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Assessment of the anticancer mechanisms of the c-MET inhibitor tivantinib in the therapy of gastrointestinal tumors

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To My Parents

Declaration

I hereby declare that the thesis is my original work and I have not received outside assistance. All the work and results presented in the thesis were performed independently. Anything from the literature was cited and listed in the reference. No unauthorized data was included.

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

The work for the thesis began from Oct. 2010 with the supervision from Prof. Dr. med. Alexander L. Gerbes, PD.Dr. med. Enrico De Toni in the laboratory of Hepatology, Med II of University Hospital Grosshadern, Ludwig-Maximilians University Munich, Germany.

Munich, on

(Shuai Lu)

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ZUSAMMENFASSUNG

Hintergrund und Ziele: Tivantinib ist ein neu entwickelter c-MET Inhibitor, welcher derzeit für die Therapie unterschiedlicher Tumorentitäten im Rahmen klinischer Studien untersucht wird. Insbesondere zeigte dieser Inhibitor in einer Phase 2 randomisierten Studie ein Überlebensvorteil in Patienten mit hepatozellulären Karzinomen (HCC) in denen eine Überexpression von c-MET nachgewiesen werden konnte. Aufgrund der prädiktiven Bedeutung von c-MET, wurde die sukzessive, derzeit laufende Phase 3 klinische Studie von Tivantinib in selektierten „c-MET-high“ HCC Patienten durchgeführt. Trotz extensiver klinischer Untersuchung, wurde die Effektivität von Tivantinb als c-MET Inhibitor – und somit die Rationale für seine Anwendung in dieser Patientensubgruppe – von vor kurzem publizierten Studien in Frage gestellt.

Methoden: In dieser Studie untersuchten wir *in vitro* die Wirkungsmechanismen von Tivantinib. Hierfür wurde ein Panel unterschiedlicher Zelllinien aus gastrointestinalen Tumoren mit unterschiedlichen c-MET Expressionsstatus verwendet, sowie c-MET-Exon 16-KO Zelllinien, in denen die Bindungsstelle von Tivantinib nicht vorhanden ist. Zellproliferation, Apoptose, Zellzyklus und die zugrundeliegenden molekularen Veränderungen wurden durch Viabilitätanalyse, FACS, Western blot, funktionelle Caspase-Aktivierung und si-RNA-Ansätze erforscht.

Ergebnisse: Die Inkubation mit Tivantinib induzierte Apoptose und Zellzyklusarrest. Zugleich konnten die Herabregulierung von p-c-MET, der antiapoptotischen Moleküle Bcl-xl und Mcl-1, sowie die Hochregulierung von Cyclin-B1 beobachtet werden. Die biologische Wirkung von Tivantinib sowie die damit assoziierte molekulare Veränderungen konnten sowohl in c-MET-exon 16-KO Zellen, als auch in c-MET Wildtyp Zellen reproduziert werden. Die Stimulation von c-MET durch HGF führte zu einer erhöhten Phosphorylierung von c-MET sowie zu der Hochregulierung von Mcl-1 und Bcl-xl. Dies hatte dennoch keinen relevanten Einfluss auf Cyclin B1.

Zusammenfassung: Die biologische Wirkung von Tivantinib wird durch die Regulierung von Mcl-1, Bcl-xl and Cyclin B1 vermittelt. Wie durch Experimente in c-MET-KO Zellen zu sehen ist, erfolgen dieser Veränderungen aber unabhängig von der Fähigkeit dieses Medikaments p-c-MET zu inhibieren. Die Tatsache dass Mcl-1, Bcl-xl – jedoch nicht Cyclin B1 – auch durch die Inhibition von c-MET beeinflusst werden können, stellt eine mögliche Erklärung für die prädiktiven Bedeutung von c-MET in klinischen Studien dar. Da Cyclin B1, Mcl-1 und Bcl-xl unabhängig von der Expression von c-MET von Tivantinib beeinflusst werden, sollten diese Moleküle als alternative, und potentiell geeignetere Prädiktoren der therapeutischen Wirkung von Tivantinib untersucht werden.

SUMMARY

Introduction: Tivantinib is a c-MET inhibitor which demonstrated clinical benefit in patients bearing tumors exhibiting elevated c-MET expression in the clinical setting. A phase-3 trial in advanced HCC patients selected according to c-MET expression has been thus initiated. Yet, surprisingly, the effectiveness of tivantinib as specific c-MET inhibitor and thus its employment in this subset of patients have very recently been questioned.

Aims and methods: In the present paper we aimed at assessing the still not fully elucidated mechanisms underlying the antitumor effects of tivantinib and their relation to the inhibitory effect of this compound on c-MET. To this aim, analysis of cell viability, apoptosis, cell cycle and of the underlying molecular changes were conducted after tivantinib administration in a panel of cell lines exhibiting different c-MET expression status and in c-MET exon 16 MET KO cell lines, which lack the binding site for Tivantinib.

Results: Tivantinib induced caspase-dependent apoptosis and cell cycle arrest accompanied by decrease of antiapoptotic Bcl-xl and Mcl-1 and increase of Cyclin B1. Cell viability and these molecular changes were affected independently of c-MET as shown by experiments in c-MET exon 16 KO cell lines. However, stimulation of c-MET by HGF induced increase of Bcl-xl and Mcl-1 revealing that these molecules are targets downstream of c-MET.

Conclusions: Tivantinib impinges on Bcl-xl, Mcl-1 and on Cyclin B1 to cause apoptosis and cell cycle arrest independently of c-MET. However, our finding that Bcl-xl and Mcl-1 are downstream targets of c-MET provides a possible explanation for the predictive effect of c-MET in clinical trials. Cyclin B1, Mcl-1 and Bcl-xl were affected by tivantinib regardless of c-MET and should be considered as possible and potentially more reliable response predictors.

1. INTRODUCTION

1.1. HGF/c-MET Axis.

1.1.1. Structure of c-MET

The N-methyl-N'-nitro-nitroso-guanidine human osteosarcoma transforming (c-MET) proto-oncogene represents a prototypic member of receptor tyrosine kinase (RKT) family. Located on chromosome 7q21-q31, c-MET was discovered in 1984 by George Van de Wounde from a human osteosarcoma cell line and identified to form a fusion protein with translocated promoter region (*TPR*) in the chromosomal rearrangement *TPR-MET* [1, 2]. The protein of c-MET is synthesized in the post-Golgi compartment of hepatocytes as a primary single chain precursor ($p170^{met}$) and undergoes a proteolytic process to form a mature glycosylated heterodimer receptor ($p190^{met}$) which is composed of an extracellular α subunit ($p50^{met}$) and a transmembrane β subunit ($p140^{met}$). Physiologically, c-MET is expressed mainly on the surface of endothelial and epithelial cells in many organs, such as liver, pancreas, kidney and bone marrow [3]. On the extracellular side, c-MET comprises three domain modes: a semaphorin (Sema) domain, which includes the complete α subunit and the N-terminal part of the β subunit, the Plexin-Semaphorin-Integrin (PSI) domain, (which contains four disulphide bonds), and four Immunoglobulin – Plexin - Transcription domains (IPT) [4, 5]. The intracellular field of c-MET contains a tyrosine kinase domain including two catalytic tyrosines (Tyr1234 and Tyr1235) which are phosphorylated upon activation of c-MET. On the contrary, the phosphorylation of a juxtamembrane tyrosine (Tyr1003) negatively modulates the activity of c-MET by promoting polyubiquitination, endocytosis and degradation of c-MET upon the recruitment of ubiquitin ligase casitas B lineage lymphoma (c-CBL). Two other pivotal tyrosines (Tyr1349 and Tyr1356) are located in the C-terminal region and are responsible for recruiting several adapter proteins when c-MET is activated. A schematic representation of the structure of c-MET is provided in Fig. 1

[4, 5].

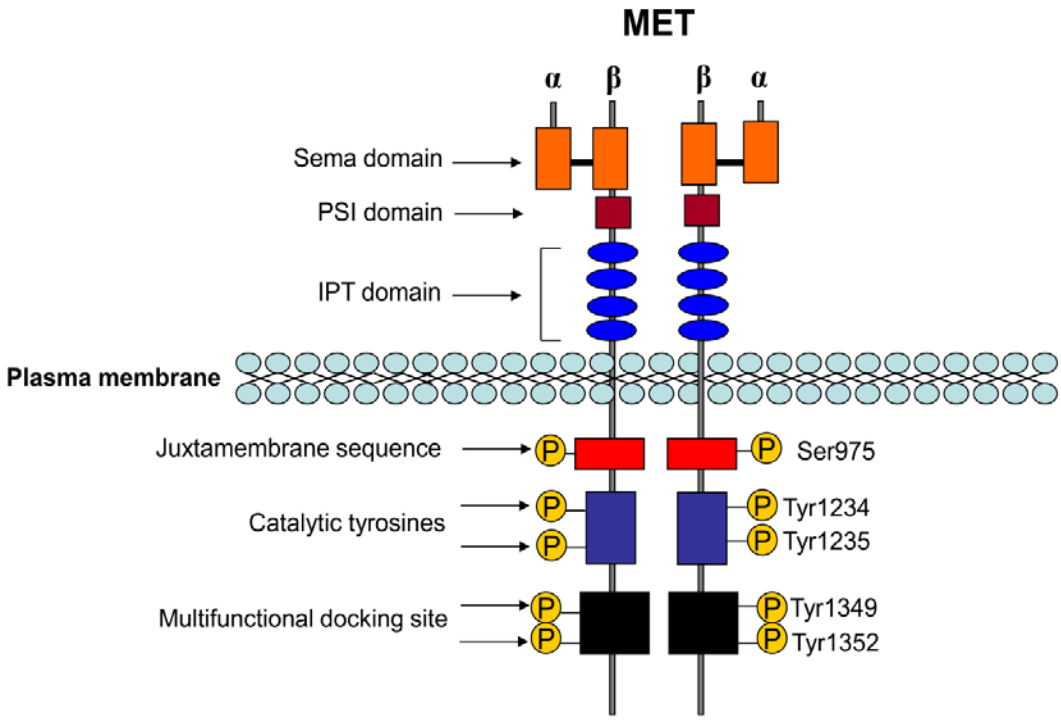


Figure 1. Schematic structure of c-MET. Mature c-MET receptor is composed of an extracellular α subunit and a transmembrane β subunit.

1.1.2. Structure of HGF

Hepatocyte growth factor (HGF - also known as "scatter factor") was discovered in 1984 and is the only known ligand for c-MET. HGF is secreted predominantly by cells of mesenchymal origin as a single chain precursor (Pro-HGF) which undergoes a proteolytic process into an active status via extracellular proteases [6-8]. The bioactive form of HGF contains an α chain including an N-terminal hairpin loop domain and four "kringle domains" (K1 to K4), and a C-terminal β chain which contains the serine protease homology (SPH) domain [9-12].

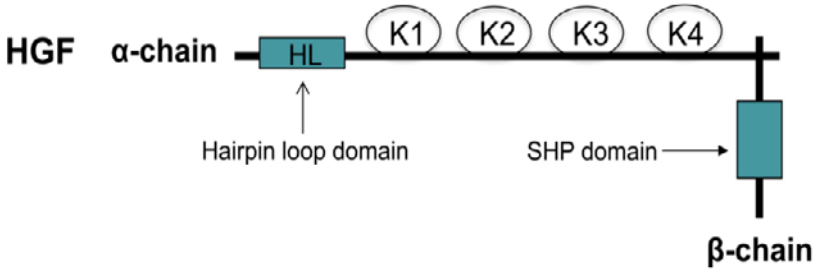


Figure 2. Schematic structure of HGF.

1.1.3. Activation of HGF/c-MET signaling pathway

Binding of HGF to the Sema domain of c-MET leads to receptor homodimerization and autophosphorylation of the two tyrosine residues Tyr1234 and Tyr1235 [13, 14]. The second step of c-MET activation implies the phosphorylation of the tyrosine sites Tyr1349 and Tyr1356 within the carboxyl terminal region of the receptor [3, 15]. Phosphorylation of these tyrosines forms a multifunctional docking site recruiting a spectrum of intracellular adaptors and effectors including adaptor proteins (growth factor-bound protein 2 - GRB2, src homology 2 domain-containing - SHC, v-crk sarcoma virus CT10 oncogene homolog - CRK and CRK-like - CRKL) and effector molecules (phosphatidylinositol 3-kinase - PI3K, phospholipase Cγ - PLCγ and SRC, the src homology 2 domian-containing 5' inositol phosphatase SHP-2 and the signal transducer and activator of transcription STAT3) [4]. Moreover, GRB2-associated binding protein 1 (GAB1), a peculiar multi-adaptor protein, binds either directly or indirectly with c-MET, recruiting more binding sites for the downstream adaptors [16-19].

Physiologically, c-MET is expressed predominantly in epithelial cells of a wide spectrum of tissues, including liver, pancreas, kidney, prostate, muscle and bone marrow, where it exerts a crucial function in embryogenesis, wound healing and tissue

repair by affecting different functions such proliferation, cell scattering, motility, mitogenesis, morphogenesis, and angiogenesis depending on the specific cell type and the microenvironment [20-24]. The importance of c-MET signaling during embryogenesis is corroborated by the fact that c-MET- or HGF-deficient mice show a severe impairment of muscle tissue formation [9, 12] and smaller liver size [25].

The effect of c-MET activation on cell proliferation, motility and morphogenesis are mediated in the different contexts by the activation of three main different signaling pathways which are schematically represented in Fig.3. These include the PI3K/Akt signaling axis, the Ras-Raf-MEK-Erk cascade and STAT3 pathway.

The PI3K/Akt signaling axis is activated upon the binding of the p85 and p110 subunits of PI3K directly to c-MET or indirectly through GAB1, activating the serine-threonine kinase Akt/protein kinase B (PKB) [26]. After its dissociation from the plasma membrane, Akt has been shown to phosphorylate the pro-apoptotic molecules Bad and Bax causing resistance to apoptosis. Furthermore, the activation of Akt leads to the phosphorylation of the Tuberous Sclerosis Complex which releases its inhibitory effect on mTOR. Activated mTOR affects cell growth and proliferation through its activity as transcription factor [27]. Activated Akt can also lead to the activation of Ras thus establishing a cross-talk between the PI3K/Akt/Tor axis and the Ras/Raf/MAPK signaling pathway [28].

The Ras-Raf-MEK-Erk cascade is activated upon binding of the adaptor protein GRB2 to the activated phosphorylated form of c-MET. This leads in turn to the recruitment of the rat sarcoma viral oncogene homolog Son of Sevenless (SOS), which causes the activation of Ras. Activated Ras, leads to the activation of the kinase B-Raf which phosphorylates and activates the MAP kinase extracellular signal-regulated kinases 1 and 2 (MEK1/MEK2) and successively the extracellular signal-regulated kinases 1 and 2 (Erk1/Erk2) which translocate into the nucleus to phosphorylate additional transcriptional factors, hereby regulating cell proliferation,

cell motility and cell cycle progression [29-32]. The Erk/MAPK axis can be additionally activated through the c-MET-mediated activation of Gab1 and SHP2 [17, 31].

The STAT3 signaling pathway is activated by c-MET through the phosphorylation of STAT which leads to its dimerization and translocation to the nucleus, which has been shown to result in tubulogenesis and invasion [33, 34].

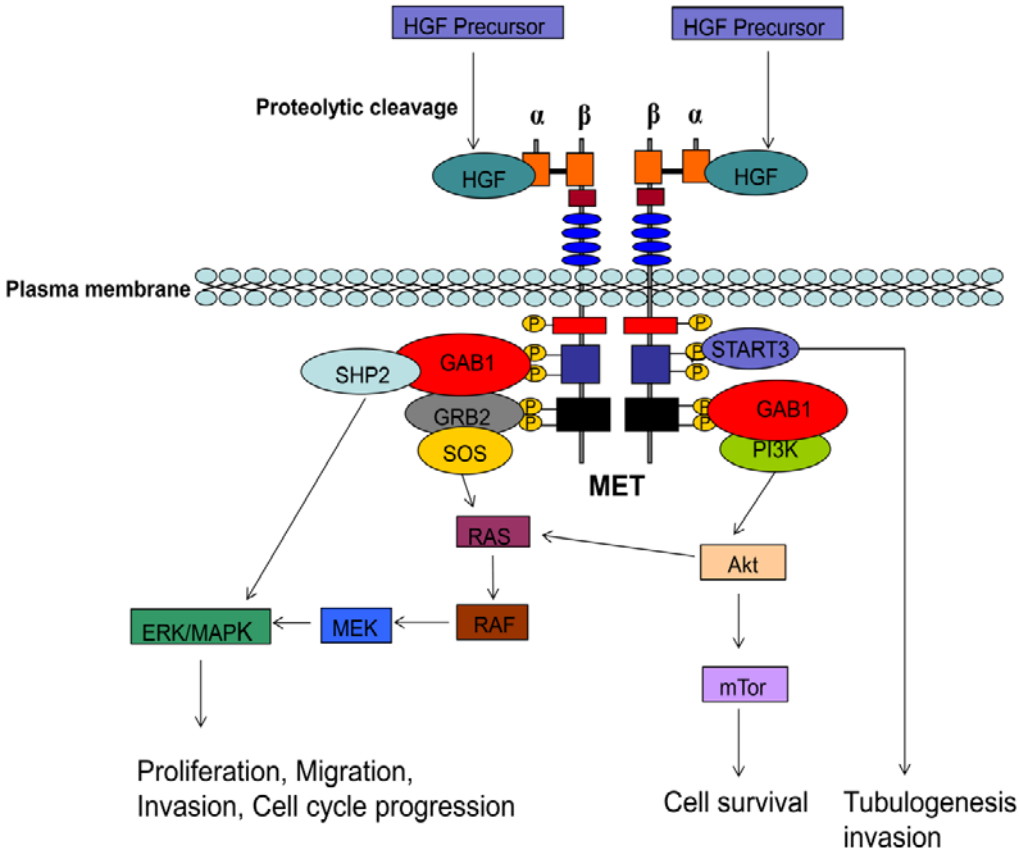


Figure 3. HGF/c-MET axis and its downstream pathways. Binding of HGF to the Sema domain of c-MET leads to autophosphorylation of Tyr1234 and Tyr1235 and subsequently the phosphorylation of the tyrosine sites Tyr1349 and Tyr1356. Phosphorylation of these tyrosines forms a multifunctional docking site recruiting a spectrum of intracellular adaptors and effectors, activating a spectrum of downstream signaling pathways, such as the PI3K/Akt-axis, the Ras-Raf-MEK-Erk cascade, and the STAT3 signaling pathway.

1.2. HGF/c-MET axis in carcinogenesis

1.2.1. Aberrant activation of HGF/c-MET

While the activation of c-MET in physiological condition is indispensable to control various aspects of cell proliferation and motility, the aberrant constitutive activation of this receptor has been shown to cause uncontrolled proliferation, cell migration and to determine the metastatic potential of cancer cells. Aberrant activation of c-MET signaling has been shown to occur due to several mechanisms which include the transcriptional upregulation of c-MET, c-MET gene mutations and amplifications, and its constitutive (HGF-dependent or –independent) autocrine or paracrine stimulation of this receptor. These different ways of c-MET aberrant activation are briefly summarized below.

Increased transcription of c-MET in the absence of c-MET amplification represents one of the major causes of constitutive activation of c-MET [35, 36]. Overexpression of c-MET has been documented in several tumor entities including hepatocellular carcinoma, colorectal cancer, cholangiocellular carcinoma, pancreatic, gastric, lung and breast carcinomas and multiple myeloma. The importance of c-MET overexpression in these tumor entities is corroborated by several studies showing a direct correlation between the overexpression of c-MET and patient's survival, an aggressive tumor phenotype, or resistance to chemotherapeutic treatment [37-40]. A summary of different reports on c-MET overexpression in different tumor entities is provided in Table 1: in hepatocellular carcinoma (HCC) patients c-MET overexpression is associated with poor differentiation, the presence of metastasis and shorter five-year survival, and with tumor recurrence after partial hepatectomy [41-44]. Correspondingly, it has been shown that transgenic mice overexpressing c-MET more frequently develop hepatocellular carcinomas and that a regression of such tumors can be achieved by pharmacological inhibition of c-MET [45]. In breast cancer, c-MET/HGF overexpression has been shown to correlate with high tumor cell

proliferation, poor differentiation, poor prognosis and higher incidence of metastases [25, 46]. High levels of c-MET and HGF expression in lung adenocarcinomas also showed a correlation with poor survival, metastases formation, resistance to radiotherapy, and to the administration of EGFR inhibitors [47-49] (Table 1).

	Expression (% or +)*		**Association**
	Met	**HGF**	**Poor prognosis**
Gastrointestinal cancers			
Hepatocellular	68-69%	+	+
Colon cancer (incl.liver Mets)	55-78%	+	+
Cholangiocarcinoma	+	+	
Pancreatic	+	+	+
Gastric carcinoma	75-90%	+	+
Other tumor types			
Breast cancer	25-60%	+	+
Lung (non-small cell)	41-72%	+	+
Multiple myeloma	48-80%		

Table.1 c-MET and HGF expression in human cancers and their correlation with prognosis. * Expression (% or +): Proportion of tumor samples that are positive for expression is shown with (%) or indicated with (+) where expression range has not been reported. Adapted from Giorgio V. Scagliotti et al. The emerging role of MET/HGF inhibitors in oncology. (From: Cancer Treatment Reviews 39 (2013), 793-801).

c-MET point mutations have been reported seldom in hepatocellular carcinoma, sporadic and inherited human papillary renal cell cancer (pRCC), NSCLC and breast carcinomas [5, 50]. The majority of these mutations occur in the c-CBL binding site of cytoplasmic juxtamembrane domain playing a role on the ubiquitination of c-MET and other receptor tyrosine kinases (RTKs) including EGFR and PDGFR. c-MET

mutations in c-CBL binding domain prevent ubiquitination which lead to c-MET overexpression and cellular transformation [51]. Missense mutations of c-MET have also been reported in the Sema and juxtamembrane domain. These mutations are very likely to play a role in determining the metastatic potential of cancer cells, since they were shown to occur more frequently in metastatic tumor tissues in comparison to their primary tumors of origin [52].

Amplification of c-MET and the consequent c-MET protein expression and activation have been found in a number of primary cancers including colon carcinoma, gastric cancers and NSCLC [53-55]. However, several reports have shown that c-MET amplification occurs more frequently in metastatic tumors, such as liver metastasis from colon carcinoma, which indicates the role of c-MET in the late phase of tumor progression [56, 57].

Autocrine and paracrine activation of c-MET. A number of neoplastic cells show intracytoplasmic positivity for HGF, indicating an autocrine pattern of c-MET stimulation through endogenous HGF secretion as possible mechanism of constitutive c-MET activation in cancer [58]. In addition to the autocrine or paracrine activation of c-MET through HGF, some evidence exists that c-MET could be constitutively activated by DCP (des-gamma-carboxy prothrombin). DCP is a well-known diagnostic and prognostic marker of hepatocellular carcinoma which is at this time undergoing extensive investigation in the screening and diagnosis of this tumor. DCP is increased in the serum of 44 to 81% of HCC patients and has been shown to bind to c-MET hereby leading to its activation and increased proliferation of HCC cells [59].

In addition to these mechanisms, activation of c-MET has been shown to occur in consequence of the interaction of c-MET with other membrane receptors like EGFR and proteinase-activated receptor-2 (PAR-2) which can interact with c-MET leading to c-MET constitutive activation and c-MET mediated cell invasion [60, 61].

1.3. The small molecule c-MET inhibitor tivantinib

The increasing understanding of the function of the HGF/c-MET axis has led to the development of several c-MET inhibitors for cancer therapy. Among these compounds, tivantinib (originally known as ARQ197) has emerged as a small molecule c-MET inhibitor with a spectrum of antitumor activity as a single agent in *in vitro* and *in vivo* investigations and clinical trials.

1.3.1. Clinical trials of tivantinib

Tivantinib has been developed as small molecule kinase inhibitor of c-MET. In recent years, this agent has been extensively studied as alternative cancer treatment in several tumor entities. After proving safe and well tolerated in phase I studies [62, 63], a phase 2 study of tivantinib as a second-line therapy was conducted in 107 advanced HCC patients after failure of a previous first-line systemic therapy with sorafenib [64]. In this study, in which patients were allocated 2:1 to receive tivantinib or placebo, tivantinib modestly improved time to progression (TTP) in the intent-to-treat population (median 1.6 vs 1.4 month; HR=0.64; p=0.04). However, in patients bearing tumors with high expression of c-MET, as judged by predetermined immunohistochemical (IHC) criteria (>2+ staining intensity in >50% of tumor cells), Overall survival (OS - median 7.2 vs 3.8 months; HR=0.64; p=0.04), time to progression (TTP - median 2.7 vs 1.4; HR=0.43; p=0.03) and progression free survival (PFS - 2.4 vs 1.5 months; HR=0.45; p=0.02) were significantly improved in comparison to placebo-treated patients. Conversely, tivantinib failed to improve OS, PFS or TTP in patients with c-MET low expression tumors. Due to these promising results, a randomized Phase III clinical trial with tivantinib as a second line therapy in patients selected according to the expression of c-MET and exhibiting a “c-MET-high” staining in tumor samples has been initiated.

A second study showing a correlation between c-MET expression and outcome of

patients treated with tivantinib is provided by a Phase II study [65] in non-small cell lung cancer (NSCLC) patients investigating the tivantinib/erlotinib vs. the placebo/erlotinib-combination. This study demonstrated a significant improvement of PFS and OS in a subset of patients with K-RAS mutations. Moreover, tivantinib in association to erlotinib delayed time to new metastasis formation in patients with non-squamous histology. However, the subsequent phase III study was terminated prematurely after an interim analysis failing to show signs of effectiveness with respect to the primary endpoint of improved OS [66]. In spite of these results in the intention-to-treat population, a successive subgroup analysis revealed a substantial improvement in the OS of the subgroup of patients carrying tumors exhibiting increased expression of c-MET [67].

Thus, tivantinib showed signs of in patients affected by different tumor entities showing overexpression of c-MET.

In addition to the clinical trials conducted with tivantinib as single agent, tivantinib has also been assessed as a combination treatment in association to the kinase inhibitor sorafenib. In particular, a phase 1 study of sorafenib and tivantinib corroborated preliminary safety and efficacy of the combination of these two agents and showed promising signs of efficacy [69].

1.3.2. Mechanisms of action of tivantinib and issues on its specificity as c-MET inhibitor

Tivantinib has been initially presented as a highly selective, orally bioavailable, small-molecule inhibitor of c-MET tyrosine kinase. In contrast to the majority of most available c-MET inhibitors, which act in an ATP-competitive way, tivantinib inhibits the binding of ATP to c-MET kinase in a non-ATP competitive manner [70].

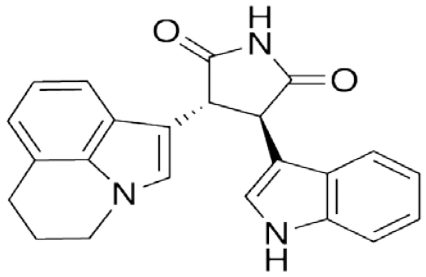


Figure 4. Schematic structure of Tivantinib. (From Eathiraj S *et al.* J Biol Chem. 2012).

Data on the specificity of tivantinib as c-MET inhibitor were provided by Munshi and colleagues [70] who found that tivantinib inhibited c-MET in a wide panel of human cancer cell lines with an IC_{50} of 355 nmol/L but had no relevant inhibitory effect on a large panel of human kinases in the same concentration range. Also, the authors reported that cancer cell lines without detectable c-MET protein expression were less responsive to tivantinib than those exhibiting high c-MET expression. In addition, tivantinib showed anti-proliferative activities and apoptosis induction in various human carcinoma cell lines with constitutive c-MET activity *in vitro*, suggesting that the antiproliferative effect of tivantinib is independent of the mode of activation of c-MET [70]. These preclinical data seemed to be confirmed by the aforementioned clinical studies showing that c-MET is an outcome predictor in patients treated with tivantinib.

However, more recently the concept of tivantinib as c-MET inhibitor has been challenged by two independent laboratories: Katayama and colleagues [71] reported

that tivantinib fails to inhibit c-MET in serveral cell lines, that this agent inhibits cell viability in both c-MET addicted and non-addicted cells and indicated a perturbation in microtubule formation as the main target of tivantinib. Another study, conducted by Basilico et al. [72], observed that tivantinib does not inhibit c-MET autophophorylation in several cancer cells but promotes microtubule stabilization independently of c-MET. These studies openly challenged the rationale underlying the use of this inhibitor in the subset of patients showing high c-MET expression. This pre-clinical evidence apparently contradicts the notion that c-MET is as predictor of efficacy of tivantinib in the clinical setting. The reasons for this discrepancy have become the object of intense debate [73-75] and the question arose of whether c-MET expression might represents a response marker rather than the actual target of tivantinib.

The actuality of this issue and the intensive clinical use of tivantinib in subsets of patients selected according to c-MET status in ongoing clinical trials also in our institution prompted us to investigate the molecular mechanisms underlying the function of tivantinib and their dependency on c-MET.

To this purpose, analysis of cell viability, apoptosis, cell cycle and of the underlying molecular changes were conducted after tivantinib administration in a panel of cell lines exhibiting different c-MET expression status and in c-MET exon 16 MET KO cell lines, which lack the binding site for tivantinib.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Cell lines

Name of cell line	Cell type	Morphology	Medium
Huh7	Hepatocellular carcinoma cell line	Adherent	DMEM
HepG2	Hepatocellular carcinoma cell line	Adherent	DMEM
Hep3B	Hepatocellular carcinoma cell line	Adherent	MEM
Chang	Hepatocellular carcinoma cell line	Adherent	DMEM
DLD1	Colon cancer cell line	Adherent	DMEM
DLD1 Wild type	Colon cancer cell line	Adherent	RPMI1640
DLD1 Met exon 16 knock out	Colon cancer cell line	Adherent	RPMI1640
PL5	Pancreatic carcinoma cell line	Adherent	DMEM
PANC1	Pancreatic carcinoma cell line	Adherent	DMEM
TFK1	Cholangiocarcinoma cell line	Adherent	RPMI1640

HepG2, DLD1, PL5 and PANC1 cells were purchased from ATCC (Rockville, MD, USA), Huh7 cells were purchased from the Japanese collection of research biosources (Osaka, Japan), Chang cells were purchased from Cell lines services (Eppelheim, Germany) and TFK1 cells were purchased from German collection of microorganisms and cell cultures (Brunswick, Germany). DLD1 wild type and MET exon 16 knockout cell lines were a kind gift from Prof. A. Bardelli (Department of Oncology, IRCC, Institute for Cancer Research and Treatment and University of Torino, Candiolo, Italy).

2.1.2. Cell culture

1. 100 mM Sodium Pyruvate Solution — PAA, Austria
2. 100 x Amino Acids Non Essential — PAA, Austria
3. 100 x L-Glutamine 200nM — PAA, Austria
4. 100 x Penicillin/Streptomyclin — PAA, Austria
5. Cell Culture Mediums: — PAA, Austria

   Dulbecco´s modified Eagle´s Medium (DMEM)

   High Glucose with L-Glutamine (4.5 g/L)

   Modified Eagle´s Medium (MEM)

   Roswell Park Memorial Institute 1640 (RPMI 1640) with L-Glutamine
6. Dimethylsulphoxid (DMSO) — Calbiochem, USA
7. Disposable Serological Pipette — Corning Incorporated, USA

   5ml, 10ml and 25ml
8. Dulbecco´s PBS without Ca & Mg — PAA, Austria
9. Falcon Tubes 15 ml and 50 ml — BD, USA
10. Fetal Bovine Serum (FBS) — PAA, Austria
11. Tissue Culture Dish (100 x 20 mm) — BD, USA
12. Tissue Culture Plate, 6 well, 12well and 96 well — Becton Dickinson, USA
13. Trypsin-EDTA (1x) 0.05%/0.02% in PBS — PAA, Austria

2.1.3. Colony Formation Assay

1. Crystal violet — Sigma, USA
2. Paraformaldehyde — Merck, Germany

2.1.4. Fluorescence Activated Cell Sorting (FACS)

1. Propidium Iodide 1mg/ml — Sigma, USA
2. Triton x-100 — Sigma, USA
3. Tri-Natriumcitra-Dihydrat — Merck, Germany
4. BD Accuri™ C6 Flow Cytometer — BD, USA

2.1.4.1. PI Staining Solution

Ingredients	Volume
Propidum Iodide	10 ml
Triton x-100	188.8 µl
Tri-Natriumcitrat-Dihydrat	227.928 mg
Dissolved in 200ml ddH_2O	

2.1.5. Materials for Western Blot

1. SDS Page Running Buffer — CLN GmbH, Germany
2. Criterion™ Cell — Bio-Rad, USA
3. Filter Paper — Whatman, UK
4. Methanol 100% — CLN GmbH, Germany
5. MicroPulser Electroporator — Bio-Rad, USA
6. Milk Powder — CLN GmbH, Germany

7. Protease Inhibitor Cocktail Set 1 — Calbiochem ◯,R, USA
8. PVDF Transfer Membrane — Millipore, USA
9. Pierce ECL ◯,R Western Blotting Substrate — Thermol Scentific, USA

   SuperSignal ◯,R West Pico — Thermol Scentific, USA

   Chemiluminescent Substrate — Thermol Scentific, USA

   SuperSignal ◯,R West Dura — Thermol Scentific, USA

   Extended Duration Substrate — Thermol Scentific, USA
10. Medical X-Ray Film — FujiFilm Corporation, Japan

2.1.5.1. Lysis Buffer

Unit	Ingredients
20 mM	Tris base, pH 7.4
1%	Triton x-100
2 mM	EDTA
1 mM	Na_3VO4
150 mM	NaCl
20 mM	Naf
2 mM	Sodium Pyrophosphate
1%	Protease Inhibitor Cocktail Set 1

2.1.5.2. Loading Buffer

Unit	Ingredients
1M Tris-HCl	1.25 ml
20% SDS	2.5 ml
Glycerol	2.5 ml
Bromphenol Blue	0.025 g
Dissolved in 10 ml ddH_2O	

2.1.5.3. Transfer Buffer (10x)

Ingredients	Volume
250mM Tris base	30.3 g
192mM Glycine	144 g

Dissolved in 1000 ml dd H_2O
Diluted 1:10 with 20% Methanol before using

2.1.5.4. TBST Buffer (10x)

Ingredients	Volume
150 mM Tris base	24.1 g
150 mM Nacl	80 g

Dissolved in 1000 ml ddH_2O. pH 7.6
Diluted 1:10 with 0.2% Tween 20 before using

2.1.5.5. Antibodies

Name	Molecular Weight (kDa)	Source	Concentration	Company
Akt1	60	Mouse	1:1000 in 5% milk	Cell signaling
Phospho-Akt (Ser473)	60	Rabbit	1:1000 in 5% BSA	Cell signaling

Met (C-28)	145	Rabbit	1:500 in 5% milk	Santa Cruz
Phospho-Met (Tyr1349)	145	Rabbit	1:1000 in 5% BSA	Cell signaling
P53 (DO-1)	53	Mouse	1:1000 in 5% milk	Santa Cruz
Phospho-p53 (Ser46)	53	Rabbit	1:1000 in 5% BSA	Cell signaling
Caspase-3	17, 19, 35	Rabbit	1:1000 in 5% milk	Cell signaling
Caspase-8	18, 43, 57	Mouse	1:1000 in 5% milk	Cell signaling
PARP	89, 116	Rabbit	1:1000 in 5% milk	Cell signaling
Bad	23	Rabbit	1:1000 in 5% milk	Cell signaling
Bax	20	Rabbit	1:1000 in 5% BSA	Cell signaling
Bcl-2 (50E3)	26	Rabbit	1:1000 in 5% milk	Cell signaling
Bcl-xL (54H6)	30	Rabbit	1:1000 in 5% milk	Cell signaling
Bid	15, 22	Rabbit	1:1000 in 5% milk	Cell signaling
Mcl-1	40	Mouse	1:1000 in 5% milk	Cell signaling
CyclinB1 (GNS1)	60	Mouse	1:1000 in 5% milk	Santa Cruz
β -actin	42	Mouse	1:10000 in 5% milk	Sigma

2.1.6. Small interfering RNA

RNAase free Water	Sigma, USA
DharmaFECT 4 Transfection Reagent	Thermo Scientific, USA
CCNB1 (CyclinB1) Smart Pool siRNA Reagent	Thermo Scientific, USA
ON-TARGETplus SMARTpool, Human MET	Thermo Scientific, USA
siGENOME Non-Targeting Control siRNAs	Thermo Scientific, USA

2.1.7. Laboratory Equipment

1. Centrifuges:

Qualitron DW-41 Microcentrifuge	Qualitron, Germany
Heraeus Biofuge Primo R	Heraeus, Germany
Hettich Rotantal centrifuge	Hettich, Germany

2. Dynatech Microtiterplate reader Mr7000 Labexchange, Germany

3. Filmprocessor.Curix 60 AGFA-Gevaert, Belgium
4. Freezer, -20 ^{o}C Liebherr, Germany

   Freezer, -80 ^{o}C Thermol Scientific, USA
5. GloMax®-Multi Microplate Multimode Reader Promega, USA
6. Heraeus B 5028 Incubator Heraeus, Germany
7. Heraeus Safe Clean Bench Heraeus, Germany
8. Hotplate Magnetic Stirrers, Type RM54 CAT, Germany
9. Microprocessor pH-mV Meter pH538 WTW, Germany
10. Microplate reader PeQLab, Germany
11. Orbital Shaker, SSL1 Stuart, Germany
12. Pipettes: 2.5µl, 10µl, 20µl, 100µl, 200µl and 1000µl

    Eppendorf, Germany
13. Power PAC200 Bio-Rad, USA
14. Thermo Electron LED GmbH Thermo Scientific, USA
15. Thermomixer comfort No.5355 24990 Eppendorf, Germany
16. Vortex Genie 2-Mixer Bender&Hobein AG, Switzerland
17. Vortex Genie2 Scientific Industries, USA
18. W22 water bath Medingen, Germany

2.1.8. Other Reagents

1. Caspase-8 Inhibitor II, Z-IETD-FMK	BD, USA
2. Hoechst 33342 dye 100 mg/ml	Sigma, USA
3. SYBR ◯,R Green I Nucleic Acid Gel Stain	Lonza, Switzerland
4. Recombinant Human HGF 50µg/ml	R&D Systems, USA
5. Recombinant Protein A 50mg/ml	Bio Vision, USA
6. Tivantinib (ARQ197) 30 mM	ArQule, USA
7. Tigatuzumab (CS-1008) 10mg/ml	Daiichi Sankyo Phama Development, USA
8. Sorafenib 10 mM	Bayer, Germany

2.2. Methods

2.2.1. Cell culture

2.2.1.1. Cell culture conditions

Cell culture procedures were performed under a laminar flow hood under sterile conditions. DMEM and RPMI 1640 media were supplemented with 10% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomyclin; MEM medium was supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1x Amino Acids and 2mML-Glutamine. All cell lines were cultivated in a humidified incubator with 5% CO2 at 37°C. Cells were plated in tissue culture dishes (100 x 20 mm) and sub-cultured every 2-3 days by incubating with 1 x Trypsin EDTA (3 min long for DLD1 wild type and MET exon 16 knockout cells; 5 min for Huh7, HepG2, Hep3B, Chang and PANC1 cells; 10 min for DLD1 and TFK1 cells; 12 min for PL5 cells).

2.2.1.2. Cell storage and recover

For long term storage, 80% confluent adherent cells per dish were released and suspended in 1.5 ml of cell culture medium with 10% DMSO. Cells were aliquoted into cell freezing containers and stored in a -80^{o}C freezer (Short term storage) or in liquid nitrogen (-196^{o}C - Long time storage). Rapid thawing of the cells in a 37^{o}C water bath is required when performing cell recovery. Cells were resuspended in cell culture medium, centrifuged for 5 min at 1200 rpm before fresh culture medium was added to remove DMSO.

2.2.2. Cell Proliferation Assays

1. Cells were washed with warm-PBS (37^{o}C) and collected after incubation with Trypsin-EDTA. After counting, cells were seeded onto 96-well plates at the indicated cell density prior to tivantinib treatment.

Cell line	Cell number/well
Huh7	2500
HepG2	3000
Hep3B	2500
Chang	1000
DLD1	1000
PL5	600
PANC1	700
TFk1	3500

2. After overnight incubation, cells were incubated with a cell culture medium solution containing different concentrations of tivantinib. 0.02% DMSO was used as control.

3. After incubation for 6 days, culture media were removed and cells were washed with ice-cold PBS. For osmotic lysis, 100µl ddH_2O was added into each well.

4. 100µl Sybr green solution (1:500 in ddH_2O) per well was added. Fluorescence was measured by using Cytofluor® Series 4000 Fluorescence Multi-well Reader. Proliferation index was calculated as a ratio to control-treated cells.

2.2.3. Colony Formation Assay

1. Different cancer cell lines were plated onto 6-well plates before incubation with tivantinib at the following cell densities.

Cell line	Cell number/well
Huh7	5000
HepG2	5000
Hep3B	5000
Chang	4000
DLD1	4000
PL5	4000
PANC1	5000
TFk1	5000

2. 24 h after cell seeding, culture media containing increasing concentration of tivantinib or DMSO were added.

3. After 24h, and subsequently every three days, media in each well were replaced with fresh culture media. Cells were cultivated for altogether 3 weeks.

4. 1 ml crystal violet staining solution was added onto each well. After 30 min the staining solution was removed and the number of colonies in each well was counted.

2.2.4. Fluorescence Activated Cell Sorting (FACS) Analysis

Cells were plated onto 12-well plates at the following cell densities.

Cell line	Cell number/well
Huh7	1×10^5

HepG2	1.25 x 10^5
Hep3B	1.5 x 10^5
Chang	1 x10^5
DLD1	0.8 x 10^5
PL5	0.8 x 10^5
PANC1	1.2 x 10^5
TFk1	1.5 x 10^5

1. After overnight incubation, cells were treated with increasing concentration of tivantinib for 24, 48 or 72 h.

2. Cells were collected in 1.5 ml tubes on ice, centrifuged for 8 min at 1500 rpm and washed with ice-cold PBS.

3. Cells were stained with 5% Propidium Iodide Staining Solution according to the method of Nicoletti et al. [76].

4. FACS analysis was performed by means of the Accuri™ C6 Flow Cytometer according to the manufacture´s instruction.

2.2.5. Western Blot Analysis

1. Cells growing in Petri dishes were collected and washed twice with ice-cold PBS and then lysed in cell lysis buffer.

2. Protein concentration was measured by the BCA assay kit (Sigma, USA). 60 µg of protein lysate were loaded and separated by 8% or 12% SDS-Page gels and transferred onto polyvinylidene difluoride (PVDF) membranes.

3. Membranes were blocked at room temperature with 5% non-fat milk or BSA for 1 h and incubated overnight at 4°C with specific antibodies in TBS-T (TBS-0.1% Tween20).

4. After washing with TBS-T, membranes were incubated at room temperature with horseradish-peroxidase-conjugated anti-rabbit/mouse secondary IgG-antibody for

2h. The bands were visualized by an enhanced chemoluminescence detection system (Thermo Scientific, USA) according to the manufacturer's instruction.

2.2.6. Use of DLD1 MET exon 16 knock out (KO) colon carcinoma cells.

To assess whether the anti-tumor efficacy of tivantinib is specifically due to its inhibitory effect on c-MET, two isogenic clones of engineered MET exon 16 knockout (KO) and wild type DLD1 cells (Fig.6) were used. These cell lines were previously described [77].

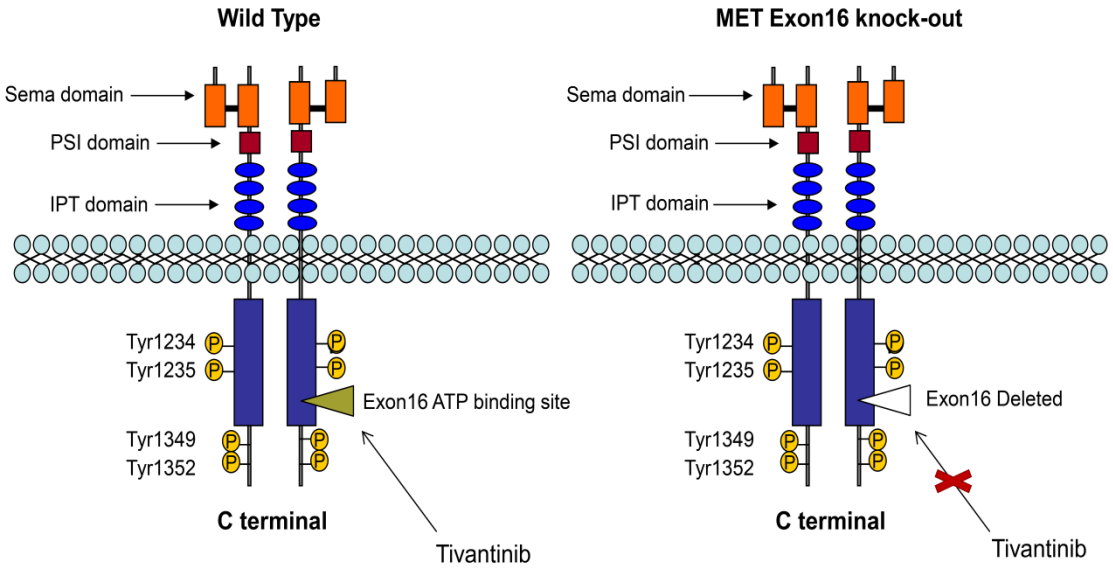


Figure 5. c-MET exon 16 knock-out DLD1 colon carcinoma cell lines. MET exon16, which encodes the ATP binding cleft including also the hydrophobic site where tivantinib binds, was deleted through homologous recombination, resulting in an inactive MET receptor which is neither able to be auto-phosphorylated nor to be activated by its ligand HGF.

Cells were plated onto 96, 12 or 6 well plates at the cell density of 3000, 8000 or 10000 cells per well respectively prior to treatment. Cell proliferation assays, FACS analysis and Western blot analysis were performed as described above.

2.2.7. Apo-ONER Homogeneous Caspase-3/7 Assay

1. 5 x 10^3 Huh7 and HepG2 cells were cultivated in 96-well plates for 24 hours.
2. Cells were treated with 533 or 4800 nM tivantinib for 24h. Cells incubated with DMSO or cell culture media alone were used as negative controls.
3. 100µl of Apo-ONE® Caspase-3/7 Reagent was added to each well.
4. Plates were placed on a plate shaker for one hour at room temperature.
5. Fluorescence was measured in each well at an excitation wavelength range of 485 ± 20nm and an emission wavelength range of 530 ± 25nm.

2.2.8. Hoechst Staining

1. 2 x 10^5 cells per well were seeded onto 6-well plates and incubated at 37℃.

2. After incubation with tivantinib for 1 day, cells were fixed with 4% formalin at 4℃ for 30 min.

3. After washing with PBS, cells were stained with Hoeschst 33 (1:1000 in TBST solution) at room temperature for 30 min and assessed by an immunofluorescence microscope.

2.2.9. Cyclin B1 small interfering RNA and transfection

To assess the functional relevance of Cyclin B1 expression in determining the G2-cell cycle arrest induced by tivanitnib, Cyclin B1 was silenced by small interfering RNA (siRNA) or non-coding siRNA before tivantinb administration. The commercially available siRNA SMARTpool® kit containing the following 4 pooled Cyclin B1-specific RNA oligonucleotides was used. Transfection procedures were performed according to the instruction of the manufacturer.

1) siGENOME SMARTpool siRNA D-003206-05, CCNB1

Target Sequence: CAACAUUACCUGUCAUAUA Mol.Wt. 13343.0 (g/mol)

2) siGENOME SMARTpool siRNA D-003206-06, CCNB1

Target Sequence: CCAAAUACCUGAUGGAACU Mol.Wt. 13373.0 (g/mol)

3) siGENOME SMARTpool siRNA D-003206-07, CCNB1

Target Sequence: GAAAUGUACCCUCCAGAAA Mol.Wt. 13373.0 (g/mol)

4) siGENOME SMARTpool siRNA D-003206-21, CCNB1

Target Sequence: ACUGUAGGGUAGCGGAAA Mol.Wt. 13388.0 (g/mol)

2.2.10. Cell cycle and viability analysis after siRNA against CyclinB1

1. Huh7 cells were seeded onto 6 or 96-well plates with a cell density of 1 x 10^5 or 5 x 10^3 per well in serum and antibiotic-free DMEM medium respectively.

2. 24 h later, 2 ml or 200µl/well 25nM CyclinB1-siRNA solution was added with equal volume of 25nM non-targeting control-siRNA or serum and antibiotic-free medium as negative or blank control respectively.

3. After incubation for 6 h, culture medium was replaced with standard cell culture medium containing 10% FCS and 1% Penicillin/Streptomyclin.

4. Cells underwent incubation with 250 or 300 nM tivantinib for 24 hours (cell cycle analysis) or 48 hours (viability assay).

2.2.11. Knock-down of c-MET by si-RNA

To assess the effect of c-MET silencing, the commercially available

ON-TARGETplus SMARTpool siRNA kit containing the following 4 pooled c-MET-specific RNA oligonucleotides were used.

1). ON-TARGETplus SMARTpool siRNA J-003156-13, MET

Target Sequence: GAACUGGUGUCCCGGAUAU Mol.Wt. 13444.8 (g/mol)

2). ON-TARGETplus SMARTpool siRNA J-003156-14, MET

Target Sequence:GAACAGCGAGCUAAAUAUA Mol.Wt. 13399.9(g/mol)

3). ON-TARGETplus SMARTpool siRNA J-003156-15, MET

Target Sequence:GAGCCAGCCUGAAUGAUGA Mol.Wt. 13444.9 (g/mol)

4). ON-TARGETplus SMARTpool siRNA J-003156-16, MET

Target Sequence: GUAAGUGCCCGAAGUGUAA Mol.Wt. 13429.7 (g/mol)

2.2.12. c-MET expression and HGF stimulation

Huh7 cells were incubated for 24 hours with two different concentrations of tivantinib (533 and 4800 nM) and then stimulated with 100 ng/ml recombinant human HGF (R&D System, USA) for 10 min. After protein quantification, cell lysates were analyzed by western blot as described above.

3. RESULTS

3.1. Tivantinib exerts a strong anti-proliferative effect in gastrointestinal cancer cell lines

To assess the effect of tivantinib on c-MET inhibition, phosphorylation of c-MET was measured in Huh7 and HepG2 cells by western blot. Due to low basal levels of phosphorylated c-MET, western blot analysis was conducted after pre-incubation with the HGF ligand c-MET. As exemplarily shown in Fig. 6, upon incubation with tivantinib both the phosphorylation of c-MET and the overall expression of this receptor were reduced in both Huh7 and HepG2 cell lines. To assess the anti-proliferative properties of tivantinib, cell proliferation assays were performed in a panel of eight cell lines from different gastrointestinal tumors exhibiting different c-MET expression status (Fig. 7A). As determined by dsDNA assessment, administration of increasing doses of tivantinib resulted in a remarkable loss of cell viability with IC_{50} values comprised between 9.9 nM (Huh7) and 448 nM (Hep3B – Fig. 7B). The strong anti-proliferative effect of tivantinib was confirmed by colony forming assays showing a remarkable reduction in the number and size of colonies (Fig. 8). As shown in Fig. 8A the expression of c-MET varied greatly across different cell lines and was expressed at high level in Hep3B or PL5 cells, whereas it was expressed at lower level in HepG2 cells. Nevertheless, no apparent correlation could be seen between c-MET expression status and the effectiveness of tivantinib in the different cell lines as judged by viability assay and colony forming assay.

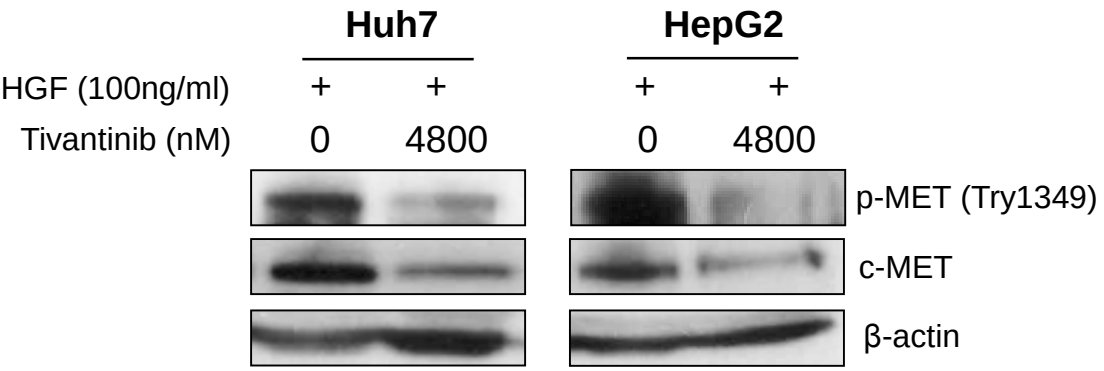


Figure 6. Western blot analysis of c-MET and phospho-c-MET upon incubation with tivantinib.

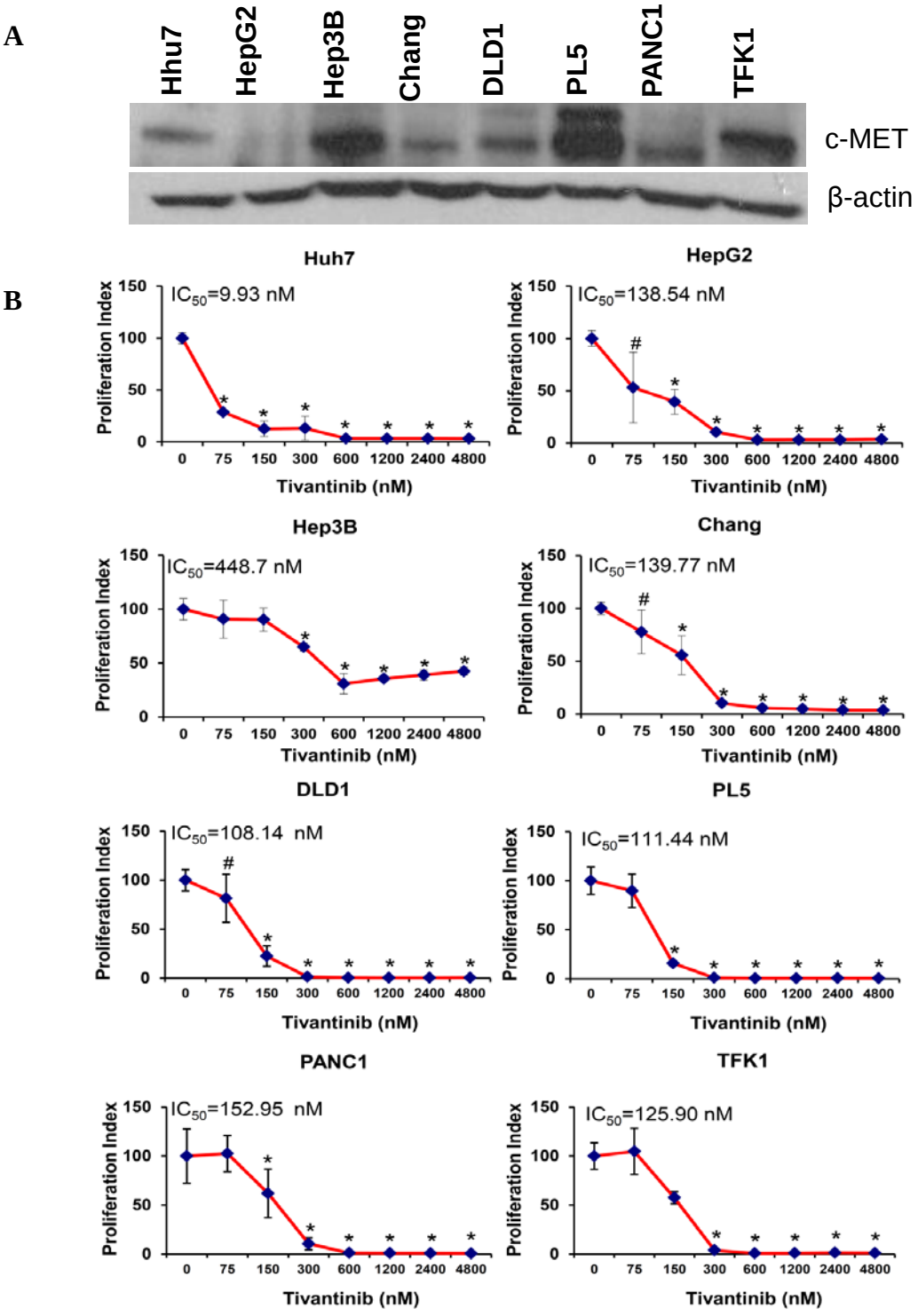


Figure 7. Effect of tivantinib on proliferation of HCC and of four additional cancer cell lines (colon cancer [DLD1], pancreatic cancer [PL5, PANC1] and cholangiocellular carcinoma [TFK1]). (**A**) Assessment of c-MET by western blot and **(B)** Cell viability assays in the different indicated cell lines. Results are expressed as mean and standard deviation of 3 independent experiments each conducted in triplicate. * $p < 0.01$; # $p < 0.05$ in comparison to control treated cells.

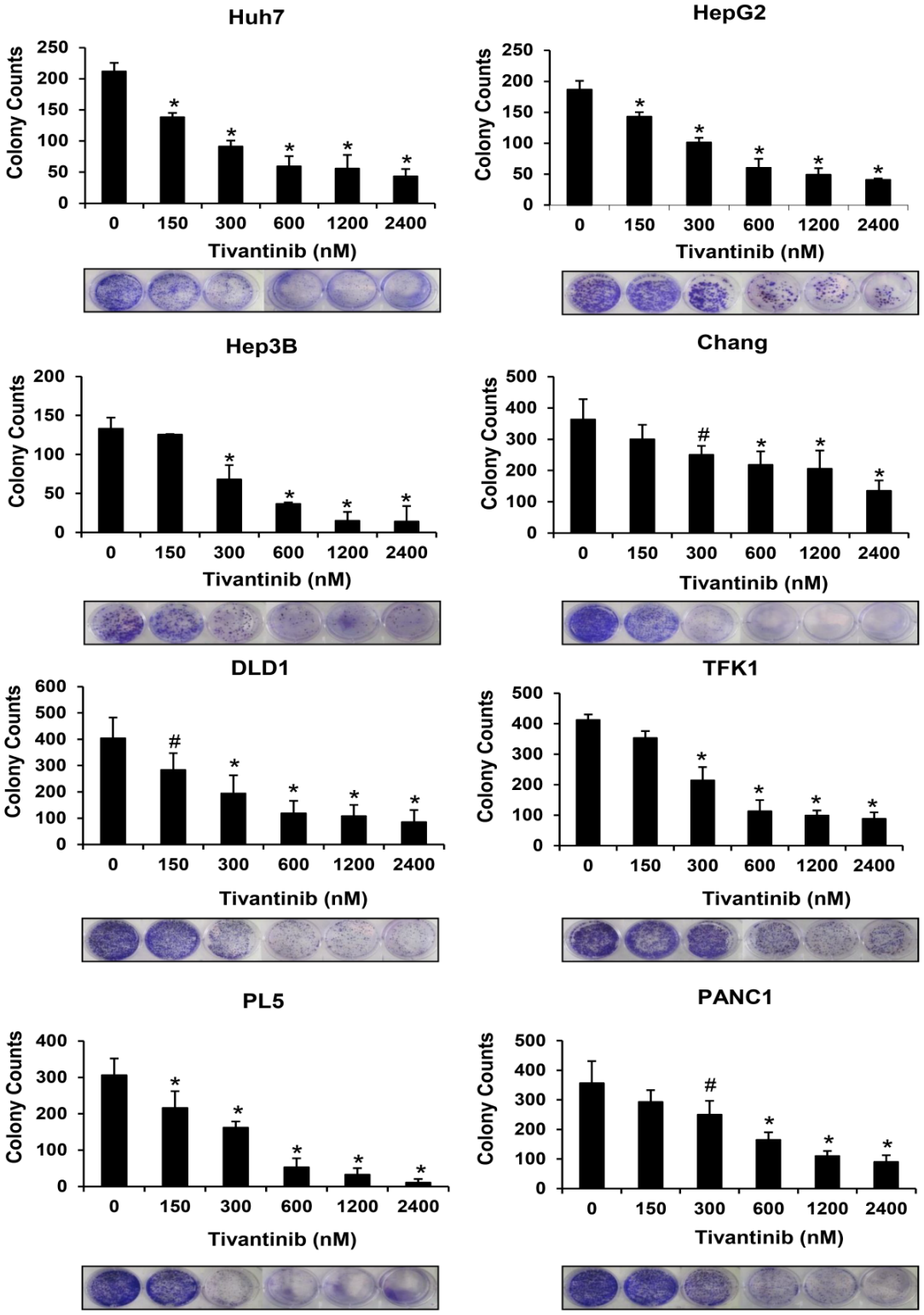


Figure 8. Effect of tivantinib on colony formation of HCC and other four gastrointestinal cancer cells. Data are representative of three independent experiments and are expressed as the mean +SEM (standard error of the mean). * $p < 0.01$; # $p < 0.05$ in comparison to control treated cells.

3.2.Tivantinib induced apoptosis in a dose and time dependent way

To further assess the events underlying the loss of cell viability in tivantinib-treated cell lines, cellular apoptosis was assessed by FACS analysis. As judged by the count of the sub-G1 cell fraction after PI staining (Fig. 9 and Fig. 10) a dose- and time-dependent increase of apoptosis could be observed upon incubation with tivantinib. The pro-apoptotic effect of tivantinib was readily observable at the concentration of 533 nM, most cells showing features of apoptosis for a concentration above 4 µM after 48 hours incubation. Concomitantly, progressive increase of caspase 3 and PARP cleavage (Fig. 11A), and caspase 3/7 activation could be observed (Fig. 11B). After Hoechst staining, cells treated with tivantinib at the concentration of 4.8 µM showed typical features of apoptosis with characteristic signs of chromatin condensation and nuclear fragmentation (Fig. 12).

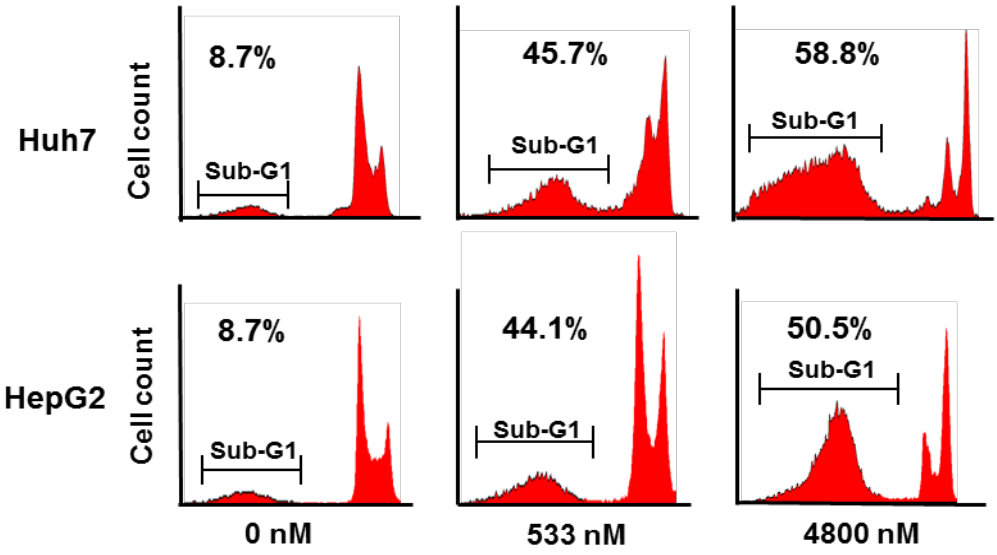


Figure 9. Typical FACS pattern of Huh7 and HepG2 cells. Typical FACS patterns showing increase of nuclear fragmentation corresponding to the increase of sub-G1 events after PI staining at FACS analysis.

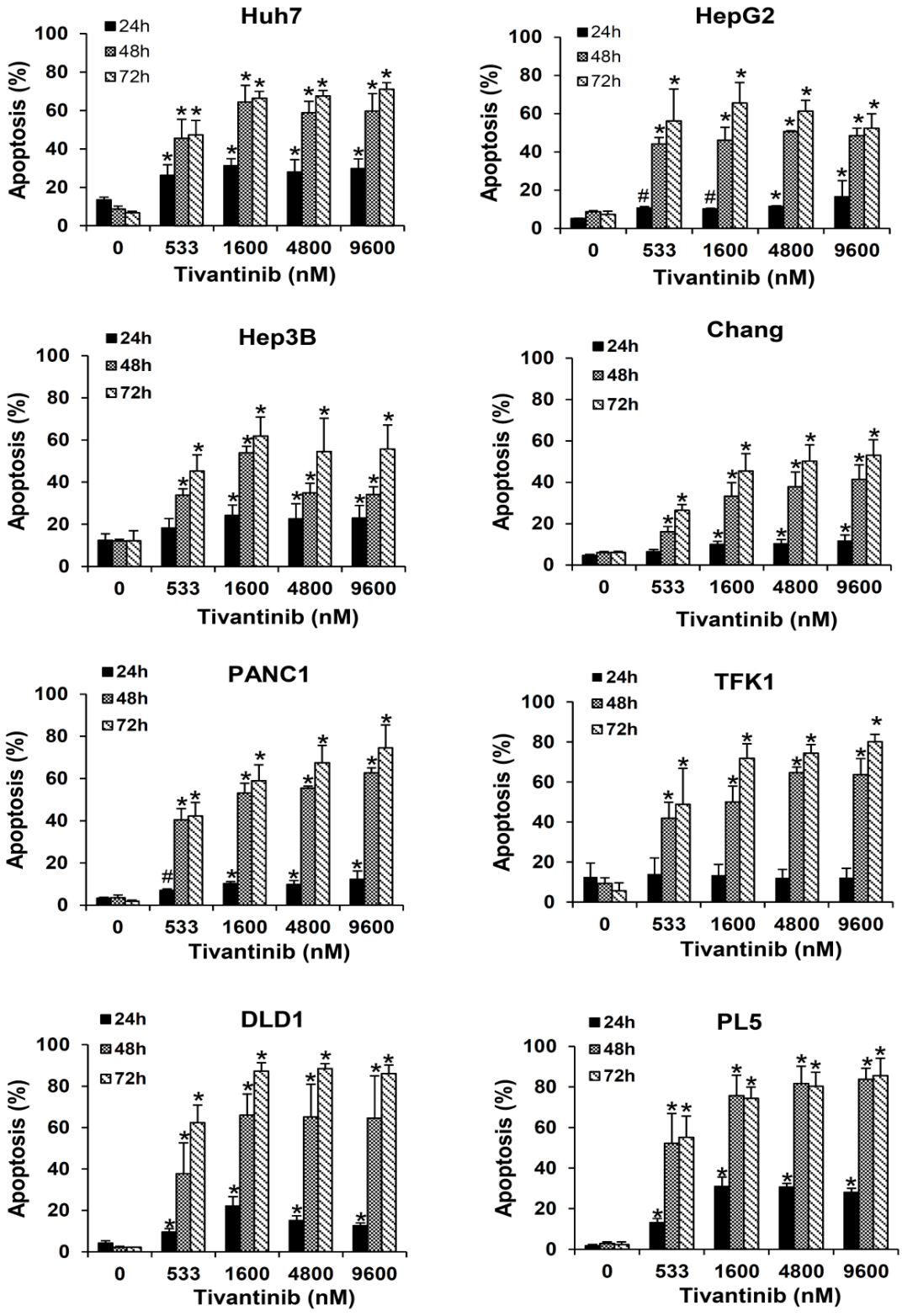
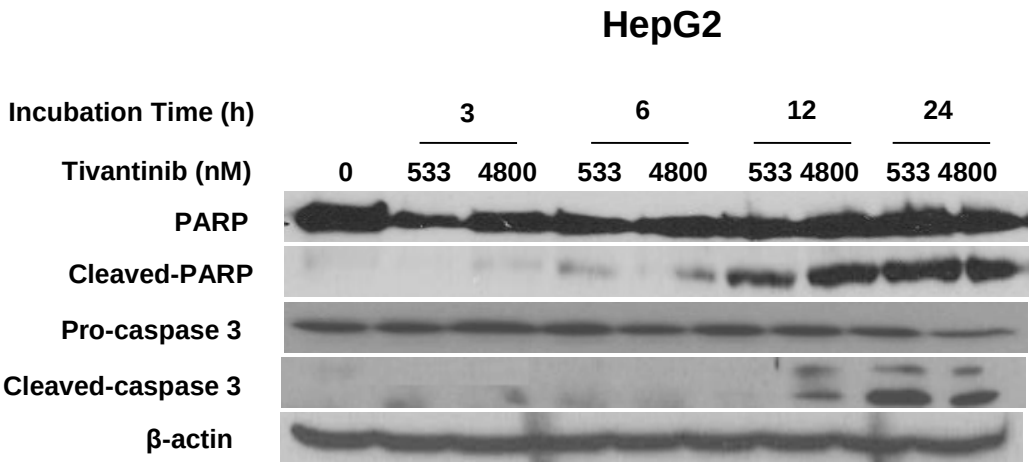


Figure 10. FACS-analysis based quantitation of apoptosis after PI-staining of tivantinib-treated cells. Count of sub-G1 events was conducted by gating the fraction of cells with subdyploid DNA content after propidium iodide (PI) staining at the indicated time points.

A



B

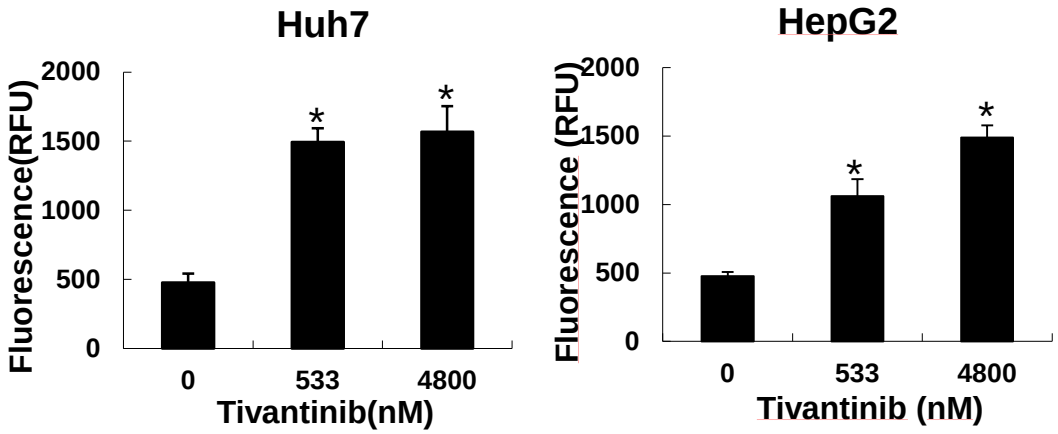


Figure 11. Assessment of caspase cleavage. Assessment of caspase cleavage by detection of fragmentation products of caspase 3 and PARP by western blot **(A)** and of caspase 3/7 activation as determined by fluorimetric analysis **(B)** 24 hours after incubation with tivantinib. Data are representative of three independent experiments and are expressed as the mean +SEM. * $p < 0.01$, in comparison to control treated cells.

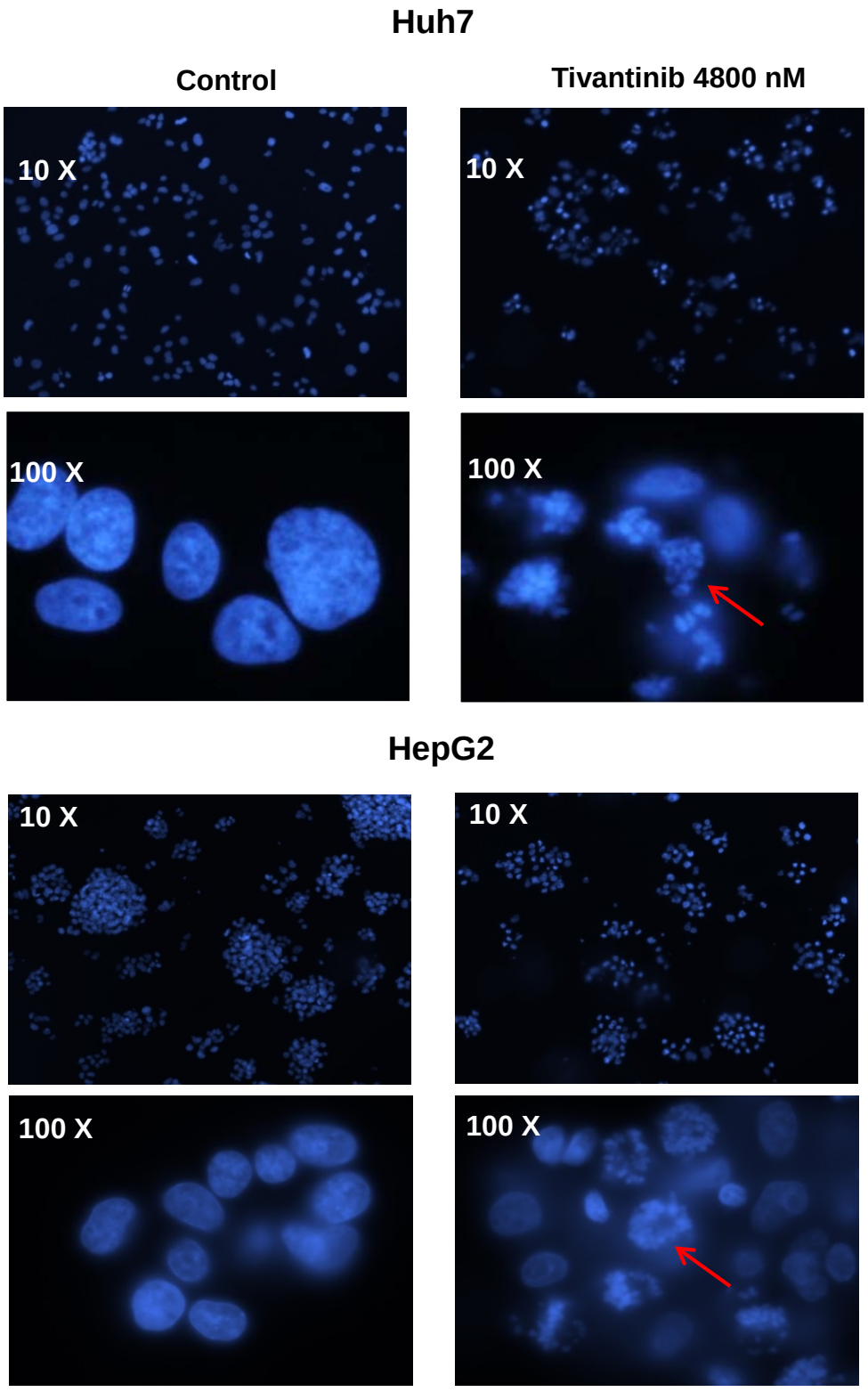


Figure 12. Tivantinib induces typical features of apoptosis with nuclear fragmentation. Fluorescence microscopy features of Huh7 and HepG2 cells after Hoechst staining showing intact cell nuclei of DMSO-treated cells and chromatin condensation and nuclear fragmentation (indicated by the arrows) after incubation with tivantinib.

3.3. Tivantinib enhances apoptosis by influencing effectors of the mitochondrial signaling pathway.

To investigate the mechanisms of apoptosis triggered by tivantinib, we assessed the role of different pro- and antiapoptotic molecules regulating the extrinsic apoptotic pathway (which is activated at the level of the “death receptors” upstream of caspase 8) and the intrinsic apoptotic pathway (which is triggered in response to mitochondrial depolarization). Incubation with tivantinib led to increased caspase 8 cleavage followed by cleavage of Bid, which indicates the activation of the extrinsic signaling pathway (Fig. 13). Assessment of the mitochondrial regulators of apoptosis showed a decrease of the antiapoptotic molecules Bcl-xl and Mcl-1, while no quantitative changes could be seen in the pro-apoptotic protein Bax and Bad and in the antiapoptotic regulator Bcl-2. These results indicate that tivantinib causes the activation of the extrinsic apoptotic pathway by recruitment of caspase 8 while simultaneously switching the balance of pro- and anti-apoptotic mitochondrial regulators of apoptosis to recruit effector caspases and cause cell death. However, preincubation of Huh7 and HepG2 cells with the caspase 8 inhibitor Z-IETD-FMK, only marginally affected cell viability upon incubation with tivantinib (Fig. 14), indicating that apoptosis triggered in response to this agent is mediated mainly by the activation of the mitochondrial pathway.

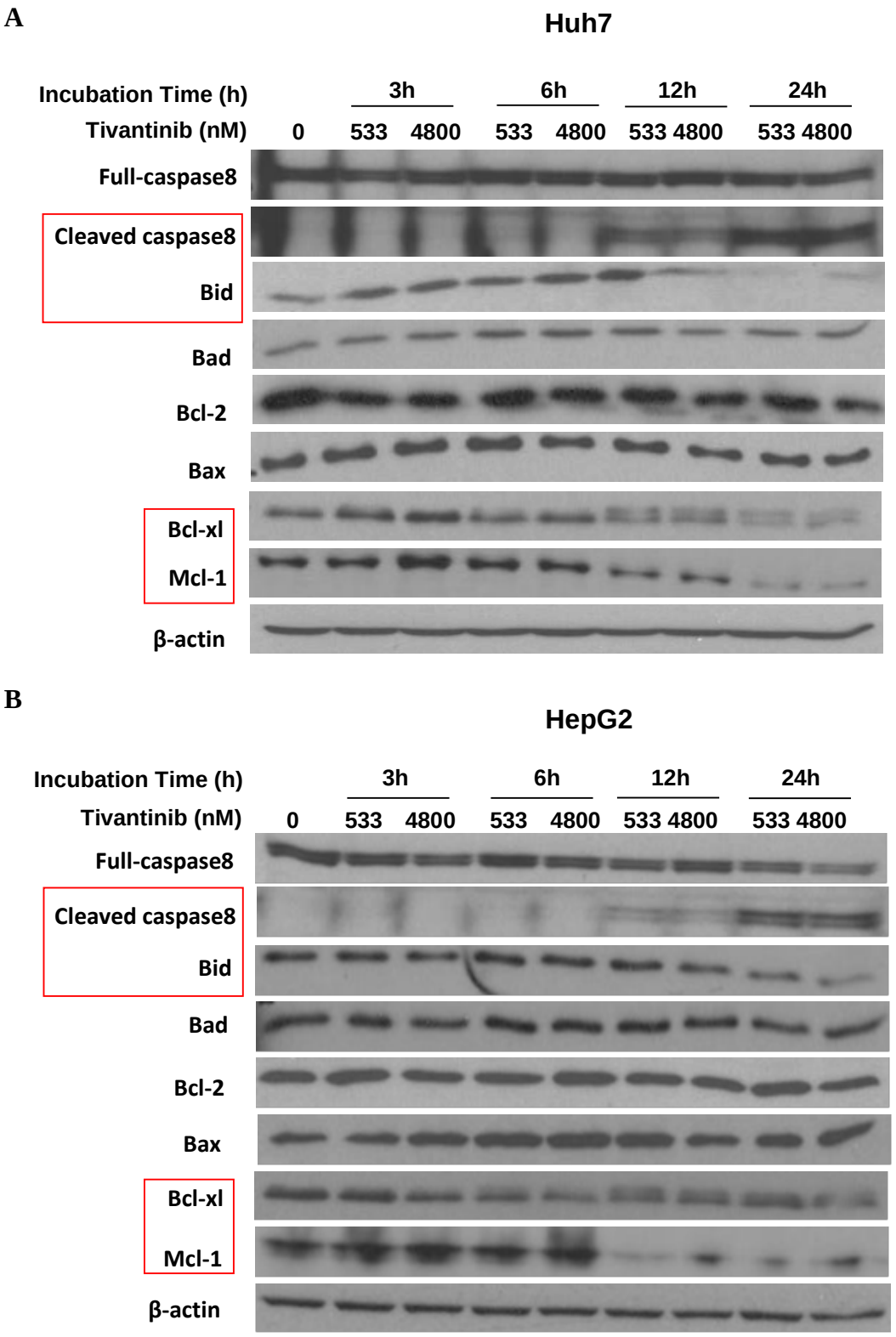


Figure 13. Effect of tivantinib on different regulators of apoptosis in HepG2 and Huh7 cancer cells. Western blot-based analysis of different regulators of apoptosis in Huh7 (A) or HepG2 (B) cell lines at the indicated time-points.

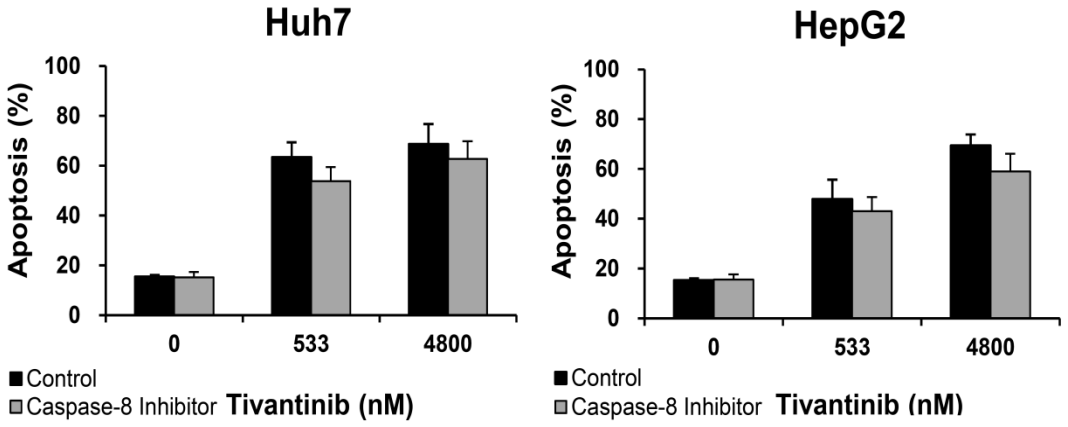


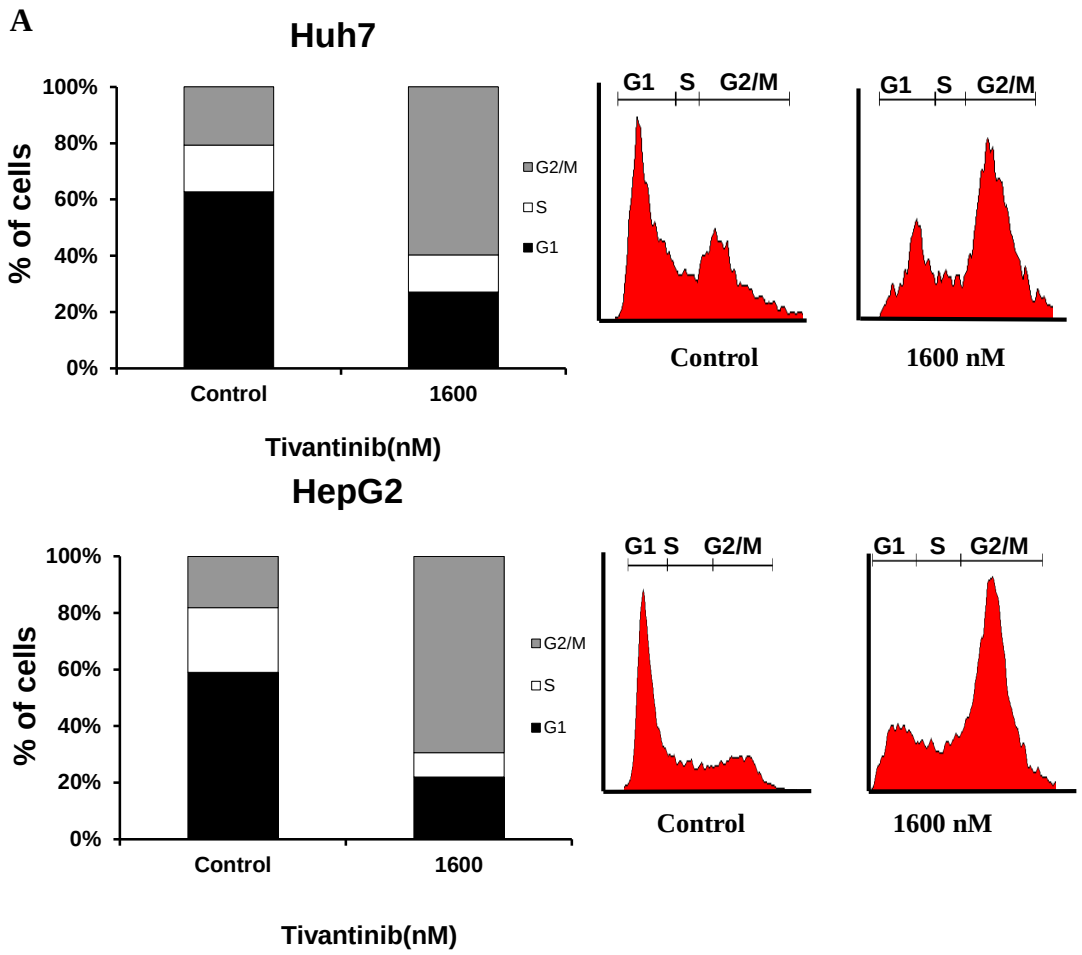
Figure 14. Effect of tivantinib administration on apoptosis after inhibition of caspase 8. FACS analysis was performed to assess nuclear fragmentation 24 hours after administration of tivantinib in the presence or absence of the Caspase 8 inhibitor Z-IETD-FMK.

3.4. Tivantinib causes a Cyclin B1-dependnet G2/M cell cycle arrest

To assess whether an effect of tivantinib on cell proliferation concurs with apoptosis induction to determine its effect on cell viability, analysis of the cell fraction in different phases of the cell cycle was performed by FACS analysis after PI staining as shown in Fig. 15. Administration of tivantinib caused a G2/M cell cycle arrest in all cell lines assessed, with a corresponding decrease of the fraction of cells in G1 and S phases. For example, the fraction of cells in G1, S and G2/M phase in vehicle-treated Huh7 cells were 57.9±4.1%, 10.4±5.9%, and 31.7±9.8% respectively. After 24 h incubation with tivantinib at the concentration of 1.6 μM the fractions of cells in the respective phases of cell cycle were 24.1%±2.8, 11.1±2.0%, 64.9±9.8% ($p<0.01$ - Fig. 16A). Similar effects were observed in several cell lines from colon, gallbladder and pancreatic cancer cells (data not shown).

To determine the mechanisms underlying the G2/M cell cycle arrest caused by tivantinib, the regulatory protein Cyclin B1, which is known to control the cellular transition at the G2/M checkpoint [78], was assessed for 24 hours. Incubation with tivantinib was associated with an early, strong increase of Cyclin B1 (Fig.15B). The

functional importance of this molecule to determine the observed cell cycle changes was confirmed by silencing of Cyclin B1 by siRNA, which led to a robust and effective downregulation of Cyclin B1 over 72 hours (Fig.16A). Silencing of Cyclin B1 prevented the G2/M cell cycle arrest induced by tivantinib and significantly reduced the loss of cell viability caused by tivantinib (Fig.16 B and C).



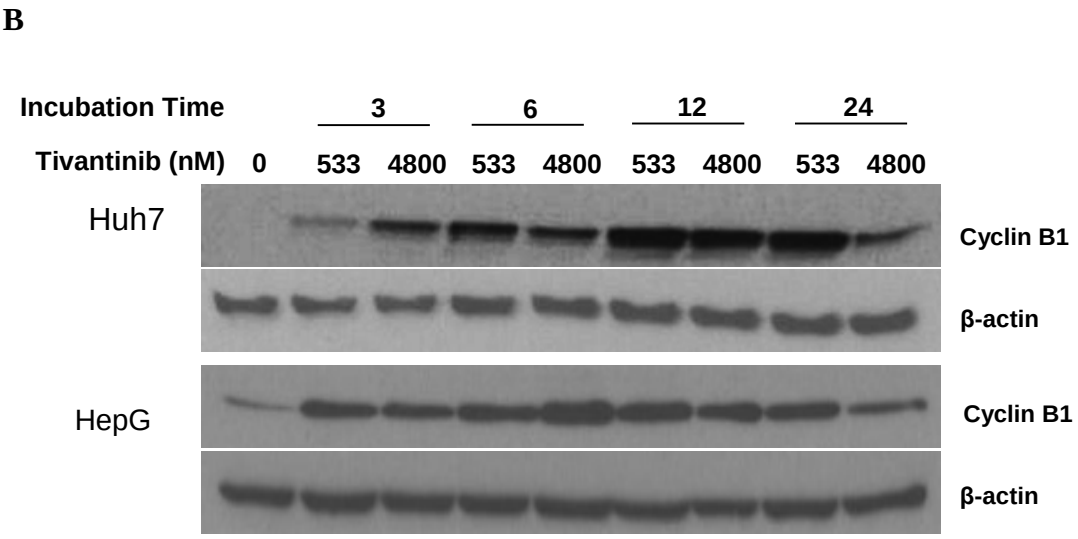


Figure 15. **Tivantinib induced significant G2/M cell cycle arrest in HCC cell lines and augmented Cyclin B1 expression. (A)** Quantification and typical flow cytometry patterns of G2/M cell cycle arrest of the indicated HCC cell lines after incubation with tivantinib for 24 hours. **(B)** Time kinetic of Cyclin B1 expression.

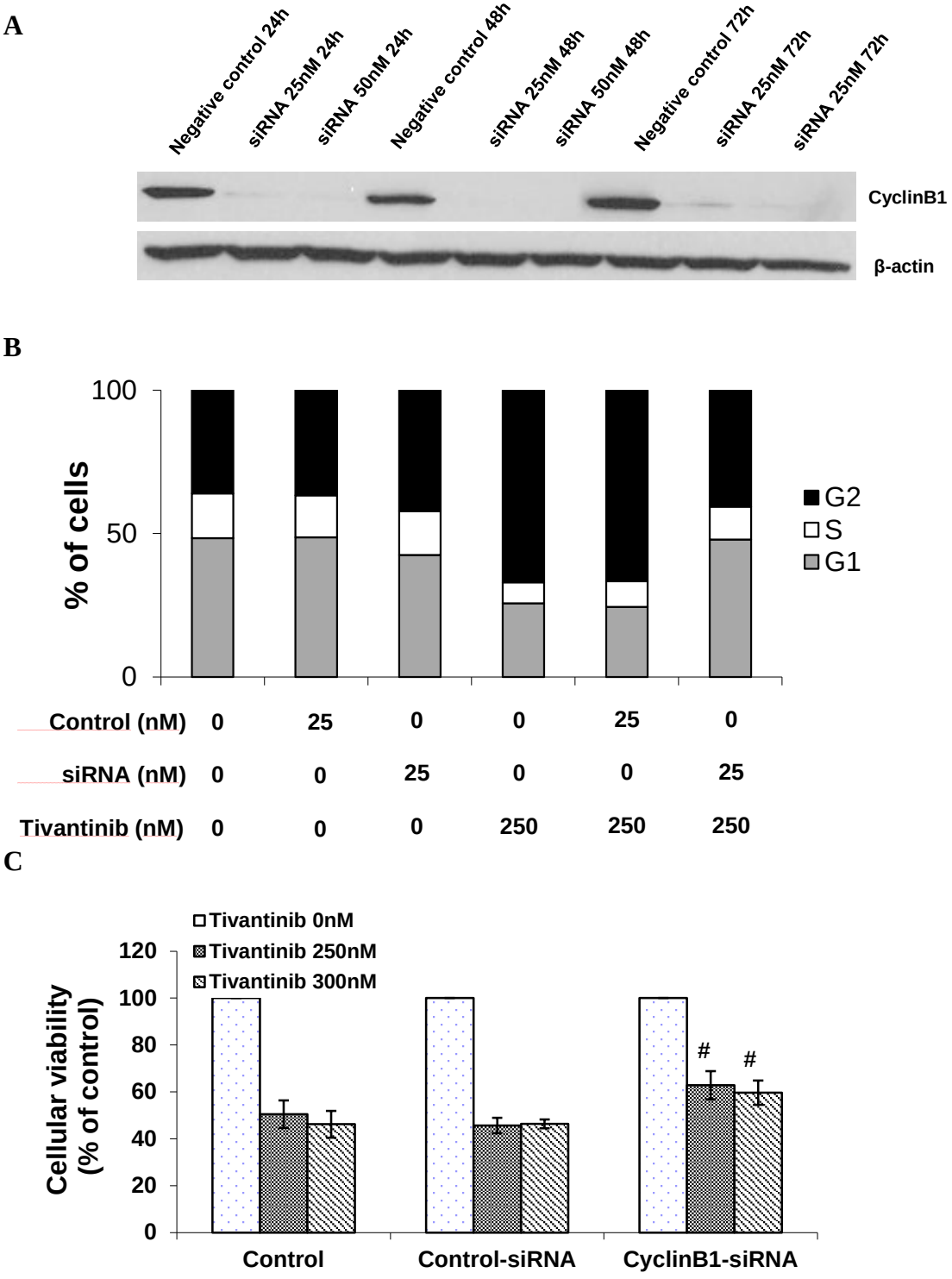


Figure 16. **Tivantinib causes a Cyclin B1-dependent G2/M cell cycle arrest. (A)** siRNA-based silencing of Cyclin B1in Huh7 cells. **(B)** Effect of tivantinib on cell cycle distribution after Cyclin B1 silencing in Huh7 cells. (**C**) Effect of tivantinib on cell viability after Cyclin B1 silencing in Huh7 cells assessed by MTS assay after 48 hours. # $p < 0.05$ compared to control (medium only) or control-siRNA (non-coding siRNA).

3.5. The antiproliferative effect of tivantinib is independent of c-MET but is caused by molecular targets downstream of c-MET.

The expression of c-MET, as judged by immunohistochemistry of human liver cancer tissue samples, was associated with a higher response rate in the clinical setting. As show in Fig. 8A, the expression of c-MET varied greatly across the eight different cell lines used in our system: c-MET was highly expressed in Hep3B or PL5 cells, whereas it was expressed at lower level in HepG2 cells. However, no apparent correlation could be seen between c-MET expression (Fig. 7A) and the biological effect of tivantinib on cell viability (Fig. 7B), colony formation (Fig. 8), apoptosis (Fig. 9 to 12) and cell cycle (Fig. 15). These results indicate that c-MET might not be the sole target of tivantinib as purported in other previous studies [71, 72].

To further explore whether the antiproliferative effect exerted by tivantinib is dependent on its inhibitory effect on c-MET, a type of engineered DLD1 c-MET exon 16 KO cell line, which lacks the binding site for tivantinib was used. As shown in Fig. 18, tivantinib caused loss of cell viability in a dose dependent manner by inducing apoptosis and cell cycle arrest in DLD1 wild type (WT) and in the two c-MET exon 16 knock out independent clones (KO1 and KO2 - Fig. 17). Furthermore, western blot analysis of these cells clones showed that the expression of Mcl-1 and Bcl-xl was reduced (Fig. 18A) and Cyclin B1 (Fig. 18B) increased in both wild type and c-MET-exon 16 knock out DLD1 cells. To rule out clonal artefacts potentially occurring in gene knockout model systems, RNA-interference experiments were additionally conducted using unselected Huh7 cells. As shown in Fig. 19A, transfection of siRNA targeting c-MET caused a robust downregulation of c-MET, but failed to induce apoptosis or cell cycle arrest (Fig.19B).Taken together, these data suggest that the cytotoxic activity of tivantinib is largely independent of c-MET.

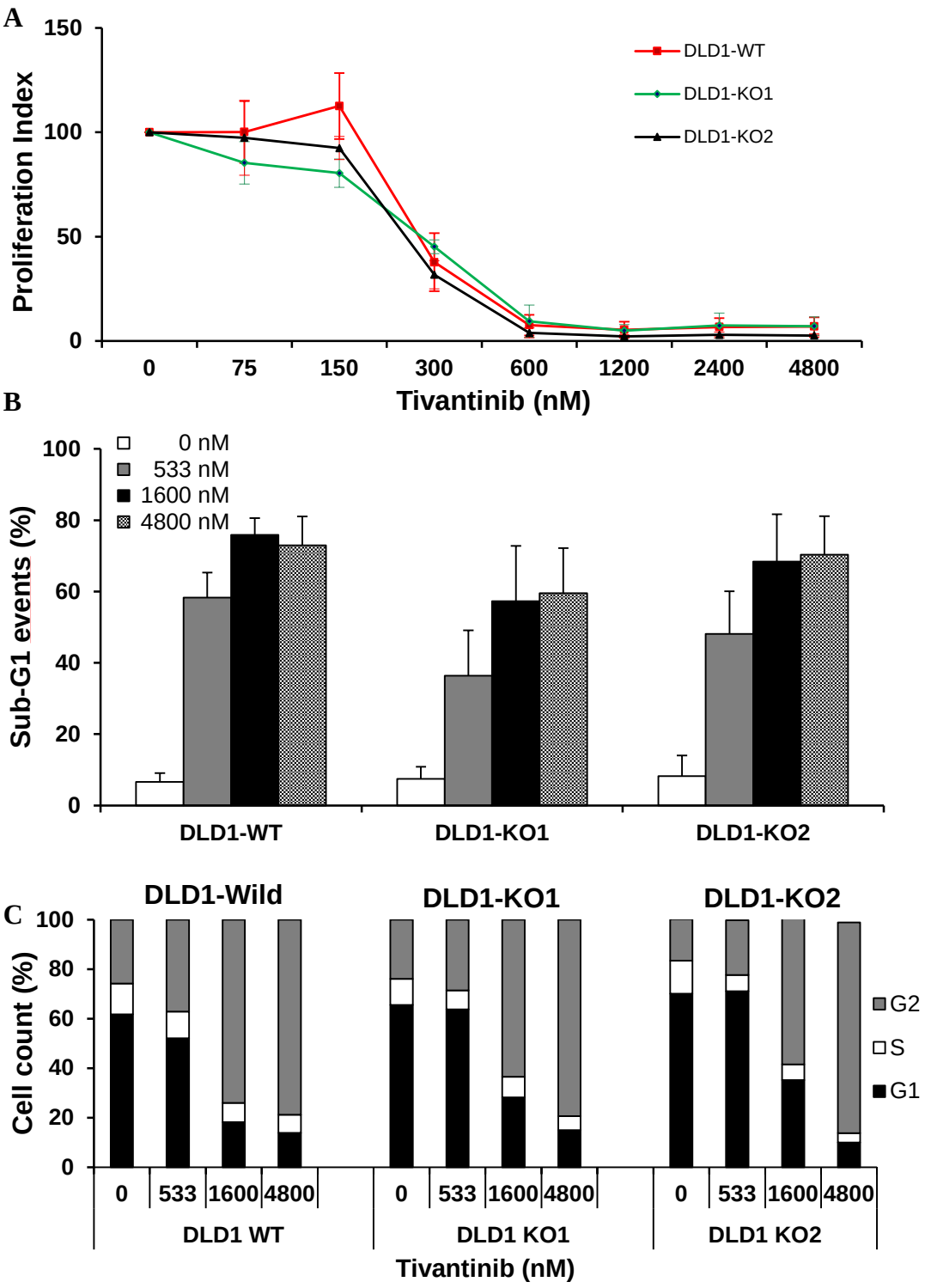
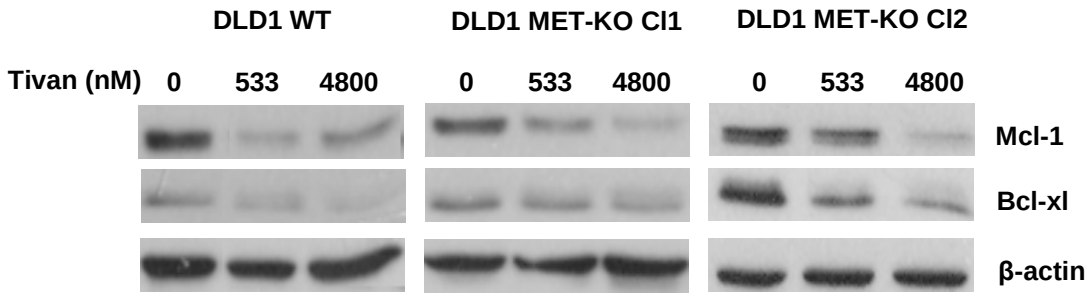


Figure 17. Effect of tivantinib on c-MET exon16 knock-out cell lines. (**A**) Viability assay upon administration of tivantinib in parental DLD1 cells, and in the two independent c-MET-exon 16 KO cells (DLD-KO1 and DLD-KO2). (**B**,**C**) FACS-based quantitation of apoptosis (**B**) and cell cycle (**C**) after PI-staining of tivantinib-treated cells. Results are expressed as mean and standard error of 3 independent experiments each conducted in triplicate.

A



B

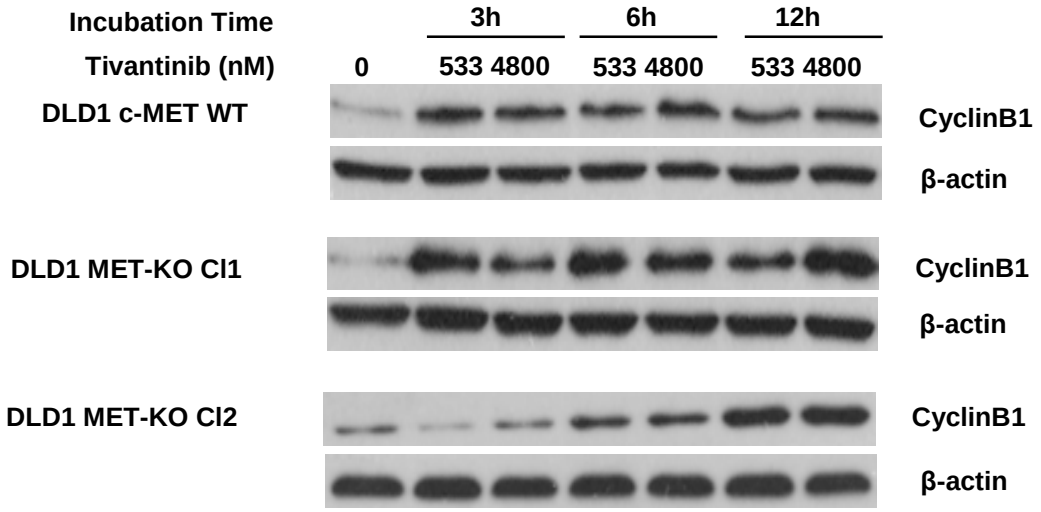


Figure 18. **Effect of tivantinib on c-MET-KO clones**. (**A**) Assessment of Mcl-1 and Bcl-xl by western blot upon administration of tivantinib at the indicated time points in parental DLD1 cells, and in the two c-MET-exon 16 KO cell clones. (**B**) Time kinetic of Cyclin B1 in the indicated cell lines. Data are representative of at least two independent experiments.

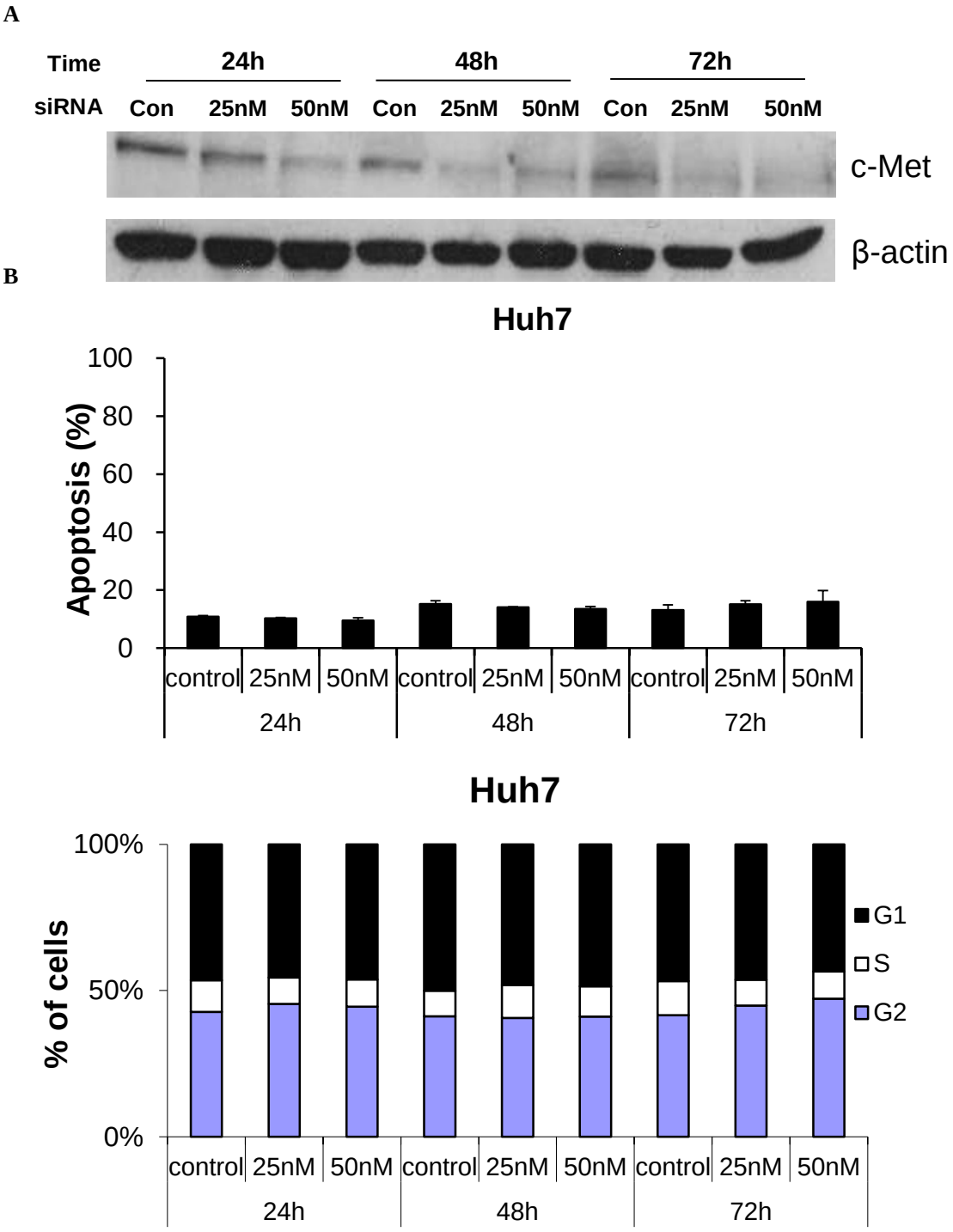


Figure 19. Effect of c-MET silencing on apoptosis and cell cycle. (A) c-MET protein expression after siRNA transfection at the indicated time points. **(B)** Effect of c-MET silencing on apoptosis and **(C)** on cell cycle distribution. Results are expressed as mean and standard error of 3 independent experiments each conducted in triplicate.

Subsequently, we hypothesized that the predictive relevance of c-MET in determining the outcome of patients on tivantinib is related to the fact that Mcl-1, Bcl-xl and Cyclin B1 are downstream targets of c-MET, and that the efficacy of tivantinib on tumors overexpressing c-MET reflects the effect of this compound on c-MET-driven overexpression of these molecules even in cells poorly responsive to the effect of tivantinib on the kinase activity of c-MET. To assess this possibility, Huh7 cells were stimulated with the c-MET ligand HGF with or without the addition of tivantinib. HGF administration resulted, as expected, in an increase of phospho-c-MET and phospho-Akt, which is known to be activated downstream of c-MET. HGF administration also caused an increase of Bcl-xl and Mcl-1 (Fig. 20). In contrast, administration of HGF caused an increase of Cyclin B1 expression levels in Huh7 cells, and the expression of this molecule was further increased by co-incubation with tivantinib. Since c-MET is the only know receptor for HGF, the data indicate that Bcl-xl and Mcl-1 are regulated downstream of c-MET, whereas Cyclin B1 is not.

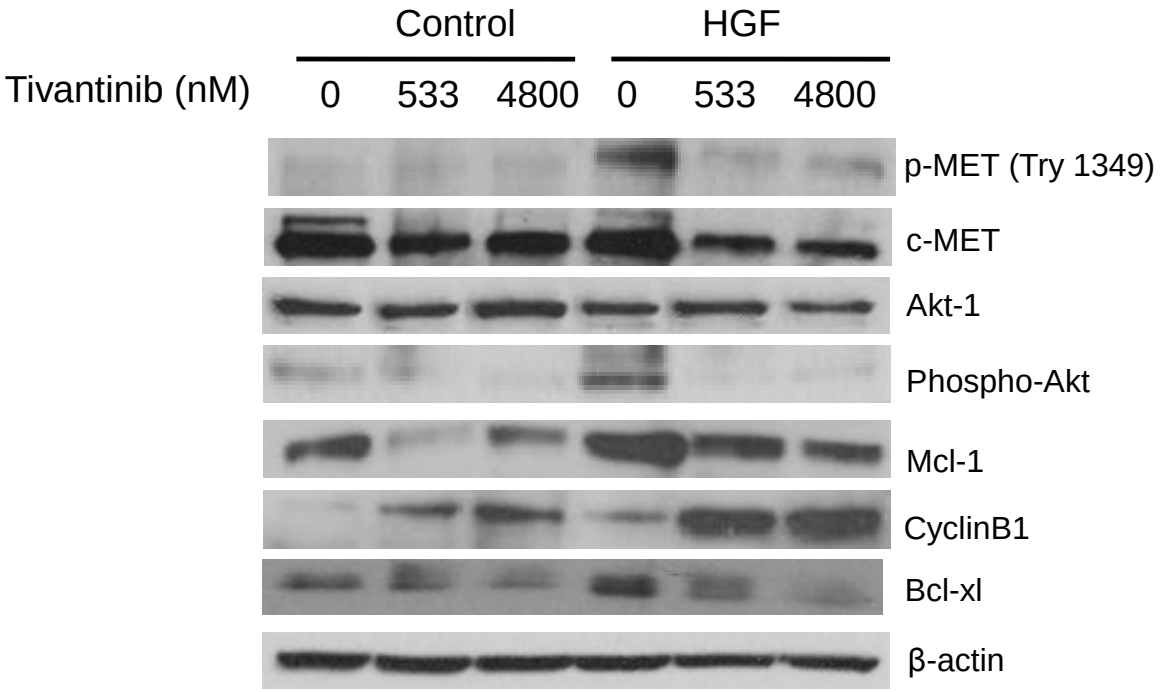


Figure 20. Effect of c-MET stimulation by HGF and tivantinib on p-c-MET and target molecules of tivantinib in Huh7 cells.

3.6. Tivantinib sensitizes cancer cells to the pro-apoptotic action of TRAIL-receptor stimulation

In a final set of experiments, to assess whether the pro-apoptotic effect of tivantinib might exert a sensitizing effect on the action of therapeutic agents targeting the pro-apoptotic membrane receptors, cells were co-incubated with tivantinib and tigatuzumab, a humanized agonistic monoclonal antibody which is capable of specifically inducing apoptosis in cancer cells by binding TRAIL-R2. For comparison, apoptosis induced by the combination of tivantinib and sorafenib was assessed. Low concentrations of tivantinib and tigatuzumab resulted in more than additive increase of apoptosis rates when compared to the administration of each agent alone: combined application of tivantinib and tigatuzumab caused a 2.9±0.7 fold increase of apoptosis in Huh7 cells and a 1.5±0.1 fold increase of HepG2 cells vs. the administration of these agents alone (Fig. 21). In contrast, the application of tivantinib and sorafenib resulted in apoptosis rates comparable to those observed after the administration of these agents individually (ratio of apoptotic cells in the combination treatment vs. the sum of each agent was 1.1±0.2 for Huh7 and 0.9±0.2 for HepG2). These data suggest that the effect of tivantinib on caspase 8 recruitment and its inhibition of antiapoptotic molecules Mcl-1 and Bcl-xl overcome the resistance of cancer cells to apoptosis, and together represent a mechanistic rationale for its combination with agents specifically capable of inducing apoptosis in cancer cells.

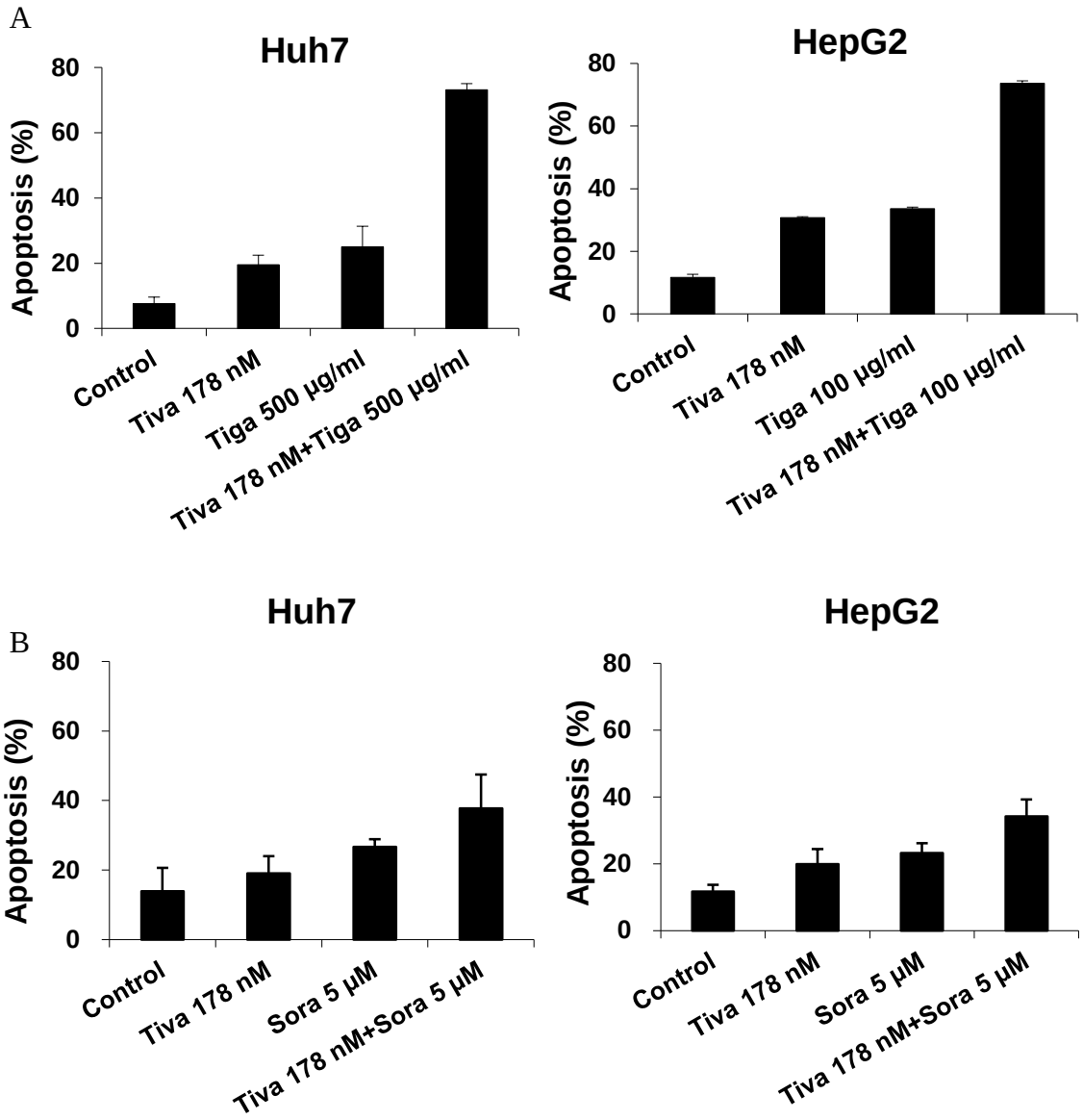


Figure 21. Tivantinib increases the sensitivity of HCC cells to the effect of tigatuzumab but not of sorafenib. FACS analysis of DNA fragmentation 24 after incubation with the respective agents. Graphs shows average rates of apoptosis and standard deviation of one representative experiment, performed in triplicate and repeated at least three times (Tiva=tivantinib, Tiga=tigatuzumab, Sora=Sorafenib).

4. DISCUSSION

The recognition of the role played by c-MET in the biology of tumors led to the establishment and extensive clinical investigation of several inhibitors of c-MET [79]. Tivantinib is a novel c-MET inhibitor which is being extensively studied in different tumor entities and which significantly prolonged time-to-progression and overall survival among “MET-high” HCC patients as a second line treatment after failure of a first line sorafenib treatment [64, 80]. Based on these phase 2 results, a phase 3 study of tivantinib in HCC patients has been initiated in several centers worldwide, including the GI-oncology unit of our institution, to enroll “c-MET-high” patients. Tivantinib also showed signs of efficacy in association with sorafenib, indicating that this agent could be effectively employed also as combination-treatment.

The clinical efficacy of tivantinib was previously attributed to its pro-apoptotic and anti-proliferative effects, to its capability to inhibit the metastatic potential of cancer cells [81] or to cause impairment of microtubule assembly or degradation [71, 72]. Nevertheless, the molecular mechanisms underlying the biological effects of tivantinib in tumor cells have yet to be thoroughly investigated. Furthermore, while it has been shown that tivantinib binds to c-MET, affects downstream signaling of c-MET pathway in patients, and provides survival benefit only in MET-High patients, the specific efficacy of tivantinib as c-MET inhibitor and the rationale for using this compound as cancer treatment in a subset of patients bearing tumors with high c-MET expression has been recently questioned [71, 72, 74], suggesting that c-MET might not represent the only target of this compound.

First, we aimed at assessing the molecular events underlying the biological effects of tivantinib, particularly focusing on apoptosis and cell cycle arrest. Secondly, we addressed the issue of whether the biological effect of tivantinib is mediated through its efficacy as c-MET inhibitor as originally postulated, or whether “off-target” molecules might be responsible for the observed biological properties as recent

studies seem to suggest. To this aim we investigated the effect of tivantinib in a panel of eight different cell lines exhibiting different c-MET status and employed additionally *MET* exon-16 KO cell lines which lack the binding site for tivantinib. These two issues and the clinical implication of our findings are separately discussed in the following paragraphs.

4.1. Molecular mechanisms underlying the effect of tivantinib on apoptosis and cell cycle.

Apoptosis can be triggered by two separate signaling pathways. The extrinsic (or receptor-mediated) pathway is characterized by the activation of caspase 8 in consequence of the stimulation of the membrane-bound "death-receptors" such as TRAIL-R1 and –R2 or CD95 [82, 83]. The intrinsic apoptotic pathway is activated by mitochondrial depolarization leading to caspase 9 cleavage and eventually to cleavage of caspase 3. This pathway is usually activated by stimuli occurring within the cell leading to a shift of the balance between pro-apoptotic molecules (such as Bax, Bak, Bim and Bid) and anti-apoptotic molecules (such as Bcl-2, Bcl-xl and Mcl-1). However, the intrinsic apoptotic pathway can also be initiated in consequence of the activation of the receptor-mediated pathway through the caspase 8-mediated cleavage of Bid [82, 83]. As we examined the time kinetic of caspase activation after administration of tivantinib, we observed a dose- and time-dependent cleavage of caspase 8 followed by the cleavage of Bid indicating a recruitment of receptor-mediated apoptosis (Fig.13). However, co-incubation of cell lines with a caspase 8 inhibitor only marginally affected the cell viability caused by tivantinib (Fig.14). As we subsequently assessed several pro- and anti-apoptotic regulators of the intrinsic apoptotic pathway by western blot, we could observe a clear downregulation of Mcl-1 and Bcl-xl (Fig.13). Mcl-1 and Bcl-xl are Bcl-2-related proteins, which bind to Bax and Bak, thereby blocking their pro-apoptotic interaction with the outer surface

of the mitochondria. These data indicate that when cells are stimulated with tivantinib alone, apoptosis is triggered mainly due to the mitochondrial pathway. It was previously shown that phosphorylation of p53 represents a mechanism by which inhibition of c-MET causes cell death by phagocytosis in lung cancer cells. Although phosphorylation of p53 was observed early after stimulation with tivantinib in HCC cells in our system (data not shown), tivantinib did not differently affect the cell viability of cell lines exhibiting different p53 status (Huh7 shows a mutant p53 phenotype and Hep3B shows a p53 gene deletion), demonstrating that the effect of tivantinib is independent of p53.

To further assess the pro-apoptotic potential of tivantinib, in a further experiment, we assessed whether the increased cleavage of caspase 8 observed after administration of this compound might affect the sensitivity of cancer cells upon stimulation of the membrane death receptors. To this aim, tivantinib was administered alone or in combination with the TRAIL-R2-binding monoclonal antibody tigatuzumab, which has been recently made available as anticancer treatment. Administration of tivantinib sensitized several HCC cell lines to the action of tigatuzumab but had marginal effect on apoptosis rates observed after the administration of sorafenib (Fig.21). Therefore, although the administration of tivantinib alone seems to cause apoptosis mainly through the activation of the mitochondria, the sensitization of HCC cell lines to the action of tigatuzumab show that tivantinib-mediated increase of caspase 8 cleavage is capable to enhance the sensibility of cancer cells to the effect of TRAIL-R-targeting compounds.

Besides these changes in the regulators of apoptosis we could detect a strong increase of Cyclin B1 after administration of tivantinib (Fig. 15B). Cyclin B1 in complex with Cdk2 controls the G2-M transition of the cell cycle [84, 85]. Also, Ito and colleagues showed that prolonged arrest at the G2-phase and abrupt entry into aberrant M-phase in the presence of accumulated Cyclin B1 are followed by cell death and that loss of

cell viability could be achieved by abrogation of cytoplasmic Cyclin B1 accumulation [86]. Furthermore, a previous report showed that elevation of Cyclin B1 correlated with the mitotic delay observed in Hela cells expressing autonomous CaMKII (Calcium/Calmodulin-dependent protein kinase II) [87]. In line with these previous reports, we could detect a strong Cyclin B1 accumulation as soon as 3 hours after administration of tivantinib. The functional relevance of Cyclin B1 in determining the effect of tivantinib on cell cycle could be confirmed by experiments using specific siRNA targeting Cyclin B1. As shown in Fig. 16, siRNA targeting Cyclin B1 but not control-siRNA reversed the fraction of cells in the G2/M phase to almost basal levels after co-administration of tivantinib. Correspondingly, silencing of Cyclin B1 significantly reduced the loss of cell viability observed after tivantinib administration. These data show that cell cycle arrest concurs with apoptosis in determining the anti-proliferative effect of tivantinib.

In their recent work, Basilico and colleagues showed accumulation of alpha-tubulin-containing microtubules 36 hours after administration of tivantinib and indicated that tivantinib-mediated apoptosis occurs as a consequence of cell cycle arrest caused by impaired microtubule formation [72]. Subsequently, Katayama and colleagues showed inhibition of microtubule polymerization as a mechanism of action of tivantinib. In agreement with these observations, we found that apoptosis progressively increased until 72 hours after administration of tivantinib. However, a time kinetic of Bcl-xl and Mcl-1 showed a strong downregulation of these antiapoptotic proteins at an early time point after administration of tivantinib and before cell cycle changes could be observed. Accordingly, the sensitizing effect of tivantinib to TRAIL-R2 stimulation led to apoptosis in the majority of cells within 24 hours. These findings, together with our data on the reversibility of cell cycle changes as a consequence of cyclin B1 inhibition, indicate that both apoptosis and cell cycle arrest occurring at an early time point might concur with cell death occurring at a later time point in consequence of microtubules perturbations to determine the

antiproliferative effect of tivantinib.

4.2. c-MET expression and response to tivantinib.

Our data showed that tivantinib exerted a remarkable anti-proliferative effect in cell lines expressing very different levels of c-MET (Fig. 7). This discrepancy between our data and the predictive effect of c-MET expression in the clinical setting might be due to differences related to the higher complexity of the mechanisms of action of tivantinib *in vivo*, e.g. those underlying invasion and metastasis formation [81], and to microenvironmental changes related to previous administration of sorafenib in these patients. Such hypothesis would be supported by the reported role of c-MET in determining the resistance to anti-angiogenic therapy [88, 89]. Nevertheless, differences in c-MET expression observed by us in cells equally responsive to tivantinib are remarkable and seem to confirm recent reports suggesting that c-MET expression might not be the only determinant of the response to tivantinib [71, 72]. As shown by western blot analysis of p-MET, our data confirm the fact that tivantinib acts as c-MET inhibitor (Fig. 6). Interestingly, we also observed a decrease of total c-MET after tivantinib treatment (Fig. 6) possibly contributing to the MET-dependent mechanism of action of this drug independently of its ability to inhibit the kinase activity of c-MET. A similar observation was reported previously and was suggested to be mediated by the ubiquitin pathway [90]. In addition, we could show that the HGF-mediated increase of antiapoptotic Bcl-xl and Mcl-1 downstream of c-MET could be reversed by tivantinib (Fig. 20). Nevertheless, as shown in Fig. 17, silencing of c-MET by specific siRNA failed to reproduce the effects of tivantinib on apoptosis and on cell cycle. Moreover, the effect of tivantinb on cell viability, apoptosis, cell cycle and the regulation of Bcl-xl, Mcl-1 and Cyclin B1 in c-MET-KO cell clones, was indistinguishable from that exerted by this agent in c-MET wild-type cells (Fig. 18 and 19). In addition, the early increase of Cyclin-B1 observed upon administration

of tivantinib, proved independent of c-MET, as shown by the activation of c-MET by HGF (Fig. 20). These results indicate that the downregulation of important regulators of apoptosis like Mcl-1 and Bcl-xl can occur through both c-MET-dependent and c-MET independent mechanisms, but are affected by tivantinib independently of c-MET, while the activation of Cyclin B1 represents a c-MET-unrelated mechanism of action of tivantinib.

4.3. Conclusions and clinical pitfalls

In summary, we provide a first report on the molecular changes underlying apoptosis and cell cycle arrest caused by tivantinb and on their relation to c-MET. Tivantinib caused increased caspase 8 recruitment and sensitized cancer cells to the pro-apoptotic effect of TRAIL-R-stimulation. Since the activation of the death receptors on the cells surface represents a crucial mechanisms for the immune-mediated clearance of tumor cells [91, 92], tivantinib might exert an additional antitumor effect in vivo by sensitizing cancer cells to the pro-apoptotic effect of endogenous TRAIL, thus favoring the clearance of metastatic cells. These data suggest also that the pro-apoptotic potential of tivantinib can be exploited by its association with pro-apoptotic agents recently made available for clinical employment [93], and that such agents should be considered as alternative to the tivantinib-sorafenib combination as second line treatment of HCC.

Experiments of c-MET stimulation with HGF showed that Mcl-1 and Bcl-xl are downstream targets of c-MET. Yet, these molecules are affected by tivantinib independently of c-MET. The efficacy of tivantinib on tumors overexpressing c-MET might therefore reflect the effect of this compound on the c-MET-driven overexpression of these molecules even in cells poorly responsive to the effect of tivantinib on the kinase activity of c-MET. These findings might account for the apparent discrepancy between the relevance of c-MET as predictor of the outcome of

patients treated with tivantinib and the recent in vitro reports questioning the activity of tivantinib as c-MET inhibitor. Since Cyclin B1, Mcl-1 and Bcl-xl were affected by tivantinib regardless of c-MET, we suggest that these molecules are considered as possible and potentially more reliable response predictors.

5. TABLES AND FIGUERS

6. ABBREVIATIONS

c-MET:	N-methyl-N'-nitro-nitroso-guanidine human osteosarcoma transforming
CRK:	v-crk sarcoma virus CT10 oncogene homolog
CRKL:	CRK-like
DCP:	Des-gamma-carboxy Prothrombin
DMEM:	Dulbecco´s Modified Eagle´s Medium
DMSO:	Dimethylsulphoxid
EGFR:	Epidermal Growth Factor Receptor
FACS:	Fluorescence Activated Cell Sorting
FBS:	Fetal Bovine Serum
GAB1:	GRB2-Associated Binding Protein 1
GRB2:	Growth Factor-Bound Protein 2
HCC:	Hepatocellular Carcinoma
HGF:	Hepatocyte Growth Factor
IHC:	Immunohistochemistry
IPT:	Immunoglobulin-Plexin-Transcription
IGF1R:	Insulin Growth Factor Receptor (IGF1R)
ITT:	Intent-to-Treat
KO:	Knock Out

MAPK:	Mitogen Activated Protein Kinase
Mcl-1:	Myeloid Cell Leukemia Sequence 1
MEM:	Modified Eagle´s Medium
NSCLC:	Non-Small Cell Lung Cancer
OS:	Overall Survival
PAR-2:	Proteinase-Activated Receptor-2
PDGFR:	Pletelet Drived Growth Factor Receptor
PFS:	Progression Free Survival
PI:	Propidium Iodide
PI3K:	Phosphatidylinositol 3-kinase
PKB:	Protein Kinase B
PLCγ:	Phospholipase Cγ
PR:	Partial Response
pRCC:	Human Papillary Renal Cell Cancer
PSI:	Plexin-Semaphorin-Integrin
Raf:	v-raf murine sarcoma viral oncogene homolog B1
RAS:	Rat Aarcoma Viral Oncogene Homolog
RPMI 1640:	Roswell Park Memorial Institute 1640
RTKs:	Receptor Tyrosine Kinases
SEM:	Standard Error of the Mean

SF:	Scatter Factor
SHC:	Src Homology 2 Domain-Containing
SHP2:	Src Homolog 2 Domian-Containing Phosphatase-2
siRNA:	small interfering RNA
SOS:	Son of Sevenless
SPH:	Serine Protease Homology
STAT3:	The Signal Tranducer and Activator of Transcription 3
TPR:	Translocated Promoter Region
TRIAL:	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TRAILR:	TRIAL Receptor
TTP:	Time To Progression
VEGFR:	Vascular Epithelial Growth Factor Receptor

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8. CURRICULUM VITAE

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Education

09/2010- present: Medizinische Klinik II, University of Munich, Munich (Germany) as doctoral student (Dr.hum biol).

09/2007-07/2010: Tongji University, Master Degree in Pathology and Pathophysiology, Master's Degree

09/2002-06/2007: Guangxi Medical University, Clinical Medicine, Bachelor´s Degree

Internships

09/2005-05/2006: First affiliated hospital of Guangxi Province, dept. of respiratory,cardiology, haematology, neurology and ophthalmology.

05/2006-11/2006: 303th Chinese People's Liberation Army Hospital dept. of urology, general surgery, orthopedics and anaesthetics.

11/2006-05/2007: Taikang people's Hospital of Henan Province. dept. of gastroenterology, contagion, pediatrics and endocrinology.

Research activity

09/2007-06/2010 Study on the mechanism underlying acute pancreatitis: Chaperonin

60 expression and its roles in the enzymatic lesion of pancreas.

09/2007-07/2010 Study on the effects of Cannabinoid agonist and antagonists on motility of colon and ileum in mouse and rat

10/2010-Present Study of the anticancer property and mechanisms of action of c-MET inhibition by the kinase inhibitor Tivantinib in gastrointestinal tumors.

Oral Presentations and attendance to congresses

27-28. Jan, 2012 -28. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber. Hamburg, Germany- Poster presentation: "Antitumor efficacy of the c-MET inhibitor tivantinib (ARQ–197) in liver cancer cells: effects on apoptosis and cell cycle".

30. May, 2012 -CSC-LMU Program auditing seminar, Munich, Germany- Presentation: "Sensitization of cancer cells to apoptosis as alternative therapeutic approach".

01-05. Nov, 2013 -64th Annual Meeting of The American Association for the Study of Liver Diseases (AASLD). Washington, USA-Poster Presentation:

1. Anti-proliferative mechanisms downstream of c-MET of the kinase inhibitor tivantinib (ARQ 197).
2. Antiproliferative and proapoptotic effect of the MLK/JNK inhibitor CEP-1347 in hepatocellular carcinoma.

Publications

1. **Shuai Lu**, Antonia Rizzani, Frank T. Kolligs, Eike Gallmeier, Sabrina Arena, Alberto Bardelli, Burkhard Göke, Alexander L. Gerbes, Enrico N. De Toni. Anti-proliferative mechanisms downstream of c-MET of the kinase inhibitor tivantinib (ARQ 197). Hepatology (Suppl; Abstract). In: 64th Annual Meeting of

American Association for the study of Liver Diseases, Washington, DC, 2013 .

2. **Shuai Lu**, Johanna Liebl, Antonia Rizzani, Burkhard Göke, Eike Gallmeier, Alexander L. Gerbes, Angelika Vollmar, Enrico N. De Toni. Antiproliferative and proapoptotic effect of the MLK/JNK inhibitor CEP-1347 in hepatocellular carcinoma. Hepatology (Suppl; Abstract). In: 64th Annual Meeting of American Association for the study of Liver Diseases, Washington, DC, 2013.

3. Yongyu Li, Birol Yuece, Hua Ming Cao, Hong Xu Lin, **Shuai Lu**, Jie Chang Chen, Stephanie Ochs, Andrej Sibaev, Elisabeth Deindl, Claus Schaefer and Martin Storr. Inhibition of p38/Mk2 signaling pathway improves the anti-inflammatory effect of WIN55 on mouse experimental colitis. Laboratory Investigation. 2013, 93, 322-333.

4. **Shuai Lu**, Antonia Rizzani, Frank T Kolligs, Burhard Göke, Alexander Gerbes, Enrico De Toni. Antitumor efficacy of the c-MET inhibitor tivantinib (ARQ-197) in liver cancer cells: effects on apoptosis and cell cycle. Z Gastroenterol 2012; 50-P5_31. (Congress abstract).

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6. **Shuai Lu**, Jia-yan Feng, Zhi-rong Gao, Kun,Li Yan-na Li, Lei-lei Fang, Yong-yu Li.LPS and cerulein induce isolated rat pancreatic tissue injury and inhibit expression of HSP60 protein. Chinese Journal of pathophysiology. 2010, 26 (6):1146-1150. (In chinese)

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8. Yongyu Li, Yanna Li,Jianbo Ni, Changjie Chen, **Shuai Lu,** Shangyu Chai, Rihan Wu, Birol Yuece, Martin Storr. Involvement of cannabinoid-1 and cannabinoid-2 receptors in septic ileus. Neurogastroenterol Motil. 2010; 22(3): 350-357.

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10. Yong-Yu Li , Xue-Jin Li, **Shuai Lu,** Kun Li,Yan-Na Li, Zhi-Rong Gao, Jia-Yan Feng ,Chang-Jie Chen , Claus Schaefer.Ascitic fluid and serum from rats with acute pancreatitis injure rat pancreatic tissues and alter the expression of heat shock protein 60. Cell Stress and Chaperones. DOI 10.1007/s12192-010-0170-5.

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