.d.	n.d.
Huh-7	1.17 ± 0.05	0.21 ± 0.10*	0.31 ± 0.12*;#	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SK-Hep1	1.43 ± 0.11*	0.75 ± 0.09*	0.89 ± 0.07#	3.21 ± 0.34*	0.80 ± 0.07*	0.82 ± 0.09#	1.87 ± 0.12*	0.27 ± 0.12*	0.35 ± 0.06*;#

Cell lysates of HCC cell lines as described for Table 1, were subjected to specific western-blots for PPARγ, COX 2 and iNOS and for β actin as equal loading control. Densitometry of specific band intensity was normalized to β-actin expression. Mean values ± SD from three independent experiments. PPARγ, COX-2 and iNOS protein expression levels were calculated in relation to the respective untreated cells. Mean values ± SD from three independent experiments. *P < 0.05 compared to the untreated control, # P < 0.05 compared to IGF-1-treated cells, n.d. - not detected.

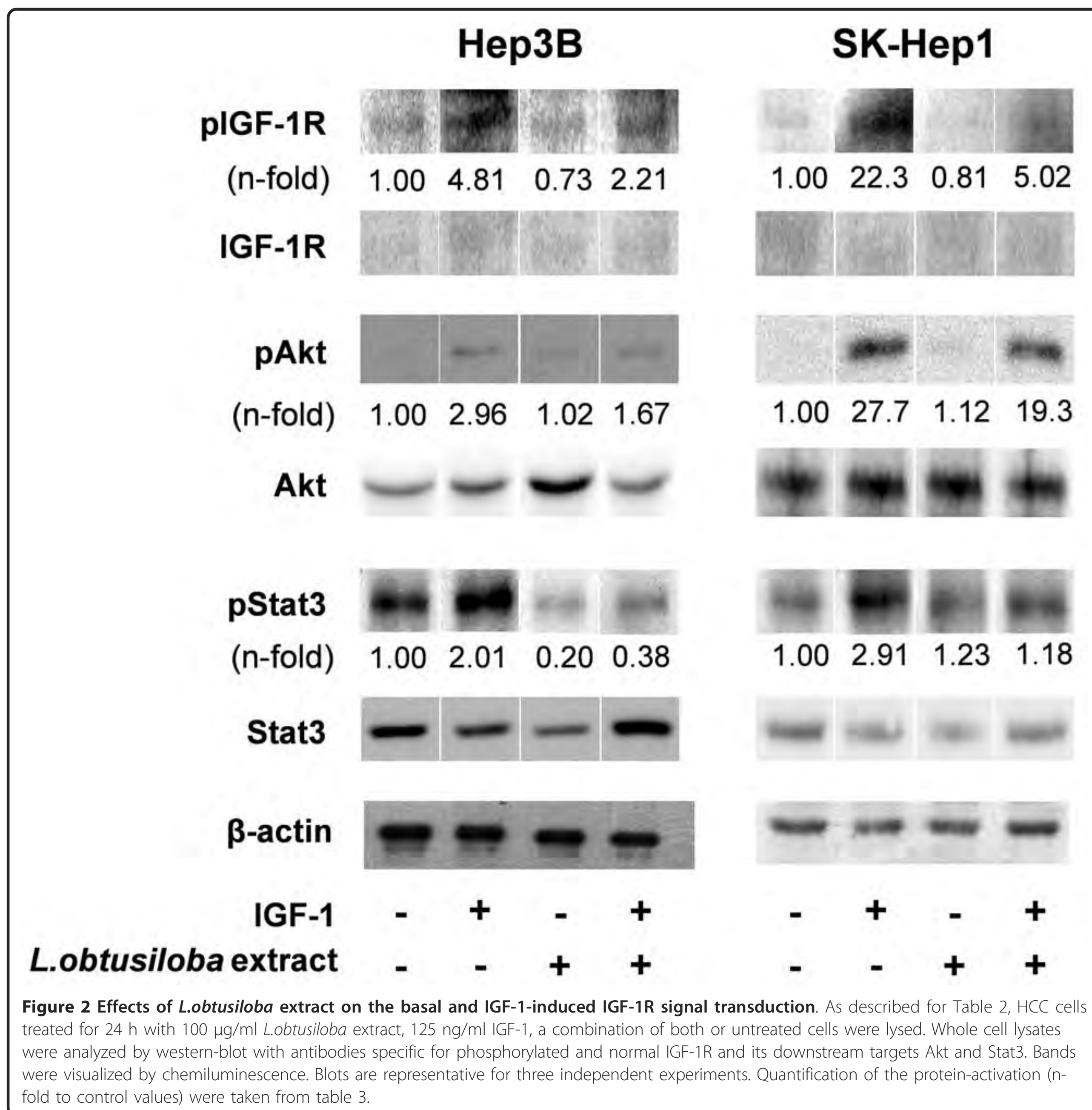

Figure 2 Effects of *L.obtusiloba* extract on the basal and IGF-1-induced IGF-1R signal transduction. As described for Table 2, HCC cells treated for 24 h with 100 μg/ml *L.obtusiloba* extract, 125 ng/ml IGF-1, a combination of both or untreated cells were lysed. Whole cell lysates were analyzed by western-blot with antibodies specific for phosphorylated and normal IGF-1R and its downstream targets Akt and Stat3. Bands were visualized by chemiluminescence. Blots are representative for three independent experiments. Quantification of the protein-activation (n-fold to control values) were taken from table 3.

the activity of NF-κB in HCC cells (Figure 3). All four HCC cell lines transfected for transient constitutive expression of NF-κB exhibited high levels of basal NF-κB transcriptional activity of about 160 260 RLU. This activity was not significantly increased by addition of TNFα. In all cell lines, treatment of transfected cells with the specific NF-κB-inhibitor 17-DMAG reduced the activity to <10% of the basal level thus approving the function of the experimental system (data not shown). Except for HepG2 cells, *L.obtusiloba* extract attenuated the transcriptional activity of NF-κB to 75% ($P < 0.05$) of the basal level in Huh-7 and to ~65% ($P < 0.001$) in Hep3B cells while in the poorly differentiated SK-Hep1 cells the high basal transcriptional activity of NF-κB was reduced to 50% ($P < 0.001$). These results at the level of regulation clearly strengthen our conclusion that *L.obtusiloba* extract directly impairs the survival and the angiogenic program in HCC cells.

Discussion

In the present study with human HCC cell lines we provide evidence that a well standardized aqueous extract

Table 3 Effects of *L.obtusiloba* extract on basal and IGF-1-induced signal transduction via IGF-1R

	Hep3B			SK-Hep1		
	IGF-1	L.obtusiloba extract	IGF-1 and L.obtusiloba extract	IGF-1	L.obtusiloba extract	IGF-1 and L.obtusiloba extract
pIGF-1R	4.81 ± 0.40*	0.73 ± 0.08*	2.21 ± 0.31*; #	22.3 ± 1.98*	0.81 ± 0.07	5.02 ± 0.60*; #
pAkt	2.96 ± 0.25*	1.02 ± 0.10	1.67 ± 0.19*; #	27.7 ± 2.84*	1.12 ± 0.09	19.3 ± 1.45*; #
pStat3	2.01 ± 0.18*	0.20 ± 0.07*	0.38 ± 0.21*; #	2.91 ± 0.22*	1.23 ± 0.20	1.18 ± 0.19#
pErk2	1.32 ± 0.14*	0.13 ± 0.03*	0.57 ± 0.07*; #	2.81 ± 0.32*	1.01 ± 0.10	1.82 ± 0.17*; #

HCC cells were treated with 100 μg/ml *L.obtusiloba* extract, 125 ng/ml IGF 1, a combination of both or remained untreated for 24 h. Phosphorylation of IGF-1R and its downstream targets Akt, Stat3 and Erk were studied in whole cell lysates by specific western-blot analysis as shown in representative blots in Fig. 3. Activation of the proteins was determined from densitometric assessment in comparison to total expression levels of the respective non-phosphorylated protein. Mean values ± SD in relation to untreated cells from three independent experiments. *P < 0.05 compared to the untreated control, # P < 0.05 compared to IGF-1-treated cells.

from wood and bark of *L.obtusiloba* exerts direct and non-direct anti-neoplastic effects via attenuated IGF-1R- and NF-κB-signaling.

Initially, we examined the effects of a standardized active extract of *L.obtusiloba* on the proliferation of well characterized human HCC cell lines with poorly differentiated SK-Hep1 considered more aggressive than the other three used. *L.obtusiloba* extract blocked the growth of the HCC cells in a dose dependent manner with a physiologically relevant IC_{50} of ~100 μg/ml (Figure 1A) [31,32]. In addition, *L.obtusiloba* extract inhibited tumor cell invasion (Figure 1C). Here, SK-Hep1 cells rather than the well differentiated HepG2, Hep3B and Huh-7 cells were more sensitive to *L.obtusiloba* extract. Thus, in conjunction with the induction of apoptosis in all four cell lines (Figure 1B), *L.obtusiloba* extract exerts three primary prerequisites for the treatment of cancer [35,36].

Aberrant growth and apoptosis regulation in carcinogenesis is mediated by growth factor receptors such as IGF-1R which therefore represents an attractive therapeutic target [8,37] and all of the four cell lines investigated are known to express the IGF-1R [38]. Since HCC is characterized by strong neo-angiogenesis [39] with VEGF as its main mediator we investigated the upstream IGF-1/IGF-1R signal transduction and the expression of VEGF via induction of HIF-1α [13]. *L. obtusiloba* extract blocked the basal and IGF-1-induced protein expression of HIF-1α and VEGF accompanied by decreased phosphorylation of Akt, Stat3 and Erk. (Figure 2, Tables 1, 2, 3). Since a forced activation of Akt, Stat3 and Erk was shown to protect from apoptosis and to induce VEGF expression [40,41], our results suggest that a decreased activation of the IGF-1/IGF-1R-axis due to *L.obtusiloba* extract treatment contributes to its apoptosis-inducing effects and might be a reason for the reduced expression of VEGF and HIF-1α in HCC cells treated with *L.obtusiloba* extract [11,12]. These findings are in accordance with studies using extracts from green tea describing a decreased expression of

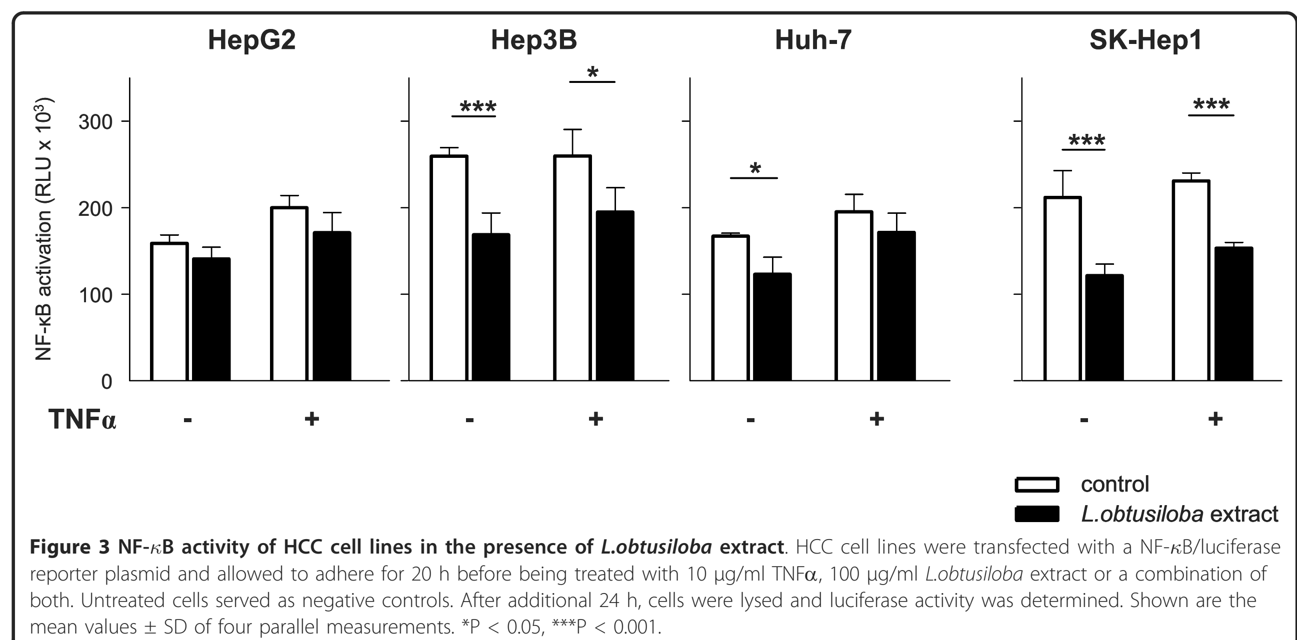

Figure 3 NF-κB activity of HCC cell lines in the presence of *L.obtusiloba* extract. HCC cell lines were transfected with a NF-κB/luciferase reporter plasmid and allowed to adhere for 20 h before being treated with 10 μg/ml TNFα, 100 μg/ml *L.obtusiloba* extract or a combination of both. Untreated cells served as negative controls. After additional 24 h, cells were lysed and luciferase activity was determined. Shown are the mean values ± SD of four parallel measurements. *P < 0.05, ***P < 0.001.

VEGF and HIF-1α accompanied by a block of PI3K/Akt-signaling in HCC cells [42].

IGF-1R signaling also impacts the expression of the transcription factor PPARγ which in turn modulates the expression of other angiogenesis-regulating proteins like COX-2 and iNOS. The implication of PPARγ in carcinogenesis is still debated. Some data show anti-tumor effects of PPARγ ligands. However, these effects could also be independent of PPARγ activation and in addition the usage of PPARγ antagonists also exerts anticancer effects [43]. In contrast to PPARγ, several studies clearly show a positive correlation between the expression of COX-2 and iNOS and HCC progression, e.g. indicated as enhanced microvessel density in HCC [44]. While COX-2 impacts growth and progression of HCC and its inhibition suppressed HCC-associated angiogenesis *in vitro* and *in vivo* [45], iNOS is a key enzyme in generating nitric oxide, thus modulating tumorigenesis by regulating tumor cell proliferation, survival and migration, as well as angiogenesis, drug resistance and DNA repair [5,46].

In line with previous reports [47,48], *L.obtusiloba* extract reduced the expression of COX-2 and iNOS (Table 2). Notably, poorly differentiated SK-Hep1 cells were susceptible to IGF-1 and inhibition of IGF-1 by *L. obtusiloba* extract. A similar result was obtained for the expression of PPARγ (Table 2). We therefore conclude that downregulation of COX 2 and iNOS by *L.obtusiloba* extract is mediated by diminished expression of PPARγ.

Beside PPARγ, IGF-R-signaling, through different upstream pathways, could trigger the activation of the transcription factor NF-κB [49] which likewise regulates COX-2 and iNOS and plays a role in viral hepatitis, chronic liver disease including fibrosis and cirrhosis and in HCC [24,50] and is spontaneously activated in HCC cells [22]. Inhibition of NF-κB reduced proliferation and invasion as well as expression of VEGF in HCC cells and sensitized the cells to sorafenib induced cell death [51].

As shown in Figure 3, *L.obtusiloba* extract markedly reduced the transcriptional activity of NF-κB in Hep3B, Huh-7 and SK-Hep1 cells and to a lesser extent in HepG2 cells. Thus, downregulation of COX-2 and iNOS by *L.obtusiloba* extract is mediated by diminished expression of PPARγ and due to a reduced transcriptional activity of NF-κB. Since NF-κB activity supports cell survival or entails anti-apoptotic effects [23,24,49], the inhibition of NF-κB by *L.obtusiloba* extract might contribute to the apoptosis inducing effects of the extract in the cancer cells (Figure 1B).

In summary, our findings *in vitro* strongly suggest *L. obtusiloba* extract as a specific compound to suppress tumor cell growth and migration and to induce apoptosis in aggressive, poorly differentiated human tumor cells via attenuation of NF-κB transcriptional activity and IGF-1R signaling. Further, the expression of key proteins in regulation of angiogenesis was reduced due to *L.obtusiloba* extract treatment. Due to its good physiological compatibility, in Korea *L.obtusiloba* extract is traditionally applied in humans to treat chronic inflammatory diseases of the liver [28]. Thus, our *in vitro* results are in line with and add more scientific strength to the traditional use of *L.obtusiloba* extract in treatment for chronic liver disease including HCC.

Regarding biologically active compounds in the extract several studies describe the isolation and structural characterization of drugs from *Lindera obtusiloba* [29,30,52]. In this line, preliminary data of us suggest that lignans such as sesamin or episesamin might contribute to the anti-fibrotic and anti-tumor effects of *L.obtusiloba* extract (not shown).

Complemental to the anti-fibrogenic, anti-inflammatory and anti-adipogenic efficacy of *L.obtusiloba* extract [31,32], our results suggest the use of an inflammation-associated tumor model of HCC to assess all aspects of the anti-tumor effects of *L.obtusiloba* extract *in vivo*.

Conclusions

Due to its potential to inhibit critical receptor tyrosine kinases involved in HCC progression via the IGF-1 signaling pathway and NF-κB, we conclude that *L.obtusiloba* extract or its active compounds represent a useful tool in a rational complementary approach e.g. with sorafenib for treatment of HCC or as cancer preventive agents.

Acknowledgements
This study was supported by the Collaborative Research Centers (SFB366 C5/C10) and (SFB633 Z1) from the Deutsche Forschungsgemeinschaft, and by the Medical Research Council, UK.

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Authors' contributions
CF participated in the design and coordination of the study, carried out the analyses and wrote the manuscript. MR and UE helped to draft the manuscript. UN and DS provided the HCC cell lines and helped to draft the manuscript. KK helped to prepare the *L.obtusiloba* extract and helped to draft the manuscript. WTK helped to prepare the *L.obtusiloba* extract and participated in the design of the study. TS designed the cell transfection experiments. MZ helped to draft the manuscript. RS participated in the data interpretation and manuscript preparation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 24 January 2011 Accepted: 12 May 2011
Published: 12 May 2011

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Pre-publication history

The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1472-6882/11/39/prepub

doi:10.1186/1472-6882-11-39
Cite this article as: Freise *et al.*: A hepatoprotective *Lindera obtusiloba* extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines. *BMC Complementary and Alternative Medicine* 2011 **11**:39.

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2.3.2 (+)-Episesamin, isoliert aus *Lindera obtusiloba*, zeigt protektive Effekte bei Fibrose und Neoplasie *in vitro*

- (Trowitzsch-Kienast W., Rühl M., Kim KY., Emmerling F., Erben U., Somasundaram R. and **Freise C.**: Absolute Configuration of Antifibrotic (+)-Episesamin Isolated from Lindera obtusiloba BLUME. Z. *Naturforsch. C* 2011 66(9-10), 460-4)
- (**Freise C.**, Ruehl M., Erben U., Seehofer D, Kim KY., Trowitzsch-Kienast W., Somasundaram R.: (+)-Episesamin exerts anti-neoplastic effects in human hepatocellular carcinoma cell lines via suppression of nuclear factor-kappa B and inhibition of MMP-9. *Invest New Drugs* 2011 30(6), 2087-95)

Um mögliche Einzelsubstanzen aus LOE zu isolieren, wurde ein 70%iges ethanolisches Extrakt aus Zweigen der Heilpflanze *Lindera obtusiloba* hergestellt. Dieses wurde in unterschiedlich polare Isolate fraktioniert, die *in vitro* hinsichtlich ihres Potenzials zur Hemmung u.a. der Proliferation von HSZ untersucht wurden. Die stärksten Effekte zeigten sich durch die Chloroform-Fraktion, welche neben der HSZ-Proliferation auch die autokrine, TGF-β1-induzierte Expression von TGF-β1 selbst hemmte. Nach chromatographischer Auftrennung und Analyse der Chloroform-Fraktion konnte eine kristalline Verbindung isoliert werden, welche mittels Kernspinresonanzspektroskopie (NMR), Schmelzpunktanalyse und der Bestimmung der optischen Aktivität als (7S,7´R,8R,8´R)-3,4:3´,4´-bis(methylenedioxy)-7,9´:7´,9-diepoxy-lignan [(+)-Episesamin; ES] identifiziert wurde. Mittels Röntgendiffraktometrie konnte so erstmals die absolute Konfiguration für ES bestimmt werden. In Zellkulturversuchen führte ES als Reinsubstanz, ähnlich wie das Chloroform-Extrakt, zu einer Hemmung der HSZ-Proliferation und der autokrinen TGF-β-Expression in HSZ ohne dabei zytotoxisch zu wirken.

Die zweite Arbeit beschreibt den erstmaligen Einsatz von ES in *in vitro*-Studien mit den humanen HCC-Tumorzelllinien HepG2, Huh-7 und SK-Hep1. ES (10 µM) hemmte deren Proliferation um ~50%, reduzierte die Invasion und induzierte Apoptose. ES-behandelte Tumorzellen zeigen eine verringerte basale und TNFα-induzierte Aktivierung von NF-κB, begleitet von einer verminderten TNFα- und Lipopolysaccharid (LPS)-induzierten Sekretion von pro-inflammatorischem IL-6. Hinweise auf hemmende Effekte von ES bezüglich Angiogenese ergaben sich durch eine deutlich verringerte VEGF-Sekretion der Tumorzellen nach ES-Behandlung. Dies war von einer verminderten Sekretion und enzymatischen Aktivität der Gelatinase MMP-9 begleitet, welche insbesondere in invasiven HCCs verstärkt exprimiert wird. Zusammengefasst zeigt dies, dass ES anti-neoplastisch wirksam ist und diese Wirkung u.a. mit einer Hemmung der Aktivierung von NF-κB, sowie verminderter VEGF- und MMP-9-Sekretion bzw. -Aktivität einhergeht. Die Ergebnisse dienen als Rationale für eine weitere Testung von ES in spezifischen Tiermodellen.

Ähnlich wie TRAM-34 sind die protektiven Effekte von ES nicht auf Leberfibrose und HCC beschränkt: Die im nachfolgenden Kapitel beschriebene Arbeit behandelt protektive Effekte von ES im *in vitro*-Modell der Entzündungs- bzw. Zytokin-induzierten VSMC-Aktivierung, einem grundlegenden Prozess im Rahmen der Atheroskleroseentstehung.

http://www.znaturforsch.com/s66c/s66c0460.pdf

http://dx.doi.org/10.1007/s10637-011-9762-x

2.3.3 Anti-atherosklerotische Effekte von (+)-Episesamin im *in vitro*-Modell in glatten Gefäßmuskelzellen

- (Freise, C. and Querfeld U.: The lignan (+)-episesamin interferes with TNF-alpha-induced activation of VSMC via diminished activation of NF-κB, ERK1/2 and AKT and decreased activity of gelatinases. *Acta Physiol (Oxf)*, 2015. **213**(3): 642-52).

Aufgrund seiner zuvor gezeigten anti-inflammatorischen Eigenschaften, wurde die Wirkung von ES auf die TNF-α-induzierte Aktivierung von VSMC untersucht, welche mit verstärkter Migration und Proliferation einhergeht und so zur Entstehung und Progression der Atherosklerose beiträgt. In humanen und murinen VSMC hemmte ES die basale sowie die TNF-α-induzierte Proliferation sowie Migration durch eine Hemmung der Aktivierung von ERK1/2 bzw. Protein Kinase B (AKT), zwei Signalmolekülen des MAPK- bzw. des Phosphatidylinositol-4,5-Bisphosphat 3-kinase (PI3K)-Signalweges. Analog zu den HCC-Zellen hemmte ES in den VSMC auch die TNF-α-induzierte Aktivierung des Transkriptionsfaktors NF-κB. Dies führte, neben einer verminderten Proliferation, auch zu einer verminderten Genexpression bzw. Sekretion von MMP-2 und MMP-9, welche maßgebliche Faktoren bei der Migration von VSMC darstellen. Neben der Leberfibrose fördert oxidativer Stress auch die Progression der Atherosklerose. ES hemmte in dieser Studie den durch H_2O_2 und durch TNF-α induzierten oxidativen Stress in VSMC, u.a. durch Induktion des anti-inflammatorischen Moleküls *heme-oxygenase-1* (HO-1). Zusammengefasst weisen die Ergebnisse damit auf ein mögliches therapeutisches Potential von ES auch bei kardiovaskulären Erkrankungen hin, welches in entsprechenden Tiermodellen verifiziert werden soll.
http://dx.doi.org/10.1111/apha.12400

3 Diskussion

Eine Fibrose oder Zirrhose der Leber und analog auch die Fibroseentstehung in anderen Organsystemen, wie z.B. im Darm, sind Resultat eines pathologischen Ungleichgewichts von Auf- und Abbau der EZM, u.a. im Rahmen (chronisch) inflammatorischer Prozesse. MMPs als proteolytischen Enzymen kommt dabei eine zentrale Bedeutung zu. Ein besseres Verständnis der Interaktion von MMPs mit ihren Substraten und Bindungspartnern und deren gezielte Modulation könnte daher perspektivisch eine Grundlage für das bessere pathophysiologische Verständnis der Fibrose sowie die Ableitung/Entwicklung von neuen - eventuell sogar nur lokal wirkenden - Therapieansätzen darstellen.

Vor diesem Hintergrund beschreiben die vorgelegten Arbeiten eine mögliche Beeinflussung der regulatorischen Funktion von Molekülen/Fragmenten der EZM bei der Pathogenese der Leberfibrose und des HCC u.a. durch Nutzung synthetischer Kollagenpeptide.

Neben Untersuchungen auf unmittelbarer Matrixebene wurden komplementär hierzu auch neue Ansätze der Beeinflussung der Fibro- und Karzinogenese auf zellulärer Ebene durch synthetische Ionenkanalinhibitoren und eines pflanzlichen Extrakts von *Lindera obtusiloba*, für das anti-fibrotische Effekte in der fernöstlichen Medizin beschrieben wurden, näher untersucht.

3.1 Moleküle und Fragmente/Peptide der extrazellulären Matrix als potentielle Modulatoren des Matrixumbaus

Ursprünglich wurde die EZM nur als passiver, stützender/einbettender Gewebsverband für Organe und Zellen angesehen. Erst seit etwa 1975 ermöglichten molekular- und zellbiologische Techniken die nähere Charakterisierung der EZM [148]. Heute ist bekannt, dass die EZM eine wichtige regulatorische Funktion bei physio- und pathologischen Vorgängen einnimmt. Zum einen dient die EZM durch ihre Bindungseigenschaften als Reservoir für Zytokine oder Enzyme [149-153]. Zum anderen können durch Degradation von Matrixmolekülen kleine, biologisch aktive Matrixfragmente (Matrikine) generiert werden, welche zelluläre Prozesse (Wachstum, Differenzierung) aktiv beeinflussen können [47, 54, 154] und auch als neue Therapeutika z.B. für Tumoren untersucht werden [155]. Ein neuer Aspekt in diesem Kontext ist die Beeinflussung nicht von zellulären Vorgängen, sondern von Matrix-degradierenden MMPs durch **Moleküle/Fragmente der EZM**.

Die hier vorgestellten Arbeiten zeigten, dass vor allem ProMMPs an kollagene Strukturen der EZM binden [156, 157]. Spezifische Bindungspartner sind dabei u.a. Kollagene, welche teilweise nicht-Substrate der MMPs darstellen (z.B. die Kollagen VI-Bindung von ProMMP-2/-9) [156, 157]. Als Folge der Bindung an die EZM stehen MMPs, im Sinne einer Speicherung in der EZM, nicht für Matrix-degradierende Prozesse zur Verfügung. Umgekehrt leitet sich daraus ein Ansatz ab um MMPs wieder vermehrt bioverfügbar zu machen: Durch Kollagen-ähnliche Peptide wie $(GPO)_{10}$ bzw. isolierte Kollagenfragmente wie α2(VI) kann die Kollagenbindung der MMPs kompetitiv gehemmt und inaktive

ProMMPs aktiviert werden, bzw. aktivierte MMPs vor Autodegradation geschützt werden. Neben einer erhöhten Bioverfügbarkeit kann so auch eine höhere bzw. längere enzymatische Aktivität der MMPs induziert werden [156-158].
Obwohl in frühen Stadien der Leberfibrose vermehrt aktivierte, eigentlich Kollagen-abbauende MMPs nachgewiesen werden [16], zeigt sich mit Fibroseprogression dennoch eine vermehrte Ablagerung der Kollagene I, III und IV. Dies wird unterschiedlich diskutiert: Einerseits wird die Kollagen-synthetisierende HSZ MMP-2-vermittelt stimuliert [17, 18], andererseits wird die Aktivität von MMP-2 durch ebenfalls vermehrte Expression von TIMP-2 gehemmt [137]. Ein MMP-2-vermittelter Stimulus der HSZ nimmt so ab, es werden aber auch weniger Kollagene degradiert. HSZ sind neben Kollagen auch die Hauptproduzenten von MMPs wie ProMMP-2. Ein Großteil der Matrix-lokalisierten ProMMP-2-Moleküle ist daher durch ihre Affinität für kollagene Strukturen im perizellulären Raum der HSZ zu erwarten. Durch eine gezielte Mobilisierung und Aktivierung der Matrix-gebundenen ProMMP-2-Moleküle könnte ein lokaler fibrolytischer Stimulus gesetzt werden. Dies setzt aber auch eine hohe Organspezifität voraus, um mögliche systemische Nebenwirkungen durch Aktivierung von ProMMP-2 zu begrenzen. Hier ist die Kopplung von $(GPO)_{10}$ oder der α2(VI) an Leber- bzw. sogar HSZ-spezifische Trägersubstanzen wie Mannose-6-Phosphat möglich [159-161].

Zentrale Bedeutung für die Fibroseresolution hat das **TIMP-MMP-Gleichgewicht**. Eine Verschiebung zugunsten der MMPs, z.B. durch adenovirale Überexpression von MMP-1 oder MMP-8, führt zu einem erhöhten Anteil an freien und proteolytisch aktiven MMPs, welche durch erhöhten Matrixabbau zur Resolution der Fibrose beitragen können [162, 163]. Eine erhöhte MMP-1- und -8-Aktivität stimuliert auch die Apoptose der HSZ, die durch Abbau perizellulärer Matrix Liganden für anti-apoptotische Adhäsionsmoleküle verlieren [21]. Dadurch könnten neben der fibrotischen Neomatrix gleichzeitig die HSZ als Haupt-Matrixproduzenten eliminiert werden. Die eigenen Arbeiten zeigten, dass Kollagen VI, bzw. im speziellen KVI-F/PR und die daraus isolierten tripelhelikalen Abschnitte der Einzelketten, potente Bindungspartner vor allem der inaktiven Proformen der Kollagenasen (inklusive der o.g. MMP-1 und -8) darstellen. Kollagen VI könnte damit eine ähnliche Speicher- und Schutzfunktion bezüglich der enzymatischen Aktivierung der Kollagenasen zukommen wie Kollagen I für die Gelatinasen. Im Umkehrschluss können Kollagen VI-Ketten wie α2(VI), bzw. daraus abgeleitete Mimetika, durch Modulation der Kollagen-MMP-Bindung ein mögliches Werkzeug zur Beeinflussung von MMP-assoziierten, pathologischen Vorgängen wie der Leberfibrose darstellen. Allerdings ist unklar, wie die Forschungsergebnisse/Zeitabläufe der Tiermodelle auf den Menschen übertragbar sind. In Tier-Fibrose-Modellen entsteht die Fibrose/Zirrhose in der Regel in einem Zeitraum von ca. vier Wochen, während der Entstehungsprozess im Menschen i.d.R. chronisch über Jahre bis Jahrzehnte verläuft, so dass auch bestimmte Aspekte des EZM-Aufbaus und damit die Einflussmöglichkeiten im Menschen verändert sind.

Insbesondere vor dem Hintergrund, dass oft die eigentliche Ursache der chronischen Lebererkrankung nicht immer beseitigt werden kann, z.B. bei der chronischen Virushepatitis B/D, gewinnen komplementäre Therapieansätze wie die Modulation der EZM bzw. des EZM-Abbaus durch Matrixfragmente eine verstärkte Bedeutung: Wird hier doch die gemeinsame „Endstrecke“ vieler chronischer Lebererkrankungen, nämlich die Fibrose, direkt moduliert.

Neben der Leberfibrose sind MMP-spezifische Wirkstoffe auch bei **Neoplasien** interessant. Tumorzellen durchdringen mit Hilfe sezernierter MMPs Gewebebarrieren, woran neben den Gelatinasen auch Kollagenasen wie MMP-13 beteiligt sind [100, 102]. Therapeutische Ansatzpunkte sind spezifische, enzymatische MMP-13-Inhibitoren sowie Inhibitoren der Genexpression von MMP-13 in Tumorzellen [164]. In diesem Zusammenhang bietet sich die Austestung von α2(VI) als potenten Inhibitor kollagenolytischer Aktivität und – konzentrationsabhängig – auch gelatinolytischer Aktivität, an [156].

Neben Zellwachstum oder Invasion (HSZ bei der Leberfibrose; Tumorzellen bei Tumoren) beeinflusst die Matrix auch die EMT [43, 44]. Durch Induktion von MMP-3, welche einen natürlichen Tumorpromotor darstellt [165], kann die EMT normaler Brust-Epithelzellen zu einem Fibroblasten-ähnlichen Phänotyp induziert werden. Zudem konnten MMP-1 und MMP-7 als fördernde Faktoren für die Ausbildung eines HCC-begünstigenden Milieus identifiziert werden [109]. Daraus ergeben sich ebenfalls rationale Ansätze um die Tumor-fördernde Aktivität der MMPs durch deren gezielte Freisetzung und/oder Inhibition, z.B. durch α2(VI), zu hemmen.

Um den tatsächlichen Nutzen von α2(VI) oder $(GPO)_{10}$ zu klären, sind weitere Versuche in spezifischen Tiermodellen nötig. Zum Beispiel mit synthetischen Peptiden aus der Aminosäuresequenz der α2(VI)-Kette, um Kollagen- und MMP-spezifische Motive zu lokalisieren. Fernziel ist es, synthetische Kollagenmimetika mit hoher MMP- und Organspezifität als therapeutische Werkzeuge zu etablieren.

Einen Hinweis auf einen therapeutischen Nutzen des Kollagenmimetikums $(GPO)_{10}$ lieferte dessen Einsatz im akuten Modell der murinen DSS-Colitis [166]. Auch wenn die intestinale Fibrose in diesem Modell im Vergleich z.B. zu der TNBS-induzierten Colitis [167, 168] oder dem TGF-β-Gentransfer-Modell [169] etwas schwächer ausgeprägt ist, zeigen sich anhand u.a. vermehrter Kollagenablagerungen, Infiltrationen von Immunzellen sowie erhöhten Expressionen von MMP-2 und -9 [170] dennoch typische Zeichen der Pathophysiologie der intestinalen Fibrose [171]. Tatsächlich bewirkte der Einsatz von $(GPO)_{10}$ im Modell der DSS-Colitis u.a. eine Verbesserung der klinischen Symptome sowie histopathologischer Veränderungen. Zymographien aus Colon-Gewebsproben von $(GPO)_{10}$-behandelten Kontroll-Mäusen zeigten, in Übereinstimmung mit Daten der *in vitro*-Vorversuche, einen erhöhten Gehalt von aktivem MMP-2 und einen verringerten MMP-9 Gehalt.

Während MMP-2 vor allem in der EZM der Submukosa des Darms lokalisiert ist, liegt MMP-9 perizellulär in der Lamina propria vor [172, 173]. Die Gewebsverteilung und Substratspezifität beider MMPs wird durch ihre CBD beeinflusst, welche die Interaktion der Enzyme mit Substraten und nicht-Substraten ermöglicht. Wie *in vitro* gezeigt, interagiert $(GPO)_{10}$ mit der CBD und setzt so kollagengebundene ProMMPs frei. ProMMP-2 wird dabei gleichzeitig aktiviert, während bei ProMMP-9 nur die Kollagenbindung gehemmt wird, ohne dass es zu einer Aktivierung kommt [157].

Aus den eigenen Ergebnissen und Literaturdaten lassen sich damit folgende Wirkhypothesen für $(GPO)_{10}$ ableiten, welche in Folgestudien überprüft werden sollen: a) $(GPO)_{10}$ setzt Matrix-gebundenes ProMMP-9 frei (ohne es zu aktivieren [174]), einem wichtigen Schritt um MMP-9-vermittelte Gewebsschäden [105, 106] zu minimieren; b) $(GPO)_{10}$ aktiviert Matrix-gebundenes ProMMP-2 und fördert so dessen regenerative Funktion auf das Epithel [107] (vgl. Kap. 1.5.4); c) $(GPO)_{10}$ induziert die Produktion anti-inflammatorischer Zytokine wie IL-10 [166] und vermindert so die intestinale Entzündungsreaktion; d) durch die Wirkung auf MMP-2 und MMP-9, zwei wichtigen Mediatoren des Tumorstroma, könnte $(GPO)_{10}$ möglicherweise die Inzidenz kolorektaler Malignome bei chronisch intestinaler Entzündung reduzieren [102, 164].

Abseits MMP-spezifischer Wirkungen von Kollagen-Fragmenten, sind auch deren vorbeschriebene **mitogene Effekte** auf Zellen von Interesse. KVI-F/PR stimuliert die Proliferation verschiedener mesenchymaler Zelltypen inklusive der HSZ [175] und wirkt zudem anti-apoptotisch [47]. Diese Effekte können durch Vorinkubation mit α2(VI) oder α3(VI) antagonisiert werden, vermutlich durch Blockade des Kollagen VI-Rezeptors [138]. Mit dem pro-mitogenen und anti-apoptotischen KVI-F/PR und der entgegengesetzt wirkenden α2(VI)-Kette stehen somit potentiell zwei weitere Regulierungsansätze zur Modulation der Fibrogenese/Fibrolyse zur Verfügung, mit denen sowohl MMPs als auch Zellen gezielt beeinflusst werden könnten. Zusammengefasst liefern die Ergebnisse der hier diskutierten Arbeiten die Grundlage für den Einsatz von $(GPO)_{10}$ und Kollagen VI-Kettenfragmenten bzw. strukturell ähnlichen Analoga in Tiermodellen der Fibrose bzw. des HCC.

3.2 Anti-fibrotische und anti-neoplastische Effekte durch Inhibition des Kalzium-aktivierten Kaliumkanals mit mittlerer Leitfähigkeit (KCa3.1) mit TRAM-34

Das Konzept der Inhibition von Ionenkanälen als anti-fibrotischen Ansatz findet in den letzten Jahren immer mehr Anwendung. So konnte z.B. durch Inhibition des Ionenkanals *transient receptor potential melastain 7* (TRPM7) die Proliferation und die Aktivierung von HSZ gehemmt werden [29, 30], wobei die TRPM7-Expression in HSZ interessanterweise durch TGF-β1 induziert werden kann [176]. Die ubiquitär exprimierten TRPM7 spielen vor allem bei der intrazellulären Magnesiumhomöostase eine Rolle, sind aber auch an vielen zellulären bzw. pathologischen Prozessen diverser Krankheiten involviert [7, 8]. Ebenso wurde der *transient receptor potential vanilloid 4* (TRPV4), welcher u.a. den Influx von

Kalziumionen in Zellen steuert, als anti-fibrotische Zielstruktur untersucht. Durch pharmakologische Inhibition oder TRPV4-*knockdown* mit siRNAs wurde die TGF-β1-induzierte Proliferation in HSZ gehemmt [12].

Die in den eigenen Arbeiten untersuchten **KCa3.1** steuern den Efflux von Kaliumionen und damit die Polarisation der Zellmembran bzw. den damit einhergehenden Influx von Kalziumionen. Da die Kalziumionenaufnahme notwendig für den Verlauf des Zellzyklusses ist, gelten KCa3.1-Inhibitoren als potentielle anti-proliferative Wirkstoffe [177, 178]. Vor dem Hintergrund einer verstärkten HSZ-Proliferation als grundlegenden Prozess der Fibrogenese in der Leber [179], stellt die in eigenen Arbeiten gezeigte Proliferationshemmung und der induzierte Zellzyklusarrest in HSZ durch Inhibition von KCa3.1 mit TRAM-34 einen grundlegenden Wirkmechanismus dar [89]. Unsere Befunde ähneln damit einer früheren Studie, in der TRAM-34 in einem *in vivo*-Modell der renalen Fibrose die Anzahl der interstitiellen renalen Myofibroblasten, den Pendants zu den HSZ in der Leber, reduzierte und wichtiger noch, die Entstehung der induzierten renalen Fibrose hemmte [180]. Ein weiterer Wirkmechanismus von TRAM-34 in den eigenen Studien war die Störung der pro-fibrotischen TGF-β1-Signaltransduktion [89]. Ein vergleichbarer Befund wurde auch im Modell der renalen Fibrose gezeigt, wo u.a. durch einen KCa3.1-*knockdown* die TGF-β1-Signaltransduktion und damit auch die Genexpressionen u.a. von Kollagen I und *alpha smooth muscle actin* unterdrückt wurde [181, 182].

Darüber hinaus führte TRAM-34 im Rattenmodell der durch Gallengangsligation induzierten Leberfibrose zu einem verminderten portalen Druck, einer typischen Komplikation bei fortgeschrittener Fibrose [89]. Maßgeblichen Einfluss auf die Entstehung von portalem Druck hat die Kontraktilität von Gefäßzellen, die auch assoziierte Komplikationen wie Aszites und Blutungen aus gastroösophagealen Varizen [183] beeinflusst. In humanen, Myofibroblasten-ähnlichen Zellen führte die Inhibition von KCa3.1 durch TRAM-34 u.a. zu einer verminderten Freisetzung intrazellulärer Kalziumdepots und damit verminderter Kontraktilität [184]. Bisher gibt es zwar keine gezielten Studien zur möglichen Assoziation von Kontraktilität und der Behandlung mit TRAM-34 in HSZ, allerdings zeigten Studien mit TRAM-34 hemmende Effekte in anderen Myofibroblasten, in denen die Regulation der Kontraktilität identisch zu den HSZ verläuft [185-188]. Dies, zusammen mit eigenen *in vivo*-Befunden, weist auf die Reduktion des portalen Drucks sowie die Reduktion der Kollagen-neosynthese als wichtige Wirkmechanismen von TRAM-34 hin.

Interessanterweise führt, im Gegensatz zu der Inhibition von KCa3.1, die Inhibition von Kalzium-aktivierten Kaliumkanälen mit <u>geringer</u> Leitfähigkeit (KCa1.1) mit Iberiotoxin zu einem erhöhten portalen Druck [189], was die Variabilität der Kalium-Kanäle und die Bedeutung von sehr selektiven Inhibitoren (für therapeutische Zwecke) veranschaulicht.

Im Gegensatz zu anderen KCa3.1-Inhibitoren wie CLT, zeigt TRAM-34 selbst in Langzeitstudien keine toxischen Effekte in verschiedenen Tiermodellen [190]. Eine jüngere Studie weist jedoch auf eine mögliche Hemmung einiger rekombinanter Cytochrom P450-Isoformen durch TRAM-34 hin [191], so das mögliche Cytochrom P450-spezifische Wirkstoffwechselwirkungen durch TRAM-34 *in vivo* in Zukunft untersucht werden sollten.

Neben anti-fibrotischen Effekten zeigt TRAM-34 auch anti-neoplastisches Potential *in vitro*. Bisher war bekannt, dass TRAM-34 das Wachstum von endometrialen und Pankreas-Tumorzellen hemmt [192, 193]. Die in eigenen Arbeiten gezeigte Wachstumshemmung in humanen HCC-Zelllinien war von keiner Apoptose-Induktion begleitet [194], analog zu Versuchsreihen in Melanomzellen [195]. Als ein möglicher Mechanismus wird, wie in den HSZ [89], die Induktion eines Zellzyklusarrestes in der G_0/G_1-Phase vermutet [192]. Als einen weiteren wichtigen Ansatzpunkt für die Wirkung von TRAM-34 in den HCC-Zellen wurde die Inhibition der Aktivierung des Transkriptionsfaktors NF-κB gefunden, welcher beim HCC spontan aktiviert ist [196] und das Überleben von Tumorzellen bzw. anti-apoptotische Effekte fördert [197-199]. Aus Versuchen mit der Brustkrebszelllinie MCF-7 war bekannt, dass TRAM-34 ERα aktiviert [200]. Unsere Studien dagegen zeigten, dass TRAM-34 die ERα-Expression in der aggressiven HCC-Zelllinie SK-Hep1 hemmt und dies, genauso wie ein siRNA-induzierter ERα-*knockdown*, zu einem verminderten Tumorzellwachstum führt [194]. Ebenso wie in HSZ, reduziert TRAM-34 auch die Genexpression von KCa3.1 in HCC-Zellen, u.a. durch die oben beschriebene NF-κB-Hemmung [194].

Durch die effektive Wachstumshemmung in den Tumorzellen und die relativ gute physiologische Verträglichkeit von TRAM-34 [190], welche besonders bei einer eingeschränkten Leberfunktion, z.B. bei vorliegender Zirrhose als Grunderkrankung, von Bedeutung ist, etablieren diese Arbeiten TRAM-34 für eine weitere Testung in spezifischen Tiermodellen des HCC.

3.3 *Lindera obtusiloba* und (+)-Episesamin als potentielle Wirkstoffe bei Fibrose und Neoplasie

In der fernöstlichen Heilkunde hat die Behandlung chronischer Lebererkrankungen mit pflanzlichen Wirkstoffen eine lange Tradition, wobei experimentelle/quantifizierbare Wirkbelege teilweise nur unzureichend sind [201, 202]. Während sich in der „westlichen Medizin“ die Suche nach neuen Wirkstoffen auf molekulare, zelluläre und pharmakologische Eigenschaften fokussiert, steht beim fernöstlichen Ansatz eher die reine empirische Effizienz im Vordergrund [203, 204]. Zur Untersuchung anti-fibrotischer Wirkungen von LOE haben wir daher den ersten Ansatz verfolgt, wobei für LOE anti-proliferative, anti-oxidative und anti-fibrotische Effekte gezeigt werden konnten [205, 206], was die überlieferte hepatoprotektive Wirkung bestätigte.

Im *in vitro*-Modell der Leberfibrose mit HSZ hemmte LOE deren Proliferation ohne die Vitalität bzw. Apoptose zu beeinflussen, was eine mögliche Induktion eines wieder quieszenten Phänotyps impliziert.

Ähnliches wurde auch für Baicalein, einem Wirkstoff aus Sho-saiko-to [207], Sho-saiko-to selbst [208] und *Compound 861* [209] gezeigt. Zudem zeigte LOE hepatoprotektive Effekte durch Inhibition von Staurosporin-induzierter Apoptose in parenchymalen Leberzellen ohne zytotoxische Nebeneffekte [205].

Während LOE keine Apoptose in aktivierten HSZ induziert, wird genau diese mögliche, durch Apoptose-induzierte, Elimination aktivierter HSZ bzw. Myofibroblasten als potentieller therapeutischer Ansatz diskutiert. Ein positiver Effekt wird durch reduzierte Kollagen- und TIMP-Synthese u.a. in Rattenlebern beschrieben, als Folge des Wegfalls der Myofibroblasten als MMP/TIMP-Quelle [210-212]. Dem entgegen steht eine neuere Arbeit, die HSZ bzw. Myofibroblasten als essentiell für die Leberintegrität beschreibt und deren Elimination, z.B. durch Apoptose, Gewebsnekrosen und Entzündungen in der Leber fördert [213].
Das stark fibrogene Zytokin TGF-β1 und dessen Rezeptoren werden von HSZ exprimiert [214], was zu einem autokrinen *loop* der Aktivierung von TGF-β1 bzw. der HSZ [215, 216] führt und im Vergleich zur Normalleber zu einer achtfach erhöhten Kollagen I-Expression bei fortgeschrittener Fibrose [217]. Nicht-toxische LOE-Konzentrationen hemmten die TGF-β1-induzierte Aktivierung von TGF-β1 selbst, die Expression von TIMP-1 und die Aktivität von MMP-2. Umgekehrt induzierte LOE die Expression von kollagenolytischem MMP-3, was, ähnlich wie bei Sho-saiko-to [218], das anti-fibrotische Potential von LOE unterstreicht.
Ein weiterer Fibrose-fördernder Faktor ist oxidativer Stress [219, 220]. Wie auch LOE [205], zeigten pflanzliche Wirkstoffe wie Glycyrrhizin und Sho-saiko-to im Rahmen der HSZ-Aktivierung protektive, anti-oxidative Effekte *in vitro* und *in vivo* durch Inhibition von Lipid-Peroxidation und einem verminderten Absinken des intrazellulären Glutathiongehaltes [20, 221, 222].

Vor dem Hintergrund der hepatoprotektiven Effekte haben wir auch anti-neoplastische Wirkungen von LOE in humanen Zelllinien des HCC untersucht. Durch die in den eigenen Studien gezeigten anti-neoplastischen sowie anti-inflammatorischen Effekte [205, 206, 223] bieten sich Folgestudien mit LOE zur Pharmakodynamik und –kinetik in entsprechenden Tiermodellen an. Zudem auch komplementäre Therapieansätze mit „etablierten" pharmakologischen Behandlungen, die fester Bestandteil gängiger onkologischer Forschung sind. So potenziert beispielsweise die Kombination von Sorafenib mit dem Cyclooxygenase-2 (COX-2)-Inhibitor Celecoxib die Sorafenib-vermittelte anti-Tumor Wirkung *in vitro* in HCC-Zellen [224, 225] und eine Kombination von Sorafenib mit dem monoklonalen VEGF-Antikörper Bevacizumab verstärkt die anti-Tumor Wirkung von Sorafenib beim Ovarialkarzinom [226].

Vor dem Hintergrund der traditionellen Anwendung von LOE im Menschen, bzw. der guten Verträglichkeit, ist bei guter Wirkung auch im Tiermodell eine Translation in die Klinik, möglicherweise als komplementäre Nahrungsergänzung, denkbar. Dafür ist jedoch auch eine sichere Standardisierung von LOE erforderlich. Ein erster Schritt dorthin ist die Kenntnis der pharmakologisch aktiven Wirkstoffe.

Ein solcher LOE-Wirkstoff ist ES, welcher analog zu LOE in *in vitro*-Modellen der Fibrose mit HSZ, und des HCC mit humanen HCC-Zelllinien eingesetzt und charakterisiert wurde [145, 146]. ES reduzierte u.a. die VEGF-Sekretion der Tumorzellen, wirkt also potentiell der Angiogenese entgegen. Wie auch bei LOE war ein Angriffspunkt von ES der Transkriptionsfaktor NF-κB. NF-κB gilt als Überlebensfaktor von Tumorzellen und fördert die Expression pro-angiogenetischer Proteine wie COX-2 und der induzierbaren NO-Synthase [197-199]. Interessanterweise führt eine Inhibition von NF-κB auch zu einer höheren Sensitivität von HCC-Zellen gegenüber Sorafenib, was ES wie auch LOE potentiell für komplementäre Studien mit Sorafenib empfiehlt [227].
Neben dem Wachstum ist die Invasion von Tumorzellen durch Matrixmembranen von Blutgefäßen ein wichtiger Schritt bei der Metastasierung, welcher die proteolytische Aktivität von MMPs erfordert [228]. Die Inhibition von rekombinantem MMP-9 durch ES sowie die verminderte gelatinolytische Aktivität in Überständen ES-behandelter Tumorzellen deutet auf potente anti-invasive Effekte hin.

Karzinogenese und Fibrose/Inflammation sind eng miteinander verknüpfte Prozesse [229]. Neben der Aktivierung von NF-κB, einem zentralen Mediator von Entzündungen, hemmte ES auch die LPS- und TNF-α-induzierte Freisetzung des pro-inflammatorischen Zytokins IL-6 in Tumorzellen und Makrophagen, was eine anti-inflammatorische Aktivität von ES belegte. In weiteren eigenen Arbeiten zeigte ES zudem anti-adipogene und anti-inflammatorische Effekte in Makrophagen-ähnlichen Präadipozyten sowie anti-oxidative Effekte in VSMC-Kulturen [144, 230]. ES stellt damit als Ergänzung zu LOE einen möglichen Kandidaten für die komplementäre Behandlung von Fibrose oder Neoplasien dar, dessen Austestung in weiteren Tiermodellen durch die hier vorgestellten Arbeiten eine rationale Basis erhält.

4 Zusammenfassung

Zielsetzung der Arbeiten war es, die Bedeutung von extrazellulärer Matrix, Kaliumkanälen sowie eines *Lindera obtusiloba* Extraktes bzw. daraus isoliertem (+)-Episesamin für eine mögliche Beeinflussung von Fibrose, Inflammation und Neoplasie zu untersuchen.

Die Arbeiten zeigten, dass Kollagene und Kollagenfragmente, als Hauptbestandteil fibrotischer Matrix, inaktive Proformen von Matrix-Metalloproteinasen (ProMMPs) speichern und zelluläre Prozesse wie Proliferation und Differenzierung beeinflussen. Die tripelhelikale Kollagensequenz „(Glycin-Prolin-Hydroxyprolin)$_n$" - (GPO)$_n$ - wurde als Konsensusbindungsstelle für ProMMPs identifiziert. Mit dem daraus abgeleiteten synthetischen Kollagenpeptid (GPO)$_{10}$ konnte die Kollagenbindung der Gelatinasen ProMMP-2 und -9 geschwächt bzw. gebundene Gelatinasen freigesetzt und im Fall von ProMMP-2 aktiviert werden. Dies führte *in vitro* zu verstärkter Proliferation und Invasion von hepatischen Sternzellen (HSZ) und Tumorzellen aufgrund erhöhter Verfügbarkeit von aktiviertem MMP-2.

Neben der Leberfibrose werden MMP-2 und -9 auch im Darm exprimiert und sind dort an der Aufrechterhaltung der strukturellen Integrität des Darmepithels beteiligt. Im akuten Modell der murinen DSS-Colitis zeigte (GPO)$_{10}$ neben anti-inflammatorischen Effekten auch protektive Effekte gegenüber der strukturellen Zerstörung des Darmepithels, wahrscheinlich durch Freisetzung und Aktivierung von Epithel-protektiver MMP-2 sowie durch Freisetzung, aber keiner Aktivierung von Epithel-schädigender MMP-9.

Mit der tripelhelikalen Domäne der alpha 2-Kette von Kollagen VI wurde ein weiteres Matrixfragment identifiziert, welches die Matrixbindung und enzymatische Aktivität vor allem der Kollagenasen hemmte, die Gelatinase ProMMP-2 dagegen aktivierte und vor Autodegradation schützte.

Aus pathophysiologischer Sicht wird bei der Fibrose die <u>lokale,</u> Matrix-abbauende Wirkung der MMPs durch überschüssiges Kollagen neutralisiert, so dass Kollagene akkumulieren. Durch gezielte Beeinflussung dieser lokalen MMP-Matrix-Interaktion, z.B. durch Freisetzung von MMPs mit o.g. MMP-spezifisch wirksamen Peptiden, ist ein lokal wirkender, fibrolytischer Stimulus denkbar.

Neben der Matrix befassten sich komplementäre Studien auf Zellebene mit der Expression und den Auswirkungen einer Inhibition von Kalzium-aktivierten Kaliumkanälen mit mittlerer Leitfähigkeit (KCa3.1) auf die Fibrose. Erstmals wurde eine KCa3.1-Expression vor allem in aktivierten HSZ sowie in humanen, fibrotischen Lebergewebsproben gezeigt. In quieszenten HSZ und gesundem Lebergewebe dagegen wurden KCa3.1 nicht bzw. nur schwach exprimiert. Eine KCa3.1-Inhibition mit dem spezifischen Inhibitor TRAM-34 hemmte zum einen die HSZ-Proliferation durch Induktion eines Zellzyklusarrestes und zum anderen die TGF-β1-Signaltransduktion in HSZ, einem wichtigen, fibrogenen Signalweg. Im Rattenmodell der durch Gallengangsligation induzierten Leberfibrose reduzierte TRAM-

34 den Fibrose-fördernden portalen Druck, was Effekte von KCa3.1 auf die Kontraktion der Gefäßzellen impliziert.

TRAM-34 zeigte auch anti-neoplastisches Potential in humanen Zelllinien des HCC, u.a. durch Hemmung der Aktivierung des Transkriptionsfaktors NF-κB, welcher beim HCC durch spontane Aktivierung als Überlebensfaktor gilt, und durch verminderte ERα-Expression. TRAM-34 ist physiologisch gut verträglich, was besonders bei eingeschränkter Leberfunktion, z.B. bei vorliegender Zirrhose als Vorerkrankung des HCC, von Bedeutung sein kann. Jüngere eigene Arbeiten weisen zudem auf eine Beteiligung von KCa3.1 bei der Kalzifizierung glatter Gefäßmuskelzellen hin, welche durch TRAM-34 gehemmt wurde.

Weitere Studien auf Zellebene befassten sich mit der Wirkung pflanzlicher Wirkstoffe auf Fibrose und Neoplasie. Im *in vitro*-Modell der Leberfibrose mit HSZ hemmte das *Lindera obtusiloba* Extrakt die Proliferation sowie die TGF-β1-induzierte Expression fibrogener Marker wie Kollagen I und inhibierte der Bildung reaktiver Sauerstoffradikale. In humanen HCC-Zelllinien zeigte das Extrakt anti-mitogene Effekte, einhergehend mit der Induktion von Apoptose sowie der Hemmung der IGF-1-Signaltransduktion und von NF-κB.
Mit (+)-Episesamin wurde ein pharmakologisch wirksamer Inhaltsstoff aus dem Extrakt isoliert und dessen absolute Konfiguration erstmals beschrieben. Ähnlich wie das Extrakt, zeigte (+)-Episesamin durch Hemmung von Proliferation und dem TGF-β1-Signalweg ebenfalls anti-fibrotische Effekte in HSZ. Zum anderen auch anti-neoplastische Effekte in HCC-Zelllinien durch MMP-Inhibition sowie durch Hemmung von Proliferation, NF-κB und der VEGF-Sekretion. Anti-inflammatorische Effekte von (+)-Episesamin wurden in Tumorzellen und Makrophagen durch Hemmung TNF-α- und LPS-induzierter IL-6-Sekretion sowie durch Hemmung der TNF-α- und PDGF-induzierten Aktivierung glatter Gefäßmuskelzellen gezeigt.

Wie in Abbildung 5 schematisch dargestellt, beschreiben die hier vorgestellten Untersuchungen zur Pathogenese von Fibrose und Karzinogenese zusammengefasst potentielle Möglichkeiten ihrer Beeinflussung auf Matrix- und Zellebene. Die Ergebnisse stellen eine Rationale für weitere Untersuchungen in spezifischen Tiermodellen dar, wobei auch die Kombination von Matrix- (z.B. $(GPO)_{10}$) und zellspezifischen Ansätzen (z.B. TRAM-34) denkbar ist.

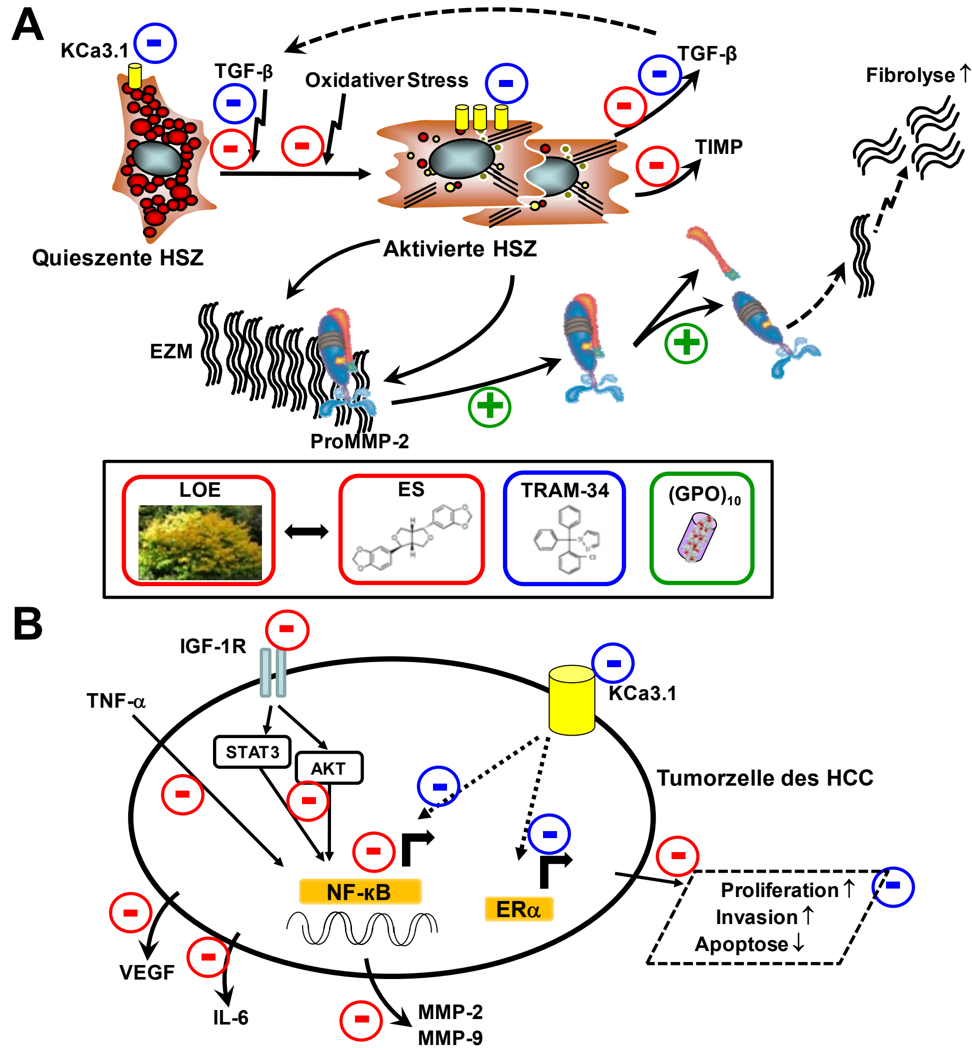

Abbildung 5: Schematische Zusammenfassung der anti-fibrotischen und anti-neoplastischen Wirkmechanismen von LOE/ES, TRAM-34 und $(GPO)_{10}$. (A - anti-fibrotische Effekte): Auf Zellebene wird die TGF-β-induzierte Aktivierung/Proliferation von HSZ durch LOE/ES sowie durch den KCa3.1-Inhibitor TRAM-34 gehemmt. Dies geht mit einer verminderten Expression von TGF-β sowie von TIMP-1 einher, was eine weitere autokrine Aktivierung durch TGF-β unterdrückt, bzw. einen Abbau der EZM durch MMPs begünstigt. TRAM-34 vermindert zudem die verstärkte KCa3.1-Expression in TGF-β-aktivierten HSZ. Auf Matrixebene zeigt sich nach Aktivierung der HSZ eine vermehrte Ablagerung von EZM sowie eine vermehrte MMP-Produktion. Die enzymatische Aktivität von MMPs wird jedoch durch eine Bindung an Kollagene effektiv gehemmt. Mit dem Kollagenpeptid $(GPO)_{10}$ kann Kollagen-gebundenes ProMMP-2 freigesetzt und aktiviert werden, was einen fibrolytischen Stimulus setzen könnte. (**B - anti-neoplastische Effekte**): LOE/ES hemmen u.a. durch Inhibition der Aktivierung bzw. der Signaltransduktion des IGF-1R und von NF-κB die Proliferation humaner HCC-Zelllinien und induzieren Apoptose. Durch eine Hemmung von Sekretion und enzymatischer Aktivität der Gelatinasen MMP-2 und -9, sowie einer verminderten VEGF-Sekretion, hemmen LOE/ES zudem die Invasion der Tumorzellen und zeigen anti-angiogenetisches Potential. Darüber hinaus zeigen beide Substanzen potente anti-inflammatorische Effekte, z.B. durch Hemmung der TNF-α-induzierten NF-κB-Aktivierung einhergehend mit verminderter IL-6-Sekretion. Eine KCa3.1-Inhibition mit TRAM-34 führt neben einer Hemmung der NF-κB-Aktivierung auch zu einer verminderten ERα-Expression, was die Proliferation der Tumorzellen hemmt. *Abkürzungen:* AKT, Protein Kinase B; ERα, *estrogen receptor alpha;* ES, (+)-Episesamin; EZM; extrazelluläre Matrix; $(GPO)_{10}$, tripelhelikales Kollagenpeptid $(Glycin\text{-}Prolin\text{-}Hydroxyprolin)_{10}$; HSZ, hepatische Sternzelle; HCC, hepatozelluläres Karzinom; IGF-1R, *insulin growth factor receptor* 1; IL-6, Interleukin-6; KCa3.1, Kalzium-aktivierter Kaliumkanal mit mittlerer Leitfähigkeit; LOE, wässriges *Lindera obtusiloba* Extrakt; MMP, Matrix-Metalloproteinase; NF-κB, *nuclear factor kappaB*; ProMMP, inaktive Proform von Matrix-Metalloproteinasen; STAT3, *signal transducer and activator of transcription* 3; TGF-β, *transforming growth factor beta*; TRAM-34, KCa3.1-Inhibitor; TNF-α, *tumor necrosis factor alpha*; VEGF, *vascular endothelial growth factor*.

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Danksagung

Besonders herzlicher Dank geht an Herrn Prof. Rajan Somasundaram, der mich seit Beginn meiner wissenschaftlichen „Karriere“ nachhaltig unterstützt und in meiner wissenschaftlichen Arbeit bestärkt hat. Ebenso herzlich bedanke ich mich bei Dr. Martin Rühl, der mich zusammen mit Prof. Somasundaram als jungen Diplomanden in seiner Arbeitsgruppe aufgenommen und stets in allen Belangen des wissenschaftlichen Alltags unterstützt hat.
Die langjährigen gemeinsamen Arbeiten zur extrazellulären Matrix und darüber hinaus bieten bis heute ein reichhaltiges Reservoir interessanter wissenschaftlicher Fragestellungen die nicht nur Grundlage dieser Habilitationsschrift, sondern bestimmt noch vieler weiterer wissenschaftlicher Abhandlungen, auch unabhängig von der Leber, sein werden.

Weiter bedanken möchte ich mich bei Dr. Ulrike Erben und allen weiteren ehemaligen Kolleginnen und Kollegen der Medizinischen Klinik I im Karl-Landsteiner Haus des CBF. Die Arbeit mit euch sowie viele gemeinsame Diskussionen, Witze und Sudokus haben für ein wundervolles Arbeitsklima (und eine tolle Ehefrau) gesorgt.

Darüber hinaus gilt mein Dank auch allen ehemaligen Kooperationspartnern wie Prof. Ki Young Kim, Prof. Ulf Neumann oder Prof. Wolfram Trowitzsch-Kienast, ohne die viele Arbeiten, Experimente und finanzielle Überbrückungen nicht möglich gewesen wären.

Ausdrücklich möchte ich mich auch bei Herrn Prof. Uwe Querfeld herzlich für meine Aufnahme in seine Arbeitsgruppe am *Center for Cardiovascular Research* der Charité sowie für sein Vertrauen und viele Freiheiten bei meiner experimentellen Forschung bedanken. Dadurch konnten viele „Leber-Projekte“ auch erfolgreich auf die nephrologische/kardiovaskuläre Ebene „übertragen“ werden.

Zu guter Letzt danke ich meiner Frau, meinen Kindern sowie meinen Eltern und Großeltern für viel Liebe und private Unterstützung während meines akademischen Weges an der Charité.

Erklärung

§ 4 Abs 3(k) der HabOMed der Charité
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Hiermit erkläre ich, dass

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- mir die geltende Habilitationsordnung bekannt ist

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Berlin, , Dr. Christian Freise