Heat shock protein 90 is a promising target for effective growth inhibition of gastrointestinal neuroendocrine tumors

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Abstract. Treatment of gastroenteropancreatic neuroendocrine tumors (GEP-NET) is still unsatisfactory and innovative therapeutic approaches are urgently needed. Heat shock protein 90 (Hsp90) is overexpressed in a wide range of tumor types and is an emerging target for the treatment of cancer. However, the potential activity of Hsp90 inhibitors in GEP-NET has not yet been investigated. We studied the antineoplastic activity of the Hsp90 inhibitor IPI-504 on GEP-NET cells, and characterized its mechanism of action. In human insulinoma (CM) and pancreatic carcinoid (BON) cells IPI-504 induced a dose-dependent growth inhibition by almost 70%. The antiproliferative effect of IPI-504 correlated with a reduction in protein levels of the IGF-1 receptor. Additionally, several proteins of the PI3K/AKT/mTOR pathway, downstream of IGF-1 receptor activation in GEP-NETs, were downregulated as a consequence of Hsp90 inhibition. Combination treatment of IPI-504 with mTOR- or AKT-inhibitors led to additive antiproliferative effects. In addition, effects of IGF-1 receptor tyrosine kinase inhibition were strongly enhanced by IPI-504. Cancer gene expression profiling and FACS analysis revealed that IPI-504 antiproliferative effects were due to both induction of cell cycle arrest and apoptosis. A modified chick chorioallantoic membrane (CAM) assay confirmed the antineoplastic activity of IPI-504 in GEP-NETs *in vivo*. In conclusion, this study showed that Hsp90 inhibition may be an attractive target for innovative GEP-NET treatment alone or in combination with either IGF-1R or mTOR inhibitors.

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Introduction

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs), often synonymously called carcinoids, are a very heterogeneous group of neoplasms. Originally thought of as representing a rather homogeneous group, advances in the knowledge of molecular changes within different tumor entities of the GEP-NETs led to the classification system of the WHO in 2000 (1). The tumors are classified as well differentiated neuroendocrine tumors, well differentiated neuroendocrine carcinomas, poorly differentiated neuroendocrine carcinomas and mixed exocrine-endocrine carcinomas. However, a variety of different nomenclature systems has been developed, causing much confusion. Therefore the system of nomenclature, grading and staging of GEP-NETs was supplemented with additional studies which confirmed the WHO classification system (2).

With a reported incidence of 2-3:100,000 these tumors are relatively rare, but the 5-year survival rate is only approximately 67% (3). For localized tumors producing an excess of biogenic amines and hormones, systemic symptoms are limited by the rapid hepatic clearance of these molecules. Patients with metastases, however, often have incapacitating symptoms, including diarrhea, flushing, wheezing and skin rashes.

The treatment of choice for localized tumors is still surgical resection but approximately 80% of patients have already developed liver or lymph node metastases upon presentation (4). In the advanced stages, the medical treatment options are poor. Although tumor-related symptoms are often well-controlled by somatostatin-analogues (i.e., lanreotide and octreotide) sometimes combined with interferon-α, an effective inhibitor of tumor growth is not available at this time. Combinations of etoposide plus cisplatin, or streptozocin plus 5-FU or doxorubicin are used in chemotherapy treatment, but response rates are generally a disappointing 0-30% (5). Thus, effective new treatment strategies are urgently needed.

Heat shock protein 90 (Hsp90), an emerging target for the treatment of cancer (6), is a highly expressed protein chaperone that associates with many 'client' proteins implicated in oncogenesis. Indeed, numerous Hsp90 client proteins are kinases or transcription factors involved in cellular proliferation, angiogenesis, invasion, and metastasis. Hsp90 has been shown to be overexpressed in a wide range of tumor types including breast, endometrial, ovarian, colon, lung and prostate. Several of the proteins that are known to be overexpressed in GEP-NET are regulated by Hsp90, including EGFR, ERbB2, IGF-1R and AKT (7). Moreover, a recently published report from Gilbert *et al* demonstrated that Hsp90 and IGF-1R are important molecular biomarkers of neuroendocrine tumor cells and potential targets for anti-GEP-NETs molecules (8).

Inhibition of Hsp90 leads to rapid degradation of client proteins through the ubiquitin-proteasome pathway (9). Studies have demonstrated the activity of Hsp90 inhibitors in multiple models of solid (e.g., lung, breast, prostate, pancreatic, melanoma) and hematologic (e.g., chronic myelogenous leukemia, multiple myeloma) cancers (10). Several Hsp90 inhibitors are currently being tested in clinical trials, including IPI-504 (retaspimycin hydrochloride), which is a derivative of the natural product geldanamycin (11). In this study we investigated the effect of IPI-504 on the growth of GEP-NET cells and its mechanism of action.

Materials and methods

Cell lines. The human pancreatic carcinoid tumor cell line BON (12) (Verto Institute, Stamford, CT, USA) in collaboration with Kjell Oberg (Uppsala, Sweden), and the cholecystokinin secreting murine neuroendocrine cell line STC-1 (30) were grown in a 1:2 mixture of DMEM and Ham's F-12 medium containing 10% (v/v) FCS (Biochrom Co., Berlin, Germany) and 1% (v/v) L-glutamine with a doubling time of 34 ± 4 h. The human insulinoma cell line CM (13) provided by Professor P. Pozzilli (University La Sapienza of Rome, Italy), the pancreatic carcinoma of islet cell line QGP-1 (Japan Health Sciences Foundation, Osaka, Japan), and the ileal carcinoid cell line HC-45 (Mayo Clinic, Rochester, NY, USA) were all cultured in RPMI-1640 supplemented with 10% (v/v) FCS. All cell lines were kept at 37°C in a humidified atmosphere (5% CO_2).

Drugs. Triciribine was purchased from Merck (Darmstadt, Germany). Doxorubicin was purchased from Sigma (St. Louis, MO, USA). NVP-AEW541 and RAD-001 (everolimus) were bought from Cayman Chemical (Ann Arbor, MI, USA) and Molcan Corp., (Toronto, ON, CA), respectively. IPI-504 was from Infinity Pharmaceuticals (Cambridge, MA, USA). Stock solutions were prepared in DMSO, stored at -20°C, and diluted to their final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration was <0.1%.

Measurement of growth inhibition. Cells were incubated with increasing concentrations of IPI-504 for up to 96 h. Viability studies were performed using Cell Titer Glow (Promega, Madison, WI, USA), a vital mitochondrial function stain. The data were normalized with respect to DMSO vehicle control to generate IC_{50} values. Drug-induced changes in cell numbers

cells were evaluated by crystal violet staining, as described previously (14).

Western blotting and ELISA. Western blotting was performed as described (15). In brief, whole-cell extracts were prepared by lysing cells. Lysates of protein were subjected to gel electrophoresis and were transferred to PVDF membrane by electroblotting. After blocking in 5% skim milk powder solution (Merck, Darmstadt, Germany) the blots were incubated at 4°C overnight with antibodies directed against the β chain of the IGF-1R (1:500; Santa Cruz Biotechnology, CA, USA), phospho-AKT, AKT, phosphoS6 and Hsp70 (Cell Signaling Technology, MA, USA). After incubation with horseradish peroxidase-coupled anti-IgG antibody (1:10.000; Amersham, Uppsala, Sweden) the blots were developed using enhanced chemiluminescent (ECL) detection (Amersham) and subsequently exposed to Hyperfilm ECL (Amersham). Experiments were performed three times, and representative experiments are shown.

BON cells were incubated with increasing concentrations of IPI-504 for 24 h. Levels of phosphorylated IGF-1R were monitored in cell lysates using a phospho-IGF-1R ELISA (R&D Systems, Minneapolis, MN).

DNA microarray (84 cancer pathway specific genes). Total cellular RNA was extracted from cells using Array Grade Total RNA Isolation Kit (SABiosciences, Frederick, MD, USA). RNA was quantified by spectrophotometer (GeneQuant, Biochrom, Cambridge, UK). The True-Labelling AMP 2.0 amplification kit (SABiosciences) was used for transcription of mRNA into cDNA, which was then converted into biotin-labelled cRNA using biotin-16-UTP (Roche) via *in vitro* transcription. Before hybridization, the cRNA probes were purified with an ArrayGrade cRNA cleanup kit (SABiosciences). The purified cRNA probes were then hybridized to the pre-treated Oligo GEArray Human Cancer Pathway Finder array (OHS-033, SABiosciences). After washing steps, array spots binding cRNA were detected by a chemiluminescence method according to the manufacturer's instructions. Image acquisition was performed using X-ray film and digital scanner. Spots were then analyzed and converted into numerical data by using the GEArray Expression Analysis Suite software (SABiosciences). Data evaluation included background correction (subtraction of minimum value) and normalization to reference genes. The cut-off for upregulation was set at a 1.4-fold increase of the ratio of genes in the treated samples, whereas downregulation was determined as the 0.8-fold expression of genes in the treated samples.

Migration assay. CM cells were allowed to grow into full confluence in 6-well plates. The monolayer was then scratched with a 10 μl pipette tip. Fresh growth medium was added and different concentrations of IPI-504 were added. Images were taken with a Kappa digital camera after 24 h of incubation at 37°C in a humidified atmosphere of 5% CO_2. The migrated cells were quantified by using the software TScratch (16).

Cell cycle analysis/apoptotic cell death by flow cytometry (FACS). Cell cycle analysis was performed by a modified method of Fried *et al* (17). Cells were seeded at a concentration of 10^5 cells/ml and treated with IPI-504 for 24 h. Cells were
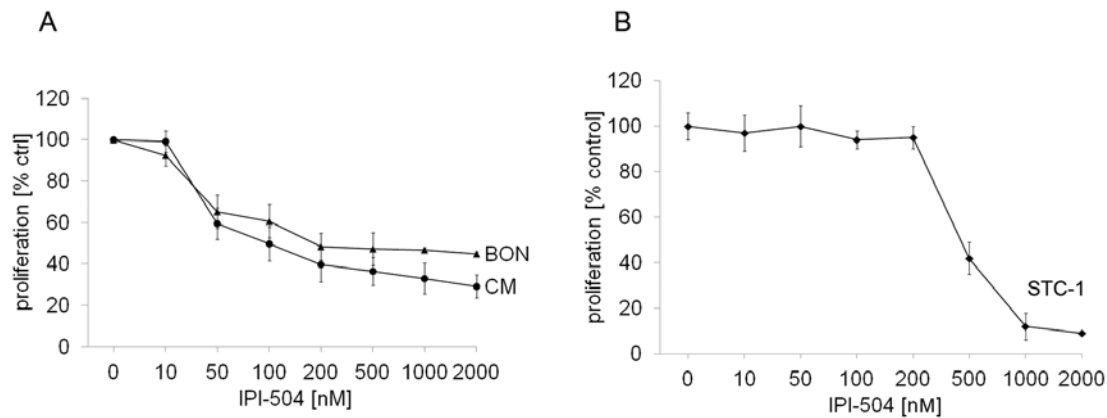
Figure 1. IPI-504 inhibits the growth of GEP-NET cells. CM, BON (A) and STC-1 cells (B) were incubated with increasing concentrations of IPI-504 for 48 h and drug-induced changes in cell numbers were evaluated by crystal violet staining. Data are given as percentage of untreated controls, which were set 100%. Means of n=3 independent experiments ± SD.

then washed with PBS and fixed in PBS/2% formaldehyde (v/v) on ice for 30 min. Afterwards cells were incubated in ethanol/PBS (2:1, v/v) for 30 min and pelleted. Resuspension in PBS containing 40 μg/ml RNase A followed. After incubation for 30 min at 37°C, cells were pelleted again and resuspended in PBS containing 50 μg/ml propidium iodide. Cells were then analyzed on a FACSCalibur flow cytometer using CellQuest Pro Software (BD Biosciences, Heidelberg, Germany) and FlowJo Software (Tree Star, Ashland, OR, USA). Apoptotic cells can be identified as a hypoploid cell population commonly called sub-G1.

Caspase-3 assay. Changes in caspase-3 activity were assessed by measuring the cleavage of the fluorogenic substrate AC-DEVD-AMC (Calbiochem-Novabiochem, Bad Soden, Germany), as described previously (30). In brief, cell lysates were incubated for 1 h at 37°C with a substrate solution containing 20 μg/ml AC-DEVD-AMC, 20 mM HEPES, 10% glycerol, 2 mM DTT with a pH adjusted to 7.5. Substrate cleavage was measured fluorometrically using a VersaFluor fluorometer (filter wavelengths: excitation: 360/40 nm, emission: 460/10 nm) from Bio-Rad (Munich, Germany).

Chorioallantoic membrane (CAM) angiogenesis assay. The chorioallantoic membrane (CAM) assay was done as described previously (18,43). Briefly, 2.5x10^{6} CM cells or 5x10^{6} BON cells were resuspended in 20 μl growth medium and mixed with 30 μl growth factor reduced Matrigel (BD Biosciences). The cell suspensions were implanted on fertilized chicken eggs on day 10 of incubation using a silicone ring of 10 mm in diameter. After two days, tumors were topically treated with either PBS (negative control) or PBS containing 2 μM IPI-504 in 100 μl PBS for 96 h. Tumor growth and viability of the embryo were controlled daily by stereo microscopy. At the end of experiments, tumors were recovered for weighing and pictures were taken using a stereomicroscope equipped with a Kappa digital camera system. For data analysis the increase in tumor weight of both treated and untreated tumors was calculated relative to the initial volume of the tumor cell suspension.

Results

IPI-504 inhibits the proliferation of neuroendocrine cell lines by induction of apoptosis and cell cycle arrest. Changes in the cell number caused by Hsp90 inhibition were studied by performing crystal violet assays. IPI-504 (0-2000 nM) time- and dose-dependently inhibited the growth of CM and BON cells (Fig. 1A). After 48 h of incubation, a decrease of almost 70% was observed. The IC_{50} values of IPI-504, determined after 48 h, amounted to 100 nM in fast-growing CM cells (doubling time: 21±1 h) and 192 nM in the moderately-growing BON cells (doubling time: 34±4 h). Additionally performed viability studies with the ileal carcinoid cell line HC-45 and the pancreatic carcinoma of islet cell line QGP-1 as well as with the slow growing intestinal neuroendocrine cell line STC-1 (doubling time: 54±6 h) confirmed the growth inhibitory potency of IPI-504 in neuroendocrine gastrointestinal tumors. In STC-1 cells, the incubation with IPI-504 time- and dose-dependently reduced the growth by >95% after 48 h, yielding an IC_{50} value of 437 nM (Fig. 1B). The IC_{50} values of IPI-504 in HC-45 and in QGP-1 cells amounted to 302 and 705 nM, respectively (data not shown).

To better understand the inhibitory effects of IPI-504 in GEP-NET cells, the induction of cell cycle arrest and apoptosis was investigated. CM cells were treated with increasing concentrations of IPI-504 for 24 h, and apoptosis was determined by measuring fragmentation of genomic DNA (hypodiploid cells) using FACS analysis. Hsp90 inhibition by IPI-504 resulted in a dose-dependent increase of apoptotic CM cells of up to 8-fold as compared to the basal level of untreated controls (Fig. 2A). To confirm the apoptotic effects of IPI-504 additional capase-3 measurements were performed. Incubation with 0-1000 nM IPI-504 led to an apoptosis specific increase in caspase-3 activation of both CM and BON cells (Fig. 2B and C).

CM cells were also analyzed for cell cycle arresting effects of IPI-504 (Fig. 2D). After 24 h of incubation with IPI-504 there was an accumulation of cells in the G0/G1-phase, and the amount of cells in S- or G2/M-phase decreased proportionally, as determined by FACS analysis. These data strongly indicate that the antineoplastic mode of action of IPI-504

Table I. IPI-504 induced alteration in gene expression.

| Symbol | Up- and down-regulated genes | |
	Product	Fold
Apoptosis		
APAF-1	Apoptotic peptidase activating factor 1	8.84
BAX	BCL2-associated X protein	13.52
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	0.30
FAS	Fas (TNF receptor superfamily, member 6)	2.56
TERT	Telomerase reverse transcriptase	1.49
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	0.68
Cell cycle related genes		
BRCA1	Breast cancer 1, early onest	2.56
CCNE1	Cyclin E1	0.17
CDK2	Cyclin-dependent kinase 2	0.57
CDK4	Cyclin-dependent kinase 4	0.68
CDKN1A	Cyclin-dependent kinase ibhibitor 1A (p21, Cip1)	2.33
CDKN1B	Cyclin-dependent kinase ibhibitor 1B (p27, Kip1)	1.40
CDKN2A	Cyclin-dependent kinase ibhibitor 2A	1.84
E2F1	E2F transcription factor 1	0.04
PTEN	Phosphatase and tensin homolog	5.56
TP53	Tumor protein p53	1.93
Signal transduction molcules and transcription factors		
AKT1	V-akt murine thymoma viral oncogene homolog 1	0.18
ERBB2	V-erb-B2 erythroblastic leukemia viral oncogene homolog 2	0.79
MAP2K1	Mitogen-activates protein kinase kinase 1	0.72
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α	1.70
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	0.24
SNCG	Synuclein γ	4.87
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	2.89
Adhesion		
CD44	CD44 molecule	4.95
EPDR1	Ependymin related protein 1	0.43
ITGA3	Integrin α3	1.63
ITGB1	Integrin β1	0.70
ITGB5	Integrin β5	0.71
MCAM	Melanoma cell adhesion molecule	0.76
NCAM1	Neural cell adhesion molecule 1	0.56
PNN	Pinin, desmosome associated protein	0.76
Angiogenesis		
IGF1	Insulin-like growth factor 1	3.57
IL8	Interleukin 8	33.59
PDGFA	Platelet-derived growth factor α polypeptide	9.70
PDGFB	Platelet-derived growth factor β polypeptide	8.03
TGFBR1	Transforming growth factor β receptor	4.35
THBS1	Thrombospondin 1	2.51
THBS2	Thrombospondin 2	5.91

Table I. Continued.

Symbol	Up- and down-regulated genes	
	Product	Fold
Invasion and metastasis		
CD82	CD82 molecule	4.09
KISS1	KiSS-1 metastasis	4.82
MMP1	Matrix metallopeptidase 1	18.32
MMP2	Matrix metallopeptidase 2	1.93
NME1	Non-metastatic cell 1, protein	0.69
NME4	Non-metastatic cell 4, protein	0.65
PLAU	Plasminogen activator, urokinase	4.88
PLAUR	Plasminogen activator, urokinase receptor	4.09
TIMP1	TIMP metallopeptidase inhibitor 1	0.69
TIMP3	TIMP metallopeptidase inhibitor 3	0.71

CM cells were treated with the IC_{50} concentration of IPI-504 (100 nM) for 48 h and a gene expression array was performed using the Oligo GEArray 'Human Cancer Pathway Finder' DNA Microarray according to the manufacturer's instructions.
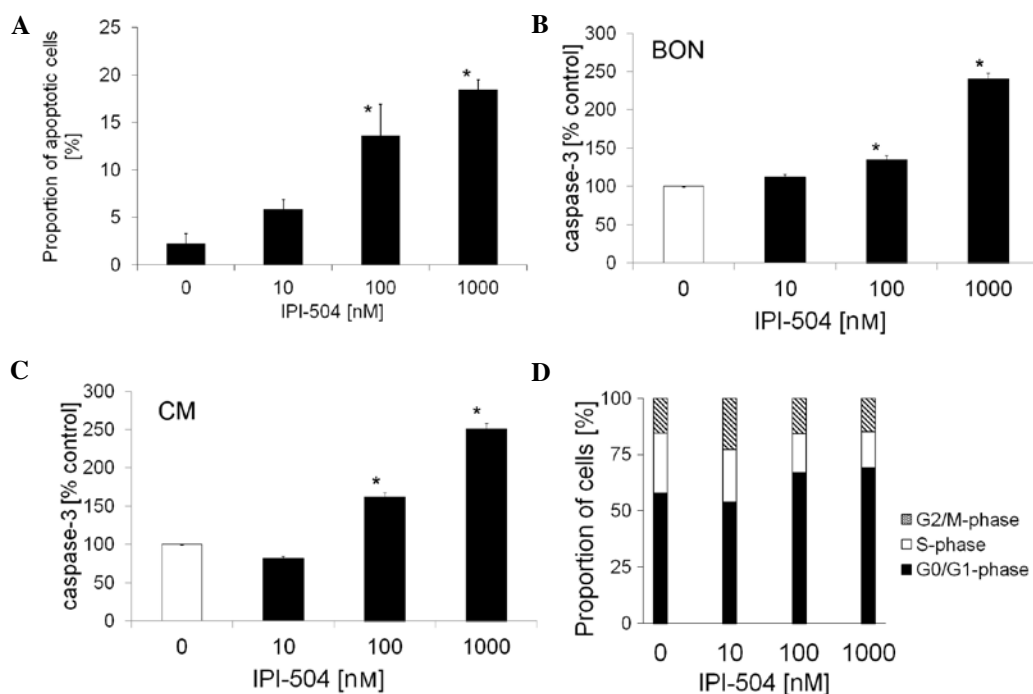

Figure 2. IPI-504 triggers apoptosis and blocks the cell cycle of GEP-NET cells. (A) CM cells were treated with rising concentrations of IPI-504 (0-1000 nM) for 24 h, fixed and analyzed by flow cytometry for apoptotic cells (sub-G1-peak) according to DNA content (PI staining). (B and C) Induction of caspase-3 in GEP-NET cells after incubation with 0-1000 nM IPI-504 for 48 h [BON; (B)] or 24 h [CM; (C)]. Data are given as percentage of untreated controls, which were set 100%. (D) CM cells were incubated for 24 h with increasing concentrations of IPI-504 and analyzed by flow cytometry for the cell cycle phase they were in. Results represent means ± SEM of three independent experiments *p<0.05 vs. control.

involves the induction of apoptosis as well as an arrest of the cell cycle.

Hsp90 inhibition alters the expression of various oncogenes. The effects of IPI-504 on the expression of 84 genes that are known to be cancer related were investigated next. CM cells were treated with the IC_{50} concentration of 100 nM of IPI-504 for 48 h and a gene expression array was performed. IPI-504 treatment led to a change of the expression levels of 48 genes compared to control (Table I). As expected, many of these were cell cycle arrest-related genes. Cyclin E and cyclin-dependent kinase 2 (CDK2), important for the G1/S-phase transition, were both downregulated after IPI-504 treatment. CDK4, which influences the G0/S-phase transition, was also downregulated.
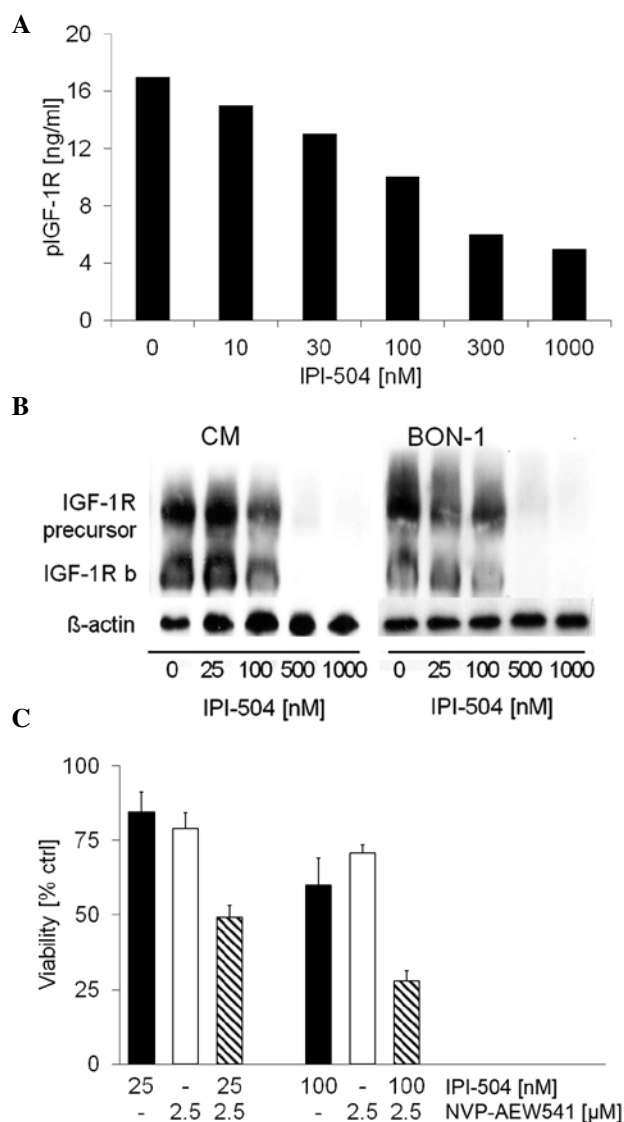
A

B

C

Figure 3. IGF1-R, an Hsp90 client protein, is expressed in GEP-NET cells. (A) BON cells were incubated with increasing concentrations of IPI-504 for 24 h. Levels of phosphorylated IGF-1R were monitored in cell lysates using a phospho-IGF-1R ELISA. (B) Expression of IGF-1 receptor and IGF-1 receptor precursor was evaluated in IPI-504-treated CM (left side) and BON (right side) cells by Western blotting. (C) CM and BON cells were incubated with IPI-504 and/or with NVP-AEW541 (2.5 μM) for 48 h. Cell number was determined by crystal violet staining. Results represent means ± SD of three independent experiments.
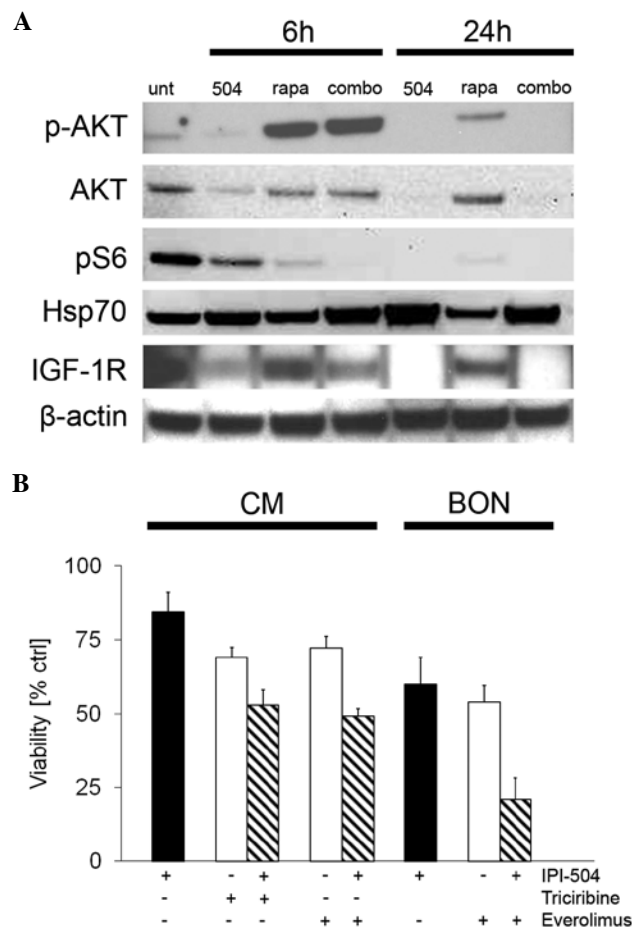

A

B

Figure 4. Additive effects of Hsp90, mTOR or Akt inhibition in neuroendocrine cell lines. (A) BON cells were left untreated (unt) or incubated for 6 h or 24 h with 1 μM IPI-504 (504), 100 nM rapamycin (rapa) or the combination of both (combo). Cell lysate (50 μg) was immunoblotted for pAKT, total AKT, pS6, IGF-1R, Hsp70, and β-actin. (B) CM and BON cells were incubated with IPI-504 alone or in combination with either 100 nM everolimus or 5 μM triciribine for 48 h. Cell numbers were determined by crystal violet staining. Means ± SD of three independent experiments.

IPI-504 inhibits the Hsp90 client protein IGF-1R. Insulin-like growth factor 1 receptor (IGF-1R) is overexpressed in GEP-NET cells and is also a client protein of Hsp90 (42). BON cells were incubated with increasing concentrations of IPI-504 for 24 h, and levels of phosphorylated IGF-1R were monitored in cell lysates using a phospho-IGF-1R ELISA. BON cells constitutively express phosphorylated insulin receptors (data not shown) and the IGF-1R receptor. By inhibiting Hsp90, IPI-504 destabilized and degraded phospho-IGF-1R in a dose-dependent manner with an IC$_{50}$ of 50 nM, suggesting that the growth inhibitory effect may be linked to the IPI-504 dephosphorylating activity on the IGF-1 receptor (Fig. 3A).

To confirm these data, Western blot analyses were performed. BON and CM cells were incubated for 24 h with increasing concentrations of IPI-504, and 30 μg of cell lysate was immunoblotted for IGF-1R. In both cell lines, IPI-504 treatment resulted in a marked decrease in IGF-1R expression (Fig. 3B).

We also investigated the effect of a combination treatment with IPI-504 and the IGF-1R specific inhibitor NVP-AEW541 on GEP-NET cell proliferation (Fig. 3C). BON and CM cells were incubated with IPI-504 and/or NVP-AEW541 (2.5 μM) for 48 h and stained with crystal violet. Results showed that

The cyclin-dependent kinase inhibitors p21, p27 and p16, which inhibit the G0/S-phase transition, were upregulated.

IPI-504 treatment also led to an upregulation of BRCA1 and p53, both negative regulators of the cell cycle progression (20,21). The E2F transcription factor 1 (E2F1), a regulator of the expression of several proteins involved in cell cycle progression, was highly downregulated (22). Additionally, there were alterations in the expression of several apoptosis and senescence-related genes. AKT was downregulated, while PTEN, the main inhibitor of AKT, was upregulated. Other apoptosis inducing genes, such as APAF1 and BAX were upregulated and the anti-apoptotic gen BIRC5 (survivin) was downregulated (23). These results confirm that IPI-504 arrests the cell cycle of neuroendocrine cells and also triggers apoptosis.
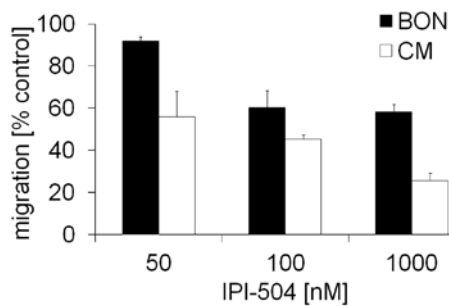
Figure 5. IPI-504 markedly decreases the migration of GEP-NET cells. Scratch assays were used to examine the effect of IPI-504 on neuroendocrine tumor cell migration. CM and BON cell monolayers were scratched with a pipette tip and treated with increasing concentrations of IPI-504 for 24 h. The scratch closure was determined by TScratch software. Data are presented as migration of IPI-504 treated cells compared to untreated control cells, whose migration after 24 h was set 100%. Means ± SD of three independent experiments.

concomitant inhibition of Hsp90 and IGF-1R leads to additive growth inhibition.

Additive effect of combined Hsp90 and mTOR or Akt inhibition in neuroendocrine cell lines. Since the PI3K/Akt/mTOR pathway is activated in neuroendocrine gastrointestinal tumors, we also studied the combination effects of IPI-504 and drugs which inhibit the AKT/mTOR pathway. BON cells were incubated for 6 or 24 h with 1 μM IPI-504, 100 nM rapamycin or the combination of both. Cell lysate (50 μg) was immunoblotted for pAKT, total AKT, pS6, IGF-1R, Hsp70, and β-actin. As previously described, rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism (19). Since IPI-504 treatment causes AKT degradation it also abrogates the above-mentioned feedback. Especially after 24 h the combination of IPI-504 and rapamycin exhibited additive effects (Fig. 4A). In a second experiment, BON and CM cells were treated for 48 h with 100 nM IPI-504 alone or in combination with an mTOR inhibitor (everolimus) or an AKT-selective small molecule inhibitor (triciribine). In CM cells IPI-504 enhanced the antiproliferative effects of triciribine and everolimus. In BON cells the combination of IPI-504 with everolimus led to additive growth inhibitory effects (Fig. 4B).

IPI-504 markedly decreases the migration of GEP-NET cells. Scratch assays were performed to examine the effect of IPI-504 on neuroendocrine tumor cell migration. CM and BON cell monolayers were scratched with a pipette tip, treated with increasing doses of IPI-504 for 24 h, and the scratch closure was measured by TScratch software. Results showed that IPI-504 markedly decreased the migration of CM and BON cells as compared to the untreated control cells (Fig. 5).

IPI-504 suppresses the growth of neuroendocrine tumor cells in vivo. The antitumor activity of IPI-504 *in vivo* was evaluated by using a modified chorioallantoic membrane (CAM) assay (43). BON and CM cells were implanted on the CAM of fertilized chicken eggs. Two days after inoculation both cell lines formed large, macroscopic tumor nodules. Tumors were topically treated with 2 μM IPI-504 for 96 h and then recovered for pathologic analysis. While the growth inhibitory effect of IPI-504 in slow-growing BON tumors was less pronounced, the treatment almost completely inhibited the growth of fast-growing CM tumors (Fig. 6).

Discussion

Thanks to the use of established drugs like somatostatin analogues, great progress has been made in controlling the often debilitating hyper-secretion syndrome encountered in patients with metastasized GEP-NETs (24). However, cytostatic therapy regimens aimed at slowing tumor progression, or inducing remission had only limited success (25). Hsp90 is known to be important for tumor survival and progression (26,27), and several proteins deregulated in GEP-NETs are at least partially controlled by Hsp90 (7,28). Currently there are about a dozen clinical trials investigating the effects of Hsp90 inhibition in different types of cancers (29). This is the first publication to report on the effects of Hsp90 inhibition in neuroendocrine tumor cells. In this study, we showed that inhibition of Hsp90 by IPI-504 may be a promising approach for novel GEP-NET treatment options.

Since GEP-NETs are very heterogeneous, and include both slow- and fast-growing aggressive tumors, it is important to look at different representatives of this tumor entity when testing new therapeutic agents. Thus we chose cell lines with different
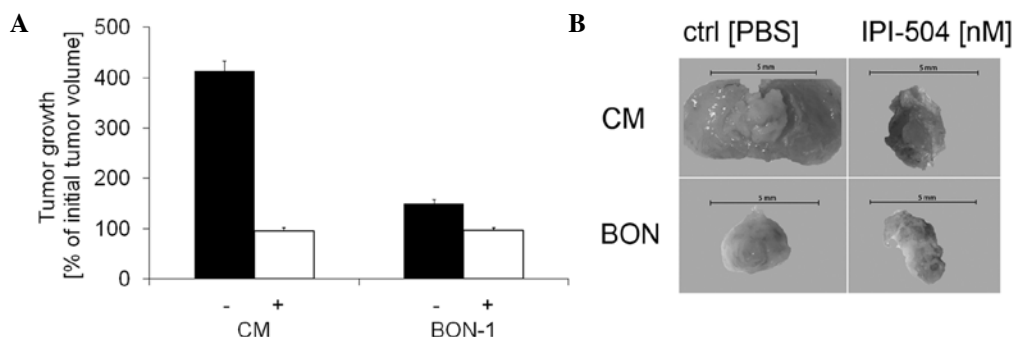

Figure 6. IPI-504 suppresses the growth of neuroendocrine tumor cells *in vivo*. A modified chorioallantoic membrane (CAM) assay was performed to analyze the antineoplastic effects of IPI-504 *in vivo*. (A) Increase in tumor weight after 96 h of incubation with IPI-504 (2 μM). Data are given as relative changes of the volume of treated and untreated tumors compared to the initial volume of the inoculated tumor cell preparation. Means ± SD of three independent experiments for each tumor entity. (B) Pictures of the tumors were taken with a stereomicroscope equipped with a Kappa digital camera system. Representative pictures of at least 3 independent preparations of BON- and CM-tumor bearing CAMs.

growth rates and origins (30) and could show that IPI-504 is highly effective in all tested GEP-NET cell lines.

Treatment of GEP-NET cells with the specific Hsp90 inhibitor IPI-504, led to a dose-dependent reduction in cell growth by inducing cell cycle arrest and/or apoptosis. In BON and CM cells the antiproliferative effect of IPI-504 correlated with a reduction in protein levels of the IGF-1 receptor. This is consistent with earlier publications where we reported that the IGF-1 receptor plays a crucial role in the survival and proliferation of GEP-NETs (15), as well as with a recently published report of Gilbert *et al* showing that Hsp90 inhibition induces a reduction of IGF-1R expression and an inhibition of constitutive IGF-1R autophosphorylation in bronchopulmonary neuroendocrine tumor cells (8). The authors concluded that Hsp90 and the IGF-1R are valuable molecular biomarkers important for neuroendocrine tumor cell growth and that they may serve as the basis for further research into novel targeted therapeutics for these tumors.

Additionally, several proteins in the PI3K/AKT/mTOR pathway, which are thought to be tightly regulated by the IGF-1 receptor in neuroendocrine tumor cells (31), were downregulated as a consequence of Hsp90 inhibition. This most likely happens not only as a consequence of IGF-1R downregulation, but also as a direct influence of Hsp90 inhibition. AKT is known to be associated with Hsp90 as its chaperone to prevent degradation (32). We showed that treating GEP-NETs with IPI-504 not only decreased the amount of active, phosphorylated AKT, but also led to a reduction of total AKT which indicates an increase in ubiquitination of this protein. Furthermore, the ribosomal protein S6 (pS6), which is further downstream in the PI3K-AKT pathway, is also downregulated and directly influenced by Hsp90 (33).

A recent phase II clinical trial concluded that everolimus was a promising therapeutic approach among patients with advanced pancreatic NETs (34), and a recently published phase III trial (Radiant-3) demonstrated the suitability of everolimus as a first-line option for patients with advanced pancreatic NETs. Compared with placebo, everolimus significantly prolonged progression-free survival among patients with progressive advanced pancreatic neuroendocrine tumors and was associated with a low rate of severe adverse events (44).

Therefore, we looked at combinations of IPI-504 with other targeted cancer therapeutics. mTORC1 is affected by the PI3K-AKT pathway, as mTOR is phosphorylated by AKT (35). Studies have shown that mTORC1/mTOR inhibition leads to upregulation of AKT via loss of feedback inhibition making mTORC1 inhibitors even more attractive in combination with drugs targeting other proteins within the PI3K/AKT pathway (36). As a consequence, several new compounds are being studied as dual PI3K/mTORC1 inhibitors (37,38). Since Hsp90 inhibition leads to rapid dephosphorylation of AKT, we combined IPI-504 with the mTORC1 inhibitor everolimus (RAD-001) and found additive antiproliferative effects. This combination effect also occurred when targeting other proteins of the PI3K/AKT pathway. The AKT inhibitor triciribine showed additive effects when used in conjunction with IPI-504. Also, the antineoplastic effect of an IGF-1R inhibitor NVP-AEW541 was strongly enhanced by addition of IPI-504. These results are important for two reasons. First, many *in vitro* and *in vivo* studies using chemotherapeutic agents for targeted therapy in cancer have only shown modest antineoplastic effects, principally slower tumor progression. This is believed to be due to escape-mechanisms of the cells, which are more pronounced when only a single molecule is targeted. Therefore, targeting a growth pathway simultaneously at multiple sites might result in a better tumor response *in vivo*.

Using cancer gene expression profiling we showed that the antineoplastic effects of IPI-504 are, at least partially, due to an induction of cell cycle arrest and apoptosis. Several genes responsible for cell cycle control and apoptosis were altered by IPI-504 treatment. Correspondingly, flow cytometry revealed an increase of cells in G0/G1-phase, while the amount of cells in S- or G2/M-phase decreased. Moreover, apoptosis-inducing effects of IPI-504 were demonstrated by measurements of caspase-3 activation and the increase of the sub-G1-peak in FACS analyses.

Recently, several studies indicated that Hsp90 may be important on an extracellular level, where it could influence cell motility (39). A monoclonal antibody selectively targeting extracellular Hsp90 has been shown to decrease the formation of metastatic lesions in different types of cancer *in vitro* (40). In this study, we showed that IPI-504 potently inhibits the migration of gastrointestinal neuroendocrine tumor cells, which suggests its role in metastatic diseases.

Angiogenesis is a crucial step in the growth of tumors (45). Hsp90 seems to amplify angiogenesis by facilitating the activation of Akt and endothelial nitric oxide synthase (eNOS). The pharmacologic inhibition of Hsp90 can reverse this effect (41). By employing a modified chick chorioallantoic membrane (CAM) assay (43) we were able to show that IPI-504 slows down and even stops the growth of GEP-NETs cells *in vivo*.

In conclusion, we demonstrated that Hsp90 inhibition may become an attractive approach in treating GEP-NETs. Combination treatments showed promising additive effects, and metastatic disease seems to be an especially promising target for this new therapeutic option.

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